Swansea University Medical School
Ysgol Feddygaeth Prifysgol Abertawe

Swansea University
Prifysgol Abertawe

# A genome guided evaluation of probiotic bacteria from the *Lactobacillus* and *Bifidobacterium* genera.

Swansea University
Prifysgol Abertawe

**A thesis submitted to Swansea University in fulfilment of the requirements for the Degree of Doctor of Philosophy.**

**By**

**Laura Baker**

**(BSc Hons)**

UNDEB EWROPEAIDD
EUROPEAN UNION
Llywodraeth Cymru
Welsh Government
Cronfa Gymdeithasol Ewrop
European Social Fund

kess2
Ysgoloriaethau Sgiliau Economi Gwybodaeth
Knowledge Economy Skills Scholarships

CULTECH
BIOSPECIALITY PRODUCTS

**School of Medicine**

**Swansea University**

**2022**

**DECLARATION**

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

**Signed**:  (candidate)

**Date**: 03/01/2022

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

**Signed**:  candidate)

**Date**: 03/01/2022

STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access approved by the Swansea University.**

**Signed:**  candidate)

**Date:** 03/01/2022

# Abstract

Manipulating the microbiota has the potential to mitigate disease. Probiotics are currently a popular approach used to promote gut health. However, beneficial attributes are often strain-specific; therefore, an in-depth classification is valuable when evaluating such products.

Here, the draft genomes of 16 bacteria (from the *Lactobacillus* and *Bifidobacterium* genera) are presented (referred to as CUL isolates), including: *L.acidophilus* (CUL21, CUL60)*, L.gasseri* (CUL09), *L.helveticus* (CUL76), *L.salivarius* (CUL61), *L.plantarum* (CUL66, CUL66N), *L.paracasei* (CUL37, CUL07, CUL08), *L.casei (*CUL06), *L.rhamnosus (*CUL63), *L.fermentum* (CUL40, CUL67), *B.bifidum* (CUL20) and *B.animalis* subsp. *lactis* (CUL34). Furthermore, multi-locus sequence analysis revealed that CUL strains are novel entries into GenBank.

Genomes were mined for beneficial and deleterious features of probiotic bacteria. As such, antibiotic resistance genes and phenotypic antibiotic resistance (ABR) profiles were established. Generally, most phenotypic resistance was linked to recognized resistance profiles e.g., kanamycin and chloramphenicol (in lactobacilli) and tetracycline (in bifidobacteria). However, ampicillin resistance was common, although a genomic basis was not established. Indeed, the correlation between genotype and phenotype was often low. In addition, when challenged with a combination of antibiotics and bile, a phenotypic shift from antibiotic-resistant to sensitive typically occurred. Interestingly, *L.helveticus* CUL76 developed enhanced resistance against chloramphenicol and vancomycin.

Beneficial traits, including adherence, bile tolerance, and host interaction properties were identified in all CUL strains. *L.helveticus* CUL76 putatively encoded five bacteriocins, offering scope for future antimicrobial studies. Bile Salt Hydrolase (genes with bile tolerance and cholesterol reduction capabilities) were identified in several CUL species. *L.plantarum* CUL66N expressed *bsh* genes when challenged with bile and could reduce the bile acid pool (including free cholesterol) *in vitro*, indicating a *bsh* mediated, cholesterol-lowering capability of CUL66N.

Here, the first in-depth genomic analysis of CUL strains is presented, allowing future research to continue using a genome-guided approach to evaluate health-promoting attributes of CUL bacteria.

## Acknowledgements

Throughout my PhD candidature I have gained invaluable experience, learning from truly great scientists. I would like to take the time to thank my industrial collaborators, who have enabled my development and provided me with an industrial application of science. Therefore, A special thank you to Dr Sue Plummer, Dr Daryn Michael, and Dr Alison Jack for all your time, support, and encouragement over the years.

Next, I would like to thank my second supervisor, Dr Ricardo Del Sol, for the support and guidance provided throughout my PhD and for always being the voice of reason in the scientific debates.

A huge thank you to my Primary Supervisor Dr Paul Facey, who took me on as his first postgraduate student. Thank you for your faith, encouragement, and teachings over the years. I am extremely grateful for all that I have learned, and I will carry the lessons I have gained with me throughout my career.

I would also like to express thanks to Dr Josie Parker, for training and use of the GC/ MS.

Additionally, many thanks to KESS II (my funding body), for providing me with the opportunity to carry out my PhD research. And finally, to my amazing family, without which I would not be where I am today. Thank you for the patience, cups of coffee and understanding that you gave me, during some incredibly challenging times. Thank you to my Mum (who probably knows just as much about this thesis as I do), for listening to all my rambling. To my Dad, for his never-ending encouragement and words of wisdom. To my sister and partner, for always being there and for their constant support and finally to my amazing Nan who always believed in me.

# Contents

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| **AA** | Auxiliary Activities |
| **AB** | Antibiotic |
| **ABR** | Antibiotic Resistance |
| **AIC** | Akaike Information Criterion |
| **AMR** | Antimicrobial Resistance |
| **AC** | Accession Number |
| **AD** | Agar Dilution |
| **ARG** | Antibiotic Resistance Gene |
| **BA** | Bile Acid |
| **BD** | Broth Dilution |
| **BIC** | Bayesian Information Criterion |
| **Bsh/BSH** | Bile Salt Hydrolase (protein) |
| ***bsh*** | Bile Salt Hydrolase (gene) |
| **BSTFA TMCS** | N,O-Bis(trimethylsilyl)trifluoroacetamide |
| **CA** | Cholic Acid |
| **CBM** | Carbohydrate Binding Molecules |
| **CDCA** | Chenodeoxycholic Acid |
| **CBA** | Conjugated Bile Acid |
| **Cbp** | Collagen Binding Protein |
| **CE** | Carbohydrate Esterase's |
| **CLSI** | Clinical and Laboratory Standards Institute |
| **CVD** | Cardiovascular Disease |
| **DBA** | De-conjugated Bile Acid |
| **DCA** | Deoxycholic Acid |
| **EFSA** | European Food and Safety Authority |
| **EPS** | Exopolysaccharide |
| **EUCAST** | European Committee on Antimicrobial Susceptibility Testing |
| **F** | Phenylalanine |
| **FAO** | Food and Agricultural Organisation |
| **FBP** | Fibronectin Binding Protein |
| **FDA** | Food and Drug Administration |
| **G + C** | Guanine and Cytosine |
| **gDNA** | Genomic DNA |
| **GCA** | Glyco-Cholic Acid |

| | |
|---|---|
| **GCDCA** | Glyco-Chenodeoxycholic Acid |
| **GC-MS** | Gas Chromatography – Mass Spectrophotometry |
| **GDCA** | Glyco-deoxycholic Acid |
| **GH** | Glycoside Hydrolases |
| **GI** | Genomic Island |
| **GI-tract** | Gastrointestinal Tract |
| **GOI** | Gene Of Interest |
| **GRAS** | Generally Regarded As Safe |
| **GT** | Glycoside Transferases |
| **h** | Hour |
| **HDL** | High-Density Lipoprotein |
| **HGT** | Horizontal Gene Transfer |
| **ISO/IDF** | International Organization of Standardization/ International Dairy Federation |
| **K80** | Kimura 2-parameter |
| **LAB** | Lactic Acid Bacteria |
| **LCA** | Lithocholic Acid |
| **LDL** | Low Density Lipoprotein |
| **LSM** | LAB Susceptibility test Medium (90% Iso-sensitest + 10% MRS) |
| **MIC** | Minimum Inhibition Concentration |
| **MGE** | Mobile Genetic Element |
| **MHB** | Mueller Hinton Broth |
| **ML** | Maximum Likelihood |
| **MLSA** | Multi Locus Sequence Analysis |
| **MRCA** | Most Recent Common Ancestor |
| **MRS** | De Man, Rogosa and Sharpe |
| **NCBI** | National Center for Biotechnology Information |
| **NGS** | Next Generation Sequencing |
| **NJ** | Neighbor Joining |
| **.nwk** | Newick file extension |
| **ORF** | Open Reading Frame |
| **PA** | Penicillin Acylase |
| **PCR** | Polymerase Chain Reaction |
| **PHAST** | PHAge Search Tool |
| **PL** | Polysaccharide Lyases |
| **PVA** | Penicillin V Acylase |
| **(q)RTPCR** | Quantitative Reverse Transcription Polymerase Chain Reaction |

| | |
|---|---|
| **QPS** | Qualified Presumption of Safety |
| **RAST** | Rapid Annotation using Subsystem Technology |
| **rRNA** | Ribosomal Ribo-deoxynucleic acid |
| **SCFA** | Short Chain Fatty Acid |
| **T** | Tyrosine |
| **TCA** | Taurocholic Acid |
| **TCDCA** | Tauro-Chenodeoxycholic Acid |
| **TDCA** | Tauro-Deoxycholic Acid |
| **TN92** | Tajima-Nei Method |
| **WGS** | Whole Genome Sequencing |
| **WHO** | World Health Organisation |
| **w/v** | Weight/Volume |

# PhD outputs

## Conference Proceedings

**Society of Microbiology Annual Conference 2018**: Poster Presentation; Birmingham, United Kingdom.
**International Microbiome Conference**: Poster Presentation and abstract published; Heidelberg, Germany 2018.

## Publication List.

Quinn, G. A., Abdelhameed, A. M., Banat, A. M., Alharbi, N. K., **Baker, L. M**., Castro, H. C., ... & Banat, I. M. (2021). Streptomyces Isolates from the Soil of an Ancient Irish Cure Site, Capable of Inhibiting Multi-Resistant Bacteria and Yeasts. *Applied Sciences*, *11*(11), 4923.

**Baker, L. M**., Webberley, T. S., Masetti, G., Hughes, T. R., Marchesi, J. R., Jack, A. A., ... & Facey, P. D. (2021). A genome guided evaluation of the Lab4 probiotic consortium. *Genomics*, *113*(6), 4028-4038. (**Appendix 6**),

Webberley, T. S., Masetti, G., **Baker, L. M.,** Dally, J., Hughes, T. R., Marchesi, J., ... & Michael, D. R. The Impact of Lab4 Probiotic Supplementation in a 90-day Study in Wistar Rats. *Frontiers in Nutrition*, 994. (**Appendix 6**).

## Outreach

**Pint of Science 2019:** Invited speaker “the secrets of the GI tract – is it more than just a gut feeling?”

## Funding

**KESS II:** Upgrade funding (MSc to PhD) 2018.
**Society of Microbiology:** Travel Grant awarded 2018.

# Chapter 1. Introduction

## 1.1. Background

The importance of microbial interactions with human hosts is unequivocal. Indeed, the historical focus has often been to understand the negative effects that pathogenic microorganisms exert on host health (Almquist, 1922; Dethlefsen et al., 2007; Maki, 1978; Maloy et al., 1996). However, more recently, the ability of microorganisms to contribute to human health has become apparent (Fan & Pedersen, 2021; Lynch & Pedersen, 2016) and the role they play in health and disease is beginning to emerge.

## 1.2. The evolution of microbial and human interactions

Members of the bacterial kingdom are thought to have outdated human life by almost 2 billion years (Lee & Mazmanian, 2010). As such, it has been proposed that the evolution of microorganisms may be linked with the development of the human body (Bordenstein & Theis, 2015; Hooper & Gordon, 2001; Karl et al., 2018). The co-evolution between bacteria and humans is not fully understood, and the subsequent effect on human development is not known (Hooper & Gordon, 2001). However, some evidence to support the co-evolution between them does exist. For example, it has been proposed that eukaryotic mitochondria and chloroplasts are descendants of bacteria, putatively suggesting a role in eukaryotic evolution (Bordenstein & Theis, 2015). In addition, the human genome encodes 223 proteins which exhibit closer sequence homology to certain bacterial proteins than to other eukaryotes, potentially indicating the acquisition of genes via Horizontal Gene Transfer (HGT(Hooper & Gordon, 2001)). Furthermore, the ability of microorganisms to colonise within eukaryotic systems, such as a human's Gastro-Intestinal (GI) tract, indicates specific habitat adaptations (which may have arisen during coevolution) to cope with the harsh conditions provided by the body, for example in acidic and bile rich regions (Bäckhed et al., 2005; Lee & Mazmanian, 2010).

## 1.3. The microbiota

The human body harbours a vast plethora of organisms, comprised of bacteria, fungi, viruses, protozoa and archaea, collectively termed the microbiota (**Figure 1.1.** (Ma et al., 2017b)). The genomic material of the microbiota is known as the microbiome. Estimates predict that the combined genomic information encoded by the microbiome is approximately 100 times greater than the human genome (Knight et al., 2017; MetaHIT Consortium et al., 2010), indicating a large impact on host physiology. Microflora colonisation within mammalian systems occurs at locations which are exposed to the environment, including the skin, oral cavity, GI tract and vagina (**Figure 1.1.** (Gareau et al., 2010)). The dominant components of the microbiota are organisms from the bacterial kingdom, which typically

colonise sites that provide suitable conditions for their growth and proliferation, for example, the digestive tract (Tlaskalová-Hogenová et al., 2011). At what point we become 'inoculated' is difficult to say and is highly debated. For example, some studies state that there is evidence of establishment during foetal development via the maternal placenta (Aagaard et al., 2014; RodrÍguez et al., 2015). However, others support the hypothesis that the foetus is sterile until birth, after which they receive a massive delivery of bacteria from the environment (Gareau et al., 2010; Turroni et al., 2022). Moreover, the composition of bacterial species within the microbiota can differ depending on the delivery method (e.g., caesarean versus natural birth) and the gestational age (full-term infants versus prenatal (Gareau et al., 2010; Kapourchali & Cresci, 2020; Turroni et al., 2020)). Indeed, Chen et al., (2007) found that the colonisation rate of bifidobacteria species was less successful in infants who were born via caesarean in comparison to those born by vaginal delivery. In addition, preterm infants also exhibit a diminished population of bifidobacteria species (Tauchi et al., 2019), as well as an overall decreased bacterial diversity when compared to full-term infants, who are characterised by having a high number of bacterial strains from the *Lactobacillus, Bifidobacterium* and *Prevotella* genera (Thursby & Juge, 2017; Tirone et al., 2019). The establishment of bacteria continues throughout early life and begins to transition into a mature form by the time the infant is two years old, from this point the microbiota is generally considered "stable" until a final shift in composition occurs when an individual reaches the approximate age of 65 (Thursby & Juge, 2017; Walsh et al., 2014).

In addition to the shifting communities of the microbiota, the composition is also variable between individuals, as it is shaped by a combination of genetics and environmental factors ( e.g., diet, antibiotic use, and hygiene), which enables each human to possess a somewhat unique microbiota (Derrien & van Hylckama Vlieg, 2015; Qin et al., 2010; Sommer & Bäckhed, 2013). Interestingly, Qin et al., (2010) showed that within their study cohort (of 124 European individuals), 40 % of the microbial genes present in each person were shared by at least 50 % of the whole study cohort. This observation led to the proposition that the gut microbes provided a conserved set of functions, which is now termed the core gut microbiome (Qin et al., 2010; Walsh et al., 2014).

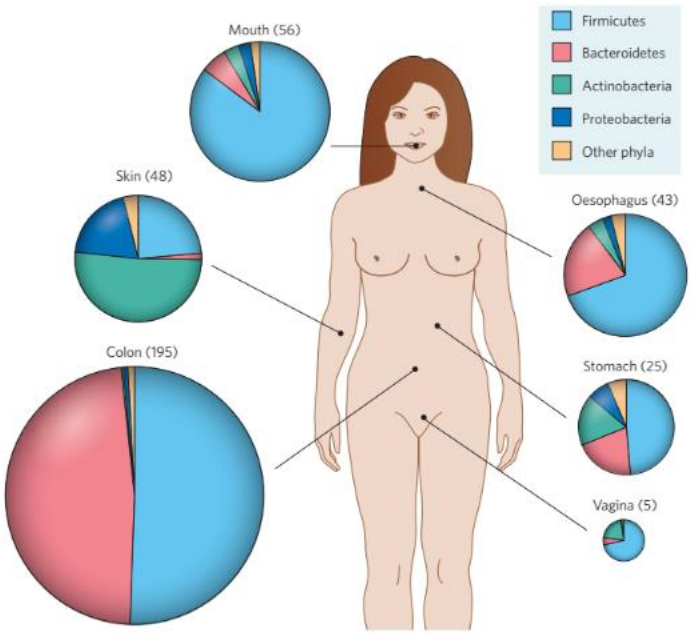



**Figure 1.1. Site-specific bacterial phyla populations in humans.** Host body site colonisations of microbial communities. A pie chart is used to depict the average number of distinct phylotypes per individual. The pie charts represent the proportion of phylotypes belonging to different bacterial phyla, where light blue represents Firmicutes; Pink, Bacteroidetes, Green, Actinobacteria; Dark Blue Proteobacteria and Yellow; additional phyla (Figure from (Dethlefsen et al., 2007)).

## 1.4. The gut microbiota

The GI tract represents the most abundant and diverse consortia of microorganisms within the human body, where the intestine alone, encompasses more than $10^{14}$ bacterial cells (Sommer & Bäckhed, 2013). The microbial composition within regions of the GI tract (incorporating the stomach, small intestine, and large intestine) is varied, reflecting the different physiological features of each section, for example, flow rates, acidity and bile concentrations (Flint et al., 2012). As such, population densities range across the gut (**Figure 1.2**), with the colon housing the largest microbial population within the body (Karl et al., 2018; Sekirov et al., 2010). Indeed, the colon has previously been described as one of the most densely populated microbial regions currently recognised on the planet (Rinninella et al., 2019).

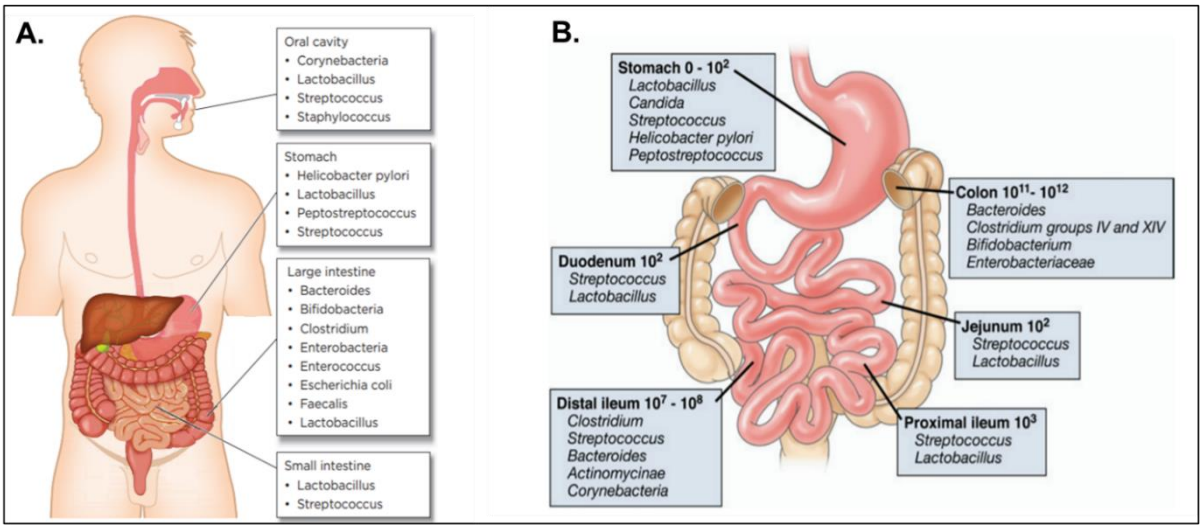



**Figure 1.2. The human gut microbiota composition. A.** Location and populations of bacterial groups in the human GI tract (figure from Knight et al., (2019)). **B.** Composition and approximate concentrations of microbial communities in the gut (Figure from Sartor, 2008)).

The gut microbes are predominately anaerobes (Hugon et al., 2015; Walsh et al., 2014), of which at least 70 % cannot currently be cultivated (Clemente et al., 2012; Cross et al., 2019; Lagier et al., 2012; Turnbaugh et al., 2007). Estimates predict that the gut houses between 500 to 1000 bacterial species (Falony et al., 2016; Human Microbiome Project Consortium, 2012; Ley et al., 2006; Qin et al., 2010), primarily from the Firmicutes and the Bacteroides phyla (Karl et al., 2018). As such, it is now proposed that each gut section is an individual habitat, with differing bacterial communities providing a variety of features and host interactions which may contribute to host health and disease (Martin et al., 2009; Thursby & Juge, 2017; Tlaskalová-Hogenová et al., 2011). Recent findings have revealed that the species richness and diversity within the GI tract are influenced by several parameters including host genetics (Kurilshikov et al., 2017), environmental pressures (Rothschild et, 2018), diet (Graf et al., 2015), illness and/or disease (Thursby & Juge, 2017). Indeed, it has been reported that microbial populations correlate with the country where their host resided, indicating a demographic role in shaping an individual's gut microbiota (Li et al., 2014).

Research on the microbiota was previously limited due to the reliance on culture-based methodologies (Moore & Holdeman, 1974). However, recently, the development of molecular techniques has enabled a more thorough understanding of the complex interactions which occur between a host and their microbiota. Indeed, 16S rRNA amplicon sequencing has become a fundamental tool in identifying microbial populations and signatures in health and disease (Chen et al., 2019b; Clemente et al., 2012; Dethlefsen et al., 2008; Human Microbiome Project Consortium, 2012; Sinclair et al., 2015). As a result, it has been possible

to examine the interactions that occur between bacteria and their host, allowing an understanding of the microbial impact on human bodily processes (Clemente et al., 2012; Fan & Pedersen, 2021).

## 1.5. Terminology

When reviewing the literature, two common terms are frequently used to describe relations observed between a host and its microbiota:

**Mutualistically:** encompassing commensalism, a relationship where neither member of the party is impacted detrimentally, where both coexist. In addition, symbiotic relations are also incorporated in this definition, where either one party or both receive a benefit from interactions with each other (Hooper & Gordon, 2001).

**Pathogenicity:** Where one species causes a detrimental effect on the other (Hooper & Gordon, 2001).

Indeed, Hooper & Gordon, (2001) state that the microbiota is a continuum between commensal, symbiotic and pathogenic bacteria and the dominant associations found within the gut are mutualistic. Pathogenicity can then arise when varying conditions (e.g., immuno-deficiency) occurs, which leads to a shift from health-promoting to disease-causing microbial communities (Tlaskalová-Hogenová et al., 2011). Bacteria gain a stable environment with a plentiful source of nutrition from associations with members of the animal kingdom, providing a reason for them to safeguard host health, hence ensuring optimal niche conditions (Lee & Mazmanian, 2010). As a result, the gut microbiota, and the genetic material it offers to a host, can provide humans with capabilities which they could not carry out independently (Cammarota et al., 2014; Mithieux, 2018; Tremaroli & Bäckhed, 2012). For example, Gill et al., (2006) compared the human genome with the average content of sequenced microbial genomes. Here, they showed that bacteria in the gut assist in the digestion of complex carbohydrates, providing a metabolic capacity to the host (Gill et al., 2006).

## 1.6. Microbiota focused research

Research focusing on attributes provided by the gut microbiota has grown exponentially, with large multidisciplinary projects such as ‘The Human Microbiome Project’, and ‘MetaHIT’ attempting to characterise the role of the human microbiome in health and disease (Cammarota et al., 2014; Li et al., 2014; MetaHIT Consortium et al., 2010; Turnbaugh et al., 2007). Today, research on the gut microbiota is primarily focused on health impacts associated with bacterial and host interactions (Michael et al., 2020; Schluter et al., 2020; Silva et al., 2020; Witkowski et al., 2020), with outcomes indicating that the consortia of

bacteria within the gut are crucial components of the gut's physiology (Sommer & Bäckhed, 2013). As such, the term 'hidden organ' is frequently encountered in the literature when referring to the gut microbiota (Guinane & Cotter, 2013; Mitev & Taleski, 2019; Yan & Charles, 2017), due to its metabolic capacity equating to that of a human liver, highlighting its importance in maintaining human health (O'Hara & Shanahan, 2006; Sommer & Bäckhed, 2013).

## 1.7. The interplay between the gut microbiota and the host

The enormity of the GI tract's microbial load, and the large degree of heterogenicity exhibited between hosts, means that the mechanistic knowledge of how the microbiota affects host performance is limited (Medlock et al., 2018). However, research is beginning to underpin how the microbiota can influence health. As such, the functional capacity of gut microbe's influence on host health has been extensively reviewed (Fan & Pedersen, 2021; Lynch & Pedersen, 2016), and reports have shown that gut microbial populations can contribute to, host immunity training (Yoo et al., 2020), nutritional metabolism, including the breakdown and digestion of complex carbohydrates (Kaoutari et al., 2013; Tremaroli & Bäckhed, 2012), the regulation of the gut's endocrine function (Clarke et al., 2014; Régnier et al., 2021), neurological signalling (Bauer et al., 2016), drug metabolism (Alexander et al., 2017; Li et al., 2016) and the production of beneficial metabolites (Jandhyala et al., 2015; Ross et al., 2010). In addition, there are accounts of the microbiota mediating host gene expression. For example, Anderson et al., (2010) showed that *Lactobacillus plantarum* MB452 could modify the expression of 19 genes in the intestine which are involved in the integrity of the barrier (e.g., tight junction formation, which is responsible for directing the permeability of the paracellular pathway) and suggested that this may contribute to an improved intestinal barrier function. A 'healthy' microbiota has also been correlated with the successful competition against pathogens, as colonisation by mutualistic organisms, reduces the nutritional capacity and space available for invading pathogenic bacteria (Sassone-Corsi & Raffatellu, 2015). In addition, intraspecific competition within genera is frequently encountered. For example, Momose et al., (2008), showed that the presence of two commensal strains of *Escherichia coli* prevented the establishment of the pathogenic *E.coli* strain 0157 (predominant causative agent of bloody diarrhoea) by competing for the amino acid proline. Furthermore, Hsiao et al., (2014) showed via a mouse model that the presence of *Ruminococcus obeum*, in the gut can deter the establishment of *Vibrio cholerae* (a diarrhoea-causing pathogen) and mutualistic organisms have also been shown to produce neurotransmitters, for example, bifidobacteria can produce serotonin precursors, which may regulate temperament and appetite (Dinan & Cryan, 2017).

## 1.8. Intestinal dysbiosis

The term dysbiosis has broadly been ascribed to a microbial deviation from what is considered a 'healthy' microbiota (Petersen & Round, 2014). Although, exactly what constitutes a healthy microbiota is almost impossible to pinpoint. In practice, this means that if alterations (in comparison to a healthy, non-disease phenotype) in the microbiota arise, communications are disrupted between the host and microbes, which could produce a disease phenotype (Hemarajata & Versalovic, 2013). Indeed, over 25 diseases have been associated with an altered intestinal flora, including irritable bowel disease (IBD (Casén et al., 2015; Ni et al., 2017)), irritable bowel syndrome (IBS (Chassard et al., 2012; Masoodi et al., 2020)), autoimmune inflammation disorders, such as celiac disease (Chen & Vitetta, 2021; Girbovan et al., 2017), neurogenerative diseases, e.g., Parkinson's disease (Pietrucci et al., 2019; Sun & Shen, 2018), cardiovascular diseases including hypertension (Li et al., 2017), obesity (Gao et al., 2018; Guirro et al., 2019), type II diabetes (Sircana et al., 2018; Sroka-Oleksiak et al., 2020) and additionally, some forms of cancer (Deng et al., 2021; Ni et al., 2019; Zou et al., 2018). From a therapeutic standpoint, manipulating the microbiota is an attractive means of promoting health or reducing the impact of dysbiosis (Walsh et al., 2014). To meet these objectives, the use of probiotics as gut modulators has become an area of interest (Hemarajata & Versalovic, 2013; Walsh et al., 2014).

## 1.9. Probiotics and their potential biotherapeutic application

Historically, microorganisms have often been utilised as a remedy to promote host health (Ozen & Dinleyici, 2015). For example, fermented milk was consumed as a treatment for gastroenteritis, as recommended by academics during the Roman Era (Schrezenmeir & de Vrese, 2001). However, in the early 1900s, Ellie Metchnikoff drew links between the extended life of Bulgarian peasants and the consumption of soured milk (Fuller, 2012; Mackowiak, 2013). From these observations, Metchnikoff isolated a bacterium from the milk (which is speculated as having been *Lactobacillus delbrueckii* subsp. *bulgaricus*) and suggested that the bacterium could reduce gut pathogens (Fuller, 2012; Schrezenmeir & de Vrese, 2001). Fascinatingly, Metchnikoff's hypothesis is regarded as the first direct utilisation of microorganisms to promote health (Anukam & Reid, 2007; Fuller, 2012), and since then, bioprospecting bacteria has developed into a massive industry which is continually expanding, as consumer demand increases (Salvetti & O'Toole, 2017).

## 1.10. The probiotic concept

The term ‘Probiotic’ has Greek origins, which translates to ‘For Life’ (Beena Divya et al., 2012). Probiotic was originally used by Lilly and Stillwell in the 1960s to describe “A microbial substance able to stimulate the growth of another microorganism” (Fuller, 2012; Narayan et al., 2010). Since then, the definition of a probiotic has continued to evolve (Fuller, 2012; Schrezenmeir & de Vrese, 2001) and has been accompanied by the terms prebiotic (non-digestible food ingredients that beneficially affect the host, by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already residing in the colon (Saad et al., 2013)), postbiotic (metabolites and/or cell-wall components released by probiotics (Aguilar-Toalá et al., 2018)), and synbiotics (a combination of probiotics and prebiotics (Pandey et al., 2015)). Most recently, the International Scientific Association of Probiotics and Prebiotics (ISAPP) have published consensus statements on these terms to guide clarity (Hill et al., 2014; Salminen et al., 2021). Even the term probiotic has undergone several revisions over the last two decades. In 2001, the World Health Organisation (WHO) defined a probiotic as “a live microorganism which when administered in adequate amounts, confers a health benefit on the host” and since then, is now the most widely accepted definition (FAO & WHO, 2001; Hill et al., 2014). In 2013, the ISAPP re-evaluated the field of probiotics (Hill & Sanders, 2013). Here, based on scientific expertise, recommendations were made to ensure that the title ‘probiotic’, was only assigned to microorganisms that have revealed health benefits in robust clinical trials (Hill et al., 2014; Hill & Sanders, 2013). The ISAPP convention also led to a probiotic product definition: “To deliver live microorganisms with a suitable viable count of well-defined strains with a reasonable expectation of delivering benefits for the well-being of the host”, emphasising the importance of taxonomy and the strain used in any products sold as a probiotic (Hill et al., 2014; Morovic et al., 2016). Yet, many of these recommendations are still not being followed and it is evident that there are glaring omissions in their enforcement within the industry (Drago et al., 2013; Huys et al., 2006; Jackson et al., 2019; Kolacek et al., 2017; Lewis et al., 2016; Marinova et al., 2019; Morovic et al., 2016).

## 1.11. The *Lactobacillus* and *Bifidobacterium* genera as important reservoirs of probiotics

### 1.11.1. *Lactobacillus*

Belonging to the Firmicute phylum, species within the *Lactobacillus* genus are the most varied group of LAB, and before the recent reclassification of the genus (Zheng et al., 2020), included approximately 200 species (Herbel et al., 2013; Zheng et al., 2015). Lactobacilli are generally anaerobic, non-mobile and rod-shaped bacteria which can colonise a variety of habitats. The species within this genus have been described as either homo-fermentative, where they produce lactic acid as the main product following the fermentation of hexose sugars, or hetero-fermentative, producing lactic acid, carbon dioxide, acetic acid, and ethanol as the end product of sugar fermentation (Herbel et al., 2013; Makarova et al., 2006; Pot et al., 2014; Zheng et al., 2015). Indeed, members of this genus are often used as starter cultures during the fermentation processes of food (Ávila et al., 2014; Bartkiene et al., 2018; Ng et al., 2011), for example, *Lactobacillus bulgaricus* is often used for yoghurt cultivation (Wasilewska et al., 2019). As such, there is long withstanding documentation of the safe consumption of lactobacilli by humans (Sun, Harris, et al., 2015a). Furthermore, several lactobacilli have been provided with a Generally Regarded as Safe (GRAS) status by the Food and Drug Association (FDA) in the USA, or a Qualified Presumption of Safety (QPS) by the European Food and Safety Authority (EFSA) designation (Arena et al., 2014; Jankovic et al., 2010).

The diversity of lactobacilli is further emphasised by the large range of G+C (guanine and cytosine) content (32 - 59 %) reported within the genus, which is twice the size of a typical range accepted for a distinct bacterial genus (Herbel et al., 2013; Pot et al., 2014; Slover & Danziger, 2008; Zheng et al., 2015). Since its designation in 1901 the *Lactobacillus* genus has been relatively unstable with numerous reclassifications of species (Huang et al., 2018a, 2020; Pot et al., 2014; Wood & Holzapfel, 1992; Zheng et al., 2020) resulting in an unstable taxonomy. For example, due to the size and diverse nature of *Lactobacillus,* the genus has been split into groups/phylogroups (Pot et al., 2014; Salvetti et al., 2018). Originally, only three groups were suggested (e.g., the *L.acidophilus* group) based on their carbohydrate metabolism (Sun et al., 2015; Wood & Holzapfel, 1992). However, following a dramatic increase in the number of described species, the number of reported phylogroups ranges from 15 (Salvetti et al., 2012) – 24 (Zheng et al., 2015), where the addition of a novel isolate can dramatically adjust phylogenetic data and signal, providing a putative reason for such a large difference in group numbers (Felis & Dellaglio, 2007; Sun, Harris, et al., 2015a; Zheng et al., 2015). As a result of the extreme heterogenicity associated with the *Lactobacillus* genus over

the last decade, Zheng et al., (2020) recently proposed the genus be split into 25 genera (23 of which are novel).

### 1.11.2*. Bifidobacterium*

The bifidobacteria genus belongs to the Actinobacteria phyla and was first discovered at the start of the 20th century, in the faeces of breast-fed children (Herbel et al., 2013; Leahy et al., 2005; Tissier, 1900; Turroni et al., 2011). Bifidobacteria species are anaerobic, nonsporulating, non-motile, Gram-positive rods (Bottacini et al., 2014; Esaiassen et al., 2017). Members of this bacterial group have a long and intertwined relationship with lactobacilli, due to their common shared attributes, such as lactate production and both being regular inhabitants of the mammalian gut, which often meant species were mistaken for each other (Lawson, 2018; Turroni et al., 2011). Indeed, bifidobacteria species represent a dominant component of breast-fed infant's gut microflora (Korpela., 2021; Turroni et al., 2012) and are highly prevalent in the mammalian GI tract (Bottacini et al., 2014). There are over 60 species and subspecies of bifidobacteria (Klimenko et al., 2020) and the reported genome sizes are greatly varied within the genus, ranging from 1.73 Mb *(Bifidobacterium indicum)* to 3.25 Mb (*Bifidobacterium biavatii* (Milani et al., 2016)). Indeed, it has been proposed that genome size variation is a result of widespread gene loss or acquisition (Lee & O'Sullivan, 2010; Milani et al., 2016). In addition, the reported G+C content of bifidobacterial genomes ranges from 59.2 % (*B.adolescentis*) to 64.6 % (*B.scardovii* (O'Callaghan & van Sinderen, 2016)). Plasmids are not a common entity within bifidobacteria and have only been identified in approximately 20 % of all the species (Lee & O'Sullivan, 2010). Bifidobacteria strains encode a unique form of carbohydrate metabolism known as the bifid shunt, which results in the fermentation of glucose and hexose sugars to lactic acid which is catalysed by the fructose--6--phosphate phosphoketolase (F6PPK) enzyme (Milani et al., 2016; O'Callaghan & van Sinderen, 2016). The gene encoding the bifid shunt metabolic function is a component of the bifidobacteria core genome, which is found uniquely in all bifidobacteria and interestingly, in one gardnerella species (Gavini et al., 1996; Milani et al., 2016).

### 1.11.3. Relevance

Strains of *Lactobacillus* and *Bifidobacterium* are increasingly being recognised for their role in human health and disease. Indeed, numerous strains from these genera have been deemed as beneficial organisms, based on their ability to confer health benefits to the host (Kobyliak et al., 2018; Koutnikova et al., 2019; Liu et al., 2017; Madsen et al., 2001). However, probiotic attributes are often strain-specific (Campana et al., 2017; McFarland et al., 2018; Ramos et al., 2013). The WHO guidelines state that proper nomenclature and strain designation is required when assigning the term probiotic to an organism (Hill et al., 2016; WHO, 2002). As such, correct taxonomic identification is a desirable trait when evaluating the roles which such organisms can have in host health.

## 1.12. Probiotic classification

To develop a probiotic product, determining the taxonomy of an isolate is highly beneficial, as it allows insight into traits which may differentiate between beneficial and non-beneficial strains (Salvetti & O'Toole, 2017). Furthermore, the accurate classification of bacterial isolates, using a robust combination of phylogenetic methodologies, is fundamental for further comparative genomic analysis. Microbial classification has continually evolved as new techniques and technologies have become available. Previously, the classification of novel microorganisms was typically accomplished by phenotypic testing (Murray & Holt, 2005; Winslow et al., 1920), incorporating parameters such as fermentation processes, biochemical signatures, and physiological traits including morphology and cell wall structure (Komagata & Suzuki, 1988; Pot & Tsakalidou, 2009). However, the sole use of phenotypic data has several pitfalls including indistinguishable morphological variations, random emergence of 'identifying' traits and the dependence on the ability to generate pure cultures (Gupta, 2016; Kirisits et al., 2005; Murray & Holt, 2005; Proctor et al., 1995; Wai et al., 1998). As such, bacterial taxonomy is still a developing field, where even well-characterised pathogenetic bacteria are being reassigned into new genera, as sequence databases grow and allow bacteria to be viewed phylogenetically (Lawson et al., 2016).

## 1.13. DNA sequencing

As genomic sequencing has become more accessible, identification and classification methods have grown to incorporate genomic data. Indeed, the field of molecular phylogenetics has emerged and involves the study of the relatedness of organisms and in turn, their genetic content (Yang & Rannala, 2012) The importance of studying phylogenetics is becoming increasingly apparent. Aside from accurate species classification, it also permits the identification of gene conservation both intra and inter-species, in addition, it may allow the putative identification of conserved virulence and/or health-promoting factors (Collins et al., 2017; Tanaseichuk et al., 2014; Wuyts et al., 2017; Yamaguchi et al., 2019; Yang & Rannala, 2012). In molecular phylogenetics, a region of the genetic code is utilised to infer relatedness – these regions are known as genetic markers (Goodnight, 1999; Queller & Goodnight, 1989). Indeed, an example of such is the ribosomal (r)RNA gene, which since its proposition as a suitable marker in the late 20$^{th}$ century (Fox et al., 1977; Woese, 1987; Woese et al., 1985), has become a gold standard when analysing microbial phylogenetics.

## 1.14. 16S rRNA classification

The 16S rRNA gene codes for the small subunit components of ribosomal molecules, which are responsible for translating mRNA into functional proteins (Byrne et al., 2018). Within the 16S rRNA gene, there are nine hypervariable regions which allow organism identification (Nguyen et al., 2016). During 16S rRNA sequencing, primers are designed to target and amplify these hypervariable regions (typically V1-V3 or V3-V5) (Nguyen et al., 2016).16S rRNA sequences are usually compared with a sequence depository or database (such as GenBank (Leray et al., 2019) or Silvr (Quast et al., 2013) to determine taxonomy. Typical guidelines are that sequences of > 95 % identity represent the same genus, and sequences of > 98.7 % identity represent the same species (Johnson et al., 2019; Rossi-Tamisier et al., 2015).

16S rRNA has several advantages as a genomic marker. First, it is ubiquitous throughout the bacterial kingdom, allowing a standardised comparative methodology (Patel, 2001; Woese et al., 1985). In addition, 16S rRNA has a universally conserved function which does not appear to have changed over time, implying that any base changes occurring at the sequence level, reflect time/evolution (Janda & Abbott, 2007; Patel, 2001). Furthermore, the evolution of 16S rRNA appears to occur slowly, thus allowing comparisons between microorganisms (Santos & Ochman, 2004; Woese, 1987). Finally, the 16S rRNA gene is also large enough to allow statistical significance in comparisons and its size (1,500 bp) also enables effective primers to be designed (Patel, 2001).

Indeed, 16S rRNA amplicon sequencing has become a powerful tool in microbial classification and taxonomy, allowing a view of microbial diversity in whole ecosystems (without the need for cultivation), and permitting the recognition of previously unidentified microorganisms (Amann et al., 1995; Benga et al., 2014; Drancourt et al., 2000; Huse et al., 2012; Ki et al., 2009; Lagier et al., 2018; Petti et al., 2005). However, there are also several limitations to the sole use of 16S rRNA as a marker in microbial phylogenetics. Indeed, short read lengths (Quince et al., 2009), low phylogenetic resolution and sensitivity (in comparison to metagenomics (Poretsky et al., 2014)), as well as the variation seen in 16S rRNA ability to resolve classification to species level (Drancourt et al., 2000; Janda & Abbott, 2007; Johnson et al., 2019; Mignard & Flandrois, 2006) are all recognisable drawbacks associated with the sole use of 16S rRNA sequencing in microbial classification. In addition, variations in reliable taxonomic classification using 16S rRNA can also arise due to errors in sequencing (Quince et al., 2011), biases selected for during amplicon region selection (Johnson et al., 2019; Wang et al., 2007; Youssef et al., 2009) and reliance on database comparisons (Janda & Abbott, 2007). Complications associated with database reliance include database selection (for example EzBioCloud (Yoon et al., 2017), Greengene (DeSantis et al., 2006) and Silva (Quast et al., 2013)), which can impact taxonomic identification due to the variation in the number of sequences deposited per database (Park & Won, 2018). A deficiency of reference sequences can also result in the misclassification of strains, due to a depletion of closest relatives. Furthermore, mis-annotation of species during sequence deposition into databases, either due to incorrect nomenclature or the re-classification of a species, can result in erroneous downstream classifications (Ashelford et al., 2005; Heikens et al., 2005; Lesack & Birol, 2018). Additional issues with database reliance and accurate species classification is the potential for species to share high similarity or even identical sequences when using one genomic marker (Fox et al., 1992). Perhaps the most important limitation of 16S rRNA, is that there can be multiple copies of the gene in each microbial genome which can differ in sequence, leading to erroneous identification (Case et al., 2007; Coenye & Vandamme, 2003).

## 1.15. Alternative markers

The importance of accurate strain identification in microbiology is well appreciated (Aktas et al., 2016; Hunt & Ballard, 2013; King et al., 2004; McFarland et al., 2018a; Mishra & Prasad, 2005). As such, additional genomic marker candidates have been suggested to offer higher taxonomic resolution than the sole use of 16S rRNA, for example, housekeeping genes such as *rpoB*, *rpoA, cpn60* and *pheS* (Case et al., 2007; Dahllöf et al., 2000; Ki et al., 2009; Mota et al., 2004; Santos & Ochman, 2004; Schellenberg et al., 2009). Moreover, it is essential to corroborate phylogenies generated from single genes, with additional phylogenetic markers (Wu & Eisen, 2008). As research has continued to recognise the limitations involved with 16S rRNA and single gene classifications, methods have been developed to improve accuracies, such as Multi-locus Sequence Analysis (MLSA) and comparative platforms (Segata et al., 2013). MLSA is thought to provide a more reliable phylogeny that depicts a more viable representation of speciation events (López-Hermoso et al., 2017). The process of MLSA involves the concatenation of a selection of protein-encoding genes which are sequenced and aligned, and evolutionary relationships inferred (López-Hermoso et al., 2017). Subsequent phylogenies have shown improved topologies in comparison to their 16S rRNA phylogeny counterparts (Bouvet et al., 2014; Chaloner et al., 2011; Pilet et al., 2019; Thompson et al., 2007).

## 1.16. The role of Whole Genome Sequencing in microbial taxonomy

In recent years, the cost of whole-genome sequencing (WGS) has dramatically declined to as little as 70 USD per genome (Sundermann et al., 2021). As such, there has been a rapid increase in the number of microbial WGS available in public databases. For example, as of September 2020, the NBCI documented 268,507 prokaryote genomes (*Genome List - Genome - NCBI*, 2020.). Indeed, the expansion in the number of available genomes has allowed microbial classification to develop, allowing techniques such as core (genes shared by all genomes) and pan (all genes from all genomes) genome comparisons to take place (Land et al., 2015; Lukjancenko et al., 2010). In addition, having a plethora of genetic data available has also allowed the identification of suitable genomic markers for MLSA (Santos & Ochman, 2004; Segata et al., 2013). Bioinformatic platforms have since been developed, allowing large-scale genomic comparisons and alignments using an optimised magnitude of genomic markers, permitting the inference of a more robust evolutionary relationship and isolate taxonomy (Ciccarelli et al., 2006; Jordan et al., 2002; Lalucat et al., 2020; Segata et al., 2013; Wu & Eisen, 2008).

## 1.17. Lactobacilli and bifidobacteria: the importance of robust classification

The importance of taxonomy in ascribing health benefits to an organism is evident, as health-promoting attributes are often strain-specific (Campana et al., 2017; McFarland et al., 2018a; Ramos et al., 2013). Indeed, 16S rRNA sequencing may not encompass enough phylogenetic signal for strain designation. Recently, WGS has been proposed as the new gold standard for strain designation (Binda et al., 2020). The importance of taxonomic identification can be seen in several studies, where inconsistent bacterial profiles in commercially available microbial supplements have been reported (Huys et al., 2006; Lewis et al., 2016; Morovic et al., 2016). Furthermore, reports have shown genus-level misidentification of commercial products (Huys et al., 2006), and a recent study reported that 42 % of commercial products tested had inconsistencies with their labels, including misidentified organisms, missing consortia members, or species not accounted for (Morovic et al., 2016), emphasising the importance of robust taxonomic identification.

## 1.18. Traits

The most prevalent bacterial genera within the probiotic industry are lactobacilli and bifidobacteria which are either sold as either an individual or as multi-strained products (Ouwehand et al., 2002; Vlasova et al., 2016; Walsh et al., 2014). In 2002 the first regulated guidelines for assessing an organism's probiotic capacity and safety were outlined by the WHO and Food and Agriculture Organisation (FAO(Araya et al., 2002; de Melo Pereira et al., 2018)) and included the following criteria. An organism should be beneficial to the host and not contain a safety risk; tolerant of stomach acid pH, bile salts and digestive enzymes (e.g., pancreatin and pepsin) in the human gut; have adhesion capabilities to intestinal epithelial and the antimicrobial activity should be assessed (**Figure 1.3**). Martinez et al., (2015) stated that probiotics should also produce antimicrobial substances (bacteriocins) against pathogens and have their safety and efficacy validated by placebo-controlled clinical trials.

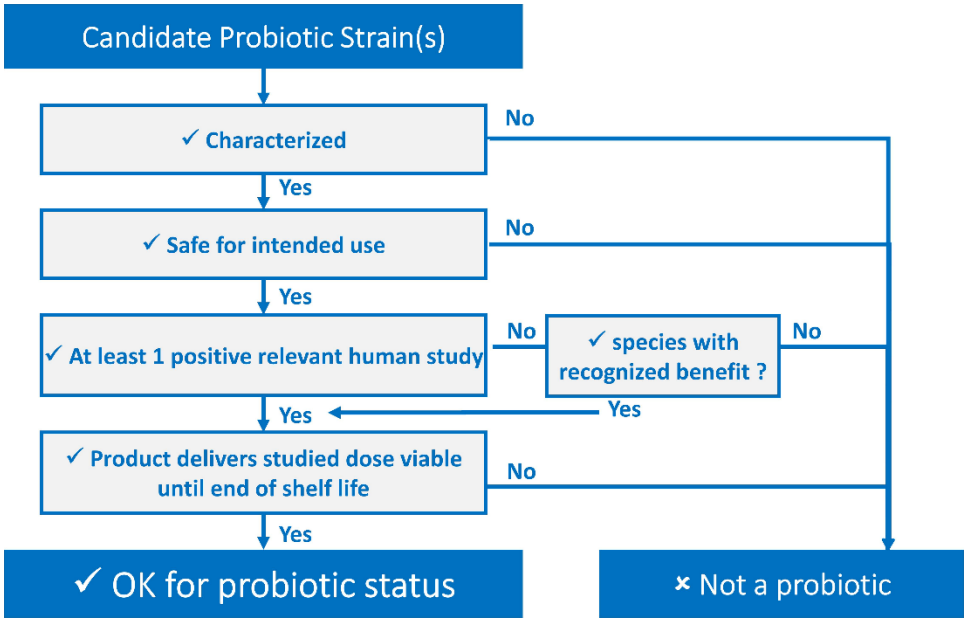



**Figure 1.3. Criteria for probiotic designation** (Figure by Binda et al., 2020).

## 1.19. Desirable probiotic attributes

### 1.19.1. *Survival during GI tract transit: acid and bile tolerance*

The human GI tract is a harsh and inhospitable environment for microorganisms, where conditions such as high concentrations of stomach acid (Amund, 2016; Mbye et al., 2020), and the digestive solution bile (Begley, Gahan, et al., 2005; Kheadr et al., 2007), can cause a great deal of physiological stress to a bacterium, reducing the viability and therefore any potential effects (**Figure 1.4** (Gómez et al., 2002; Sahadeva et al., 2011; Szulińska et al., 2018)). Bacteria from both lactobacilli and bifidobacteria are often associated with the gut (**Figure 1.2** (Flint et al., 2012; Rinninella et al., 2019; Turroni et al., 2012), suggesting a genomic basis for tolerance in some species. Indeed, numerous studies have reported such tolerances (Begley et al., 2006; Mulaw et al., 2019; Pan et al., 2009) and genomic traits have been described for such attributes (Begley et al., 2006; Desriac et al., 2013; Goel et al., 2020; Hamon et al., 2011). However, given the diversity that is seen within species, WGS can enhance the reliability of phenotypic observations by enabling putative correlation with genomic regions.

### 1.19.2. *Probiotics: adherence within the gut lumen or transient?*

A desirable trait of a probiotic is its ability to adhere to host cells (e.g., such as epithelium) to prolong the effects it may exert on the host (**Figure 1.4** (McNaught & MacFie, 2001)). However, despite numerous reports of the *in vitro* adherence capacity of lactobacilli and bifidobacteria (Bhat et al., 2019; Falah et al., 2019; Juntunen et al., 2001; Wang et al., 2010), some studies have shown that probiotics can only persist in a host for a short duration, remaining transient rather than establishing a population (Alander et al., 1999; Giatsis et al., 2016; Skjermo et al., 2015). Indeed, Ciorba, (2012) states that continued consumption of probiotics is required to maintain the health attributes received from the product, emphasising the importance of bacterial dose and supplementation duration in clinical testing.

### 1.19.3. *Probiotic formulations*

To exert a beneficial effect, probiotics should remain viable and be delivered at a minimum concentration of $10^7$ CFU/mL (Binda et al., 2020; Corcoran et al., 2008; Ding & Shah, 2009). In addition, the effects of probiotic supplementation can also be influenced by the product delivery and the number of species present (Ding & Shah, 2009)**.** Indeed, products can either be mono (single) or multi-strain (>1) and the effect can differ depending on the purpose of the treatment**.** For example, the probiotic *Lactobacillus rhamnosus* GG has been shown to reduce rotavirus infection and diarrhoea (Wu et al., 2013). In contrast, other reports have shown that probiotic effects are greater in multi-strain products (Dale et al., 2019; Kobyliak et al., 2018).

When producing a multi-strain product, it is beneficial to evaluate its genomic potential to interact with other members of the consortia. For example, it has been reported that *L.delbrueckii* and *Streptococcus thermophilus*, two organisms frequently combined in fermentation starter cultures, have a symbiotic relationship, each offering metabolites to help the other prosper (also known as protocooperation (Guchte et al., 2006; Liu et al., 2016)). In contrast, *L.acidophilus* have been shown to inhibit *L.delbrueckii* strains via bacteriocin production (Gaspar et al., 2018), highlighting the importance of genomics when designing a probiotic product, as it may enable targeted downstream phenotypic testing for such traits.

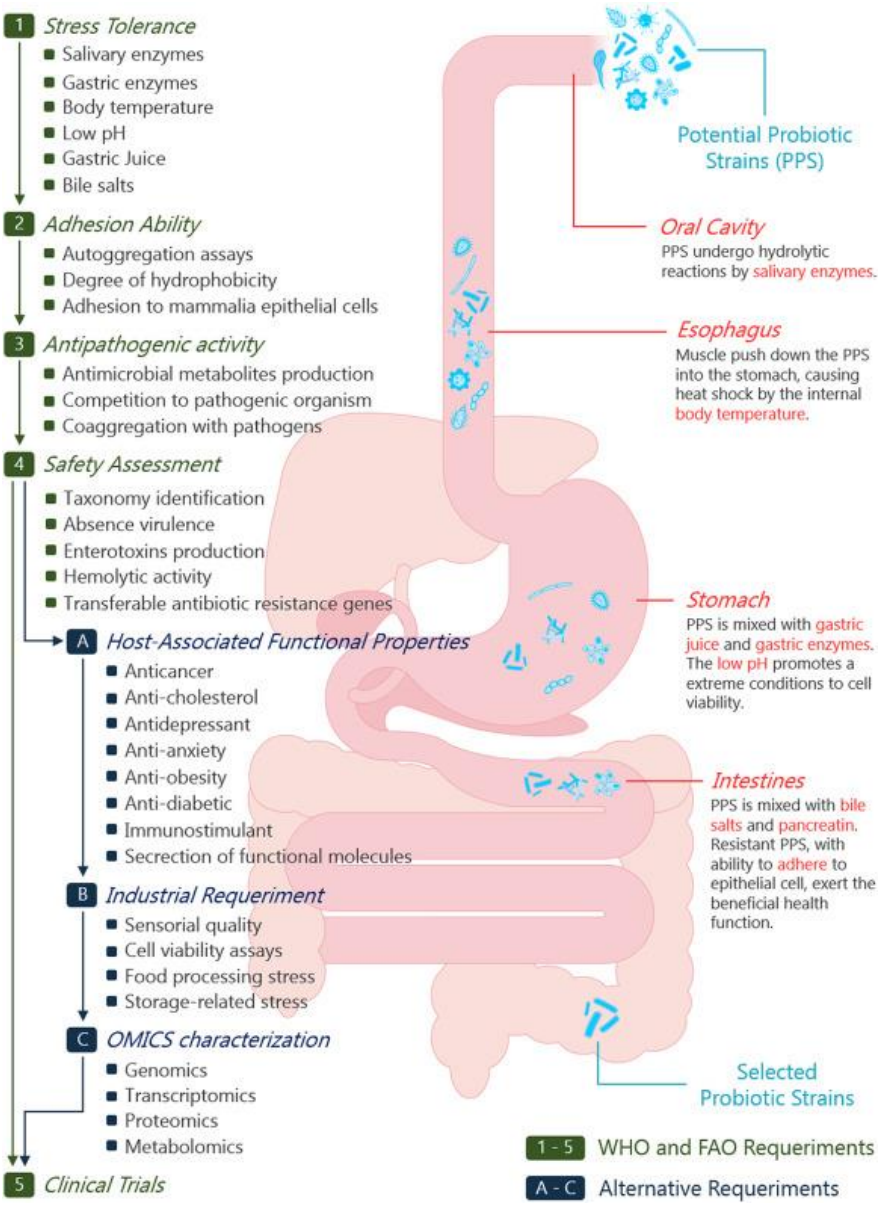



**Figure 1.4. Screening approaches that are used for the characterization of probiotic strains** (according to WHO/FAO and additional recommendations (de Melo Pereira et al., 2018))**.** Physiological features of the host that may exert stress on a candidate probiotic are described in red. WHO criteria for a candidate probiotic are outlined in navy. Additional beneficial requirements when describing a probiotic are highlighted in blue (figure and recommendations from de Melo Pereira et al., 2018).

## 1.20. Demonstrable health effects of probiotics

Characterising health effects associated with probiotic use is notoriously difficult (Skonieczna-Żydecka et al., 2020), exasperated by the number of covariables that can influence outcomes, including, host physiology (Shenderov, 2013), existing microbiota composition and environmental factors (Grześkowiak et al., 2012; Ohashi & Ushida, 2009) and incorrect strain use (Morovic et al., 2016; Zyrek et al., 2007). Furthermore, the absence of the universal regulation of probiotic products worldwide has led to the term being misused, making the validation of products difficult (Salvetti & O'Toole, 2017). In fact, in Europe, probiotics are only recognised as nutritional supplements and the EFSA does not permit the association of health claims with such products (Trush et al., 2020). In contrast, in other countries (e.g., Canada), probiotics are prescribed to promote health and aid in disease therapy (Trush et al., 2020). Therefore, it is apparent from evaluating the literature that the use of probiotics as a healthcare aid is not universally accepted, which can be inferred from their general title of dietary supplements (Ciorba, 2012; Herbel et al., 2013). Issues can arise when strain-level identification is not carried out, making the validation of health claims difficult, as health-related outcomes are typically both strain and disease-specific (Gareau et al., 2010; McFarland et al., 2018b). For example, *L.reuteri* SD2112 is capable of producing reuterin (a metabolite associated with gut pathogen inhibition (Cadieux et al., 2008; Langa et al., 2014)), and in contrast, a strain within the same species (*L.reuteri* RC14), was able to produce biosurfactants (linked with the inhibition of uropathogens (Reid, 2016; Velraeds et al., 1998)), depicting functions that are not universal across species (Reid, 2016). Each probiotic product requires a precise criterion (e.g., dose, strain and duration of administration), which it must achieve to deliver health benefits. Deviations in the criteria have the potential to affect the experimental significance (Hill et al., 2014). In addition, translating results from animal studies, for comparison with human trials may also contribute to contradictions associated with probiotic health benefits (Farnworth, 2008; Herbel et al., 2013; O'Callaghan & van Sinderen, 2016). Therefore, it is important to fully characterise all mechanisms of a probiotic product.

Attributable to the number of confounding variables that can impact the assessment of probiotic effects, systematic reviews and meta-analyses are imperative when evaluating microbial-mediated gut modulation. Indeed, numerous studies have associated the administration of LAB bacteria with health benefits including, a reduction in blood pressure (Lewis-Mikhael et al., 2020; Qi et al., 2020), immune modulation (Kazemi et al., 2021; Khalesi et al., 2018; Milajerdi et al., 2020; Roshan et al., 2019), inflammation reduction (Shu et al., 2020; Thongprayoon et al., 2019), cardiovascular diseases (Dixon et al., 2020) including total cholesterol reduction (Mo et al., 2019; Wang, Guo, et al., 2018a), obesity (Michael et al., 2020; Pontes et al., 2021), digestive issues (Oak & Jha, 2019; Qiu et al., 2019; Zhang et al., 2020),

intestinal disorders (Derwa et al., 2017; Jia et al., 2018; Zhang et al., 2021), and diabetes (Tao et al., 2020). Additionally, reports on pathogen inhibition (Jeng et al., 2020; Su et al., 2020; Wan et al., 2019; Wang et al., 2021; Zhang et al., 2020), and oral health promotion (Seminario-Amez et al., 2017) are also present.

## 1.21. Probiotic mechanisms of action

The most characterised mechanisms proposed for probiotic function are the production of antimicrobials, competing with pathogens for space and nutrients, modulating immune responses and the degradation of toxins (Pandey et al., 2015). Indeed, probiotic strains have been shown to produce bacteriocins (a form of an antimicrobial peptide), which is thought to be an evolutionary metabolite that enables competitive inhibition and may aid in the prevention of pathogen colonisation (Riley & Wertz, 2002). For example, (Gaspar et al., 2018), demonstrated that a bacteriocin produced by *L.acidophilus* KS400 was capable of inhibiting *Gardnerella vaginalis*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa* and *Lactobacillus delbrueckii in vitro.* In addition, Corr et al., (2007), demonstrated that when mice were fed the probiotic strain *Lactobacillus salivarius* UCC118, bacteriocin production (Abp118) led to significant protection against the pathogen *Listeria monocytogenes*, effectively preventing its colonisation. Additionally, probiotics have also been associated with the modulation of the host's immune system, for example by enhancing the gut mucosal barrier (Plaza-Diaz et al., 2019; Ukena et al., 2007). Moreover, the production of metabolites which result in immunomodulatory and anti-inflammatory functions have also been implicated as a mode of action of probiotic products (Beck et al., 2015; Cano et al., 2013; Plaza-Diaz et al., 2019). Probiotic strains have also been shown to modify the intestinal microflora (Fang et al., 2020; Wang, Ji, et al., 2018b).

## 1.22. Bioprospecting

Identifying natural products from biological organisms is known as bioprospecting (Rausser, 2000). Designing probiotic administration for specific disease states is a current interest amongst researchers, due to the number of health-promoting attributes that have been correlated with their consumption (Kaushik et al., 2009; Nagpal et al., 2012; Pavlović et al., 2012).

### 1.22.1. Antimicrobial peptide (AMP) production

Both lactobacilli (Choi et al., 2021; Collins et al., 2017; Deraz et al., 2007; Gaspar et al., 2018) and bifidobacteria (Cheikhyoussef et al., 2010; Liu et al., 2015; Yildirim & Johnson, 1998) species are capable of producing bacteriocins. Bacteriocins can have either a broad or narrow range (Cotter et al., 2013). It is hypothesised that the function of bacteriocin production is to aid in niche competition, providing a selective advantage to acquire resources (Hassan et al., 2012). Gram-positive bacteria produce bacteriocins which are divided into two classifications: I (lantibiotics) and II (non-lantibiotics (Hassan et al., 2012). One of the earliest reports of bacteriocin production in *Lactobacillus* was the discovery of lactoactin, produced by *Lactobacillus acidophilus*, which exhibited pathogen inhibitory effects against *Salmonella* and *Escherichia* (Barefoot & Klaenhammer, 1983). In addition, bifidobacteria bacteriocins have been reported since the 1980s and are associated with the inhibition of a variety of pathogens including *E.coli* and *Clostridium* (Martinez et al., 2013). It is thought that bacteriocins could be a potential biotherapeutic route for treating antibiotic-resistant infections (Hassan et al., 2012).

### 1.22.2. Cholesterol reduction

An elevation above the desired homeostasis level of blood (serum) cholesterol is a condition known as hypercholesterolemia (Sudha et al., 2009). The hypercholesterolemia state arises when there is an increased abundance of low-density lipoprotein (LDL) in comparison to high-density lipoprotein (HDL), which subsequently leads to the hardening and narrowing of blood vessels (Sudha et al., 2009). Major health concerns are associated with the development of hypercholesterolemia, as it is a widely acknowledged risk factor associated with the onset of numerous cardiovascular diseases (CVD (Anandharaj et al., 2014)). Indeed, the risk of heart attacks is three times higher in patients with hypercholesterolemia (Anandharaj et al., 2014). The WHO states that approximately 17.9 million people per year die from CVD, accounting for 32 % of deaths worldwide (WHO, 2021b), highlighting the major health burden CVD inflicts across the globe.

Cholesterol is the precursor molecule for the aqueous digestive solution known as bile in humans (Begley et al., 2006). Bile Acids (BA's) account for 50 % of the composition of bile (Begley, Gahan, et al., 2005). Following conjugation with amino acids and enzymatic modification in the liver, results in at least six derivatives of human conjugated BA's, including taurocholic acid (TCA), taurodeoxycholic acid (TDCA); taurochenodeoxycholic acid (TCDCA); glycocholic acid (GCA); glycodeoxycholic acid (GDCA); and glycochenodeoxycholic acid (GCDCA (Fang et al., 2009b)). Of these BA derivatives conjugated deoxycholic acid is observed in the largest quantity (Ijare et al., 2005). Research on cholesterol-lowering properties of probiotics has grown exponentially over the last few decades, with many

confirmed reports of their ability to lower serum cholesterol (Bendali et al., 2017; Fuentes et al., 2016; Tsai et al., 2014).

Deconjugation, is the enzymatic hydrolysis of the amide bond between the steroid ring and the amino acid side chain of conjugated bile acid (CBA), liberating taurine or glycine (Long et al., 2017), catalysed by the Bile Salt Hydrolase (BSH; E.C.3.5.1.24) enzymes (Elkins et al., 2001). BSH is typically associated with Gram-positive bacteria and belongs to the cholylglycine hydrolase family of enzymes, or the Ntn-hydrolase superfamily of proteins (Long et al., 2017; Pavlović et al., 2012). The Ntn-hydrolase superfamily also contains Penicillin Acylases (PA (E.C. 3.5.1.11)), enzymes that share large sequence similarity, as well as the same primary residue (Cys) of the mature protein, with the BSH enzymes (Kumar et al., 2006; Lambert, Bongers, et al., 2008). The high level of homology exhibited between these two groups has often led to the mis-annotation of the proteins (Long et al., 2017). Multiple copies of BSH protein-encoding genes have been reported in lactobacilli and bifidobacteria strains, for example, *bsh* 1 – 4 in *L.plantarum* (Lambert, Bongers, et al., 2008), *bshA* and *bshB* in *L.acidophilus* (McAuliffe et al., 2005), and *bsh1* and *bsh2* in *L.salivarius* (Fang et al., 2009b), which interestingly have a large sequence diversity between homologs within the same genome (McAuliffe et al., 2005). As such, many *in vitro* studies have shown that a probiotic capable of deconjugation via BSH production can reduce cholesterol levels (Costabile et al., 2017; Pereira et al., 2003; Zhang et al., 2008). Several *in vivo* experiments have also confirmed a correlation between probiotic consumption and host cholesterol reduction, providing putative validation of *in vitro* studies (Bendali et al., 2017; Costabile et al., 2017; Michael et al., 2017; Park et al., 2008; Wang et al., 2019). For example, Bendali et al., (2017) treated hypercholesterolemic induced rabbits with the probiotic *L.pentosus* KF92370, in comparison to the control group, hypercholesteremic rabbits exhibited an 18 % decrease in the total serum cholesterol without affecting HDL levels (Bendali et al., 2017). Likewise, Nguyen et al., (2007) treated hypercholesteraemic mice with *L.plantarum* PH04 and recorded a 7 % reduction in serum cholesterol, highlighting the variety of probiotic species which are capable of serum cholesterol reduction, and the number of hosts under which they can function within. Human trials utilising strains such as *L.acidophilus* La5 and *L.reuteri* NCIMB 30242 have validated the *in vivo* ability of probiotic bacteria to reduce serum cholesterol (Ejtahed et al., 2011; Jones et al., 2012). Indeed, a recent meta-analysis incorporating 15 studies, with a collated cohort of approximately 950 subjects, highlighted the use of probiotics to reduce cholesterol (Wu et al., 2017). The study found that lactobacilli species *L.plantarum* and *L.reuteri* were the most efficient at reducing LDL cholesterol (Wu et al., 2017).

There are several proposed mechanisms of BSH-mediated deconjugation of BAs, which can result in the removal of cholesterol (**Figure 1.5**).

i) When deconjugated, BAs are less soluble and hence are not reabsorbed during enterohepatic circulation (Costabile et al., 2017). As a result, the BA is excreted in the faeces, resulting in serum cholesterol being absorbed in the liver to replenish the BA pool, lowering blood cholesterol (Kumar et al., 2006).

ii) Cholesterol can also be incorporated into the bacterial cell membrane (known as cholesterol assimilation), which prohibits the formation of cholesterol micelles and prevents the transport of fatty acids to the surface of the intestine for absorption, reducing cholesterol further (Choi et al., 2018; Lye et al., 2010).

iii) Cholesterol and free BAs are coprecipitated and then excreted in the faeces (Liong & Shah, 2005; Oner, Aslim, & Babaoğlu Aydaş, 2013).

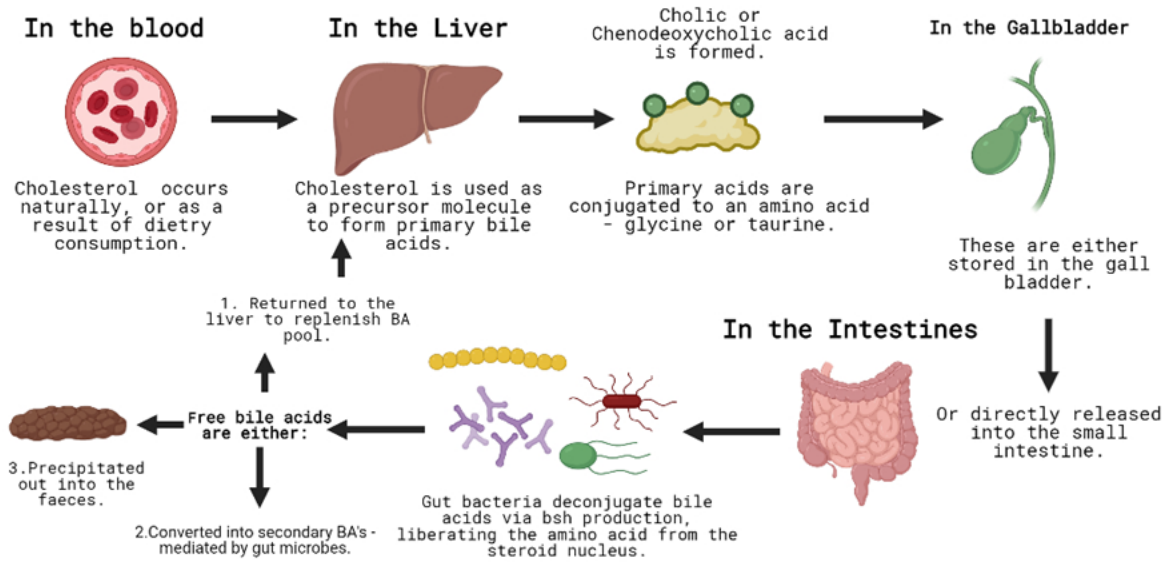



**Figure 1.5. Schematic of cholesterol metabolism.** Visual representation of enterohepatic recirculation (Liong & Shah, 2005; Oner, Aslim, & Aydaş, 2013).

## 1.23. Genomic risk factors associated with probiotic supplementation

Ventura et al., (2009) state that probiogenomic studies (the genomic study of probiotic bacteria) allows insight into the mechanisms behind the probiotic function and therefore may further or dispute the use of probiotics for disease prevention and/or treatment. As previously mentioned, both lactobacilli and bifidobacteria are typically associated with the human gut, as natural components of the microbiota (Rinninella et al., 2019) and numerous species from these genera have a QPS (Hazards (BIOHAZ) et al., 2020) or GRAS designation (Giraffa, 2014). However, it is of great benefit to fully characterise the molecular mechanisms behind the expressed phenotypes of the bacterial species that are consumed worldwide, not only to evaluate their exact function whilst inside the human system but to also determine any safety issues they may possess, such as the presence of virulence factors or mobile antibiotic resistance (ABR) genes (Gueimonde et al., 2013).

Bacteria can transfer genes intra and interspecifically, which drives their evolution and adaptation capabilities (Lerner et al., 2017). HGT is the focus of microbial gene exchange, where the lateral exchange of genes is heavily attributed to mobile genetic elements (MGE) such as plasmids, insertion sequences and introns (Lerner et al., 2017; van Reenen & Dicks, 2011). The nature of probiotics means that they encounter commensal bacteria within the gut, suggesting a potential risk of HGT to pathogenic organisms (Imperial & Ibana, 2016). Indeed, prophages (regions of viral DNA incorporated into a bacterial genome) have been shown to facilitate HGT in *L.gasseri* strains *in vitro* (Baugher et al., 2014), suggesting *in vivo* potential. Virulence factors are typically defined as components of an organism which contribute to its pathogenicity (Hill, 2012), and will therefore pose a threat if they are encoded on MGE, which may facilitate transfer to other organisms (van Reenen & Dicks, 2011). However, traits that may be beneficial to a probiotic, can also translate to a virulence factor in pathogenic organisms. For example, adhesion properties promote microbial to host cell interactions (Wassenaar & Klein, 2008). Therefore, efforts should be made to deduce the genomic risk of transfer (Colautti et al., 2022), such as the examination of genomes for plasmids or genomic islands.

### 1.23.1. The global threat of Antibiotic Resistance (ABR)

The term antibiotic is ascribed to a molecule which possesses the ability to obstruct or kill a microorganism by interacting with a variety of bacterial targets (Davies & Davies, 2010). The importance of antibiotics to global health is unequivocal. Indeed, since the discovery of penicillin in the 1920s and the subsequent boom in antibiotic development that followed, millions of lives have been saved from otherwise fatal bacterial infections (Blair et al., 2015; Levy, 1998). Antibiotics can be classified in several ways, including chemical compositions, activity spectrum, or mode of action (Etebu & Arikekpar, 2016). For example, in terms of their mechanisms, antibiotics such as chloramphenicol, aminoglycosides, macrolides, streptothricin and tetracyclines act by protein modification, while others such as β-lactam and glycopeptides modify the cell wall of the bacteria leading to cell death (**Table 1.1** (van Hoek et al., 2011). Other mechanisms of AB action include interaction with the bacteria's DNA/RNA and modification of their cellular metabolism (van Hoek et al., 2011).

Antimicrobial resistance (AMR) is when a bacterium undergoes a genetic or biological change which enables its survival in the presence of a previously effective antibiotic (Prestinaci et al., 2015). ABR is a more specified definition of AMR, focusing on resistance to antibiotics, and for this thesis, AMR and ABR will be used interchangeably. Today, due to the selective pressure created by antibiotic overuse (and/or misuse) in the medical, veterinary, and agricultural fields, the emergence of AMR in bacteria is becoming increasingly frequent (Ventola, 2015). The proliferation of resistant infectious strains has resulted in a healthcare crisis, which continually threatens the lives of millions of people, due to a depletion of effective therapeutics (Munita & Arias, 2016). Indeed, the WHO has previously classified ABR as one of the top ten most important health threats in the 21st century (WHO, 2021a). It's predicted that 10 million lives will be lost per annum due to AMR-related infections by 2050 (Álvarez-Cisneros & Ponce-Alquicira, 2018). Thus, preventing and controlling the risk of ABR is of paramount importance, with practices such as government interventions and individual education on effective antibiotic use, representing a few of the suggested mechanisms to combat the threat of ABR (WHO, 2021a).

| Drug Class | Antibiotic | Use of Treatment | Isolated from | Mode of Action |
|---|---|---|---|---|
| Aminoglycoside | Kanamycin | Broad spectrum aerobic Gram-negative bacteria including: *Pseudomonas* , *Acinetobacter* and *Enterobacter* . | *Streptomyces kanamyceticus* | Ribosomal modification leading to lack of protein synthisis. |
| | Neomycin | Enteric bacteria and other eubacteria, *Escherichia coli, Klebsiella* and *Enterobacter*. | *Streptomyces fradiae* | |
| | Streptomycin | Mild to moderate infections. | *Streptomyces griseus* | |
| | Gentamicin | Moderate to severe Gram-negative infections. | *Micromonospora purpurea* and related species | |
| Macrolide | Erythromycin | Broad spectrum Gram-positive and Gram-negative infections. | *Saccharopolyspora erythraea* | |
| Amphenicols | Chloramphenicol | Cholera, tetracycline-resistant vibrios and bacterial conjunctivitis. | *Streptomyces venequelae* now synthetically. | |
| Lincosamide | Clindamycin | Broad spectrum: Gram-positive and Gram-negative bacteria. | Semisynthetic | |
| Beta-lactam | Ampicillin | Broad spectrum: Gram-positive and Gram-negative infections. | Semisynthetic aminopenicillin | Binds to penicillin-binding proteins in the bacterial cell wall, blocking the synthesis of peptidoglycan inhibiting cell growth and causing cell lysis. |
| | Amoxicillin | Moderate-spectrum antibiotic: wide range of Gram-positive, and a limited range of Gram-negative bacteria. | | |
| | Oxacillin | Moderate to severe infections: e.g. penicillinase resistant staphylococcal infections. | | |
| | Penicillin G | narrow spectrum antibiotic: typically susceptible Gram positive aerobic organisms e.g. *Streptococcus pneumoniae* . | Naturally occurring | |
| Glycopeptide | Vancomycin | *Listeria monocytogenes* , *Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus agalactiae* and *Actinomyces* species. | *Amycolatopsis orientalis* | Vancomycin binds to the D-alanyl-D-alanine in bacterial cell wall precursors, interfering with bacterial cell wall synthesis. Vancomycin may also alter the permeability of bacterial cytoplasmic membranes and may inhibit RNA synthesis. |
| Tetracyclines | Tetracycline | Common Gram-positive infections. | Produced semisynthetically from chlortetracycline | Inhibits bacterial growth by inhibiting translation. Additionally tetracycline may alter the cytoplasmic membrane of bacteria, causing leakage. |

**Table 1.1. Antibiotic classifications**. Classification of antibiotics used in this study including their typical use in clinical practice, their antimicrobial role, and the origin of the compound (Data from PubChem(https://pubchem.ncbi.nlm.nih.gov) and Drugbank (https://go.drugbank.com/drugs/DB01053) last accessed September 2021).

### 1.23.2. Molecular mechanisms of ABR

ABR is an ancient natural phenomenon (D'Costa et al., 2011). Most antibiotics used today are natural products (albeit some recreated synthetically) of microorganisms, which are manufactured to enhance their survivability against species competing for the same resources (Blair et al., 2015; Fischbach, 2009). Bacteria evolve rapidly and as a result so does the development of ABR (Croll & McDonald, 2012; Dong et al., 2019). Staying true to Darwin's theory of natural selection, the development of ABR can emerge in a survival of the fittest scenario (Read & Woods, 2014). In brief, an antibiotic will interact with and kill most of a bacterial target, however, if a heterologous individual with a genetic capacity of surviving in the presence of the antibiotic is present, it will remain post-antibiotic treatment (Alanis et al., 2005). Hence the 'fittest bacteria' can go forth and replicate, allowing the continuation of a resistant genotype (Alanis, 2005; Holmes et al., 2016).

ABR mechanisms have been identified in a variety of commensal and pathogenic bacteria against most antibiotics (Allen et al., 2010; Ventola, 2015). It is generally accepted that the basis of AMR occurs at the genetic level, which results in a biological adaptation to reducing the efficacy of an antibiotic (Alanis, 2005). Intrinsic resistance is typically characterised when a gene responsible for the resistant phenotype or the 'Antibiotic Resistant Gene' (ARG) is encoded on the chromosome and is commonly identified within the same species (Ammor, Flórez, Hoek, et al., 2008). In addition, the rapid evolution experienced by microbes allows for fast adaptation to new pressures, and resistance can arise because of genetic mutation (leading to a change in protein function) or as a result of gene duplication (Yelin & Kishony, 2018). Intrinsic and mutational resistance is not generally regarded as a public health concern, as these are considered to possess a lower risk of gene transfer to other species (EFSA, 2012; Sandner-Miranda et al., 2018). However, resistance can also be acquired (EFSA, 2012) and is driven by the acquisition of novel genetic information via HGT (Culyba et al., 2015), which is often mediated by the processes of transformation, transduction, and conjugation (Giedraitienė et al., 2011; Thomas & Nielsen, 2005). Perhaps most importantly, acquired resistance often results in the transfer of resistance genes between unrelated bacterial species. Genes acquired via HGT are usually located on MGE including transposons, plasmids, genomic islands (Dobrindt et al., 2004) and integrons (Alanis, 2005; Sandner-Miranda et al., 2018). Resistance that has arisen by HGT is considered far more insidious, as this is suggestive of a mobilised pool of genetic resistance that can be passed on. Indeed, numerous examples of AMR transfer are documented (Frazão et al., 2019; Lester et al., 2006; Nawaz et al., 2011; Oladeinde et al., 2019; Porse et al., 2017; Shoemaker et al., 2001). For example, a Multi-Drug Resistant (MDR) *Acinetobacter baumannii* (strain XDR-AB) was capable of transferring ticarcillin and kanamycin resistance to environmental (ticarcillin and

kanamycin sensitive) *Acinetobacter* strain via plasmid transfer (Heuer et al., 2011). Similarly, two pathogenic species; *Neisseria meningitidis* and *Neisseria gonorrhoeae* could obtain an alternative gene from commensal *Neisseria* species via transformation, resulting in enhanced resistance to penicillin (Spratt et al., 1992).

Several biochemical pathways have been described as an ABR phenotype:

1. Antibiotic modification via enzyme degradation, for example, staphylococci produce penicillinase which degrades penicillin (Alekshun & Levy, 2007).

2. Removal of the antibiotic via efflux pumps, that pump the antibiotic out of the cell until it reaches sublethal concentrations (Blair et al., 2015). If multiple efflux pumps are present this confers multiple drug resistance e.g., multiple drug-resistant efflux pumps (Blair et al., 2015).

3. Modification of antibiotic targets, changing receptor structures reducing the antibiotic's ability to target the bacteria. For example, by modifying Penicillin Binding Proteins (PBP's) so penicillin cannot bind (Munita & Arias, 2016).

4. Modifying cell membrane permeability, decreasing the risk of antibiotics reaching intracellular regions e.g., the development of vancomycin resistance in enterococci is caused by changes in the peptidoglycan structure of the cell wall (Munita & Arias, 2016).

Therefore, as we enter the brink of a post-antibiotic era, it is of paramount importance that all risks of HGT of ARGs are monitored and restricted when possible.

### 1.23.3. Probiotics and Antibiotic Resistance

When assessing genomic safety and the mobility of probiotic organisms, ARGs are typically focused upon (Colautti et al., 2022). ARGs can be identified in a bacterium either as part of their chromosome DNA (intrinsic/innate) or their plasmid DNA (Ashraf & Shah, 2011; Guo et al., 2017). There are three types of recognised ABR: intrinsic, acquired and mutational (Ashraf & Shah, 2011). When the resistance is intrinsic or brought around by a mutation it is generally not a safety concern for a probiotic species and has even been associated with health benefits such as restoring the microflora of the gut after antibiotic treatment (Gueimonde et al., 2013). The presence of resistant genes is only regarded as a serious safety issue when there is a possibility that the gene can be transferred by the MGEs (Ammor, Flórez, Hoek, et al., 2008; Gueimonde et al., 2013). For example, Egervärn et al., (2009) found that *L.plantarum* strains with atypical phenotypic resistance to tetracycline carried a *tetM* gene on a plasmid. Projects such as EUSAFE recommend that probiotic species should not contain transferable ABR genes, highlighting the importance of fully characterising species genomes, promoting full product safety and depicting any risks and/or benefits associated with a probiotic product (Aires et al., 2007; Gueimonde et al., 2013; Štšepetova et al., 2017). As ARGs have already been discovered in strains of *Lactobacillus* and *Bifidobacterium* (Gueimonde et al., 2013), it is desirable to ensure novel isolates do not contain plasmids encoding ABR, as well as virulence factors. Indeed, as a QPS candidate, ABR profiles should be distinguished before a QPS designation (EFSA, 2008; Gueimonde et al., 2013). In addition, the guidelines set by the FDA and the WHO state that before a probiotic can be used in food for human consumption, a risk assessment of its ABR patterns must first be conducted (Araya et al., 2002). Indeed, with the popularity of probiotic consumption continually expanding (as shown by the global market worth predicted to exceed USD 64 billion by 2023), the ability of a probiotic to act as an ARG reservoir for pathogenic bacteria should be robustly characterised (Ammor, Flórez, Hoek, et al., 2008; Gotcheva et al., 2018). Despite regulatory obligations, there is a surprising lack of standardised methods when assessing the ABR mechanisms in probiotic bacteria (Dong et al., 2019; Guo et al., 2017; Mayrhofer et al., 2010; Zhang et al., 2018).

## 1.24. A polyphasic (two-pronged) approach

Despite advances in microbial phylogenetics, there are arguments against the sole use of genomics to assign taxonomy and a functional capacity of a bacterium. For example, the presence of a gene does not always mean that it is expressed and translated into a phenotypic function (Kämpfer & Glaeser, 2012). In addition, limitations in gene annotation such as incorrect gene nomenclature and similarities in gene sequence but differences in function (homology) could also impact genomic classification (Falcão Salles et al., 2012; Komárek, 2016; Lomsadze et al., 2018). The concept is known as 'polyphasic taxonomy' was first recognised in the 1970s by Colwell, (1970), employing a combination of both genomic and phenotypic traits to assign taxonomy. Likewise, it is also imperative to use a similar approach following taxonomic classification, when predicting a functional capacity of an organism. As such, utilising both genomic and phenotypic analysis (or a genome-guided approach) is highly desirable when analysing the safety and functionality of microbial food supplements.

## 1.25. CUL organisms

Cultech is a probiotic manufacturing firm based in South Wales, United Kingdom. Here, they hold a bank of food supplements from the lactobacilli and bifidobacteria genera (**Table 1.2**). Previous taxonomic identification of strains has been conducted by Amplified Fragment Length Polymorphism (AFLP) and API analysis. Indeed, certain health benefits have already been associated with Cultech's primary supplements including, Lab4 (Salvi et al., 2019), Lab4B (Davies et al., 2018) and Lab4P (Michael, 2021; Michael et al., 2017, 2020; O'Morain, 2019).

**Table 1.2. Putative taxonomic classification of CUL isolates.** Putative taxonomy is assigned according to the strain's manufacturers. A summary of phenotypic characteristics for such species is provided, including morphology, Gram type, cell wall composition, lactic acid product, carbohydrate fermentation process and whether isolates are already marketed as a LAB4/B probiotic product (phenotypic properties according to (Pot & Tsakalidou, 2009).

| Genus | Species | Strain | Morphology | Gram +/- | Cell Wall Peptidoglycan | Lactic Acid Isomer | Oxygen Tolerance | Carbohydrate Fermentation | LAB4/LAB4B consortium |
|---|---|---|---|---|---|---|---|---|---|
| ***Lactobacillus*** | *paracasei* | CUL37 | Non-motile bacillus. smooth/non transparent/Cream - light yellow. | + | L-Lys-D-Asp | L | Facultatively anaerobic | Facultatively heterofermentative | No |
| | *paracasei* | CUL07 | | + | | | | | No |
| | *paracasei* | CUL08 | | + | | | | | Yes |
| | *casei* | CUL06 | Non-motile bacillus/smooth/white. | + | L-Lys-D-Asp | L | Facultatively anaerobic | Facultatively heterofermentative | No |
| | *rhamnosus* | CUL63 | Non-motile bacillus. | + | L-Lys-D-Asp | L | Facultatively anaerobic | Facultatively heterofermentative | No |
| | *fermentum* | CUL40 | Non-motile bacillus/smooth/white. | + | Orn-D-Asp | DL | Facultatively anaerobic | Obligately heterofermentative | No |
| | *fermentum* | CUL67 | | + | | | | | No |
| | *salivarius* | CUL61 | Non-motile bacillus. | + | L-Lys-D-Asp | L | Facultatively anaerobic | Obligately homofermentative | Yes |
| | *helveticus* | CUL76 | Non-motile bacillus single cell or paired cell. | + | L-Lys-D-Asp | DL | Facultatively anaerobic | Obligately homofermentative | No |
| | *gasseri* | CUL09 | Non-motile bacillus. | + | L-Lys-D-Asp | DL | Anaerobic | Obligately homofermentative | No |
| | *acidophilus* | CUL60 | Non-motile, short-chain bacillus smooth/white. | + | L-Lys-D-Asp | DL | Facultatively anaerobic | Obligately homofermentative | Yes |
| | *acidophilus* | CUL21 | | + | | | | | Yes |
| | *plantarum* | CUL66 | Non-motile bacillus, smooth creamy/white. | + | DAP | DL | Facultatively anaerobic | Facultatively heterofermentative | No |
| | *plantarum* | CUL66N | | + | | | | | No |
| ***Bifidobacterium*** | *bifidum* | CUL20 | Branched bacillus. | + | Murein L-Orn-D-Ser-D-Asp | L | Anaerobe | N.A. | Yes |
| | *animalis* | CUL34 | Branched-bacillus (bone like structure). | + | Murein L-Lys-(L-orn)L-Ser-(L-Ala)-L.Ala2 | L | Anaerobe | | Yes |

## 1.26. Aims and Objectives

This research project sets out to provide a robust taxonomic profile of CUL strains using a multifaceted approach. Initially, genomic (g)DNA extractions from Gram-positive bacteria will be optimised. Following, Sanger sequencing and WGS will be conducted on CUL strains, and the efficacy of single vs. multi-panel of genomic markers will be assessed when ascertaining strain level taxonomic resolution from lactobacilli and bifidobacteria species. Using MLSA the evolutionary lineage of CUL strains will be determined with a scope including all available genomes from members of their respective genera. Strain level designation will thus be provided. WGS will facilitate CUL genomes annotation and enable the subsequent mining for known regions of interest, allowing a safety and probiotic functionality assessment. Following, the putative determination of traits that confer a 'safety risk' or a 'health benefit', subsequent chapters will aim at using a genome-guided approach to correlate genotype with phenotype, enabling a multifaceted view of function (**Figure 1.7**). In summary, this thesis will provide the first robust taxonomic assessment of the CUL bacteria, which are marketed as probiotics. WGS will then allow a genome-guided approach to investigate the functionality and safety of CUL isolates (**Figure 1.6**).

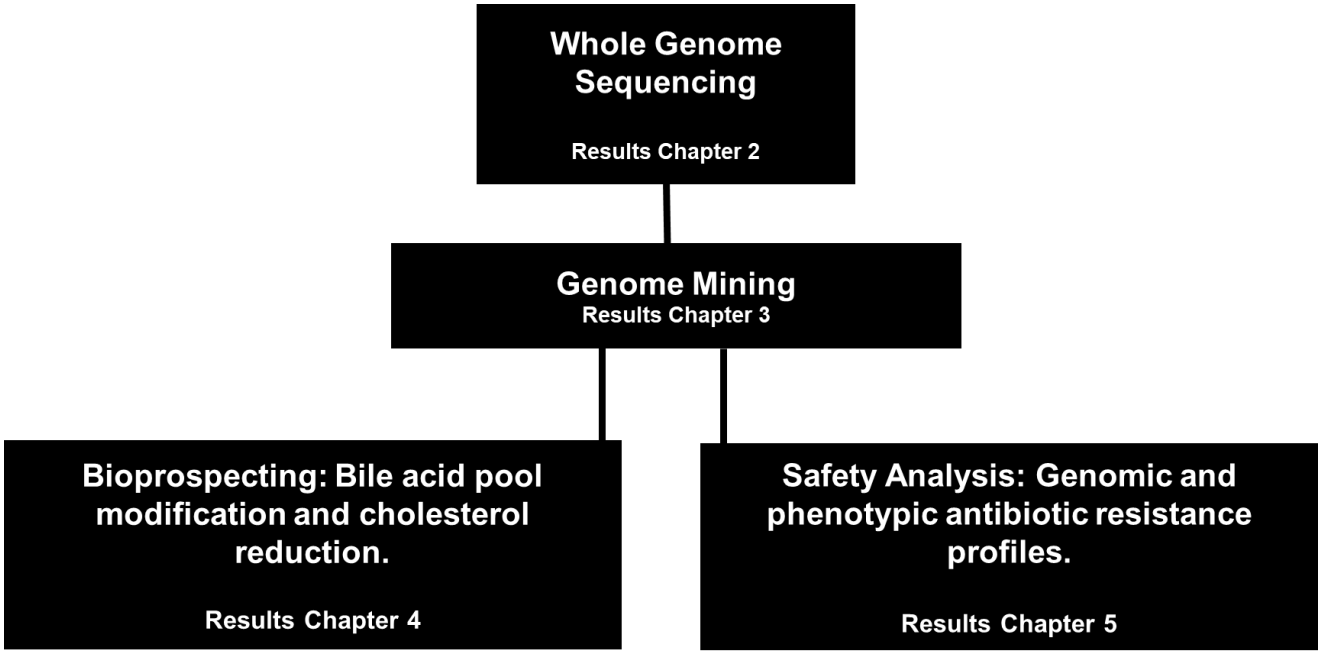



**Figure 1.6. The scaffold of the thesis, including an overview of the aims of subsequent chapters.**

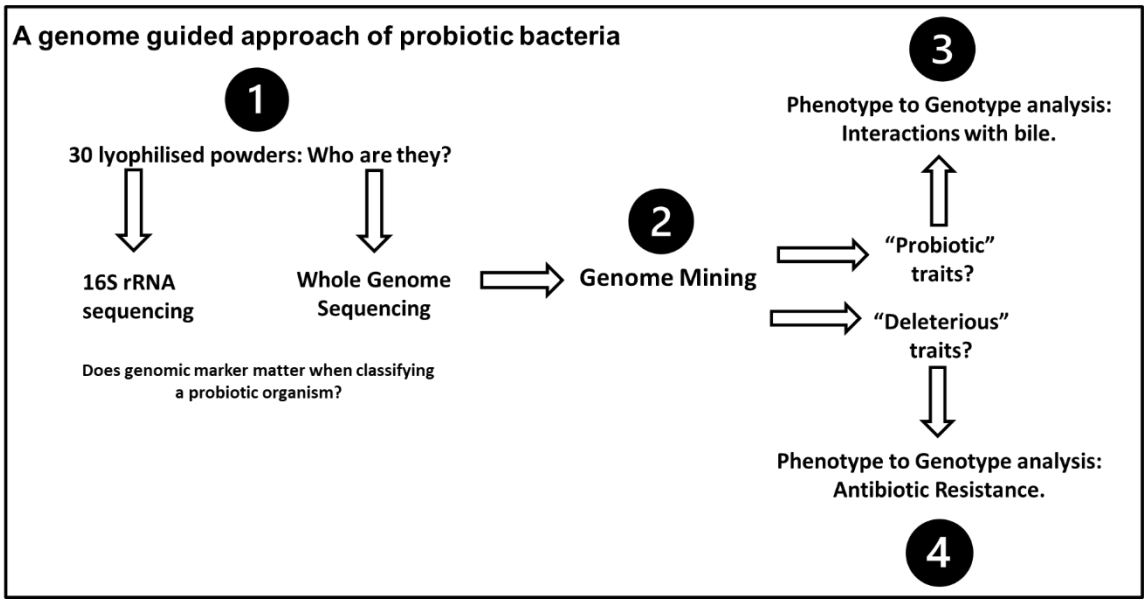



**Figure 1.7. Thesis chapter schematic, highlighting the workflow undertaken throughout this project.** Numbers are used to represent the steps of the workflow taken throughout this project.

# Chapter 2.

# The taxonomic classification of CUL isolates.

## 2.1. Introduction

The accurate classification of bacterial isolates, using a robust phylogenetic framework, is paramount for downstream genomic analysis. However, it is evident from the literature that many studies still rely solely on a single gene (often 16S rRNA) based phylogenies that may fail to accurately portray strain genealogies (Bazireh et al., 2020; Foschi et al., 2017; Lee et al., 2021). This leads to either an accumulation of incorrectly assigned taxonomies in publicly available databases or poorly resolved bacterial lineages, often compromising the reliability of comparative genomic analysis and prediction of evolutionary pathways amongst related isolates. Indeed, it has been demonstrated several times (Drancourt et al., 2000; Janda & Abbott, 2007; Johnson et al., 2019; Mignard & Flandrois, 2006) that classifications are based solely on 16S rRNA sequences do not always accurately capture the true species genealogy. Moreover, this issue is further exacerbated in genera (such as *Lactobacillus*) that have many closely related species. An example of such can be seen in the *L.casei* phylogroup which encompasses *L.casei*, *L.paracasei*, *L.rhamnosus* and *L.zeae*, all of which have pairwise 16S rRNA gene sequence percentage similarity of around 99 % (Huang et al., 2018b). Further emphasising the uncertain taxonomy, the genus *Lactobacillus* during the last census in 2020, was split into several new genera; in addition to the historical major revisions the genus has undergone, as new strains have been identified (Bull et al., 2013; Dicks et al., 1996.; Felis et al., 2001; Pang et al., 2012; Vancanneyt et al., 2006; Zheng et al., 2020b). Given the limitations of 16S rRNA phylogenies, there is a need to implement a higher resolution when assigning taxonomy, such as MLSA phylogenies. Overall, MLSA appears to provide more reliable phylogenetic divergences (due to the increased phylogenetic signal offered by multiple genomic markers), which increases the likelihood that true speciation events are represented (López-Hermoso et al., 2017).

### 2.1.2. Taxonomic difficulties in probiotic rich genera

*Lactobacillus* and *Bifidobacterium* are well-established probiotic-rich genera whose species have been exploited significantly in the probiotic market. Numerous strains from these genera have been deemed as beneficial organisms, based on their putative ability to confer health benefits to a host (Kobyliak et al., 2018; Koutnikova et al., 2019; Liu et al., 2017; Madsen et al., 2001). However, there is growing evidence that probiotic traits are strain specific (Campana et al., 2017; McFarland et al., 2018a; Ramos et al., 2013). In addition, the genus *Lactobacillus* is highly heterogenous, consisting of over 200 species that possess the largest GC% range of any other bacterial genera and encompasses a large metabolic diversity (Duar et al., 2017; Harris et al., 2017; Sun, Zhang, et al., 2015). Yet surprisingly, many studies are still bioprospecting novel probiotic candidates using 16S rRNA-based phylogenies (Awd et al.,

2020; Fredua-Agyeman et al., 2020; Lashani et al., 2020). This confounds WHO guidelines, which state that proper nomenclature and strain designation is required when assigning the term probiotic to an organism (Hill et al., 2016; WHO, 2002). Additionally, EFSA has now recommended that the genomes of all probiotic strains be sequenced to help ascertain genomic risks (EFSA, 2012; Rychen et al., 2018). Certainly, a misclassified organism marketed as safe for consumption, could not only impact consumer health but also lead to inconsistency in the literature regarding the relevance and functionality of such isolates as probiotic strains. Indeed, several studies have shown discrepancies between the probiotic product label and its taxonomy, even at the genus level (Huys et al., 2006; Morovic et al., 2016; Temmerman et al., 2003). In addition to consumer safety, accurate strain identification can also drive bioprospecting through genomic mining (Collins et al., 2017; Harris et al., 2017; Spinler et al., 2014; Wuyts et al., 2017). Thus, WGS and robust phylogenetic placement of any bacterial isolate intended for human consumption is paramount.

### 2.1.3. Aims and Objectives

Candidate probiotic strains provided by Cultech Ltd (Herein referred to as CUL isolates) have been classified using Amplified Fragment Length Polymorphism (AFLP) and API analysis. As legislation has changed to reflect the development and accessibility of genomic tools, the need to provide a more in-depth classification of microorganisms sold for consumption is desirable. Therefore, this chapter aims to robustly classify up to 30 bacterial isolates using a multifaceted approach. Using a combination of 16S rRNA and MLSA, this study will assess the resolution that different taxonomic markers offer when assigning taxonomy, therefore allowing a robust taxonomic designation to be given to all CUL strains. Additionally, due to the ongoing taxonomic uncertainties that still exist in the *Lactobacillus* genus, this study will also conduct a genus-wide analysis, evaluating the phylogroups provided by MLSA in comparison to previous reports (Pot & Tsakalidou, 2009; Salvetti et al., 2018; Zheng et al., 2020b), enabling the evaluation of groups housing CUL strains. This chapter will therefore confirm the taxonomic identity of CUL isolates, which will enable studies presented in future chapters to undertake a genome-guided analysis to evaluate the safety and functionality of CUL bacteria.

**Note. April 2020: the reclassification of the *Lactobacillus* genus.**

At this point, it is worth noting that in April 2020, the *Lactobacillus* genus was reanalysed and reclassified (**Supp. Table S2.12**), work presented in this thesis predates this reclassification. Many authors conclude that as additional species are being continually added to the genus, nomenclature should change, to reflect true taxonomy (Claesson et al., 2008; Salvetti et al., 2018; Salvetti & O'Toole, 2017b; Sun, Zhang, et al., 2015; Wittouck et al., 2019). As such, in the latest revision, the *Lactobacillus* genus was split into 25 genera (23 of which are novel), following an extensive genomic review (Zheng et al., 2020b). There is now moderate uptake of the new nomenclature described by (Zheng et al., 2020b). However, data analysis for this study was conducted before this technical change, and to avoid confusion, the new nomenclature is detailed in **Table 2.1**.

**Table 2.1. Nomenclature key for CUL isolates.** The new nomenclature proposed for the isolates focused on within this study.

| Species | 2020 Reclassification* |
|---|---|
| *Lactobacillus paracasei* | *Lacticaseibacillus paracasei* |
| *Lactobacillus casei* | *Lacticaseibacillus casei* |
| *Lactobacillus rhamnosus* | *Lacticaseibacillus rhamnosus* |
| *Lactobacillus zeae* | *Lacticaseibacillus casei* |
| *Lactobacillus fermentum* | *Limosilactobacillus fermentum* |
| *Lactobacillus salivarius* | *Ligilactobacillus salivarius* |
| *Lactobacillus helveticus* | No Change |
| *Lactobacillus gasseri* | No Change |
| *Lactobacillus acidophilus* | No Change |
| *Lactobacillus plantarum* | *Lactiplantibacillus plantarum* |

* (Zheng et al., 2020b).

## 2.2. Materials and Methods

### 2.2.1. Lyophilised probiotic preparations

Probiotic bacterial isolates (**Table 1.2**) were provided by Cultech Ltd in the form of lyophilised powders. Powders were prepared by Cultech from overnight cultures grown in De-Man Rogosa Sharpe (MRS) medium (CM0361, Oxoid, Basingstoke, UK) and inoculated from a single colony. Cultures were centrifuged at 2500 *g* for 20 minutes, and the supernatant was discarded. The resultant cell pellet was washed three times in 0.9 % NaCl and finally resuspended in 10 mL of 0.9 % NaCl and 60 % maltodextrin. Solutions were freeze-dried at -40 °C for one hour and then for 2 days at -20 °C. The purity of these powders was tested by resuspending a small amount of lyophilised powder in 10 mL of MRS broth and growing overnight. Streak plates were produced from the overnights on MRS agar to confirm the purity of the powders.

### 2.2.2. Culturing of *Lactobacillus* and *Bifidobacterium*

Cultures of lactobacilli strains were grown as follows; a sterile loop was used to inoculate lyophilised powder stocks on MRS agar. Plates were incubated anaerobically (10 % carbon dioxide, 10 % hydrogen and 80 % nitrogen) at 37 °C in an anaerobic workstation (Don Whitley, DG250, United Kingdom) for 48 h. A single colony was transferred to 20 mL of MRS broth for 24 h overnight incubation. Cultures were grown anaerobically (as previously described) for 24 h with gentle shaking.

For bifidobacteria strains, growth was conducted using the same workflow as lactobacilli, with some modifications. Strains were grown on modified, pre-reduced MRS (MRSX) containing 0.001 % lithium chloride (w/v), 0.0015 % sodium propionate (w/v) and 0.00025 % L-cysteine hydrochloride (w/v), for 72 h on agar. Cultures were prepared in MRSX broth and grown anaerobically (as previously described) for 48 h with gentle shaking. Cultures were set up from single colonies, produced by streak plating on MRSX to check for purity. Glycerol stocks (final glycerol concentration 15 %) of each strain were stored at – 20 °C. Bacterial concentrations were estimated according to the Miles & Misra, (1938) method. In brief, overnight cultures were diluted to 0.2 OD and further diluted 1:200 times in MRS broth and were utilised as a starting concentration. Following, dilutions were prepared in a 96-well plate. 1: 10 dilutions were initially conducted, where 1 represents the bacterial culture and 9 parts represent Maximum Recovery Dilutant (MRD). MRS plates were divided into 3 segments and 5 x 5 µL (total 25 µL) drops per dilution were inoculated per plate, representing each dilution (incorporating 1, 10, 100, and 1000). Plates were incubated anaerobically for 48 h (lactobacilli)

or 72 h (bifidobacteria). CFUs were counted by visual scoring. The following calculation was then conducted:

CFU per mL = (average number of colonies for a dilution x 40) x dilution factor. Experiments were conducted in triplicate and a mean average was calculated.

### 2.2.3. Assigning putative species identity via 16S rRNA amplicon sequencing

From streak plates, a single colony was transferred directly to a pre-chilled PCR tube, containing 1 X Mangomix (Meridian Bioscience, 25033) and 0.5 µM of each of the following oligonucleotide: U1 5'-ACGCGTCGACAGAGTTTGATCCTGGCT-3', U1R 5'-GGGACTACCAGGGTATCTAAT-3' (for lactobacilli), or 27F 5'-AGAGTTTGATCMTGGCTCAG- 3', 1492R TACGGYTACCTTGTTACGACTT-3', for bifidobacteria.

Polymerase Chain Reactions (PCR) were performed in 20 µL volumes and cycled in a C100 thermocycler (Bio-Rad, UK). Thermocycling profiles were as follows for lactobacilli: 3 minutes initial denaturing at 95 °C, followed by 30 seconds denaturing, 30 seconds annealing at 50 °C and extension at 72 °C for 30 seconds, for 35 cycles. The final extension step was at 72 °C for 5 minutes. For bifidobacteria: 2 minutes initial denaturing at 94 °C, followed by 30 seconds annealing at 55 °C and extension at 72 °C for 30 seconds, for 35 cycles. The final extension step was at 72 °C for 5 minutes. Annealing temperatures (TA) were chosen as 5 °C less than those calculated by Primer3 (Untergasser et al., 2012). PCRs were checked on a 1 % agarose gel (105 V for 20 minutes) alongside a λ HindIII molecular weight marker. For Sanger sequencing, PCR products were precipitated overnight by adding 1/10 volume of 5 M sodium acetate (pH 5.0), and 2 X starting volume of 100 % ethanol. Incubations were conducted overnight at -20 °C. Precipitated nucleic acids were desalted with 70 % ethanol and resuspended in 25 µL of nuclease-free water. Sanger sequencing of PCR products was undertaken at LGC Genomics (Berlin, Germany) using the PCR primers listed above.

16S rRNA amplicon sequencing traces were initially observed in Chromas (version 2.1), to determine per-base sequence quality. Sequences were manually trimmed at either end or when base calls were ambiguous. Sequences were then used as queries for BLASTn (using default parameters). The top retrieved BLASTn (Chen et al., 2015) hits were subsequently used to determine putative taxonomic identity.

### 2.2.4. Type strain phylogeny

The 16S rRNA nucleotide sequences of lactobacilli and bifidobacteria type strains were identified on https://www.bacterio.net/ and retrieved from NCBI (accession numbers and species names (**Supp. Table S2.1 & S2.2**)). In total, 228 sequences were retrieved for lactobacilli and 61 for bifidobacteria (from the date of access, April 2019). These sequences, together with those obtained from CUL strains were aligned using CLUSTALW (Li, 2003). Alignments were manually trimmed to remove gap-rich regions at either end of the sequences. A substitution model for phylogeny reconstruction was determined using a MRMODEL test (Nei & Kumar, 2000; Tamura et al., 2013). The model was selected according to the lowest Bayesian Information Criterion (BIC) and the highest Akaike Information Criterion (AIC) scores. Both phylogenies were reconstructed using the distance-based Neighbor-Joining algorithm (Saitou & Nei, 1987) including all codon positions. Multiple substitutions were corrected using the Kimura 2-parameter method (K80 (Kimura, 1980)), for *Lactobacillus* and the Tajima-Nei method (TN92 (Tajima & Nei, 1984)) for *Bifidobacterium*. Rate variation amongst sites was modelled with a gamma distribution (shape parameter = 0.52); as determined by the ModelTest (Nylander, 2004) for the *Lactobacillus* phylogeny and a gamma distribution with a shape parameter of 0.37 for the *Bifidobacterium* phylogeny. The robustness of each node (on both phylogenies) was assessed using bootstrap analysis (Horowitz, 2001 (for 1000 pseudoreplicates)).

### 2.2.5. Genomic gDNA extraction

For lactobacilli, genomic DNA (gDNA) was isolated from anaerobically grown overnight cultures using the DNeasy Blood and Tissue DNA isolation kit (Qiagen, Germany) as per the manufacturer's recommendations with the following modifications: Pelleted bacteria was washed three times with NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0 (Alimolaei & Golchin, 2017)). Lysis was performed enzymatically for 1 h at 37 °C, with 20 mg/mL of lysozyme (Sigma-Aldrich) and 100 units of mutanolysin (Sigma, M4782). This was followed by the addition of 10 mg/mL proteinase K (Sigma, P2308) and incubated for 1 h at 56 °C. For bifidobacteria, gDNA isolation was performed as above, with slight modifications. 30 mL of culture (incubated for 48 h) was utilised for the extraction. Cultures were resuspended in 20 mg/mL of lysozyme and incubated overnight on an orbital rotator, at 37 °C**.** Following, mechanical lysis was performed by applying a bead-beating step (Matrix E (MPBio, United Kingdom)) for 20 s at speed 6. Cultures were then processed through the DNA spin columns and the resulting gDNA was eluted in 25 µL of autoclaved MilliQ (nuclease-free) water.

### 2.2.6. Assessing the quality and quantity of isolated DNA

The quality of intact gDNA was assessed via agarose gel electrophoresis (0.7 % agarose gel). Isolated double-stranded DNA was quantified using a Qubit fluorometric analyser (Invitrogen, V.2.0), in accordance with the manufacturer's instructions. gDNA yields were recorded as ng/µL.

### 2.2.7. WGS and genome assembly

Draft genome sequences were supplied by either LGC Genomics (Berlin, Germany) or Microbes NG (Birmingham, UK). SpAdes (Bankevich et al., 2012), is a *de novo* (without the use of a reference genome) bacterial assembly tool, whose assembly method is based on de Bruijn graphs. CUL sequences were subsequently assembled in spAdes (Bankevich et al., 2012) by the genome providers. During the process, sequence reads were overlapped into continuous sequences (Contigs) *de novo*. Upon genome retrieval, assembled sequences were annotated via NCBI's PGAAP pipeline, which allowed contamination from sequencing processes to be removed. Genomic annotation was further supplied by Rapid Annotation using Subsystem Technology (RAST (Aziz et al., 2008)) server, which allowed an overview of genomic metrics. Median reported genome sizes and %GC were retrieved from NCBI GenBank (Leray et al., 2019) acting as an assessment marker of draft genome quality.

### 2.2.8. Multi Locus Sequence Analysis and phylogenetic classification

Maximum likelihood (ML) phylogenies were generated by conducting a multi-locus sequence alignment in PhyloPhlAn (Segata et al., 2013). Briefly, WGSs were autonomously retrieved for (i) the genera classified as Lactic Acid Bacteria (LAB), including *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Weissella*, *Bacillales*, *Dolosigranulum*, *Abiotrophia*, *Aerococcus*, *Camobacterium*, *Leuconostoc* and *Oenococcus* (ii). *Lactobacillus*, *Pediococcus* and *Lactococcus*, and (iii) all bifidobacteria and *Aeriscardovia aeriphila* LMG 21773 from the GenBank FTP site using the WGET script (database last accessed August 2019). WGSs were annotated in PROKKA (Seemann, 2014). The translated coding sequences were then used for the identification, alignment, and concatenation of 400-core protein sequences in PhyloPhlAn (using the "-u" command). The ML phylogenies were reconstructed from the concatenated alignments in FastTree MP (Price et al., 2010 (JTT+CAT)) implemented in the Cipres Science Gateway Server (Miller et al., 2010). The robustness of the phylogeny was assessed using 1000 bootstrap pseudoreplicates. To determine tree polarity (direction) and allow for inference of evolutionary events, trees were outgroup rooted. Suitable outgroup roots were determined by reconstructing a Lactic Acid Bacteria phylogeny for lactobacilli due to the documented paraphyletic nature of the genus (Makarova & Koonin, 2007). Final rooted trees (using *Lactococcus* due to its non-paraphyletic relationship with *Lactobacillus*) as an out-group for *Lactobacillus,* as determined by initial MLSA and *Aeriscardovia* for *Bifidobacterium* were rendered as circular phylogenies in iTOL (https://itol.embl.de/).

### 2.2.9. Phylogenetic analysis of the *Lactobacillus* and *Bifidobacterium* genera

To determine the correct phylogroup allocations for each CUL isolate a literature review was conducted to establish previous species groupings. Each species was tallied according to its annotated name, and a phylogroup was assigned and compared to previous designations for the entire *Lactobacillus* and *Bifidobacterium* genera (Holzapfel & Wood, 2014; Lugli et al., 2018; Salvetti et al., 2018; Sun, Zhang, et al., 2015; Zheng et al., 2020b). In some cases, genomes retrieved for the construction of the MLSA analysis were not recorded in any established phylogroup, and as such, after clade position scrutiny, they were assigned to the group they were either (i) located in or (ii) a new group was designated (typically named after the first species in the clade) if the clade was located outside of any other recognised phylogroups. In addition, disagreement often arose in the literature in terms of species phylogenetic assignment. As such, differences were documented, and clade positioning was inspected to establish the best annotation. During analysis, numerous genomes were retrieved either unlabelled or mislabelled. MLSA allowed the reannotation of species to truly reflect their taxonomy based on their clade position and bootstrap reliability.

## 2.3. Results

### 2.3.1. Optimisation of Genomic DNA extraction procedures

Initial extractions of gDNA from lactobacilli resulted in low yield and low molecular weight DNA (evidenced by smearing below the 2,027 bp marker, in lane 2 of **Figure 2.1A**). Incorporation of several wash steps, using NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0), improved the recovery rate of gDNA, leading to increased yields of high molecular weight DNA (**Figure 2.1A**, lanes 3 - 20). When recovered DNA was of a significantly high yield, it was evidenced by the presence of DNA within the wells (lanes 8, 9, 10, 15 and 20), in addition to travelling through the gel (**Figure 2.1A**). Isolating high-quality genomic DNA provided a basis to conduct further molecular analysis of CUL isolates, including Sanger sequencing, where DNA was used to amplify the 16S rRNA gene of lactobacilli and bifidobacteria strains (**Figure 2.1B**).

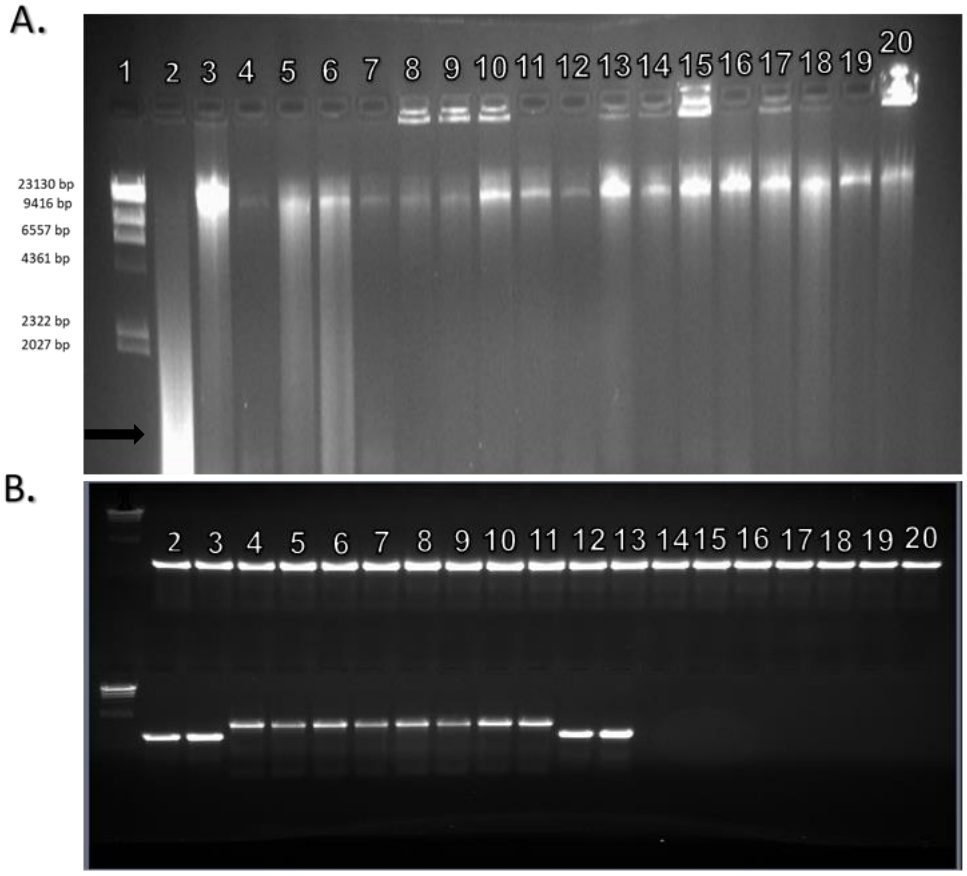



**Figure 2.1. Assessing the quality of isolated gDNA and success of 16S rRNA PCR.**
**A. *Lactobacillus* gDNA integrity comparison )with or without NaCl wash step before lysis).** 0.7 % agarose gel, comparing gDNA methods. Lane 2 – *Lactobacillus* gDNA without wash step, where the sample is sheared, as indicated by a bright smear of fluorescence within the lane (specified by an arrow). Lane 3 – 20: *Lactobacillus* gDNA after cellular pellet was washed 3 times with NaCl + EDTA (pH 8.0) before lysis. Intact, non-sheared gDNA is indicated by the solid band appearing at approximately 20 Kb. Florescence within wells (lanes 8, 9,10,15,17 and 20) suggests the presence of intact, high molecular weight gDNA. The size was determined by comparison to the λHindIII ladder (lane 1). **B. 16S rRNA PCR products. Top Row**: *Lactobacillus* amplification using primers U1F and U1R. **Bottom Row:** lanes 2 and 3 *Lactobacillus* amplified from U1 and U1R (approx. 1000 bp). Lanes 4 - 11, *Bifidobacteria* amplified by 27F and 1492R (approx. 1500 bp). Lanes 12 and 13 *Lactobacillus* U1, U1R (lane 1 λHindIII ladder).

### 2.3.2. Initial taxonomic identification of CUL isolates using 16S rRNA colony PCR

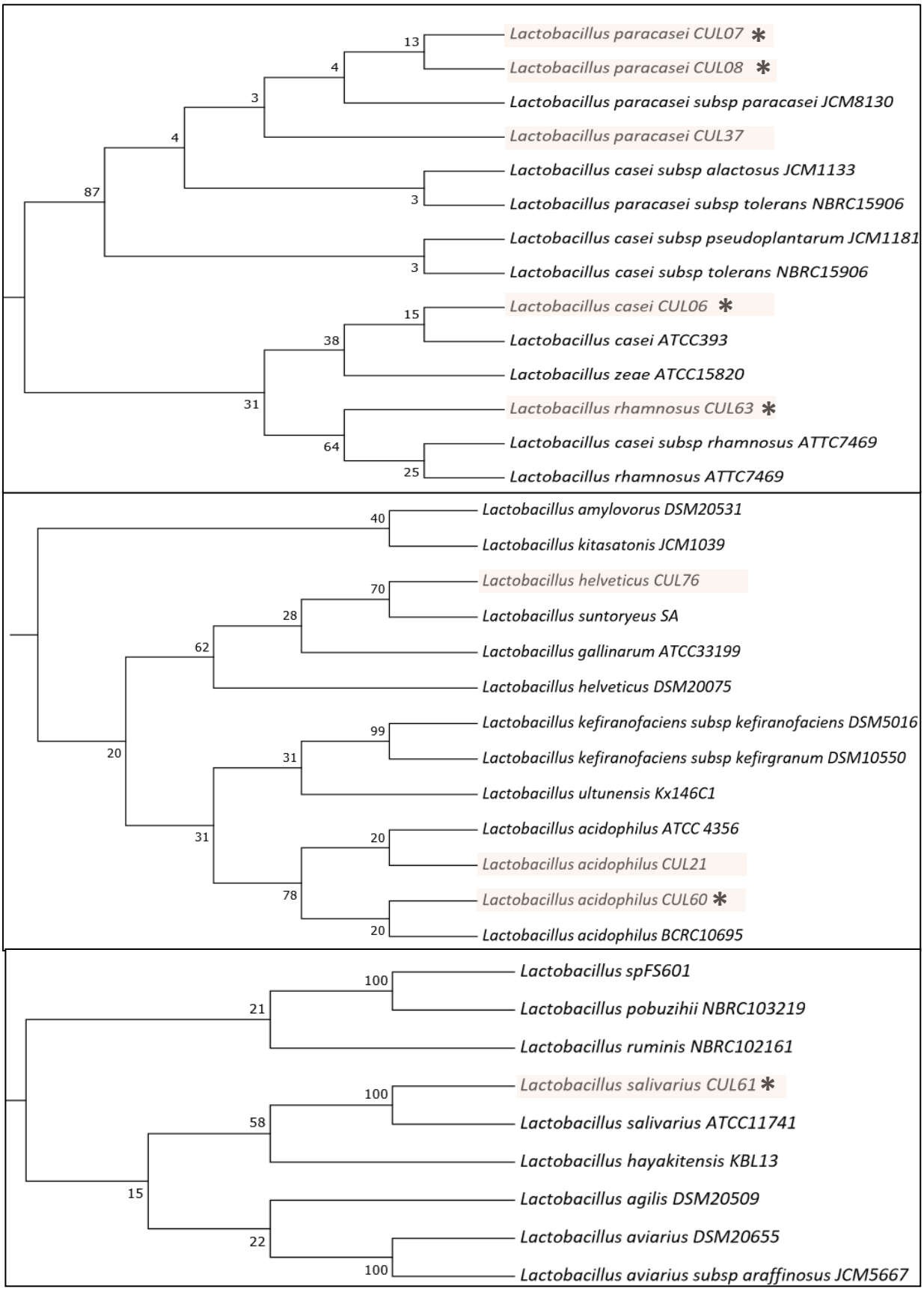
Sanger sequencing of 16S rRNA PCR amplicons supported the genus-level identification of all CUL isolates, with (in most cases) greater than 98 % sequence similarity to strains within the same genus and species (using top BLASTn hits; **Table 2.2**). However, in comparison, both bifidobacteria isolates, *B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34, *Lactobacillus plantarum* CUL66N and *Lactobacillus gasseri* CUL09 had percentage similarities slightly lower (96 – 97 %) than the top BLASTn hit. To visualise evolutionary relationships, a distance-based, Neighbor-Joining (NJ), type-strain 16S rRNA phylogeny was reconstructed using all recognised type strains from the *Lactobacillus* and *Bifidobacterium* genera (as of September 2019 (**Figure 2.2, panels A - E**)). Due to the large heterogeneity of the *Lactobacillus* genus, phylogenies were left unrooted to ensure topologies were not skewed due to the paraphyletic nature of the genus (Sun et al., 2015a). According to the *Lactobacillus* type strain phylogeny, most CUL isolates grouped as per their expected genealogy with no unresolved, polytomous splits. This observation was consistent for all *Lactobacillus* CUL isolates, except for *L.helveticus* CUL76 grouped with *L.suntoryeus* strain SA and *L.gallinarum* strain ATCC33199 (but had *L.helveticus* DSM20075 as a sister grouping). In support of the *Lactobacillus* Type-strain phylogeny, the topology recovered all known phylogroups previously reported(Pot et al., 2014). Interestingly, despite manual trimming and alignment of gap-rich regions, bootstrap values throughout the phylogeny were low, with several branches displaying less than 50 % confidence. In contrast, the genus *Bifidobacterium* is significantly smaller, with only 61 type-strain sequences available. The topology of the *Bifidobacterium* Neighbour-joining phylogeny (**Figure 2.3**) shows slightly higher nodal support. However, some nodes still possessed low bootstrap values (< 50 %), indicating that in some cases there was not enough phylogenetic signal to confidently reconstruct splits. That said, both *B.animalis* subsp. *lactis* CUL34 and *B.bifidum* CUL20 are grouped within the *B.animalis sensu lato* and *B.bifidum* clades, respectively.

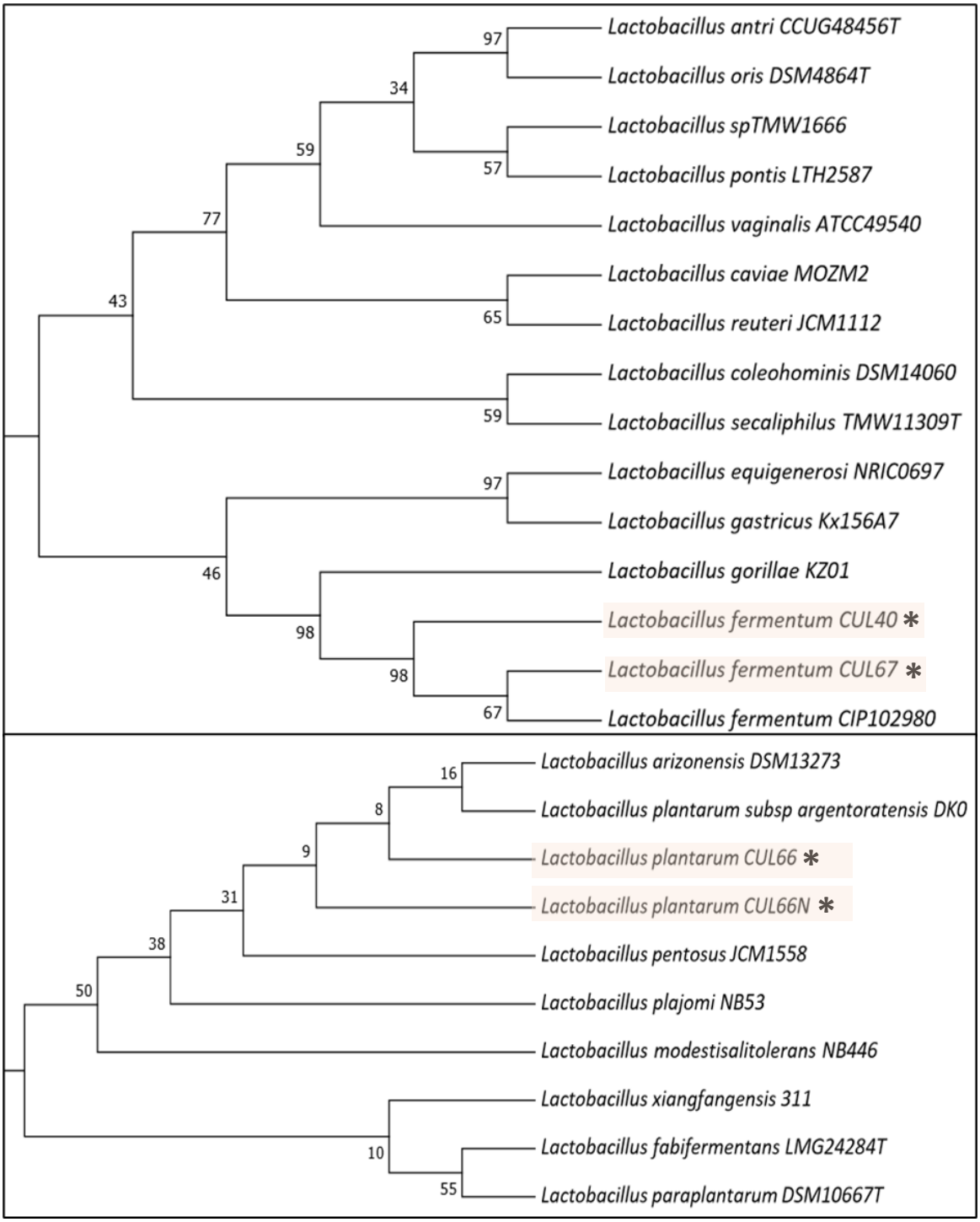
**Table 2.2. The putative identification of CUL isolates determined by Sanger sequencing.**

| CUL ID | Classification[1] | 16S rRNA BLASTn top hit | % ID[2] |
|---|---|---|---|
| CUL37 | *Lactobacillus paracasei* | *Lactobacillus casei strain LC1* | 99 |
| CUL07 | *Lactobacillus paracasei* | *Lactobacillus casei strain NWAFU1574* | 99 |
| CUL08 | *Lactobacillus paracasei* | *Lactobacillus paracasei Sh144* | 99 |
| CUL06 | *Lactobacillus casei* | *Lactobacillus casei strain TM1* | 99 |
| CUL63 | *Lactobacillus rhamnosus* | *Lactobacillus rhamnosus AF3G* | 99 |
| CUL40 | *Lactobacillus fermentum* | *Lactobacillus fermentum DRZ87* | 98 |
| CUL67 | *Lactobacillus fermentum* | *Lactobacillus fermentum NS9* | 99 |
| CUL61 | *Lactobacillus salivarius* | *Lactobacillus salivarius DJ-sa-01* | 99 |
| CUL76 | *Lactobacillus helveticus* | *Lactobacillus helveticus 27170* | 99 |
| CUL09 | *Lactobacillus gasseri* | *Lactobacillus gasseri R12.4* | 97 |
| CUL60 | *Lactobacillus acidophilus* | *Lactobacillus acidophilus HM1* | 98 |
| CUL21 | *Lactobacillus acidophilus* | *Lactobacillus acidophilus zrx02* | 99 |
| CUL66 | *Lactobacillus plantarum* | *Lactobacillus plantarum Sh352* | 99 |
| CUL66N | *Lactobacillus plantarum* | *Lactobacillus plantarum HBUAS51002* | 96 |
| CUL20 | *Bifidobacterium bifidum* | *Bifidobacterium bifidum TMC 3115* | 96 |
| CUL34 | *Bifidobacterium animalis* subsp. *lactis* | *Bifidobacterium animalis BL3* | 96 |

[1] Classification previously provided.

[2] Percentage identify across the complete amplicon.


Lactobacillus paracasei CUL07 *
Lactobacillus paracasei CUL08 *
Lactobacillus paracasei subsp paracasei JCM8130
Lactobacillus paracasei CUL37
Lactobacillus casei subsp alactosus JCM1133
Lactobacillus paracasei subsp tolerans NBRC15906
Lactobacillus casei subsp pseudoplantarum JCM1181
Lactobacillus casei subsp tolerans NBRC15906
Lactobacillus casei CUL06 *
Lactobacillus casei ATCC393
Lactobacillus zeae ATCC15820
Lactobacillus rhamnosus CUL63 *
Lactobacillus casei subsp rhamnosus ATTC7469
Lactobacillus rhamnosus ATTC7469
13
4
3
4
87
3
3
15
38
31
64
25
Lactobacillus amylovorus DSM20531
Lactobacillus kitasatonis JCM1039
Lactobacillus helveticus CUL76
Lactobacillus suntoryeus SA
Lactobacillus gallinarum ATCC33199
Lactobacillus helveticus DSM20075
Lactobacillus kefiranofaciens subsp kefiranofaciens DSM5016
Lactobacillus kefiranofaciens subsp kefirgranum DSM10550
Lactobacillus ultunensis Kx146C1
Lactobacillus acidophilus ATCC 4356
Lactobacillus acidophilus CUL21
Lactobacillus acidophilus CUL60 *
Lactobacillus acidophilus BCRC10695
40
70
28
62
20
99
31
31
20
78
20
Lactobacillus spFS601
Lactobacillus pobuzihii NBRC103219
Lactobacillus ruminis NBRC102161
Lactobacillus salivarius CUL61 *
Lactobacillus salivarius ATCC11741
Lactobacillus hayakitensis KBL13
Lactobacillus agilis DSM20509
Lactobacillus aviarius DSM20655
Lactobacillus aviarius subsp araffinosus JCM5667
100
21
100
58
15
22
100


Lactobacillus antri CCUG48456T
Lactobacillus oris DSM4864T
Lactobacillus spTMW1666
Lactobacillus pontis LTH2587
Lactobacillus vaginalis ATCC49540
Lactobacillus caviae MOZM2
Lactobacillus reuteri JCM1112
Lactobacillus coleohominis DSM14060
Lactobacillus secaliphilus TMW11309T
Lactobacillus equigenerosi NRIC0697
Lactobacillus gastricus Kx156A7
Lactobacillus gorillae KZ01
Lactobacillus fermentum CUL40 *
Lactobacillus fermentum CUL67 *
Lactobacillus fermentum CIP102980
97
34
59
57
77
43
65
59
97
46
98
98
67
Lactobacillus arizonensis DSM13273
Lactobacillus plantarum subsp argentoratensis DK0
Lactobacillus plantarum CUL66 *
Lactobacillus plantarum CUL66N *
Lactobacillus pentosus JCM1558
Lactobacillus plajomi NB53
Lactobacillus modestisalitolerans NB446
Lactobacillus xiangfangensis 311
Lactobacillus fabifermentans LMG24284T
Lactobacillus paraplantarum DSM10667T
16
8
9
31
38
50
10
55

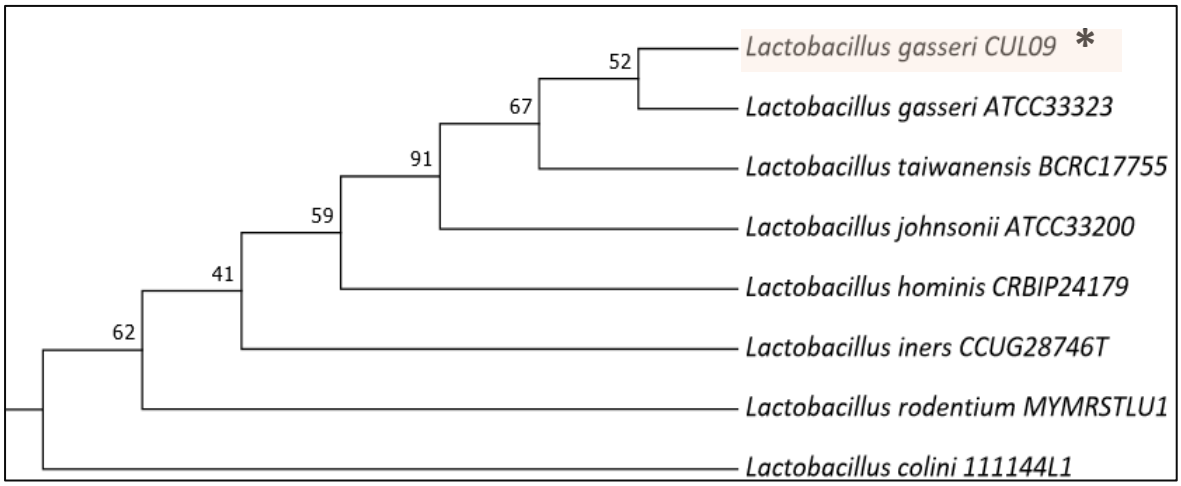



**Figure 2.2. 16S rRNA phylogenetic reconstruction of the *Lactobacillus* genus**.
The phylogeny was reconstructed using 228 *Lactobacillus* 16S rRNA type-strain sequences retrieved from Bacterio.net (last accessed Sept 2019). The phylogeny was calculated using the distance-based, Neighbor-Joining algorithm, including all codon positions. Multiple substitutions were corrected by using the Kimura 2-parameter method (K80 (Kimura, 1980)). Rate variation amongst sites was modelled with a gamma distribution (shape parameter = 0.52); as determined by the MrModelTest. All positions containing gaps and missing data were eliminated. The robustness of each node was assessed using bootstrap analysis (1000 pseudoreplicates). Numbers at nodes represent percentages. CUL strains are indicated with an asterisk.

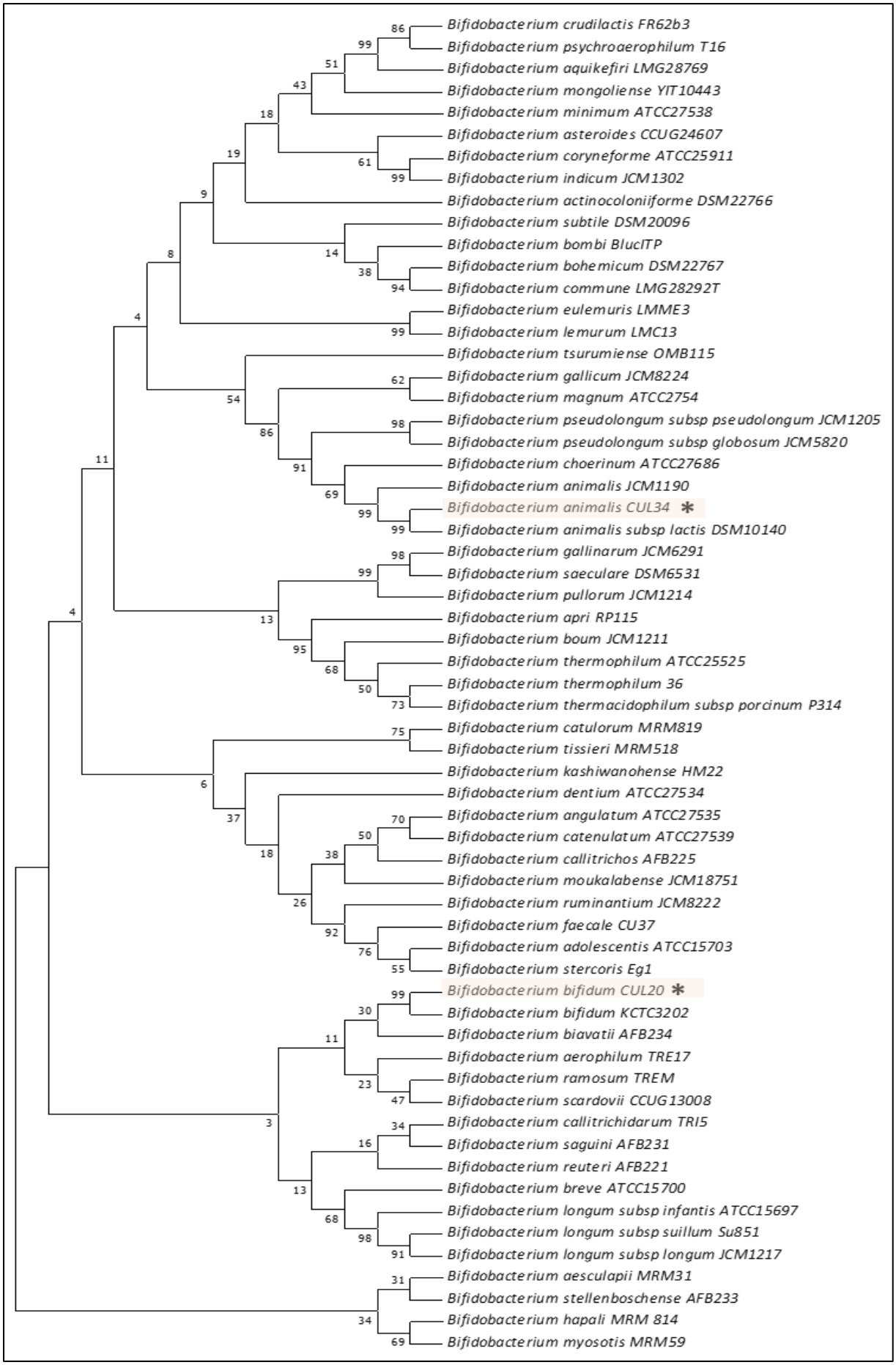

Bifidobacterium crudilactis FR62b3
Bifidobacterium psychroaerophilum T16
Bifidobacterium aquikefiri LMG28769
Bifidobacterium mongoliense YIT10443
Bifidobacterium minimum ATCC27538
Bifidobacterium asteroides CCUG24607
Bifidobacterium coryneforme ATCC25911
Bifidobacterium indicum JCM1302
Bifidobacterium actinocoloniiforme DSM22766
Bifidobacterium subtile DSM20096
Bifidobacterium bombi BlucITP
Bifidobacterium bohemicum DSM22767
Bifidobacterium commune LMG28292T
Bifidobacterium eulemuris LMME3
Bifidobacterium lemurum LMC13
Bifidobacterium tsurumiense OMB115
Bifidobacterium gallicum JCM8224
Bifidobacterium magnum ATCC2754
Bifidobacterium pseudolongum subsp pseudolongum JCM1205
Bifidobacterium pseudolongum subsp globosum JCM5820
Bifidobacterium choerinum ATCC27686
Bifidobacterium animalis JCM1190
Bifidobacterium animalis CUL34 *
Bifidobacterium animalis subsp lactis DSM10140
Bifidobacterium gallinarum JCM6291
Bifidobacterium saeculare DSM6531
Bifidobacterium pullorum JCM1214
Bifidobacterium apri RP115
Bifidobacterium boum JCM1211
Bifidobacterium thermophilum ATCC25525
Bifidobacterium thermophilum 36
Bifidobacterium thermacidophilum subsp porcinum P314
Bifidobacterium catulorum MRM819
Bifidobacterium tissieri MRM518
Bifidobacterium kashiwanohense HM22
Bifidobacterium dentium ATCC27534
Bifidobacterium angulatum ATCC27535
Bifidobacterium catenulatum ATCC27539
Bifidobacterium callitrichos AFB225
Bifidobacterium moukalabense JCM18751
Bifidobacterium ruminantium JCM8222
Bifidobacterium faecale CU37
Bifidobacterium adolescentis ATCC15703
Bifidobacterium stercoris Eg1
Bifidobacterium bifidum CUL20 *
Bifidobacterium bifidum KCTC3202
Bifidobacterium biavatii AFB234
Bifidobacterium aerophilum TRE17
Bifidobacterium ramosum TREM
Bifidobacterium scardovii CCUG13008
Bifidobacterium callitrichidarum TRI5
Bifidobacterium saguini AFB231
Bifidobacterium reuteri AFB221
Bifidobacterium breve ATCC15700
Bifidobacterium longum subsp infantis ATCC15697
Bifidobacterium longum subsp suillum Su851
Bifidobacterium longum subsp longum JCM1217
Bifidobacterium aesculapii MRM31
Bifidobacterium stellenboschense AFB233
Bifidobacterium hapali MRM 814
Bifidobacterium myosotis MRM59

**Figure 2.3. 16S rRNA phylogenetic reconstruction of the *Bifidobacterium genus***.
The phylogeny was reconstructed using 61 *Bifidobacterium* type-strain 16S rRNA sequences retrieved from Bacterio.net (last accessed Sept 2019). The phylogeny was calculated using the distance-based Neighbor-Joining algorithm, including all codon positions. Multiple substitutions were corrected, using the Tajima-Nei method (K80 (Tajima & Nei, 1984)). Rate variation among sites was modelled with a gamma distribution (shape parameter = 0.37); as determined by the MrModelTest. All positions containing gaps and missing data were eliminated. The robustness of each node was assessed using bootstrap analysis (1000 pseudoreplicates). Numbers at each node represent percentages. CUL strains are indicated with an asterisk.

### 2.3.3. Whole Genome Sequencing

Following assembly, CUL genomes were uploaded to RAST, and genome metrics were retrieved (**Table 2.3**). The number of contigs (greater than 1000 bp) ranged from 20 (*L.acidophilus* CUL21 and *B.animalis* subsp. *lactis* CUL34) to 142 (*L.casei* CUL06). Seven strains had contig counts between 1 – 49, *L.salivarius* CUL61, *L.acidophilus* CUL21, and CUL60, *L.plantarum* strains CUL66 and, CUL66N, *B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34. Five strains had between 50 – 100 contigs including *L.paracasei* strains CUL37, CUL07, CUL08, *L.rhamnosus* CUL63 and *L.gasseri* CUL09. Four strains had between 101 – 150 contigs: *L.fermentum* CUL40 and CUL67, *L.casei* CUL06 and *L.helveticus* CUL76. All sequenced genomes possessed a %GC content and genome size that were commensurate with their related species (when compared with the median reported values from GenBank; **Table 2.3**).

**Table 2.3. Genome metrics of CUL strains.**

| Genus | Species | Strain | Contig count >1000bp | Contig GC content (%) | Sequenced Genome Size (bp) | Median reported genome size (Mb) | Median reported % GC |
|---|---|---|---|---|---|---|---|
| *Lactobacillus* | *paracasei* | CUL37LB | 86 | 46.3 | 3,072,860 | | |
| | *paracasei* | CUL07 | 82 | 46.3 | 3,076,839 | 2.98 | 46.3 |
| | *paracasei* | CUL08 | 81 | 46.3 | 3,047,450 | | |
| | *casei* | CUL06 | 142 | 47.8 | 2,868,013 | 3 | 46.4 |
| | *rhamnosus* | CUL63 | 58 | 46.6 | 2,996,596 | 2.93 | 46.7 |
| | *fermentum* | CUL40LB | 133 | 51.1 | 2,114,335 | 1.99 | 51.9 |
| | *fermentum* | CUL67 | 105 | 51.6 | 2,065,028 | | |
| | *salivarius* | CUL61 | 31 | 32.6 | 2,012,069 | 2.03 | 32.8 |
| | *helveticus* | CUL76 | 132 | 36.6 | 2,076,906 | 2.05 | 36.8 |
| | *gasseri* | CUL09 | 65 | 34.9 | 1,965,205 | 1.96 | 34.9 |
| | *acidophilus* | CUL60 | 29 | 34.6 | 1,984,526 | 1.99 | 34.7 |
| | *acidophilus* | CUL21 | 20 | 34.6 | 1,966,526 | | |
| | *plantarum* | CUL66 | 23 | 44.5 | 3,216,058 | 3.28 | 44.4 |
| | *plantarum* | CUL66N | 22 | 44.5 | 3,248,614 | | |
| *Bifidobacterium* | *bifidum* | CUL20 | 36 | 62.6 | 2,199,325 | 2.21 | 62.7 |
| | *animalis* subsp. *lactis* | CUL34 | 20 | 60.4 | 1,931,696 | 1.93 | 60.5 |

### 2.3.4. Phylogenetic reconstruction of the Lactic Acid Bacteria: Determining a suitable outgroup root for the *Lactobacillus* MLSA

A previous analysis conducted by the PDF group (Colledge, R., 2019, unpublished) determined the phylogenetic relationship between the Lactic Acid Bacteria (LAB) group (**Figure 2.4**). Bacillales appear to encompass a high degree of heterogenicity at the species level, with the presence of three large subclades. The remaining LAB genera appear to diverge from the third Bacillales clade, with a step-like divergence of each species throughout the phylogeny. The genus *Lactobacillus* is highly heterogeneous in comparison to other members of LAB and contains a known paraphyly with *Pediococcus*. Moreover, *Enterococcus* and *Weissella* are close neighbours to *Lactobacillus,* and as such were excluded as candidate roots for the *Lactobacillus* MLSA. In comparison, *Lactococcus* occurs as a discrete clade, which appears to share a last common ancestor with lactobacilli, thus making an ideal outgroup root for use in further analysis.

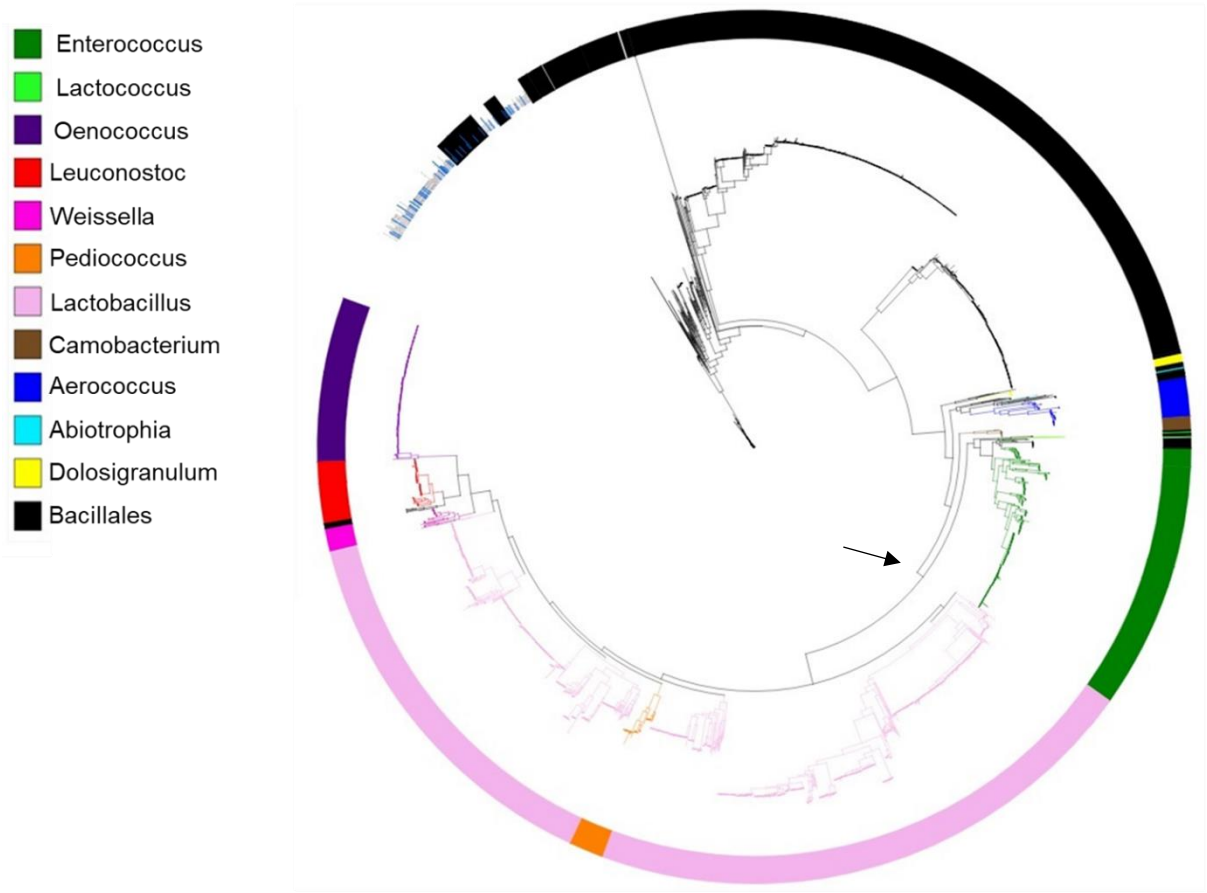



**Figure 2.4. MLSA phylogenetic reconstruction of the Lactic Acid Bacteria group.**

The amino acid sequences of all available LAB genome sequences were aligned in PhyloPhlAn using 400 ubiquitous proteins. The tree is unrooted.

The phylogeny incorporates the following species and subsequent number of strains, *Enterococcus* - 2,210, *Lactococcus* - 202, *Weissella* - 52, *Lactobacillus* - 1,876, *Dolosigranulum* - 12, *Leuconostoc* - 144, *Pediococcus* - 65, *Camobacterium* - 45, *Aerococcus* - 64, *Abiotrophia* - 2, *Oenococcus*- 227. *Streptococcus* (number of assemblies - > 12,000) was not included to aid in topological clarity. The phylogeny highlights the paraphyletic nature of *Pediococcus* and *Lactobacillus*. The last common ancestor of *Lactobacillus* and *Lactococcus* is indicated by an arrow.

### 2.3.5. Genus-wide MLSA phylogenetic reconstructions of *Lactobacillus* and *Bifidobacterium*, including an appraisal of species clades

The topologies of both multi-locus ML phylogenies (***Lactobacillus* Figures 2.5 and *Bifidobacterium*, Figure 2.7**), further support the standing nomenclature of the CUL strains. The topology seen in the *Lactobacillus* phylogeny is consistent with previous taxonomic assessments of the genus (Salvetti et al., 2018) and contains well-defined homogenous clades. Bootstrap values for all major divisions were always greater than 90 %, however, the annotations on the phylogeny were omitted for visual clarity, due to the size of the phylogeny, but can be viewed via the supplementary .nwk file. It is worth noting that the bootstrap values should be and therefore treated with caution as recommended for large phylogenies with multiple concatenated markers (Lemoine et al., 2018). As evidenced by the phylogenies, all CUL probiotic isolates represent novel entries into GenBank, that being, all isolates occupied their branch in the phylogenies. Of note is that the *Pediococcus* clade was positioned as a subclade within the genus *Lactobacillus,* a known paraphyly (Makarova & Koonin, 2007). Of particular interest, is the clear dichotomous split separating the phylogroups into two large clades. Group A consists of, *L.coryniformis, L.sakeii, L.casei, L.alimentarius* and *L.delbrueckii*, and Group B, incorporates, *L.salivarius, Pediococcus, L.plantarum, L.reuteri, L.collinoides, L.brevis, L.buchneri, L.fructivorans* and *L.kunkeei.*

CUL strains were spread well between these two groups with *L.paracasei* CUL37, CUL07 and CUL08, *L.casei* CUL06, *L.rhamnosus* CUL63, *L.helveticus* CUL76, *L.gasseri* CUL09 and *L.acidophilus* CUL21 and CUL60 grouping with the appropriate clades within group A, and *L.salivarius* CUL61*, L.plantarum* CUL66 and CUL66N, and *L.fermentum* CUL40 and CUL67 being spread well throughout group B, indicating a highly diverse consortium of bacteria.

After an extensive analysis of the species' evolutionary positioning, in accordance with **Figure 2.5**, phylogroups were designated according to the species' evolutionary lineages **(Supp. Table S2.3 and Table S2.6**). As such, this study recognises 17 distinct phylogroups **(Supp. Table S2.3**). The number of strains encompassed within the phylogroups ranges from 2 (*L.composti* and *L.floricola*) to 526 (*L.delbrueckii* (**Supp. Table S2.6**)). *Lactobacillus delbrueckii* and *Lactobacillus plantarum* are the most dominant phylogroups with 526 and 496 strains within their clades respectively (**Figure 2.5, Table 2.4**). Throughout the tree, several misidentified, or undesignated species were present **(Supp. Table S2.4 – S2.5**). Interestingly, the majority of misidentified or unclassified species occurred either within the *L.casei* phylogroup (28 strains) or *L.delbrueckii* (51 strains (**Table 2.4**)).

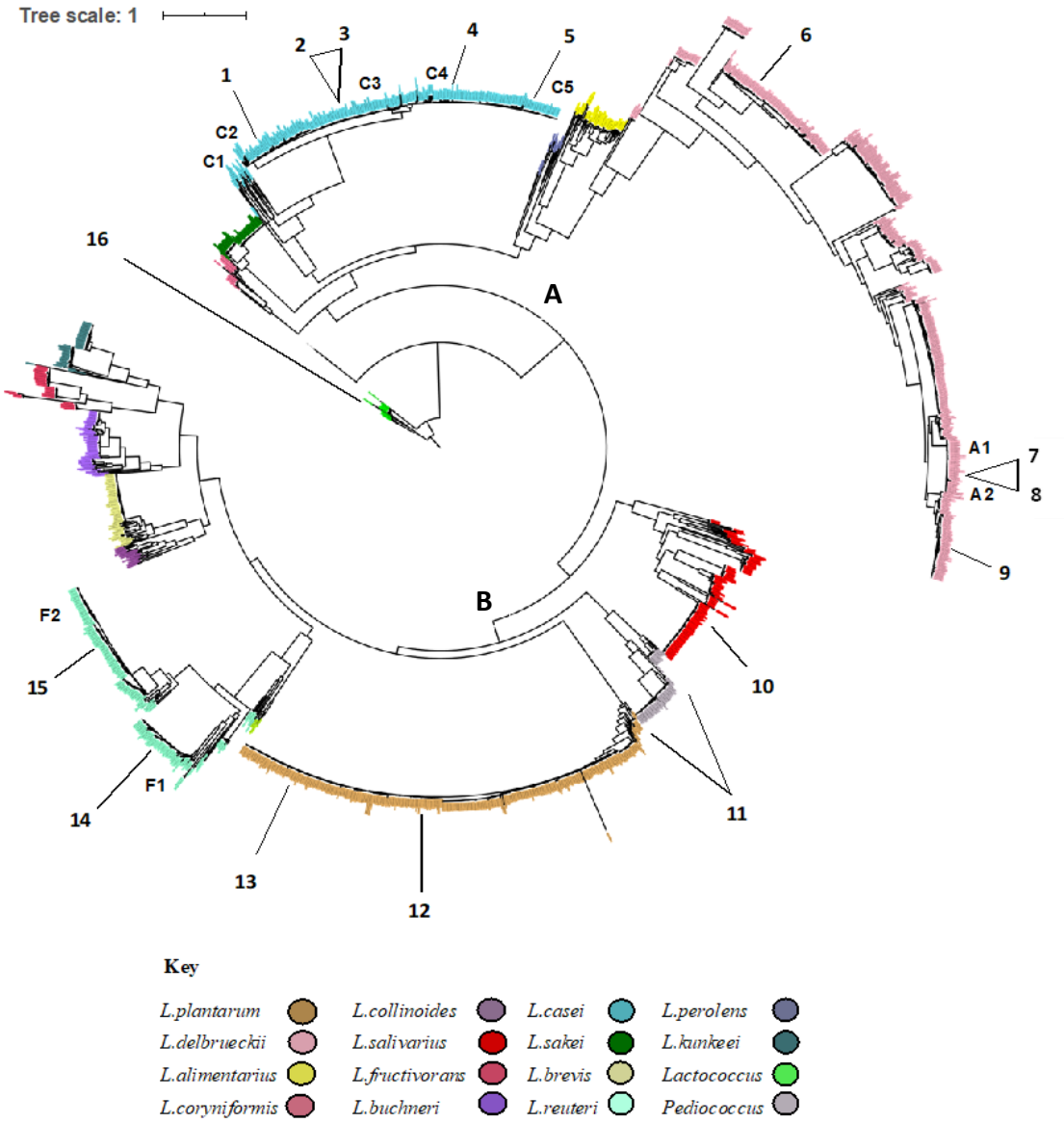
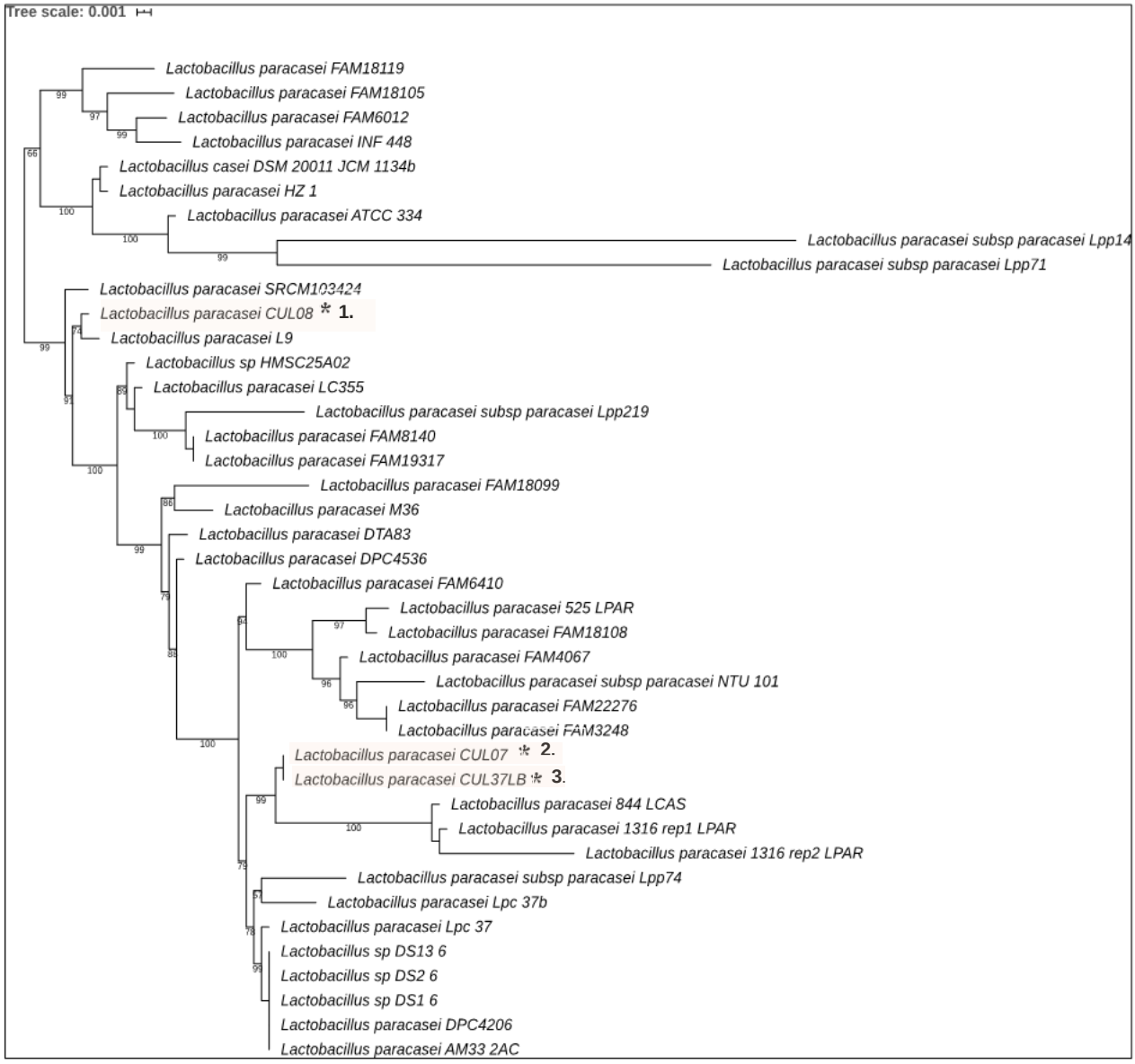
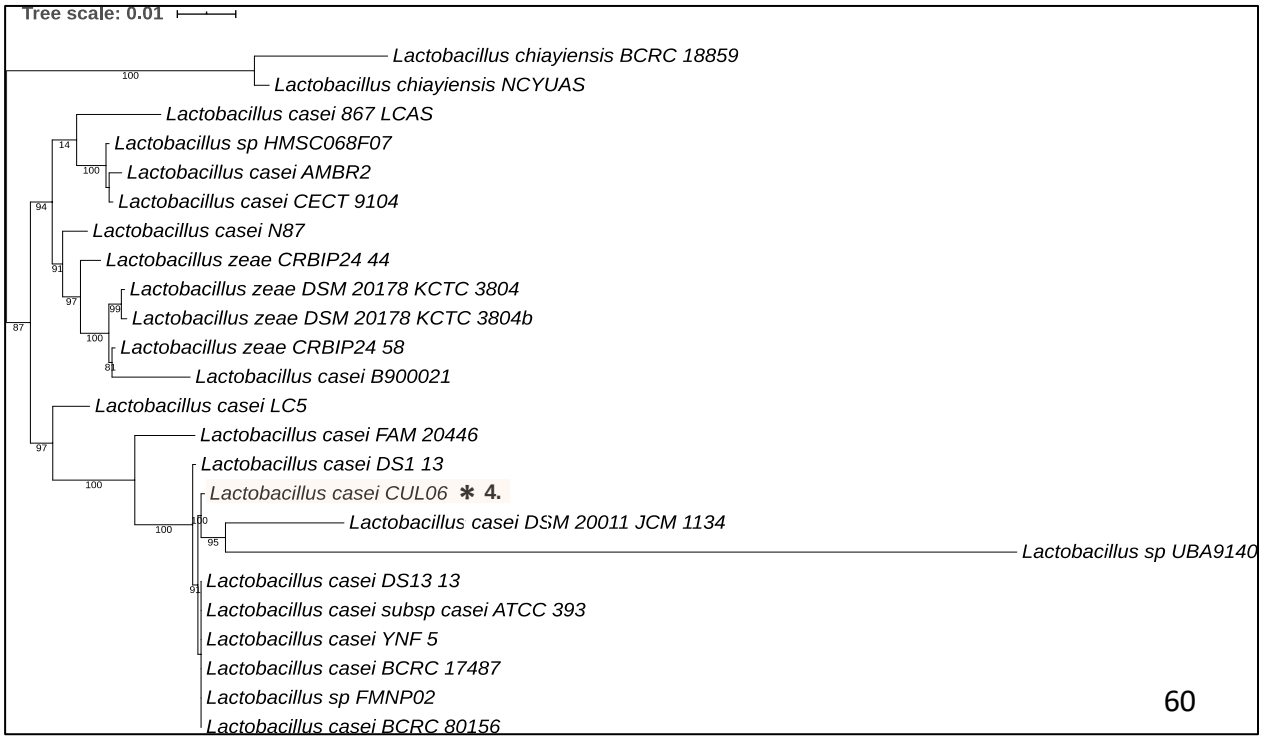
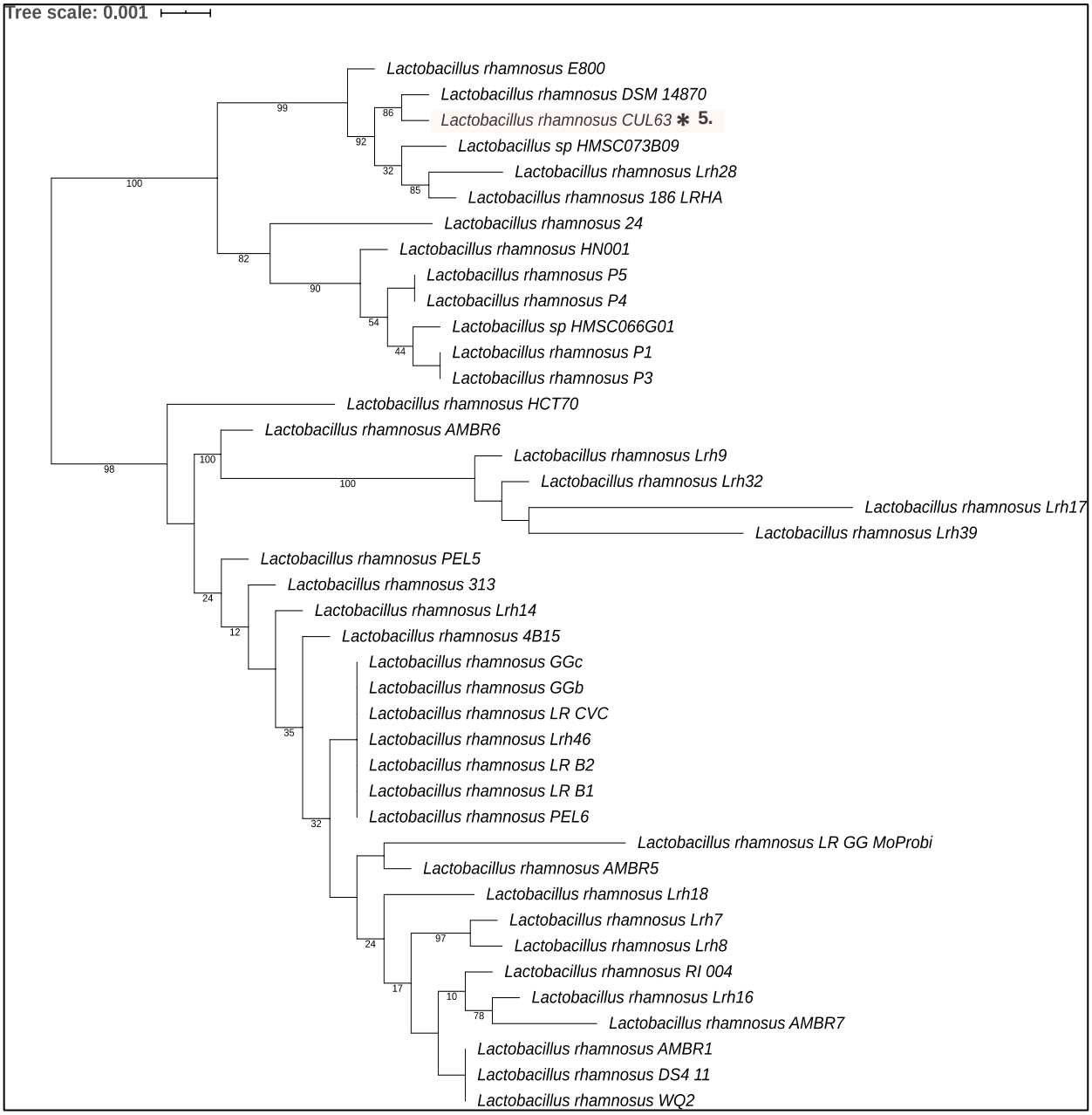
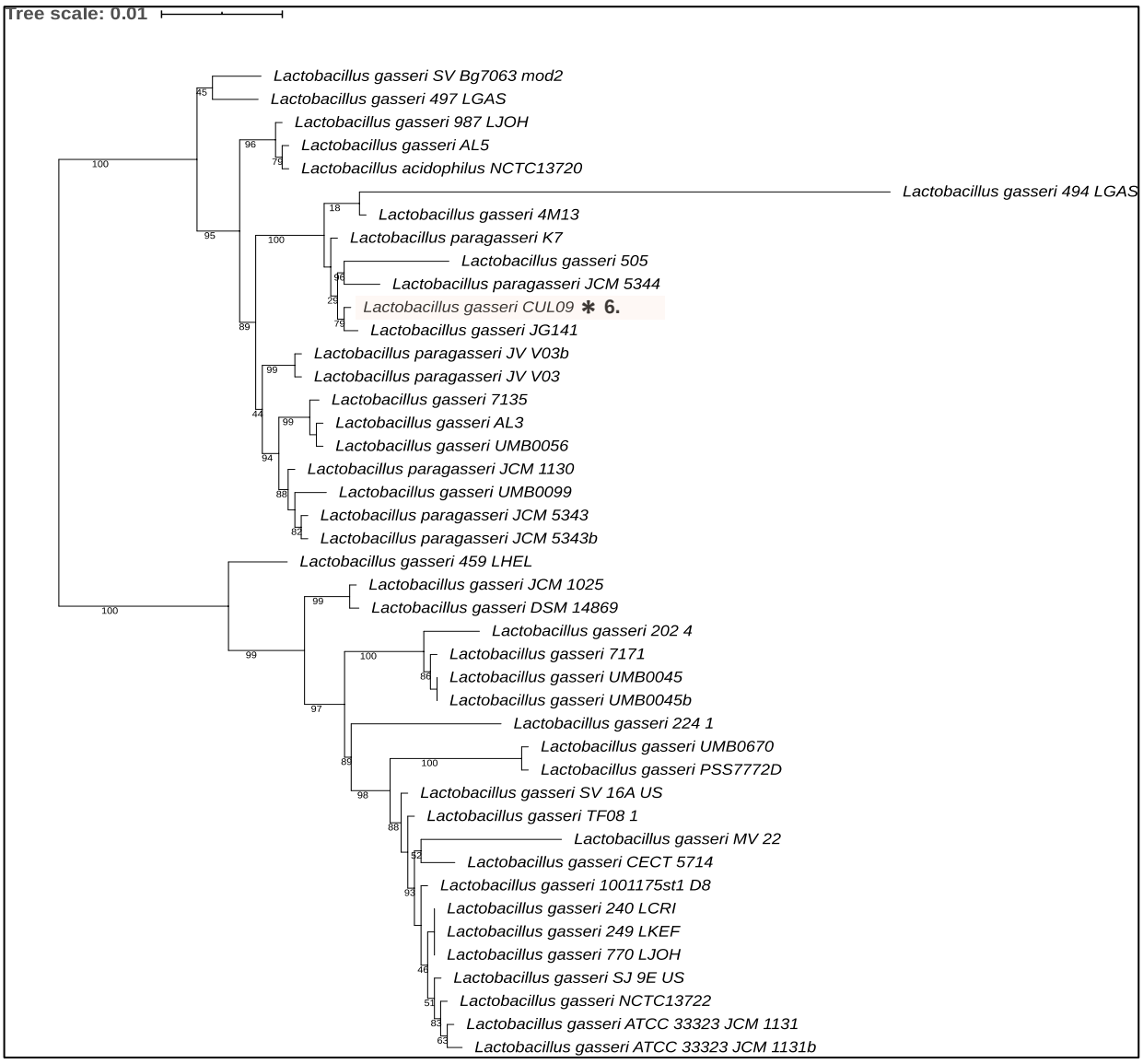


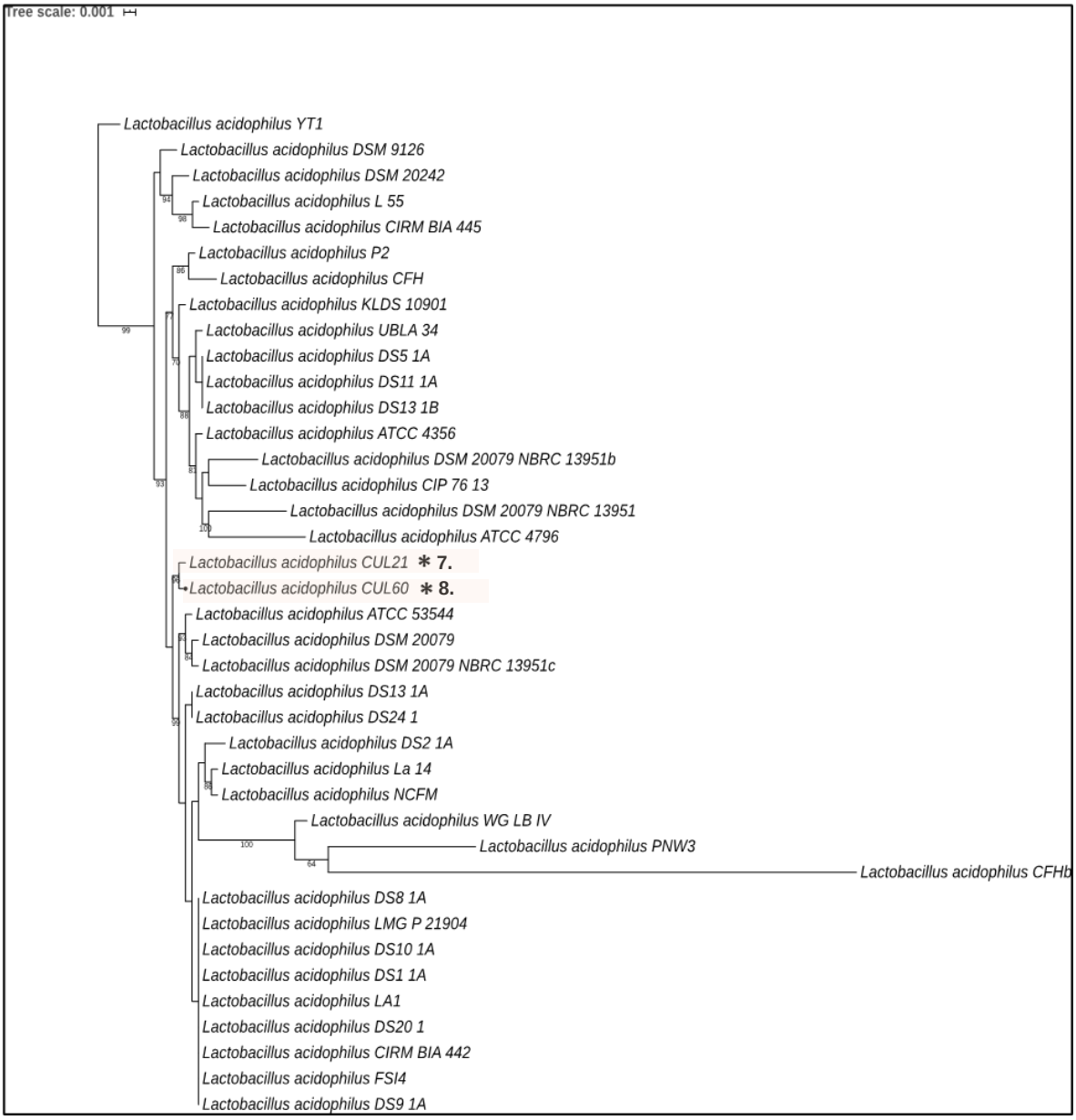

**Figure 2.5. Multi-Locus phylogenetic reconstruction of the *Lactobacillus* genus**. Rooted Maximum likelihood multi-locus phylogeny of *Lactobacillus.* The circular phylogeny was created using a concatenation of 400 core proteins throughout all available *Lactobacillus* genomes retrieved from NCBI (2194 genomes, last accessed in September 2020). *Lactobacillus* phylogeny is rooted along the lineage leading to *Lactococcus* (including 16 genomes) and includes the paraphyletic clade *Pediococcus* (11 strains). The phylogeny was created in PhyloPhlAn and rendered in iTOL. Position of CUL isolates are indicated with sequential numbers: *L.paracasei* CUL08 (1), *L.paracasei* CUL07 (2), *L.paracasei* CUL37 (3), *L.casei* CUL06 (4), *L.rhamnosus* CUL63 (5), *L.gasseri* CUL09 (6), *L.acidophilus* CUL21 (7), *L.acidophilus* CUL60 (8), *L.helveticus* CUL76 (9), *L.salivarius* CUL61 (10), *L.plantarum* CUL66 (12), *L.plantarum* CUL66N (13), *L.fermentum* CUL67 (14) and *L.fermentum* CUL40 (15). Large phylogenetic groups are indicated by the letters A and B. Subclades of interest are labelled as follows: *L.casei* (C1 – C5), *L.acidophilus* (A1 and A2) and *L.fermentum* (F1 and F2). The scale bar represents branch length. Clade colours represent species phylogroups.

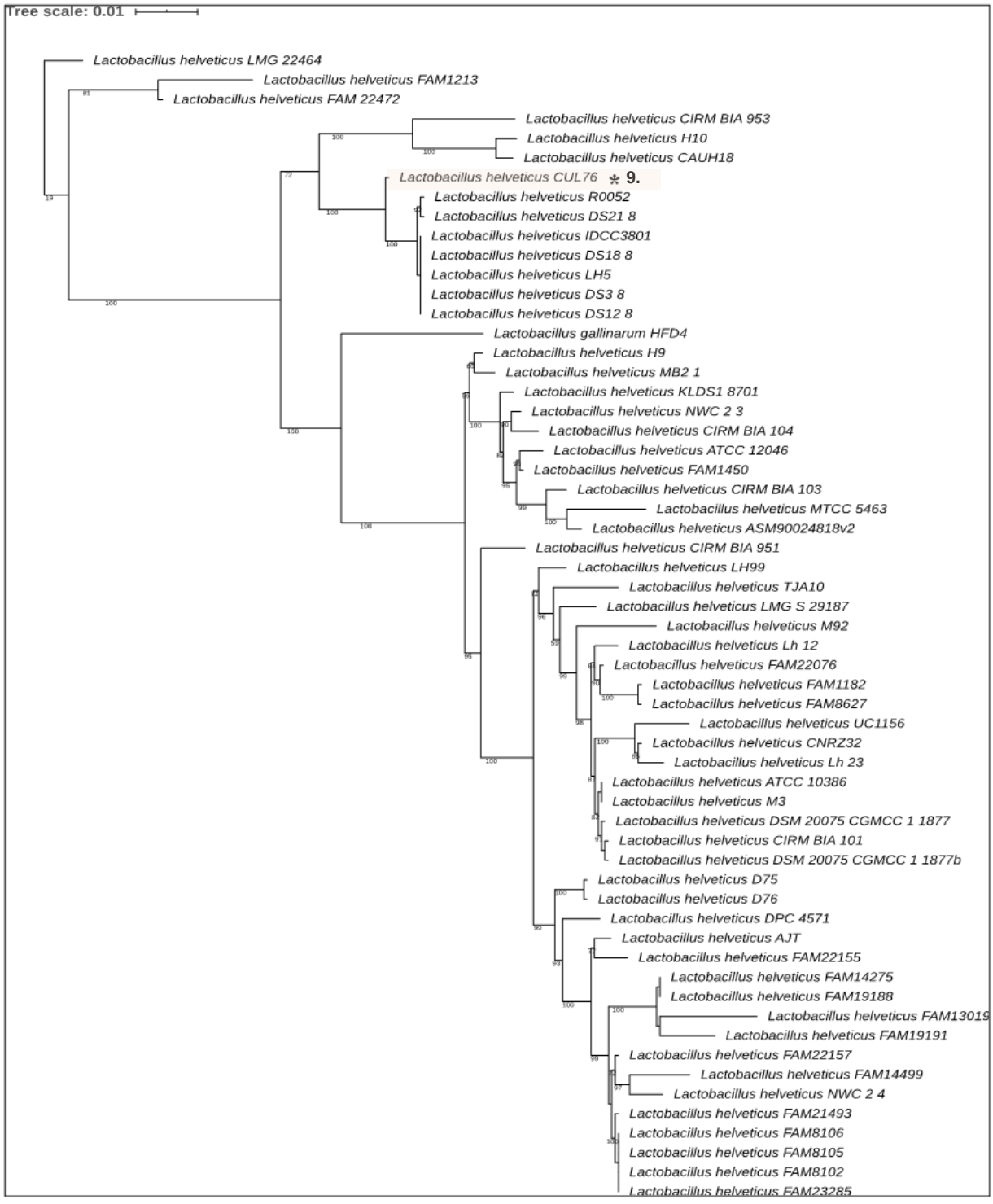

Tree scale: 0.001
Lactobacillus paracasei FAM18119
Lactobacillus paracasei FAM18105
Lactobacillus paracasei FAM6012
Lactobacillus paracasei INF 448
Lactobacillus casei DSM 20011 JCM 1134b
Lactobacillus paracasei HZ 1
Lactobacillus paracasei ATCC 334
Lactobacillus paracasei subsp paracasei Lpp14
Lactobacillus paracasei subsp paracasei Lpp71
Lactobacillus paracasei SRCM103424
Lactobacillus paracasei CUL08 * 1.
Lactobacillus paracasei L9
Lactobacillus sp HMSC25A02
Lactobacillus paracasei LC355
Lactobacillus paracasei subsp paracasei Lpp219
Lactobacillus paracasei FAM8140
Lactobacillus paracasei FAM19317
Lactobacillus paracasei FAM18099
Lactobacillus paracasei M36
Lactobacillus paracasei DTA83
Lactobacillus paracasei DPC4536
Lactobacillus paracasei FAM6410
Lactobacillus paracasei 525 LPAR
Lactobacillus paracasei FAM18108
Lactobacillus paracasei FAM4067
Lactobacillus paracasei subsp paracasei NTU 101
Lactobacillus paracasei FAM22276
Lactobacillus paracasei FAM3248
Lactobacillus paracasei CUL07 * 2.
Lactobacillus paracasei CUL37LB * 3.
Lactobacillus paracasei 844 LCAS
Lactobacillus paracasei 1316 rep1 LPAR
Lactobacillus paracasei 1316 rep2 LPAR
Lactobacillus paracasei subsp paracasei Lpp74
Lactobacillus paracasei Lpc 37b
Lactobacillus paracasei Lpc 37
Lactobacillus sp DS13 6
Lactobacillus sp DS2 6
Lactobacillus sp DS1 6
Lactobacillus paracasei DPC4206
Lactobacillus paracasei AM33 2AC

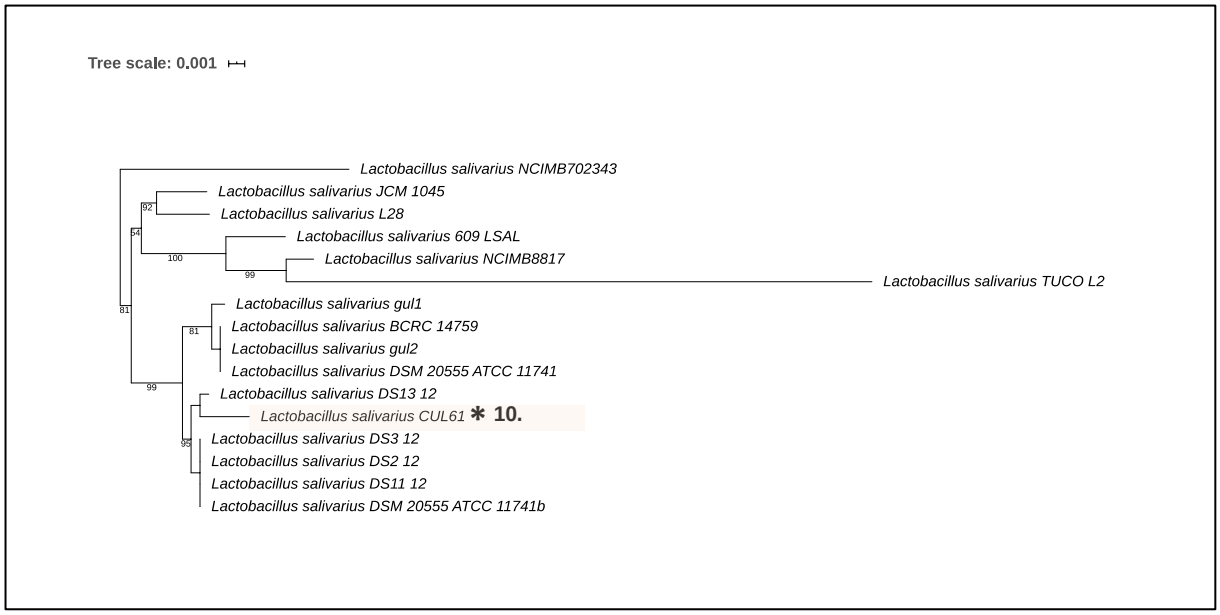

Tree scale: 0.01
Lactobacillus chiayiensis BCRC 18859
Lactobacillus chiayiensis NCYUAS
Lactobacillus casei 867 LCAS
Lactobacillus sp HMSC068F07
Lactobacillus casei AMBR2
Lactobacillus casei CECT 9104
Lactobacillus casei N87
Lactobacillus zeae CRBIP24 44
Lactobacillus zeae DSM 20178 KCTC 3804
Lactobacillus zeae DSM 20178 KCTC 3804b
Lactobacillus zeae CRBIP24 58
Lactobacillus casei B900021
Lactobacillus casei LC5
Lactobacillus casei FAM 20446
Lactobacillus casei DS1 13
Lactobacillus casei CUL06 * 4.
Lactobacillus casei DSM 20011 JCM 1134
Lactobacillus sp UBA9140
Lactobacillus casei DS13 13
Lactobacillus casei subsp casei ATCC 393
Lactobacillus casei YNF 5
Lactobacillus casei BCRC 17487
Lactobacillus sp FMNP02
Lactobacillus casei BCRC 80156

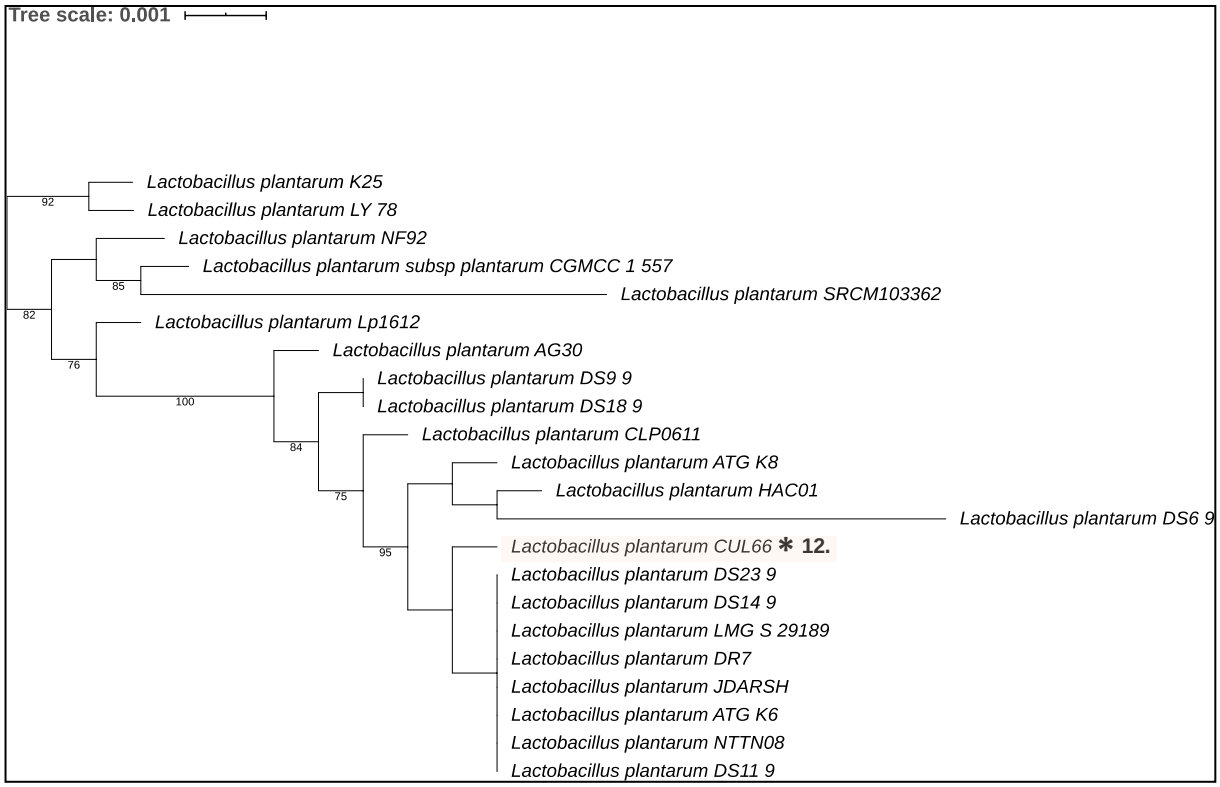

Tree scale: 0.001
Lactobacillus rhamnosus E800
Lactobacillus rhamnosus DSM 14870
Lactobacillus rhamnosus CUL63 * 5.
Lactobacillus sp HMSC073B09
Lactobacillus rhamnosus Lrh28
Lactobacillus rhamnosus 186 LRHA
Lactobacillus rhamnosus 24
Lactobacillus rhamnosus HN001
Lactobacillus rhamnosus P5
Lactobacillus rhamnosus P4
Lactobacillus sp HMSC066G01
Lactobacillus rhamnosus P1
Lactobacillus rhamnosus P3
Lactobacillus rhamnosus HCT70
Lactobacillus rhamnosus AMBR6
Lactobacillus rhamnosus Lrh9
Lactobacillus rhamnosus Lrh32
Lactobacillus rhamnosus Lrh17
Lactobacillus rhamnosus Lrh39
Lactobacillus rhamnosus PEL5
Lactobacillus rhamnosus 313
Lactobacillus rhamnosus Lrh14
Lactobacillus rhamnosus 4B15
Lactobacillus rhamnosus GGc
Lactobacillus rhamnosus GGb
Lactobacillus rhamnosus LR CVC
Lactobacillus rhamnosus Lrh46
Lactobacillus rhamnosus LR B2
Lactobacillus rhamnosus LR B1
Lactobacillus rhamnosus PEL6
Lactobacillus rhamnosus LR GG MoProbi
Lactobacillus rhamnosus AMBR5
Lactobacillus rhamnosus Lrh18
Lactobacillus rhamnosus Lrh7
Lactobacillus rhamnosus Lrh8
Lactobacillus rhamnosus RI 004
Lactobacillus rhamnosus Lrh16
Lactobacillus rhamnosus AMBR7
Lactobacillus rhamnosus AMBR1
Lactobacillus rhamnosus DS4 11
Lactobacillus rhamnosus WQ2

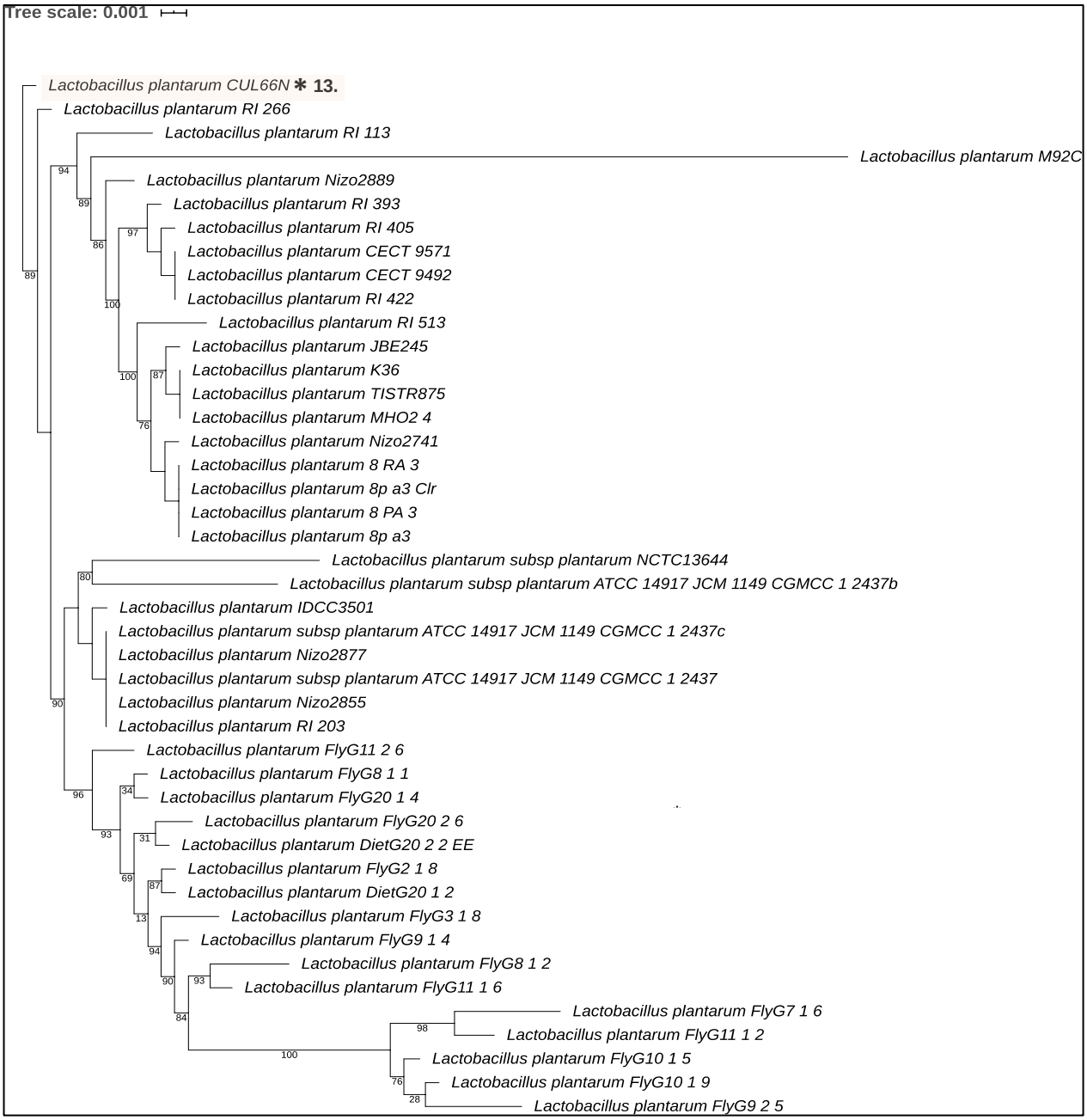

Tree scale: 0.01
Lactobacillus gasseri SV Bg7063 mod2
Lactobacillus gasseri 497 LGAS
Lactobacillus gasseri 987 LJOH
Lactobacillus gasseri AL5
Lactobacillus acidophilus NCTC13720
Lactobacillus gasseri 494 LGAS
Lactobacillus gasseri 4M13
Lactobacillus paragasseri K7
Lactobacillus gasseri 505
Lactobacillus paragasseri JCM 5344
Lactobacillus gasseri CUL09 ✱ 6.
Lactobacillus gasseri JG141
Lactobacillus paragasseri JV V03b
Lactobacillus paragasseri JV V03
Lactobacillus gasseri 7135
Lactobacillus gasseri AL3
Lactobacillus gasseri UMB0056
Lactobacillus paragasseri JCM 1130
Lactobacillus gasseri UMB0099
Lactobacillus paragasseri JCM 5343
Lactobacillus paragasseri JCM 5343b
Lactobacillus gasseri 459 LHEL
Lactobacillus gasseri JCM 1025
Lactobacillus gasseri DSM 14869
Lactobacillus gasseri 202 4
Lactobacillus gasseri 7171
Lactobacillus gasseri UMB0045
Lactobacillus gasseri UMB0045b
Lactobacillus gasseri 224 1
Lactobacillus gasseri UMB0670
Lactobacillus gasseri PSS7772D
Lactobacillus gasseri SV 16A US
Lactobacillus gasseri TF08 1
Lactobacillus gasseri MV 22
Lactobacillus gasseri CECT 5714
Lactobacillus gasseri 1001175st1 D8
Lactobacillus gasseri 240 LCRI
Lactobacillus gasseri 249 LKEF
Lactobacillus gasseri 770 LJOH
Lactobacillus gasseri SJ 9E US
Lactobacillus gasseri NCTC13722
Lactobacillus gasseri ATCC 33323 JCM 1131
Lactobacillus gasseri ATCC 33323 JCM 1131b


Tree scale: 0.001
Lactobacillus acidophilus YT1
Lactobacillus acidophilus DSM 9126
Lactobacillus acidophilus DSM 20242
Lactobacillus acidophilus L 55
Lactobacillus acidophilus CIRM BIA 445
Lactobacillus acidophilus P2
Lactobacillus acidophilus CFH
Lactobacillus acidophilus KLDS 10901
Lactobacillus acidophilus UBLA 34
Lactobacillus acidophilus DS5 1A
Lactobacillus acidophilus DS11 1A
Lactobacillus acidophilus DS13 1B
Lactobacillus acidophilus ATCC 4356
Lactobacillus acidophilus DSM 20079 NBRC 13951b
Lactobacillus acidophilus CIP 76 13
Lactobacillus acidophilus DSM 20079 NBRC 13951
Lactobacillus acidophilus ATCC 4796
Lactobacillus acidophilus CUL21 * 7.
Lactobacillus acidophilus CUL60 * 8.
Lactobacillus acidophilus ATCC 53544
Lactobacillus acidophilus DSM 20079
Lactobacillus acidophilus DSM 20079 NBRC 13951c
Lactobacillus acidophilus DS13 1A
Lactobacillus acidophilus DS24 1
Lactobacillus acidophilus DS2 1A
Lactobacillus acidophilus La 14
Lactobacillus acidophilus NCFM
Lactobacillus acidophilus WG LB IV
Lactobacillus acidophilus PNW3
Lactobacillus acidophilus CFHb
Lactobacillus acidophilus DS8 1A
Lactobacillus acidophilus LMG P 21904
Lactobacillus acidophilus DS10 1A
Lactobacillus acidophilus DS1 1A
Lactobacillus acidophilus LA1
Lactobacillus acidophilus DS20 1
Lactobacillus acidophilus CIRM BIA 442
Lactobacillus acidophilus FSI4
Lactobacillus acidophilus DS9 1A


Tree scale: 0.01
Lactobacillus helveticus LMG 22464
Lactobacillus helveticus FAM1213
Lactobacillus helveticus FAM 22472
Lactobacillus helveticus CIRM BIA 953
Lactobacillus helveticus H10
Lactobacillus helveticus CAUH18
Lactobacillus helveticus CUL76
Lactobacillus helveticus R0052
Lactobacillus helveticus DS21 8
Lactobacillus helveticus IDCC3801
Lactobacillus helveticus DS18 8
Lactobacillus helveticus LH5
Lactobacillus helveticus DS3 8
Lactobacillus helveticus DS12 8
Lactobacillus gallinarum HFD4
Lactobacillus helveticus H9
Lactobacillus helveticus MB2 1
Lactobacillus helveticus KLDS1 8701
Lactobacillus helveticus NWC 2 3
Lactobacillus helveticus CIRM BIA 104
Lactobacillus helveticus ATCC 12046
Lactobacillus helveticus FAM1450
Lactobacillus helveticus CIRM BIA 103
Lactobacillus helveticus MTCC 5463
Lactobacillus helveticus ASM90024818v2
Lactobacillus helveticus CIRM BIA 951
Lactobacillus helveticus LH99
Lactobacillus helveticus TJA10
Lactobacillus helveticus LMG S 29187
Lactobacillus helveticus M92
Lactobacillus helveticus Lh 12
Lactobacillus helveticus FAM22076
Lactobacillus helveticus FAM1182
Lactobacillus helveticus FAM8627
Lactobacillus helveticus UC1156
Lactobacillus helveticus CNRZ32
Lactobacillus helveticus Lh 23
Lactobacillus helveticus ATCC 10386
Lactobacillus helveticus M3
Lactobacillus helveticus DSM 20075 CGMCC 1 1877
Lactobacillus helveticus CIRM BIA 101
Lactobacillus helveticus DSM 20075 CGMCC 1 1877b
Lactobacillus helveticus D75
Lactobacillus helveticus D76
Lactobacillus helveticus DPC 4571
Lactobacillus helveticus AJT
Lactobacillus helveticus FAM22155
Lactobacillus helveticus FAM14275
Lactobacillus helveticus FAM19188
Lactobacillus helveticus FAM13019
Lactobacillus helveticus FAM19191
Lactobacillus helveticus FAM22157
Lactobacillus helveticus FAM14499
Lactobacillus helveticus NWC 2 4
Lactobacillus helveticus FAM21493
Lactobacillus helveticus FAM8106
Lactobacillus helveticus FAM8105
Lactobacillus helveticus FAM8102
Lactobacillus helveticus FAM23285


Tree scale: 0.001
Lactobacillus salivarius NCIMB702343
Lactobacillus salivarius JCM 1045
Lactobacillus salivarius L28
Lactobacillus salivarius 609 LSAL
Lactobacillus salivarius NCIMB8817
Lactobacillus salivarius TUCO L2
Lactobacillus salivarius gul1
Lactobacillus salivarius BCRC 14759
Lactobacillus salivarius gul2
Lactobacillus salivarius DSM 20555 ATCC 11741
Lactobacillus salivarius DS13 12
Lactobacillus salivarius CUL61 * 10.
Lactobacillus salivarius DS3 12
Lactobacillus salivarius DS2 12
Lactobacillus salivarius DS11 12
Lactobacillus salivarius DSM 20555 ATCC 11741b


Tree scale: 0.001
Lactobacillus plantarum K25
Lactobacillus plantarum LY 78
Lactobacillus plantarum NF92
Lactobacillus plantarum subsp plantarum CGMCC 1 557
Lactobacillus plantarum SRCM103362
Lactobacillus plantarum Lp1612
Lactobacillus plantarum AG30
Lactobacillus plantarum DS9 9
Lactobacillus plantarum DS18 9
Lactobacillus plantarum CLP0611
Lactobacillus plantarum ATG K8
Lactobacillus plantarum HAC01
Lactobacillus plantarum DS6 9
Lactobacillus plantarum CUL66 * 12.
Lactobacillus plantarum DS23 9
Lactobacillus plantarum DS14 9
Lactobacillus plantarum LMG S 29189
Lactobacillus plantarum DR7
Lactobacillus plantarum JDARSH
Lactobacillus plantarum ATG K6
Lactobacillus plantarum NTTN08
Lactobacillus plantarum DS11 9


Tree scale: 0.001
Lactobacillus plantarum CUL66N * 13.
Lactobacillus plantarum RI 266
Lactobacillus plantarum RI 113
Lactobacillus plantarum M92C
Lactobacillus plantarum Nizo2889
Lactobacillus plantarum RI 393
Lactobacillus plantarum RI 405
Lactobacillus plantarum CECT 9571
Lactobacillus plantarum CECT 9492
Lactobacillus plantarum RI 422
Lactobacillus plantarum RI 513
Lactobacillus plantarum JBE245
Lactobacillus plantarum K36
Lactobacillus plantarum TISTR875
Lactobacillus plantarum MHO2 4
Lactobacillus plantarum Nizo2741
Lactobacillus plantarum 8 RA 3
Lactobacillus plantarum 8p a3 Clr
Lactobacillus plantarum 8 PA 3
Lactobacillus plantarum 8p a3
Lactobacillus plantarum subsp plantarum NCTC13644
Lactobacillus plantarum subsp plantarum ATCC 14917 JCM 1149 CGMCC 1 2437b
Lactobacillus plantarum IDCC3501
Lactobacillus plantarum subsp plantarum ATCC 14917 JCM 1149 CGMCC 1 2437c
Lactobacillus plantarum Nizo2877
Lactobacillus plantarum subsp plantarum ATCC 14917 JCM 1149 CGMCC 1 2437
Lactobacillus plantarum Nizo2855
Lactobacillus plantarum RI 203
Lactobacillus plantarum FlyG11 2 6
Lactobacillus plantarum FlyG8 1 1
Lactobacillus plantarum FlyG20 1 4
Lactobacillus plantarum FlyG20 2 6
Lactobacillus plantarum DietG20 2 2 EE
Lactobacillus plantarum FlyG2 1 8
Lactobacillus plantarum DietG20 1 2
Lactobacillus plantarum FlyG3 1 8
Lactobacillus plantarum FlyG9 1 4
Lactobacillus plantarum FlyG8 1 2
Lactobacillus plantarum FlyG11 1 6
Lactobacillus plantarum FlyG7 1 6
Lactobacillus plantarum FlyG11 1 2
Lactobacillus plantarum FlyG10 1 5
Lactobacillus plantarum FlyG10 1 9
Lactobacillus plantarum FlyG9 2 5

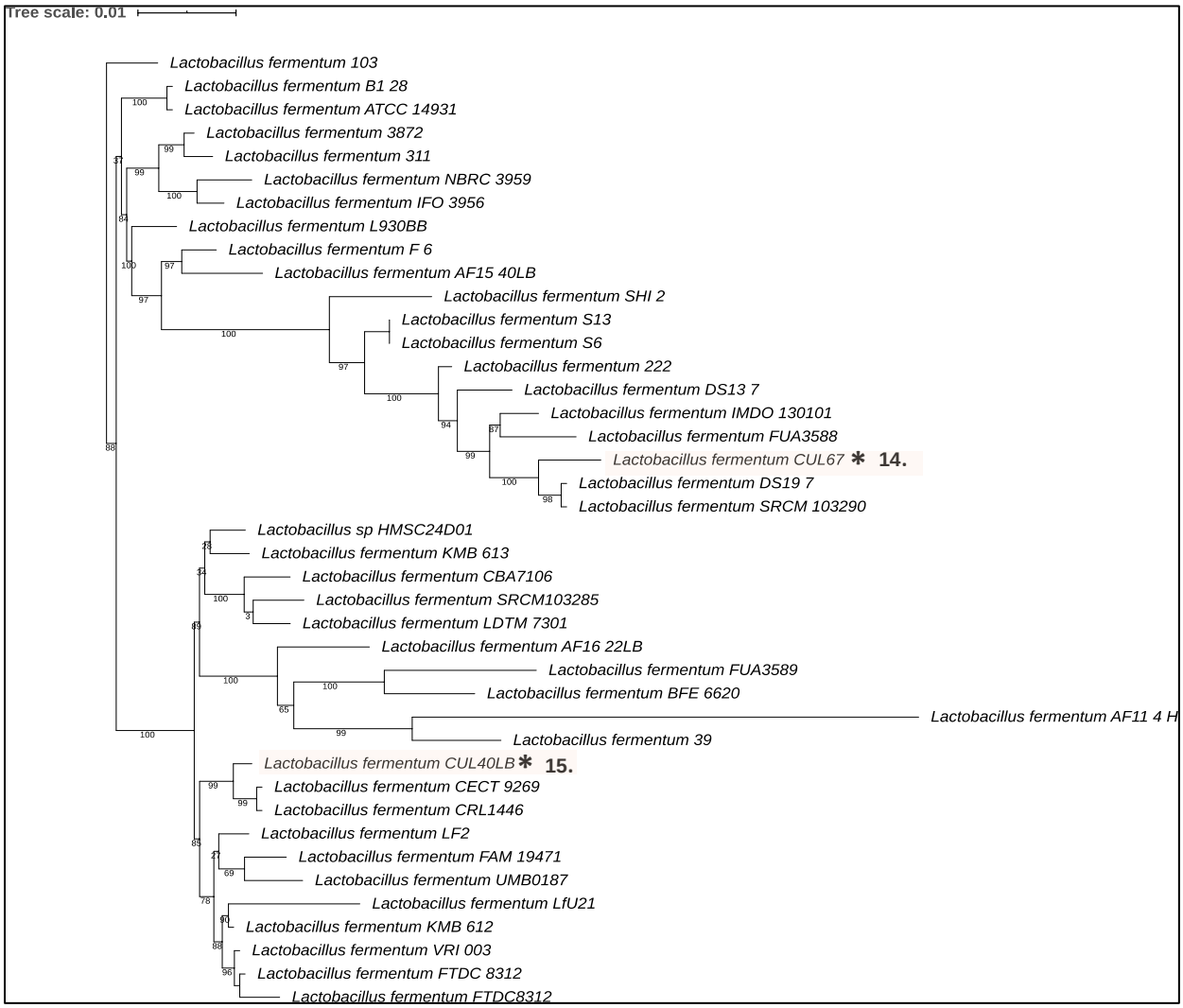



**Figure 2.6. Subclade images of the MLSA *Lactobacillus* phylogeny, highlighting the taxonomic position of CUL isolates.** Rooted maximum likelihood multi-locus phylogenies of *Lactobacillus.* Circular phylogenies were created using a concatenation of 400 core proteins throughout all available *Lactobacillus* genomes retrieved from GenBank (Sept 2020). PhyloPhlAn generated .nwk files were rendered in ITOL. The robustness of each node was assessed using bootstrap analysis (1000 pseudoreplicates). Numbers at nodes represent percentages. CUL strains are indicated by an asterisk. 1. *L.paracasei* CUL08, 2. *L.paracasei* CUL07, 3. *L.paracasei* CUL37, 4. *L.casei* CUL06 5. *L.rhamnosus* CUL63, 6. *L.gasseri* CUL09, 7. *L.acidophilus* CUL21, 8. *L.acidophilus* CUL60, 9. *L.helveticus* CUL76, 10. *L.salivarius* CUL61,12. *L.plantarum* CUL66, 13. *L.plantarum* CUL66N, 14. *L.fermentum* CUL67 and 15. *L.fermentum* CUL40. Tree scale bars represent branch length.

### 2.3.6. *Lactobacillus* taxonomy

*Lactobacillus* is an extremely diverse genus encompassing over 200 species. Indeed, several studies have attempted to phylogenetically classify the group, using a variety of taxonomic methodologies (Salvetti et al., 2018; Sun et al., 2015a; Zheng et al., 2020b). However, the most recent analysis, which resulted in several *Lactobacillus* species being assigned to entirely new genera, conducted such evaluations using 101 identified protein-encoding genes in a subset of genomes (297 (Zheng et al., 2020b)). Here we report a variation on such taxonomic analysis of the *Lactobacillus* (**A2, Table S2.3 – S2.6**) and *Bifidobacterium* (**A2, Table S2.7 – S2.11**) genera using 400 ubiquitous protein markers, including all draft and complete genomes available on NCBI at the time of analysis. As a result, a phylogeny was reconstructed in ITOL, allowing a strain-level taxonomic analysis (**Figure 2.5**). Phylogrouping comparisons were made from previous studies and a consensus was drawn on several parameters including phylogroup, species counts and mis-annotation (**A2**, **Table S2.3 – S2.9**). Overall, the species included in the *L.plantarum* phylogroup remain consistent throughout the literature including, *L.plantarum, L.plantarum* subsp. *plantarum, L.plantarum* subsp. *argentoratensis, L.pentosus, L.herbarum, L.paraplantarum, L.fabifermentans* and *L.xiangfangensis*, in addition to *L.mudanjiangensis, L.modestisalitolerans* and *L.plajomi,* all of which were not reported in the 2014 (Holzapfel & Wood, 2014) and 2018 (Salvetti et al., 2018), phylogroup assessment, however in this study and the 2020 analysis (Zheng et al., 2020b), all three species were clear members of the *L.plantarum* phylogroup and did not influence the topology within this clade. In addition, two previously unidentified strains, *Lactobacillus sp.* D1501 and *Lactobacillus* ATCC 15578 were reclassified in this study as novel *L.plantarum* strains and *L.acidophilus* NCTC1407 was reannotated as an *L.plantarum* strain**.** For the *Lactobacillus rossiae/siliginis* phylogroup, out of the three studies used as comparators, there was a high degree of consensus on the phylogroup designation, except for the 2018 (Salvetti et al., 2018) study, where no designation was provided (Holzapfel & Wood, 2014; Salvetti et al., 2018; Zheng et al., 2020b). The *Lactobacillus reuteri* phylogroup is somewhat more complex when comparing species phylogroup designations. In previous studies, the addition of new strains (for example *Lactobacillus timonensis*, which was only recognised in the 2020 classification) has left gaps and changes possible within this phylogroup. Indeed, the following species*, Lactobacillus oligofermantans, Lactobacillus suebicus, Lactobacillus vaccinostercus, Lactobacillus hokkaidonensis* and *Lactobacillus wasatchensis* were originally classified as members of the *L.reuteri* phylogroup in 2014 (Holzapfel & Wood, 2014) and 2018 (Salvetti et al., 2018). However, in 2020 (Zheng et al., 2020b), identified a new phylogroup *Lactobacillus vaccinostercus,* in addition to the not previously known *Lactobacillus nenjiangensis (*due to the divergence seen from other members of *L.reuteri)*, which is in accordance with this study. The largest number of available genomes for this phylogroup was seen for *L.reuteri* (144), and

*L.fermentum* (66), with most other species being represented by > 5 genomes. Several misclassified or unannotated genomes were reclassified as *L.reuteri* (increasing from 139 to 144), *Lactobacillus mucosae* (from 6 to 8) *L.fermentum* (from 65 to 66) and *L.vasccinostercus* (from 1 to 2 (**A2, Table S2.4 - 2.5**)).

The proposed phylogroups *L.collinoides, L.buchneri and L.brevis* have been used to incorporate several species over the last decade. However, species seem to move between groups depending on the study reporting the grouping. For example*, Lactobacillus oryzae, Lactobacillus malefermentans, Lactobacillus kimchicus, Lactobacillus collinoides, Lactobacillus paracollinoides, Lactobacillus similis* and *Lactobacillus odoratitofui* were initially classified as *L.collinoides* in 2014 (Holzapfel & Wood, 2014)*,* then *L.buchneri* in 2018 (Salvetti et al., 2018) and back to *L.collinoides* in 2020 (Zheng et al., 2020b), in agreement with this study. Similar trends are also seen for members of the *L.brevis* phylogroup, which were previously designated as *L.buchneri* in 2018 (Salvetti et al., 2018), including, *L.acidifarinae, L.zymae, L.spicheri, L.namurensis, L.senmaizukei, L.paucivorans, L.hammesii, L.parabrevis* and *L.koreensis*. In contrast, the species designated as *L.buchneri* phylogroup members appear to be more conserved in the literature destinations.

Initially, in 2014 (Holzapfel & Wood, 2014), the following species were classified as members of the *L.buchneri* group, *L.fructivorans, L.florum, L.lindneri* and *L.sanfranciscensis.* However, from 2018 (Salvetti et al., 2018; Zheng et al., 2020b) onwards, these species were classified as members of the *L.fructivorans* phylogroup, in agreement with the results presented in this study. Likewise, the following species were either classified as *L.buchneri* or not present in the 2014 analysis (Holzapfel & Wood, 2014): *L.kunkeei, L.ozensis, L.apinorum, L.quenuiae, L.timberlakei, L.kosoi and L.micheneri.* However, here and in agreement with later analyses (2018 and 2020) these species were designated as members of the *Lactobacillus kunkeei* and *L.ozensis* phylogroup.

In comparison with other phylogroup designations, the following species generally remain consistent members of the *L.salivarius* group throughout the studies analysed, including the results presented here, *L.ceti, L.saerimneri, L.pobuzihii, L.acidipiscis, L.aviarius* subsp. *araffinosus, L.aviarius* subsp. *aviaries, L.aviaries, L.ruminis, L.apodeme, L.animalis, L.murinus, L.equi, L.agilis, L.hayakitensis* and *L.salivarius*. Some species, including, *L.algidus, L.nagelii, L.ghanensis, L.vini, L.capillatus, L.sucicola, L.aquaticus, L.uvarum, L.oeni, L.satsumensis, L.cacaonum, L.hordei* and *L.mali* were classified as members of the *L.mali* phylogroup in 2018 (Salvetti et al., 2018) however, they were classified as *L.salivarius* in 2020 (Zheng et al., 2020b) and as *L.salivarius* in this study.

The species designated in the phylogroups *Lactobacillus coryniformis, Lactobacillus sakei and Lactobacillus casei* agreed throughout the literature and were also recaptured in this study (Holzapfel & Wood, 2014; Salvetti et al., 2018; Zheng et al., 2020b).

The species designated as members of the *L.perolens* phylogroup are typically in agreement across studies, where applicable (Holzapfel & Wood, 2014; Salvetti et al., 2018; Zheng et al., 2020b). However, *Lactobacillus concavus* and *Lactobacillus dextrinicus* were initially proposed to be members of the *Lactobacillus delbreuckii/ Lactobacillus plantarum* in 2014 (Holzapfel & Wood, 2014) and *Lactobacillus dextrinicus* in 2018 (Salvetti et al., 2018), whereas in this study, are proposed to be members of the *L.perolens* phylogroup.

Overall, where applicable, most of the species designated as members of the *L.alimentarius* phylogroup in this study remained consistent across the literature (Holzapfel & Wood, 2014; Salvetti et al., 2018; Zheng et al., 2020b).

Initially, both *Lactobacillus floricola and L.composti* were encompassed in the *Lactobacillus delbrueckii* clade, however, due to the evolutionary distance observed between these two species and the rest of the *L.delbrueckii* group in the MLSA presented here, it is proposed that *Lactobacillus floricola* and *L.composti* should be recognised as a separate phylogroup as first suggested in 2014 (Holzapfel & Wood, 2014), and agreement with the new species designation allocated by (Zheng et al., 2020b). The MLSA phylogroup findings are reported in **Table 2.4.**

**Table 2.4. Phylogroup metrics for the *Lactobacillus* genus.**

| Designated phylogroup | Number of Species | Number of Strains * | Number of Strains** | Change (+/-) in strain numbers following taxonomic analysis |
|---|---|---|---|---|
| *Lactobacillus plantarum* | 11 | 493 | 496 | 0 |
| *Lactobacillus rossiae/siliginis* | 2 | 4 | 4 | 0 |
| *Lactobacillus vaccinostercus* | 6 | 9 | 11 | 2 |
| *Lactobacillus reuteri* | 17 | 236 | 244 | 8 |
| *Lactobacillus collinoides* | 11 | 26 | 26 | 0 |
| *Lactobacillus brevis* | 14 | 86 | 93 | 7 |
| *Lactobacillus buchneri* | 15 | 74 | 76 | 2 |
| *Lactobacillus fructivorans* | 4 | 41 | 41 | 0 |
| *Lactobacillus kunkeei and ozensis* | 7 | 58 | 58 | 0 |
| *Lactobacillus salivarius* | 28 | 198 | 207 | 9 |
| *Lactobacillus coryniformis* | 7 | 21 | 22 | 1 |
| *Lactobacillus sakei* | 6 | 75 | 75 | 0 |
| *Lactobacillus casei* | 19 | 390 | 418 | 28 |
| *Lactobacillus perolens* | 6 | 10 | 11 | 1 |
| *Lactobacillus alimentarius* | 24 | 49 | 50 | 1 |
| *L.composti and floricola* | 1 | 2 | 2 | 0 |
| *Lactobacillus delbrueckii* | 41 | 515 | 566 | 51 |

*The total number of strains annotated (per phylogroup) before taxonomic analysis.
**The total number of strains per phylogroup after taxonomic analysis.

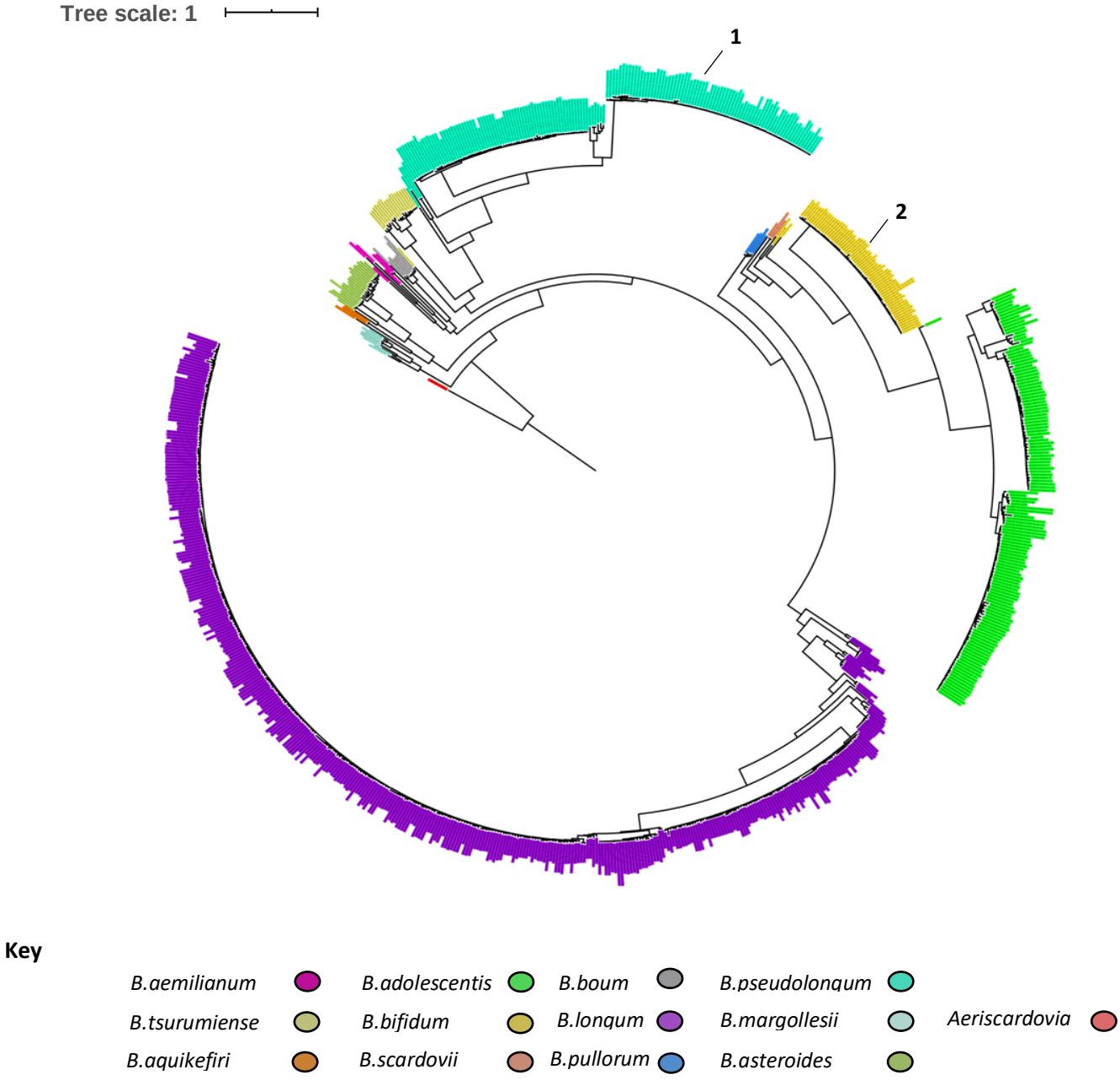



**Figure 2.7. Multi-locus phylogenetic reconstruction of the *Bifidobacterium* genus.** Rooted Maximum likelihood multi-locus phylogeny of the *Bifidobacterium* genus. The Circular phylogeny was created using a concatenation of 400 core proteins throughout all available *Bifidobacterium* genomes retrieved from GenBank (551 genomes, last accessed in Sept 2020). The phylogeny is rooted along the lineage leading to *Aeriscardovia aeriphila*. The phylogeny was created in PhyloPhlAn and rendered in iTOL. The position of CUL strains is indicated with numbers: 1. *B.animalis* subsp. *lactis* CUL34, 2. *B.bifidum* CUL20. The scale bar represents branch length. Clade colours represent species phylogroups.

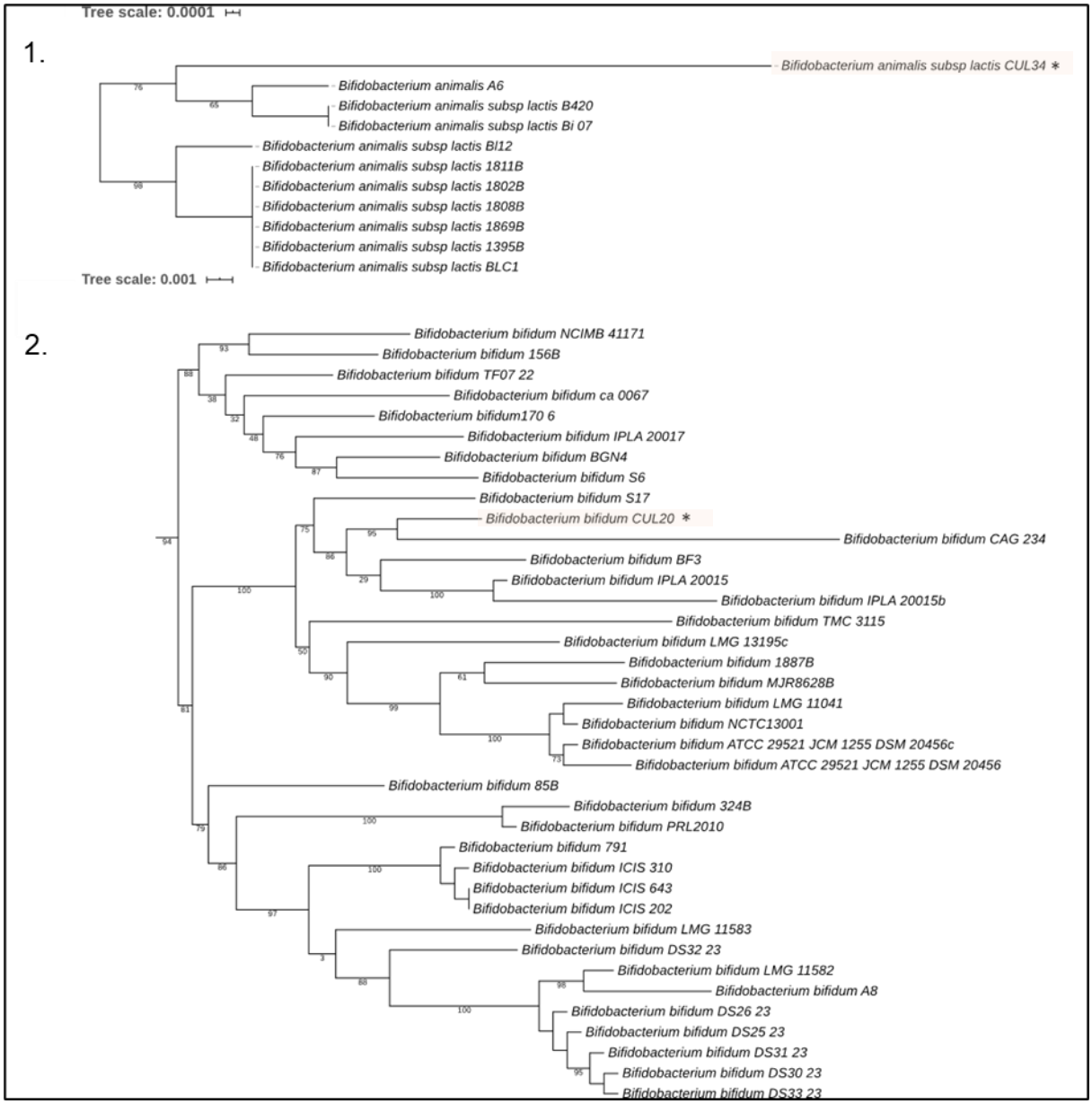



**Figure 2.8. Subclade images of the MLSA *Bifidobacterium* phylogeny, highlighting the taxonomic position of CUL isolates.** Rooted maximum likelihood multi-locus phylogenies of *Bifidobacteria.* Circular phylogenies were created using a concatenation of 400 core proteins throughout all available *Bifidobacterium* genomes retrieved from GenBank (Sept 2020). PhyloPhlAn generated .nwk files were rendered in ITOL. The robustness of each node was assessed using Bootstrap analysis (1000 pseudoreplicates). Numbers at nodes represent percentages. (1) *B.animalis* clade containing CUL34. (2) *B.bifidum* clade containing CUL20. CUL strains are indicated by an *. Tree scale bars represent branch length.

### 2.3.7. Bifidobacteria phylogroup analysis

The phylogenetic position of clades in the *Bifidobacterium* genus was far less ambiguous in comparison to *Lactobacillus*. From this study, the phylogenetic grouping of species was typically in concordance with previous reports (Lugli et al., 2018; Sun, Zhang, et al., 2015). However, several new species, which were not previously reported, were included in this study, and therefore a new phylogroup designation was proposed for such clades, with annotation based on the first-named species of the group (**Table 2.5**).

**Table 2.5. Phylogroup metrics for the *Bifidobacterium* genus.**

| Phylogroup Designation | Number of Species | Number of Strains* | Number of Strains** | Change (+/-) in strain numbers following taxonomic analysis |
|---|---|---|---|---|
| *Bifidobacterium margollesii* | 6 | 6 | 7 | 1 |
| *Bifidobacterium aquikefiri* | 2 | 3 | 3 | 0 |
| *Bifidobacterium asteroides* | 5 | 19 | 21 | 2 |
| *Bifidobacterium aemilianum* | 5 | 6 | 6 | 0 |
| *Bifidobacterium boum* | 5 | 8 | 8 | 0 |
| *Bifidobacterium tsurumiense* | 1 | 1 | 1 | 0 |
| *Bifidobacterium pseudolongum* | 13 | 152 | 152 | 0 |
| *Bifidobacterium pullorum* | 3 | 4 | 4 | 0 |
| *Bifidobacterium scardovii* | 1 | 2 | 2 | 0 |
| *Bifidobacterium bifidum* | 3 | 68 | 68 | 0 |
| *Bifidobacterium adolescentis* | 8 | 129 | 136 | 7 |
| *Bifidobacterium longum* | 19 | 380 | 387 | 7 |

*The total number of strains annotated (per phylogroup) before taxonomic analysis.
**The total number of strains per phylogroup after taxonomic analysis.

.

## 2.4. Discussion

A comprehensive classification of a bacterium is essential, especially when deliberately introducing a live microorganism into the food chain. Indeed, for probiotic products, accurate classification is not only paramount to promoting the safety of the consumer but is also crucial for bioprospecting, where genomic mining may aid in the identification of potential beneficial attributes. This is perhaps more important for lactobacilli and bifidobacteria where beneficial traits are generally species and even strain specific (Campana et al., 2017; McFarland et al., 2018a; Ramos et al., 2013). In this study, the draft genomes of 16 strains of lactobacilli and bifidobacteria (CUL strains) are reported, with a robust multifaceted taxonomic analysis. A variety of genomic markers were compared, and a detailed taxonomic identification was proposed for each CUL strain.

### 2.4.1. Optimisation of nucleic acid purification

The initial optimisation of gDNA isolation from *Lactobacillus* strains supported the importance of washing the cell pellet before the lysis step. Indeed, Gram-positive bacteria, especially *Lactobacillus* species, are notoriously difficult to lyse (Scornec et al., 2014). Some species, such as *L.helveticus* strains are resistant to the effects of lysozyme (Kido et al., 2021; Neviani et al., 1991). It has been previously shown that the addition of a high-salt wash may enhance cellular lysis (Alimolaei & Golchin, 2017) and the results presented in this chapter supported this. Certainly, the wash step aided in the extraction of high molecular weight, intact gDNA in comparison to extractions without. In DNA extractions without a wash step, bands appeared as a ‘smear’, which is likely from “older” cells within the culture that have naturally lysed and released cytoplasmic nucleases. Alimolaei & Golchin, (2017) suggest that salt washes soften the peptidoglycan wall and facilitate lysis by lysozyme. In addition, we assume that such wash steps may also remove lactic acid (generated by LAB during growth) and thus provide a more optimum pH (neutral) for more efficient enzymatic lysis. Furthermore, the use of multiple enzymes together (lysozyme and mutanolysin) also seemed to enhance cellular lysis.

### 2.4.2. Classification of CUL strains using a 16S rRNA type strain NJ phylogeny

Taxonomic assignment based solely on 16S rRNA is generally ambiguous (Drancourt et al., 2000; Janda & Abbott, 2007; Johnson et al., 2019; Mignard & Flandrois, 2006) and it has been shown that classifications do not always recapitulate the true species genealogy; hence differentiation of strains based on 16S rRNA is not recommended (Ashelford et al., 2005; Fox et al., 1977, 1992; Heikens et al., 2005; Lesack & Birol, 2018).

To evaluate the use of the 16S rRNA marker when classifying CUL strains, isolates were initially classified via Sanger sequencing of the 16S rRNA gene. This resolved the taxonomic identity of all CUL strains to the genus level and these identities were commensurate with standing nomenclature. In brief, the majority of CUL strains had a nucleotide sequence similarity of > 98 % to a member of the same species according to the GenBank 16S rRNA database. In the literature, cut-off values of > 95 % similarity along the 16S rRNA amplicon generally support genus taxonomic resolution, whilst > 98.7 % correspond to species level (Johnson et al., 2019; Rossi-Tamisier et al., 2015), suggesting the initial taxonomic assignment was correct. Moreover, the topology of the *Lactobacillus* type strain phylogeny, reconstructed all known phylogroups previously reported (Pot et al., 2014), suggesting that for most *Lactobacillus* species, assigning taxonomy with a 16S rRNA type-strain phylogeny may provide sufficient resolution to designate species level nomenclature. In addition, despite the well-established hypothesis that 16S rRNA as a sole marker is not always effective at discriminating between strains (Claesson et al., 2008; Huang et al., 2018b; Naser et al., 2005; Singh et al., 2009; Xie et al., 2019), when CUL isolates of the same species, for example in the cases of, *L.plantarum* (CUL66N and CUL66), *L.acidophilus* (CUL21 and CUL60) and *L.paracasei* (CUL07, CUL08 and CUL37) were analysed via a type strain phylogeny, 16S rRNA appeared to provide enough resolution to identify each isolate as a separate strain, shown by the presence of a dichotomous split between each CUL strain (in both the *Lactobacillus* and *Bifidobacterium* type strain phylogenies). However, there was one exception amongst the CUL strains, as *L.helveticus* CUL76 grouped with *L.suntoryeus* SA and *L.gallinarum* ATCC 33199 (but had *L.helveticus* DSM20075 as a sister grouping), indicating that 16S rRNA has a lower resolution for this species. This is likely due to the close relation between *L.helveticus* and other lactobacilli. Indeed, it has been previously shown that *L.helveticus* strains shared a 16S rRNA sequence similarity of 98.4 % with the probiotic *L.acidophilus* NCFM (Claesson et al., 2008), suggesting, in this case, 16S rRNA is too conserved and unreliable for the resolution of novel *L.helveticus* strains. In addition, it has recently been reported that *L.gallinarum* and *L.helveticus* shared 99.17 % sequence similarity, with the authors concluding that members of the *Lactobacillus acidophilus* group were difficult to identify using 16S rRNA sequences (You & Kim, 2020), in agreement with the findings reported here. Certainly, closely related species can share a high similarity or even identical

16S rRNA sequences, which can significantly impact taxonomic identification (Ashelford et al., 2005; Fox et al., 1977, 1992; Heikens et al., 2005; Lesack & Birol, 2018). An example of this is shown in the highly intermixed phylogroup of *L.casei* (encompassing species: *L.casei*, *L.paracasei*, *L.rhamnosus*). However, in the 16S rRNA type strain phylogeny presented in this chapter, species, and strain level phylogenetic resolution of all CUL isolates within the *L.casei* phylogroup was achieved, suggesting that the sole use of the 16S rRNA marker provided a strong basis for assigning putative taxonomy to such species. The bootstrap values throughout the *Lactobacillus* type-strain phylogeny were low, with several branches displaying less than 50 % confidence. Low bootstrap values for lactobacilli have been reported previously (Juwana et al., 2020), and could be explained by the insufficient signal in the 16S rRNA amplicon, resulting in, species being randomly assigned to different clades. For bifidobacteria strains (CUL34 and CUL20), 16S rRNA offered sufficient phylogenetic signal to distinguish between species and strains (again evidenced by a dichotomous split). Bootstrap values for the bifidobacteria phylogeny were higher than the lactobacilli, however, were still relatively low. Despite the more stable nature of the bifidobacteria taxonomy in comparison to lactobacilli, the use of 16S rRNA as a genomic marker is also controversial for species designation (Duranti et al., 2017b; Hu et al., 2017; Johnson et al., 2019; Ventura et al., 2006). As such, there are arguments for and against the use of 16S rRNA to assign taxonomy to candidate probiotics within this study. For certain species such as *L.plantarum, L.casei, L.acidophilus, and B.animalis* subsp. *lactis and B.bifidum,* 16S rRNA appears to be diverse enough to designate strain identification. However, a combination of low bootstrap values in the 16S rRNA phylogenies, in addition to the lower resolution of 16S rRNA when identifying *L.helveticus,* suggests that the use of 16S rRNA may provide inconsistent results across species, and to provide a robust analysis of strains, additional genomic markers should be incorporated.

### 2.4.3. Identifying an outgroup root for the *Lactobacillus* MLSA phylogeny

Due to the reported paraphyletic nature of the *Lactobacillus* genus (Makarova & Koonin, 2007; Sun, Harris, et al., 2015a), an MLSA-based phylogeny was constructed for the entire LAB group, to identify the most suitable outgroup for the *Lactobacillus* phylogeny. The resulting topology was in agreement with previous reports, where the genera *Leuconostoc*, *Weissella*, *Oenococcus* and *Fructobacillus* shared a Most Recent Common Ancestor (MRCA) with *Lactobacillus*, which has also been coined the *Lactobacillus* genus complex (Wittouck et al., 2019). In addition, the paraphyly exhibited between *Pediococcus* and *Lactobacillus* has also been previously described, utilising fewer genomic markers (Salvetti et al., 2012), and the results in this chapter, therefore, support such findings. As such, based on the resultant topology supported by the literature, *Lactococcus* was identified as the

most suitable outgroup for the *Lactobacillus*-specific MLSA, due to its evolutionary distance from *Lactobacillus* and its non-paraphyletic nature.

### 2.4.4. A taxonomic reassessment of the *Lactobacillus* genus

The topology of the *Lactobacillus* MLSA phylogeny presented within this chapter was consistent with previous taxonomic assessments of the genus (Salvetti et al., 2018; Zheng et al., 2020b) and contains well-defined homogenous clades. *Lactobacillus* encompasses an enormous metabolic diversity (Duar et al., 2017; Harris et al., 2017; Sun, Zhang, et al., 2015). As such, the taxonomy of *Lactobacillus* has undergone extensive debate, analysis and change over the last decade (Salvetti et al., 2012, 2018; Sun, Harris, et al., 2015a; Zheng et al., 2020b), which has recently resulted in the proposition for the genus to be split into 23 novel genera (Zheng et al., 2020b). However, uptake of such changes is currently moderate (Han et al., 2021; Peng et al., 2021; Petrova et al., 2021; Zhao et al., 2021), with arguments stating that changing the nomenclature of such an economically important group is logistically difficult, due to the numerous patents that currently exist for "*Lactobacillus*" and the confusion it would generate in databases, research papers and consumers (Pot et al., 2019). The scale of heterogenicity exhibited by *Lactobacillus* strains is highlighted in the MLSA phylogeny presented here, where two distinct groups of species are visible and separated by a large evolutionary distance. Group A consists of: *L.coryniformis, L.sakeii, L.casei, L.alimentarius* and *L.delbrueckii*, and Group B, incorporates *L.salivarius, Pediococcus, L.plantarum, L.reuteri, L.collinoides, L.brevis, L.buchneri, L.fructivorans* and *L.kunkeei,* a phenomenon previously described (Salvetti et al., 2018). Previous taxonomic analyses on the *Lactobacillus* genera have attempted to resolve the genus into distinct phylogroups (Holzapfel & Wood, 2014; Salvetti et al., 2018; Zheng et al., 2020b). However, such studies are typically focused on a subset of representative species (encompassing approx. 220 species) and utilise a range of genomic markers, from 29 (Salvetti et al., 2018), 73 (Sun, Harris, et al., 2015a) and 114 core genes (Zheng et al., 2020b). Here we present a phylogeny based on all annotated *Lactobacillus* strains in NCBI, encompassing over 2100 genomes using 400 core proteins ubiquitous to the entire bacteria kingdom, building on such studies. As a result, a detailed phylogenetic analysis was enabled for all genomes currently available. For strains that were either mislabelled or that were not annotated, the taxonomic position was inferred, and a putative nomenclature was assigned (**Supp. S2.4-5**). Most reassigned species were either members of the *L.delbrueckii* or *L.casei* phylogroups, which was to be expected given the size of these groups.

In addition, phylogroups were also reviewed and compared with previous designations (Holzapfel & Wood, 2014; Salvetti et al., 2018; Zheng et al., 2020b). Overall, discrepancies between group designation were typical with earlier taxonomic evaluations (Holzapfel & Wood,

2014; Salvetti et al., 2018), whereas when the number of genomic markers was increased, the phylogroup and taxonomic position were typically in agreement between Zheng et al., (2020b) and the MLSA presented in this study. The *Lactobacillus* genus encompasses a vast degree of controversy, with arguments for (Zheng et al., 2020b) and against (Pot et al., 2019) the reassignment of species into new genera, and more granularly, based on phylogroup assignment (Wittouck et al., 2019).

### 2.4.5. CUL taxonomy

All CUL probiotic isolates were identified as novel strains, characterized by the occupation of individual branches within the MLSA phylogenies.

#### 2.4.5.1. *Lactobacillus acidophilus*

Two CUL strains were identified in the *L.acidophilus* clade, CUL21 and CUL60. Of interest is the split within the *L.acidophilus* clade, which separates the *L.acidophilus* strains into two subclades (A1 and A2). Previous reports have suggested dichotomous splits in *Lactobacillus* clades are a result of habitat (Duar et al., 2017), however, there were no discernible trends observed from the isolation source of the *L.acidophilus* strains in this study. For CUL strains, both CUL21 and CUL60 were positioned in sub-clade 2 and exhibited a close relationship with each other, sharing a MRCA. Within their designated subclade, the presence of at least two well-documented probiotic strains; *L.acidophilus* ATCC 53544, which has shown to produce bacteriocins within secreted mobile vessels that can kill *L.delbrueckii* (Dean et al., 2017, 2019, 2020) and *L.acidophilus* DSM 20079 which, also has a documented capability of producing bacteriocins (Deraz et al., 2005). In addition, DSM 20079 has also exhibited the potential to competitively inhibit oral pathogens (Tahmourespour & Kermanshahi, 2011) and induce anti-inflammatory effects (Hidalgo-Cantabrana et al., 2018). The close relation between CUL isolates and strains with documented probiotic capabilities offers the potential to bio-prospect CUL strains to determine whether they possess similar properties, which can be explored with comparative genomics.

### 2.4.5.2. *Lactobacillus fermentum*

The *L.fermentum* clade in the MLSA phylogeny, reveals two distinct groupings (demarcated as F1 and F2). The *L.fermentum* species has been subjected to evaluation and reclassifications previously. Indeed, two species, *L.fermentum* and *L.cellobiosus* have been deemed too genetically similar and were, therefore, collapsed into one species *L.fermentum*, offering a putative explanation for such diversity (Dellaglio et al., 2004). Both *L.fermentum* CUL strains, CUL67 and CUL40, clustered with other *L.fermentum* strains on the phylogeny. Curiously, each CUL strain was located within the two different clades. For CUL67, the closest genomic neighbours are strains isolated from several habitats including *L.fermentum* IMDO130101 isolated from sourdough, DS13 isolated from the cocoa bean, and SHI 2 isolated from human saliva (Brandt et al., 2020). CUL40LB resides in a subclade with *L.fermentum* VRI 003, a probiotic product shown to reduce respiratory illness (Cox et al., 2010), LF2 which has been shown to produce exopolysaccharides (Ale et al., 2016) and CRL1446, which has displayed probiotic potential in mice (Mukdsi et al., 2012).

### 2.4.5.3. *Lactobacillus gasseri*

CUL09 falls within the *L.gasseri* clade of the ML phylogeny within the *L.delbrueckii* phylogroup. It also clusters with several strains with putative probiotic potential, such as *L.gasseri* 505 (Lee et al., 2018), and *L.gasseri* K7, which have been shown to inhibit pathogen adhesion, and produce bacteriocins (Matijašić et al., 2006; Peternel et al., 2010), and *L.gasseri* 4M13, which has also been identified as a probiotic candidate (Kim et al., 2019; Oh et al., 2018), offering scope to bioprospect CUL09 for similar traits.

### 2.4.5.4. *Lactobacillus helveticus*

*Lactobacillus helveticus* CUL76 fell within the *L.helveticus* clade on a lone branch, strongly indicating a novel strain deposition into NCBI.

*L.helveticus* strains are extremely important starter cultures in the dairy industry, due to their high protease activity (Giraffa, 2014; Yamashita et al., 2014). As such, strains are often isolated from a variety of dairy products (Bian et al., 2016; Miyamoto et al., 2015; Moser et al., 2017). Of interest, *L.helveticus* CUL76 closest relative, *L.helveticus* R0052 is a well-established isolate that is marketed as a probiotic (Arseneault-Bréard et al., 2012; Messaoudi et al., 2011; Tompkins et al., 2012), indicating a strong basis of probiotic function for CUL76.

### 2.4.5.5. *Lactobacillus plantarum*

Of all the clades, the phylogroup *L.plantarum* was the second largest (encompasses 496 strains). CUL66 and CUL66N, two putative *L.plantarum* strains, fall within the *L.plantarum* phylogroup, with considerable genetic distance from each other. CUL66 was located within a subclade, along with 6 strains that appear to be genetically identical at the core protein level, suggesting that they are in fact, the same strain. CUL66 shares a MRCA with *L.plantarum* CLP- 0611, a strain that has been documented to ameliorate colitis (Jang et al., 2014). CUL66N, lies as an outgroup to a separate subclade, suggesting significant variation in genomic features between both *L.plantarum* CUL strains.

### 2.4.5.6. *Lactobacillus casei phylogroup*

*The Lactobacillus casei* phylogroup consists of several species, including *L.casei*, *L.paracasei* and *L.rhamnosus*, which have previously undergone extensive taxonomic scrutiny, due to the close relation of such species (Huang et al., 2018b; Wuyts et al., 2017).

From the MLSA phylogeny, it appears that within this phylogroup, there are five distinct subclades. Subclade C1 consists of the following species, *L.selangorensis, L.songhuajiangensis, L.sharpeae, L.thialandensis, L.pantheris, L.camelliae, L.nasuensis, L.porcinae, L.manihotivorans, L.brante,* and *L.saniviri,* and is separated by a greater evolutionary distance in comparison to C2 – C5.

In contrast C2, C3 and C4 appear to show a higher degree of relation to each other in comparison to C1, represented by the shorter branch lengths between each subclade. Interestingly, C2 is made up entirely of *L.paracasei* subsp. *paracasei* strains. C3 generally contains *L.paracasei strains,* C4 *L.zeae* and *L.casei* and C5, mainly *L.rhamnosus*. However, admixture between *L.casei*, *L.paracasei*, *L.rhamnosus* and *L.zeae*is frequently exhibited. CUL isolates were in subclades containing species of their expected taxonomy. *L.casei* CUL06 groups together with other annotated *L.casei* strains (C4), despite the *L.casei* phylogroup being in admixture suggesting a true *L.casei* designation.

In terms of bioprospecting, CUL06 is located within a subclade along with *L.casei* ATCC 393, an isolate that has documented probiotic potential (Saxami et al., 2012; Sidira et al., 2010) and anticancer properties (Ethiraj et al., 2019). All putative *L.paracasei* strains grouped with other *L.paracasei* strains in subclade C3, providing strong evidence for correct taxonomic classification. CUL08 was positioned within a separate subclade to CUL07 and CUL37LB. Of particular interest, is that despite 16S rRNA showing all three isolates as distinct species, ML analysis positioned CUL07 and CUL37 on a linear branch, supported by a high bootstrap value, suggesting that they are likely genetically identical. CUL08 shares a MRCA with *L.paracasei* L9, a strain which has been suggested to have probiotic properties (Jiang et al.,

2015; Wang et al., 2017; Zhu et al., 2016). Similarly, *L.rhamnosus* CUL67 also shares a MRCA with a strain that has documented probiotic potential, *L.rhamnosus* DSM 14870 (Dardmeh et al., 2017; Marcotte et al., 2017).

#### 2.4.5.7. *Lactobacillus salivarius*

The *L.salivarius* phylogroup is the first group that appears after the deep divergence between group A and group B in the MLSA phylogeny. 28 species fall within the *L.salivarius* group. Each species generally clusters together in small subclades. *L.salivarius* is the most abundant species and is in a large subclade which appears closely related to the *Pediococcus* paraphyly. *L.salivarius* CUL61, falls within the large *L.salivarius* clade, indicating that the CUL strain was correctly identified at the species level during its initial classification.

#### 2.4.5.8. *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum*

The Number of *Bifidobacterium* species is significantly lower than that of *Lactobacillus*. At the time of writing, there are 71 recognised species within the bifidobacteria genera, which subsequently reduces the amount of heterogeneity in the genus and phylogroups (in comparison to *Lactobacillus*). However, as NGS has become more accessible, so has the number of genomes available (Turroni et al., 2011). Therefore, this study updated the recently allocated phylogroups designated by Lugli et al., (2018) to encompass newly identified or unassigned species, for example, *Bifidobacterium aemilianum*. As such we were able to designate 11 phylogroups based on the ML taxonomic positioning. The number of strains within each phylogroup ranges from 7 (*Bifidobacterium margollesii*) to 385 (*Bifidobacterium longum*). In addition, mislabelled and unassigned species were also annotated with a suggested species designation, in accordance with their phylogenetic groupings. The topology of the bifidobacteria ML phylogeny is in agreement with that shown previously (Lugli et al., 2018). Of interest, is the separation of the *B.longum* phylogroup from the others, offering an insight into the evolutionary divergence of the group.

Both *B.animalis* subsp. *lactis* CUL34 and *B.bifidum* CUL20 were grouped within the *B.animalis* and *B.bifidum* clades respectively, indicating that a correct taxonomic classification has been assigned. It is worth noting that *B.bifidum* CUL20 has recently diverged from a well-characterised strain *B.bifidum* S17 (Sun et al., 2014; Wei et al., 2016; Westermann et al., 2012). Similarly, *B.animalis* subsp. *lactis* CUL34 is closely related to *B.animalis* A6, which has previously shown probiotic potential (Huo et al., 2020; Sun et al., 2015), providing scope and rationale for the use of these organisms to guide comparative genomics, to bio-prospect for probiotic traits in CUL isolates.

### 2.4.6. Strain potential

Despite the large genomic and phenotypic variation documented within the *Bifidobacterium* and *Lactobacillus* genera (Claesson et al., 2008; Duar et al., 2017; Zheng et al., 2020b), health-promoting traits have been documented for several species within these groups (Capurso, 2019; Ma et al., 2013; Michael et al., 2020; Morovic et al., 2016; O'Morain, 2019; Ritchie & Romanuk, 2012). *Lactobacillus* species are widespread and occupy a vast variety of habitats (Collins et al., 2017; Dias et al., 2013; Duar et al., 2017; Kumar & Kumar, 2015; Liu et al., 2020; Melo et al., 2016) which has resulted in trait selection and genome evolution: either to lose redundant functions or gain novel adaptations to allow survival (Callanan et al., 2008; Mendes-Soares et al., 2014). In addition, bifidobacteria are commonly identified as key members of the human gut microbiota, indicating a host selection for a relationship with such bacteria (Chaplin et al., 2015; Feng et al., 2019b; Yang et al., 2019). Therefore, classifying and genetically characterising each new strain from either *Lactobacillus* or *Bifidobacteria* is essential to deduce the evolution of such isolates, infer the lack of virulence and identify potential health-promoting attributes. Here, the genomes of 16 novel probiotics are presented, positively identified as lactobacilli and bifidobacteria species.

### 2.4.7. Limitations

This study provides the first in-depth genomic analysis of CUL strains. However, genomes are presented as draft versions and therefore have limitations associated with incomplete genome assembly. For example, the presence of gaps between contigs, gene fragmentation (Klassen & Currie, 2012) and disproportionately missing certain genes (Shay, 2016), which may impact downstream genomic analysis.

Genomes were assembled without the use of a reference genome (a more detailed description of how these process works have been included in the methods). Reference genomes were not used as they may influence taxonomic identification and it is not a standard assembly method for bacterial genomes. Indeed, CUL genome sizes were comparable with the median size reported for their designated species, indicating that our genomes were commensurate with the rest of the genus. However, gaps may limit genome annotation, due to a depletion in sequences. To combat such effects genomes were aligned and mapped to their closest relative (identified on the MLSA phylogeny), to reorder contigs and potentially reduce the impact of gaps on genome mining (methods presented in subsequent chapters). Even so, future work should aim at closing the gaps to produce complete genomes, which will offer a more robust capacity for genome mining.

### 2.4.8. Importance of the study

Probiotic products are not regulated in the same way as therapeutic drugs, as they are live entities, that are difficult to validate the health effects of (Salvetti & O'Toole, 2017b). Therefore, products are typically regulated as food supplements (Caselli et al., 2013; Salvetti & O'Toole, 2017b). As such, legislation has previously not required detailed sequencing of organisms marketed as probiotics, which is noteworthy, given the ambiguous nature of these probiotic rich genera. A misclassified organism marketed as safe for consumption, could not only impact consumer health when taxonomy is wrong at the genus level (Huys et al., 2006) but may also lead to inconsistencies in the literature regarding the relevance and functionality of probiotic strains. Indeed, several studies have shown discrepancies between the probiotic product label and its taxonomy even at the genus level (Huys et al., 2006; Morovic et al., 2016; Temmerman et al., 2003), furthering the motive to undertake a more detailed genomic analysis.

### 2.4.9. Summary

This chapter outlines the foundation of the thesis. Here, the genomes of 16 bacterial strains are presented along with robust taxonomic analysis. All strains were identified to species level via 16S rRNA sequencing, and MLSA phylogenies aided in the classification of CUL isolates to strain level. It was revealed that all CUL strains were novel entries into the NCBI GenBank, providing a strong argument to continue bioprospecting for probiotic traits.

## 2.5. Appendix 2.

**Supplementary tables**

https://docs.google.com/spreadsheets/d/11iQp7b_tTZLWik2MtMyR3IlC_anLeTNf/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true

Contains the following tables:

**Table S2.1.** Lactobacilli 16S rRNA type strain sequences and accession numbers.

**Table S2.2.** Bifidobacteria 16S rRNA type strain sequences and accession numbers.

**Table S2.3.** Lactobacilli phylogroup assessment.

**Table S2.4.** Lactobacilli genus reassigned species.

**Table S2.5.** Lactobacilli genus reclassified species following analysis.

**Table S2.6.** Lactobacilli genus phylogroup summary.

**Table S2.7.** Bifidobacteria genus phylogroup assessment.

**Table S2.8.** Bifidobacteria genus reassigned species.

**Table S2.9.** Bifidobacteria mislabelled species.

**Table S2.10-S2.11.** Bifidobacteria phylogroup summary.

**Table S2.12.** Lactobacilli new species names 2020 (Zheng et al., 2020a).

**Figure S2.1.** nwk file for *Lactobacillus* MLSA.

https://drive.google.com/file/d/15aHdV4mT5s3sgNlbpmF8KdqfEftVEno/view?usp=sharing

**Figure S2.2.** nwk file for *Bifidobacterium* MLSA.

https://drive.google.com/file/d/1G48BQKHbbcgjedyKx1KnCyQb288AQqeE/view?usp=sharing

# Chapter 3.
# A genomic evaluation of CUL strains: a safety and functional assessment.

## 3.1. Background

The development of high throughput NGS has offered new insight when characterising microorganisms (Wittouck et al., 2019; Zheng et al., 2020b). Indeed, comparative genomics has become a prominent aspect of microbiology, as it allows in-depth genomic mining for beneficial or harmful traits (Fontana et al., 2019; Salvetti & O'Toole, 2018; Sun, Harris, et al., 2015a) in bacteria. Comparative genomics/genome mining is achievable as a result of specific databases such as NCBI GenBank (Benson et al., 2000), RAST (SEED framework (Aziz et al., 2008)), and so on. Such databases harbour an enormous amount of genomic information, which facilitates comparisons between organisms, allowing a putative understanding of its evolution and relation to others (Binnewies et al., 2006). Conducting genome annotation and mining, enables the prediction of genes of interest (GOI), for example in the case of pathogens, virulence factors (Campedelli et al., 2019; Özkan et al., 2021; Yamaguchi et al., 2019) or probiotics 'health promoting' attributes, which can then be used to target specific phenotypic testing (Fontana et al., 2019; Guo et al., 2020a; Jun et al., 2013).

Certain traits are considered desirable in probiotic products, including survival throughout the GI tract, mediated by acid (Han et al., 2017; Mishra & Prasad, 2005) and bile tolerance (Begley et al., 2006; Hu et al., 2018a), adherence to host cells/mucosa (Buck et al., 2005; Jung et al., 2019), antimicrobial peptide production (bacteriocins (Hegarty et al., 2016)) and microbe-host interactions (Choi et al., 2021; LeBlanc et al., 2017; Pietzke et al., 2020). In addition, the absence of transferable virulence factors, including biogenic amines and ARGs (Campedelli et al., 2019) is also considered beneficial when designating a probiotic (George Kerry et al., 2018)**.** Genome sequencing allows increased accuracy in taxonomic classifications and provides means to assess bacteria's probiotic potential, via a genome mining approach. Doing so allows targeted phenotypic testing which can ultimately lead to the safety and functionality of bacterial strains being classified via a two-prong or polyphasic approach (Lehri et al., 2017).

### 3.1.2. Aims

Following the taxonomic classification of CUL strains in the previous chapter, this chapter sets out to mine the genomes of CUL isolates for features of ‘interest’ including traits considered beneficial, such as bile and acid tolerance, adhesion, production of beneficial molecules and pathogen exclusion. In addition, genomes of CUL strains will also be analysed for the presence of traits deemed “deleterious” in organisms intended for consumption. Such traits include the potential to produce virulence factors, and the presence of ARG and mobile elements. This chapter is designed to allow targeted phenotypic testing based on initial genomic findings.

## 3.2. Materials and methods

### 3.2.1. Genome annotation

Initially, genomes were deposited in NCBI and subsequently processed through its Prokaryotic Genome Automated Annotation Pipeline (PGAAP (Tatusova et al., 2016)) for putative genome annotation, using a combination of HMM-based gene predictions and a pan-genome approach for protein prediction, in addition to generating accession numbers for strains. PGAAP also allowed the identification and removal of any human contamination from sequencing. Following, the contigs of CUL genomes were mapped against the closest relative (identified as the closest relative with a complete WGS on their respective PhyloPhlAn trees, **Table 3.1**) using Mauve (Darling et al., 2004). Contigs were reordered using this method, to potentially minimize the presence of gaps in the draft genome sequences.

**Table 3.1. Reference strains for contig reordering**. The closest relative (with a WGS) of each CUL strain was identified on the respective ML phylogeny genomes were retrieved and used for CUL contig reordering via Mauve.

| Species | CUL | Reference Strain |
|---|---|---|
| *L.paracasei* | CUL37 | *Lactobacillus paracasei* subsp. *paracasei* JCM 8130 (AP012541) |
| *L.paracasei* | CUL07 | *Lactobacillus paracasei* subsp. *paracasei* JCM 8130 (AP012541) |
| *L.paracasei* | CUL08 | *Lactobacillus paracasei* subsp. *paracasei* JCM 8130 (AP012541) |
| *L.casei* | CUL06 | *Lactobacillus casei* subsp. *casei* ATCC 393 (AP012544) |
| *L.rhamnosus* | CUL63 | *Lactobacillus rhamnosus* GG (NC013198) |
| *L.fermentum* | CUL40 | *Lactobacillus fermentum* IFO 3956 (NC010610) |
| *L.fermentum* | CUL67 | *Lactobacillus fermentum* IFO 3956 (NC010610) |
| *L.salivarius* | CUL61 | *Lactobacillus salivarius* UCC118 (NC007929) |
| *L.helveticus* | CUL76 | *Lactobacillus helveticus* CAUH18 (CP012381) |
| *L.gasseri* | CUL09 | *Lactobacillus gasseri* ATCC 33323 (NC008530) |
| *L.acidophilus* | CUL60 | *Lactobacillus acidophilus* NCFM (NC006814) |
| *L.acidophilus* | CUL21 | *Lactobacillus acidophilus* NCFM (NC006814) |
| *L.plantarum* | CUL66 | *Lactobacillus plantarum* WCFS1 (NC004567) |
| *L.plantarum* | CUL66N | *Lactobacillus plantarum* WCFS1 (NC004567) |
| *B.bifidum* | CUL20 | *Bifidobacterium bifidum* S17 (NC014616) |
| *B.animalis* subsp. *lactis* | CUL34 | *Bifidobacterium animalis* subsp. *lactis* B420 (NC017866) |

### 3.2.2. Broad genome mining

Utilising the reordered contigs, further genomic features and gene annotations were generated in the RAST server (Rapid Annotation using Subsystems Technology (Aziz et al., 2008)). An in-depth evaluation of genes within the Virulence and Defence subsystem was conducted and the number of genes predicted was tallied. A heatmap of putative virulence factors was constructed in R using both ggplots (Wickham, 2006) and the heatmap plus package (Day, 2012). In addition, RAST annotation was also utilised to predict the AMR profiles of CUL strains. The number of genes within the resistance subcategory was tallied and the results are presented via a stacked graph.

### 3.2.3. Prophage presence in CUL strains

The presence of prophage elements within CUL draft genome sequences was predicted via the PHAST pipeline (Zhou et al., 2011). In brief, CUL genomes were uploaded onto the PHAST database (Last accessed March 2019), which first predicts Open Reading Frames (ORFs) using GLIMMER3 (Delcher et al., 2007). Phages were then predicted by performing a BLAST search against PHAST's sequence database. Putative phages are described as incomplete or intact depending on the predicted presence of the gene cluster.

### 3.2.4. Genomic island prediction

Genomic islands (GI) were predicted using IslandViewer4 (Bertelli et al., 2017) and manually curated by comparing the genomic neighbourhoods amongst closely related species. Reference strains used for GI prediction are shown in **Table 3.2**. GIs were then manually curated and mined for GOI.

**Table 3.2. The closest relative identified on the multi-locus phylogeny.** The closest relative with a complete WGS is designated the reference strain and is presented with its respective NCBI accession number. Such strains were used as a reference for CUL genomic island prediction via Islandviewer4.

| Species | Strain | Reference Strain |
|---|---|---|
| *L.paracasei* | CUL37 | *Lactobacillus paracasei* L9 (CP012148) |
| *L.paracasei* | CUL07 | |
| *L.paracasei* | CUL08 | |
| *L.casei* | CUL06 | *Lactobacillus casei* subsp. *Casei* ATCC 393 (AP012544) |
| *L.rhamnosus* | CUL63 | *Lactobacillus rhamnosus* DSM 14870 (CP006804) |
| *L.fermentum* | CUL40 | *Lactobacillus fermentum* FTDC 8312 (CP021104) |
| *L.fermentum* | CUL67 | *Lactobacillus fermentum* 3872 (CP011536) |
| *L.salivarius* | CUL61 | *Lactobacillus salivarius* UCC118 (007929) |
| *L.helveticus* | CUL76 | *Lactobacillus helveticus* R0052 (018528) |
| *L.gasseri* | CUL09 | *Lactobacillus gasseri* 4M13 (CP021427) |
| *L.acidophilus* | CUL60 | *Lactobacillus acidophilus* DSM 20079 (CP020620) |
| *L.acidophilus* | CUL21 | |
| *L.plantarum* | CUL66 | *Lactobacillus plantarum* WCFS1 (004567) |
| *L.plantarum* | CUL66N | |
| *B.bifidum* | CUL20 | *Bifidobacterium bifidum* (ATCC29521) |
| *B.animalis* subsp. *lactis* | CUL34 | *Bifidobacterium animalis* subsp. *lactis* BL04 (SD5219) |

#### 3.2.5.1. Probio-genomic analysis: predicting the presence of 'Genes of Interest'

Initial genome mining of CUL strains was undertaken via RAST subsystem analysis. Following, a more in-depth analysis was conducted to predict the presence of genomic regions that have previously been reported to encode features from the following categories: health-promoting, microbe-host interactions and virulence.

#### 3.2.5.2. *Identifying Genes of Interest (GOI)*

The current literature was screened to allow the putative identification of genomic features which may contribute toward functional traits or virulence factors. GOI were identified, and the subsequent proteins were retrieved from NCBI. Protein sequences were next subjected to individual BLASTp analysis via the RAST server into CUL genomes. If a hit was predicted, sequence homology was > 50 %, the e value was < 0, and annotation was the same, it was considered that the GOI was present in the genome. If the e-value was less than 0, but the sequence homology was < 50 %, The CUL protein sequence was retrieved and reciprocally analysed by BLASTp via the NCBI GenBank, to retrieve a greater resolution for protein similarity and annotation.

#### 3.2.5.3. *Targeted mining*

Following the establishment of an evolutionary lineage of CUL strains in **Chapter 2**, characterised traits in closely related strains were identified in the literature. Following, amino acid or nucleotide sequences were retrieved for the gene/protein of interest and were manually BLASTn/BLASTp into the relevant CUL strain. A gene was considered present if sequence similarity was > 98 %.

### 3.2.6. Genotype analysis of carbohydrate metabolism

Carbohydrate-active enzyme profiles (CAZy) were predicted using dbCAN2 (Zhang et al., 2018), a meta server for automated carbohydrate-active enzyme annotation (Last accessed November 2021). Results were manually filtered for profiles, which were predicted using the maximum three methods; (i) HMMER search against the dbCAN HMM (Hidden Markov Model) database (Finn et al., 2011); (ii) DIAMOND (Buchfink et al.,2015) search against the CAZy pre-annotated CAZyme sequence database and (iii) Hotpep (Busk et al., 2017) search against the conserved CAZyme short peptide database. A heatmap was constructed in R using both ggplots (Wickham, 2006) and the heatmap plus package (Day, 2012).

### 3.2.7. Prediction of bacteriocins

The genomes of CUL strains were individually uploaded to BAGEL4, a webserver used to predict Post translationally modified Peptides (RiPPs) and bacteriocins (van Heel et al., 2018). Bacteriocins were allocated to groups according to the classification schemes, defined by BAGEL using default parameters. Sequence homology was evaluated by analysis of the E-value, and the amino acid sequence percent identity (%).

## 3.3. Results

### 3.3.1. Genome annotation: RAST outputs

The genomes of CUL isolates were aligned to their closest relative according to the MLSA detailed in Chapter 2. Contigs were reordered to reduce gaps in the draft WGS and uploaded to RAST for further annotation. Genome size ranged from 1.99 Mb (*B.animalis* subsp. *lactis*) to 3.2 Mb, *L.plantarum* CUL66 (**Table 3.3**). Of the lactobacilli species, CUL strains previously identified as members of the *L.casei* and *L.plantarum* phylogroup (**Chapter 2**) possessed the largest genomes ranging from 2.9 - 3.2 Mb (**Table 3.3**), whereas members of the *L.delbrueckii* (including *L.acidophilus*, *L.gasseri* and *L.helveticus*), *L.fermentum* and *L.salivarius* phylogroups were typically smaller, ranging from 2.0 - 2.2 Mb (**Table 3.3**). Genome sizes of bifidobacteria strain CUL20 and CUL34 were 2.1 Mb and 1.9 Mb respectively (**Table 3.3**).

The number of predicted subsystems followed a similar trend according to genome size (**Table 3.3**). For lactobacilli, CUL strains members of *L.delbrueckii* phylogroup were predicted to have the lowest number of subsystems, 269 – 282 in *L.acidophilus* (CUL21) and *L.helveticus* (CUL76) respectively (**Table 3.3**). The greatest number of subsystems were predicted in the largest genomes, within the *L.casei* and *L.plantarum* phylogroups, ranging from 330 (*L.casei* CUL06) to 343 (*L.paracasei* CUL07). Bifidobacteria strains CUL20 and CUL34 were predicted to encode the smallest number of subsystems, 198 and 267 respectively (**Table 3.3**).

In addition, the number of predicted coding sequences was also largest within the *L.plantarum* (3079 in CUL66 and 3081 in CUL66N) and the *L.casei* phylogroup (2764 in CUL06 to 3042 in CUL07 (**Table 3.3**)). Whereas *L.acidophilus* strains (CUL21 and CUL60), *L.gasseri, L.fermentum* and *L.helveticus* strains were predicted to encode between 1864 (CUL21) to 2152 (CUL76) coding sequences (**Table 3.3**). Bifidobacteria strains were also predicted to encode under 2000 coding sequences, 1903 in *B.bifidum* CUL20 and 1630 in *B.animalis* subsp. *lactis* CUL34 (**Table 3.3**).

Following deposition, RAST organises gene annotation into categories of functional roles or subsystems (**Table 3.4**). Subsystems including, I) Carbohydrates II) Protein Metabolism III) Amino Acids and Derivatives IV) Cell Wall and Capsule and V) Cofactors, Vitamins, Prosthetic Groups, and Pigments, were the most frequently predicted protein-encoding genes in CUL strains. In contrast, genes with predicted roles in Photosynthesis, Motility and Chemotaxis, Iron acquisition and metabolism, Secondary Metabolism, Metabolism of Aromatic Compounds and Nitrogen Metabolism were less frequently predicted within CUL strains (**Table 3.4**).

RAST annotation putatively suggested that CUL strains encoded genetic determinants for potential health-promoting attributes, such as vitamin synthesis, amino acid production and carbohydrate interactions (**Table 3.4**). In addition, all strains were predicted to encode virulence factors and mobile elements (such as phage/plasmid elements and insertion

sequences) except for bifidobacteria strains (CUL20 and CUL34) and *L.helveticus* CUL76 (**Table 3.4**). Several protein-encoding genes (PEGs) related to stress response were predicted in all CUL strains, which offered rationale to investigate such genes and their potential role in probiotic function (**Table 3.4**).

**Table 3.3. Genomic information of CUL strains following RAST annotation.**

| Species | Strain | Size (bp) | %GC | Number of Subsystems | Number of Coding Sequences | Number of RNAs |
|---|---|---|---|---|---|---|
| *Lactobacillus paracasei* | CUL37 | 3167005 | 46.1 | 341 | 3042 | 105 |
| *Lactobacillus paracasei* | CUL07 | 3145586 | 46.2 | 343 | 3043 | 88 |
| *Lactobacillus paracasei* | CUL08 | 3090450 | 46.2 | 336 | 2992 | 87 |
| *Lactobacillus casei* | CUL06 | 2943575 | 47.6 | 330 | 2764 | 93 |
| *Lactobacillus rhamnosus* | CUL63 | 3064027 | 46.5 | 334 | 3016 | 91 |
| *Lactobacillus fermentum* | CUL40 | 2206994 | 51.1 | 318 | 2095 | 90 |
| *Lactobacillus fermentum* | CUL67 | 2133894 | 51.4 | 305 | 1998 | 91 |
| *Lactobacillus salivarius* | CUL61 | 2080688 | 32.9 | 297 | 1940 | 118 |
| *Lactobacillus helveticus* | CUL76 | 2267783 | 37 | 282 | 2152 | 100 |
| *Lactobacillus gasseri* | CUL09 | 2108946 | 35.4 | 269 | 1943 | 91 |
| *Lactobacillus acidophilus* | ATCC 4356 | 1956698 | 34.6 | 186 | 1834 | 63 |
| *Lactobacillus acidophilus* | CUL60 | 2137415 | 35.4 | 273 | 1899 | 86 |
| *Lactobacillus acidophilus* | CUL21 | 2034996 | 34.8 | 269 | 1864 | 105 |
| *Lactobacillus plantarum* | CUL66 | 3292808 | 44.4 | 335 | 3079 | 104 |
| *Lactobacillus plantarum* | CUL66N | 3291001 | 44.4 | 336 | 3081 | 100 |
| *Bifidobacterium bifidum* | CUL20 | 2199325 | 62.6 | 198 | 1903 | 56 |
| *Bifidobacterium animalis* subsp. *lactis* | CUL34 | 1995553 | 59.9 | 267 | 1630 | 89 |

## Table 3.4. Predicted genomic subsystem composition of CUL strains, designated by RAST annotation.

| Subsystem Feature Count | *L.casei* | | | | | *L.fermentum* | | *L.salivarius* | *L.helveticus* | *L.gasseri* | *L.acidophilus* | | | *L.plantarum* | | *Bifdidobacteria* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **CUL37** | **CUL07** | **CUL08** | **CUL06** | **CUL63** | **CUL40** | **CUL67** | **CUL61** | **CUL76** | **CUL09** | **CUL60** | **CUL21** | **ATCC 4356** | **CUL66** | **CUL66N** | **CUL20** | **CUL34** |
| Cofactors, Vitamins, Prosthetic Groups, Pigments | 102 | 105 | 101 | 78 | 94 | 144 | 141 | 66 | 77 | 58 | 65 | 64 | 45 | 129 | 134 | 59 | 79 |
| Cell Wall and Capsule | 107 | 117 | 116 | 109 | 106 | 86 | 85 | 104 | 97 | 109 | 82 | 82 | 30 | 145 | 143 | 16 | 56 |
| Virulence, Disease and Defense | 63 | 65 | 60 | 56 | 57 | 58 | 44 | 43 | 51 | 48 | 42 | 40 | 33 | 56 | 48 | 38 | 28 |
| Potassium metabolism | 4 | 4 | 4 | 6 | 4 | 10 | 10 | 5 | 15 | 9 | 12 | 12 | 6 | 11 | 12 | 2 | 11 |
| Photosynthesis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Miscellaneous | 25 | 26 | 24 | 23 | 23 | 26 | 29 | 12 | 21 | 11 | 23 | 22 | 7 | 36 | 32 | 7 | 13 |
| Phages, Prophages, Transposable elements, Plasmids | 11 | 11 | 7 | 68 | 21 | 16 | 16 | 4 | 0 | 20 | 1 | 1 | 0 | 24 | 17 | 0 | 0 |
| Membrane Transport | 69 | 68 | 69 | 41 | 63 | 17 | 20 | 30 | 53 | 55 | 53 | 53 | 23 | 61 | 61 | 10 | 30 |
| Iron acquisition and metabolism | 1 | 1 | 1 | 1 | 1 | 8 | 1 | 0 | 0 | 0 | 0 | 0 | 4 | 1 | 1 | 0 | 0 |
| RNA Metabolism | 103 | 103 | 103 | 99 | 104 | 88 | 85 | 104 | 80 | 58 | 57 | 57 | 31 | 120 | 118 | 23 | 63 |
| Nucleosides and Nucleotides | 99 | 98 | 94 | 95 | 95 | 109 | 99 | 84 | 85 | 75 | 79 | 79 | 80 | 103 | 103 | 41 | 69 |
| Protein Metabolism | 216 | 215 | 221 | 217 | 215 | 191 | 188 | 208 | 206 | 213 | 208 | 207 | 115 | 184 | 185 | 165 | 178 |
| Cell Division and Cell Cycle | 47 | 47 | 48 | 47 | 49 | 45 | 42 | 50 | 46 | 48 | 43 | 46 | 4 | 52 | 51 | 0 | 24 |
| Motility and Chemotaxis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 5 |
| Regulation and Cell signaling | 43 | 42 | 39 | 41 | 42 | 18 | 12 | 19 | 23 | 25 | 27 | 28 | 8 | 46 | 40 | 5 | 12 |
| Secondary Metabolism | 4 | 4 | 4 | 1 | 4 | 0 | 0 | 0 | 9 | 1 | 1 | 1 | 1 | 4 | 4 | 0 | 1 |
| DNA Metabolism | 96 | 94 | 98 | 106 | 114 | 87 | 79 | 138 | 118 | 98 | 73 | 73 | 46 | 89 | 89 | 52 | 60 |
| Fatty Acids, Lipids, and Isoprenoids | 76 | 76 | 76 | 73 | 76 | 79 | 82 | 61 | 70 | 51 | 49 | 46 | 23 | 81 | 85 | 12 | 36 |
| Nitrogen Metabolism | 5 | 6 | 5 | 0 | 4 | 10 | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 8 |
| Dormancy and Sporulation | 6 | 6 | 6 | 5 | 6 | 5 | 5 | 1 | 5 | 5 | 5 | 5 | 6 | 6 | 6 | 1 | 1 |
| Respiration | 21 | 21 | 23 | 23 | 26 | 28 | 14 | 18 | 3 | 12 | 11 | 9 | 12 | 27 | 25 | 8 | 11 |
| Stress Response | 63 | 63 | 61 | 45 | 56 | 55 | 52 | 32 | 38 | 33 | 26 | 26 | 5 | 64 | 63 | 6 | 28 |
| Metabolism of Aromatic Compounds | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 4 | 2 | 1 | 0 | 0 | 0 | 10 | 10 | 3 | 2 |
| Amino Acids and Derivatives | 169 | 169 | 157 | 137 | 141 | 183 | 162 | 124 | 124 | 52 | 98 | 98 | 86 | 222 | 228 | 158 | 187 |
| Sulfur Metabolism | 9 | 9 | 9 | 28 | 9 | 8 | 8 | 7 | 5 | 6 | 9 | 9 | 4 | 14 | 13 | 4 | 19 |
| Phosphorus Metabolism | 28 | 28 | 34 | 28 | 28 | 32 | 32 | 22 | 15 | 15 | 17 | 15 | 0 | 40 | 36 | 25 | 24 |
| Carbohydrates | 452 | 456 | 439 | 366 | 538 | 159 | 164 | 213 | 225 | 190 | 250 | 246 | 97 | 392 | 423 | 92 | 162 |
| **Total** | **1821** | **1836** | **1801** | **1695** | **1878** | **1465** | **1387** | **1349** | **1369** | **1194** | **1232** | **1220** | **666** | **1918** | **1928** | **735** | **1107** |

### 3.3.2. Investigation of RAST predicted virulence factors in CUL genomes

Following initial subsystem analysis, the virulence defence and disease category was further mined to investigate the virulence profiles of CUL strains (**Figure 3.1**). Overall, four main subcategories were predicted in CUL strains including, phages and prophages, toxins and superantigens, invasion and adhesion, and *S.pyrogenes* recombination zone (**Figure 3.1**).

The subcategory *S.pyrogenes* recombination zone consisted of two proteins, fibronectin-binding protein and chaperonin heat shock protein 33 (**Figure 3.1**). At least one of these genes was predicted within the genomes of all lactobacilli CUL strains (with none predicted within bifidobacteria isolates (**Figure 3.1**)).

Generally, *L.casei* CUL06 was predicted to encode the largest number of virulence factors (89), primarily consisting of phage elements (**Figure 3.1**). In contrast, both bifidobacteria strains *B.animalis* subsp. *lactis* CUL34 and *B.bifidum* CUL20, in addition to both *L.acidophilus* strains (CUL21 and CUL60) putatively encoded the smallest amount of virulence factors (12), with hits only predicting genes involved with invasion and intracellular interactions (**Figure 3.1**). Various components of phage elements, for example, phage major tail protein or phage capsid protein, were predicted in most CUL strains with high frequencies observed in *L.plantarum* CUL66 and CUL66N, *L.fermentum* CUL40 and CUL67, *L.rhamnosus* CUL63 and *L.gasseri* CUL09 (**Figure 3.1**). Interestingly, members of the *L.delbrueckii* group, including *L.acidophilus* strains CUL21 and CUL60, and *L.helveticus* CUL76 were predicted to encode fewer phage components in comparison to the *L.plantarum* group. However, *L.gasseri* CUL09 was an exception to this trend, as it appears to encode a larger number of phage components (**Figure 3.1**).

Overall, the most frequently predicted virulence gene in CUL strains, was a DNA-directed RNA polymerase beta subunit 1 (predicted 28 times in total). In contrast, individual phage elements (in several strains), streptolysin biosynthesis proteins (*L.helveticus* CUL76), quinolinate synthetase (*B.bifidum* CUL20), L-aspartate oxidase (CUL20) and quinolinate phosphoribosyltransferase (CUL20) were less frequently predicted, and typically unique to an individual CUL strain (**Figure 3.1**). Of interest, the protein DEDA, which is considered to have a putative role in invasion, is heavily predicted in *L.paracasei* strains in comparison to the other CUL strains. In addition, *L.helveticus* CUL76 was the only strain predicted to encode genes categorised as functional members of the toxin and superantigen subsystem, with protein annotations comprising of streptolysin biosynthesis proteins B, C and D (**Figure 3.1**).

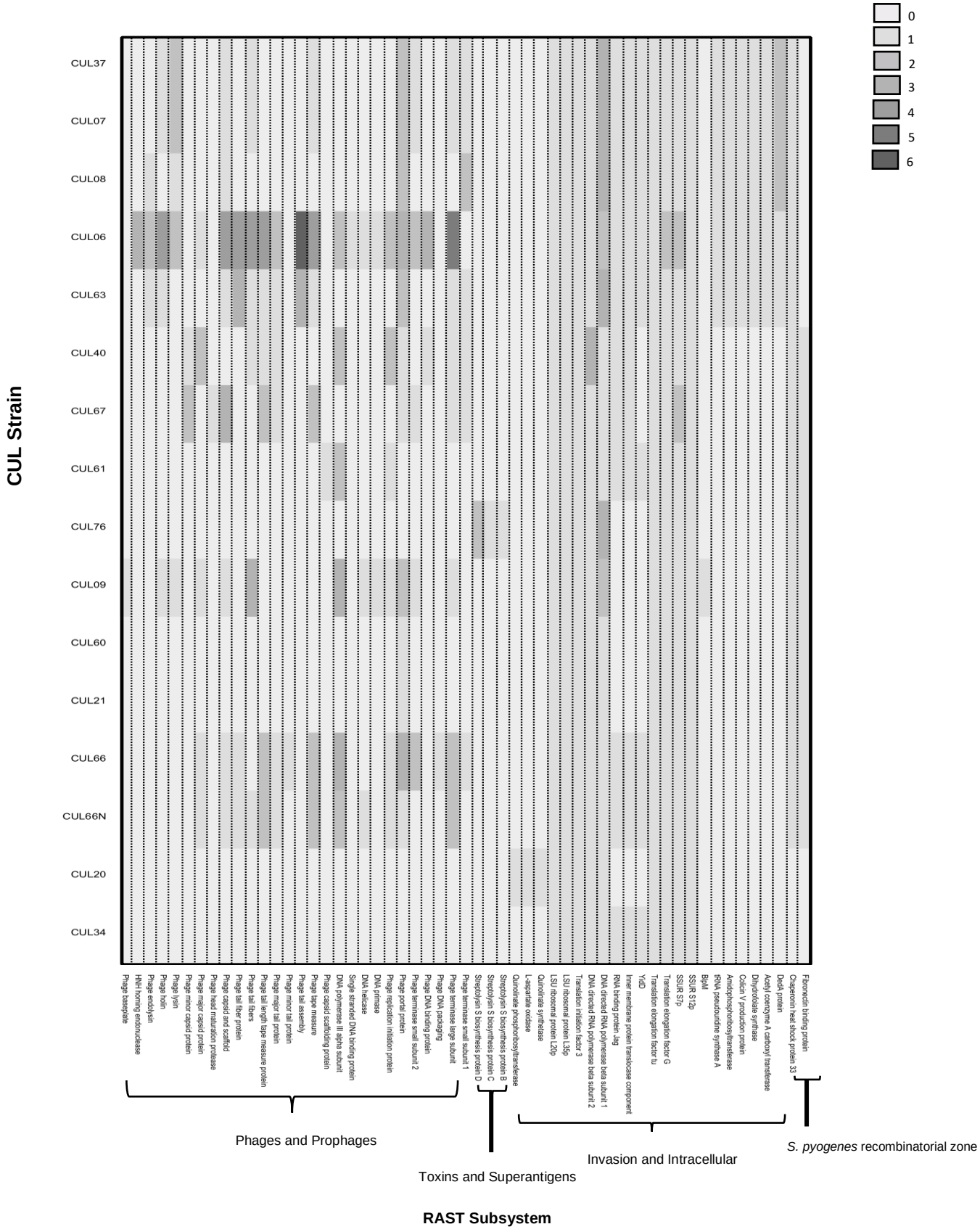



**Figure 3.1. RAST predicted virulence factors.** The number of protein-encoding genes that are functionally classified as virulence or defence protein by the RAST server within CUL strains. Genomic annotation is provided, and outer groups represent the subcategory to which proteins are allocated by RAST. An increase in colour density correlates with the number of predicted genes. Where the lightest grey equates to 0 predicted protein-encoding genes, and dark grey represents the maximum of 6 predicted protein-encoding genes.

### 3.3.3. Putative identification of Antibiotic Resistance Genes (ARG)

Initial genome mining via RAST subsystem profiling, revealed several putative ARGs (between 7 and 17) within each CUL genome (**Figure 3.2 A-B**). ARG profiles conferring specific ABR were grouped according to their antibiotic classification. Genes associated with tetracycline resistance were identified in all CUL genomes (**Figure 3.2A**) apart from the reference strain *L.acidophilus* ATCC4356. Specifically, most strains tentatively encoded a tet-like-2 ribosomal protection protein, except for members of the *L.casei* group (**Figure 3.2B**). The ARG composition of the *L.casei* group was predicted to encode identical drug resistance groups including beta-lactamase, fluoroquinolones, and multidrug-resistant efflux pumps. Of interest is the identification of multidrug efflux transporters in *L.paracasei* CUL07, which is the only anomaly in ARG composition profiles seen between the three *L.paracasei* strains (**Figure 3.2B**). The remaining CUL isolates putatively encode for similar ABR profiles, including tetracycline, fluoroquinolones, beta-lactamase (except for ATCC4356) and multidrug efflux pumps **(Figure 3.2A**). Additionally, genes involved with streptothricin resistance were also described in *L.helveticus* CUL76, and all *L.acidophilus* strains (CUL21, CUL60 and ATCC4356). The tentative ARG profiling in *L.acidophilus* CUL strains produced identical genetic profiles (**Figure 3.2 A-B**), however, for *L.acidophilus* ATCC4356 (type strain) genome mining revealed no genes associated with beta-lactamase resistance in comparison to CUL21 and CUL60 (**Figure 3.2 A-B**). Overall, the highest abundance of putative ARGs was identified in both *L.plantarum* strains CUL66 and CUL66N (both predicted to encode 17 ARGs). In contrast, *B.animalis* subsp. *lactis* CUL34 and type strain *L.acidophilus* ATCC4356 (which is a complete genome), presented with the lowest abundance of ARGs (7 and 8 respectively). Of interest is the vague and non-specific nature of the subsystem annotations in RAST which result in a virulence designation, such as *Streptococcus pneumonia* vancomycin tolerance locus, which suggests a specific ARG origin (**Figure 3.2A**).

The predicted ARG composition in bifidobacteria CUL isolates is highly varied. For *B.bifidum* CUL20, the total ARGs reported are split into 5 components: tetracycline and fluoroquinolones resistance, copper tolerance, vancomycin tolerance locus and multidrug efflux pumps. In contrast, *B.animalis* subsp. *lactis* CUL34's ARG profile was smaller and was only predicted to encode tetracycline and fluoroquinolone resistance.

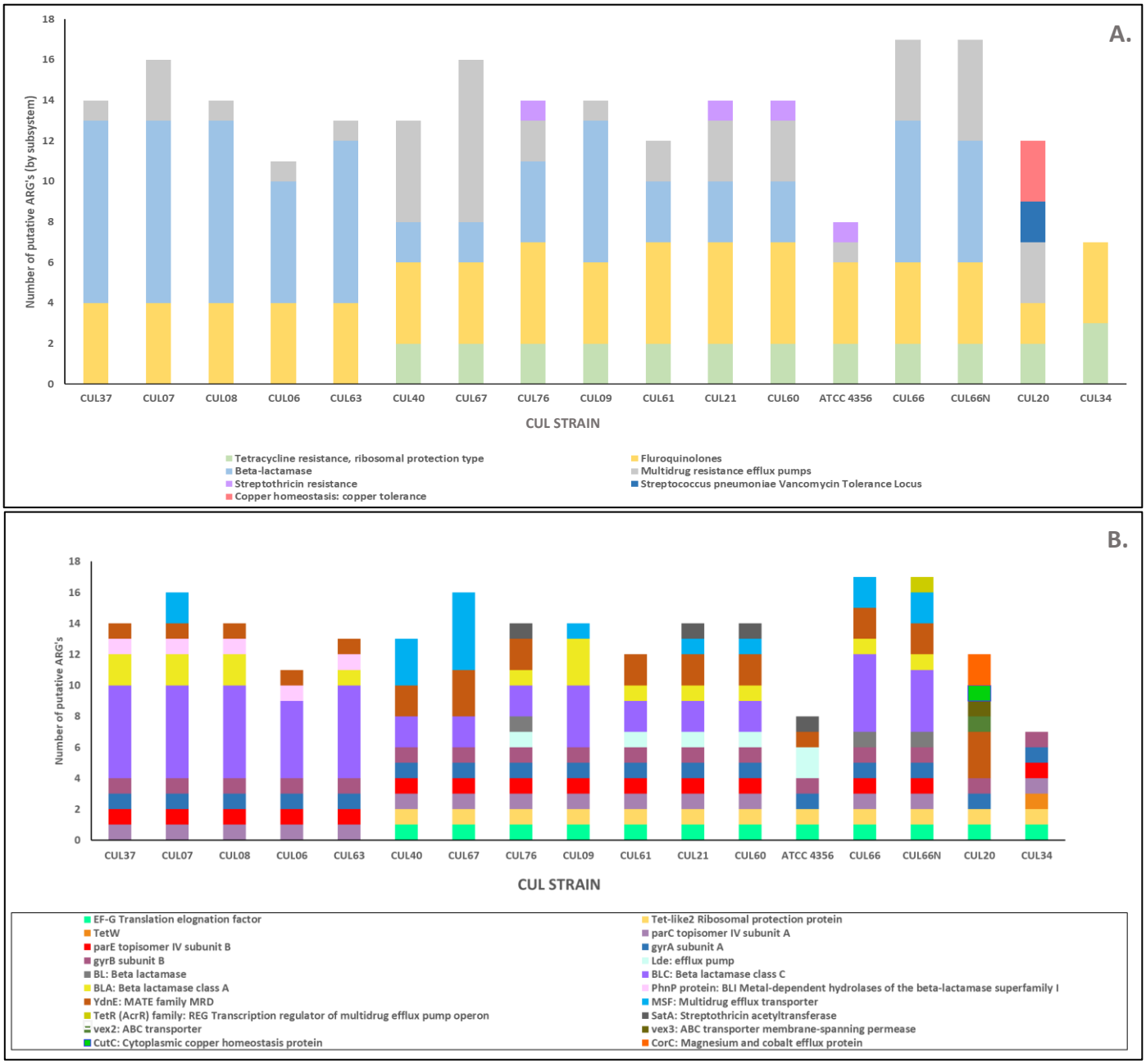



**Figure 3.2. ARG prediction in CUL isolates.** CUL strains were annotated in RAST where proteins were grouped into subsystems according to their physiological function. **A.** Proteins from the virulence subsystem relating to antibiotic resistance were grouped and tallied. **B.** The protein composition of the subsystems identified in A was then tallied in B to provide a more detailed overview of genomic function.

### 3.3.4. Determining phage presence in CUL strains

Following phage prediction in RAST and its categorisation as virulence factors (**Figure 3.1**), a more targeted analysis was conducted to deduce the presence of phages within CUL strains (**Table 3.5**). The Phage Search Tool (PHAST) database was employed to predict the presence of intact phages within CUL genomes. Of the 16 strains, 11 were predicted to encode intact prophages, including the maximum of 4 phages within *L.casei* (CUL06), and the minimum of 1 phage within *L.gasseri* CUL09, *L.helveticus* CUL76 and *L.rhamnosus* CUL63 (**Table 3.5**). *L.paracasei* strains were predicted to possess 2 (CUL37 and CUL08) or 3 (CUL07) phages, *L.plantarum* strains CUL66 and CUL66N were both predicted to encode 2 phages, whilst *L.salivarius* CUL61, *L.acidophilus* strains CUL21 and CUL60 and bifidobacteria isolates (CUL20 and CUL34) were not predicted to encode any intact phage's (**Table 3.5**).

All intact phages predicted in CUL06 were previously identified as lactobacilli phages (**Table 3.5**). All were putatively annotated as a *Lactobacillus* phage phiAT3. In contrast, *L.plantarum* strains had different phage predictions in comparison to each other (**Table 3.5**). *L.plantarum* CUL66 was predicted to encode phages; *Lactobacillus* Sha1 and *Enterococcus* EcZZ2, whereas CUL66N putatively revealed the presence of a *Bacillus* phage (VBBHas-171) and *Oenococcus* phage (phiS13) within its genome (**Table 3.5**). In fact, out of the 23 predicted phages in CUL genomes, 10 were matched with phages previously identified in other bacterial genera (**Table 3.5**).

**Table 3.5. Phage prediction in CUL genomes.** The presence of phages was analysed within CUL genomes via the PHAST pipeline. The completeness and annotation of the predicted phage are described, in addition to the region length.

| Species | CUL | Completeness | Region length (Kb) | Phage Name | GC (%) | Accession Number |
|---|---|---|---|---|---|---|
| *Lactobacillus paracasei* | CUL37 | intact | 42.8 | Streptococcus phage phiARI0746 | 44.7 | NC031907 |
| | | intact | 45.8 | Lactobacillus phage iLp84 | 44.92 | NC028783 |
| | | incomplete | 11.5 | Enterococcus phage Ec-ZZ2 | 45.71 | NC031260 |
| *Lactobacillus paracasei* | CUL07 | intact | 40.2 | Lactobacillus phage phiAT3 | 45.16 | NC005893 |
| | | intact | 45.8 | Lactobacillus phage iLp84 | 44.92 | NC028783 |
| | | intact | 40.8 | Staphylococcus phage SPbeta-like | 40.28 | NC029119 |
| | | incomplete | 14 | Enterococcus phage Ec-ZZ2 | 45.27 | NC031260 |
| *Lactobacillus paracasei* | CUL08 | intact | 36.1 | Streptococcus phage phiARI0746 | 45.09 | NC031907 |
| | | intact | 26 | Staphylococcus phage SPbeta-like | 43.45 | NC029119 |
| | | questionable | 18.1 | Lactobacillus phage J-1 | 45.5 | NC022756 |
| | | questionable | 13.8 | Enterococcus phage IME-EFm5 | 45.37 | NC028826 |
| | | incomplete | 6.4 | Staphylococcus phage SPbeta-like | 44.89 | NC029119 |
| *Lactobacillus casei* | CUL06 | intact | 33.1 | Lactobacillus phage phiAT3 | 45.22 | NC005893 |
| | | intact | 30.7 | Lactobacillus phage phiAT3 | 44.41 | NC005893 |
| | | intact | 36.1 | Lactobacillus phage phiAT3 | 42.05 | NC005893 |
| | | intact | 41 | Lactobacillus phage phiAT3 | 42.91 | NC005893 |
| | | questionable | 14.1 | Lactobacillus phage CL2 | 46.94 | NC028835 |
| | | questionable | 5.3 | Lactobacillus phage phiAT3 | 43.73 | NC005893 |
| | | incomplete | 35.3 | Bacillus phage Stahl | 48.23 | NC028856 |
| | | incomplete | 8.4 | Streptococcus phage phiARI0923 | 47.11 | NC030946 |
| | | incomplete | 7.4 | Synechococcus phage S-CBP1 | 42.26 | NC025456 |
| *Lactobacillus rhamnosus* | CUL63 | intact | 33.2 | Lactobacillus phage Lc-Nu | 41.79 | NC007501 |
| | | questionable | 13.7 | Staphylococcus phage tp310-3 | 45.08 | NC009763 |
| *Lactobacillus fermentum* | CUL40 | intact | 54.4 | Lactobacillus phage LF1 | 46.74 | NC019486 |
| | | intact | 16.8 | Staphylococcus phage SPbeta-like | 42.49 | NC029119 |
| | | intact | 16.6 | Staphylococcus phage SPbeta-like | 41.24 | NC029119 |
| | | questionable | 39 | Staphylococcus phage SPbeta-like | 49.17 | NC029119 |
| | | incomplete | 7.4 | Bacillus phage SP-15 | 52.69 | NC031245 |
| | | incomplete | 20.8 | Lactobacillus phage LF1 | 43.13 | NC019486 |
| *Lactobacillius fermentum* | CUL67 | intact | 39.7 | Lactobacillus phage LF1 | 46.11 | NC019486 |
| | | intact | 26.5 | Staphylococcus phage SPbeta-like | 41.85 | NC029119 |
| | | questionable | 29.1 | Bacillus phage WBeta | 46.69 | NC007734 |
| | | questionable | 8.9 | Staphylococcus phage SPbeta-like | 44.23 | NC029119 |
| | | questionable | 10 | Staphylococcus phage SPbeta-like | 37.08 | NC029119 |
| *Lactobacillius salivarius* | CUL61 | incomplete | 17.1 | Geobacillus virus E3 | 31.33 | NC029073 |
| | | incomplete | 9.7 | Staphylococcus phage phiSa119 | 29.81 | NC025460 |
| | | incomplete | 5.9 | Bacillus phage SPBc2 | 42.21 | NC001884 |
| *Lactobacillus helveticus* | CUL76 | intact | 8.7 | Lactobacillus phage Lb338-1 | 41.05 | NC012530 |
| | | incomplete | 7.8 | Lactococcus phage 1358 | 36.23 | NC027120 |
| *Lactobacillus gasseri* | CUL09 | intact | 57.1 | Lactobacillus phage KC5a | 35.7 | NC007924 |
| | | questionable | 41.5 | Gordonia phage Wizard | 40.58 | NC030913 |
| | | incomplete | 21.8 | Bacillus phage G | 32.42 | NC023719 |
| | | incomplete | 8.2 | Enterococcus phage phiEF24C | 34.08 | NC009904 |
| *Lactobacillus acidophilus* | CUL60 | incomplete | 26 | Lactobacillus prophage Lj965 | 33.41 | NC005355 |
| *Lactobacillus acidophilus* | CUL21 | incomplete | 26 | Lactococcus phage 50101 | 33.41 | NC031040 |
| *Lactobacillus plantarum* | CUL66 | intact | 54.9 | Lactobacillus Sha1 | 40.68 | NC019489 |
| | | intact | 83.9 | Enterococcus EcZZ2 | 41.49 | NC031260 |
| *Lactobacillus plantarum* | CUL66N | intact | 39.8 | Bacillus phage vBBhaS-171 | 40.85 | NC030904 |
| | | intact | 34.2 | Oenococcus phage phiS13 | 42.93 | NC023560 |
| | | incomplete | 8.8 | Oenococcus phage phiS13 | 37.53 | NC023560 |
| | | incomplete | 16.4 | Lactobacillus phage phig1e | 39.76 | NC004305 |
| *Bifidobacterium bifidum* | CUL20 | - | - | - | - | - |
| *Bifidobacteria animalis subsp lactis* | CUL34 | incomplete | 6.7 | Gordonia phage Gsput1 | 61.54 | NC030932 |

### 3.3.5. Genomic islands (GIs)

To investigate the fluidity of CUL genomes, Genomic Islands were predicted via islandviewer4 (**Table 3.6**). The number of predicted GIs in CUL strains ranged from 6 (*L.acidophilus* CUL21) to 27 (*L.fermentum* CUL67). GIs were subsequently manually analysed for regions of 'interest'.

*L.paracasei* strains (CUL37, CUL07 and CUL08) were predicted to encode 23, 19 and 18 GIs respectively (**Table 3.6**). Within *L.paracasei* CUL37 the size of the genomic islands varied from 2 genes – 126 genes (**A3 Table S3.1**). Interestingly, GI 18 was the largest and encoded several genes which may aid in host colonisation, including, chitinase, DPS, collagen adhesion protein, sortease A, sialic acid regulation and fosmidomycin resistance. In addition, two transposon elements were also predicted within GI 18. For *L.paracasei* CUL07 the size of GIs ranged from 4 – 123 genes (**A3 Table S3.2**). Likewise, the largest genomic island, GI 15, also encoded similar host colonisation properties to CUL37, including chitinase, collagen adhesion proteins, DPS, sortease A and transposon elements (TrsK and Tn5252). Two large islands were predicted to encode several phage elements (GI 5 and GI 7) corresponding with the two phages' predicted by PHAST. Interestingly GI 19 was predicted to contain lactate monooxygenase (**A3 Table S3.3**). For *L.paracasei* CUL08, GI sizes ranged from 5 – 79 genes. GI 4 and GI 5 were both predicted to encode phage elements corresponding with the number of predicted phages via PHAST. Two large genomic islands GI 1 (61 genes) and GI 16 (79 genes) were also predicted to encode host colonisation traits such as DPS, chitinase and D-lactate dehydrogenase (**A3 Table S3.3**). *L.casei* CUL06 was predicted to encode 23 (**Table 3.6**), GIs ranging from 4 (GI 10) to 90 (GI 1) genes in size. 9 GIs (GI 1 - 4, 6, 9,16, 21 and 22) consisted of mainly phage elements. Interestingly GI 19 and GI 20 were both predicted to encode a D-lactate dehydrogenase and in GI 11, genes involved in bacteriocins, transposable elements (Tn916) and P60 (**A3 Table S3.4**).

*L.rhamnosus* CUL63 was predicted to encode 16 GIs (**Table 3.6**) ranging from 3 (GI 11) to 65 (GI 10) genes in size (**A3 Table S3.5**). GI 10 typically consisted of adhesion proteins including surface anchor, sortease A and FPXTG motifs. In addition, GI 10 also putatively encoded a Na+ H+ antiporter which has been associated with acid tolerance. Two GIs were predicted to encode phage elements (GI 1 and GI 12) and an additional acid tolerance-related gene was identified on GI 13 (**A3 Table S3.5**).

*L.fermentum* CUL40 and CUL67 were predicted to contain the largest number of GIs out of all the CUL strains (33 and 27 respectively (**Table 3.6**)). The size of *L.fermentum* CUL40's GIs ranged from 2 (GI 19) to 100 (GI 27) genes (**A3 Table S3.6**). Several resistance genes were identified on GIs, including drug-resistant transporter (*ermB*), ABC transporter (GI 8), arsenic resistance (GI 13 and 14) and *tetR* (GI 27). In addition, many islands were predicted to contain

mobile elements including GI 1, 5, 23, 27, and 29). Interestingly, D-lactate dehydrogenase was encoded on GI 25 and a heat-inducible chaperone on GI 9 (**A3 Table S3.6**).

The GIs predicted in *L.fermentum* CUL67 ranged from 2 (GI 20) to 141 genes (GI 18). Interestingly, the largest GI (18) was predicted to encode several mobile elements and transporters (**A3 Table S3.7**). GI (27) was also predicted to encode an exopolysaccharide biosynthesis protein. Intriguingly, despite *L.fermentum* CUL strains a large number of GIs and the prediction of 3 and 2 phages within CUL40 and CUL67 genomes respectively, only one GI (GI 5) seemed to encode mainly phage-related elements in CUL67 and one in CUL40 (GI 22).

Members of the *Lactobacillus delbrueckii* phylogroup *L.gasseri* (CUL09) and *L.acidophilus* (CUL21 and CUL60) typically encoded a smaller number of GIs (15, 9 and 10 respectively) in comparison to other lactobacilli CUL species, except for *L.helveticus* (CUL76) which was predicted to possess 25 GIs (**Table 3.6**). For *L.gasseri* (CUL09) the size of the GIs ranged from 2 (GI 13) to 66 (GI 9) genes. Four GIs mainly consisted of phage elements (GI 1, 2, 5 and 6). Interestingly, pyruvate oxidase was identified on GI 14 (**A3 Table S3.9**). In *L.acidophilus* CUL21 GIs ranged from 6 (GI 1) to 38 (GI 5) genes. A penicillin-binding protein was identified on GI 2. In *L.acidophilus* CUL60, GIs ranged from 2 (GI 3) to 37 (GI 6) genes. GI 2 also putatively encoded a penicillin-binding protein (**A3 Table S3.12**). *L.helveticus* CUL76 GI sizes ranged from 2 (GI 21, 22 and 23) to 73 (GI 18). In agreement with the putative identification via RAST subsystem analysis (**Figure 3.1**), a streptolysin S biosynthesis protein was identified on GI 6. GI 13 generally encoded host interaction mechanisms including biotin, niacin transport and fatty acid synthesis (**A3 Table S3.8**). The largest GI 18 identified several putative AMR proteins, including *tetR*, cobalt-zinc resistance, and beta-lactamase. Phage elements were predicted on GI3, 18 and 19. GI 25 mainly consisted of genes associated with mobile elements.

*L.salivarius* CUL61 was predicted to encode 11 GIs (**Table 3.6**), ranging from 2 (GI 10) to 35 (GI 9) genes. The largest GIs mainly consisted of hypothetical proteins. In addition, a cholylglycine hydrolase protein was also identified on GI 3 (**A3 Table S3.10**).

*L.plantarum* strains CUL66 and CUL66N were predicted to encode 14 and 22 GIs respectively (**Table 3.6**). For CUL66, GI size ranged in size from 2 (GI 13) to 76 (GI 11) genes. Phage elements were predicted within 3 GIs (6, 8 and 9). Genes with predicted arsenic resistance function were in the largest GI (11) (**A3 Table S3.13**). For *L.plantarum* CUL66N, GI size ranged from 1 (GI 16) to 71 (GI 15) genes (**A3 Table S3.14**). Phage proteins were identified on GI 15, 17 and 18. GI 19 a relatively large GI, was made up of 48 genes which putatively encoded several hosts – microbe interaction factors including folate transporters, HK, clpL, lactate – 2 – monooxygenase, pyruvate oxidase and chitinase (**A3 Table S3.14**).

*B.bifidum* CUL20 was predicted to encode 11 GIs ranging from 7 (GI 7 and 8) to 17 (GI 6) genes (**Table 3.6**). GI 1 is comprised of 26 genes with several transposes and ABC transporters predicted. GI 4 is made up of 17 genes and interestingly identifies a cholylglycine hydrolase within its genomic architecture (**A3 Table S3.15**). *B.animalis* subsp. *lactis* CUL34 was predicted to encode 9 GIs, ranging from 3 (GI 4) to 15 (GI 1) genes in size. Interestingly *tetW* was recognised within GI 1 and a CRISPR region in GI 3. No phages were identified within any GIs identified in bifidobacteria CUL strains (**A3 Table S3.16**).

**Table 3.6. The number of intact phages and genomic islands predicted within CUL strains.** Phage prediction was conducted via the PHAST pipeline and the number of intact phages is summarised. GI prediction was performed by islandviewer4 and manually curated to infer the number of GI regions.

| Genus | Species | Strain | N of GIs | N of Phages |
|---|---|---|---|---|
| *Lactobacillus* | *paracasei* | CUL37 | 23 | 2 |
| | *paracasei* | CUL07 | 19 | 3 |
| | *paracasei* | CUL08 | 18 | 2 |
| | *casei* | CUL06 | 21 | 4 |
| | *rhamnosus* | CUL63 | 16 | 1 |
| | *fermentum* | CUL40 | 33 | 3 |
| | *fermentum* | CUL67 | 27 | 2 |
| | *salivarius* | CUL61 | 11 | 0 |
| | *helveticus* | CUL76 | 25 | 1 |
| | *gasseri* | CUL09 | 15 | 1 |
| | *acidophilus* | CUL60 | 10 | 0 |
| | *acidophilus* | CUL21 | 9 | 0 |
| | *plantarum* | CUL66 | 14 | 2 |
| | *plantarum* | CUL66N | 22 | 2 |
| *Bifidobacterium* | *bifidum* | CUL20 | 9 | 0 |
| | *animalis* subsp. *lactis* | CUL34 | 12 | 0 |

### 3.3.6. Investigating the presence of GOI within CUL strains

Following an investigation of the literature for genes which may translate to functional attributes for host colonisation, beneficial attributes and safety features, genomes of CUL strains were mined for GOI. The GOI is broken down into the proposed relevant function/safety concern including the following: acid tolerance, bile tolerance/interaction, stress response, adherence, virulence, including AMR, mobile elements and host-microbe interactions (**Table 3.7**).

#### 3.3.6.2. *Acid and bile tolerance*

All CUL strains putatively encoded multiple copies of L-lactate dehydrogenase, ranging from 2 (*B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34) to 6 in *L.plantarum* strains (CUL66 and CUL66N (**Table 3.7**)). In addition, all lactobacilli strains possessed genes encoding for the optical isomer D-lactate dehydrogenase (Desriac et al., 2013). Whilst mining for such traits, lactate oxidase was also recognised in several lactobacilli CUL strains (including all members of the *L.casei* group, *L.gasseri* CUL09 and both *L.plantarum* strains CUL66 and CUL66N), a gene with a putative role in hydrogen peroxide production (Seki et al., 2004). All CUL strains (except for *L.gasseri* CUL09, which was not intact) possessed multiple genes coding for F0F1-type ATP synthase operon, which has a suggested role in acid tolerance (according to RAST). Furthermore, bifidobacteria strains (*B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34) additionally encoded a gene relating to a Na+/H+ NhaA antiporter (Desriac et al., 2013).

Genes *dltD* and *dltA* have been shown to have functional capabilities in acid and defensin resistance (Goel et al., 2020). All lactobacilli CUL strains were predicted to encode at least one copy of *dltD*, and a maximum of 2 (*L.salivarius* CUL61 (**Table 3.7**)). Additionally, all strains except for *L.acidophilus* CUL21, *B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34 were predicted to encode *dltA*. Furthermore, *clpA* reportedly has an active role in both acid and bile tolerance (Goel et al., 2020). All strains were predicted to encode multiple copies of *clp*, with a minimum copy number of 2 (*L.plantarum* CUL66) and a maximum of 7 (*L.salivarius* CUL61 (**Table 3.7**)).

The cholylglycine hydrolase family have a putative role in bile tolerance (Begley et al., 2006). *L.fermentum* strains CUL67 and CUL40 possessed 1 and 2 copies respectively (**Table 3.7**). *L.salivarius* CUL61 2 copies (corresponding to the previous prediction of one cholylglycine hydrolase encoded on a GI), *L.gasseri* CUL09 3 copies, *L.helveticus* CUL76 1 copy, *L.acidophilus* CUL21 and CUL60, 2 copies and *L.plantarum* CUL66 and CUL66N 3 copies. A single copy of cholylglycine hydrolase was predicted in both bifidobacteria strains (**Table 3.7**). Additionally, L-linear amide CN was predicted in *L.plantarum* strains (CUL66 and CUL66N)

and conjugated bile salt hydrolase-related amidase (all *L.casei* phylogroup (**Table 3.7**)). Glucosamine -6- phosphate deaminase has a putative role in bile tolerance (Alcántara & Zúñiga, 2012). All CUL strains apart from *L.fermentum* strains (CUL67 and CUL40) and *B.animalis* subsp. *lactis* CUL34 were predicted to encode a copy of this gene (**Table 3.7**).

#### 3.3.6.3. *Stress tolerance*

All CUL strains possessed universal stress-related protein (Gomes et al., 2011), ranging from 1 (in bifidobacteria strains) to 4 copies (in *L.fermentum* strains CUL40 and CUL67) **Table 3.7**). In addition, DPS proteins related to stress tolerance (Facey et al., 2009, 2011), were also predicted in all lactobacilli CUL strains, ranging from 1 copy to two copies (in *L.paracasei* strains CUL07 and CUL08, *L.rhamnosus* CUL63, *L.helveticus* CUL76 and *L.plantarum* CUL66N (**Table 3.7**)).

#### 3.3.6.4. *Adherence*

Several adhesion factors were predicted in CUL strains, including enolase, *strA* (Buck et al., 2005) and Fibronectin Binding Proteins (FBP) Buck et al., 2005; Hymes et al., 2016) in all lactobacilli strains (**Table 3.7**). In addition, a collagen-binding protein (CBP (Yadav et al., 2013)) was identified in *L.paracasei* CUL37 and *L.plantarum* CUL66N. Furthermore, slpA (Buck et al., 2005) was predicted in *L.helveticus* CUL76, *L.acidophilus* CUL21 and CUL60 and MSA (Buck et al., 2005) were also predicted in several strains. Interestingly, *L.paracasei* CUL07 and *L.casei* CUL06 were the only two of the *L.casei* group to encode MSA (**Table 3.7**).

#### 3.3.6.5. *Specific virulence traits*

The RAST subsystem analysis predicted the presence of a mycobacterium virulence operon in all CUL strains. A genetic basis of biogenic amines production, *panD* (Evanovich et al., 2019) was not identified in any CUL strains (**Table 3.7**). Genes related to ABR, such as tetracycline resistance, were putatively identified including *tetW* in *B.animalis* subsp. *lactis* CUL34 (in agreement with RAST analysis **Figure 3.2**). Phage portal proteins are associated with mobile elements. No homologs to such genes were identified in CUL strains.

### 3.3.6.6. *Health-promoting attributes*

All CUL strains were shown to encode genes involved with short-chain fatty acid production (Choi et al., 2021). Of interest, all *L.paracasei* strains CUL37, CUL07 and CUL08 encoded genes involved in the generation of acetate, butyrate, and formate (**Table 3.7**).

All CUL strains harboured at least one gene encoding an enzyme involved in antioxidant production (Glutathione peroxide, glutathione catalase and glutathione reductase (Choi et al., 2021)), except for *L.salivarius* CUL61 and both bifidobacteria strains *B.animalis* subsp. *lactis* CUL34 and *B.bifidum* CUL20 (**Table 3.7**)**.** Of interest, *L.plantarum* CUL66 encoded all three of the antioxidant enzymes that were used as queries (**Table 3.7**). *L.plantarum* CUL66 and CUL66N, and *L.fermentum* CUL40 (interestingly absent from *L.fermentum* CUL67) were shown to carry glutamate decarboxylate homologs (Choi et al., 2021).

Both *L.acidophilus* strains CUL21 and CUL60 were predicted to carry copies of lactocepin encoding genes (**Table 3.7**). Lactocepin S-layer protein is an extracellular protease with documented anti-inflammatory capabilities (Salvetti & O'Toole, 2018).

The protein P40 has been associated with maintaining host intestinal immunological homeostasis (Salvetti & O'Toole, 2018). All the CUL strains within the *L.casei* phylogroup were predicted to encode P40 homologs (**Table 3.7**). Endopeptidase P60 has been proposed as a contributing protein that aids in intestinal survival (Heo et al., 2018). The majority of CUL strains were predicted to encode copies of P60, except for *L.fermentum* strains CUL40 and CUL76, *L.plantarum* strains CUL66 and CUL66N and *L.helveticus* CUL76 (**Table 3.7**).

**Table 3.7. Gene of Interest prediction in CUL strains.** Genomic traits identified in the literature were mined via a combination of BLASTp and BLASTn.

| Category/Role | EC Number* | Proposed Relevance | CUL37 | CUL07 | CUL08 | CUL06 | CUL63 | CUL40 | CUL67 | CUL61 | CUL09 | CUL76 | CUL21 | CUL60 | CUL66 | CUL66N | CUL20 | CUL34 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L-Lactate dehydrogenase | 1.1.1.27 | Acid tolerance | 4 | 4 | 4 | 4 | 4 | 5 | 4 | 3 | 3 | 3 | 3 | 3 | 6 | 6 | 2 | 2 |
| L-Lactate dehydrogenase (Glycolate oxidase) | 1.1.2.3 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| D-Lactate dehydrogenase | 1.1.1.28 | Acid tolerance | 2 | 2 | 2 | 2 | 2 | 4 | 4 | 3 | 1 | 2 | 1 | 1 | 2 | 2 | 0 | 0 |
| L-Lactate oxidase | | pyruvate and H2O2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| F0F1-type ATP synthase: | | Acid tolerance | | | | | | | | | | | | | | | | |
| α-chain | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| β-chain | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| γ-chain | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| δ-chain | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| ε-chain | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| F0 sector subunit a | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| F0 sector subunit b | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| F0 sector subunit c | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Na+/H+ NhaA antiporter | | Acid tolerance | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| DLTd | | Acid and defensin resistance | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| DLTa | | Acid and defensin resistance | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |
| clpL (clpA) | | Acid and Bile tolerance | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 7 | 3 | 4 | 3 | 3 | 2 | 6 | 3 | 3 |
| Choloylglycine hydrolase | 3.5.1.24 | Bile tolerance | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 2 | 3 | 1 | 2 | 2 | 3 | 3 | 1 | 1 |
| L-linear amide C-N | | Bile interactions | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Conjugated bile salt hydrolase related amidase | | Bile interactions | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Glucosamine-6-phosphate deaminase | 3.5.99.6 | Bile tolerance | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Non-specific DNA-binding protein (DPS) | | DNA binding/Stress response | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | 0 | 0 |
| Universal stress protein | | Stress response | 3 | 3 | 3 | 2 | 2 | 4 | 4 | 3 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 |
| Enolase | 4.2.1.11 | Adherence | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 3 | 1 | 1 |
| Fibronectin-binding protein | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 0 | 0 |
| mucus binding protein (gram positive anchor domain) | | | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 |
| strA | | | 1 | 1 | 2 | 1 | 4 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 2 |
| cbP | | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| msA | | | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 1 | 0 | 1 | 1 | 2 | 2 | 0 | 0 |
| slpA | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 2 | 0 | 0 | 0 | 0 |
| Mycobacterium virulence operons: | | Virulence | | | | | | | | | | | | | | | | |
| SSU ribosomal protein S7p (S5e) | | | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| SSU ribosomal protein S12p (S23e) | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| LSU ribosomal protein L35p | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| LSU ribosomal protein L20p | | | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| DNA-directed RNA polymerase β-subunit | 2.7.7.6 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Translation initiation factor 3 | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Translation elongation factor Tu | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Translation elongation factor G | | Virulence/Antibiotic resistance | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| panD | | Biogenic amines | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ribosome protection-type tetracycline resistance related proteins | | Antibiotic resistance | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 |
| Tetracycline Resistance (tetW) | | Antibiotic resistance | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| nsr | | Nisin-resistance protein | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Phage portal protein | | Mobile element | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| formate C-acetyltransferase | 2.3.1.54 | short chain fatty acid production | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 2-acetate kinase | | | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 2 | 2 | 5 | 5 | 4 | 3 | 3 | 1 | 1 |
| butyrate kinase | | | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| glutathione peroxidase (POD) | | Antioxidant production | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| glutathione reductase | | | 2 | 2 | 2 | 2 | 1 | 1 | 2 | 0 | 1 | 2 | 1 | 1 | 4 | 5 | 0 | 0 |
| glutathione catalase | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| glutamate decarboxylase | 4.1.115 | Host-microbe interactions | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| lactocepin proteins | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| p40 | | | 2 | 2 | 2 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| p60 | | | 1 | 1 | 2 | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 3 | 4 | 0 | 0 | 1 | 1 |
| hk7 | | Bacteriocin regulation | 1 | 2 | 2 | 2 | 2 | 4 | 2 | 2 | 4 | 2 | 4 | 4 | 1 | 5 | - | - |
| hk6 | | | 0 | 1 | 1 | 1 | 2 | 1 | 0 | 2 | 0 | 0 | 1 | 1 | 1 | 1 | - | - |
| hpk11 | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | - | - |

* - Gene function may not be conserved in the *Bifidobacterium* genus.

## Table 3.8. Specific genome mining. Traits were identified and selected from close relatives of CUL strains (via MLSA).

| CUL | ML Neighbour | Traits for Mining | AC. Number | Locus Tag | Proposed Relevance | P/A | Reference |
|---|---|---|---|---|---|---|---|
| *Lactobacillus gasseri* CUL09 | *Lactobacillus gasseri* K7 | *Lactobacillus gasseri* gassericin K7 A | EF392861.1 | | Bacterocin production | ✓ | Peternel, Metoda Zorič, Andreja Čanžek Majhenič, Helge Holo, Ingolf F. Nes, Zhian Salehian, Aleš Berlec, and Irena Rogelj. "Wide-inhibitory spectra bacteriocins produced by Lactobacillus gasseri K7." Probiotics and antimicrobial proteins 2, no. 4 (2010): 233-240. |
| | | *Lactobacillus gasseri* gassericin K7 B | AY307382.1 | | | ✓ | |
| *Lactobacillus acidophilus* CUL21 | Whole species | NADH-dependent flavin reductase subunit 1 | Q74HL7.1 | LJ 1853 | Hydrogen peroxide production | ✓ | Hertzberger, R., Arents, J., Dekker, H.L., Pridmore, R.D., Gysler, C., Kleerebezem, M. and de Mattos, M.J.T., 2014. H2O2 production in species of the Lactobacillus acidophilus group: a central role for a novel NADH-dependent flavin reductase. Applied and environmental microbiology, 80(7), pp.2229-2239. |
| | | Pyruvate oxidase | EC 1.2.3.3 | | | ✓ | |
| *Lactobacillus acidophilus* CUL60 | Whole species | NADH-dependent flavin reductase subunit 1 | Q74HL7.1 | | Hydrogen peroxide production | ✓ | Hertzberger, R., Arents, J., Dekker, H.L., Pridmore, R.D., Gysler, C., Kleerebezem, M. and de Mattos, M.J.T., 2014. H2O2 production in species of the Lactobacillus acidophilus group: a central role for a novel NADH-dependent flavin reductase. Applied and environmental microbiology, 80(7), pp.2229-2239. |
| | | Pyruvate oxidase | EC 1.2.3.3 | LJ 1853 | | ✓ | |
| *Lactobacillus helveticus* CUL76 | *Lactobacillus helveticus* R0052 | Mucus binding protein precursor Mub | AFR22220.1 | R0052 07110 | Adhesion | ✓ | Tompkins, T.A., Barreau, G. and Broadbent, J.R., 2012. Complete genome sequence of Lactobacillus helveticus R0052, a commercial probiotic strain.hat this particular strain is able to persist in the gut..e00725-14 |
| | | Mucus binding protein | AFR22221.1 | R0052 07115 | | ✓ | |
| | | Mucus binding protein | AFR22068.1 | R0052 06155 | | ✓ | |
| | | Mucus binding protein precursor | AFR21758.1 | R0052 04290 | | ✓ | |
| | | Mucus binding protein precursor | AFR21759.1 | R0052 04295 | | ✓ | |
| | | Protein precursor | AFR21760.1 | R0052 04300 | | ✓ | |
| *Lactobacillus fermentum* CUL40 | *Lactobacillus fermentum* CRL1446 | Alpha/beta hydrolase | PNV58462.1 | | Biocatalysts | ✓ | Abeijón Mukdsi, María C., Lucila Saavedra, María P. Gauffin Cano, Elvira M. Hebert, and Roxana B. Medina. "Draft Genome Sequence of the Feruloyl Esterase-Producing Strain Lactobacillus fermentum CRL1446, a Probiotic for Malnutrition." Genome announcements 6, no. 21 (2018): e00225-18. |
| | | Feruloyl esterase | AOR52356.1 | | Antioxidant | ✓ | |
| *Lactobacillus paracasei* CUL08 | *Lactobacillus paracasei* L9 | Malolactic enzyme protein | AKU58851.1 | LPL9 0797 | Bile tolerance | ✓ | Rema, Xiayin, Guohong Wang, Zhengyuan Zhai, Pengyu Zhou, and Yanling Hao. "Global transcriptomic analysis and function identification of malolactic enzyme pathway of Lactobacillus paracasei L9 in response to bile stress." Frontiers in microbiology 9 (2018): 1978. |
| | | Malate transporter protein | AKU58852.1 | LPL9 0798 | Bile tolerance | ✓ | |
| | | Collagen-binding protein | AKU58700 | LPL9 0646 | Adhesion | ✓ | |
| | | Fibronectin-binding protein A | AKU59633 | LPL9 1579 | Adhesion | ✓ | |
| | | Surface antigen | AKU58074 | LPL9 0020 | host immune regulation | ✓ | |
| | | Hypothetical protein | AKU58079 | LPL9 0025 | Adhesion | ✓ | |
| | | Surface antigen | AKU60169 | LPL9 2115 | host immune regulation | ✓ | |
| *Bifidobacterium bifidum* CUL20 | *Bifidobacterium bifidum* S17 | Peptide/nickel transport system, permease protein | ADO52838.1 | BBIF_0633 | Adhesion | ✓ | Gleinser, Marita, Verena Grimm, Daria Zhurina, Jing Yuan, and Christian U. Riedel. "Improved adhesive properties of recombinant bifidobacteria expressing the Bifidobacterium bifidum-specific lipoprotein BopA." Microbial cell factories 11, no. 1 (2012): 1-14. |
| | | Peptide/nickel transport system, permease protein | ADO52839.1 | BBIF_0634 | | ✓ | |
| | | Peptide/nickel transport system, ATP-binding protein | ADO52840.1 | BBIF_0635 | | ✓ | |
| | | Peptide/nickel transport system, extracellular solute-binding protein | ADO52841.1 | BBIF_0636 | | ✓ | |
| | | Bleomycin hydrolase C | ADO52842.1 | BBIF_0637 | | ✓ | |
| Bifidobacterium animalis subsp *lactis* CUL34 | *Bifidobacterium animalis* subsp. *lactis* A6 | Aminotransferase class I/II-fold pyridoxal phosphate-dependent enzyme | WP004268678 | BAA6 0439 | Acid Tolerance | ✓ | Sun, Erna, Liang Zhao, Fazheng Ren, Songling Liu, Ming Zhang, and Huiyuan Guo. "Complete genome sequence of Bifidobacterium animalis subsp. lactis A6, a probiotic strain with high acid resistance ability." Journal of biotechnology 200 (2015): 8-9. |
| | | Ystathionine gamma-synthase | WP004218806 | BAA6 0571c | | ✓ | |
| | | Pyridoxal-phosphate dependent enzyme | WP004218807 | BAA6 0572 | | ✓ | |
| | | Basic amino acid/polyamine antiporter | WP004218183 | BAA6 1282b | | ✓ | |
| | | Formyl-CoA transferase | WP004219152 | BAA6 1441 | | ✓ | |
| | | Oxalyl-CoA decarboxylase | WP004219148 | BAA6 1444 | | ✓ | |

### 3.3.7. Phylogenetically targeted genome mining

The literature was mined for putative traits which may be conserved within the evolutionary lineage (identified in Chapter 2). *L.gasseri* CUL09 was shown to share a common bacteriocin with *L.gasseri* K9 ((Peternel et al., 2010) **Table 3.8**). Both *L.acidophilus* strains were predicted to encode genes that confer hydrogen peroxide-producing capabilities (**Table 3.8**), which is shared with the whole clade (Hertzberger et al., 2014). Predicted cellular adhesion genes were recognised in *L.helveticus* R0052 (Tompkins et al., 2012) including several mucus binding proteins, which appear to be conserved in the lineage from which CUL76 emerges (**Table 3.8**). *L.paracasei* CUL08 shares a MRCA with *L.paracasei* L9. Functions including adhesion, bile tolerance and host immune regulation were identified in L9 (Ma et al., 2018) and predicted for in *L.paracasei* CUL08 (**Table 3.8**). An intact Exopolysaccharide (EPS) operon consisting of 18 genes was also conserved in *L.paracasei* L9 and CUL08 (**Figure 3.3**). In addition, a close relative of *B.bifidum* CUL20, *B.bifidum* S17 has documented operon encoding adhesion properties, which are also intact within CUL20 (**Table 3.8**). Similarly, *B.animalis* subsp. *lactis* A6 (which shares a MRCA with *B.animalis* subsp. *lactis* CUL34) encodes an operon which confers high acid tolerance capabilities (Sun et al., 2015), comparative BLASTp analysis revealed that this operon was conserved in *B.animalis* subsp. *lactis* CUL34 (**Table 3.8, Figure 3.4**).

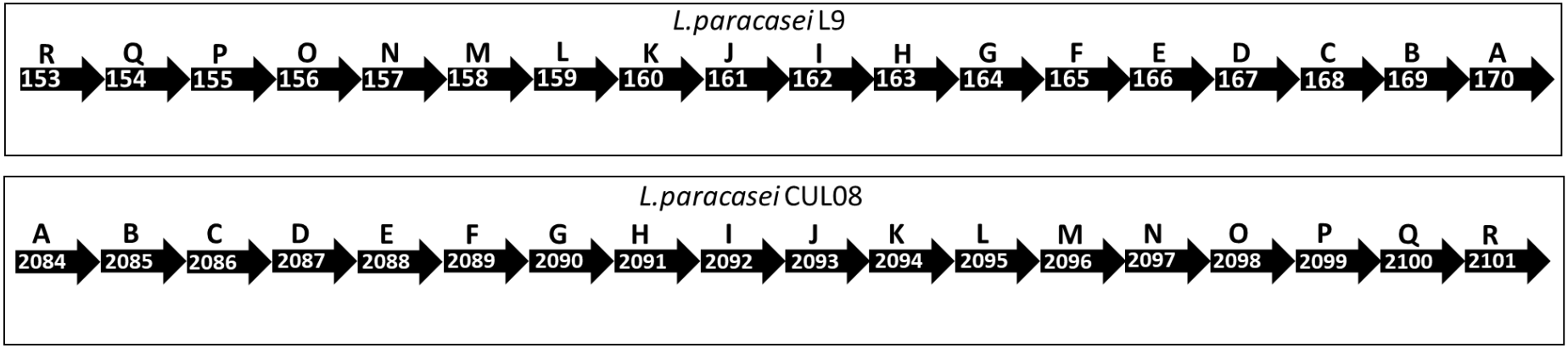



**Figure 3.3. Conserved EPS operon within *Lactobacillus paracasei* strains CUL08 and L9** (Ma et al., 2018)**.** The numbers on arrows indicate genomic position either reported as locus tag for reference strain or protein-encoding gene number for CUL strain. Letters correspond with protein annotation. A: teichoic acid/polysaccharide phospho-glycerol transferase, B. hypothetical protein, C: exopolysaccharide tyrosine-protein kinase, D: lipopolysaccharide biosynthesis protein, E: polysaccharide biosynthesis protein, F: glycosyltransferase, G: glycosyltransferase, H: hypothetical protein, I: hypothetical protein, J: lysozyme M1, K: hypothetical protein, L: acyltransferase 3, M: Glycosyltransferase, N: UDP-N-acetylglucosamine 2-epimerase, O: Hypothetical protein, P: lipopolysaccharide synthesis sugar transferase, Q: glycosyltransferase and R: glycosyltransferase.

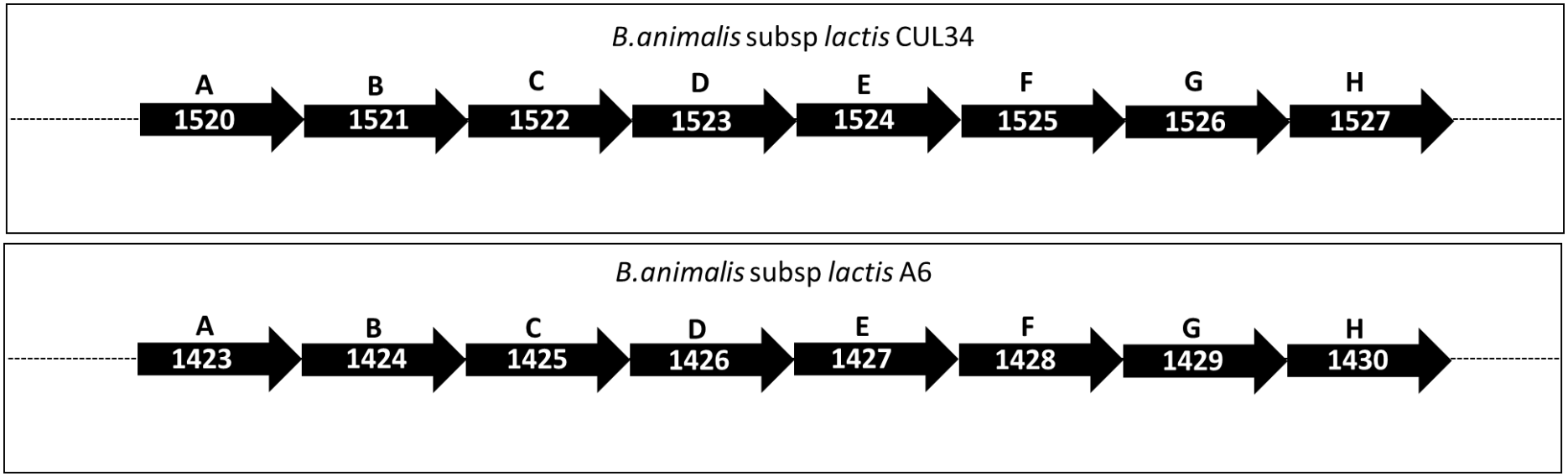



**Figure 3.4. Conserved EPS operon within *Bifidobacterium animalis* subsp. *lactis* strains A6 and CUL34** (Sun et al., 2015)**.** The numbers on arrows indicate genomic position either reported as locus tag for reference strain or protein-encoding gene number for CUL strain. Letters correspond with protein annotation, A: F0F1 ATP synthase subunit A, B: ATP synthase F0 subunit C, C: F0F1 ATP synthase subunit B, D: F0F1 ATP synthase subunit delta, E: F0F1 ATP synthase subunit alpha, F: F0F1 ATP synthase subunit gamma, G: F0F1 ATP synthase subunit beta, H: F0F1 ATP synthase subunit epsilon.

### 3.3.8. The carbohydrate-active enzyme profiles of CUL strains

The Cazyzome prediction of CUL strains revealed the presence of 6 active enzyme families within the consortia (**Figure 3.5**). Auxiliary Activities (AA) and Polysaccharide Lyases (PL) are less common, with only 0 – 5 predicted per strain. In contrast, Glycoside Hydrolases (GH) are highly represented in all strains with a minimum of 20 and a maximum of 50 predicted subfamilies per strain (**Figure 3.5**). Glycoside Transferases (GTs) and Carbohydrate Binding Molecules (CBM) are moderately represented across isolates whereas Carbohydrate Esterase (CE) are only moderately predicted in *L.paracasei* CUL07, which contrasts with the other *L.paracasei* strains (**Figure 3.5**).

### 3.3.9. Bacteriocin prediction

*L.paracasei* CUL37, CUL07 and CUL08 were all predicted to encode two bacteriocins, LSEI 2386 (91.7 % similarity) a previously reported *L.casei* bacteriocin, and camocin CP52 (71.6 % similarity) originally described in *Camobacterium piscicola* (**Table 3.9**). Interestingly, only one bacteriocin was putatively predicted in *L.casei* CUL06, enterocin (originally described in *Enterococcus faecium*) however with a low bit score of 46. *L.rhamnosus* CUL63 was also predicted to encode one bacteriocin, Camocin CP52 (originally described in *Camobacterium piscicola*) with a bit score of 72 (**Table 3.9**). *L.fermentum* strains CUL40 and CUL67 and *L.salivarius* CUL61 were each predicted to encode the bacteriocin enterolysin A, without a reported bit score (**Table 3.9**). Members of the *Lactobacillus delbrueckii* group were predicted to encode the highest abundance of bacteriocins (up to 5) with *L.helveticus* CUL76 encoding two helveticin J, one enterolysin A and two LAPs (**Table 3.9**). *L.gasseri* CUL09, putatively encoding 3 bacteriocins, acidocin B (169-bit score), gassericin T (142-bit score) and helveticin J (no reported bit score). Both *L.acidophilus* strains CUL21 and CUL60 putatively encoded three bacteriocins, acidocin J1132, enterolysin J and helveticin J (**Table 3.9**). *L.plantarum* strains CUL66 and CUL66N were both predicted to encode plantaricin E (originally described in *L.plantarum*).

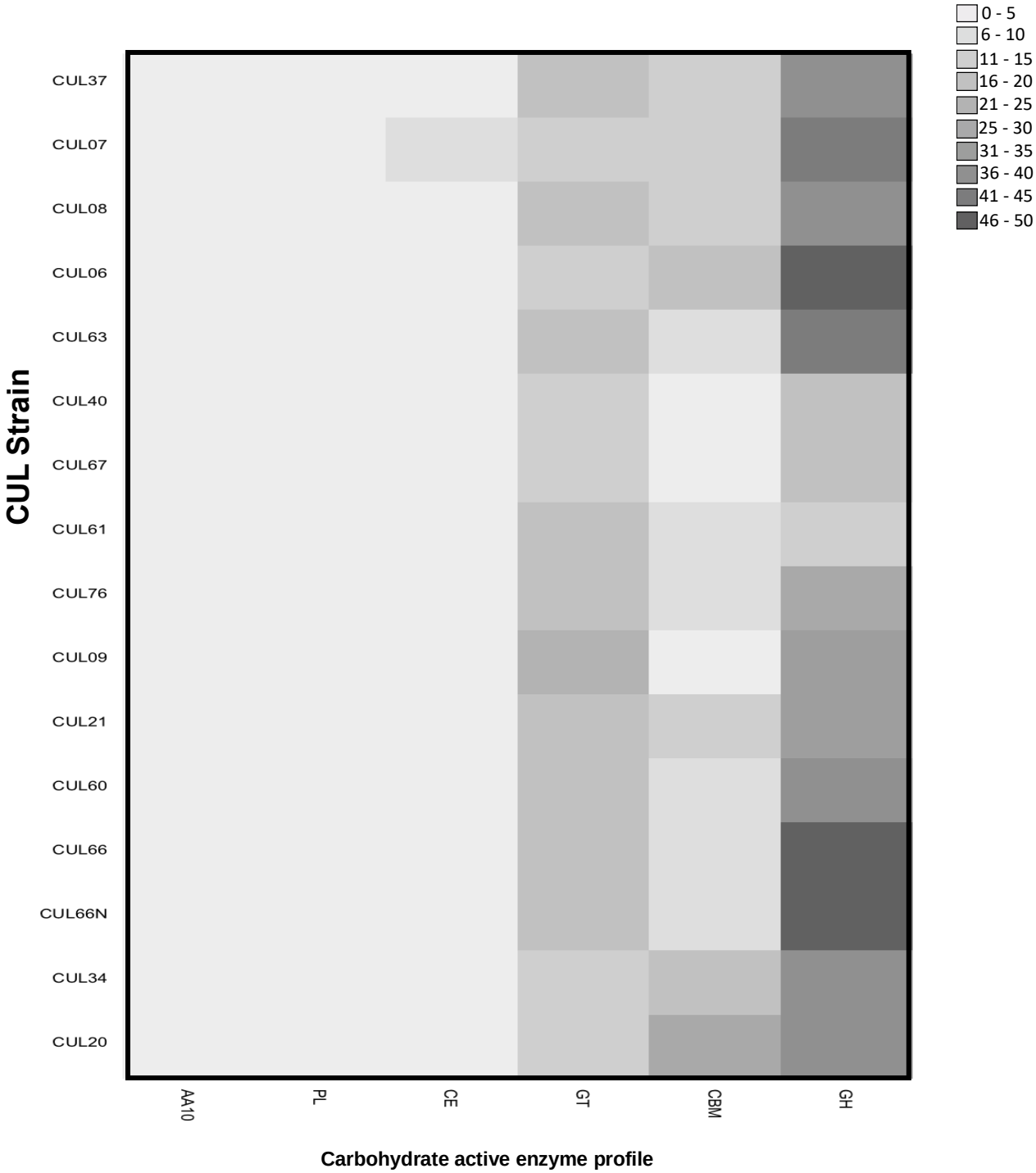



**Figure 3.5. Carbohydrate active enzyme profiles of CUL isolates**. Profiles were predicted using the dbCAN2 meta server and results were filtered for enzymes predicted by three methods: HMMer, Hotpep and DIAMOND. The Colour gradient represents the number of predicted enzymes, 0 – 5 is indicated by light grey whereas the maximum 46 – 50 is indicated by dark grey. Enzyme classes are as follows; GH: Glycoside Hydrolases, CBM: Carbohydrate-Binding Molecules, GT: Glycoside Transferases, CE: Carbohydrate Esterase, PL: Polysaccharide Lyases, AA: Auxiliary Activities.

**Table 3.9. Bacteriocin prediction in CUL strains.** BAGEL4 was utilised to determine the genomic potential of CUL strains to produce bacteriocins. Region length is reported in base pairs (bp) and bit score indicates similarity to reference sequence.

| Strain | Predicted Bacterocin Name | Start (bp) | End (bp) | Size (bp) | Bit Score | Reference Sequence Organism | E- value |
|---|---|---|---|---|---|---|---|
| *L.paracasei* CUL37 | 143.2 LSEI 2386 | 1030640 | 1050772 | 20132 | 91.663 | *Lactobacillus casei* ATCC334 | 2.87E-30 |
| | 51.2 Carnocin CP52 | 1342493 | 1365052 | 22559 | 71.633 | *Carnobacterium piscicola* | 1.62E-20 |
| *L.paracasei* CUL07 | 143.2 LSEI 2386 | 973544 | 993676 | 20132 | 91.663 | *Lactobacillus casei* ATCC334 | 2.87E-30 |
| | 51.2 Carnocin CP52 | 1282238 | 1304797 | 22559 | 71.633 | *Carnobacterium piscicola* | 1.62E-20 |
| *L.paracasei* CUL08 | 143.2 LSEI 2386 | 1031024 | 1051156 | 20132 | 93.589 | *Lactobacillus casei* ATCC334 | 5.96E-31 |
| | 51.2 Carnocin CP52 | 74837 | 97225 | 22388 | 74.329 | *Carnobacterium piscicola* | 1.64E-21 |
| *L.casei* CUL06 | 97.2 Enterocin X chain beta | 2288675 | 2308810 | 20135 | 46.210 | *Enterococcus faecium* KU-B5 | 4.93E-12 |
| *L.rhamnosus* CUL63 | 51.2 Carnocin CP52 | 1485533 | 1509706 | 24173 | 72.403 | *Carnobacterium piscicola* | 1.08E-20 |
| *L.fermentum* CUL40 | 62.3 Enterolysin A | 1205582 | 1226041 | 20459 | | | 1.31E-23 |
| *L.fermentum* CUL67 | 63.3 Enterolysin A | 1102814 | 1123255 | 20441 | | | 2.81E-25 |
| *L.salvarius* CUL61 | 62.3 Enterolysin A | 1708859 | 1729366 | 20507 | - | - | 6.13E-24 |
| *L.gasseri* CUL09 | 158.1 Acidocin B | 1198208 | 1218433 | 20225 | 169.088 | *Lactobacillus acidophilus* | 9.63E-22 |
| | 104.2 Gassericin T | 1371455 | 1391860 | 20405 | 142.895 | *Lactobacillus gasseri* strain EV1461 | 2.94E-49 |
| | 6.3 Bacteriocin helveticin J | 1419200 | 1439935 | 20735 | - | *Lactobacillus helveticus* | 1.86E-61 |
| *L.helveticus* CUL76 | 6.3 Bacteriocin helveticin J | 759164 | 779680 | 20516 | | | 9.18E-59 |
| | 64.3 Enterolysin A | 1018760 | 1039222 | 20462 | | | 1.89E-65 |
| | 70.3 Helveticin J | 1232843 | 1253503 | 20660 | | | 4.16E-178 |
| | LAPs | 1488965 | 1508965 | 20000 | | | 4.58E-07 |
| | LAPs | 1555313 | 1575313 | 20000 | | | 1.74E-17 |
| *L.acidophilus* CUL60 | 6.2 Acidocin J1132 beta peptide N-terminal | 879095 | 904027 | 24932 | 51.603 | *Lactobacillus acidophilus* JCM1132 | 6.30E-14 |
| | 64.3 Enterolysin A | 620078 | 640561 | 20483 | - | - | 2.19E-64 |
| | 6.3 Bacteriocin helveticin J | 1501913 | 1522876 | 20963 | - | - | - |
| *L.acidophilus* CUL21 | 6.2 Acidocin J1132 beta peptide N-terminal | 876068 | 900805 | 24737 | 51.603 | *Lactobacillus acidophilus* JCM1132 | 6.30E-14 |
| | 64.3 Enterolysin A | 619310 | 639793 | 20483 | - | - | 2.19E-64 |
| | 6.3 Bacteriocin helveticin J | 1494986 | 1515949 | 20963 | - | - | 0 |
| *L.plantarum* CUL66 | 170.2 Plantaricin E | 1423448 | 1447309 | 23861 | 112.464 | *Lactobacillus plantarum* | 3.77E-38 |
| *L.plantarum* CUL66N | 170.2 Plantaricin E | 2602715 | 2627080 | 24365 | 90.508 | *Lactobacillus plantarum* | 9.28E-30 |
| *B.bifidum* CUL20 | - | - | - | - | - | - | - |
| *B.animalis* subsp *lactis* CUL34 | - | - | - | - | - | - | - |

## 3.4. Discussion

Genome mining was employed to identify putative virulence and potential 'probiotic' traits within CUL genomes. As such, a genomic profile is presented for each CUL strain. Subsequent genomic profiles will allow for targeted phenotypic testing and provide the basis for a polyphasic (multifaceted) overview of the CUL organisms.

### 3.4.1. CUL virulence profiles

In Europe, the EFSA have a 'Qualified Presumption of Safety' or QPS designation for organisms intended for human consumption (EFSA, 2008). The QPS is designed to provide a safety assessment for such organisms. Specifically, it is stated that the virulence profiles and potential to contribute to ABR should be defined. ABR in pathogenic bacteria is a major health concern worldwide (Andersson et al., 2020; Guo et al., 2020b). As such, identifying ABR profiles of microorganisms deliberately introduced into the food chain is necessary to assess virulence potential. RAST annotation allowed the putative identification of the CUL ABR resistome. Resistant profiles were predicted to encode between 7 (*B.animalis* subsp. *lactis* CUL34) – 17 (*L.plantarum* strains CUL66 and CUL66N) genes within the CUL strains. Of interest, is the vague and non-specific nature of the subsystem annotations provided by RAST which results in a virulence designation. For example, a subcategory is named *Streptococcus pneumonia* vancomycin tolerance locus, which suggests a specific ARG origin from that species. In terms of annotation, only five ABR-specific protein-encoding genes were designated by RAST; *TetW*, conferring tetracycline resistance in *B.animalis* subsp. *lactis* CUL34, a well-established trait in *B.animalis* strains (Gueimonde et al., 2010; Rozman et al., 2020; Sharma et al., 2021), satA, which has been related to streptothricin resistance in *Enterococcus* (Werner et al., 2000) and *Bacillus* (Burckhardt & Escalante-Semerena, 2017) was predicted in *L.helveticus* CUL76 and *L.acidophilus* strains CUL21 and CUL60, and beta-lactamase genes (*bl*, *blA* and *blC*) were predicted in all genomes, despite not being well reported in lactobacilli species (Campedelli et al., 2019). The lack of specificity and the vague nature of annotations suggested a need for a more detailed ARG analysis with a specific pipeline/database.

RAST annotation additionally offered a breakdown of genomic features within CUL genomes. From this broad overview, the presence of virulence factors was predicted in each genome, ranging from 18 genes in *B.animalis* subsp. *lactis* to 89 genes within *L.casei* CUL06. In general lactobacilli ,strains are typically considered a non-pathogenetic organisms to human hosts (Bernardeau et al., 2008; Choudhary et al., 2019; Salvetti & O'Toole, 2017). However, there are some reports of lactobacilli becoming opportunistic pathogens within immunocompromised patients (Aaron et al., 2017; Campagne et al., 2020; Chery et al., 2013;

Wallet et al., 2002), albeit these reports are scarce. When evaluating the virulence profiles of CUL strains, four main subcategories were presented. Phage components were most frequently predicted in the majority of CUL strains, followed by traits associated with invasion and intracellular resistance. However, the functional annotations provided are of genes/proteins typically associated with standard cellular mechanisms such as DNA-directed RNA polymerase subunits and ribosomal proteins. Indeed, it has recently been highlighted that depletion in safety-related databases for non-pathogenic bacteria such as LAB (Colautti et al., 2022), may lead to ambiguous virulence predictions, as traits are often species (Kilian et al., 2014; Salvadori et al., 2019), if not, strain-specific (Christoffersen et al., 2012; Köhler & Dobrindt, 2011). Providing further evidence of such is that a virulence subcategory was named *S.pyrogenes* recombination zone, suggesting that the genes designated within this group are specific to *S.pyrogenes*. Certainly, within this subcategory fibronectin-binding proteins (Fbp) were reported within most CUL strains and therefore ascribed as a pathogenic function. However, fibronectin is a component of the extracellular matrix of intestinal epithelial cells and lactobacilli have been shown to confer adherence capabilities as a result of Fbp production (Hymes et al., 2016). The ability to adhere to host cells is considered a desirable trait for probiotic bacteria (Kadlec & Jakubec, 2014; Monteagudo-Mera et al., 2019), leading to conflicting trait categorisation.

*L.helveticus* CUL76 was predicted to encode three streptolysin S proteins, B, C and D. Streptolysin S is a cytolytic toxin typically produced by *Streptococcus* (Molloy et al., 2015), which has been implicated in the lysis of red blood cells (Markley et al., 2012). However, it has been demonstrated that for streptolysin S synthesis to function, the entire operon (consisting of 9 genes) is required (Datta et al., 2005), indicating that in *L.helveticus* CUL76, such proteins are not functional virulence components. Furthermore, streptolysin S is a ribosomal produced peptide and its enzymatic modifications are like bacteriocins (Flaherty et al., 2014), implying a potential for bacteriocin production within *L.helveticus* CUL76, which was initially predicted to be a virulence factor during early genome mining.

The largest group of predicted virulence traits within CUL genomes was phage elements. Phages are segments of viral DNA, that have invaded a bacterial cell and are established as part of the bacteria's genome (Zhou et al., 2011). Temperate phages can lie benign, but when induced, they can lyse and kill the host cell, which in turn, releases viral genetic material capable of infecting new cells (Zhou et al., 2011). The potential spread of genetic material encoding attributes (such as adhesion and ABR) to pathogenic bacteria, poses safety concerns (Casjens, 2003; Lekunberri et al., 2017). The phage search tool PHAST (Zhou et al., 2011), was employed to further investigate the presence of intact phages within CUL genomes. Following, 11 out of 16 strains were predicted to encode at least one intact phage.

The largest abundance of intact phages (four) was seen in the *L.casei* CUL06 genome. All were identified as the well-characterised *L.casei* phage phi AT3, a siphophage which has previously been reported to encode an IS element, ISLC3 (Lo et al., 2005; Villion & Moineau, 2009). A recent analysis of prophages in over 1000 lactobacilli strains, reported a maximum of 15 intact phages within *L.paracasei* EG9 (Pei et al., 2021). Indeed, the presence of phages within bacterial genomes is not unusual and is generally widespread across a plethora of bacterial genera, including high occurrences in *Salmonella* (Mottawea et al., 2018), *Streptococcus* (Javan et al., 2019) and *Campylobacter* (Connerton et al., 2011). Specifically, a recent study reported the presence of intact prophages within 64.1 % of the lactobacilli strains analysed (Pei et al., 2021), emphasising a putative reassurance when discovering intact phages in strains, when assessing safety.

Bacteriophages are well reported in lactobacilli which are utilised as starter cultures in the dairy industry (Binetti et al., 2008; Brüssow, 2001; Garneau & Moineau, 2011; Zaburlin et al., 2017; Zago et al., 2017) and in organisms used during food fermentation processes (Chen et al., 2019a; Park et al., 2022; Samson & Moineau, 2013). Reports typically focus on the capacity of phages to lyse host cells and prevent effective fermentation. Therefore, the virulence capacity of lactobacilli phages within human hosts is not well established, limiting the depth of the safety evaluation required when assessing the impact of phage prediction. However, a recent report has suggested the presence of ARGs within prophage elements, which has an associated HGT risk (Pei et al., 2021). Interestingly, several predicted CUL phages were initially identified in other genera, including, *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Bacillus* and *Oenococcus*, suggesting the movement of genetic material between lactobacilli and such species. Phage movement has previously been described between *Salmonella* strains (Diard et al., 2017) and reports of phage-facilitated ABR gene transmission have also been reported elsewhere (Balcazar, 2014; Modi et al., 2013; Quirós et al., 2014) suggesting that phage composition and behaviour should be investigated further.

Genomic islands are regions within bacterial genomes that likely arose as a result of HGT (Bertelli et al., 2017). Indeed, GIs often carry mobile genetic elements (Dobrindt et al., 2004) and are recognised as an important component of microbial evolution and genome plasticity. Furthermore, GIs can be expelled from the bacterial genome and received by a new host facilitating HGT (Dobrindt et al., 2004). This can either be positive, increasing adaptations to cope with environmental stresses (Rodriguez-Valera et al., 2016), or negative facilitating the spread of ‘deleterious’ traits, for example, ARGs (Hall, 2010; Rivard et al., 2020) or adherence properties (Juhas et al., 2009), which may increase the pathogenicity of the recipient organism (Becq et al., 2007; Sui et al., 2009).

To further assess CUL strains' virulence profiles, the presence of GIs was predicted using islandviewer 4.0. Genomes contained between 9 (*B.bifidum* CUL20 and *L.acidophilus* CUL21)

– 33 (*L.fermentum* CUL40) GIs. Scrutiny of the gene composition of the predicted GIs showed that they were mainly composed of hypothetical proteins, recombinases, proteins related to transport, glycosyltransferases, endopeptidases and phage elements. Several possible environmentally beneficial traits were identified on GIs within CUL strains. For example, islands with adhesion factors were identified in most of the *L.casei* phylogroup, including protein-encoding genes such as collagen adhesion protein (Yadav et al., 2013) and sortease A (Wu et al., 2020) which are beneficial traits in lactobacilli. However, the transmission of such traits to pathogenic bacteria could potentially be a safety hazard. In addition, *L.rhamnosus* CUL63 was predicted to encode several genes associated with acid tolerance, such as a Na+ H+ antiporter on GI 10 (Lucas et al., 2003; Montijo-Prieto et al., 2019). Furthermore, the protein-encoding gene, cholylglycine hydrolase was predicted in *L.salivarius* CUL61 and *B.bifidum* CUL20 on GI 3 and GI 4 respectively. Cholylglycine hydrolase is a group of enzymes which encompass BSH, which may have a putative role in bile tolerance (Begley et al., 2006; Begley, Gahan, et al., 2005). Supporting an accurate GI designation, *L.salivarius* strains have been reported to encode a *bsh* gene on a megaplasmid elsewhere (Fang et al., 2009).

In contrast, less desirable traits were also identified within some CUL strains. For example, *L.fermentum* CUL40 had several resistance genes identified on GIs, including drug-resistant transporter (*ermB*), ABC transporter, arsenic resistance, and *tetR*. Furthermore, *L.helveticus* CUL76, in agreement with the putative identification via RAST subsystem analysis, identifies a streptolysin S biosynthesis protein (Molloy et al., 2015) on GI 6, suggesting a putative genomic transfer between *Streptococcus* and *L.helveticus* (Liu et al., 2009). Although evidence of HGT events within lactobacilli species is limited. GI 1 of *B.animalis* subsp. *lactis* CUL34 was found to harbour a tetracycline resistance (*tet*(W)) gene. However, *tet(W)* has been described as ubiquitous in *B.animalis* subsp. *lactis* (Aires et al., 2007; Gueimonde et al., 2010). Furthermore, the genomic region presented here is homologous to that of other strains of bifidobacteria (Ammor, Flórez, Álvarez-Martín, et al., 2008; Gueimonde et al., 2010). Therefore, it is likely that this genomic region is a false prediction of an acquired island. Additionally, the transfer of *tet* genes from bifidobacteria to other bacterial species has usually been unsuccessful (Polit et al., 2018). Indeed, recent phylogenetic analysis has shown that *tet(W*) within the *B.animalis* subsp. *lactis* is a component of an 'ancient resistome' and therefore the risk of transfer is minimal (Nøhr-Meldgaard et al., 2021). Several predicted GIs encoded less than 5 genes. GIs are typically large regions and predicting single gene acquisition is difficult, therefore many methods use a cut-off criterion of 8 genes (Langille et al., 2010), further supporting the potential of erroneous GI prediction in CUL strains.

### 3.4.2. Genes of Interest

#### 3.4.2.1. *Acid and bile tolerance*

All CUL strains were shown to encode several genes associated with acid tolerance. For example, the presence of multiple copies of L – lactate dehydrogenase was identified in all strains (Desriac et al., 2013). LDH enzymes have been previously correlated with acid tolerance, as it facilitates the conversion of pyruvate to lactate, which allows the removal of acidic compounds from the cell (Desriac et al., 2013). Additionally, bifidobacteria strains (*B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34) were also predicted to encode a Na+/H+ NhaA antiporter (Desriac et al., 2013) and all lactobacilli CUL strains were predicted to encode at least one copy of *dltD*, both of which are genomic traits associated with acid tolerance, providing putative evidence of a functional acid tolerance capacity in CUL strains.

CUL strains were predicted to encode a range of cholylglycine hydrolase copies which may play a role in bile tolerance (Begley, Gahan, et al., 2005). For example, three copies were predicted in *L.plantarum* strains CUL66 and CUL66N. Interestingly *L.salivarius* was predicted to encode two copies, where one was predicted to occur on a GI, following island viewer analysis. Previous reports of *bsh* activity in *L.salivarius* strains have shown the presence of one chromosomally encoded *bsh* gene and one *bsh* copy on a mega pmegaplasmid et al., 2009), providing the rationale for the presence of *bsh* on a GI in *L.salivarius* CUL61 and suggesting the presence of a mega plasmid within CUL61's genome. A high degree of homology is described between members of the cholylglycine hydrolase family (Kumar et al., 2006), therefore additional variations were also mined for and recorded, including, L-linear amide CN (in *L.plantarum* strains CUL66 and CUL66N) and conjugated bile salt hydrolase related amidase (in all *L.casei* phylogroup). Interestingly 4 BSH proteins have been reported in *L.plantarum* strains, with divergence occurring between *bsh1* and *bsh* 2-4 (Lambert, Bongers, et al., 2008), suggesting an accurate prediction in CUL *L.plantarum* strains, which were also predicted to encode 4 BSH copies. All CUL strains (apart from *L.fermentum* strains CUL67 and CUL40 and *B.animalis* subsp. *lactis* CUL34) were predicted to encode a homolog of Glucosamine -6- phosphate deaminase an additional genomic marker of bile tolerance (Alcántara & Zúñiga, 2012), suggesting that genomic features conferring bile tolerance were present in all strains.

#### 3.4.2.2. *Adherence*

Several genomic features have been recognised as factors that aid in host adhesion. Cellular adhesion is considered a desirable trait of a probiotic as it allows survival and proliferation within the gut (Kadlec & Jakubec, 2014). Fbp (Buck et al., 2005; Hymes et al., 2016) mediates adhesion to mucin and fibronectin and was identified in all lactobacilli CUL strains. In addition, collagen-binding proteins (Cbp) can interact with extracellular matrix component proteins and bind to collagen in a host's tissue (Sillanpää et al., 2000; Yadav et al., 2013) and were also identified in *L.paracasei* CUL37 and *L.plantarum* CUL66N, providing putative evidence of desirable probiotic traits in CUL strains.

#### 3.4.2.3. *Virulence*

Biogenic amine production is associated with virulence in bacteria (Beatrice et al., 2018). A genetic basis of such production is *panD* (Evanovich et al., 2019). Following mining, *panD* was not identified in any CUL strains. In addition, phage portal proteins are associated with mobile elements (as designated by RAST). No homologs to such genes were identified in CUL strains providing a positive outcome in terms of safety assessment (Baker et al., 2021).

#### 3.4.2.4. *Microbe – Host interactions*

All CUL strains were shown to encode genes involved with short-chain fatty acid (SCFA) production (Choi et al., 2021). Of interest, all *L.paracasei* strains CUL37, CUL07 and CUL08 were shown to encode both SCFA's acetate and butyrate. SCFA production is associated with beneficial effects on a host's metabolic function (LeBlanc et al., 2017). Furthermore, SCFA has also demonstrated potential in promoting a healthy intestine via inflammation reduction (Parada Venegas et al., 2019) and improving mucosal barrier function (van der Beek et al., 2017). Additionally, the production of formate also contributes to energy metabolism and genetic determinants of production were also identified in CUL *L.paracasei* strains (Pietzke et al., 2020).

Glutathione peroxide, glutathione catalase and glutathione reductase are enzymes associated with antioxidant production (Choi et al., 2021). All CUL strains encoded at least one of these enzymes, except for *L.salivarius* CUL61 and both bifidobacteria strains CUL34 and CUL20**.** Of interest *L.plantarum* CUL66 encoded all three enzymes, suggesting strong antioxidant production potential. Glutamate decarboxylase facilitates the production of GABA from glutamate, an inhibitory neurotransmitter, which may aid in cognitive function (Choi et al., 2021). *L.plantarum* CUL66 and CUL66N, and *L.fermentum* CUL40 (interestingly absent from CUL67) were shown to carry glutamate decarboxylate homologs.

lactocepin S-layer protein is an extracellular protease with documented anti-inflammatory capabilities (Salvetti & O'Toole, 2018). Both *L.acidophilus* CUL21 and CUL60 were predicted to carry copies of lactocepin encoding genes. Lactate oxidase was also predicted in several lactobacilli CUL strains, a gene with a putative role in hydrogen peroxide production (Seki et al., 2004). Hydrogen peroxide production has associated antimicrobial properties and has been shown to inhibit some pathogenic bacteria (Pridmore et al., 2008; Xu et al., 2008), leading to the proposition that such a trait may aid in microbiota regulation (Felten et al., 1999; Pascual et al., 2006).

### 3.4.3. Specific genome mining

Conducting a MLSA of all available *Lactobacillus* and *Bifidobacteria* genomes in **Chapter 2,** allowing the putative identification of the phylogenetically closest related strains to CUL isolates. Following identification, the literature was mined for putative traits which may be conserved within the evolutionary lineage. As such, *L.gasseri* CUL09 was shown to share a common antimicrobial peptide (bacteriocin) with *L.gasseri* K9 (Peternel et al., 2010), which may indicate an inhibitory trait towards pathogenic bacteria.

Both *L.acidophilus* strains were predicted to encode genes that confer hydrogen peroxide-producing capabilities, a shared attribute with the whole clade (Hertzberger et al., 2014). Indeed, hydrogen peroxide-producing lactobacilli have been shown to inhibit *Salmonella enterica in vitro* (Pridmore et al., 2008), indicating putative anti-pathogenic traits within CUL21 and CUL60.

Predicted cellular adhesion genes were recognised in *L.helveticus* R0052 (Tompkins et al., 2012) including several mucus binding proteins, which appear to be conserved in the lineage from which CUL76 emerges. As previously stated, adhesion to gut epithelial cells is a desired trait of a probiotic, as it allows establishment within the host, providing putative genomic evidence of probiotic traits.

*L.paracasei* CUL08 shares a MRCA with *L.paracasei* L9. Genes involved in potential probiotic function were identified within the L9 genome including adhesion, bile tolerance and host immune regulation (Ma et al., 2018). Such genes were also identified in *L.paracasei* CUL08. In addition, an intact EPS operon consisting of 18 genes was also conserved in L9 and CUL08. EPS clusters have been implicated in several putative probiotic functions such as adhesion (Živković et al., 2016), colonization (Kanmani et al., 2013; Tulumoglu et al., 2013), stress resistance (Gauri et al., 2009; Lebeer et al., 2008; Nguyen et al., 2020; Seesuriyachan, 2012), host-bacteria interactions (Bengoa et al., 2020; Bhat & Bajaj, 2019; Dertli et al., 2013). In addition, there is also scope to utilise EPS as novel drug delivery vectors (Laubach et al., 2021), highlighting a plethora of putative beneficial traits associated with *L.paracasei* CUL08.

In addition, a close relative of *B.bifidum* CUL20, *B.bifidum* S17 has an operon encoding adhesion traits, which is also intact within CUL20. Indeed, *B.bifidum* S17 has been shown to exhibit strong adherence capabilities to intestinal epithelial cells (Preising et al., 2010; Riedel et al., 2006; Zhurina et al., 2011). Likewise, *B.bifidum* CUL20 has been shown to have the greatest adherence capability of the Lab4 consortia, when cultured with Caco-2 enterocytes (Baker et al., 2021), suggesting a genomic basis for such attributes. Similarly, *B.animalis* subsp. *lactis* A6 (which shares a MRCA with *B.animalis* subsp. *lactis* CUL34) encodes an operon which confers high acid tolerance capabilities (Sun et al., 2015), comparative BLASTp analysis revealed that this operon was conserved between the two strains.

### 3.4.4. Mining for beneficial traits

When mining for health-promoting attributes the CAZYome predictions for all CUL strains indicate an overrepresentation of glycoside hydrolase (GH) genes in all genomes. GH genes have been implicated in binding to mucin (Tailford et al., 2015), aiding in the bioprospecting potential for adherence traits.

Pathogen inhibition is a desirable trait of probiotic bacteria (Tuo et al., 2018). Bacteriocins are ribosomally synthesised peptides with antimicrobial properties (Castro et al., 2011; da Silva Sabo et al., 2014), which have shown a capacity to inhibit pathogenic microorganisms (Ghanbari et al., 2013; Messi et al., 2001; Todorov & Dicks, 2005; Zahid, 2015). Therefore, CUL strains were mined for a genomic capability of bacteriocin production, using the bacteriocin-specific platform BAGEL4. As such, several strains were predicted to encode bacteriocins.

*L.acidophilus* CUL60 and CUL21 were predicted to encode 3 putative bacteriocins, acidosin J1132, helveticin J and enterolysin A. As previously described, both CUL *L.acidophilus* strains are situated in a clade with *L.acidophilus* DSM 20079 (Chapter 2) and this strain has also demonstrated an ability to produce acidosin 20079 (Deraz et al., 2005, 2007), validating the use of MLSA to search for conserved traits. Indeed, several bacteriocins coined acidosin have been described in several *L.acidophilus* strains (Chumchalová et al., 2004; Deraz et al., 2005; Modiri et al., 2020; Tahara et al., 1996). Each bacteriocin varies in structure and has different antimicrobial capacities, restricting the sole use of genomics to predict functionality. For example, *L.acidophilus* CUL21 and CUL60 shared homology with acidosin J1132, which has been previously shown to have a restricted antimicrobial potential in non-pathogenic strains (Deraz et al., 2005; Tahara et al., 1996). In contrast, acidosin 4356 production has displayed an ability to combat *in vivo Pseudomonas aeruginosa* infections (Modiri et al., 2020). As the homology of acidosin J1132 was only 50 % with the bacteriocins predicted in CUL strains, may suggest a divergence in function with a varied antimicrobial potential. In addition,

*L.gasseri* CUL09 was also predicted to encode acidosin B, which again may encode varied antimicrobial action.

Helveticin J was predicted in all CUL genomes which were members of the *L.delbrueckii* phylogroup. Indeed, it has been previously reported that helveticin J tends to cluster within the *L.acidophilus* species, with authors suggesting that clustering indicates bacteriocin production is an ancestral trait for this group (Collins et al., 2017). A recent study reported the isolation of a bacteriocin NX371 which shared 98.15 % homology to helveticin J and had strong antimicrobial action against an abundance of pathogens (Meng et al., 2021).

The bacteriocin Enterolysin A was also predicted in several CUL genomes including *L.acidophilus* strains CUL21 and CUL60, *L.fermentum* strains CUL40 and CUL67, *L.salivarius* CUL61 and *L.helveticus* CUL76. Enterolysin A is a well-described bacteriocin of *Enterococcus* species (Franz et al., 2007). Interestingly, no bit score or reference organism was provided during BAGEL annotation of enterolysin A, suggesting either an erroneous annotation or if true, a potential indicator of HGT between lactobacilli and enterococci species, resulting in enterolysin A identification in CUL strains.

Plantaricins are a group of bacteriocins which belong to the species *L.plantarum* (Meng et al., 2017) and have documented antimicrobial effects against pathogenic and LAB strains (Meng et al., 2017; Pal & Srivastava, 2014). Plantaricin E was identified in both *L.plantarum* strains CUL66 and CUL66N, indicating bioprospecting potential for antimicrobial capabilities.

Gassericin T was predicted in *L.gasseri* CUL09. In contrast, specific genome mining earlier in the study indicated a putative prediction of the bacteriocin Gassericin A. Bacteriocin grouping is broken down into two classes: class I, the lantibiotics and class II, the non-lantibiotics. Class II has four subgroups: class IIa pediocin-like bacteriocins, IIb two-peptide bacteriocins, IIc cyclic bacteriocins, and IId linear non-pediocin-like one-peptide bacteriocins (Maldonado-Barragán et al., 2016). Interestingly, Gassericin A and Gassericin T are thought to be members of different subclasses, class IIc (circular) and class IIb (two-peptide) respectively (Maldonado-Barragán et al., 2016). The varied annotation presented using two search methods reflects the ambiguous nature the sole use of genomic mining can have when bioprospecting for probiotic traits. However, homology has been reported between the active peptide of gassericin K7 B and the complementary peptide of gassericin T, offering a putative explanation for the varied annotations across methodologies (Peternel et al., 2010). Additionally, gassericin A has shown wide inhibition spectrum of pathogenic organisms, for example in the case of; *Listeria monocytogenes, Bacillus cereus,* and *S. aureus* (Kawai et al., 2001; Pandey et al., 2013; Peternel et al., 2010), suggesting the capability for *L.gasseri* CUL09 to behave similarly.

Overall, *L.helveticus* CUL76 was predicted to encode the largest number of bacteriocins (5) which were from a variety of classes. *L.acidophilus* strains CUL21 and CUL60 and *L.gasseri*

CUL09 were each predicted to encode 3 bacteriocins each, suggesting that these strains would be a good starting point to focus antimicrobial capability assays on. Interestingly, the presence of bacteriocins within the bifidobacteria strains was not predicted, despite previous reports suggesting an ability to encode for such antimicrobial peptides, for example, bifidocin in *B.bifidum* strains (Martinez et al., 2013), highlighting the importance of strain designation when ascribing probiotic attributes.

### 3.4.5. Limitations

Genomic mining can be a proficient tool to predict microbial behaviour and metabolic pathways (Fontana et al., 2019; Salvetti & O'Toole, 2018; Sun, Harris, et al., 2015a). However, utilising draft genomes for mining can be seen as a limitation, due to the 'gaps' left in the genome sequencing (Ricker et al., 2012). Closing out the CUL genomes will enable a more detailed and reliable annotation. Aligning the genomes to a known complete reference sequence may enable the reduction of gaps in this analysis and therefore enhance the mining potential presented here. It is also worth noting that genomic mining is also limited by the reliance on databases and subsequent comparisons to the sequences that are currently available. Therefore, as research continues to expand, so will sequence annotation, which may over time, result in more specific and reliable annotations.

### 3.4.6. Summary

The purpose of this chapter was to provide an initial overview of the CUL strain's genomic profiles, investigating their safety and bioprospecting potential. As such, a putative AMR profile is presented per strain, with *tet*((W)) appearing on a well-established genomic island (Gueimonde et al., 2010; Rozman et al., 2020; Sharma et al., 2021), in *B.animalis* subsp. *lactis* CUL34. RAST analysis was considered too vague to deduce ARG profiles of CUL strains. In addition, the presence of mobile elements including bacteriophages and genomic islands were evaluated and discovered in all strains, with notice of several ARG predictions within a GI in *L.fermentum* CUL40, providing a rationale for a more in-depth ABR analysis. Several putative health-promoting traits were identified across CUL strains, including genomic markers for, acid and bile tolerance, cellular adherence, microbial-host interactions, carbohydrate metabolism and bacteriocin production. An interesting trend was noted between the occurrence of the *bsh* genes and the number of gene copies in lactobacilli CUL strains, with the additional observation of a cholylglycine hydrolase predicted on a genomic island in *L.salivarius* CUL61. Cholylglycine hydrolase has been implicated in bile tolerance and the promotion of health (Begley et al., 2006) and such interactions should be a focus for future work.

Genome mining has identified several traits which each deserve more detailed downstream analysis to correlate gene prediction and phenotype presentation. To begin such work, the following chapters will focus on the safety aspect of AMR and the bioprospecting potential of *bsh* genes in CUL strains.

**3.5 Appendix 3.**

**One drive link for genomic island predictions Table S3.1-S3.16.**

**https://docs.google.com/spreadsheets/d/1nMXITUbpaqNWbBQfqJ0gNVltA3554Bn8/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true**

**Supplementary Table S3.1**. Genomic Island prediction for CUL37.

**Supplementary Table S3.2**. Genomic Island prediction for CUL07.

**Supplementary Table S3.3**. Genomic Island prediction for CUL08.

**Supplementary Table S3.4**. Genomic Island prediction for CUL06.

**Supplementary Table S3.5**. Genomic Island prediction for CUL63.

**Supplementary Table S3.6**. Genomic Island prediction for CUL40.

**Supplementary Table S3.7**. Genomic Island prediction for CUL67.

**Supplementary Table S3.8**. Genomic Island prediction for CUL76.

**Supplementary Table S3.9**. Genomic Island prediction for CUL09.

**Supplementary Table S3.10**. Genomic Island prediction for CUL61.

**Supplementary Table S3.11**. Genomic Island prediction for CUL21.

**Supplementary Table S3.12**. Genomic Island prediction for CUL60.

**Supplementary Table S3.13**. Genomic Island prediction for CUL66.

**Supplementary Table S3.14**. Genomic Island prediction for CUL66N.

**Supplementary Table S3.15**. Genomic Island prediction for CUL20.

**Supplementary Table S3.16**. Genomic Island prediction for CUL34.

**Supplementary Table S3.17-33.** Cazyzome predictions for CUL strains.

**https://docs.google.com/spreadsheets/d/10z4Bgz1t7nn5e3K3uw0uDkN6B0gvNmbi/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true**

**Supplementary Table S3.34**. Accession numbers for genes of interest.

**https://docs.google.com/spreadsheets/d/1d4XQiicdkmGCloamu32u1syeV8V04eyt/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true**

**Supplementary Table S3.35.** RAST subsystem breakdown for CUL strains.

**https://docs.google.com/spreadsheets/d/1KCyBUSZbBVjYfFhKpEOJ_TNTPt_tDlIT/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true**

# Chapter 4.
# Investigating the bile response in CUL strains.

## 4.1. Background

### 4.1.1. The importance of bile tolerance in probiotics

Survival throughout the gastro-intestinal tract is a desirable trait for a candidate probiotic bacterium. To be effective, a product must remain viable throughout its transit in the harsh and unfavourable environment generated by the body (Fijałkowski et al., 2016). As such, BA resistance or bile tolerance was identified by the WHO as a required attribute of any product marketed as a probiotic (Ganguly et al., 2011; WHO, 2002). In addition, certain microbial-bile interactions have been shown to facilitate the depletion of the BA pool, which has demonstrated several links with host serum cholesterol reduction (Costabile et al., 2017; Kumar et al., 2006). Therefore, such interactions should be investigated, to determine potential health-promoting attributes. One such approach is the screening of genome sequences for the presence of genes facilitating said functions. However, ascribing a genomic character is difficult as the mechanisms are often multifaceted: involving efflux pumps (Pfeiler & Klaenhammer, 2009; Piddock, 2006), enzymatic reactions (BSH) and in the case of tolerating the antimicrobial properties of bile, attributes such as cell wall architecture (Bustos et al., 2018). Thus, genotypic assessments must be performed alongside phenotypic screening to validate the survivability of the microorganisms throughout gut transit and to deduce the mechanisms which may aid in promoting gut health.

BSH are a group of enzymes produced by several microorganisms, which facilitate BA deconjugation (Begley et al., 2006; Moser et al., 2001). The physiological purpose of BSH production is unclear, however, bile detoxification is a popular hypothesis (Begley et al., 2006; Fang et al., 2009; Ridlon et al., 2015), albeit with conflicting results (Arnold et al., 2018; Hamon et al., 2011; Moser et al., 2001; Oh et al., 2012). In addition, BSH production is crucial, as it acts in a symbiotic nature with the host, facilitating the release of the steroid ring from the amino acid of the BA, allowing further oxidation and de-hydroxylation steps to take place, which leads to the production of secondary BA's (such as lithocholic and deoxycholic acid (Lambert, Bongers, et al., 2008)).

The confusion between BSH proteins with the structurally similar enzyme Penicillin V Acylases (PVA) is well established (Kumar et al., 2006). Indeed, Penicillin Acylases exhibit a high level of sequence similarity with BSH proteins (Kumar et al., 2006; Lambert, Bongers, et al., 2008), which often leads to erroneous annotation and conflicting evidence of *bsh* function (Long et al., 2017). To further exacerbate this, there is also a large degree of sequence dissimilarity within the BSH protein family. For example, BSH proteins often exhibit different substrate specificities (e.g., taurine or glycine conjugated BAs), which can potentially be attributed to specific amino acids located throughout the primary sequence (Xu et al., 2019). However,

these observations are drawn from a limited number of studies and may not reflect a protein-wide consensus, emphasising the difficulties in identifying BSH proteins and ascribing any activity to them, emphasizing the importance of phenotype to genotype correlation.

### 4.1.2. Limitations and Future Scope

Evaluating whether a candidate probiotic can tolerate physiological bile concentrations and deconjugate secondary BAs is difficult. Most studies focus on uncovering the deconjugation of BAs using agar plate assays and scoring the presence of a white precipitate around the bacterial inoculum (Dong et al., 2012; Kumar et al., 2012; Ren et al., 2011). Indeed, despite the support of this method in the literature, this chapter highlights its unreliable nature of it and offers a multifaceted approach to bioprospecting BSH activity in candidate probiotics.

### 4.1.3. Aims and objectives

This chapter sets out to investigate the interactions between CUL isolates and bile, based on the WHO guidelines (FAO/WHO, 2002), to evaluate bile tolerance and the BSH potential of probiotic strains. Bile tolerance profiles of all CUL strains will be determined using several techniques that encompass a broad range of bile concentrations. From **Chapter 3**, genes related to BSH function were putatively described in some CUL strains. Therefore, phenotypic BSH production will be correlated with a predicted genomic basis. Furthermore, targeted gene expression will be used to validate the capability of using a genome-guided approach to predict functional probiotic traits. Data generated in this chapter will provide an in-depth basis for the presence or absence of certain probiotic traits and functional capacities, with scope for bioprospecting identified.

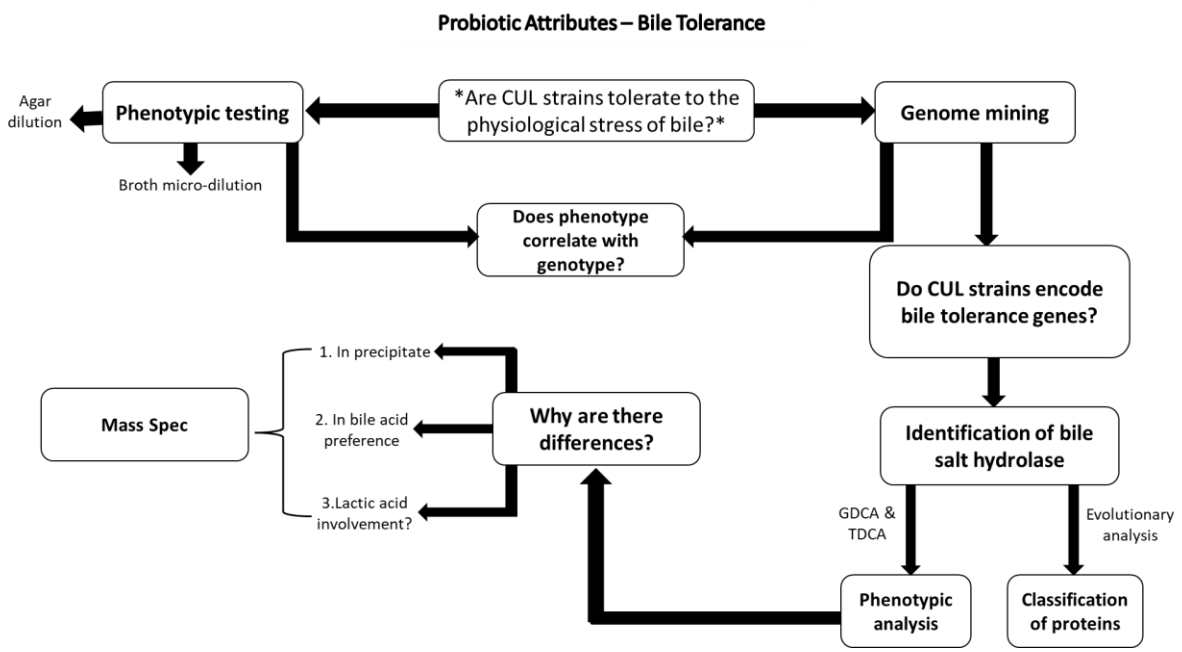



**Figure 4.1. A schematic of chapter 4's workflow.** The figure depicts the main questions focused on within this chapter and the experimental routes taken in the attempt to answer them.

## 4.2. Materials and Methods

### 4.2.1. Genome mining for genes involved with bile tolerance: phylogenetic analysis of cholylglycine hydrolase in CUL strains

As previously described (**Chapter 3, Table 3.7**), CUL genomes were mined using the RAST annotation server (Aziz et al., 2008) to identify genes associated with bile tolerance. Further, BSH protein sequences were identified and retrieved from CUL strains using BLASTp. In this case, known BSH proteins were used as queries for BLASTp with default BLAST parameters. PVA proteins, identified by (O'Flaherty et al., 2018), were also included in the phylogeny to guide the correct identification and annotation of BSH proteins. PVA proteins from O'Flaherty et al., (2018) were manually retrieved. Amino acid BSH sequences were retrieved from NCBI (accessed on 18.12.20). Sequences were only collected from strains that had complete genomes available. Sequences were then manually curated to remove erroneous gaps. Proteins were named with taxonomic and genomic positioning, to allow inference of species-level protein conservation. Multiple sequence alignment was performed in ClustalW ( Thompson et al., 2003) and a maximum likelihood phylogeny was constructed in MEGA7 ( Kumar et al., 2016), using the LG amino acid model (Le & Gascuel, 2008) + G and 1000 bootstrap replicates to assess the robustness of the reconstructed topology. BSH clades demarcated on the phylogeny with well-supported (bootstrap value > 50 %) nodes were assigned and labelled.

### 4.2.2. Determining Bile Tolerance

#### 4.2.2.1 *Semi-quantitative analysis using 2-fold dilutions in MRS agar*

Overnight cultures of CUL strains were grown anaerobically at 37 °C, as described in section **2.2.1. - 2.2.2**. Bile tolerance was assessed using 2-fold dilutions (between 6.4 mM to 0.4 mM) of bovine bile (Sigma, B3883) in MRS agar.

Bile is made up of several organic and inorganic substrates. Of the substrate pool, approximately 50% are bile acids. When determining probiotic tolerance of bile acids, numerous reports cite 0.3 % Oxgall bile as the most physiological representation, without further context or rationale (Hu et al., 2018b; Lee et al., 2011; Lin et al., 2007). However, bile acid concentrations in the body can fluctuate (Gunn, 2000; Hu et al., 2018b). For example, ranging from 4 mM (pre-prandial) to 14 mM (postprandial (Humbert et al., 2018; Northfield & McColl, 1973). Therefore, to assess bile tolerance here, a 2-fold dilution series (6.4 mM to 0.4 mM) of bovine bile (Sigma, B3883) was performed in MRS agar. This brand of bovine bile was chosen as it has been shown to resemble most closely human bile (Hu et al., 2018b). the concentration range (0.4 mM to 6.4 mM) was chosen to approximate post-prandial total BA concentrations in the gut (Northfield & McColl, 1973). Such concentrations were calculated based on the frequently reported physiological concentration of bile (0.3 % bile diluted in MRS broth), which was used as a starting concentration. From there, the respective concentrations of bile acids within the Oxgall pool were converted into mM (0.3 % = 0.4 mM) using the Oxgall composition identified and reported by Hu et al., (2018b).

Briefly, overnight cultures of each strain were pelleted by centrifugation (3000 *g* for 10 minutes), washed once with fresh MRS broth and resuspended to 0.1 $OD_{600}$ (approximately 1 McFarland standard) in fresh MRS broth; providing approximately 5.56 x $10^8$ ± 4.67 x $10^8$/ mL (depending on the species) CFUs (as per **method 2.2.2**). This dilution was chosen to ensure that clear, reproducible growth was observed on plates. 5 µL of diluted culture ( ~ 1 x $10^7$) was used to spot inoculate agar plates containing bovine bile. MRS agar plates, without bile, were used as growth controls. Plates were incubated anaerobically between 48 - 72 h at 37 °C. Survivability under each bile concentration was determined by the presence or absence of growth in 3 independent experiments.

#### *4.2.2.2. Broth Microdilution*

Independent overnight cultures of CUL strains were grown anaerobically at 37 °C (as described in **2.2.2**). Bile tolerance was determined for lactobacilli CUL isolates using a 2-fold dilution series of bovine bile (Sigma, B3883) prepared in sterile MRS broth. A maximum stock solution of bovine bile was prepared at 13.8 mM and serial diluted two-fold to a minimum concentration of 0.86 mL. Following, 100 µL of each dilution of bovine bile was added to each well of a 96-well plate. Previously grown overnight cultures of CUL strains were pelleted by centrifugation (3000 *g* for 10 minutes), washed once with fresh MRS broth and resuspended at 0.2 $OD_{600.}$ Final concentrations of 0.1 $OD_{600}$ were achieved by adding 100 µL of bacterial culture to each well. The addition of the bacterial cultures resulted in bovine bile concentrations being diluted a further 1:2 times, providing a final concentration gradient ranging from 0.4 mM to 6.4 mM. Controls included wells with MRS media only, to assess for contamination, wells with MRS broth + bacteria as a growth standard and MRS + bovine bile. Plates were incubated anaerobically for 18 h and read at 600 nm spectrophotometrically, using a Multiskan FC (Thermo Scientific). Bile tolerance was inferred by calculating the difference in absorbance (after subtracting the OD value of bile), between the growth control and growth in the presence of bile. The assay was performed independently 3 times, each with triplicate samples (n = 3).

### 4.2.3. Assessing the ability of CUL isolates to deconjugate conjugated bile acids

Independent overnight cultures of CUL strains were grown anaerobically at 37 °C. The ability to deconjugate CBA's (TDCA and GDCA) was assessed using a 2-fold dilution series of both TDCA (Sigma, T0875) and GDCA (Sigma, G9910) diluted in MRS agar (independently). Concentrations ranged from 0.48 % (as suggested in(Elkins et al., 2001)) to 0.0075 %, to determine concentration-dependent reactions. This method was adapted from Shehata et al., (2016). Briefly, overnight cultures were pelleted by centrifugation (3000 *g* for 10 minutes), washed once in fresh MRS broth and resuspended to 0.1 $OD_{600}$ (approximately 1 McFarland standard / 5.56 x $10^8$ (($\pm$ 4.67 x $10^8$ dependant on species) CFUs/ mL). 5 µL of diluted culture was spot inoculated onto agar plates containing either TDCA or GDCA. MRS agar without BA's was used as negative controls. Plates were incubated anaerobically between 48 - 72 h at 37°C. Survivability of each CUL strain was determined by the presence or absence of growth and the ability to deconjugate conjugated BAs was scored based on the presence of a white precipitate (deoxycholic/cholic acid) surrounding the inoculated spot in 3 independent experiments. If variations in precipitate type occurred, these were also noted and imaged for analysis.

### 4.2.4. Assessing the ability of lactic acid to deconjugate secondary bile acids

To determine whether the acidification of the agar (due to the CUL strain's ability to produce lactic acid), had any influence on the development of the precipitate, agar plates containing TDCA and GDCA were also inoculated with 5 µL of L- lactic acid (Merck, L1750) or an equimolar mixture of both lactic acid optical isomers, DL - lactic acid (Merck, 69785) at a neat maximum concentration of 98 % (pH 1.2) and 90 % (approximately pH 1.2) respectively. Precipitates were photographed after 24 h. In all cases, where a precipitate was formed in the agar, these were cored out using a sterile blade and stored at - 80 °C in an airtight container for further downstream analysis.

To aid with visualising the formation of precipitates, all agar plates were imaged under both natural lighting and with illumination from underneath (using a lightbox).

For the TDCA/GDCA plate assay, a score was assigned to precipitate formation based on a visual comparison to control growth. Scores ranged between 0 – 5, where 0: indicates standard growth with no precipitate, 1. Weak growth; 2. Precipitation on agar surface; 3. Precipitation plug, scattering in the agar; 4. Precipitation plug-in agar, and 5. No growth. Following, a heatmap was constructed in R, using packages GGPLOT (Wickham, 2006), and heatmap. plus (Day, 2012) and Rcolorbrewer (Neuwirth & Neuwirth, 2011), to visualise the trends between CUL strains and their phenotypes when challenged with concentrations of BA and varying BAs substrates. The survivability of each CUL strain was determined by the presence or absence of growth.

### 4.2.5. The quantification of bile deconjugation via Gas Chromatography/Mass Spectrophotometry (GC/MS)

To quantify BA deconjugation, samples were prepared for GC/MS as follows. Overnight broth cultures (in MRS) were prepared as previously stated (**2.2.2**). Cultures were split into equal volumes (2 x 10 mL per culture) and cells were pelleted via centrifugation (3000 *g* for 10 mins). Supernatants were removed and cultures were resuspended in either 10 mL MRS broth, or 10 mL 0.43 mM bovine bile (diluted in MRS broth). Cultures were incubated for 3 hours anaerobically, after which, cell-free extracts were collected by centrifugation (3000 *g* for 10 mins) and the supernatant was filtered through a 0.22 µM syringe filter. Three independent biological replicates for each strain and condition were collected. To putatively identify in-agar precipitates**,** two strains (*L.acidophilus* CUL60 and *L.gasseri* CUL09) were spot-inoculated onto the agar plates (MRS + GDCA) as described by the method outlined in **4.2.3**. Plates were incubated anaerobically for 48 h and precipitates were cored out using a sterile blade. Samples were stored in an airtight container at 4 °C before GC/MS analysis.

### 4.2.6. Extraction of bile acids from cell-free supernatants and agar-plugs *(conducted by Dr Josie Parker, Swansea University).*

All procedures were carried out in glass tubes. BAs were extracted from cell-free supernatants or agar plugs as follows, initially, agar plugs were dissolved in 2 mL dd$H_2O$ at 80 °C for 30 min with occasional vortexing. Before BA extraction an internal standard of 5 µL of 2 mg/mL (10 µg) 5α-Cholestanol (Merck, D6128) was added to each sample. BAs were extracted from either 1 mL dissolved agar or 0.25 mL filtered culture media by the addition of 3 mL of ethyl acetate followed by vortexing (30 seconds). The ethyl acetate layer was removed and transferred to a clean glass tube. Extractions were repeated with two volumes of ethyl acetate pooled and dried in a vacuum centrifuge. Samples were then analysed by the method reported by Parker et al. (2013). Briefly, samples were derivatised by the addition of 0.1 mL N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA TMCS) (99:1,Merck,10255) and 0.3 mL anhydrous pyridine (Merck, 270970) and heated at 80 °C for 2 h. TMS-derivatised BAs were analysed and identified using GC/MS (Thermo 1300 GC coupled to a Thermo ISQ mass spectrometer, Thermo Scientific) and Xcalibur software (Thermo Scientific). The retention times and mass spectra for the following known standards were used to identify BAs. 5α-Cholestanol (retention time 19.21 minutes), Cholesterol (retention time, 20.0 minutes), Glyocodeoxycholic acid (retention time, 19.76 minutes), Taurodeoxycholic acid (retention time, 19.78 minutes), Chenodeoxycholic acid (21.75 minutes), Lithocholic acid (retention time. 21.33 minutes), Deoxycholic acid (retention time, 21.54 minutes), Taurocholic acid (retention time, 20.34 minutes), and Gylcocholic acid (retention time, 20.78 minutes).

### 4.2.7. Data analysis

BAs were quantified by calculating the peak area of each component in a 0.3 % bile + MRS standard, relative to the loading control 5α-Cholestanol (n = 3). Peak area relative to the loading control was calculated as a percentage of the bile standard and averaged (n = 3), Data were presented as a percentage increase or decrease compared to control.

### 4.2.8. BSH gene expression

Overnight cultures of *L.plantarum* CUL66N were prepared as described in section **2.2.2**. Following, cultures were separated into two 10 mL aliquots (A and C), vortexed (5 seconds) and centrifuged (3000 *g* for 10 minutes), to pellet the cells. The supernatant was discarded. 2 % bovine bile was prepared in sterile MRS broth. Pellets were either resuspended in 10 mL of 2 % bile or fresh MRS as a control. Cultures were incubated for 1 h. Following, cultures were centrifuged (3000 *g* for 10 minutes), resuspended in 5 mL RNA protect (Qiagen, 76506 (to stabilise RNA)) and incubated overnight, at room temperature. Cultures were centrifuged

(3000 *g* for 10 minutes) to remove RNA protect and washed in 200 µL of NaCl + EDTA (pH 8.0) three times. Pellets were resuspended in 200 µL of lysozyme (20 mg/mL) and incubated at 37 °C for 1 h. Following, 100 units of mutanolysin, 40 µL of proteinase K and 350 µL of RTL buffer (Qiagen, 79216) were added to aid lysis and incubate at 56 °C for 1 h. The total RNA was isolated using the RNeasy Mini Kit (Qiagen, United Kingdom). RNA was finally resuspended in 25 µL of milli water. The concentration and purity of the total RNA extracted were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and the integrity was checked by gel electrophoresis (on 1 % agarose gel). Residual DNA was removed by treating RNA with RNase-free DNase I, as per the manufacturer's instructions (Thermo Fisher, EN0521). RNA was precipitated with 2.5 M lithium chloride, overnight, at -20°C. Precipitated nucleic acids were desalted with 70 % ethanol and resuspended in 25 µL of nuclease-free water. Following, RNA was diluted to a standard working concentration of 100 ng/µL. Ten micrograms of RNA were reverse transcribed to cDNA with cDNA Ultrascript (PCR Biosystems, United Kingdom) according to the manufacturer's protocol.

### 4.2.9. Quantitative PCR

Specific gene primers were designed for the three predicted *bsh* genes in *L.plantarum* CUL66N and the housekeeping gene (*ldlH*), using Beacon Designer (**Table 4.1** (Thornton & Basu, 2011)), specifically for CUL66N and synthesised by Eurofins Genomics (Germany). Quantitative Polymerase Chain Reactions (qPCR) were performed in 20 µL volumes and cycled in an IQ5 thermocycler (Bio-Rad, UK) with FastStart Universal SYBR Green Master (Roche, Germany). The total reaction volume was 20 µL, which was comprised of: 10 µL of SYBR, 2.5 µL of a forward primer, 2.5 µL of a reverse primer and 2 µg of cDNA template. Thermocycling profiles were measured for 35 cycles. Annealing temperatures (TA) were chosen as 5 °C less than those calculated by beacon (Thornton & Basu, 2011). A dilution gradient of gDNA was utilised to calculate the primer efficiencies (**Supplementary Figures S4.8 – S4.11**). Fold expression was calculated according to the (Pfaffl, 2001) method. Experiments were conducted in triplicate and results are reported ± SD.

**Table 4.1. *bsh* and housekeeping primers designed for *L.plantarum* CUL66N gene expression analysis.** Primers were calculated by Beacon Designer (Thornton & Basu, 2011).

| Primer | Sequence | TA °C |
|---|---|---|
| ldhDF | CCGTGCTGCCATCGATATTT | 52.5 |
| ldhDR | CGTCCAAGGTGTCAACGTAC | 52.5 |
| PEG40F | AGAGTGGTTCTACTTACG | 55.3 |
| PEG40R | ATTCTTGAGATGCCATTC | 55.3 |
| PEG1210F | AACTATCGTGCCTTATCAA | 54.6 |
| PEG1210R | CAAGTCTCCAGGTAATCC | 54.6 |
| PEG1331F | TACCTGGTGACTATACTT | 55.6 |
| PEG1331R | TTGACTGTATCTGTTGTT | 55.6 |

## 4.3. Results

### 4.3.1. Phylogenetic analysis and annotation of CUL cholylglycine hydrolases

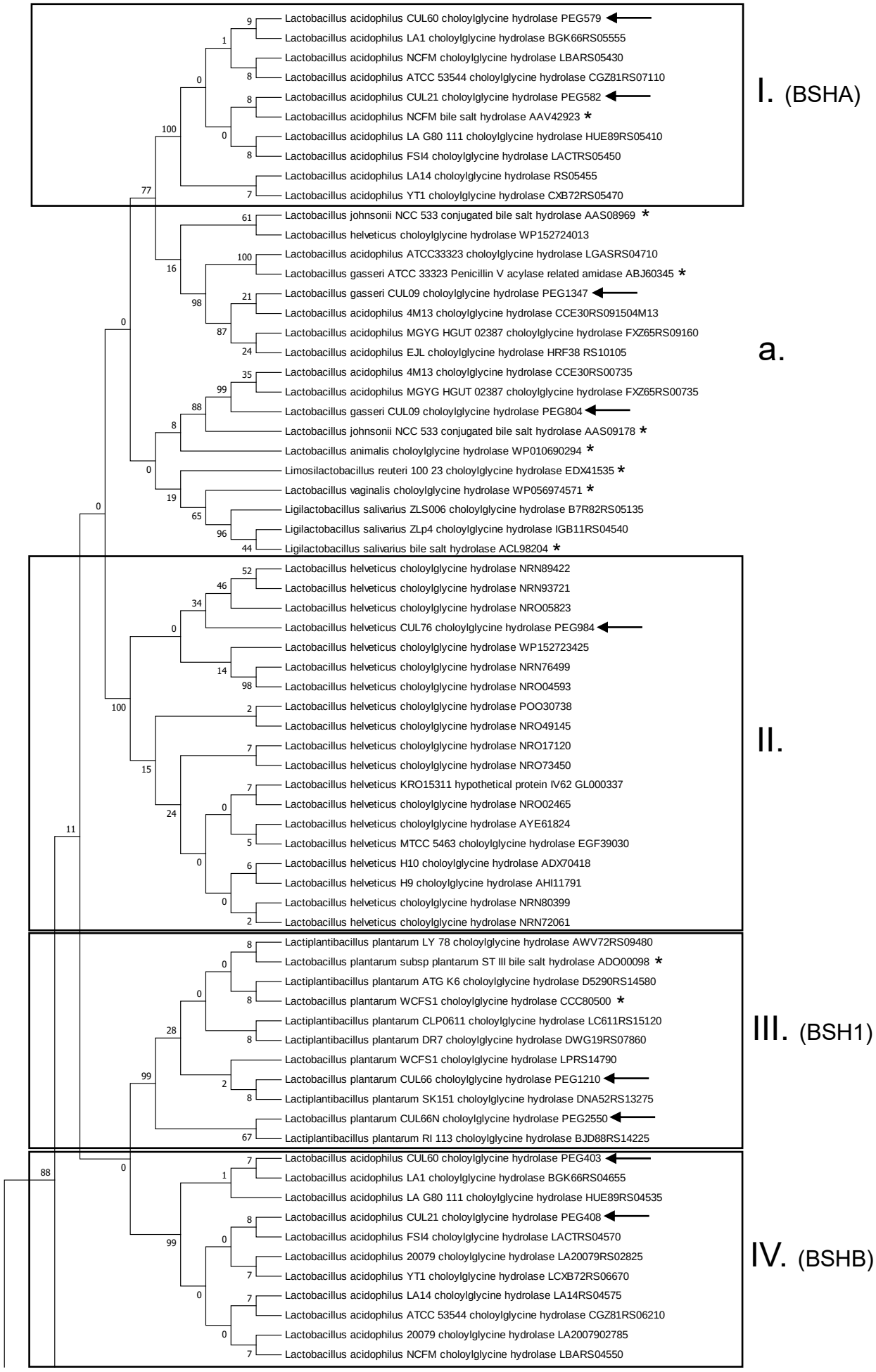
The phylogenetic reconstruction of the evolutionary lineage of BSH and PVA proteins revealed the presence of ten distinct homogenous clades of proteins, which were subsequently labelled (**I – X, Figure 4.1**). The location of the reference PVA and BSH sequences in the phylogeny provided the rationale for similar gene annotations to be given to predicted BSH sequences identified in CUL strains (**Chapter 3, Table 3.7**). Clade annotation was determined by the presence of well described BSH proteins in the literature. For example, clade I and clade IV contain BSHA and BSHB respectively, that have been previously described in *L.acidophilus* NCFM (McAuliffe et al., 2005). In these clades, proteins from the same species were grouped and annotated similarly.

Smaller, non-homogenous clusters with lower bootstrap values were labelled a, b, and c; **Figure 4.1.** Species composition in group a is like that of group b, with pairs of proteins retrieved from the same genome, distributed across these two sub-groups (for example, the genome *L.johnsonii* NC533 contained two proteins annotated as conjugated bile salt hydrolases which separated into subgroups a and b). Both sub-groups *a* and *b* are comprised of proteins with varying annotations (e.g., PVA-related amidase, conjugated bile salt hydrolase and cholylglycine hydrolase) and both a and b form sister groups to well-supported clades of *L.acidophilus* proteins. That said, the species that outgroup the *L.acidophilus* clades (subgroup a and b), are typically other members of the phylogroup *L.delbrueckii* (such as *L.gasseri* and *L.johnsonii*). Neither cluster a nor b contain any of the reference sequences (identified by O'Flaherty et al., 2018) as PVAs. Two cholylglycine hydrolase proteins found in *L.gasseri* CUL09 separated between these two subgroups.

Clade I and Clade IV (**Figure 4.1**) were comprised of protein pairs retrieved from the same genome, where each pair separates between Clade I and Clade IV, except for *L.acidophilus* 20079, as both proteins encoded in this genome grouped together within Clade V. For CUL genomes, *L.acidophilus* CUL21 PEG582 and *L.acidophilus* CUL60 PEG579 were annotated as BSHB, whereas *L.acidophilus* CUL21 PEG408 and *L.acidophilus* CUL60 PEG403 were recognised as BSHA proteins. It is noteworthy that there appears to be some separation between proteins from the same genomes. Likewise, Clade II and VI, are solely comprised of protein sequences retrieved from *L.helveticus* strains. Clade II is comprised of *L.helveticus* proteins annotated as linear amide C-N hydrolase and proteins within Clade VI are all annotated as cholylglycine hydrolase. *L.helveticus* CUL76 PEG984 is located within Clade VI (**Figure 4.2**). Both *L.helveticus* clades share a most recent common ancestral protein with two well-supported and characterised *L.salivarius* BSH clades, Clade V and IX respectively (**Figure 4.2**).

Between one to two protein sequences were predicted per *L.salivarius* genome. Within Clade V, the predicted BSH sequences from individual *L.salivarius* strains were grouped together (**Figure 4.2**). All the *L.salivarius* strains within Clade V, had two genes, with the second copy separating into Clade IX, splitting the homologs by a large dichotomy. Moreover, Clade V was comprised of protein-encoding genes identified on *L.salivarius* mega plasmids, whereas all BSH proteins in Clade IX were predicted to be chromosomally encoded (**Figure 4.2**). Two copies of cholylglycine hydrolase proteins were predicted in *L.salivarius* CUL61 (**Chapter 3, Table 3.7**), the first PEG1728 was found within Clade V, clustering with plasmid-encoded genes, suggesting a potential for CUL61 to encode a mobile element. Further BLAST analysis provided a putative clade annotation of BSH1. *L.salivarius* CUL61 PEG435 was in Clade IX and groups with other chromosomal encoded proteins from *L.salivarius.* BLASTp analysis revealed proteins within this clade are generally annotated as BSH2 for *L.salivarius* species. Clade X is closely related to Clade IX and is made up of sequences from *L.fermentum* genomes. Intriguingly, two PVA reference strains designated by O'Flaherty et al., (2018) were in Clade V (**Figure 4.2**).

Previously there have been between 3 to 4 *bsh* copies (*bsh*1, 2, 3, and 4) described in *L.plantarum* genomes (Lambert, Bongers, et al., 2008). Three copies of cholylglycine hydrolase were retrieved from both CUL *L.plantarum* strains (CUL66 and CUL66N (**Chapter 3, Table 3.7**)). The presence of three distinct *L.plantarum* protein groups emerged in the maximum likelihood phylogeny (as seen in Clades III, VII and VIII). From each genome, the three proteins retrieved were spread equally across the three clades. Further BLAST analysis provided tentative gene annotations as Clade III - BSH1, Clade VII - BSH2 and Clade VIII - BSH3. Interestingly, one *L.plantarum* strain that fell outside of Clade IX (*Lactobacillus plantarum* STIII ADN99333) was described as BSH4. This protein was clustered with additional sequences from the PVA reference dataset (indicated with **). For the two CUL *L.plantarum* strains, both encoded three protein copies, which were also equally distributed throughout the three clades. CUL66 PEG1210 and CUL66N PEG2550 were located within clade III and were therefore annotated as BSH1. CUL66 PEG1331 and CUL66N PEG2345 were grouped with clade VII and were designated as BSH2 proteins and finally, CUL66 PEG40 and CUL66N PEG2416 clustered with Clade IX and were therefore annotated as BSH3 (**Figure 4.2**).


Lactobacillus acidophilus CUL60 choloylglycine hydrolase PEG579
Lactobacillus acidophilus LA1 choloylglycine hydrolase BGK66RS05555
Lactobacillus acidophilus NCFM choloylglycine hydrolase LBARS05430
Lactobacillus acidophilus ATCC 53544 choloylglycine hydrolase CGZ81RS07110
Lactobacillus acidophilus CUL21 choloylglycine hydrolase PEG582
Lactobacillus acidophilus NCFM bile salt hydrolase AAV42923 *
Lactobacillus acidophilus LA G80 111 choloylglycine hydrolase HUE89RS05410
Lactobacillus acidophilus FSI4 choloylglycine hydrolase LACTRS05450
Lactobacillus acidophilus LA14 choloylglycine hydrolase RS05455
Lactobacillus acidophilus YT1 choloylglycine hydrolase CXB72RS05470
I. (BSHA)
Lactobacillus johnsonii NCC 533 conjugated bile salt hydrolase AAS08969 *
Lactobacillus helveticus choloylglycine hydrolase WP152724013
Lactobacillus acidophilus ATCC33323 choloylglycine hydrolase LGASRS04710
Lactobacillus gasseri ATCC 33323 Penicillin V acylase related amidase ABJ60345 *
Lactobacillus gasseri CUL09 choloylglycine hydrolase PEG1347
Lactobacillus acidophilus 4M13 choloylglycine hydrolase CCE30RS091504M13
Lactobacillus acidophilus MGYG HGUT 02387 choloylglycine hydrolase FXZ65RS09160
Lactobacillus acidophilus EJL choloylglycine hydrolase HRF38 RS10105
Lactobacillus acidophilus 4M13 choloylglycine hydrolase CCE30RS00735
Lactobacillus acidophilus MGYG HGUT 02387 choloylglycine hydrolase FXZ65RS00735
Lactobacillus gasseri CUL09 choloylglycine hydrolase PEG804
Lactobacillus johnsonii NCC 533 conjugated bile salt hydrolase AAS09178 *
Lactobacillus animalis choloylglycine hydrolase WP010690294 *
Limosilactobacillus reuteri 100 23 choloylglycine hydrolase EDX41535 *
Lactobacillus vaginalis choloylglycine hydrolase WP056974571 *
Ligilactobacillus salivarius ZLS006 choloylglycine hydrolase B7R82RS05135
Ligilactobacillus salivarius ZLp4 choloylglycine hydrolase IGB11RS04540
Ligilactobacillus salivarius bile salt hydrolase ACL98204 *
a.
Lactobacillus helveticus choloylglycine hydrolase NRN89422
Lactobacillus helveticus choloylglycine hydrolase NRN93721
Lactobacillus helveticus choloylglycine hydrolase NRO05823
Lactobacillus helveticus CUL76 choloylglycine hydrolase PEG984
Lactobacillus helveticus choloylglycine hydrolase WP152723425
Lactobacillus helveticus choloylglycine hydrolase NRN76499
Lactobacillus helveticus choloylglycine hydrolase NRO04593
Lactobacillus helveticus choloylglycine hydrolase POO30738
Lactobacillus helveticus choloylglycine hydrolase NRO49145
Lactobacillus helveticus choloylglycine hydrolase NRO17120
Lactobacillus helveticus choloylglycine hydrolase NRO73450
Lactobacillus helveticus KRO15311 hypothetical protein IV62 GL000337
Lactobacillus helveticus choloylglycine hydrolase NRO02465
Lactobacillus helveticus choloylglycine hydrolase AYE61824
Lactobacillus helveticus MTCC 5463 choloylglycine hydrolase EGF39030
Lactobacillus helveticus H10 choloylglycine hydrolase ADX70418
Lactobacillus helveticus H9 choloylglycine hydrolase AHI11791
Lactobacillus helveticus choloylglycine hydrolase NRN80399
Lactobacillus helveticus choloylglycine hydrolase NRN72061
II.
Lactiplantibacillus plantarum LY 78 choloylglycine hydrolase AWV72RS09480
Lactobacillus plantarum subsp plantarum ST III bile salt hydrolase ADO00098 *
Lactiplantibacillus plantarum ATG K6 choloylglycine hydrolase D5290RS14580
Lactobacillus plantarum WCFS1 choloylglycine hydrolase CCC80500 *
Lactiplantibacillus plantarum CLP0611 choloylglycine hydrolase LC611RS15120
Lactiplantibacillus plantarum DR7 choloylglycine hydrolase DWG19RS07860
Lactobacillus plantarum WCFS1 choloylglycine hydrolase LPRS14790
Lactobacillus plantarum CUL66 choloylglycine hydrolase PEG1210
Lactiplantibacillus plantarum SK151 choloylglycine hydrolase DNA52RS13275
Lactobacillus plantarum CUL66N choloylglycine hydrolase PEG2550
Lactiplantibacillus plantarum RI 113 choloylglycine hydrolase BJD88RS14225
III. (BSH1)
Lactobacillus acidophilus CUL60 choloylglycine hydrolase PEG403
Lactobacillus acidophilus LA1 choloylglycine hydrolase BGK66RS04655
Lactobacillus acidophilus LA G80 111 choloylglycine hydrolase HUE89RS04535
Lactobacillus acidophilus CUL21 choloylglycine hydrolase PEG408
Lactobacillus acidophilus FSI4 choloylglycine hydrolase LACTRS04570
Lactobacillus acidophilus 20079 choloylglycine hydrolase LA20079RS02825
Lactobacillus acidophilus YT1 choloylglycine hydrolase LCXB72RS06670
Lactobacillus acidophilus LA14 choloylglycine hydrolase LA14RS04575
Lactobacillus acidophilus ATCC 53544 choloylglycine hydrolase CGZ81RS06210
Lactobacillus acidophilus 20079 choloylglycine hydrolase LA2007902785
Lactobacillus acidophilus NCFM choloylglycine hydrolase LBARS04550
IV. (BSHB)

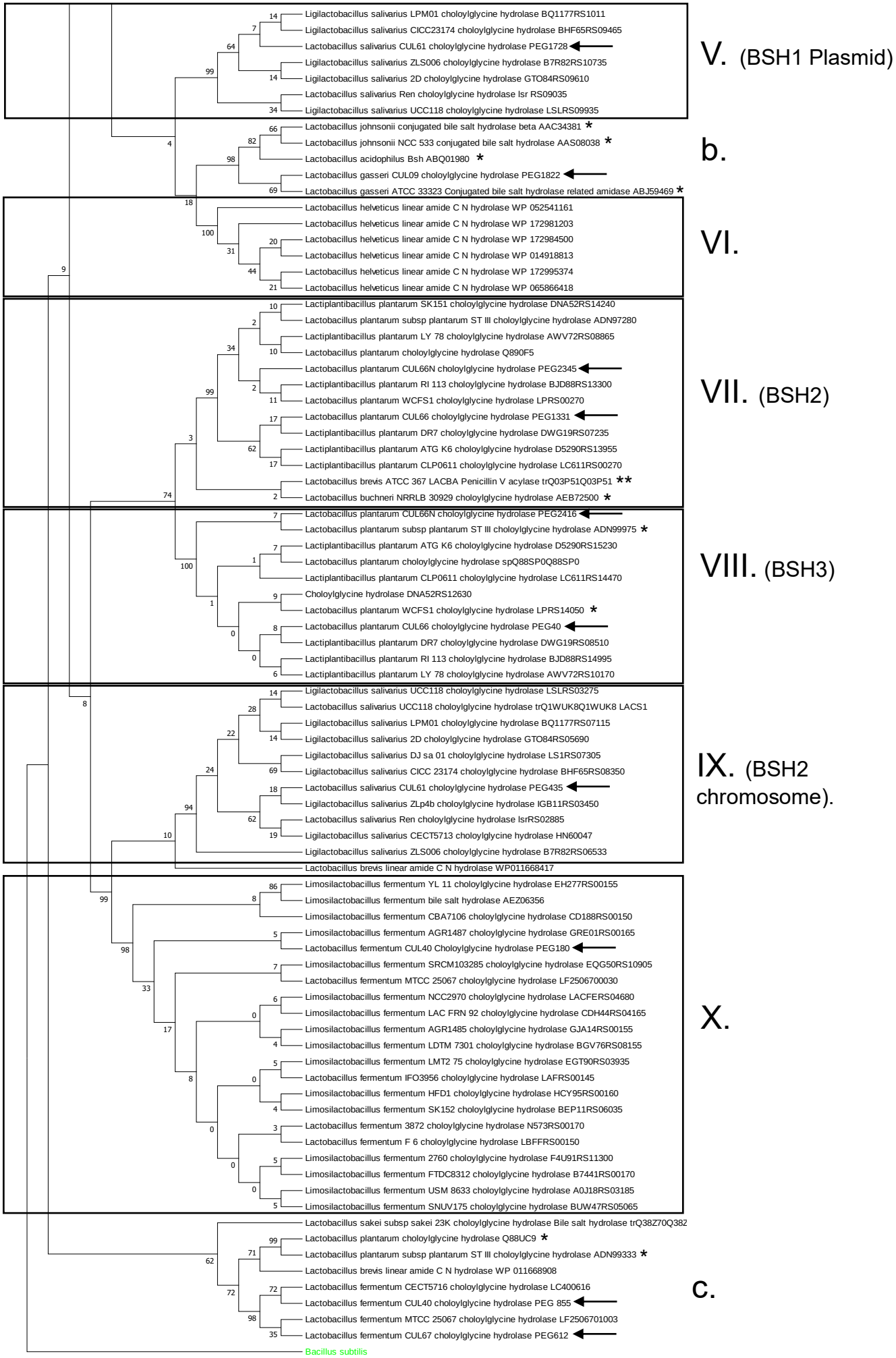

Ligilactobacillus salivarius LPM01 choloylglycine hydrolase BQ1177RS1011
Ligilactobacillus salivarius ClCC23174 choloylglycine hydrolase BHF65RS09465
Lactobacillus salivarius CUL61 choloylglycine hydrolase PEG1728
Ligilactobacillus salivarius ZLS006 choloylglycine hydrolase B7R82RS10735
Ligilactobacillus salivarius 2D choloylglycine hydrolase GTO84RS09610
Lactobacillus salivarius Ren choloylglycine hydrolase lsr RS09035
Ligilactobacillus salivarius UCC118 choloylglycine hydrolase LSLRS09935
V. (BSH1 Plasmid)
Lactobacillus johnsonii conjugated bile salt hydrolase beta AAC34381 *
Lactobacillus johnsonii NCC 533 conjugated bile salt hydrolase AAS08038 *
Lactobacillus acidophilus Bsh ABQ01980 *
Lactobacillus gasseri CUL09 choloylglycine hydrolase PEG1822
Lactobacillus gasseri ATCC 33323 Conjugated bile salt hydrolase related amidase ABJ59469 *
b.
Lactobacillus helveticus linear amide C N hydrolase WP 052541161
Lactobacillus helveticus linear amide C N hydrolase WP 172981203
Lactobacillus helveticus linear amide C N hydrolase WP 172984500
Lactobacillus helveticus linear amide C N hydrolase WP 014918813
Lactobacillus helveticus linear amide C N hydrolase WP 172995374
Lactobacillus helveticus linear amide C N hydrolase WP 065866418
VI.
Lactiplantibacillus plantarum SK151 choloylglycine hydrolase DNA52RS14240
Lactobacillus plantarum subsp plantarum ST III choloylglycine hydrolase ADN97280
Lactiplantibacillus plantarum LY 78 choloylglycine hydrolase AWV72RS08865
Lactobacillus plantarum choloylglycine hydrolase Q890F5
Lactobacillus plantarum CUL66N choloylglycine hydrolase PEG2345
Lactiplantibacillus plantarum RI 113 choloylglycine hydrolase BJD88RS13300
Lactobacillus plantarum WCFS1 choloylglycine hydrolase LPRS00270
Lactobacillus plantarum CUL66 choloylglycine hydrolase PEG1331
Lactiplantibacillus plantarum DR7 choloylglycine hydrolase DWG19RS07235
Lactiplantibacillus plantarum ATG K6 choloylglycine hydrolase D5290RS13955
Lactiplantibacillus plantarum CLP0611 choloylglycine hydrolase LC611RS00270
Lactobacillus brevis ATCC 367 LACBA Penicillin V acylase trQ03P51Q03P51 **
Lactobacillus buchneri NRRLB 30929 choloylglycine hydrolase AEB72500 *
VII. (BSH2)
Lactobacillus plantarum CUL66N choloylglycine hydrolase PEG2416
Lactobacillus plantarum subsp plantarum ST III choloylglycine hydrolase ADN99975 *
Lactiplantibacillus plantarum ATG K6 choloylglycine hydrolase D5290RS15230
Lactobacillus plantarum choloylglycine hydrolase spQ88SP0Q88SP0
Lactiplantibacillus plantarum CLP0611 choloylglycine hydrolase LC611RS14470
Choloylglycine hydrolase DNA52RS12630
Lactobacillus plantarum WCFS1 choloylglycine hydrolase LPRS14050 *
Lactobacillus plantarum CUL66 choloylglycine hydrolase PEG40
Lactiplantibacillus plantarum DR7 choloylglycine hydrolase DWG19RS08510
Lactiplantibacillus plantarum RI 113 choloylglycine hydrolase BJD88RS14995
Lactiplantibacillus plantarum LY 78 choloylglycine hydrolase AWV72RS10170
VIII. (BSH3)
Ligilactobacillus salivarius UCC118 choloylglycine hydrolase LSLRS03275
Lactobacillus salivarius UCC118 choloylglycine hydrolase trQ1WUK8Q1WUK8 LACS1
Ligilactobacillus salivarius LPM01 choloylglycine hydrolase BQ1177RS07115
Ligilactobacillus salivarius 2D choloylglycine hydrolase GTO84RS05690
Ligilactobacillus salivarius DJ sa 01 choloylglycine hydrolase LS1RS07305
Ligilactobacillus salivarius ClCC 23174 choloylglycine hydrolase BHF65RS08350
Lactobacillus salivarius CUL61 choloylglycine hydrolase PEG435
Ligilactobacillus salivarius ZLp4b choloylglycine hydrolase IGB11RS03450
Lactobacillus salivarius Ren choloylglycine hydrolase lsrRS02885
Ligilactobacillus salivarius CECT5713 choloylglycine hydrolase HN60047
Ligilactobacillus salivarius ZLS006 choloylglycine hydrolase B7R82RS06533
IX. (BSH2 chromosome).
Lactobacillus brevis linear amide C N hydrolase WP011668417
Limosilactobacillus fermentum YL 11 choloylglycine hydrolase EH277RS00155
Limosilactobacillus fermentum bile salt hydrolase AEZ06356
Limosilactobacillus fermentum CBA7106 choloylglycine hydrolase CD188RS00150
Limosilactobacillus fermentum AGR1487 choloylglycine hydrolase GRE01RS00165
Lactobacillus fermentum CUL40 Choloylglycine hydrolase PEG180
Limosilactobacillus fermentum SRCM103285 choloylglycine hydrolase EQG50RS10905
Lactobacillus fermentum MTCC 25067 choloylglycine hydrolase LF2506700030
Limosilactobacillus fermentum NCC2970 choloylglycine hydrolase LACFERS04680
Limosilactobacillus fermentum LAC FRN 92 choloylglycine hydrolase CDH44RS04165
Limosilactobacillus fermentum AGR1485 choloylglycine hydrolase GJA14RS00155
Limosilactobacillus fermentum LDTM 7301 choloylglycine hydrolase BGV76RS08155
Limosilactobacillus fermentum LMT2 75 choloylglycine hydrolase EGT90RS03935
Lactobacillus fermentum IFO3956 choloylglycine hydrolase LAFRS00145
Limosilactobacillus fermentum HFD1 choloylglycine hydrolase HCY95RS00160
Limosilactobacillus fermentum SK152 choloylglycine hydrolase BEP11RS06035
Lactobacillus fermentum 3872 choloylglycine hydrolase N573RS00170
Lactobacillus fermentum F 6 choloylglycine hydrolase LBFFRS00150
Limosilactobacillus fermentum 2760 choloylglycine hydrolase F4U91RS11300
Limosilactobacillus fermentum FTDC8312 choloylglycine hydrolase B7441RS00170
Limosilactobacillus fermentum USM 8633 choloylglycine hydrolase A0J18RS03185
Limosilactobacillus fermentum SNUV175 choloylglycine hydrolase BUW47RS05065
X.
Lactobacillus sakei subsp sakei 23K choloylglycine hydrolase Bile salt hydrolase trQ38Z70Q382
Lactobacillus plantarum choloylglycine hydrolase Q88UC9 *
Lactobacillus plantarum subsp plantarum ST III choloylglycine hydrolase ADN99333 *
Lactobacillus brevis linear amide C N hydrolase WP 011668908
Lactobacillus fermentum CECT5716 choloylglycine hydrolase LC400616
Lactobacillus fermentum CUL40 choloylglycine hydrolase PEG 855
Lactobacillus fermentum MTCC 25067 choloylglycine hydrolase LF2506701003
Lactobacillus fermentum CUL67 choloylglycine hydrolase PEG612
Bacillus subtilis
c.

**Figure 4.2. Maximum Likelihood phylogenetic analysis of proteins annotated as BSH, Cholylglycine hydrolase and PVA.** The evolutionary history was deduced using the Maximum likelihood method (Le Cam, 1990). The bootstrap consensus tree was inferred from 1000 replicates (Horowitz, 2001) to represent the evolutionary history of the proteins analysed and is indicated along tree branches. The evolutionary distances were computed using the LG + G method (Le & Gascuel, 2008) and units are the number of amino acid substitutions per site. The analysis involved 151 amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

### 4.3.2. *in vitro* phenotypic capability of CUL isolates to tolerate bile

Using an agar dilution assay, all CUL strains were resistant to Oxgall bile at 0.4 mM, except for both *L.fermentum* strains, which exhibited lethality at the minimum bile concentration (**Table 4.2**). All other species (except *L.paracasei* CUL07 and *B.animalis* subsp. *lactis* CUL34) demonstrated bile tolerance up to 6.4 mM. Of particular interest, is the observed difference in survivability between the three strains of *L.paracasei* tested. Both CUL08 and CUL37 survived at bile concentrations 2-fold higher than CUL07. This was also evident among *B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34. A comparison of agar dilution and broth micro-dilution assays was employed to validate bile tolerance (**Table 4.3**). Bile tolerance phenotypes were identical between assays. However, the same assays were used to assess the tolerance of the bifidobacteria strains (CUL20 and CUL34). In the case of the micro-broth dilution assay, neither strain exhibited growth (in bile or the control MRS).

**Table 4.2. The ability of CUL strains to tolerate bile stress: determined via agar dilution**.

| Isolate | | Total bile acid concentration (mM) | | | | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Strain** | **0†** | **0.4** | **0.8** | **1.6** | **3.2** | **6.4** |
| *L.paracasei* | CUL37 | + | + | + | + | + | + |
| *L.paracasei* | CUL07 | + | + | + | - | - | - |
| *L.paracasei* | CUL08 | + | + | + | + | + | + |
| *L.casei* | CUL06 | + | + | + | + | + | + |
| *L.rhamnosus* | CUL63 | + | + | + | + | + | + |
| *L.salivarius* | CUL61 | + | + | + | + | + | + |
| *L.gasseri* | CUL09 | + | + | + | + | + | + |
| *L.helveticus* | CUL76 | + | + | + | + | + | + |
| *L.fermentum* | CUL40 | + | - | - | - | - | - |
| *L.fermentum* | CUL67 | + | - | - | - | - | - |
| *L.acidophilus* | CUL60 | + | + | + | + | + | + |
| *L.acidophilus* | CUL21 | + | + | + | + | + | + |
| *L.plantarum* | CUL66 | + | + | + | + | + | + |
| *L.plantarum* | CUL66N | + | + | + | + | + | + |
| *B.bifidum* | CUL20 | + | + | + | + | + | + |
| *B.animalis* subsp. *lactis* | CUL34 | + | + | + | - | - | - |

† MRS agar without bile; “+” = growth; “-“ = no growth

**Table 4.3. The ability of CUL strains to tolerate bile stress: determined via broth micro-dilution.**

| Isolate | | Total bile acid concentration (mM) | | | | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Strain** | **0** | **0.4** | **0.8** | **1.6** | **3.2** | **6.4** |
| *L.paracasei* | CUL37 | + | + | + | + | + | + |
| *L.paracasei* | CUL07 | + | + | + | - | - | - |
| *L.paracasei* | CUL08 | + | + | + | + | + | + |
| *L.casei* | CUL06 | + | + | + | + | + | + |
| *L.rhamnosus* | CUL63 | + | + | + | + | + | + |
| *L.salivarius* | CUL61 | + | + | + | + | + | + |
| *L.gasseri* | CUL09 | + | + | + | + | + | + |
| *L.helveticus* | CUL76 | + | + | + | + | + | + |
| *L.fermentum* | CUL40 | + | - | - | - | - | - |
| *L.fermentum* | CUL67 | + | - | - | - | - | - |
| *L.acidophilus* | CUL60 | + | + | + | + | + | + |
| *L.acidophilus* | CUL21 | + | + | + | + | + | + |
| *L.plantarum* | CUL66 | + | + | + | + | + | + |
| *L.plantarum* | CUL66N | + | + | + | + | + | + |

† MRS without bile; “+” = growth; “-“ = no growth

### 4.3.3. Analysis of precipitates formation when CUL strains are challenged on a concentration gradient of Tauro-deoxycholic Acid (TDCA)

Following identification of putative BSH proteins in CUL genomes (**Chapter 3, Table 3.7**). Plate assays were conducted to determine the phenotypic behaviour of CUL strains.

*L.salivarius* CUL61 transitions through several phenotypes along the TDCA concentration gradient. In this strain, no precipitate was seen until 0.03 % where an in-agar precipitate developed. Interestingly the in-agar precipitate changes at 0.48 % TDCA, to a white, crusty surface precipitate and the in-agar (plug-like) precipitate disappears (**Table 4.4, Figure 4.4**). A similar trend is also observed in *L.gasseri* CUL09 which shows no reaction to TDCA at lower concentrations. However, at 0.12 % a white, crusty precipitate develops on the agar surface, which disappears and changes to an in-agar (plug-like) precipitate for the remaining TDCA concentrations. *L.helveticus* CUL76 and *L.fermentum* CUL67 had no visible precipitates on any concentration of TDCA. *L.fermentum* strains varied in their phenotypic behaviour when challenged with TDCA. CUL40 had diminished growth from 0.24 % onwards, whereas CUL67 grew consistently across all concentrations (**Table 4.4, Figure 4.4**). Even though both *L.acidophilus* strains (CUL21 and CUL60) are (genetically) almost identical (**Chapter 2, Figure 2.6**), their response to TDCA differs. A crusty, white, surface precipitate emerges in CUL60 at 0.015 % which changes to an in agar plug from 0.24 % TDCA onwards. In contrast, no precipitate is seen with CUL21 until 0.12 %, which is 3-fold higher than CUL60 (**Table 4.4, Figure 4.4**). In CUL21, this precipitate remains as an on-the-surface phenotype throughout, suggesting an alternative mechanism in CUL60 that is driving a phenotypic change (**Table 4.4, Figure 4.4**). Both *L.plantarum* strains (CUL66 and CUL66N) produced identical precipitates throughout the concentration series. In these cases, a crusty white surface precipitate is first observed at 0.06 %. This precipitate remains the same until the top of the concentration gradient (0.48 %); similarly, the two bifidobacteria strains (CUL20 and CUL34) also display identical precipitates on TDCA (**Table 4.4, Figure 4.4**). Activity is first seen at 0.015 % TDCA as a dense in-agar plug. From 0.03 % the precipitate changes to a less-dense, scattered-like appearance which was not seen in any of the *Lactobacillus* species (**Table 4.4, Figure 4.4**).

Interestingly, significant differences were observed in the types of precipitates formed when CUL isolates were incubated on a concentration gradient of TDCA. The precipitate phenotypes varied depending on (i) the bacterial species/strain, (ii) the bile acid and (iii) the bile acid concentration (**Figure 4.3**). Phenotypes ranged from “normal” to “weaker” and no growth as determined by referencing a media-only growth control. Additionally, several different precipitates were observed, that ranged from a crusty white precipitate on the agar surface (surrounding the bacterial inoculum), a scattered precipitate within the agar and a dense (plug-

like) precipitate within the agar (**Figure 4.3, 4.4** and **4.5)** Variations in the precipitate were observed along the concentration gradients of TDCA and GDCA and between CUL strains (**Figure 4.4 and 4.5**). When challenged with TDCA, isolates from the *L.casei* phylogroup, which includes, *L.casei*, *L.paracasei* and *L.rhamnosus*, all grew consistently throughout the concentration gradient. At 0.24 % TDCA, *L.casei (*CUL06*)* and *L.paracasei (*CUL37, CUL07 and CUL08*)* exhibited weaker growth and at 0.48 % all members of the *L.casei* phylogroup showed diminished growth (**Table 4.4, Figure 4.4**). No on-agar or in-agar precipitates were present in these strains.

**Table 4.4. The phenotypic determination of BSH activity when simulated with the bile acid TDCA, utilizing an agar dilution methodology**.

| | | TDCA % | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Species** | **CUL** | **0** | **0.0075** | **0.015** | **0.03** | **0.06** | **0.12** | **0.24** | **0.48** |
| *L.paracasei* | CUL37LB | | | | | | | W* | W* |
| *L.paracasei* | CUL07 | | | | | | | W* | W* |
| *L.paracasei* | CUL08 | | | | | | | W* | W* |
| *L.casei* | CUL06 | | | | | | | W* | W* |
| *L.rhamnosus* | CUL63 | | | | | | | | W* |
| *L.salivarius* | CUL61 | | | | $P^3$ | | $P^3$ | $P^3$ | $P^1$ | $P^1$ |
| *L.gasseri* | CUL09 | | | | | | $P^1$ | $P^3$ | $P^3$ |
| *L.helveticus* | CUL76 | | | | | | | | |
| *L.fermentum* | CUL40 | | | | | | | W* | W* |
| *L.fermentum* | CUL67 | | | | | | | | |
| *L.acidophilus* | CUL60 | | | $P^1$ | $P^1$ | $P^1$ | $P^1$ | $P^3$ | $P^3$ |
| *L.acidophilus* | CUL21 | | | | | | $P^1$ | $P^1$ | $P^1$ |
| *L.plantarum* | CUL66 | | | | | $P^1$ | $P^1$ | $P^1$ | $P^1$ |
| *L.plantarum* | CUL66N | | | | | $P^1$ | $P^1$ | $P^1$ | $P^1$ |
| *B.bifidum* | CUL20 | | | $P^3$ | $P^2$ | $P^2$ | $P^2$ | $P^2$ | $P^2$ |
| *B.animalis* subsp. *lactis* | CUL34 | | | $P^3$ | $P^2$ | $P^2$ | $P^2$ | $P^2$ | $P^2$ |

$P^1$ (Precipitation on agar surface), $P^2$ (Scattering precipitate within the agar), $P^3$ (Precipitation plug within the agar), W* (weaker growth than control), blank (growth, no precipitate).

CUL isolates were inoculated on MRS agar supplemented with the BA TDCA. Concentrations of TDCA were increased in a dose-dependent manner, to identify whether concentration impacts the phenotype. MRS agar was used as a growth control for all strains.

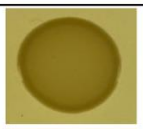
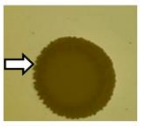
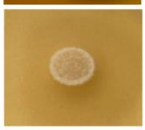
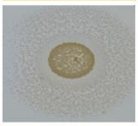
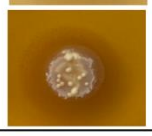
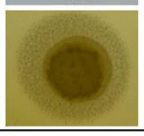
| Abbreviation | Definition | Example 1 | Example 2 |
|---|---|---|---|
| Blank | Standard growth |  |  |
| W* | Weak or less growth |  |  |
| P¹ | Precipitate on agar surface |  |  |
| P² | Precipitate in agar (scattering) |  |  |
| P³ | Precipitate in agar (plug) |  |  |

**Figure 4.3. An example of the variations of growth and precipitations observed on MRS agar + bile acid substrate. The variation of growth and precipitation phenotypes was observed when CUL strains were challenged with BA substrates, utilising two visualisation techniques.** Here shows the phenotype representations of the abbreviations used in subsequent tables. Blank represents a standard bacterial growth, where no difference is observed between control and assay. Weak (W) is used to describe a diminished growth when compared to a control. $P^1$ represents an on-agar precipitate typically expected for a BSH assay. $P^2$ is assigned to precipitates that scatter throughout the agar in a unique "firework" like manner. $P^3$ encompasses precipitates that form within the agar in a solid plug-like structure. Example 1 depicts precipitate examples visualised using standard laboratory lighting conditions. Example 2 is the same cultures visualised on a lightbox. Varying the light used to observe precipitation formation, provided different resolutions at assigning a positive/negative result, therefore both conditions were used to allow visualisation biases to be observed.

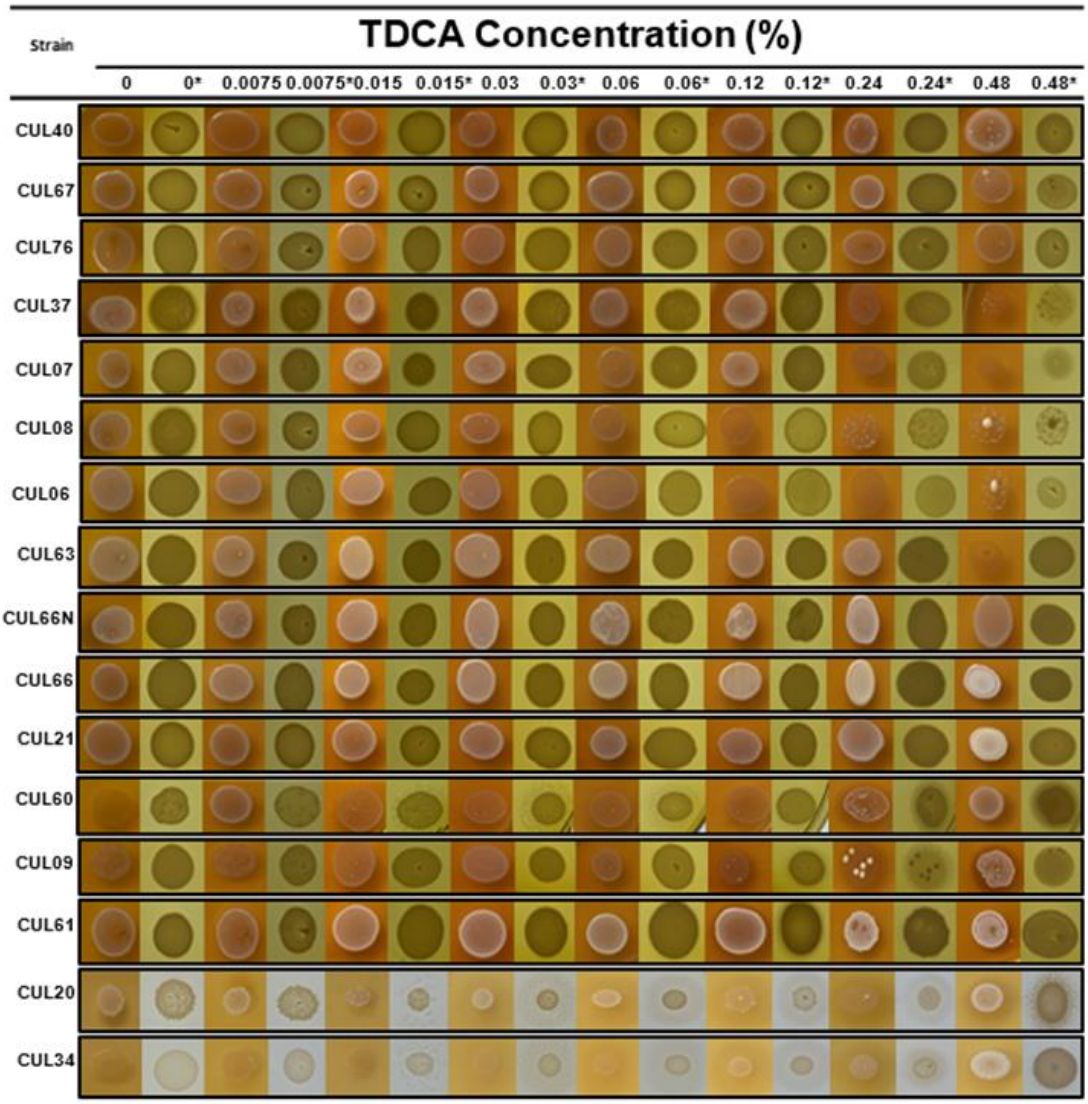



**Figure 4.4. The phenotypic determination of BSH activity when simulated with the bile acid TDCA, utilizing an agar dilution methodology - images**.

CUL isolates were inoculated on MRS agar supplemented with the bile acid TDCA. Concentrations of TDCA were increased in a dose-dependent manner, to identify whether the concentration affects the observed phenotype. MRS agar was used as a growth control for all strains. *The same inoculum visualised under a light box in comparison to the standard “lab bench” conditions.

### 4.3.4. Analysis of precipitate formation when CUL strains are incubated in a concentration gradient of Glyco-deoxycholic Acid (GDCA)

GDCA was also used as a substrate to determine whether substrate selection can also affect *bsh* characterisation (**Table 4.5, Figure 4.5**). Strains from *L.casei* and *L.paracasei* both had a diminished growth from 0.06 % - 0.48 % GDCA (**Table 4.5, Figure 4.5**), similarly *L.rhamnosus* CUL63 experienced a reduction in growth at 0.48 %. *L.salivarius* CUL61 also exhibits weaker growth from 0.06 % onwards. Several of the strain's tested did not exhibit any activity on GDCA (as determined by a lack of precipitate formation). It was noted that some species also experienced a decrease in growth below 0.12 % (*L.helveticus* CUL76 and *L.fermentum* CUL67). Strain level variation in activity on GDCA is seen in *L.fermentum* CUL40 (in comparison to CUL67), which produces a series of different precipitates across the GDCA concentration series. Growth of CUL40 is categorised as weak at 0.06 %, it remains weak but begins to precipitate in the agar from 0.12 - 0.24 % and then dies at 0.48 %, highlighting a dependence on the BA concentration for observing BSH activity (**Table 4.5, Figure 4.5**). *L.gasseri* CUL09 displays BSH activity from 0.03 % GDCA onwards and solely presents an in-agar precipitate.

Strain level variation in BSH activity is also seen between the two *L.acidophilus* strains. CUL60 begins by displaying an in agar, *bsh* active phenotype, from 0.015 % GDCA until 0.12 % and then dies. CUL21 starts by growing weaker at 0.06 % and then precipitates in agar at 0.24 %. *L.plantarum* strains present identical BSH profiles, where activity is observed on an on the surface precipitate between 0.12 – 0.48 %. Similarly, both bifidobacteria strains also present identical BSH profiles, whereas an in the agar scatter develops at 0.015 and remains until 0.48 % (**Table 4.5, Figure 4.5**).

From studying the *bsh* activity patterns, several factors were identified that had the potential to influence the outcome. First, is the substrate selected and the concentration used for the initial analysis, as precipitate formation varies depending on these parameters. The second is the visualisation and characterisation of the precipitation. For example, in **Figures 4.4** and **4.5,** there is a noticeable variation in the phenotype observed under different light. Concentrations marked with an * highlight normal lab bench conditions. Without the * represent the same plates imaged and analysed on a lightbox. Of interest is that the varying light conditions reveal phenotypes that would be otherwise missed. For example, in **Figure 4.5**, in the case of *L.acidophilus* CUL21 at 0.24 %, the in-agar precipitate would be missed under natural lighting but is detected using a lightbox The reverse is also true for *L.gasseri* CUL09 at 0.48 % TDCA (**Figure 4.4**), where a clear in agar plug exists under normal lighting but is not detected with enhanced lighting, highlighting the experimental biases that can be present when solely using this method to identify BSH activity. To understand the effects that

concentration and substrate selection has on the characterisation of *bsh*, phenotypic results were compared and can be visualised in **Figure 4.6**.

**Table 4.5. The phenotypic determination of BSH activity when simulated with the bile acid GDCA, utilizing an agar dilution methodology**.

| | | GDCA % | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Species** | **CUL** | **0** | **0.0075** | **0.015** | **0.03** | **0.06** | **0.12** | **0.24** | **0.48** |
| *L.paracasei* | CUL37 | | | | | W* | W* | W* | W* |
| *L.paracasei* | CUL07 | | | | | W* | W* | W* | W* |
| *L.paracasei* | CUL08 | | | | | W* | W* | W* | W* |
| *L.casei* | CUL06 | | | | | W* | W* | W* | W* |
| *L.rhamnosus* | CUL63 | | | | | | | | W* |
| *L.salivarius* | CUL61 | | | | | W* | W* | W* | W* |
| *L.gasseri* | CUL09 | | | $P^3$ | $P^3$ | $P^3$ | $P^3$ | $P^3$ | $P^3$ |
| *L.helveticus* | CUL76 | | | | W* | X | X | X | X |
| *L.fermentum* | CUL40 | | | | | W* | $P^3$ | $P^3$ | W* |
| *L.fermentum* | CUL67 | | | | W* | W* | X | X | X |
| *L.acidophilus* | CUL60 | | | $P^3$ | $P^3$ | $P^3$ | $P^3$ | W* | W* |
| *L.acidophilus* | CUL21 | | | | | W* | W* | $P^3$ | W* |
| *L.plantarum* | CUL66 | | | | | | $P^1$ | $P^1$ | $P^1$ |
| *L.plantarum* | CUL66N | | | | | | $P^1$ | $P^1$ | $P^1$ |
| *B.bifidum* | CUL20 | | | $P^2$ | $P^2$ | $P^2$ | $P^2$ | $P^3$ | $P^3$ |
| *B.animalis* subsp. *lactis* | CUL34 | | | $P^2$ | $P^2$ | $P^2$ | $P^2$ | $P^3$ | $P^3$ |

*$P^1$ (Precipitation on agar surface), $P^2$ (Scattering precipitate within the agar), $P^3$ (Precipitation plug within the agar), W* (weaker growth than control), blank (growth, no precipitate).

CUL isolates were inoculated on MRS agar supplemented with the BA GDCA. Concentrations of GDCA were increased in a dose-dependent manner, to identify whether the concentration affects the observed phenotype. MRS agar was used as a growth control for all strains.

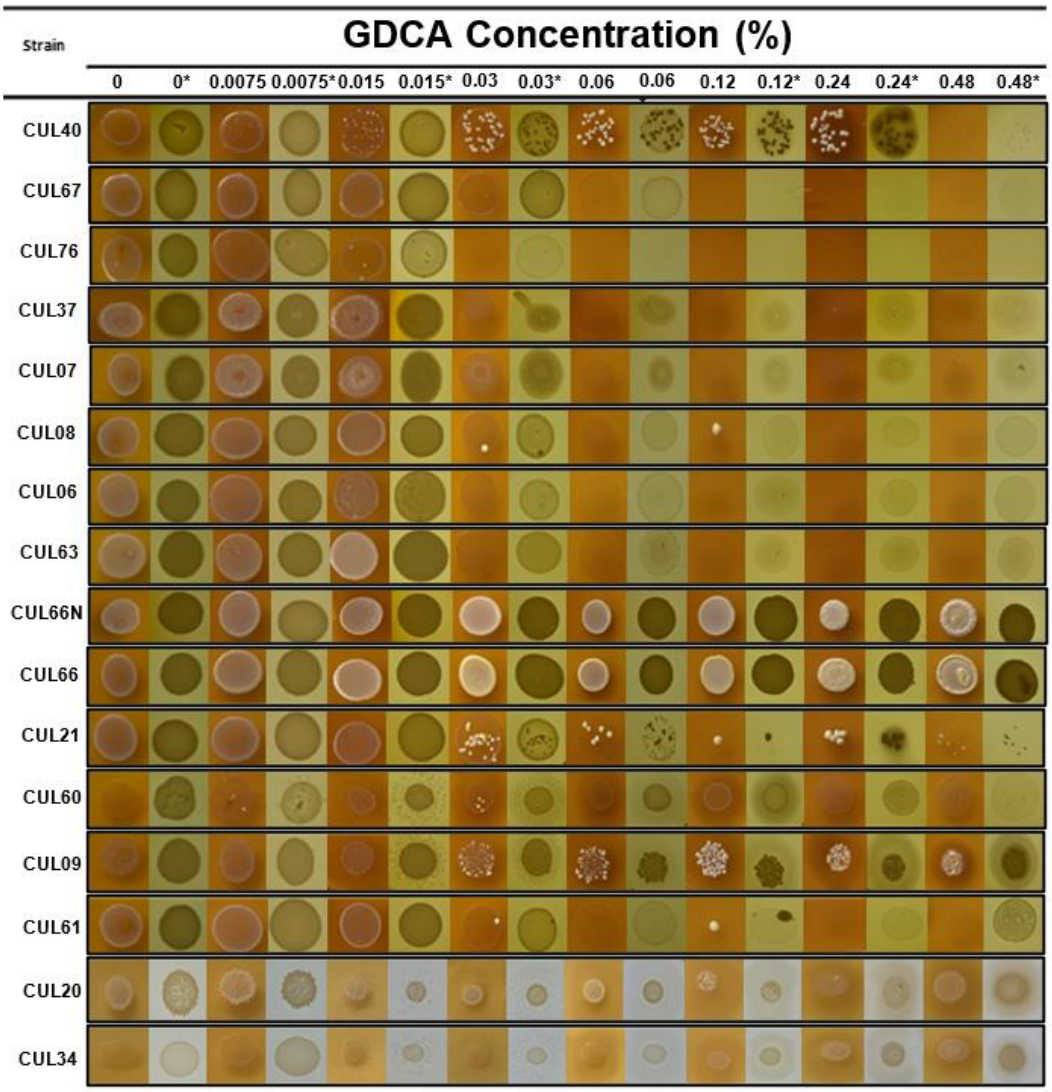



**Figure 4.5. The phenotypic determination of BSH activity when simulated with the bile acid GDCA utilizing an agar dilution methodology - images**. CUL isolates were inoculated on MRS agar supplemented with the bile acid GDCA. Concentrations of GDCA were increased in a dose-dependent manner, to identify whether the concentration affects the observed phenotype. MRS agar was used as a growth control for all strains. *The same inoculum was visualised under a light box in comparison to the standard 'lab bench' conditions.

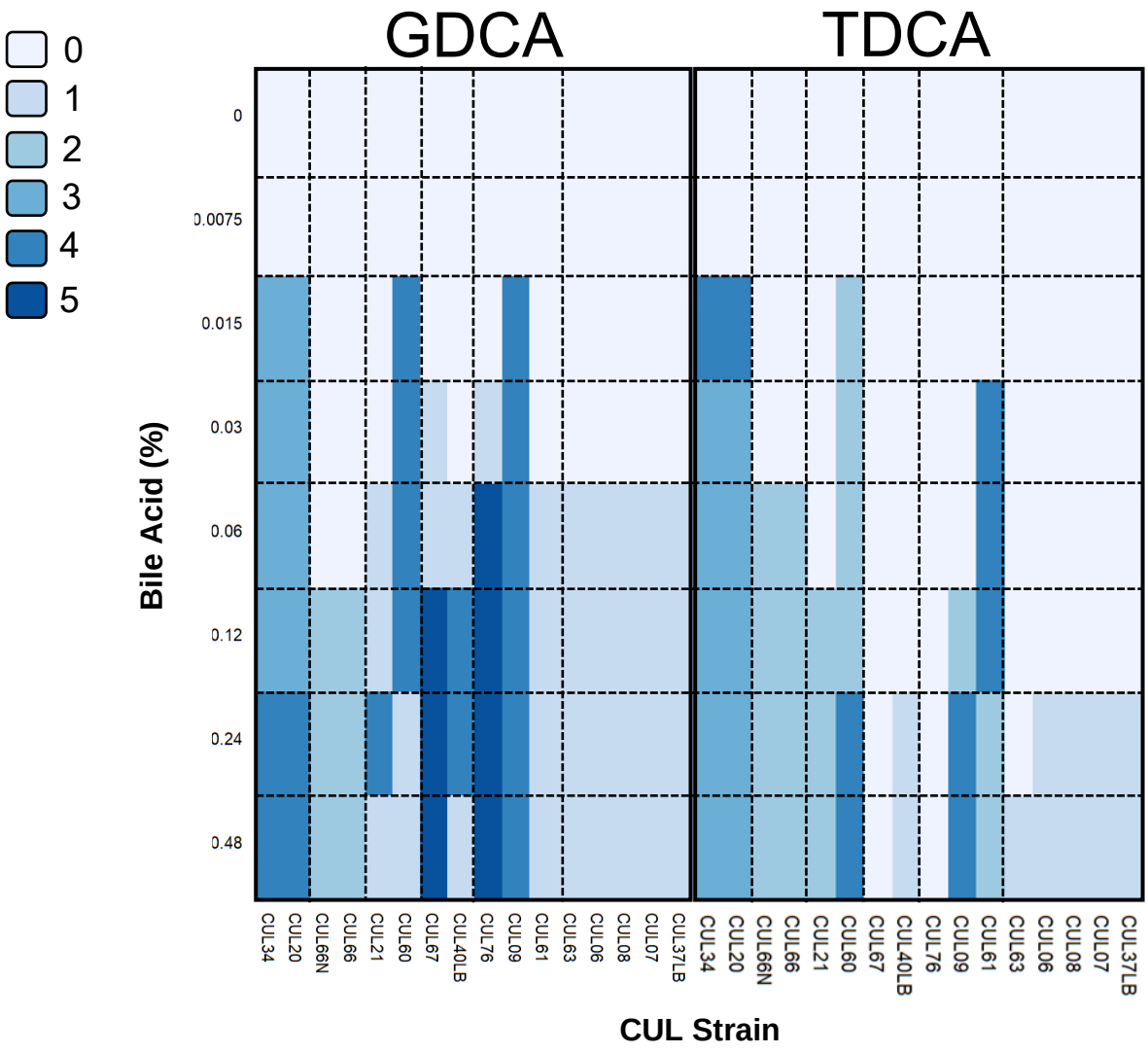



**Figure 4.6. Phenotypic variation of CUL strains BSH activity when challenged on different bile acid substrates.** A heatmap was constructed to highlight the phenotypic variation, ranging from growth to precipitation type CUL strains produced when exposed to concentration gradients of two bile acids TDCA and GDCA utilising an agar dilution method. Key numbers represent a documented phenotype. 0. Standard growth; 2. Weaker Growth; 3. Precipitation on agar surface; 4. Precipitation plug Scattering in Agar; 5. Precipitation Plug in agar; 5. No growth.

### 4.3.5. The lactic acid hypothesis

Due to the variation in precipitate phenotypes (**Figure 4.4 & 4.5**) and the large abundances of lactic acid that lactobacilli are known to produce, the effects of lactic acid on precipitate formation were determined. Lactic acid isomers including DL and L+ were inoculated in the same volume as bacteria cultures (at a maximum neat concentration of 90 % and 98 % respectively) and monitored for the presentation of a precipitate. Of interest, was that a reaction only seemed to occur in the presence of high concentrations of glycine conjugated bile acids (GDCA). The strength of the reaction (evaluated by the visual concentration and density of the phenotype presented) increases as the concentration of GDCA increases with both lactic acid isomers (**Table 4.6**), suggesting a strong interaction between GDCA and lactic acid independent of bacterial modulation.

**Table 4.6. The phenotypic effect of lactic acid inoculation on agar containing bile acid, utilizing an agar dilution methodology.**

| Lactic acid isomer | GDCA Concentration (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 0.0075 | 0.015 | 0.03 | 0.06 | 0.12 | 0.24 | 0.48 |
| L+ | - | - | - | - | - | | | |
| DL | - | - | - | - | - | | | |

Lactic acid isomers were inoculated on MRS agar supplemented with either the bile acid GDCA or TDCA. Concentrations of bile acids were increased in a dose-dependent manner, to identify whether the concentration affects the observed phenotype. MRS agar was used as a positive control. (–) indicates no reaction, whereas an image depicts precipitation observed.

### 4.3.6. Polyphasic analysis of BSH activity

All members of the *L.casei* clade, have no phenotypic BSH activity on either BA substrate and possessed one conjugated bile salt hydrolase-related amidase (**Table 4.7**). *L.salivarius* CUL61 only presented a phenotype on TDCA and was found to encode two *bsh* genes. *L.gasseri* was phenotypically active and presented a *bsh* positive phenotype on both BA substrates, with RAST identifying three *bsh* genes and one conjugated bile salt transporter (**Table 4.7**). *L.helveticus* showed no BSH activity on either BA substrate and genome mining did not reveal the presence of any genes encoding BSH traits, highlighting a good correlation between genotype and phenotype. Of interest is the variation between the two *L.fermentum* strains CUL67 and CUL40. CUL67 has one gene annotated as a Cholylglycine hydrolase in contrast with CUL40, which was predicted to encode two Cholylglycine hydrolases (**Table 4.7**). CUL67 showed no phenotypic activity against either BA substrate, whereas CUL40 was BSH positive on GDCA, suggesting that at least one copy is a *bsh* gene. CUL strains from the species *L.acidophilus*, *L.plantarum*, *B.bifidum* and *B.animalis* subsp. *lactis* contains 2, 3, 1, and 1 *bsh* homologs respectively and are all phenotypically active on both BA substrates (**Table 4.7**). It is hypothesised that the number of *bsh* genes confers a substrate-based advantage. However, bifidobacteria strains were predicted to encode one copy of *bsh* but were active on both substrate types.

**Table 4.7. A genotype to phenotype analysis of bile salt hydrolase activity in CUL isolates**.

| Species | CUL | Bile Salt Hydrolase | Conjugated bile salt transporter | Conjugated BSH-related amidase | Cholylglycine hydrolase | Activity | |
|---|---|---|---|---|---|---|---|
| | | | | | | TDCA | GDCA |
| *L.paracasei* | CUL37LB | | | 1 | | - | - |
| *L.paracasei* | CUL07 | | | 1 | | - | - |
| *L.paracasei* | CUL08 | | | 1 | | - | - |
| *L.casei* | CUL06 | | | 1 | | - | - |
| *L.rhamnosus* | CUL63 | | | 1 | | - | - |
| *L.salivarius* | CUL61 | 2 | | | | + | - |
| *L.gasseri* | CUL09 | 3 | 1 | | | + | + |
| *L.helveticus* | CUL76 | | | | | - | - |
| *L.fermentum* | CUL40LB | | | | 2 | - | + |
| *L.fermentum* | CUL67 | | | | 1 | - | - |
| *L.acidophilus* | CUL60 | 2 | | | | + | + |
| *L.acidophilus* | CUL21 | 2 | | | | + | + |
| *L.plantarum* | CUL66 | 3 | | | | + | + |
| *L.plantarum* | CUL66N | 3 | | | | + | + |
| *B.bifidum* | CUL20 | 1 | | | | + | + |
| *B.animalis* subsp. *lactis* | CUL34 | 1 | | | | + | + |

BSH activity observed on either TDCA or GDCA is highlighted with the presence of a (+) and no activity is indicated with a (-). Gene annotations are as designated by RAST. CUL genomes were mined and genes associated with bile salt hydrolase functionality (assigned by RAST or manual BLASTp) were tallied for each CUL strain. RAST identified several gene sequences encoding proteins associated with the cholylglycine hydrolase family and each was documented to detect putative Penicillin V Acylases (PVA).

### 4.3.7. Semi-quantitative analysis of CUL strains bile salt deconjugation capabilities

GC/MS analysis was conducted to deduce a semi-quantitative measure of BSH action, allowing the identification of deconjugation efficient isolates. Following incubation with 0.3 % bile, samples were centrifuged to collect cellular material and the supernatant was subsequently removed and filter sterilised for downstream GC/MS analysis of the total BA pool profile with/without bacterial mediation.

5-α cholestanol, a well-established standard used in GC/MS analysis was selected and added at a known concentration to each sample as a loading control, providing a reference for quantification (**Figure S4.1 A - B**). The mass spectra were generated to create a standard reference library for peak identification, comprised of the following: A total BA pool (from Oxgall bile at 0.3 %), purified bile components > 99 % including the bile precursor molecule cholesterol (**Figure S4.1 C - D**), conjugated primary bile acids TCA (**Figure S4.4 A-C**) and GCA (**Figure S4.5 A - D**). Unconjugated primary bile acid CDCA (**Figure S4.3 A - B**). Conjugated secondary bile acids TDCA (**Figure S4.2 D - F**) and GDCA (**Figure S4.2 A-C**) and unconjugated secondary bile acids LCA (**Figure S4.3 C - D**) and DCA (**Figure S4.3 E - F**). Of note, when analysing the reference spectra, it was expected that purified bile acids would produce a single peak and this was the case for 5-α cholestanol and cholesterol. However, GDCA and TDCA both presented one large peak (19.76 min and 19.78 min respectively) and a smaller peak both occurring at approximately 21.35 min which suggests that traces of deoxycholic acid are also present within the reference sample (**Figure S4.2. A - F**). Interestingly, the TCA and GCA standards also had several peaks that are similar to that of the bile acids CA and DCA (**Figure S4.4 A - C**: **Figure S4.5 A – D**).

Following the preparation of the reference library, several CUL isolates were selected to deduce the capability of lactobacilli strains to influence the makeup of the BA pool. To produce a general overview, a panel of strains were selected based on the number of *bsh* genes they encoded: *L.casei* CUL06, *bsh* negative (**Figure 4.7 B**), *L.acidophilus* CUL21 - 2 *bsh* homologs (**Figure 4.7 C**), *L.gasseri* CUL09 - two *bsh* homologs (**Figure 4.7 D**), *L.plantarum* CUL66N and CUL66 – 3 *bsh* homologs (**Figure 4.7 E and F** respectively). From the reference library, several bile components could be identified in the Oxgall bile pool including Cholesterol, 5α-cholestanol, GDCA/TDCA and TCA/GCA. deoxycholic acid, cholic acid and CDCA. Of note, both GDCA and TDCA (**Figure S4.2 A - F** & **Figure 4.7 A**) show peaks at the same retention time and mass spectra pattern, which is also the case for TCA and GCA, making it impossible to identify the two conjugate variations using GC/MS.

Strains were incubated with 0.3 % bile (physiological concentration). When compared with the internal standard, *L.plantarum* strains CUL66 and CUL66N and *L.gasseri* CUL09 exhibit a

sharp increase in the primary BAs, cholic and chenodeoxycholic acid. *L.gasseri* CUL09 also appears to increase the abundance of DCA in the BA pool (**Figure 4.10**). *L.acidophilus* CUL21 and CUL60 increase in concentrations of CA, CDCA and DCA in much smaller amounts (**Figure 4.10**). All strains showed a decreased abundance of cholesterol and TDCA/GDCA, with *L.plantarum* strains causing the largest depletion within the BA pool. GCA and TCA are also reduced in samples incubated with *L.plantarum* CUL66, CUL66N and *L.gasseri* CUL09. Interestingly, *L.acidophilus* CUL21 appears to increase slightly in TCA/GCA concentration (**Figure 4.10**). Furthermore, *L.casei* CUL06 which is a *bsh* negative strain has a decreased abundance of cholesterol and GDCA/TDCA and increased abundances of CA, CDCA, DCA and GCA/TCA (**Figure 4.10**).

### 4.3.8. Agar precipitation variation and the effects of lactic acid

Driven by the ability of the *bsh* negative strain *L.casei* CUL06 to manipulate the BA pool and by the variation seen in the precipitate phenotypes (**Figures 4.4 and 4.5**), the effect of lactic acid on BA composition was analysed (**Figure 4.8**) using the established reference library (**Figure S4.7**). Two precipitates (*L.acidophilus* CUL60 – ON agar and *L.gasseri* CUL09 – IN agar) from the BSH agar plate assay were selected to determine whether the difference in phenotype generated was due to a variation in bile conversion. Plates were composed of GDCA at 0.48 % concentration. Both strains despite the phenotypic disparity showed no variation following GC/MS analysis (**Figure 4.9 A - D**), but the analysis did allow the inference that GDCA is deconjugated to DCA by both species.

Lactic acid production is a well-established characteristic of lactobacilli species. To deduce whether lactic acid isomers could affect BA components, lactic acid was incubated on 0.48 % GDCA agar. Interestingly, a second peak emerges from the side of the GDCA peak at a retention time of 19.98 mins. This phenomenon was only observed in samples incubated with lactic acid (**Figure 4.8**).

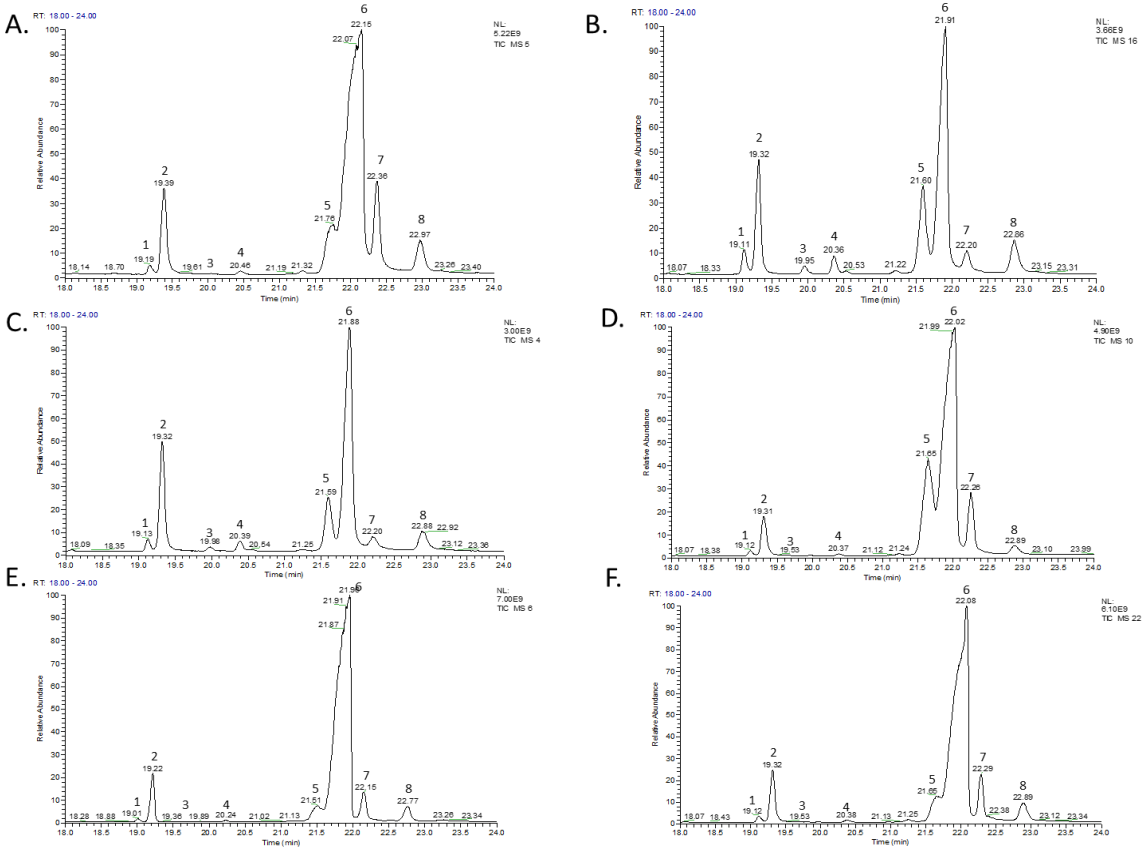



**Figure 4.7. GC/MS spectra of bile acid profiles**. Spectra profiles of the bile acid composition in 0.3 % Oxgall bile (suspended in MRS broth), following 3 h of anaerobic incubation. Letters represent bacterial species incubated in the presence of bile, to deduce the effects probiotic isolates have on the composition of bile. **A**. 0.3 % bile standard. **B**. *L.casei* (CUL06) - *bsh* negative. **C**. *L.acidophilus* (CUL21) – two *bsh* homologs. **D**. *L.gasseri* (CUL09) - two *bsh* homologs. **E**. *L.plantarum* (CUL66N) – 3 *bsh* homologs. **F**. *L.plantarum* (CUL66) – 3 *bsh* homologs. Numbers represent distinct peaks identified as various components of the bile acid pool. Peaks were identified via the Excalibur library and validated via compound standard analysis (1. Cholesterol; 2. 5α-Cholestanol; 3. GDCA/TDCA; 4.GCA/TCA; 5.Deoxycholic acid; 6. Cholic acid; 7. CDCA; 8. Unknown C.

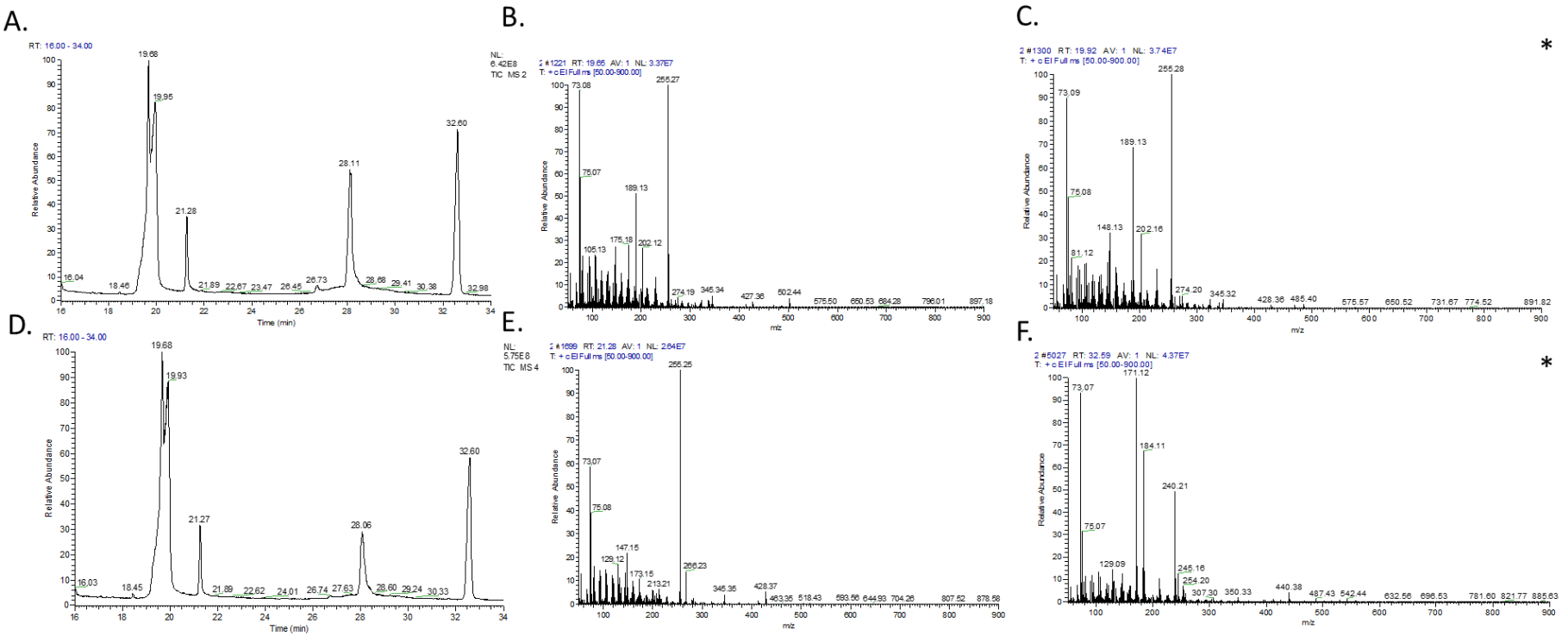



**Figure 4.8. The effects of lactic acid isomers on the composition of MRS + GDCA agar: determined via GC/MS. A.** 0.48 % GDCA + DL lactic acid spectrum. **B**. 0.48 % GDCA + DL lactic acid mass spectrum (19.68 min). **C**. 0. 48 % GDCA + DL lactic acid mass spectrum * Only seen in presence of lactic acid (19.95 min). **D**. 0.48 % GDCA + L lactic acid spectrum. **E**. 0.48 % GDCA + L lactic acid mass spectrum (21.28 min). **F**. 0.48 % GDCA + L lactic acid mass spectrum * Only seen in presence of lactic acid (32.59 min).

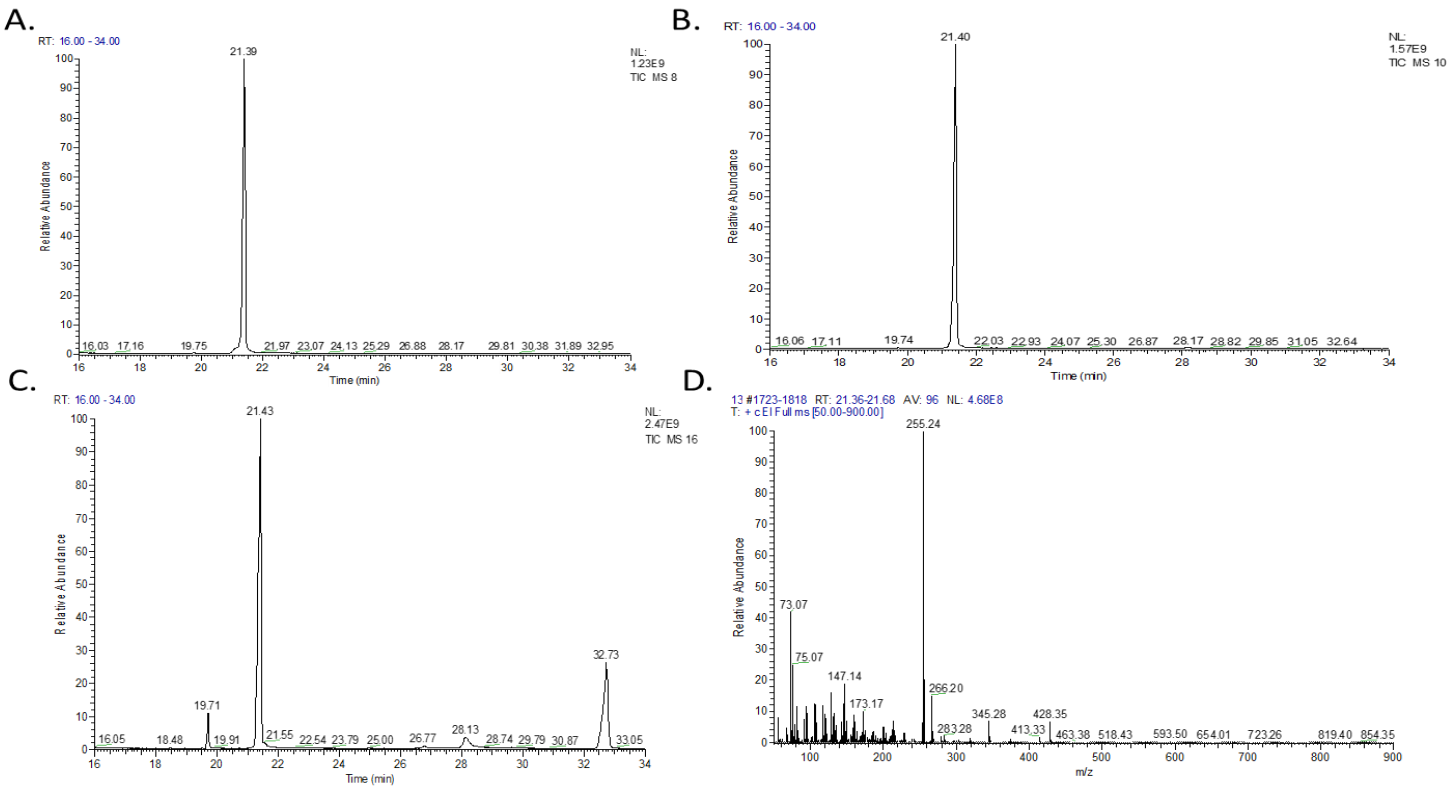



**Figure 4.9. The effects of bacterial incubation on the composition of MRS + GDCA agar: determined via GC/MS. A.** Spectrum of the precipitate produced by *L.gasseri* CUL09 on 0.48 % GDCA agar. **B**. Spectrum of the precipitate produced by *L. acidophilus* CUL60 on 0.48 % GDCA agar. **C**. Spectrum of the precipitate produced by *L.gasseri* CUL09 on 0.48 % GDCA agar showing GDCA peaks, not complete conversion to DCA. **D**. Retention Time and fragmentation DCA std showing GDCA is deconjugated to DCA by CUL09 and CUL60.

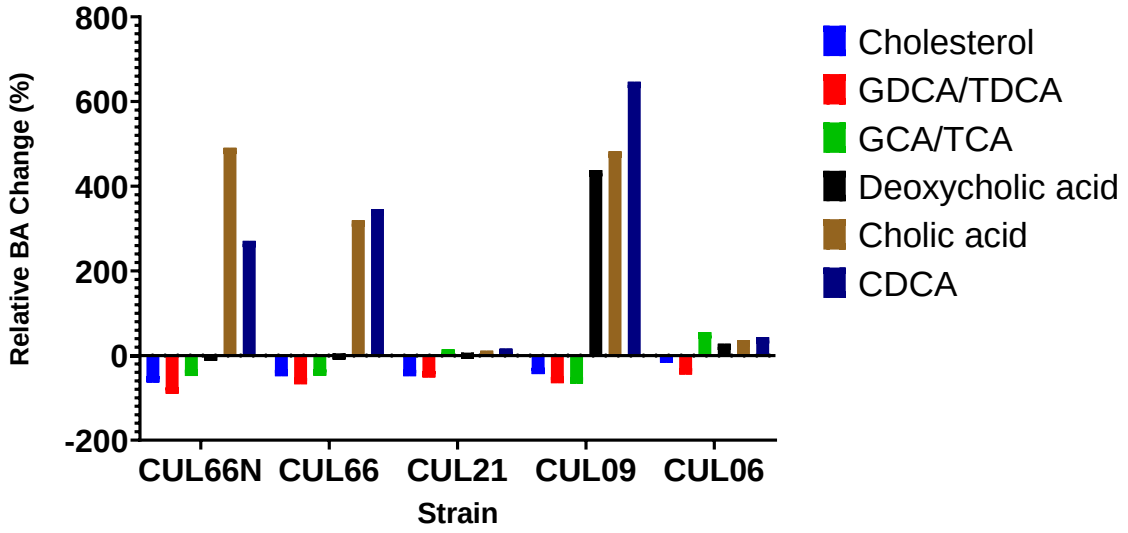



**Figure 4.10. Semi-quantitative analysis of *Lactobacillus* CUL isolates deconjugation capabilities: determined via GC-MS. micro broth** Bile acids were quantified by calculating the peak area of each component in a 0.3 % bile + MRS standard, relative to the loading control 5α-Cholestanol (N=3). CUL strains were incubated with Oxgall bile and the peak area relative to the loading control was calculated as a percentage of the bile standard and averaged. The percentage increase was deduced in comparison to the MRS standard. Both strains of *L.plantarum* (CUL66 and CUL66N) have a percentage decrease in cholesterol, GDCA/TDCA and GCA/TCA, with increases in concentrations of cholic and chenodeoxycholic acid, with CUL66N being more efficient. *L.acidophilus* CUL21 has a percentage decrease in cholesterol and GDCA/TDCA, with a small increase in cholic and chenodeoxycholic acid. *L.gasseri* CUL09 increases by a large amount in cholic acid, deoxycholic acid and chenodeoxycholic acid, decreases are observed in cholesterol, GCA/TCA and GDCA/TDCA. *L.casei* showed marginal decreases in cholesterol and GDCA/TDCA, with small increases in cholic acid, chenodeoxycholic acid, GCA/TCA and deoxycholic acid. Experiments were performed in triplicate.

### 4.3.9. Gene expression analysis

The relative expression of the predicted *bsh* genes in *L.plantarum* CUL66N (PEG1210, PEG1331 and PEG40) were upregulated 1.6, 2.7 and 3.7 fold respectively, following incubation with 2 % bile salt concentration for 1 h at 37 °C (**Figure 4.11**). Interestingly, PEG40 saw the largest fold change followed by PEG1331. From earlier analysis (**Figure 4.1**) PEG40 was predicted to be a homolog annotated as *bsh*3 in accordance with previously identified *bsh* copies in the *L.plantarum* species, whereas PEG1210 and PEG1331 were predicted to be copies of *bsh1* and *bsh2* respectively.

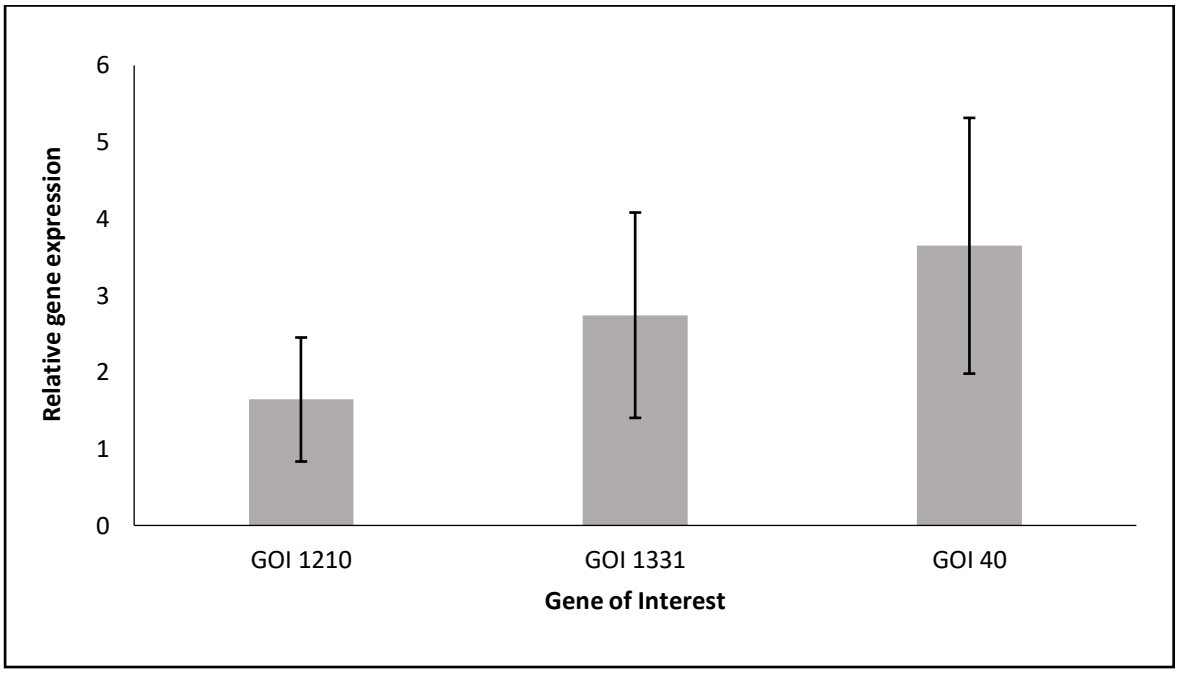



**Figure 4.11. Relative expression of putative *bsh* genes.** Putative *bsh* genes identified in *L.plantarum* CUL66N when exposed to 2 % Oxgall bile for 1 h. Cultures grown without the presence of bile were used as a control. Gene expression was calculated using the Pfaffl method (Pfaffl, 2001) with the housekeeping gene *ldhD* (± SD).

## 4.4. Discussion

Understanding the complex nature of microbial interactions with bile is of interest when designing a probiotic product, particularly those targeting cholesterol reduction. Indeed, throughout intestinal transit, probiotic bacteria are exposed to BAs that can be highly toxic to microorganisms that are not adapted to survive under such conditions (Begley, Gahan, et al., 2005), emphasising the desirability of bile tolerance for probiotic functionality. However, BA pools are highly heterogeneous, not only between mammalian species (Dawson & Karpen, 2015; Lefebvre et al., 2009) but also between individuals of the same species (Lourenco & Camilo, 2002), reflecting the difficulty of identifying true bile tolerance and deducing microbial to bile interactions.

### 4.4.1. Phylogenetics

Following genome mining, a variation in the abundance of *bsh* homologs putatively encoded in CUL isolates was found, ranging from 1 - 3 per genome. However, this is not an uncommon finding for lactobacilli species. For example, *L.acidophilus* NCFM was found to encode for two *bsh* copies, *bsh*A and *bsh*B (McAuliffe et al., 2005) and *L.johnsonii* PF01 three copies: *bshA, bshB* and *bshC* (Chae et al., 2013), *L.plantarum* WCFM four copies: *bsh1*, *bsh*2*, bsh3* and *bsh4* (Lambert, Bongers, et al., 2008) and *L.gasseri* FR4 a single *bsh* copy (Rani et al., 2017). Indeed, genetic analysis has revealed that the number of *bsh* homologs is also strain dependent, for example (Lambert, Bongers, et al., 2008) found that *L.plantarum* WCFS1 had four homologs (*bsh 1- 4*). Whereas Gu et al., (2014) only identified three copies of (*bsh 2 - 4)* in *L.plantarum* CGMCC8198. Such variations are not limited to *L.plantarum* but have also been documented in other species such as *L.salivarius* (Fang et al., 2009), where all 33 strains analysed encoded *bsh1* on a mega-plasmid, however, two strains also encoded an additional *bsh* gene, which the authors concluded provided further substrate coverage (Fang et al., 2009).

The physiological purpose of BSH production is not well understood, despite extensive work focusing on these enzymes for decades (Begley et al., 2006; Foley et al., 2021; Smet et al., 1995). A popular conclusion is that multiple BSH proteins may be an evolutionary development to allow substrate preference, for example, taurine vs. glycine (Kumar et al., 2012; McAuliffe et al., 2005; Pavlović et al., 2012). However, it is of particular interest that bifidobacteria CUL strains only possess one copy of *bsh* but are active on both substrate types, a phenomenon previously noted (Jarocki et al., 2014; Kim et al., 2004; Liong & Shah, 2005; Tanaka et al., 1999). However, different “types” of BSH protein have been identified in bifidobacteria strains which may provide a putative explanation for this phenomenon (Jarocki et al., 2014; Kim et al., 2004).

As such, an extensive phylogenetic analysis of all species designated as *bsh* positive was undertaken, to aid in the understanding of the vast variations seen within BSH production and the number of *bsh* homologs present per genome. Ten distinct, homogenous, clusters of proteins were identified within the BSH ML phylogeny. Clade I and Clade IV contain BSHA and BSHB respectively, previously described in *L.acidophilus* NCFM (McAuliffe et al., 2005) and additional *L.acidophilus* strains (Horackova et al., 2020). CUL21 PEG582 and CUL60 PEG579 were putatively annotated as BSHB, whereas CUL21 PEG408 and CUL60 PEG403 as BSHA. It is noteworthy that there appears to be a large divergence between proteins from the same genomes. Indeed, it has been previously shown that *bshA* and *bshB* in *L.acidophilus* NCFM only share 57 % sequence identity (McAuliffe et al., 2005). It was also highlighted that *bshA* and *bshB* activity is dictated by the composition of the BA substrate. *bshA* activity is reliant on the steroid nucleus of the conjugated bile salt (having a reduced affinity for bile salts containing chenodeoxycholic acid as the steroid moiety), while *bshB* is inactive against taurine conjugated bile salts, suggesting a selection for glycine specific deconjugation for this homolog (McAuliffe et al., 2005). The capability of *L.acidophilus* strains to deconjugate both taurine and glycine conjugated BAs with a greater affinity for glycine CBA has also been reported elsewhere (Jiang et al., 2010). It has been previously hypothesised that altering the substrate preference of *bsh* may be an evolved trait to alter the ratio of glycine/ taurine conjugated BA exposure, to modify the toxicity of pooled BAs (Foley et al., 2021). Interestingly Horackova et al., (2020), found that phenotypically *bsh* negative *L.acidophilus* strains did encode *bshA* and *bshB* genes. However, these were not induced in the presence of bile, suggesting that an additional promoter is responsible for *bsh* expression. Thus, indicating that when solely testing for BSH activity phenotypically, encompassing both glycine and taurine CBA is crucial when determining true *bsh* potential.

From the ML phylogeny, it is apparent that both BSHA and BSHB proteins share a closer evolutionary relationship with proteins from different species than they do with each other. Such divergences suggest that the two genes did not arise from a gene duplication event and may indicate HGT (Begley, Sleator, et al., 2005; Daly et al., 2021; Elkins et al., 2001). Indeed, inconsistent promoter regions for *bsh* (Elkins et al., 2001; Ridlon et al., 2006), dissimilarity in flanking regions of *bsh* genes (McAuliffe et al., 2005) and genes associated with MGE (Kumar et al., 2014) have been identified in *Lactobacillus* species. Typically, the number and phylogenetic position of *bsh* homologs within a species remained consistent between clades, which may suggest an ancient acquisition of *bsh* genes, that has evolved in the gut niche (Jones et al., 2008). Multiple *bsh* copies per genome suggest that the functions encoded by such genes incur an evolutionary advantage to the bacteria. Indeed, a previous phylogenetic analysis revealed the presence of *bsh* homologs specifically in gut isolated bacteria and PVA's

in environmental isolates (Jones et al., 2008; O'Flaherty et al., 2018). Interestingly *L.acidophilus* 20079 lacked a *bshA* homolog and was predicted to encode two *bshB* copies (Clade V). Such a finding putatively suggests a gene duplication event which was only observed in one strain of *L.acidophilus,* further emphasising strain level variations when characterising probiotic functions.

Two copies of cholylglycine hydrolase proteins were identified in *L.salivarius* CUL61, the first, PEG1728 is found within Clade III, which was solely comprised of genes identified on plasmids, suggesting the potential for CUL61 to encode a mobile element. Indeed, the presence of mega plasmids that encode *bsh* genes has been previously reported, supporting such findings (Fang et al., 2009; Li et al., 2007). In fact, for CUL61, a genomic island encoding a cholylglycine hydrolase was previously predicted (**Chapter 3, Table 3.6**) supporting an accurate BSH annotation. Further BLAST analysis led to the protein being annotated as BSH1. CUL61 PEG435 is in Clade VII and groups with other chromosomal encoded proteins from *L.salivarius.* BLASTp analysis revealed that protein-encoding genes within this clade are generally annotated as *bsh2* for *L.salivarius* species (Fang et al., 2009), however, protein homology modelling has shown that some chromosomally encoded BSH sequences are mis-annotated and could in-fact be a PVA (Fang et al., 2009; Lambert, Siezen, et al., 2008). Furthermore, Clade VII is made up of two subclades, the first is a cluster of *L.salivarius* proteins and the second is sequences from the species *L.fermentum*. CUL40 PEG180 was the only predicted *L.fermentum* BSH sequence from CUL isolates to fall within Clade VII. Intriguingly, two PVA reference sequences (designated by O'Flaherty et al., 2018) were also located in clade VII, providing a further rationale that this clade is comprised of mis-annotated PVA proteins. The remaining *L.fermentum* CUL BSH sequences were all located in subgroup C, suggesting that CUL40 PEG855 and CUL67 PEG612 are the functional *bsh* genes responsible for the precipitate seen when CUL40 was challenged with GDCA.

Previous phylogenetic analysis has revealed the presence of between 1 – 4 *bsh* homologs within the *L.plantarum* species (Lambert, Bongers, et al., 2008). Here, three putative *bsh* protein-encoding genes were identified and subsequently separated into 3 distinct clades in the ML phylogeny (Clades IV, VIII and IX). From each genome, the three proteins retrieved were spread equally across the three clades. Further BLASTp analysis provided tentative gene annotations as Clade IV as BSH1, Clade VIII as BSH2 and Clade IX as BSH3. Interestingly, an *L.plantarum* strain fell outside of Clade IX (*Lactobacillus plantarum* STIII ADN99333) and was putatively annotated as BSH4, which grouped with additional sequences from the PVA reference dataset. Both *L.plantarum* CUL strains (66 and 66N), possessed three protein copies each, which also were equally distributed throughout the three clades. CUL66 PEG1210 and CUL66N PEG2550 were located within clade IV and were therefore annotated

as BSH1. CUL66 PEG1331 and CUL66N PEG2345 were grouped with clade VIII and designated as BSH2 and finally CUL66 PEG40 and CUL66N PEG2416 clustered with Clade IX and were therefore annotated as BSH3.

Within *L.plantarum,* it has previously been reported that *bsh1* is highly variable, whereas (despite the long genetic distance between them), the homologs *bsh2*, *bsh3* and *bsh4* are highly conserved (Gu et al., 2014). Such findings agree with the results presented here, where BSH1 (clade III) exhibited greater homology to *L.acidophilus* BSHB (which both share a MRCA) than to the additional homologs from the same genome BSH2 (clade VII), BSH3 (clade VIII) and BSH4 (subgroup C). BSH1 appears to have diverged a lot earlier in the gene's evolution from BSH2 and BSH3, suggested by the vast separation of the proteins on the phylogeny. In contrast, BSH2 and BSH3 appeared to have undergone a recent divergence event sharing a greater degree of homology and a MRCA. The large divergence between BSH1 and a closer relation of BSH2 and BSH3 in *L.plantarum* has been described previously (Lambert, Bongers, et al., 2008), boosting the confidence in the topology accuracy of the phylogeny presented here and the correct annotation of BSH proteins within *L.plantarum* CUL strains.

The mis-annotation of *bsh* genes has been documented, leading to further unresolved uncertainty around *bsh* function (Fang et al., 2009; O'Flaherty et al., 2018). For example, in the case of *L.plantarum* WCFM, Lambert, Bongers, et al., (2008) found that *bsh1* is correlated with BSH activity in *L.plantarum* strains and is, therefore, the main contributor of BSH activity for this species. Phylogenetic analysis also revealed that *bsh2*, *bsh3* and *bsh4* shared significant sequence homology with Penicillin Acylase genes and when analysed revealed that the overexpression of *bsh3* (and *bsh2* and *bsh4* to a smaller degree) triggered an increase in acylase activity of penicillin V, providing putative evidence for a mis-annotation of the said gene (Lambert, Bongers, et al., 2008). Indeed, based on the ML phylogeny presented here, *bsh2* and *bsh3* appear to be closer related to putative PVA's than well-defined *bsh* homologs. It has been shown that PVA genes facilitate penicillin V resistance and as such we suggest that when putative *bsh* genes are annotated, due to the high sequence homology documented between the gene families, a phenotypic test for penicillin V resistance should be conducted, dismiss the possibility of a mis-annotation (Begley, Sleator, et al., 2005). In addition (Liang et al., 2018), recommends utilising their subtype criteria of BSH proteins before annotation, whilst suggesting based on their sequence analysis, that the presence of Gln82 and Asn82 are two indicating amino acids in the protein, which would suggest that the BSH protein has high bile acid hydrolase capability. However, it has been shown that BSH enzymes can utilize penicillin as a substrate adding to the complexity of *bsh* designation (Daly et al., 2021). Such

complexities highlight the importance of fully characterising BSH when attempting to determine the cholesterol-reducing mechanisms of probiotics.

### 4.4.2. Bile tolerance of CUL isolates

In this study, the capacity for CUL strains to persist in the presence of Oxgall bile was determined *in vitro*. Previous reports suggest that survival at 0.3 % Oxgall bile represents a bile tolerant profile (Hu et al., 2018b; Lee et al., 2011; Lin et al., 2007). However, bile concentrations in the gut rarely remain constant and have been shown to fluctuate depending on dietary intake (Gunn, 2000; Hu et al., 2018b). Indeed, BA concentrations in the duodenum can range from 4 mM (pre-prandial) to 14 mM (postprandial (Humbert et al., 2018; Northfield & McColl, 1973)). Here CUL strains were challenged across a concentration gradient of bile to provide a comprehensive overview of tolerance across the length of the GI tract. All strains were deemed as bile tolerant using the traditional guidelines of 0.3 % (0.4 mM), except for both *L.fermentum* strains CUL40 and CUL67, which were inhibited by the minimum concentration of Oxgall (0.4 mM / 0.3 %). Strain level variation in bile tolerance profiles within *L.fermentum* has been previously reported (Bao et al., 2010; Lin et al., 2007; Zeng et al., 2011), where some strains exhibit high sensitivity when exposed to bile (Lin et al., 2007) and others thrive in high concentrations, for example, 2 % (Zoumpopoulou et al., 2008). Given the almost ubiquitous nature of lactobacilli species, with species (Ahirwar et al., 2017; Karami et al., 2017; Yin & Zheng, 2005) and strains (Siezen et al., 2010) often isolated from a range of habitats, strain level variation is not surprizing. Indeed, *L.fermentum* strains isolated from poultry were shown to have low tolerance, whereas swine isolates retained high survivability in the presence of bile (Lin et al., 2007).

The emergence of strain dependant traits is often thought to arise as a consequence of environmental selection (Lemay et al., 2000), where species evolve specialised traits to acclimatise to their habitat, eliminating deleterious attributes and maintaining functions that aid survival (Martino et al., 2016). Therefore, the emergence of bile tolerance and mechanisms associated with such characteristics would presumably be driven by frequent bile exposure. However, previous reports have described bile tolerance in *L.fermentum* strains isolated from dairy products (Bao et al., 2010) and sensitive strains from animal hosts (Lin et al., 2007). Indicating that bile tolerance may not be a specialist adaptation. Indeed, for other species of *Lactobacillus* such as *L.plantarum*, phylogenetic analysis has revealed a lack of significance when comparing clade-specific genomic clusters with isolation sources (Choi et al., 2018; Martino et al., 2016).

All *L.paracasei* strains within this study were classified as bile tolerant, but also exhibited strain level variation when comparing their bile tolerance profiles. CUL08 and CUL37 exhibited a

two-fold higher rate of tolerance in comparison to CUL07. Such variation has been shown for other members of the *L.paracasei* species (Reale et al., 2015). Interestingly, the strains identified with the highest tolerance in such studies were isolated from; wine, cheese and human faeces (Reale et al., 2015), again indicating that frequent bile exposure may not influence the emergence of bile tolerance.

It is important to note that reporting true bile tolerance is difficult, not only due to the experimental variations in testing seen within the literature, for example, bile substrate (Reale et al., 2015; Zhang et al., 2008), incubation time (Solieri et al., 2014; Wu et al., 2009), bacterial concentration (Verdenelli et al., 2009) and general methodology but also given the number of stimuli encountered *in vivo* which may influence subsequent tolerance profiles. To attempt to combat experimental bias and determine the effects method selection had on the bile susceptibility profiles of CUL strains, a second method was applied, moving from agar to broth-based assays and tolerance designation shifting from visual (subjective) to a more accurate spectrophotometric analysis. Tolerance profiles for lactobacilli CUL strains were consistent across methodologies, suggesting that the bile tolerance profiles in this study were as robust as currently possible via *in vitro* conditions. However, neither bifidobacteria strain grew at any concentration using the MIC method, suggesting that this may not be suitable for such slow-growing, strict anaerobic microorganisms.

### 4.4.3. Interactions between CUL strains and bile acids

When investigating the interactions between probiotic candidates and bile, several proteins have been identified as coping strategies, including BSH (Hamon et al., 2011). Despite conflicting opinions regarding the relation between BSH production and bile tolerance (Arnold et al., 2018; Begley, Sleator, et al., 2005; Bron, 2004; Duary et al., 2012; Fang et al., 2009; Hamon et al., 2011; Jarocki et al., 2014; Oner, Aslim, & Aydaş, 2013; Smet et al., 1995; Vizoso Pinto et al., 2006), the focus has largely centred on probiotic production of BSH, due to its potential role in host cholesterol reduction (Costabile et al., 2017; Pereira et al., 2003; Zhang et al., 2008). Indeed, BSH production is a desirable trait and is now included in the WHO recommended criteria for the designation of new probiotics (Begley et al., 2006; Dong et al., 2012).

Typically, BSH activity is characterised via a plate assay, where bacterial strains are incubated on an agar substrate containing a known concentration of BA. BSH activity is then assigned based on the emergence of a white precipitate. As such, CUL isolates were independently challenged across a concentration gradient of either TDCA or GDCA, to evaluate the effect that the concentration of a specific BA has on the development of a precipitate.

#### 4.4.3.1. *Substrate and concentration variations alter the phenotype*

In the literature, when phenotypically testing for BSH production, experimental parameters are often varied. Indeed, comparisons between studies are difficult given the number of variations in experimental design reported. For example, the use of different bile salts (Casarotti et al., 2017; Kumar et al., 2012; Lafy & Alash, 2018; Moser et al., 2001; Nuhwa et al., 2019; Solieri et al., 2014; Zhang et al., 2008), pH (Archer & Halami, 2015; Vizoso Pinto et al., 2006), incubation time (Huang et al., 2020), percentage of bile used (Grill et al., 2000; Mallappa et al., 2019; Oh et al., 2008; Shehata et al., 2016; Zhang et al., 2021) and general methodology (González-Vázquez et al., 2015; Kusada et al., 2021). A correlation between the activity of *bsh* genes and substrate specificity has been drawn repeatedly (Foley et al., 2021; McAuliffe et al., 2005) and perhaps more importantly, BAs in the human gut are both conjugated with glycine and taurine. Thus, testing the ability of a candidate probiotic against either TDCA or GDCA singularly does not fully capture the ability of these strains to deconjugate BAs. Scrutiny of the literature also reveals that many studies use BA concentrations (such as 0.5 % TDCA) without context to physiological relevance (Archer & Halami, 2015; Bustos et al., 2012; Caggia et al., 2015; Fang et al., 2009; Panicker et al., 2018; Shehata et al., 2016). In addition, to combat pH, some studies add substrates such as calcium carbonate to agar plates to act as pH buffers (Kumar et al., 2012; Panicker et al., 2018; Vizoso Pinto et al., 2006). However, lactobacilli when grown will produce abundances of lactic acid that can react with calcium carbonate to produce calcium lactate (Yao & Toda, 1990), which is visually white, making this method unreliable**.**

#### 4.4.3.2. *Phenotypic analysis of BSH activity in CUL isolates*

To attempt to combat the issues mentioned previously, the BSH activity of CUL isolates was analysed on a concentration gradient of BA (to incorporate physiological relevance) and stimulated by a representative of both conjugated BA's, GDCA and TDCA. Several interesting trends were revealed when correlating the varied experimental conditions. For example, as the concentration of CBA increases, members of the *L.casei* group presented with no BSH activity (as evidenced by the absence of a visual precipitate) but begin to show diminished growth on both BA substrates at the upper end of the gradient, suggesting toxicity at high concentrations of CBA. Furthermore, tolerance to GDCA appears to be reduced in comparison to TDCA across most CUL isolates. Indeed, glycine-conjugated BA has previously been shown to be significantly more inhibitory than taurine-conjugates (Ridlon et al., 2016), which is thought to arise in a pH-dependent manner (Grill et al., 2000, offering a putative explanation for trends seen in this study. In addition, none of the CUL strains experienced lethality under the concentrations of TDCA tested here. In contrast, *L.fermentum* CUL67 and *L.helveticus*

CUL76 both experience toxic effects between 0.06 – 0.12 % GDCA. Furthermore, *L.salivarius* CUL61 displayed no BSH activity on GDCA but is BSH positive on TDCA and demonstrates a decreased growth capacity at higher concentrations, again highlighting the toxic effects of GDCA, in agreement with previous reports (Wang et al., 2015). It is important to note, that such observations were made using a solid agar assay and therefore any toxicity observed was not influenced by physiological effects, such as enterohepatic recirculation and gut motility.

Modifying experimental parameters generated several variables for all strains tested. For example, *L.fermentum* CUL40 exhibits no BSH activity on TDCA, only diminished growth when challenged with strong concentrations ( e.g., 0.24 - 0.48 %). CUL40 also experiences reduced growth on GDCA, but produces an in-agar precipitate between 0.12 – 0.24 % and dies at 0.48 %. demonstrating a bile substrate preference that would be missed utilising one substrate and a concentration-dependent reaction. Such a trend was not only seen in *L.fermentum* strains but also in *L.salivarius* CUL61, highlighting flaws within the current methodology.

Interestingly, both *L.plantarum* strains (CUL66 and CUL66N), do not appear to be influenced by the CBA tested and are BSH positive. BSH activity is a well-documented phenotype in *L.plantarum* strains and the presence of multiple *bsh* homologs has been suggested to provide a broader substrate coverage (Duary et al., 2012; Lambert, Bongers, et al., 2008; McAuliffe et al., 2005). Likewise, both bifidobacterial strains (CUL20 and CUL34), also appear to be uninfluenced by the amino acid side chain and present activity on both substrates in agreement with previous reports (Bordoni et al., 2013; Jarocki et al., 2014). Both CUL20 and CUL34 produced two precipitate phenotypes on both BA substrates, with the in-agar precipitate. and in-agar scattering (which was only seen in bifidobacteria strains) and at the time of writing and to the author's knowledge is not something that has been previously described/investigated. *L.acidophilus* strains (CUL21 and CUL60) also exhibit BSH activity on both BA substrates. However, the precipitation varies between strains, concentration and BA substrate, suggesting an impact on concentration and substrate selection when assessing BSH activity in *L.acidophilus*.

Indeed, phylogenetic analysis has revealed the presence of up to 57 distinct clusters of BSH proteins, which may dictate the efficiency of deconjugation and perhaps offer a putative explanation for phenotypic variation in the precipitate (O'Flaherty et al., 2018). However, clusters were typically species-related and variations here are exhibited at the strain level. The number of variations observed when experimental parameters were modified generated several important questions.

**1.** What causes the precipitate phenotype variation and how does concentration affect the phenotype observed? For example, *L.acidophilus* CUL60 presents an in-agar precipitate and a white surface precipitate.

**2.** Does phenotype variation represent different reactions?

**3.** Does the difference in precipitate represent the strength of BSH activity or reflect the number of *bsh* homologs within a genome?

#### 4.4.3.3. *The lactic acid hypothesis*

As CUL isolates produced several phenotypic precipitations when stimulated by CBA's, which seemed to be independent of trend (except for bifidobacteria which were the only isolates to produce a scatter-like precipitate in agar), it was decided that the effect of lactic acid isomers (produced by lactobacilli) should be evaluated independently. Intriguingly, a reaction was seen (as designated by the emergence of a white precipitate) when lactic acid was inoculated on plates containing GDCA and not on TDCA, suggesting that lactic acid may have the capability to deconjugate high concentrations of glycine CBA. Indeed, in previous years co-polymers have been developed containing glycine and DL lactic acid and it has been shown that higher concentrations of glycine, escalate the decomposition of the polymer (Schakenraad et al., 1989), supporting the phenotypic reaction observed here. At the time of writing, this is the only link that could be found between glycine and lactic acid and future efforts should attempt to characterise this interaction further. Such phenomena led to a subsequent analysis via GC/MS to deduce the effect lactic acid exerted on BA composition. Interestingly, a second peak emerges from the side of the GDCA peak at a retention time of 19.98 mins. This phenomenon was only observed in samples incubated with lactic acid, suggesting that lactic acid does contribute to the structural alteration of GDCA.

In addition, the biochemical profiles of bacteria-mediated precipitate formation, when incubated on 0.48 % GDCA, using two variations: *L.acidophilus* CUL60 (on agar) and *L.gasseri* CUL09 (in agar), were selected to evaluate if lactic acid modification may provide a putative explanation for precipitate phenotype variation. However, both variants showed a capability to deconjugate GDCA to DCA with no additional modifications. It is worth noting that due to the derivatisation process when conducting GC/MS, some resolution is lost when characterising mass ions. Therefore, to further distinguish the structural changes induced by lactic acid and to identify a broader range of compounds, Liquid Chromatography/Mass Spectrometry should be utilised to enhance the dataset.

#### 4.4.3.4. *Genetic basis for CUL deconjugation capabilities*

The prevalence of *bsh* genes in the *Lactobacillus* genera is highly variable, not only between species but even at strain level (Fang et al., 2009; Gu et al., 2014; Lambert, Bongers, et al., 2008). Indeed, it seems that *bsh* genes are predominantly observed in species which occupy the GI tract and not in species from other ecological niches (Jones et al., 2008; O'Sullivan et al., 2009). In addition, the protein sequence similarity of BSH inter-species can range between 20.33 % to 99.69 % (Dong & Lee, 2018) and strains containing multiple homologs have shown to have greater sequence homology to *bsh* genes from other species, than they do to other *bsh* genes in the same genome (Chae et al., 2013; Jayashree et al., 2014; McAuliffe et al., 2005).

Following genomic mining, it was putatively revealed that CUL isolates belonging to the *L.casei* clade, had no phenotypic BSH activity on either BA substrate, but were all predicted to possess one conjugated bile salt hydrolase-related amidase protein. The annotation is non-specific and could encompass a BSH or a penicillin acylase function, given the high degree of similarity observed between these groups (Kumar et al., 2006). It has often been reported that members of the *L.casei* group do not possess BSH capabilities, in agreement with our findings (Caggia et al., 2015; Maragkoudakis et al., 2006; Muñoz-Quezada et al., 2013; Solieri et al., 2014; Xiong et al., 2017). Lack of BSH in *L.casei* is perhaps to be expected, given that they are typically isolated from dairy products (Hill et al., 2018). However, in contrast, some studies have reported BSH activity in *L.casei* (Zhang et al., 2009; Zhang et al., 2021) despite a general understanding that *L.casei* do not possess *bsh* genes (Wang et al., 2011; Xiong et al., 2017), further emphasising the importance of robustly characterising both isolate and BSH activity in a polyphasic manner.

*L.salivarius CUL61* produced a precipitate solely when challenged with TDCA and was found to encode two *bsh* genes, which is consistent with the literature (Fang et al., 2009; Neville & O'Toole, 2010). Interestingly, it has been reported that *bsh*1 in *L.salivarius* is typically encoded on a mega-plasmid (Claesson et al., 2006; Fang et al., 2009), suggesting a potential plasmid prediction within CUL61 and supporting the prediction of a GI in CUL61's genome in earlier work (**Chapter 3, Table 3.6).**

*L.gasseri* was phenotypically active and presented a BSH positive phenotype on both BA substrates. In addition, RAST annotation identified three *bsh* genes and one conjugated bile salt transporter, suggesting a gene copy mediated substrate preference. Indeed, a recent study has identified a substrate preference for glycine CBAs in comparison to taurine (Rani et al., 2017). Furthermore, two *bsh* homologs have been shown to encode an amino acid substrate preference, where *bshA* preferentially deconjugated taurine and *bshB* glycine conjugates (Foley et al., 2021). *L.helveticus* showed no BSH activity on either BA substrate,

in agreement with weak BSH activity in dairy isolates reported elsewhere (Fontana et al., 2019; Tanaka et al., 1999).

Of interest is the variation between the two *L.fermentum* strains CUL67 and CUL40. CUL67 was predicted to encode a single cholylglycine hydrolase, which could encompass any proteins within that enzyme family. CUL40 in contrast, putatively encodes two cholylglycine hydrolases, again raising a similar issue with annotation. When correlating genotype to phenotype, CUL67 showed no phenotypic activity against either BA substrate, but CUL40 revealed activity on GDCA, suggesting that at least one copy is a functional *bsh* gene. The *L.fermentum* species are another example of a good correlation between phenotypic and genomic data, demonstrating successful use of the polyphasic approach.

#### 4.4.3.5. *Bioprospecting CUL isolates*

Elevated cholesterol levels have been correlated to several morbidities (Buckley & Ramji, 2015; Houston et al., 2011; Jamnagerwalla et al., 2018; McLaren et al., 2011) and subsequent mortality (Anandharaj et al., 2014; Nabel, 2003), with strong links existing between elevated cholesterol and the development of CVD (Nabel, 2003). Although treatments currently exist to aid in cholesterol reduction, these are flawed and come with their own detrimental impact to host health (Culver et al., 2012). Research on cholesterol-lowering properties of probiotics has grown exponentially over the last few decades, with many confirmed reports of their ability to lower serum cholesterol levels (Bendali et al., 2017; Fuentes et al., 2016; Tsai et al., 2014).

Due to the qualitative nature of the BSH plate assay and the limitations discussed previously, strains deemed to have the greatest potential to deconjugate BA were selected for in-depth analysis via GC/MS. Despite incorporating both taurine and glycine conjugates within the plate assay, human bile is comprised of 67 % BA's, 22 % phospholipids, 5 % cholesterol, 5 % proteins and 0.3 % bilirubin (Esteller, 2008). 96 % of the BAs in the human GI tract consist of GCA, GCDCA, GDCA, TCA, TCDCA and TDCA in a molar ratio of ~ 6:6:4:3:3:2 (Hu et al., 2018b). Therefore, a total BA pool was selected to provide increased physiological conditions *in vitro*.

In a recent study by Hu et al., (2018b), it was concluded that Oxgall bile produced by Sigma, has the highest degree of similarity to human bile and was therefore selected for this analysis. The species *Lactobacillus plantarum* has repeatedly been linked to *bsh* production and cholesterol-lowering capabilities (Costabile et al., 2017; Dong et al., 2012; Huang et al., 2020; Michael et al., 2016; Nguyen et al., 2007; Wu et al., 2017). Previous analysis of *L.plantarum* CUL66 (Michael et al., 2016, 2017, 2020), provided a strong basis for a need to further characterise the molecular interactions seen between CUL *L.plantarum* strains and bile, due to recent reports of CUL66's high efficacy at contributing to weight loss in clinically obese

patients (Michael et al., 2020) and wild-type mice on a High Fat Diet (HFD). Here, we show a strong effective relationship between *L.plantarum* CUL strains and bile modification. Following a three-hour incubation with bovine bile, *L.plantarum* CUL66 and CUL66N, in addition to *L.gasseri* CUL09 appear to stimulate a sharp increase of the primary BA's (and *L.acidophilus* CUL21 to a smaller extent), cholic and chenodeoxycholic acid within the BA pool, indicating effective deconjugation. Furthermore, *L.gasseri* CUL09 also appears to additionally increase the abundance of deoxycholic acid. All three strains saw a depletion in the concentrations of TCA/GCA and TDCA/GDCA further indicating an efficient capability at deconjugating BA's. Moreover, free cholesterol in the bile pool was also reduced in *L.plantarum* strains and *L.gasseri*, suggesting *L.gasseri* CUL09 should be a focus for further bioprospecting *in vivo*. In addition, during targeted gene expression analysis, the three predicted *bsh* homologs in *L.plantarum* CUL66N were upregulated in the presence of 0.3 %, suggesting an active role for these genes in the interaction with bile. Indeed, such findings offer significant scope for bioprospecting and potentially provides mechanistic support for previously described cholesterol lowering capacities of *L.plantarum* CUL strains (Michael et al., 2016, 2017, 2020). Certainly, many *in vitro* (Costabile et al., 2017; Dong et al., 2012; Pereira et al., 2003), *in vivo* (Aminlari et al., 2019; Bendali et al., 2017; Liang et al., 2020; Michael et al., 2017) and combined meta-analysis (Guo et al., 2011; Wang, Guo, et al., 2018a) studies have correlated probiotic consumption with reduced cholesterol levels. BSH production is thought to stimulate cholesterol reduction by the following mechanism: deconjugated BAs are less soluble and hence are not reabsorbed during enterohepatic circulation (Costabile et al., 2017). As a result, DBA's are precipitated and excreted in the faeces, resulting in serum cholesterol being absorbed and recruited to the liver to replenish the BA pool, hence lowering blood cholesterol (Kumar et al., 2006). In addition, cholesterol can also be incorporated into the bacterial cell membrane (via cholesterol assimilation), which prohibits the formation of cholesterol micelles and prevents the transport of fatty acids to the surface of the intestine for absorption, reducing the cholesterol further (Lye et al., 2010). Several studies have cited *bsh*-related mechanisms for probiotic-mediated cholesterol reduction (Oner, Aslim, & Aydaş, 2013; Pavlović et al., 2012; Pereira et al., 2003; Shehata et al., 2016; Taranto et al., 1997; Tomaro-Duchesneau et al., 2014; Tsai et al., 2014). Interestingly, *L.casei* CUL06 which is a *bsh* negative strain has a decreased abundance of cholesterol and GDCA/TDCA and increased abundances of CA, CDCA, DCA and GCA/TCA. However, previous reports have shown that probiotic supplementation can lower cholesterol levels by regulating cholesterol transport and metabolism (Huang & Zheng, 2010; Michael et al., 2017; Yoon et al., 2012), offering an alternative bioprospecting pathway that could be investigated for *bsh* negative strains.

### 4.4.4. Summary and future work

The majority of CUL isolates (except for *L.fermentum* strains) are suitable probiotic candidates, evidenced by their ability to tolerate bile across a concentration gradient of physiological relevance. In addition, the ability to lower host serum cholesterol levels was putatively analysed and *L.plantarum* strains revealed the great potential to modify BA pools via a *bsh*-mediated response. Furthermore, *L.gasseri* CUL09 revealed a strong capacity to interact with bile, suggesting future *in vivo* studies should be conducted to deduce if *in vitro* behaviours translate *in vivo*.

Future work should focus on global transcriptomics of *L.plantarum* CUL66N's response to bile stimulation, to determine whether additional factors aid in BA pool modification and subsequently contribute to host cholesterol reduction. In addition, LC/MS should be performed to establish whether lactic acid mediates the modification of GDCA.

## 4.5. Appendix 4.

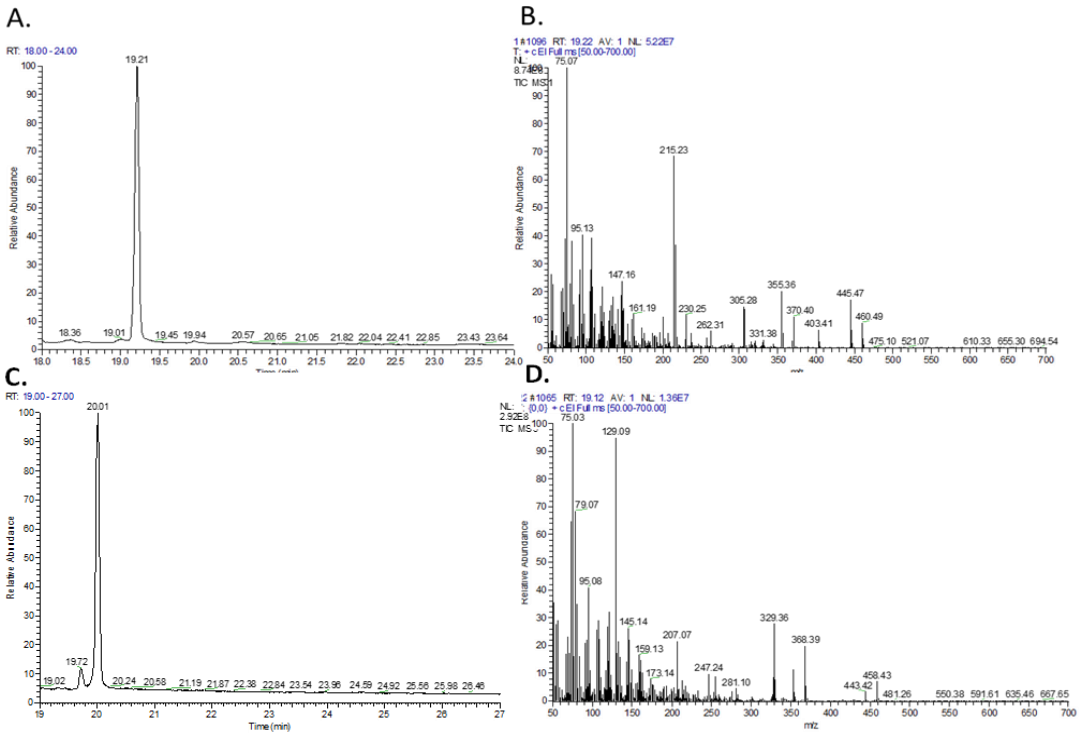



**Figure S4.1. Control GC/MS spectra for bile acid composition analysis**. **A**. 5a-Cholestanol loading control GC/MS spectrum. **B**. 5a-Cholestanol mass spectrum. **C**. Cholesterol GC/MS spectrum. **D**. Control cholesterol GC/MS mass spectrum, for downstream identification. 5a-Cholestanol was loaded with each test sample as a loading control, to allow downstream quantification.

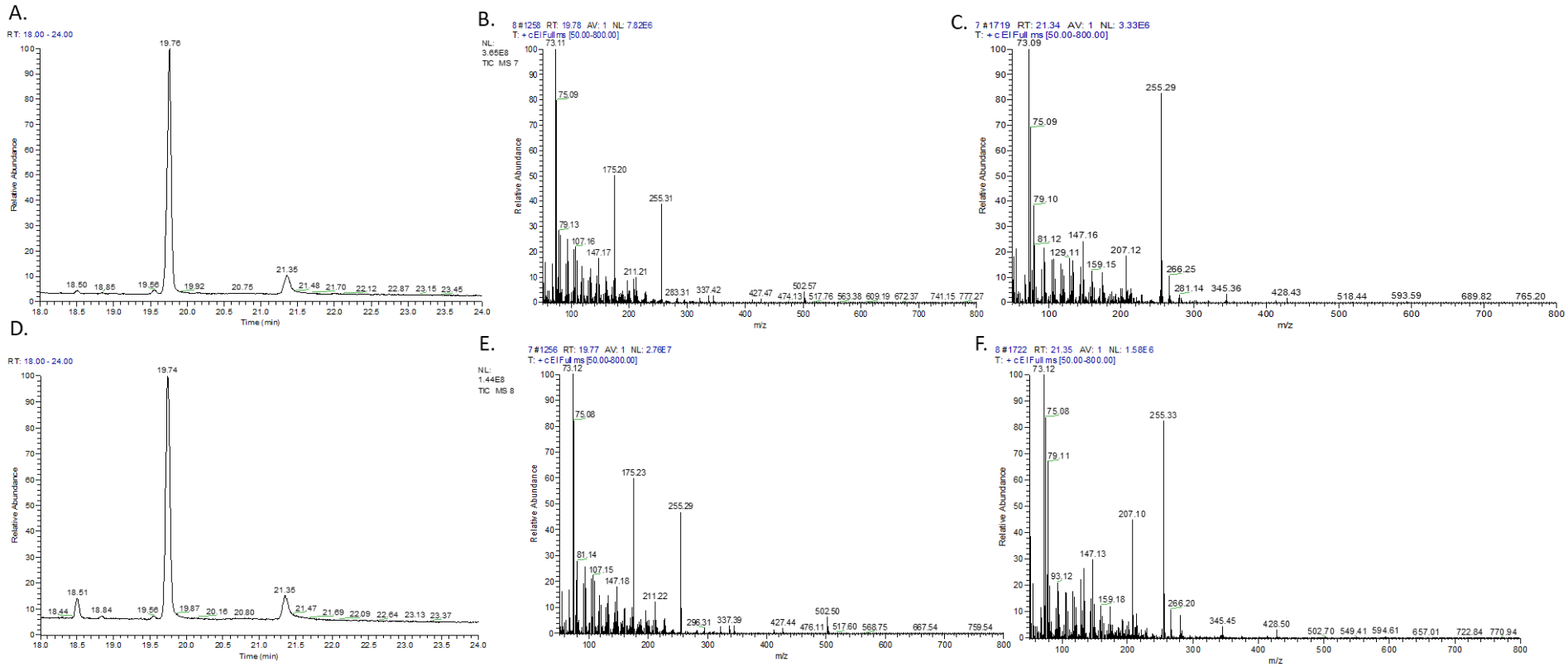



**Figure S4.2. Single bile acid GC/MS profiles and fragmentation spectra for known standards used to identify bile acids**. **A**. GDCA spectrum. **B**. GDCA mass spectrum (main peak 19.76 min). **C**. GDCA mass spectrum (21.34 min – possibly DCA).
**D**. TDCA spectrum. **E**. TDCA mass spectrum (main peak 19.78 min). **F**. TDCA mass spectrum (21.35 min – possibly DCA).
GDCA and TDCA (99 %) purity, both produced one main peak (19.76 min and 19.78 min respectively) and a smaller subpeak at 21.35 which has the potential to be deoxycholic acid.

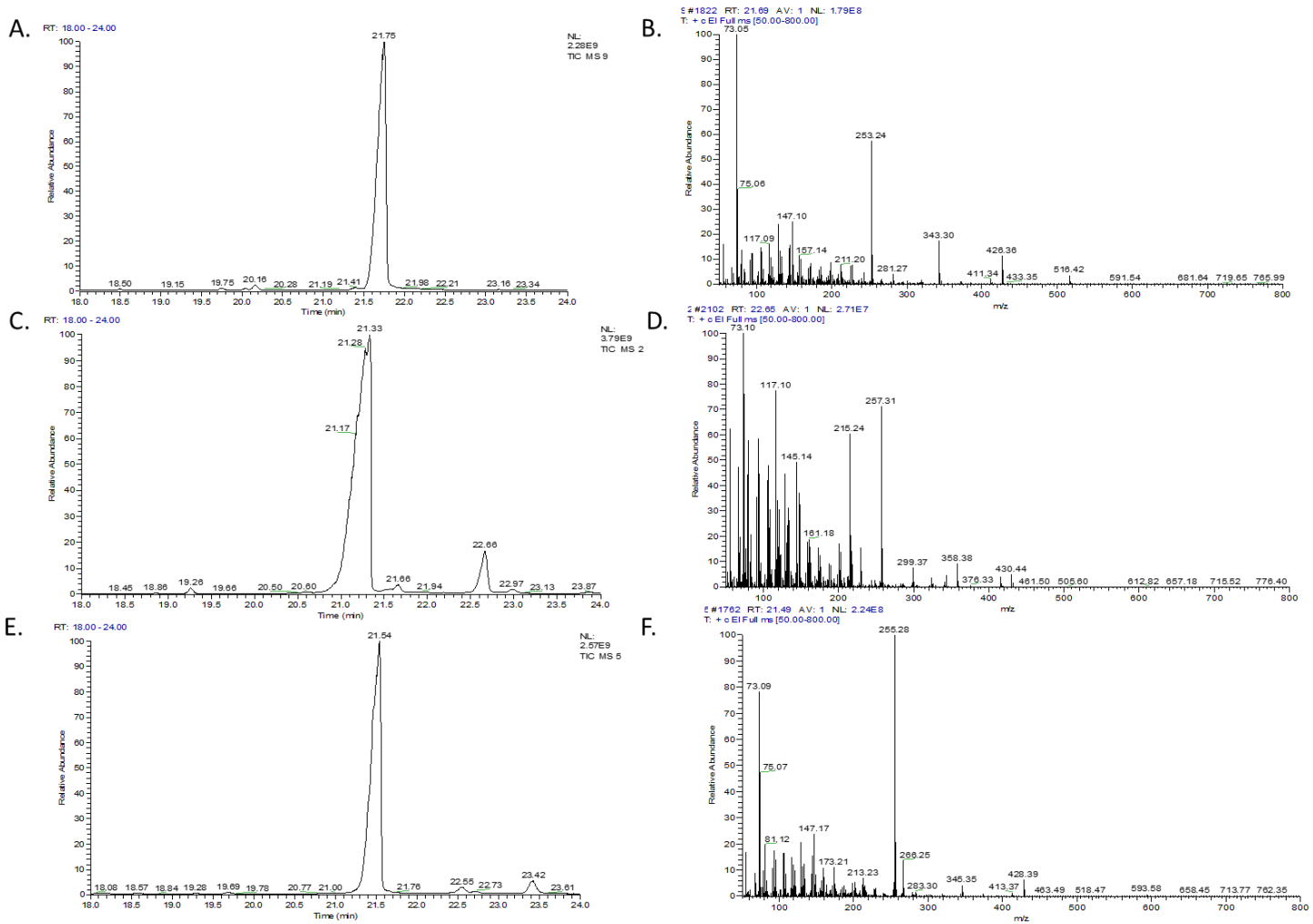



**Figure S4.3. Single bile acid GC/MS profiles and fragmentation spectrum for known standards used to identify bile acids**. **A**. Chenodeoxycholic acid (CDCA) mass spectrum. **B**. CDCA mass spectrum. **C**. Lithocholic acid (LCA) mass spectrum. **D**. LCA mass spectrum (main peak 21.33 min). **E**. Deoxycholic acid (DCA) spectrum **F**. DCA mass spectrum.

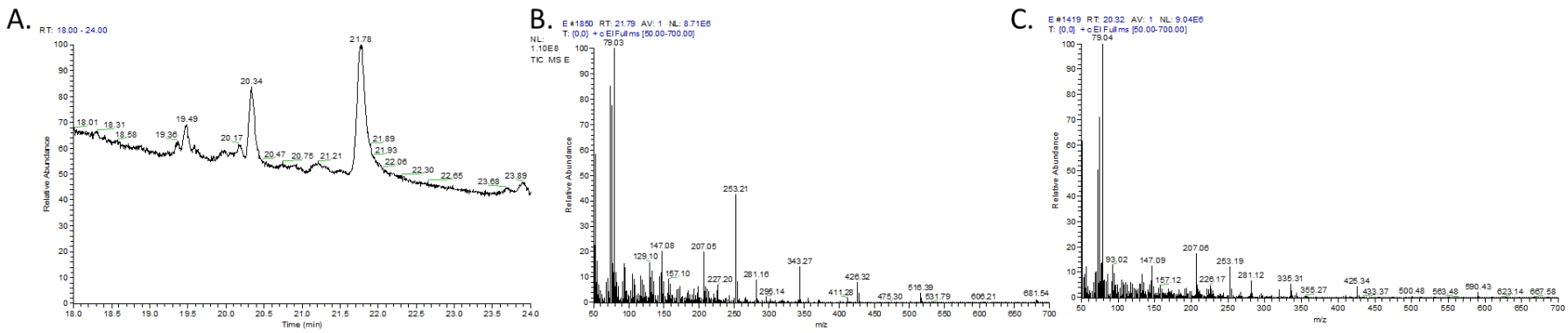



**Figure S4.4. Single bile acid GC/MS profiles and fragmentation spectra for known standards used to identify bile acids** **A**. Taurocholic acid (TCA spectrum). **B**. TCA mass spectrum (main peak 20.79 min – potentially CA). **C**. TCA mass spectrum (20.34 min).

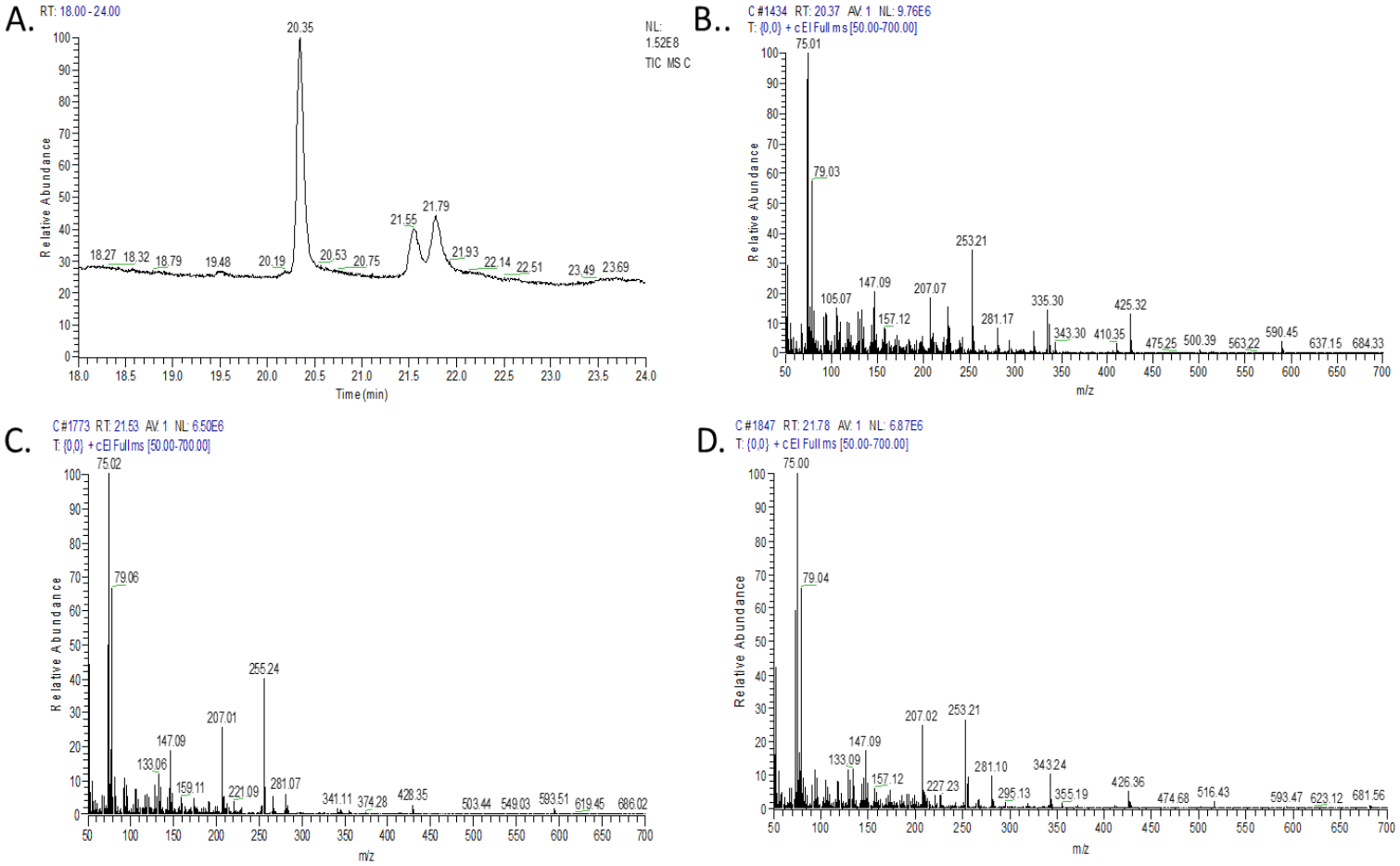



**Figure S4.5. Single bile acid GC/MS profiles and fragmentation spectra for known standards used to identify bile acids**. **A**. Gylcocholic acid (GCA) spectrum. **B**. GCA mass spectrum (main peak 20.35 min). **C**. GCA mass spectrum (main peak 20.53 min). **D.** GCA mass spectrum (main peak 20.78 min – possibly CA).

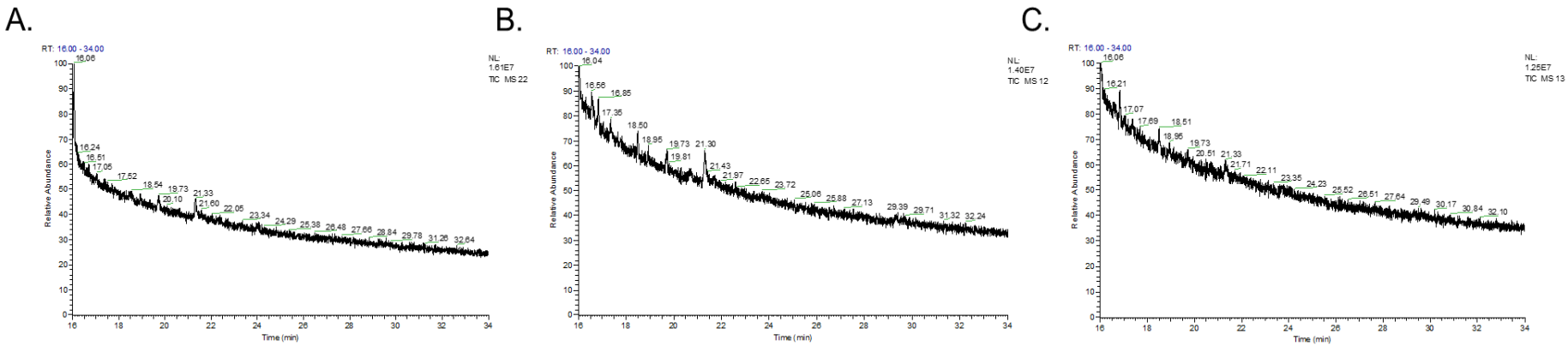



**Figure S4.6. GC/MS MRS agar control spectra for bile acid analysis**. **A**. MRS Agar. **B**. MRS Agar + DL lactic acid. **C**. MRS Agar + L+ Lactic acid. No bile acids or other peaks were present in MRS or MRS + lactic acid controls.

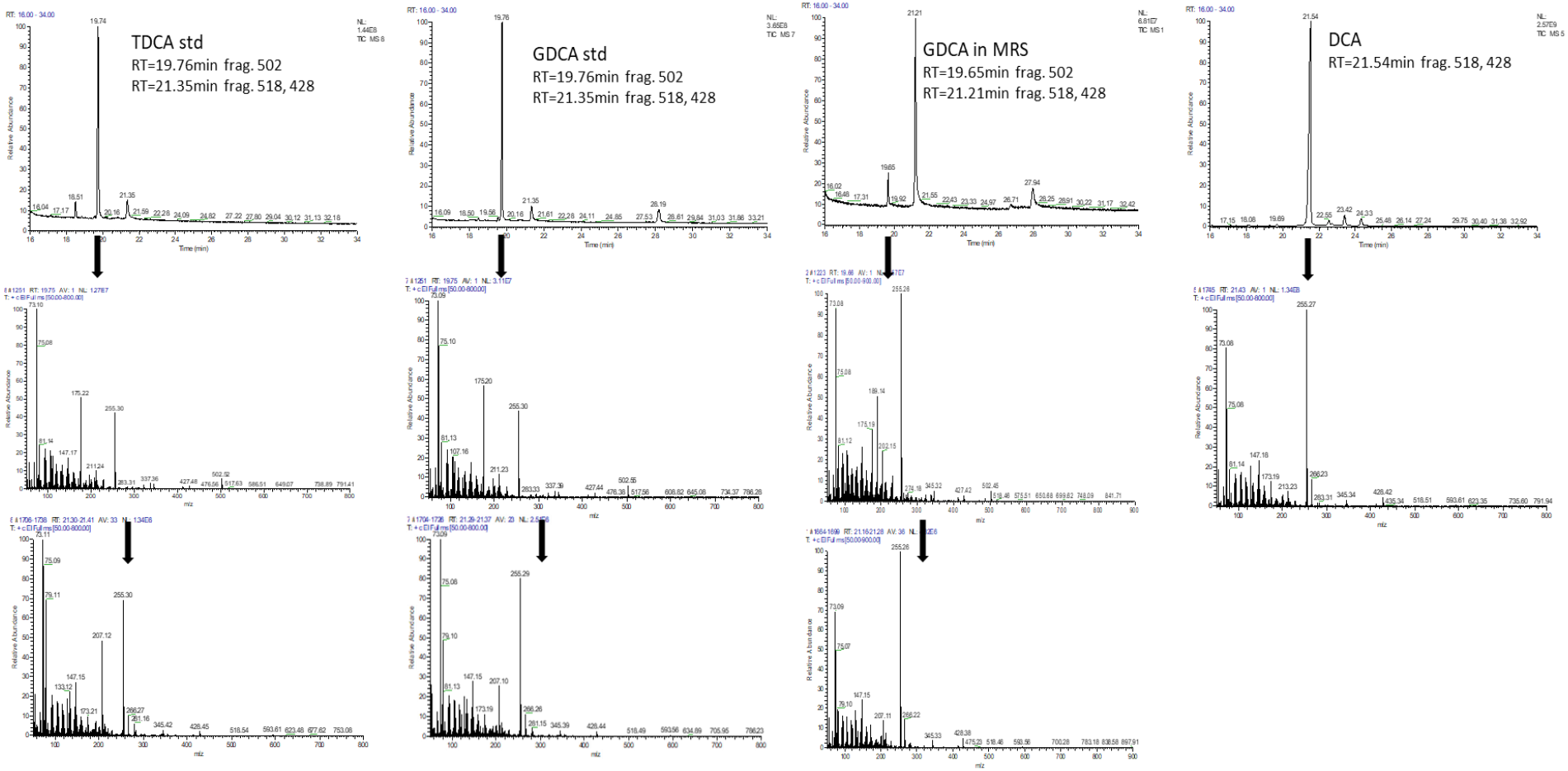
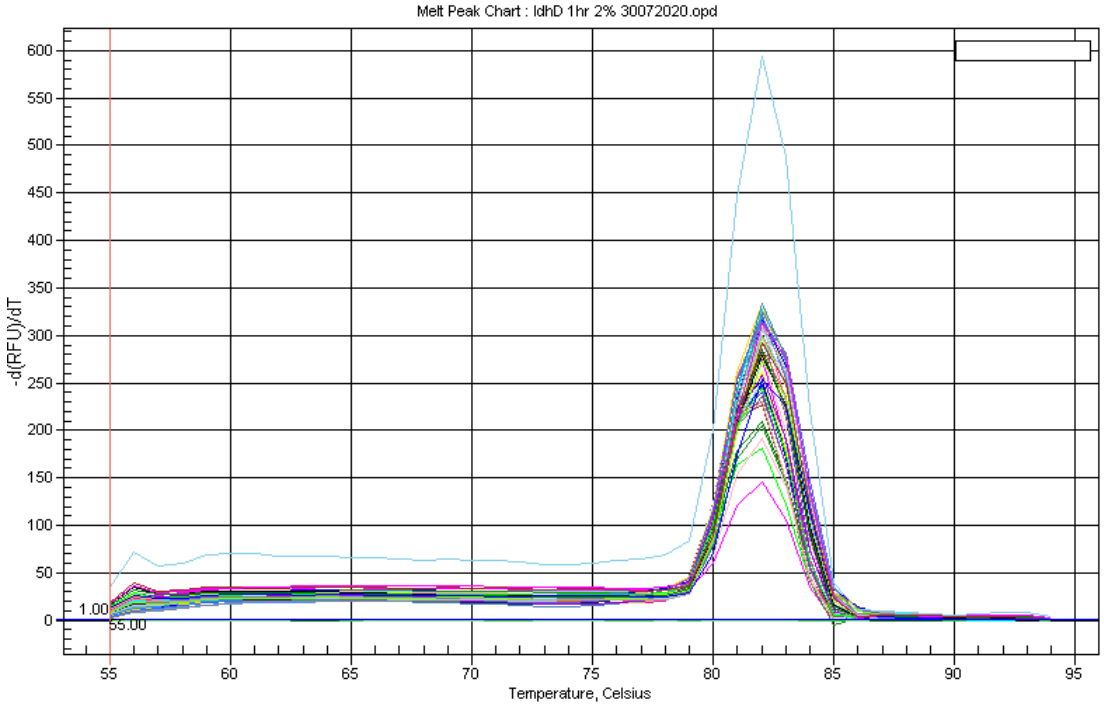
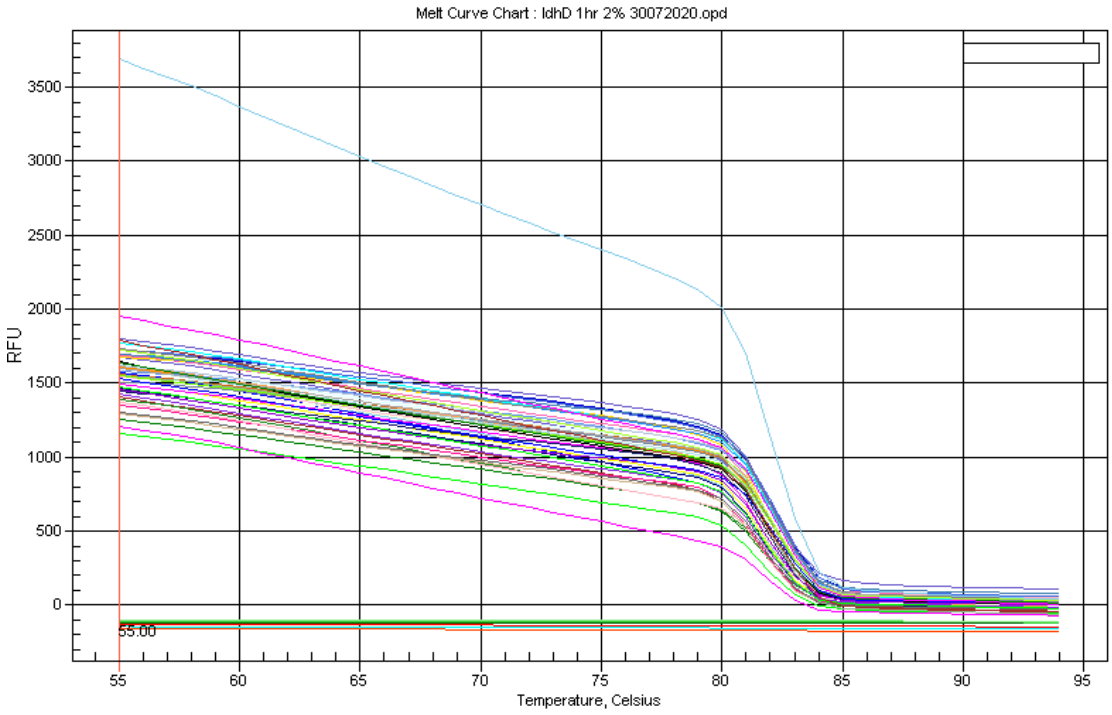



**Figure S4.7. GC/MS MRS agar standards for downstream bile acid profiling. A**(i-iii). TDCA standard spectrum and peak mass spectrum as a control reference. **B** (i-iii). GDCA standard spectrum and peak mass spectrum as a control reference. **C**(i-iii). GDCA in MRS agar standard spectrum and peak mass spectrum as a control reference. **D**. DCA standard spectrum and peak mass spectrum as a control reference.

## Primer efficiency for QPCR



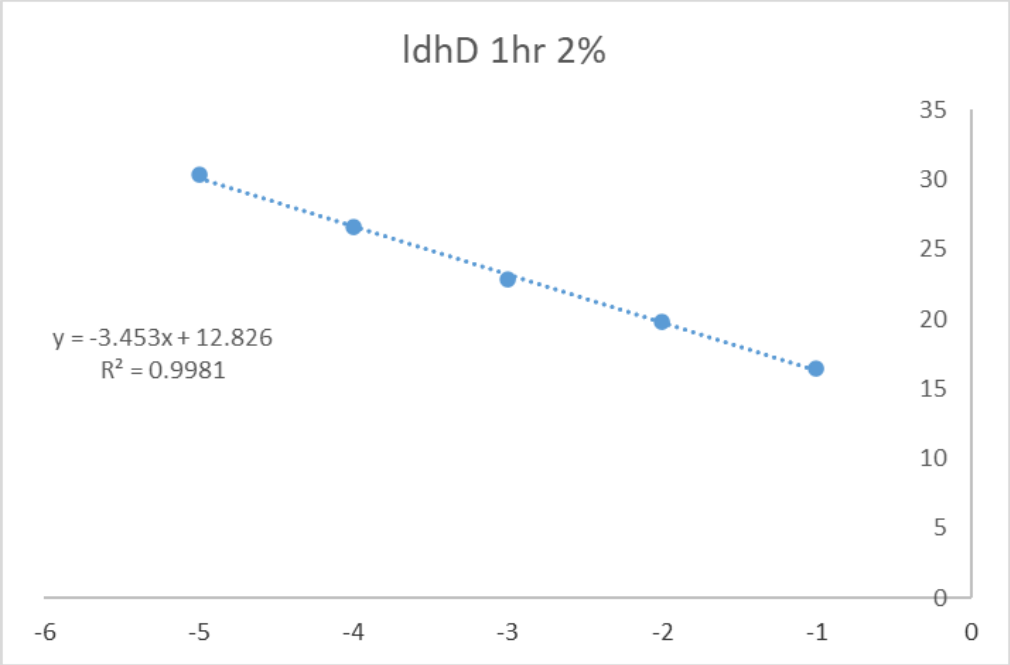
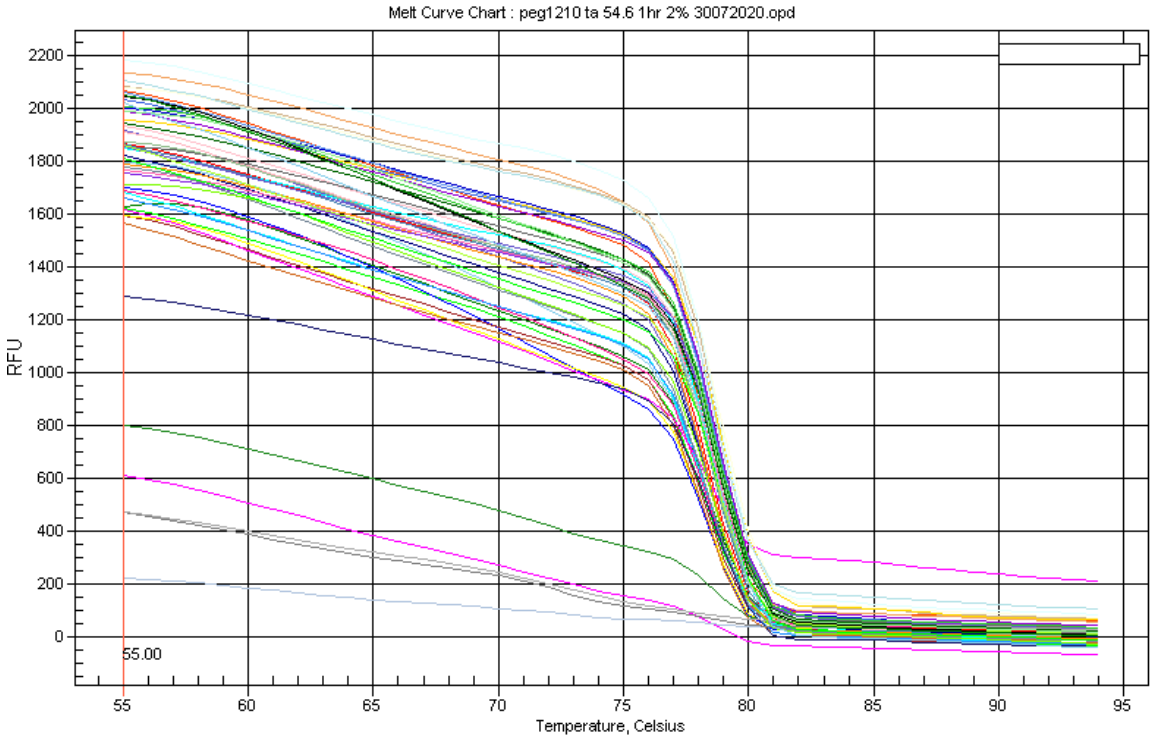
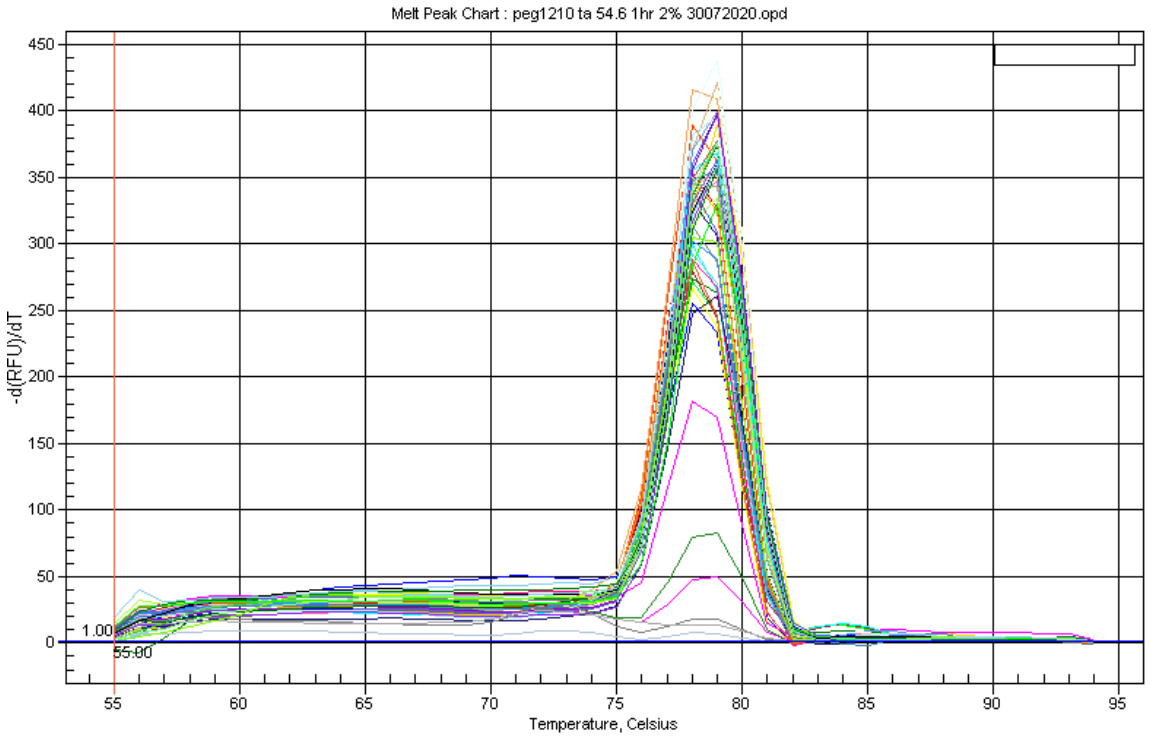



**Figure S4.8.** Primer efficiency for *ldhD* including, melt peak, melt curve and efficiency plot. Efficacy was calculated from the line equation and was approximately 94.8 %.


Melt Curve Chart : peg1210 ta 54.6 1hr 2% 30072020.opd
RFU
Temperature, Celsius
55.00


Melt Peak Chart : peg1210 ta 54.6 1hr 2% 30072020.opd
-d(RFU)/dT
Temperature, Celsius
1.00
55.00

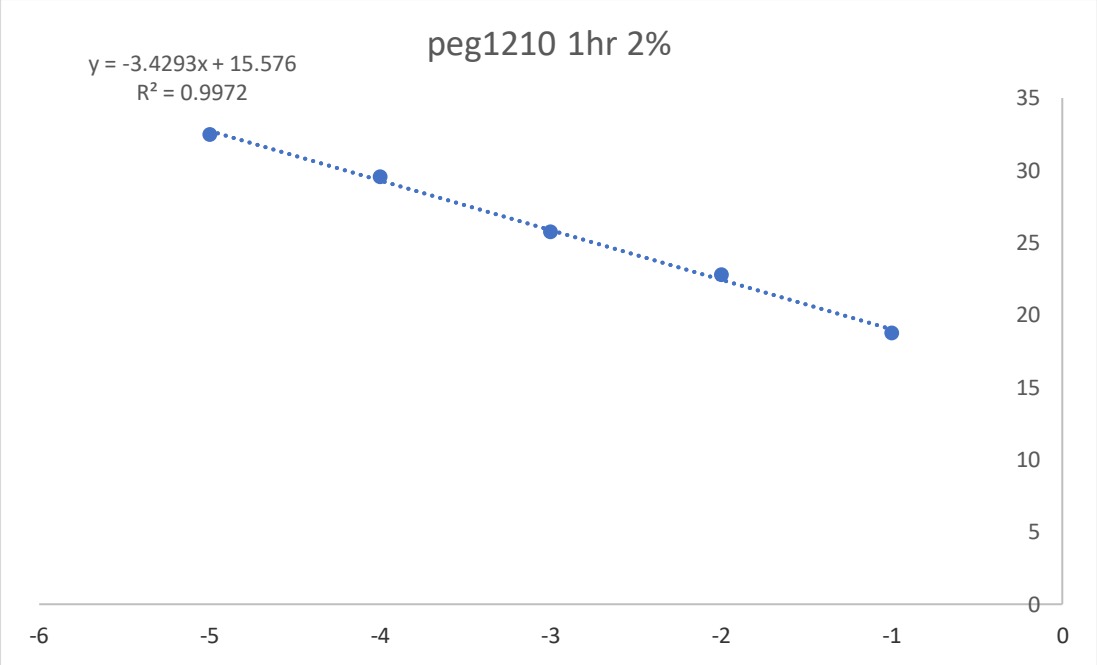
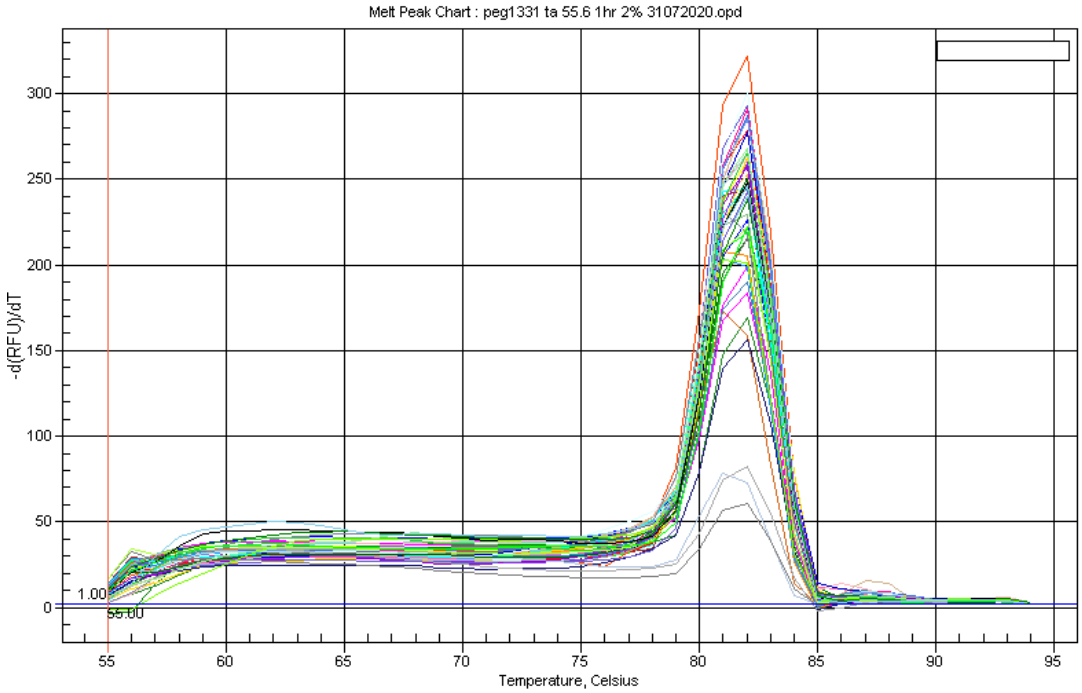
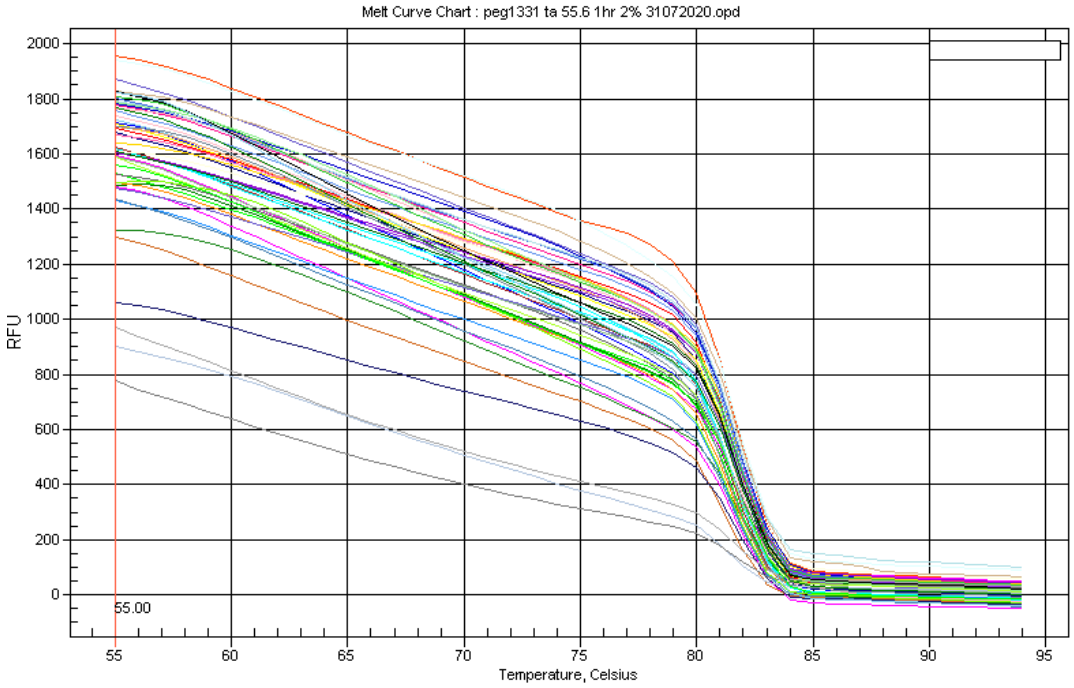



**Figure S4.9.** Primer efficiency for PEG1210 including, melt peak, melt curve and efficiency plot. Efficacy was calculated from the line equation and was approximately 95.7 %.


Melt Peak Chart : peg1331 ta 55.6 1hr 2% 31072020.opd
-d(RFU)/dT
Temperature, Celsius
1.00
55.00


Melt Curve Chart : peg1331 ta 55.6 1hr 2% 31072020.opd
RFU
Temperature, Celsius
55.00

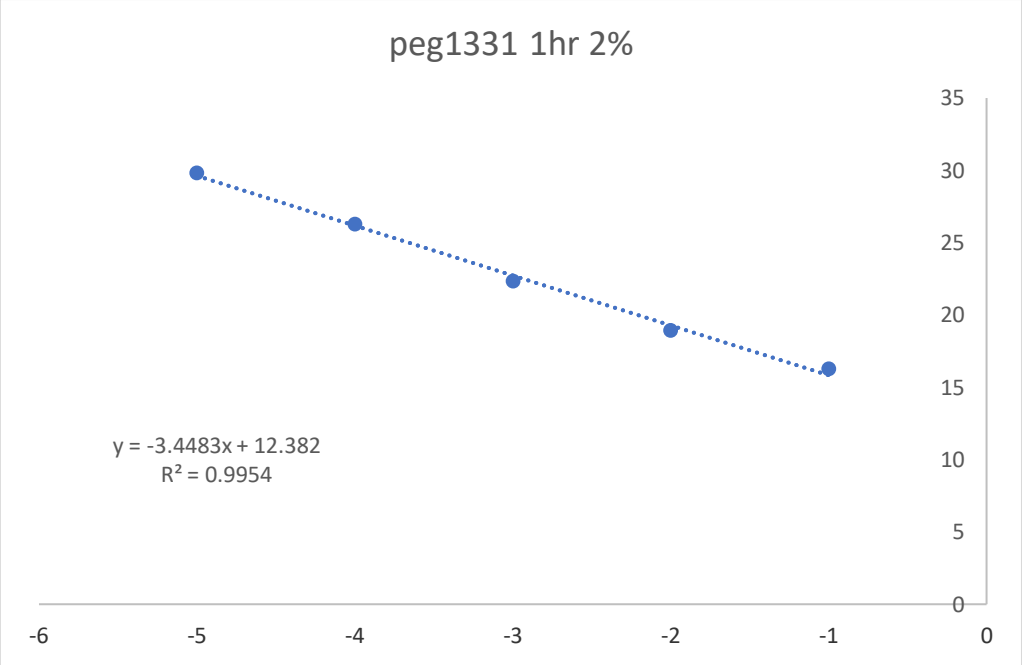



**Figure S4.10.** Primer efficiency for PEG1331 including, melt peak, melt curve and efficiency plot. Efficacy was calculated from the line equation and was approximately 95 %.

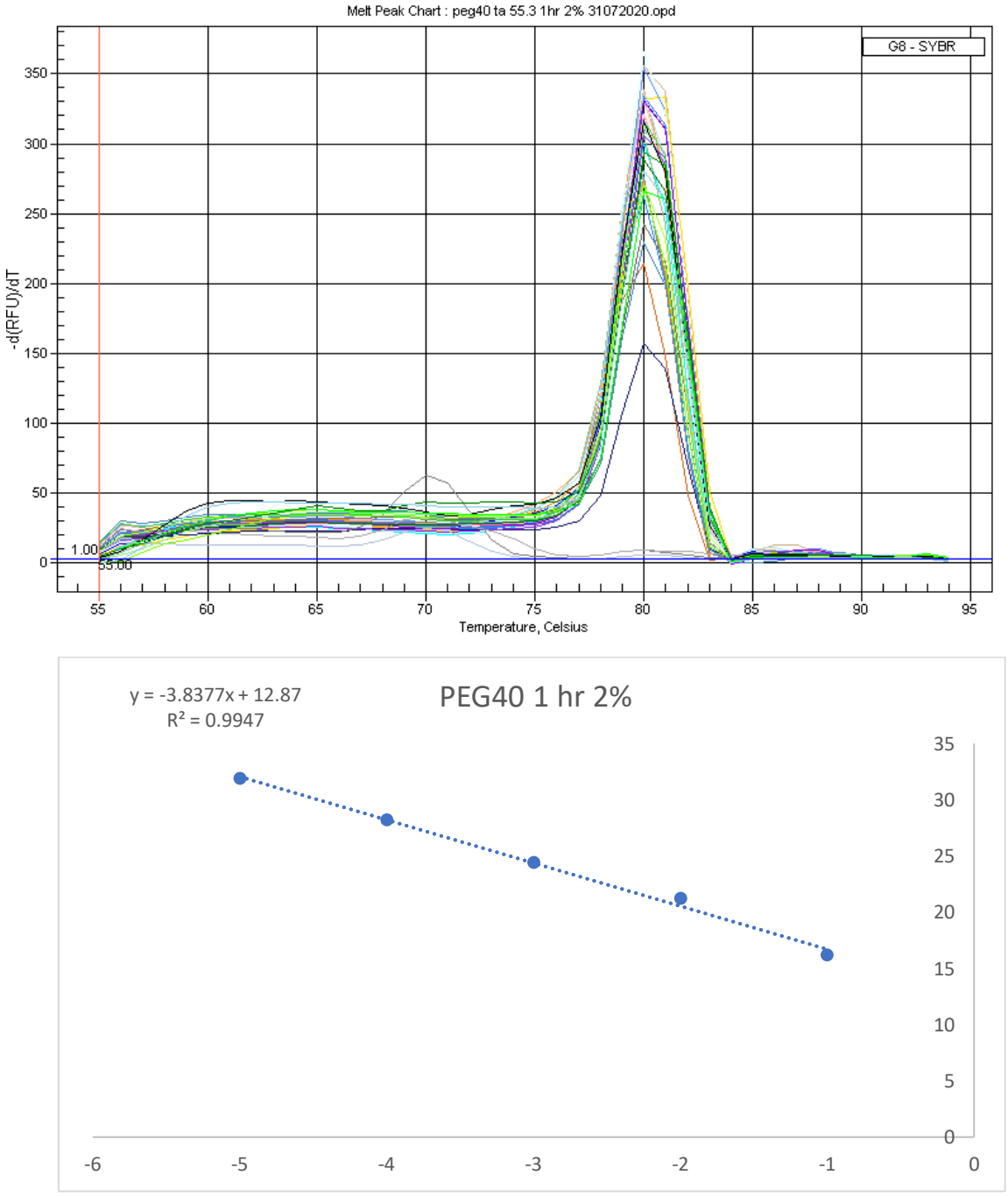



**Figure S4.11.** Primer efficiency for PEG40 including, melt peak, melt curve and efficiency plot. Efficacy was calculated from the line equation and was approximately 82.2 %.

**Supplementary Tables**

**Table S4.1.** pfaffl gene expression.
https://docs.google.com/spreadsheets/d/1pZGgUks42-VHq5e1d4kKjmNFJYpXqLny/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true

# Chapter 5.
# Evaluating the Antibiotic Resistome in CUL isolates: a safety analysis.

## 5.1. Background

### 5.1.1. Antimicrobial Resistance

The rapid emergence of AMR is unequivocable and poses a significant threat to human health (Christaki et al., 2020; Laxminarayan et al., 2020; San Millan, 2018). The proliferation of resistant and infectious bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (Turner et al., 2019) and *Pseudomonas aeruginosa* (Bassetti et al., 2018), has resulted in a healthcare crisis, due to a depletion of effective therapeutics (Horcajada et al., 2019; Hutchings et al., 2019; Munita & Arias, 2016; Turner et al., 2019; Zhang et al., 2017). Thus, AMR is now considered to be one of the most important health threats of the 21st century (WHO., 2014) and efforts to prevent further escalation of the AMR crisis are paramount.

Historically, characterising the AMR profiles of pathogenic bacterial strains has been the objective of many studies; primarily due to the implications that resistance has on the development of effective disease treatment (Anderson, 1968; Aoki et al., 1971; Bryan et al., 1985; Marín et al., 2021; Shankar et al., 2020; von Wintersdorff et al., 2016). That said, AMR mechanisms have now been identified in both pathogenic and commensal bacteria, resulting in antibiotic-resistant phenotypes being characterised in a magnitude of bacteria, for most antibiotics currently available (Allen et al., 2010; Forsberg et al., 2012; Ventola, 2015). As research has continued to characterise the gut microbiota, several *in silico* predictions have outlined an HGT network within the gut (Broaders et al., 2013; Kent et al., 2020; Li et al., 2020). Indeed, factors such as gut inflammation have demonstrated a capability to initiate transient enterobacterial blooms, which then facilitate an unprecedented rate of conjugative transfer (Stecher et al., 2012) and frequent transmission of an extended-spectrum beta-lactamase encoding plasmid in the infant gut microbiota has also been experimentally shown (Hagbø et al., 2020). Additionally, outbreaks of carbapenem-resistant *E. coli* have been linked with a conjugative transfer of resistance genes between *Klebsiella pneumonia* and *E. coli* in hospital patients (Borgia et al., 2012; Broaders et al., 2013; Mulvey et al., 2011).

### 5.1.2. Antibiotic resistance in probiotic bacteria

It is paramount that bacteria manufactured for consumption, do not contribute to the resistance of pathogens. Indeed, safety legislation and guidance are provided to manufacturers to communicate risks, outline standards and minimise the sales of harmful bacteria (EFSA, 2012, 2016; FDA, 2021; Hill et al., 2014, 2016; Hoffmann et al., 2014; US Food and Drug Administration, 2016). Moreover, A QPS candidate should be subjected to ABR testing before their QPS designation (EFSA, 2008; Gueimonde et al., 2013). In addition, the guidelines set out by the FDA and the WHO, state that before a probiotic can be used in food for human consumption, a risk assessment of its ABR patterns must first be conducted (Araya et al., 2002).

### 5.1.3. European Food Safety Authority (EFSA) guidelines on probiotic ABR

In 2012, the EFSA released detailed guidelines on the antibiotic susceptibility profiles that should be identified in probiotic bacteria (EFSA, 2012). Nine antibiotics were deemed of significant importance by EFSA including ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol (EFSA, 2012). In addition, the report also describes a set of microbiological cut-off values (breakpoints), to categorise resistant and sensitive strains of commensal bacteria to such antibiotics. However, there are several confines to EFSA guidance, such as the limited panel of antibiotics and breakpoints provided and the vague nature of the species/grouping given for microbial breakpoints, for example, the breakpoints provided for bifidobacteria are only for genus level and not for individual species, despite resistance variation occurring at strain level (Anisimova & Yarullina, 2018; Campedelli et al., 2019; Duranti et al., 2017a). Furthermore, a recent analysis of antibiotic susceptibility in lactobacilli type strains revealed that 88 % of the breakpoints identified, failed to meet the criteria set by EFSA, emphasising the need for cut-offs to be re-evaluated (Campedelli et al., 2019). In addition, there is also room for the interpretation of methodology, which states, “that the existing body of scientific literature should be used to infer a protocol including dilution method, growth media and incubation conditions” (EFSA, 2012). Indeed, media selection, inoculum size and incubation times, are some examples of parameters which can influence resistance profiles (Danielsen & Wind, 2003; Egervärn et al., 2007; Huys et al., 2002). Furthermore, the genomic analysis of the ARG landscape is only required if the MIC profile exceeds the breakpoint value (Anisimova & Yarullina, 2018; Aymerich et al., 2006; Dias et al., 2013; Kõll et al., 2010; Sorokulova et al., 2008). The requirement to establish a robust protocol, which considers the variations that can arise in the resistance profile, in addition to characterising the genetic profile of microorganisms, is necessary to truly establish safety.

### 5.1.4. A critical analysis of current procedures: are variations in methodologies occluding true phenotypes and genotypes?

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) are an organisation responsible for synchronising antimicrobial breakpoints in Europe (Kahlmeter et al., 2006). When determining a resistance phenotype, EUCAST-recommended methodologies and breakpoint values are often utilised to ensure a standardised operating procedure (Espinel-Ingroff et al., 2005; European Committee on Antimicrobial Susceptibility Testing, 2013; Kassim et al., 2016). However, for many facultative and obligate anaerobic bacteria, (e.g., *Lactobacillus* and *Bifidobacterium*), there is a depletion in the availability of such guidelines in EUCAST. As a result, two procedures have been published by two independent organisations: the Clinical and Laboratory Standards Institute (CLSI) and the International Organization of Standardization (ISO)/International Dairy Federation (IDF (ISO 10932:2010)) for the determination of probiotic resistance profiles, which have been adopted by researchers (Campedelli et al., 2019; CLSI, 2006, 2010; ISO and IDF, 2010; Guo et al., 2017; Jorgensen et al., 2007; Ledina et al., 2018; Zhou et al., 2012). However, variations in methodology occur. For example, CLSI recommends the use of Mueller–Hinton broth (MHB) supplemented with 10 % lysed horse blood, whereas the ISO state susceptibility testing should be conducted in LSM (iso-sensitest + 10 % MRS) broth (CLSI, 2010; ISO and IDF, 2010). Research on the comparability of these two methods focusing on the resistance profiles of 22 *Lactobacillus* species (Mayrhofer et al., 2014), did not find parity and highlighted a significant variation in the results generated (Mayrhofer et al., 2014). Additionally, the characterisation of a MIC is typically assessed visually (Anisimova & Yarullina, 2018; Campedelli et al., 2019; Dec et al., 2017; Mayrhofer et al., 2010) and may influence the MIC ascribed, in contrast to breakpoints determined spectrophotometrically (due to the low inoculum used within the assay). Further complexities arise when switching from liquid to solid media, as this could also aid in result variation, masking the true resistance profile (Balouiri et al., 2016; Gajdács et al., 2017; Luber et al., 2003). Therefore, considering the variations of MIC profiles reported, it is prudent that AMR testing is performed by both methods to obtain a true phenotype (Mayrhofer et al., 2014). Furthermore, studies have identified broth microdilution and agar dilution as the best methods for MIC determination (Gajdács et al., 2017). In addition, due to the differences in the growth patterns between bacterial species, it has been suggested that specific protocols are developed to encompass potential biofilm formation, which may enhance MIC values (Salas-Jara et al., 2016)**.**

### 5.1.5. Determining the genetic AR profiles in probiotic bacteria

There are currently no standard guidelines available to identify the genetic basis of ABR and its subsequent risk of transferability (Zhang et al., 2018). With the decreased cost associated with high throughput sequencing, the availability of WGS is providing a more enhanced capability for identifying genes that cause resistance (Dong et al., 2019; Wang et al., 2017). Databases such as the Comprehensive ABR Database (CARD; (McArthur et al., 2013)), have also allowed users with whole genomes to access a plethora of ABR coding proteins, to be used as queries, for the putative annotation of ARGS (McArthur et al., 2013; Zankari et al., 2012). However, the identification of an ARG does not necessarily mean that the gene is expressed and correlations between genotype and phenotype are weak (Anisimova & Yarullina, 2018; Campedelli et al., 2019). However, in contrast a single amino acid change in the DDL protein has been shown to confer vancomycin resistance in lactobacilli (Campedelli et al., 2019). Indeed, the difficulty in correlating positive ABR phenotypes with a genetic basis, highlights the importance of performing both phenotypic and genotypic analyses, to robustly determine ABR profiles.

### 5.1.6. Aims and objectives

Considering the variation in AMR profiles when determined phenotypically, the lack of guidance for the genomic characterisation of ARGs and the nonspecific nature of the EFSA breakpoints, there is a need to robustly characterise ABR profiles via a multifaceted platform. This chapter will focus on the use of in-depth genomic and robust phenotypic methodologies, to define a reliable set of methods to assess the phenotypic ABR profile of CUL isolates. The resistance profiles of CUL isolates when challenged with 13 clinically relevant (for human and veterinary use) antibiotics will be evaluated and putative ARGs will be predicted, allowing a phenotype-genotype correlation to be executed, providing a robust ABR profile for CUL strains. Finally, the effect that gut physiological conditions (such as bile) will have on subsequent ABR profiles will be determined.

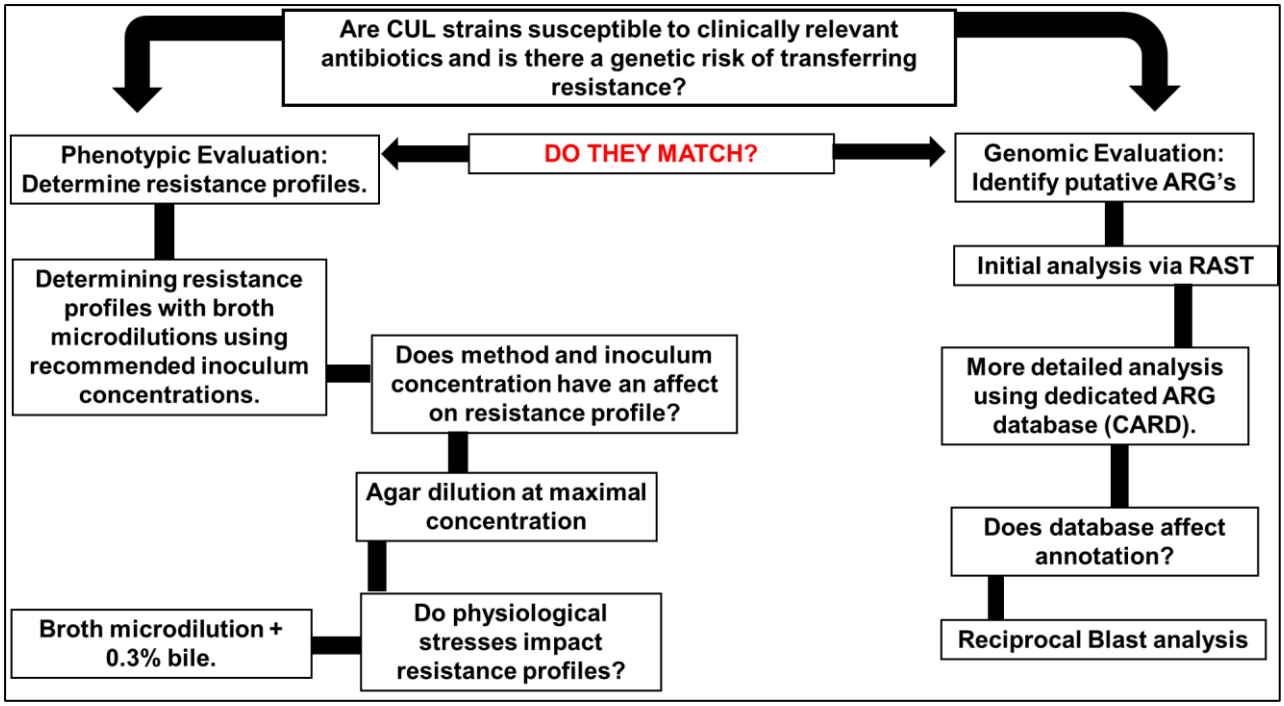



**Figure 5.1. Schematic representing the chapter goals and the workflow followed to characterize antibiotic resistance in CUL isolates.**

## 5.2. Materials and Methods

### 5.2.1. Growth of CUL isolates

As described in **Chapter 2, section 2.2.1 – 2.2.2.**

### 5.2.2. Phenotypic analysis of antibiotic resistance

#### 5.2.2.1. *Antibiotics*

The antibiotic susceptibility profiles of CUL isolates when challenged with the following clinically relevant antibiotics: kanamycin sulphate, neomycin sulphate, streptomycin sulphate, ampicillin sodium salt, amoxicillin, oxacillin, erythromycin hydrate, clindamycin hydrochloride, vancomycin hydrochloride, gentamycin sulphate, tetracycline, chloramphenicol and penicillin G was determined by both broth micro-dilution (CLSI, 2010; ISO and IDF, 2010) and agar dilution (Gajdács et al., 2017).

Antibiotic stocks were prepared as follows: antibiotic powders were dissolved in their relevant dilutant (**Table 5.1**) at a concentration of 20 mg/mL, filter sterilized and stored at -20 °C. For broth micro dilutions, stocks were prepared in LSM medium (90 % ISO: 10 % MRS+/- cys) to concentrations of either 64 µg/mL or 128 µg/mL and subsequently serially diluted 2-fold to 0.0625 µg/mL. 100 µL of each concentration was aliquoted into each well of a 96-well plate (Nunc, Nunclon). For agar dilution, antibiotic stock preparation and dilutions were carried out as previously described, with the substitution of LSM agar for LSM broth.

#### 5.2.2.2. *Broth microdilution*

Broth MICS were determined according to the method suggested by the Clinical and Laboratory Standards Institute (CLSI; www.clsi.org) and ISO standards (ISO 10932/IDF 223., 2010) with some modifications. A stock solution of each antibiotic (see **Table 5.1**) was prepared at 500 µg/mL in autoclaved iso-sensitest + 10 % MRS broth. A 2-fold dilution series was performed to obtain a concentration gradient of each antibiotic to a minimum concentration of 0.125 µg/mL. Cultures were prepared (as described in **Chapter 2, 2.2.2**) and incubated at 37 °C, anaerobically (80 % nitrogen, 10 % hydrogen, 10 % carbon dioxide) overnight. Cell pellets were collected by centrifugation (3000 *g* for 10 minutes) and resuspended in 5 mL of fresh Iso-sensitest (10 % MRS media) broth to remove supernatant (containing lactic acid). Cultures were diluted to an OD of 0.2 (600nm) and then further diluted 1:100 times in LSM broth (approx. **$1.39 \times 10^6$**). Cultures were further 1:2 diluted into a 96-well plate, giving a final dilution of 1:200 (i.e., 100 µL of each antibiotic concentration and 100 µL of culture were added to a 96-well plate to provide a final concentration range of 250 - 0.0625

µg/mL). Dilutions were used to provide consistent growth between replicates. Controls included: a positive control (without antibiotic) for each strain, a negative (no bacteria) control to ensure contamination-free media and a control strain *L.acidophilus* ATCC 4356 (Campedelli et al., 2019) was also included to ensure the functionality of the antibiotic stock. Plates were incubated at 37 °C, anaerobically for at least 24 h (or until visible growth was seen in the control well). Following incubation, the MIC was initially scored visually as recommended by Dec et al., (2017) and then read on Multiskan FC (Thermo Scientific) at 600 nm. The minimum inhibitory concentration (MIC) was defined as the lowest antibiotic concentration where no growth was present when the optical density was equal to the control well (determined spectrophotometrically). Experiments were performed in triplicate. MIC values were compared against the microbiological breakpoint values assigned by EFSA (EFSA, 2012). Where no EFSA guidelines were available for the tested antibiotic (neomycin, amoxicillin, oxacillin and penicillin G) breakpoints were assigned according to Ammor et al., (2007); Danielsen & Wind, (2003) and Felten et al., (1999).

#### 5.2.2.3. *Agar dilution*

A stock solution of each antibiotic (**Table 5.1**) was prepared at 250 µg/mL in sterile iso-sensitest + 10 % MRS agar. Plates were poured in a 2-fold dilution series, from 250 µg/mL to 0.0625 µg/mL. Cultures were prepared as previously described. Cell pellets were collected by centrifugation (3000 *g* for 10 minutes) and resuspended in 1 mL of fresh LSM to approx. 4.8 x $10^{10}$ CFU/mL. Plates containing antibiotics were spot inoculated with 5 µL of bacterial culture and incubated at 37 °C, anaerobically, for 48 h and 72 h for lactobacilli and bifidobacteria isolates, respectively. Plates were scored by the presence or absence of growth. The MIC value was determined as the concentration which supported no growth. Breakpoints were then compared to the EFSA recommended breakpoints. Where no EFSA guidelines were available for the tested antibiotic (neomycin, amoxicillin, oxacillin and penicillin G) breakpoints were assigned according to Ammor et al., (2007); Danielsen & Wind, (2003) and Felten et al., (1999). Experiments were performed in triplicate.

**Table 5.1.** Antibiotic agents and the dilutants used to produce antibiotic stocks of 20 mg /mL.

| Antibiotic Class | Antibiotic | Concentration (mg/mL) | Dilutant |
|---|---|---|---|
| Aminoglycoside | Kanamycin sulphate | 20 | Water |
| | Neomycin sulphate | 20 | |
| | Streptomycin sulphate | 20 | |
| | Gentamycin sulphate | 20 | |
| Beta-lactam | Ampicillin sodium salt | 20 | |
| | Amoxicillin | 20 | |
| | Oxacillin | 20 | |
| | Penicillin G | 20 | |
| Lincosamide | Clindamycin hydrochloride | 20 | |
| Glycopeptide | Vancomycin hydrochloride | 20 | |
| Macrolide | Erythromycin hydrate | 20 | 100 % EtOH |
| Tetracycline | Tetracycline | 20 | |
| Amphenicols | Chloramphenicol | 20 | |

### 5.2.3. The impact of physiological stresses on CUL isolates MIC profiles

To determine whether *in vitro* testing of antibiotic susceptibilities could be influenced by physiological stresses microorganisms would encounter within the host, a representative of each species from CUL isolates including *L.plantarum* CUL66, *L.casei* CUL06, *L.paracasei* CUL08, *L.rhamnosus* CUL63, *L.fermentum* CUL40, *L.helveticus* CUL76, *L.gasseri* CUL09 and *L.salivarius* CUL61 were selected to determine the impact bile would have on the subsequent MIC profile. Antibiotics that exhibited high resistance levels in initial MIC testing were selected including vancomycin, kanamycin and chloramphenicol. Broth micro dilutions were performed as previously described, with the following modifications: bovine bile (Oxgall, Sigma) was prepared in LSM broth to a concentration of 1.2 %. Following, 50 µL of the 1.2 % bile was seeded into a 96-well plate and diluted 1:4 with antibiotic and either (*L.paracasei* CUL08, *L.casei* CUL06, *L.rhamnosus* CUL63, *L.fermentum* CUL40, *L.helveticus* CUL76, *L.gasseri* CUL09, *L.salivarius* CUL61 and *L.plantarum* CUL66), generating a final bile concentration of 0.3 %. Final antibiotic concentrations in the wells ranged from 250 – 0.0625 µg/mL. CUL strains were prepared and diluted as previously described and 100 µL of culture was inoculated into each well. Controls for each strain tested included: a growth control (bacteria but no antibiotic nor bile), a second growth control (bacteria, no antibiotic and 0.3 % bile) and a negative, control (0.3 % bile), the latter was also used to correct for the effect bile had on the optical density of the cultures. Plates were incubated at 37 °C anaerobically for at least 24 h (or until visible growth was seen in the control well). Following incubation, MIC break points were determined visually, with plates also read at 600 nm spectrophotometrically Multiskan FC (Thermo Scientific). The MIC was defined as the lowest antibiotic concentration where no growth was seen in comparison to the growth control OD reading, subtracting the bile OD reading. Experiments were performed in triplicate. MIC values were compared to the breakpoint values assigned by EFSA (European Food Safety Authority, 2012).

### 5.2.4. Genetic characterization of ARGs

CUL genomes were sequenced and annotated by RAST (**Chapter 3, method 3.2.1**). Initially, a RAST subsystem analysis was conducted to putatively identify ARGs. Following identification, a more in-depth ARG analysis was performed per the protocol outlined by Campedelli et al., (2019). Briefly, all protein sequences of ARGs identified on the CARD database (https://card.mcmaster.ca/home) were retrieved and a local *de novo* BLASTp database was created using BLAST+ (version 1.3). The database generated consisted of 4807 ARG protein sequences. ARGs were identified using translated Coding Domain Sequences (CDS) from CUL isolates as queries for BLASTp. ARGs were identified as the top BLASTp hit with an amino acid sequence identity of > 30 % and query coverage of > 70 % as suggested by Campedelli *et al*., (2019). The data were manually curated to ensure single protein-encoding genes (PEGs) were not annotated as multiple ARGs. Following identification, ARGs were also manually annotated by querying the NCBI non-redundant (nr) protein database and the bacterial ABR reference gene database (last accessed in August 2019). A heatmap was reconstructed in R (Ihaka & Gentleman, 1996), to allow the visualization and comparison of the presence of ARGs in CUL strains, using the following packages: ggplots (Wickham, 2006), ggplots2 (Wickham, 2011) and Rcolorbrewer (Neuwirth & Neuwirth, 2011).

### 5.2.5. Phenotype to genotype correlation

The correlation between an ABR phenotype and the identification of an ARG related to specific antibiotic drug classes was determined and analyzed on a scale of 0 – 4 where: 0 = Sensitive strain, no ARG, 1 = Resistant, no ARG, 2 = Sensitive, ≥ 1 ARG, 3 = Resistant, ≥ 1 ARG, 4 = Resistance (not required by EFSA) ≥ 1 ARG. A heatmap was reconstructed in R (Ihaka & Gentleman, 1996) using the following packages: ggplots (Wickham, 2006), ggplots2 (Wickham, 2011) and Rcolorbrewer (Neuwirth & Neuwirth, 2011) to allow visualization of trends.

### 5.2.6. DDL protein gene alignment

Previous studies have shown that a single amino acid change in the active site of the DDL protein (261) results in vancomycin resistance in lactobacilli species (Campedelli et al., 2019). Indeed, Campedelli et al., (2019) showed that if phenylalanine (F) was present in the DDL protein, there was a correlation with vancomycin resistance, whereas when phenylalanine was replaced with tyrosine (Y), strains were sensitive. The D-alanine - D-alanine ligase amino acid FASTA sequence for *Lactobacillus acidophilus ATCC 700396 (*accession number Q5FMN6)

was used as a query to search CUL genomes via BLASTp. Top hit amino acid sequences were retrieved and aligned via CLUSTALW (Kumar et al., 2016; Thompson et al., 2007).

## 5.3. Results

### 5.3.1. Phenotypic evaluation of antibiotic susceptibilities in CUL strains

For initial phenotypic observations, guidelines set out by EFSA (EFSA, 2012), CLSI (CLSI, 2010) and ISO (ISO and IDF, 2010) were followed, however, bacterial concentrations were inoculated at a 5-fold higher (1:200) concentration than recommended (1:1000). This dilution was chosen to ensure consistent growth among replicates and to reduce the chance of false positives (i.e., observed no/or weak growth when the phenotype is resistant). In brief, a range of MICs was exhibited across all phylogroups for all antibiotics tested. Out of the 13 antibiotics, all CUL strains were sensitive to clindamycin and gentamycin (**Table 5.2**). Where breakpoints were described, all strains were sensitive to vancomycin (**Table 5.2** and **Figure 5.2**). For streptomycin and erythromycin, only one strain was resistant to each (*L.acidophilus* CUL21 and *L.acidophilus* CUL60 respectively). The majority of CUL isolates were sensitive to tetracycline, however *L.paracasei* CUL07, *L.acidophilus* CUL21, CUL60 and *B.animalis* subsp. *lactis* CUL34 displayed resistance profiles. Using breakpoints identified in the literature, 47 % (8) of strains exhibited resistance profiles to oxacillin. Despite no breakpoints being described for penicillin G, a wide range of sensitivities are documented (0.0625 - 32 µg/mL). Resistance was also identified in 59 % (10) of CUL strains when challenged with chloramphenicol, 59 % (10) against kanamycin, 71 % (12) for neomycin and 82 % (14) for ampicillin.

#### 5.3.1.1. *Kanamycin*

In the *L.casei* phylogroup susceptibility to kanamycin varied. *L.paracasei* strains (CUL37, CUL07 and CUL08) produced MIC values above the breakpoint set out by EFSA. However, in comparison, both *L.casei* (CUL06*)* and *L.rhamnosus (*CUL63*)* were both sensitive (**Table 5.2**).

Resistance to kanamycin was common in most lactobacilli strains tested, with *L.fermentum* (CUL40 and CUL67), *L.helveticus* (CUL76), *L.gasseri* (CUL09), *L.salivarius* (CUL61) and both *L.plantarum* strains (CUL66 and CUL66N) achieving breakpoints above the resistance cut-offs set out by EFSA. In contrast, *L.acidophilus* strains (CUL21, CUL60 and ATCC4356) were all sensitive to kanamycin (breakpoint < 64 µg/mL). With regards to the bifidobacteria CUL strains, there are currently no designated breakpoints for kanamycin resistance available. However*, B.animalis* subsp. *lactis* CUL34 and *B.bifidum* CUL20 were tolerant to kanamycin at concentrations > 32 µg/mL (**Table 5.2**).

### 5.3.1.2. *Gentamycin, streptomycin and neomycin*

For other aminoglycoside antibiotics such as gentamycin and streptomycin, all CUL isolates were sensitive, except for *L.acidophilus* CUL21, which produced a resistant phenotype to streptomycin, contrasting with the sensitive profile seen in *L.acidophilus* CUL60 (**Table 5.2**). For neomycin, EFSA does not provide guidelines for resistance and as such, previously reported cut-offs were used to deduce resistance profiles. Neomycin resistance was not consistent for the *Lactobacillus* CUL strains tested. For example, in the case of *L.paracasei*, CUL37 and CUL07 were resistant but CUL08 was sensitive. *L.fermentum* (CUL40, CUL67), *L.helveticus* (CUL76), *L.salivarius* (CUL61), *L.gasseri* (CUL09) and both *L.plantarum* strains (CUL66 and CUL66N) were resistant. *L.acidophilus* strains were predominantly sensitive (CUL21 and ATCC4356) but interestingly, CUL60 was resistant.

### 5.3.1.3. *Beta–lactams*

Strain-specific resistance was also seen when CUL isolates are challenged with a variety of beta-lactam antibiotics (**Table 5.2**). For example, when challenged with oxacillin, all members of the *L.casei* phylogroup were deemed resistant, however *L.paracasei* CUL08 was susceptible. The same strain variation is seen within the two *L.fermentum* strains, where CUL40 was shown to be sensitive to oxacillin and CUL67 was resistant and for the *L.acidophilus* isolates where CUL60 is resistant compared with CUL21 and ATCC4356 were sensitive. *L.plantarum* strains presented with resistant phenotypes to oxacillin. For amoxicillin, the majority of CUL isolates had MIC values classed as intermediate in terms of AMR in the literature. However, *L.paracasei* CUL37 and CUL07 and *L.rhamnosus* CUL63 were deemed as resistant and *L.acidophilus* CUL21 and ATCC4356 were classified as sensitive. Ampicillin was the only beta-lactam tested with EFSA breakpoint guidelines. The majority of *Lactobacillus* CUL strains were resistant. Of interest, *L.fermentum* CUL67 produced a sensitive profile in comparison to CUL40. Additionally, *L.salivarius* CUL61 was the only other *Lactobacillus* strain to exhibit sensitivity to ampicillin**.**

### 5.3.1.4. *Erythromycin*

All lactobacilli CUL strains were sensitive to erythromycin, except for *L.acidophilus* CUL60, which was resistant (**Table 5.2**).

### 5.3.1.5. *Vancomycin*

EFSA only describe/require vancomycin breakpoints for certain lactobacilli species (**Table 5.2**). Of the species where no breakpoint is described, high levels of tolerance are observed at > 32 µg/mL. For species where cut-offs are established, such *as L.acidophilus*, *L.helveticus* and *L.gasseri,* all CUL strains produced sensitive profiles, exhibiting no resistance to vancomycin. The sensitive strains (*L.acidophilus* CUL21, CUL60 and ATCC4356, *L.helveticus* CUL76 and *L.gasseri* CUL09) all encoded a tyrosine (Y+261) amino acid within their respective DDL proteins (**Table 5.2, Figure 5.2**)**.**

### 5.3.1.6. *Chloramphenicol*

Resistance to chloramphenicol was not consistent within phylogroups or even species. For example, within the *L.casei* phylogroup, *L.paracasei* strains were split between a sensitive (CUL07 & CUL08) and resistant phenotype (CUL37). Chloramphenicol resistance was additionally recorded in *L.fermentum* CUL40 and CUL67, *L.gasseri* CUL09, *L.acidophilus* CUL21 and CUL60 and *L.plantarum* strains CUL66 and CUL66N. Strains inhibited by chloramphenicol include *L.salivarius* CUL61, *L.helveticus* CUL76 and type strain *L.acidophilus* ATCC4356 (**Table 5.2**).

### 5.3.1.7. *Tetracycline*

Strain level resistance was also exhibited when strains were challenged with tetracycline, where only one out of three *L.paracasei* strains (CUL07) showed resistance and two out of the three *L.acidophilus* strains (CUL21 and CUL60) surviving in tetracycline concentrations that suggest resistance (**Table 5.2**).

### 5.3.1.8. *Clindamycin*

All lactobacilli strains were sensitive to clindamycin (**Table 5.2**).

### 5.3.1.9. *Bifidobacteria broth MICs*

Both bifidobacteria strains were sensitive to all antibiotics tested, except for *B.bifidum* CUL20, which displayed resistance to ampicillin and *B.animalis* subsp. *lactis* CUL34 presents a tetracycline-resistant phenotype (**Table 5.2**).

**Table 5.2. The antibiotic resistance profiles of CUL isolates when challenged with clinically relevant antibiotics: determined by micro-broth dilution.**

| Strain | Strain MIC (µg/mL) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aminoglycoside | | | | Beta-lactam | | | | Macrolide | Glycopeptide | Amphenicols | Tetracyclines | Lincosamide |
| | Kanamycin | Gentamycin | Streptomycin | Neomycin | Oxacillin | Amoxicillin | Ampicillin | Penicillin G | Erythromycin | Vancomycin | Chloramphenicol | Tetracycline | Clindamycin |
| *L.paracasei* CUL37 | 125 R | 16 S | 32 S | > 32 R[a] | 32 R[b] | 16 R[bc] | 8 R | 1 * | 0.125 S | >32 * F | 8 R | 1 S | 0.0625 S |
| *L.paracasei* CUL07 | 125 R | 16 S | 32 S | > 32 R[a] | > 32 R[a] | 16 R[bc] | 32 R | 4 * | 0.125 S | >32 * F | 4 S | 16 R | 0.0625 S |
| *L.paracasei* CUL08 | 125 R | 8 S | 32 S | 8 S[a] | 4 S[b] | 8 I[bc] | 8 R | 0.5 * | 0.0625 S | >32 * F | 4 S | 2 S | 0.125 S |
| *L.casei* CUL06 | 62.5 S | 2 S | 16 S | > 32 R[a] | >32 R[b] | 8 I[bc] | > 32 R | 8 * | 0.5 S | >32 * F | 16 R | 1 S | 1 S |
| *L.rhamnosus* CUL63 | 62.5 S | 8 S | 16 S | > 32 R[a] | >32 R[b] | 16 R[bc] | > 32 R | > 32 * | 0.25 S | >32 * F | 16 R | 2 S | 0.25 S |
| *L.fermentum* CUL40 | 125 R | 8 S | 32 S | > 32 R[a] | 8 S[b] | 4 I[bc] | 1 S | 0.25 * | 0.125 S | >32 * F | 8 R | 4 S | 0.0625 S |
| *L.fermentum* CUL67 | 62.5 R | 2 S | 16 S | > 32 R[a] | 16 R[b] | 4 I[bc] | 4 R | 2 * | 0.25 S | >32 * F | >32 R | 8 S | 0.25 S |
| *L.helveticus* CUL76 | 62.5 R | 4 S | 2 S | > 32 R[a] | 1 S[b] | 4 I[bc] | 2 R | 1 * | 0.0625 S | 1 S Y | 4 S | 2 S | 0.25 S |
| *L.gasseri* CUL09 | 125 R | 8 S | 16 S | 32 R[a] | 2 S[b] | 4 I[bc] | 4 R | 0.25 * | 0.0625 S | 2 S Y | 4 R | 4 S | 1 S |
| *L.salivarius* CUL61 | 250 R | 4 S | 8 S | > 32 R[a] | 4 S[b] | 4 I[bc] | 4 S | 0.5 * | 0.0625 S | >32 * F | 4 S | 1 S | 0.125 S |
| *L .acidophilus* CUL21 | 32 S | 16 S | > 32 R | 16 S[a] | 8 S[b] | 4 I[bc] | 4 R | 0.5 * | 0.125 S | 2 S Y | 8 R | 16 R | 0.5 S |
| *L.acidophilus* CUL60 | 32 S | 4 S | 8 S | > 32 R[a] | 32 R[b] | 0.5 S[bc] | 4 R | 0.5 * | 2 R | 1 S Y | 8 R | 16 R | 1 S |
| *L.acidophilus* ATCC4356 | 8 S | 2 S | 4 S | 4 S[a] | 0.5 S[b] | 1 S[bc] | 4 R | < 0.625 * | < 0.625 S | 1 S Y | 4 S | 2 S | 1 S |
| *L.plantarum* CUL66 | 125 R | 4 S | 32 * | > 32 R[a] | > 32 R[b] | 8 I[bc] | 8 R | 8 * | 0.5 S | >32 * F | 16 R | 32 S | 2 S |
| *L.plantarum* CUL66N | 125 R | 8 S | > 32 * | > 32 R[a] | > 32 R[b] | 8 I[bc] | > 32 R | 32 * | 0.5 S | >32 * F | 16 R | 32 S | 0.25 S |
| *B.bifidum* CUL20 | > 32 * | 8 S | 16 S | 8 * | 4 * | 8 * | 8 R | 0.5 * | 0.125 S | 1 S | 0.5 S | 2 S | 0.5 S |
| *B.animalis* CUL34 | > 32 * | 16 S | 1 S | 8 * | 2 * | 2 * | 1 S | 0.125 * | 0.125 S | 1 S | 2 S | 32 R | 0.0625 S |

Resistance was designated per the epidemiological cut-off values outlined by EFSA (EFSA, 2012). Resistant strains with MIC values higher than the designated cut-offs are highlighted in grey and sensitive strains are highlighted in green. Strains which produce intermediate values are shaded in blue and antibiotics without described breakpoints are shaded in pink. Resistant, intermediate and sensitive strains are also accompanied by R, I and S respectively. Antibiotics are grouped into their drug classes as indicated. * = Not required by EFSA a. Ammor, M.S., Flórez, A.B. and Mayo, B., (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology, 24(6), pp.559-570. B. Danielsen, M. and Wind, A. (2003) Susceptibility of *Lactobacillus* spp. To antimicrobial agents. International Journal of Food Microbiology. 82(1): 1-11c. Felten, A., Barrau, C., Bizet, C., Lagrange, P.H., Philippon, A., (1999). *Lactobacillus* species identification, H2O2 production and antibiotic resistance and correlation with human clinical status. J. Clin. Microbiol. 37, 729–733.

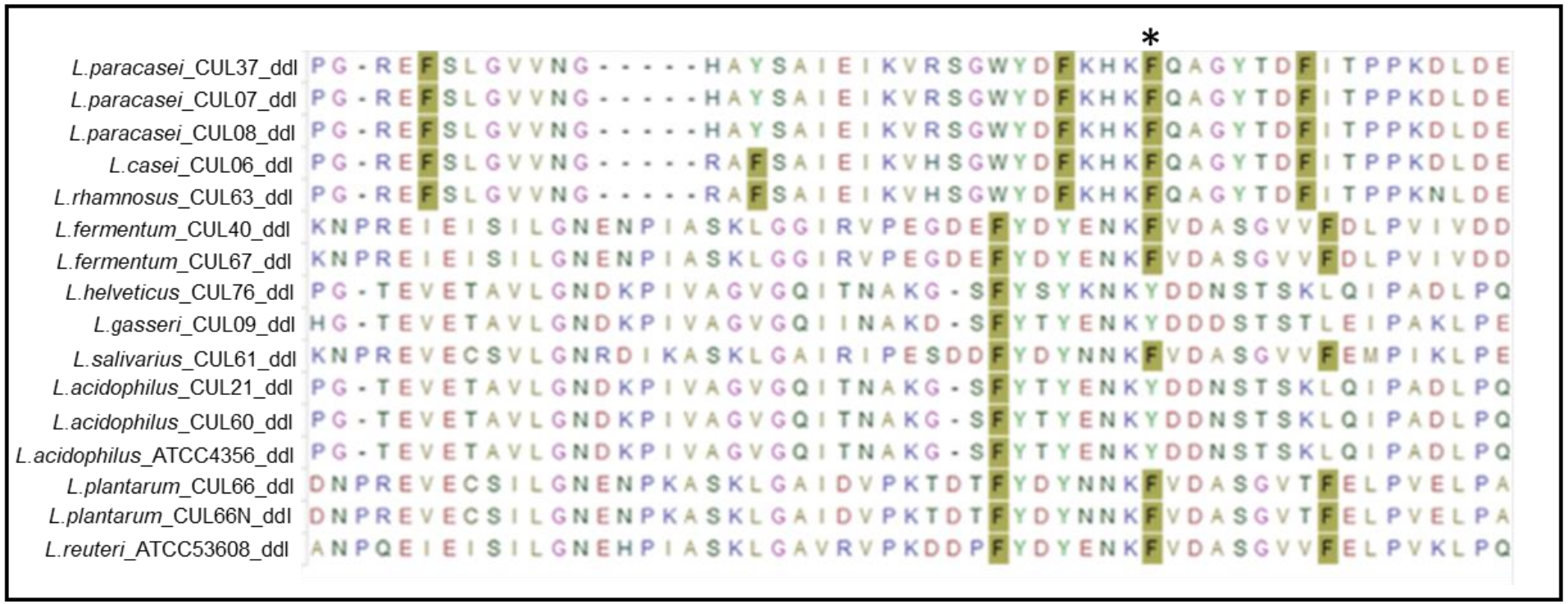



**Figure 5.2. Amino acid substitution in the DDL protein, putatively conferring vancomycin tolerance**. The amino acid sequences for the d-ala-d-ala protein were retrieved via BLASTp identification in CUL isolates. Sequences were aligned via clustalW and the identification of an amino acid substitution in the active site of the enzyme was analysed (position F-261-Y marked with an *).

### 5.3.2. The effect bacterial dose has on the ABR profiles of CUL isolates

The determination of an accurate MIC can be affected by several experimental parameters including, the medium used for testing, incubation time and bacterial concentration. Therefore, agar dilution with a maximal cellular concentration (defined as a concentrated overnight culture) was selected to determine the utmost AMR profile CUL isolates could produce (**Table 5.3**).

#### 5.3.2.1. *Aminoglycosides*

The *L.casei* phylogroup exhibited high levels of resistance to aminoglycoside antibiotics, with all members displaying resistance profiles when challenged with kanamycin, streptomycin and neomycin (**Table 5.3**). Interestingly, two *L.paracasei* strains CUL37 and CUL08 were sensitive to gentamycin, whereas the third *L.paracasei* strain CUL07 was resistant, in addition to *L.acidophilus* CUL06 and *L.rhamnosus* CUL63. Both *L.fermentum* strains were resistant to kanamycin and neomycin, however, of interest was that CUL67 was sensitive to gentamycin and streptomycin whereas CUL40 was resistant. Similarly, *L.gasseri* CUL09, *L.salivarius* CUL61 and *L.helveticus* CUL76 also showed resistance profiles to all aminoglycosides except for CUL09, which displayed sensitivity to gentamycin. *L.acidophilus* strains were both resistant to all aminoglycosides tested, except for CUL21, which interestingly exhibits a much lower MIC value in comparison to CUL60 (0.5 µg/mL and 125 µg/mL respectively).

Resistance to aminoglycosides was also the case for *L.plantarum* strains (CUL66 and CUL66N), where breakpoints are described. In contrast, bifidobacteria isolates were sensitive to all aminoglycosides tested (**Table 5.3**).

#### 5.3.2.2. *Beta-lactams*

CUL strains produced variable MIC profiles when challenged by a variety of beta-lactam antibiotics (**Table 5.3**).

**(I)** ***Oxacillin***

In general, most strains were susceptible to oxacillin, except for *L.paracasei* CUL37 (whereas CUL08 and CUL07 were both sensitive), *L.casei* CUL06, *L.rhamnosus* CUL63 and both *L.plantarum* strains (CUL66, CUL66N), which presented a resistant phenotype (**Table 5.3**).

### (II) *Amoxicillin*

All *Lactobacillus* strains displayed either a resistant or intermediate phenotype to amoxicillin, except for *L.acidophilus* CUL60, whose MIC breakpoint (0.5 µg/mL), was classified as sensitive. Of interest, is *L.acidophilus* CUL21's MIC breakpoint of 16 µg/mL, which is significantly higher than *L.acidophilus* CUL60 and designated as resistant (**Table 5.3**).

### (III) *Ampicillin*

All bifidobacteria and lactobacilli strains presented a resistant phenotype to ampicillin with the sole exception of *L.fermentum* CUL40 which was sensitive (**Table 5.3**).

### (IV) *Penicillin* G

A wide range of MIC values was reported when CUL isolates were challenged with penicillin. despite no breakpoints being described for this antibiotic, high levels of resistance (which exceeded 250 µg/mL), were documented for strains such as *L.acidophilus* CUL21 and *L.plantarum* CUL66N (**Table 5.3**). *L.acidophilus* CUL60 was extremely sensitive to penicillin G, with an inhibition value of 0.5 µg/mL. The variation in MIC values within the *L.casei* group is also noteworthy, ranging from 0.5 µg/mL (*L.paracasei* CUL37 and CUL08) to 125 µg/mL (*L.rhamnosus* CUL63).

## 5.3.2.3. *Macrolides*

Bifidobacteria strains were both sensitive to erythromycin with MIC breakpoints below 1 µg/mL (**Table 5.3**). The majority of *Lactobacillus* CUL isolates presented with extremely high MIC values (>250 µg/mL), achieving resistance status to erythromycin (**Table 5.3**). However, *L.paracasei* CUL08 was sensitive to low concentrations of erythromycin, with an MIC value of 1 µg/mL.

## 5.3.2.4. *Glycopeptide*

High MIC values were reported for all strains challenged with vancomycin (16 – 250 µg/mL). Where MIC breakpoints are described, all strains are classified as resistant (*L.acidophilus* CUL21 and CUL60, *L.gasseri* CUL09, *L.helveticus* CUL76, *B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34). There is an interestingly large variation in MIC profiles when comparing *L.acidophilus* CUL21 and CUL60 (16 µg/mL and 250 µg/mL respectively (**Table 5.3**)).

#### 5.3.2.5. *Amphenicols*

All CUL isolates were resistant to chloramphenicol, with the exception of *L.paracasei* CUL08 and *B.bifidum* CUL20, which were both classified as sensitive (**Table 5.3**).

#### 5.3.2.6. *Tetracycline*

A wide range of MIC values was produced for CUL isolates when challenged with tetracycline (from 8 µg/mL to 250 µg/mL). However, all strains were classified as resistant (**Table 5.3**), except for *B.bifidum* CUL20, which had an MIC value below the cutoff (8 µg/mL).

#### 5.3.2.7. *Lincosides*

A range of MIC values is reported when strains were challenged with clindamycin (**Table 5.3**). For members of the *L.casei* clade, the majority presented a resistant phenotype. However, *L.paracasei* CUL08 falls under the breakpoint and is deemed sensitive. The variation in MICs seen for *L.paracasei* strains is noteworthy, with CUL37 and CUL07 producing MIC values of 64 µg/mL and 32 µg/mL respectively. Whereas CUL08 was inhibited by clindamycin at concentrations of 0.125 µg/mL. Both *L.plantarum* strains (CUL66 and CUL66N) presented resistant phenotypes (both with MIC values of 16 µg/mL) in addition to *L.gasseri* CUL09 (250 µg/mL) and *B.bifidum* (8 µg/mL). the remaining CUL strains were classified as sensitive.

**Table 5.3. The antibiotic resistance profiles of CUL isolates when challenged with clinically relevant antibiotics: determined by agar dilution**.

| Strain | Strain MIC (µg/mL) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aminoglycoside | | | | Beta-lactam | | | | Macrolide | Glycopeptide | Amphenicols | Tetracyclines | Lincosamide |
| | Kanamycin | Gentamycin | Streptomycin | Neomycin | Oxacillin | Amoxicillin | Ampicillin | Penicillin G | Erythromycin | Vancomycin | Chloramphenicol | Tetracycline | Clindamycin |
| *L.paracasei* CUL37 | >250 R | 32 S | >250 R | > 250 R[a] | > 250 R[b] | 125 R[bc] | 8 R | 0.5 * | >250 R | > 250 * | 16 R | >250 R | 32 R |
| *L.paracasei* CUL07 | >250 R | 62.5 R | >250 R | > 250 R[a] | 16 S[b] | 32 R[bc] | 32 R | 4 * | >250 R | > 250 * | 32 R | 16 R | 62.5 R |
| *L.paracasei* CUL08 | >250 R | 16 S | >250 R | 125 R[a] | 8 S[b] | 8 I[bc] | 8 R | 0.5 * | 1 S | > 250 * | 4 S | 16 R | 0.125 S |
| *L.casei* CUL06 | >250 R | 250 R | >250 R | 125 R[a] | > 250 R[b] | 32 R[bc] | 125 R | 8 * | >250 R | > 250 * | 16 R | >125 R | 250 R |
| *L.rhamnosus* CUL63 | >250 R | 62.5 R | >250 R | 125 R[a] | > 250 R[b] | 32 R[bc] | 125 R | 125 * | >250 R | > 250 * | 16 R | >125 R | >250 R |
| *L.fermentum* CUL40 | >250 R | 32 R | 125 R | 32 R[a] | 8 S[b] | 8 I[bc] | 2 S | 0.5 * | >250 R | > 250 * | 16 R | >250 R | 0.0625 S |
| *L.fermentum* CUL67 | >250 R | 4 S | 16 S | > 250 R[a] | 8 S[b] | 8 I[bc] | 4 R | 4 * | >250 R | > 250 * | 125 R | >125 R | 0.5 S |
| *L.helveticus* CUL76 | >250 R | 32 R | >250 R | 125 R[a] | 1 S[b] | 8 I[bc] | 4 R | 1 * | >250 R | >250 R | 16 R | 16 R | 0.25 S |
| *L.gasseri* CUL09 | >250 R | 16 S | 62.5 R | > 250 R[a] | 8 S[b] | 32 R[bc] | 4 R | 1 * | >250 R | >250 R | 16 R | 16 R | 250 R |
| *L.salivarius* CUL61 | >250 R | 250 R | 250 R | >250 R[a] | 16 S[b] | 8 I[bc] | 8 R | 4 * | >250 R | > 250 * | 8 R | 16 R | 0.125 S |
| *L .acidophilus* CUL21 | >250 R | 32 R | >250 R | 0.5 S[a] | 16 S[b] | 16 R[bc] | 32 R | > 250 * | >250 R | 16 R | 16 R | 8 R | 0.125 S |
| *L.acidophilus* CUL60 | >250 R | 62.5 R | 125 R | 125 R[a] | 8 S[b] | 0.5 S[bc] | 8 R | 0.5 * | >250 R | >250 R | 16 R | 32 R | 0.5 S |
| *L.plantarum* CUL66 | >250 R | 32 R | > 250 * | >250 R[a] | > 250 R[b] | 250 R[bc] | 8 R | 8 * | >250 R | > 250 * | 16 R | >125 R | 16 R |
| *L.plantarum* CUL66N | >250 R | >250 R | > 250 * | >250 R[a] | > 250 R[b] | 250 R[bc] | 4 R | >250 * | >250 R | > 250 * | 32 R | >125 R | 16 R |
| *B.bifidum* CUL20 | 125 * | 64 S | 62.5 S | 125 * | 8 * | 8 * | 8 R | 1 * | 0.125 S | > 250 R | 2 S | 8 S | 8 R |
| *B.animalis* CUL34 | 250 * | 64 S | 62.5 S | 125 * | 16 * | 8 * | 4 R | 0.5 * | 0.5 S | > 250 R | 8 R | 125 R | 0.5 S |

Resistance was designated in accordance with the epidemiological cut-off values provided by EFSA (EFSA, 2012) and is represented by the following: Resistant strains with MIC values higher than the designated cut-off are highlighted in grey and sensitive strains are highlighted in green. Strains which produce intermediate values are shaded in blue and antibiotics without described breakpoints are shaded in pink. Resistant, intermediate and sensitive strains are also accompanied by an R, I and S respectively. Antibiotics are grouped into their drug classes as indicated.* = Not required by EFSA a. Ammor, M.S., Flórez, A.B. and Mayo, B., (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology, 24(6), pp.559-570. b. Danielsen, M. and Wind, A. (2003) Susceptibility of *Lactobacillus* spp. To antimicrobial agents. International Journal of Food Microbiology. 82(1): 1-11c. Felten, A., Barrau, C., Bizet, C., Lagrange, P.H., Philippon, A., (1999). *Lactobacillus* species identification, H2O2 production and antibiotic resistance and correlation with human clinical status. J. Clin. Microbiol. 37, 729–733.

### 5.3.3. A methodology comparison

When comparing methodology, using broth or agar dilution can generate a variation in results due to the nature of the assays. In addition, the concentration difference can also impact the identified MIC. Therefore, to deduce the minimum to maximum resistance profile of CUL isolates, a comparison of resistance profiles utilising two methods was conducted (**Table 5.4**).

#### 5.3.3.1. *Kanamycin*

In general, the resistance profiles of CUL isolates when challenged with kanamycin were consistent when comparing results generated by broth microdilution (BMD) and high inoculum agar dilution (AD (**Table 5.4**)). Indeed, 10 strains both exhibited resistant profiles when challenged with kanamycin in broth and on agar. However, method dependant variations are seen in the *L.casei* phylogroup where *L.casei* and *L.rhamnosus* present sensitive profiles when tested using the micro broth dilution method (62.5 µg/mL) but were deemed resistant using the agar dilution method (>250 µg/mL). Such variations were also demonstrated in the *L.acidophilus* strains, CUL21 and CUL60, where the resistance profile increased from 32 µg/mL (micro-broth) to >250 µg/mL (agar), highlighting the phenotype variations that can occur.

#### 5.3.3.2. *Gentamycin*

The majority of CUL strains saw an increased MIC value arise when utilising AD in comparison to BMD (10 out 17). Interestingly, only one of the three *L.paracasei* strains developed a resistant profile on agar (*L.paracasei* CUL07, increased from 16 – 62.5 µg/mL). Similarly, one of the two *L.fermentum* strains, CUL40 also developed a resistant phenotype on agar in comparison to in broth (increasing from 8 – 32 µg/mL). Bifidobacteria strains saw an increase in MIC value when tested by AD, however, they both maintained their sensitive designation (**Table 5.4**).

#### 5.3.3.3. *Streptomycin*

Most of the *Lactobacillus* strains saw an increase in their MIC values when challenged via antibiotic agar dilution in comparison to broth microdilution (**Table 5.4**). Indeed, MICs increased from a minimum of 2-fold to a maximum of 7-fold when tested on agar as opposed to in broth. *L.fermentum* CUL67 was the only *Lactobacillus* strain to maintain a consistent MIC value (16 µg/mL) across both methods, in comparison to CUL40 where the MIC increased from 32 µg/mL in BMD to 125 µg/mL in AD. Despite large increases in the MIC value between methods for both *Bifidobacterium* strains (8 – 62.5 µg/mL) both maintained a sensitive profile.

#### 5.3.3.4. *Neomycin*

Variation in testing methodology appears to have minimal influence when testing CUL isolates against neomycin, where strains appear to produce consistent resistance profiles (**Table 5.4**). *L.paracasei* CUL08 however, is an exception to this trend, where its MIC increases from 8 – 125 µg/mL (BMD – AD) changing its designation from sensitive to resistant when utilising AD. Of particular interest is *L.acidophilus* CUL21's response to neomycin when challenged via the two methods. Despite the increased cell density starting volume when testing via AD, the MIC decreases on agar compared to in broth (from 16 µg/mL – 0.5 µg/mL). For *B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34, there are currently no breakpoints described for assigning resistance to neomycin. However, the MIC is raised 4-fold when tested on AD in comparison to BMD.

#### 5.3.3.5. *Oxacillin*

MIC profiles for CUL isolates show similar trends to that seen when challenged with neomycin (**Table 5.4**). Oxacillin appears to maintain consistent AMR profiles when assigning resistance and sensitivity to strains when tested via two different methodologies. However, *L.paracasei* CUL07, *L.fermentum* CUL67 and *L.acidophilus* CUL60 saw a reduction in AMR when tested via AD (32 – 16 µg/mL, 16-8 µg/mL, 32 – 8 µg/mL respectively), reducing their resistant designation under BMD, to sensitive.

#### 5.3.3.6. *Amoxicillin*

AD and BMD appear to provide similar profiles when assigning levels of resistance in CUL isolates. Four strains, however, do have a variation in profile, including *L.gasseri* CUL09. where resistance increased from 4 µg/mL (intermediate) in BMD to 32 µg/mL resistant in AD. *L.acidophilus* CUL21 saw a similar increase from intermediate to resistant when testing on AD (4-16 µg/mL). In addition, both *L.plantarum* strains CUL66 and CUL66N, also transitioned from intermediate resistance profiles to complete resistance when challenged with amoxicillin in solid media (8 - 250 µg/mL).

### 5.3.3.7. *Ampicillin*

When testing for ampicillin resistance, whether CUL strains are challenged in liquid or solid media seems to make minimal difference to the resistance profile generated. All isolates except for *B.animalis* subsp. *lactis* CUL34 produced the same resistance profile across both methodologies. CUL34 however, did transition from a sensitive (1 µg/mL) to a resistant (4 µg/mL) profile when switching from broth to agar, respectively (**Table 5.4**)**.**

### 5.3.3.8. *Penicillin G*

There are no breakpoints reported for penicillin G and a variation of MICs is reported for all CUL strains across both testing conditions (**Table 5.4**). In certain examples, MIC values remain similar in broth and in agar (for example within the *L.casei* phylogroup). However dramatic increases in MIC values are seen when moving from broth to agar, for example, *L.acidophilus* CUL21 was inhibited by penicillin G at 0.5 µg/mL in broth, which increased to 250 µg/mL on agar.

### 5.3.3.9. *Erythromycin*

Perhaps one of the antibiotics most heavily affected by method choice is erythromycin. When challenged with erythromycin, CUL strains produced significantly higher MIC values on agar than they did in broth, in some cases over 10-fold higher (for example *L.salivarius* CUL61, which increased from 0.625 µg/mL in broth to >250µg/mL on agar (**Table 5.4**)). The only exception within the *Lactobacillus* CUL strains was *L.paracasei* CUL08, which despite an increased MIC value on agar (from 0.0625 µg/mL to 1 µg/mL) maintained its sensitive designation, in comparison to the other *L.paracasei* strains (CUL07 and CUL37), which both produced MIC values > 250µg/mL via AD. Interestingly, the two *Bifidobacterium* strains (CUL20 and CUL34) both increased their MIC value when challenged with erythromycin via AD, however, they both maintained their sensitive status.

### 5.3.3.10. *Vancomycin*

*Lactobacillus* CUL strains typically had high vancomycin MIC values regardless of methodology (**Table 5.4**). However, for the strains where breakpoints are described (*L.helveticus* CUL76, *L.gasseri* CUL09, *L.acidophilus* CUL21 and CUL60, *B.bifidum* CUL20 and *B.animalis* subsp. *lactis* CUL34) resistant profiles shifted from sensitive to resistant when using AD, with MIC's increasing from 1 – 250 µg/mL in some cases (*L.helveticus* CUL76, *B.bifidum* CUL20, *B.animalis* subsp. *lactis* CUL34 and *L.acidophilus* CUL60).

#### 5.3.3.11. *Chloramphenicol*

The impact on methodology is less obvious for chloramphenicol, where the majority of resistance profiles remained consistent regardless of culture strategy (**Table 5.4**). However, some strains from a variety of species present as outliers to this trend, increasing from a sensitive designation in broth to a resistant strain when challenged on agar, including *L.paracasei* CUL07 (4 - 32 µg/mL), *L.helveticus* CUL76 (4 - 16 µg/mL), *L.salivarius* CUL61 (4 - 8 µg/mL) *B.bifidum* CUL20 (2 - 8 µg/mL).

#### 5.3.3.12. *Tetracycline*

The majority of CUL strains subjected to MIC testing against tetracycline exhibited a shift from sensitive to resistant when comparing broth and agar methodologies (**Table 5.4**). Strains identified as resistant via BMD maintained that designation when subjected to AD testing (*L.paracasei* CUL07, *L.acidophilus* CUL21, CUL60 and *B.animalis* subsp. *lactis* CUL34). Of interest is that *B.bifidum* CUL20 maintained a sensitive profile to tetracycline regardless of the methodology selected.

#### 5.3.3.13. *Clindamycin*

CUL strains presented a variety of sensitive and resistant phenotypes utilising both methods when challenged with clindamycin (**Table 5.4**). Eight strains transitioned from a sensitive profile in broth to resistant in agar including: *L.paracasei* CUL37 (0.0625 – 32 µg/mL), *L.paracasei* CUL07 (0.0625 – 62.5 µg/mL), *L.casei* CUL06 (1 – 250 µg/mL), *L.rhamnosus* CUL63 (0.25 - 250 µg/mL), *L.gasseri* CUL09 (1 – 250 µg/mL), *L.plantarum* CUL66 (2 - 16 µg/mL), *L.plantarum* CUL66N (0.25 – 16 µg/mL) and *B.bifidum* CUL20 (0.5 – 8 µg/mL). The remaining eight isolates maintained a sensitivity to clindamycin across both methods.

## Table 5.4. Method comparison when designating antimicrobial resistance profiles.

| Drug class | MIC method | Strain MIC (µg/mL) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***L.paracasei*** **CUL37** | ***L.paracasei*** **CUL07** | ***L.paracasei*** **CUL08** | ***L.casei*** **CUL06** | ***L.rhamnosus*** **CUL63** | ***L.fermentum*** **CUL40** | ***L.fermentum*** **CUL67** | ***L.helveticus*** **CUL76** | ***L.gasseri*** **CUL09** | ***L.salivarius*** **CUL61** | ***L .acidophilus*** **CUL21** | ***L.acidophilus*** **CUL60** | ***L.plantarum*** **CUL66** | ***L.plantarum*** **CUL66N** | ***B.bifidum*** **CUL20** | ***B.animalis*** **CUL34** |
| Aminoglycoside | Kanamycin | 125 R | 125 R | 125 R | 62.5 S | 62.5 S | 125 R | 62.5 R | 62.5 R | 125 R | 250 R | 32 S | 32 S | 125 R | 125 R | > 32 * | > 32 * |
| | Kanamycin Agar | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | >250 R | > 250 R | > 250 R | 125 * | > 250 * |
| | EFSA | 64 | 64 | 64 | 64 | 64 | 32 | 32 | 16 | 16 | 16 | 64 | 64 | 64 | 64 | nr | nr |
| | Gentamycin | 16 S | 16 S | 8 S | 2 S | 8 S | 8 S | 2 S | 4 S | 8 S | 4 S | 16 S | 4 S | 4 S | 8 S | 8 S | 16 S |
| | Gentamycin Agar | 32 S | 62.5 R | 16 S | 250 R | 62.5 R | 32 R | 4 S | 32 R | 16 S | 250 R | 32 R | 62.5 R | 32 R | > 250 R | 64 S | 64 S |
| | EFSA | 32 | 32 | 32 | 32 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 64 | 64 |
| | Streptomycin | 32 S | 32 S | 32 S | 16 S | 16 S | 32 S | 16 S | 2 S | 16 S | 8 S | > 32 R | 8 S | 32 * | > 32 * | 16 S | 1 S |
| | Streptomycin Agar | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | 125 R | 16 S | > 250 R | 62.5 R | 250 R | > 250 R | 125 R | > 250 * | > 250 * | 62.5 S | 62.5 S |
| | EFSA | 64 | 64 | 64 | 64 | 32 | 64 | 64 | 16 | 16 | 16 | 16 | 16 | nr | nr | 128 | 128 |
| | Neomycin | > 32 R[a] | > 32 R[a] | 8 S[a] | > 32 R[a] | > 32 R[a] | > 32 R[a] | > 32 R[a] | > 32 R[a] | 32 R[a] | >32 R[a] | 16 S[a] | > 32 R[a] | >32 R[a] | >32 R[a] | 8 * | 8 * |
| | Neomycin agar | > 250 R[a] | > 250 R[a] | 125 R[a] | 125 R[a] | 125 R[a] | 32 R[a] | > 250 R[a] | 125 R[a] | > 250 R[a] | >250 R[a] | 0.5 S[a] | 125 R[a] | >250 R[a] | >250 R[a] | 125 * | 125 * |
| | EFSA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Beta-lactam | Oxacillin | 32 R[b] | > 32 R[b] | 4 S[b] | > 32 R[b] | > 32 R[b] | 8 S[b] | 16 R[b] | 1 S[b] | 1 S[b] | 4 S[b] | 8 S[b] | 32 R[b] | >32 R[b] | >32 R[b] | 4 * | 2 * |
| | Oxacillin Agar | > 250 R[b] | 16 S[b] | 8 S[b] | > 250 R[b] | > 250 R[b] | 8 S[b] | 8 S[b] | 1 S[b] | 8 S[b] | 16 S[b] | 16 S[b] | 8 S[b] | >250 R[b] | >250 R[b] | 8 * | 16 * |
| | EFSA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | Amoxicillin | 16 R[bc] | 16 R[bc] | 8 I[bc] | 8 I[bc] | 16 R[bc] | 4 I[bc] | 4 I[bc] | 4 I[bc] | 4 I[bc] | 4 I[bc] | 4 I[bc] | 0.5 S[bc] | 8 I[bc] | 8 I[bc] | 8 * | 2 * |
| | Amoxicillin agar | 125 R[bc] | 32 R[bc] | 8 I[bc] | 32 R[bc] | 32 R[bc] | 8 I[bc] | 8 I[bc] | 8 I[bc] | 32 R[bc] | 8 I[bc] | 16 R[bc] | 0.5 S[bc] | 250 R | 250 R | 8 * | 8 * |
| | EFSA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ampicillin | 8 R[b] | 32 R[b] | 8 R[b] | >32 R[b] | >32 R[b] | 1 S | 4 R | 2 R | 4 R | 4 R | 4 R | 4 R | 8 R | > 32 R | 8 R | 1 S |
| | Ampicillin Agar | 8 R[b] | 32 R[b] | 8 R[b] | 125 R[b] | 125 R[b] | 2 S | 4 R | 4 R | 4 R | 8 R | 32 R | 8 R | 8 R | 4 R | 8 R | 4 R |
| | EFSA | 4 | 4 | 4 | 4 | 4 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 |
| | Penicillin G | 1 * | 4 * | 0.5 * | 8 * | > 32 * | 0.25 * | 2 * | 1 * | 0.25 * | 0.5 * | 0.5 * | 0.5 * | 8 * | 32 * | 0.5 * | 0.125 * |
| | Penicillin G Agar | 0.5 | 4 * | 0.5 * | 8 * | 125 * | 0.5 * | 4 * | 1 * | 1 * | 4 * | > 250 * | 0.5 * | 8 * | > 250 * | 1 * | 0.5 * |
| | EFSA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Macrolide | Erythromycin | 0.125 S | 0.125 S | 0.0625 S | 0.5 S | 0.25 S | 0.125 S | 0.25 S | 0.0625 S | 0.0625 S | 0.0625 S | 0.125 S | 2 R | 0.5 S | 0.5 S | 0.125 S | 0.125 S |
| | Erythromycin Agar | > 250 R | > 250 R | 1 S | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | > 250 R | >250 R | >250 R | 0.125 S | 0.5 S |
| | EFSA | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Glycopeptide | Vancomycin | > 32 * F | > 32 * F | > 32 * F | > 32 * F | > 32 * F | > 32 * F | > 32 * F | 1 S Y | 2 S Y | >32 R F | 2 Y S | 1 Y S | > 32 * F | > 32 * F | 1 S | 1 S |
| | Vancomycin Agar | > 250 * | > 250 * | > 250 * | > 250 * | > 250 * | > 250 * | > 250 * | > 250 R | > 250 R | > 250 R F | 16 R | > 250 R | > 250 * | > 250 * | > 250 R | > 250 R |
| | EFSA | nr | nr | nr | nr | nr | nr | nr | 2 | 2 | 2 | 2 | 2 | nr | nr | 2 | 2 |
| Amphenicols | Chloramphenicol | 8 R | 4 S | 4 S | 16 R | 16 R | 8 R | > 32 R | 4 S | 4 R | 4 S | 8 R | 8 R | 16 R | 16 R | 0.5 S | 2 S |
| | Chloramphenicol Agar | 16 R | 32 R | 4 S | 16 R | 16 R | 16 R | 125 R | 16 R | 16 R | 8 R | 16 R | 16 R | 16 R | 32 R | 2 S | 8 R |
| | EFSA | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 8 | 8 | 4 | 4 |
| Tetracyclines | Tetracycline | 1 S | 16 R | 2 S | 1 S | 2 S | 4 S | 8 S | 2 S | 4 S | 1 S | 16 R | 16 R | 32 S | 32 S | 2 S | 32 R |
| | Tetracycline Agar | >250 R | 16 R | 16 R | > 125 R | > 125 R | > 250 R | > 125 R | 16 R | 16 R | 16 R | 8 R | 32 R | >125 R | >125 R | 8 S | 125 R |
| | EFSA | 4 | 4 | 4 | 4 | 8 | 8 | 8 | 4 | 4 | 4 | 4 | 4 | 32 | 32 | 8 | 8 |
| Lincosamide | Clindamycin | 0.0625 S | 0.0625 S | 0.125 S | 1 S | 0.25 S | 0.0625 S | 0.25 S | 0.25 S | 1 S | 0.125 S | 0.5 S | 1 S | 2 S | 0.25 S | 0.5 S | 0.0625 S |
| | Clindamycin Agar | 32 R | 62.5 R | 0.125 S | 250 R | >250 R | 0.0625 S | 0.5 S | 0.25 S | 250 R | 0.125 S | 0.125 S | 0.5 S | 16 R | 16 R | 8 R | 0.5 S |
| | EFSA | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 |

Summary of broth and agar MIC values and respective resistance designations in comparison to EFSA's MIC cut-offs (indicated in yellow). Resistant, intermediate and sensitive strains are also accompanied by an R, I and S respectively. Antibiotics are grouped into their drug classes as indicated. When methods both produce a resistance profile is indicated in grey. When both produce a sensitive profile or breakpoints are not described is highlighted in white. Red depicts a variation of resistance profiles between methods. * = Not required by EFSA a. Ammor, M.S., Flórez, A.B. and Mayo, B., (2007). ABR in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology, 24(6), pp.559-570. b. Danielsen, M. and Wind, A. (2003) Susceptibility of *Lactobacillus* spp. To antimicrobial agents. International Journal of Food Microbiology. 82(1): 1-11c. Felten, A., Barrau, C., Bizet, C., Lagrange, P.H., Philippon, A., (1999). *Lactobacillus* species identification, H2O2 production and antibiotic resistance and correlation with human clinical status. J. Clin. Microbiol. 37, 729–733.

### 5.3.4. The influence of physiological stresses on lactobacilli AB resistance profiles

A panel of CUL strains were subjected to additional ABR profiling in the presence of physiologically relevant 0.3 % bile (**Table 5.5**). The impact on CUL strains MICs was variable in the presence of bile, with factors such as strain and antibiotic tested influencing the outcome. Indeed, in the presence of bile. some strains increased their MIC, some decreased their MIC and others maintained their AB tolerance regardless.

#### 5.3.4.1. *L.paracasei* CUL08

When MIC values were determined for CUL08 in the presence of bile, a mixed response was seen depending on the antibiotic (**Table 5.5**). For vancomycin, the MIC increased in the presence of bile from 32 – 250 µg/mL, however, there are no breakpoints recommended and therefore no impact was seen on the resistance profile of the isolate. For kanamycin, the MIC fell greatly in the presence of bile, resulting in a shift from resistant to sensitive in strain designation. In the case of chloramphenicol, the addition of bile saw a reduced MIC value (from 4 – 1 µg/mL) however values generated were still below EFSA guidelines and CUL08 maintained its sensitive profile for chloramphenicol.

#### 5.3.4.2. *L.casei* CUL06

For *L.casei*, the addition of bile to the antibiotic susceptibility assay caused a decrease in MIC in all antibiotics tested, which resulted in chloramphenicol moving from a resistant to a sensitive susceptibility profile in the presence of bile (16 - < 0.0625 µg/mL) **Table 5.5**)).

#### 5.3.4.3. *L.rhamnosus* CUL63

The antibiotic susceptibility profiles of CUL63 were impacted in a variety of ways when additionally challenged with bile (**Table 5.5**). Interestingly, for both vancomycin and chloramphenicol, MIC values were raised in the presence of bile, however, this had no impact on the subsequent resistant profiles to these antibiotics. Nevertheless, for kanamycin the MIC value dropped from 62.5 – 16 µg/mL when challenged with bile, shifting its MIC below the cut-off, producing a sensitive profile.

#### 5.3.4.4. *L.helveticus* CUL76

The physiological stress of bile on CUL76 had a variety of effects on MIC profile, depending on the antibiotic under investigation (**Table 5.5**). For vancomycin and chloramphenicol, AMR susceptibility profiles shifted from sensitive to resistant in the presence of bile (1 - 4 µg/mL, 4 – 32 µg/mL, respectively). However, the addition of bile reduced CUL76's susceptibility to kanamycin with a MIC reduction of 62.5 - < 0.0625 µg/mL, changing its designation to sensitive.

#### 5.3.4.5. *L.gasseri* CUL09

The addition of bile to AMR testing saw a reduction in MIC profiles across all antibiotics tested against *L.gasseri* CUL09 (**Table 5.5**). The decrease in MIC values did not influence the resistance profiles for vancomycin and chloramphenicol. However, kanamycin transitioned from resistant to sensitive in the presence of bile.

#### 5.3.4.6. *L.salivarius* CUL61

A mixed response was also seen with CUL61, with the addition of bile to MIC testing (**Table 5.5**). For vancomycin and chloramphenicol, MIC values increased in the presence of bile, which resulted in a shift from a sensitive to a resistant classification for chloramphenicol. In contrast, CUL61's MIC to kanamycin was reduced in the presence of bile (250 – 8 µg/mL) indicating that the presence of bile reduced CUL61's tolerance to kanamycin resulting in a sensitive AMR profile (**Table 5.5**).

#### 5.3.4.7. *L.plantarum* CUL66

Bile reduced CU66's MIC values for both kanamycin and chloramphenicol, moving it from a resistant to a sensitive phenotype (**Table 5.5**). In the case of vancomycin, the MIC was unaffected by the presence of bile and CUL66 maintained high levels of resistance.

**Table 5.5. The antimicrobial resistance profiles of CUL strains in the presence of 0.3 % bovine bile.** Determined by broth microdilution.

| Species | Method | Strain MIC (µg/mL) | | |
|---|---|---|---|---|
| | | Vancomycin | Kanamycin | Chloramphenicol |
| *L.paracasei* CUL08 | MIC | >32 nbpr | 125 R | 4 S |
| | MIC + 0.3 % | >250 nbpr | 8 S | 1 S |
| *L.casei* CUL06 | MIC | >32 nbpr | 62.5 S | 16 R |
| | MIC + 0.3 % | < 0.0625 nbpr | <0.0625 S | <0.0625 S |
| *L.rhamnosus* CUL63 | MIC | >32 nbpr | 62.5 S | 16 R |
| | MIC + 0.3 % | >250 nbpr | 16 S | >32 R |
| *L.helveticus* CUL76 | MIC | 1 S | 62.5 R | 4 S |
| | MIC + 0.3 % | 4 R | <0.0625 S | >32 R |
| *L.gasseri* CUL09 | MIC | 2 S | 125 R | 4 S |
| | MIC + 0.3 % | <0.0625 S | 8 S | 1 S |
| *L.salivarius* CUL61 | MIC | >32 R | 250 R | 4 S |
| | MIC + 0.3 % | >250 R | 8 S | >32 R |
| *L.plantarum* CUL66 | MIC | >32 nbpr | 125 R | 16 R |
| | MIC + 0.3 % | >250 nbpr | 16 S | 8 S |

A panel of CUL strains were selected to represent each species and antibiotics which provided that the most resistant profiles in the *Lactobacillus* strains (vancomycin, kanamycin and chloramphenicol) were chosen to determine the effects the physiological concentration of bile would have on the subsequent resistance profile. MIC values previously determined are included for comparative purposes. Resistant and sensitive strains are accompanied by an R and S respectively. Values are highlighted in green when previously resistant strains present a sensitive profile in the presence of bile. Values highlighted in grey depict sensitive strains but produce a resistant profile in the presence of bile.

### 5.3.5. An *in silico* prediction of the CUL Resistome

In-depth genomic mining utilising ARG-specific databases for annotation and downstream analysis, including CARD and NCBI (**Appendix 5, Table S5.1**), provided a more detailed prediction of the resistome in CUL isolates in comparison to the initial screening via RAST, eliminating factors such as duplicated Protein Encoding Genes (PEGS) and ambiguous annotations. A local *de novo* BLASTp database of curated proteins involved with ABR was produced and CUL genomes were used as a query to putatively ID PEGs (of note is that CARD as a web-based platform provided no strict hits when initial ARG mining was conducted). BLASTp was performed with the following flags makeblastdb.dbtype prot –parse_serds to generate the database, then - blastp – query -evalue 0.01, to predict ARGS.

Hits were filtered and PEGs were curated to ensure duplicate annotations were not carried over for further analysis. During manual curation, the absence of *Lactobacillus*-specific proteins of interest was apparent (**Appendix 5, Table S5.1**). Indeed, the majority of BLASTp hits were sequences previously identified in pathogenic bacteria, highlighting a bias in specialised annotation databases. Following sequence curation, manual functional annotation was conducted to organise PEGs based on the antibiotic or the antibiotic class that they confer resistance to. Hits identified as Single Nucleotide Variants (SNVs) in distantly related organisms or as a mutation were discarded due to their strain-specific nature. A tally was generated to allow visualisation of the abundance of ARGs within each genome and the relative abundances of ARG classes predicted in each CUL isolate (**Figure 5.3**). When ARGs predicted in CUL strains were related to specific antibiotics phenotypically tested, these were labelled specifically outside of their broader drug class in **Figure 5.3**, to allow putative phenotypic to genotypic correlation**.**

A total of 97 putative ARGs were recorded across the 17 genomes analysed, which were predicted to encode resistance to chloramphenicol (9 sequences), kanamycin (1 sequence), macrolide (18 sequences), aminoglycosides (4), tetracycline (64), vancomycin (129), trimethoprim (16), mupirocin (11), sulphonamide (5), streptogramin (3), bacitracin (11), in addition to a magnitude of efflux pumps and ABC transporters (>300 (**Figure 5.3**)).

#### 5.3.5.1. *Chloramphenicol-related ARGs*

Genes encoding chloramphenicol acetyltransferase were putatively identified in the genomes of eight CUL strains, including: *L.fermentum* strains (*catB10*), *L.salivarius* CUL61 (*catS*), *L.plantarum* CUL66 (*catS* & *cmlv*), *L.plantarum* CUL66N (*cmlv*), *L.helveticus* CUL76 (*cat*), *B.bifidum* CUL20 (*cmlv*) and *B.animalis* subsp. *lactis* CUL34 (*cmlv*). Interestingly, *L.fermentum* and *L.plantarum* strains presented a resistant phenotype and CUL76, CUL61, CUL20 and CUL34 were sensitive (**Figure 5.3**).

#### 5.3.5.2. *Kanamycin-related ARG*

Only one sequence (*kat*) was associated with kanamycin resistance, identified in *B.animalis* subsp. *lactis* CUL34 (**Figure 5.3**). Breakpoints are not described for *B.animalis* resistance to kanamycin, however high tolerance levels were seen when CUL34 was challenged with this antibiotic (**Table 5.2**).

#### 5.3.5.3. *Macrolide-related ARGs*

All predicted macrolide related ARGs were variants of ribosomal methyltransferases. All CUL strains were predicted to have at least one *erm* variant, with CUL21 being the only exception. *L.acidophilus* ATCC4356 was predicted to encode three *erm* variants: *erm68*, *ermD* and *ermF*, however, it did not produce a resistant phenotype (**Table 5.2, Figure 5.3 & 5.4**). Of interest is the lack of resistance documented at the phenotypic level, where only CUL60 presented with a resistant MIC profile despite most strains having a predicted macrolide resistance gene (**Table 5.2, Figure 5.3**).

#### 5.3.5.4. *Aminoglycoside-related ARGs*

Only two strains were predicted to encode genes related to aminoglycoside resistance (disregarding kanamycin as discussed previously). Both *L.acidophilus* strains (CUL21 and CUL60) were predicted to encode 2 copies of *aacc*7 (aminoglycoside acyltransferase) and 2 copies of *alph'2lips* (aminoglycoside phosphorylation). Aminoglycoside resistance is variable across CUL strains, however, CUL21 exhibits a streptomycin-resistant profile and CUL60 a neomycin-resistant profile (**Table 5.2, Figure 5.3, Figure 5.4**).

### 5.3.5.5. *Tetracycline-related ARGs*

All strains were predicted to encode at least one gene associated with tetracycline resistance, with abundances of genes varying from 1( e.g., CUL40) – 6 ( e.g., CUL66N). Tetracycline resistant ARGs were mainly ribosomal protection proteins (*tet36, tet44, tetBP* and *tetW*), PEG subunits that form an ABC transporter (*tetAB48*, *tetAB60* and *otrA*) and a tetracycline efflux pump ((*otrB*) **Figure 5.3**)).

### 5.3.5.6. *Ribosomal protection proteins*

*L.paracasei* strains CUL07, CUL08 and CUL37 (tet34), *L.acidophilus* CUL21 (*tet36*), CUL60 (*tetBP*) and ATCC4356 (*tetBP*). *L.plantarum* CUL66 (*tetW*) and CUL66N (*tet44*), *L.gasseri* CUL09 (*tetBP*), *L.salivarius* CUL61 (*tetBP*) *B.bifidum* CUL20 (*tetBP*) and *B.animalis* CUL34 (*tetBP* and *tetW*) both *L.fermentum* strains CUL40, CUL67 putatively encode the ribosomal protection protein *otrA*. Strains that do not possess ribosomal protection proteins include *L.casei* CUL06, *L.rhamnosus* CUL63 and *L.helveticus* CUL76. All members of the *L.casei* phylogroup are predicted to encode *otrB*, with two copies annotated in *L.rhamnosus* CUL63. The most abundant tetracycline-related genes are two PEGs that form a heterodimeric ABC transporter, however, not all are complete (**Figure 5.3**). All the *L.casei* phylogroup putatively encoded one complete *tetAB* ATP binding cassette. *L.acidophilus* CUL21, ATCC4356 and *L.gasseri* CUL09 were also predicted to encode a complete *tetAB60* cassette. Both *L.plantarum* strains CUL66 and CUL66N encode two copies of the ATP binding cassette (*tetAB48* and *tetAB60*) and *B.animalis* subsp. *lactis* CUL34, encodes 1 copy (*tetAB*). Both *L.fermentum* strains CUL40 and CUL67, in addition to *L.helveticus* CUL76, *L.acidophilus* CUL60 and *B.bifidum* CUL20 only have half of the cassette (either *tetA* or *tetB*). In summary, several tetracycline resistant mechanisms have been putatively assigned to the majority of the CUL strains, with *L.plantarum* strains encoding two ABC transporters and a ribosomal protection protein associated with tetracycline resistance. *B.animalis* subsp. *lactis* CUL34 also encoded two ribosomal protection proteins and one ABC transporter. *L.paracasei* strains encoded a ribosomal protection protein an efflux pump and an ABC transporter (**Figure 5.3**). Interestingly, only one *L.paracasei* strain CUL07, presents a resistant phenotype to tetracycline (**Table 5.2, Figure 5.3**), in addition to both *L.acidophilus* strains (which encode for less tetracycline related ARGs than *L.paracasei*, **Figure 5.3**) and *B.animalis* subsp. *lactis* CUL34. Noteworthy, is the consistent identification of *tetW* in CUL34, using both RAST annotation and CARD analysis (**Figure 3.2, Figure 5.3**).

### 5.3.5.7. *Vancomycin-related ARGs*

All CUL strains were predicted to encode at least one gene related to vancomycin resistance (**Figure 5.3**).

*L.casei/L.paracasei* strains (CUL06, CUL37, CUL07 and CUL08) were predicted to encode: 3 to 4 dehydrogenase genes (*vanH*), 2-3 transcriptional activators (*vanR* and *vanU*) and 1 regulatory response regulator (*vanS*). *L.rhamnosus* CUL63 putatively encoded: 1 dehydrogenase gene (*vanH*), 3 transcriptional activators (*vanR*) 1 response regulator (*vanS*) and an accessory protein (*vanZ*).

*L.fermentum* strains CUL40 and CUL67 were predicted to have: 1 ligase (*vanD*), 8-9 dehydrogenase PEG's (*vanH*), 1-2 transcriptional activators (*vanR*) and 1 response regulator (*vanS*).

*L.helveticus* CUL76 and *L.acidophilus* strains (CUL21, CUL60) were predicted to have: 1 ligase PEG (*vanB*), between 2-3 dehydrogenase (*vanH*), 1-2 transcriptional activator (*vanR* and *vanU*), 1 response regulator (*vanS*) and a resistance accessory protein (*vanZ*).

*L.gasseri* CUL09: 1 ligase (*vanB*), 1 dehydrogenase (*vanH*), 2 transcriptional activators (*vanR* and *vanU*) and 1 response regulator (*vanS*).

*L.salivarius* CUL61 predicted van profile was: 1 ligase PEG (*vanD*), 2 dehydrogenases (*vanH*), 2 transcriptional activators (*vanR*), 1 response regulator (vanS), 1 racemase component (*vanTrL*) and 1 D, D, dipeptidase (*vanX*).

*L.acidophilus* ATCC4356: 1 ligase (*vanB*), 2 dehydrogenase (*vanH*), 1 transcriptional activator (*vanU*), 1 response regulator (*vanS*) and 1 resistance accessory protein (*vanZ*).

*L.plantarum* CUL66 and CUL66N: 1 ligase PEG (*vanC*), 1 dehydrogenase (*vanH*), 1 transcriptional activator (*vanR*), 1 response regulator (*vanS*), 1 D, D, dipeptidase (*vanX*) and 1 D, D, carboxypeptidase (*vanXY*).

*B.bifidum* CUL20 is thought to encode: 1 ligase PEG (*vanB*), 2 transcriptional activators (*vanU*), 1 response regulator (*vanS*) and 1 D, D, dipeptidase and D, D, carboxypeptidase (*vanXYL*) gene. *B.animalis* subsp*. lactis* CUL34 was only thought to encode for 1 van related ligase gene (*vanL*).

### 5.3.5.8. *Lincosamide-related ARGs*

Lincosamide ABC efflux pumps *(lmrB, lmrC* and *lmrD*) were predicted in all CUL genomes, with up to 5 copies predicted per genome. *B.animalis* subsp. *lactis* CUL34 was the only strain that had no copies predicted within its genome. Additional transporters related to clindamycin resistance were also identified in most of the *L.casei* phylogroup (except for CUL08), all *L.acidophilus* strains, *L.salivarius* (CUL61) and *L.helveticus* CUL76 (*lsaA* and *lsaC* (**Figure 5.3**)). Of note, is that there was no resistance designation to clindamycin during phenotypic testing (**Table 5.2**).

### 5.3.5.9. *Additional specific ARGs*

All isolates putatively encoded one dihydrofolate reductase, associated with trimethoprim resistance (**Figure 5.3**). In addition, *L.fermentum* strains CUL40 and CUL67, *L.plantarum* strains CUL66 and CUL66N and *B.bifidum* CUL20 were predicted to encode a sulphonamide-resistant dihydropteroate synthase (*sul3*). ARGs involved with streptogramin resistance were less prevalent in CUL strains, with only *L.plantarum* (CUL66 and CUL66N) and *L.helveticus* CUL76 putatively encoding an acyltransferase (*vatD* and *vatE* respectively) linked to streptogramin resistance. PEGs associated with bacitracin resistance (*bacA*) were predicted in several genomes, including all members of the *L.casei* phylogroup, *L.plantarum* strains (CUL66, CUL66N), *L.fermentum* strains (CUL40, CUL67) and *Bifidobacterium* isolates (CUL20, CUL34). PEGs suspected of conferring mupirocin resistance were also predicted in 11 genomes (*ileS* and *mupA*). ABC transporters linked to macrolide resistance were predicted in *L.gasseri* CUL09 (*msrE*), *L.acidophilus* ATCC4356 (*carA*) and *B.animalis* subsp*. lactis* CUL34 (*carA*).

### 5.3.5.10. *Multidrug transporters/efflux pumps*

The most abundant ARG class identified in all CUL strains was the multidrug transporters (MDT) and efflux pumps, accounting for over 300 sequences (**Figure 5.3**). Indeed, in terms of non-specific ARG prediction, the largest abundance of hits for all genes were for the two components of the efflux pump *EfrAB* (*EfrA* and *EfrB*). However, the complex must be complete to confer resistance and in many cases, multiple copies do not translate into an *EfrAB* complex (for example there are up to 7 copies of *EfrB* in one genome which will not confer resistance without its *EfrA* counterpart). The maximum number of copies for a complete *EfrAB* efflux pump predicted in CUL genomes is 2 (*L.paracasei* CUL37 and CUL07, *L.casei* CUL06 and *B.bifidum* CUL20). The remaining strains all code for one copy, with the exceptions of *L.helveticus* CUL76 and type strain *L.acidophilus* ATCC4356 which are only predicted to code for half of the complex.

*PatA* and *PatB* (ABC transporters), are proteins linked with fluoroquinolone resistance and are frequently predicted in CUL genomes, with the following predicted to encode both subunits: *L.casei* phylogroup (CUL06, CUL07, CUL08, CUL37 and CUL63), *L.acidophilus* strains (CUL21 and CUL60), *L.helveticus* CUL76 and *L.plantarum* CUL66N.

An efflux pump (*bmr*) associated with chloramphenicol resistance was predicted in *L.fermentum* CUL40 and *EfmA*, a major facilitator superfamily (MFS) transporter which confers macrolide and fluoroquinolones resistance, was also putatively identified in all *L.paracasei* strains. Additionally, *poxtA* an ATP-binding cassette protein, that confers resistance to tetracycline, -phenicol and oxazolidinone, was also putatively identified within the *L.casei* phylogroup (1-2 copies per genome), *L.helveticus* CUL76 and all *L.acidophilus* strains.

Overall, the number of complete MDR transporters predicted in CUL strains ranged from 1 (*L.gasseri* CUL09) to 6 (*L.paracasei*, CUL37 and CUL07). Of interest is that CUL08 was only predicted to encode 4 MDR transporters in comparison to its *L.paracasei* counterparts.

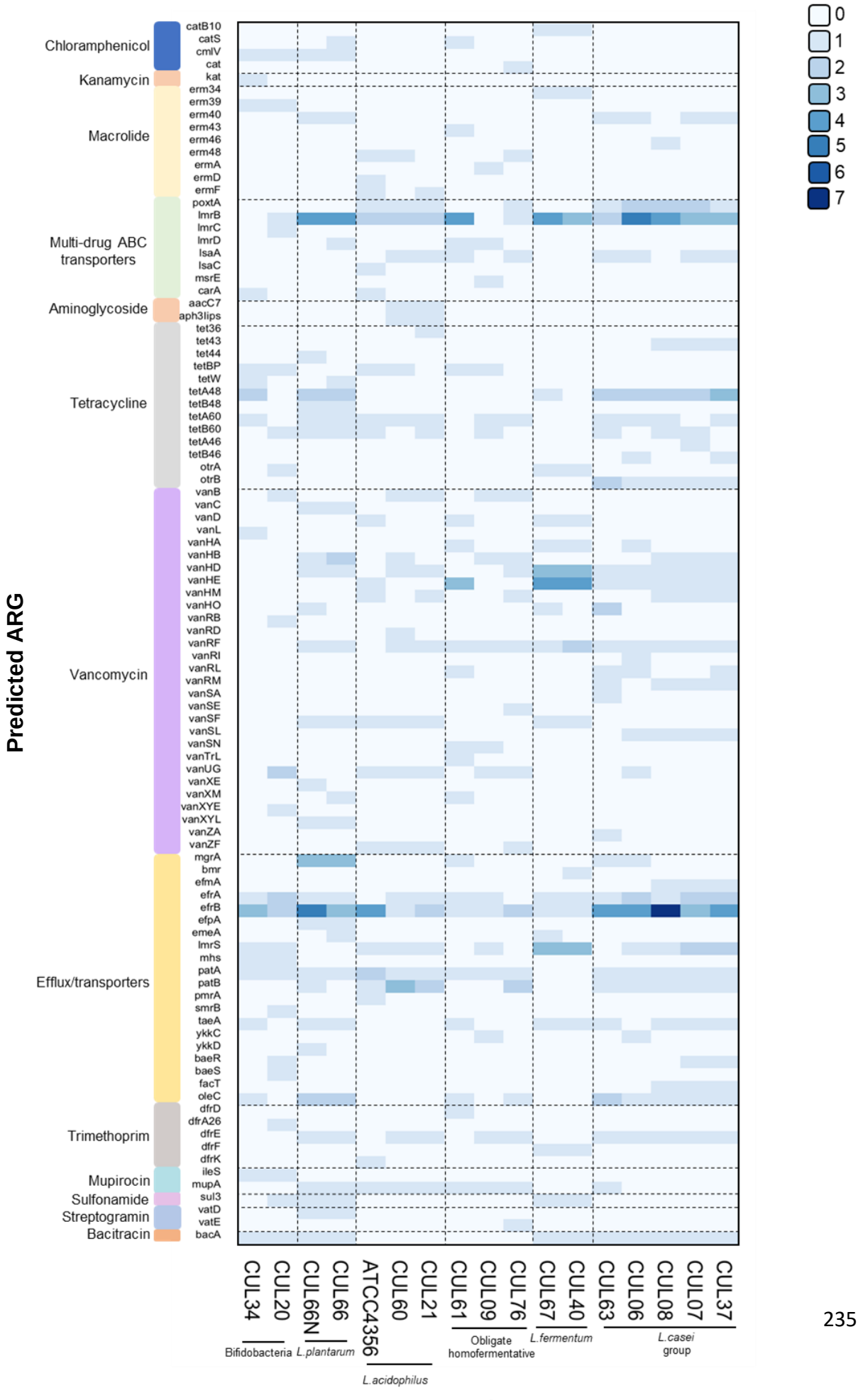

Predicted ARG
Chloramphenicol
catB10
catS
cmlV
cat
Kanamycin
kat
Macrolide
erm34
erm39
erm40
erm43
erm46
erm48
ermA
ermD
ermF
Multi-drug ABC transporters
poxtA
lmrB
lmrC
lmrD
lsaA
lsaC
msrE
carA
Aminoglycoside
aacC7
aph3lips
Tetracycline
tet36
tet43
tet44
tetBP
tetW
tetA48
tetB48
tetA60
tetB60
tetA46
tetB46
otrA
otrB
Vancomycin
vanB
vanC
vanD
vanL
vanHA
vanHB
vanHD
vanHE
vanHM
vanHO
vanRB
vanRD
vanRF
vanRI
vanRL
vanRM
vanSA
vanSE
vanSF
vanSL
vanSN
vanTrL
vanUG
vanXE
vanXM
vanXYE
vanXYL
vanZA
vanZF
Efflux/transporters
mgrA
bmr
efmA
efrA
efrB
efpA
emeA
lmrS
mhs
patA
patB
pmrA
smrB
taeA
ykkC
ykkD
baeR
baeS
facT
oleC
Trimethoprim
dfrD
dfrA26
dfrE
dfrF
dfrK
Mupirocin
ileS
mupA
Sulfonamide
sul3
Streptogramin
vatD
vatE
Bacitracin
bacA
0
1
2
3
4
5
6
7
CUL34
CUL20
CUL66N
CUL66
ATCC4356
CUL60
CUL21
CUL61
CUL09
CUL76
CUL67
CUL40
CUL63
CUL06
CUL08
CUL07
CUL37
Bifidobacteria
L.plantarum
L.acidophilus
Obligate homofermentative
L.fermentum
L.casei group
CUL Strain

**Figure 5.3. Heatmap representation of CUL resistome.** Prediction of putative ARGs was performed using BLASTp against a *de novo* BLAST database of ARGs, downloaded from the CARD database. ARG-related drug class/classes are indicated on the left side of the heatmap. The number of gene hits is represented by the gradient of colour: 0 (white) - 7 (dark blue).

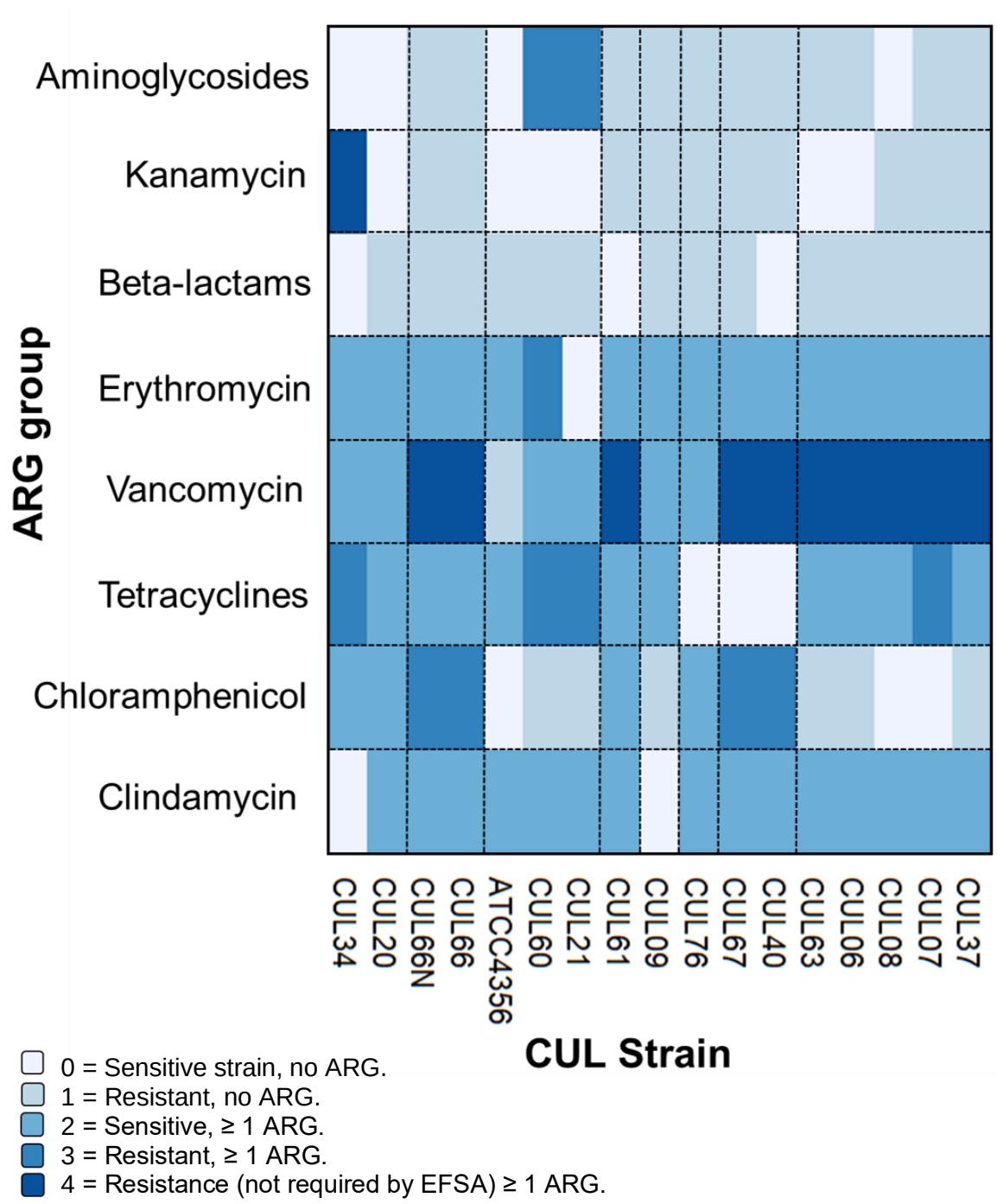



**Figure 5.4. The correlation between antimicrobial resistance phenotypes and the identification of antibiotic resistance genes.** Correlation is analysed on a scale of 0 – 4 depending on the genetic and phenotypic variation. 0 = Sensitive strain, no ARG. 1 = Resistant, no ARG. 2 = Sensitive, ≥ 1 ARG. 3 = Resistant, ≥ 1 ARG. 4 = Resistance (not required by EFSA) ≥ 1 ARG.

## 5.4. Discussion

The primary goal of any candidate probiotic is to promote the health of the consumer and not have a detrimental impact to host health. Considering the escalating AMR healthcare crisis, which is currently rising at an alarming rate, ensuring probiotic products do not burden the healthcare system by intensifying pathogen resistance is of utmost importance. The focus of this chapter was primarily to identify the risk that AMR in CUL strains may inflict on a consumer, however as the study progressed, it became increasingly apparent that fundamental flaws persist in the current methodology recommended to characterise AMR resistance in probiotic strains and as such an evaluation was undertaken.

### 5.4.1. The antibiotic resistome of CUL strains

Given the breadth of the *Lactobacillus* genus, it is difficult to summarise all phenotypic AMR profiles identified at the species, let alone the strain level. Indeed, when reporting the AMR profiles in both lactobacilli and bifidobacteria, The methodology varies enormously throughout the literature, with common discrepancies including media use (Anisimova & Yarullina, 2018; Belletti et al., 2009; Fukao & Yajima, 2012; Klare et al., 2007), inoculum concentration (Anisimova & Yarullina, 2018; Pan et al., 2011; Saleem, 2018; Shao et al., 2015), incubation time (Dušková et al., 2020) test conducted, for example by E-test (Georgieva et al., 2015), microdilution (Dec et al., 2017), macro-dilution (Shao et al., 2015), disk diffusion (Erginkaya et al., 2018; Sharma et al., 2016) and agar dilution (Ma et al., 2017a) and an overall vague description of the methodology (Thumu & Halami, 2012), making direct comparisons extremely difficult. Indeed, previous work has shown that AMR method selection impacts the MIC profiles for *L.acidophilus* strains (Mayrhofer et al., 2010).

In this study, broth microdilution was conducted at a fivefold higher concentration than recommended by guidelines (CLSI, 2010; ISO and IDF, 2010), to stimulate better growth, to potentially reveal more representative resistance profiles. A probiotic supplement should have a minimum concentration range of $10^6 - 10^9$ CFU/mL$^{-1}$ (Georgieva et al., 2009). According to the recommended guidelines for susceptibility testing, the inoculum should be visually adjusted to 1 McFarland standard and diluted 1: 1000 times (Campedelli et al., 2019; CLSI, 2010; ISO and IDF, 2010), giving an inoculum of approximately $3 \times 10^4$ CFU/mL (Egervärn et al., 2007; Mayrhofer et al., 2014), therefore underrepresenting a supplement concentration. When diluted 1:200 times this value typically increased to $1.39 \times 10^6$ (+/- $1.20 \times 10^6$) providing a concentration more relevant to the product consumed. In addition, the IDO protocol also states that cells should be adjusted to the relevant concentration in a saline solution. Salt is a osmotic stress, which can lyse cells or influence the bacterial cell wall (Piuri et al., 2005). In fact, during a DNA extraction optimisation experiment, effective lysis was achieved by

including a 30 mM NaCl + EDTA (pH 8.0) wash step, emphasizing the potential loss of viable cells, which could occlude true MIC profiles.

To conclude the impact that the inoculum concentration would have on the MIC profile of CUL isolates, in addition to analysing the differences that could be exhibited across methodologies, a maximum concentration of an overnight culture of each CUL strain (approximately 4.8 x $10^{10}$ CFU/mL) were challenged with antibiotics via agar dilution, a method that has been classified as a gold standard, when deciphering AMR resistance in anaerobic bacteria (Gajdács et al., 2017; Rychen et al., 2018). Agar dilution allows a maximum inoculum to be applied to the assay without the visual limitations that could arise via broth micro-dilutions (i.e., not limited by the maximum OD allowance by spectrophotometry). Moreover, a solid surface assay may also provide a more physiologically relevant environment, by simulating adherence of the probiotic to the gut epithelia. As anticipated, discrepancies were seen when comparing results between the two methodologies. In general, a higher level of resistance was reported when using the agar dilution technique, although for some antibiotics resistance profiles between the two methods were consistent (for example when challenged with clindamycin). However, the variations seen were often strain and antibiotic dependant, as shown by the ability of *L.fermentum* CUL67 to maintain a consistent MIC value (16 µg/mL) across both methods when challenged with streptomycin, in addition, *L.paracasei* CUL07 was the only *L.paracasei* strain to develop a resistant profile on agar when challenged with gentamycin (increased from 16 – 62.5 µg/mL). Such variations are crucial to acknowledge when evaluating the antibiotic susceptibility of lactobacilli and bifidobacteria isolates, providing further evidence for the necessity of species/strain-specific AST guidelines to maintain consistency across the literature, additionally allowing a more reliable identification of transferable AMR in commensal bacteria. It is worth noting, that drawbacks are also associated with the agar dilution methodology, such as the potential to mask the true MIC due to certain antibiotics (e.g., chloramphenicol, macrolides, tetracyclines and clindamycin) bacteriostatic effects (Hancock, 2005).

### 5.4.2. A safety analysis of CUL isolates using traditional methodology

In this study, a range of MICs was exhibited across all phylogroups for all antibiotics tested. Indeed, for a vast majority of antibiotics, variations were present at the strain level, which has additionally been reported in the type strains of lactobacilli elsewhere (Campedelli et al., 2019). For example, it has been shown that members of the *L.coryniformis* phylogroup have a 50:50 ratio of resistant to sensitive strains when challenged with erythromycin (Campedelli et al., 2019). Here we demonstrate a similar trend, where inconsistent resistance profiles were present for several species analysed in this study. For example, *L.acidophilus* CUL21 was classified as resistant to streptomycin, in comparison to a sensitive designation for CUL60 and resistance to erythromycin was documented for *L.acidophilus* CUL60, whereas the two other *L.acidophilus* strains were sensitive (CUL21 and ATCC4356). The heterogenicity of resistance profiles is of extreme importance when considering MIC breakpoints. Cut-off values described by EFSA, are at best, only designated to species level, where in some cases, for example, in bifidobacteria, cut-offs are only described for the genus as a whole (EFSA, 2012). The need for values to be reconsidered and modified to reflect the variations that can arise is evident, to encompass a range of MIC breakpoints per species.

#### 5.4.2.1. *Vancomycin resistance*

When discussing AMR, the evolutionary nature of such traits is important to consider. The rise of resistant profiles is ultimately a natural progression during the evolution of microorganisms (Davies & Davies, 2010). Most antibiotics currently in use were initially isolated from microbes (Hutchings et al., 2019; Kumar et al., 2010), where the producing organism would manufacture the compound as a warfare mechanism to compete against other species for space and resources (Amabile-Cuevas, 2016). Indeed, genetic determinants of AMR have been found in bacteria isolated from habitats untouched by humans and in historic samples before the widespread use of antibiotics (D'Costa et al., 2011). Therefore, in terms of resistance profiles in LAB, widespread and natural resistance to antibiotics such as vancomycin has been commonly reported and is not atypical. Indeed, the intrinsic capability of lactobacilli to tolerate vancomycin is a well-documented trait within the literature (Blair et al., 2015; Das et al., 2020; Goldstein et al., 2015). Vancomycin interacts with the D-Ala-D-Ala terminus on the peptidoglycan cell wall. In many species of *Lactobacillus*, D-Ala is replaced with either a D-lactate or a D-serine residue, which prevents vancomycin from binding to the cell wall (Fukao & Yajima, 2012; Gueimonde et al., 2013). High tolerance to vancomycin was documented for most lactobacilli CUL strains when challenged by broth microdilution, except for *L.acidophilus*, *L.helveticus* and *L.gasseri* which all presented sensitive phenotypes. It has been previously reported that *L.acidophilus* has lost its ability to tolerate vancomycin, as a result of a plasmid-

mediated transfer (Das et al., 2020; Johnson et al., 1990). However, this hypothesis contradicts the intrinsic nature of vancomycin resistance. Furthermore, other members of the *L.delbrueckii* phylogroup have shown sensitivity to vancomycin in concordance with the resistance seen for CUL76 and CUL09 in this study (Devriese & Butaye, 1998). Indeed, research focused on the DDL dipeptide ligase enzyme, which incorporates d-Ala-d-Ala, has shown that modification in the active site (F261Y) impacts the resistance phenotype seen when challenged with vancomycin (Campedelli et al., 2019; Zhang et al., 2018). An amino acid substitution from phenylalanine to tyrosine has been shown to predict vancomycin sensitivity in lactobacilli strains (Zhang et al., 2018). To validate the sensitive designation of *L.acidophilus* strains and other members of the *L.delbrueckii* clade (CUL76 and CUL09), a multi-sequence amino acid alignment was conducted to correlate resistance with the amino acid present at position 258. The substitution of F (phenylalanine) for Y (tyrosine) is related to the sensitivity seen during phenotypic testing. Vancomycin is a potent antibiotic, typically used to treat Gram-positive infections, including MDR *Staphylococcus aureus* (Jeffres, 2017) and *Clostridium difficle* infections (Surawicz et al., 2013). Therefore, the intrinsic nature of vancomycin resistance in these putative probiotics provides a potential biotherapeutic route, where patients with infections, such as *C.difficile*, who often see a depletion in their microbiota (Zhang et al., 2015), could be treated with an antibiotic and probiotic combination approach, to aid in gastrointestinal disease. Benefits of this two-pronged approach include bacterial competition for resources against pathogens, pathogen exclusion and the potential aid in the maintenance of healthier gut microbiota during AB therapy (Hickson, 2011; Wang et al., 2017). Albeit the positive use of ABR microbes remains controversial.

Despite the intrinsic basis for vancomycin resistance, all genomes were predicted to encode vancomycin-related ARGs. The presence of *van* operons in *Enterococcus* are well described (Ahmed & Baptiste, 2018). These operons (typically *vanA* or *vanB*) replace d-alanyl-d-alanine, with d-alanyl-d-lactate or d-alanyl-d-serine, both of which cause glycopeptides to bind poorly to the cell wall (Amyes, 2007). The operon is composed of two regulation genes, *vanR* and *vanS,* which induce transcription of three genes known to confer glycopeptide resistance, for example, *vanH*, *vanA* and *vanX* in the case of the *vanA* operon (Amyes, 2007). CUL strains were all predicted to encode variants of these genes, although operons were not always complete. However, *Lactobacillus* has previously been reported to lack *vanA*, *vanB* and *vanC* genes (Klein et al., 2000). Such findings indicate either a mis-annotation of genes or the transfer of genes from enterococci, suggesting they should be further characterised.

#### 5.4.2.2. *Aminoglycoside resistance*

Aminoglycosides are a group of broad-spectrum antibiotics typically used as a last resort treatment (Feng et al., 2019a). The intrinsic capability of lactobacilli bacteria to confer

resistance to aminoglycosides has been well described (Abriouel et al., 2015; Gu et al., 2014; Patel et al., 2012; Rozman et al., 2020). Numerous mechanisms for intrinsic aminoglycoside resistance have been reported, such as enzymatic inactivation of the antibiotic (via acetyltransferases, nucleotidyltransferases and phosphotransferases (Shaw et al., 1993)), decreased cell permeability and altered ribosome binding sites (Chaudhary & Payasi, 2014; Shao et al., 2015). In this study, variations in aminoglycoside susceptibilities were demonstrated, depending on the drug tested. For example, all strains were classed as sensitive to gentamicin, in agreement with sensitivity profiles in lactobacilli reported elsewhere (Campedelli et al., 2019; Shao et al., 2015). Indeed, it has been proposed that an enhanced ability of gentamycin to cross the cell wall of lactobacilli can account for lower MIC values for this aminoglycoside (Danielsen & Wind, 2003; Egervärn et al., 2007; Elkins & Mullis, 2004; Mayrhofer et al., 2010). In addition, the majority of CUL strains were highly susceptible to streptomycin in agreement with some previous reports (de Souza et al., 2019), with resistance only demonstrated in *L.acidophilus* CUL21. However, variations in streptomycin tolerance at strain level have previously been described (Mayrhofer et al., 2010; Shao et al., 2015) and high levels of streptomycin resistance in *Lactobacillus* strains have also been reported (Mayrhofer et al., 2010). The incongruities within strain level resistance profiles stress the importance of MIC breakpoints being reviewed and a range to be encompassed. Strain variation is often considered to arise when HGT has taken place and genes are acquired (Rychen et al., 2018), putatively suggesting the potential for CUL21 to contribute to the ARG reservoir within the gut (McInnes et al., 2020), however, acyltransferases were predicted in both *L.acidophilus* strains, suggesting a chromosomal basis of resistance. Nevertheless, the lack of resistance in CUL60 in comparison to CUL21 suggests a variation in expression requirements for each strain. The genes related to aminoglycoside resistance were only identified in *L.acidophilus* strains (CUL21 and CUL60). Each was predicted to encode 2 copies of *aacc*7 (aminoglycoside acetyltransferase) and 2 copies of alph'2lips (aminoglycoside phosphorylation) which have previously been described in *L.salivarius* (Dec et al., 2017) and *L.casei/paracasei* (Ouoba et al., 2008). In contrast, high levels of tolerance were observed in CUL strains when challenged with kanamycin (66 % resistant) and neomycin (80 % resistant). Kanamycin resistance is widely reported (Abriouel et al., 2015; Štšepetova et al., 2017), as an intrinsic capability of lactobacilli species due to cell membrane in-permeability (Abriouel et al., 2015; Elkins & Mullis, 2004; Nawaz et al., 2011; Patel et al., 2012). However, in this study, *L.casei* CUL06 was susceptible to kanamycin. For other *L.casei* strains, high levels of kanamycin resistance have been reported (de Souza et al., 2019; Shin et al., 2021). Indeed, in a recent evaluation of AMR in *Lactobacillus* species, kanamycin resistance was documented in approximately 80 % of the *L.casei* phylogroup (Campedelli et al., 2019). All

*L.acidophilus* strains also demonstrated a sensitivity to kanamycin, which is interesting due to the proposed intrinsic nature of kanamycin resistance. Certainly, Campedelli et al., (2019) showed that a wide range of resistance profiles was seen in the *L.delbrueckii* phylogroup, where only 50 % of strains were resistant to kanamycin. In addition, Mayrhofer et al., (2010) reported an MIC range of 0.5 – 8 µg/mL for *L.acidophilus* strains, further supporting the sensitivity seen in CUL strains. Similarly, an MIC analysis on the type of strain used in this study (although using MRS instead of LSM), reported an MIC value of 0.125 µg/mL for *L.acidophilus* ATCC 4356, emphasising the enormous heterogeneity exhibited at the intra-strain level for AMR If different methods are used.

Neomycin MIC breakpoints are not described by EFSA, presumably due to the inference that resistance is intrinsic. The AMR profiles for neomycin are not consistent for *Lactobacillus* CUL strains. For example, in the case of *L.paracasei*, CUL37 and CUL07 are resistant but CUL08 is sensitive. *L.acidophilus* strains are predominantly sensitive (CUL21 and ATCC4356). However, CUL60 was resistant, indicating a strain-specific resistance profile. Previous studies have also conducted MIC analysis of *Lactobacillus* strains, coining all as sensitive but presenting a range of 1 to 32 µg/mL (Guo et al., 2017). the range exhibited by CUL strains was 4 – 32 µg/mL, however, using previously reported breakpoints resistance was classified in CUL strains (Ammor et al., 2007). The confusion surrounding what is resistant and what is sensitive is apparent, as aminoglycoside resistance is described as an intrinsic trait for *Lactobacillus* species (Nawaz et al., 2011; Patel et al., 2012). In terms of 'designated' resistance profiles, it appears that most studies will cite that resistance is intrinsic (Nawaz et al., 2011; Patel et al., 2012), resulting in a paper trail dating back to the 1990s (Teuber et al., 1999), with a little discussion regarding the evidence of intrinsic resistant (Elkins & Mullis, 2004), emphasising the importance in re-evaluating the basis for resistance in bacteria deliberately introduced into the food chain.

### 5.4.2.3. *Tetracycline, clindamycin and erythromycin*

It is commonly acknowledged that lactobacilli are sensitive to drugs which inhibit protein synthesis including erythromycin (Ammor et al., 2007; Hummel et al., 2007; Sharma et al., 2016), clindamycin (Sharma et al., 2016), chloramphenicol (Coppola et al., 2005) and tetracyclines (Gueimonde et al., 2013; Guo et al., 2017; Nawaz et al., 2011).

This study largely follows a similar trend, with all CUL strains presenting a sensitive phenotype to clindamycin, erythromycin (excluding *L.acidophilus* CUL60) and tetracycline (except for *L.paracasei* CUL07 and *L.acidophilus* CUL21 and CUL60). Sensitivity to tetracycline has previously been reported for lactobacilli species (Ammor et al., 2007; Danielsen & Wind, 2003; Štšepetova et al., 2017) in concordance with the results reported here. However, there is also a significant number of studies that describe resistance to tetracycline in *Lactobacillus* (Anisimova & Yarullina, 2018; Hoque et al., 2010; Pan et al., 2011; Thumu & Halami, 2012). Interestingly, genome mining predicted the presence of at least one gene involved with tetracycline resistance in all CUL strains, including ribosomal protection proteins (*tet36*, *tet44*, *tetBP* and *tetW*), ABC transporters (*tetAB48, tetAB60* and *otrA*) and tetracycline efflux pumps (*otrB*). In addition, all CUL strains were predicted to have at least one *erm* variant (ribosomal RNA methyltransferase) with the following annotations: *erm34, erm39*, *erm40, erm43, erm46, erm48, ermA, ermD* and *ermF.* The genetic basis of tetracycline (Huys et al., 2006; Ledina et al., 2018; Mayrhofer et al., 2010; Thumu & Halami, 2012) and erythromycin resistance (Das et al., 2020; Patel et al., 2012; Štšepetova et al., 2017) has been frequently reported in *Lactobacillus*. Indeed, in a review of AMR profiles in LAB bacteria, Patel et al., (2012) highlighted *tetM*, *ermB* and *cat* as the most abundant ARGs in LAB. Genes associated with tetracycline, macrolide and chloramphenicol were amongst the most frequently predicted ARGs in CUL genomes in accordance with previous findings (Campedelli et al., 2019; Patel et al., 2012). Furthermore, several tetracycline ribosomal protection proteins have been recognised in the literature, with *tetM* being the most prevalent annotation, a gene typically associated with transposons (Campedelli et al., 2019; Ledina et al., 2018). Botina et al., (2011) identified *tetM* on conjugative transposons in *L.casei/paracasei* and *L.helveticus* strains, which shared high homology with the *tetM* genes of *Enterococcus faecalis* and *Streptococcus pneumoniae*. Moreover, numerous authors have concluded that tetracycline resistance is an acquired trait in *Lactobacillus* (Anisimova & Yarullina, 2018; Danielsen & Wind, 2003; Klare et al., 2007; Mayrhofer et al., 2010). Here we report the phenotypic tolerance shown by two *L.acidophilus* strains CUL21 and CUL60, which both displayed resistance towards tetracycline and were predicted to have a tetracycline ribosomal protection protein (*tet36* and *tetBP* respectively). In addition, CUL21 was suggested to encode a tetracycline efflux pump, *tetAB*. The presence of *tet* genes does not always translate to phenotypic resistance (Ledina et al.,

2018). For example, *L.acidophilus* ATCC4356 was predicted to encode *tetBP* and a *tetAB60* efflux pump but showed no phenotypic tolerance to tetracycline. Interestingly, not only was this strain phenotypically sensitive, but in a previous study was predicted to contain *tetM* (Campedelli et al., 2019), suggesting potential acquired resistance and highlighting the importance of not relying on an annotation to validate a genotype. In addition, there has been some evidence that lactobacilli strains can transfer *tetM* to *Enterococcus* and *Lactococcus* isolates *in vitro* (Devirgiliis et al., 2009; Gevers et al., 2003), however, others have contradicted such findings (Klare et al., 2007). *L.paracasei* CUL07 also showed resistance to tetracycline and encoded a ribosomal protection protein (*tet34*), an efflux pump (tetAB) and an ABC transporter (*otrB*). In agreement with the prediction of *tet* genes within CUL *L.paracasei* strains, previous work has identified a conjugative transposon tn916 encoding *tetM*, in strains of *L.paracasei* capable of transferring to *Enterococcus* species *in vitro* (Devirgiliis et al., 2009). Despite *tetM* being unaccounted for within CUL isolates, the potential of mis-annotation should be highlighted. Annotation designation can differ depending on the ARG identification method. For example, several studies designate *tetM* based on PCR amplification, whereas, as WGS becomes more readily and databases have expanded, so does the potential in recognising new annotations, new genes and subsequent resistance profiles in the literature (Roberts & Schwarz, 2016). A recent study using WGS prediction methodology showed the presence of *tetBP* and the efflux pump *otrA* in *Lactobacillus* isolates, which is reportedly the first study to identify these genes in *Lactobacillus* (Zhang & Zhang, 2019). Utilizing WGS, we validate these gene annotations in CUL *Lactobacillus* strains.

All CUL strains were predicted to encode at least one *erm* variant, with CUL21 having the only macrolide-resistant phenotype. CUL60 was predicted to encode *erm48,* a ribosomal methyltransferase. Typically, *ermB* is the most widely reported macrolide ARG in lactobacilli (Anisimova & Yarullina, 2018; Campedelli et al., 2019; Comunian et al., 2010; Drago et al., 2011; Dušková et al., 2020; Nawaz et al., 2011). The presence of *erm* genes is generally thought to be acquired (Dušková et al., 2020). Indeed, the successful transfer of *ermB* from *Lactobacillus* to *Enterococcus* has been described (Nawaz et al., 2011). However, despite *ermB* not being predicted in CUL isolates, variants of *erm* were present. For example, *L.acidophilus* ATCC4356 was predicted to encode three erm variants: *erm68*, *ermD* and *ermF*, however, it did not produce a resistant phenotype. Indeed, previous studies have shown that lactobacilli have a greater heterogenicity of *erm* variants in comparison to other genera, such as bifidobacteria, including *ermA*, *ermB*, *ermC* and *ermT* (Hoek et al., 2008). In terms of safety, it is important to note that several *erm* variants have been identified on plasmids in lactobacilli genomes (Feld et al., 2008; Gfeller et al., 2003; Tannock et al., 1994), highlighting the caution required when identifying *erm* genes in lactobacilli.

#### 5.4.2.4. *Chloramphenicol*

Of the *Lactobacillus* CUL strains, 67 % exhibited a resistant profile when challenged with chloramphenicol. However**,** sensitivity against chloramphenicol is generally anticipated (Anisimova & Yarullina, 2018; Das et al., 2020). Nevertheless, resistant strains have previously been identified (Pan et al., 2011; Zhou et al., 2012). In fact, for the entire *Lactobacillus* genus, an MIC range of 2 – 16 µg/mL was demonstrated when strains were challenged with chloramphenicol, where 49 % of the type strains were designated as resistant (Campedelli et al., 2019). Here, despite an increased inoculum, we report similar ranges (between 4 – 32 µg/mL). It is difficult to locate information as to why chloramphenicol resistance is not anticipated when nearly 50 % of type strains are resistant. Chloramphenicol resistance is thought to be mediated by the presence of *cat* genes, that convert chloramphenicol to inactive diacetyl chloramphenicol (Dec et al., 2017; Hummel et al., 2007). Indeed, the subsequent analysis predicted 9 chloramphenicol acyltransferases (*cat*) within the CUL genomes including *catB10*, *catS*, *cmlV* and *cat*. *L.plantarum* CUL66 was the only strain predicted to encode two copies and presented with a resistant phenotype. Interestingly, only four out of the ten strains phenotypically designated as resistant to chloramphenicol had the presence of at least one *cat* gene (*L.plantarum* CUL66, CUL66N and *L.fermentum* CUL40, CUL67). Therefore 60 % of resistant strains were not predicted to encode an acyltransferase and 100 % of the sensitive stains were predicted to have copies of the *cat* gene. Similar, inconsistencies between phenotype and genotype have been previously reported for chloramphenicol resistance suggesting that there is a mutational basis for expression (Campedelli et al., 2019; Hummel et al., 2007). Although *cat* genes have been identified on plasmids (Hou et al., 2014) and on the chromosomes of *Lactobacillus* species (Abriouel et al., 2015). Indeed, in a recent analysis of the evolutionary history of *cat* genes in *Lactobacillus*, *Enterococcus* and *Staphylococcus* shares high homology between chromosomal encoded genes (Abriouel et al., 2015). *Lactobacillus cat* genes located on plasmids shared a greater degree of similarity with *cat* genes from *Enterococcus* and *Staphylococcus aureus*, as such the authors conclude HGT may be the origin of *Lactobacillus* plasmid-borne *cat* genes (Abriouel et al., 2015). In agreement, it has also been shown that the amino acid sequence of the *L.acidophilus* BFE 7429 *cat* gene shared 100 % identity with the corresponding region of the *cat* gene in plasmids pIP501 of *S. agalactiae* and pRE25 of *E.faecalis* RE25 (Hummel et al., 2007). The *cat* nucleotide sequences of *L.delbrueckii* subsp. *bulgaricus* BFE 7430 and *S. thermophilus* BFE 7420 also shared 100 % identity with the *cat* gene from plasmid pC221 of *S.aureus* pTZ12 of *B.subtilis* (Hummel et al., 2007), highlighting a definitive role for HGT in the migration of *cat* genes. Therefore, the robust classification of cat genes and their genomic

architecture is of great importance when determining the safety of probiotic candidates. However, for strains that were phenotypically resistant but were not predicted to encode *cat* genes, increased tolerance to chloramphenicol may be explained by the presence of efflux pumps and ABC transporters which were frequently predicted in CUL isolates.

#### 5.4.2.5. *Beta-lactams*

Beta-lactam antibiotics such as penicillin, target and inhibit bacterial cell wall synthesis (Ammor et al., 2007). For beta-lactam antibiotics, CUL strains presented varied MIC patterns. In general, it is widely reported that *Lactobacillus* strains are sensitive to beta-lactams (D'Aimmo et al., 2007; Klare et al., 2007; Sharma et al., 2016). Despite widespread reports of sensitivity to a penicillin (Caggia et al., 2015; D'Aimmo et al., 2007; Danielsen & Wind, 2003), which has resulted in the lack of microbiological breakpoints for *Lactobacillus* (EFSA, 2012), CUL strains MICs ranged from 0.0625-32 µg/mL when challenged with penicillin G. Previous assessment of AMR in the *Lactobacillus* genus revealed, that despite an overall sensitivity to penicillin G, certain subgroups including *L.zymae* and *L.plantarum* presented with MIC's >16 µg/mL (Campedelli et al., 2019). Here we validate this finding, as the only two strains presenting with > 16 µg/mL were part of the same phylogroups, *L.rhamnosus* CUL63 and *L.plantarum* CUL66N, following previous reports, despite a larger starting inoculum.

Ampicillin is the only beta-lactam antibiotic tested with available EFSA breakpoints. Most of the *Lactobacillus* CUL strains tested presented with resistant phenotypes when challenged with ampicillin. Interestingly, ampicillin resistance is not widely reported in LAB bacteria (Botta et al., 2014; D'Aimmo et al., 2007; Danielsen & Wind, 2003; Dec et al., 2020; Kirtzalidou et al., 2011). However, in this study, when increasing the starting inoculum, ampicillin resistance was present for 82 % of strains tested. With only *L.fermentum* CUL40, *L.salivarius* CUL61 and *B.animalis* subsp*. lactis* CUL34 displaying sensitive profiles. Nevertheless, reports of ampicillin resistance in *Lactobacillus* are not unheard of and have been described elsewhere (Argyri et al., 2013; Caggia et al., 2015; Dušková et al., 2020). A recent study involving an adaptative laboratory experiment increased the MIC profiles of *L.plantarum* strains when continually exposed to ampicillin (Cao et al., 2020). Moreover, utilizing a proteomics-guided approach, proteins were identified that when inactivated resulted in a phenotypic reversion (including, ATP-dependent Clp protease/the ATP-binding subunit ClpL and a small heat shock protein (Cao et al., 2020)). In addition, genome mining did not reveal any MGE associated with genes conferring ampicillin tolerance. Therefore, it was concluded that even after continually exposing and eliciting an ampicillin tolerant phenotype in *Lactobacillus* strains, the risk of HGT was low (Cao et al., 2020). Point mutations in chromosomal *pbp* genes (Pbp1a, Pbp2a and/or Pbp2x) have also been shown to aid in ampicillin resistance

in *L.reuteri* (Rosander et al., 2008). Studies such as these, provide reassurance that despite the increased ampicillin resistance shown in this study, the risk to host health is low. However, it is interesting that this is the first real divergence in phenotype – generated by increasing the bacteria concentration, suggesting alternative mechanisms of tolerance. Currently, there are calls to evaluate the roles bacterial biofilm formation may have on AMR (Bowler et al., 2020), driven by reports that HGT is 700 times more efficient within biofilms than amongst free-living cells (Flemming et al., 2016) and reports of biofilms enhancing AMT (Hennequin et al., 2012). *Lactobacillus* has been shown to form biofilms at certain concentrations (Jones & Versalovic, 2009; Kubota et al., 2009). Resistance to environmental stresses has been shown to increase when isolates develop biofilms which may explain increased ampicillin resistance (Bowler et al., 2020).

Oxacillin tolerance is the most described beta-lactam resistance within the *Lactobacillus* genus (Coppola et al., 2005; Danielsen & Wind, 2003). Nevertheless, EFSA has no set breakpoints to designate a resistant phenotype. Adopting previously reported breakpoints, Oxacillin resistance was putatively assigned to 53 % of *Lactobacillus* CUL strains.

For amoxicillin, the majority of CUL isolates had MIC values classed as intermediate in terms of AMR in the literature. However, CUL37, CUL07 and CUL63 were deemed as resistant. Indeed, enhanced resistance to amoxicillin has been shown in other members of the *L.casei* phylogroup, where proteomics revealed resistance was associated with alterations in carbohydrate and amino acid metabolism, as well as certain components involved in membrane metabolism, were activated indicating an intrinsic nature for resistance in CUL strains (Wang, Guo, et al., 2018a), suggesting a reduced risk of HGT.

#### 5.4.2.6. *Antimicrobial resistance in bifidobacteria CUL strains*

When challenged with a panel of clinically relevant antibiotics, bifidobacteria CUL strains were overall susceptible, except for *B.bifidum* CUL20 when tested with ampicillin and *B.animalis* CUL34 with tetracycline. Of interest, is the variation in resistance profiles described for the bifidobacteria isolates when compared with the literature. Members of the *Bifidobacteria* genera are known to possess intrinsic factors that enable their resistance to antibiotics including aminoglycosides (Mayrhofer et al., 2011; Vlková et al., 2015), polypeptides (Kim et al., 2018; Wei et al., 2012), quinolones (Ouoba et al., 2008; Xiao et al., 2010) and mupirocin (Kim et al., 2018; Vlková et al., 2015). In general, it is thought that *Bifidobacterium* species are sensitive to penicillin (Zhou et al., 2005), chloramphenicol (Mayrhofer et al., 2011; Wei et al., 2012), clindamycin (D'Aimmo et al., 2007) and erythromycin (Kim et al., 2018; Ouoba et al., 2008). However, as with lactobacilli, discrepancies appear in the literature in the case of certain antibiotics, with some authors concluding that *Bifidobacterium* species have intrinsic

resistance to vancomycin (Charteris et al., 1998; Kheadr et al., 2007; Patel et al., 2012) and tetracycline (Bottacini et al., 2018; Mättö et al., 2007; Xiao et al., 2010), whilst others report sensitivity (D'Aimmo et al., 2007; Mättö et al., 2007; Ouoba et al., 2008; Wei et al., 2012). However, no phenotypic resistance to aminoglycosides was designated for the antibiotics which had EFSA breakpoints available. Nevertheless, both CUL20 and CUL34 exhibited high tolerances to kanamycin (MIC >32 µg/mL) and neomycin (MIC = 8 µg/mL). Indeed, ARG predictions displayed potential for CUL34 to encode for a kanamycin acyltransferase (*kat*), which may aid in aminoglycoside tolerance. Tetracycline and erythromycin ARGs have been well described (Ammor, Flórez, Hoek, et al., 2008; Cao et al., 2020; Gueimonde et al., 2013; Wang et al., 2017). In agreement with previous findings, Both CUL20 and CUL34 were predicted to encode several genes conferring tetracycline resistance. Copies of *tetBP* and tetracycline efflux pumps were identified in both strains (*tetAB* for CUL34 and *otrA* for CUL20). Furthermore, CUL34 was predicted to encode the ribosomal protection protein *tetW,* which is a ubiquitous trait in *B.animalis* strains and is generally considered responsible for tetracycline resistance in the species (Cao et al., 2020; Gueimonde et al., 2010). Despite being recognised as a potential transferable gene (Hu et al., 2016), *tetW* is thought to have been acquired early in the evolutionary history of *B.animalis* (Cao et al., 2020), suggesting a diminished risk of transfer. Macrolide resistance genes are also typical for bifidobacteria (Bottacini et al., 2018; Li et al., 2020). However, despite the prediction of *erm39* in both CUL20 and CUL34, neither presented a phenotypic resistance to erythromycin. Interestingly a variation (*erm49)*, has been shown to aid in erythromycin tolerance in *B.breve,* suggesting the potential for *erm39* to confer erythromycin resistance (Martínez et al., 2018). Regardless of the lack of AMR phenotypes, several other ARGs were predicted in bifidobacteria CUL genomes. Here, we identified a chloramphenicol acyltransferase *clmv* in both CUL20 and CUL34. However, no chloramphenicol resistance was presented, suggesting such genes were inactive during AB exposure. It is important to consider that previous reports have found similar genes, such as *cmX*, on a plasmid when scrutinising ARGs in bifidobacteria, providing a reasonable incentive to investigate the genomic regions here (Cao et al., 2020). Furthermore, mupirocin-resistant isoleucyl-tRNA synthetase (*ileS*) was also identified in the majority of bifidobacterial strains, in agreement with this study's findings (Cao et al., 2020). Reports of ampicillin resistance in bifidobacteria are scarce (D'Aimmo et al., 2007; Li et al., 2020; Zhou et al., 2005), however when testing with a larger starting inoculum, *B.bifidum* presented an ampicillin tolerant phenotype. Several multidrug efflux pumps were also reported and may explain an increased resistance, which has previously been demonstrated with erythromycin resistance in bifidobacteria (Gueimonde et al., 2013).

The EFSA only provide breakpoints at the genus level for *Bifidobacterium*, with values based on only a few strains (EFSA, 2012). Overall, in bifidobacteria CUL strains, sensitivities were following EFSA, however, discrepancies were present, as previously mentioned. Recently, Duranti et al., (2017a) investigated the antibiotic susceptibility of all *Bifidobacteria* type strains (91 in total). They found that most strains had a larger MIC breakpoint than the recommended EFSA criteria and concluded that the EFSA breakpoints should be reviewed, to encompass more of the species within the genus.

There is limited information available on the transferability of ARGs in *Bifidobacteria*, however, the consensus indicates that the risk of transfer is low (Xiao et al., 2010, Taft et al., 2018). However, Kazimierczak et al., (2006) showed that a *tetW* containing transposon was transferable between two *Bifidobacterium* strains *in vitro*, although this was at low frequencies and transferability has not been shown to occur in other species (Gueimonde et al., 2013). In contrast, Moubareck et al., (2007) demonstrated the transfer of the *bla* gene (coding beta-lactamase inhibition) was halted in the presence of bifidobacteria, suggesting a role in alleviating HGT of ARGs. Indeed, a recent clinical trial showed that infant supplementation with *Bifidobacterium longum* subsp. *infantis* EVC001, reduced the number of ARGs within the infant microbiota by 87 % (Casaburi et al., 2019). Similar trends have also been reported elsewhere (Taft et al., 2018). Lactobacilli (Lê et al., 2013) and bifidobacteria (Turroni et al., 2011) are anticipated endogenous members of the gut microbiota and have shown to reduce the incidence of pathogenic bacteria (Fijan et al., 2019; Woo & Ahn, 2013), which ultimately could reduce the incidence of HGT amongst pathogenic organisms. When determining the risk to host health, it is important to weigh up such factors.

#### *5.4.2.7. A generalised summary*

Overall, the method selection for analysing CUL strains MIC when challenged with clinically relevant antibiotics appears to be of great importance to the subsequent results. When comparing methods there was no true consistency where all isolates produced the same MIC regardless of method. For kanamycin resistant profiles were different in 4 out of 16 isolates, for gentamycin 10 out of 16, streptomycin 10 out of 16, neomycin 1 out of 16, oxacillin 3 out of 16, amoxicillin 4 out of 16, ampicillin 1 out of 16, erythromycin 13 out of 16, vancomycin 6 out of 16, chloramphenicol 4 out of 16, tetracycline 11 out of16 and finally 8 out of 16 for clindamycin.

### 5.4.3. The impact of physiological stresses on AMR profiles

The target site for a typical probiotic is the host's GI tract. Numerous physiological stresses are encountered throughout the transit of the body, including bile. Bile acts as a detergent in the gut, with an antimicrobial function (Begley et al., 2006). Therefore, a candidate probiotic should be able to tolerate exposure to such molecules. However, when suggesting the treatment of gastrointestinal diseases with an AB and probiotic combination, evaluating the effects that physiological stresses (such as bile), can have on the AMR profiles is fundamental. In this study, interactions between CUL strains, antibiotics and bile were highly varied. Strains were selected, that were previously identified as bile tolerant and had a high tolerance to antibiotics. The intrinsic nature of vancomycin resistance was not affected by the additional pressure of 0.3 % bile and all strains challenged maintained their high MIC profiles, which have also been reported elsewhere (Charteris et al., 2000). Interestingly, *L.helveticus* CUL76 developed resistance to vancomycin in the presence of bile. For kanamycin, the addition of bile caused all strains to shift from a resistant profile to a sensitive one, suggesting that kanamycin and probiotic simultaneous supplementation would not be an effective treatment. The reduction in aminoglycoside tolerance in the presence of bile has been previously reported (Elkins & Mullis, 2004). In addition, decreased resistance to aminoglycosides was thought to be due to an increased cell membrane permeability, which would allow the antibiotics to penetrate more efficiently (Elkins & Mullis, 2004).

The most interesting modifications to phenotypic AMR profiles were seen when CUL strains were challenged with chloramphenicol and bile. *L.casei* CUL06, *L.fermentum* CUL40 and *L.plantarum* CUL66 all experienced a decreased MIC value in the presence of bile, shifting from a resistant to a sensitive profile. However, *L.helveticus* CUL76 and *L.salivarius* CUL61 demonstrated an increased MIC profile in the presence of bile, transitioning from a sensitive to resistant profile when exposed to chloramphenicol. Increased antibiotic tolerance in the presence of bile has previously been reported elsewhere (Charteris et al., 2000; Kheadr et al., 2007).

For *L.helveticus*, bile seems to aid in antibiotic tolerance, with two out of three AMR profiles shifting from sensitive to resistant. No clear mechanism for such a phenomenon is currently available, however, a two-component signal transduction system (typically comprised of a sensor kinase and a response regulator) has been shown to monitor environmental signals (e.g., stress) and prompt a change in activity, resulting in *Lactobacillus* increasing or decreasing tolerance to certain ABs (Alcántara et al., 2011), highlighting a potential process for such an occurrence. Alternatively, in other species of bacteria, bile deconjugation has been associated with energy generation (Begley, Gahan, et al., 2005), where the potential for increased energy, could translate into enhanced gene expression. In addition, bile efflux

pumps may aid in the tolerance seen towards vancomycin and chloramphenicol in the case of *L.helveticus*, due to their role as a coping mechanism to physiological stresses (Pfeiler & Klaenhammer, 2009).

### 5.4.4. Genome mining

The identification of the genomic origin of resistance is crucial when determining the risk of transferring AMR to pathogenic bacteria. The consequence of deliberately introducing microorganisms into the food chain is the potential to contribute to the ARG reservoir. As such, the focus is now shifting to the genetic characterisation of AMR, with new guidance beginning to emerge that requests the genomic characterisation of food microorganisms (Rozman et al., 2020; Rychen et al., 2018). However, such actions are yet to be routinely used, which is emphasized within this study, where all putative ARGs identified on CARD, a specific ARG depository, did not contain one *Lactobacillus* specific hit, suggesting a depletion/ underrepresentation of ARG classification within the genus. Indeed, several genes associated with ABR have been specifically identified within the *Lactobacillus* genus, which is highlighted in the reciprocal BLASTp analysis performed here, where *Lactobacillus* specific hits with a % identity of 100 % were found in most cases. Certainly, database selection, criteria used and deposited annotation are all factors that may influence the genomic identification of genes involved with ABR. For example, the sole use of the RAST annotation server did not offer as high of a resolution as found when using CARD, providing vague annotations such as *streptococcus* vancomycin-resistant locus and identifying genes associated with beta-lactam resistance (*bL, blA. blB* and *blC)* which were not detected via CARD analysis, nor is such genomic resistance well documented in the literature. However, some agreement was seen when comparing both genome mining platforms, such as the high abundance of tetracycline ARGS and specifically the presence of *tetW* in *B.animalis* subsp. *lactis* CUL34.

The Comprehensive Antibiotic Resistance Database (McArthur et al., 2013), is a platform specifically designed for the identification of genes involved in ABR. Typically, CARD is used via a web interface, where a genome is uploaded and filtered via strict or loose hits of ARGs. using CARD the traditional way (online), no strict or loose hits were predicted in CUL genomes. The generation of a *de novo* BLASTp database (using the protein sequences from CARD), was the only way to achieve enhanced precision and prediction of AMR genomic factors in CUL isolates, further adding to the complexity of scrutinising the safety of bacteria intended for consumption.

### 5.4.5. Genotype to phenotype correlation

Overall, there was little correlation exhibited between the genetic and phenotypic profiles presented within CUL strains. Indeed, previous studies have also recognised a lack of correlation between gene prediction and expressed phenotype (Campedelli et al., 2019). In total 97 putative ARGS were predicted in CUL genomes, however, the correlation was only seen in genotype and characterised phenotype in the case of *L.fermentum* (CUL40 and CUL67) and *L.plantarum* (CUL66 and CUL66N) strains, which were both predicted to encode ARGs associated with chloramphenicol resistance and presented resistant phenotypes.

*B.animalis* subsp. *lactis* CUL34 was predicted to encode a *kat* gene related to kanamycin resistance and exhibited high tolerance to kanamycin phenotypically. *L.acidophilus* strains were each predicted to encode four genes related to aminoglycoside resistance, which interestingly correlated to a resistance phenotype related to different drugs from the aminoglycoside class (streptomycin resistance for CUL21 and neomycin resistance for CUL60). Genes linked with tetracycline resistance were predicted in all CUL genomes but only correlated with a resistant phenotype in *L.acidophilus* strains CUL21 and CUL60, *L.paracasei* CUL07 and *B.animalis* subsp. *lactis* CUL34.

For vancomycin, where EFSA resistance breakpoints were provided, all strains (*L.acidophilus* CUL21 and CUL60, *L.gasseri* CUL09 and *L.helveticus* CUL76) were phenotypically sensitive and encoded the amino acid change which reportedly confers vancomycin sensitivity (Campedelli et al., 2019). Interestingly, copies of the *Enterococcus* vancomycin operon were predicted in some CUL isolates and further work should seek to validate the annotation and deduce whether the operons are intact. *Van* genes were not identified on any GI's previously (**Chapter 3, Table 3.6**) and it is therefore likely that these have been mis-annotated, especially given that previous analyses have reported the lack of van operons in lactobacilli (Klein et al., 2000).

Additional resistance profiles were also designated across the antibiotic panel tested; however, no specific genomic mining could be assigned to such phenotypes. The vast abundance of efflux pumps and transporters predicted may provide a putative genomic origin of such phenotypes. The minimal correlation between genotype and phenotype displayed here highlights the utmost importance of using a polyphasic approach when determining the ABR profiles of products designed for consumption.

### 5.4.6. Limitations

An important limitation to recognise is the lack of breakpoints supplied by the EFSA for certain antibiotics tested (including, penicillin g, amoxicillin, oxacillin and neomycin). As such, previously suggested breakpoints were utilised, including those suggested by: Ammor, Flórez, Hoek, et al., (2008), Danielsen & Wind, (2003) and Felten et al., (1999). More recently, authors who have encountered similar issues with a depletion in available breakpoints, have adopted the same references (Campedelli et al., 2019; Rozman et al., 2020). However, methodologies vary in the determination of breakpoints, for example, Ammor, Flórez, Hoek, et al., (2008) and Danielsen & Wind, (2003) conducted MIC testing using E-test strips, whereas Felten et al., (1999) utilised agar dilution (which is somewhat more relevant to the EFSA guidance). As such, without an updated MIC evaluation from an authorized body, this is the only means to deduce antibiotic susceptibility for bacteria when challenged with such antibiotics.

### 5.4.7. Conclusions and future work

In today's era of widespread AMR and the health crisis that it results in, it is extremely important to design specific platforms for the in-depth classification of AMR profiles in symbiotic bacteria.

Results presented within this chapter highlight the number of methodological variations that can influence the phenotypic emergence of ABR, where modifying from broth to agar and increasing bacterial load can translate to a positive ABR phenotype. Given that microbial supplements can be purchased to incorporate billions of CFUs, deducing the effect dose can have on antibiotic susceptibility is important, to evaluate *in vivo* ABR potential.

Utilising recommended methodology, we show that CUL strains exhibit phenotypic resistance to several antibiotics, however intraspecies variation is seen (a phenomenon that has previously been described (Campedelli et al., 2019)). An example in this study was seen in the case of tetracycline resistance, where *L.paracasei* CUL08 and CUL37 were sensitive and CUL07 was classed as resistant, highlighting a need for breakpoints to be re-evaluated to encompass an MIC range.

As initial genome mining (**Chapter 3, Figure 3.2**) revealed the potential for CUL strains to encode ARGs, a more in-depth evaluation was undertaken using the CARD database. 97 specific ARGs were identified in CUL strains, in addition to a magnitude of efflux pumps and transporters. Many "hits" were not initially characterised in both lactobacilli and bifidobacteria, revealing a database bias towards pathogen-characterised ARGs.

In terms of correlating genotype and phenotype, *L.fermentum* (CUL40 and CUL67) and *L.plantarum (*CUL66 and CUL66N) strains both presented phenotypic chloramphenicol resistance and were predicted to encode ARGs conferring such resistance. *B.animalis* subsp.

*lactis* CUL34 was predicted to encode a *kat* gene related to kanamycin resistance and exhibited high tolerance to kanamycin phenotypically. *L.acidophilus* strains were each predicted to encode 4 genes related to aminoglycoside resistance, which interestingly correlated to resistance in different drugs from the aminoglycoside class. Genes linked with tetracycline resistance were predicted in all CUL genomes but only correlated with a resistant phenotype in *L.acidophilus* strains CUL21 and CUL60, *L.paracasei* CUL07 and *B.animalis* subsp. *lactis* CUL34. *L.acidophilus* CUL21 and CUL60, *L.gasseri* CUL09 and *L.helveticus* CUL76 were sensitive to vancomycin and encoded the amino acid change in the DDL protein, which has been correlated with vancomycin sensitivity (Campedelli et al., 2019).

In conclusion, it is suggested that specific databases for genetic resistance determinants need to be created as a matter of priority. Furthermore, biological breakpoints should be re-evaluated to encompass strain variation and cover a greater breadth of antibiotics to proactively reduce the potential of contributing to the ARG reservoir. Indeed, a robust pipeline such as the one set out here, where several antibiotics, several methods and deep comprehensive genome mining should be put forward as a mandatory action before the deliberate use of any microorganism. Further analysis should also include gene expression and where possible RNA sequencing, to attempt to identify undiscovered genomic regions involved in AMR, generating more specific commensal bacteria data for such organisms.

## 5.5. Appendix 5.

**Supplementary Table S5.1.** Manual CARD curation.

**https://docs.google.com/spreadsheets/d/1juZ-BzuYhM3wKAFGz9hEm6uz6EgWT_6v/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true**

**Supplementary Table S5.2.** CARD ARG analysis for CUL strains.

**https://docs.google.com/spreadsheets/d/1tTu9LNopuW_t8kRON5HrwxN_aTZaU9Md/edit?usp=sharing&ouid=101492219108741881971&rtpof=true&sd=true**

# Chapter 6. Discussion

## 6.1. Discussion

The data presented in this thesis allows a comprehensive view of the CUL strains which are marketed as probiotics. Before this work, these strains were initially classified using AFLP and API, however, given the recommendations that microorganisms deliberately introduced into the food chain should be genetically characterised (Binda et al., 2020; Hill et al., 2014; Morovic et al., 2016) and the enhanced accessibility to genome sequencing (Köser et al., 2012), has enabled a robust taxonomic classification to be presented for all CUL strains. Indeed, the importance of conducting such work is seen over the years where several independent studies have reported misidentification, missing and non-labelled species in commercially available supplements (Huys et al., 2006; Lewis et al., 2016; Morovic et al., 2016) and perhaps most worrying are reports which have shown genus level misidentification in 30 % of commercial products (Huys et al., 2006), impacting consumer trust and carrying a safety risk if misidentified species are opportunistic pathogens (Shioya et al., 2011).

Using a multifaceted approach, all CUL strains were confirmed as members of the lactobacilli and bifidobacteria genera (**Chapter 2**). The resolution of genomic markers at deducing strain level taxonomy was evaluated, given the frequently cited notion that 16S rRNA sequencing does not provide enough phylogenetic signal to evaluate strain level taxonomy (Drancourt et al., 2000; Janda & Abbott, 2007; Johnson et al., 2019; Mignard & Flandrois, 2006 Claesson et al., 2008; Huang et al., 2018; Naser et al., 2005; Singh et al., 2009; Xie et al., 2019).16S rRNA provided species level taxonomic resolution for both bifidobacteria and most lactobacilli CUL strains, except for *L.helveticus* CUL76 (**Chapter 2, Figures 2.2 – 2.3, Table 2.2**). Indeed, previous reports have shown that *L.helveticus* 16S rRNA shares high sequence homology with other members of the *L.delbrueckii* clade (Claesson et al., 2008). Furthermore, 16S rRNA Neighbor-joining phylogenies had low bootstrap values, indicating an unstable topology which may shift when additional gene sequences are added. Therefore, despite both type strain phylogenies indicating that all CUL strains were novel and the BLASTn result demonstrating that species level designation was correct, given the variation seen with *L.helveticus* CUL76 and the low bootstraps in the phylogenies, 16S rRNA was deemed insufficient at providing a robust phylogenetic and taxonomic classification for CUL strains. High molecular weight, intact gDNA was extracted from strains (using an improved lysis step, consisting of 3 x NaCl + EDTA, pH 8.0, washes, before enzymatic lysis) and sent for WGS. Draft genomes were assembled and contamination was removed following submission to the PGAAP pipeline (Tatusova et al., 2016). Genomes were subsequently annotated in PROKKA (Seemann, 2014) for further downstream analysis. Genome metrics from annotation platforms were compared with median species reports from NCBI and all CUL strains were within the described genome size and %GC range for their species assignment.

MLSA has been shown to enhance taxonomic classifications, due to increased phylogenetic signal provided by additional genomic markers (Segata et al., 2013).

Given the highly unstable nature of the *Lactobacillus* genus (Huang et al., 2018, 2020; Pot et al., 2014; Wood & Holzapfel, 1992; Zheng et al., 2020), all genomes available in NCBI from the LAB group were retrieved and aligned using PhyloPhlAn (Segata et al., 2013), with increased number of genomic markers (400 ubiquitous bacterial proteins) enabling an enhanced phylogenetic signal. A known paraphyly with *Pediococcus* was replicated in the LAB phylogeny (Makarova & Koonin, 2007), providing confidence in the topology achieved. Evolutionary analysis revealed *Lactococcus* to be the most suitable root for subsequent lactobacilli-specific analysis, due to the greater evolutionary distance observed in comparison to *Enterococcus* and *Weissella* and the lack of paraphyly, which was observed with *Pediococcus*. Following, over 2194 genomes of lactobacilli and the entire genome collection available for *Lactococcus* was aligned in PhyloPhlAn and a phylogeny was reconstructed using *Lactococcus* as the root (**Chapter 2, Figure 2.5**). Numerous points of interest were apparent from the resultant lactobacilli MLSA, however many deviates from the purpose of this study, to provide a robust classification of CUL isolates. Nevertheless, to do so, evaluating the entire evolutionary lineage of lactobacilli and bifidobacteria was important, to assess the accuracy of their phylogroup designations. For example, historically, lactobacilli have been subjected to numerous taxonomic modifications (Huang et al., 2018, 2020; Pot et al., 2014; Wood & Holzapfel, 1992; Zheng et al., 2020), where genera have been merged and even recently, separated into 25 separate genera (23 of which are novel (Zheng et al., 2020)). In conducting such analysis, several strains deposited in GenBank were noticeably mis-annotated or unidentified and therefore, this study provides a taxonomic classification to such isolates. In addition, to assess the accuracy of the topology produced, the phylogroups and the species they encompass were compared with previous reports (Holzapfel & Wood, 2014; Salvetti et al., 2018; Zheng et al., 2020). The lactobacilli MLSA produced here correlated with designations provided by Zheng et al., (2020), the most recent analysis of the *Lactobacillus* genus, therefore enhancing the reliability of the topology generated and expanding on the novel designations/phylogroups formed by Zheng et al., (2020), using an increased number of genomes (all available, not only type strains) and an increased number of genomic markers (114 (Zheng et al., 2020) – 400 (this study)).

The bifidobacteria genera were processed through the same analysis pipeline and the resultant topology was in accordance with previous reports (Lugli et al., 2018; Sun, Zhang, et al., 2015) expanding on the previous works by encompassing all genomes and allowing insight into new clades, with putative suggestions of novel phylogroups that have not been previously described (**Chapter 2, Figure 2.7 Table 2.5** (Lugli et al., 2018; Sun, Zhang, et al., 2015)).

All CUL strains were represented on singular branches within their respective genus phylogenies, indicating that they were all novel entries into GenBank at the time of submission (**Chapter 2, Figures 2.5 and 2.7**). Each strain appeared as novel entries into the following species: *L.acidophilus* (CUL21, CUL60), *L.gasseri* (CUL09), *L.helveticus* (CUL76), *L.casei* (CUL06), *L.paracasei* (CUL08), *L.plantarum* (CUL66 and CUL66N), *L.fermentum* (CUL40 and CUL67), *L.salivarius* (CUL61), *B.bifidum* (CUL20) and *B.animalis* subsp. *lactis* (CUL34). The only exception was seen in two *L.paracasei* strains, CUL37 and CUL07, which represented novel entries (indicated by their position on an independent branch on the topology) but were located on a linear branch with each other, putatively suggesting that they were the same strain.

Large divergences were seen between both the *L.plantarum* (CUL66 and CUL66N) and *L.fermentum* (CUL40 and CUL67) strains, suggesting such a variation in probiotic function. The distribution of CUL strains within the *Lactobacillus* phylogeny highlighted a diverse consortium of organisms from across the breadth of the genus, as strains were identified in subgroups A and B on the MLSA. It was therefore concluded that MLSA using WGS was accurate and provided strain level designation for CUL strains, allowing the inference that each strain was a novel entity in NCBI at the time of the analysis.

Following the robust taxonomic classification of CUL strains and given their putative probiotic status, genome sequences were employed to deduce regions of 'interest' predicted in CUL isolates. As such, sequences were aligned in Mauve (Darling et al., 2004) to a complete reference sequence (identified as the closest neighbour with a complete genome on the MLSA phylogeny) to attempt to reduce gaps between contigs.

Genomes were additionally annotated in RAST which allowed a subsystem analysis to be undertaken (**Chapter 3, Table 3.3**). Initially, the virulence subsystem was mined to deduce the safety of these organisms intended for human consumption (**Chapter 3, Figure 3.1**). The putative prediction of ABR determinants (including *tetW* within *B.animalis* subsp. *lactis* CUL34) and phage-related proteins, were recognised in all CUL genomes (**Chapter 3, Figure 3.2 and 3.3**). RAST appeared to offer a vague annotation when describing virulence factors, such as, *S.pyrogenes* recombination zone and adhesion proteins (e.g., fbp), emphasising the depletion in safety-related databases for non-pathogenic bacteria such as LAB (Colautti et al., 2022).

Generally, *L.casei* CUL06 was predicted to encode the largest number of virulence factors (89, primarily consisting of phage elements) and bifidobacteria strains (CUL20 and CUL34) were shown to encode the lowest (12 per genome).

As phages were primarily identified as virulence markers in RAST analysis and given that phages have been implicated with the facilitation of HGT (Casjens, 2003; Lekunberri et al., 2017; Pei et al., 2021), PHAST analysis was undertaken to determine the presence of intact

phage's within CUL genomes (**Chapter 3, Table 3.5**). *L.casei* CUL06 was predicted to encode 4 intact phages, previously identified in lactobacilli strains. Interestingly, 44 % of phages predicted in CUL genomes were initially identified in different genera, including *Enterococcus* EcZZ2 (*L.plantarum* CUL66), *Bacillus* VBBHas-171 and *Oenococcus* phage (*L.plantarum* CUL66N), which may suggest the acquisition of such elements. To further deduce the virulence capacity of CUL strains, genomes were mined for putative mobile elements (**Chapter 3, Table 3.6**), including genomic islands. The number of predicted genomic islands in CUL strains ranged from 6 (*L.acidophilus* CUL21) to 27 (*L.fermentum* CUL67). Following, GIs were manually analysed for regions of 'interest'. Generally, GIs identified were mainly composed of hypothetical proteins, recombinases, proteins related to transport, glycosyltransferases, endopeptidases and phage elements. Some GIs were potentially beneficial for a CUL organism, encoding traits such as collagen adhesion proteins (Yadav et al., 2013) and sortase A (Wu et al., 2020), which may have a role in adhesion for lactobacilli strains. However, the transmission of such traits to pathogenic bacteria could potentially be a safety hazard. In addition, *L.rhamnosus* CUL63 was predicted to encode several genes associated with acid tolerance, for example, a Na+ H+ antiporter (Lucas et al., 2003; Montijo-Prieto et al., 2019). Interestingly, a cholylglycine hydrolase homolog was predicted as a component of a GI in *L.salivarius* CUL61 and *B.bifidum* CUL20.

Manual curation of GI's also identified traits considered 'deleterious' if encoded on an MGE. For example, ARGs were identified on GIs including a tetracycline resistance gene (*tetW)* in *B.animalis* subsp. *lactis* CUL34 and several genes in *L.fermentum* strains (CUL40 and CUL67). Based on such findings, an in-depth focus was taken to deduce the AMR profiles of CUL strains assessing any potential to contribute to the gut resistome.

EUCAST guidelines are typically followed when determining the AMR profiles of pathogenic organisms (Espinel-Ingroff et al., 2005; European Committee on Antimicrobial Susceptibility Testing, 2013; Kassim et al., 2016). However, there is a depletion of such guidelines for non-pathogenic anaerobic bacteria. As such, in 2012 the EFSA released guidelines on how to assess the AMR profiles of microbial food supplements (EFSA, 2012). However, the methods were vague and allowed room for variations, for example, using agar or broth testing which can lead to differences in the phenotype observed (Campedelli et al., 2019; EFSA, 2012). Furthermore, the breakpoints reported for MIC values were often nonspecific, for example, breakpoints were only presented at genus level for bifidobacteria and 'lactobacilli homofermentative' in lactobacilli. Previous reports have indicated that MIC breakpoints should be re-evaluated to encompass a broader range of MIC values and species-specific cut-offs, as resistance can vary between strains (Campedelli et al., 2019). In addition, method-specific breakpoints should also be provided, to ensure comparability between studies, as is provided

by EUCAST. Indeed, the EFSA recommended starting inoculum is relatively low (0.1 OD (1:1000 dilution)), as such, when initially trialled, CUL strains failed to stimulate growth. Therefore, phenotypic antibiotic testing was conducted at a 5-fold higher concentration (1:200 dilution) than is typically recommended by EFSA (1:1000 dilution), to ensure reliable growth and a more physiological representation of concentrations that would be consumed as a supplement (**Chapter 5, Table 5.2**).

The EFSA recommend a panel of 9 clinically relevant antibiotics which should be used when determining AMR profiles (EFSA, 2012). In this study, 9 was increased to 13, incorporating a greater diversity of drug classes. However, as such, there was a depletion of resistance breakpoints available. Therefore, previously recommended breakpoints predating EFSA guidance were adopted to provide a general overview of resistance levels for these antibiotics (Ammor et al., 2007; Danielsen & Wind, 2003; Felten et al., 1999). In brief, a range of MICs was exhibited across all phylogroups, for all antibiotics tested (**Chapter 5, Table 5.2**). Out of the 13 antibiotics all CUL strains were sensitive to clindamycin and gentamycin. Where breakpoints were described, all strains were sensitive to vancomycin. Further analysis revealed a substitution in the DDL protein (from F to Y), differentiated tolerant and sensitive strains, a phenomenon reported elsewhere (**Chapter 5, Figure 5.2** (Campedelli et al., 2019)). For streptomycin and erythromycin, only one strain was deemed resistant (*L.acidophilus* CUL21 and *L.acidophilus* CUL60 respectively). The majority of CUL isolates were sensitive to tetracycline, however *L.paracasei* CUL07, *L.acidophilus* CUL21 and CUL60 and *B.animalis* subsp. *lactis* CUL34 displayed resistance profiles. The phenotypic resistance profile exhibited by CUL34 correlates well the prediction that a tetracycline resistance gene (*tetW*) was encoded within the genome (**Chapter 3, Figure 3.2**), indicating this gene is functional and activated in response to tetracycline exposure. Using breakpoints identified in the literature (Ammor et al., 2007; Danielsen & Wind, 2003; Felten et al., 1999), 47 % (8 out of 17 tested) of strains exhibited resistance profiles to oxacillin. Despite no breakpoints being described for penicillin G a wide range of sensitivities are documented (0.0625 - 32 µg/mL). High MIC values were present for chloramphenicol 59 % (10/17 resistant), 57 % for kanamycin (10/17 resistant), 71 % for neomycin (12/17 resistant) and 82 % for ampicillin (14/17 resistant).

However, given the variations in the methodology it is suggested that these breakpoints are reanalysed and issued by EFSA, to enable a more reliable evaluation of ABR for these antibiotics.

Agar and broth microdilution are often referred as ‘the gold standard’ when evaluating ABR profiles of anaerobic bacteria (Gajdács et al., 2017; Rychen et al., 2018), however, comparability between these methods is difficult. In addition, starting concentrations may also play a role in the ability of a microorganism to tolerate antibiotic stress. Therefore, a crude

analysis, where overnight cultures were spun down, concentrated into 1 mL of MRS broth and inoculated on LSM agar, was conducted to observe the effects such modifications would have on the AMR profiles (**Chapter 5, Table 5.3**). Overall the method selection for MIC determination appears to be of great importance to the subsequent results. When comparing methods there was no true consistency where all isolates produced the same MIC regardless of method (**Chapter 5, Table 5.4**). For kanamycin resistant profiles were different in 4/16 isolates. gentamycin 10/16, streptomycin 10/16, neomycin 1/16, oxacillin 3/16, amoxicillin 4/16, ampicillin 1/16, erythromycin 13/16, vancomycin 6/16, chloramphenicol 4/16, tetracycline 11/16 and finally 8/16 for clindamycin. Interestingly, variations were not always an increase in resistance as a result of higher bacterial concentrations. For example, *L.acidophilus* CUL21's response to neomycin when challenged via the two methods (despite the increased cell density starting volume when testing via AD), exhibited a decreased MIC on agar compared with in broth (from 16 µg/mL – 0.5 µg/mL), highlighting the scope of variations that can be seen between methods and the test organism.

Given the nonspecific and ambiguous annotations provided by initial virulence mining, an ABR-specific database was employed to identify additional ARGs in CUL genomes. 97 specific ARGs were identified in CUL strains, in addition to a magnitude of efflux pumps and transporters. When correlating genotype and phenotype, *L.fermentum* CUL40 and CUL67 and *L.plantarum* strains CUL66 and CUL66N both presented phenotypic chloramphenicol resistance and were predicted to encode chloramphenicol related ARGs (**Chapter 5, Figure 5.3**). *B.animalis* subsp. *lactis* CUL34 was predicted to encode a *kat* gene related to kanamycin resistance and exhibited high tolerance to kanamycin phenotypically. *L.acidophilus* strains were each predicted to encode 4 genes related to aminoglycoside resistance, which correlated to resistance in different drugs from the aminoglycoside class (streptomycin resistance for CUL21 and neomycin resistance for CUL60). Genes linked with tetracycline resistance were predicted in all CUL genomes but only correlated with a resistant phenotype in *L.acidophilus* strains CUL21 and CUL60, *L.paracasei* CUL07 and *B.animalis* subsp. *lactis* CUL34.

Bile is a physiological stress a bacteria will encounter within the gut, even if tolerant, the antimicrobial properties of bile will likely induce stress mechanisms within an organism. Therefore, to deduce the physiological AMR profiles of CUL strains, antibiotic assays were conducted in the presence of 0.3 % bile (**Chapter 5, Table 5.5**). Interestingly in some cases, the presence of bile resulted in an increased AMR profile, seen in *L.helveticus* CUL76 when challenged with vancomycin and chloramphenicol and *L.salivarius* CUL61 when challenged with chloramphenicol, suggesting a potential beneficial interaction between some CUL strains and bile. Indeed, previous reports have suggested that deconjugating BAs liberates the amino acid, which can then be incorporated by a bacterium and used as an energy source (Begley,

Sleator, et al., 2005), indicating that in some cases bile can increase the fitness of a microorganism.

Genome mining also led to the prediction of several beneficial traits in CUL strains (**Chapter 3, Table 3.7 & 3.8**).

Following taxonomic classification and analysis in **Chapter 2**, close relatives of CUL strains were identified. As such, the literature was mined for putative probiotic traits associated with CUL relatives, to determine whether such functions were conserved within the evolutionary lineage.

By utilising this approach, *L.gasseri* CUL09 was predicted to share a common antimicrobial peptide (bacteriocin) with *L.gasseri* K9 (Peternel et al., 2010), suggesting antimicrobial potential. Both *L.acidophilus* strains were predicted to encode genes that confer hydrogen peroxide-producing capabilities, a shared attribute with the whole clade (Hertzberger et al., 2014). The hydrogen peroxide-producing lactobacilli have been shown to inhibit *Salmonella enterica in vitro* (Pridmore et al., 2008), providing putative anti-pathogenic traits within CUL21 and CUL60 (**Chapter 3, Table 3.8**).

Adhesion genes were recognised in *L.helveticus* R0052 (Tompkins et al., 2012), including several mucus binding proteins which appear to be conserved in the lineage from which CUL76 emerges. *L.paracasei* CUL08 was predicted to encode genes involved in; adhesion, bile tolerance and host immune regulation (Ma et al., 2018). In addition, an intact EPS operon consisting of 18 genes was also conserved in *L.paracasei* CUL08 (**Chapter 3, Figure 3.3**). EPS clusters have been implicated in several putative probiotic functions such as adhesion (Živković et al., 2016), colonization (Kanmani et al., 2013; Tulumoglu et al., 2013), stress resistance (Gauri et al., 2009; Lebeer et al., 2008; Nguyen et al., 2020; Seesuriyachan, 2012) and host-bacteria interactions (Bengoa et al., 2020; Bhat & Bajaj, 2019; Dertli et al., 2013), highlighting a plethora of putative beneficial traits associated with *L.paracasei* CUL08. In addition, *B.bifidum* CUL20 was predicted to encode an adhesion operon. In support of such findings, *B.bifidum* CUL20 has displayed the greatest adherence capability of the Lab4 consortia, when cultured with Caco-2 enterocytes (Baker et al., 2021), suggesting a genomic basis for such attributes. Similarly, *B.animalis* subsp. *lactis* CUL34 was predicted to encode an operon which confers high acid tolerance capabilities (Sun et al., 2015).

This genome-led approach highlighted focus points for further downstream analysis. As such CUL genomes were mined with the bacteriocin search tool BAGEL4 (**Chapter 3, Table 3.9** (van Heel et al., 2018)). Bacteriocins were predicted in 11 out of the 16 CUL strains sequenced, which may confer antimicrobial effects against pathogenetic bacteria *in vivo* (Gaspar et al., 2018). Initial mining led to the annotation of Gassericin A in *L.gasseri* CUL09. However, BAGEL4 produced the annotation Gassericin T. Gassericin A and Gassericin T are

thought to be members of different subclasses, class IIc (circular) and class IIb (two-peptide) respectively (Maldonado-Barragán et al., 2016). The varied annotation presented using two search methods reflects the ambiguous nature that the sole use of genomic mining can have when bioprospecting for probiotic traits. Gassericin A has presented a wide inhibition capability of pathogenic organisms, for example in the case of; *Listeria monocytogenes, Bacillus cereus* and *S. aureus* (Kawai et al., 2001; Pandey et al., 2013; Peternel et al., 2010), suggesting a capacity for *L.gasseri* CUL09 to behave similarly.

Genome mining also led to the prediction of putative probiotic traits in CUL strains, including the genomic basis for adherence, acid and bile tolerance (**Chapter 3, Table 3.7**). Indeed, several CUL strains were predicted to encode cholylglycine hydrolases, a protein with a suggested role in bile tolerance and host cholesterol reduction (Begley et al., 2006). The objective of this study was to provide a safety overview and identify functional traits t bioprospect CUL strains. Therefore, given the potential dual role of the BSH protein as a functional (bile tolerance) and health-promoting (cholesterol reduction) attribute (Begley et al., 2006), this genomic trait was selected for further downstream analysis (**Chapter 4**).

Initially, all CUL strains were subjected to bile tolerance testing, utilising bovine bile, which has shown to be the closest representation of human bile for *in vitro* testing (**Chapter 4, Table 4.2 - 4.3** (Hu et al., 2018b)). From evaluating the literature, numerous reports state that 0.3 % bovine bile is the equivalent to a physiological representation of the gut (Hu et al., 2018b; J. Lee et al., 2011; Lin et al., 2007). However, post-prandial concentrations can reach 2 % in the small intestine (Northfield & McColl, 1973). As such, CUL strains were challenged across a concentration gradient of bile to represent a 'truer' physiological picture of their bile tolerance capacity *in vivo*.

All CUL strains were tolerant to 0.3 % (which is the equivalent of 0.4 mM), Oxgall bile, except for *L.fermentum* CUL40 and CUL67. Interestingly in **Chapter 3** (**Table 3.7**) the gene Glucosamine -6- phosphate deaminase was reported as a bile tolerance trait in lactobacilli (Alcántara & Zúñiga, 2012) and was not predicted in either *L.fermentum* genomes but was identified in the rest of the lactobacilli CUL strains, putatively suggesting a genomic basis for tolerance.

All other species (except *L.paracasei* CUL07 and *B.animalis* subsp. *lactis* CUL34, who both experienced bile lethality at 0.8 mM) exhibited bile tolerance up to 6.4 mM. suggesting a strong potential for strains to survive transit throughout the gut.

Given that the initial genome, mining revealed multiple cholylglycine hydrolase homologs within the same genome and that previous reports suggested that multiple copies may confer a substrate preference (McAuliffe et al., 2005), phenotypic analysis of BSH production was conducted on both TDCA and GDCA to deduce the effect that the amino acid conjugate would

have on the designation of a BSH positive phenotype (**Chapter 4, Table 4.4 – 4.7**). In addition, often when testing for BSH activity, a typical concentration of 0.5 % TDCA is used without an explanation of the physiological relevance of the concentration (Archer & Halami, 2015; Bustos et al., 2012; Caggia et al., 2015; Fang et al., 2009; Panicker et al., 2018; Shehata et al., 2016). Therefore, a concentration range was also utilised on both acids to incorporate physiological relevance and determine the effects that the concentration would have on the emergence of a BSH phenotype. BSH activity was considered positive using the plate assay in several CUL strains including *L.acidophilus* CUL21 and CUL60*, L.plantarum* CUL66 and CUL66N*, L.gasseri* CUL09*, L.salivarius* CUL61, one strain of *L.fermentum* CUL40 and both bifidobacteria isolates (CUL20 and CUL34). No BSH activity was detected in *L.casei* CUL06*, L.paracasei* CUL37, CUL07 and CUL08*, L.rhamnosus* CUL63*, L.helveticus* CUL76 and *L.fermentum* CUL67.

Numerous factors influenced the designation of a BSH positive phenotype including, substrate, concentration and the lighting used (**Chapter 4, Figure 4.3).** Interesting trends emerged, including disappearing positive phenotypes and variations in the phenotype presented on the plate. Indeed, three precipitate variations were noted, including in the agar plug, an on-the-surface precipitate and a scattering precipitate. The detected differences led to questioning the reliability of the assay when designating BSH activity. One assumption was that the lactic acid may interact with the BA and in turn, generate a phenotype. Indeed, when lactic acid isomers were challenged with GDCA at high concentrations, precipitation did emerge (**Chapter 4, Figure 4.8**), suggesting a deconjugation capacity of lactic acid. GC/MS was therefore applied to provide a semi-quantitative overview of the most phenotypically active CUL strains and their interactions with a total BA pool.

Indeed, *L.plantarum* strains CUL66 and CUL66N and *L.gasseri* CUL09 showed strong capabilities to deplete the conjugated BA pool, indicated by a sharp rise in CA and DCA and a fall in TCA, GCA, TDCA and GDCA (**Chapter 4, Figure 4.10**). In addition, the free cholesterol in the BA pool was also depleted, suggesting a cholesterol-reducing capability of CUL strains. To further characterise the interactions seen between lactic acid and GDCA and determine whether lactic acid played a role in the emergence of phenotype disparities in the plate assay, two variations of bacterial mediated precipitation were cored out of the agar and subjected to GC/MS. Lactic acid incubated solely on GDCA was also cored out as a control. Both precipitate types showed the deconjugation of GDCA, as DCA rose in abundance, indicating no variation in mechanistic deconjugation between in agar and on agar precipitation. However, lactic acid incubated with GDCA, modified the composition of GDCA, indicated by the emergence of a second peak on the GC/MS trace, which was not present in the MRS +

GDCA control, putatively suggesting that lactic acid plays a role in modifying the GDCA compound (**Chapter 4, Figure 4.8 - 4.9**).

To ascertain the molecular mechanisms behind such interactions, the cholylglycine hydrolase sequences identified during initial genome mining (**Chapter 3**) were retrieved, in addition to a reference dataset of known BSH and PVA sequences (O'Flaherty et al., 2018). An ML phylogeny was reconstructed to determine the relationship between CUL protein sequences and other known proteins (**Chapter 4, Figure 4.2**). All RAST predicted cholylglycine hydrolases grouped within their species homologs, indicating a robust topology. Following BLAST analysis, putative annotations were obtained for each protein clade, where *L.plantarum* CUL66 and CUL66N were predicted to encode *bsh* 1-3, *L.acidophilus* CUL21 and CUL60*, bshA* and *bshB*, *L.gasseri* CUL09 *bsh1*, *L.salivarius* CUL61 *bsh 1-2.*

Retrieving such sequences and reconstructing the phylogeny, enabled the identification of *L.salivarius bsh1* which grouped with other *L.salivarius bsh* genes that had previously been predicted on a mega-plasmid, corroborating the earlier finding of a cholylglycine hydrolase protein encoded on a GI in CUL61 and suggesting that this GI is a well-described mega-plasmid of the *L.salivarius* species (Fang et al., 2009).

To fully evaluate the genotype-phenotype relationship between predicted BSH proteins and BSH activity, CUL66N was incubated for 1 h with bovine bile and *bsh* gene expression was quantified by qPCR. Indeed, each predicted homolog was induced in the presence of bile, indicating a well-correlated genome-guided analysis of *bsh* activity in CUL strains.

# Chapter 7. Conclusions and Future Work

## 7.1. Summary

In summary, the work presented in this thesis provides a robust classification and genomic overview of CUL strains.

16S rRNA appears to be suitable for genus-level designation of members of the lactobacilli and bifidobacteria genera. However, low bootstrap values indicate the need for a more robust multi-locus approach to supply strain-level taxonomy. Probiotic functions are often strain-specific and therefore the need for WGS of CUL isolates was evident. MLSA using 400 ubiquitous protein markers and all available genomes for each CUL genera allowed the taxonomic resolution to be increased from the initial 16S rRNA analysis and indicated that each CUL strain was a novel entry into the NCBI database.

Furthermore, detailed genome mining revealed a strong probiotic potential of CUL strains, where beneficial traits including bile and acid tolerance, cellular adhesion and host interactions were identified and described.

In addition, using a clade-guided approach, probiotic traits described in relatives of CUL strains also enabled targeted scrutiny of CUL genomes, including bacteriocin prediction in *L.gasseri* CUL09, hydrogen peroxide production potential in *L.acidophilus* CUL21 and CUL60, adhesion capabilities in *L.helveticus* CUL76 and an intact acid tolerance operon in *B.animalis* subsp. *lactis* CUL34 and a magnitude of genomic regions encoding traits such as adherence, acid and bile tolerance in *L.paracasei* CUL08. In addition, CUL08 was also predicted to encode an intact EPS cluster, with a drug transporting potential (Raveendran et al., 2013). BAGEL4 analysis facilitated the prediction of bacteriocins with potential antimicrobial properties in 11 out of the 16 CUL strains. *L.helveticus* CUL76 was predicted to encode 5 bacteriocins, indicating that this strain should be further characterised to deduce its antimicrobial potential. Several cholylglycine hydrolase proteins were identified in CUL genomes, offering a genome-guided route to analyse bile tolerance functionality and cholesterol-reducing capabilities of CUL strains, given its putative role in both functions (Begley et al., 2006). All CUL strains were phenotypically tolerant across a physiological gradient of bile, except for *L.fermentum* strains. Interestingly, all CUL strains were predicted to encode Glucosamine -6- phosphate deaminase, a gene with a previously documented role in bile tolerance (Alcántara & Zúñiga, 2012). *L.fermentum* strains were the only exception to this trend, indicating that this gene may contribute to the bile tolerance seen in CUL strains and future work should aim to corroborate such links. Following, phenotypic testing revealed putative BSH activity in CUL strains, although several variables were present including, substrate preference, concentration preference and visual bias depending on how precipitates are manually visualised (light box vs. lab bench light). Furthermore, it was evident that utilising the typically reported concentration of TDCA (0.5 %) was an over-representation of concentrations seen *in vivo.*

Indeed, in some cases BSH phenotypes generated by CUL strains were seen earlier in a concentration gradient and disappeared at 0.5 %, highlighting an issue in the current methodology, as BSH reactions may be missed when utilizing the wrong concentration. The presence of multiple *bsh* homologs was predicted in some CUL strains including, *L.plantarum (*CUL66 and CUL66N*)*, *L.salivarius (*CUL61*)* and *L.acidophilus* (CUL21 and CUL60*)*. Indeed, in the case of *L.salivarius* CUL61, the presence of a BSH protein-encoding gene on a GI was initially predicted. Following phylogenetic analysis, one *L.salivarius bsh* copy was clustered with other *L.salivarius* plasmid copies, indicating *L.salivarius* CUL61 encodes a well-described mega-plasmid (Fang et al., 2009), which should be extracted and sequenced to deduce its genomic composition.

Previous reports have correlated the presence of multiple *bsh* copies with a BA substrate preference. Therefore, both TDCA and GDCA were employed in all assays and revealed a greater toxicity in comparison to its counterpart, shown by the death of CUL strains. Interestingly, precipitation phenotypes varied and included three versions, in agar plug, an on the agar precipitate and a scattering in agar precipitate. However, GC/MS did not reveal any variations in BA profiles between the in agar and on agar precipitations, only allowing the identification of a deconjugation event. The ability of lactic acid to influence precipitation was also analysed, given lactobacilli strains well established lactic acid production capabilities. A precipitate phenotype was observed when lactic acid isomers were challenged with GDCA (interestingly not TDCA) indicating an interaction between lactic acid and GDCA. Indeed, GC/MS revealed a lactic acid modification of GDCA which was absent from the control (visualised by an additional peak), which should be further characterised, with improved methodology (such as Liquid Chromatography/ Mass Spectrophotometry).

*L.plantarum* strains CUL66 and CUL66N and *L.gasseri* CUL09 showed a capacity to reduce conjugated BAs from a BA pool and reduce free cholesterol as revealed by GC/MS analysis. *L.plantarum* strains were initially predicted to encode 3 *bsh* sequences and were both phenotypically active on plate assays. Targeted gene expression revealed that the genes identified during genome mining were induced in the presence of bile, indicating a successful genome-guided approach to bioprospecting CUL strains and the potential cholesterol-reducing capabilities of CUL strains, which should be further investigated by global transcriptomics.

Additionally, traits that may be considered deleterious were also identified during initial genome mining. The presence of phages and genomic islands were predicted in all genomes, both of which have associated HGT mechanisms. Indeed, *L.fermentum* strains (CUL67 and CUL40) were predicted to encode several putative ARGS on GIs. In addition, *L.salivarius* CUL61 putatively encodes a mega-plasmid. Phage elements are frequently encountered

within lactobacilli genomes (Villion & Moineau, 2009) and as many lactobacilli species have QPS status (EFSA, 2008), provides putative reassurance when assessing the safety of phages. RAST annotation revealed nonspecific ARG annotation in CUL genomes.

AMR is a rising global health concern and as putative ARGs were predicted MIC testing was conducted to evaluate the potential of CUL organisms to contribute to the gut resistome. Phenotypic antibiotic testing was conducted 5-fold higher (1:200 dilution) than typically recommended by EFSA (1:1000 dilution), to ensure reliable growth and a more physiological representation of the concentrations consumed as a supplement.

The MIC of CUL strains when challenged with 13 antibiotics was evaluated. As such, all strains were sensitive to clindamycin and gentamycin. Where breakpoints were described, all strains were sensitive to vancomycin. Further analysis revealed a substitution in the DDL protein(from an F to a Y), allowed the identification of vancomycin sensitive strains from the tolerant strains, a phenomenon reported elsewhere (Campedelli et al., 2019) For streptomycin and erythromycin, only one strain was resistant to each (*L.acidophilus* CUL21 and *L.acidophilus* CUL60 respectively). The majority of CUL isolates were sensitive to tetracycline, however *L.paracasei* CUL07, *L.acidophilus* CUL21 and CUL60 and *B.animalis* subsp. *lactis* CUL34 displayed resistance profiles. The phenotypic resistance profile exhibited by CUL34 correlates well with predictions that a tetracycline resistance gene (*tetW*) was encoded within the genome from previous analysis, indicating this gene is functional and is switched on in response to tetracycline exposure.

Using breakpoints identified in the literature, 47 % (8 out of 17 tested) of strains exhibited resistance profiles to oxacillin. A wide range of sensitivities was documented for penicillin G (0.0625 - 32 µg/mL). 59 % of CUL strains were resistant to chloramphenicol, 57 % to kanamycin, 71 % to neomycin and 82 % to ampicillin.

Overall, the method selection made when analysing the MIC appears to be of great importance to the subsequent results. When comparing methods there was no true consistency where all isolates produced the same MIC regardless of method. Interestingly, variations were not always an increased resistance, at high bacterial concentrations. For example, in the case of *L.acidophilus* CUL21's response to neomycin when challenged via the two methods. Despite the increased cell density starting volume when testing via AD, the MIC decreases on agar compared to in broth (from 16 µg/mL – 0.5 µg/mL).

When correlating genotype and phenotype, *L.fermentum* CUL40 and CUL67 and *L.plantarum* strain CUL66 and CUL66N both presented phenotypic chloramphenicol resistance and were predicted to encode ARGs conferring such resistance. *B.animalis* subsp. *lactis* CUL34 was predicted to encode a *kat* gene related to kanamycin resistance and exhibited high tolerance to kanamycin phenotypically. *L.acidophilus* strains were each predicted to encode 4 genes

related to aminoglycoside resistance, which correlated to resistance in different drugs from the aminoglycoside class (streptomycin resistance for CUL21 and neomycin resistance for CUL60). Genes linked with tetracycline resistance were predicted in all CUL genomes but only correlated with a resistant phenotype in *L.acidophilus* strains CUL21 and CUL60, *L.paracasei* CUL07 and *B.animalis* subsp. *lactis* CUL34. However future work should aim at validating the correlation of genotype and phenotype with targeted gene expression analysis.

To deduce the impact of physiological stress would have on AMR profiles, antibiotic assays were conducted in the presence of 0.3 % bile. Interestingly in some cases, the presence of bile resulted in an increased AMR profile, as seen in *L.helveticus* CUL76 when challenged with vancomycin and chloramphenicol and in *L.salivarius* CUL61 when challenged with chloramphenicol, suggesting a potential beneficial interaction between some CUL strains and bile.

## 7.2. Conclusion

This study provides a robust multifaceted taxonomic analysis of CUL strains. Each CUL strain was shown to be a novel member of the lactobacilli and bifidobacteria genera. MLSA analysis revealed strong evolutionary relationships between CUL strains and previously described health-promoting organisms. Genome mining enabled a broad overview and genomic profile of features considered beneficial and deleterious to probiotic candidates, which allowed further targeted mining and phenotypic testing.

The ABR profiles of CUL strains were established, however the correlation between phenotype and genotype was relatively low, a phenomenon previously described (Campedelli et al., 2019). Nevertheless, the presence of a *tetW* gene in *B.animalis* subsp. *lactis* CUL34 was predicted via a magnitude of methods, including RAST annotation, GI prediction and CARD and correlated with a phenotypic resistance profile. Generally, most resistance characterised in lactobacilli by EFSA guidance was linked to well-established lactobacilli resistance profiles, for example, kanamycin (Abriouel et al., 2015; Štšepetova et al., 2017). Perhaps most surprisingly was a high degree of resistance in lactobacilli CUL strains to ampicillin, however, a genomic basis for such tolerance was not established. The substitution of F to Y in the DDL protein was also shown to be a good indicator for vancomycin-sensitive lactobacilli strains. The impact of bile on ABR profiles generally caused a shift from resistant to sensitive, however, intriguingly *L.helveticus* CUL76 and *L.salivarius* CUL61 saw enhanced resistance capabilities, suggesting a positive interaction with bile.

Genome mining presented an overview of putative health promoting and functional traits of probiotic bacteria. Indeed, several genes involved in adherence, acid and bile tolerance and host interactions were predicted in all CUL genomes, which provides a strong basis for future work to characterise such traits. Furthermore, several bacteriocins were predicted in the majority of CUL strains. Indeed, *L.helveticus* CUL76 putatively encoded five bacteriocins, again offering scope for future antimicrobial studies on this strain. Cholylglycine hydrolase homologs were identified in several CUL species and offered the potential to use a genome-guided approach to characterise bile tolerance and cholesterol-lowering capabilities. *L.plantarum* CUL66N revealed a strong capacity to express *bsh* genes and reduce the BA pool generated *in vitro*. In addition, free cholesterol was also diminished in the bile pool, strongly suggesting a *bsh*-mediated cholesterol lowering capability of CUL strains.

In summary, the bacteria analysed in this study were all members of the lactobacilli and bifidobacteria genera. WGS enabled the robust classification of isolates and facilitated a genome-guided approach to evaluate the safety and functionality of strains. Many traits were revealed that could each uniquely be focused on for future work, such as phage compositions, ARG gene expression and transcriptome analysis of CUL66N when challenged with bile.

The research presented here provides a strong basis to facilitate a full evaluation of the safety and functionality of CUL strains as probiotic bacteria.

**Appendix 6.**

**Link 1.**

Baker et al 2021.pdf

**Link 2.**

Webberley et al 2021.pdf

## References

**Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J**. (2014). The Placenta Harbors a Unique Microbiome. *Science Translational Medicine*, *6*(237), 237ra65. https://doi.org/10.1126/scitranslmed.3008599

**Aaron, J. G., Sobieszczyk, M. E., Weiner, S. D., Whittier, S., & Lowy, F. D.** (2017). Lactobacillus rhamnosus Endocarditis After Upper Endoscopy. *Open Forum Infectious Diseases*, *4*(2). https://doi.org/10.1093/ofid/ofx085

**Abriouel, H., Casado Muñoz, M. del C., Lavilla Lerma, L., Pérez Montoro, B., Bockelmann, W., Pichner, R., Kabisch, J., Cho, G.S., Franz, C. M. A. P., Gálvez, A., & Benomar, N.** (2015). New insights in antibiotic resistance of Lactobacillus species from fermented foods. *Food Research International*, *78*, 465–481. https://doi.org/10.1016/j.foodres.2015.09.016

**Aguilar-Toalá, J. E., Garcia-Varela, R., Garcia, H. S., Mata-Haro, V., González-Córdova, A. F., Vallejo-Cordoba, B., & Hernández-Mendoza, A.** (2018). Postbiotics: An evolving term within the functional foods field. *Trends in Food Science & Technology*, *75*, 105–114. https://doi.org/10.1016/j.tifs.2018.03.009

**Ahirwar, S., Gupta, M., Gupta, G., & Singh, V.** (2017). Screening, Isolation and Identification of Lactobacillus Species from Dental Caries of Children. *International Journal of Current Microbiology and Applied Sciences*, *6*, 497–503. https://doi.org/10.20546/ijcmas.2017.601.059

**Ahmed, M. O., & Baptiste, K. E.** (2018). Vancomycin-Resistant Enterococci: A Review of Antimicrobial Resistance Mechanisms and Perspectives of Human and Animal Health. *Microbial Drug Resistance*, *24*(5), 590–606. https://doi.org/10.1089/mdr.2017.0147

**Aires, J., Doucet-Populaire, F., & Butel, M. J.** (2007). Tetracycline Resistance Mediated by tet(W), tet(M), and tet(O) Genes of Bifidobacterium Isolates from Humans. *Applied and Environmental Microbiology*, *73*(8), 2751–2754. https://doi.org/10.1128/AEM.02459-06

**Aktas, B., Wolfe, T. J. D., Safdar, N., Darien, B. J., & Steele, J. L**. (2016). The Impact of Lactobacillus casei on the Composition of the Cecal Microbiota and Innate Immune System Is Strain Specific. *PLOS ONE*, *11*(5), e0156374. https://doi.org/10.1371/journal.pone.0156374

**Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., & Wright, A. von.** (1999). Persistence of Colonization of Human Colonic Mucosa by a Probiotic Strain, Lactobacillus rhamnosus GG, after Oral Consumption. *Applied and Environmental Microbiology*, *65*(1), 351.

**Alanis, A. J.** (2005). Resistance to Antibiotics: Are We in the Post-Antibiotic Era? *Archives of Medical Research*, *36*(6), 697–705. https://doi.org/10.1016/j.arcmed.2005.06.009

**Alcántara, C., Revilla-Guarinos, A., & Zúñiga, M.** (2011). Influence of Two-Component Signal Transduction Systems of Lactobacillus casei BL23 on Tolerance to Stress Conditions. *Applied and Environmental Microbiology*, *77*(4), 1516–1519. https://doi.org/10.1128/AEM.02176-10

**Alcántara, C., & Zúñiga, M.** (2012). Proteomic and transcriptomic analysis of the response to bile stress of Lactobacillus casei BL23. *Microbiology*, *158*(5), 1206–1218. https://doi.org/10.1099/mic.0.055657-0

**Ale, E. C., Perezlindo, M. J., Burns, P., Tabacman, E., Reinheimer, J. A., & Binetti, A. G.** (2016). Exopolysaccharide from Lactobacillus fermentum Lf2 and its functional characterization as a yogurt additive. *Journal of Dairy Research*, *83*(4), 487–492. https://doi.org/10.1017/S0022029916000571

**Alekshun, M. N., & Levy, S. B.** (2007). Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell*, *128*(6), 1037–1050. https://doi.org/10.1016/j.cell.2007.03.004

**Alexander, J. L., Wilson, I. D., Teare, J., Marchesi, J. R., Nicholson, J. K., & Kinross, J. M**. (2017). Gut microbiota modulation of chemotherapy efficacy and toxicity. *Nature Reviews Gastroenterology & Hepatology*, *14*(6), 356–365. https://doi.org/10.1038/nrgastro.2017.20

**Alimolaei, M., & Golchin, M.** (2017). A comparison of methods for extracting plasmids from a difficult to lyse bacterium: Lactobacillus casei. *Biologicals*, *45*, 47–51. https://doi.org/10.1016/j.biologicals.2016.10.001

**Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., & Handelsman, J.** (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nature Reviews Microbiology*, *8*(4), 251–259. https://doi.org/10.1038/nrmicro2312

**Almquist, E.** (1922). Variation and Life Cycles of Pathogenic Bacteria. *The Journal of Infectious Diseases*, *31*(5), 483–493.

**Álvarez-Cisneros, Y. M., & Ponce-Alquicira, E.** (2018). *Antibiotic resistance in lactic acid bacteria.* IntechOpen.

**Amabile-Cuevas, C. F.** (2016). *Antibiotics and Antibiotic Resistance in the Environment*. CRC Press. https://doi.org/10.1201/b19549

**Amann, R. I., Ludwig, W., & Schleifer, K. H.** (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, *59*(1), 143–169.

**Aminlari, L., Shekarforoush, S. S., Hosseinzadeh, S., Nazifi, S., Sajedianfard, J., & Eskandari, M. H.** (2019). Effect of Probiotics Bacillus coagulans and Lactobacillus plantarum on Lipid Profile and Feces Bacteria of Rats Fed Cholesterol-Enriched Diet. *Probiotics and Antimicrobial Proteins*, *11*(4), 1163–1171. https://doi.org/10.1007/s12602-018-9480-1

**Ammor, M. S., Belén Flórez, A., & Mayo, B**. (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology*, *24*(6), 559–570. https://doi.org/10.1016/j.fm.2006.11.001

**Ammor, M. S., Flórez, A. B., Álvarez-Martín, P., Margolles, A., & Mayo, B.** (2008). Analysis of tetracycline resistance tet(W) genes and their flanking sequences in intestinal Bifidobacterium species. *Journal of Antimicrobial Chemotherapy*, *62*(4), 688–693. https://doi.org/10.1093/jac/dkn280

**Ammor, M. S., Flórez, A. B., Hoek, A. H. A. M. van, Reyes-Gavilán, C. G. de los, Aarts, H. J. M., Margolles, A., & Mayo, B.** (2008). Molecular Characterization of Intrinsic and Acquired Antibiotic Resistance in Lactic Acid Bacteria and Bifidobacteria. *Microbial Physiology*, *14*(1–3), 6–15. https://doi.org/10.1159/000106077

**Amund, O. D.** (2016). Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of lactobacilli and bifidobacteria. *Canadian Journal of Microbiology*, *62*(9), 715–725. https://doi.org/10.1139/cjm-2016-0186

**Amyes, S. G. B.** (2007). Enterococci and streptococci. *International Journal of Antimicrobial Agents*, *29*, S43–S52. https://doi.org/10.1016/S0924-8579(07)72177-5

**Anandharaj, M., Sivasankari, B., & Parveen Rani, R**. (2014). Effects of Probiotics, Prebiotics and Synbiotics on Hypercholesterolemia: A Review. *Chinese Journal of Biology*, *2014*, 1–7. https://doi.org/10.1155/2014/572754

**Anderson, E. S.** (1968). Drug Resistance in Salmonella Typhimurium and its Implications. *Br Med J*, *3*(5614), 333–339. https://doi.org/10.1136/bmj.3.5614.333

**Anderson, R. C., Cookson, A. L., McNabb, W. C., Park, Z., McCann, M. J., Kelly, W. J., & Roy, N. C.** (2010). Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiology*, *10*(1), 316. https://doi.org/10.1186/1471-2180-10-316

**Andersson, D. I., Balaban, N. Q., Baquero, F., Courvalin, P., Glaser, P., Gophna, U., Kishony, R., Molin, S., & Tønjum, T.** (2020). Antibiotic resistance: Turning evolutionary principles into clinical reality. *FEMS Microbiology Reviews*, *44*(2), 171–188. https://doi.org/10.1093/femsre/fuaa001

**Anisimova, E., & Yarullina, D.** (2018). Characterization of Erythromycin and Tetracycline Resistance in Lactobacillus fermentum Strains. *International Journal of Microbiology*, *2018*, e3912326. https://doi.org/10.1155/2018/3912326

**Anukam, K. C., & Reid, G.** (2007). *Probiotics: 100 years (1907-2007) after Elie Metchnikoff's Observation*.

**Aoki, T., Egusa, S., Ogata, Y., & Watanabe, T. Y.** (1971). Detection of Resistance Factors in Fish Pathogen Aeromonas liquefaciens. *Microbiology*, *65*(3), 343–349. https://doi.org/10.1099/00221287-65-3-343

**Araya, M., Morelli, L., Reid, G., Sanders, M., Stanton, C., Pineiro, M., & Embarek, P.** (2002). Joint FAO/WHO Working Group report on drafting guidelines for the evaluation of probiotics in food. London, Canada: World Health Organization, *Food and Agriculture Organization of the United Nations.*

**Archer, A. C., & Halami, P. M.** (2015). Probiotic attributes of Lactobacillus fermentum isolated from human feces and dairy products. *Applied Microbiology and Biotechnology*, *99*(19), 8113–8123. https://doi.org/10.1007/s00253-015-6679-x

**Arena, M. P., Fiocco, D., Massa, S., Capozzi, V., Russo, P., & Spano, G.** (2014). Lactobacillus plantarum as a strategy for an in situ production of vitamin B2. *J. Food. Nutr. Disord*, *1*, 4. http://dx.doi.org/10.4172/2324-9323.S1-004

**Argyri, A. A., Zoumpopoulou, G., Karatzas, K.-A. G., Tsakalidou, E., Nychas, G.-J. E., Panagou, E. Z., & Tassou, C. C.** (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiology*, *33*(2), 282–291. https://doi.org/10.1016/j.fm.2012.10.005

**Arnold, J. W., Simpson, J. B., Roach, J., Kwintkiewicz, J., & Azcarate-Peril, M. A.** (2018). Intra-species Genomic and Physiological Variability Impact Stress Resistance in Strains of Probiotic Potential. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.00242

**Arseneault-Bréard, J., Rondeau, I., Gilbert, K., Girard, S.-A., Tompkins, T. A., Godbout, R., & Rousseau, G.** (2012). Combination of Lactobacillus helveticus R0052 and Bifidobacterium longum R0175 reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. *British Journal of Nutrition*, *107*(12), 1793–1799. https://doi.org/10.1017/S0007114511005137

**Ashelford, K. E., Chuzhanova, N. A., Fry, J. C., Jones, A. J., & Weightman, A. J.** (2005). At Least 1 in 20 16S rRNA Sequence Records Currently Held in Public Repositories Is Estimated to Contain Substantial Anomalies. *Applied and Environmental Microbiology*, *71*(12), 7724–7736. https://doi.org/10.1128/AEM.71.12.7724-7736.2005

**Ashraf, R., & Shah, N.** (2011). Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. *International Food Research Journal*, *18*, 837–853.

**Ávila, C. L. S., Carvalho, B. F., Pinto, J. C., Duarte, W. F., & Schwan, R. F.** (2014). The use of Lactobacillus species as starter cultures for enhancing the quality of sugar cane silage. *Journal of Dairy Science*, *97*(2), 940–951. https://doi.org/10.3168/jds.2013-6987

**Awd, A., Ismail, A., & Abdelfatah Ahmed Khater, K.** (2020). Identification and Phylogenetic Studies of a New Probiotic Lactobacillus spp. Egyptian Isolate Based on 16S rRNA Gene. *Acta Scientific Nutritional Health*, *4*. https://doi.org/10.31080/ASNH.2020.04.0654

**Aymerich, T., Martin, B., Garriga, M., Vidal-Carou, M. C., Bover-Cid, S., & Hugas, M.** (2006). Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *Journal of Applied Microbiology*, *100*(1), 40–49. https://doi.org/10.1111/j.1365-2672.2005.02772.x

**Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., … Zagnitko, O.** (2008). The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics*, *9*(1), 75. https://doi.org/10.1186/1471-2164-9-75

**Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I.** (2005). Host-Bacterial Mutualism in the Human Intestine. *Science*, *307*(5717), 1915–1920. https://doi.org/10.1126/science.1104816

**Baker, L. M., Webberley, T. S., Masetti, G., Hughes, T. R., Marchesi, J. R., Jack, A. A., Joyce, T. S. C., Allen, M. D., Plummer, S. F., Michael, D. R., Ramanathan, G., Del Sol, R., & Facey, P. D.** (2021). A genome guided evaluation of the Lab4 probiotic consortium. *Genomics*, *113*(6), 4028–4038. https://doi.org/10.1016/j.ygeno.2021.08.007

**Balcazar, J. L.** (2014). Bacteriophages as Vehicles for Antibiotic Resistance Genes in the Environment. *PLOS Pathogens*, *10*(7), e1004219. https://doi.org/10.1371/journal.ppat.1004219

**Balouiri, M., Sadiki, M., & Ibnsouda, S. K.** (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

**Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A.** (2012). SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *Journal of Computational Biology*, *19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

**Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., Wang, Y., & Zhang, H.** (2010). Screening of potential probiotic properties of Lactobacillus fermentum isolated from traditional dairy products. *Food Control*, *21*(5), 695–701. https://doi.org/10.1016/j.foodcont.2009.10.010

**Barefoot, S. F., & Klaenhammer, T. R.** (1983). Detection and activity of lactacin B, a bacteriocin produced by Lactobacillus acidophilus. *Applied and Environmental Microbiology*, *45*(6), 1808–1815. https://doi.org/10.1128/aem.45.6.1808-1815.1983

**Bartkiene, E., Zavistanaviciute, P., Lele, V., Ruzauskas, M., Bartkevics, V., Bernatoniene, J., Gallo, P., Tenore, G. C., & Santini, A.** (2018). Lactobacillus plantarum LUHS135 and paracasei LUHS244 as functional starter cultures for the food fermentation industry: Characterisation, mycotoxin-reducing properties, optimisation of biomass growth and sustainable encapsulation by using dairy by-products. *LWT*, *93*, 649–658. https://doi.org/10.1016/j.lwt.2018.04.017

**Bassetti, M., Vena, A., Croxatto, A., Righi, E., & Guery, B.** (2018). How to manage Pseudomonas aeruginosa infections. *Drugs in Context*, *7*, 212527. https://doi.org/10.7573/dic.212527

**Bauer, K. C., Huus, K. E., & Finlay, B. B.** (2016). Microbes and the mind: Emerging hallmarks of the gut microbiota–brain axis. *Cellular Microbiology*, *18*(5), 632–644. https://doi.org/10.1111/cmi.12585

**Baugher, J. L., Durmaz, E., & Klaenhammer, T. R.** (2014). Spontaneously Induced Prophages in Lactobacillus gasseri Contribute to Horizontal Gene Transfer. *Applied and Environmental Microbiology*, *80*(11), 3508. https://doi.org/10.1128/AEM.04092-13

**Bazireh, H., Shariati, P., Azimzadeh Jamalkandi, S., Ahmadi, A., & Boroumand, M. A.** (2020). Isolation of Novel Probiotic Lactobacillus and Enterococcus Strains from Human Salivary and Fecal Sources. *Frontiers in Microbiology*, *0*. https://doi.org/10.3389/fmicb.2020.597946

**Beatrice, T., Francesca, P., Barbara, T., Filippo, F., & Roberta, N.** (2018). Qualitative and quantitative evaluation of biogenic amines in vitro production by bacteria isolated from ewes' milk cheeses. *European Food Research and Technology*, *244*(4), 721–728. https://doi.org/10.1007/s00217-017-2992-1

**Beck, B. R., Kim, D., Jeon, J., Lee, S.-M., Kim, H. K., Kim, O.-J., Lee, J. I., Suh, B. S., Do, H. K., Lee, K. H., Holzapfel, W. H., Hwang, J. Y., Kwon, M. G., & Song, S. K.** (2015). The effects of combined dietary probiotics Lactococcus lactis BFE920 and Lactobacillus plantarum FGL0001 on innate immunity and disease resistance in olive flounder (Paralichthys olivaceus). *Fish & Shellfish Immunology*, *42*(1), 177–183. https://doi.org/10.1016/j.fsi.2014.10.035

**Becq, J., Gutierrez, M. C., Rosas-Magallanes, V., Rauzier, J., Gicquel, B., Neyrolles, O., & Deschavanne, P.** (2007). Contribution of Horizontally Acquired Genomic Islands to the Evolution of the Tubercle Bacilli. *Molecular Biology and Evolution*, *24*(8), 1861–1871. https://doi.org/10.1093/molbev/msm111

**Beena Divya, J., Kulangara Varsha, K., Madhavan Nampoothiri, K., Ismail, B., & Pandey, A.** (2012). Probiotic fermented foods for health benefits. *Engineering in Life Sciences*, *12*(4), 377–390. https://doi.org/10.1002/elsc.201100179

**Begley, M., Gahan, C. G. M., & Hill, C.** (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews*, *29*(4), 625–651. https://doi.org/10.1016/j.femsre.2004.09.003

**Begley, M., Hill, C., & Gahan, C. G. M**. (2006). Bile Salt Hydrolase Activity in Probiotics. *Applied and Environmental Microbiology*, *72*(3), 1729–1738. https://doi.org/10.1128/AEM.72.3.1729-1738.2006

**Begley, M., Sleator, R. D., Gahan, C. G. M., & Hill, C.** (2005). Contribution of Three Bile-Associated Loci, bsh, pva, and btlB, to Gastrointestinal Persistence and Bile Tolerance of Listeria monocytogenes. *Infection and Immunity*, *73*(2), 894–904. https://doi.org/10.1128/IAI.73.2.894-904.2005

**Bellentani, S., Pecorari, M., Cordoma, P., Marchegiano, P., Manenti, F., Bosisio, E., De Fabiani, E., & Galli, G.** (1987). Taurine increases bile acid pool size and reduces bile saturation index in the hamster. *Journal of Lipid Research*, *28*(9), 1021–1027. https://doi.org/10.1016/S0022-2275(20)38617-X

**Belletti, N., Gatti, M., Bottari, B., Neviani, E., Tabanelli, G., & Gardini, F.** (2009). Antibiotic Resistance of Lactobacilli Isolated from Two Italian Hard Cheeses. *Journal of Food Protection*, *72*(10), 2162–2169. https://doi.org/10.4315/0362-028X-72.10.2162

**Bendali, F., Kerdouche, K., Hamma-Faradji, S., & Drider, D.** (2017). In vitro and in vivo cholesterol lowering ability of Lactobacillus pentosus KF923750. *Beneficial Microbes*, *8*(2), 271–280. https://doi.org/10.3920/BM2016.0121

**Benga, L., Benten, W. P. M., Engelhardt, E., Köhrer, K., Gougoula, C., & Sager, M**. (2014). 16S rRNA ribosomal DNA sequence-based identification of bacteria in laboratory rodents: A practical approach in laboratory animal bacteriology diagnostics. *Laboratory Animals*, *48*(4), 305–312. https://doi.org/10.1177/0023677214538240

**Bengoa, A. A., Dardis, C., Gagliarini, N., Garrote, G. L., & Abraham, A. G**. (2020). Exopolysaccharides From Lactobacillus paracasei Isolated From Kefir as Potential Bioactive Compounds for Microbiota Modulation. *Frontiers in Microbiology*, *11*, 2567. https://doi.org/10.3389/fmicb.2020.583254

**Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., Rapp, B. A., & Wheeler, D. L.** (2000). GenBank. *Nucleic Acids Research*, *28*(1), 15–18. https://doi.org/10.1093/nar/28.1.15

**Bernardeau, M., Vernoux, J. P., Henri-Dubernet, S., & Guéguen, M.** (2008). Safety assessment of dairy microorganisms: The Lactobacillus genus. *International Journal of Food Microbiology*, *126*(3), 278–285. https://doi.org/10.1016/j.ijfoodmicro.2007.08.015

**Bertelli, C., Laird, M. R., Williams, K. P., Fraser University Research Computing Group, S., Lau, B. Y., Hoad, G., Winsor, G. L., & Brinkman, F. S.** (2017). IslandViewer 4: Expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Research*, *45*(W1), W30–W35. https://doi.org/10.1093/nar/gkx343

**Bhat, B., & Bajaj, B. K.** (2019). Hypocholesterolemic potential and bioactivity spectrum of an exopolysaccharide from a probiotic isolate Lactobacillus paracasei M7. *Bioactive Carbohydrates and Dietary Fibre*, *19*, 100191. https://doi.org/10.1016/j.bcdf.2019.100191

**Bhat, M. I., Singh, V. K., Sharma, D., Kapila, S., & Kapila, R.** (2019). Adherence capability and safety assessment of an indigenous probiotic strain Lactobacillus rhamnosus MTCC-5897. *Microbial Pathogenesis*, *130*, 120–130. https://doi.org/10.1016/j.micpath.2019.03.009

**Bian, X., Evivie, S. E., Muhammad, Z., Luo, G.-W., Liang, H.Z., Wang, N.N., & Huo, G.C.** (2016). In vitro assessment of the antimicrobial potentials of Lactobacillus helveticus strains isolated from traditional cheese in Sinkiang China against food-borne pathogens. *Food & Function*, *7*(2), 789–797. https://doi.org/10.1039/C5FO01041A

**Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., Tremblay, A., & Ouwehand, A. C.** (2020). Criteria to Qualify Microorganisms as "Probiotic" in Foods and Dietary Supplements. *Frontiers in Microbiology*, *11*, 1662. https://doi.org/10.3389/fmicb.2020.01662

**Binetti, A. G., Capra, M. L., Álvarez, M. A., & Reinheimer, J. A.** (2008). PCR method for detection and identification of Lactobacillus casei/paracasei bacteriophages in dairy products. *International Journal of Food Microbiology*, *124*(2), 147–153. https://doi.org/10.1016/j.ijfoodmicro.2008.03.006

**Binnewies, T. T., Motro, Y., Hallin, P. F., Lund, O., Dunn, D., La, T., Hampson, D. J., Bellgard, M., Wassenaar, T. M., & Ussery, D. W.** (2006). Ten years of bacterial genome sequencing: Comparative-genomics-based discoveries. *Functional & Integrative Genomics*, *6*(3), 165–185. https://doi.org/10.1007/s10142-006-0027-2

**Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. V.** (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, *13*(1), 42–51. https://doi.org/10.1038/nrmicro3380

**Bogovič Matijašić, B., Narat, M., Zorič Peternel, M., & Rogelj, I.** (2006). Ability of Lactobacillus gasseri K7 to inhibit Escherichia coli adhesion in vitro on Caco-2 cells and ex vivo on pigs' jejunal tissue. *International Journal of Food Microbiology*, *107*(1), 92–96. https://doi.org/10.1016/j.ijfoodmicro.2005.08.017

**Bordenstein, S. R., & Theis, K. R.** (2015). Host Biology in Light of the Microbiome: Ten Principles of Holobionts and Hologenomes. *PLOS Biology*, *13*(8), e1002226. https://doi.org/10.1371/journal.pbio.1002226

**Bordoni, A., Amaretti, A., Leonardi, A., Boschetti, E., Danesi, F., Matteuzzi, D., Roncaglia, L., Raimondi, S., & Rossi, M.** (2013). Cholesterol-lowering probiotics: In vitro selection and in vivo testing of bifidobacteria. *Applied Microbiology and Biotechnology*, *97*(18), 8273–8281. https://doi.org/10.1007/s00253-013-5088-2

**Borgia, S., Lastovetska, O., Richardson, D., Eshaghi, A., Xiong, J., Chung, C., Baqi, M., McGeer, A., Ricci, G., Sawicki, R., Pantelidis, R., Low, D. E., Patel, S. N., & Melano, R. G.** (2012). Outbreak of Carbapenem-Resistant Enterobacteriaceae Containing blaNDM-1, Ontario, Canada. *Clinical Infectious Diseases*, *55*(11), e109–e117. https://doi.org/10.1093/cid/cis737

**Botina, S. G., Poluektova, E. U., Glazova, A. A., Zakharevich, N. V., Koroban, N. V., Zinchenko, V. V., Babykin, M. M., Zhilenkova, O. G., Amerkhanova, A. M., & Danilenko, V. N.** (2011). Antibiotic resistance of potential probiotic bacteria of the genus Lactobacillus from human gastrointestinal microbiome. *Microbiology*, *80*(2), 164–171. https://doi.org/10.1134/S0026261711020032

**Botta, C., Langerholc, T., Cencič, A., & Cocolin, L.** (2014). In Vitro Selection and Characterization of New Probiotic Candidates from Table Olive Microbiota. *PLOS ONE*, *9*(4), e94457. https://doi.org/10.1371/journal.pone.0094457

**Bottacini, F., Morrissey, R., Esteban-Torres, M., James, K., van Breen, J., Dikareva, E., Egan, M., Lambert, J., van Limpt, K., Knol, J., O'Connell Motherway, M., & van Sinderen, D.** (2018). Comparative genomics and genotype-phenotype associations in Bifidobacterium breve. *Scientific Reports*, *8*(1), 10633. https://doi.org/10.1038/s41598-018-28919-4

**Bottacini, F., Ventura, M., van Sinderen, D., & O'Connell Motherway, M.** (2014). Diversity, ecology and intestinal function of bifidobacteria. *Microbial Cell Factories*, *13*(1), S4. https://doi.org/10.1186/1475-2859-13-S1-S4

**Bouvet, P., Ferraris, L., Dauphin, B., Popoff, M.-R., Butel, M. J., & Aires, J.** (2014). 16S rRNA Gene Sequencing, Multi locus Sequence Analysis, and Mass Spectrometry Identification of the Proposed New Species "Clostridium neonatale". *Journal of Clinical Microbiology*, *52*(12), 4129–4136. https://doi.org/10.1128/JCM.00477-14

**Bowler, P., Murphy, C., & Wolcott, R.** (2020). Biofilm exacerbates antibiotic resistance: Is this a current oversight in antimicrobial stewardship? *Antimicrobial Resistance & Infection Control*, *9*(1), 162. https://doi.org/10.1186/s13756-020-00830-6

**Brandt, K., Nethery, M. A., O'Flaherty, S., & Barrangou, R.** (2020). Genomic characterization of Lactobacillus fermentum DSM 20052. *BMC Genomics*, *21*, 1–13. Broaders, E., Gahan, C. G. M., & Marchesi, J. R. (2013). Mobile genetic elements of the human gastrointestinal tract. *Gut Microbes*, *4*(4), 271–280. https://doi.org/10.4161/gmic.24627

**Bron, P. A.** (2004). *The molecular response of Lactobacillus plantarum to intestinal passage and conditions*.

**Brüssow, H.** (2001). Phages of Dairy Bacteria. *Annual Review of Microbiology*, *55*(1), 283–303. https://doi.org/10.1146/annurev.micro.55.1.283.

**Bryan, C. S., John, J. F., Jr, Pai, M. S., & Austin, T. L.** (1985). Gentamicin vs Cefotaxime for Therapy of Neonatal Sepsis: Relationship to Drug Resistance. *American Journal of Diseases of Children*, *139*(11), 1086–1089. https://doi.org/10.1001/archpedi.1985.02140130024022

**Buchfink, B., Xie, C., & Huson, D. H.** (2015). Fast and sensitive protein alignment using DIAMOND. *Nature Methods*, *12*(1), 59–60. https://doi.org/10.1038/nmeth.3176

**Buck, B. L., Altermann, E., Svingerud, T., & Klaenhammer, T. R.** (2005). Functional Analysis of Putative Adhesion Factors in Lactobacillus acidophilus NCFM. *Applied and Environmental Microbiology*, *71*(12), 8344–8351. https://doi.org/10.1128/AEM.71.12.8344-8351.2005

**Buckley, M. L., & Ramji, D. P.** (2015). The influence of dysfunctional signaling and lipid homeostasis in mediating the inflammatory responses during atherosclerosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1852*(7), 1498–1510. https://doi.org/10.1016/j.bbadis.2015.04.011

**Bull, M., Plummer, S., Marchesi, J., & Mahenthiralingam, E**. (2013). The life history of Lactobacillus acidophilus as a probiotic: A tale of revisionary taxonomy, misidentification and commercial success. *FEMS Microbiology Letters*, *349*(2), 77–87. https://doi.org/10.1111/1574-6968.12293

**Burckhardt, R. M., & Escalante-Semerena, J. C**. (2017). In Bacillus subtilis, the SatA (Formerly YyaR) Acetyltransferase Detoxifies Streptothricin via Lysine Acetylation. *Applied and Environmental Microbiology*, *83*(21), e01590-17. https://doi.org/10.1128/AEM.01590-17

**Busk, P. K., Pilgaard, B., Lezyk, M. J., Meyer, A. S., & Lange, L**. (2017). Homology to peptide pattern for annotation of carbohydrate-active enzymes and prediction of function. *BMC Bioinformatics*, *18*(1), 214. https://doi.org/10.1186/s12859-017-1625-9

**Bustos, A. Y., Font de Valdez, G., Fadda, S., & Taranto, M. P.** (2018). New insights into bacterial bile resistance mechanisms: The role of bile salt hydrolase and its impact on human health. *Food Research International*, *112*, 250–262. https://doi.org/10.1016/j.foodres.2018.06.035

**Bustos, A. Y., Saavedra, L., de Valdez, G. F., Raya, R. R., & Taranto, M. P.** (2012). Relationship between bile salt hydrolase activity, changes in the internal pH and tolerance to bile acids in lactic acid bacteria. *Biotechnology Letters*, *34*(8), 1511–1518. https://doi.org/10.1007/s10529-012-0932-5

**Byrne, S. J., Butler, C. A., Reynolds, E. C., & Dashper, S. G**. (2018). Chapter 7—Taxonomy of Oral Bacteria. In V. Gurtler & J. T. Trevors (Eds.), *Methods in Microbiology* (Vol. 45, pp. 171–201). Academic Press. https://doi.org/10.1016/bs.mim.2018.07.001

**Cadieux, P., Wind, A., Sommer, P., Schaefer, L., Crowley, K., Britton, R. A., & Reid, G.** (2008). Evaluation of Reuterin Production in Urogenital Probiotic Lactobacillus reuteri RC-14. *Applied and Environmental Microbiology*, *74*(15), 4645–4649. https://doi.org/10.1128/AEM.00139-08

**Caggia, C., De Angelis, M., Pitino, I., Pino, A., & Randazzo, C. L.** (2015). Probiotic features of Lactobacillus strains isolated from Ragusano and Pecorino Siciliano cheeses. *Food Microbiology*, *50*, 109–117. https://doi.org/10.1016/j.fm.2015.03.010

**Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAuliffe, O., Sangrador-Vegas, A., Slattery, L., Fitzgerald, G. F., Beresford, T., & Ross, R. P.** (2008). Genome Sequence of Lactobacillus helveticus, an Organism Distinguished by Selective Gene Loss and Insertion Sequence Element Expansion. *Journal of Bacteriology*, *190*(2), 727–735. https://doi.org/10.1128/JB.01295-07

**Cammarota, G., Ianiro, G., Bibbò, S., & Gasbarrini, A.** (2014). Gut microbiota modulation: Probiotics, antibiotics or fecal microbiota transplantation? *Internal and Emergency Medicine*, *9*(4), 365–373. https://doi.org/10.1007/s11739-014-1069-4

**Campagne, J., Guichard, J. F., Moulhade, M. C., Kawski, H., & Maurier, F.** (2020). Lactobacillus endocarditis: A case report in France and literature review. *IDCases*, *21*, e00811. https://doi.org/10.1016/j.idcr.2020.e00811

**Campana, R., van Hemert, S., & Baffone, W.** (2017). Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. *Gut Pathogens*, *9*. https://doi.org/10.1186/s13099-017-0162-4

**Campedelli, I., Mathur, H., Salvetti, E., Clarke, S., Rea, M. C., Torriani, S., Ross, R. P., Hill, C., & O'Toole, P. W.** (2019). Genus-Wide Assessment of Antibiotic Resistance in Lactobacillus spp. *Applied and Environmental Microbiology*, *85*(1), e01738-18. https://doi.org/10.1128/AEM.01738-18

**Cano, P. G., Santacruz, A., Trejo, F. M., & Sanz, Y.** (2013). Bifidobacterium CECT 7765 improves metabolic and immunological alterations associated with obesity in high-fat diet-fed mice. *Obesity*, *21*(11), 2310–2321. https://doi.org/10.1002/oby.20330

**Cao, C., Wang, J., Liu, Y., Kwok, L.-Y., Zhang, H., & Zhang, W.** (2020). Adaptation of Lactobacillus plantarum to Ampicillin Involves Mechanisms That Maintain Protein Homeostasis. *MSystems*, *5*(1), e00853-19. https://doi.org/10.1128/mSystems.00853-19

**Capurso, L.** (2019). Thirty Years of Lactobacillus rhamnosus GG: A Review. *Journal of Clinical Gastroenterology*, *53*, S1. https://doi.org/10.1097/MCG.0000000000001170

**Casaburi, G., Smilowitz, J., Underwood, M., Vance, D., Duar, R., Contreras, L., & Frese, S. A.** (2019). Metagenomic analysis reveals reduction in antibiotic resistance genes in breastfed infants fed Bifidobacterium longum subsp. Infantis EVC001. *Pediatrics*, *144*(2), 659–659. https://doi.org/10.1542/peds.144.2_MeetingAbstract.659

**Casarotti, S. N., Carneiro, B. M., Todorov, S. D., Nero, L. A., Rahal, P., & Penna, A. L. B.** (2017). In vitro assessment of safety and probiotic potential characteristics of

Lactobacillus strains isolated from water buffalo mozzarella cheese. *Annals of Microbiology*, *67*(4), 289–301. https://doi.org/10.1007/s13213-017-1258-2

**Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F., & Kjelleberg, S.** (2007). Use of 16S rRNA and rpoB Genes as Molecular Markers for Microbial Ecology Studies. *Applied and Environmental Microbiology*, *73*(1), 278–288. https://doi.org/10.1128/AEM.01177-06

**Caselli, M., Cassol, F., Calò, G., Holton, J., Zuliani, G., & Gasbarrini, A.** (2013). Actual concept of 'probiotics': Is it more functional to science or business? *World Journal of Gastroenterology : WJG*, *19*(10), 1527–1540. https://doi.org/10.3748/wjg.v19.i10.1527

**Casén, C., Vebø, H. C., Sekelja, M., Hegge, F. T., Karlsson, M. K., Ciemniejewska, E., Dzankovic, S., Frøyland, C., Nestestog, R., Engstrand, L., Munkholm, P., Nielsen, O. H., Rogler, G., Simrén, M., Öhman, L., Vatn, M. H., & Rudi, K.** (2015). Deviations in human gut microbiota: A novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Alimentary Pharmacology & Therapeutics*, *42*(1), 71–83. https://doi.org/10.1111/apt.13236

**Casjens, S.** (2003). Prophages and bacterial genomics: What have we learned so far? *Molecular Microbiology*, *49*(2), 277–300. https://doi.org/10.1046/j.1365-2958.2003.03580.x

**Castro, M. P., Palavecino, N. Z., Herman, C., Garro, O. A., & Campos, C. A.** (2011). Lactic acid bacteria isolated from artisanal dry sausages: Characterization of antibacterial compounds and study of the factors affecting bacteriocin production. *Meat Science*, *87*(4), 321–329. https://doi.org/10.1016/j.meatsci.2010.11.006

**Chae, J. P., Valeriano, V. D., Kim, G.B., & Kang, D.K.** (2013). Molecular cloning, characterization and comparison of bile salt hydrolases from Lactobacillus johnsonii PF01. *Journal of Applied Microbiology*, *114*(1), 121–133. https://doi.org/10.1111/jam.12027

**Chaloner, G. L., Ventosilla, P., & Birtles, R. J.** (2011). Multi-Locus Sequence Analysis Reveals Profound Genetic Diversity among Isolates of the Human Pathogen Bartonella bacilliformis. *PLOS Neglected Tropical Diseases*, *5*(7), e1248. https://doi.org/10.1371/journal.pntd.0001248

**Chaplin, A. V., Efimov, B. A., Smeianov, V. V., Kafarskaia, L. I., Pikina, A. P., & Shkoporov, A. N.** (2015). Intraspecies Genomic Diversity and Long-Term Persistence of Bifidobacterium longum. *PLOS ONE*, *10*(8), e0135658. https://doi.org/10.1371/journal.pone.0135658

**Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K.** (1998). Antibiotic susceptibility of potentially probiotic Bifidobacterium isolates from the human gastrointestinal tract. *Letters in Applied Microbiology*, *26*(5), 333–337. https://doi.org/10.1046/j.1472-765x.1998.00342.x

**Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K.** (2000). Effect of Conjugated Bile Salts on Antibiotic Susceptibility of Bile Salt–Tolerant Lactobacillus and Bifidobacterium Isolates. *Journal of Food Protection*, *63*(10), 1369–1376. https://doi.org/10.4315/0362-028X-63.10.1369

**Chassard, C., Dapoigny, M., Scott, K. P., Crouzet, L., Del'homme, C., Marquet, P., Martin, J. C., Pickering, G., Ardid, D., Eschalier, A., Dubray, C., Flint, H. J., & Bernalier-Donadille, A.** (2012). Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome. *Alimentary Pharmacology & Therapeutics*, *35*(7), 828–838. https://doi.org/10.1111/j.1365-2036.2012.05007.x

**Chaudhary, M., & Payasi, A.** (2014). *Resistance Patterns and Prevalence of the Aminoglycoside Modifying Enzymes in Clinical Isolates of Gram-Negative Pathogens*. 7.

**Cheikhyoussef, A., Cheikhyoussef, N., Chen, H., Zhao, J., Tang, J., Zhang, H., & Chen, W.** (2010). Bifidin I – A new bacteriocin produced by Bifidobacterium infantis BCRC 14602: Purification and partial amino acid sequence. *Food Control*, *21*(5), 746–753. https://doi.org/10.1016/j.foodcont.2009.11.003

**Chen, J., Cai, W., & Feng, Y.** (2007). Development of intestinal bifidobacteria and lactobacilli in breast-fed neonates. *Clinical Nutrition*, *26*(5), 559–566. https://doi.org/10.1016/j.clnu.2007.03.003

**Chen, J., & Vitetta, L.** (2021). Intestinal dysbiosis in celiac disease: Decreased butyrate production may facilitate the onset of the disease. *Proceedings of the National Academy of Sciences*, *118*(41). https://doi.org/10.1073/pnas.2113655118

**Chen, X., Guo, J., Liu, Y., Chai, S., Ma, R., & Munguntsetseg, B.** (2019a). Characterization and adsorption of a Lactobacillus plantarum virulent phage. *Journal of Dairy Science*, *102*(5), 3879–3886. https://doi.org/10.3168/jds.2018-16019

**Chen, Y., Ye, W., Zhang, Y., & Xu, Y.** (2015). High speed BLASTN: An accelerated MegaBLAST search tool. *Nucleic Acids Research*, *43*(16), 7762–7768. https://doi.org/10.1093/nar/gkv784

**Chen, Z., Hui, P. C., Hui, M., Yeoh, Y. K., Wong, P. Y., Chan, M. C. W., Wong, M. C. S., Ng, S. C., Chan, F. K. L., & Chan, P. K. S.** (2019b). Impact of Preservation Method and 16S rRNA Hypervariable Region on Gut Microbiota Profiling. *MSystems*, *4*(1). https://doi.org/10.1128/mSystems.00271-18

**Chery, J., Dvoskin, D., Morato, F. P., & Fahoum, B.** (2013). Lactobacillus fermentum, a pathogen in documented cholecystitis. *International Journal of Surgery Case Reports*, *4*(8), 662–664. https://doi.org/10.1016/j.ijscr.2013.04.034

**Choi, S., Baek, M., Chung, M.-J., Lim, S., & Yi, H.** (2021). Distribution of bacteriocin genes in the lineages of Lactiplantibacillus plantarum. *Scientific Reports*, *11*(1), 20063. https://doi.org/10.1038/s41598-021-99683-1

**Choi, S., Jin, G.D., Park, J., You, I., & Kim, E. B.** (2018). Pan-Genomics of Lactobacillus plantarum Revealed Group-Specific Genomic Profiles without Habitat Association. *Journal of Microbiology and Biotechnology*, *28*(8), 1352–1359. https://doi.org/10.4014/jmb.1803.03029

**Choudhary, J., Dubey, R. C., Sengar, G., & Dheeman, S.** (2019). Evaluation of Probiotic Potential and Safety Assessment of Lactobacillus pentosus MMP4 Isolated From Mare's Lactation. *Probiotics and Antimicrobial Proteins*, *11*(2), 403–412. https://doi.org/10.1007/s12602-018-9431-x

**Christaki, E., Marcou, M., & Tofarides, A.** (2020). Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence. *Journal of Molecular Evolution*, *88*(1), 26–40. https://doi.org/10.1007/s00239-019-09914-3

**Christoffersen, T. E., Jensen, H., Kleiveland, C. R., Dørum, G., Jacobsen, M., & Lea, T.** (2012). In vitro comparison of commensal, probiotic and pathogenic strains of Enterococcus faecalis. *British Journal of Nutrition*, *108*(11), 2043–2053. https://doi.org/10.1017/S0007114512000220

**Chumchalová, J., Stiles, J., Josephsen, J., & Plocková, M.** (2004). Characterization and purification of acidocin CH5, a bacteriocin produced by Lactobacillus acidophilus CH5. *Journal of Applied Microbiology*, *96*(5), 1082–1089. https://doi.org/10.1111/j.1365-2672.2004.02237.x

**Ciccarelli, F. D., Doerks, T., Mering, C. von, Creevey, C. J., Snel, B., & Bork, P.** (2006). Toward Automatic Reconstruction of a Highly Resolved Tree of Life. *Science*, *311*(5765), 1283–1287. https://doi.org/10.1126/science.1123061

**Ciorba, M. A.** (2012). A Gastroenterologist's Guide to Probiotics. *Clinical Gastroenterology and Hepatology : The Official Clinical Practice Journal of the American Gastroenterological Association*, *10*(9), 960. https://doi.org/10.1016/j.cgh.2012.03.024

**Claesson, M. J., Li, Y., Leahy, S., Canchaya, C., Pijkeren, J. P. van, Cerdeño-Tárraga, A. M., Parkhill, J., Flynn, S., O'Sullivan, G. C., Collins, J. K., Higgins, D., Shanahan, F., Fitzgerald, G. F., Sinderen, D. van, & O'Toole, P. W.** (2006). Multireplicon genome architecture of Lactobacillus salivarius. *Proceedings of the National Academy of Sciences*, *103*(17), 6718–6723. https://doi.org/10.1073/pnas.0511060103

**Claesson, M. J., van Sinderen, D., & O'Toole, P. W. (2008). Lactobacillus phylogenomics—Towards a reclassification of the genus**. *International Journal Of Systematic And Evolutionary Microbiology*, *58*(12), 2945–2954. https://doi.org/10.1099/ijs.0.65848-0

**Clarke, G., Stilling, R. M., Kennedy, P. J., Stanton, C., Cryan, J. F., & Dinan, T. G.** (2014). Minireview: Gut Microbiota: The Neglected Endocrine Organ. *Molecular Endocrinology*, *28*(8), 1221–1238. https://doi.org/10.1210/me.2014-1108

**Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R.** (2012). The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell*, *148*(6), 1258–1270. https://doi.org/10.1016/j.cell.2012.01.035

**CLSI.** (2006). *Clinical and Laboratory Standards Institute (CLSI). . Methods for antimicrobial dilution and disc susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline.* M45-A, 1st ed., CLSI, Wayne, PA.

**CLSI.** (2010). *Clinical and Laboratory Standards Institute (CLSI). Methods for antimicrobial dilution and disc susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline.* M45-A2, 2nd ed., CLSI, Wayne, PA.

**Coenye, T., & Vandamme, P.** (2003). Intragenomic heterogeneity between multiple 16S rRNA ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiology Letters*, *228*(1), 45–49. https://doi.org/10.1016/S0378-1097(03)00717-1

**Colautti, A., Arnoldi, M., Comi, G., & Iacumin, L.** (2022). Antibiotic resistance and virulence factors in lactobacilli: Something to carefully consider. *Food Microbiology*, *103*, 103934. https://doi.org/10.1016/j.fm.2021.103934

**Collins, F. W. J., O'Connor, P. M., O'Sullivan, O., Gómez-Sala, B., Rea, M. C., Hill, C., & Ross, R. P.** (2017). Bacteriocin Gene-Trait matching across the complete Lactobacillus Pan-genome. *Scientific Reports*, *7*(1), 3481. https://doi.org/10.1038/s41598-017-03339-y

**Colwell, R. R.** (1970). Polyphasic taxonomy of bacteria. *Culture Collections of Microorganisms*, 421–436.

**Comunian, R., Daga, E., Dupré, I., Paba, A., Devirgiliis, C., Piccioni, V., Perozzi, G., Zonenschain, D., Rebecchi, A., Morelli, L., De Lorentiis, A., & Giraffa, G.** (2010). Susceptibility to tetracycline and erythromycin of Lactobacillus paracasei strains isolated from traditional Italian fermented foods. *International Journal of Food Microbiology*, *138*(1), 151–156. https://doi.org/10.1016/j.ijfoodmicro.2009.11.018

**Connerton, P. L., Timms, A. R., & Connerton, L. F.** (2011). Campylobacter bacteriophages and bacteriophage therapy. *Journal of Applied Microbiology*, *111*(2), 255–265. https://doi.org/10.1111/j.1365-2672.2011.05012.x

**Coppola, R., Succi, M., Tremonte, P., Reale, A., Salzano, G., & Sorrentino, E.** (2005). Antibiotic susceptibility of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. *Le Lait*, *85*(3), 193–204. https://doi.org/10.1051/lait:2005007

**Corcoran, B. M., Stanton, C., Fitzgerald, G., & Ross, R. P.** (2008). Life Under Stress: The Probiotic Stress Response and How it may be Manipulated. *Current Pharmaceutical Design*, *14*(14), 1382–1399. http://dx.doi.org/10.2174/138161208784480225

**Corr, S. C., Li, Y., Riedel, C. U., O'Toole, P. W., Hill, C., & Gahan, C. G. M.** (2007). Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. *Proceedings of the National Academy of Sciences*, *104*(18), 7617–7621. https://doi.org/10.1073/pnas.0700440104

**Costabile, A., Buttarazzi, I., Kolida, S., Quercia, S., Baldini, J., Swann, J. R., Brigidi, P., & Gibson, G. R.** (2017). An in vivo assessment of the cholesterol-lowering efficacy of Lactobacillus plantarum ECGC 13110402 in normal to mildly hypercholesterolaemic adults. *PLOS ONE*, *12*(12), e0187964. https://doi.org/10.1371/journal.pone.0187964

**Cotter, P. D., Ross, R. P., & Hill, C.** (2013). Bacteriocins—A viable alternative to antibiotics? *Nature Reviews Microbiology*, *11*(2), 95–105. https://doi.org/10.1038/nrmicro2937

**Cox, A. J., Pyne, D. B., Saunders, P. U., & Fricker, P. A**. (2010). Oral administration of the probiotic Lactobacillus fermentum VRI-003 and mucosal immunity in endurance athletes. *British Journal of Sports Medicine*, 44(4), 222-226. http://dx.doi.org/10.1136/bjsm.2007.044628

**Croll, D., & McDonald, B. A.** (2012). The Accessory Genome as a Cradle for Adaptive Evolution in Pathogens. *PLOS Pathogens*, *8*(4), e1002608. https://doi.org/10.1371/journal.ppat.1002608

**Cross, K. L., Campbell, J. H., Balachandran, M., Campbell, A. G., Cooper, C. J., Griffen, A., ... & Podar, M.** (2019). Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. *Nature biotechnology*, 37(11), 1314-1321. https://doi.org/10.1038/s41587-019-0260-6

**Culver, A. L., Ockene, I. S., Balasubramanian, R., Olendzki, B. C., Sepavich, D. M., Wactawski-Wende, J., Manson, J. E., Qiao, Y., Liu, S., Merriam, P. A., Rahilly-Tierny, C., Thomas, F., Berger, J. S., Ockene, J. K., Curb, J. D., & Ma, Y.** (2012). Statin Use and Risk of Diabetes Mellitus in Postmenopausal Women in the Women's Health Initiative. *Archives of Internal Medicine*, *172*(2), 144–152. https://doi.org/10.1001/archinternmed.2011.625

**Culyba, M. J., Mo, C. Y., & Kohli, R. M.** (2015). Targets for Combating the Evolution of Acquired Antibiotic Resistance. *Biochemistry*, *54*(23), 3573–3582. https://doi.org/10.1021/acs.biochem.5b00109

**da Silva Sabo, S., Vitolo, M., González, J. M. D., & Oliveira, R. P. de S.** (2014). Overview of Lactobacillus plantarum as a promising bacteriocin producer among lactic acid bacteria. *Food Research International*, *64*, 527–536. https://doi.org/10.1016/j.foodres.2014.07.041

**Dahllöf, I., Baillie, H., & Kjelleberg, S.** (2000). RpoB-Based Microbial Community Analysis Avoids Limitations Inherent in 16S rRNA Gene Intraspecies Heterogeneity. *Applied and Environmental Microbiology*, *66*(8), 3376–3380. https://doi.org/10.1128/AEM.66.8.3376-3380.2000

**D'Aimmo, M. R., Modesto, M., & Biavati, B.** (2007). Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. Isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology*, *115*(1), 35–42. https://doi.org/10.1016/j.ijfoodmicro.2006.10.003

**Dale, H. F., Rasmussen, S. H., Asiller, Ö. Ö., & Lied, G. A.** (2019). Probiotics in Irritable Bowel Syndrome: An Up-to-Date Systematic Review. *Nutrients*, *11*(9), 2048. https://doi.org/10.3390/nu11092048

**Daly, J. W., Keely, S. J., & Gahan, C. G. M.** (2021). Functional and Phylogenetic Diversity of BSH and PVA Enzymes. *Microorganisms*, *9*(4), 732. https://doi.org/10.3390/microorganisms9040732

**Danielsen, M., & Wind, A.** (2003). Susceptibility of Lactobacillus spp. To antimicrobial agents. *International Journal of Food Microbiology*, *82*(1), 1–11. https://doi.org/10.1016/S0168-1605(02)00254-4

**Dardmeh, F., Alipour, H., Gazerani, P., van der Horst, G., E Brandsborg, & Nielsen, H. I.** (2017). Lactobacillus rhamnosus PB01 (DSM 14870) supplementation affects markers of sperm kinematic parameters in a diet-induced obesity mice model. *PLoS ONE*, *12*(10), e0185964. https://doi.org/10.1371/journal.pone.0185964

**Darling, A. C. E., Mau, B., Blattner, F. R., & Perna, N. T.** (2004). Mauve: Multiple Alignment of Conserved Genomic Sequence with Rearrangements. *Genome Research*, *14*(7), 1394–1403. https://doi.org/10.1101/gr.2289704

**Das, D. J., Shankar, A., Johnson, J. B., & Thomas, S.** (2020). Critical insights into antibiotic resistance transferability in probiotic Lactobacillus. *Nutrition*, *69*, 110567. https://doi.org/10.1016/j.nut.2019.110567

**Datta, V., Myskowski, S. M., Kwinn, L. A., Chiem, D. N., Varki, N., Kansal, R. G., Kotb, M., & Nizet, V.** (2005). Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. *Molecular Microbiology*, *56*(3), 681–695. https://doi.org/10.1111/j.1365-2958.2005.04583.x

**Davies, J., & Davies, D.** (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*, *74*(3), 417–433. https://doi.org/10.1128/MMBR.00016-10

**Davies, T. S., Plummer, S. F., & Jack, A. A.** (2018). *Lactobacillus and Bifidobacterium Promote Antibacterial and Antiviral Immune Response in Human Macrophages*. *6*(1), 7. https://doi.org/10.4172/2329-8901.1000195

**Dawson, P. A., & Karpen, S. J.** (2015). Intestinal transport and metabolism of bile acids. *Journal of Lipid Research*, *56*(6), 1085–1099. https://doi.org/10.1194/jlr.R054114

**Day, A.** (2012). Heatmap. Plus: Heatmap with more sensible behavior. *R Package*, *1*.

**D'Costa, V., King, C., Kalan, L., Morar, M., Sung, W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G., Poinar, H., & Wright, G.** (2011). Antibiotic Resistance is Ancient. *Nature*, *477*, 457–461. https://doi.org/10.1038/nature10388

**de Melo Pereira, G. V., de Oliveira Coelho, B., Magalhães Júnior, A. I., Thomaz-Soccol, V., & Soccol, C. R.** (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnology Advances*, *36*(8), 2060–2076. https://doi.org/10.1016/j.biotechadv.2018.09.003

**de Souza, B. M. S., Borgonovi, T. F., Casarotti, S. N., Todorov, S. D., & Penna, A. L. B.** (2019). Lactobacillus casei and Lactobacillus fermentum Strains Isolated from Mozzarella Cheese: Probiotic Potential, Safety, Acidifying Kinetic Parameters and Viability under Gastrointestinal Tract Conditions. *Probiotics and Antimicrobial Proteins*, *11*(2), 382–396. https://doi.org/10.1007/s12602-018-9406-y

**Dean, S. N., Leary, D. H., Sullivan, C. J., Oh, E., & Walper, S. A.** (2019). Isolation and characterization of Lactobacillus -derived membrane vesicles. *Scientific Reports*, *9*(1), 877. https://doi.org/10.1038/s41598-018-37120-6

**Dean, S. N., Rimmer, M. A., Turner, K. B., Phillips, D. A., Caruana, J. C., Hervey, W. J. I., Leary, D. H., & Walper, S. A.** (2020). Lactobacillus acidophilus Membrane Vesicles as a Vehicle of Bacteriocin Delivery. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.00710

**Dean, S. N., Vora, G. J., & Walper, S. A.** (2017). Complete Genome Sequence of Lactobacillus acidophilus Strain ATCC 53544. *Genome Announcements*, *5*(42). https://doi.org/10.1128/genomeA.01138-17

**Dec, M., Stępień-Pyśniak, D., Nowaczek, A., Puchalski, A., & Urban-Chmiel, R.** (2020). Phenotypic and genotypic antimicrobial resistance profiles of fecal lactobacilli from domesticated pigeons in Poland. *Anaerobe*, *65*, 102251. https://doi.org/10.1016/j.anaerobe.2020.102251

**Dec, M., Urban-Chmiel, R., Stępień-Pyśniak, D., & Wernicki, A.** (2017). Assessment of antibiotic susceptibility in Lactobacillus isolates from chickens. *Gut Pathogens*, *9*(1), 54. https://doi.org/10.1186/s13099-017-0203-z

**Delcher, A. L., Bratke, K. A., Powers, E. C., & Salzberg, S. L.** (2007). Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics*, *23*(6), 673–679. https://doi.org/10.1093/bioinformatics/btm009

**Dellaglio, F., Torriani, S., & Felis, G. E. (**2004). Reclassification of Lactobacillus cellobiosus Rogosa et al. 1953 as a later synonym of Lactobacillus fermentum Beijerinck 1901. *International Journal of Systematic and Evolutionary Microbiology*, *54*(3), 809–812. https://doi.org/10.1099/ijs.0.02947-0

**Deng, Y., Tang, D., Hou, P., Shen, W., Li, H., Wang, T., & Liu, R.** (2021). Dysbiosis of gut microbiota in patients with esophageal cancer. *Microbial Pathogenesis*, *150*, 104709. https://doi.org/10.1016/j.micpath.2020.104709

**Deraz, S. F., Karlsson, E. N., Hedström, M., Andersson, M. M., & Mattiasson, B.** (2005). Purification and characterisation of acidocin D20079, a bacteriocin produced by Lactobacillus acidophilus DSM 20079. *Journal of Biotechnology*, *117*(4), 343–354. https://doi.org/10.1016/j.jbiotec.2005.02.005

**Deraz, S. F., Karlsson, E. N., Khalil, A. A., & Mattiasson, B.** (2007). Mode of action of acidocin D20079, a bacteriocin produced by the potential probiotic strain, Lactobacillus acidophilus DSM 20079. *Journal of Industrial Microbiology & Biotechnology*, *34*(5), 373–379. https://doi.org/10.1007/s10295-007-0206-8

**Derrien, M., & van Hylckama Vlieg, J. E. T.** (2015). Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends in Microbiology*, *23*(6), 354–366. https://doi.org/10.1016/j.tim.2015.03.002

**Dertli, E., Colquhoun, I. J., Gunning, A. P., Bongaerts, R. J., Gall, G. L., Bonev, B. B., Mayer, M. J., & Narbad, A.** (2013). Structure and Biosynthesis of Two Exopolysaccharides Produced by Lactobacillus johnsonii FI9785. *Journal of Biological Chemistry*, *288*(44), 31938–31951. https://doi.org/10.1074/jbc.M113.507418

**Derwa, Y., Gracie, D. J., Hamlin, P. J., & Ford, A. C.** (2017). Systematic review with meta-analysis: The efficacy of probiotics in inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics*, *46*(4), 389–400. https://doi.org/10.1111/apt.14203

**DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., & Andersen, G. L.** (2006). Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

**Desriac, N., Broussolle, V., Postollec, F., Mathot, A.-G., Sohier, D., Coroller, L., & Leguerinel, L.** (2013). Bacillus cereus cell response upon exposure to acid environment: Toward the identification of potential biomarkers. *Frontiers in Microbiology*, *4*, 284. https://doi.org/10.3389/fmicb.2013.00284

**Dethlefsen, L., Huse, S., Sogin, M. L., & Relman, D. A.** (2008). The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. *PLOS Biology*, *6*(11), e280. https://doi.org/10.1371/journal.pbio.0060280

**Dethlefsen, L., McFall-Ngai, M., & Relman, D. A.** (2007). An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature*, *449*(7164), 811–818. https://doi.org/10.1038/nature06245

**Devirgiliis, C., Coppola, D., Barile, S., Colonna, B., & Perozzi, G.** (2009). Characterization of the Tn916 Conjugative Transposon in a Food-Borne Strain of Lactobacillus paracasei. *Applied and Environmental Microbiology*, *75*(12), 3866–3871. https://doi.org/10.1128/AEM.00589-09

**Devriese, L. A., & Butaye, P.** (1998). Vancomycin Susceptibility as an Aid to the Identification of Lactobacilli. *Letters in Applied Microbiology*, *27*(2), 121–121. https://doi.org/10.1046/j.1472-765X.1998.t01-1-00400.x

**Diard, M., Bakkeren, E., Cornuault, J. K., Moor, K., Hausmann, A., Sellin, M. E., Loverdo, C., Aertsen, A., Ackermann, M., De Paepe, M., Slack, E., & Hardt, W.D.** (2017). Inflammation boosts bacteriophage transfer between Salmonella spp. *Science*, *355*(6330), 1211–1215. https://doi.org/10.1126/science.aaf8451

**Dias, F. S., Duarte, W. F., Santos, M. R. R. M., Ramos, E. M., & Schwan, R. F**. (2013). Screening of Lactobacillus Isolated from Pork Sausages for Potential Probiotic Use and Evaluation of the Microbiological Safety of Fermented Products. *Journal of Food Protection*, *76*(6), 991–998. https://doi.org/10.4315/0362-028X.JFP-12-491

**Dicks, L. M. T., Plessis, E. M. D., & Dellagl, F.** (1996). *Reclassification of Lactobacillus casei subsp. Casei ATCC 393 and Lactobacillus rhamnosus ATCC 15820 as Lactobacillus zeae norn. Rev., Designation of ATCC 334 as the Neotype of L. casei subsp. Casei, and Rejection of the Name Lactobacillus paracasei*. 4. https://doi.org/10.1099/00207713-46-1-337

**Dinan, T. G., & Cryan, J. F.** (2017). The Microbiome-Gut-Brain Axis in Health and Disease. *Gastroenterology Clinics*, *46*(1), 77–89. https://doi.org/10.1016/j.gtc.2016.09.007

**Ding, W. K., & Shah, N. P.** (2009). Effect of Various Encapsulating Materials on the Stability of Probiotic Bacteria. *Journal of Food Science*, *74*(2), M100–M107. https://doi.org/10.1111/j.1750-3841.2009.01067.x

**Dixon, A., Robertson, K., Yung, A., Que, M., Randall, H., Wellalagodage, D., Cox, T., Robertson, D., Chi, C., & Sun, J.** (2020). Efficacy of Probiotics in Patients of Cardiovascular Disease Risk: A Systematic Review and Meta-analysis. *Current Hypertension Reports*, *22*(9), 74. https://doi.org/10.1007/s11906-020-01080-y

**Dobrindt, U., Hochhut, B., Hentschel, U., & Hacker, J.** (2004). Genomic islands in pathogenic and environmental microorganisms. *Nature Reviews Microbiology*, *2*(5), 414–424. https://doi.org/10.1038/nrmicro884

**Dong, Y., Zhang, F., Wang, B., Gao, J., Zhang, J., & Shao, Y.** (2019). Laboratory Evolution Assays and Whole-Genome Sequencing for the Development and Safety Evaluation of Lactobacillus plantarum With Stable Resistance to Gentamicin. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01235

**Dong, Z., & Lee, B. H.** (2018). Bile salt hydrolases: Structure and function, substrate preference, and inhibitor development. *Protein Science*, *27*(10), 1742–1754. https://doi.org/10.1002/pro.3484

**Dong, Z., Zhang, J., Lee, B., Li, H., Du, G., & Chen, J.** (2012). A bile salt hydrolase gene of Lactobacillus plantarum BBE7 with high cholesterol-removing activity. *European Food Research and Technology*, *235*(3), 419–427. https://doi.org/10.1007/s00217-012-1769-9

**Drago, L., Mattina, R., Nicola, L., Rodighiero, V., & De Vecchi, E.** (2011). Macrolide resistance and In Vitro selection of resistance to antibiotics in Lactobacillus isolates. *The Journal of Microbiology*, *49*(4), 651. https://doi.org/10.1007/s12275-011-0470-1

**Drago, L., Rodighiero, V., Celeste, T., Rovetto, L., & Vecchi, E. D.** (2013). Microbiological Evaluation of Commercial Probiotic Products Available in the USA in 2009. *Journal of Chemotherapy*. https://doi.org/10.1179/joc.2010.22.6.373

**Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.P., & Raoult, D.** (2000). 16S rRNA Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates. *Journal of Clinical Microbiology*, *38*(10), 3623–3630. https://doi.org/10.1128/JCM.38.10.3623-3630.2000

**Duar, R. M., Lin, X. B., Zheng, J., Martino, M. E., Grenier, T., Pérez-Muñoz, M. E., Leulier, F., Gänzle, M., & Walter, J.** (2017). Lifestyles in transition: Evolution and natural history of the genus Lactobacillus. *FEMS Microbiology Reviews*, *41*(Supp_1), S27–S48. https://doi.org/10.1093/femsre/fux030

**Duary, R. K., Batish, V. K., & Grover, S.** (2012). Relative gene expression of bile salt hydrolase and surface proteins in two putative indigenous Lactobacillus plantarum strains under in vitro gut conditions. Molecular biology reports, 39(3), 2541-2552. https://doi.org/10.1099/00207713-46-1-337

**Duranti, S., Lugli, G. A., Mancabelli, L., Turroni, F., Milani, C., Mangifesta, M., Ferrario, C., Anzalone, R., Viappiani, A., van Sinderen, D., & Ventura, M.** (2017a). Prevalence of Antibiotic Resistance Genes among Human Gut-Derived Bifidobacteria. *Applied and Environmental Microbiology*, *83*(3), e02894-16. https://doi.org/10.1128/AEM.02894-16

**Duranti, S., Mangifesta, M., Lugli, G. A., Turroni, F., Anzalone, R., Milani, C., Mancabelli, L., Ossiprandi, M. C., & Ventura, M. 2017.** (2017b). Bifidobacterium vansinderenii sp. Nov., isolated from faeces of emperor tamarin (Saguinus imperator). *International Journal of Systematic and Evolutionary Microbiology*, *67*(10), 3987–3995. https://doi.org/10.1099/ijsem.0.002243

**Dušková, M., Morávková, M., Mrázek, J., Florianová, M., Vorlová, L., & Karpíšková, R**. (2020). Assessment of antibiotic resistance in starter and non-starter lactobacilli of food origin. *Acta Veterinaria Brno*, *89*(4), 401–411. https://doi.org/10.2754/avb202089040401

**EFSA.** (2008). The maintenance of the list of QPS microorganisms intentionally added to food or feed—Scientific Opinion of the Panel on Biological Hazards. *EFSA Journal*, *6*(12), 923. https://doi.org/10.2903/j.efsa.2008.923

**EFSA.** (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal*, *10*(6). https://doi.org/10.2903/j.efsa.2012.2740

**EFSA.** (2016). General scientific guidance for stakeholders on health claim applications. *EFSA Journal*, *14*(1), 4367. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2016.4367

**Egervärn, M., Lindmark, H., Roos, S., Huys, G., & Lindgren, S.** (2007). Effects of Inoculum Size and Incubation Time on Broth Microdilution Susceptibility Testing of Lactic Acid Bacteria. *Antimicrobial Agents and Chemotherapy*, *51*(1), 394–396. https://doi.org/10.1128/AAC.00637-06

**Egervärn, M., Roos, S., & Lindmark, H.** (2009). Identification and characterization of antibiotic resistance genes in Lactobacillus reuteri and Lactobacillus plantarum. *Journal of Applied Microbiology*, *107*(5), 1658–1668. https://doi.org/10.1111/j.1365-2672.2009.04352.x

**Ejtahed, H. S., Mohtadi-Nia, J., Homayouni-Rad, A., Niafar, M., Asghari-Jafarabadi, M., Mofid, V., & Akbarian-Moghari, A.** (2011). Effect of probiotic yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium lactis* on lipid profile in individuals with type 2 diabetes mellitus. *Journal of Dairy Science*, *94*(7), 3288–3294. https://doi.org/10.3168/jds.2010-4128

**Elkins, C. A., Moser, S. A., & Savage, D. C. Y.** (2001). Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species. *Microbiology*, *147*(12), 3403–3412. https://doi.org/10.1099/00221287-147-12-3403

**Elkins, C. A., & Mullis, L. B.** (2004). Bile-Mediated Aminoglycoside Sensitivity in *Lactobacillus* Species Likely Results from Increased Membrane Permeability Attributable to Cholic Acid. *Applied and Environmental Microbiology*, *70*(12), 7200–7209. https://doi.org/10.1128/AEM.70.12.7200-7209.2004

**Erginkaya, Z., Turhan, E. U., & Tatlı, D.** (2018). Determination of antibiotic resistance of lactic acid bacteria isolated from traditional Turkish fermented dairy products. *Iranian Journal of Veterinary Research*, *19*(1), 53–56.

**Esaiassen, E., Hjerde, E., Cavanagh, J. P., Simonsen, G. S., Klingenberg, C., & Norwegian Study Group on Invasive Bifidobacterial Infections.** (2017). Bifidobacterium Bacteremia: Clinical Characteristics and a Genomic Approach To Assess Pathogenicity. *Journal of Clinical Microbiology*, *55*(7), 2234–2248. https://doi.org/10.1128/JCM.00150-17

**Espinel-Ingroff, A., Barchiesi, F., Cuenca-Estrella, M., Pfaller, M. A., Rinaldi, M., Rodriguez-Tudela, J. L., & Verweij, P. E.** (2005). International and Multicenter Comparison of EUCAST and CLSI M27-A2 Broth Microdilution Methods for Testing Susceptibilities of Candida spp. To Fluconazole, Itraconazole, Posaconazole, and Voriconazole. *Journal of Clinical Microbiology*, *43*(8), 3884–3889. https://doi.org/10.1128/JCM.43.8.3884-3889.2005

**Esteller, A.** (2008). Physiology of bile secretion. *World Journal of Gastroenterology : WJG*, *14*(37), 5641. https://doi.org/10.3748/wjg.14.5641

**Etebu, E., & Arikekpar, L.** (2016). *Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives*. 12.

**Ethiraj, S., Suresh, S. A., Sellamuthu, I., & Rajnish, N.** (2019). Recombinant l-Asparaginase II from Lactobacillus casei subsp. Casei ATCC 393 and Its Anticancer Activity. *Indian Journal of Microbiology*, *59*, 313–320. https://doi.org/10.1007/s12088-019-00806-0

**European Committee on Antimicrobial Susceptibility Testing.** (2013). EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. *EUCAST*, *2013*.

**Evanovich, E., de Souza Mendonça Mattos, P. J., & Guerreiro, J. F.** (2019). Comparative Genomic Analysis of Lactobacillus plantarum: An Overview. *International Journal of Genomics*, *2019*, e4973214. https://doi.org/10.1155/2019/4973214

**Facey, P. D., Hitchings, M. D., Saavedra-Garcia, P., Fernandez-Martinez, L., Dyson, P. J., & Del Sol, R.** (2009). Streptomyces coelicolor Dps-like proteins: Differential dual roles in response to stress during vegetative growth and in nucleoid condensation during reproductive cell division. *Molecular Microbiology*, *73*(6), 1186–1202. https://doi.org/10.1111/j.1365-2958.2009.06848.x

**Facey, P. D., Sevcikova, B., Novakova, R., Hitchings, M. D., Crack, J. C., Kormanec, J., Dyson, P. J., & Sol, R. D.** (2011). The dpsA Gene of Streptomyces coelicolor: Induction of Expression from a Single Promoter in Response to Environmental Stress or during Development. *PLOS ONE*, *6*(9), e25593. https://doi.org/10.1371/journal.pone.0025593

**Falah, F., Vasiee, A., Behbahani, B. A., Yazdi, F. T., Moradi, S., Mortazavi, S. A., & Roshanak, S.** (2019). Evaluation of adherence and anti-infective properties of probiotic Lactobacillus fermentum strain 4-17 against Escherichia coli causing urinary tract infection in humans. *Microbial Pathogenesis*, *131*, 246–253. https://doi.org/10.1016/j.micpath.2019.04.006

**Falcão Salles, J., Le Roux, X., & Poly, F.** (2012). Relating Phylogenetic and Functional Diversity among Denitrifiers and Quantifying their Capacity to Predict Community Functioning. *Frontiers in Microbiology*, *3*. https://doi.org/10.3389/fmicb.2012.00209

**Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., Kurilshikov, A., Bonder, M. J., Valles-Colomer, M., Vandeputte, D., Tito, R., Chaffron, S., Rymenans, L., Verspecht, C., Sutter, L., Lima-Mendez, G., D'hoe, K., Jonckheere, K., Homola, D., & Raes, J.** (2016). Population-level analysis of gut microbiome variation. *Science*, *352*, 560–564. https://doi.org/10.1126/science.aad3503

**Fan, Y., & Pedersen, O.** (2021). Gut microbiota in human metabolic health and disease. *Nature Reviews Microbiology*, *19*(1), 55–71. https://doi.org/10.1038/s41579-020-0433-9

**Fang, F., Li, Y., Bumann, M., Raftis, E. J., Casey, P. G., Cooney, J. C., Walsh, M. A., & O'Toole, P. W.** (2009). Allelic Variation of Bile Salt Hydrolase Genes in Lactobacillus salivarius Does Not Determine Bile Resistance Levels. *Journal of Bacteriology*, *191*(18), 5743. https://doi.org/10.1128/JB.00506-09

**Fang, Z., Lu, W., Zhao, J., Zhang, H., Qian, L., Wang, Q., & Chen, W.** (2020). Probiotics modulate the gut microbiota composition and immune responses in patients with atopic dermatitis: A pilot study. *European Journal of Nutrition*, *59*(5), 2119–2130. https://doi.org/10.1007/s00394-019-02061-x

**FAO, & WHO.** (2001). *Joint FAO/WHO Expert Consultation on*. 34.

**FAO/WHO.** (2002). Guidelines for the Evaluation of Probiotics in Food. Food and Agriculture Organization of the United Nations and. *World Health Organization Working Group Report.*

**Farnworth, E. R.** (2008). The Evidence to Support Health Claims for Probiotics. *The Journal of Nutrition*, *138*(6), 1250S-1254S. https://doi.org/10.1093/jn/138.6.1250S

**FDA**.(2021).*GRAS Notices*. https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices

**Feld, L., Schjørring, S., Hammer, K., Licht, T. R., Danielsen, M., Krogfelt, K., & Wilcks, A.** (2008). Selective pressure affects transfer and establishment of a Lactobacillus plantarum resistance plasmid in the gastrointestinal environment. *Journal of Antimicrobial Chemotherapy*, *61*(4), 845–852. https://doi.org/10.1093/jac/dkn033

**Felis, G. E., & Dellaglio, F.** (2007). *Taxonomy of Lactobacilli and Bifidobacteria*. 19.

**Felis, G. E., Dellaglio, F., Mizzi, L., & Torriani, S.** (2001). Comparative sequence analysis of a recA gene fragment brings new evidence for a change in the taxonomy of the Lactobacillus casei group. *International Journal of Systematic and Evolutionary Microbiology, 51*(6), 2113–2117. https://doi.org/10.1099/00207713-51-6-2113

**Felten, A., Barreau, C., Bizet, C., Lagrange, P. H., & Philippon, A.** (1999). *Lactobacillus* Species Identification, H2O2 Production, and Antibiotic Resistance and Correlation with Human Clinical Status. *Journal of Clinical Microbiology*, *37*(3), 729–733. https://doi.org/10.1128/JCM.37.3.729-733.1999

**Feng, C., Zhang, F., Wang, B., Gao, J., Wang, Y., & Shao, Y.** (2019a). Evaluation of kanamycin and neomycin resistance in *Lactobacillus plantarum* using experimental evolution and whole-genome sequencing. *Food Control*, *98*, 262–267. https://doi.org/10.1016/j.foodcont.2018.11.030

**Feng, Y., Duan, Y., Xu, Z., Lyu, N., Liu, F., Liang, S., & Zhu, B.** (2019b). An examination of data from the American Gut Project reveals that the dominance of the genus Bifidobacterium is associated with the diversity and robustness of the gut microbiota. *MicrobiologyOpen*, *8*(12), e939. https://doi.org/10.1002/mbo3.939

**Fijałkowski, K., Peitler, D., Rakoczy, R., & Żywicka, A.** (2016). Survival of probiotic lactic acid bacteria immobilized in different forms of bacterial cellulose in simulated gastric juices and bile salt solution. *LWT - Food Science and Technology*, *68*, 322–328. https://doi.org/10.1016/j.lwt.2015.12.038

**Fijan, S., Frauwallner, A., Langerholc, T., Krebs, B., ter Haar (née Younes), J. A., Heschl, A., Mičetić Turk, D., & Rogelj, L.** (2019). Efficacy of Using Probiotics with Antagonistic Activity against Pathogens of Wound Infections: An Integrative Review of Literature. *BioMed Research International*, *2019*, e7585486. https://doi.org/10.1155/2019/7585486

**Finn, R. D., Clements, J., & Eddy, S. R.** (2011). HMMER web server: Interactive sequence similarity searching. *Nucleic Acids Research*, *39*, W29–W37. https://doi.org/10.1093/nar/gkr367

**Fischbach, M. A.** (2009). Antibiotics from microbes: Converging to kill. *Current Opinion in Microbiology*, *12*(5), 520–527. https://doi.org/10.1016/j.mib.2009.07.002

**Flaherty, R. A., Freed, S. D., & Lee, S. W.** (2014). The Wide World of Ribosomally Encoded Bacterial Peptides. *PLOS Pathogens*, *10*(7), e1004221. https://doi.org/10.1371/journal.ppat.1004221

**Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S.** (2016). Biofilms: An emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563–575. https://doi.org/10.1038/nrmicro.2016.94

**Flint, H., Louis, P., & Duncan, S.** (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews. Gastroenterology & Hepatology*, *9*, 577–589. https://doi.org/10.1038/nrgastro.2012.156

**Foley, M. H., O'Flaherty, S., Allen, G., Rivera, A. J., Stewart, A. K., Barrangou, R., & Theriot, C. M.** (2021). *Lactobacillus* bile salt hydrolase substrate specificity governs bacterial fitness and host colonization. *Proceedings of the National Academy of Sciences*, *118*(6), e2017709118. https://doi.org/10.1073/pnas.2017709118

**Fontana, A., Falasconi, I., Molinari, P., Treu, L., Basile, A., Vezzi, A., Campanaro, S., & Morelli, L.** (2019). Genomic Comparison of Lactobacillus helveticus Strains Highlights Probiotic Potential. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01380

**Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O. A., & Dantas, G.** (2012). The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. *Science*, *337*(6098), 1107–1111. https://doi.org/10.1126/science.1220761

**Foschi, C., Laghi, L., Parolin, C., Giordani, B., Compri, M., Cevenini, R., Marangoni, A., & Vitali, B.** (2017). Novel approaches for the taxonomic and metabolic characterization of lactobacilli: Integration of 16S rRNA gene sequencing with MALDI-TOF MS and 1H-NMR. *PLOS ONE*, *12*(2), e0172483. https://doi.org/10.1371/journal.pone.0172483

**Fox, G. E., Magrum, L. J., Balch, W. E., Wolfe, R. S., & Woese, C. R.** (1977). Classification of methanogenic bacteria by 16S rRNA ribosomal RNA characterization. *Proceedings of the National Academy of Sciences*, *74*(10), 4537–4541. https://doi.org/10.1073/pnas.74.10.4537

**Fox, G. E., Wisotzkey, J. D., & Jurtshuk, P.** (1992). How Close Is Close: 16S rRNA Sequence Identity May Not Be Sufficient to Guarantee Species Identity. *International Journal of Systematic and Evolutionary Microbiology, 42*(1), 166–170. https://doi.org/10.1099/00207713-42-1-166

**Franz, C. M. A. P., Van Belkum, M. J., Holzapfel, W. H., Abriouel, H., & Gálvez, A.** (2007). Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. *FEMS Microbiology Reviews*, *31*(3), 293–310. https://doi.org/10.1111/j.1574-6976.2007.00064.x

**Frazão, N., Sousa, A., Lässig, M., & Gordo, L.** (2019). Horizontal gene transfer overrides mutation in Escherichia coli colonizing the mammalian gut. *Proceedings of the National Academy of Sciences*, *116*(36), 17906–17915.

**Fredua-Agyeman, M., Ofosu-Boateng, M., Offei, A. O., Mehta, M., Gaisford, S., & Limayem, A.** (2020). Study on the functional properties of potential indigenous probiotics isolated from human samples in West Africa. *LWT*, *133*, 109895. https://doi.org/10.1016/j.lwt.2020.109895

**Fuentes, M. C., Lajo, T., Carrión, J. M., & Cuñé, J.** (2016). A randomized clinical trial evaluating a proprietary mixture of Lactobacillus plantarum strains for lowering cholesterol. *Mediterranean Journal of Nutrition and Metabolism*, *9*(2), 125–135. https://doi.org/10.3233/MNM-160065

**Fukao, M., & Yajima, N.** (2012). Assessment of antibiotic resistance in probiotic lactobacilli. *In Antibiotic Resistant Bacteria—A Continuous Challenge in the New Millennium; InTech*, 503–512.

**Fuller, R.** (2012). *Probiotics: The scientific basis*. Springer Science & Business Media.

**Gajdács, M., Spengler, G., & Urbán, E.** (2017). Identification and Antimicrobial Susceptibility Testing of Anaerobic Bacteria: Rubik's Cube of Clinical Microbiology? *Antibiotics*, *6*(4), 25. https://doi.org/10.3390/antibiotics6040025

**Ganguly, N. K., Bhattacharya, S. K., Sesikeran, B., Nair, G. B., Ramakrishna, B. S., Sachdev, H. P. S., Batish, V. K., Kanagasabapathy, A. S., Muthuswamy, V., Kathuria, S. C., Katoch, V. M., Satyanarayana, K., Toteja, G. S., Rahi, M., Rao, S., Bhan, M. K., Kapur, R., & Hemalatha, R.** (2011). ICMR-DBT Guidelines for Evaluation of Probiotics in Food. *The Indian Journal of Medical Research*, *134*(1), 22–25.

**Gao, R., Zhu, C., Li, H., Yin, M., Pan, C., Huang, L., Kong, C., Wang, X., Zhang, Y., Qu, S., & Qin, H.** (2018). Dysbiosis Signatures of Gut Microbiota Along the Sequence from Healthy, Young Patients to Those with Overweight and Obesity. *Obesity*, *26*(2), 351–361. https://doi.org/10.1002/oby.22088

**Gareau, M. G., Sherman, P. M., & Walker, W. A.** (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nature Reviews. Gastroenterology & Hepatology*, *7*(9), 503. https://doi.org/10.1038/nrgastro.2010.117

**Garneau, J. E., & Moineau, S.** (2011). Bacteriophages of lactic acid bacteria and their impact on milk fermentations. *Microbial Cell Factories*, *10*(1), S20. https://doi.org/10.1186/1475-2859-10-S1-S20

**Gaspar, C., Donders, G. G., Palmeira-de-Oliveira, R., Queiroz, J. A., Tomaz, C., Martinez-de-Oliveira, J., & Palmeira-de-Oliveira, A**. (2018). Bacteriocin production of the probiotic Lactobacillus acidophilus KS400. *AMB Express*, *8*(1), 153. https://doi.org/10.1186/s13568-018-0679-z

**Gauri, S. S., Mandal, S. M., Mondal, K. C., Dey, S., & Pati, B. R.** (2009). Enhanced production and partial characterization of an extracellular polysaccharide from newly isolated Azotobacter sp. SSB81. *Bioresource Technology*, *100*(18), 4240–4243. https://doi.org/10.1016/j.biortech.2009.03.064

**Gavini, F., Esbroeck, M. V., Touzel, J. P., Fourment, A., & Goossens, H.** (1996). Detection of Fructose-6-phosphate Phosphoketolase (F6PPK), a Key Enzyme of the Bifid-Shunt, in Gardnerella vaginalis. *Anaerobe*, *3*(2), 191–193. https://doi.org/10.1006/anae.1996.0025

***Genome List—Genome—NCBI***. (2021). Retrieved 2 October 2020, from https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/

**George Kerry, R., Patra, J. K., Gouda, S., Park, Y., Shin, H.-S., & Das, G.** (2018). Benefaction of probiotics for human health: A review. *Journal of Food and Drug Analysis*, *26*(3), 927–939. https://doi.org/10.1016/j.jfda.2018.01.002

**Georgieva, R., Iliev, I., Haertlé, T., Chobert, J.-M., Ivanova, I., & Danova, S**. (2009). Technological properties of candidate probiotic Lactobacillus plantarum strains. *International Dairy Journal*, *19*(11), 696–702. https://doi.org/10.1016/j.idairyj.2009.06.006

**Georgieva, R., Yocheva, L., Tserovska, L., Zhelezova, G., Stefanova, N., Atanasova, A., Danguleva, A., Ivanova, G., Karapetkov, N., Rumyan, N., & Karaivanova, E.** (2015). Antimicrobial activity and antibiotic susceptibility of Lactobacillus and Bifidobacterium spp. Intended for use as starter and probiotic cultures. *Biotechnology & Biotechnological Equipment*, *29*(1), 84–91. https://doi.org/10.1080/13102818.2014.987450

**Gevers, D., Huys, G., & Swings, J.** (2003). In vitro conjugal transfer of tetracycline resistance from Lactobacillus isolates to other Gram-positive bacteria. *FEMS Microbiology Letters*, *225*(1), 125–130. https://doi.org/10.1016/S0378-1097(03)00505-6

**Gfeller, K. Y., Roth, M., Meile, L., & Teuber, M.** (2003). Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from Lactobacillus fermentum ROT1. *Plasmid*, *50*(3), 190–201. https://doi.org/10.1016/j.plasmid.2003.08.001

**Ghanbari, M., Jami, M., Kneifel, W., & Domig, K. J.** (2013). Antimicrobial activity and partial characterization of bacteriocins produced by lactobacilli isolated from Sturgeon fish. *Food Control*, *32*(2), 379–385. https://doi.org/10.1016/j.foodcont.2012.12.024

**Giatsis, C., Sipkema, D., Ramiro-Garcia, J., Bacanu, G. M., Abernathy, J., Verreth, J., Smidt, H., & Verdegem, M.** (2016). Probiotic legacy effects on gut microbial assembly in tilapia larvae. *Scientific Reports*, *6*(1), 33965. https://doi.org/10.1038/srep33965

**Giedraitienė, A., Vitkauskienė, A., Naginienė, R., & Pavilonis, A. (**2011). Antibiotic Resistance Mechanisms of Clinically Important Bacteria. *Medicina*, *47*(3), 19. https://doi.org/10.3390/medicina47030019

**Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., & Nelson, K. E.** (2006). Metagenomic Analysis of the Human Distal Gut Microbiome. *Science*, *312*(5778), 1355–1359. https://doi.org/10.1126/science.1124234

**Giraffa, G.** (2014). Lactobacillus helveticus: Importance in food and health. *Frontiers in Microbiology*, *5*, 338. https://doi.org/10.3389/fmicb.2014.00338

**Girbovan, A., Sur, G., Samasca, G., & Lupan, I.** (2017). Dysbiosis a risk factor for celiac disease. *Medical Microbiology and Immunology*, *206*(2), 83–91. https://doi.org/10.1007/s00430-017-0496-z

**Goel, A., Halami, P. M., & Tamang, J. P.** (2020). Genome Analysis of Lactobacillus plantarum Isolated From Some Indian Fermented Foods for Bacteriocin Production and Probiotic Marker Genes. *Frontiers in Microbiology*, *11*, 40. https://doi.org/10.3389/fmicb.2020.00040

**Goldstein, E. J. C., Tyrrell, K. L., & Citron, D. M**. (2015). Lactobacillus Species: Taxonomic Complexity and Controversial Susceptibilities. *Clinical Infectious Diseases*, *60*, S98–S107. https://doi.org/10.1093/cid/civ072

**Gomes, C. S., Izar, B., Pazan, F., Mohamed, W., Mraheil, M. A., Mukherjee, K., Billion, A., Aharonowitz, Y., Chakraborty, T., & Hain, T.** (2011). Universal Stress Proteins Are Important for Oxidative and Acid Stress Resistance and Growth of Listeria monocytogenes EGD-e In Vitro and In Vivo. *PLoS ONE*, *6*(9). https://doi.org/10.1371/journal.pone.0024965

**Gómez Zavaglia, A., Kociubinski, G., Pérez, P., Disalvo, E., & De Antoni, G.** (2002). Effect of bile on the lipid composition and surface properties of bifidobacteria. *Journal of Applied Microbiology*, *93*(5), 794–799. https://doi.org/10.1046/j.1365-2672.2002.01747.x

**González-Vázquez, R., Azaola-Espinosa, A., Mayorga-Reyes, L., Reyes-Nava, L. A., Shah, N. P., & Rivera-Espinoza, Y.** (2015). Isolation, Identification and Partial Characterization of a Lactobacillus casei Strain with Bile Salt Hydrolase Activity from Pulque. *Probiotics and Antimicrobial Proteins*, *7*(4), 242–248. https://doi.org/10.1007/s12602-015-9202-x

**Goodnight, K. F.** (1999). *Short Communication*.

**Gotcheva, V., Petrova, G., Petkova, M., Kuzmanova, Y., & Angelov, A.** (2018). Molecular and in vitro assessment of some probiotic characteristics of amylolytic Lactobacillus plantarum strains from Bulgarian fermented products. *Engineering in Life Sciences*, *18*(11), 820–830. https://doi.org/10.1002/elsc.201800054

**Graf, D., Di Cagno, R., Fåk, F., Flint, H. J., Nyman, M., Saarela, M., & Watzl, B.** (2015). Contribution of diet to the composition of the human gut microbiota. *Microbial Ecology in Health and Disease*, *26*(1), 26164. https://doi.org/10.3402/mehd.v26.26164

**Grill, J. P., Cayuela, C., Antoine, J. M., & Schneider, F.** (2000). Isolation and characterization of a Lactobacillus amylovorus mutant depleted in conjugated bile salt hydrolase activity: Relation between activity and bile salt resistance. *Journal of Applied Microbiology*, *89*(4), 553–563. https://doi.org/10.1046/j.1365-2672.2000.01147.x

**Grześkowiak, Ł., Grönlund, M.-M., Beckmann, C., Salminen, S., von Berg, A., & Isolauri, E.** (2012). The impact of perinatal probiotic intervention on gut microbiota: Double-blind placebo-controlled trials in Finland and Germany. *Anaerobe*, *18*(1), 7–13. https://doi.org/10.1016/j.anaerobe.2011.09.006

**Gu, X.C., Luo, X.G., Wang, C.X., Ma, D.Y., Wang, Y., He, Y.Y., Li, W., Zhou, H., & Zhang, T.C.** (2014). Cloning and analysis of bile salt hydrolase genes from Lactobacillus plantarum CGMCC No. 8198. *Biotechnology Letters*, *36*(5), 975–983. https://doi.org/10.1007/s10529-013-1434-9

**Guchte, M. van de, Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., Robert, C., Oztas, S., Mangenot, S., Couloux, A., Loux, V., Dervyn, R., Bossy, R., Bolotin, A., Batto, J.M., Walunas, T., Gibrat, J.-F., Bessières, P., Weissenbach, J., … Maguin, E.** (2006). The complete genome sequence of Lactobacillus bulgaricus reveals extensive and ongoing reductive evolution. *Proceedings of the National Academy of Sciences*, *103*(24), 9274–9279. https://doi.org/10.1073/pnas.0603024103

**Gueimonde, M., Flórez, A. B., van Hoek, A. H. A. M., Stuer-Lauridsen, B., Strøman, P., de los Reyes-Gavilán, C. G., & Margolles, A.** (2010). Genetic Basis of Tetracycline Resistance in Bifidobacterium animalis subsp. Lactis. *Applied and Environmental Microbiology*, *76*(10), 3364–3369. https://doi.org/10.1128/AEM.03096-09

**Gueimonde, M., Sánchez, B., de los Reyes-Gavilán, C. G., & Margolles, A.** (2013). Antibiotic resistance in probiotic bacteria. *Frontiers in Microbiology*, *4*. https://doi.org/10.3389/fmicb.2013.00202

**Guinane, C. M., & Cotter, P. D.** (2013). Role of the gut microbiota in health and chronic gastrointestinal disease: Understanding a hidden metabolic organ. *Therapeutic Advances in Gastroenterology*, *6*(4), 295–308. https://doi.org/10.1177/1756283X13482996

**Guirro, M., Costa, A., Gual-Grau, A., Herrero, P., Torrell, H., Canela, N., & Arola, L**. (2019). Effects from diet-induced gut microbiota dysbiosis and obesity can be ameliorated by fecal microbiota transplantation: A multiomics approach. *PLOS ONE*, *14*(9), e0218143. https://doi.org/10.1371/journal.pone.0218143

**Gunn, J. S.** (2000). Mechanisms of bacterial resistance and response to bile. *Microbes and Infection*, *2*(8), 907–913. https://doi.org/10.1016/S1286-4579(00)00392-0

**Guo, H., Pan, L., Li, L., Lu, J., Kwok, L., Menghe, B., Zhang, H., & Zhang, W.** (2017). Characterization of Antibiotic Resistance Genes from Lactobacillus Isolated from Traditional Dairy Products. *Journal of Food Science*, *82*(3), 724–730. https://doi.org/10.1111/1750-3841.13645

**Guo, X., Chen, J., Sun, H., Luo, L., Gu, Y., Yi, Y., Wang, X., Shan, Y., Liu, B., Zhou, Y., & Lü, X.** (2020). Mining, heterologous expression, purification, and characterization of 14 novel bacteriocins from Lactobacillus rhamnosus LS-8. *International Journal of Biological Macromolecules*, *164*, 2162–2176. https://doi.org/10.1016/j.ijbiomac.2020.08.067

**Guo, Y., Song, G., Sun, M., Wang, J., & Wang, Y.** (2020). Prevalence and Therapies of Antibiotic-Resistance in Staphylococcus aureus. *Frontiers in Cellular and Infection Microbiology*, *10*, 107. https://doi.org/10.3389/fcimb.2020.00107

**Guo, Z., Liu, X. M., Zhang, Q. X., Shen, Z., Tian, F. W., Zhang, H., Sun, Z. H., Zhang, H. P., & Chen, W.** (2011). Influence of consumption of probiotics on the plasma lipid profile: A meta-analysis of randomised controlled trials. *Nutrition, Metabolism and Cardiovascular Diseases*, *21*(11), 844–850. https://doi.org/10.1016/j.numecd.2011.04.008

**Gupta, R. S.** (2016). Impact of genomics on the understanding of microbial evolution and classification: The importance of Darwin's views on classification. *FEMS Microbiology Reviews*, *40*(4), 520–553. https://doi.org/10.1093/femsre/fuw011

**Hagbø, M., Ravi, A., Angell, I. L., Sunde, M., Ludvigsen, J., Diep, D. B., Foley, S. L., Vento, M., Collado, M. C., Perez-Martinez, G., & Rudi, K.** (2020). Experimental support for multidrug resistance transfer potential in the preterm infant gut microbiota. *Pediatric Research*, *88*(1), 57–65. https://doi.org/10.1038/s41390-019-0491-8

**Hall, R. M.** (2010). Salmonella genomic islands and antibiotic resistance in Salmonella enterica. *Future Microbiology*, *5*(10), 1525–1538. http://dx.doi.org/10.2217/fmb.10.122

**Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoudé-Werner, D., & Ennahar, S.** (2011a). Comparative proteomic analysis of Lactobacillus plantarum for the identification of key proteins in bile tolerance. *BMC Microbiology*, *11*(1), 63. https://doi.org/10.1186/1471-2180-11-63

**Han, Q., Kong, B., Chen, Q., Sun, F., & Zhang, H.** (2017). In vitro comparison of probiotic properties of lactic acid bacteria isolated from Harbin dry sausages and selected probiotics. *Journal of Functional Foods*, *32*, 391–400. https://doi.org/10.1016/j.jff.2017.03.020

**Han, S.K., Shin, Y.J., Lee, D.Y., Kim, K. M., Yang, S.J., Kim, D. S., Choi, J.W., Lee, S., & Kim, D.H.** (2021). Lactobacillus rhamnosus HDB1258 modulates gut microbiota-mediated immune response in mice with or without lipopolysaccharide-induced systemic inflammation. *BMC Microbiology*, *21*(1), 146. https://doi.org/10.1186/s12866-021-02192-4

**Hancock, R. E.** (2005). Mechanisms of action of newer antibiotics for Gram-positive pathogens. *The Lancet Infectious Diseases*, *5*(4), 209–218. https://doi.org/10.1016/S1473-3099(05)70051-7

**Harris, H. M. B., Bourin, M. J. B., Claesson, M. J., & O'Toole, P. W.** (2017). Phylogenomics and comparative genomics of Lactobacillus salivarius, a mammalian gut commensal. *Microbial Genomics*, *3*(8). https://doi.org/10.1099/mgen.0.000115

**Hassan, M., Kjos, M., Nes, I. f., Diep, D. b., & Lotfipour, F.** (2012). Natural antimicrobial peptides from bacteria: Characteristics and potential applications to fight against antibiotic resistance. *Journal of Applied Microbiology*, *113*(4), 723–736. https://doi.org/10.1111/j.1365-2672.2012.05338.x

**Hazards (BIOHAZ), E. P. on B., Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Fernández Escámez, P. S., … Herman, L.** (2020). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). *EFSA Journal*, *18*(2), e05966. https://doi.org/10.2903/j.efsa.2020.5966

**Hegarty, J. W., Guinane, C. M., Ross, R. P., Hill, C., & Cotter, P. D.** (2016). Bacteriocin production: A relatively unharnessed probiotic trait? *F1000Research*, *5*. https://doi.org/10.12688/f1000research.9615.1

**Heikens, E., Fleer, A., Paauw, A., Florijn, A., & Fluit, A. C.** (2005). Comparison of Genotypic and Phenotypic Methods for Species-Level Identification of Clinical Isolates of Coagulase-Negative Staphylococci. *Journal of Clinical Microbiology*, *43*(5), 2286–2290. https://doi.org/10.1128/JCM.43.5.2286-2290.2005

**Hemarajata, P., & Versalovic, J.** (2013). Effects of probiotics on gut microbiota: Mechanisms of intestinal immunomodulation and neuromodulation. *Therapeutic Advances in Gastroenterology*, *6*(1), 39–51. https://doi.org/10.1177/1756283X12459294

**Hennequin, C., Aumeran, C., Robin, F., Traore, O., & Forestier, C.** (2012). Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing Klebsiella pneumoniae isolate. *Journal of Antimicrobial Chemotherapy*, *67*(9), 2123–2130. https://doi.org/10.1093/jac/dks169

**Heo, J., Shin, D., Chang, S. Y., Bogere, P., Park, M. R., Ryu, S., Lee, W. J., Yun, B., Lee, H. K., Kim, Y., & Oh, S.** (2018). Comparative Genome Analysis and Evaluation of Probiotic Characteristics of Lactobacillus plantarum Strain JDFM LP11. *Korean Journal for Food Science of Animal Resources*, *38*(5), 878–888. https://doi.org/10.5851/kosfa.2018.e21

**Herbel, S. R., Vahjen, W., Wieler, L. H., & Guenther, S.** (2013). Timely approaches to identify probiotic species of the genus Lactobacillus. *Gut Pathogens*, *5*(1), 27. https://doi.org/10.1186/1757-4749-5-27

**Hertzberger, R., Arents, J., Dekker, H. L., Pridmore, R. D., Gysler, C., Kleerebezem, M., & de Mattos, M. J. T.** (2014). H2O2 Production in Species of the Lactobacillus acidophilus Group: A Central Role for a Novel NADH-Dependent Flavin Reductase. *Applied and Environmental Microbiology*, *80*(7), 2229–2239. https://doi.org/10.1128/AEM.04272-13

**Heuer, H., Schmitt, H., & Smalla, K.** (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Current Opinion in Microbiology*, *14*(3), 236–243. https://doi.org/10.1016/j.mib.2011.04.009

**Hickson, M.** (2011). Probiotics in the prevention of antibiotic-associated diarrhoea and Clostridium difficile infection. *Therapeutic Advances in Gastroenterology*, *4*(3), 185–197. https://doi.org/10.1177/1756283X11399115

**Hidalgo-Cantabrana, C., Moro-García, M. A., Blanco-Míguez, A., Fdez-Riverola, F., Lourenço, A., Riestra, S., Alonso-Arias, R., & Sánchez García, B.** (2018). *Extracellular proteins of Lactobacillus acidophilus DSM 20079 downregulates pro-inflammatory mediators in IBD patients*. https://digital.csic.es/handle/10261/187333
Hill, C. (2012). Virulence or Niche Factors: What's in a Name? *Journal of Bacteriology*, *194*(21), 5725. https://doi.org/10.1128/JB.00980-12

**Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E.** (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

**Hill, C., & Sanders, M. E.** (2013). Rethinking "probiotics". *Gut Microbes*, *4*(4), 269–270. https://doi.org/10.4161/gmic.25143

**Hill, C., Scott, K., Klaenhammer, T. R., Quigley, E., & Sanders, M. E.** (2016). Probiotic nomenclature matters. *Gut Microbes*, *7*(1), 1–2. https://doi.org/10.1080/19490976.2015.1127484

**Hill, D., Sugrue, I., Tobin, C., Hill, C., Stanton, C., & Ross, R. P.** (2018). The Lactobacillus casei Group: History and Health Related Applications. *Frontiers in Microbiology*, *9*, 2107. https://doi.org/10.3389/fmicb.2018.02107

**Hoek, A. H. A. M. van, Margolles, A., Domig, K. J., Korhonen, J. M., Zycka-Krzesinska, J., Bardowski, J. K., Danielsen, M., Huys, G., Morelli, L., & Aarts, H. J. M.** (2008). Molecular assessment of erythromycin and tetracycline resistance genes in lactic acid bacteria and bifidobacteria and their relation to the phenotypic resistance. *International Journal of Probiotics and Prebiotics*, *3*(4), 271–280.

**Hoffmann, D. E., Fraser, C. M., Palumbo, F., Ravel, J., Rowthorn, V., & Schwartz, J.** (2014). Probiotics: Achieving a Better Regulatory Fit. *Food and Drug Law Journal*, *69*(2), 237–ii.

**Holmes, A. H., Moore, L. S. P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J., & Piddock, L. J. V.** (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *The Lancet*, *387*(10014), 176–187. https://doi.org/10.1016/S0140-6736(15)00473-0

**Holzapfel, W. H., & Wood, B. J. B.** (2014). *Lactic Acid Bacteria: Biodiversity and Taxonomy*. John Wiley & Sons.

**Hooper, L. V., & Gordon, J. I.** (2001). Commensal Host-Bacterial Relationships in the Gut. *Science*, *292*(5519), 1115–1118. https://doi.org/10.1126/science.1058709

**Horackova, S., Vesela, K., Klojdova, I., Bercikova, M., & Plockova, M.** (2020). Bile salt hydrolase activity, growth characteristics and surface properties in Lactobacillus acidophilus. *European Food Research and Technology*, *246*(8), 1627–1636. https://doi.org/10.1007/s00217-020-03518-8

**Hoque, M. Z., F, A., Hossain, K., MSM, R., & Islam, K. M. D.** (2010). Isolation, Identification and Analysis of Probiotic properties of Lactobacillus Spp. From selective regional yoghurts. *World Journal of Dairy & Food Sciences*, *5*, 39–46.

**Horcajada, J. P., Montero, M., Oliver, A., Sorlí, L., Luque, S., Gómez-Zorrilla, S., Benito, N., & Grau, S.** (2019). Epidemiology and Treatment of Multidrug-Resistant and Extensively Drug-Resistant Pseudomonas aeruginosa Infections. *Clinical Microbiology Reviews*, *32*(4), e00031-19. https://doi.org/10.1128/CMR.00031-19

**Horowitz, J. L.** (2001). Chapter 52—The Bootstrap. In J. J. Heckman & E. Leamer (Eds.), *Handbook of Econometrics* (5), 3159–3228. https://doi.org/10.1016/S1573-4412(01)05005-X

**Hou, C., Wang, Q., Zeng, X., Yang, F., Zhang, J., Liu, H., Ma, X., & Qiao, S.** (2014). Complete genome sequence of Lactobacillus reuteri I5007, a probiotic strain isolated from healthy piglet. *Journal of Biotechnology*, *179*, 63–64. https://doi.org/10.1016/j.jbiotec.2014.03.019

**Houston, D. K., Ding, J., Lee, J. S., Garcia, M., Kanaya, A. M., Tylavsky, F. A., Newman, A. B., Visser, M., & Kritchevsky, S. B.** (2011). Dietary fat and cholesterol and risk of cardiovascular disease in older adults: The Health ABC Study. *Nutrition, Metabolism and Cardiovascular Diseases*, *21*(6), 430–437. https://doi.org/10.1016/j.numecd.2009.11.007

**Hsiao, A., Ahmed, A. M. S., Subramanian, S., Griffin, N. W., Drewry, L. L., Petri, W. A., Haque, R., Ahmed, T., & Gordon, J. I.** (2014). Members of the human gut microbiota involved in recovery from Vibrio cholerae infection. *Nature*, *515*(7527), 423–426. https://doi.org/10.1038/nature13738

**Hu, L., Lu, W., Wang, L., Pan, M., Zhang, H., Zhao, J., & Chen, W.** (2017). Assessment of Bifidobacterium Species Using groEL Gene on the Basis of Illumina MiSeq High-Throughput Sequencing. *Genes*, *8* (11), 336. https://doi.org/10.3390/genes8110336

**Hu, P.L., Yuan, Y.H., Yue, T.L., & Guo, C.F.** (2018a). A new method for the in vitro determination of the bile tolerance of potentially probiotic lactobacilli. *Applied Microbiology and Biotechnology*, *102*(4), 1903–1910. https://doi.org/10.1007/s00253-018-8742-x

**Hu, P.L., Yuan, Y.H., Yue, T.L., & Guo, C.F.** (2018b). Bile acid patterns in commercially available oxgall powders used for the evaluation of the bile tolerance ability of potential probiotics. *PLoS ONE*, *13*(3). https://doi.org/10.1371/journal.pone.0192964

**Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., Lin, I. Y. C., Wu, N., Weimer, B. C., Gao, G. F., Liu, Y., & Zhu, B.** (2016). The Bacterial Mobile Resistome Transfer Network Connecting the Animal and Human Microbiomes. *Applied and Environmental Microbiology*, *82*(22), 6672–6681. https://doi.org/10.1128/AEM.01802-16

**Huang, C.H., Chen, C.C., Liou, J.S., Lee, A.-Y., Blom, J., Lin, Y.C., Huang, L., & Watanabe, K. 2020.** (2020). Genome-based reclassification of Lactobacillus casei: Emended classification and description of the species Lactobacillus zeae. *International Journal of Systematic and Evolutionary Microbiology*, *70*(6), 3755–3762. https://doi.org/10.1099/ijsem.0.003969

**Huang, C.H., Li, S.W., Huang, L., & Watanabe, K.** (2018). Identification and Classification for the Lactobacillus casei Group. *Frontiers in Microbiology*, *0*. https://doi.org/10.3389/fmicb.2018.01974

**Huang, W., Wang, G., Xia, Y., Xiong, Z., & Ai, L.** (2020). Bile salt hydrolase-overexpressing Lactobacillus strains can improve hepatic lipid accumulation in vitro in an NAFLD cell model. *Food & Nutrition Research*, *64*. https://doi.org/10.29219/fnr.v64.3751

**Huang, Y., & Zheng, Y.** (2010). The probiotic Lactobacillus acidophilus reduces cholesterol absorption through the down-regulation of Niemann-Pick C1-like 1 in Caco-2 cells. *British Journal of Nutrition*, *103*(4), 473–478. https://doi.org/10.1017/S0007114509991991

**Hugon, P., Dufour, J.C., Colson, P., Fournier, P.E., Sallah, K., & Raoult, D.** (2015). A comprehensive repertoire of prokaryotic species identified in human beings. *The Lancet Infectious Diseases*, *15*(10), 1211–1219. https://doi.org/10.1016/S1473-3099(15)00293-5

**Human Microbiome Project Consortium.** (2012). Structure, Function and Diversity of the Healthy Human Microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

**Humbert, L., Rainteau, D., Tuvignon, N., Wolf, C., Seksik, P., Laugier, R., & Carrière, F.** (2018). Postprandial bile acid levels in intestine and plasma reveal altered biliary circulation in chronic pancreatitis patients. *Journal of Lipid Research*, *59*(11), 2202–2213. https://doi.org/10.1194/jlr.M084830

**Hummel, A. S., Hertel, C., Holzapfel, W. H., & Franz, C. M. A. P.** (2007). Antibiotic Resistances of Starter and Probiotic Strains of Lactic Acid Bacteria. *Applied and Environmental Microbiology*, *73*(3), 730–739. https://doi.org/10.1128/AEM.02105-06

**Hunt, J. J., & Ballard, J. D.** (2013). Variations in Virulence and Molecular Biology among Emerging Strains of Clostridium difficile. *Microbiology and Molecular Biology Reviews*, *77*(4), 567–581. https://doi.org/10.1128/MMBR.00017-13

**Huo, Y., Lu, X., Wang, X., Wang, X., Chen, L., Guo, H., Zhang, M., & Li, Y**. (2020). Bifidobacterium animalis subsp. Lactis A6 Alleviates Obesity Associated with Promoting Mitochondrial Biogenesis and Function of Adipose Tissue in Mice. *Molecules*, *25*(7), 1490. https://doi.org/10.3390/molecules25071490

**Huse, S. M., Ye, Y., Zhou, Y., & Fodor, A. A.** (2012). A Core Human Microbiome as Viewed through 16S rRNA Sequence Clusters. *PLOS ONE*, *7*(6), e34242. https://doi.org/10.1371/journal.pone.0034242

**Hutchings, M. I., Truman, A. W., & Wilkinson, B.** (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology*, *51*, 72–80. https://doi.org/10.1016/j.mib.2019.10.008

**Huys, G., D'Haene, K., & Swings, J.** (2002). Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. *Letters in Applied Microbiology*, *34*(6), 402–406. https://doi.org/10.1046/j.1472-765X.2002.01109.x

**Huys, G., Vancanneyt, M., D'Haene, K., Vankerckhoven, V., Goossens, H., & Swings, J.** (2006). Accuracy of species identity of commercial bacterial cultures intended for probiotic or nutritional use. *Research in Microbiology*, *157*(9), 803–810. https://doi.org/10.1016/j.resmic.2006.06.006

**Hymes, J. P., Johnson, B. R., Barrangou, R., & Klaenhammer, T. R.** (2016). Functional Analysis of an S-Layer-Associated Fibronectin-Binding Protein in Lactobacillus acidophilus NCFM. *Applied and Environmental Microbiology*, *82*(9), 2676–2685. https://doi.org/10.1128/AEM.00024-16

**Ihaka, R., & Gentleman, R.** (1996). R: A Language for Data Analysis and Graphics. *Journal of Computational and Graphical Statistics*, *5*(3), 299–314. https://doi.org/10.1080/10618600.1996.10474713

**Ijare, O. B., Somashekar, B. S., Jadegoud, Y., & Gowda, G. A. N.** (2005). 1H and 13C NMR characterization and stereochemical assignments of bile acids in aqueous media. *Lipids*, *40*(10), 1031. https://doi.org/10.1007/s11745-005-1466-1

**Imperial, I. C. V. J., & Ibana, J. A.** (2016). Addressing the Antibiotic Resistance Problem with Probiotics: Reducing the Risk of Its Double-Edged Sword Effect. *Frontiers in Microbiology*, *7*, 1983. https://doi.org/10.3389/fmicb.2016.01983

**ISO and IDF.** (2010). International Organization of Standardization/International Dairy Federation (ISO 10932/IDF 223). Milk and milk products. Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). *1st ed.*

**Jackson, S. A., Schoeni, J. L., Vegge, C., Pane, M., Stahl, B., Bradley, M., Goldman, V. S., Burguière, P., Atwater, J. B., & Sanders, M. E**. (2019). Improving End-User Trust in the Quality of Commercial Probiotic Products. *Frontiers in Microbiology*, *10*, 739. https://doi.org/10.3389/fmicb.2019.00739

**Jamnagerwalla, J., Howard, L. E., Allott, E. H., Vidal, A. C., Moreira, D. M., Castro-Santamaria, R., Andriole, G. L., Freeman, M. R., & Freedland, S. J.** (2018). Serum cholesterol and risk of high-grade prostate cancer: Results from the REDUCE study. *Prostate Cancer and Prostatic Diseases*, *21*(2), 252–259. https://doi.org/10.1038/s41391-017-0030-9

**Janda, J. M., & Abbott, S. L.** (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *Journal of Clinical Microbiology*, *45*(9), 2761–2764. https://doi.org/10.1128/JCM.01228-07

**Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D. N.** (2015). Role of the normal gut microbiota. *World Journal of Gastroenterology : WJG*, *21*(29), 8787. https://doi.org/10.3748/wjg.v21.i29.8787

**Jang, S.E., Han, M. J., Kim, S.Y., & Kim, D.H.** (2014). Lactobacillus plantarum CLP-0611 ameliorates colitis in mice by polarizing M1 to M2-like macrophages. *International Immunopharmacology*, *21*(1), 186–192. https://doi.org/10.1016/j.intimp.2014.04.021

**Jankovic, I., Sybesma, W., Phothirath, P., Ananta, E., & Mercenier, A**. (2010). Application of probiotics in food products—Challenges and new approaches. *Current Opinion in Biotechnology*, *21*(2), 175–181. https://doi.org/10.1016/j.copbio.2010.03.009

**Jarocki, P., Podleśny, M., Glibowski, P., & Targoński, Z.** (2014). A New Insight into the Physiological Role of Bile Salt Hydrolase among Intestinal Bacteria from the Genus Bifidobacterium. *PLoS ONE*, *9*(12), e114379. https://doi.org/10.1371/journal.pone.0114379

**Jayashree, S., Pooja, S., Pushpanathan, M., Rajendhran, J., & Gunasekaran, P.** (2014). Identification and characterization of bile salt hydrolase genes from the genome of Lactobacillus fermentum MTCC 8711. *Applied Biochemistry and Biotechnology*, *174*(2), 855–866. https://doi.org/10.1007/s12010-014-1118-5

**Jeffres, M. N.** (2017). The Whole Price of Vancomycin: Toxicities, Troughs, and Time. *Drugs*, *77*(11), 1143–1154. https://doi.org/10.1007/s40265-017-0764-7

**Jeng, H.S., Yan, T.R., & Chen, J.Y.** (2020). Treating vaginitis with probiotics in non-pregnant females: A systematic review and meta-analysis. *Experimental and Therapeutic Medicine*, *20*(4), 3749–3765. https://doi.org/10.3892/etm.2020.9090

**Jia, K., Tong, X., Wang, R., & Song, X.** (2018). The clinical effects of probiotics for inflammatory bowel disease: A meta-analysis. *Medicine*, *97*(51). https://doi.org/10.1097/MD.0000000000013792

**Jiang, J., Hang, X., Zhang, M., Liu, X., Li, D., & Yang, H**. (2010). Diversity of bile salt hydrolase activities in different lactobacilli toward human bile salts. *Annals of Microbiology*, *60*(1), 81–88. https://doi.org/10.1007/s13213-009-0004-9

**Jiang, Y., Li, Z., Ren, F., Liu, S., Zhao, L., Sun, E., Zhang, M., Guo, H., Zhang, H., Jiang, L., & Hou, C.** (2015). Complete genome sequence of Lactobacillus paracasei L9, a new probiotic strain with high lactic acid-producing capacity. *Journal of Biotechnology*, *216*, 127–128. https://doi.org/10.1016/j.jbiotec.2015.09.027

**Johnson, A. P., Uttley, A. H., Woodford, N., & George, R. C.** (1990). Resistance to vancomycin and teicoplanin: An emerging clinical problem. *Clinical Microbiology Reviews*, *3*(3), 280–291.

**Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M.** (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1), 5029. https://doi.org/10.1038/s41467-019-13036-1

**Jones, B. V., Begley, M., Hill, C., Gahan, C. G. M., & Marchesi, J. R.** (2008). Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proceedings of the National Academy of Sciences*, *105*(36), 13580–13585. https://doi.org/10.1073/pnas.0804437105

**Jones, M. L., Martoni, C. J., Parent, M., & Prakash, S.** (2012). Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active Lactobacillus reuteri NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. *British Journal of Nutrition*, *107*(10), 1505–1513. https://doi.org/10.1017/S0007114511004703

**Jones, S. E., & Versalovic, J.** (2009). Probiotic Lactobacillus reuteri biofilms produce antimicrobial and anti-inflammatory factors. *BMC Microbiology*, *9*(1), 35. https://doi.org/10.1186/1471-2180-9-35

**Jordan, I. K., Rogozin, I. B., Wolf, Y. I., & Koonin, E. V.** (2002). Essential Genes Are More Evolutionarily Conserved Than Are Nonessential Genes in Bacteria. *Genome Research*, *12*(6), 962–968. https://doi.org/10.1101/gr.87702

**Jorgensen, J. H., Hindler, J. F., Reller, L. B., & Weinstein, M. P.** (2007). New Consensus Guidelines from the Clinical and Laboratory Standards Institute for Antimicrobial Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. *Clinical Infectious Diseases*, *44*(2), 280–286. https://doi.org/10.1086/510431

**Juhas, M., van der Meer, J. R., Gaillard, M., Harding, R. M., Hood, D. W., & Crook, D. W.** (2009). Genomic islands: Tools of bacterial horizontal gene transfer and evolution. *FEMS Microbiology Reviews*, *33*(2), 376–393. https://doi.org/10.1111/j.1574-6976.2008.00136.x

**Jun, C., Sa, Y. S., Gu, S.A., Joo, J. C., Kim, S., Kim, K.J., & Kim, Y. H.** (2013). Discovery and characterization of a thermostable d-lactate dehydrogenase from Lactobacillus jensenii through genome mining. *Process Biochemistry*, *48*(1), 109–117. https://doi.org/10.1016/j.procbio.2012.11.013

**Jung, J.H., Kim, S.J., Lee, J. Y., Yoon, S.R., You, S.Y., & Kim, S. H.** (2019). Multifunctional properties of Lactobacillus plantarum strains WiKim83 and WiKim87 as a starter culture for fermented food. *Food Science & Nutrition*, *7*(8), 2505–2516. https://doi.org/10.1002/fsn3.1075

**Juntunen, M., Kirjavainen, P. V., Ouwehand, A. C., Salminen, S. J., & Isolauri, E.** (2001). Adherence of Probiotic Bacteria to Human Intestinal Mucus in Healthy Infants and during Rotavirus Infection. *Clinical Diagnostic Laboratory Immunology*, *8*(2), 293–296. https://doi.org/10.1128/CDLI.8.2.293-296.2001

**Juwana, A., Seno, B. A., Lindayani, & Hartayanie, L.** (2020). Identification of Probiotic Potential Lactobacillus from Mandai Using Molecular Technique. *Digital Press Life Sciences*, *2*, 00001. https://doi.org/10.29037/digitalpress.22324

**Kadlec, R., & Jakubec, M.** (2014). The effect of prebiotics on adherence of probiotics. *Journal of Dairy Science*, *97*(4), 1983–1990. https://doi.org/10.3168/jds.2013-7448

**Kahlmeter, G., Brown, D. F. J., Goldstein, F. W., MacGowan, A. P., Mouton, J. W., Odenholt, I., Rodloff, A., Soussy, C.J., Steinbakk, M., Soriano, F., & Stetsiouk, O.** (2006). European Committee on Antimicrobial Susceptibility Testing (EUCAST) Technical Notes on antimicrobial susceptibility testing. *Clinical Microbiology and Infection*, *12*(6), 501–503. https://doi.org/10.1111/j.1469-0691.2006.01454.x

**Kämpfer, P., & Glaeser, S. P.** (2012). Prokaryotic taxonomy in the sequencing era – the polyphasic approach revisited. *Environmental Microbiology*, *14*(2), 291–317. https://doi.org/10.1111/j.1462-2920.2011.02615.x

**Kanmani, P., Satish Kumar, R., Yuvaraj, N., Paari, K. A., Pattukumar, V., & Arul, V.** (2013). Probiotics and Its Functionally Valuable Products—A Review. *Critical Reviews in Food Science and Nutrition*, *53*(6), 641–658. https://doi.org/10.1080/10408398.2011.553752

**Kaoutari, A. E., Armougom, F., Gordon, J. I., Raoult, D., & Henrissat, B.** (2013). The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nature Reviews Microbiology*, *11*(7), 497–504. https://doi.org/10.1038/nrmicro3050

**Kapourchali, F. R., & Cresci, G. A. M.** (2020). Early-Life Gut Microbiome—The Importance of Maternal and Infant Factors in Its Establishment. *Nutrition in Clinical Practice*, *35*(3), 386–405. https://doi.org/10.1002/ncp.10490

**Karami, S., Roayaei, M., Hamzavi, H., Bahmani, M., Hassanzad-Azar, H., Leila, M., & Rafieian-Kopaei, M.** (2017). Isolation and identification of probiotic Lactobacillus from local dairy and evaluating their antagonistic effect on pathogens. *International Journal of Pharmaceutical Investigation*, *7*(3), 137. https://doi.org/10.4103/jphi.JPHI_8_17

**Karl, J. P., Hatch, A. M., Arcidiacono, S. M., Pearce, S. C., Pantoja-Feliciano, I. G., Doherty, L. A., & Soares, J. W.** (2018). Effects of Psychological, Environmental and Physical Stressors on the Gut Microbiota. *Frontiers in Microbiology*, *9*, 2013. https://doi.org/10.3389/fmicb.2018.02013

**Kassim, A., Omuse, G., Premji, Z., & Revathi, G.** (2016). Comparison of Clinical Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing guidelines for the interpretation of antibiotic susceptibility at a University teaching hospital in Nairobi, Kenya: A cross-sectional study. *Annals of Clinical Microbiology and Antimicrobials*, *15*(1), 21. https://doi.org/10.1186/s12941-016-0135-3

**Kaushik, J. K., Kumar, A., Duary, R. K., Mohanty, A. K., Grover, S., & Batish, V. K.** (2009). Functional and Probiotic Attributes of an Indigenous Isolate of *Lactobacillus plantarum*. *PLOS ONE*, *4*(12), e8099. https://doi.org/10.1371/journal.pone.0008099

**Kawai, Y., Ishii, Y., Uemura, K., Kitazawa, H., Saito, T., & Itoh, T.** (2001). Lactobacillus reuteri LA6 and Lactobacillus gasseri LA39 isolated from faeces of the same human infant produce identical cyclic bacteriocin. *Food Microbiology*, *18*(4), 407–415. https://doi.org/10.1006/fmic.2001.0412

**Kazemi, A., Soltani, S., Nasri, F., Clark, C. C. T., & Kolahdouz-Mohammadi, R.** (2021). The effect of probiotics, parabiotics, synbiotics, fermented foods and other microbial forms on immunoglobulin production: A systematic review and meta-analysis of clinical trials. *International Journal of Food Sciences and Nutrition*, *72*(5), 632–649. https://doi.org/10.1080/09637486.2020.1857710

**Kazimierczak, K. A., Flint, H. J., & Scott, K. P.** (2006). Comparative Analysis of Sequences Flanking tet(W) Resistance Genes in Multiple Species of Gut Bacteria. *Antimicrobial Agents and Chemotherapy*, *50*(8), 2632–2639. https://doi.org/10.1128/AAC.01587-05

**Kent, A. G., Vill, A. C., Shi, Q., Satlin, M. J., & Brito, I. L.** (2020). Widespread transfer of mobile antibiotic resistance genes within individual gut microbiomes revealed through bacterial Hi-C. *Nature Communications*, *11*(1), 4379. https://doi.org/10.1038/s41467-020-18164-7

**Khalesi, S., Johnson, D. W., Campbell, K., Williams, S., Fenning, A., Saluja, S., & Irwin, C.** (2018). Effect of probiotics and synbiotics consumption on serum concentrations of liver function test enzymes: A systematic review and meta-analysis. *European Journal of Nutrition*, *57*(6), 2037–2053. https://doi.org/10.1007/s00394-017-1568-y

**Kheadr, E., Dabour, N., Le Lay, C., Lacroix, C., & Fliss, I.** (2007). Antibiotic Susceptibility Profile of Bifidobacteria as Affected by Oxgall, Acid, and Hydrogen Peroxide Stress. *Antimicrobial Agents and Chemotherapy*, *51*(1), 169–174. https://doi.org/10.1128/AAC.00261-06

**Ki, J.S., Zhang, W., & Qian, P.Y.** (2009). Discovery of marine Bacillus species by 16S rRNA and rpoB comparisons and their usefulness for species identification. *Journal of Microbiological Methods*, *77*(1), 48–57. https://doi.org/10.1016/j.mimet.2009.01.003

**Kido, Y., Maeno, S., Tanno, H., Kichise, Y., Shiwa, Y., & Endo, A.** (2021). Niche-specific adaptation of Lactobacillus helveticus strains isolated from malt whisky and dairy fermentations. *Microbial genomics*, 7(4). https://doi.org/10.1099/mgen.0.000560

**Kilian, M., Riley, D. R., Jensen, A., Brüggemann, H., & Tettelin, H.** (2014). Parallel Evolution of Streptococcus pneumoniae and Streptococcus mitis to Pathogenic and Mutualistic Lifestyles. *MBio*, *5*(4), e01490-14. https://doi.org/10.1128/mBio.01490-14

**Kim, D. H., Chun, S.H., Oh, N. S., Lee, J. Y., & Lee, K.W.** (2019). Anti-inflammatory activities of Maillard reaction products from whey protein isolate fermented by Lactobacillus gasseri 4M13 in lipopolysaccharide-stimulated RAW264.7 cells. *Journal of Dairy Science*, *102*(9), 7707–7716. https://doi.org/10.3168/jds.2018-15774

**Kim, G.B., Yi, S.H., & Lee, B. H.** (2004). Purification and Characterization of Three Different Types of Bile Salt Hydrolases from Bifidobacterium Strains. *Journal of Dairy Science*, *87*(2), 258–266. https://doi.org/10.3168/jds.S0022-0302(04)73164-1

**Kim, M. J., Ku, S., Kim, S. Y., Lee, H. H., Jin, H., Kang, S., Li, R., Johnston, T. V., Park, M. S., & Ji, G. E.** (2018). Safety Evaluations of Bifidobacterium bifidum BGN4 and Bifidobacterium longum BORI. *International Journal of Molecular Sciences*, *19*(5), 1422. https://doi.org/10.3390/ijms19051422

**Kim, S.K., Matsunari, H., Takeuchi, T., Yokoyama, M., Murata, Y., & Ishihara, K.** (2007). Effect of different dietary taurine levels on the conjugated bile acid composition and growth performance of juvenile and fingerling Japanese flounder Paralichthys olivaceus. *Aquaculture*, *273*(4), 595–601. https://doi.org/10.1016/j.aquaculture.2007.10.031

**Kimura, M.** (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111–120. https://doi.org/10.1007/BF01731581

**King, T., Ishihama, A., Kori, A., & Ferenci, T.** (2004). A Regulatory Trade-Off as a Source of Strain Variation in the Species Escherichia coli. *Journal of Bacteriology*, *186*(17), 5614–5620. https://doi.org/10.1128/JB.186.17.5614-5620.2004

**Kirisits, M. J., Prost, L., Starkey, M., & Parsek, M. R.** (2005). Characterization of Colony Morphology Variants Isolated from Pseudomonas aeruginosa Biofilms. *Applied and Environmental Microbiology*, *71*(8), 4809–4821. https://doi.org/10.1128/AEM.71.8.4809-4821.2005

**Kirtzalidou, E., Pramateftaki, P., Kotsou, M., & Kyriacou, A.** (2011). Screening for lactobacilli with probiotic properties in the infant gut microbiota. *Anaerobe*, *17*(6), 440–443. https://doi.org/10.1016/j.anaerobe.2011.05.007

**Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W., & Goossens, H.** (2007). Antimicrobial susceptibilities of Lactobacillus, Pediococcus and Lactococcus human isolates and cultures intended for probiotic or nutritional use. *The Journal of Antimicrobial Chemotherapy*, *59*(5), 900–912. https://doi.org/10.1093/jac/dkm035

**Klassen, J. L., & Currie, C. R.** (2012). Gene fragmentation in bacterial draft genomes: Extent, consequences, and mitigation. *BMC Genomics*, *13*(1), 14. https://doi.org/10.1186/1471-2164-13-14

**Klein, G., Hallmann, C., Casas, I. A., Abad, J., Louwers, J., & Reuter, G.** (2000). Exclusion of vanA, vanB and vanC type glycopeptide resistance in strains of Lactobacillus reuteri and Lactobacillus rhamnosus used as probiotics by polymerase chain reaction and hybridization methods. *Journal of Applied Microbiology*, *89*(5), 815–824. https://doi.org/10.1046/j.1365-2672.2000.01187.x

**Klimenko, E. S., Pogodina, A. V., Rychkova, L. V., & Belkova, N. L.** (2020). The Ability of Taxonomic Identification of Bifidobacteria Based on the Variable Regions of 16S rRNA Gene. *Russian Journal of Genetics*, *56*(8), 926–934. https://doi.org/10.1134/S1022795420080074

**Knight, J., Weston, B., & Nigam, Y.** (2019). Gastrointestinal tract 6: The effects of gut microbiota on human health. *Nursing Times*, *115*, 11–46.

**Knight, R., Callewaert, C., Marotz, C., Hyde, E. R., Debelius, J. W., McDonald, D., & Sogin, M. L**. (2017). The Microbiome and Human Biology. *Annual Review of Genomics and Human Genetics*, *18*(1), 65–86. https://doi.org/10.1146/annurev-genom-083115-022438

**Kobyliak, N., Falalyeyeva, T., Mykhalchyshyn, G., Kyriienko, D., & Komissarenko, I.** (2018). Effect of alive probiotic on insulin resistance in type 2 diabetes patients: Randomized clinical trial. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, *12*(5), 617–624. https://doi.org/10.1016/j.dsx.2018.04.015

**Köhler, C.D., & Dobrindt, U.** (2011). What defines extraintestinal pathogenic Escherichia coli? *International Journal of Medical Microbiology*, *301*(8), 642–647. https://doi.org/10.1016/j.ijmm.2011.09.006

**Kolacek, S., Hojsak, I., Berni Canani, R., Guarino, A., Indrio, F., Orel, R., Pot, B., Shamir, R., Szajewska, H., Vandenplas, Y., van Goudoever, J., Weizman, Z., E. W. G.** (2017). Commercial Probiotic Products: A Call for Improved Quality Control. A Position Paper by the ESPGHAN Working Group for Probiotics and Prebiotics. *Journal of Pediatric Gastroenterology and Nutrition*, *65*(1), 117–124. https://doi.org/10.1097/MPG.0000000000001603

**Kõll, P., Mändar, R., Smidt, I., Hütt, P., Truusalu, K., Mikelsaar, R.-H., Shchepetova, J., Krogh-Andersen, K., Marcotte, H., Hammarström, L., & Mikelsaar, M**. (2010). Screening and Evaluation of Human Intestinal Lactobacilli for the Development of Novel Gastrointestinal Probiotics. *Current Microbiology*, *61*(6), 560–566. https://doi.org/10.1007/s00284-010-9653-y

**Komagata, K., & Suzuki, K.I.** (1988). 4 Lipid and Cell-Wall Analysis in Bacterial Systematics. In R. R. Colwell & R. Grigorova (Eds.), *Methods in Microbiology* (Vol. 19, pp. 161–207). Academic Press. https://doi.org/10.1016/S0580-9517(08)70410-0

**Komárek, J.** (2016). A polyphasic approach for the taxonomy of cyanobacteria: Principles and applications. *European Journal of Phycology*, *51*(3), 346–353. https://doi.org/10.1080/09670262.2016.1163738

**Korpela, K.** (2021). Impact of delivery mode on infant gut microbiota. *Annals of Nutrition and Metabolism*, 77(3), 11-19. https://doi.org/10.1159/000518498

**Köser, C. U., Ellington, M. J., Cartwright, E. J. P., Gillespie, S. H., Brown, N. M., Farrington, M., Holden, M. T. G., Dougan, G., Bentley, S. D., Parkhill, J., & Peacock, S. J.** (2012). Routine Use of Microbial Whole Genome Sequencing in Diagnostic and Public Health Microbiology. *PLoS Pathogens*, *8*(8). https://doi.org/10.1371/journal.ppat.1002824

**Koutnikova, H., Genser, B., Monteiro-Sepulveda, M., Faurie, J.-M., Rizkalla, S., Schrezenmeir, J., & Clément, K.** (2019). Impact of bacterial probiotics on obesity, diabetes and non-alcoholic fatty liver disease related variables: A systematic review and meta-analysis of randomised controlled trials. *BMJ Open*, *9*(3), e017995. https://doi.org/10.1136/bmjopen-2017-017995

**Kubota, H., Senda, S., Tokuda, H., Uchiyama, H., & Nomura, N. (**2009). Stress resistance of biofilm and planktonic Lactobacillus plantarum subsp. Plantarum JCM 1149. *Food Microbiology*, *26*(6), 592–597. https://doi.org/10.1016/j.fm.2009.04.001

**Kumar, A., & Kumar, D.** (2015). Characterization of Lactobacillus isolated from dairy samples for probiotic properties. *Anaerobe*, *33*, 117–123. https://doi.org/10.1016/j.anaerobe.2015.03.004

**Kumar, N., Singh, R., Mishra, S., Singh, A., & Pachouri, U. C.** (2010). Isolation and screening of soil Actinomycetes as source of antibiotics active against bacteria. *International Journal of Microbiology Research, 2*, 12–16. https://doi.org/10.9735/0975-5276.2.2.12-16

**Kumar, R., Grover, S., & Batish, V. K.** (2012). Bile Salt Hydrolase (Bsh) Activity Screening of Lactobacilli: In Vitro Selection of Indigenous Lactobacillus Strains with Potential Bile Salt Hydrolysing and Cholesterol-Lowering Ability. *Probiotics and Antimicrobial Proteins*, *4*(3), 162–172. https://doi.org/10.1007/s12602-012-9101-3

**Kumar, R., Grover, S., Kaushik, J. K., & Batish, V. K.** (2014). IS30-related transposon mediated insertional inactivation of bile salt hydrolase (bsh1) gene of Lactobacillus plantarum strain Lp20. *Microbiological Research*, *169*(7), 553–560. https://doi.org/10.1016/j.micres.2013.10.006

**Kumar, R. S., Brannigan, J. A., Prabhune, A. A., Pundle, A. V., Dodson, G. G., Dodson, E. J., & Suresh, C. G.** (2006). Structural and Functional Analysis of a Conjugated Bile Salt Hydrolase from Bifidobacterium longum Reveals an Evolutionary Relationship with Penicillin V Acylase. *Journal of Biological Chemistry*, *281*(43), 32516–32525. https://doi.org/10.1074/jbc.M604172200

**Kumar, S., Stecher, G., & Tamura, K.** (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

**Kurilshikov, A., Wijmenga, C., Fu, J., & Zhernakova, A.** (2017). Host Genetics and Gut Microbiome: Challenges and Perspectives. *Trends in Immunology*, *38*(9), 633–647. https://doi.org/10.1016/j.it.2017.06.003

**Kusada, H., Morinaga, K., & Tamaki, H.** (2021). Identification of Bile Salt Hydrolase and Bile Salt Resistance in a Probiotic Bacterium Lactobacillus gasseri JCM1131T. *Microorganisms*, *9*(5), 1011. https://doi.org/10.3390/microorganisms9051011

**Lafy, A., & Alash, S.** (2018). *Screening test of Bile Salt Hydrolase activity produced by Lactobacillus spp. by qualitative and quantitative methods*. *6*, 1567–1571.

**Lagier, J.C., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C., Bittar, F., Fournous, G., Gimenez, G., Maraninchi, M., Trape, J.F., Koonin, E. V., La Scola, B., & Raoult, D.** (2012). Microbial culturomics: Paradigm shift in the human gut microbiome study. *Clinical Microbiology and Infection*, *18*(12), 1185–1193. https://doi.org/10.1111/1469-0691.12023

**Lagier, J.C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., Levasseur, A., Rolain, J.M., Fournier, P.E., & Raoult, D.** (2018). Culturing the human microbiota and culturomics. *Nature Reviews Microbiology*, *16*(9), 540–550. https://doi.org/10.1038/s41579-018-0041-0

**Lalucat, J., Mulet, M., Gomila, M., & García-Valdés, E**. (2020). Genomics in Bacterial Taxonomy: Impact on the Genus Pseudomonas. *Genes*, *11*(2), 139. https://doi.org/10.3390/genes11020139

**Lambert, J. M., Bongers, R. S., Vos, W. M. de, & Kleerebezem, M**. (2008). Functional Analysis of Four Bile Salt Hydrolase and Penicillin Acylase Family Members in Lactobacillus plantarum WCFS1. *Applied and Environmental Microbiology*, *74*(15), 4719–4726. https://doi.org/10.1128/AEM.00137-08

**Lambert, J. M., Siezen, R. J., de Vos, W. M., & Kleerebezem, M.** (2008). Improved annotation of conjugated bile acid hydrolase superfamily members in Gram-positive bacteria. *Microbiology*, *154*(8), 2492–2500. https://doi.org/10.1099/mic.0.2008/016808-0

**Land, M., Hauser, L., Jun, S.R., Nookaew, I., Leuze, M. R., Ahn, T.-H., Karpinets, T., Lund, O., Kora, G., Wassenaar, T., Poudel, S., & Ussery, D. W.** (2015). Insights from 20 years of bacterial genome sequencing. *Functional & Integrative Genomics*, *15*(2), 141–161. https://doi.org/10.1007/s10142-015-0433-4

**Langa, S., Martín-Cabrejas, I., Montiel, R., Landete, J. M., Medina, M., & Arqués, J. L**. (2014). Short communication: Combined antimicrobial activity of reuterin and diacetyl against foodborne pathogens. *Journal of Dairy Science*, *10*(97), 6116–6121. https://doi.org/10.3168/jds.2014-8306

**Langille, M. G. I., Hsiao, W. W. L., & Brinkman, F. S. L**. (2010). Detecting genomic islands using bioinformatics approaches. *Nature Reviews Microbiology*, *8*(5), 373–382. https://doi.org/10.1038/nrmicro2350

**Lashani, E., Davoodabadi, A., & Dallal, M. M. S**. (2020). Some probiotic properties of Lactobacillus species isolated from honey and their antimicrobial activity against foodborne pathogens. *Veterinary Research Forum*, *11*(2), 121. https://doi.org/10.30466/vrf.2018.90418.2188

**Laubach, J., Joseph, M., Brenza, T., Gadhamshetty, V., & Sani, R. K.** (2021). Exopolysaccharide and biopolymer-derived films as tools for transdermal drug delivery. *Journal of Controlled Release*, *329*, 971–987. https://doi.org/10.1016/j.jconrel.2020.10.027

**Lawson, P. A.** (2018). The phylum Actinobacteria. In *The Bifidobacteria and related organisms. Biology, Taxonomy and Applications.* (1st ed., pp. 1–7). Academic Press.

**Lawson, P. A., Citron, D. M., Tyrrell, K. L., & Finegold, S. M.** (2016). Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. *Anaerobe*, *40*, 95–99. https://doi.org/10.1016/j.anaerobe.2016.06.008

**Laxminarayan, R., Van Boeckel, T., Frost, I., Kariuki, S., Khan, E. A., Limmathurotsakul, D., Larsson, D. G. J., Levy-Hara, G., Mendelson, M., Outterson, K., Peacock, S. J., & Zhu, Y.G.** (2020). The Lancet Infectious Diseases Commission on antimicrobial resistance: 6 years later. *The Lancet Infectious Diseases*, *20*(4), e51–e60. https://doi.org/10.1016/S1473-3099(20)30003-7

**Le Cam, L.** (1990). Maximum Likelihood: An Introduction. *International Statistical Review / Revue Internationale de Statistique*, *58*(2), 153–171. https://doi.org/10.2307/1403464

**Lê, K,A., Li, Y., Xu, X., Liu, T., Yang, W., He, F., Su, K., Cai, D. H., Go, V. L. W., Pandol, S., & Hui, H.** (2013). Alterations in fecal Lactobacillus and Bifidobacterium species in type 2 diabetic patients in Southern China population. *Frontiers in Physiology*, *0*. https://doi.org/10.3389/fphys.2012.00496

**Le, S. Q., & Gascuel, O.** (2008). An Improved General Amino Acid Replacement Matrix. *Molecular Biology and Evolution*, *25*(7), 1307–1320. https://doi.org/10.1093/molbev/msn067

**Leahy, S. C., Higgins, D. G., Fitzgerald, G. F., & van Sinderen, D**. (2005). Getting better with bifidobacteria. *Journal of Applied Microbiology*, *98*(6), 1303–1315. https://doi.org/10.1111/j.1365-2672.2005.02600.x

**Lebeer, S., Vanderleyden, J., & De Keersmaecker, S. C. J.** (2008). Genes and Molecules of Lactobacilli Supporting Probiotic Action. *Microbiology and Molecular Biology Reviews*, *72*(4), 728–764. https://doi.org/10.1128/MMBR.00017-08

**LeBlanc, J. G., Chain, F., Martín, R., Bermúdez-Humarán, L. G., Courau, S., & Langella, P.** (2017). Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial Cell Factories*, *16*(1), 79. https://doi.org/10.1186/s12934-017-0691-z

**Ledina, T., Mohar-Lorbeg, P., Golob, M., Djordjevic, J., Bogovič-Matijašić, B., & Bulajic, S.** (2018). Tetracycline resistance in lactobacilli isolated from Serbian traditional raw milk cheeses. *Journal of Food Science and Technology*, *55*(4), 1426–1434. http://dx.doi.org/10.1007/s13197-018-3057-6

**Lee, J. M., Park, S. H., Jin, C.Z., Kang, M.K., Park, D.J., & Kim, C.J**. (2021). The groESL ISR sequence-based species-specific identification of GRAS and non-GRAS Lactiplantibacillus as an alternative to 16S rRNA sequencing. *LWT*, *147*, 111504. https://doi.org/10.1016/j.lwt.2021.111504

**Lee, J. Y., Kim, S. G., Shin, Y. K., & Oh, N. S.** (2018). Immunomodulatory Effects of Fermented Milk based on Synbiotic Interaction between Cudrania tricuspidata Leaf Extract and Lactobacillus gasseri 505. Journal of Dairy Science and Biotechnology; Korean Society of Dairy Science and Biotechnology. https://doi.org/10.22424/jmsb.2018.36.1.39

**Lee, J., Yun, H. S., Cho, K. W., Oh, S., Kim, S. H., Chun, T., Kim, B., & Whang, K. Y**. (2011). Evaluation of probiotic characteristics of newly isolated Lactobacillus spp.: Immune modulation and longevity. *International Journal of Food Microbiology*, *148*(2), 80–86. https://doi.org/10.1016/j.ijfoodmicro.2011.05.003

**Lee, J.H., & O'Sullivan, D. J.** (2010). Genomic Insights into Bifidobacteria. *Microbiology and Molecular Biology Reviews*, *74*(3), 378–416. https://doi.org/10.1128/MMBR.00004-10

**Lee, Y. K., & Mazmanian, S. K.** (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science (New York, N.Y.)*, *330*(6012), 1768–1773. https://doi.org/10.1126/science.1195568

**Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., & Staels, B.** (2009). Role of Bile Acids and Bile Acid Receptors in Metabolic Regulation. *Physiological Reviews*, *89*(1), 147–191. https://doi.org/10.1152/physrev.00010.2008

**Lehri, B., Seddon, A. M., & Karlyshev, A. V.** (2017). Potential probiotic-associated traits were revealed from the completed high quality genome sequence of Lactobacillus fermentum 3872. *Standards in Genomic Sciences*, *12*, 19. https://doi.org/10.1186/s40793-017-0228-4

**Lekunberri, I., Subirats, J., Borrego, C. M., & Balcázar, J. L.** (2017). Exploring the contribution of bacteriophages to antibiotic resistance. *Environmental Pollution*, *220*, 981–984. https://doi.org/10.1016/j.envpol.2016.11.059

**Lemay, M.J., Rodrigue, N., Gariépy, C., & Saucier, L.** (2000). Adaptation of Lactobacillus alimentarius to environmental stresses. *International Journal of Food Microbiology*, *55*(1), 249–253. https://doi.org/10.1016/S0168-1605(00)00181-1

**Lemoine, F., Entfellner, J.B. D., Wilkinson, E., Correia, D., Felipe, M. D., Oliveira, T. D., & Gascuel, O.** (2018). Renewing Felsenstein's Phylogenetic Bootstrap in the Era of Big Data. *Nature*, *556*(7702), 452. https://doi.org/10.1038/s41586-018-0043-0

**Leray, M., Knowlton, N., Ho, S.L., Nguyen, B. N., & Machida, R. J.** (2019). GenBank is a reliable resource for 21st century biodiversity research. *Proceedings of the National Academy of Sciences*, *116*(45), 22651–22656. https://doi.org/10.1073/pnas.1911714116

**Lerner, A., Matthias, T., & Aminov, R**. (2017). Potential Effects of Horizontal Gene Exchange in the Human Gut. *Frontiers in Immunology*, *8*, 1630. https://doi.org/10.3389/fimmu.2017.01630

**Lesack, K., & Birol, I.** (2018). Nomenclature Errors in Public 16S rRNA Gene Reference Databases. *BioRxiv*, 441576. https://doi.org/10.1101/441576

**Lester, C. H., Frimodt-Møller, N., Sørensen, T. L., Monnet, D. L., & Hammerum, A. M.** (2006). In Vivo Transfer of the vanA Resistance Gene from an Enterococcus faecium Isolate of Animal Origin to an E. faecium Isolate of Human Origin in the Intestines of Human Volunteers. *Antimicrobial Agents and Chemotherapy*, *50*(2), 596–599. https://doi.org/10.1128/AAC.50.2.596-599.2006

**Levy, S. B.** (1998). The Challenge of Antibiotic Resistance. *Scientific American*, *278*(3), 46–53.

**Lewis, Z. T., Shani, G., Masarweh, C. F., Popovic, M., Frese, S. A., Sela, D. A., Underwood, M. A., & Mills, D. A.** (2016). Validating bifidobacterial species and subspecies identity in commercial probiotic products. *Pediatric Research*, *79*(3), 445–452. https://doi.org/10.1038/pr.2015.244

**Lewis-Mikhael, A.M., Davoodvandi, A., & Jafarnejad, S.** (2020). Effect of Lactobacillus plantarum containing probiotics on blood pressure: A systematic review and meta-analysis. *Pharmacological Research*, *153*, 104663. https://doi.org/10.1016/j.phrs.2020.104663

**Ley, R. E., Peterson, D. A., & Gordon, J. I.** (2006). Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine. *Cell*, *124*(4), 837–848. https://doi.org/10.1016/j.cell.2006.02.017

**Li, C., Chen, J., & Li, S. C.** (2020). Understanding Horizontal Gene Transfer network in human gut microbiota. *Gut Pathogens*, *12* (1), 33. https://doi.org/10.1186/s13099-020-00370-9

**Li, H., He, J., & Jia, W.** (2016). The influence of gut microbiota on drug metabolism and toxicity. *Expert Opinion on Drug Metabolism & Toxicology*, *12*(1), 31–40. https://doi.org/10.1517/17425255.2016.1121234

**Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S., Arumugam, M., Kultima, J. R., Prifti, E., Nielsen, T., Juncker, A. S., Manichanh, C., Chen, B., Zhang, W., Levenez, F., Wang, J., Xu, X., Xiao, L., Liang, S., … Wang, J.** (2014). An integrated catalog of reference genes in the human gut microbiome. *Nature Biotechnology*, *32*(8), 834–841. https://doi.org/10.1038/nbt.2942

**Li, J., Zhao, F., Wang, Y., Chen, J., Tao, J., Tian, G., Wu, S., Liu, W., Cui, Q., Geng, B., Zhang, W., Weldon, R., Auguste, K., Yang, L., Liu, X., Chen, L., Yang, X., Zhu, B., & Cai, J.** (2017). Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome*, *5*(1), 14. https://doi.org/10.1186/s40168-016-0222-x

**Li, K.B**. (2003). ClustalW-MPI: ClustalW analysis using distributed and parallel computing. *Bioinformatics*, *19*(12), 1585–1586. https://doi.org/10.1093/bioinformatics/btg192

**Li, T., Teng, D., Mao, R., Hao, Y., Wang, X., & Wang, J.** (2020). A critical review of antibiotic resistance in probiotic bacteria. *Food Research International*, *136*, 109571. https://doi.org/10.1016/j.foodres.2020.109571

**Li, Y., Canchaya, C., Fang, F., Raftis, E., Ryan, K. A., van Pijkeren, J.P., van Sinderen, D., & O'Toole, P. W.** (2007). Distribution of Megaplasmids in Lactobacillus salivarius and Other Lactobacilli. *Journal of Bacteriology*, *189*(17), 6128–6139. https://doi.org/10.1128/JB.00447-07

**Liang, L., Yi, Y., Lv, Y., Qian, J., Lei, X., & Zhang, G**. (2018). A Comprehensive Genome Survey Provides Novel Insights into Bile Salt Hydrolase (BSH) in Lactobacillaceae. *Molecules*, *23*(5), 1157. https://doi.org/10.3390/molecules23051157

**Liang, X., Zhang, Z., Zhou, X., Lu, Y., Li, R., Yu, Z., Tong, L., Gong, P., Yi, H., Liu, T., & Zhang, L.** (2020). Probiotics improved hyperlipidemia in mice induced by a high cholesterol diet via downregulating FXR. *Food & Function*, *11*(11), 9903–9911. https://doi.org/10.1039/D0FO02255A

**Lin, W.H., Yu, B., Jang, S.H., & Tsen, H.Y.** (2007). Different probiotic properties for Lactobacillus fermentum strains isolated from swine and poultry. *Anaerobe*, *13*(3), 107–113. https://doi.org/10.1016/j.anaerobe.2007.04.006

**Liong, M. T., & Shah, N. P.** (2005). Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. *International Dairy Journal*, *15*(4), 391–398. https://doi.org/10.1016/j.idairyj.2004.08.007

**Liu, E., Zheng, H., Shi, T., Ye, L., Konno, T., Oda, M., Shen, H., & Ji, Z.S.** (2016). Relationship between Lactobacillus bulgaricus and Streptococcus thermophilus under whey conditions: Focus on amino acid formation. *International Dairy Journal*, *56*, 141–150. https://doi.org/10.1016/j.idairyj.2016.01.019

**Liu, G., Ren, L., Song, Z., Wang, C., & Sun, B.** (2015). Purification and characteristics of bifidocin A, a novel bacteriocin produced by Bifidobacterium animals BB04 from centenarians' intestine. *Food Control*, *50*, 889–895. https://doi.org/10.1016/j.foodcont.2014.10.049

**Liu, J., Wang, Y., Li, A., Iqbal, M., Zhang, L., Pan, H., Liu, Z., & Li, J.** (2020). Probiotic potential and safety assessment of Lactobacillus isolated from yaks. *Microbial Pathogenesis*, *145*, 104213. https://doi.org/10.1016/j.micpath.2020.104213

**Liu, M., Siezen, R. J., & Nauta, A.** (2009). In Silico Prediction of Horizontal Gene Transfer Events in Lactobacillus bulgaricus and Streptococcus thermophilus Reveals Protocooperation in Yogurt Manufacturing. *Applied and Environmental Microbiology*, *75*(12), 4120–4129. https://doi.org/10.1128/AEM.02898-08

**Liu, P. C., Yan, Y. K., Ma, Y. J., Wang, X. W., Geng, J., Wang, M. C., Wei, F. X., Zhang, Y. W., Xu, X. D., & Zhang, Y. C.** (2017). *Probiotics Reduce Postoperative Infections in Patients Undergoing Colorectal Surgery: A Systematic Review and Meta-Analysis* [Review Article]. Gastroenterology Research and Practice; Hindawi. https://doi.org/10.1155/2017/6029075

**Lo, T.C., Shih, T.C., Lin, C.F., Chen, H.W., & Lin, T.H**. (2005). Complete genomic sequence of the temperate bacteriophage ΦAT3 isolated from Lactobacillus casei ATCC 393. *Virology*, *339*(1), 42–55. https://doi.org/10.1016/j.virol.2005.05.022

**Lomsadze, A., Gemayel, K., Tang, S., & Borodovsky, M.** (2018). Modeling leaderless transcription and atypical genes results in more accurate gene prediction in prokaryotes. *Genome Research*, *28*(7), 1079–1089. https://doi.org/10.1101/gr.230615.117

**Long, S. L., Gahan, C. G. M., & Joyce, S. A.** (2017). Interactions between gut bacteria and bile in health and disease. *Molecular Aspects of Medicine*, *56*, 54–65. https://doi.org/10.1016/j.mam.2017.06.002

**López-Hermoso, C., de la Haba, R. R., Sánchez-Porro, C., Papke, R. T., & Ventosa, A.** (2017). Assessment of Multi Locus Sequence Analysis As a Valuable Tool for the Classification of the Genus Salinivibrio. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01107

**Lourenco, R., & Camilo, M.** (2002). Taurine: A conditionally essential amino acid in humans? An overview in health and disease. *Nutrición Hospitalaria : Organo Oficial de La Sociedad Española de Nutrición Parenteral y Enteral*, *17*, 262–270.

**Luber, P., Bartelt, E., Genschow, E., Wagner, J., & Hahn, H.** (2003). Comparison of Broth Microdilution, E Test, and Agar Dilution Methods for Antibiotic Susceptibility Testing of Campylobacter jejuni and Campylobacter coli. *Journal of Clinical Microbiology*, *41*(3), 1062–1068. https://doi.org/10.1128/JCM.41.3.1062-1068.2003

**Lucas, P., Landete, J., Coton, M., Coton, E., & Lonvaud-Funel, A.** (2003). The tyrosine decarboxylase operon of Lactobacillus brevis IOEB 9809: Characterization and conservation in tyramine-producing bacteria. *FEMS Microbiology Letters*, *229*(1), 65–71. https://doi.org/10.1016/S0378-1097(03)00787-0

**Lugli, G. A., Milani, C., Duranti, S., Mancabelli, L., Mangifesta, M., Turroni, F., Viappiani, A., Sinderen, D. van, & Ventura, M.** (2018). Tracking the Taxonomy of the Genus Bifidobacterium Based on a Phylogenomic Approach. *Applied and Environmental Microbiology*, *84*(4). https://doi.org/10.1128/AEM.02249-17

**Lukjancenko, O., Wassenaar, T. M., & Ussery, D. W**. (2010). Comparison of 61 sequenced Escherichia coli genomes. *Microbial Ecology*, *60*(4), 708–720. https://doi.org/10.1007/s00248-010-9717-3

**Lye, H.S., Rusul, G., & Liong, M.T.** (2010). Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol. *Journal of Dairy Science*, *93*(4), 1383–1392. https://doi.org/10.3168/jds.2009-2574

**Lynch, S. V., & Pedersen, O.** (2016). The Human Intestinal Microbiome in Health and Disease. *New England Journal of Medicine*, *375*(24), 2369–2379. https://doi.org/10.1056/NEJMra1600266

**Ma, Q., Fu, Y., Sun, H., Huang, Y., Li, L., Yu, Q., Dinnyes, A., & Sun, Q**. (2017a). Antimicrobial resistance of Lactobacillus spp. From fermented foods and human gut. *LWT*, *86*, 201–208. https://doi.org/10.1016/j.lwt.2017.07.059

**Ma, W., Zhang, L., Zeng, P., Huang, C., Li, J., Geng, B., Yang, J., Kong, W., Zhou, X., & Cui, Q.** (2017b). An analysis of human microbe–disease associations. *Briefings in Bioinformatics*, *18*(1), 85–97. https://doi.org/10.1093/bib/bbw005

**Ma, X., Wang, G., Zhai, Z., Zhou, P., & Hao, Y.** (2018). Global Transcriptomic Analysis and Function Identification of Malolactic Enzyme Pathway of Lactobacillus paracasei L9 in Response to Bile Stress. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.01978

**Ma, Y.Y., Li, L., Yu, C.H., Shen, Z., Chen, L.H., & Li, Y.M.** (2013). Effects of probiotics on nonalcoholic fatty liver disease: A meta-analysis. *World Journal of Gastroenterology : WJG*, *19*(40), 6911. https://doi.org/10.3748/wjg.v19.i40.6911

**Mackowiak, P.** (2013). Recycling Metchnikoff: Probiotics, the Intestinal Microbiome and the Quest for Long Life. *Frontiers in Public Health*, *1*, 52. https://doi.org/10.3389/fpubh.2013.00052

**Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L., & De Simone, C.** (2001). Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology*, *121*(3), 580–591. https://doi.org/10.1053/gast.2001.27224

**Makarova, K. S., & Koonin, E. V.** (2007). Evolutionary Genomics of Lactic Acid Bacteria. *Journal of Bacteriology*, *189*(4), 1199. https://doi.org/10.1128/JB.01351-06

**Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D. M., Hawkins, T., Plengvidhya, V., … Mills, D.** (2006). Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences*, *103*(42), 15611–15616. https://doi.org/10.1073/pnas.0607117103

**Maki, D. G.** (1978). Control of Colonization and Transmission of Pathogenic Bacteria in the Hospital. *Annals of Internal Medicine*, *89*, 777–780. https://doi.org/10.7326/0003-4819-89-5-777

**Maldonado-Barragán, A., Caballero-Guerrero, B., Martín, V., Ruiz-Barba, J. L., & Rodríguez, J. M.** (2016). Purification and genetic characterization of gassericin E, a novel co-culture inducible bacteriocin from Lactobacillus gasseri EV1461 isolated from the vagina of a healthy woman. *BMC Microbiology*, *16*(1), 37. https://doi.org/10.1186/s12866-016-0663-1

**Mallappa, R. H., Singh, D. K., Rokana, N., Pradhan, D., Batish, V. K., & Grover, S**. (2019). Screening and selection of probiotic Lactobacillus strains of Indian gut origin based on assessment of desired probiotic attributes combined with principal component and heatmap analysis. *LWT*, *105*, 272–281. https://doi.org/10.1016/j.lwt.2019.02.002

**Maloy, S. R., Stewart, V. J., & Taylor, R. K.** (1996). *Genetic analysis of pathogenic bacteria: A laboratory manual*. Cold Spring Harbor Laboratory Press.

**Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., & Tsakalidou, E.** (2006). Probiotic potential of Lactobacillus strains isolated from dairy products. *International Dairy Journal*, *16*(3), 189–199. https://doi.org/10.1016/j.idairyj.2005.02.009

**Marcotte, H., Krogh Andersen, K., Lin, Y., Zuo, F., Zeng, Z., Larsson, P. G., Brandsborg, E., Brønstad, G., & Hammarström, L.** (2017). Characterization and complete genome sequences of L. rhamnosus DSM 14870 and L. gasseri DSM 14869 contained in the EcoVag® probiotic vaginal capsules. *Microbiological Research*, *205*, 88–98. https://doi.org/10.1016/j.micres.2017.08.003

**Marín, J. E. O., Villatoro, E., Luna, M. J., Barrientos, A. M., Mendoza, E., Lemos, A. P. S., Camargo, C. H., Sacchi, C. T., Cunha, M. P. V., Galas, M., & Gabastou, J.M.** (2021). Emergence of MDR invasive Neisseria meningitidis in El Salvador, 2017–19. *Journal of Antimicrobial Chemotherapy*, *76*(5), 1155–1159. https://doi.org/10.1093/jac/dkab010

**Marinova, V. Y., Rasheva, I. K., Kizheva, Y. K., Dermenzhieva, Y. D., & Hristova, P. K.** (2019). Microbiological quality of probiotic dietary supplements. *Biotechnology & Biotechnological Equipment*, *33*(1), 834–841. https://doi.org/10.1080/13102818.2019.1621208

**Markley, A. L., Jensen, E. R., & Lee, S. W.** (2012). An Escherichia coli-based bioengineering strategy to study streptolysin S biosynthesis. *Analytical Biochemistry*, *420*(2), 191–193. https://doi.org/10.1016/j.ab.2011.09.020

**Martin, F.P. J., Sprenger, N., Yap, I. K. S., Wang, Y., Bibiloni, R., Rochat, F., Rezzi, S., Cherbut, C., Kochhar, S., Lindon, J. C., Holmes, E., & Nicholson, J. K**. (2009). Pan organismal Gut Microbiome−Host Metabolic Crosstalk. *Journal of Proteome Research*, *8*(4), 2090–2105. https://doi.org/10.1021/pr801068x

**Martinez, F. A. C., Balciunas, E. M., Converti, A., Cotter, P. D., & de Souza Oliveira, R. P.** (2013). Bacteriocin production by Bifidobacterium spp. A review. *Biotechnology Advances*, *31*(4), 482–488. https://doi.org/10.1016/j.biotechadv.2013.01.010

**Martínez, N., Luque, R., Milani, C., Ventura, M., Bañuelos, O., & Margolles, A.** (2018). A Gene Homologous to rRNA Methylase Genes Confers Erythromycin and Clindamycin Resistance in Bifidobacterium breve. *Applied and Environmental Microbiology*, *84*(10), e02888-17. https://doi.org/10.1128/AEM.02888-17

**Martinez, R. C. R., Bedani, R., & Saad, S. M. I.** (2015). Scientific evidence for health effects attributed to the consumption of probiotics and prebiotics: An update for current perspectives and future challenges. *British Journal of Nutrition*, *114*(12), 1993–2015. https://doi.org/10.1017/S0007114515003864

**Martino, M. E., Bayjanov, J. R., Caffrey, B. E., Wels, M., Joncour, P., Hughes, S., Gillet, B., Kleerebezem, M., Hijum, S. A. F. T. van, & Leulier, F.** (2016). Nomadic lifestyle of Lactobacillus plantarum revealed by comparative genomics of 54 strains isolated from different habitats. *Environmental Microbiology*, *18*(12), 4974–4989. https://doi.org/10.1111/1462-2920.13455

**Masoodi, I., Alshanqeeti, A. S., Alyamani, E. J., AlLehibi, A. A., Alqutub, A. N., Alsayari, K. N., & Alomair, A. O.** (2020). Microbial dysbiosis in irritable bowel syndrome: A single-center metagenomic study in Saudi Arabia. *JGH Open*, *4*(4), 649–655. https://doi.org/10.1002/jgh3.12313

**Mättö, J., van Hoek, A. H. A. M., Domig, K. J., Saarela, M., Floréz, A. B., Brockmann, E., Amtmann, E., Mayo, B., Aarts, H. J. M., & Danielsen, M**. (2007). Susceptibility of human and probiotic Bifidobacterium spp. To selected antibiotics as determined by the Etest method. *International Dairy Journal*, *17*(9), 1123–1131. https://doi.org/10.1016/j.idairyj.2007.01.008

**Mayrhofer, S., Mair, C., Kneifel, W., & Domig, K. J**. (2011). Susceptibility of Bifidobacteria of Animal Origin to Selected Antimicrobial Agents. *Chemotherapy Research and Practice*, *2011*, 1–6. https://doi.org/10.1155/2011/989520

**Mayrhofer, S., van Hoek, A. H. A. M., Mair, C., Huys, G., Aarts, H. J. M., Kneifel, W., & Domig, K. J.** (2010). Antibiotic susceptibility of members of the Lactobacillus acidophilus group using broth microdilution and molecular identification of their resistance determinants. *International Journal of Food Microbiology*, *144*(1), 81–87. https://doi.org/10.1016/j.ijfoodmicro.2010.08.024

**Mayrhofer, S., Zitz, U., Birru, F. H., Gollan, D., Golos, A. K., Kneifel, W., & Domig, K. J.** (2014). Comparison of the CLSI Guideline and ISO/IDF Standard for Antimicrobial Susceptibility Testing of Lactobacilli. *Microbial Drug Resistance*, *20*(6), 591–603. http://dx.doi.org/10.1089/mdr.2013.0189

**Mbye, M., Baig, M. A., AbuQamar, S. F., El-Tarabily, K. A., Obaid, R. S., Osaili, T. M., Al-Nabulsi, A. A., Turner, M. S., Shah, N. P., & Ayyash, M. M**. (2020). Updates on understanding of probiotic lactic acid bacteria responses to environmental stresses and highlights on proteomic analyses. *Comprehensive Reviews in Food Science and Food Safety*, *19*(3), 1110–1124. https://doi.org/10.1111/1541-4337.12554

**McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., Bhullar, K., Canova, M. J., De Pascale, G., Ejim, L., Kalan, L., King, A. M., Koteva, K., Morar, M., Mulvey, M. R., O'Brien, J. S., Pawlowski, A. C., Piddock, L. J. V., Spanogiannopoulos, P., … Wright, G. D.** (2013). The Comprehensive Antibiotic Resistance Database. *Antimicrobial Agents and Chemotherapy*, *57*(7), 3348–3357. https://doi.org/10.1128/AAC.00419-13

**McAuliffe, O., Cano, R. J., & Klaenhammer, T. R.** (2005). Genetic Analysis of Two Bile Salt Hydrolase Activities in Lactobacillus acidophilus NCFM. *Applied and Environmental Microbiology*, *71*(8), 4925–4929. https://doi.org/10.1128/AEM.71.8.4925-4929.2005

**McFarland, L. V., Evans, C. T., & Goldstein, E. J. C.** (2018). Strain-Specificity and Disease-Specificity of Probiotic Efficacy: A Systematic Review and Meta-Analysis. *Frontiers in Medicine*, *5*. https://doi.org/10.3389/fmed.2018.00124

**McInnes, R. S., McCallum, G. E., Lamberte, L. E., & van Schaik, W**. (2020). Horizontal transfer of antibiotic resistance genes in the human gut microbiome. *Current Opinion in Microbiology*, *53*, 35–43. https://doi.org/10.1016/j.mib.2020.02.002

**McLaren, J. E., Michael, D. R., Ashlin, T. G., & Ramji, D. P.** (2011). Cytokines, macrophage lipid metabolism and foam cells: Implications for cardiovascular disease therapy. *Progress in Lipid Research*, *50*(4), 331–347. https://doi.org/10.1016/j.plipres.2011.04.002

**McNaught, C. E., & MacFie, J.** (2001). Probiotics in clinical practice: A critical review of the evidence. *Nutrition Research*, *21*(1), 343–353. https://doi.org/10.1016/S0271-5317(00)00286-4

**Medlock, G. L., Carey, M. A., McDuffie, D. G., Mundy, M. B., Giallourou, N., Swann, J. R., Kolling, G. L., & Papin, J. A.** (2018). Inferring Metabolic Mechanisms of Interaction within a Defined Gut Microbiota. *Cell Systems*, *7*(3), 245-257.e7. https://doi.org/10.1016/j.cels.2018.08.003

**Melo, T. A., dos Santos, T. F., de Almeida, M. E., Junior, L. A. G. F., Andrade, E. F., Rezende, R. P., Marques, L. M., & Romano, C. C.** (2016). Inhibition of Staphylococcus aureus biofilm by Lactobacillus isolated from fine cocoa. *BMC Microbiology*, *16*(1), 250. https://doi.org/10.1186/s12866-016-0871-8

**Mendes-Soares, H., Suzuki, H., Hickey, R. J., & Forney, L. J.** (2014). Comparative Functional Genomics of Lactobacillus spp. Reveals Possible Mechanisms for Specialization of Vaginal Lactobacilli to Their Environment. *Journal of Bacteriology*, *196*(7), 1458–1470. https://doi.org/10.1128/JB.01439-13

**Meng, F., Zhu, X., Lu, F., Bie, X., & Lu, Z.** (2017). Functional Analysis of Plantaricin E and Its Mutant by Heterologous Expression in Escherichia coli. *Applied Biochemistry and Biotechnology*, *182*(1), 311–323. https://doi.org/10.1007/s12010-016-2328-9

**Meng, F., Zhu, X., Zhao, H., Nie, T., Lu, F., Lu, Z., & Lu, Y.** (2021). A class Ⅲ bacteriocin with broad-spectrum antibacterial activity from Lactobacillus acidophilus NX2-6 and its preservation in milk and cheese. *Food Control*, *121*, 107597. https://doi.org/10.1016/j.foodcont.2020.107597

**Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., Bisson, J.F., Rougeot, C., Pichelin, M., Cazaubiel, M., & Cazaubiel, J.M.** (2011). Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *British Journal of Nutrition*, *105*(5), 755–764. https://doi.org/10.1017/S0007114510004319

**Messi, P., Bondi, M., Sabia, C., Battini, R., & Manicardi, G.** (2001). Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a Lactobacillus plantarum strain. *International Journal of Food Microbiology*, *64*(1), 193–198. https://doi.org/10.1016/S0168-1605(00)00419-0

**MetaHIT Consortium, Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., … Wang, J.** (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, *464*(7285), 59–65. https://doi.org/10.1038/nature08821

**Michael, D. R., Davies, T. S., Jack, A. A., Masetti, G., Marchesi, J. R., Wang, D., ... & Plummer, S. F.** (2021). Daily supplementation with the Lab4P probiotic consortium induces significant weight loss in overweight adults. *Scientific reports*, *11*(1), 1-8. https://doi.org/10.1038/s41598-020-78285-3

**Michael, D. R., Davies, T. S., Moss, J. W. E., Calvente, D. L., Ramji, D. P., Marchesi, J. R., Pechlivanis, A., Plummer, S. F., & Hughes, T. R.** (2017). The anti-cholesterolaemic effect of a consortium of probiotics: An acute study in C57BL/6J mice. *Scientific Reports*, *7*(1), 2883. https://doi.org/10.1038/s41598-017-02889-5

**Michael, D. R., Jack, A. A., Masetti, G., Davies, T. S., Loxley, K. E., Kerry-Smith, J., Plummer, J. F., Marchesi, J. R., Mullish, B. H., McDonald, J. A. K., Hughes, T. R., Wang, D., Garaiova, I., Paduchová, Z., Muchová, J., Good, M. A., & Plummer, S. F.** (2020). A randomised controlled study shows supplementation of overweight and obese adults with lactobacilli and bifidobacteria reduces bodyweight and improves well-being. *Scientific Reports*, *10*(1), 4183. https://doi.org/10.1038/s41598-020-60991-7

**Michael, D. R., Moss, J. W. E., Calvente, D. L., Garaiova, I., Plummer, S. F., & Ramji, D. P.** (2016). Lactobacillus plantarum CUL66 can impact cholesterol homeostasis in Caco-2 enterocytes. *Beneficial Microbes*, *7*(3), 443–451. https://doi.org/10.3920/BM2015.0146

**Mignard, S., & Flandrois, J. P.** (2006). 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. *Journal of Microbiological Methods*, *67*(3), 574–581. https://doi.org/10.1016/j.mimet.2006.05.009

**Milajerdi, A., Mousavi, S. M., Sadeghi, A., Salari-Moghaddam, A., Parohan, M., Larijani, B., & Esmaillzadeh, A.** (2020). The effect of probiotics on inflammatory biomarkers: A meta-analysis of randomized clinical trials. *European Journal of Nutrition*, *59*(2), 633–649. https://doi.org/10.1007/s00394-019-01931-8

**Milani, C., Turroni, F., Duranti, S., Lugli, G. A., Mancabelli, L., Ferrario, C., van Sinderen, D., & Ventura, M.** (2016). Genomics of the Genus Bifidobacterium Reveals Species-Specific Adaptation to the Glycan-Rich Gut Environment. *Applied and Environmental Microbiology*, *82*(4), 980–991. https://doi.org/10.1128/AEM.03500-15

**Miles, A. A., & Misra, S. S.** (1938). Miles, A. A. & Misra, S. S. *J. Hyg., Camb., 38*, 732.

**Mishra, V., & Prasad, D. N.** (2005). Application of in vitro methods for selection of Lactobacillus casei strains as potential probiotics. *International Journal of Food Microbiology*, *103*(1), 109–115. https://doi.org/10.1016/j.ijfoodmicro.2004.10.047

**Mitev, K., & Taleski, V.** (2019). Association between the Gut Microbiota and Obesity. *Open Access Macedonian Journal of Medical Sciences*, *7*(12), 2050. https://doi.org/10.3889/oamjms.2019.586

**Mithieux, G.** (2018). Gut Microbiota and Host Metabolism: What Relationship. *Neuroendocrinology*, *106*(4), 352–356. https://doi.org/10.1159/000484526

**Miyamoto, M., Ueno, H. M., Watanabe, M., Tatsuma, Y., Seto, Y., Miyamoto, T., & Nakajima, H.** (2015). Distinctive proteolytic activity of cell envelope proteinase of Lactobacillus helveticus isolated from airag, a traditional Mongolian fermented mare's milk. *International Journal of Food Microbiology*, *197*, 65–71. https://doi.org/10.1016/j.ijfoodmicro.2014.12.012

**Mo, R., Zhang, X., & Yang, Y.** (2019). Effect of probiotics on lipid profiles in hypercholesterolaemic adults: A meta-analysis of randomized controlled trials. *Medicina Clínica 152*(12), 473–481. https://doi.org/10.1016/j.medcle.2018.09.013

**Modi, S. R., Lee, H. H., Spina, C. S., & Collins, J. J.** (2013). Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature*, *499*(7457), 219–222. https://doi.org/10.1038/nature12212

**Modiri, S., Kasra Kermanshahi, R., Soudi, M. R., Arab, S. S., Khammari, A., Cousineau, B., Vali, H., Zahiri, H. S., & Noghabi, K. A.** (2020). Multifunctional Acidocin 4356 Combats Pseudomonas aeruginosa through Membrane Perturbation and Virulence Attenuation: Experimental Results Confirm Molecular Dynamics Simulation. *Applied and Environmental Microbiology*, *86*(10), e00367-20. https://doi.org/10.1128/AEM.00367-20

**Molloy, E. M., Casjens, S. R., Cox, C. L., Maxson, T., Ethridge, N. A., Margos, G., Fingerle, V., & Mitchell, D. A.** (2015). Identification of the minimal cytolytic unit for streptolysin S and an expansion of the toxin family. *BMC Microbiology*, *15*(1), 141. https://doi.org/10.1186/s12866-015-0464-y

**Momose, Y., Hirayama, K., & Itoh, K.** (2008). Competition for proline between indigenous Escherichia coli and E. coli O157:H7 in gnotobiotic mice associated with infant intestinal microbiota and its contribution to the colonization resistance against E. coli O157:H7. *Antonie van Leeuwenhoek*, *94*(2), 165–171. https://doi.org/10.1007/s10482-008-9222-6

**Monteagudo-Mera, A., Rastall, R. A., Gibson, G. R., Charalampopoulos, D., & Chatzifragkou, A.** (2019). Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. *Applied Microbiology and Biotechnology*, *103*(16), 6463–6472. https://doi.org/10.1007/s00253-019-09978-7

**Montijo-Prieto, S. D., Castro, D. J., Reina, J. C., Jimenez-Valera, M., Ruiz-Bravo, A.** (2019). Draft genome sequence of Lactobacillus plantarum C4 (CECT 9567), a potential probiotic strain isolated from kefir. *Archives of Microbiology*, *201*(3), 409–414. http://dx.doi.org/10.1007/s00203-019-01629-9

**Moore, W. E. C., & Holdeman, L. V.** (1974). Human Fecal Flora: The Normal Flora of 20 Japanese Hawaiians. *Applied Microbiology*, *27*(5), 961–979. https://doi.org/10.1128/am.27.5.961-979.1974

**Morovic, W., Hibberd, A. A., Zabel, B., Barrangou, R., & Stahl, B. (**2016). Genotyping by PCR and High-Throughput Sequencing of Commercial Probiotic Products Reveals Composition Biases. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01747

**Moser, A., Berthoud, H., Eugster, E., Meile, L., & Irmler, S.** (2017). Detection and enumeration of Lactobacillus helveticus in dairy products. *International Dairy Journal*, *68*, 52–59. https://doi.org/10.1016/j.idairyj.2016.12.007

**Moser, S. A., Elkins, C. A., & Savage, D. C**. (2001). Genes encoding bile salt hydrolases and conjugated bile salt transporters in Lactobacillus johnsonii 100-100 and other Lactobacillus species. *Microbiology*, *147*(12), 3403–3412. https://doi.org/10.1099/00221287-147-12-3403

**Mota, F. F. D., Gomes, E. A., Paiva, E., Rosado, A. S., & Seldin, L.** (2004). Use of rpoB gene analysis for identification of nitrogen-fixing Paenibacillus species as an alternative to the 16S rRNA gene. *Letters in Applied Microbiology*, *39*(1), 34–40. https://doi.org/10.1111/j.1472-765X.2004.01536.x

**Mottawea, W., Duceppe, M.-O., Dupras, A. A., Usongo, V., Jeukens, J., Freschi, L., Emond-Rheault, J.G., Hamel, J., Kukavica-Ibrulj, I., Boyle, B., Gill, A., Burnett, E., Franz, E., Arya, G., Weadge, J. T., Gruenheid, S., Wiedmann, M., Huang, H., Daigle, F., … Ogunremi, D**. (2018). Salmonella enterica Prophage Sequence Profiles Reflect Genome Diversity and Can Be Used for High Discrimination Subtyping. *Frontiers in Microbiology*, *9*, 836. https://doi.org/10.3389/fmicb.2018.00836

**Moubareck, C., Lecso, M., Pinloche, E., Butel, M. J., & Doucet-Populaire, F.** (2007). Inhibitory Impact of Bifidobacteria on the Transfer of β-Lactam Resistance among Enterobacteriaceae in the Gnotobiotic Mouse Digestive Tract. *Applied and Environmental Microbiology*, *73*(3), 855–860. https://doi.org/10.1128/AEM.02001-06

**Mukdsi, M. C. A., Cano, M. P. G., González, S. N., & Medina, R. B**. (2012). Administration of Lactobacillus fermentum CRL1446 increases intestinal feruloyl esterase activity in mice. *Letters in Applied Microbiology*, *54*(1), 18–25. https://doi.org/10.1111/j.1472-765X.2011.03166.x

**Mulaw, G., Sisay Tessema, T., Muleta, D., & Tesfaye, A.** (2019). In Vitro Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from Some Traditionally Fermented Ethiopian Food Products. *International Journal of Microbiology*, *2019*, e7179514. https://doi.org/10.1155/2019/7179514

**Mulvey, M. R., Grant, J. M., Plewes, K., Roscoe, D., & Boyd, D. A**. (2011). New Delhi Metallo-β-Lactamase in Klebsiella pneumoniae and Escherichia coli, Canada. *Emerging Infectious Diseases*, *17*(1), 103. https://doi.org/10.3201/eid1701.101358

**Munita, J. M., & Arias, C. A.** (2016). Mechanisms of Antibiotic Resistance. In *Virulence Mechanisms of Bacterial Pathogens* 481–511. https://doi.org/10.1128/9781555819286.ch17

**Muñoz-Quezada, S., Chenoll, E., Vieites, J. M., Genovés, S., Maldonado, J., Bermúdez-Brito, M., Gomez-Llorente, C., Matencio, E., Bernal, M. J., Romero, F., Suárez, A., Ramón, D., & Gil, A.** (2013). Isolation, identification, and characterisation of three novel probiotic strains (Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036) from the faeces of exclusively breast-fed infants. *British Journal of Nutrition*, *109*(S2), S51–S62. https://doi.org/10.1017/S0007114512005211

**Murray, R. G. E., & Holt, J. G.** (2005). The History of Bergey's Manual. In D. J. Brenner, N. R. Krieg, J. T. Staley, & G. M. Garrity (Eds.), *Bergey's Manual® of Systematic Bacteriology: Volume Two: The Proteobacteria, Part A Introductory Essays* (1–14). Springer US. https://doi.org/10.1007/0-387-28021-9_1

**Nabel, E. G.** (2003). Cardiovascular Disease. *New England Journal of Medicine*, *349*(1), 60–72. https://doi.org/10.1056/NEJMra035098

**Nagpal, R., Kumar, A., Kumar, M., Behare, P. V., Jain, S., & Yadav, H.** (2012). Probiotics, their health benefits and applications for developing healthier foods: A review. *FEMS Microbiology Letters*, *334*(1), 1–15. https://doi.org/10.1111/j.1574-6968.2012.02593.x

**Narayan, S., Jalgaonkar, S., Shahani, S., & Kulkarni, V.** (2010). Probiotics: Current trends in the treatment of diarrhea. *Hong Kong Medical Journal*, *16*, 213–218.

**Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., & Swings, J.** (2005). Application of multi locus sequence analysis (MLSA) for rapid identification of Enterococcus species based on rpoA and pheS genes. *Microbiology*, *151*(7), 2141–2150. https://doi.org/10.1099/mic.0.27840-0

**Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J. E., Cherie Millar, B., & Xu, J.** (2011). Characterization and Transfer of Antibiotic Resistance in Lactic Acid Bacteria from Fermented Food Products. *Current Microbiology*, *62*(3), 1081–1089. https://doi.org/10.1007/s00284-010-9856-2

**Nei, M., & Kumar, S.** (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press.

**Neuwirth, E., & Neuwirth, M., E.** (2011). *2011. Package 'RColorBrewer*. CRAN 2011-06-17 08: 34: 00. Apache License 2.0.

**Neviani, E., Carminati, D., Veaux, M., Hermier, J., & Giraffa, G.** (1991). Characterization of *Lactobacillus helveticus* strains resistant to lysozyme. *Le Lait*, *71*(1), 65–73. https://doi.org/10.1051/lait:199115

**Neville, B. A., & O'Toole, P. W.** (2010). Probiotic properties of Lactobacillus salivarius and closely related Lactobacillus species. *Future Microbiology*, *5*(5), 759–774. http://dx.doi.org/10.2217/fmb.10.35

**Ng, E. W., Yeung, M., & Tong, P. S.** (2011). Effects of yogurt starter cultures on the survival of Lactobacillus acidophilus. *International Journal of Food Microbiology*, *145*(1), 169–175. https://doi.org/10.1016/j.ijfoodmicro.2010.12.006

**Nguyen, N.P., Warnow, T., Pop, M., & White, B.** (2016). A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity. *Npj Biofilms and Microbiomes*, *2*(1), 1–8. https://doi.org/10.1038/npjbiofilms.2016.4

**Nguyen, P.T., Nguyen, T.T., Bui, D.C., Hong, P.T., Hoang, Q.K., & Nguyen, H.T.** (2020). Exopolysaccharide production by lactic acid bacteria: The manipulation of environmental stresses for industrial applications. *AIMS Microbiology*, *6*(4), 451. https://doi.org/10.3934/microbiol.2020027

**Nguyen, T. D. T., Kang, J. H., & Lee, M. S.** (2007). Characterization of Lactobacillus plantarum PH04, a potential probiotic bacterium with cholesterol-lowering effects. *International Journal of Food Microbiology*, *113*(3), 358–361. https://doi.org/10.1016/j.ijfoodmicro.2006.08.015

**Ni, J., Huang, R., Zhou, H., Xu, X., Li, Y., Cao, P., Zhong, K., Ge, M., Chen, X., Hou, B., Yu, M., Peng, B., Li, Q., Zhang, P., & Gao, Y.** (2019). Analysis of the Relationship Between the Degree of Dysbiosis in Gut Microbiota and Prognosis at Different Stages of Primary Hepatocellular Carcinoma. *Frontiers in Microbiology*, *10*, 1458. https://doi.org/10.3389/fmicb.2019.01458

**Ni, J., Wu, G. D., Albenberg, L., & Tomov, V. T.** (2017). Gut microbiota and IBD: Causation or correlation? *Nature Reviews Gastroenterology & Hepatology*, *14*(10), 573–584. https://doi.org/10.1038/nrgastro.2017.88

**Nøhr-Meldgaard, K., Struve, C., Ingmer, H., & Agersø, Y.** (2021). The Tetracycline Resistance Gene, tet(W) in Bifidobacterium animalis subsp. Lactis Follows Phylogeny and Differs From tet(W) in Other Species. *Frontiers in Microbiology*, *12*, 1623. https://doi.org/10.3389/fmicb.2021.658943

**Northfield, T. C., & McColl, I.** (1973). Postprandial concentrations of free and conjugated bile acids down the length of the normal human small intestine. *Gut*, *14*(7), 513–518. https://doi.org/10.1136/gut.14.7.513

**Nuhwa, R., Tanasupawat, S., Taweechotipatr, M., Sitdhipol, J., & Savarajara, A.** (2019). Bile salt hydrolase activity and cholesterol assimilation of lactic acid bacteria isolated from flowers. *Journal of Applied Pharmaceutical Science*, *9*, 106–110. https://doi.org/10.7324/JAPS.2019.90615

**Nylander**, **J.** (2004). MrModeltest v25. *Program Distributed by the Author*. https://ci.nii.ac.jp/naid/10031040605

**Oak, S. J., & Jha, R.** (2019). The effects of probiotics in lactose intolerance: A systematic review. *Critical Reviews in Food Science and Nutrition*, *59*(11), 1675–1683. https://doi.org/10.1080/10408398.2018.1425977

**O'Callaghan, A., & van Sinderen, D.** (2016). Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Frontiers in Microbiology*, *7*, 925. https://doi.org/10.3389/fmicb.2016.00925

**O'Flaherty, S., Crawley, A. B., Theriot, C. M., & Barrangou, R.** (2018). The Lactobacillus Bile Salt Hydrolase Repertoire Reveals Niche-Specific Adaptation. *MSphere*, *3*(3). https://doi.org/10.1128/mSphere.00140-18

**Oh, H.K., Lee, J.Y., Lim, S.J., Kim, M.J., Kim, G.B., Kim, J.H., Hong, S.K., & Kang, D.K.** (2008). Molecular Cloning and Characterization of a Bile Salt Hydrolase from Lactobacillus acidophilus PF01. *Journal of Microbiology and Biotechnology*, *18*(3), 449–456.

**Oh, N. S., Joung, J. Y., Lee, J. Y., & Kim, Y.** (2018). Probiotic and anti-inflammatory potential of Lactobacillus rhamnosus 4B15 and Lactobacillus gasseri 4M13 isolated from infant feces. *PLOS ONE*, *13*(2), e0192021. https://doi.org/10.1371/journal.pone.0192021

**Oh, S.J., Chai, C.H., Kim, S.H., Kim, Y.J., Kim, H.S., & Worobo, R. W.** (2012). Comparison of Acid and Bile Tolerances, Cholesterol Assimilation, and CLA Production in Probiotic Lactobacillus acidophilus Strains. *Korean Journal for Food Science of Animal Resources*, *32*(4), 409–413. https://doi.org/10.5851/kosfa.2012.32.4.409

**O'Hara, A. M., & Shanahan, F.** (2006). The gut flora as a forgotten organ. *EMBO Reports*, *7*(7), 688–693. https://doi.org/10.1038/sj.embor.7400731

**Ohashi, Y., & Ushida, K.** (2009). Health-beneficial effects of probiotics: Its mode of action. *Animal Science Journal*, *80*(4), 361–371. https://doi.org/10.1111/j.1740-0929.2009.00645.x

**Oladeinde, A., Cook, K., Lakin, S. M., Woyda, R., Abdo, Z., Looft, T., Herrington, K., Zock, G., Lawrence, J. P., Thomas, J. C., Beaudry, M. S., & Glenn, T.** (2019). Horizontal Gene Transfer and Acquired Antibiotic Resistance in Salmonella enterica Serovar Heidelberg following In Vitro Incubation in Broiler Ceca. *Applied and Environmental Microbiology*, *85*(22), e01903-19. https://doi.org/10.1128/AEM.01903-19

**O'Morain, V.** (2019). *The beneficial effects of the Lab4 probiotic consortium in atherosclerosis* [Phd, Cardiff University]. https://orca.cardiff.ac.uk/124825/

**Oner, O., Aslim, B., & Aydaş, S.** (2013). Mechanisms of Cholesterol-Lowering Effects of Lactobacilli and Bifidobacteria Strains as Potential Probiotics with Their bsh Gene Analysis. *Journal of Molecular Microbiology and Biotechnology*, *24*, 12–18. https://doi.org/10.1159/000354316

**O'Sullivan, O., O'Callaghan, J., Sangrador-Vegas, A., McAuliffe, O., Slattery, L., Kaleta, P., Callanan, M., Fitzgerald, G. F., Ross, R. P., & Beresford, T.** (2009). Comparative genomics of lactic acid bacteria reveals a niche-specific gene set. *BMC Microbiology*, *9*(1), 50. https://doi.org/10.1186/1471-2180-9-50

**Ouoba, L. I. I., Lei, V., & Jensen, L. B.** (2008). Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: Determination and transferability of the resistance genes to other bacteria. *International Journal of Food Microbiology*, *121*(2), 217–224. https://doi.org/10.1016/j.ijfoodmicro.2007.11.018

**Ouwehand, A. C., Salminen, S., & Isolauri, E.** (2002). Probiotics: An overview of beneficial effects. In R. J. Siezen, J. Kok, T. Abee, & G. Schasfsma (Eds.), *Lactic Acid Bacteria: Genetics, Metabolism and Applications,* 279–289. Springer Netherlands. https://doi.org/10.1007/978-94-017-2029-8_18

**Ozen, M., & Dinleyici, E. C.** (2015). The history of probiotics: The untold story. *Beneficial Microbes*, *6*(2), 159–165. https://doi.org/10.3920/BM2014.0103

**Özkan, E. R., Öztürk, H. İ., Demirci, T., & Akın, N.** (2021). Detection of biofilm formation, virulence factor genes, antibiotic resistance, adherence properties, and some beneficial properties of cheese origin S. infantarius, S. gallolyticus, and S. lutetiensis strains belonging to the S. bovis/S. equinus complex. *LWT*, *150*, 112077. https://doi.org/10.1016/j.lwt.2021.112077

**Pal, G., & Srivastava, S.** (2014). Cloning and heterologous expression of plnE, -F, -J and -K genes derived from soil metagenome and purification of active plantaricin peptides. *Applied Microbiology and Biotechnology*, *98*(3), 1441–1447. https://doi.org/10.1007/s00253-013-5097-1

**Pan, L., Hu, X., & Wang, X.** (2011). Assessment of antibiotic resistance of lactic acid bacteria in Chinese fermented foods. *Food Control*, *22*(8), 1316–1321. https://doi.org/10.1016/j.foodcont.2011.02.006

**Pan, X., Chen, F., Wu, T., Tang, H., & Zhao, Z.** (2009). The acid, bile tolerance and antimicrobial property of Lactobacillus acidophilus NIT. *Food Control*, *20*(6), 598–602. https://doi.org/10.1016/j.foodcont.2008.08.019

**Pandey, Kavita. R., Naik, Suresh. R., & Vakil, Babu. V.** (2015). Probiotics, prebiotics and synbiotics - a review. *Journal of Food Science and Technology*, *52*(12), 7577–7587. https://doi.org/10.1007/s13197-015-1921-1

**Pandey, N., Malik, R. K., Kaushik, J. K., & Singroha, G.** (2013). Gassericin A: A circular bacteriocin produced by Lactic acid bacteria Lactobacillus gasseri. *World Journal of Microbiology and Biotechnology*, *29*(11), 1977–1987. https://doi.org/10.1007/s11274-013-1368-3

**Pang, H., Kitahara, M., Tan, Z., Wang, Y., Qin, G., Ohkuma, M., & Cai, Y.** (2012). Reclassification of Lactobacillus kimchii and Lactobacillus bobalius as later subjective synonyms of Lactobacillus paralimentarius. *International Journal of Systematic and Evolutionary Microbiology,* *62*(Pt_10), 2383–2387. https://doi.org/10.1099/ijs.0.035329-0

**Panicker, A. S., Ali, S. A., Anand, S., Panjagari, N. R., Kumar, S., Mohanty, A. K., & Behare, P. V.** (2018). Evaluation of some in vitro probiotic properties of Lactobacillus fermentum Strains. *Journal of Food Science and Technology*, *55*(7), 2801–2807. https://doi.org/10.1007/s13197-018-3197-8

**Parada Venegas, D., De la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., Harmsen, H. J. M., Faber, K. N., & Hermoso, M. A.** (2019). Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Frontiers in Immunology*, *10*, 277. https://doi.org/10.3389/fimmu.2019.00277

**Park, D.W., Kim, S.H., & Park, J.H.** (2022). Distribution and characterization of prophages in Lactobacillus plantarum derived from kimchi. *Food Microbiology*, *102*, 103913. https://doi.org/10.1016/j.fm.2021.103913

**Park, S.C., & Won, S.** (2018). Evaluation of 16S rRNA Databases for Taxonomic Assignments Using a Mock Community. *Genomics & Informatics*, *16*(4). https://doi.org/10.5808/GI.2018.16.4.e24

**Park, Y. H., Kim, J. G., Shin, Y. W., Kim, H. S., Kim, Y.J., Chun, T., Kim, S. H., & Whang, K. Y.** (2008). Effects of *Lactobacillus acidophilus* 43121 and a Mixture of *Lactobacillus casei* and *Bifidobacterium longum* on the Serum Cholesterol Level and Fecal Sterol Excretion in Hypercholesterolemia-Induced Pigs. *Bioscience, Biotechnology, and Biochemistry*, *72*(2), 595–600. https://doi.org/10.1271/bbb.70581

**Parker, J. E., Warrilow, A. G., Cools, H. J., Fraaije, B. A., Lucas, J. A., Rigdova, K., ... & Kelly, S. L.** (2013). Prothioconazole and prothioconazole-desthio activities against Candida albicans sterol 14-α-demethylase. Applied and environmental microbiology, 79(5), 1639-1645. https://doi.org/10.1128/AEM.03246-12

**Pascual, L. M., Daniele, M. B., Pájaro, C., & Barberis, L.** (2006). Lactobacillus species isolated from the vagina: Identification, hydrogen peroxide production and nonoxynol-9 resistance. *Contraception*, *73*(1), 78–81. https://doi.org/10.1016/j.contraception.2005.06.066

**Patel, A., Shah, N., & Prajapati, J.** (2012). Antibiotic Resistance Profile of Lactic Acid Bacteria and Their Implications in Food Chain. *World Journal of Dairy & Food Sciences*, *7*, 202–211. https://doi.org/10.5829/idosi.wjdfs.2012.7.2.1113

**Patel, J.** (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnosis*, *6*(4), 313–321. https://doi.org/10.1054/modi.2001.29158

**Pavlović, N., Stankov, K., & Mikov, M.** (2012). Probiotics—Interactions with Bile Acids and Impact on Cholesterol Metabolism. *Applied Biochemistry and Biotechnology*, *168*. https://doi.org/10.1007/s12010-012-9904-4

**Pei, Z., Sadiq, F. A., Han, X., Zhao, J., Zhang, H., Ross, R. P., Lu, W., & Chen, W.** (2021). Comprehensive Scanning of Prophages in Lactobacillus: Distribution, Diversity, Antibiotic Resistance Genes, and Linkages with CRISPR-Cas Systems. *MSystems*, *6*(3), e01211-20. https://doi.org/10.1128/mSystems.01211-20

**Peng, W., Meng, D., Yue, T., Wang, Z., & Gao, Z.** (2021). Effect of the apple cultivar on cloudy apple juice fermented by a mixture of Lactobacillus acidophilus, Lactobacillus plantarum, and Lactobacillus fermentum. *Food Chemistry*, *340*, 127922. https://doi.org/10.1016/j.foodchem.2020.127922

**Pereira, D. I. A., McCartney, A. L., & Gibson, G. R.** (2003). An In Vitro Study of the Probiotic Potential of a Bile-Salt-Hydrolyzing Lactobacillus fermentum Strain, and Determination of Its Cholesterol-Lowering Properties. *Applied and Environmental Microbiology*, *69*(8), 4743–4752. https://doi.org/10.1128/AEM.69.8.4743-4752.2003

**Peternel, M., Čanžek Majhenič, A., Holo, H., Salehian, Z., Berlec, A., & Rogelj, I.** (2010). Wide-Inhibitory Spectra Bacteriocins Produced by Lactobacillus gasseri K7. *Probiotics and Antimicrobial Proteins*, *2*, 233–240. https://doi.org/10.1007/s12602-010-9044-5

**Petersen, C., & Round, J. L.** (2014). Defining dysbiosis and its influence on host immunity and disease. *Cellular Microbiology*, *16*(7), 1024–1033. https://doi.org/10.1111/cmi.12308

**Petrova, M. I., Reid, G., & ter Haar, J. A.** (2021). Lacticaseibacillus rhamnosus GR-1, a.k.a. Lactobacillus rhamnosus GR-1: Past and Future Perspectives. *Trends in Microbiology*, *29*(8), 747–761. https://doi.org/10.1016/j.tim.2021.03.010

**Petti, C. A., Polage, C. R., & Schreckenberger, P.** (2005). The Role of 16S rRNA Gene Sequencing in Identification of Microorganisms Misidentified by Conventional Methods. *Journal of Clinical Microbiology*, *43*(12), 6123–6125. https://doi.org/10.1128/JCM.43.12.6123-6125.2005

**Pfaffl, M. W.** (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, *29*(9), e45. https://doi.org/10.1093/nar/29.9.e45

**Pfeiler, E. A., & Klaenhammer, T. R.** (2009). Role of Transporter Proteins in Bile Tolerance of Lactobacillus acidophilus. *Applied and Environmental Microbiology*, *75*(18), 6013–6016. https://doi.org/10.1128/AEM.00495-09

**Piddock, L.** (2006). Multidrug-resistance efflux pumps? Not just for resistance. *Nature Reviews. Microbiology*, *4*, 629–636. https://doi.org/10.1038/nrmicro1464

**Pietrucci, D., Cerroni, R., Unida, V., Farcomeni, A., Pierantozzi, M., Mercuri, N. B., Biocca, S., Stefani, A., & Desideri, A.** (2019). Dysbiosis of gut microbiota in a selected population of Parkinson's patients. *Parkinsonism & Related Disorders*, *65*, 124–130. https://doi.org/10.1016/j.parkreldis.2019.06.003

**Pietzke, M., Meiser, J., & Vazquez, A.** (2020). Formate metabolism in health and disease. *Molecular Metabolism*, *33*, 23–37. https://doi.org/10.1016/j.molmet.2019.05.012

**Pilet, F., Quaicoe, R. N., Osagie, I. J., Freire, M., & Foissac, X.** (2019). Multi locus Sequence Analysis Reveals Three Distinct Populations of "Candidatus Phytoplasma palmicola" with a Specific Geographical Distribution on the African Continent. *Applied and Environmental Microbiology*, *85*(8). https://doi.org/10.1128/AEM.02716-18

**Piuri, M., Sanchez-Rivas, C., & Ruzal, S. M.** (2005). Cell wall modifications during osmotic stress in Lactobacillus casei. *Journal of Applied Microbiology*, *98*(1), 84–95. https://doi.org/10.1111/j.1365-2672.2004.02428.x

**Plaza-Diaz, J., Ruiz-Ojeda, F. J., Gil-Campos, M., & Gil, A.** (2019). Mechanisms of Action of Probiotics. *Advances in Nutrition*, *10*, S49–S66. https://doi.org/10.1093/advances/nmy063

**Polit, A., Yang, H., & Amund, D.** (2018). Investigating the transmissibility of *tet*(W) in bifidobacteria exposed to acid and bile stress. *Bioscience of Microbiota, Food and Health*, *37*(2), 39–43. https://doi.org/10.12938/bmfh.17-017

**Pontes, K. S. da S., Guedes, M. R., Cunha, M. R. da, Mattos, S. de S., Barreto Silva, M. I., Neves, M. F., Marques, B. C. A. A., & Klein, M. R. S. T.** (2021). Effects of probiotics on body adiposity and cardiovascular risk markers in individuals with overweight and obesity: A systematic review and meta-analysis of randomized controlled trials. *Clinical Nutrition*, *40*(8), 4915–4931. https://doi.org/10.1016/j.clnu.2021.06.023

**Poretsky, R., Rodriguez-R, L. M., Luo, C., Tsementzi, D., & Konstantinidis, K. T.** (2014). Strengths and Limitations of 16S rRNA Gene Amplicon Sequencing in Revealing Temporal Microbial Community Dynamics. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0093827

**Porse, A., Gumpert, H., Kubicek-Sutherland, J. Z., Karami, N., Adlerberth, I., Wold, A. E., Andersson, D. I., & Sommer, M. O. A.** (2017). Genome Dynamics of Escherichia coli during Antibiotic Treatment: Transfer, Loss, and Persistence of Genetic Elements In situ of the Infant Gut. *Frontiers in Cellular and Infection Microbiology*, *7*. https://doi.org/10.3389/fcimb.2017.00126

**Pot, B., Felis, G. E., Bruyne, K. D., Tsakalidou, E., Papadimitriou, K., Leisner, J., & Vandamme, P.** (2014). The genus Lactobacillus. In *Lactic Acid Bacteria* (pp. 249–353). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118655252.ch19

**Pot, B., Salvetti, E., Mattarelli, P., & Felis, G. E.** (2019). The potential impact of the Lactobacillus name change: The results of an expert meeting organised by the Lactic Acid Bacteria Industrial Platform (LABIP). *Trends in Food Science & Technology*, *94*, 105–113. https://doi.org/10.1016/j.tifs.2019.07.006

**Pot, B., & Tsakalidou, E.** (2009). *Taxonomy and metabolism of Lactobacillus*, 3–58.

**Preising, J., Philippe, D., Gleinser, M., Wei, H., Blum, S., Eikmanns, B. J., Niess, J.H., & Riedel, C. U.** (2010). Selection of Bifidobacteria Based on Adhesion and Anti-Inflammatory Capacity In Vitro for Amelioration of Murine Colitis. *Applied and Environmental Microbiology*, *76*(9), 3048–3051. https://doi.org/10.1128/AEM.03127-09

**Prestinaci, F., Pezzotti, P., & Pantosti, A.** (2015). Antimicrobial resistance: A global multifaceted phenomenon. *Pathogens and Global Health*, *109*(7), 309–318. https://doi.org/10.1179/2047773215Y.0000000030

**Price, M. N., Dehal, P. S., & Arkin, A. P.** (2010). FastTree 2 – Approximately Maximum likelihood Trees for Large Alignments. *PLOS ONE*, *5*(3), e9490. https://doi.org/10.1371/journal.pone.0009490

**Pridmore, R. D., Pittet, A.C., Praplan, F., & Cavadini, C.** (2008). Hydrogen peroxide production by Lactobacillus johnsonii NCC 533 and its role in anti-Salmonella activity. *FEMS Microbiology Letters*, *283*(2), 210–215. https://doi.org/10.1111/j.1574-6968.2008.01176.x

**Proctor, R. A., van Langevelde, P., Kristjansson, M., Maslow, J. N., & Arbeit, R. D**. (1995). Persistent and Relapsing Infections Associated with Small-Colony Variants of Staphylococcus aureus. *Clinical Infectious Diseases*, *20*(1), 95–102. https://doi.org/10.1093/clinids/20.1.95

**Qi, D., Nie, X.L., & Zhang, JJ.** (2020). The effect of probiotics supplementation on blood pressure: A systemic review and meta-analysis. *Lipids in Health and Disease*, *19*(1), 79. https://doi.org/10.1186/s12944-020-01259-x

**Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., … Wang, J.** (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, *464*(7285), 59–65. https://doi.org/10.1038/nature08821

**Qiu, G., Yu, Y., Wang, Y., & Wang, X.** (2019). The significance of probiotics in preventing radiotherapy-induced diarrhea in patients with cervical cancer: A systematic review and meta-analysis. *International Journal of Surgery*, *65*, 61–69. https://doi.org/10.1016/j.ijsu.2019.03.015

**Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O.** (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*, D590–D596. https://doi.org/10.1093/nar/gks1219

**Queller, D. C., & Goodnight, K. F.** (1989). Estimating Relatedness Using Genetic Markers. *Evolution*, *43*(2), 258–275. https://doi.org/10.1111/j.1558-5646.1989.tb04226.x

**Quince, C., Lanzén, A., Curtis, T. P., Davenport, R. J., Hall, N., Head, I. M., Read, L. F., & Sloan, W. T.** (2009). Accurate determination of microbial diversity from 454 pyrosequencing data. *Nature Methods*, *6*(9), 639–641. https://doi.org/10.1038/nmeth.1361

**Quince, C., Lanzen, A., Davenport, R. J., & Turnbaugh, P. J.** (2011). Removing noise from pyrosequenced amplicons. *BMC Bioinformatics*, *12*, 38. https://doi.org/10.1186/1471-2105-12-38

**Quirós, P., Colomer-Lluch, M., Martínez-Castillo, A., Miró, E., Argente, M., Jofre, J., Navarro, F., & Muniesa, M.** (2014). Antibiotic Resistance Genes in the Bacteriophage DNA Fraction of Human Fecal Samples. *Antimicrobial Agents and Chemotherapy*, *58*(1), 606–609. https://doi.org/10.1128/AAC.01684-13

**Ramos, C. L., Thorsen, L., Schwan, R. F., & Jespersen, L.** (2013). Strain-specific probiotics properties of Lactobacillus fermentum, Lactobacillus plantarum and Lactobacillus brevis isolates from Brazilian food products. *Food Microbiology*, *36*(1), 22–29. https://doi.org/10.1016/j.fm.2013.03.010

**Rani, R. P., Anandharaj, M., & Ravindran, A. D.** (2017). Characterization of Bile Salt Hydrolase from Lactobacillus gasseri FR4 and Demonstration of Its Substrate Specificity and Inhibitory Mechanism Using Molecular Docking Analysis. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01004

**Rausser, G. C.** (2000). Valuing Research Leads: Bioprospecting and the Conservation of Genetic Resources. *Journal of Political Economy*, 34.

**Raveendran, S., Poulose, A. C., Yoshida, Y., Maekawa, T., & Kumar, D. S.** (2013). Bacterial exopolysaccharide-based nanoparticles for sustained drug delivery, cancer chemotherapy and bioimaging. *Carbohydrate Polymers*, *91*(1), 22–32. https://doi.org/10.1016/j.carbpol.2012.07.079

**Read, A. F., & Woods, R. J.** (2014). Antibiotic resistance management. *Evolution, Medicine, and Public Health*, *2014*(1), 147. https://doi.org/10.1093/emph/eou024

**Reale, A., Di Renzo, T., Rossi, F., Zotta, T., Iacumin, L., Preziuso, M., Parente, E., Sorrentino, E., & Coppola, R.** (2015). Tolerance of Lactobacillus casei, Lactobacillus paracasei and Lactobacillus rhamnosus strains to stress factors encountered in food processing and in the gastro-intestinal tract. *LWT - Food Science and Technology*, *60*(2, Part 1), 721–728. https://doi.org/10.1016/j.lwt.2014.10.022

**Régnier, M., Hul, M. van, Knauf, C., & Cani, P.** (2021). Gut microbiome, endocrine control of gut barrier function and metabolic diseases. *Journal of Endocrinology*, *248*(2), R67. https://doi.org/10.1530/JOE-20-0473

**Reid, G.** (2016). Probiotics: Definition, scope and mechanisms of action. *Best Practice & Research Clinical Gastroenterology*, *30*(1), 17–25. https://doi.org/10.1016/j.bpg.2015.12.001

**Ren, J., Sun, K., Wu, Z., Yao, J., & Guo, B.** (2011). All 4 Bile Salt Hydrolase Proteins Are Responsible for the Hydrolysis Activity in Lactobacillus plantarum ST-III. *Journal of Food Science*, *76*(9), M622–M628. https://doi.org/10.1111/j.1750-3841.2011.02431.x

**Rezaei Javan, R., Ramos-Sevillano, E., Akter, A., Brown, J., & Brueggemann, A. B.** (2019). Prophages and satellite prophages are widespread in Streptococcus and may play a role in pneumococcal pathogenesis. *Nature Communications*, *10*(1), 4852. https://doi.org/10.1038/s41467-019-12825-y

**Ricker, N., Qian, H., & Fulthorpe, R. R.** (2012). The limitations of draft assemblies for understanding prokaryotic adaptation and evolution. *Genomics*, *100*(3), 167–175. https://doi.org/10.1016/j.ygeno.2012.06.009

**Ridlon, J. M., Kang, D.J., & Hylemon, P. B.** (2006). Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research*, *47*(2), 241–259. https://doi.org/10.1194/jlr.R500013-JLR200

**Ridlon, J. M., Kang, D.J., Hylemon, P. B., & Bajaj, J. S.** (2015). Gut Microbiota, Cirrhosis, and Alcohol Regulate Bile Acid Metabolism in the Gut. *Digestive Diseases*, *33*(3), 338–345. https://doi.org/10.1159/000371678

**Riedel, C. U., Foata, F., Goldstein, D. R., Blum, S., & Eikmanns, B. J.** (2006). Interaction of bifidobacteria with Caco-2 cells-adhesion and impact on expression profiles. *International Journal of Food Microbiology*, *110*(1), 62–68. https://doi.org/10.1016/j.ijfoodmicro.2006.01.040

**Riley, M. A., & Wertz, J. E.** (2002). Bacteriocins: Evolution, Ecology, and Application. *Annual Review of Microbiology*, *56*(1), 117–137. https://doi.org/10.1146/annurev.micro.56.012302.161024

**Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., & Mele, M. C.** (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms*, *7*(1), 14. https://doi.org/10.3390/microorganisms7010014

**Ritchie, M. L., & Romanuk, T. N.** (2012). A Meta-Analysis of Probiotic Efficacy for Gastrointestinal Diseases. *PLOS ONE*, *7*(4), e34938. https://doi.org/10.1371/journal.pone.0034938

**Rivard, N., Colwell, R. R., & Burrus, V.** (2020). Antibiotic Resistance in Vibrio cholerae: Mechanistic Insights from IncC Plasmid-Mediated Dissemination of a Novel Family of Genomic Islands Inserted at trmE. *MSphere*, *5*(4), e00748-20. https://doi.org/10.1128/mSphere.00748-20

**Roberts, M. C., & Schwarz, S.** (2016). Tetracycline and Phenicol Resistance Genes and Mechanisms: Importance for Agriculture, the Environment, and Humans. *Journal of Environmental Quality*, *45*(2), 576–592. https://doi.org/10.2134/jeq2015.04.0207

**RodrÍguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M. C., Marchesi, J. R., & Collado, M. C.** (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease*, *26*(s2), 26050. https://doi.org/10.3402/mehd.v26.26050

**Rodriguez-Valera, F., Martin-Cuadrado, A.B., & López-Pérez, M.** (2016). Flexible genomic islands as drivers of genome evolution. *Current Opinion in Microbiology*, *31*, 154–160. https://doi.org/10.1016/j.mib.2016.03.014

**Rosander, A., Connolly, E., & Roos, S.** (2008). Removal of Antibiotic Resistance Gene-Carrying Plasmids from Lactobacillus reuteri ATCC 55730 and Characterization of the Resulting Daughter Strain, L. reuteri DSM 17938. *Applied and Environmental Microbiology*, *74*(19), 6032–6040. https://doi.org/10.1128/AEM.00991-08

**Roshan, H., Ghaedi, E., Rahmani, J., Barati, M., Najafi, M., Karimzedeh, M., & Nikpayam, O.** (2019). Effects of probiotics and synbiotic supplementation on antioxidant status: A meta-analysis of randomized clinical trials. *Clinical Nutrition ESPEN*, *30*, 81–88. https://doi.org/10.1016/j.clnesp.2019.02.003

**Ross, R. P., Mills, S., Hill, C., Fitzgerald, G. F., & Stanton, C.** (2010). Specific metabolite production by gut microbiota as a basis for probiotic function. *International Dairy Journal*, *20*(4), 269–276. https://doi.org/10.1016/j.idairyj.2009.12.003

**Rossi-Tamisier, M., Benamar, S., Raoult, D., & Fournier, P.E.** (2015). Cautionary tale of using 16S rRNA gene sequence similarity values in identification of human-associated bacterial species. *International Journal of Systematic and Evolutionary Microbiology, 65*, 1929–1934. https://doi.org/10.1099/ijs.0.000161

**Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P. I., Godneva, A., Kalka, I. N., Bar, N., Shilo, S., Lador, D., Vila, A. V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B. C., … Segal, E.** (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature*, *555* (7695), 210–215. https://doi.org/10.1038/nature25973

**Rozman, V., Mohar Lorbeg, P., Accetto, T., & Bogovič Matijašić, B.** (2020). Characterization of antimicrobial resistance in lactobacilli and bifidobacteria used as probiotics or starter cultures based on integration of phenotypic and in silico data. *International Journal of Food Microbiology*, *314*, 108388. https://doi.org/10.1016/j.ijfoodmicro.2019.108388

**Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. de L., Bories, G., Chesson, A., Cocconcelli, P. S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., Puente, S. L., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R. E., … Galobart, J.** (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal*, *16*(3), e05206. https://doi.org/10.2903/j.efsa.2018.5206

**Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M., & Bressollier, P.** (2013). An overview of the last advances in probiotic and prebiotic field. *LWT - Food Science and Technology*, *50*(1), 1–16. https://doi.org/10.1016/j.lwt.2012.05.014

**Sahadeva, R. P. K., Leong, S. F., Chua, K. H., Tan, C. H., Chan, H. Y., Tong, E. V., Wong, S. Y. W., & Chan, H. K.** (2011). Survival of commercial probiotic strains to pH and bile. *International Food Research Journal*, *18*(4), 1515–1522.

**Saitou, N., & Nei, M.** (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees*, *4*(4), 406–425.

**Salas-Jara, M. J., Ilabaca, A., Vega, M., & García, A.** (2016). Biofilm Forming Lactobacillus: New Challenges for the Development of Probiotics. *Microorganisms*, *4*(3), 35. https://doi.org/10.3390/microorganisms4030035

**Saleem, N.** (2018). Phenotypic and Molecular Analysis of Antibiotic Resistance in Lactobacilli of Poultry Origin from Lahore, Pakistan. *Pakistan Veterinary Journal*, *38*(04), 409–413. https://doi.org/10.29261/pakvetj/2018.084

**Salminen, S., Collado, M. C., Endo, A., Hill, C., Lebeer, S., Quigley, E. M. M., Sanders, M. E., Shamir, R., Swann, J. R., Szajewska, H., & Vinderola, G.** (2021). The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nature Reviews Gastroenterology & Hepatology*, *18*(9), 649–667. https://doi.org/10.1038/s41575-021-00440-6

**Salvadori, G., Junges, R., Morrison, D. A., & Petersen, F. C.** (2019). Competence in Streptococcus pneumoniae and Close Commensal Relatives: Mechanisms and Implications. *Frontiers in Cellular and Infection Microbiology*, *9*, 94. https://doi.org/10.3389/fcimb.2019.00094

**Salvetti, E., Harris, H. M. B., Felis, G. E., & O'Toole, P. W.** (2018). Comparative Genomics of the Genus *Lactobacillus* Reveals Robust Phylogroups That Provide the Basis for Reclassification. *Applied and Environmental Microbiology*, *84*(17), e00993-18. https://doi.org/10.1128/AEM.00993-18

**Salvetti, E., & O'Toole, P. W.** (2017). When regulation challenges innovation: The case of the genus Lactobacillus. *Trends in Food Science & Technology*, *66*, 187–194. https://doi.org/10.1016/j.tifs.2017.05.009

**Salvetti, E., & O'Toole, P. W.** (2018). The Genomic Basis of Lactobacilli as Health-Promoting Organisms. In *Bugs as Drugs* (pp. 49–71). John Wiley & Sons, Ltd. https://doi.org/10.1128/9781555819705.ch2

**Salvetti, E., Torriani, S., & Felis, G. E.** (2012). The Genus Lactobacillus: A Taxonomic Update. *Probiotics and Antimicrobial Proteins*, *4*(4), 217–226. https://doi.org/10.1007/s12602-012-9117-8

**Salvi, M., Colucci, G., Masetti, G., Covelli, D., Muller, I., Koehling, H., Garaiova, I., Michael, D., Zhang, L., Ludgate, M., Marchesi, J., & Biscarini, F.** (2019). The randomised probiotic trial of indigo study, investigation of novel biomarkers and definition of role of the microbiome in Graves. *Endocrine Abstracts*, *63*. https://doi.org/10.1530/endoabs.63.GP71

**Samson, J. E., & Moineau, S.** (2013). Bacteriophages in Food Fermentations: New Frontiers in a Continuous Arms Race. *Annual Review of Food Science and Technology*, *4*(1), 347–368. https://doi.org/10.1146/annurev-food-030212-182541

**San Millan, A.** (2018). Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context. *Trends in Microbiology*, *26*(12), 978–985. https://doi.org/10.1016/j.tim.2018.06.007

**Sandner-Miranda, L., Vinuesa, P., Cravioto, A., & Morales-Espinosa, R.** (2018). The Genomic Basis of Intrinsic and Acquired Antibiotic Resistance in the Genus Serratia. *Frontiers in Microbiology*, *9*, 828. https://doi.org/10.3389/fmicb.2018.00828

**Santos, S. R., & Ochman, H.** (2004). Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology*, *6*(7), 754–759. https://doi.org/10.1111/j.1462-2920.2004.00617.x

**Sartor, R. B.** (2008). Microbial Influences in Inflammatory Bowel Diseases. *Gastroenterology*, *134*(2), 577–594. https://doi.org/10.1053/j.gastro.2007.11.059

**Sassone-Corsi, M., & Raffatellu, M.** (2015). No Vacancy: How Beneficial Microbes Cooperate with Immunity to Provide Colonization Resistance to Pathogens. *The Journal of Immunology*, *194*(9), 4081–4087. https://doi.org/10.4049/jimmunol.1403169

**Saxami, G., Ypsilantis, P., Sidira, M., Simopoulos, C., Kourkoutas, Y., & Galanis, A.** (2012). Distinct adhesion of probiotic strain Lactobacillus casei ATCC 393 to rat intestinal mucosa. *Anaerobe*, *18*(4), 417–420. https://doi.org/10.1016/j.anaerobe.2012.04.002

**Schakenraad, J. M., Nieuwenhuis, P., Molenaar, I., Helder, J., Dijkstra, P. J., & Feijen, J.** (1989). In vivo and in vitro degradation of glycine/DL-lactic acid copolymers. *Journal of Biomedical Materials Research*, *23*(11), 1271–1288. https://doi.org/10.1002/jbm.820231105

**Schellenberg, J., Links, M. G., Hill, J. E., Dumonceaux, T. J., Peters, G. A., Tyler, S., Ball, T. B., Severini, A., & Plummer, F. A.** (2009). Pyrosequencing of the Chaperonin-60 Universal Target as a Tool for Determining Microbial Community Composition. *Applied and Environmental Microbiology*, *75*(9), 2889–2898. https://doi.org/10.1128/AEM.01640-08

**Schluter, J., Peled, J. U., Taylor, B. P., Markey, K. A., Smith, M., Taur, Y., Niehus, R., Staffas, A., Dai, A., Fontana, E., Amoretti, L. A., Wright, R. J., Morjaria, S., Fenelus, M., Pessin, M. S., Chao, N. J., Lew, M., Bohannon, L., Bush, A., … Xavier, J. B.** (2020). The gut microbiota is associated with immune cell dynamics in humans. *Nature*, *588*(7837), 303–307. https://doi.org/10.1038/s41586-020-2971-8

**Schrezenmeir, J., & de Vrese, M.** (2001). Probiotics, prebiotics, and synbiotics—Approaching a definition. *The American Journal of Clinical Nutrition*, *73*(2), 361s–364s. https://doi.org/10.1093/ajcn/73.2.361s

**Scornec, H., Tichit, M., Bouchier, C., Pédron, T., Cavin, J.F., Sansonetti, P. J., & Licandro-Seraut, H.** (2014). Rapid 96-well plates DNA extraction and sequencing procedures to identify genome-wide transposon insertion sites in a difficult to lyse bacterium: Lactobacillus casei. *Journal of Microbiological Methods*, *106*, 78–82. https://doi.org/10.1016/j.mimet.2014.08.001

**Seemann, T.** (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics*, *30*(14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

**Seesuriyachan, P.** (2012). Statistical modeling and optimization for exopolysaccharide production by Lactobacillus confusus in submerged fermentation under high salinity stress. *Food Science and Biotechnology*, *21*(6), 1647–1654. https://doi.org/10.1007/s10068-012-0219-6

**Segata, N., Börnigen, D., Morgan, X. C., & Huttenhower, C.** (2013). PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. *Nature Communications*, *4*(1), 2304. https://doi.org/10.1038/ncomms3304

**Seki, M., Iida, K., Saito, M., Nakayama, H., & Yoshida, S.** (2004). Hydrogen Peroxide Production in Streptococcus pyogenes: Involvement of Lactate Oxidase and Coupling with Aerobic Utilization of Lactate. *Journal of Bacteriology*, *186*(7), 2046–2051. https://doi.org/10.1128/JB.186.7.2046-2051.2004

**Sekirov, I., Russell, S. L., Antunes, L. C. M., & Finlay, B. B.** (2010). Gut Microbiota in Health and Disease. *Physiological Reviews*, *90*(3), 859–904. https://doi.org/10.1152/physrev.00045.2009

**Seminario-Amez, M., López-López, J., Estrugo-Devesa, A., Ayuso-Montero, R., & Jané-Salas, E.** (2017). Probiotics and oral health: A systematic review. *Medicina Oral, Patología Oral y Cirugía Bucal*, *22*(3), e282. https://doi.org/10.4317/medoral.21494

**Shankar, C., Jacob, J. J., Vasudevan, K., Biswas, R., Manesh, A., Sethuvel, D. P. M., Varughese, S., Biswas, I., & Veeraraghavan, B.** (2020). Emergence of Multidrug Resistant Hypervirulent ST23 Klebsiella pneumoniae: Multidrug Resistant Plasmid Acquisition Drives Evolution. *Frontiers in Cellular and Infection Microbiology*, *10*. https://doi.org/10.3389/fcimb.2020.575289

**Shao, Y., Zhang, W., Guo, H., Pan, L., Zhang, H., & Sun, T.** (2015). Comparative studies on antibiotic resistance in Lactobacillus casei and Lactobacillus plantarum. *Food Control*, *50*, 250–258. https://doi.org/10.1016/j.foodcont.2014.09.003

**Sharma, M., Wasan, A., & Sharma, R. K**. (2021). Recent developments in probiotics: An emphasis on Bifidobacterium. *Food Bioscience*, *41*, 100993. https://doi.org/10.1016/j.fbio.2021.100993

**Sharma, P., Tomar, S. K., Sangwan, V., Goswami, P., & Singh, R.** (2016). Antibiotic Resistance of Lactobacillus sp. Isolated from Commercial Probiotic Preparations. *Journal of Food Safety*, *36*(1), 38–51. https://doi.org/10.1111/jfs.12211

**Shaw, K. J., Rather, P. N., Hare, R. S., & Miller, G. H.** (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews*, *57*(1), 138–163. https://doi.org/10.1128/mr.57.1.138-163.1993

**Shay, J. A.** (2016). *Analysis of Genomic Islands and Other Features in Draft Versus Complete Bacterial Genomes* [Thesis, Science: Department of Molecular Biology and Biochemistry]. http://summit.sfu.ca/item/16643

**Shehata, M. G., El Sohaimy, S. A., El-Sahn, M. A., & Youssef, M. M.** (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Annals of Agricultural Sciences*, *61*(1), 65–75. https://doi.org/10.1016/j.aoas.2016.03.001

**Shenderov, B. A.** (2013). Metabiotics: Novel idea or natural development of probiotic conception. *Microbial Ecology in Health and Disease*, *24*(1), 20399. https://doi.org/10.3402/mehd.v24i0.20399

**Shin, M., Ban, O.H., Jung, Y. H., Yang, J., & Kim, Y.** (2021). Genomic characterization and probiotic potential of Lactobacillus casei IDCC 3451 isolated from infant faeces. *Letters in Applied Microbiology*, *72*(5), 578–588. https://doi.org/10.1111/lam.13449

**Shioya, K., Michaux, C., Kuenne, C., Hain, T., Verneuil, N., Budin-Verneuil, A., Hartsch, T., Hartke, A., & Giard, J.C.** (2011). Genome-Wide Identification of Small RNAs in the Opportunistic Pathogen Enterococcus faecalis V583. *PLOS ONE*, *6*(9), e23948. https://doi.org/10.1371/journal.pone.0023948

**Shoemaker, N. B., Vlamakis, H., Hayes, K., & Salyers, A. A.** (2001). Evidence for Extensive Resistance Gene Transfer among Bacteroides spp. And among Bacteroides and Other Genera in the Human Colon. *Applied and Environmental Microbiology*, *67*(2), 561–568. https://doi.org/10.1128/AEM.67.2.561-568.2001

**Shu, Z., Li, P., Yu, B., Huang, S., & Chen, Y.** (2020). The effectiveness of probiotics in prevention and treatment of cancer therapy-induced oral mucositis: A systematic review and meta-analysis. *Oral Oncology*, *102*, 104559. https://doi.org/10.1016/j.oraloncology.2019.104559

**Sidira, M., Galanis, A., Ypsilantis, P., Karapetsas, A., Progaki, Z., Simopoulos, C., & Kourkoutas, Y.** (2010). Effect of Probiotic-Fermented Milk Administration on gastrointestinal Survival of Lactobacillus casei ATCC 393 and Modulation of Intestinal Microbial Flora. *Microbial Physiology*, *19*(4), 224–230. https://doi.org/10.1159/000321115

**Siezen, R. J., Tzeneva, V. A., Castioni, A., Wels, M., Phan, H. T. K., Rademaker, J. L. W., Starrenburg, M. J. C., Kleerebezem, M., Molenaar, D., & Vlieg, J. E. T. V. H.** (2010). Phenotypic and genomic diversity of Lactobacillus plantarum strains isolated from various environmental niches. *Environmental Microbiology*, *12*(3), 758–773. https://doi.org/10.1111/j.1462-2920.2009.02119.x

**Sillanpää, J., Martínez, B., Antikainen, J., Toba, T., Kalkkinen, N., Tankka, S., Lounatmaa, K., Keränen, J., Höök, M., Westerlund-Wikström, B., Pouwels, P. H., & Korhonen, T. K.** (2000). Characterization of the Collagen-Binding S-Layer Protein CbsA of Lactobacillus crispatus. *Journal of Bacteriology*, *182*(22), 6440–6450. https://doi.org/10.1128/JB.182.22.6440-6450.2000

**Silva, Y. P., Bernardi, A., & Frozza, R. L.** (2020). The Role of Short-Chain Fatty Acids from Gut Microbiota in Gut-Brain Communication. *Frontiers in Endocrinology*, *11*, 25. https://doi.org/10.3389/fendo.2020.00025

**Sinclair, L., Osman, O. A., Bertilsson, S., & Eiler, A.** (2015). Microbial Community Composition and Diversity via 16S rRNA Gene Amplicons: Evaluating the Illumina Platform. *PLOS ONE*, *10*(2), e0116955. https://doi.org/10.1371/journal.pone.0116955

**Singh, S., Goswami, P., Singh, R., & Heller, K. J.** (2009). Application of molecular identification tools for Lactobacillus, with a focus on discrimination between closely related species: A review. *LWT - Food Science and Technology*, *42*(2), 448–457. https://doi.org/10.1016/j.lwt.2008.05.019

**Sircana, A., Framarin, L., Leone, N., Berrutti, M., Castellino, F., Parente, R., De Michieli, F., Paschetta, E., & Musso, G.** (2018). Altered Gut Microbiota in Type 2 Diabetes: Just a Coincidence? *Current Diabetes Reports*, *18*(10), 98. https://doi.org/10.1007/s11892-018-1057-6

**Skjermo, J., Bakke, I., Dahle, S. W., & Vadstein, O**. (2015). Probiotic strains introduced through live feed and rearing water have low colonizing success in developing Atlantic cod larvae. *Aquaculture*, *438*, 17–23. https://doi.org/10.1016/j.aquaculture.2014.12.027

**Skonieczna-Żydecka, K., Kaźmierczak-Siedlecka, K., Kaczmarczyk, M., Śliwa-Dominiak, J., Maciejewska, D., Janda, K., Stachowska, E., Łoniewska, B., Malinowski, D., Borecki, K., Marlicz, W., & Łoniewski, I.** (2020). The Effect of Probiotics and Synbiotics on Risk Factors Associated with Cardiometabolic Diseases in Healthy People—A Systematic Review and Meta-Analysis with Meta-Regression of Randomized Controlled Trials. *Journal of Clinical Medicine*, *9*(6), 1788. https://doi.org/10.3390/jcm9061788

**Slover, C. M., & Danziger, L.** (2008). Lactobacillus: A Review. *Clinical Microbiology Newsletter*, *30*(4), 23–27. https://doi.org/10.1016/j.clinmicnews.2008.01.006

**Smet, I. D., Hoorde, L. V., Woestyne, M. V., Christiaens, H., & Verstraete, W.** (1995). Significance of bile salt hydrolytic activities of lactobacilli. *Journal of Applied Bacteriology*, *79*(3), 292–301. https://doi.org/10.1111/j.1365-2672.1995.tb03140.x

**Solieri, L., Bianchi, A., Mottolese, G., Lemmetti, F., & Giudici, P.** (2014). Tailoring the probiotic potential of non-starter Lactobacillus strains from ripened Parmigiano Reggiano cheese by in vitro screening and principal component analysis. *Food Microbiology*, *38*, 240–249. https://doi.org/10.1016/j.fm.2013.10.003

**Sommer, F., & Bäckhed, F.** (2013). The gut microbiota—Masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227–238. https://doi.org/10.1038/nrmicro2974

**Sorokulova, I. B., Pinchuk, I. V., Denayrolles, M., Osipova, I. G., Huang, J. M., Cutting, S. M., & Urdaci, M. C.** (2008). The Safety of Two Bacillus Probiotic Strains for Human Use. *Digestive Diseases and Sciences*, *53*(4), 954–963. https://doi.org/10.1007/s10620-007-9959-1

**Spinler, J. K., Sontakke, A., Hollister, E. B., Venable, S. F., Oh, P. L., Balderas, M. A., Saulnier, D. M. A., Mistretta, T.A., Devaraj, S., Walter, J., Versalovic, J., & Highlander, S. K.** (2014). From Prediction to Function Using Evolutionary Genomics: Human-Specific Ecotypes of Lactobacillus reuteri Have Diverse Probiotic Functions. *Genome Biology and Evolution*, *6*(7), 1772–1789. https://doi.org/10.1093/gbe/evu137

**Spratt, B. G., Bowler, L. D., Zhang, Q.-Y., Zhou, J., & Smith, J. M.** (1992). Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal Neisseria species. *Journal of Molecular Evolution*, *34*(2), 115–125. https://doi.org/10.1007/BF00182388

**Sroka-Oleksiak, A., Młodzińska, A., Bulanda, M., Salamon, D., Major, P., Stanek, M., & Gosiewski, T.** (2020). Metagenomic Analysis of Duodenal Microbiota Reveals a Potential Biomarker of Dysbiosis in the Course of Obesity and Type 2 Diabetes: A Pilot Study. *Journal of Clinical Medicine*, *9*(2), 369. https://doi.org/10.3390/jcm9020369

**Stecher, B., Denzler, R., Maier, L., Bernet, F., Sanders, M. J., Pickard, D. J., Barthel, M., Westendorf, A. M., Krogfelt, K. A., Walker, A. W., Ackermann, M., Dobrindt, U., Thomson, N. R., & Hardt, W.D.** (2012). Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proceedings of the National Academy of Sciences*, *109*(4), 1269–1274. https://doi.org/10.1073/pnas.1113246109

**Štšepetova, J., Taelma, H., Smidt, I., Hütt, P., Lapp, E., Aotäht, E., & Mändar, R.** (2017). Assessment of phenotypic and genotypic antibiotic susceptibility of vaginal Lactobacillus sp. *Journal of Applied Microbiology*, *123*(2), 524–534. https://doi.org/10.1111/jam.13497

**Su, M., Jia, Y., Li, Y., Zhou, D., & Jia, J.** (2020). Probiotics for the Prevention of Ventilator-Associated Pneumonia: A Meta-Analysis of Randomized Controlled Trials. *Respiratory Care*, *65*(5), 673–685. https://doi.org/10.4187/respcare.07097

**Sudha, M., Chauhan, A., Dixit, K., Babu, S., & Jamil, K.** (2009). Probiotics as complementary therapy for hypercholesterolemia. *Biology and Medicine*, *1*.

**Sui, S. J. H., Fedynak, A., Hsiao, W. W. L., Langille, M. G. I., & Brinkman, F. S. L.** (2009). The Association of Virulence Factors with Genomic Islands. *PLOS ONE*, *4*(12), e8094. https://doi.org/10.1371/journal.pone.0008094

**Sun, E., Zhao, L., Ren, F., Liu, S., Zhang, M., & Guo, H.** (2015). Complete genome sequence of Bifidobacterium animalis subsp. Lactis A6, a probiotic strain with high acid resistance ability. *Journal of Biotechnology*, *200*, 8–9. https://doi.org/10.1016/j.jbiotec.2015.02.016

**Sun, M.F., & Shen, Y.Q.** (2018). Dysbiosis of gut microbiota and microbial metabolites in Parkinson's Disease. *Ageing Research Reviews*, *45*, 53–61. https://doi.org/10.1016/j.arr.2018.04.004

**Sun, Z., Harris, H. M. B., McCann, A., Guo, C., Argimón, S., Zhang, W., Yang, X., Jeffery, I. B., Cooney, J. C., Kagawa, T. F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M. C., O'Sullivan, O., Ritari, J., … O'Toole, P. W.** (2015a). Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications*, *6*(1), 8322. https://doi.org/10.1038/ncomms9322

**Sun, Z., Westermann, C., Yuan, J., & Riedel, C.** (2014). Experimental determination and characterization of the gap promoter of Bifidobacterium bifidum S17. *Bioengineered*, *5*. https://doi.org/10.4161/bioe.34423

**Sun, Z., Zhang, W., Guo, C., Yang, X., Liu, W., Wu, Y., Song, Y., Kwok, L. Y., Cui, Y., Menghe, B., Yang, R., Hu, L., & Zhang, H.** (2015). Comparative Genomic Analysis of 45 Type Strains of the Genus Bifidobacterium: A Snapshot of Its Genetic Diversity and Evolution. *PLoS ONE*, *10*(2). https://doi.org/10.1371/journal.pone.0117912

**Sundermann, A. J., Chen, J., Kumar, P., Ayres, A. M., Cho, S. T., Ezeonwuka, C., ... & Harrison, L. H.** (2021). Whole-Genome Sequencing Surveillance and Machine Learning of the Electronic Health Record for Enhanced Healthcare Outbreak Detection. *Clinical Infectious Diseases.* https://doi.org/10.1093/cid/ciab946

**Surawicz, C. M., Brandt, L. J., Binion, D. G., Ananthakrishnan, A. N., Curry, S. R., Gilligan, P. H., McFarland, L. V., Mellow, M., & Zuckerbraun, B. S.** (2013). Guidelines for Diagnosis, Treatment, and Prevention of Clostridium difficileInfections. *Official Journal of the American College of Gastroenterology ACG*, *108*(4), 478–498. https://doi.org/10.1038/ajg.2013.4

**Szulińska, M., Łoniewski, I., Van Hemert, S., Sobieska, M., & Bogdański, P.** (2018). Dose-Dependent Effects of Multispecies Probiotic Supplementation on the Lipopolysaccharide (LPS) Level and Cardiometabolic Profile in Obese Postmenopausal Women: A 12-Week Randomized Clinical Trial. *Nutrients*, *10*(6), 773. https://doi.org/10.3390/nu10060773

**Taft, D. H., Liu, J., Maldonado-Gomez, M. X., Akre, S., Huda, M. N., Ahmad, S. M., Stephensen, C. B., & Mills, D. A.** (2018). Bifidobacterial Dominance of the Gut in Early Life and Acquisition of Antimicrobial Resistance. *MSphere*, *3*(5), e00441-18. https://doi.org/10.1128/mSphere.00441-18

**Tahara, T., Oshimura, M., Umezawa, C., & Kanatani, K.** (1996). Isolation, partial characterization, and mode of action of Acidocin J1132, a two-component bacteriocin produced by Lactobacillus acidophilus JCM 1132. *Applied and Environmental Microbiology*, *62*(3), 892–897. https://doi.org/10.1128/aem.62.3.892-897.1996

**Tahmourespour, A., & Kermanshahi, R. K.** (2011). The effect of a probiotic strain (Lactobacillus acidophilus) on the plaque formation of oral Streptococci. *Bosnian Journal of Basic Medical Sciences*, *11*(1), 37–40.

**Tailford, L. E., Crost, E. H., Kavanaugh, D., & Juge, N.** (2015). Mucin glycan foraging in the human gut microbiome. *Frontiers in Genetics*, *6*, 81. https://doi.org/10.3389/fgene.2015.00081

**Tajima, F., & Nei, M.** (1984). Estimation of evolutionary distance between nucleotide sequences. *Molecular Biology and Evolution*, *1*(3), 269–285. https://doi.org/10.1093/oxfordjournals.molbev.a040317

**Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S.** (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

**Tanaka, H., Doesburg, K., Iwasaki, T., & Mierau, I.** (1999). Screening of Lactic Acid Bacteria for Bile Salt Hydrolase Activity. *Journal of Dairy Science*, *82*(12), 2530–2535. https://doi.org/10.3168/jds.S0022-0302(99)75506-2

**Tanaseichuk, O., Borneman, J., & Jiang, T.** (2014). Phylogeny-based classification of microbial communities. *Bioinformatics*, *30*(4), 449–456. https://doi.org/10.1093/bioinformatics/btt700

**Tannock, G. W., Luchansky, J. B., Miller, L., Connell, H., Thode-Andersen, S., Mercer, A. A., & Klaenhammer, T. R.** (1994). Molecular Characterization of a Plasmid-Borne (pGT633) Erythromycin Resistance Determinant (ermGT) from Lactobacillus reuteri 100-63. *Plasmid*, *31*(1), 60–71. https://doi.org/10.1006/plas.1994.1007

**Tao, Y.W., Gu, Y.L., Mao, X.Q., Zhang, L., & Pei, Y.F.** (2020). Effects of probiotics on type II diabetes mellitus: A meta-analysis. *Journal of Translational Medicine*, *18* (1), 30. https://doi.org/10.1186/s12967-020-02213-2

**Taranto, M. P., Sesma, F., Pesce de Ruiz Holgado, A., & de Valdez, G. F.** (1997). Bile salts hydrolase plays a key role on cholesterol removal by Lactobacillus reuteri. *Biotechnology Letters*, *19*(9), 845–847. https://doi.org/10.1023/A:1018373217429

**Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., Lomsadze, A., Pruitt, K. D., Borodovsky, M., & Ostell, J.** (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research*, *44*(14), 6614–6624. https://doi.org/10.1093/nar/gkw569

**Tauchi, H., Yahagi, K., Yamauchi, T., Hara, T., Yamaoka, R., Tsukuda, N., Watanabe, Y., Tajima, S., Ochi, F., Iwata, H., Ohta, M., Ishii, E., Matsumoto, S., & Matsuki, T.** (2019). Gut microbiota development of preterm infants hospitalised in intensive care units. *Beneficial Microbes*, *10*(6), 641–651. https://doi.org/10.3920/BM2019.0003

**Temmerman, R., Scheirlinck, I., Huys, G., & Swings, J.** (2003). Culture-Independent Analysis of Probiotic Products by Denaturing Gradient Gel Electrophoresis | Applied and Environmental Microbiology. *Applied and Environmental Microbiology*, *61*(1), 220–226.

**Teuber, M., Meile, L., & Schwarz, F.** (1999). Acquired antibiotic resistance in lactic acid bacteria from food. In W. N. Konings, O. P. Kuipers, & J. H. J. H. In 't Veld (Eds.), *Lactic Acid Bacteria: Genetics, Metabolism and Applications* 115–137. Springer Netherlands. https://doi.org/10.1007/978-94-017-2027-4_5

**Thomas, C., & Nielsen, K.** (2005). Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Micro 3: 711-721. *Nature Reviews. Microbiology*, *3*, 711–721. https://doi.org/10.1038/nrmicro1234

**Thompson, F. L., Gomez-Gil, B., Vasconcelos, A. T. R., & Sawabe, T.** (2007). Multi locus Sequence Analysis Reveals that Vibrio harveyi and V. campbellii Are Distinct Species. *Applied and Environmental Microbiology*, *73*(13), 4279–4285. https://doi.org/10.1128/AEM.00020-07

**Thompson, J. D., Gibson, Toby. J., & Higgins, D. G.** (2003). Multiple Sequence Alignment Using ClustalW and ClustalX. *Current Protocols in Bioinformatics*, *00*(1), 2.3.1-2.3.22. https://doi.org/10.1002/0471250953.bi0203s00

**Thongprayoon, C., Kaewput, W., Hatch, S. T., Bathini, T., Sharma, K., Wijarnpreecha, K., Ungprasert, P., D'Costa, M., Mao, M. A., & Cheungpasitporn, W.** (2019). Effects of Probiotics on Inflammation and Uremic Toxins Among Patients on Dialysis: A Systematic Review and Meta-Analysis. *Digestive Diseases and Sciences*, *64*(2), 469–479. https://doi.org/10.1007/s10620-018-5243-9

**Thornton, B., & Basu, C.** (2011). Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education: A Bimonthly Publication of the International Union of Biochemistry and Molecular Biology*, *39*(2), 145–154. https://doi.org/10.1002/bmb.20461

**Thumu, S. C. R., & Halami, P. M.** (2012). Presence of erythromycin and tetracycline resistance genes in lactic acid bacteria from fermented foods of Indian origin. *Antonie van Leeuwenhoek*, *102*(4), 541–551. https://doi.org/10.1007/s10482-012-9749-4

**Thursby, E., & Juge, N.** (2017). Introduction to the human gut microbiota. *Biochemical Journal*, *474*(11), 1823–1836. https://doi.org/10.1042/BCJ20160510

**Tirone, C., Pezza, L., Paladini, A., Tana, M., Aurilia, C., Lio, A., D'Ippolito, S., Tersigni, C., Posteraro, B., Sanguinetti, M., Di Simone, N., & Vento, G.** (2019). Gut and Lung Microbiota in Preterm Infants: Immunological Modulation and Implication in Neonatal Outcomes. *Frontiers in Immunology*, *10*, 2910. https://doi.org/10.3389/fimmu.2019.02910

**Tissier.** (1900). Recherchers sur la flora intestinale normale et pathologique du nourisson. *Thesis*, *University of Paris, Paris, France.*

**Tlaskalová-Hogenová, H., Štěpánková, R., Kozáková, H., Hudcovic, T., Vannucci, L., Tučková, L., Rossmann, P., Hrnčíř, T., Kverka, M., Zákostelská, Z., Klimešová, K., Přibylová, J., Bártová, J., Sanchez, D., Fundová, P., Borovská, D., Šrůtková, D., Zídek, Z., Schwarzer, M., … Funda, D. P.** (2011). The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: Contribution of germ-free and gnotobiotic animal models of human diseases. *Cellular & Molecular Immunology*, *8*(2), 110–120. https://doi.org/10.1038/cmi.2010.67

**Todorov, S. D., & Dicks, L. M. T.** (2005). Lactobacillus plantarum isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enzyme and Microbial Technology*, *36*(2), 318–326. https://doi.org/10.1016/j.enzmictec.2004.09.009

**Tomaro-Duchesneau, C., Jones, M. L., Shah, D., Jain, P., Saha, S., & Prakash, S.** (2014). Cholesterol Assimilation by Lactobacillus Probiotic Bacteria: An In Vitro Investigation. *BioMed Research International*, *2014*, e380316. https://doi.org/10.1155/2014/380316

**Tompkins, T. A., Barreau, G., & Broadbent, J. R.** (2012). Complete Genome Sequence of Lactobacillus helveticus R0052, a Commercial Probiotic Strain. *Journal of Bacteriology*, *194*(22), 6349–6349. https://doi.org/10.1128/JB.01638-12

**Tremaroli, V., & Bäckhed, F.** (2012). Functional interactions between the gut microbiota and host metabolism. *Nature*, *489*(7415), 242–249. https://doi.org/10.1038/nature11552

**Trush, E. A., Poluektova, E. A., Beniashvilli, A. G., Shifrin, O. S., Poluektov, Y. M., & Ivashkin, V. T.** (2020). The Evolution of Human Probiotics: Challenges and Prospects. *Probiotics and Antimicrobial Proteins*, *12*(4), 1291–1299. https://doi.org/10.1007/s12602-019-09628-4

**Tsai, C.C., Lin, P.P., Hsieh, Y.M., Zhang, Z., Wu, H.C., & Huang, C.C.** (2014). Cholesterol-Lowering Potentials of Lactic Acid Bacteria Based on Bile-Salt Hydrolase Activity and Effect of Potent Strains on Cholesterol Metabolism In Vitro and In Vivo. *The Scientific World Journal*, *2014*, e690752. https://doi.org/10.1155/2014/690752

**Tulumoglu, S., Yuksekdag, Z. N., Beyatli, Y., Simsek, O., Cinar, B., & Yaşar, E.** (2013). Probiotic properties of lactobacilli species isolated from children's feces. *Anaerobe*, *24*, 36–42. https://doi.org/10.1016/j.anaerobe.2013.09.006

**Tuo, Y., Song, X., Song, Y., Liu, W., Tang, Y., Gao, Y., Jiang, S., Qian, F., & Mu, G.** (2018). Screening probiotics from Lactobacillus strains according to their abilities to inhibit pathogen adhesion and induction of pro-inflammatory cytokine IL-8. *Journal of Dairy Science*, *101*(6), 4822–4829. https://doi.org/10.3168/jds.2017-13654

**Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I.** (2007). The Human Microbiome Project. *Nature*, *449*(7164), 804–810. https://doi.org/10.1038/nature06244

**Turner, N. A., Sharma-Kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., Holland, T. L., & Fowler, V. G.** (2019). Methicillin-resistant Staphylococcus aureus: An overview of basic and clinical research. *Nature Reviews Microbiology*, *17*(4), 203–218. https://doi.org/10.1038/s41579-018-0147-4

**Turroni, F., Milani, C., Duranti, S., Lugli, G. A., Bernasconi, S., Margolles, A., Di Pierro, F., van Sinderen, D., & Ventura, M.** (2020). The infant gut microbiome as a microbial organ influencing host well-being. *Italian Journal of Pediatrics*, *46*(1), 16. https://doi.org/10.1186/s13052-020-0781-0

**Turroni, F., Milani, C., Ventura, M., & van Sinderen, D.** (2022). The human gut microbiota during the initial stages of life: Insights from bifidobacteria. *Current Opinion in Biotechnology*, *73*, 81–87. https://doi.org/10.1016/j.copbio.2021.07.012

**Turroni, F., Peano, C., Pass, D. A., Foroni, E., Severgnini, M., Claesson, M. J., Kerr, C., Hourihane, J., Murray, D., Fuligni, F., Gueimonde, M., Margolles, A., Bellis, G. D., O'Toole, P. W., Sinderen, D. van, Marchesi, J. R., & Ventura, M.** (2012). Diversity of Bifidobacteria within the Infant Gut Microbiota. *PLOS ONE*, *7*(5), e36957. https://doi.org/10.1371/journal.pone.0036957

**Turroni, F., van Sinderen, D., & Ventura, M.** (2011). Genomics and ecological overview of the genus Bifidobacterium. *International Journal of Food Microbiology*, *149*(1), 37–44. https://doi.org/10.1016/j.ijfoodmicro.2010.12.010

**Ukena, S. N., Singh, A., Dringenberg, U., Engelhardt, R., Seidler, U., Hansen, W., Bleich, A., Bruder, D., Franzke, A., Rogler, G., Suerbaum, S., Buer, J., Gunzer, F., & Westendorf, A. M.** (2007). Probiotic Escherichia coli Nissle 1917 Inhibits Leaky Gut by Enhancing Mucosal Integrity. *PLOS ONE*, *2*(12), e1308. https://doi.org/10.1371/journal.pone.0001308

**Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G.** (2012). Primer3—New capabilities and interfaces. *Nucleic Acids Research*, *40*(15), e115. https://doi.org/10.1093/nar/gks596

**US Food and Drug Administration.** (2016). US Food and Drug Administration, 2016. New Dietary Ingredients Notification Process. *Washington, DC*.

**van der Beek, C. M., Dejong, C. H. C., Troost, F. J., Masclee, A. A. M., & Lenaerts, K.** (2017). Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. *Nutrition Reviews*, *75*(4), 286–305. https://doi.org/10.1093/nutrit/nuw067

**van Hoek, A. H. A. M., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., & Aarts, H. J. M.** (2011). Acquired Antibiotic Resistance Genes: An Overview. *Frontiers in Microbiology*, *2*. https://doi.org/10.3389/fmicb.2011.00203

**van Reenen, C. A., & Dicks, L. M. T.** (2011). Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: What are the possibilities? A review. *Archives of Microbiology*, *193*(3), 157–168. https://doi.org/10.1007/s00203-010-0668-3

**Vancanneyt, M., Naser, S. M., Engelbeen, K., De Wachter, M., Van der Meulen, R., Cleenwerck, I., Hoste, B., De Vuyst, L., & Swings, J.** (2006). Reclassification of Lactobacillus brevis strains LMG 11494 and LMG 11984 as Lactobacillus parabrevis sp. Nov. *International Journal of Systematic and Evolutionary Microbiology, 56*(7), 1553–1557. https://doi.org/10.1099/ijs.0.64215-0

**van Heel, A. J., de Jong, A., Song, C., Viel, J. H., Kok, J., & Kuipers, O. P.** (2018). BAGEL4: A user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Research*, *46*(W1), W278–W281. https://doi.org/10.1093/nar/gky383

**Velraeds, M. M. C., Van De Belt-Gritter, B., Van Der Mei, H. C., Reid, G., & Busscher, H. J. Y.** (1998). Interference in Initial Adhesion of Uropathogenic Bacteria and Yeasts to Silicone Rubber by A Lactobacillus Acidophilus Biosurfactant. *Journal of Medical Microbiology*, *47*(12), 1081–1085. https://doi.org/10.1099/00222615-47-12-1081

**Ventola, C. L.** (2015). The Antibiotic Resistance Crisis. *Pharmacy and Therapeutics*, *40*(4), 277–283.

**Ventura, M., Canchaya, C., Casale, A. D., Dellaglio, F., Neviani, E., Fitzgerald, G. F., & van Sinderen, D.** (2006). Analysis of bifidobacterial evolution using a multi locus approach. *International Journal of Systematic and Evolutionary Microbiology*, *56*(12), 2783–2792. https://doi.org/10.1099/ijs.0.64233-0

**Ventura, M., O'Flaherty, S., Claesson, M. J., Turroni, F., Klaenhammer, T. R., van Sinderen, D., & O'Toole, P. W.** (2009). Genome-scale analyses of health-promoting bacteria: Probiogenomics. *Nature Reviews Microbiology*, *7*(1), 61–71. https://doi.org/10.1038/nrmicro2047

**Verdenelli, M. C., Ghelfi, F., Silvi, S., Orpianesi, C., Cecchini, C., & Cresci, A.** (2009). Probiotic properties of Lactobacillus rhamnosus and Lactobacillus paracasei isolated from human faeces. *European Journal of Nutrition*, *48*(6), 355–363. https://doi.org/10.1007/s00394-009-0021-2

**Villion, M., & Moineau, S.** (2009). *Bacteriophages of Lactobacillus*. https://doi.org/10.2741/3332

**Vizoso Pinto, M. G., Franz, C. M. A. P., Schillinger, U., & Holzapfel, W. H.** (2006). Lactobacillus spp. Within vitro probiotic properties from human faeces and traditional fermented products. *International Journal of Food Microbiology*, *109*(3), 205–214. https://doi.org/10.1016/j.ijfoodmicro.2006.01.029

**Vlasova, A. N., Kandasamy, S., Chattha, K. S., Rajashekara, G., & Saif, L. J.** (2016). Comparison of probiotic lactobacilli and bifidobacteria effects, immune responses and rotavirus vaccines and infection in different host species. *Veterinary Immunology and Immunopathology*, *172*, 72–84. https://doi.org/10.1016/j.vetimm.2016.01.003

**Vlková, E., Salmonová, H., Bunešová, V., Geigerová, M., Rada, V., & Musilová, Š.** (2015). A new medium containing mupirocin, acetic acid, and norfloxacin for the selective cultivation of bifidobacteria. *Anaerobe*, *34*, 27–33. https://doi.org/10.1016/j.anaerobe.2015.04.001

**von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M., & Wolffs, P. F. G.** (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00173

**Wai, S. N., Mizunoe, Y., Takade, A., Kawabata, S.I., & Yoshida, S.I.** (1998). Vibrio cholerae O1 Strain TSI-4 Produces the Exopolysaccharide Materials That Determine Colony Morphology, Stress Resistance, and Biofilm Formation. *Applied and Environmental Microbiology*, *64*(10), 3648–3655. https://doi.org/10.1128/AEM.64.10.3648-3655.1998

**Wallet, F., Dessein, R., Armand, S., & Courcol, R. J.** (2002). Molecular Diagnosis of Endocarditis Due to Lactobacillus casei subsp. Rhamnosus. *Clinical Infectious Diseases*, *35*(10), e117–e119. https://doi.org/10.1086/344181

**Walsh, C. J., Guinane, C. M., O'Toole, P. W., & Cotter, P. D.** (2014). Beneficial modulation of the gut microbiota. *FEBS Letters*, *588*(22), 4120–4130. https://doi.org/10.1016/j.febslet.2014.03.035

**Wan, M. L. Y., Forsythe, S. J., & El-Nezami, H.** (2019). Probiotics interaction with foodborne pathogens: A potential alternative to antibiotics and future challenges. *Critical Reviews in Food Science and Nutrition*, *59*(20), 3320–3333. https://doi.org/10.1080/10408398.2018.1490885

**Wang, F., Pan, B., Xu, S., Xu, Z., Zhang, T., Zhang, Q., Bao, Y., Wang, Y., Zhang, J., Xu, C., & Xue, X.** (2021). A meta-analysis reveals the effectiveness of probiotics and prebiotics against respiratory viral infection. *Bioscience Reports*, *41*(3), BSR20203638. https://doi.org/10.1042/BSR20203638

**Wang, G., Huang, W., Xia, Y., Xiong, Z., & Ai, L.** (2019). Cholesterol-lowering potentials of Lactobacillus strain overexpression of bile salt hydrolase on high cholesterol diet-induced hypercholesterolemic mice. *Food & Function*, *10*(3), 1684–1695. https://doi.org/10.1039/C8FO02181C

**Wang, G., Li, D., Ma, X., An, H., Zhai, Z., Ren, F., & Hao, Y**. (2015). Functional role of oppA encoding an oligopeptide-binding protein from Lactobacillus salivarius Ren in bile tolerance. *Journal of Industrial Microbiology and Biotechnology*, *42*(8), 1167–1174. https://doi.org/10.1007/s10295-015-1634-5

**Wang, G., Yin, S., An, H., Chen, S., & Hao, Y.** (2011). Coexpression of bile salt hydrolase gene and catalase gene remarkably improves oxidative stress and bile salt resistance in Lactobacillus casei. *Journal of Industrial Microbiology and Biotechnology*, *38*(8), 985–990. https://doi.org/10.1007/s10295-010-0871-x

**Wang, J., Guo, H., Cao, C., Zhao, W., Kwok, L.Y., Zhang, H., & Zhang, W.** (2018). Characterization of the Adaptive Amoxicillin Resistance of Lactobacillus casei Zhang by Proteomic Analysis. *Frontiers in Microbiology. 9*, 292. https://doi.org/10.3389/fmicb.2018.00292

**Wang, J., Ji, H., Wang, S., Liu, H., Zhang, W., Zhang, D., & Wang, Y**. (2018). Probiotic Lactobacillus plantarum Promotes Intestinal Barrier Function by Strengthening the Epithelium and Modulating Gut Microbiota. *Frontiers in Microbiology*, *9*, 1953. https://doi.org/10.3389/fmicb.2018.01953

**Wang, L., Guo, M.J., Gao, Q., Yang, J.F., Yang, L., Pang, X.L., & Jiang, X.J.** (2018). The effects of probiotics on total cholesterol: A meta-analysis of randomized controlled trials. *Medicine*, *97*(5). https://doi.org/10.1097/MD.0000000000009679

**Wang, L.Q., Meng, X.C., Zhang, B.R., Wang, Y., & Shang, Y.L.** (2010). Influence of cell surface properties on adhesion ability of bifidobacteria. *World Journal of Microbiology and Biotechnology*, *26*(11), 1999–2007. https://doi.org/10.1007/s11274-010-0384-9

**Wang, N., Hang, X., Zhang, M., Liu, X., & Yang, H.** (2017). Analysis of newly detected tetracycline resistance genes and their flanking sequences in human intestinal bifidobacteria. *Scientific Reports*, *7*(1), 6267. https://doi.org/10.1038/s41598-017-06595-0

**Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R.** (2007). Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

**Wang, X., Hui, Y., Zhao, L., Hao, Y., Guo, H., & Ren, F.** (2017). Oral administration of Lactobacillus paracasei L9 attenuates PM2.5-induced enhancement of airway hyperresponsiveness and allergic airway response in murine model of asthma. *PLoS ONE*, *12*(2). https://doi.org/10.1371/journal.pone.0171721

**Wang, Y., Liu, L., Moore, D. J., Shen, X., Peek, R. M., Acra, S. A., Li, H., Ren, X., Polk, D. B., & Yan, F.** (2017). An LGG-derived protein promotes IgA production through upregulation of APRIL expression in intestinal epithelial cells. *Mucosal Immunology*, *10*(2), 373–384. https://doi.org/10.1038/mi.2016.57

**Wasilewska, E., Zlotkowska, D., & Wroblewska, B.** (2019). Yogurt starter cultures of Streptococcus thermophilus and Lactobacillus bulgaricus ameliorate symptoms and modulate the immune response in a mouse model of dextran sulfate sodium-induced colitis. *Journal of Dairy Science*, *102*(1), 37–53. https://doi.org/10.3168/jds.2018-14520

**Wassenaar, T. M., & Klein, G.** (2008). Safety Aspects and Implications of Regulation of Probiotic Bacteria in Food and Food Supplements. *Journal of Food Protection*, *71*(8), 1734–1741. https://doi.org/10.4315/0362-028X-71.8.1734

**Wei, X., Wang, S., Zhao, X., Wang, X., Li, H., Lin, W., Lu, J., Zhurina, D., Li, B., Riedel, C. U., Sun, Y., & Yuan, J.** (2016). Proteomic Profiling of Bifidobacterium bifidum S17 Cultivated Under In Vitro Conditions. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00097

**Wei, Y.X., Zhang, Z.Y., Liu, C., Malakar, P. K., & Guo, X.K.** (2012). Safety assessment of Bifidobacterium longum JDM301 based on complete genome sequences. *World Journal of Gastroenterology : WJG*, *18*(5), 479–488. https://doi.org/10.3748/wjg.v18.i5.479

**Werner, G., Klare, I., Heier, H., Hinz, K.H., Böhme, G., Wendt, M., & Witte, W.** (2000). Quinupristin/Dalfopristin-Resistant Enterococci of the satA (vatD) and satG (vatE) Genotypes from Different Ecological Origins in Germany. *Microbial Drug Resistance*, *6*(1), 37–47. https://doi.org/10.1089/mdr.2000.6.37

**Westermann, C., Zhurina, D. S., Baur, A., Shang, W., Yuan, J., & Riedel, C. U.** (2012). Exploring the genome sequence of Bifidobacterium bifidum S17 for potential players in host-microbe interactions. *Symbiosis*, *58*(1), 191–200. https://doi.org/10.1007/s13199-012-0205-z

**WHO.** (2021a). *Antimicrobial resistance*. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

**WHO.** (2021b). *Cardiovascular diseases*. https://www.who.int/westernpacific/health-topics/cardiovascular-diseases

**WHO, J.** (2002). *WHO working group report on drafting guidelines for the evaluation of probiotics in food*. 30.

**Wickham, H.** (2006). *An introduction to ggplot: An implementation of the grammar of graphics in R*.

**Winslow, C.E. A., Broadhurst, J., Buchanan, R. E., Krumwiede, C., Rogers, L. A., & Smith, G. H.** (1920). The Families and Genera of the Bacteria. *Journal of Bacteriology*, *5*(3), 191–229.

**Witkowski, M., Weeks, T. L., & Hazen, S. L.** (2020). Gut Microbiota and Cardiovascular Disease. *Circulation Research*, *127*(4), 553–570. https://doi.org/10.1161/CIRCRESAHA.120.316242

**Wittouck, S., Wuyts, S., & Lebeer, S.** (2019). Towards a Genome-Based Reclassification of the Genus *Lactobacillus*. *Applied and Environmental Microbiology*, *85*(3), e02155-18. https://doi.org/10.1128/AEM.02155-18

**Woese, C. R.** (1987). Bacterial evolution. *Microbiological Reviews*, *51*(2), 221–271.

**Woese, C. R., E, S., T, M., & Ge, F.** (1985). *A phylogenetic definition of the major eubacterial taxa*. Systematic and Applied Microbiology; Syst Appl Microbiol. https://doi.org/10.1016/s0723-2020(85)80047-3

**Woo, J., & Ahn, J.** (2013). Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. *Letters in Applied Microbiology*, *56*(4), 307–313. https://doi.org/10.1111/lam.12051

**Wood, B. J. B., & Holzapfel, W. H. N.** (1992). *The Genera of Lactic Acid Bacteria*. Springer Science & Business Media.

**Wu, M., & Eisen, J. A.** (2008). A simple, fast, and accurate method of phylogenomic inference. *Genome Biology*, *9*(10), R151. https://doi.org/10.1186/gb-2008-9-10-r151

**Wu, R., Wang, L., Wang, J., Li, H., Menghe, B., Wu, J., Guo, M., & Zhang, H.** (2009). Isolation and preliminary probiotic selection of lactobacilli from koumiss in Inner Mongolia. *Journal of Basic Microbiology*, *49*(3), 318–326. https://doi.org/10.1002/jobm.200800047

**Wu, S., Yuan, L., Zhang, Y., Liu, F., Li, G., Wen, K., Kocher, J., Yang, X., & Sun, J.** (2013). Probiotic Lactobacillus rhamnosus GG mono-association suppresses human rotavirus-induced autophagy in the gnotobiotic piglet intestine. *Gut Pathogens*, *5*(1), 22. https://doi.org/10.1186/1757-4749-5-22

**Wu, Y., Zhang, Q., Ren, Y., & Ruan, Z.** (2017). Effect of probiotic Lactobacillus on lipid profile: A systematic review and meta-analysis of randomized, controlled trials. *PLOS ONE*, *12*(6), e0178868. https://doi.org/10.1371/journal.pone.0178868

**Wu, Z., Wu, J., Lang, F., Cai, Z., Zeng, X., Guo, Y., Liu, X., & Pan, D.** (2020). Characterization of the sortase A from Lactobacillus acidophilus ATCC 4356 involved in adherence to intestinal cells. *Future Microbiology*, *15*(7), 485–496. https://doi.org/10.2217/fmb-2019-0219

**Wuyts, S., Wittouck, S., Boeck, I. D., Allonsius, C. N., Pasolli, E., Segata, N., & Lebeer, S.** (2017). Large-Scale Phylogenomics of the Lactobacillus casei Group Highlights Taxonomic Inconsistencies and Reveals Novel Clade-Associated Features. *MSystems*, *2*(4). https://doi.org/10.1128/mSystems.00061-17

**Xiao, J., Takahashi, S., Odamaki, T., Yaeshima, T., & Iwatsuki, K.** (2010). Antibiotic Susceptibility of Bifidobacterial Strains Distributed in the Japanese Market. *Bioscience, Biotechnology, and Biochemistry*, *74*(2), 336–342. https://doi.org/10.1271/bbb.90659

**Xie, M., Pan, M., Jiang, Y., Liu, X., Lu, W., Zhao, J., Zhang, H., & Chen, W.** (2019). GroEL Gene-Based Phylogenetic Analysis of Lactobacillus Species by High-Throughput Sequencing. *Genes*, *10*(7), 530. https://doi.org/10.3390/genes10070530

**Xiong, Z.Q., Wang, Q.H., Kong, L.H., Song, X., Wang, G.Q., Xia, Y.J., Zhang, H., Sun, Y., & Ai, L.Z.** (2017). Short communication: Improving the activity of bile salt hydrolases in Lactobacillus casei based on in silico molecular docking and heterologous expression. *Journal of Dairy Science*, *100*(2), 975–980. https://doi.org/10.3168/jds.2016-11720

**Xu, H.Y., Tian, W.H., Wan, C.-X., Jia, L.J., Wang, L.Y., Yuan, J., Liu, C.M., Zeng, M., & Wei, H.** (2008). Antagonistic Potential against Pathogenic Microorganisms and Hydrogen Peroxide Production of Indigenous Lactobacilli Isolated from Vagina of Chinese Pregnant Women. *Biomedical and Environmental Sciences*, *21*(5), 365–371. https://doi.org/10.1016/S0895-3988(08)60056-2

**Yadav, A. K., Tyagi, A., Kaushik, J. K., Saklani, A. C., Grover, S., & Batish, V. K.** (2013). Role of surface layer collagen binding protein from indigenous Lactobacillus plantarum 91 in adhesion and its anti-adhesion potential against gut pathogen. *Microbiological Research*, *168*(10), 639–645. https://doi.org/10.1016/j.micres.2013.05.003

**Yamaguchi, M., Goto, K., Hirose, Y., Yamaguchi, Y., Sumitomo, T., Nakata, M., Nakano, K., & Kawabata, S.** (2019). Identification of evolutionarily conserved virulence factor by selective pressure analysis of Streptococcus pneumoniae. *Communications Biology*, *2*. https://doi.org/10.1038/s42003-019-0340-7

**Yamashita, M., Ukibe, K., Uenishi, H., Hosoya, T., Sakai, F., & Kadooka, Y.** (2014). Lactobacillus helveticus SBT2171, a cheese starter, regulates proliferation and cytokine production of immune cells. *Journal of Dairy Science*, *97*(8), 4772–4779. https://doi.org/10.3168/jds.2014-8041

**Yan, J., & Charles, J. F.** (2017). Gut Microbiome and Bone: To Build, Destroy, or Both? *Current Osteoporosis Reports*, *15*(4), 376–384. https://doi.org/10.1007/s11914-017-0382-z

**Yang, B., Chen, Y., Stanton, C., Ross, R. P., Lee, Y.K., Zhao, J., Zhang, H., & Chen, W.** (2019). Bifidobacterium and Lactobacillus Composition at Species Level and Gut Microbiota Diversity in Infants before 6 Weeks. *International Journal of Molecular Sciences*, *20*(13), 3306. https://doi.org/10.3390/ijms20133306

**Yang, Z., & Rannala, B.** (2012). Molecular phylogenetics: Principles and practice. *Nature Reviews Genetics*, *13*(5), 303–314. https://doi.org/10.1038/nrg3186

**Yao, P. X., & Toda, K.** (1990). Lactic acid production in electrodialysis culture. *The Journal of General and Applied Microbiology*, *36*(2), 111–125.

**Yelin, I., & Kishony, R.** (2018). Antibiotic Resistance. *Cell*, *172*(5), 1136-1136.e1. https://doi.org/10.1016/j.cell.2018.02.018

**Yildirim, Z., & Johnson, M., G.** (1998). Characterization and Antimicrobial Spectrum of Bifidocin B, a Bacteriocin Produced by Bifidobacterium bifidum NCFB 1454. *Journal of Food Protection*, *61*(1), 47–51. https://doi.org/10.4315/0362-028X-61.1.47

**Yin, Q., & Zheng, Q.** (2005). Isolation and identification of the dominant Lactobacillus in gut and faeces of pigs using carbohydrate fermentation and 16S rRNA rDNA analysis. *Journal of Bioscience and Bioengineering*, *99*(1), 68–71. https://doi.org/10.1263/jbb.99.68

**Yoo, J. Y., Groer, M., Dutra, S. V. O., Sarkar, A., & McSkimming, D. I.** (2020). Gut Microbiota and Immune System Interactions. *Microorganisms*, *8*(10), 1587. https://doi.org/10.3390/microorganisms8101587

**Yoon, H., Ju, J.H., Kim, H.N., Park, H., Ji, Y., Lee, J., Shin, H.K., Do, M.S., & Holzapfel, W.** (2012). Reduction in cholesterol absorption in Caco-2 cells through the down-regulation of Niemann-Pick C1-like 1 by the putative probiotic strains Lactobacillus rhamnosus BFE5264 and Lactobacillus plantarum NR74 from fermented foods. *International Journal of Food Sciences and Nutrition*, *64*. https://doi.org/10.3109/09637486.2012.706598

**Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J.** (2017). Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology*, *67*(5), 1613–1617. https://doi.org/10.1099/ijsem.0.001755

**You, I., & Kim, E. B.** (2020). Genome-based species-specific primers for rapid identification of six species of Lactobacillus acidophilus group using multiplex PCR. *PLOS ONE*, *15*(3), e0230550. https://doi.org/10.1371/journal.pone.0230550

**Youssef, N., Sheik, C. S., Krumholz, L. R., Najar, F. Z., Roe, B. A., & Elshahed, M. S.** (2009). Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. *Applied and Environmental Microbiology*, *75*(16), 5227–5236. https://doi.org/10.1128/AEM.00592-09

**Zaburlin, D., Mercanti, D. J., & Quiberoni, A.** (2017). A fast PCR-based method for the characterization of prophage profiles in strains of the Lactobacillus casei group. *Journal of Virological Methods*, *248*, 226–233. https://doi.org/10.1016/j.jviromet.2017.07.016

**Zago, M., Orrù, L., Rossetti, L., Lamontanara, A., Fornasari, M. E., Bonvini, B., Meucci, A., Carminati, D., Cattivelli, L., & Giraffa, G.** (2017). Survey on the phage resistance mechanisms displayed by a dairy Lactobacillus helveticus strain. *Food Microbiology*, *66*, 110–116. https://doi.org/10.1016/j.fm.2017.04.014

**Zahid, M.** (2015). Antimicrobial Activity of Bacteriocins Isolated from Lactic Acid Bacteria Against Resistant Pathogenic Strains. *International Journal of Nutrition and Food Sciences*, *4*(3), 326. https://doi.org/10.11648/j.ijnfs.20150403.20

**Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F., & Larsen, M.** (2012). Identification of Acquired Antimicrobial Resistance Genes. *The Journal of Antimicrobial Chemotherapy*, *67*, 2640–2644. https://doi.org/10.1093/jac/dks261

**Zeng, X. Q., Pan, D. D., & Zhou, P. D.** (2011). Functional Characteristics of Lactobacillus fermentum F1. *Current Microbiology*, *62*(1), 27–31. https://doi.org/10.1007/s00284-010-9669-3

**Zhang, F., Gao, J., Wang, B., Huo, D., Wang, Z., Zhang, J., & Shao, Y.** (2018). Whole-genome sequencing reveals the mechanisms for evolution of streptomycin resistance in Lactobacillus plantarum. *Journal of Dairy Science*, *101*(4), 2867–2874. https://doi.org/10.3168/jds.2017-13323

**Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., Busk, P. K., Xu, Y., & Yin, Y.** (2018). dbCAN2: A meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Research*, *46*(W1), W95–W101. https://doi.org/10.1093/nar/gky418

**Zhang, J., Wu, H. M., Wang, X., Xie, J., Li, X., Ma, J., Wang, F., & Tang, X.** (2020). Efficacy of prebiotics and probiotics for functional dyspepsia: A systematic review and meta-analysis. *Medicine*, *99*(7). https://doi.org/10.1097/MD.0000000000019107

**Zhang, L., Dong, D., Jiang, C., Li, Z., Wang, X., & Peng, Y.** (2015). Insight into alteration of gut microbiota in Clostridium difficile infection and asymptomatic C. difficile colonization. *Anaerobe*, *34*, 1–7. https://doi.org/10.1016/j.anaerobe.2015.03.008

**Zhang, M., Hang, X., Fan, X., Li, D., & Yang, H.** (2008). Characterization and selection of Lactobacillus strains for their effect on bile tolerance, taurocholate deconjugation and cholesterol removal. *World Journal of Microbiology and Biotechnology*, *24*(1), 7–14. https://doi.org/10.1007/s11274-007-9431-6

**Zhang, M., Zhang, C., Zhao, J., Zhang, H., Zhai, Q., & Chen, W.** (2020). Meta-analysis of the efficacy of probiotic-supplemented therapy on the eradication of H. pylori and incidence of therapy-associated side effects. *Microbial Pathogenesis*, *147*, 104403. https://doi.org/10.1016/j.micpath.2020.104403

**Zhang, R., Liu, L., Zhou, H., Chan, E. W., Li, J., Fang, Y., Li, Y., Liao, K., & Chen, S.** (2017). Nationwide Surveillance of Clinical Carbapenem-resistant Enterobacteriaceae (CRE) Strains in China. *EBioMedicine*, *19*, 98–106. https://doi.org/10.1016/j.ebiom.2017.04.032

**Zhang, S., Oh, J.H., Alexander, L. M., Özçam, M., & van Pijkeren, J.P.** (2018). D-Alanyl-d-Alanine Ligase as a Broad-Host-Range Counterselection Marker in Vancomycin-Resistant Lactic Acid Bacteria. *Journal of Bacteriology*, *200*(13), e00607-17. https://doi.org/10.1128/JB.00607-17

**Zhang, W. Y., Wu, R. N., Sun, Z. H., Sun, T. S., Meng, H., & Zhang, H. P.** (2009). Molecular cloning and characterization of bile salt hydrolase in Lactobacillus casei Zhang. *Annals of Microbiology*, *59*(4), 721–726. https://doi.org/10.1007/BF03179214

**Zhang, X.F., Guan, X.X., Tang, Y.J., Sun, J.F., Wang, X.K., Wang, W.D., & Fan, J.M.** (2021). Clinical effects and gut microbiota changes of using probiotics, prebiotics or synbiotics in inflammatory bowel disease: A systematic review and meta-analysis. *European Journal of Nutrition*, *60*(5), 2855–2875. https://doi.org/10.1007/s00394-021-02503-5

**Zhang, Y., & Zhang, Q.** (2019). Relationship between tetracycline antibiotic susceptibility and genotype in oral cavity Lactobacilli clinical isolates. *Antimicrobial Resistance & Infection Control*, *8*(1), 27. https://doi.org/10.1186/s13756-019-0483-9

**Zhang, Z., Zhou, H., Zhou, X., Sun, J., Liang, X., Lv, Y., Bai, L., Zhang, J., Gong, P., Liu, T., Yi, H., Wang, J., & Zhang, L.** (2021). *Lactobacillus casei* YRL577 ameliorates markers of non-alcoholic fatty liver and alters expression of genes within the intestinal bile acid pathway. *British Journal of Nutrition*, *125*(5), 521–529. https://doi.org/10.1017/S0007114520003001

**Zhao, W., Peng, C., Sakandar, H. A., Kwok, L.Y., & Zhang, W.** (2021). Meta-Analysis: Randomized Trials of Lactobacillus plantarum on Immune Regulation Over the Last Decades. *Frontiers in Immunology*, *12*, 728. https://doi.org/10.3389/fimmu.2021.643420

**Zheng, J., Ruan, L., Sun, M., & Gänzle, M.** (2015). A Genomic View of Lactobacilli and Pediococci Demonstrates that Phylogeny Matches Ecology and Physiology. *Applied and Environmental Microbiology*, *81*(20), 7233. https://doi.org/10.1128/AEM.02116-15

**Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'Toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S.** (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International Journal of Systematic and Evolutionary Microbiology, 70*(4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107

**Zhou, J. S., Pillidge, C. J., Gopal, P. K., & Gill, H. S.** (2005). Antibiotic susceptibility profiles of new probiotic Lactobacillus and Bifidobacterium strains. *International Journal of Food Microbiology*, *98*(2), 211–217. https://doi.org/10.1016/j.ijfoodmicro.2004.05.011

**Zhou, N., Zhang, J. X., Fan, M. T., Wang, J., Guo, G., & Wei, X. Y.** (2012). Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts. *Journal of Dairy Science*, *95*(9), 4775–4783. https://doi.org/10.3168/jds.2011-5271

**Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., & Wishart, D. S.** (2011). PHAST: A Fast Phage Search Tool. *Nucleic Acids Research*, *39*, W347–W352. https://doi.org/10.1093/nar/gkr485

**Zhu, Y., Zhu, J., Zhao, L., Zhang, M., Guo, H., & Ren, F.** (2016). Effect of oral administration of Lactobacillus paracasei L9 on mouse systemic immunity and the immune response in the intestine. *Archives of Biological Sciences*, *68*(2), 311–318.

**Zhurina, D., Zomer, A., Gleinser, M., Brancaccio, V. F., Auchter, M., Waidmann, M. S., Westermann, C., van Sinderen, D., & Riedel, C. U.** (2011). Complete Genome Sequence of Bifidobacterium bifidum S17. *Journal of Bacteriology*, *193*(1), 301–302. https://doi.org/10.1128/JB.01180-10

**Živković, M., Miljković, M. S., Ruas-Madiedo, P., Markelić, M. B., Veljović, K., Tolinački, M., Soković, S., Korać, A., & Golić, N.** (2016). EPS-SJ Exopolisaccharide Produced by the Strain Lactobacillus paracasei subsp. Paracasei BGSJ2-8 Is Involved in Adhesion to Epithelial Intestinal Cells and Decrease on E. coli Association to Caco-2 Cells. *Frontiers in Microbiology*, *7*, 286. https://doi.org/10.3389/fmicb.2016.00286

**Zorič Peternel, M., Čanžek Majhenič, A., Holo, H., Nes, I. F., Salehian, Z., Berlec, A., & Rogelj, I.** (2010). Wide-Inhibitory Spectra Bacteriocins Produced by Lactobacillus gasseri K7. *Probiotics and Antimicrobial Proteins*, *2*(4), 233–240. https://doi.org/10.1007/s12602-010-9044-5

**Zou, S., Fang, L., & Lee, M.H.** (2018). Dysbiosis of gut microbiota in promoting the development of colorectal cancer. *Gastroenterology Report*, *6*(1), 1–12. https://doi.org/10.1093/gastro/gox031

**Zoumpopoulou, G., Foligne, B., Christodoulou, K., Grangette, C., Pot, B., & Tsakalidou, E.** (2008). Lactobacillus fermentum ACA-DC 179 displays probiotic potential in vitro and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and Salmonella infection in murine models. *International Journal of Food Microbiology*, *121*(1), 18–26. https://doi.org/10.1016/j.ijfoodmicro.2007.10.013

**Zyrek, A. A., Cichon, C., Helms, S., Enders, C., Sonnenborn, U., & Schmidt, M. A.** (2007). Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKC redistribution resulting in tight junction and epithelial barrier repair. *Cellular Microbiology*, *9*(3), 804–816. https://doi.org/10.1111/j.1462-5822.2006.00836.x