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Potential of Cheddar Cheese to Impact on the Gut Microbiota

Thesis presented by

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Table of Contents

Declaration 4

Author Contributions 5

Abstract 6

List of Publications and Conference Contributions 8

Chapter 1 10

1.1 Summary 11

1.2 A Brief Overview of Cheese Microbiology 12

1.3 The Gut Microbiome 21

1.4 *In Vitro* Testing for Potential Probiotics 33

1.5 Project Aims 60

References 61

Chapter 2 84

Abstract 85

2.1 Introduction 86

2.2 Fermented Food 89

2.3 Health Benefits of Fermented Foods 98

2.4 Evidence of Fermented Foods that Modulate the Gut Microbiome 103

2.5 Nutrients from Fermented Foods that Modulate the Gut Microbiome 108

2.6 Potential of Fermented Food Microbiota to Survive and Modulate the Gut Microbiome 113

2.7 Conclusions 120

References 122

Chapter 3 140

Abstract 141

Graphical Abstract 142

3.1 Introduction 143

3.2 Materials and Methods 146

3.3 Results 154

3.4 Discussion 170

3.5 Conclusion 175

3.6 Supplementary Material 176

References 179

Chapter 4 186

Abstract 187

Graphical Abstract 188

4.1 Introduction 189
4.2 Materials and Methods 192
4.3 Results 198
4.4 Discussion 209
4.5 Conclusion 214
4.6 Supplementary Material 215
References 222
Chapter 5 226
Abstract 227
5.1 Introduction 228
5.2 Materials and Methods 232
5.3 Results 236
5.4 Discussion 256
5.5 Conclusion 263
References 264
Chapter 6 269
Abstract 270
6.1 Introduction 271
6.2 Materials and Methods 274
6.3 Results 277
6.4 Discussion 283
6.5 Conclusion 285
References 286
Chapter 7 291
References 297
Appendices 300
Appendix I 300
Appendix II 302
Appendix III 304

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

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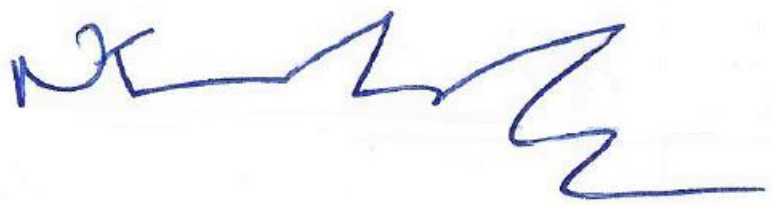


Author Contributions

All of the work conducted in this thesis was performed independently by the author, with the following exceptions:

Chapter 4: Joanne J. Hayes aided in the growth of the starter lactic acid bacteria cultures. Tom P. Beresford aided in the production of the Cheddar cheeses.

Chapter 5: Kanishka N. Nilaweera aided with various aspects of the mouse feeding trial, including culling and dissection of the animals. Raul C. Rubio carried out the bioinformatic analysis and related statistics for the sequencing data.

Chapter 6: Raul C. Rubio carried out the bioinformatic analysis and related statistics for the sequencing data.

Abstract

There is growing evidence in the scientific literature relating to the potential of food to programme gut microbiota and, in so doing, impact positively on gut-health. Fermented foods and in particular cheese, support a rich microbial population and are a well-recognized source of indigenous lacticaseibacilli, referred to as Non-Starter Lactic Acid Bacteria (NSLAB) that grow to high numbers in the cheese during ripening. Species such as *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* are common members of the NSLAB microbiota and are also recognised as an important component of a healthy gut microbiome. Indeed, many of the commercially successful probiotic bacteria include these species. Fermented foods are also highly nutritious and offer the potential to influence directly the growth of the indigenous gut microbiota. The hypothesis tested in this project was that cheese, along with its natural NSLAB populations, has the potential to positively affect the gut microbiota and thus be considered a food with added human health benefits.

To explore this hypothesis, the potential of NSLAB to survive gastric transit was investigated by exposing the bacterial populations separated from the cheese matrix to Simulated Stomach and Duodenum Passage (SSDP) followed by plating on MRS medium. Populations of NSLAB, up to 10^7 CFU/g of cheese were recovered following this treatment suggesting that significant populations may survive gastric transit and thus, gain entry to the small and large intestine. A selection of 240 isolates surviving SSDP were characterised including examination for a number of typical probiotic characteristics. Arising from this, two strains, *Lacticaseibacillus paracasei* DPC 7150 and *Lacticaseibacillus rhamnosus* DPC 7102, were selected that demonstrated probiotic potential. To further investigate how these strains survive in cheese and to explore whether a cheese or fermented milk matrix provided best protection during simulated digestion, these strains were added during the manufacture of both products. Potential to survive gastric transit was investigated using the INFOGEST2.0 static digestion model and strains were identified using Pulse-Field Gel Electrophoresis (PFGE). The data obtained demonstrated that *Lb. paracasei* DPC 7150 and *Lb. rhamnosus* DPC 7102 persisted in the cheese during ripening and that the Cheddar cheese matrix provided significantly more protection during simulated gastric transit.

In order to determine the effects of these lacticaseibacilli-containing Cheddar cheeses *in vivo*, a short-term feeding study using Black 6 mice was designed to assess cheese intake on the gut microbiome, as well as other parameters of interest. While groups consuming cheese did not differ from the control chow diet in body weight, organ mass, adipose tissue mass, or total plasma cholesterol, differences were observed in plasma triglyceride levels (with those of the Cheddar-consuming groups being significantly higher). The alpha and beta diversity of the gut microbiome, including between certain dietary groups and when compared with their microbial populations prior to commencement of the feeding trial differed significantly. Thus, consumption of Cheddar cheese containing the lacticaseibacilli as adjuncts caused shifts in intestinal microbial populations in comparison in mice even when administered for only 3 weeks. However, in a human study whereby a pre-diabetic cohort was instructed to consume 120 g of commercial Cheddar cheese not containing the lacticaseibacilli adjuncts per day for a 6-week period, no significant differences were observed between the gut microbiome groups prior and post cheese-enriched diet.

The overall conclusions from this study are that Cheddar cheese contains a diverse NSLAB population, a significant portion of which can survive gastric transit and display probiotic characteristics. These strains survive during manufacture and ripening of Cheddar cheese and fermented milk and the Cheddar cheese matrix offers good protection during gastric transit. Cheese containing selected strains of lacticaseibacilli as adjuncts can affect the mouse gut microbiome.

List of Publications and Conference Contributions

Peer-reviewed Papers

Leeuwendaal, N., Stanton, C., O'Toole, P.W. and Beresford, T.P. (2021). The potential of non-starter lactic acid bacteria from Cheddar cheese to colonise the gut. *Journal of Functional Foods* 83, 104425

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. (2022). Protection of candidate probiotic lactobacilli by Cheddar cheese matrix during simulated gastrointestinal digestion. *Journal of Functional Foods* 92, 105042

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. (2022). Fermented Foods, Health and the Gut Microbiome. *Nutrients* 14(7), 1527

Oral Presentations

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. Identification of *Lactobacillus* strains from Cheddar Cheese with the Potential to Impact the Human Gut. *Food Programme, Walsh Fellowship Regional Competition 2018*. Teagasc Food Research Centre, Ashtown, Co. Dublin. September 18th, 2018.

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. The Potential of Cheddar Cheese *Lactobacillus* Isolates to Impact the Gut. *47th Annual Food Science and Technology Conference, Institute of Food Science & Technology of Ireland*. University College Cork, Cork City, Co. Cork. December 6th-7th, 2018.

Poster Presentations

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. Cluster Analysis of Acid and Bile Resistant *Lactobacillus* Isolates from Irish Cheddar Cheeses. *School of Microbiology Research Day 2016*. University College Cork, Cork City, Co. Cork. April 2016.

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. The Potential of NSLAB to Survive Gastric Transit. *School of Microbiology Research Day 2017*. University College Cork, Cork City, Co. Cork. April 2017.

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. Ability of Non-starter Lactic Acid Bacteria Isolated from Cheddar Cheese to Inhibit Binding of Pathogenic *Escherichia coli* in a Mammalian Intestinal Model. *School of Microbiology Research Day 2018*. University College Cork, Cork City, Co. Cork. April 2018.

Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. and Beresford, T.P. Probiotic Potential of *Lactobacillus* Isolates from Irish Cheddar Cheese. *Annual International Scientific Association for Probiotics and Prebiotics Students and Fellows Association (ISAPP-SFA)*. Antwerp, Belgium. May 14th-16th, 2019.

Chapter 1

General Introduction

1.1 Summary

Microbiota was defined by Lederberg and McCray (2001) as the collection of microorganisms in a distinct environment, while a microbiome refers to the resident microbiota, their genomes and the environmental conditions of that habitat (Lederberg & McCray, 2001; Marchesi & Ravel, 2015). While microbiota have adapted to numerous niches, two microbiomes are of particular interest to this work, those being fermented food and human gut microbiomes. The main phyla comprising the gut microbiota in healthy adults are usually restricted to Firmicutes and Bacteroidetes, and Actinobacteria and Proteobacteria to a lesser extent (Mota de Carvalho et al., 2018). The microbiota of fermented foods will vary greatly depending on the food, but common phyla include Firmicutes, Actinobacteria and Ascomycota (Tamang et al., 2016). Lactic acid bacteria (members of the Firmicutes phylum) are important in fermented foods as they are often used as starter cultures in cheese production (Beresford et al., 2001). In particular, the *Lactobacillaceae* family features in both fermented food and gut microbiomes, and some have been classified as health-providing probiotics (Segers & Lebeer, 2014). Existing *Lactobacillaceae* species are extremely diverse and contains more than 200 members so far (Salvetti et al., 2018). The following review will primarily investigate the role of *Lactobacillaceae* in both fermented food and gut microbiota, as well as their potential health effects on human hosts.

1.2 A Brief Overview of Cheese Microbiology

1.2.1 Cheese and Starter Lactic Acid Bacteria

Since the practical origins of cheese making approximately 8000 years ago, stemming from the need to preserve both the shelf life and nutritional value of milk, it is estimated that over 1000 different cheese types exist today (Fox et al., 1996, 2017). Cheese production involves a myriad of microbiological and biochemical events, which differ depending on the location or variety of the cheese in question (Fox et al., 2004). The majority of cheesemaking requires four key ingredients: milk, rennet, salt and microorganisms (Beresford et al., 2001). The milk used in cheesemaking can be either raw or pasteurised and sourced from cows, goats, sheep and more uncommon species that have cultural significance or are chosen due to climatic conditions .i.e., Yak's milk in the Sikkim Himalayas, and camel milk in Pakistan (Ahmed & Kanwal, 2004; Ghatani & Tamang, 2017). The nutrients available in milk are essential for the growth and metabolic activity of cheese microbes, both those intentionally added and those that involuntarily become part of the finished product (Fox et al., 2004).

Milk must curdle (form a gel) for cheese formation, which is achieved either through acid and/or the addition of a proteinase enzyme such as rennet. Rennet is the collective term given to the enzyme mixture extracted from the stomachs of ruminant animals that brings about this coagulation process, with the principal component being the protease enzyme, chymosin (Fox et al., 2004). The addition of salt has various effects on cheese, including chemical, microbiological and biochemical, and different cheese varieties' salt content can be significantly different (Guinee, 2004). In general, salt helps extends the shelf life of the cheese (for example, by creating an environment which adversely affects unwanted microbes) and it contributes to cheese flavour (Guinee, 2004). A ripening step usually ensues once the cheese has been formed, which differs between different cheese types (with 2-3 weeks for Mozzarella and 6 months to 2 years for certain Cheddars).

The microbes found in cheese are grouped according to their function, with some being added during the initial stages of cheese production and others being necessary

for the ripening stage of the cheese. An increase in acidity is a crucial step in the manufacture of most cheese varieties as it promotes curd formation and expulsion of moisture (whey) from the curd particles by a process known as syneresis (Beresford et al., 2001). This is accomplished through the addition of so-called 'starter' or 'primary' cultures, which are responsible for the production of lactic acid from the milk sugar, lactose, and the decline in pH (Fox et al., 2004). Bacteria capable of lactic acid production are classified as lactic acid bacteria (LAB), and primary cheese cultures are often referred to as Starter LAB (SLAB) (Fox et al., 2004).

The SLAB composition of different cheeses varies, but *Lactococcus lactis, Streptococcus thermophilus* and *Lactobacillus helveticus* are the most commonly used (Fox et al., 2004). *Lactobacillus delbrueckii* subsp*. lactis* and *Lb. delbrueckii* subsp. *bulgaricus* are also used in some cheese varieties (Fox et al., 2004). The types of starter cultures used are influenced by the temperatures required when cooking the cheese, and can be divided into either mesophilic (preferring moderate temperatures) or thermophilic (preferring higher temperatures) (Fox et al., 2004). Commercial mesophilic starters include *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *cremoris*, and are required in Edam, Blue, Camembert, Gouda and (more importantly) Cheddar production (Fox et al., 2004). Thermophilic SLAB are required for the popular Italian Parmigiano Reggiano cheese, Emmental cheeses and Mozzarella, where temperatures of 37° C or higher (but no higher than 52° C) are commonplace during initial phases of cheese production (Fox et al., 2004). While mesophilic starters are not used in cheesemaking with a cooking temperature exceeding 40° C, many cheeses contain a mixture of both types of starters, rendering this means of distinction somewhat outmoded (Fox et al., 2004). Thus, starter cultures are often classified according to strain diversity (Fox et al., 2004). Natural starter cultures contain mixtures of undefined starter species in unknown compositions, and reproducibility in cheese factories sometimes relies on older batches of fermented products being used for inoculation of new batches (a process known as 'backslopping') (Fox et al., 2004). Defined strains, usually consisting of a number of known strains, selected from traditional undefined starters, are now commonly used for the industrial-scale production of many cheeses such as Cheddar, Mozzarella, etc. (Fox et al., 2004).

Starter cultures that provide functional advantages such as benefits to consumer health, shelf life and the organoleptic property of the fermented food product have been investigated (Leroy & de Vuyst, 2004). For example, autolysis in SLAB has been shown to contribute to cheese flavour (Fox et al., 2004). Autolysis of the microbes results in a release of enzymes including peptidases and amino acid catabolising enzymes into the cheese matrix, where they contribute to the production of aromatic compounds from amino acids. These aromatic compounds contribute to the flavour of the cheese, making autolysis a very important factor to consider when designing starter cultures (Fox et al., 2004; Leroy & de Vuyst, 2004). Certain SLAB such as *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* produce vitamins such as folic acid, a B vitamin that humans are unable to produce that is essential for DNA synthesis and repair (Leroy & de Vuyst, 2004). Some SLAB also inhibit growth of potentially harmful bacteria through production of organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins (Fox et al., 1996; Leroy & de Vuyst, 2004; O'Sullivan et al., 2002).

Bacteriocins are of particular interest. These compounds have antimicrobial properties directed against gram positive bacteria, and are produced by SLAB to give them a competitive advantage (Fox et al., 2004). *L. lactis*, for example, produces the bacteriocin, Nisin, which is used commercially as a food preservative due to its capacity to prevent growth of unwanted food spoilage organisms (Fox et al., 2004; O'Sullivan et al., 2002). Nisin's strengths include the broad spectrum of pathogens both inhibited and killed by it, taking effect even at very low concentrations (nM range), and its ability to remain active within a variety of food environments, including low pH conditions (such as those found in fermented dairy) (O'Sullivan et al., 2002). The genes for Nisin production and immunity can be transferred in a food grade manner between strains due to it being encoded on a transmissible transposon (O'Sullivan et al., 2002).

A number of studies have been reported that involve the transfer of bacteriocin between strains of SLAB. One such study involves the transfer of enterocin A to determine its effects on the pathogen *Listeria monocytogenes* (Liu et al., 2008). The enterocin A bacteriocin is produced by *Enterococcus faecium,* and has a reputation for being one of the best antilisterial bacteriocins identified (Liu et al., 2008). *Listeria* is a concern for some cheese types, especially soft cheeses, due to its adaptations for high

salt, low pH and low temperature environments (Fox et al., 2004). Liu et al. (2007) introduced the genes responsible for enterocin A production into *L. lactic* (Liu et al., 2008). They found that the recombinant bacteriocin was capable of inhibiting *L. monocytogenes* in both normal media and in milk, suggesting a potential role in food preservation (Liu et al., 2008). In the case of nisin, it can be incorporated into various foods as a concentrated, dry powder, or alternatively, the live producing organism itself could be introduced into the food for *in situ* bacteriocin production (O'Sullivan et al., 2002; Liu et al., 2008). Either way, both methods can be considered as a desirable alternative to the addition of chemical food preservatives, which are conceived by the majority of consumers as undesirable (Leroy & de Vuyst, 2004). Mixed starter cultures containing bacteriocin- and acid-producing strains are used for production of various different cheeses in an effort to increase bacteriocin production while generating sufficient acid for cheese formation (O'Sullivan et al., 2002). Studies in 1993 and 1994 accomplished this by mixing two *L. lactis* strains capable of normal acid formation for Cheddar cheese production, while also producing sufficient bacteriocin to inhibit or kill the common food pathogens *L. monocytogenes, Clostridium sporogenes* and *Staphylococcus aureus* (Roberts & Zottola, 1993; Zottola et al., 1994). This highlights the importance of SLAB in cheese production, as careful assimilation of the best cultures for the cheese of interest can result in a myriad of additional benefits (practically and from a health perspective), on top of providing the consumer with the desired cheese product.

As stated above cultures are necessary for curdling of milk through their conversion of lactose to lactic acid and are sometimes referred to as 'Primary' cultures. Secondary cultures do not produce acid and are added to enhance flavour, texture, and aroma and carry out various other biochemical modifications inside or on the surface of the cheese (Fox et al., 2004).They are also known as adjunct cultures and include yeasts, moulds and bacteria, including Propionic Acid Bacteria (PAB) and Non-Starter Lactic Acid Bacteria (NSLAB) (Fox et al., 2004). PAB and NSLAB are unusual in that they are found inside the cheese core while most cultures of this group tend to remain primarily on the cheese surface (Fox et al., 2004).

1.2.2 Propionic Acid Bacteria

There are four species of PAB associated with dairy, but the most commonly used is *Propionibacterium freudenreichii* (Fox et al., 2004).It is found in Swiss type cheeses, in particular, Emmental, Jarlsberg and Maasdam (Fox et al., 2004). As PAB are part of the resident flora of raw milk, they can also be found in some semi hard cheeses, and will grow well at low temperatures (Fox et al., 2004). However, widespread use of pasteurisation now means that PAB must be added at the beginning of the manufacturing process to increase their population, allowing for approximately 10^3 cfu/g of bacteria after the manufacturing process is complete (Beresford et al., 2001).

PAB are mesophilic, gram positive, anaerobic (accounting for their presence in the cheese core) to aerotolerant bacteria (Fox et al., 2004). They contribute to the formation of the 'eyes' in Swiss cheeses via propionic acid fermentation, whereby they metabolise lactate to produce carbon dioxide, which gets trapped in the cheese and forms the characteristic holes associated with these cheese types (Fox et al., 2004). In addition, propionic acid and acetate are formed, which are important for preservation of the cheese and contribute to flavour (Fox et al., 2004; McSweeney, 2004). This fermentation process is initiated during the ripening process whereby the temperature is raised to 18°C to 22° C for a time, causing an increase in the abundance of the microbe (10^8 to 10^9 cfu/g) (Beresford et al., 2001; Burns et al., 2012; Fox et al., 2004).The rate at which the carbon dioxide is produced is important, with faster rates contributing to appealing, properly formed and 'shiny' eyes (Fox et al., 2004). The carbon dioxide will diffuse through the cheese matrix, and will collect at weak points in the curd, where an eye will form provided there is sufficient CO_2 pressure (McSweeney, 2004). Once a satisfactory amount of eyes have formed, further metabolism and growth of the PAB is discouraged by storing the cheese at a lower temperature (Beresford et al., 2001).

P. freudenreichii are also important in terms of lipolysis, in which lipids become hydrolysed or 'broken down' to release short chain fatty acids that significantly affect the flavour of the cheese (McSweeney, 2004). *P. freudenreichii* contain an intracellular lipase that predominantly targets these short chain fatty acids, and have higher lipolytic potential than LAB (McSweeney, 2004). This species also contains peptidases, which contribute to both texture (by making the cheese softer through casein hydrolysis and decreasing water activity) and flavour (e.g. the liberation of amino acids

and shorter peptides) during cheese ripening (McSweeney, 2004). PAB growth appears to be encouraged by the SLAB cultures *Lb. helveticus* and *S. thermophilus* through production of PAB metabolic substrates but discouraged by *L. lactis* subsp. *lactis* (Beresford et al., 2001; Canon et al., 2020; Fox et al., 2004). Their metabolism of the amino acid aspartate and overproduction of CO_2 is also known to cause a late blowing defect in aged Emmental cheese, resulting in imperfect cheese eyes and cracks through the matrix (Beresford et al., 2001; Fox et al., 2004).

1.2.3 Non-starter Lactic Acid Bacteria

NSLAB are defined as LAB 'found in cheese which do not form part of the starter culture, i.e., do not contribute to acid production during the cheese manufacturing process' (Beresford, 2003). They are not added deliberately, but originate from the milk, the utensils used during the cheesemaking process, or the factory in which the cheese is made (Irlinger et al., 2017). NSLAB fall into one of four groups: pediococci, enterococci, *Leuconostoc* and mesophilic *Lactobacillaceae* (Casey et al., 2006). The latter group, mesophilic *Lactobacillaceae*, are Gram positive, catalase-negative, non-motile, rod-shaped bacteria and will be the primary focus for this thesis. Thus, this section will focus on mesophilic *Lactobacillaceae,* with an emphasis on Cheddar cheese.

Initially, *Lactobacillaceae* populations in cheese are low in comparison to their SLAB counterparts, but ripening allows the NSLAB microbes to attain populations of $>10^7$cfu/g while the surviving SLAB numbers dwindle (Peterson & Marshall, 2010). The *Lactobacillaceae* that comprises NSLAB populations in cheese are highly heterogeneous, with the majority comprising heterofermentative *Lactobacillaceae* which ferment sugars to produce a variety of end products (Pintado et al., 2014). Variability of NSLAB populations exists between different types of cheeses, but has also been shown to differ between cheeses of the same variety and sometimes even between cheeses prepared in the same plant (Williams et al., 2002). Although the species will vary between different types of cheeses, *Lacticaseibacillus paracasei* and *Lactiplantibacillus plantarum* are the most commonly isolated NSLAB, with *Lc. casei*, *Latilactobacillus curvatus*, *Lc. rhamnosus* and *Levilactobacillus brevis* being less common but still prevalent (Banks & Williams, 2004).

The environmental parameters during cheese maturation generally inhibit starter culture growth while allowing the adventitious *Lactobacillaceae* populations to flourish. During manufacture, levels of water are plentiful, and salt concentration is low, giving starter cultures optimal conditions for their metabolism and survival (Beresford et al., 2001). Once the curds have formed and the whey is drained, water levels decrease and salt is added, conditions for SLAB become unfavourable. This, coupled with the decreasing pH and low ripening temperatures inhibits further proliferation of starter cultures and allows growth of NSLAB due to their ability to tolerate these harsher conditions (Beresford et al., 2001). NSLAB can also utilize various substrates within the cheese matrix for energy, although this is highly strain dependant, including those from the milk, bacterial cell components and other metabolites (Williams et al., 2000). These include lactose, ribose, various amino acids, lactic acid and peptone (Williams et al., 2000).

NSLAB populations are important as their presence contributes to flavour development and overall quality of the cheese product, although not always in a positive manner. In particular, *Lactobacillaceae* have complex proteolytic systems that enables them to catabolise casein to peptides and free amino acids (Settanni & Moschetti, 2010).While the amino acids themselves can affect cheese flavour, they can be modified or further catabolised to produce acids, alcohols, amines, aldehydes, ammonia and keto acids, which all contribute to the final organoleptic properties of the cheese (Settanni & Moschetti, 2010). These traits appear to be strain specific. A paper in 2006 screened NSLAB *Lactobacillaceae* for glutamate dehydrogenase activity which produces α-ketoglutaric acid, which can be further converted into flavour and aromatic compounds (Williams et al., 2004, 2006). Cheddar cheeses made with the glutamate dehydrogenase positive strains were associated with flavour profiles of mature Cheddars, indicating that the addition of the strains accelerated the production of flavour compounds usually detected in much older cheeses (Williams et al., 2006). A similar study identified that soft cheeses containing a *Lp. plantarum* or *Lc. paracasei* NSLAB strain had higher levels of free amino acids than the control cheeses with no *Lactobacillaceae* adjuncts, with the flavour of the *Lp. plantarum* cheese in particular being favoured by recruited consumers (Burns et al., 2012).

A separate study found that exclusion of mesophilic *Lactobacillaceae* from the cores of uncooked cheeses resulted in certain flavour compounds (butyric and hexanoic acids) being absent from sensory profiles, further implying the importance of NSLAB in flavour development (Callon et al., 2011). The same study also investigated the capacity of some NSLAB to inhibit pathogen growth in cheese (Gao et al., 2019; Montel et al., 2014). Although the results could not definitively demonstrate a causal effect, Callon and colleagues showed that *L. monocytogenes* populations decreased as *Lactobacillaceae* populations increased in Saint-Nectaire-type cheese (Callon et al., 2011). A separate study looked at *L. monocytogenes* growth in Ultra-filtered White cheese (a type of white brined cheese common in Iran) and found that the addition of *Lp. plantarum* eradicated the pathogen during the 60-day storage period (Ahmadzadeh Nia & Hanifian, 2017). *Lc. rhamnosus* LC705 inhibits the growth of *Clostridium tyrobutyricum*, a food spoilage microorganism, and is often used as an adjunct in Emmental and Gouda cheeses (Irlinger et al., 2017).

While NSLAB are important from a technological point of view, many have been screened for their probiotic potential and ability to confer health benefits *in vitro*. Different groups employ different assays, but the ability to tolerate gastric conditions, including low pH, the presence of digestive enzymes and bile, is common during this screening (Papanikolaou et al., 2012; Pisano et al., 2014; Solieri et al., 2014). Additionally, food proteins can be broken down by *Lactobacillaceae* into biologically active peptides (BAP), peptides that benefit the host organism beyond that of its nutritional value (Solieri et al., 2015). Milk proteins, casein in particular, are the main nitrogen source available in the habitat of dairy-dwelling LAB and have received much attention as a source of BAP (Pessione & Cirrincione, 2016; Solieri et al., 2015). NSLAB have the ability to produce BAP via their own proteolytic systems (Solieri et al., 2015). Different strains of *Lactobacillaceae* express different proteolytic enzymes, resulting in the generation of different peptide profiles from a given protein substrate (Pessione & Cirrincione, 2016). Therefore, BAP produced in a cheese will depend on both the cheese composition and its microbial population. Such benefits include the lowering of cholesterol and blood pressure, and the inhibition of pathogen growth (Park & Nam, 2015). The ability of NSLAB to produce one such BAP, Angiotensin Converting Enzyme (ACE) -inhibiting bioactive peptide, has been examined by various researchers (Park & Nam, 2015). These BAP inhibit the ACE hormone responsible for the conversion of

angiotensin I to angiotensin II (Beltrán-Barrientos et al., 2016; Tunick & Van Hekken, 2015). Angiotensin II is a vasoconstrictor (narrows blood vessels) which also inactivates other vasodilators, resulting in increased blood pressure (Beltrán-Barrientos et al., 2016; Tunick & Van Hekken, 2015). Therefore, inhibition of ACE leads to dilation of blood vessels and lower blood pressure (Beltrán-Barrientos et al., 2016; Tunick & Van Hekken, 2015). An example of the production of ACE-inhibition BAP by NSLAB in cheese was reported by Solieri *et al*. in 2015 who investigated the production of two ACE-inhibiting peptides, Ile-Pro-Pro and Val-Pro-Pro, by NSLAB from Italian cheeses, and discovered that the *Lc. casei* PRA205 strain produced high levels of both peptides (Solieri et al., 2015). It is important that these peptides remain intact during digestion and adsorption in order to have the desired effect on the host. The ability of cheese to transport ACE-inhibiting BAP through digestion has been investigated *in vitro,* with concentrations of residual BAP varying depending on the cheese (Basiricò et al., 2015; Stuknyte et al., 2015).

It is interesting to speculate that NSLAB which express traits that positively correlate with human health *in vitro* may impart health benefits once consumed. NSLAB populations in cheese are generally high and, assuming they can survive gastric transit, have a very real chance of expressing those beneficial traits once they reach the small intestines. In addition, once ingested, the surviving NSLAB population may interact with the gut microbiome and bring about changes in it due to competitive or synergistic effects.

1.3 The Gut Microbiome

1.3.1 Gut Microbes and Health

The human body hosts many communities of microorganisms, the composition of which varies dramatically depending on the anatomical site and health of each individual (Cho & Blaser, 2012). These microbes and their metabolites interact with host cells and potentially confer benefits such as bolstering the immune system, inhibiting the growth or colonisation of deleterious microbes or providing vitamins that humans are unable to synthesise (Zhang et al., 2015). The human gut microbiome consists of a vast number and diversity of microorganisms, which increase in density from the stomach (least populated at approximately 10^1-10^3 CFU/mL) to the colon (highest microbial density at approximately 10^{11} CFU/mL) (**Figure 1**) (Donaldson et al., 2015; Sender et al., 2016).The enormous diversity of these microbial communities has been more fully realised over the past decade due to the development of Next Generation Sequencing, and this is particularly the case with the gut microbiome (Sweeney & Morton, 2013).

The composition of the gut microbiome varies between individuals, but generally follows an age-determined pattern in humans where it is highly variable during the first 2-3 years of life, after which it stabilizes and remains so until it again changes due to aging or health associated issues (Voreades et al., 2014). In healthy adults, the dominant phyla are generally *Bacteroidetes* and *Firmicutes* with others (e.g. *Actinobacteria*) being present at lower levels (Lozupone et al., 2012). Much effort has focused on determining a healthy or 'normal' gut microbiome and it has been suggested that, while the microbial compositions of individuals can show high variability, the majority of people can be categorised into three distinct 'enterotypes' (Power et al., 2014). Each of these enterotypes is dominated by one of either the *Bacteroides*, *Prevotella* or *Ruminococcus* genera, although evidence for this is not wholly supported by the literature with some researchers indicating that gene composition of the overall microbiome is of greater importance than the composition of microbes themselves (Power et al., 2014). While stable in adulthood, a paper in 2011 indicated that the gut microbiome is affected by diet to different extents, with

long-term diet being strongly associated with specific enterotypes but susceptible to changes in microbiota composition in response to dietary changes (Wu et al., 2011). Additionally, researchers are interested in perturbations of the gut microbiota that result in or are linked with a disease/disorder phenotype in humans. While healthy humans and their microbiome co-exist in a symbiotic state, dysbiosis of the gut microbiome was eloquently referred to by Doré and Blottière as 'an alteration of symbiosis, which drives a detrimental distortion of microbe-host homeostasis' (Doré & Blottière, 2015).

Dysbiosis of the gut microbiome is commonly associated with metabolic disorders. This has been the subject of much research in recent years, much of which has focused on Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). IBD is a chronic inflammatory disease which cycles between remission and relapse, and shares many symptoms with IBS, including bloating, altered bowel habits and abdominal pain (Spiller & Major, 2016). However, the inflammation and ulcers often present on the mucous membrane in IBD during the diseases acute phase are absent in IBS (Spiller & Major, 2016). In both cases, differences are seen between the diseases state microbiome and those of healthy individuals (Presti et al., 2019).

The gut microbiome of patients suffering from IBS was investigated by Rajilić-Stojanović and colleagues and its composition was found to be significantly different from the healthy controls (Rajilić-Stojanović et al., 2011). They implicated groups of *Proteobacteria* and *Firmicutes*, and noted that the ratio of *Firmicutes* to *Bacteroidetes* was higher in IBS samples due to an increase in *Dorea*, *Ruminococcus* and *Clostridium* and a decrease in *Bifidobacterium* and *Faecalibacterium* species, and methanogens (Rajilić-Stojanović et al., 2011). Following this particular study, other researchers have attempted to discern groups associated with IBS but, when compared with each other, the 'guilty' bacteria are not so easy to pinpoint (Labus et al., 2017; Zeber-Lubecka et al., 2016; Zhu et al., 2019). However, a recent systematic review examined sixteen articles which utilised 16S rRNA targeted sequencing to detect alterations in the gut microbiome in IBS patients and determined that there was a relative decrease in α-diversity and, in general, an increase in the Firmicutes to Bacteroidetes ratio, supporting the Rajilić-Stojanović et al. study described above (Duan et al., 2019).

The gut microbiome has also been studied with regards to its potential role in ulcerative colitis (UC) and Crohn's disease (CD), two different forms of Inflammatory Bowel Disease (IBD), which are also affected by diet, age and genetics relating to the immune system (Kostic et al., 2014). IBD is associated with an altered microbiota in patients in which levels of diversity of the *Firmicutes* and *Bacteroidetes* is decreased while numbers of *Enterobacteriaceae* is increased (Maloy & Powrie, 2011). This shift in composition results in increases in sulphate reduction and epithelial permeability, leading to inflammation of the gut (Das & Nair, 2019). Additionally, a study in 2017 examined faecal samples from IBD sufferers for a 2-year period and determined that the microbial composition of IBD patients tended to fluctuate and show less stability than healthy patients (Halfvarson et al., 2017). While many papers have confirmed the presence of dysbiosis in IBD models, Ni and colleagues (2017) suggested that this altered microbiome phenotype may be a result of the disease itself rather than the primary cause, with the chronic inflammation modulating microbiota changes and not the other way around, although they acknowledge that this relationship is complex and may be influenced by multiple factors (Ni et al., 2017). While further research is necessary to fully understand the role of the gut microbiome in IBD, changes observed between sufferers and healthy subjects do imply a relationship.

Dysbiosis is not only involved in metabolic disorders, but neurological ones as well. Communication between the brain and the gut occurs through the central nervous systems' (CNSs) connection to the gut via both the sympathetic and parasympathetic nerves, referred to as the gut-brain axis (Ghaisas et al., 2016). Individuals with the neurological autism spectrum disorder (ASD), the severity of which can vary greatly between affected individuals, exhibit microbiomes with higher abundances of *Clostridium*, *Lactobacillaceae* and *Desulfovibrio* bacteria and a decreased *Bacteroidetes* to *Firmicutes* ratio (Vuong & Hsiao, 2017). A study in 2016 examined faecal samples of patients suffering from bipolar disorder and discovered that patients exhibited a significantly lower fractional representation of *Faecalibacterium* in comparison to their healthy counterparts, a genus with proven anti-inflammatory effects which is also known to be less abundant in other conditions (including IBD) (Evans et al., 2017). This correlates with the results of a separate group examining microbial composition of bipolar patients, whereby healthy controls had higher concentrations of *Faecalibacterium* in their stool, additionally observing that bipolar samples contained

greater levels of *Actinobacteria* and *Coriobacteriaceae* (Painold et al., 2019). A metanalysis of 16 studies on gut populations in patients with Parkinson's disease determined that, while certain groups of microbes were noted to be consistently different from their healthy equivalents across the 16 studies, a great amount of variation exists in the pooled results, which may be the result of inconsistent experimental design, and thus an accurate consensus for what defines the microbiota of a Parkinson's sufferer does not exist currently (Boertien et al., 2019).

With dysbiosis occurring in many common disorders, one form of treatment could be the correction of the microbial composition to one associated with a healthier phenotype. Dietary strategies with varying levels of success have been employed for IBD/IBS treatment (de Filippis et al., 2018). The Specific Carbohydrate Diet (SCD) excludes certain complex carbohydrates and all refined sugar on the basis that these are commonly malabsorbed, resulting in dysbiosis and inflammation in the gut (Cohen et al., 2014). Low-fermentable oligo-, di- and monosaccharide and polyols (FODMAPs) diets, as the same suggests, limits the intake of these carbohydrates due to their hypothesized ability to trigger digestive discomfort associated with IBS (bloating, pain) when fermented by gut microorganisms (McIntosh et al., 2017). Children (and, to a lesser extent, adults) with CD have been shown to benefit from an exclusive enteral nutrition (EEN) diet, a liquid formula of monomeric or polymeric units (or a mixture of both) that is ingested orally or administered through a tube (Day, 2019). Other studies attempt to increase the presence of fibre-degrading bacteria in the gut via addition of a prebiotic (a non-digestible food component) to the diet, which is reported to improve symptoms of the metabolic disorder in some cases (de Filippis et al., 2018).

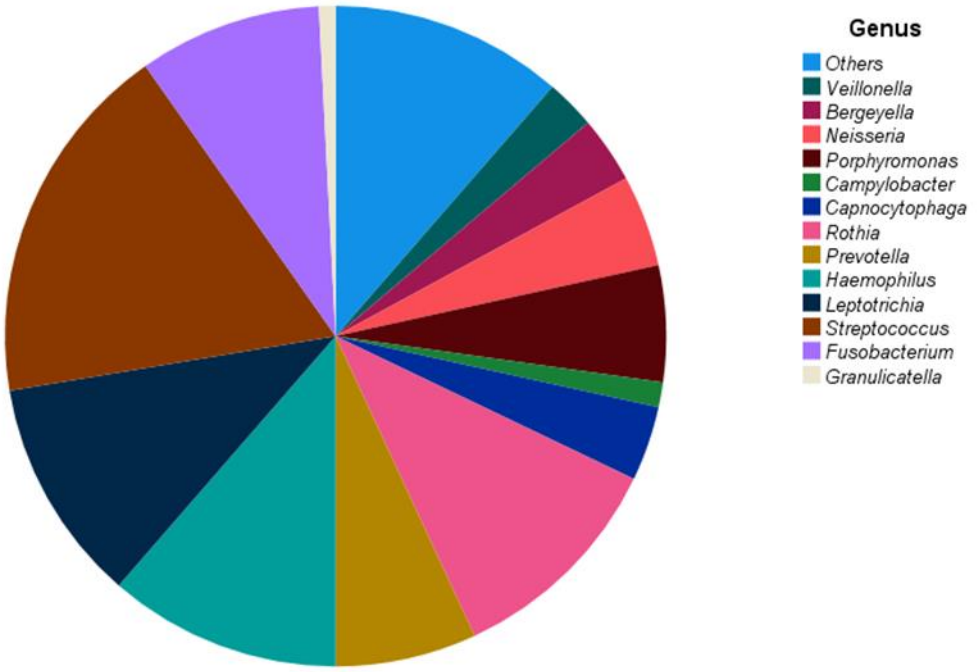
Other approaches to correcting disorders through the gut include ingestion of a live microorganism that has been shown to relieve patients of one or many symptoms. The administration of *Lactobacillaceae* strains to children with ASD has been tested by several different groups with varying degrees of success, including improvement in social and behavioural interactions and improved cognitive function in children (Umbrello & Esposito, 2016). Pärrty *et al*. (2011) administered a *Lactobacillaceae* strain to babies over the first 6 months of their lives (their mothers also ingested the microbe for 4 weeks prior to their given delivery date) and health was subsequently evaluated at 2 and 13 years of age (Pärtty et al., 2015). Pärtty and colleagues found that ingestion

of the *Lactobacillaceae* decreased the risk of children developing Asperger Syndrome (AS) and attention deficit hyperactivity disorder (ADHD) when compared to the control group who received a placebo, despite overall microbial composition of the children not being significantly different (Pärtty et al., 2015).

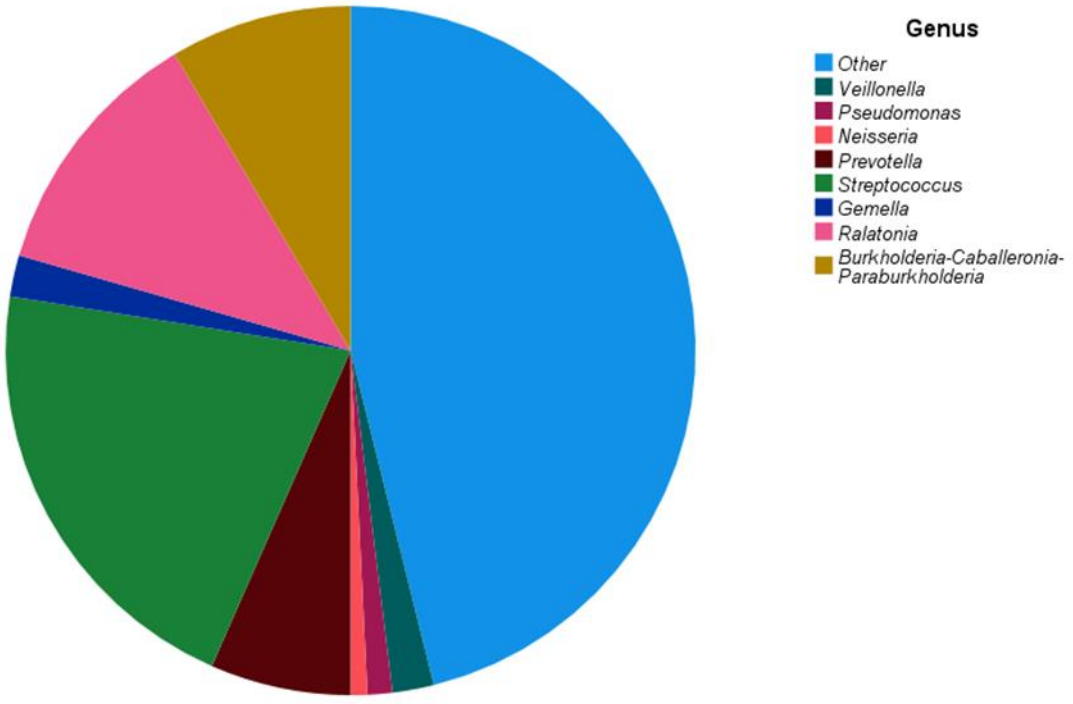
Continuing on from neurobiological disorders, the gut microbiome is crucial for development and correct function of the CNS, communicating with the brain via immune, endocrine and nervous response mechanisms and being either directly or indirectly involved in many important neurogenerative processes including synthesis of the myelin sheaths coating neurons (myelination) and microglia maturation (Martin et al., 2018; Sharon et al., 2016). Germ-free (GF) mice (those lacking a gut microbiome), relative to normal mice, exhibited microglia cells (which are an important component of the immune system associated with the CNS) that expressed an immature phenotype, altered cellular proportions and ultimately resulted in an innate immune response that is defective (Erny et al., 2015). Interestingly, seeding of the same GF mice with a diverse microbiome restored microglia to an extent (Erny et al., 2015).

The gut microbiota forms a necessary symbiotic relationship with their animal host that is essential for extraction of various nutrients, protection against disease and imbalance, and is responsible for development and maintenance of important neurological processes. Thus, the gut microbiota's role in health is prominent and can be targeted as a means of correcting certain disorders and conditions.

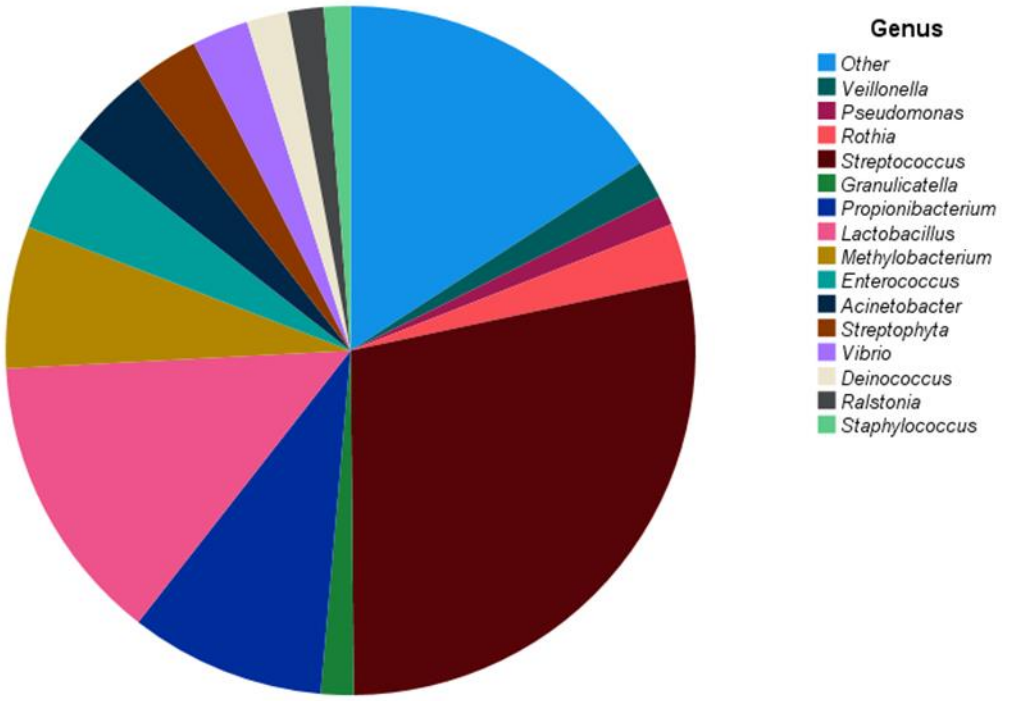
A.


Genus
Others
Veillonella
Bergeyella
Neisseria
Porphyromonas
Campylobacter
Capnocytophaga
Rothia
Prevotella
Haemophilus
Leptotrichia
Streptococcus
Fusobacterium
Granulicatella

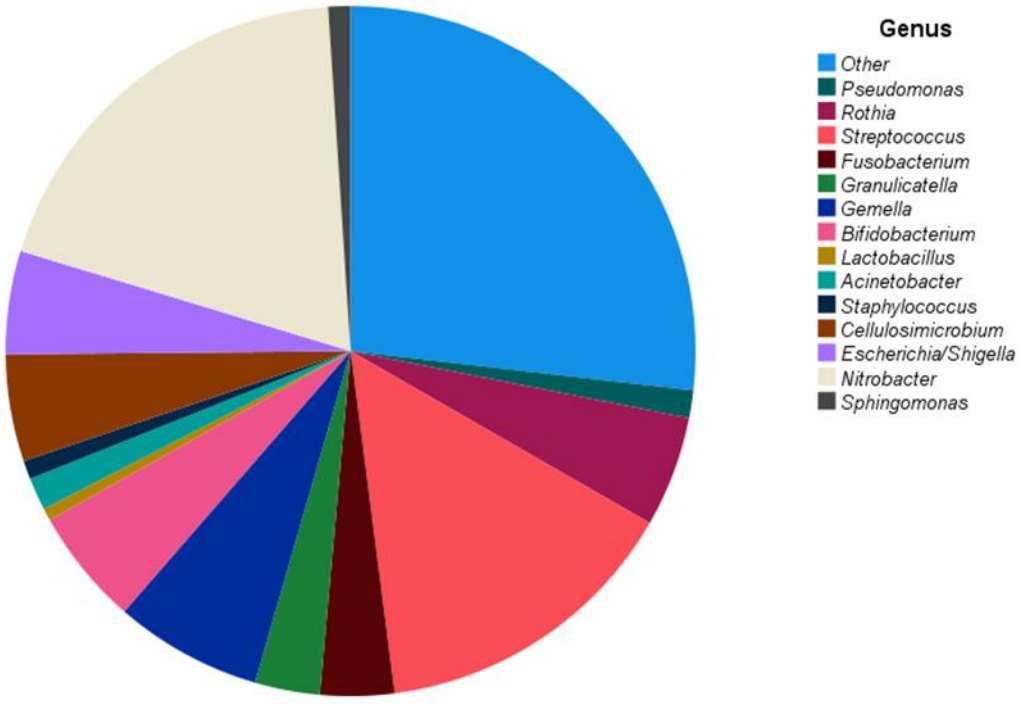
B.


Genus
Other
Veillonella
Pseudomonas
Neisseria
Prevotella
Streptococcus
Gemella
Ralatonia
Burkholderia-Caballeronia-Paraburkholderia

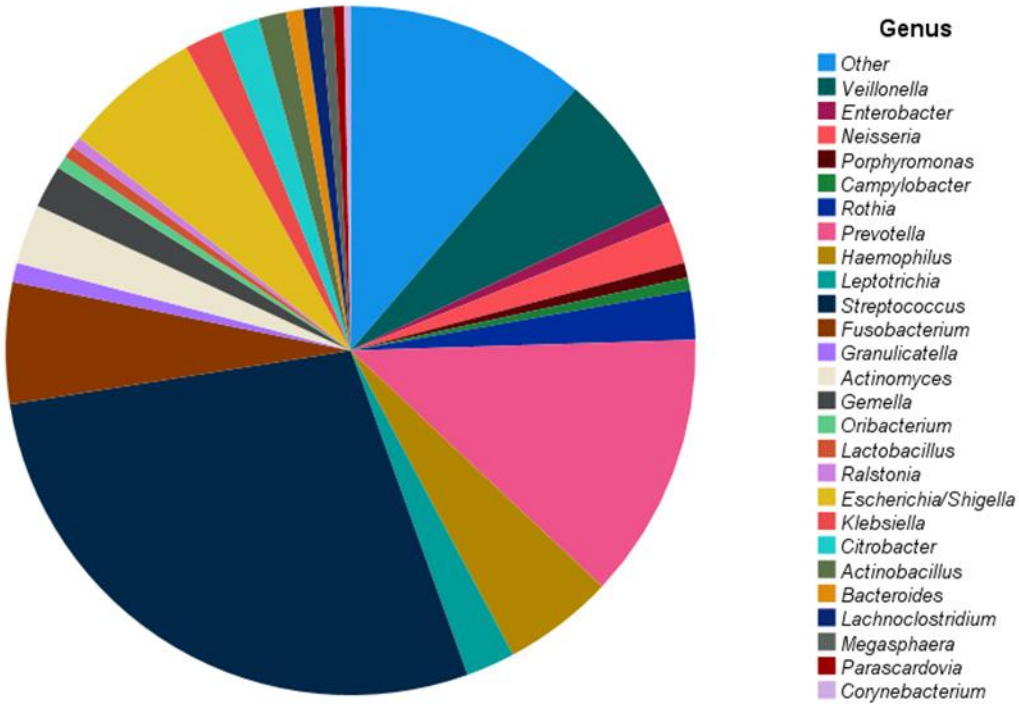
C.


Genus
Other
Veillonella
Pseudomonas
Rothia
Streptococcus
Granulicatella
Propionibacterium
Lactobacillus
Methylobacterium
Enterococcus
Acinetobacter
Streptophyta
Vibrio
Deinococcus
Ralstonia
Staphylococcus

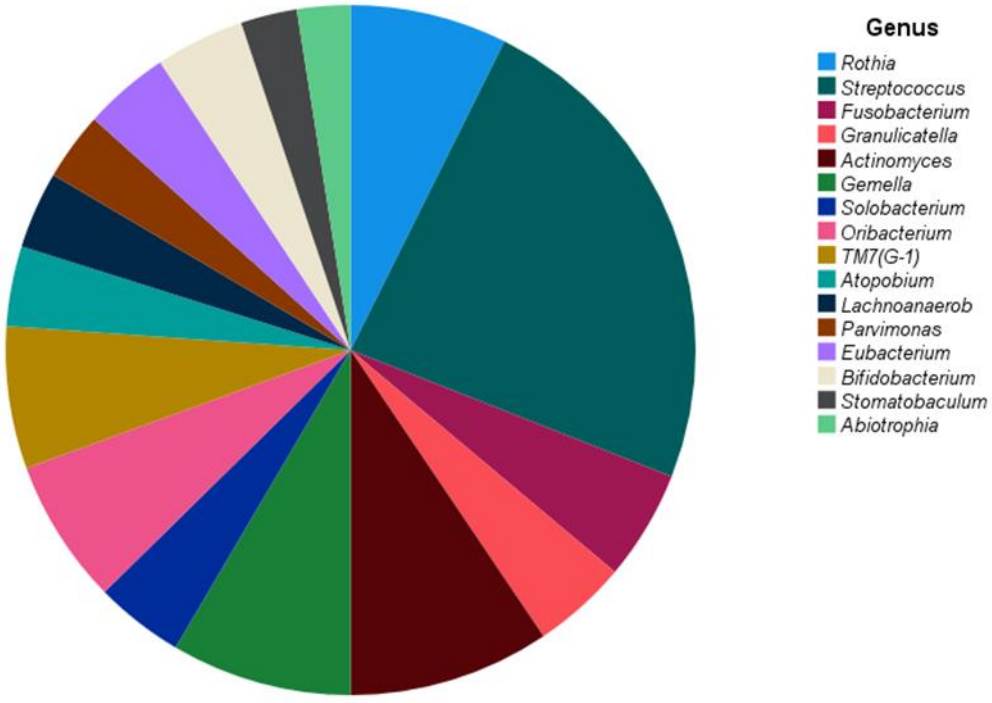
D.


Genus
Other
Pseudomonas
Rothia
Streptococcus
Fusobacterium
Granulicatella
Gemella
Bifidobacterium
Lactobacillus
Acinetobacter
Staphylococcus
Cellulosimicrobium
Escherichia/Shigella
Nitrobacter
Sphingomonas

E.


Genus
Other
Veillonella
Enterobacter
Neisseria
Porphyromonas
Campylobacter
Rothia
Prevotella
Haemophilus
Leptotrichia
Streptococcus
Fusobacterium
Granulicatella
Actinomyces
Gemella
Oribacterium
Lactobacillus
Ralstonia
Escherichia/Shigella
Klebsiella
Citrobacter
Actinobacillus
Bacteroides
Lachnoclostridium
Megasphaera
Parascardovia
Corynebacterium

F.


Genus
Rothia
Streptococcus
Fusobacterium
Granulicatella
Actinomyces
Gemella
Solobacterium
Oribacterium
TM7(G-1)
Atopobium
Lachnoanaerob
Parvimonas
Eubacterium
Bifidobacterium
Stomatobaculum
Abiotrophia

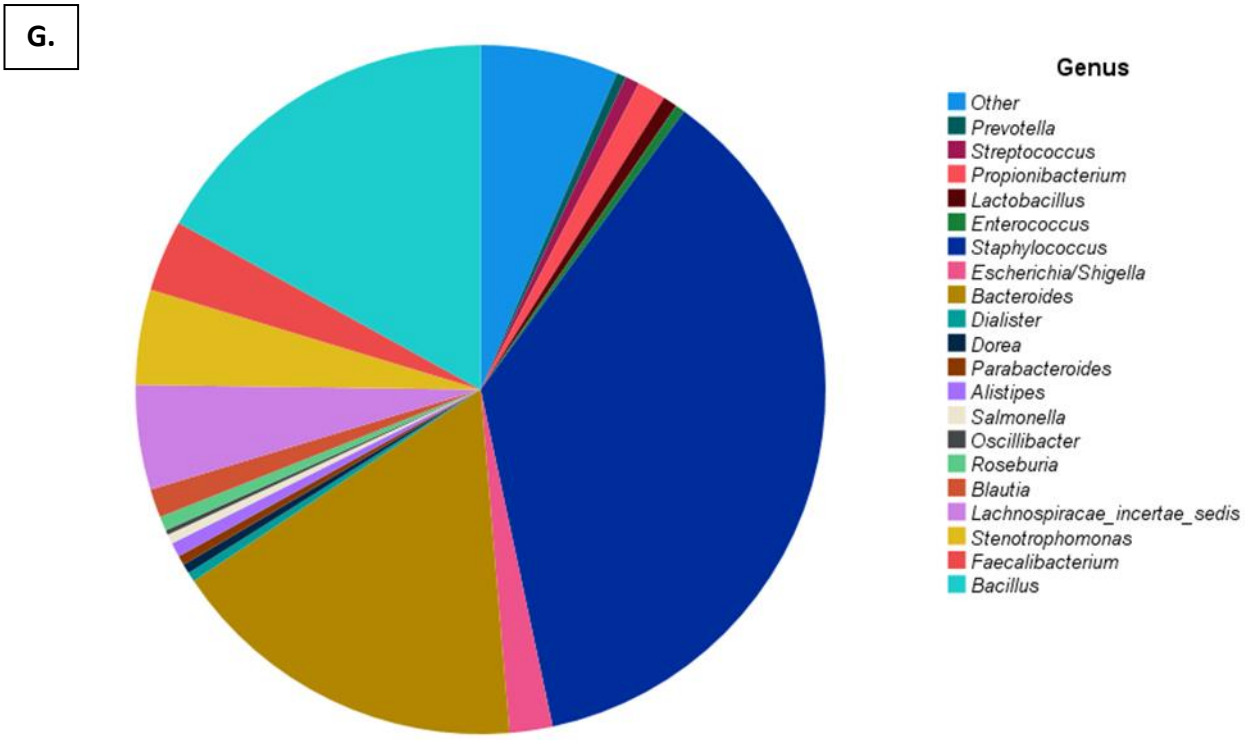


Figure 1. Composition at genus level of the Digestive Tract Microbiomes. Pie charts illustrating percentages of the dominant genera identified via 16S rRNA gene sequencing of the **A.** Oral **B.** Oesophageal **C.** Gastric **D.** Duodenal **E.** Jejunal **F.** Ileal and **G.** Colonic microbiomes in healthy individuals. Data was compiled from multiple sources (Al-Hebshi et al., 2017; Delgado et al., 2013; Li et al., 2020; Mira-Pascual et al., 2015; Sroka-oleksiak et al., 2020; Sundin et al., 2017; Villmones et al., 2018). While the outdated genus '*Lactobacillus*' has been used by the aforementioned authors, it now refers to the family Lactobacillaceae and should be read as such (Zheng et al., 2020).

1.3.2 Gut vs. Dairy *Lactobacillaceae*

Due to the genetic diversity and broad range of niches inhabited by *Lactobacillaceae* they are often used as functional genomic models to understand and identify genes required for survival within a specific niche (Cai et al., 2009; Callanan et al., 2008; Siezen et al., 2010). This is of particular interest with regard to species and strains that are unique to or capable of growth in food and/or the gut as such studies would assist in the selection of potential probiotics and indicate how food as a source of microbes could influence the gut microbiome (Azcarate-Peril et al., 2008; Li et al., 2016). With regards the screening for probiotic *Lactobacillaceae*, the hypothesis is that strains that share similar genetic traits to those specific for the gut would likely possess an adaptive advantage to the gut habitat. Genes present in *Lactobacillaceae* from one habitat may be absent or less frequent in another. For example, annotation of the genomes of typical dairy *Lactobacillaceae* *Lb. helveticus* (used in cheese production) and *Lb. bulgaricus* (used in yogurt production) revealed proteins involved in metabolism of sugars and carbohydrates commonly found in milk, while Mucus-binding (MUB) domains which play a role in aiding adherence of bacteria to the mucus layer of the gut were more common in *Lactobacillaceae* found in the gastrointestinal tract (Boekhorst et al., 2006; Ventura et al., 2009, 2012).

One such study attempted to define gut-specific genes by investigating the genome sequences of two closely related lactobacillus species which occupy very different niches (O'Sullivan et al., 2009). Sequencing revealed a 98.4% 16S ribosomal RNA identity between the dairy strain *Lactobacillus helveticus* DPC4571 and the gut strain *Lb. acidophilus* NCFM, and O'Sullivan et al. (2009) hypothesised that identification of the genes encompassing the remaining 1.6% would reveal a set of genes specific for the niche under investigation, going so far as to call these genes a 'barcode' by which niche could be identified through presence or absence in a *Lactobacillaceae* under investigation (O'Sullivan et al., 2009). Upon further investigation, they identified nine genes which they classified as niche-specific (O'Sullivan et al., 2009). By analysing their presence in a variety of *Lactobacillaceae* from different niches, the authors were able to predict which bacteria were specific to which niche, and those that traversed multiple habitats (O'Sullivan et al., 2009). For example, they identified the maltose-6-phosphate glycosidase gene (Iba_1689) in *Lb. acidophilus*, *Lc. casei*, and *Lb. johnsonii*,

which are all reported commensal gut strains (O'Sullivan et al., 2009). Glycosidase enzymes are required for the conversion of maltose to glucose, are not commonly present in LAB, and encoding such enzymes (especially those targeted at breakdown of plant-origin di- and oligosaccharides) appears to be an adaptation for *Lactobacillaceae* normally resident in the gut (O'Sullivan et al., 2009). On the other hand, *Lactobacillaceae* of dairy origin are often only capable of metabolising the milk sugar lactose, and are considered highly fastidious (O'Sullivan et al., 2009). The study by O'Sullivan et al. (2009) also indicated that the divergence of the dairy *Lb. helveticus* DPD4571 strain appeared to have been a recent event and involved the depletion of many glycosidase genes (O'Sullivan et al., 2009).

O'Sullivan et al. (2009) also identified two bile salt hydrolase genes (Iba_0892 and Iba_1078) that could be used to identify gut-specific *Lactobacillaceae* (O'Sullivan et al., 2009). This was established on the grounds that these two genes were found together only in those microbes that were gut specific (O'Sullivan et al., 2009). A non-functional copy of one of the aforementioned *bsh* genes was identified in *Lb. helveticus* DPD4571, which shares a common ancestor with *Lb. acidophilus*, a gut-specific *Lactobacillaceae* species (O'Sullivan et al., 2009). However, identifying niche organisms based on the presence of *bsh* genes may be more complex, due to variations in genetic organisation (Elkins et al., 2001). For example, a study in 2001 identified the location of BSH activity in a gut-specific *Lactobacillaceae* strain, *Lb. johnsonii* 100-100, and found three genes arranged in an apparent operon, while *bsh* genes in *Lb. plantarum* 80 were attributed to a monocistronic transcript (Elkins et al., 2001). Other *Lactobacillaceae* from varying niches were screened for the *Lb. johnsonii* locus (the *cbsHβ* locus) and while many displayed BSH activity, only a small proportion of those screened tested positive for this particular locus (Elkins et al., 2001). These included *Lb. gasseri*, *Lb. johnsonii*, and *Lb. acidophilus* from human sources, with *Lb. delbrueckii* subsp. *bulgaricus* being the only known strain of non-human origin (originated from Bulgarian yoghurt) identified to contain the locus (Elkins et al., 2001). Many other *Lactobacillaceae* species were screened for this operon but none were found to contain it, including species often associated with the human gastro-intestinal tract, such as *Limosilactobacillus reuteri* and *Lb. ruminis* (Elkins et al., 2001; Walter, 2008).The findings of Elkins et al. (2001) suggest that *Lactobacillaceae* acquired their *bsh* genes by horizontal gene transfer (HGT), and the presence of this locus and phenotype in human strains suggests its

importance for gut colonization (Elkins et al., 2001).

However, a subsequent study that examined whole genome sequences for 20 *Lactobacillaceae* species (including the two strains in O'Sullivan et al. [2009]) found that, while strains could be grouped into sets which shared genes not present in other sets, these were not origin specific (Kant et al., 2011). In fact, the dairy strain *Lb. helveticus* DPC4571 and the gut strain *Lb. acidophilus* NCFM ended up in the same phylogenetic group, separated by only a single node, with the nine niche-specific genes identified previously not being confined to any niche in particular (Kant et al., 2011; O'Sullivan et al., 2009). Another study compared both genotypic and phenotypic information of 100 *Lb. rhamnosus* strains and found that strains could generally be classified as either of two groups A or B, with group A being frequently isolated from cheeses while group B strains were more frequently associated with a gut habitat (Douillard et al., 2013). Features of group A included metabolism of lactose and loss of SpaCBA pili (which aids mucus-binding), while B strains had the ability to metabolise L-fucose (available in the gut), bile resistance and adherence to epithelial cells via the production of various pili (Douillard et al., 2013). Various studies have speculated that adaptations to certain niches, such as the dairy niche, results in gene decay in *Lactobacillaceae* whereby coding regions not required for growth in milk are 'lost' but this leaves such *Lactobacillaceae* at a disadvantage in other environments (Stefanovic et al., 2017). In addition, dairy *Lactobacillaceae* genomes can contain high levels of pseudogenes while their gut counterpart's pseudogene levels are much lower or absent in some cases (Stefanovic et al., 2017).

These studies demonstrate that differences in genomes between dairy and intestinal *Lactobacillaceae* exist, although inconsistencies between studies make it difficult to establish a clear "bar code" at this time. However, such genetic information is still useful when screening *Lactobacillaceae* as they provide some direction as to the potential of a strain to be suitable to dairy and/or gut applications.

1.4 *In Vitro* Testing for Potential Probiotics

The definition of a probiotic, as stated by the World Health Organisation (WHO) and Food and Agricultural Organization (FAO), is a "Live microorganism which when administered in adequate amounts confers a health benefit on the host" (Araya et al., 2002). A profound interest in the genus *Lactobacillaceae* exists with regards the discovery of novel probiotics strains (Maragkoudakis et al., 2006). Contributing factors to the targeting of these bacteria specifically is prolonged use in dairy and other fermented foods and their presence as a significant proportion of the gut microbiome population in healthy individuals. Indeed, *Lactobacillaceae* are members of the phylum *Firmicutes*, the largest bacterial group found in the human intestine (Kleerebezem & Vaughan, 2009). In order for an organism to be considered a 'probiotic', it must display a range of specific characteristics that focus on the ability of the organism to survive transit through the upper GIT (Fijan, 2014). The WHO/FAO in 2002 summarised the most important characteristics required for declaration of a strain as a probiotic which include: resistance to gastric acidity; bile acid resistance; adherence to mucus and/or human epithelial cells; ability to reduce pathogen adhesion to GIT surfaces and bile salt hydrolase activity (Araya et al., 2002). These tests will be discussed below, to determine the differences and most efficient ways of *in vitro* probiotic potential determination.

1.4.1 Resistance to Gastric Acidity

Bacteria with probiotic potential must have the ability to survive the harsh acidic conditions experienced in the GIT in sufficient numbers if they are to colonise the intestine (either temporarily or permanently) and confer a health benefit to their host. The most common approach to investigate this is to expose the bacteria to "simulated gastric digestion" (SGD) conditions which mimic the environmental conditions of the stomach and ileum *in vitro*. Such tests expose bacteria to low pH in the range of 1-3, in the presence of gastric enzymes (pepsin) and temperature at 37 °C (Guo et al., 2009; Haller et al., 2001). These acidity levels reflect the fluctuations experienced in the stomach, with a low of pH 1 during fasting to a high of 4.5 after ingestion (Guo et al., 2009). The exposure times used differ between researchers. For example, Pisano et al.

(2014) exposed their strains to SGD conditions for 1.5 hours, while Guo et al. (2009) evaluated survival following exposure for 1, 2, 3 and 4 hours (Pisano et al., 2014; Guo et al., 2009). The latter ascribed this logic to food often remaining in the stomach for up to 3 hours. However, Pisano et al. (2014) included shaking in a water bath to mimic peristalsis, adding to a more realistic gastric experience for the microbes (Pisano et al., 2014). Variations of these methods exist that include screening microbes on suitable laboratory media (for *Lactobacillaceae*, this usually refers to de Man, Rogosa and Sharpe (MRS) or Lactobacillus Selective (LBS) agar) or in low pH buffers. For example, Maragkoudakis et al. (2006) screened 29 *Lactobacillus* strains of dairy origin by suspension in Phosphate Buffered Saline (PBS) that had been adjusted to pH1 and pH3, while Balamurugan et al. (2014) used a similar tactic on MRS agar plates (Balamurugan et al., 2014; Maragkoudakis et al., 2006). As probiotic bacteria are usually consumed within a food, SGD models that begin with a food matrix, rather than simple bacterial cultures, are considered to provide a more accurate measure of the potential of a bacterium to survive GIT transit as the food matrix has been found to often afford the bacteria some level of protection (Minekus et al., 2014; Saxelin et al., 2010).

Cheese in particular is considered as being particularly efficient at protecting its indigenous bacteria during GIT transit due to its compact protein matrix (Sharp et al., 2008). Minekus et al. attempted to mimic, to the highest degree possible, true digestion via the simulation of steps, enzymes and materials often overlooked by other authors (Minekus et al., 2014). In addition, this study also differs in that the low pH 'Gastric' phase was maintained at pH 3 throughout the allotted 2 hour timeframe, and recommends that the pH of the digestion mixture be tested and adjusted back to pH 3 if it becomes higher (Minekus et al., 2014). This re-adjustment is justified as the food matrix's buffering capacity can lead to an increase in pH (depending on the food matrix, this could potentially increase to as high as pH 5), while a lower pH is required for optimum gastric enzyme functionality (Minekus et al., 2014). This model is referred to as the INFOGEST model and has recently been updated to also include lipase during the gastric phase, especially when dealing with matrices with high fat content (Brodkorb et al., 2019). As complex as this system may appear, it is still recognized as a static model such as those mentioned earlier. Many researchers argue in favour of more complex dynamic systems which more accurately reflect the *in vivo* reality. An example of a complex, dynamic system is the Simulator of the Human Intestinal

Microbiota System (SHIME®), originally developed by Molly et al. in 1993 (Molly et al., 1993). This consists of a multicomponent system, with each vessel mimicking a stage and/or section of human digestion, including the stomach, small intestines, and various stages of the colon (Molly et al., 1993). In this system, the pH of the 'stomach' region is controlled via programming to reflect the pH fluctuations that occur naturally during digestion (Molly et al., 1993).

Various attributes and genes have been implicated in the survival of *Lactobacillaceae* at low pH, with varying degrees of evidence. Broad statements regarding acid tolerance have been made, such as acid tolerance being linked to the pH profiles of *Lactobacillaceae* membrane H^+-ATPase and their cytoplasmic membrane design (Ljungh & Wadström, 2006)(Madureira et al., 2011). The cell membrane and wall, collectively termed the cell envelope, have both been demonstrated as important for acid resistance in different *Lactobacillaceae* (Lebeer et al., 2008). The cell envelope of *Lc. casei* appeared granular where in contact with an acidic environment when viewed under a transmission electron microscope (TEM), presumably due to increased expression of surface proteins necessary to combat the acid stress (Hossein Nezhad et al., 2010). A study by Wu et al. (2012) examined the effect of acid stress on a wild type (WT) and an acid-tolerant mutant of *Lc. casei* (Wu et al., 2012). A hundred-fold difference was seen between survival rates of the two strains, with the mutant proving more robust in the acidic environment (C. Wu, Zhang, Wang, et al., 2012). This was ascribed to lower membrane viscosity (associated with membrane fluidity), higher unsaturated/saturated membrane fatty acid ratio, and higher cell envelope integrity of the acid-tolerant mutant (C. Wu, Zhang, Wang, et al., 2012).

Information has also come to light implicating proteins, which have a DNA/protein protective or repairing function, in acid stress tolerance (Lebeer et al., 2008). For example, the RecO protein, involved in DNA repair, has been shown to be up-regulated in *Lb. casei* during acid stress (Wu et al., 2013b; Wu et al., 2012). LAB can also rely on two-component and other regulatory systems for general relief from stresses, including low pH, and rely on other, more precise strategies for the removal of the stress factors themselves. These include ATPases (enzymes), amino acid decarboxylation-antiporter reactions, and the arginine deiminase (ADI) pathway (Lebeer et al., 2008). Acid tolerance of bacteria as a result of these systems was

reported in a study done in 2013 (Wu et al., 2013a). The *atpD* gene (located on an ATPase operon and coding for the beta ATPase subunit) has been found to combat acid stress via maintaining a neutral cell pH, and exhibited significant upregulation in 2 strains of *Lp. plantarum* (Chandran et al., 2013; Duary et al., 2010). Amino acid decarboxylation-antiporter reactions increases the intracellular pH of the bacteria in response to acid stress via importing of amino acids into the cell, followed by their decarboxylation whereby a proton is expended, and the end product is expelled from the cell via an antiporter (Lebeer et al., 2008). This was tested in a study using a *Lc. casei* WT and an acid-resistant mutant, and the amino acid, aspartate (Wu et al., 2013a). Bacteria were grown in aspartate-enriched MRS media adjusted to sub-lethal pH values (6.5-4.3), followed by resuspension in MRS adjusted to pH3.3 to determine their tolerance to acid shock (Wu et al., 2013a). Exposure to the acidic conditions resulted in the accumulation of the exogenous aspartate in both strains, and was shown to increase growth and survival of both strains and aided in the maintenance of cellular pH homeostasis (Wu et al., 2013a).

Many groups have attempted to identify the exact genes that confer acid tolerance, as a means of distinguishing, through a genotypic approach alone, which *Lactobacillaceae* strains are suitable for low pH environments. Research has demonstrated that *Lactobacillaceae* and other bacteria can be pre-adapted to sub-lethal stress conditions, by exposing them to low levels of the stress of interest prior to further, more intense stress environments (van Bokhorst-van de Veen et al., 2012). In order to establish genes involved in acid tolerance, a mechanism called acid tolerance response (ATR) has been exploited by many researchers (Huang et al., 2015; Wu et al., 2014). This involves initially exposing bacteria to a mildly acidic pH to enable them to start the adaption process, followed by exposure to more intense conditions at which stage gene expression is monitored. For many bacterial species, the extent of ATR is strain specific. A paper by Wu et al. (2014) investigated this phenomenon, and detailed the genes whose expressions fluctuated during these trials (Wu et al., 2014). This work followed on from a previous study by the same research group who found that the DNA repair gene *recO* was up-regulated in *Lc. casei* during acid exposure, as mentioned previously (Wu et al., 2012). The 2014 study showed that, following mild acid exposure for 20 minutes (pH 4.5), *Lb. casei* ATCC 334 showed enhanced survival at a higher acidic condition of pH 2 for 140 minutes as opposed to control batches of ATCC 344

which were not pre-exposed to mild acidic conditions (Wu et al., 2014). Their results also showed that the *Lc. casei* used was capable of regulating its internal pH, which was done upon reaching a specific pH through proton-translocating ATPases, highlighting the importance of these proteins in acid stress response of LAB (Wu et al., 2014). In addition to this, they found that membrane permeability did not increase to the same extent when ATR was induced, which corresponds to their previous work in which acid-tolerant mutant *Lactobacillaceae* had lower membrane permeability than their WT counterparts under acid stress (Wu et al., 2014; Wu et al., 2012). They also identified proteins that were involved in carbohydrate metabolism, implicating enhanced glycolysis following acid exposure for increased ATP production and regulation of internal pH.

This work coincided with work done later by Huang et al. (2015), who analysed genes involved in the survival of *Lp. plantarum* in acidic conditions (and stress in general) and found that they could be grouped into 3 distinct categories: cell membrane fatty acid (CMFA) synthesis; cellular metabolism and energy production and Class I and II stress response pathways (Huang et al., 2015). Papers published on many *Lactobacillaceae* species with regard to acid survival genes appear to fit into these categories, for example, Wu et al. (2013) examined the *Lc. casei* Zhang strain and its Lbz-2 mutant acid-resistant counterpart (Wu et al., 2013a). This study deduced that intracellular levels of the amino acid aspartate can offer protection against acid stress, and focused on the genes *argG* and *argH*, both of whose expression increased with decreasing pH (Wu et al., 2013a). A study using *Lb. reuteri* 100-23, an autochthonous strain of the murine stomach, revealed that certain genes known to produce ammonia and amines were upregulated in this strain under acid stress conditions (Wilson et al., 2014). These genes were specifically involved in urea hydrolysis, glutamate decarboxylation and glutamine metabolism (Wilson et al., 2014). This could be an interesting approach to take when ascertaining important genes for potential probiotics, as those with genes shared by autochthonous gut *Lactobacillaceae* would indicate a higher capability of tolerating low pH, bile salts, or even the ability to colonise the gut wall.

Induction of the ATR on different *Lactobacillaceae* sp. has identified various mechanisms and proteins required for acid tolerance, which include the F_1F_0-ATPase, the glutamate decarboxylase enzyme for export of protons (H^+), intracellular pH

homeostasis (increased via urease or arginine deaminase), cell envelope adjustments, and cell density changes, as well as the generic responses that all bacteria have to stress, such as the upregulation of general shock proteins and chaperones or transcriptional proteins (Zhai et al., 2014). The sub-lethal pH used for inducing ATR range from 6.5 – 4.0, and these are then lowered to a lethal pH range of between 3.5 – 2.5 (Huang et al., 2015; Wu et al., 2014; Wu et al., 2011; Zhai et al., 2014). In the search for sources of *Lactobacillaceae* with probiotic potential, cheese is one promising environment as the pH values of many varieties falling neatly into the ATR sub-lethal range, between 6.0 – 4.6. It follows logically then that *Lactobacillaceae* consumed in a cheese matrix will have a better chance of surviving gastric pH due to prior upregulation of proteins involved in acid tolerance.

A group of researchers examined the transcription profiles of *Lc. paracasei* ATCC 334 when added either alone or in conjunction with the starter culture, *L. lactis* subsp. *cremoris* SK11, in a Cheddar cheese model (Desfossés-Foucault et al., 2014). This was done using γ-irradiation to inactivate all microbes present in the cheese, followed by production of cheese slurry containing the separate strains or both combined followed by a ripening period of 12 days (Desfossés-Foucault et al., 2014). In total, 42 genes were examined, with 34 common to both species and 8 unique to each and were grouped into functional categories (Desfossés-Foucault et al., 2014). It was noted that the genes of the *Lactobacillaceae* strain involved in stress response were overexpressed when the lactococcal strain was present (Desfossés-Foucault et al., 2014). This may be due to the lower pH of the cheese slurry in the presence of *Lactococcus* (which fell from pH 5.0 to pH 4.5) which did not occur in the slurry containing only the *Lactobacillaceae* species (Desfossés-Foucault et al., 2014). Additionally, genes involved in regulating stress of *Lactobacillaceae* (e.g., *clpC*, *dnaJ*, *groEL*, *groES*, and *htrA*) have all been identified as upregulated as a result of acid stress in other studies (Herbel et al., 2013; Lebeer et al., 2008; Wu et al., 2014; Zhai et al., 2014).

This literature supports the notion that cheese pH may induce ATR in *Lactobacillaceae*, an advantage to consider when choosing a food matrix for probiotic delivery. A gap exists in the literature concerning gene/protein expression of *Lactobacillaceae* sp. within cheese, the addressing of which could also unearth genes required for acid

tolerance. In fact, the same group mentioned earlier performed rRNA transcriptional activity tests on NSLAB in Cheddar, and postulated that their study gave a convenient approach for showing which species are capable of producing proteins during ripening (Desfossés-Foucault et al., 2013). This could also include the production of ATR-induced proteins to protect from acid stress. A paper in 2012 examined *Lc. rhamnosus* strains isolated from Parmigiano Reggiano, in an attempt to determine adaptation features of this species during cheese ripening (Bove et al., 2012). This was accomplished by growth of strains on either MRS media or cheese broth (CB, for cultivation under cheese-like conditions), followed by examination of proteins that showed decreased expression when grown on the latter (Bove et al., 2012). In contrast to the previous studies described above, this paper reported a decrease in protein expression of those involved in stress response, such as DnaK and GroEL (Bove et al., 2012). However, the initial pH of the CB was 7.5 and, after inoculation of the strains, an increase was seen in the pH following growth which may explain the downregulation of these acid tolerance-related proteins (Bove et al., 2012; Neviani et al., 2009). In this manner, it appears that the study did not fully emulate the conditions found in Parmigiano Reggiano, which has a ripened pH of 5.4 (Fox et al., 2004).

An alternative approach to identifying genes which confer acid resistance is to investigate the genomes of *Lactobacillaceae* autochthonous to the human gut. A paper in 2015 examined *Ls. reuteri* 100-23, and initially chose seven proteins that were predicted to confer acid tolerance, such as arginine deiminase (involved in the ADI Pathway mechanism) and others involved in two-component regulatory pathways (Krumbeck et al., 2015). This study made use of mutant *Ls. reuteri* strains, in which each strain had a single gene/protein of interest inactivated via insertional mutagenesis, which were then exposed to simulated gastric juice at pH 1.5 and 2 (Krumbeck et al., 2015). As expected, all mutant strains had an impaired acid tolerance compared to the WT 100-23 strain (Krumbeck et al., 2015). A study done in 2008 examined the gut-sourced *Lb. gasseri* ATCC 33323, however, unlike Krumbeck et al. (2015), this paper instead examined the whole annotated genome and grouped genes together based on a function that would aid survival in the GIT (Azcarate-Peril et al., 2008). This study found a number of chaperon proteins which are known to function during stress (such as low pH) by preventing cytoplasmic proteins from irreversible aggregation under these conditions (Azcarate-Peril et al., 2008). Similar studies were

done on autochthonous *Lactobacillaceae* from the murine forestomach involved in formation of biofilms, despite the highly acidic environment. A study by Schwab and co-workers in 2014 compared the gene expression profiles of *Lactobacillaceae* in the forestomach and hindgut of mice, as those expressed more abundantly in the former might reveal proteins involved in acid tolerance as well as biofilm formation (Schwab et al., 2014). One observation from this paper was that the forestomach populations were predominantly composed of *Lactobacillaceae* sp., while the hindgut *Lactobacillaceae* populations were significantly smaller (Schwab et al., 2014). This highlights the capacity of *Lactobacillaceae* to colonize niches often too extreme for other genera. In accordance with previous studies mentioned above, genes predominantly overexpressed (with regards acid tolerance) were those involved in cell envelope integrity and intracellular pH maintenance (Schwab et al., 2014). Additionally, genes necessary for the ADI pathway were highly expressed in the forestomach, an important mechanism for acid tolerance (Schwab et al., 2014). Lastly, they mentioned high expression of the *dlt* operon and the cyclopropane-fatty acyl-phospholipid synthase protein, which are required during exposure to low pH to alter and maintain the integrity of the cell envelope (Schwab et al., 2014). This information may aid future research by supplying a list of genes/proteins from autochthonous *Lactobacillaceae* against which potential probiotic *Lactobacillaceae* from external sources can be tested.

Acid tolerance is a necessary property when seeking *Lactobacillaceae* capable of conferring health benefits to human or animals, and a wide variety of methods exist that target phenotypic analysis for screening of potential candidates. These range from simple pH-adjusted media or saline solutions to full working *in vitro* models of the upper GIT. Work has already begun on elucidating the genes and proteins necessary for such a trait, whether through the targeting of acid-resistant mutants or the examination of those whose natural niche include low pH environments. Overall, a mixture of both will be required to determine the presence of useful gene adaptations, and low pH exposure to ensure that gene expression is high enough to confer a phenotypic benefit.

1.4.2 Bile Resistance and the Production of Bile Salt Hydrolase Enzymes

Bile is produced in the liver by pericentral hepatocytes, and in humans approximately 600 to 800 ml of bile is produced every day (Bahar & Stolz, 1999). In most vertebrates, including humans, bile is comprised of C_{24} bile salts which are synthesized through multi-enzymatic process from cholesterol (Hofmann, 1999). This involves oxidative cleavage of a cholesterol side chain, and the addition of between one and two hydroxyl groups to the core of the molecule (Hofmann, 1999). The actual composition of bile varies between individuals, as a result of different nutrition, with its main components consisting of the bile acids/salts (cholic and chenodeoxycholic acids in humans), cholesterol, phospholipids, the pigment biliverdin and inorganic salts (Patel et al., 2010; Farina et al., 2009). Usually, bile salt concentration in bile is 0.8% (Patel et al., 2010). Concentrated bile is released from the gall bladder (where it is stored) through the common bile duct into the duodenum following cholecystokinin stimulation after ingestion of food (Farina et al., 2009). Bile salts play a major role in fat emulsification and solubilisation, aided by their amphipathic and detergent-like attributes, as well as being essential co-factors for pancreatic lipases (Farina et al., 2009). Their interactions with lipids results in formation of soluble mixed micelles, allowing the fats to be degraded by enzymes and absorbed into the intestinal epithelial cells (Farina et al., 2009). Bile also plays a role in excretion of both exogenous and endogenous compounds including cholesterol (Farina et al., 2009).

During digestion, bile salts are deconjugated which enables enterohepatic recirculation via the hepatocytes, ensuring that the bile salt molecules are utilized multiple times over the course of a single digestion event (Patel et al., 2010). This involves absorption of the deconjugated bile salts along the entire gut and in the terminal ileum via passive diffusion and active transport, respectively (Begley et al., 2006). Once transported to the liver via the blood stream, deconjugated bile salts are taken up by hepatocytes where they are re-conjugated, and re-secreted into bile (Begley et al., 2006). Deconjugation can also be catalysed by bacterial BSH, and *Lactobacillaceae* that encode BSH have an obvious advantage during transit through the duodenum. Additionally, approximately 5% of the total bile acid pool is not recirculated but instead passes on through the intestines, where the indigenous bacteria may utilise it (Begley et al., 2006). Therefore, bile tolerance is an important adaptation for *Lactobacillaceae* selected for probiotic applications. Through mutant analysis, various *Lactobacillaceae* genes involved with bile adaptations have been identified (Lebeer et al., 2008).

Tolerance to bile requires protein systems similar to those involved in acid tolerance, and include general stress response proteins, efflux pumps, the ability to restructure the cell surface and activation of alternate carbohydrate metabolism pathways in addition to the ability to produce BSH enzymes (Bustos et al., 2018).

BSH hydrolyses the amide bond between the amino acid side chain and steroid moiety of bile acids, resulting in deconjugation and neutralization of the acid's adverse effects (Lebeer et al., 2008). BSH belong to the cholylglycine hydrolase family (Enzyme 3.5.1.24 on the Kyoto Encyclopaedia of Genes and Genomes database) and have previously been misannotated in some cases as penicillin-V acylases (which belong to the same family but hydrolyse penicillin-V) due to high sequence similarity (Kumar et al., 2006; O'Flaherty et al., 2018). Comparative analysis revealed that, among *Lactobacillus*, the presence of BSH genes appears to be niche specific and associated with vertebrate-adapted habitats such as the gut (O'Flaherty et al., 2018; O'Sullivan et al., 2009). Evidence reported suggests that HGT plays a role in BSH gene acquisition, with some data indicating *Lactobacillaceae* may have acquired BSH genes from *L. monocytogenes* (Begley et al., 2006; McAuliffe et al., 2005; van Reenen & Dicks, 2011). The range of intestinal bacteria with the ability to deconjugate bile is broad and includes both Gram negative and positive bacteria (Ridlon et al., 2006). In addition to the existence of different isoforms, BSH also differ in substrate specificity, catalytic efficiency and can be transcribed as monocistronic mRNA or can be arranged as an operon comprising functionally related proteins (Chand et al., 2017). A study by Lambert and colleagues identified that growth phase may be important for BSH activity as a *Lp. plantarum* strain expressed high BSH activity during exponential growth which declined during stationary phase growth (Lambert et al., 2008). Conversely, a separate study determined that bile tolerance was higher during stationary growth phase than logarithmic phase in *Lactobacillus*, although BSH itself was not investigated here, thus the potential role of growth phase on BSH remains unclear (Haller et al., 2001).

Conjugated bile acids (CBA) affect bacteria in a similar manner to organic acids, whereby exposure leads to a decrease in intracellular pH and inhibition of nutrient transport through proton motive force collapse (Bustos et al., 2012). Research by Bustos and colleagues (2012) observed that when various species of *Lactobacillaceae* were exposed to two different CBA, BSH positive strains exhibited elevated internal pH

and higher cell viability, while the internal pH of BSH negative strains decreased and viability was low in comparison, indicating a clear relationship between the presence of BSH and bile tolerance (Bustos et al., 2012). Conversely, McAuliffe and colleagues observed that inactivation of two different BSH alleles in a *Lb. acidophilus* strain did not affect their bile tolerance when grown in the presence of the conjugated acids, with the exception of taurochenodeoxycholic acid (McAuliffe et al., 2005). Additionally, other groups have concluded that bile resistance of *Lactobacillaceae* is not determined by or is not related to BSH expression (Fang et al., 2009; Moser & Savage, 2001; Panicker et al., 2018). Thus, more work in understanding the links between BSH and bile tolerance is required for a more reliable hypothesis concerning this matter.

Evidence has recently emerged that suggests that the expression of BSH can have an additional advantage for the expressing bacteria. A study by Yang and colleagues examined the ability of *Lp. plantarum* expressing BSH to adhere to an *in vitro* epithelial model (Yang et al., 2019). Two different strains were modified with plasmids containing *bsh* genes, and both exhibited higher adhesion to Caco-2 cells than their WT counterpart, with the strain with the highest BSH activity exhibiting the highest adhesion (Yang et al., 2019).

BSH expression is also of interest due to the claim that it can result in lowering blood cholesterol levels. Many studies have investigated the ability of *Lactobacillaceae* BSH enzymes to assimilate cholesterol *in vitro* (Ghatani & Tamang, 2017; Kumar et al., 2012; Shehata et al., 2016). *In vivo* studies also exist that focus on the ability of these *Lactobacillaceae* to lower cholesterol in human and animal tirals (Kumar et al., 2012). A study in hypercholesterolemic mice fed a BSH positive *Lp. plantarum* strain resulted in a 7 and 10% lower serum cholesterol and triglycerides, respectively, than their control counterparts (Nguyen et al., 2007). A separate study in which rats suffering from hypercholesteremia were fed isolated BSH from an *Lentilactobacillus buchneri* strain exhibited a reduction in serum cholesterol and triglyceride levels by 50 and 15%, respectively, when administered at a 10 ID/kg and 58 and 45%, respectively, when administered at a 20 IU/kg (Sridevi et al., 2009). In a human study, significant reductions in total cholesterol, triacylglyceride and low-density lipoprotein levels were seen in normal to mildly hypercholesterolaemic adults, as well as a significant decrease in systolic blood pressure (Costabile et al., 2017).

Thus, the ability to tolerate bile and produce BSH are important factors for consideration when screening *Lactobacillaceae* for probiotics, as these traits will impact their survivability once ingested and may have further impacts on the human host which may be beneficial in certain cases.

1.4.3 Antibiotic Resistance

Antibiotic resistance (AR) of pathogenic microorganisms has been reported to cause between 23,000-25,000 deaths a year in both the USA and Europe and is becoming a growing concern worldwide (Colson et al., 2019). While AR poses an obvious threat when exhibited by a pathogen, benign microbes, such as those found in food, may possess similar adaptations. AR is classified as being either intrinsic or acquired (Mathur & Singh, 2005). Intrinsic resistance, as the name suggests, is an inherent ability of a microbe to resist an antimicrobial agent via natural tolerance mechanisms and is seen as less of a concern due to its inability of being transferred to other, potentially dangerous microbes (Mathur & Singh, 2005). Acquired resistance arises from either mutations or through HGT, a process in which mobile genetic elements containing AR genes from one bacterium enter and integrate into the genetic makeup of a different bacterium (van Hoek et al., 2011). Plasmids and transposons (via conjugation) are typical examples of mobile elements responsible for HGT, but AR genes can also be transferred to bacteria through viral vectors (via transduction) or even through the uptake of free DNA in the environment by competent bacteria (via transformation) (van Hoek et al., 2011). These genes can often be screened for using PCR and sophisticated bioinformatic pipelines have been developed for identification of all AR genes present in whole genome sequencing (WGS) data already available (Zankari et al., 2012).

Lactobacillaceae with AR are a concern, as it is possible for food and food grade bacteria to act as reservoirs for acquired resistance genes that may then be passed on to pathogens (Franz et al., 2005). This may occur through direct interactions with pathogens present in food, or indirectly through transmission of AR genes to gut bacteria which in turn transfer them to ingested pathogens (Aarestrup et al., 2008; Capita & Alonso-Calleja, 2013). For this reason, the European Food Safety Authority

(EFSA) issued a report regarding the addition of beneficial *Lactobacillaceae* strains to food products which sets out minimum inhibitory concentration values for nine antibiotics in order to limit the amount of AR *Lactobacillaceae* being added to the food chain (Aquilina et al., 2012). If an isolate's growth is inhibited at values equal to or below those set out by this document, the bacteria is termed as 'susceptible' to antimicrobial agents, is thus unlikely to add to acquired AR to bacterial populations and is deemed 'safe' for addition to food (Aquilina et al., 2012). However, if growth of *Lactobacillaceae* isolates is seen at the specified concentrations or higher, they are classified as 'resistant' and can only be added to food products if the exhibited AR can be proven as intrinsic (Aquilina et al., 2012).

AR patterns of different *Lactobacillaceae* species have been investigated, using both genotypic and phenotypic methods, by several different groups and lists of AR genes have been compiled for this genus. Various *Lactobacillaceae* appear intrinsically resistant to vancomycin due to a mutation in the *vanX* gene which prevents binding of the antimicrobial to the cell wall, negating its effects (Gueimonde et al., 2013). Thus, vancomycin sensitivity is not an EFSA requirement for the majority of *Lactobacillaceae* species (Aquilina et al., 2012). Hummel and colleagues identified that discrepancies existed between expressed resistance phenotypes and AR genes found following genome sequencing (Hummel et al., 2007). They examined starter and probiotic strains, including 13 *Lactobacillaceae*, and found that some strains had the genes necessary for antibiotic resistance but did not express them, while others exhibited a resistant phenotype, but PCR could not detect the presence of the corresponding resistance gene (Hummel et al., 2007). Thus, determining true AR can be difficult when only using the EFSA guidelines as some bacteria may have the capacity for AR but not express the phenotype, while others may be capable of tolerating antibiotics through mechanisms and genes not previously tied to AR. These results corresponded to those of a later study by Guo and colleagues whereby >70% of the strains examined were resistant to gentamicin and streptomycin (aminoglycosides) whereas the majority of strains were susceptible to ampicillin, chloramphenicol, penicillin and tetracycline (Guo et al., 2017). This study, as with the last, could not detect all the genes involved with PCR despite a clear resistance phenotype being expressed (Guo et al., 2017). Both papers also investigated whether specific resistance genes were transferable to other bacteria through filter mating experiments, with both observing no gene transfer (Guo

et al., 2017; Hummel et al., 2007). Unfortunately, potentially acquired AR genes have been identified in other studies, such as the acquired resistance to tetracycline and erythromycin observed in *Lactobacillus* isolates from fermented Indian and Italian foods (Comunian et al., 2010; Thumu & Halami, 2012). While *Lactobacillaceae* isolates usually express a susceptibility phenotype to these antibiotics, both studies detected the presence of certain transferred genes in resistant phenotypes, including the *tetM* gene for tetracycline resistance and *ermB* for erythromycin resistance (Comunian et al., 2010; Thumu & Halami, 2012).

While most research groups limit their AR research to probiotic or food strains, Campedelli and colleagues examined 182 *Lactobacillaceae* type strains from a variety of sources and compared observed resistance phenotypes with annotated resistance genes identified in available genomes (Campedelli et al., 2019). Of the nine antibiotics of interest, the highest percentages of resistant type strains were seen in vancomycin (77%), kanamycin (61%), tetracycline (50%) and chloramphenicol (49%) (Campedelli et al., 2019). Strains showing resistance to aminoglycosides gentamicin and streptomycin were much fewer than those seen in other studies, but this could be due to this study encompassing a greater diversity of *Lactobacillaceae* species while previous studies have focused on limited probiotic/fermented food *Lactobacillaceae* (Campedelli et al., 2019; Gueimonde et al., 2013). Another interesting observation from this study was the identification of genes conferring acquired resistance, which included the previously identified *tetM* and *ermB* genes flanked by mobile genetic elements in two closely related *Lactobacillus* species, and the *ant6* gene (conferring streptomycin resistance) with a sequence 99% similar to the that of *Streptococcus suis* and *Clostridium difficile ant6* gene (Campedelli et al., 2019). This study also utilised VetMIC plates, which have been used by other research teams, and comprise two 96 well plates (classified as Lact-1 and Lact-2, for *Lactobacillaceae*) with eight antibiotics per plate at (Campedelli et al., 2019; Manzoor et al., 2016; Muñoz-Atienza et al., 2013). These plates require resuspension of overnight *Lactobacillaceae* cultures into broth consisting of part Iso Sensitest Broth and part MRS in a ratio of 9:1, followed by distribution into the VetMIC plates. As with most antibiotic sensitivity tests, VetMIC plates are minimum inhibitory concentration tests, and contain wells with the EFSA-recommended cut-off concentrations for all nine antibiotics for ease of use. While VetMIC plates will not disclose the nature of the resistance, if any is present, it is still a

useful tool to ensure *Lactobacillaceae* isolates are chosen that do not express resistant phenotypes and that are compliant with EFSA regulations.

1.4.4 Adherence to Mucus and/or Human Epithelial Cells and Cell Lines

The ability for probiotic bacteria to persist in the GIT is important in terms of conferring medium- to long-term health benefits on the host and its intestinal microbiome. Therefore, probiotics that are capable of adherence to the epithelium of the digestive tract have the advantage of a longer persistence in the gut, and thus a greater period of time over which they can deliver potential benefits. The ability to colonise specific regions of the gut, however, depends also on how well adapted the probiotic bacteria is to that area, with distinct microbial habitats being acknowledged throughout the lower GIT (Donaldson et al., 2015). For example, the small intestine is generally considered as a more hostile environment than the colon with a lower pH, higher oxygen and antimicrobial peptide concentrations, as well as the presence of bile salts (Donaldson et al., 2015). This results in the small intestines containing a reduced number and variety of microbes, when compared with the highly dense and diverse microbial ecosystems of the colon and caecum (Donaldson et al., 2015). In addition, the transit time of the small intestines is much shorter than that of the colon and caecum, making adherence in the small intestines important (Donaldson et al., 2015; Tallon et al., 2007). *In vitro* tests that measure this ability are important, and have been acknowledged by the WHO as a trait to be evaluated when assessing bacteria for probiotic potential (Araya et al., 2002).

The epithelial layer in the intestines is composed of goblet cells and enterocytes interconnected via tight junctions (Hansson, 2012). While the enterocytes are largely focused on uptake and processing of nutrients and antigens, goblet cells produce mucins (glycoproteins) that coat the epithelial layer in a material called mucus (Hansson, 2012; Snoeck et al., 2005). Mucus provides the initial protection against invading pathogens (Hansson, 2012). Different parts of the digestive system house varying layers of mucus, while the mucins themselves differ between digestive organs (Hansson, 2012). For example, the mucus in the small intestines consists of a single layer, that is not anchored to the epithelium, which coats the villi, while the colon

exhibits two layers of mucus, with only the inner layer in contact with the epithelium and the outer layer extending into the lumen (Hansson, 2012).

Testing for adherence of *Lactobacillaceae* isolates normally involve Caco-2 and/or HT-29 cell lines, continuous cell lines originally derived from separate human epithelial colorectal adenocarcinoma cells that were both created by Jorgen Fogh (Rousset, 1986). These two cell lines share many characteristics. They are both heterogeneous lines that, upon reaching confluence, differentiate to form polarized monolayers with apical brush borders with microvilli (Lea, 2015; Martínez-Maqueda et al., 2015). Adjacent cells are connected via tight junctions, and both cell lines resemble enterocytes of small intestinal origin (Lea, 2015; Martínez-Maqueda et al., 2015). Both also express proteins characteristic of enterocytes, such as sucrase-isomaltase, dipeptidylpeptidase IV and aminopeptidase N, although Caco-2 cells also produce lactase, mimicking more strongly the human enterocytes that they are modelling (Lea, 2015; Martínez-Maqueda et al., 2015).

In addition to the presence of lactase, other differences of note include the method by which differentiation of each is induced. While Caco-2 cells will begin to differentiate as soon as they reach confluence, growth on permeable filter supports improves their resemblance to human enterocytes, both from a functional and morphological perspective (Lea, 2015). On the other hand, differentiation of HT-29 cells depends on the presence of certain sugars, with glucose-rich media yielding multi-layered, undifferentiated, non-polarised cells and glucose-free (usually substituted by galactose) media leading to differentiation upon confluence into polarized, enterocyte-resembling monolayers (Huet et al., 1987). Caco-2 cells take up to 21 days to fully differentiate, while up to 30 days are required for full differentiation of HT-29 (Lea, 2015; Martínez-Maqueda et al., 2015). In addition to the absence of lactase, HT-29 cells also exhibit lower enzyme activity than their Caco-2 counterparts, and sucrase-isomaltase is only produced in 30-40% of all HT-29 cells (Lea, 2015; Martínez-Maqueda et al., 2015). Polarized Caco-2 monolayers also have a transepithelial electrical resistance (TEER) that is four times higher than polarized HT-29 cells, indicating that the permeability and integrity of the Caco-2 monolayer is greater than that of the HT-29 monolayer (Chen et al., 2015; Lea, 2015).

Differences exist between these model systems and the organ on which they are based. The first and most obvious is that the small intestine is composed of many different types of cells, while the models differentiate into only enterocytes. In addition to this, both Caco-2 and HT-29 cells were originally taken from malignant colon cells, and both retain characteristics of their source. Caco-2 cells produce certain biomarkers of colonocytes, while the concentration of the villin protein (essential for microvilli) expressed in HT-29 monolayers is closer to that seen in human colonocytes than small bowel enterocytes (Lea, 2015; Martínez-Maqueda et al., 2015). Despite differing protein expression patterns, the similarities to enterocytes are significant, and both cell lines have been successfully used in adhesion studies concerning probiotics and pathogens, including identification of potential probiotic *Lactobacillaceae* strains. Caco-2 and HT-29 cells are routinely grown in either Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640, with 10% Foetal Bovine Serum (FBS) and an antibiotic solution to avoid contamination (usually containing penicillin and streptomycin) (Duary et al., 2011; Pisano et al., 2014; Song et al., 2015; Turpin et al., 2012).

During an adhesion assay, it is most common to use a fixed number of cells to seed the wells used for the assay, with this number being dependant on the size of the wells and the number of days to reach confluency, and many papers opt for a 6-well plate with $1*10^5$ cells per plate (Duary et al., 2011; Pisano et al., 2014). Just prior to the adhesion assay, media lacking antibiotics replaces the old media, but this differs between authors with the reasons being unclear. For example, Pisano et al. (2014) initiates this step and then adds the *Lactobacillaceae* in PBS one hour afterwards, while Duary et al. (2011) changed to the antibiotic-free media a full 24 hours before the assay (Duary et al., 2011; Pisano et al., 2014). Some groups prefer to suspend the *Lactobacillaceae* in the antibiotic-free media rather than PBS, but the time added following suspension differ, as with Turpin et al. (2012) adding their bacterial suspension following a 24-hour incubation period, while Song et al. (2015) do not specify (Song et al., 2015; Turpin et al., 2012). While the number of *Lactobacillaceae* added range from 10^5-10^9 colony forming units (cfu) per millilitre, the amount of time they are exposed to the eukaryotic monolayers also differs between groups, with some preferring 30 minutes, others 1-3 hours and some even opting for a longer 24-hour period (Duary et al., 2011; Lavilla-Lerma et al., 2013; Maragkoudakis et al., 2006; Pisano et al., 2014; Song et al., 2015; Turpin et al., 2012). Unfortunately, this raises the

question of whether data between groups is comparable, when so many conditions can differ between adhesion assays. In addition, many papers do not disclose the passage numbers of the Caco-2 or HT-29 cells. An exception to this was the paper by Duary et al., who indicated that the Caco-2 used in their experiments were between 40-70 passages while the HT-29 cells were between 60-90 passages (Duary et al., 2011). Turpin and colleagues also state that the HT-29 cells used were between passages 58 and 63 (Turpin et al., 2012). It has been shown that Caco-2 cells have the ability to grow in multiple layers as their passage number increases, although experiments were carried out on HT-29 which showed genetic stability after 100 passages (Lea, 2015; Martínez-Maqueda et al., 2015). It is recommended that experiments should be carried out in earlier passages to avoid any genetic changes, and the passages should not vary greatly between experiments to ensure comparability.

The results of these tests are usually enumerated through either microscopic or plating methods, or both. In both cases, the monolayers are washed several times to ensure any non-adhesive *Lactobacillaceae* cells are washed off. The microscopic approach entails fixing the eukaryotic and adhered prokaryotic cells to the well bottoms and staining with Giemsa stain. This is followed by measuring the quantity of *Lactobacillaceae* adhering to the enterocytic cells, and may include categorisation of the bacteria depending on their binding status i.e. non-adhesive, adhesive, strongly adhesive, etc. (Duary et al., 2011). A set number of microscopic fields are used, but this is arbitrary and differs between groups (Duary et al., 2011; Tallon et al., 2007). Additionally, the categorization of *Lactobacillaceae* depending on their "level" of adherence will inevitably differ between individuals, therefore it can be considered subjective. The plating method involves selected lysing of the mammalian cells leading to detachment of adhered bacteria, followed by a simple serial dilution and spread plating on a suitable bacterial media (MRS is commonly used), and calculation of cfu/ml following a suitable incubation period (Maragkoudakis et al., 2006). A paper published in 2017 documented a novel method of measuring adhesion of probiotics to HT-29 using dual-colour flow cytometry (FCM) (Mukherjee & Ramesh, 2017). This method involves labelling the *Lactobacillaceae* cells with a dye (in this case, cFDA-SE), performing an adhesion assay as per normal with washes to remove non-adherent bacteria, lysing the eukaryotic cells and then subjecting the remaining *Lactobacillaceae*

to FCM via flow cytometry (Mukherjee & Ramesh, 2017). This method is advantageous over the microscopic staining method due to all adherent bacteria being counted, rather than a few microscopic fields which may not be representative of overall adhesion status, as well as eliminating the subjectivity of assigning adhesion categories to the bacteria.

Although both Caco2 and HT-29 cell lines are popular choices for *in vitro* adherence studies involving potential probiotics, many studies have also investigated the binding capabilities of *Lactobacillaceae* to mucus or its primary component, mucin (Gagnon et al., 2013). While the role of the mucus is to inhibit pathogenic bacteria from attaching to epithelial cell walls as part of the invasion process, it is also possible for the mucus to anchor bacteria to a particular location in the gastrointestinal tract. Therefore, it is important to consider any role the mucus might play in aiding prolonged exposure of beneficial bacteria within the GIT. In addition, binding to mucus may also provide a means for potential probiotic microbes to interact with host cells and thus elicit an immune response.

Mucins are comprised of a protein component (referred to as MUC proteins) produced by the goblet cells, which is modified by affixing oligosaccharide chains via membrane-bound transferases (Nishiyama et al., 2016; Van Tassell & Miller, 2011). These oligosaccharides also serve as food sources for commensal bacteria (Donaldson et al., 2015). Certain mucins can be found bound to the membranes of the goblet cells (e.g. MUC1, MUC3, and MUC4), while others are secreted into the mucus matrix (e.g. MUC2, MUC5B, and MUC5AC) (Gouyer et al., 2001). Although the adhesion of *Lactobacillaceae* varies between strains, it is thought to involve surface proteins of the bacteria interacting with the various oligosaccharide chains of mucin. Certain strains of *Lactobacillaceae* produce proteins with Mub domains, which are repeated functional domains that facilitate binding to mucin via its oligosaccharide chains (Devi & Halami, 2017; Van Tassell & Miller, 2011). These domains were named after the MUB protein identified in *Lb. reuteri* 1063, which enabled adhesion to both pig and hen mucus and contained two repeat regions (Mub1 and Mub2), each capable of binding to the same components of the mucus (Roos & Jonsson, 2002). Mub domains are members of the MUCin-Binding Protein (MucBP) family (Van Tassell & Miller, 2011). Proteins with these domains usually contain a C-terminal sortase recognition motif (LPXTG) (sortases

cleave and modify cell surface proteins) for anchorage to cell walls, domains of repeat sequences for adhesion and an N-terminal with an YSIRK motif to signal secretion (Lebeer et al., 2008; Nishiyama et al., 2016).

Interestingly, LAB solely appear to harbour these MucBP domains, with a few exceptions, and they are particularly associated with intestinal *Lactobacillaceae* (Van Tassell & Miller, 2011). Other proteins thought to have a role in adhesion of *Lactobacillaceae* to mucus include surface layer proteins (Slp), mucus adhesion promoting protein (MapA), GroEL and anchorless multifunctional protein elongation factor Tu (EF-Tu) (Nishiyama et al., 2016; Ramiah et al., 2009). Surface layer proteins form non-covalent bonds with cell wall polymers and usually coat the entirety of the surface in an ordered manner, and are themselves usually small (40-60 kDa) (Lebeer et al., 2008). The probiotic *Lb. acidophilus* NCFM was used to show how disruption of the *slpA* gene resulted in decreased adhesion, however this may also be due to the disruption of other surface proteins (Buck et al., 2005). Unfortunately, most work on S-layer proteins is done on enterocyte-like mammalian cells (i.e. HT-29, Caco-2) without mucus, or on the mammalian extracellular matrix (X. Chen et al., 2007). The MapA protein (26 kDa in size) has been shown to promote adhesion in *Ls. reuteri* 104R through both mucus and the epithelial cell line, Caco-2 (Miyoshi et al., 2006; Rojas et al., 2002). It is a component of an ABC transporter system, and proteins of a similar size have been found in other *Lactobacillaceae* species (for example, the 32-kDa mucus- and mucin-binding protein [32-Mmubp] expressed in *Ls. fermentum* BCS87) which share these functions (Nishiyama et al., 2016).

A paper in 2006 demonstrated a role for the *Lb. johnsonii* La1 (NCC 533) chaperone protein, GroEL, in binding to mucus and epithelial cells, the efficiency of which was shown to be pH dependant (with high rates of adherence at pH 5) (Bergonzelli et al., 2006). The same group also showed that the binding of EF-Tu to mucins and Caco-2 and HT-29 cells was pH dependant and implicated its importance in mucin binding in the *Lb. johnsonii* La1 (NCC 533) strain (Granato et al., 2004). It is important to note that MapA, GroEL and EF-Tu are all considered moonlighting proteins, as adhesion is not their primary function (Nishiyama et al., 2016). While MapA is usually expressed on the surface of bacteria due to their functions in ABC transporter systems, GroEL and EF-Tu are unusual in that they are actually cytoplasmic proteins with roles in mediating

proficient protein folding and synthesis, respectively (Bergonzelli et al., 2006; Granato et al., 2004; Nishiyama et al., 2016). Despite the absence of a secretion signal sequence, both GroEL and EF-Tu have been shown to be present at the cell surface of the *Lb. johnsonii* La1 (NCC 533) strain and have been shown to play a role in mucin-binding, with higher affinity at pH 5 which may accurately represent physiological conditions due to the lumen being more acidic where mucus is present. This drop in pH at the normally neutral lumen is believed to occur because of the mucin oligosaccharide side chains being predominantly acidic, stemming from sialic acid residues and sulphate acid groups (Granato et al., 2004; Van Tassell & Miller, 2011). Therefore, many proteins are expressed by *Lactobacillaceae* that have been shown to bind to the mucus constituent produced by mammalian intestinal cells.

Due to the importance of the mucus layer, a useful *in vitro* model is the use of the HT29-MTX cell line. This line was established in 1990, whereby a group discovered that exposure to increasingly high concentrations of the anticancer drug, methotrexate (MTX), resulted in a differentiated HT-29 phenotype which produced mucus (Lesuffleur et al., 1990). This difference in mucus production (whereby only 0.2% of parental HT-29 will differentiate into goblet cells upon reaching confluence, compared to 100% of HT29-MTX cells) results in mucin expression between the cell lines differing (Lesuffleur et al., 1990, 1993). For example, the mucin MUC1 is expressed in only a few HT-29 cells, while it is expressed universally in MTX cells 14 days after confluency (Lesuffleur et al., 1993). In addition, the expression of MUC2, MUC3 and MUC5 mRNA is high in MTX cells in comparison to its parental line, with the exception being MUC4, which is expressed at low levels in both lines (Lesuffleur et al., 1993). Another study confirmed expression of these genes, although not all results are in agreement with the previous study reported by Lesuffleur et al. in 1993 (Gouyer et al., 2001). Gouyer and colleagues were able to specify exactly when expression levels of mucin mRNA were initiated prior to confluence, and were able to additionally detect MUC11 mRNA in the MTX cell line, which was a novel finding, although levels of MUC11 transcripts were very low (Gouyer et al., 2001). Certain mucins are also absent in MTX cells, with MUC6, MUC7 or MUC12 expression not being detected (Gouyer et al., 2001). The HT29-MTX cell line and its production of these mucins have been exploited by various groups for adhesion assays as an *in vitro* model that is considered to be a better mimic of intestinal conditions than its parental cell line. In addition, MTX cells can also be co-cultured with

Caco-2 cells, as the inclusion of both goblet and enterocytic cells provides a more physiologically accurate model with a monolayer of intestinal cells and an overlay of mucus (Kleiveland, 2015). The ratios used when seeding the co-culture fall between (inclusive) 90:10 and 75:25 (Caco-2/HT29-MTX) as the most accurate ratios for their enterocyte and goblet cell counterparts in the gastrointestinal tract, and cell adhesion assays (Kleiveland, 2015; Laparra & Sanz, 2009).

1.4.5 Ability to Inhibit Pathogenic Bacterial Adhesion

The WHO has listed the ability of probiotics to inhibit or reduce the binding of pathogenic bacteria to the gut epithelium as a trait of interest when considering the potential of a bacterium to be used as a probiotic (Araya et al., 2002). This has led to the development of a number of *in vitro* assays that, in general, are modified versions of adherence assays (described in the previous section) with the inclusion of an enteropathogen. Common enteropathogens used include but are not limited to virulent strains of *Salmonella typhimurium*, *E. coli*, *Listeria monocytogenes*, and *Campylobacter jejuni* (Wan et al., 2019).

Several studies examined the role S-layer proteins from different *Lactobacillus* species in the inhibition of pathogen binding (Chen et al., 2007; Johnson-Henry et al., 2007; Li et al., 2012). Chen and colleagues (2007) extracted S-layer proteins from a *Lb. crispatus* strain isolated from pig intestines and observed loss of adhesion for both *E. coli* and *S. typhimurium* onto HeLa cell monolayers (Chen et al., 2007). Another study published in the same year observed the ability of S-layer proteins from *Lb. helveticus* to inhibit binding of the same *E. coli* strain to two human epithelial cell lines (HEp-2 and T84), while a 2012 study found that adhesion of *S. typhimurium* to Caco-2 cells was significantly lower in the presence of S-layer proteins isolated from *Lb. acidophilus* (Johnson-Henry et al., 2007; Li et al., 2012). Zhang et al. (2013) took a different approach whereby, rather than use only the proteins, five *Lactobacillaceae* strains (a single *Ligilactobacillus salivarius*, and four *Ls. reuteri*) were tested for pathogen adherence inhibition against an *E. coli* and S*. enteritidis* both with and without an S-layer (which can be accomplished using a 5 M LiCl solution on cells in suspension) (Zhang et al., 2013). In all cases, when the S-layer was absent, the ability of the *Lactobacillaceae* to inhibit pathogen binding to Caco-2 monolayers decreased

significantly, indicating an important role for the S-layer in this probiotic trait (Zhang et al., 2013). Zhang et al. (2013) and Li et al. (2012) used three distinct types of adhesion assays: Competition, Displacement and Exclusion (Li et al., 2012; Zhang et al., 2013).

Studies in 2016 and 2017 by the same group investigated the effects of probiotic strains of *Ls. reuteri* (previously isolated from human infant faeces) and their S-layers on adhesion capabilities of four pathogens to Caco-2 cells through the use of Competition, Displacement and Exclusion assays (Singh et al., 2016, 2017). In Competition Assays both the probiotic and the pathogen are added to the mammalian cells/adhesive scaffold at the same time, thus they are forced to compete for potential binding sites (Singh et al., 2016). Displacement assays add the pathogen first and allow time for binding followed by the addition of the probiotic, while Exclusion assays apply the bacteria in the opposite order (Singh et al., 2016). Both studies confirmed a significant decrease in inhibition activities for all *Lactobacillaceae* for all three types of assay when S-layers were absent (Singh et al., 2016, 2017).

While the above studies highlight the potential of *Lactobacillaceae* S-layer proteins to inhibit pathogen binding in cell culture, the mucus layer of the gut epithelium was absent from the models used. In some studies, the ability of *Lactobacillaceae* to inhibit adherence of pathogens to mucus without mammalian cells was examined, with various levels of success (Collado et al., 2007; Li et al., 2008; Van den Abbeele et al., 2016). Due to the important role of mucus in the GIT (as described above), assays that use mucus-producing epithelium cells should provide a more accurate model of *in vivo* GIT conditions. Therefore, the use of the Caco-2/HT29-MTX co-culture model and pathogen adhesion inhibition used in the current study should serve as a novel and more appropriate model to monitor the potential of a bacterial strain to inhibit adhesion by a pathogen.

1.4.6 Bacterial Polysaccharide Production

While the ability of bacteria to produce polysaccharides is not listed by the WHO as a main *in vitro* test for probiotics, these high-molecular weight polymers have various applications from both a commercial and health perspective (De Vuyst & Degeest, 1999; Food and Agriculture Organization of the United Nations, 2006; Zhou et al.,

2019). Bacterial polysaccharides can take several forms, including capsular polysaccharides (CPS) and exopolysaccharides (EPS), the former of which is tethered to the cell while the latter is secreted into the external environment (Boels et al., 2001). Both CPS and EPS affect how microbes interact with environmental parameters, such as providing protection against stresses (e.g. desiccation, osmolarity, metal ions) as well as sustaining structural integrity and cell shape (Zeidan et al., 2017). EPS can further be divided into homopolysaccharides (HoPSs), such as dextran, and heteropolysaccharides (HePSs), which are more complex structures and are often exploited commercially as they provide dairy products with texture that appeals to consumers (De Vuyst & Degeest, 1999; Galle et al., 2011).

The type of EPS produced will depend on the biosynthesis pathway employed, of which four main pathways have been identified (Zhou et al., 2019). In the case of *Lactobacillaceae*, both HoPS and HePS can be biosynthesised, with HoPSs being exclusively produced through the extracellular synthesis pathway and HePSs often being produced through more complex pathways involving numerous enzymes, such as the Wzx/Wzy-dependant pathway (Deo et al., 2019). In *Lactobacillaceae*, extracellular glycosyltransferases are generally responsible for production of HoPSs in the extracellular matrix (Zeidan et al., 2017). For the production of HePSs, many proteins are necessary, and their genes are usually found as a cluster (organised as an operon) on either a chromosome or plasmid (Zeidan et al., 2017). Genes necessary for EPS biosynthesis identified in different species were found to vary in a study published by Deo et al. (2019), which indicated that some elements of EPS gene composition were correlated with habitat (Deo et al., 2019). While some host/environment adapted *Lactobacillaceae* members primarily encode genes associated with a single habitat (e.g. dairy, gut), nomadic *Lactobacillaceae*, which grow in a number of different environments, harbour genes which support survival in a variety of different habitats (Martino et al., 2016). Overall, this research showed that EPS protein families were least similar between nomadic and host-adapted *Lactobacillaceae* species, but that free-living (bacteria that don't rely on a eukaryotic host and live 'free' on plants/in the environment) *Lactobacillaceae* shared protein families from both of these groups (Deo et al., 2019; Duar et al., 2017).

Potential probiotics may benefit from the production of EPS, as different studies have revealed that EPS production may afford protection against stresses associated with the GIT. However, these benefits are largely strain dependant. The production of EPS by *Bifidobacterium,* a genus often associated with probiotic potential, provides protection against bile (Ruiz et al., 2013). Indeed, exposure to bile induced EPS production by *B. animalis* IPLA4549 (Ruiz et al., 2013). In terms of *Lactobacillus*, conflicting information exists. A study examined the separate effects of low pH (3.0, 2.5 and 2.0) and bile salts (0.15% and 0.3%) on four different EPS-producing *Lb. delbruckii* subsp*. bulgaricus* strains (Boke et al., 2010). EPS production by each strain was quantified, with two of the strains producing ≥175 mg/L EPS, while the others produced <30 mg/L (Boke et al., 2010). For both acid tolerance and bile salt stress, higher EPS production resulted in higher survival levels (with the exception of exposure to pH 3.0) (Boke et al., 2010).

A separate study transformed a *Lc. paracasei* strain to express a glycosyltransferase which, in turn, biosynthesised the HoPS, β glucan (Stack et al., 2010). The recombinant bacteria were then subjected to acid (pH 2.0), bile (0.7%) and simulated gastric juice (containing salts, bile, lysozyme and pepsin) stress and, in all cases, showed a significantly higher survival rate when compared with their non-EPS-producing counterparts (Stack et al., 2010). Similar to Boke et al. (2010), the EPS-producing strains showed higher viability in the low acid conditions than in the presence of bile, although the conditions used between the two studies were not identical (Boke et al., 2010; Stack et al., 2010). Conversely, a study examined protein expression of the well-characterized *Lc. rhamnosus* GG probiotic and determined that EPS production was downregulated during bile stress (Koskenniemi et al., 2011). As Boke et al. (2010) and Stack et al. (2010) did not examine protein expression, the EPS biosynthesis pathways are not known in either and, therefore, more research is required with regards the mechanisms connecting EPS production and bile tolerance.

EPS produced by *Lactobacillaceae* strains have also shown various degrees of success in terms of providing health benefits towards a host once ingested (Korcz et al., 2018). EPS of *Lp. plantarum* WLPL04, a strain originally isolated from human breast milk, was purified and incubated with a pathogenic *E. coli* strain on HT-29 epithelial cells (Liu et al., 2017). Regardless of whether the purified EPS was added with, prior to, or after the

addition of the pathogen, *E. coli* adhesion was significantly reduced by the presence of EPS (Liu et al., 2017). Additionally, the same EPS was found to inhibit biofilm formation of several enteropathogenic bacteria (Liu et al., 2017). This could potentially give this strain a competitive advantage during gut colonisation, as well as confer a health benefit to the host. Again, this appears to be strain dependant as another study indicated that purified EPS of *Lc. rhamnosus* GG increased adhesion of three enteric pathogens to human mucus samples (Ruas-Madiedo et al., 2006).

The ability of EPS to lower serum cholesterol levels has been investigated by several research groups, with some indicating that EPS-producing *Lactobacillaceae* possess this ability. A study in 2010 found a significant correlation between EPS production and cholesterol removal, whereby strains which produced high levels of EPS removed a greater amount of cholesterol from their growth medium and the greater the amount of cholesterol produced (Tok & Aslim, 2010). A separate *in vivo* study found that EPS-producing *Lactobacillaceae* decreased total cholesterol and triglyceride serum levels in $Apoe^{tm1Unc}$/J mice (which show elevated plasma cholesterol) when compared with a *Lactobacillaceae* strains incapable of EPS production (London et al., 2014). An earlier study by a separate group investigated EPS-producing *Lb. kefiranofaciens* in SHRSP/Hos rats fed a high fat diet and discovered that serum and liver lipid levels were lower than that of the controls (Maeda et al., 2004). Therefore, evidence exists which implicates certain EPSs produced by *Lactobacillaceae* in providing health benefits once ingested.

EPS (and CPS, in some cases) production can be tested for *in vitro* by investigating the expression of a 'mucoid', 'ropy' (in which the colony can be stretched a few mm before snapping/breaking) or 'slimy' phenotype in certain media (Zeidan et al., 2017). This can cause some confusion as bacteria that do not produce 'ropy' phenotypes can still be EPS/CPS producers with a 'mucoid'/'slimy' phenotype (Cirrincione et al., 2018). The dye ruthenium red has the capacity to stain the bacterial cell wall and has been used to investigate EPS or CPS production (Stingele et al., 1996). In the presence of ruthenium red, colonies will grow as either red/pink or white, with red/pink growth indicating that they are 'non-ropy' as the dye stains the cell wall and white colonies indicating that bacteria are EPS/CPS-positive as the polysaccharides produced prevent the dye from coming into contact with the cell wall (Stingele et al., 1996). Therefore, this simple test can be used to quickly determine EPS/CPS production, which can be further

investigated for associated health benefits or conferring of stress resistance once ingested.

1.5 Project Aims

The literature above has identified various members of the *Lactobacillaceae* family capable of exhibiting probiotic traits. Likewise, *Lactobacillaceae* are commonly found in cheese and other fermented foods as NSLAB. This project will focus on identifying one or several NSLAB from commercially available Cheddar cheeses with the aforementioned probiotic traits. These strains of interest will then be used as adjunct cultures for production of Cheddar, with survival of the *Lactobacillaceae* being tracked throughout ripening to ensure sufficiently high survival for potential use as a functional food in an *in vivo* feeding trail. This final step will determine whether Cheddar prepared with such strains can affect gut microbiome composition, and whether the potential probiotics are capable of exerting any secondary health benefits. In this manner, I hope to tackle my hypothesis, and determine whether Cheddar can impact the gut microbiome.

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Chapter 2

Fermented Foods, Health, and the Gut Microbiome

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Abstract

Fermented foods have been a part of the human diet for almost 10,000 years, and their level of diversity in the 21st century is substantial. The health benefits of fermented foods have been intensively investigated; identification of bioactive peptides and microbial metabolites in fermented foods that can positively affect human health has consolidated this interest. Each fermented food typically hosts a distinct population of microorganisms. Once ingested, nutrients and microorganisms from fermented foods may survive to interact with the gut microbiome, which can now be resolved at the species and strain level by metagenomics. Transient or long-term colonization of the gut by fermented food strains or impacts of fermented foods on indigenous gut microbes can therefore be determined. This review considers the primary food fermentation pathways and microorganisms involved, the potential health benefits, and the ability of these foodstuffs to impact the gut micro-biome once ingested either through compounds produced during the fermentation process or through interactions with microorganisms from the fermented food that are capable of surviving in the gastro-intestinal transit. This review clearly shows that fermented foods can affect the gut microbiome in both the short and long term, and should be considered an important element of the human diet.

2.1 Introduction

Fermented foods have been a component of the human diet from ancient times. Among the earliest evidence for the deliberate application of fermentation has been found in pottery vessels discovered in China dating from 7000 BC that were used to ferment rice, honey, and fruit (McGovern et al., 2004). However, it is likely that inadvertent production and consumption of fermented foods significantly predate this, as foods must have undergone spontaneous fermentation during storage (Tamang et al., 2020). While microorganisms, first discovered in the 1670s by Antoni van Leeuwenhoek, originally received much attention as agents of food spoilage and disease, useful applications, including their ability to produce antibiotics against pathogenic bacteria and to positively impact human health, were soon discovered (Vitorino & Bessa, 2017). However, probably the most important function that microbes have played throughout human history is their involvement in food preservation via fermentation (Macori & Cotter, 2018; Vitorino & Bessa, 2017). Fermentation is the process whereby alcohols, carbon dioxide, and/or organic acids are produced by microorganisms, primarily from sugars and under mostly anaerobic conditions, for production of energy (Chilton et al., 2015). The accumulation of alcohol and organic acids and the associated increase in acidity of the food substrates inhibits the growth of other microorganisms and the activity of enzymes in the food system, thus reducing the rate of spoilage and resulting in foods with extended shelf-life.

However, while the primary function of food fermentation revolves around enhanced food safety and extended shelf-life, fermented foods have become associated with health benefits as well. One of the earliest proponents of this hypothesis was Élie Metchnikoff, who was interested in the potential of food in promoting the elongation of life. In Metchnikoff's essay entitled 'Lactic Acid and Putrefaction', he attributes the long lives of Bulgarian peasants to the staple foods of the country at the time, in particular soured milk. In his experiments, he found that lactic acid bacteria (LAB) cultures in the fermented food produced 'disinfecting bodies' beneficial to their human host (Metchnikoff, 1907). Thus, fermented foods have been linked positively with human health beginning in the early 1900s; the mechanisms subsequently postulated by which these foods can benefit health include one or a combination of

the following: (i) the direct nutritional value of fermented foods, including bioactive compounds, produced as a consequence of the fermentation process; (ii) provision of nutrients to promote growth of indigenous gut microbes; and (iii) the capacity of the microbes in fermented foods to survive gastric transit and to either become a component of the gut microbiome or to inhibit/compete with existing members of the gut microbiome.

Consequently, researchers have proposed that fermented foods be included as part of dietary guidelines. Chilton and colleagues argue that due to their long role as part of the human diet and their established and emerging nutritional and therapeutic benefits (which will be discussed in detail below), brought about in part by their own microbiome, they deserve to be considered for regular consumption and to be included in food consumption guidelines (Chilton et al., 2015). In addition, probiotic bacteria, defined as 'live microorganisms, which when consumed in adequate amounts confer a health benefit on the host', can be a part of the fermented food microbiome (Food and Agriculture Organization of the United Nations, 2006; Gilliland et al., 2001). Many fermented foods contain bacteria with probiotic potential, either added during the manufacturing process or adventitious bacteria, such as non-starter lactic acid bacteria (NSLAB) found in cheese, that are able to grow and thrive in fermented foods (Leroy & De Vuyst, 2004). 'Functional Foods' is a legal term coined in Japan that was defined in Europe in 1999 as a food that has "satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Diplock et al., 1999; Shimizu, 2003). The potential for fermented foods to positively impact the gut microbiome suggests that further research is required on this topic in order to establish whether fermented foods can in fact be defined as functional foods.

In this review, a brief introduction to fermented foods and their potential health benefits is followed by a discussion of the ability of fermented foods to impact the gut microbiome, either through fermented food communities surviving gastric transit and becoming established transiently or permanently as part of the gut microbiome, or through provision of nutrients that support the growth of indigenous gut microbiota;

in addition, we explore the potential nutritional and health benefits associated with such interactions.

2.2 Fermented Food

The earliest evidence of food fermentation dates from the Neolithic period, ~7000 BC, in China (McGovern et al., 2004), a period that coincides with the development of agriculture and thus of seasons in which an abundance of food was available followed by periods of relative scarcity. Cultivation of crops started in the Fertile Crescent of Southwest Asia ~9000 BC, and there is evidence that goat, sheep, and cattle were domesticated by 7000 BC (Kindstedt, 2012). Domestication of animals provided a source of meat and milk, which in its raw form has a very short storage time before spoiling. However, rudimentary processes to convert milk to cheese by fermentation had emerged by 6500 BC, the key benefit of which was an extended shelf life; a mechanism was thereby found to save food available in a time of plenty for one of relative scarcity. The process of making cheese had the additional advantages of (i) concentrating the fat, protein, and mineral content of the milk thus producing an energy-dense and nutritious food, and (ii) removing lactose, making the cheese accessible to adults, who at that time were mostly lactose intolerant and could not consume milk post-infancy (Kindstedt, 2012).

The process of fermentation has been exploited by almost all peoples and has been applied to plant materials (including fruits, seeds, tubers and other vegetative and non-vegetative materials) and animal materials (including meat, milk, fish, and eggs), reflecting the base foods available in different regions (Tamang et al., 2020; Voidarou et al., 2021).

2.2.1 Diversity of Fermented Food Types

It is difficult to definitively establish the number of fermented foods produced globally; most estimates suggest that there are in excess of 5000 different kinds (Tamang, 2010). However, when local and regional variations are considered, this figure is likely to expand significantly. For example, different classification schemes can be used to classify cheese, and while certain approaches suggest as few as eighteen primary cheese types, when local modifications based on variations in procedures, microorganisms, and milk types are taken into account it is generally accepted that in

excess of 1000 cheese varieties are available globally (Beresford et al., 2001). Different approaches have been used to characterize or group fermented foods, the most common of which are based on the raw/non-fermented food substrate, resulting in groupings of fermented foods made from (1) cereals, (2) vegetables, (3) legumes, (4) roots/tubers, (5) milk, (6) meat, (7) fish, (8) alcoholic beverages, and (9) miscellaneous (Tamang et al., 2016).

As a consequence of their ancient origins, there is a strong regional bias in the distribution and popularity of different fermented foods. For example, throughout much of East and South Asia and the southern regions of India fermented legumes, in particular soyabeans, as well as vegetables, fish, and meat are common components of the diet. In West Asia, Northern India, Europe, and North America, fermented cereals, including wheat, barley, and oats, are used to make bread, and fermented dairy and meat products are more common. In Africa and South America fermented seeds, including sorghum, maize, millet, cassava, and legumes, are an important component of the diet, as are fermented milk and meat products (Tamang et al., 2016). A recent review by Tamang and colleagues (2020) expertly demonstrates these differences in the origins of fermented foods around the world (Tamang et al., 2020).

2.2.2 Primary Food Fermentation Pathways

Fermentation is a process whereby substrates are converted to alcohols, carbon dioxide, and/or organic acids, predominantly anaerobically (Chilton et al., 2015). From the microorganisms' perspective, the purpose of fermentation is energy production (Madigan et al., 2009). Microbes adapted to diverse environments exhibit a range of different fermentation pathways; the pathways most relevant to fermented foods are listed in **Table 1**.

The substrate for both lactic acid and ethanol fermentation consists of the sugars available in the food material, for example (though not limited to) lactose in milk and glucose from the breakdown of starch in plant products; the products of both pathways, in addition to influencing the sensory characteristics of the fermented food, play a critical role in extending its shelf life, as the accumulated lactic acid or ethanol inhibits the growth of undesirable spoilage and/or pathogenic microorganisms.

Propionic or acetic acid fermentation is effectively secondary fermentation, the substrate being the product of lactic or ethanol fermentation, respectively. This contributes to extended shelf life and is critically important in impacting the sensory characteristics of the final product. Citric and malolactic fermentations use substrates available in the raw food material, which are converted into products that impact the sensory characteristics of the fermented food. It is important to note that during the food fermentation process, the microorganisms present can release amino acids and encrypted bioactive peptides from proteins, convert fats to more healthy formats such as conjugated linoleic acid, and produce a wide diversity of metabolites, including short chain fatty acids (SCFA), vitamins, exopolysaccharides, and gamma-aminobutyric acid; these impact the sensory characteristics and health-promoting potential of the final fermented food product (Beermann & Hartung, 2013).

The lactic acid fermentation pathway is arguably the most important fermentation pathway in terms of fermented foods. It is the basis of all dairy fermentation as well as the majority of fermentation processes using plant or animal source materials. The bacteria responsible are LAB, which are grouped depending on whether they are homo-, hetero-, or facultative fermenters (Madigan et al., 2009). While homofermentation solely produces two moles of lactate per one mole of glucose, heterofermentation produces ethanol and carbon dioxide in conjunction with lactate (Madigan et al., 2009). This is due to homofermentative LAB harbouring the aldolase enzyme and heterofermentative LAB using phosphoketolase, resulting in divergent catabolic pathways (Müller, 2001). Facultative LAB can ferment by either the homo- or the heterofermentation pathway depending on environmental conditions and substrate availability. The homofermentative LAB most commonly associated with fermented foods include *Lactococcus lactis, Streptococcus thermophilus*, homofermentative *Lactobacillaceae* including *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Lactobacillus acidophilus*, and *Lactobacillus helveticus*, and members of the *Pediococcus* and *Enterococcus* genera (Tamang et al., 2014). Heterofermentative LAB includes *Leuconostoc* spp. and several lactobacilli, including *Fructilactobacillus sanfranciscensis*, *Levilactobacillus brevis*, *Limosilactobacillus fermentum*, and *Limosilactobacillus reuteri*. The most commonly encountered facultative LAB include *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, and *Latilactobacillus curvatus* (Tamang et al., 2014).

Ethanol fermentation is responsible for the production of wines from fermented fruits, beers from cereals such as wheat, barley and rye, and other stronger liquors involving distillation following fermentation such as sake from rice, vodka from potatoes, and rum from sugar cane (Walker & Stewart, 2016). The organism most commonly responsible for ethanol fermentation is the yeast *Saccharomyces cerevisiae*, although *Zymomonas mobilis* (a gram-negative bacterium) is used for, among other products, the production of the fermented Mexican beverage Pulque (Escalante et al., 2008; Madigan et al., 2009). While a mole of glucose is converted to two moles of both ethanol and carbon dioxide regardless of whether yeast or *Zymomonas mobilis* is used, the pathways differ (Müller, 2001). While both involve the enzyme pyruvate decarboxylase and the intermediates pyruvate and acetaldehyde and both lead to the same metabolites, ethanol fermentation by yeasts yields two moles of ATP per mole of glucose while *Zymomonas* generates only one mole of ATP (Müller, 2001). This difference is because yeasts utilize the glycolytic pathway and *Zymomonas* uses a variation of this, the Entner–Doudoroff pathway (Madigan et al., 2009). It is worth noting that in fermented foods ethanol can be produced, albeit at low concentrations, by heterolactic fermentation (Müller, 2001). Kefir, which can contain up to 2% ethanol, is a good example of this; the ethanol is produced by both the yeast and heterofermentative LAB present during the fermentative process (Baschali et al., 2017).

Acetic acid is an important component in vinegar and is derived from fermenting alcoholic beverages such as wine, cider, and beer; the end product differs depending on the region in which it is produced and the alcoholic beverage used (Raspor & Goranovič, 2008). As discussed above, in alcoholic fermentations glucose is converted to ethanol, which is followed by oxidative fermentation to produce acetic acid (Saichana et al., 2015). Where homoacetic fermentation occurs, one mole of acetate is produced from each mole of ethanol consumed. Acetic Acid Bacteria (AAB) are responsible for this process (Müller, 2001). In terms of commercial vinegar production, aerobic species of *Acetobacter* and *Komagataeibacter* are of particular interest due to their high tolerance of both the substrate (ethanol) and product (acetate) present in their growth media (Saichana et al., 2015).

Emmental and other Swiss-type cheeses rely on the ability of Propionic Acid Bacteria (PAB) to ferment lactate to propionic acid, with the coproduction of acetate and CO_2 giving rise to the characteristic flavour and holes or “eyes” associated with these cheese types (Beresford et al., 2001). Propionate can be produced through either the acrylate or methylmalonyl–CoA pathway, although both produce two moles of propionate, one mole of acetate, and one mole of CO_2 for every three moles of lactate catabolized (Müller, 2001). PAB used in the production of Swiss-type cheeses utilize the latter methylmalonyl–CoA pathway (Beresford et al., 2001).

Citrate fermentation is an important metabolic pathway for certain fermented foods, in particular in the dairy sector (Laëtitia et al., 2014). This fermentation can be undertaken by a limited range of homo- and heterolactic LAB, the most important being *L. lactis* subsp. *lactis* biovar *diacetylactis* and certain *Leuconostoc* species, respectively. The fermentation pathway and the end products generated vary depending on the LAB involved as well as environmental factors, including the availability of fermentable carbohydrate and the pH. Potential end products include acetate, formate, ethanol, 2,3-butanediol, diacetyl, acetoin, carbon dioxide, and lactate (Quintans et al., 2008), of which diacetyl and acetoin are known to exhibit nutty and buttery aromatic notes. Citric acid fermentation is thus important in the production of a number of dairy products, such as cottage cheese, where diacetyl confers a buttery aroma, as well as in Gouda and other cheese varieties where in addition to impacting on aroma notes, the production of carbon dioxide is responsible for eye formation (Quintans et al., 2008).

Lactic acid bacteria are responsible for the malolactic fermentation process that is involved in the production of wine; this predominantly takes place after alcohol fermentation by yeast (Betteridge et al., 2018). This reaction converts L-malic acid to L-lactic acid and carbon dioxide and results in the production of other flavour compounds of interest such as diacetyl (Davis et al., 1985), leads to deacidification of the wine, and promotes microbial stability (Kunkee, 1991). The predominant LAB associated with this process is *Oenococcus oeni*, although other *Lactobacillaceae* and *Pediococcus* species can be used (Liu, 2002).

Fermentation Type	Substrate	End Products	Microorganisms Responsible	Reference
Lactic Acid *	Sugar			
Homo lactic		Lactic acid	*Lactococcus lactis* *Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus acidophilus* *Lactobacillus helveticus* *Pediococcus* *Enterococcus*	(Wang et al., 2021)
Hetero lactic		Lactic acid, ethanol, CO_2	*Leuconostoc* *Fructilactobacillus sanfranciscensis* *Levilactobacillus brevis* *Limosilactobacillus fermentum* *Limosilactobacillus reuteri* *Lacticaseibacillus casei* *Lactiplantibacillus plantarum* *Latilactobacillus curvatus*	(Wang et al., 2021)
Ethanol	Sugar	Ethanol, CO_2	*Saccharomyces cerevisiae* *Zymomonas mobilis*	(Eram & Ma, 2013)
Acetic Acid	Ethanol	Acetic acid	*Acetobacter* *Komagataeibacter*	(Lynch et al., 2019)
Propionic Acid	Lactic Acid	Propionic acid, acetic acid, CO_2	*Propionibacterium freudenreichii* *Propionibacterium jensenii* *Propionibacterium thoenii* *Propionibacterium acidipropionici* *Propionibacterium cyclohexanicum*	(Gonzalez-Garcia et al., 2017)
Citric Acid	Citric Acid	Acetate Formate Ethanol 2,3-butanediol Diacetyl Acetoin CO_2 Lactate	*Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* *Leuconostoc* ** *Enterococcus* ** *Lactobacillus* ** *Oenococcus oeni*	(Behera et al., 2021)
Malolactic	Malic Acid	Lactic acid, CO_2	*Oenococcus oeni* *Lactobacillaceae* *** *Pediococcus* **	(Lasik-Kurdys et al., 2018)

Table 1. A summary of the primary fermentation pathways of relevance to fermented foods, including the main fermentation end products and the microorganisms responsible for their production. * Certain LAB, referred to as facultative heterofermentative LAB, can ferment by either the homo- or heterolactic fermentation pathway depending on environmental conditions or substrate availability. ** Certain members of this genus. *** Certain members of this family.

2.2.3 Microbiome of Fermented Foods

The primary microorganisms responsible for the main fermentation pathways that occur in food are discussed above and are listed in **Table 1**. Traditionally, food fermentation has relied on the microbiota naturally occurring in the raw food material or obtained by transferring the microbiome from previously-fermented products. However, under modern large-scale commercial food production systems well--characterized or defined starter cultures are now widely used to ensure reproducible products capable of consistently meeting high consumer standards, and the safety status of these microbes are established to ensure the safety of consumers (Hill et al., 2017).

However, even under modern food processing systems most of the ingredients used in the preparation of fermented foods and the processing equipment used in their manufacture are not sterilized; thus, in addition to known starter microbiota most fermented foods and beverages contain an indigenous microbiome, potentially consisting of a wide variety of microorganisms. Traditional microbiological methods demonstrate the existence of these indigenous microorganisms, often referred to as Non-Starter organisms; however, with the development and widespread application of affordable and reliable high-throughput DNA sequencing technologies numerous fermented foods have undergone metagenomic screening, revealing highly diverse and previously unrecognized populations of adventitious Non-Starter microorganisms (Coeuret et al., 2003; Macori & Cotter, 2018; Quigley et al., 2012; Tamang et al., 2016). The diversity of microorganisms includes bacteria, yeast, and filamentous moulds. In addition to the bacteria listed in Table 1, species of *Arthrobacter*, *Bacillus*, *Bifidobacterium*, *Brachybacterium*, *Brevibacterium*, *Enterobacter*, *Hafnia*, *Haloanaerobium*, *Halobacterium*, *Halococcus*, *Klebsiella*, *Kocuria*, *Micrococcus*, *Pseudomonas*, and *Staphylococcus* have been recorded in different fermented foods (Tamang et al., 2017). The genera of yeast identified include *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Galactomyces*, *Geotrichum*, *Hansenula*, *Hanseniaspora*, *Hyphopichia*, *Issatchenkia*, *Kazachstania*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Rhodosporidium*, *Saccharomyces*, *Saccharomycodes*, *Saccharomycopsis*, *Schizosaccharomyces*, *Sporobolomyces*, *Torulaspora*, *Torulopsis*, *Trichosporon*, *Yarrowia*, and *Zygosaccharomyces*, while the filamentous moulds include *Actinomucor*, *Amylomyces*, *Aspergillus*, *Monascus*, *Mucor*,

Neurospora, *Parcilomyces*, *Penicillium*, *Rhizopus*, and *Ustilago* (Tamang et al., 2016). By assessing the microbial diversity of fermented foods, a more reliable understanding of their fermentation potential can be determined, thus allowing for a more complete understanding of each fermented food and its potential to impact human health.

The diversity that exists among fermented food microbiomes is exemplified by cheese. The microbiome that develops in cheese during ripening arises from the milk and other ingredients used for cheese manufacture, the starter cultures added at the beginning of manufacture, and the cheesemaking and ripening room environments (Cotter et al., 2017). While the majority of cheeses are produced from cow's milk, cheese from sheep, goats, buffalo, camels, and even donkeys is manufactured (Bittante et al., 2022). Genera common to all raw milk include *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus*, although certain varieties are more strongly associated with one type or another, which impacts the microbiome of a final product (Nikoloudaki et al., 2021; Quigley et al., 2013; Salazar et al., 2021). For example, while the predominant genera in cow's milk include *Pseudomonas*, *Bacillus*, *Lactococcus*, and *Acinetobacter*, the genera most common to raw camel's milk include *Enterococcus*, *Lactococcus*, and *Pediococcus* (Li et al., 2018; Rahmeh et al., 2019). The starters and adjunct cultures added to the milk at the beginning of the cheese-making process impact the food microbiome, with both bacterial and yeast/mold species sometimes being necessary for development of characteristic organoleptic properties (Lane & Fox, 1996). For compilations of popular starter and adjunct cultures used in cheese preparation, González-González et al. (2022) and Bintsis (2021) have written comprehensive reviews on bacteria and yeast/mold cultures, respectively (Bintsis, 2021; González-González et al., 2022). NSLAB are another important component of cheese; while they are present at low numbers immediately following cheese production (10^2–10^3 colony-forming units (CFU)/g), they proliferate and outcompete the starter cultures to become the dominant microbial populations during cheese ripening and are often responsible for the development of characteristic organoleptic properties (Blaya et al., 2018).

Certain cheese microbiomes can have the added complexity of maintaining distinct microbial communities, such as smear cheese. Smear cheese is produced by rubbing or 'smearing' microorganisms onto the surface of the newly formed cheese, resulting in

the development of a rind with a distinct flavour and a surface microbiome that differs greatly from the microbiome present in the cheese core (Mounier & Coton, 2021; S. Ritschard et al., 2018). Stilton, defined as a blue cheese due to veins of *Penicillium roqueforti* culture that emanate from the core outwards, has a very diverse microbiome that includes an outer rind that develops from different molds (Ercolini et al., 2003; Gkatzionis et al., 2014).

In addition, many fermented foods are eaten without further processing or preparation, and thus contain microbial populations of up to 10^8 CFU/g; these can potentially gain entry to the human gastrointestinal tract, where they may interact with or become established as part of the gut microbiome (Marco et al., 2017; Rezac et al., 2018).

2.3 Health Benefits of Fermented Foods

As discussed above, while fermented foods have a long history of safe use, there is evidence that consumption of fermented foods results in positive health effects. Much of this is driven by popular observations that fermented foods in general use unprocessed raw ingredients, contain little or no added preservatives, colours or flavourings, and are made using long-established, sustainable, and in many cases traditional technologies. Consumers may be attracted to the concept that these are "live foods" containing natural and diverse microbiota.

2.3.1 Human Dietary Studies

It is incumbent upon the relevant research community to generate data to clarify and identify the benefits of such foods, if any exist. In support of this, a number of controlled human dietary studies have recently been undertaken which sustain these popular perceptions of health benefits (Marco et al., 2017). These studies include investigations that revealed strong associations between weight management and consumption of fermented dairy products (Mozaffarian et al., 2011), reduced risk of cardiovascular disease, type 2 diabetes, and mortality associated with consumption of yoghurt (Chen et al., 2014; Eussen et al., 2016; Soedamah-Muthu et al., 2013; Tapsell, 2015), and enhanced glucose metabolism and reduced muscle soreness following acute resistance exercise as a consequence of consuming fermented milk (Iwasa et al., 2013). Consumption of kimchi was linked to anti-diabetic and anti-obesity effects (An et al., 2013; Han et al., 2015), while consumption of different fermented foods was associated with alterations in mood and brain activity (Hilimire et al., 2015; Omagari et al., 2014; Tillisch et al., 2013) and in the gut microbiome (Taylor et al., 2020). However, reports have identified a lack of sufficient clinical trials, variation in the different fermented foods being investigated, and inconsistencies among ethnic groups in suggesting that more studies need to be undertaken in order to confirm the potential benefits of fermented food consumption (Sivamaruthi et al., 2018).

2.3.2 Transformations in Food Arising from Fermentation

It well established that fermentation can enhance the digestibility of complex carbohydrates and proteins through the breakdown of starch to oligosaccharides and polypeptides to amino acids (Çabuk et al., 2018; Yadav & Khetarpaul, 1994). Fermentation allows for the destabilization of the casein micelle by bacteria present in milk, enhancing milk protein digestibility (Beermann & Hartung, 2013; Jardin et al., 2012). Fermentation, in particular with respect to cheese, facilitates the concentration of key nutrients through removal of water and enhances the bioavailability of calcium, which is important for skeletal health (Rozenberg et al., 2015).

Additionally, fermentation can facilitate transformations in raw foods that allow these foods to be tolerated by consumers that are intolerant of the original raw product. A good example of this is the ability of lactose-intolerant individuals to consume fermented dairy products, in particular ripened cheeses such as Cheddar. The reason for this is that during fermentation and cheese ripening, the LAB metabolize the lactose, significantly reducing the level of lactose in the resulting fermented food product. In addition, the presence of the lactase enzyme produced by bacteria present in the fermented matrix can help to further remove any residual lactose during ingestion and digestion (Granato et al., 2010). A similar example involves decrease in the concentration of anti-nutritional components in raw food, such as the partial eradication of harmful trypsin inhibitors during soy bean fermentation (Hong et al., 2004).

In addition, bioactive compounds can be produced through protein, lipid, and carbohydrate catabolism during the fermentation process, and can result from the range of microbial metabolites produced during the process (Leroy & De Vuyst, 2014). Indeed, production of vitamins and antioxidants during food fermentation has been reported for many LAB species, in particular members of the newly-rebranded *Lactobacillaceae* family (Kamal-Eldin, 2012; Samaranayaka & Li-Chan, 2011; Şanlier et al., 2017; Zheng et al., 2020). Bioactivities linked to lowering of blood pressure and cholesterol, improvement in metabolic syndromes, anti-cancer effects, and improvement in immune function have all been described (Şanlier et al., 2017). Vitamins B7, B11, and B12 are produced in fermented dairy products by *Lactobacillaceae* (e.g., *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii*, *Limosilactobacillus reuteri*), *Propionibacterium*, *Bifidobacterium*, and several species of

Streptococcus (Fernández et al., 2015). Folic Acid (B11) is necessary for development, and reproduction and prevents against certain disorders including some cancers and cardiovascular diseases, while many metabolic processes require B12 as a cofactor, including nucleic and amino acid metabolism (Stanton et al., 2005). Non-dairy fermented foods contain microbes which synthesize vitamins (Baschali et al., 2017). Shalgam is a Turkish fermented beverage consisting of black carrots and turnips; its resident microbiota consists predominantly of *Lactobacillaceae* along with *Leuconostoc* and *Pediococcus* (Altay et al., 2013). This beverage is an abundant source of vitamins A, B, and C as well as other minerals and polyphenols (Altay et al., 2013; Baschali et al., 2017). Antioxidants exert beneficial functions in food and protect against the damaging effects of free radicals, and are produced in fermented foods by microbial esterases (Kamal-Eldin, 2012). Kombucha, a fermented sweetened tea, contains many antioxidants with positive effects on health, including antagonistic effects towards progression of neurodegenerative diseases, diabetes, and certain cancers (Baschali et al., 2017). Many other fermented foods have been shown to positively contribute to different aspects of human health, such as kimchi, a fermented cabbage, which is capable of anti-atherogenic effects that are triggered by the active compound 3-(4'-Hydroxyl-3',5'-dimethoxyphenyl) propionic acid (HDMPPA) (Kim et al., 2017).

2.3.3 Release of Bioactive Peptides

The release of bioactive peptides as a result of protein hydrolysis during fermentation has been investigated by multiple groups. A well-studied example of bioactive peptide release as a consequence of catabolism of protein involves angiotensin-1-converting enzyme (ACE)-inhibitory peptides, a group of peptides with hypertension-lowering capabilities (Şanlier et al., 2017). LAB from both dairy and non-dairy sources can produce ACE-inhibiting peptides during fermentation of milk (Solieri et al., 2015). They are primarily produced in fermented dairy foods such as yogurt and cheese, although different casein molecules can be targeted depending on the starter LAB cultures used (Rai et al., 2017). Two lactotripeptides, isoleucine–proline–proline (IPP) and valine–proline–proline (VPP), which are not digested and remain intact and able to cross the mucosal surface to confer their antihypertensive benefits, have received much attention (Solieri et al., 2015). For example, in one study, spontaneously hypertensive rats were fed a fermented milk beverage containing a *Lactobacillus helveticus* strain, a

species known for its ability to release ACE-inhibitory peptides from milk protein, which significantly lowered blood pressure when compared with controls (Chen et al., 2014). Human trials have yielded promising results as well. A meta-analysis of the published literature on human intervention trials confirmed the efficacy of IPP and VPP, including those in functional foods; Asian patients in particular reported a decrease in blood pressure which appeared to be independent of age, lactotripeptide dosage, length of treatment, or initial blood pressure readings at a level that was statistically significant (Cicero et al., 2011). ACE-inhibitory peptides have been identified in non-dairy products such as fermented meat sausages made using strains of *Latilactobacillus sakei* and *Latilactobacillus curvatus* (Takeda et al., 2017).

2.3.4 Production of Exopolysaccharide

Many food fermentation microbes are capable of producing high molecular weight exopolysaccharides (EPS) from simple sugars present in the raw food product. EPS can be produced by *Zymomonas*, *Leuconostoc*, *Pediococcus* and *Streptococcus* species, and members of the *Lactobacillaceae* family (Kamal-Eldin, 2012). Examples of EPS production include acetan, xanthan, and kefiran, several of which are important from a food manufacturing point of view. Xanthan is used for its desirable rheological characteristics when added to fermented milk products (De Vuyst & Degeest, 1999). In terms of health, EPS-producing LAB have been found to have a role in immunomodulation, which can be either stimulatory or suppressive depending on various factors (Ryan et al., 2015). Dendritic cells bind to ingested microbial EPS via their pathogen recognition receptors through epithelial cell tight junctions, migrate with the EPS 'antigen' to other lymphoid tissues, and further communicate with other immunomodulatory cells such as Natural Killer cells, which can invoke inflammatory responses when appropriate (Ryan et al., 2015). EPS production in fermented foods has been examined in terms of cardiovascular disease (CVD) due to their ability to bind cholesterol, such as the ability of *Pediococcus*-produced β-glycans to lower serum cholesterol levels (Ryan et al., 2015). While prebiotics and probiotic bacteria are capable of lowering cholesterol through various mechanisms, EPS appears to lower cholesterol by binding bile (of which cholesterol is a constituent) from the intestines to the bacterial cell envelope, thus reducing bile reabsorption and recycling. Consequently, de novo synthesis of bile in the liver results in lowered serum

cholesterol levels (Tok & Aslim, 2010). A paper in 2002 reported that EPS-producing starter cultures were able to bind the bile salt cholic acid, while another study in 2010 demonstrated the ability of EPS-producing bacteria to bind bile from liquid growth media and that the number of EPS produced was correlated with a decrease in broth cholesterol levels (Pigeon et al., 2002; Tok & Aslim, 2010). An EPS-producing *Lactiplantibacillus paraplantarum* strain isolated from dahi (fermented milk) could reduce the cholesterol concentration in supernatant in vitro (Afreen et al., 2023). An in vivo study investigating the ability of β-glucan-producing *Lacticaseibacillus paracasei* to lower serum cholesterol in mice found that a significant decrease was observed when compared to control mice that were not given the EPS-producing *Lacticaseibacillus* strain (London et al., 2014). Additionally, this study showed that the gut microbiome was modified as a result of receiving the EPS-producing *Lacticaseibacillus paracasei* (London et al., 2014). A similar study was performed on humans with elevated serum cholesterol levels who were fed fermented oat-based products containing an EPS-producing *Pediococcus damnosus* strain (Mårtensson et al., 2005). This study concurrently noted that a statistically significant reduction in total cholesterol was seen in the group who were given the fermented product, along with a significant increase in the relative abundance of faecal *Bifidobacterium* species (which are beneficial gut microbes) and total faecal bacterial counts (Mårtensson et al., 2005). Thus, fermented foods containing EPS can positively influence gut health.

2.4 Evidence of Fermented Foods that Modulate the Gut Microbiome

The ability of fermented foods to modulate the gut microbiome has been documented by several groups with varying degrees of success. Changes are generally recorded as overall shifts in microbial populations, and do not necessarily reflect the microbial content of the fermented foods in question. A recent study evaluated the effect of general fermented plant intake on microbial and metabolomic differences in consumers versus non-consumers (nearly 7000 participants total), and determined that beta diversity was significantly different between consumers versus non-consumers (Taylor et al., 2020). Microbiomes of consumers of such fermented products were associated with *Bacteroides* spp., *Pseudomonas* spp., *Dorea* spp., *Lachnospiraceae*, *Prevotella* spp., *Alistipes putredinis*, *Oscillospira* spp., *Enterobacteriaceae*, *Fusobacterium* spp., *Actinomyces* spp., *Achromobacter* spp., *Clostridium clostridioforme*, *Faecalibacterium prausnitzii*, *Bacteroides uniformis*, *Clostridiales*, and *Delftia* spp. (Taylor et al., 2020). In parallel, 115 separate participants who consumed fermented foods at various frequencies were investigated for a four-week duration, and it was found that microbes associated with the consumers of fermented foods consisted of both fermented food-associated microbes (e.g., *Lactobacillus acidophilus*, *Levilactobacillus brevis*, *Lactobacillus kefiranofaciens*, *Lentilactobacillus parabuchneri*, *Lactobacillus helveticus*, and *Latilactobacillus sakei)* and microbes not associated with fermented foods (including *Streptococcus dysgalactiae*, *Prevotella melaninogenica*, *Enorma massiliensis*, *Prevotella multiformis*, *Enterococcus cecorum*, and *Bacteroides paurosaccharolyticus*) (Taylor et al., 2020).

In a similar fashion to the previous study, Wastyk and colleagues (2021) examined the effects of a diet rich in fermented foods (including fermented dairy products, vegetables and non-alcoholic beverages) on eighteen healthy adults over a period of seventeen weeks in parallel to a diet high in fibre (Wastyk et al., 2021). The dietary intervention consisted of an initial four-week period whereby the quantity of fermented food in the diet was increased, followed by a six-week 'maintenance' period of very high fermented food intake, and closed with a 'choice' period of four weeks in which participants could maintain whatever level of fermented food intake they desired. The fermented food-rich diet resulted in an increase in alpha diversity of the

gut microbiome that was not observed with the fibre diet. Interestingly, the increase in microbiome diversity remained during the designated 'choice' period despite intake being higher during the 'maintenance' period, with a strong relationship between time and diversity (Wastyk et al., 2021).

Different groups have reported changes in gut microbe populations following ingestion of fermented milk, although parameters vary between studies (Unno et al., 2015; Veiga et al., 2014; Yılmaz et al., 2019). A fermented milk product with defined starters and adjuncts was able to significantly increase SCFA levels in vivo, especially butyrate; when administered to irritable bowel syndrome (IBS) sufferers, it led to a decrease in *Bilophila wadsworthia* (a so-called pathobiont, that is, a bacterium negatively associated with health) and an increase in two *Clostridiales* isolates (uncharacterized genera MGS203 and MGS126) known for butyrate production (Veiga et al., 2014). A separate study examined the effects of kefir on inflammatory bowel disease (IBD) patients; a significant increase in faecal *Lactobacillaceae* abundance was observed following consumption of the fermented milk for one month (Yılmaz et al., 2019). Zeng et al. (2021) administered either milk or kefir to a colorectal cancer mouse model and observed that the kefir group experienced gut microbiome perturbations that were absent in the milk counterparts, including a relative increase and decrease in probiotic-associated and pathogenic bacteria, respectively, as well as decreased *Firmicutes*/*Bacteroidetes* and *Ascomycota*/*Basidiomycota* ratios at the phylum level (Zeng et al., 2021).

Healthy females receiving synbiotic fermented milk experienced an increase in the relative abundance of faecal *Bacteroidetes* (in particular, the *Bacteroidaceae* and *Prevotellaceae* families) species and a decrease in *Firmicutes* abundance (in particular, the *Ruminococcaceae* and *Lachnospiraceae*) that reversed when the product was no longer being ingested (Unno et al., 2015). This result is interesting, as the fermented product contained high levels of *Firmicutes* and yet a decrease in this phylum was observed, highlighting the varying and complex relationship between fermented foods and the gut microbiome. In contrast, Tillisch and colleagues (2013) reported no significant changes to the microbial composition of stool samples following ingestion of a probiotic-containing fermented milk (Tillisch et al., 2013). Children infected with *Helicobacter pylori* exhibited lower stool *Bifidobacterium* than their healthy

counterparts, which could be restored partially through ingestion of a probiotic yogurt that led to a significant increase in their stool *Bifidobacterium* spp./*Escherichia coli* ratio (Yang & Sheu, 2012). The effects of yogurt consumption on the gut microbiome of healthy subjects after 42 days was found to induce changes in overall composition and diversity, although this varied between individuals and no significant fluctuations were observed (Lisko et al., 2017).

Firmesse and colleagues examined the effects of Camembert consumption in healthy subjects over a four-week period, focusing on faecal *Enterococcus* populations and the ability of the cheese microbiome to be detected in faecal samples (Firmesse et al., 2007, 2008). *Enterococcus faecalis* populations in stool samples were significantly increased post-Camembert ingestion, while cheese populations of *L. lactis* and *Ln. mesenteroides* were detected in faeces during the trial (Firmesse et al., 2007, 2008). Le Roy and colleagues (2022) compared the gut microbial compositions of people who did or did not consume yogurt, using both whole shotgun metagenomic sequencing (*n* = 544) and 16S rRNA sequencing (*n* = 1457, participants overlap with shotgun cohort) (Le Roy et al., 2022). For the larger 16S cohort, yogurt eaters had a significantly higher alpha diversity than those that did not consume yogurt, and seven genera (specifically, four belonging to the *Ruminococcaceae* family, one *Streptococcus*, one unidentified genus belonging to the *Lachnospiraceae* family, and one belonging to the *Christensenellacaea* family) had significantly increased relative abundance (Le Roy et al., 2022). Shotgun sequencing did not mirror these results, instead revealing a significantly positive association between *S. thermophilus* and *B. animalis* and yogurt consumption, whereby greater levels of both species were present in participants who consumed higher quantities of yogurt in a dose-dependent manner, although this increased colonisation appears to be transient (Le Roy et al., 2022). Both of these species are markers of a diet rich in fermented milk products (Le Roy et al., 2022).

The ability of fermented foods to impact gut microbial populations is not limited to fermented dairy products. The effect of both fresh and fermented kimchi on the gut microbiome of obese patients was examined in (Han et al., 2015). Although both types of Kimchi caused shifts in microbial populations, namely, an increase in both Proteobacteria and Actinobacteria over the eight week period, certain changes were specific to only one group, such as the increase in Actinobacteria observed in the group

receiving fermented kimchi, which was negatively correlated with body fat (Han et al., 2015). Additionally, the fermented kimchi group saw a relative increase in *Bacteroides* and *Prevotella* and a relative decrease in *Blautia* (Han et al., 2015). A study with pasteurized versus unpasteurized sauerkraut intake in IBS patients showed significant improvements in symptoms and a significant change in the composition of the gut microbiota (specifically, reduced operational taxonomic units of the *Clostridiales* order), although the unpasteurized group showed significantly greater numbers of sauerkraut-associated LAB (*Lactiplantibacillus plantarum* and *Levilactobacillus brevis*) than their pasteurized counterparts (Nielsen et al., 2018). Two separate groups investigating the effects of fermented soy milk on healthy adult populations observed significant increases in both bifidobacteria and lactobacilli faecal populations along with decreases in clostridia, with these shifts being partially attributed to the ability of bifidobacteria and not clostridia to utilize certain soybean oligosaccharides (Cheng et al., 2005; Inoguchi et al., 2012).

Ingestion of Cha-Koji, a green tea fermented with *Aspergillus luchuensis*, and its effects on perturbations in the gut microbiota was investigated in mouse caecal and human faecal samples, showing significant increases in *Clostridium* clusters XI and XIVa (members of which are established butyrate producers), respectively (Yamamoto et al., 2018). Faecal *Bifidobacterium* spp. were significantly increased following three weeks of coffee consumption by healthy adult volunteers, although inconsistencies between subjects were observed (Jaquet et al., 2009). Fermented plant extract was found to significantly shift microbial populations in stool samples of mildly hypercholesterolemic patients by increasing bifidobacteria and lactobacilli and decreasing harmful *E. coli* and *Clostridium perfringens* (Chiu et al., 2017). The effects of raspberry juice fermented with *Lacticaseibacillus casei* on in vitro and in vivo microbiome populations were assessed by Wu and colleagues (2021), with differences being seen in both instances (Wu et al., 2021). When the fermented juice was put through artificial digestion followed by colonic fermentation, significant increases in abundance were observed for *Lactobacillus*, *Akkermansia*, *E. coli*, and butyric acid-producing bacteria, while *Bacteroides* and *Ruminococcus* decreased significantly. For their in vivo murine trial, the fermented beverage was freeze dried and fed to male Kun Ming mice in several concentrations, and was found to alter beta diversity and various genera depending on the concentration (Wu et al., 2021).

Thus, fermented foods have been shown to have the capacity to modify gut microbiome populations, although it can often be unclear as to how these changes are brought about. While this has been shown in the above examples for a myriad of different fermented food types, the results cannot be directly compared due to highly variable parameters, including the use of both healthy and disease models and the various methods by which microbes are quantified. Fermented foods may interfere with the gut microbiome through its own microbiome or through the nutrients present in its matrix. Therefore, in an effort to provide objective evidence that clearly demonstrates whether or not fermented foods can modulate the human gut microbiome, more in-depth and well-defined human feeding studies need to be undertaken. In these studies, the microbiomes of both the fermented food and the human gut need to be established using the most advanced and sensitive tools available in order to permit changes at both the genus and the species level to be determined.

2.5 Nutrients from Fermented Foods that Modulate the Gut Microbiome

In addition to the benefits of fermented foods to the host, certain chemicals present in fermented foods can affect the host's gut microbiome directly. The production of two types of chemicals in particular have come under scrutiny due to their documented effects on microbial populations, polyphenols and dietary fibre, the latter of which leads to the in vivo production of short-chain fatty acids (Voreades et al., 2014).

Fermentation can lead to increases in polyphenol bioavailability in fermented foods (Annunziata et al., 2020; Cardona et al., 2013; Shiferaw Terefe & Augustin, 2020). Polyphenols are a group of heterogenous chemicals found in plant-based foods comprising both flavonoids and non-flavonoids. In terms of the human diet, flavonoids and phenolic acids comprise the majority of dietary polyphenols and are sought after for their antioxidant properties, and have been demonstrated to directly impact on the gut microbiome (Zhang et al., 2015; Zheng et al., 2017). A recent study examined the effect of fermentation on polyphenol content in a mix of eight legumes commonly consumed in China (Gan et al., 2016). The beans were allowed to ferment either naturally (without added microorganisms) or with added lactobacilli, with both soluble and bound total phenolic content (TPC) measured following 48 h of incubation (Gan et al., 2016). While antioxidant capacity differed between the two fermentation methodologies used, significant increases in soluble TPC were seen in both when compared to non-fermented samples, while varying increases were also seen in the bound TPC fraction (Gan et al., 2016). Similar studies involving other vegetables have been reported. The free polyphenol content (FPP) of unfermented corn and corn fermented by two strains of fungi (*Agaricus* sp.) was compared; both fermented corns showed an increase in FPP content compared to the unfermented control, with one of the strains having an FPP content 88 times greater than the control (Zhai et al., 2018). Unsurprisingly, this does not appear to work for all fermented plant products. A study in 1994 investigated polyphenol levels in de-hulled black-gram dhal slurry and found that the fermented product had significantly lower levels of polyphenols in comparison to its raw material, whereby fermentation for 18 h resulted in loss of almost 50% of total polyphenol content (Yadav & Khetarpaul, 1994). Thus, each fermented plant

product should be investigated before claims can be made regarding polyphenol content.

Various studies have shown that polyphenols can impact bacteria found in the gut, although the majority of these studies focus on pathogen inhibition (Duda-Chodak et al., 2015; Hervert-Hernández & Goñi, 2011; Wu et al., 2021; Zhou et al., 2021). Tea is a commonly consumed beverage in most countries and advantageously contains a plethora of flavonoids, making it an accessible source of polyphenols (Lee et al., 2006). A study in 2006 examined the effects of tea-extracted flavonoids on gut bacteria in vitro and showed that the majority of pathogenic bacteria were inhibited, one of a number of studies that confirms the ability of tea polyphenols to inhibit pathogens (Hervert-Hernández & Goñi, 2011; Lee et al., 2006). On the other hand, the ability of polyphenols to inhibit putatively beneficial bacteria (such as commensal gut bacteria, lactobacilli, and bifidobacteria) has been studied, with many studies concluding that in most cases these microbes are not inhibited (Hervert-Hernández et al., 2009; Nash et al., 2018). While the mechanisms by which polyphenols in general stimulate beneficial gut microbes and inhibit pathogens are unclear, specific properties of each group of microbes have been highlighted as potential explanations. For example, polyphenols can be tolerated by gut microbes and not by pathogens due to their ability to reduce them to less harmful substances; certain gut microbes such as lactobacilli are even capable of utilizing polyphenols as a nutritional substrate (Aura, 2008; Parkar et al., 2013). In contrast, polyphenols have shown inhibitory effects on virulence factors of pathogenic bacteria, such as the suppression of the *H. pylori* urease enzyme required for its ability to neutralize gastric acid (Hervert-Hernández & Goñi, 2011).The microbiota themselves influence the bioavailability of ingested polyphenols through bacterial enzymes (e.g., esterases, demethylases), transforming them into forms capable of absorption through the intestinal wall (Duda-Chodak et al., 2015; Murota et al., 2018).

Wine is a rich source of polyphenols, and manipulation of various factors during grape growth and processing can increase total polyphenol content (Champ & Kundu-Champ, 2019). Red wine polyphenols have been shown to significantly alter gut microbiota groups, including an increase in overall microbial diversity, and to significantly lower total cholesterol and blood pressure (Barroso et al., 2017; Clemente-Postigo et al.,

2013; Moreno-Indias et al., 2016; Queipo-Ortuño et al., 2012). While Barroso et al. (2017) only observed an overall increase in diversity and could not identify a consensus between healthy wine-consuming individuals, Queipo-Ortuño and colleagues (2012) noted significant increases in *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Eggerthella lenta*, and *Blautia coccoides–Eubacterium rectale* groups in similarly healthy individuals who consumed wine once a day, although the dominant groups shifted throughout the duration of the study (Barroso et al., 2017; Queipo-Ortuño et al., 2012). A potential benefit of wine polyphenol consumption may be the lowering of lipopolysaccharide (LPS) or LPS-producing bacteria, as Clemente-Postigo and colleagues (2013) found a significant increase in both *Bifidobacterium* and *Prevotella* with beverage consumption that was negatively correlated with LPS (Clemente-Postigo et al., 2013). Similarly, Moreno-Indias and colleagues (2016) noted that consumption of red wine increased beneficial *Bifidobacterium* and Lactobacillaceae levels, increased the levels of butyrate-producing *Faecalibacterium prausnitzii* and *Roseburia*, and decreased LPS-producing *E. coli* and *Enterobacter cloacae* (Moreno-Indias et al., 2016). Two separate studies examined the effects of quercetin and resveratrol, two polyphenols found in red wine, on gut dysbiosis in rats fed high-fat diets (Etxeberria et al., 2015; Zhao et al., 2017). Both studies found that the polyphenols were able to reduce the *Firmicutes/Bacteroidetes* ratio linked with high-fat diets, although Etxeberria et al. found that only quercetin was able to decrease microbial groups previously associated with diet-induced obesity, while Zhao et al. concluded that a combination of quercetin and resveratrol was capable of the same effect (Etxeberria et al., 2015; Zhao et al., 2017). As polyphenols can inhibit pathogenic bacteria and potentially benefit advantageous bacteria, the consumption of fermented foods with high levels of polyphenols has the potential to impact gut bacteria.

Short chain fatty acids (SCFA) arise through the catabolism of carbohydrate fibres by microbes. This is important in human nutrition, as microbes indigenous to the colon are capable of this fermentative process and the SCFA thus produced are used as an energy source by colon cells; their formation benefits the human host, as it enables energy to be extracted from an otherwise-indigestible complex carbohydrate (Korcz et al., 2018; Marco et al., 2017). Well known examples of SCFA include acetate, propionate, and butyrate; Lactobacillaceae and *Bifidobacterium* are established producers of these valuable components in vitro (Korcz et al., 2018; Voreades et al.,

2014). While SCFA are known to play important roles in host metabolism and the central nervous system, they are important in terms of their impact on the gut microbiota as well (De Vadder et al., 2014; Korcz et al., 2018). As already stated, acetate production by bacteria in any niche will increase the level of acidification of the environment, inhibiting the proliferation of less acid-tolerant bacteria, which is advantageous in the intestines as LAB usually inhibit the growth of pathogens through this mechanism (Pessione, 2012). Acetate has been shown to modulate gut microbiota and reverse gut dysbiosis to a degree (Marques et al., 2017). More importantly, SCFA have been demonstrated to stimulate production of mucus (primarily composed of mucin proteins) by host epithelial goblet cells (Burger-van Paassen et al., 2009). Mucus coats the intestinal epithelium, with the mucosal layer being thickest in the colon. The majority of gut microbes are found in this layer, and the mucus acts as an energy source for these bacteria (Sicard et al., 2017; Van Tassell & Miller, 2011). A study in rats in 2000 confirmed that the presence of certain SCFA (acetate, butyrate and propionate) increased mucus production in the colon, while a different group in 2003 used a tissue culture model to demonstrate that SCFA stimulated the expression of the mucin-2 protein via prostaglandin production (Shimotoyodome et al., 2000; Willemsen et al., 2003). Thus, the presence of SCFA will affect the gut microbiome through influence over pH and mucus concentration.

Certain fermented foods have been shown to contain high levels of readily accessible SCFA (van Hylckama Vlieg et al., 2011). As stated above, vinegar contains high levels of acetate, which is capable of influencing the host gut microbiome, although due to its strong taste direct consumption is difficult for most individuals (Darzi et al., 2014). SCFA levels in cheeses vary and are often expressed as free fatty acids (FFA), which are important components for the organoleptic properties of cheese and encompass fatty acids of varying lengths (Fuente et al., 2005). The levels of desirable FFA differ between cheeses, with high levels leading to a rancid off-flavour in varieties such as Cheddar and Gouda (Collins et al., 2003). Italian hard cheeses are considered good sources of SCFA, with short and medium-chain fatty acids comprising 25% or more of total triglyceride content in Parmigiano Reggiano and Grana Padano (Summer et al., 2017). As stated previously, propionibacteria found in Swiss-type cheeses ferment the lactose into both acetate and propionate SCFA, resulting in products rich in these compounds (Moslemy et al., 2016). A study in 2007 examined the efficiency of *Propionibacterium*

freudenreichii, a species commonly used in the production of Swiss-type cheeses, to produce SCFA in rats; one strain in particular was found to significantly enhance the presence of these compounds within the caecum (Lan et al., 2007).

Thus, fermented foods have the potential to impact gut microbiota by modifying levels of particular compounds within food. Polyphenols have been shown to directly impact the microbial composition of the gut, while SCFA can induce a more favourable environment for the growth of beneficial gut microbes or impact mucus concentration, which acts as both an energy source and binding site for gut microbes.

2.6 Potential of Fermented Food Microbiota to Survive and Modulate the Gut Microbiome

As discussed above, fermented foods contain large and diverse microbiomes, many species of which are to be found within the gut microbiota as well; thus, it is reasonable to propose that fermented foods could be a source of these microorganisms. However, in order for this to occur the fermented food microbiota must have the capacity to survive the environmental stresses of the digestive tract. This requires tolerance to low pH and bile, traits used in the selection of probiotic bacteria. Many groups have investigated the microbiota of fermented foods as a source of new probiotic bacteria, and many have found bacteria that are capable of surviving gastric transit, implying that this property may be common among the microbiota found in fermented foods. Potential to survive gastric transit can be investigated using a range of in vitro models and using either purified bacterial cultures grown in laboratory media or by incorporating the test organism into the model food matrix.

A study in 2011 screened *Lactiplantibacillus plantarum* isolates from both Argentinean and Italian cheeses for potential probiotic characteristics (Zago et al., 2011). The most promising isolates were screened for their potential to survive gastric transit by exposure to gastric conditions (pH 2.2 and pepsin) for 90 min followed by 150 min exposure to synthetic duodenal juice (pH 8.0, pancreatin and bile) (Zago et al., 2011). Survival rates for simulated gastric juice were based on the percentages of live cell counts after varying time points relative to initial counts prior to exposure, while bile tolerance was expressed as the proportional cell count increase in differing bile concentrations compared to a bile-free control (Zago et al., 2011). The *Lactiplantibacillus plantarum* strains tested showed excellent resistance to digestive stresses and on average about 40% tolerance to bile; the data reported were the average of three different bile concentrations, including one more than three times higher than physiological conditions, in which case their survival level in vivo may be much higher (Zago et al., 2011). These data imply that these bacteria are capable of surviving gastrointestinal track (GIT) passage, which would then make it possible for them to colonize the gut either transiently or long-term and to interact with the host gut microbiome (Zago et al., 2011). In this case, the bacteria were removed from the

protective matrix of the fermented food, and should these tests be repeated with intact cheese, the results might differ. It is worth noting that *Lactiplantibacillus plantarum* is present in a myriad of fermented foods made from vegetables, meats and dairy products, and is found in the human intestine as well (de Vries et al., 2006). While survival remains strain-dependent, *Lactiplantibacillus plantarum* has been investigated by other authors and has been found to survive well under gastric conditions. Haller and colleagues investigated a number of strains both from fermented foods and of intestinal origin and determined that, while the strains from the intestines performed the best under digestive stresses, the *Lactiplantibacillus plantarum* strains survival rates that are used to ferment fruit were broadly similar (Haller et al., 2001). A study in 2014 involved isolated strains of different Lactobacillaceae genera responsible for fermentation of Croatian white cabbage for the production of sauerkraut (Beganović et al., 2014). While initial LAB counts were approximately 5.95 log CFU/mL, the group identified four *Lactiplantibacillus plantarum*, one *Ln. mesenteroides* ssp. *mesenteroides*/*dextranicum*, and one *Levilactobacillus brevis* strain, all of which were capable of surviving simulated digestion, including low pH, bile salts and digestive enzymes, at rates of above 5 log CFU/mL, resulting in only approximately 1 log CFU/mL reduction following exposure to simulated GIT conditions (Beganović et al., 2014).

Mishra and Prasad exposed *Lacticaseibacillus casei* strains from fermented dairy products to low pH (1.0, 2.0 and 3.0) and high bile (1%–2%), revealing three acid-tolerant strains (although no strains survived at pH 1.0) and strains with varying levels of bile tolerance (Mishra & Prasad, 2005). While the *Lacticaseibacillus casei* strains appear to be less tolerant of simulated digestion, it should be taken into account that they would normally be found in a fermented food matrix, and the bile concentrations used were very high in comparison to physiological conditions (Mishra & Prasad, 2005). While most reports to date have focused on studying bacterial strains isolated from fermented foods, a recent study exposed Cheddar cheese to simulated gastric digestion prior to isolation of NSLAB in an effort to understand whether significant populations of this group of bacteria naturally present in most cheeses could potentially survive gastric transit [160]. The observation from this work was that a population of 10^7 CFU/g could survive simulated gastric transit, a sufficient number to potentially impact the gut microbiome. This research demonstrates the in vitro

potential of Lactobacillaceae strains from fermented foods to survive gastric transit, potentially allowing them to influence the gut microbiota. However, the importance of the fermented food itself to protect the bacteria during digestion needs more consideration.

In other cases, fermented foods are used as a delivery vehicle for probiotic strains that may not be autochthonous for that environment. In 2005, a group investigated the effectiveness of fermented olive varieties (in particular, their skin) at carrying the established probiotic *Lacticaseibacillus paracasei* IMPC2.1 into the intestines of five human subjects, who were given 10–15 olives coated with 10^9–10^{10} CFU of lactobacilli (Lavermicocca et al., 2005). Faecal samples were taken prior to consumption of olives (subjects were told not to ingest any lactobacilli-containing edibles for one week prior to the trial) and at days 10 and 15 post-consumption (Lavermicocca et al., 2005). While the *Lacticaseibacillus* strain was absent in the faecal samples prior to ingestion of the olives, it was detected in four of five of the subjects post-consumption using a combination of Vancomycin-containing agar plates and PCR-Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Lavermicocca et al., 2005). Therefore, this study indicates that the olives were effective fermented food vehicles for safely delivering probiotics through gastric transit (Lavermicocca et al., 2005). A study by Saxelin and colleagues in 2010 investigated the potential survival of the probiotic strains *Lacticaseibacillus rhamnosus* GG and LC705, *Propionibacterium freudenreichii* subsp. *shermanii* JS, and *Bifidobacterium animalis* subsp. *lactis* Bb12 when administered as either capsules or interred in yogurt or cheese (Saxelin et al., 2010). In this study, while no matrix effect was seen for either *Lacticaseibacillus rhamnosus* strain based on counts in faeces, both the *P. freudenreichii* and *B. animalis* strains showed higher survival levels when administered in the yogurt matrix, although counts in the other matrices were still high (log10) (Saxelin et al., 2010). This study indicates that certain matrices may be more suited to certain probiotics than others.

In 2008, yogurt and low-fat cheese matrices were compared as barriers against acid exposure for the probiotic *Lacticaseibacillus casei* 334e, with the added stage of refrigerating both matrices for time periods reflecting their shelf-life (up to three weeks and three months for the yogurt and cheese, respectively) in order to test their ability to sustain probiotics during storage (Sharp et al., 2008). The *Lacticaseibacillus*

strain was inoculated into both fermented foods during the manufacturing process at 10^7 CFU/g and remained at this viable count after storage in both cases (Sharp et al., 2008). This study demonstrated that the low-fat cheese was the better option, as numbers of *Lacticaseibacillus casei* remained higher following exposure to pH 2.0, stabilizing at approximately 10^4 CFU/g after 120 min of exposure in cheese, as opposed to less than 10^1 CFU/g in yogurt after only 30 min (Sharp et al., 2008). A recent study used the INFOGEST 2.0 simulated gastric digestion model to investigate the survival of *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* strains embedded in two dairy matrices, Cheddar cheese and fermented milk (Leeuwendaal et al., 2022). The bacterial strains were added to the two fermented dairy foods during manufacture and thus were distributed, embedded, and protected as would occur naturally. Cheese offered greater protection to the two strains than fermented milk, with log reductions in the range 0.88–1.93 as opposed to 2.36–3.00 following treatment by INFOGEST 2.0. It is relevant to note, however, that 5.38–6.85 log CFU/g of the test bacteria survived the treatment from both dairy matrices, indicating that both are potentially suitable carrier foods for delivery of lacticaseibacillii strains to the intestine. *Limosilactobacillus reuteri* LR92, a strain with known potential probiotic properties, was tested for its ability to survive gastric transit using a fermented vegetable/fruit blended beverage as a matrix (Mauro et al., 2016). Unlike the study by Sharp et al. in 2008, storage in this manner resulted in varying levels of probiotic survival when the blend was run through acid exposure and bile salt tolerance assays in parallel (Mauro et al., 2016; Sharp et al., 2008). While initial levels were about 10 log CFU/mL in both, these decreased by about 5 log cycles when exposed to pH1.5 for 2 h, and only decreased by about 1 log cycle when exposed to bile salts at pH 7.4 for 150 min (Mauro et al., 2016). Thus, the fermented juice blend appears to be better at protecting against stresses present in the small intestines than against those present in the stomach (Mauro et al., 2016). Fermented vegetable/fruit beverages have the added advantage of appealing to vegetarian consumers and those with lactose intolerance issues (Shori, 2016). Therefore, fermented foods can be successfully deployed as vehicles to sustain high numbers of probiotic bacteria during digestion in order to have an effect on the gut microbiota and health.

Few studies that examine the microbial consequences of ingesting fermented foods have been reported in the literature, and those that are available are usually grouped

together with a diet (such as cheese) or with the focus on a probiotic of interest that is present already or more likely has been added in high cell numbers, with the fermented food acting as a vehicle for said strain. It has previously been established that diets dominated by specific macronutrients and deficient in others effect the composition of the gut microbiome, with enterotypes (defining microbiotas based on their dominating taxa) developing when these eating patterns are long-term (Scott et al., 2013; Wu et al., 2011). In general, a diet high in animal protein leads to high numbers of *Bacteroides* and other groups with high bile tolerance being present in the intestines of the human host due to the greater concentration of bile excreted to deal with animal fat digestion, while a high-carbohydrate diet is associated with the *Prevotella* enterotype (David et al., 2014; Wu et al., 2011). Protein fermentation by gut microorganisms has been linked with the production of precursors for toxic and in some cases carcinogenic substances as well as those associated with immune syndromes such as inflammatory bowel disease (Scott et al., 2013). A study performed in Japan by Odamaki et al. attempted to observe the effect of a yogurt drink that had been fortified with the probiotic *Bifidobacterium longum* on a cohort of volunteers who were put onto an animal-based diet for five days, followed by a 'recovery' balanced diet for fourteen days (Odamaki et al., 2016). Three different groups were established, those who were given the fortified yogurt for the duration of the trial (YAB group), those who were only given the yogurt during the fourteen-day 'recovery' period (YB), and those who did not receive any yogurt (CTR) (Odamaki et al., 2016). Following the animal-based diet, the gut microbiome of both YB and CTR groups experienced an increase in the genera *Bilophila*, *Odoribacter*, *Dorea*, and *Ruminococcus*, all of which are associated with either a metabolic disorder (Metabolic Syndrome, Inflammatory Bowel Disease, or Crohn's Disease) or other illness, including colon cancer (Joossens et al., 2011; Natividad et al., 2018; Rajilić-Stojanović et al., 2011; Zackular et al., 2013). Additionally, the relative abundance of *Bifidobacterium* decreased in these two groups (Odamaki et al., 2016). However, the gut microbiomes of the YAB group did not experience the same alterations in abundance as the previously-mentioned bacterial groups (apart from the *Dorea* group), indicating that the probiotic yogurt may have had a stabilizing capacity on the gut microbiota, although the authors stated that this may be attributed to the higher carbohydrate levels ingested in the YAB group (Odamaki et al., 2016). As the strain of *Bifidobacterium* used in this study was known to produce bile salt hydrolase enzymes,

it was proposed that this might limit the growth of the bile-metabolizing groups *Bilophila* and *Odoribacter* (Devkota & Chang, 2015; Göker et al., 2011; Odamaki et al., 2016). While this study did indicate a degree of crosstalk between a strain from the fermented food and the gut microbiota, the other starter and possible adventitious strains present in the yogurt were not mentioned as possible contributing factors.

In 2011, McNulty and colleagues used controlled conditions to ascertain the effects of a specific set of five known bacteria belonging to the family Lactobacillaceae and the genera *Bifidobacterium*, *Lactococcus* and *Streptococcus* on the microbiome of seven sets of female monozygotic human twins while, in a parallel study, gnotobiotic mice were seeded with a model human gut microbiome of known species the genomic sequences of which were available (McNulty et al., 2011). The purpose of using such a model was to lower the potential variability associated with genetic makeup, environment, and diet. With regard to the human trials, monozygotic twins were used due to their near-identical genetic makeup and the fact that their early dietary and environmental experiences are very similar to each other. Additionally, unique microbial subsets have been identified in monozygotic twins that result from long-term exposure to similar environmental factors, reinforcing their usefulness when investigating the effect of food microbes on existing gut populations (Faith et al., 2013). The five bacteria of interest were used as starter cultures to produce a fermented milk beverage which was given to the individuals, who then supplied faecal samples for analysis (McNulty et al., 2011). The mice were not fed the fermented milk beverage, and instead orally received the five strains used to culture it, negating any protective effects the fermented food may have afforded the microbes (McNulty et al., 2011). In any case, the beverage did not alter the human gut microbiome to a significant extent, although the *Bifidobacterium* strain was recovered in the highest amounts from the faecal samples in comparison to its companion starter strains (McNulty et al., 2011). The authors reported that the five strains were no longer detected following cessation of fermented beverage consumption, indicating that detection of the strains of interest during this period was a result of the beverage and not related autochthonous gut strains (McNulty et al., 2011). However, the most interesting results were the metatranscriptome profile changes that occurred in both the mice and human subjects, the majority of which resulted in upregulation of genes involved in plant polysaccharide metabolism (McNulty et al., 2011). This may indicate

that the microbiome of the fermented milk impacted the gut microbiome despite not being retained once consumption of the beverage stopped. Thus, the food and gut microbiome exhibited a degree of interaction that allowed a change in gene expression.

Another study reported the impact of yogurt containing deep sea water (DSW, a food supplement) on the gut microbiome of clinical subjects (Kang et al., 2015). This trial contained three groups: one which received the DSW-fortified yogurt, one which received non-fortified yogurt, and a control group that received only normal water (Kang et al., 2015). In terms of microbiome perturbation, investigation of the intestines of the mice revealed an increase in LAB and total bacteria counts in both yogurt groups that was significantly higher than the water-only control group (Kang et al., 2015). While this does seem to indicate that the fermented food had an impact on the gut microbiome, it is worth mentioning that the actual strains used as starter cultures in the yogurt (a *Lactiplantibacillus pentosus* strain and a *Pediococcus pentosaceus* strain) were not tracked through the experiment, and it is unclear whether these specific strains comprised a proportion of the LAB counts or whether they were even able to survive the initial gastric transit (Kang et al., 2015). Additionally, the addition of an unfermented milk control would have been useful to confirm that the microbiome changes were not contributed by nutrients already present in the milk.

Although the relative abundance of fermented food microbiota is not uniform, the gut microbiome is exposed to and interacts with these allochthonous bacteria following ingestion either transiently or on a more long-term basis (Lang et al., 2014; Plé et al., 2015). The above studies suggest that it is possible for the microbiome of fermented food products to have varied effects on the host gut microbiome, with many of the resident bacteria present in the food microbiome associated with beneficial responses in the gut and able to harbour potentially probiotic microorganisms. However, in order to establish with greater confidence that the microbiome of fermented foods can survive gastric transit, more consistent and standardized models of the human gastrointestinal tract, such as the INFOGEST 2.0 model, need to be applied and the results confirmed in human feeding studies.

2.7 Conclusions

Fermented foods have an important place in human history, and while their primary function was originally shelf life extension of seasonal foods, health benefits associated with their consumption have long been recognized (Metchnikoff, 1907). Almost all the primary foodstuffs consumed by humans can be subjected to fermentation (Tamang et al., 2016). Many of these have regional origins linked to the prominent foods produced in different localities. Local modification and adaptation of the fermentation process have greatly added to the diversity of fermented foods, as in the case of cheese; while cheese is made using milk from a limited number of mammal species, variations in fermentation technology have resulted in >1000 cheese varieties (Beresford et al., 2001). As described above and summarized in Table 1, there are six primary food fermentation pathways, and the starter microorganisms responsible for these are well characterized. However, a diverse range of non-starter microbes are associated with most fermented foods, the full extent of which is only now becoming apparent with the application of high-throughput DNA sequencing technologies (Macori & Cotter, 2018). Recently, a freely accessible online database has been established that functions as an archive for the annotated genomic, metagenomic, transcriptomic, and metataxonomic information of microbes associated with fermented foods, enabling evaluation of individual strains as potential starter cultures (Whon et al., 2021). This tool has the potential to reveal the full diversity of fermented food-associated microbes, and has useful practical applications in the fermented food industry.

As originally documented by Metchnikoff, fermented foods are being investigated for their ability to exert health benefits. There is growing scientific evidence to support this contention, including data demonstrating that fermented foods can be more easily digested due to partial protein digestion during fermentation as well as indications that they can be enriched in certain vitamins and antioxidants. It has been demonstrated that fermentation can result in the release of bioactive peptides, for example the well documented ACE inhibitory peptides and the production of bacterial EPS, which can help to reduce cholesterol. Controlled human clinical trials are the gold standard used to confirm benefits associated with foods or pharmaceuticals; however, while a number of studies have demonstrated health benefits associated with

fermented foods (for a detailed review, see Marco et al., 2017), more such studies are required.

The human gut microbiome has received much attention in recent years, with increasing evidence that it impacts both physical and mental health (Selhub et al., 2014; Valdes et al., 2018) and that many metabolic disorders are associated with disruption to the gut microbiome (Hsiao et al., 2013; Kostic et al., 2014). Lifestyle, including diet, can impact the gut microbiome, and there is increasing interest in the potential of exploiting foods to positively modulate it (De Filippis et al., 2016; De Filippis et al., 2018; Singh et al., 2017). Fermented foods offer an opportunity to positively impact the gut microbiome by either (I) providing nutrients to promote or inhibit members of the gut microbiome, or (II) members of the food microbiome establishing residence in the gut and/or interacting with the resident gut microbiome. However, few studies to date have specifically investigated the impact of fermented food consumption on the gut microbiome. Certain bioactive compounds produced by the microbes within food, including polyphenols and SCFAs, can have beneficial effects when consumed. Certain microbial strains found in food are capable of surviving digestion, and fermented foods can act as useful vehicles to carry probiotic strains safely into the gut. However, there are very few studies which focus on the potential of microbiota from fermented foods to impact the gut microbiome; one study investigating the consequences of yoghurt consumption observed higher levels of LAB associated with the gut contents (Kang et al., 2015). In addition, most studies of the gut microbiome focus on the microbiota of the large intestine, due primarily to ease of sampling; however, it is likely that fermented foods have more influence on the microbiome in the small intestine, as this location exhibits a higher proportion of LAB and other more aerotolerant bacteria often associated with fermented foods. The possibility that fermented foods impact the gut microbiome is intriguing, and merits more study and additional efforts to include investigations of the small intestine.

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Chapter 3

The Potential of Non-starter Lactic Acid Bacteria from Cheddar Cheese to Colonise the Gut

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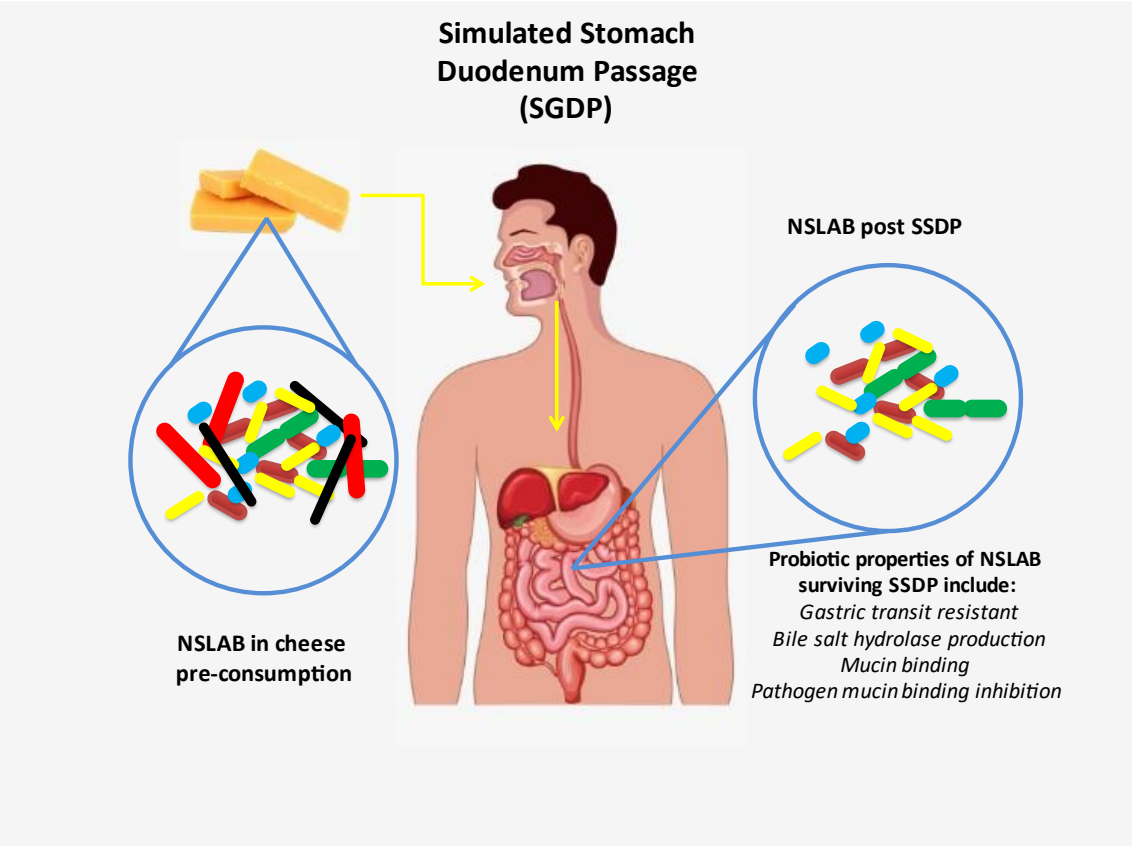
Abstract

This study was undertaken to assess the potential of Non-Starter Lactic Acid Bacteria (NSLAB) from Cheddar cheese to survive gastric transit and display probiotic-related traits including bile salt hydrolase activity, the ability to adhere to the gut epithelium and inhibition of enteropathogen binding. Populations of NSLAB, up to 10^7 CFU/g per cheese were recovered following exposure of cheese to Simulated Stomach Duodenum Passage (SSDP) conditions. A total of 240 isolates were randomly selected from twelve Cheddar cheeses and assessed probiotic traits. Two strains *Lactobacillus paracasei* DPC 7150 and *Lactobacillus rhamnosus* DPC 7102 showed the most probiotic potential. The *Lb. paracasei* and *Lb. rhamnosus* strains displayed adhesion rates of 64% and 79%, respectively and inhibited binding of pathogenic *Escherichia coli* by >20%. This research demonstrates that Cheddar cheese harbours potentially beneficial bacteria, a large portion of which can survive simulated digestion and potentially exhibit health beneficial effects once ingested.

Graphical Abstract



3.1 Introduction

Cheese is manufactured using a mixture of four major ingredients (milk, rennet, microorganisms and salt), with the assortment of cheese types available being a result of variations in this mix, such as the concentration of salt used, the source of the milk (cow, sheep or goat's milk) or the actual species of microbes added, together with variations in the manufacturing and ripening protocols (Beresford et al., 2001). While specific microbial cultures, referred to as 'starter' bacteria, are introduced during cheesemaking for the necessary production of acid, this is not the case for all bacteria present (Fox et al., 2004). Non-starter lactic acid bacteria (NSLAB) are microorganisms that grow within cheese during ripening but are not deliberately added and are not required for acid production in the initial cheese manufacturing process (Beresford, 2003). The sources of NSLAB include autochthonous milk microbes capable of surviving the pasteurisation process and those found in the manufacturing plant, with NSLAB populations eventually outcompeting the starter cultures and typically reaching between $10^7 – 10^9$ colony forming units (cfu) per gram of cheese at the end of the ripening period (Gobbetti et al., 2015). The biochemical reactions undertaken by these NSLAB are responsible for various flavour compounds present in maturing cheeses and are, therefore, responsible for important organoleptic characteristics of cheese (Settanni & Moschetti, 2010).

As cheese may contain high NSLAB populations upon ingestion, the fate of these microorganisms is of interest due to their ability to potentially impact human health or the human gut microbiome. Therefore, the capacity of cheese NSLAB populations to survive upper gastrointestinal environments should be taken into consideration when evaluating health implications of cheese intake. The cheese matrix itself has been found to promote survival of deliberately added strains of potential probiotic bacteria, providing a buffering effect against acid stresses encountered during digestion and supplying a dense, high-fat protective barrier for the probiotic strains against the unfavourable digestive tract environment (Gomes da Cruz et al., 2009). Particular attention has been focussed on the *Lactobacillus* genus, which are the dominant NSLAB found in Cheddar and most other varieties of long ripened cheese and which have also had several strains granted probiotic status (Fijan, 2014; Swearingen et al.,

2001). Probiotics have been defined by the Food and Agricultural Organisation of the United Nations (FAO)/World Health Organisation (WHO) as being 'live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host' (Araya et al., 2002).

Various *in vitro* and *in vivo* assays have been used to determine the capability of selected NSLAB strains to survive gastric transit, most of which involve the ability to survive acid stress and bile tolerance, as well as their potential to express characteristics once within the gut that have health benefits for the host (Haller et al., 2001; Maragkoudakis et al., 2006; Papanikolaou et al., 2012). As well as bile tolerance, the ability of strains to deconjugate bile via bile salt hydrolase (BSH) enzymes is a desirable trait as, as well as potentially conferring a competitive advantage to these organisms within gut environment, BSH enzymes have also been linked to reduced serum cholesterol levels in the host organism (Begley et al., 2006). The ability of the surviving strains to colonise the gut, either transiently or on a more long-term basis, is also considered a useful quality as it allows longer exposure of the host to any beneficial effects that these strains may express. The ability of strains to adhere to the intestinal lining once ingested is, therefore, a highly sought-after quality, with either whole cell lines or intestinal mucus used as the binding surface (Servin & Coconnier, 2003). Additionally, the ability of strains to inhibit the binding of food-borne pathogens can also be tested for *in vitro*, with *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* being typical antagonistic targets (Balamurugan et al., 2014; Yu et al., 2011). In addition, some bacteria, including specific strains of NSLAB are also capable of producing exopolysaccharides (EPS), high molecular-weight polymers produced from sugars, which can affect their host by modulating immune responses (Ryan et al., 2015).

To the best of our knowledge, all studies to date that have sought to elucidate the potential of NSLAB isolates to survive gastric transit and display established probiotics traits have started with individual isolates selected from cheese following conventional plating techniques which do not include any step to select for the capacity to survive gastric exposure. These techniques start by separating the bacteria from the cheese matrix by blending with a buffer followed by serial dilution and plating. As the aim of this study was to evaluate the ability of NSLAB of the *Lactobacillus* genus isolated from

Cheddar cheeses to survive gastric passage and to determine if strains capable of doing so expressed traits beneficial to the host, the first step in our selection involved exposure of NSLAB populations separated from the cheese matrix to simulated stomach duodenum passage (SSDP) that includes exposure to high acidic conditions and bile acids, prior to selective plating. Strains surviving this selection procedure were then investigated via subtractive screening for their probiotic potential using the *in vitro* tests listed in the Food and Agriculture Organisation (FAO)-guidelines (2002) for screening of potential probiotics (Food and Agriculture Organization of the United Nations, 2006).

3.2 Materials and Methods

3.2.1 Bacterial Strain Source and Storage

Twelve Irish Cheddar Cheeses were selected for this study produced from both raw and pasteurised bovine milk. Nine of these were commercial brands and three were sampled from Cheddar produced at pilot scale (Moorepark Technology Limited, Teagasc Moorepark) for a separate study. All NSLAB isolated from these cheeses were stored in MRS broth (BD Difco™) with 25% Glycerol (Sigma Aldrich). Long-term stocks were stored at -80 °C. Working stocks were kept at -20 °C, and were propagated twice in MRS broth, then twice on MRS plates for activation and to ensure purity prior to testing. All MRS broth/agar was prepared with 0.05% (w/v) L-Cysteine-HCl.

Enteropathogenic *E. coli* (EPEC) 0111:H2 (strain NCTC 8007) was obtained from the National Collection of Type Cultures (NCTC; London, UK) and was stored at -20 °C in BHI broth with 50% glycerol (v/v). Prior to the Pathogen Exclusion Assays (2.10 below), *E. coli* was grown overnight in BHI broth at 37 °C, aerobically.

3.2.2 Screening for NSLAB that Survive Simulated Stomach Duodenum Passage

The ability of NSLAB to survive passage through the upper gastrointestinal tract was tested *in vitro* via Simulated Stomach Duodenum Passage (SSDP), as reported by Pisano et al. (Pisano et al., 2014). This protocol exposes the NSLAB to the acidic conditions of the stomach (pH 3.0) followed by contact with bile acids as would be experienced in the duodenum, the two primary tests identified by the FAO for the selection of strains with probiotic potential. 5-10g of each cheese (obtained with a sterile cheese trier) was diluted 1:10 in sterile 2% (w/v) trisodium citrate (VWR™ 27833.260), followed by 5 minutes of maceration with a stomacher (BagMixer® 400 P, Interscience). 10 mL of the resultant slurry was centrifuged at 4000 rpm for 5 minutes, followed by aspiration of the supernatant. The pellet was resuspended in 10 mL of simulated gastric juice (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$, 0.3% pepsin, at pH 3.0) and incubated for 90 minutes at 37 °C, with shaking for peristalsis simulation. 17.5 mL of synthetic duodenum juice (6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, and 0.1% pancreatin, at pH 7.4) and 4 mL of 10% (w/v) Ox gall powder

(Sigma) containing bile acids were then added to the cell suspension, simulating passage into the upper intestinal tract, and incubation was continued for a further 90 minutes at 37 °C, with shaking. The suspension was then centrifuged, the supernatant discarded, and the pellet was resuspended in maximum recovery diluent (MRD) (Oxoid CM0733). Survival rates of the bacteria following SSDP were determined via plating serial dilutions in MRD of the samples in duplicate prior to (Time 0, before addition of simulated gastric juice) and following (Time 180, following the full 180 minutes of exposure to both simulated gastric and duodenum juice) exposure to SSDP conditions. Samples were plated on MRS, in duplicate, and incubated for 48 hours at 37 °C, anaerobically. Screening on each cheese was performed in triplicate.

3.2.3 Pulsed Field Gel Electrophoresis

Following SSDP, 20 colonies from countable MRS plates for each cheese were randomly selected, purified and DNA fingerprint profiles were generated using Pulsed Field Gel Electrophoresis (PFGE) as described by Stefanovic et al. (Stefanovic et al., 2017), with modifications. Bacterial cultures were inoculated at 1% and grown overnight in 10 mL MRS broth containing 0.02 M threonine, anaerobically. 0.5mL of each overnight culture was centrifuged at 12000 rpm for 5 minutes, the supernatant aspirated and the pellet resuspended in 0.5 mL of Buffer 1 (0.01 M Tris-HCl, 1 M NaCl, pH 7.6). The cell suspension was re-centrifuged, the supernatant aspirated and the pellet resuspended in 0.3 mL Buffer 1, which was then mixed with an equal volume of 2% low melting point agarose in 0.125 M EDTA (pH 7.6) and pipetted into PFGE plug moulds and allowed to solidify at room temperature. Plugs were left overnight (16-24 hours), rocking, at 37 °C in 1 mL of previously prepared EC Buffer (0.1 M EDTA, 1. 0 M NaCl, 1% [w/v] N-Lauroylsarcosine sodium salt, 0.006 M Tris-HCl, pH 7.6) with 10 mg/mL Lysozyme and 20 Units/mL Mutanolysin. Subsequently, the EC buffer was aspirated and replaced with 1 mL of Buffer 2 (0.5 M EDTA, 1% (w/v) N-Lauroylsarcosine sodium salt, pH 8.0) with 0.5 mg/mL Proteinase K, and left overnight (stationary) at 55 °C. Plugs were washed twice in TE 10/1 Buffer (0.001 M EDTA, 0.01 Tris-HCl, pH 8.0) with 0.001 M phenylmethylsulphonyl fluoride (PMSF) for 1 hour each at 37 °C (stationary) and were then stored in Eppendorf tubes containing 1 mL TE 10/100 Buffer (0.1 M EDTA, 0.01 Tris-HCl, pH 8.0) at 4 °C until required. Plugs were then cut into 1-2 mm slices and washed thrice in 1 mL TE 10/0.1 (0.0001 M EDTA, 0.01 Tris-HCl,

pH 8.0) at room temperature for 30 minutes each with gentle rocking. Slices were subsequently transferred to Eppendorf tubes containing 0.1 mL 1X Buffer Tango (Thermo Scientific) for minimum 30 minutes at 4 °C, followed by the addition of 0.4 µL of *Asc*I restriction enzyme (Thermo Scientific) and incubation continued for 24 hours at 37 °C (stationary). 0.5 mL of 0.5 M EDTA was added to the slices to deactivate the enzyme, followed by loading of the slices into wells of a 200 mL 1% agarose Pulsed Field Certified™ (Bio-Rad) gel prepared with 0.5X dilution Tris-borate EDTA (TBE) buffer (55 g/L Boric Acid), 40 mL/L 0.5 M EDTA [pH 8.0], 108 g/L Tris). Gels were run using a CHEF-DR® II PFGE apparatus (Bio-Rad) in 2.3 L of the same 0.5X TBE Buffer using the following parameters: Initial Switch Time 1 Second, Final Switch Time 20 Seconds, 6 V/cm, for 16 hours, at 14 °C. Resulting gels were stained with 10 mg/mL ethidium bromide (E1510 Merck) for 1 hour, followed by 2 destaining washes in distilled water for 40 minutes each. Gels were photographed using an Alpha Imager® 3400 (Alpha Innotech Corp). Lambda PFG Ladder (New England BioLabs® Inc.) was loaded with every gel as a reference marker.

3.2.4 PFGE Fingerprint Profile Analysis and Dendrogram Assembly

BioNumerics® 7.5 Software (Applied Maths) was used to analyse the PFGE images, as per the method described by Stefanovic et al. (Stefanovic et al., 2017). Dendrograms were assembled using the Unweighted Pair Group Method Using Average Linkage (UPGMA) distance matrix method and curve-based Pearson correlation. Profiles that had ≥ 95% similarity were grouped as the same strain (Shutt et al., 2005).

3.2.5 16S rRNA Gene Sequencing

Cultures were sent to GENEWIZ (Hope End, Takeley, Essex, CM22 6TA, United Kingdom) for DNA extraction, amplification and 16S rRNA gene sequencing. Isolated colonies previously grown on MRS agar for 48 hours at 37 °C were transferred to cryotubes (Thermo Scientific) containing 1 mL MRS agar via an inoculation needle. Following 24 hours growth at 37 °C, the cultures in the cryotubes were sent to GENEWIZ. Universal 16S rRNA primers 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-ACGGCTACCTTGTTACGACTT-3' (reverse) were used to generate PCR products of approximately 1.4 KB, the DNA sequence of which were then obtained and sequenced

following GENEWIZ DNA sequencing instructions. FASTA sequencing data was analysed using Lasergene 8 software (DNAStar Inc., Madison, WI), specifically the SeqMan and EditSeq tools, and resulting sequences were compared with pre-existing genomic data using the nucleotide basic local alignment search tool (BLASTn Suite) on the National Centre for Biotechnology Information (NCBI) server.

3.2.6 Bile Salt Hydrolase Activity

A modified version of the method described by Pisano et al. (2014) was used for this analysis (Pisano et al., 2014). NSLAB strains were grown overnight in each of (i) MRS broth, (ii) 0.3% (w/v) bile modified (m) MRS broth and (iii) 0.5% (w/v) mMRS broth. Modified MRS containing two levels of physiologically relevant bile concentrations were used in parallel with regular MRS to determine whether BSH activity could be induced (Ruiz et al., 2013). Following overnight incubation, BSH activity was screened for by loading 0.025 mL of each of the three variants of overnight NSLAB culture into wells (5 mm in diameter) bored in mMRS agar plates containing 0.03% (w/v) bile (Difco™ Oxgall, BD), 0.375 g/L $CaCl_2$ and 1% agar, in duplicate. The resulting bile plates were incubated for 72 hours at 37 °C, anaerobically. The presence of opaque haloes of deconjugated bile surrounding wells identified strains expressing BSH enzyme. *Lactobacillus reuteri* NCBI 30242 cultures were used in parallel as a positive control due to their documented ability to produce BSH enzymes.

3.2.7 Antibiotic Resistance Profile

VetMIC™ plates (National Veterinary Institute of Sweden, Uppsala, Sweden) were used to determine the resistance/susceptibility of BSH-producing strains to antibiotics of importance as listed by the European Food Safety Authority in 2012: Ampicillin, Vancomycin, Gentamicin, Kanamycin, Streptomycin, Erythromycin, Clindamycin, Tetracycline and Chloramphenicol (Aquilina et al., 2012). Fresh *Lactobacillus* cultures were streaked on MRS plates and incubated for 48 hours at 37 °C, anaerobically. Colonies were then suspended in MRD, and a portion of this was transferred to 10 mL ISO-MRS broth (90% Iso-Sensitest broth [Thermo Scientific], 10% MRS broth) to achieve a final inoculum of ≈$5x10^5$ cfu/mL. Wells of both VetMIC™ Lact-1 and Lact-2 plates were seeded with 0.1 mL from the ISO-MRS cultures, sealed with clear film (as

provided with the VetMIC™ plates) and incubated anaerobically at 37 °C for 48 hours. Growth of *Lactobacillus* cultures in the form of pellets at the bottom of the wells was examined using a backlight (colony counter) and the minimum inhibitory concentration (MIC) was recorded as the lowest concentration able to completely inhibit visible growth. Experiments were repeated 3 times.

3.2.8 Cell Culture and Establishment of Co-culture

Caco-2 and mucus-producing HT29-MTX cells were grown in parallel and a co-culture was established as an intestinal model using the method reported by Yi et al., with modifications (Li et al., 2017). Caco-2 and HT29-MTX cultures were obtained from Istituto Zooprofilattico Sperimentale di Brescia (Italy) and Public Health England General Cell Collection (ECACC 12040401), respectively. Both cells lines were resuscitated from Liquid N_2 and maintained separately in Dulbecco's Modified Eagle's Medium (DMEM) medium (Merck D5796) supplemented with 10% Foetal Bovine Serum (FBS) (Merck F7524), 1% non-essential amino acids (NEAA) (M7145), 1% antibiotic solution (100 U/mL penicillin, 100 mg/mL streptomycin) (Merck P0781) and 2 mM L-glutamine (Merck 59202C) and were incubated at 37 °C in 5% CO_2 in a humidified atmosphere. Cells were sub-cultured into 75-cm^2 tissue culture flasks (Merck C7231) every 2-3 days, once a confluence of ≥80% was reached. For co-cultures, Caco-2 and HT29-MTX were inoculated at a ratio of 3:1 onto the apical membrane of the Corning® Transwell® Polyester Membrane Cell Culture Inserts (Merck CLS3460) with a total of $6x10^4$ cells per well. Co-cultures were allowed to reach confluence, differentiate and (in the case of the HT29-MTX cells) produce mucus for 21 days. Media was changed for both apical and basolateral layers every 2-3 days, and transepithelial electrical resistance (TEER) measurements were taken prior to media changes to monitor the integrity of the developing monolayer. One day prior to Bacterial Adhesion Assays and Pathogen Exclusion Assays, media in the Transwells was replaced with antibiotic-free, complete DMEM.

3.2.9 Adhesion Assays

Mucus adhesion assays were carried out as per Morrin et al. (2019), with modifications (Morrin et al., 2019). Caco-2 cells were used between passages 41-53, while HT29-MTX

cells were used between passages 66-79. *Lactobacillus* cultures were grown overnight in MRS broth and the O.D. 600 measurement was taken via a spectrophotometer. This reading was used to determine the necessary volume required for an O.D. 600 reading of 0.4 in 10 mL, followed by centrifugation of the appropriate volume per strain at 5000 rpm for 5 minutes. Cultures were resuspended in 10 mL un-supplemented DMEM and incubated anaerobically at 37 °C for 2 hours, to reach a final O.D. of 0.5 (≈ $1x10^8$ cfu/ml). Quantification of the exact CFU/mL per bacterial suspension was enumerated via serial dilutions and spread-plating onto MRS, followed by anaerobic incubation for 48 hours at 37 °C. Media from apical and basolateral layers of Caco-2/HT29-MTX co-culture wells were aspirated and two phosphate buffered saline (PBS) (Merck D8537) washes were performed, followed by complete aspiration of the used PBS in both cases. Subsequently, 0.5 mL (≈ $5x10^7$ cfu/ml) of each bacterial culture was added to separate co-culture wells (in triplicate) and were incubated for 2 hours at 37 °C, anaerobically. Post incubation, wells were washed thrice with PBS for removal of loosely or non-adherent bacteria and 0.5 mL 0.1% Triton X100 (Merck T8787) was added to each well and left at room temperature for 15 minutes to allow for lysis of the eukaryotic cells. Serial dilutions of the lysates were prepared and plated via spread-plating on MRS. Following anaerobic incubation for 48 hours at 37 °C, CFU/mL were enumerated and % adherence of each bacterial culture was determined using CFU/mL of the original suspensions calculated previously ([CFU/mL of adherent bacteria ÷ CFU/mL of total initial bacteria] x 100). Adhesion assays were performed in triplicate. *Lb. rhamnosus* GG (ATCC 53103) cultures were used in parallel as a positive control.

3.2.10 Pathogen Exclusion Assays

The ability of two NSLAB strains to inhibit binding of an enteric pathogen to cocultured cells was examined using an exclusion model. Pathogen Exclusion Assays were carried using the Adhesion Assay method (2.9 above), with minor modifications. Caco-2 cells were used between passages 39-43, while HT29-MTX cells were used between passages 61-66. Overnight *E. coli* cultures in BHI broth were adjusted to an O.D. 600 of 0.01 (≈$8x10^6$ CFU/mL), centrifuged at 10,000 rpm for 5 minutes and resuspended in DMEM. Quantification of the exact CFU/mL per bacterial suspension was enumerated by serial dilutions and spread-plating onto Eosin Methylene Blue (EMB) Agar (Oxoid

0069), followed by aerobic incubation for 24 hours at 37 °C. NSLAB strains were incubated anaerobically at 37 °C with the co-cultures for 1 hour before being washed twice with PBS (for removal of free or loosely bound *Lactobacillus*) followed by the addition of 0.5 mL (≈4x10^6 CFU/mL) of the *E. coli* suspension and another hour of incubation under the same conditions. The cells were lysed using the same procedure as before, with serial dilutions being plated on EMB agar for *E. coli* enumeration. Controls included wells with exclusively *Lactobacillus* or *E. coli* cultures that were incubated for 2 hours. Percentage exclusion of *E. coli* was calculated by comparing the CFU/mL of *E. coli* that adhered to the co-culture when exclusively present in the wells versus when present in conjunction with the NSLAB strain (100 – {[CFU/mL of adherent *E. coli* in the presence of NSLAB ÷ CFU/mL of adherent *E. coli* exclusively] x 100}). Pathogen Exclusion Assays were performed in triplicate.

3.2.11 Exopolysaccharide Production

Ruthenium red milk agar was used to determine whether *Lactobacillus* strains were capable of producing EPS, as per the method by Stefanovic and McAuliffe (Stefanovic & McAuliffe, 2018). Freshly grown cultures were streaked on ruthenium red milk agar plates (10% reconstituted skim milk, 0.5% yeast extract, 0.08% ruthenium red [Sigma R2751], 1.5% agar), in triplicate, and incubated for 48 hours at 37 °C, under anaerobic conditions. White colonies indicated an EPS-producing strain, while colonies that remained pink indicated a negative result. The *Lactobacillus paracasei* DPC1116 strain, an established EPS-producer, was used in parallel as a positive control. EPS production experiments were performed in triplicate.

3.2.12 Whole Genome Sequencing and Annotation

Genomic DNA from *Lb. rhamnosus* DPC7102 and *Lb. paracasei /casei* DPC 7150 was isolated from overnight cultures grown in MRS broth at 37 °C using the DNeasy UltraClean Microbial Kit (Qiagen), as per the included protocol, and the quantity was measured using the NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific). Contig construction was provided by Microbes NG (http://www.microbesng.uk). Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol, with two modifications: 2

nanograms of DNA were used as input and PCR elongation time was set to 1 minute. Quantification and library preparation of DNA was carried out on a Hamilton Microlab STAR automated liquid handling system. Kapa Biosystems Library Quantification Kit for Illumina was used for quantification of pooled libraries on a Roche light cycler 96 qPCR machine. The Illumina HiSeq was used to sequence genomic libraries using a 250bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger et al., 2014). *De novo* assembly was performed on samples using SPAdes version 3.7 (Bankevich et al., 2012). Contigs were annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Haft et al., 2018). Average Nucleotide Identity (ANI) and OrthoANI values for both strains were calculated using the online tools from Kostas Lab and EZ BioCloud, respectively (Rodriguez-R & Konstantinidis, 2016; Yoon et al., 2017). ResFinder 3.2, VirulenceFinder 2.0, and PathogenFinder 1.1 databases were used for *in silico* analysis of potential virulence and acquired antimicrobial resistance genes (Cosentino et al., 2013; Joensen et al., 2014; Zankari et al., 2012). Where necessary, *Lb. rhamnosus* GG (NC_013198.1) and *Lb. paracasei* ATCC 334 (NC_008526.1) were used as reference strains for *Lb. rhamnosus* 7102 and *Lb. paracasei* 7150, respectively.

3.2.13 Statistical Analysis

Statistical analysis and graphs were generated using the IBM SPSS® software platform for Windows (Ver. 26). Adhesion results are expressed as a mean ±SD of the results of three independent assays conducted in triplicate. Tukey's Honest Significant Difference test was used to determine statistically significant differences between the control and test strains, where $p \leq 0.05$ was considered significant.

3.3 Results

3.3.1 Selection and Identification of NSLAB Demonstrating Ability to Survive SSDP

Screening of NSLAB from 12 Irish Cheddar cheeses was carried out based on the ability of the bacteria to survive SSDP which includes exposure to gastric pH levels and bile acids, the two primary tests identified by the FAO for the selection of strains with probiotic potential. NSLAB populations ranged from 2.26×10^5 to 1.04×10^8 CFU/g prior to SSDP. Exposure to SSDP resulted in population reductions in all cases with log reduction ranging from 0.03 to 2.84 being observed (**Table 1**).

In an effort to select a representative population of acid resistant, bile acid tolerant NSLAB, twenty colonies were randomly selected and purified from the post SSDP MRS plates for each cheese resulting in 240 isolates. These were then subjected to a subtractive screening methodology the first step of which was the generation of PFGE profiles for each isolate in an effort to elucidate how many unique strains were present in each cheese (Pisano et al., 2014). Dendrograms were generated, one for each cheese, and any isolates with a ≥ 95% similarity in the same cheese were grouped as a single strain and only one isolate from each cluster was picked for subsequent analysis. Using this approach, 76 unique strains were identified (**Table S1**). The number of individual strains identified in each Cheddar cheese varied considerably, with the Bionumerics software identifying up to 12 different bacterial strains in some cheeses. Conversely, Cheddar cheeses 5, 10 and 12 were found to only contain one strain each capable of surviving SSDP. A representative isolate from each strain cluster was selected for further study and each was assigned a unique Teagasc DPC culture collection number (7081-7156, inclusively). 16S rRNA gene sequence analysis identified the strains as constituting *Lb. paracasei/casei* (38 strains), *Lb. curvatus* (13 strains), *Lb. plantarum* (12 strains), *Lb. helveticus* (6 strains), *Lb. rhamnosus* (4 strains), *Lb. coryniformis* (2 strains) and *Streptococcus thermophilus* (1 strain). While a common NSLAB species was not isolated from all cheeses, *Lb. casei*/*paracasei* isolates were isolated from ten of the twelve cheeses. *Lb. plantarum* was the second most common species of NSLAB capable of surviving SSDP, with isolates being found in three of the twelve cheeses. *Lb. curvatus* and *Lb. helveticus* strains were found in two cheeses,

while all the *Lb. coryniformis* and *Lb. rhamnosus* strains originated from cheese 8 and cheese 6, respectively (**Table S1**).

		NSLAB CFU/g		
Cheese	Milk	Average CFU/g		Log Reduction
		Pre SSDP	Post SSDP	
1	Raw	6.56E+06	6.13E+06	0.03
2	Raw	1.45E+07	8.66E+06	0.22
3	Pasteurized	9.10E+06	5.22E+06	0.24
4	Pasteurized	2.26E+05	1.26E+05	0.25
5	Pasteurized	1.40E+08	1.19E+07	1.08
6	Pasteurized	6.30E+07	5.83E+07	0.03
7	Pasteurized	6.61E+07	1.60E+05	2.62
8	Pasteurized	2.84E+07	1.75E+05	2.21
9	Pasteurized	4.23E+07	7.30E+04	2.74
10	Pasteurized	5.91E+07	2.53E+06	1.37
11	Pasteurized	1.08E+07	1.55E+04	2.84
12	Pasteurized	6.18E+06	2.44E+06	0.40

Table 1. NSLAB population numbers in Cheddar cheese pre- and post- SSDP.

3.3.2 Bile Salt Hydrolase Screening

BSH activity was detected in 30 of the 76 strains, although not all tested positive for activity under all three culture conditions (**Table 2**). All 30 BSH-positive strains produced BSH when incubated overnight in normal MRS, although the diameters of the haloes surrounding the wells differed. None of the *Lb. curvatus* or *Lb. helveticus* strains produced deconjugated bile salt haloes, and neither did the one *S. thermophilus* strain tested. In contrast, only 1 of the 12 *Lb. plantarum* strains was incapable of BSH production. All 11 of the BSH positive *Lb. plantarum* strains were capable of producing deconjugated bile haloes with diameters of ≥ 2.0 cm when previously incubated in normal MRS and the majority reproduced haloes of equal size or greater when previously inoculated in MRS broth containing bile (with the exceptions being 7094 and 7095). Of the 38 *Lb. casei/paracasei* strains tested, only 17 exhibited BSH activity. All BSH positive *Lb. casei/paracasei* strains were capable of producing haloes when previously incubated in standard MRS, but this activity was not always retained when previously grown in broth containing bile. A single strain each of *Lb. rhamnosus* (out of a possible 4 strains) and *Lb. coryniformis* (1 of 2 strains) also produced BSH activity, both of which showed consistent BSH activity regardless of which culture conditions were used.

		Bile Salt Hydrolase Activity		
Species	Strain	Incubation Conditions		
		MRS	MRS + 0.3% Bile	MRS + 0.5% Bile
Lb. casei/paracasei	7086	+++	+++	+++
	7087	++	+	-
	7091	++	-	-
	7100	++	++	++
	7104	++	+	++
	7110	++	++	++
	7111	++	++	++
	7143	++	+	++
	7145	+	+	++
	7149	++	++	+
	7150	++	-	-
	7151	++	-	-
	7152	+	-	++
	7153	++	++	-
	7154	++	-	-
	7155	++	-	-
	7156	++	-	-
Lb. plantarum	7081	+++	++++	+++
	7083	++++	++++	+++
	7094	+++	++	++
	7095	+++	+++	++
	7096	+++	+++	+++
	7097	+++	+++	+++
	7126	++++	+++	+++
	7132	++++	+++	+++
	7133	+++	+++	+++
	7134	++++	+++	+++
	7141	+++	++++	++++
Lb. rhamnosus	7102	++	++	++

Lb. coryniformis	7127	++++	+++	+++
Positive Control*	30242	++++	++++	++++

Table 2. BSH activity of selected NSLAB strains in absence of or post exposure to physiological levels of bile. + Deconjugated bile halo with diameter of < 1.0 cm, ++ Deconjugated bile halo with diameter of ≥ 1.0 cm, +++ Deconjugated bile halo with diameter of 1.0-2.0 cm, ++++ Deconjugated bile halo with diameter of 2.0-3.0 cm, * *Lb. reuteri* NCIMB 30242

3.3.3 Antibiotic Resistance Profiles

The 30 strains displaying BSH activity were tested for antibiotic sensitivity. Ten were found to be resistant to antibiotics at levels higher than those specified by the EFSA in 2012 (**Table 3**). All tested strains exhibited resistance to vancomycin (which is common among lactobacilli) at the highest concentration (128 μg/mL) provided in the assay; however, susceptibility levels to this antibiotic are not required by the EFSA and were, therefore, recorded as such (**Table 3**). The MICs of the other 20 *Lactobacillus* strains were always equal to or lower than the acceptable cut-off values and thus, these were taken forward to the next stage of the subtractive screening protocol. The most prominent resistance phenotype expressed in the resistant lactobacilli was the ability to grow in the presence of high concentrations of chloramphenicol, with 8 of the 10 isolates having MIC values greater than the EFSA cut-off values. Tetracycline resistance was observed in 5 resistant strains, while kanamycin resistance was observed in 3 strains (all of which belonged to the *Lb. casei/paracasei* species). In 6 cases, resistance to chloramphenicol and 1 other antibiotic was observed.

	Minimum Inhibitory Concentration (MIC) Values of Resistant Lactobacilli to Antibiotics of Interest								
Species	**Strain**	**Antibiotic* (MIC as µg/mL)**							
		Va	**Am**	**Gm**	**Km**	**Sm**	**Em**	**Cl**	**Tc**	**Cm**
Lb. casei/paracasei	7086	n.r.[1]	<2	<2	<64	<16	<0.25	<0.25	**<32**	**<16**
	7100	n.r.	<2	<2	<64	<16	<0.25	<0.25	<4	**<16**
	7104	n.r.	<1	<8	**<128**	<64	<0.06	<0.12	<2	<4
	7143	n.r.	<2	<4	**<128**	<16	<0.06	<0.12	<1	**<8**
	7145	n.r.	<2	<4	**<128**	<16	<0.06	<0.25	<1	**<8**
	7152	n.r.	<1	<4	<64	<8	<0.06	<0.12	<2	**<8**
Lb. plantarum	7081	n.r.	<2	<2	<64	n.r.	<0.25	<0.5	**>64**	**<16**
	7083	n.r.	<1	<2	<64	n.r.	<0.25	<0.5	**>64**	**<16**
	7141	n.r.	<1	<2	<32	n.r.	<0.25	<0.5	**>64**	<8
Lb. coryniformis	7127	n.r.	<0.5	<1	<16	<8	<0.12	<0.5	**<32**	**<8**

Table 3. Antibiotic sensitivity profiles of NSLAB strains that failed the acceptable antibiotic susceptibility limits. * **Va**, Vancomycin; **Am**, Ampicillin; **Gm**, Gentamicin; **Km**, Kanamycin; **Sm**, Streptomycin; **Em**, Erythromycin; **Cl**, Clindamycin; **Tc**, Tetracycline; **Cm**, Chloramphenicol. [1] not required as per EFSA Guidelines. MIC (µg/mL) values in **bold** indicate presence of antibiotic resistance as per the EFSA Guidelines.

3.3.4 *Lactobacillus* Adherence to a Mucus-Producing Cell Model

Seven of the twenty *Lactobacillus* strains that were sensitive to antibiotics were selected, and their ability to adhere to a mucus-producing Caco-2/HT29-MTX co-culture was assessed. The criteria used to select strains for screening were that at least one strain per species was selected and the number of strains chosen per species was based on their overall abundance during the original isolation. *Lb. rhamnosus* GG was used as a positive control due to its established role as a probiotic with excellent adherence to epithelial models (Segers & Lebeer, 2014). Following three independent replicates, *Lb. casei/paracasei* DPC7110, *Lb. casei/paracasei* DPC7149, *Lb. plantarum* DPC7126 and *Lb. plantarum* DPC7096 were found to have adherence values that were statistically significantly different ($p<0.05$) from and less than the positive control (**Figure 1**). The remaining *Lb. casei/paracasei* (7150, 7087) and *Lb. rhamnosus* (7102) strains' demonstrated adherence values that were not statistically significantly different from the positive control. While the control strain exhibited the highest adherence value (90.6 ± 8.2%), *Lb. rhamnosus* strain DPC7102 showed the highest adherence of the strains of interest, at 79.2 ± 8.1%. The other 2 *Lb. casei/paracasei* strains of interest that performed well were strain 7150 and strain 7087, which had adherence values of 64.0 ± 9.1% and 58.0 ± 11.5%, respectively. Adherence to the mucus-producing mammalian co-culture varied greatly among strains, often accompanied with high standard deviations.

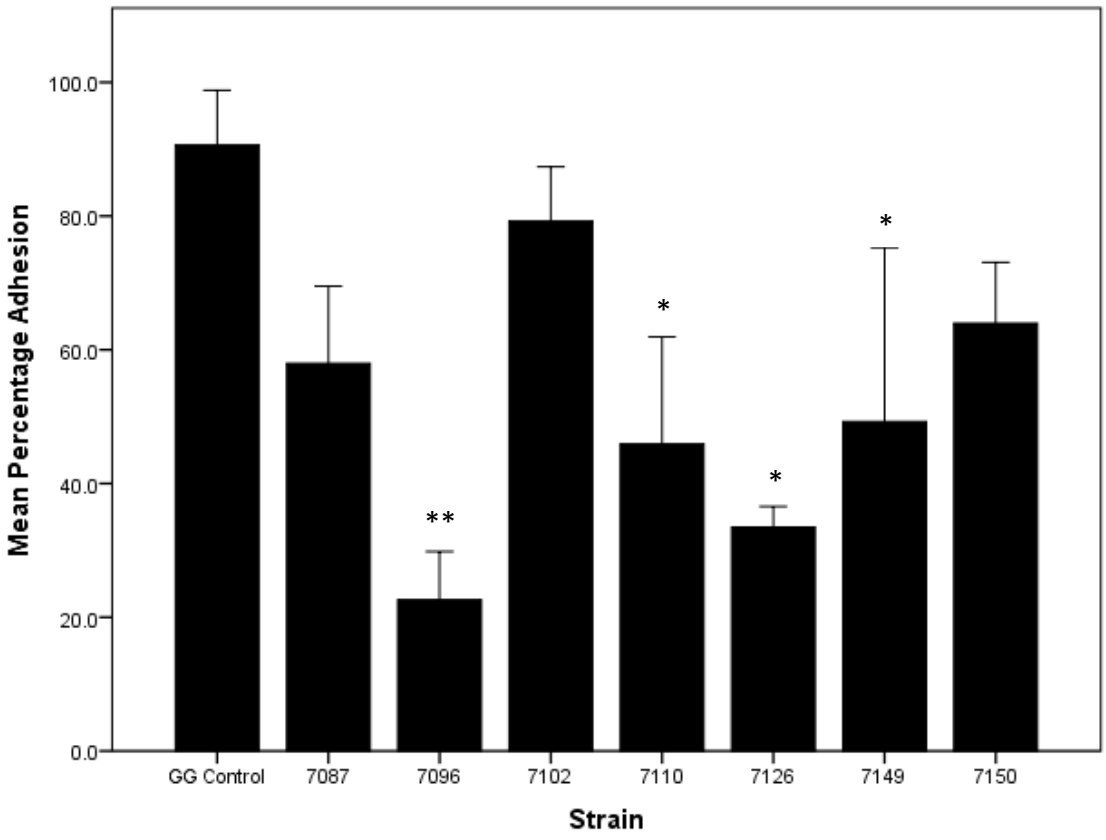


Figure 1. Ability of *Lactobacillus* strains to adhere to Caco-2/HT29-MTX co-culture. *Lactobacillus rhamnosus* GG was used as a control. Results are expressed as the mean percentage of 3 independent experiments performed in triplicate, with error bars representing standard deviation. * Statistically significant differences occurred compared to the control (GG Control) strain ($p < 0.05$). ** Highly statistically significant differences occurred compared to the control (GG Control) strain ($p < 0.001$).

3.3.5 Ability of *Lactobacillus* Strains to Inhibit Enteropathogenic *E. coli* Binding to Mucus-Producing Cells

In order to assess further probiotic potential, the two *Lactobacillus* strains with the highest rates of adherence *in vitro*, representing two species of *Lactobacillus* namely *Lb. rhamnosus* DPC7102 and *Lb. casei/paracasei* DPC7150, were tested for their ability to inhibit the binding of a known enteropathogenic *E. coli* (EPEC) strain to mucus (**Figure 2**). The experiment performed used an inhibition model whereby the lactobacilli were added to the Caco-2/HT29-MTX co-culture an hour prior to the addition of the pathogen, thereby examining the lactobacilli's ability to 'exclude' binding of the pathogen to the mucins and mammalian cells. *Lb. rhamnosus* DPC7102 was able to exclude 44.2 ± 9.6% of the total *E. coli* from adhering to the mucus co-culture, while *Lb. casei/paracasei* DPC7150 excluded 25.7 ± 2.1% of total *E. coli*.

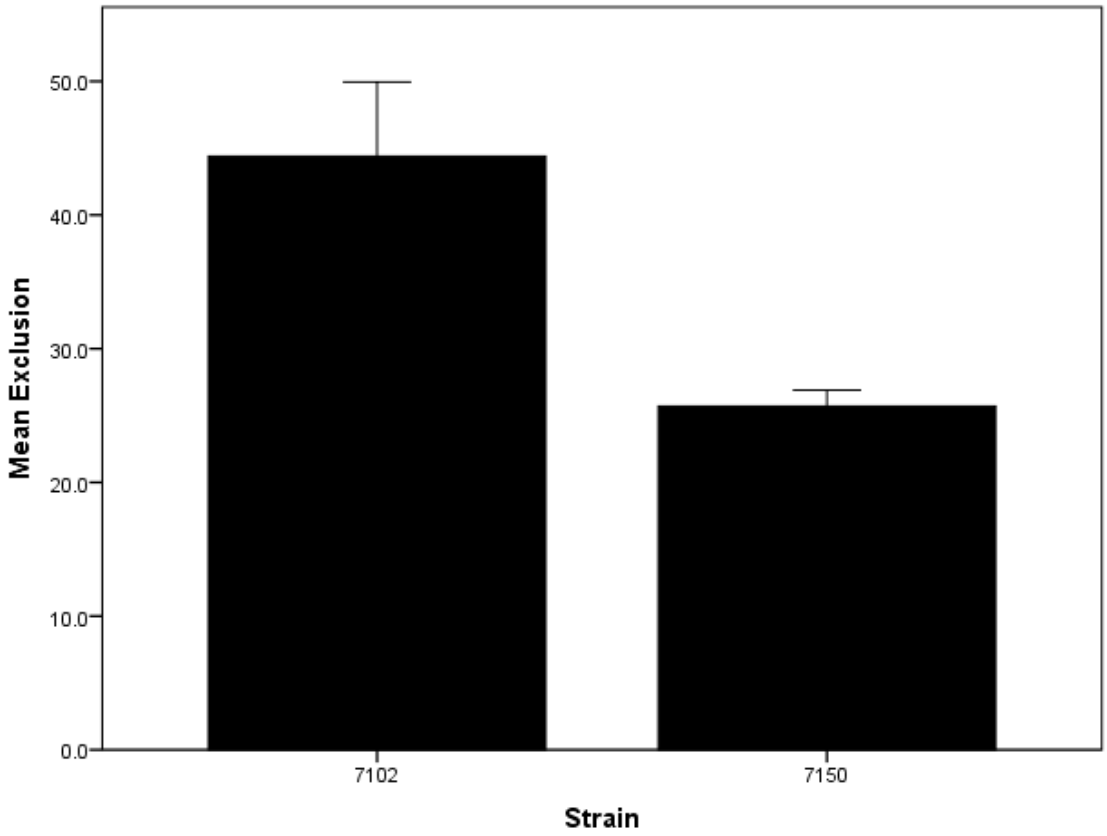


Figure 2. Ability of *Lactobacillus* strains to inhibit the binding of Enteropathogenic *E. coli* 0111:H2 to Caco-2/HT29-MTX co-culture. Results are expressed as the mean percentage of 3 independent experiments performed in triplicate, with error bars representing standard deviation.

3.3.6 Exopolysaccharide Production by Selected *Lactobacillus* Strains

The ability of lactic acid bacteria (LAB) to produce EPS is seen as a benefit, from both a commercial and health point of view. Therefore, DPC7102 and DPC7150 were grown on skim milk plates containing ruthenium red agar, in parallel with a known EPS-producing positive control (DPC1116). Both strains of interest and positive control strain produced white colonies on the pink plates, demonstrating that they each produce EPS when grown on skim milk (**Figure 3**).

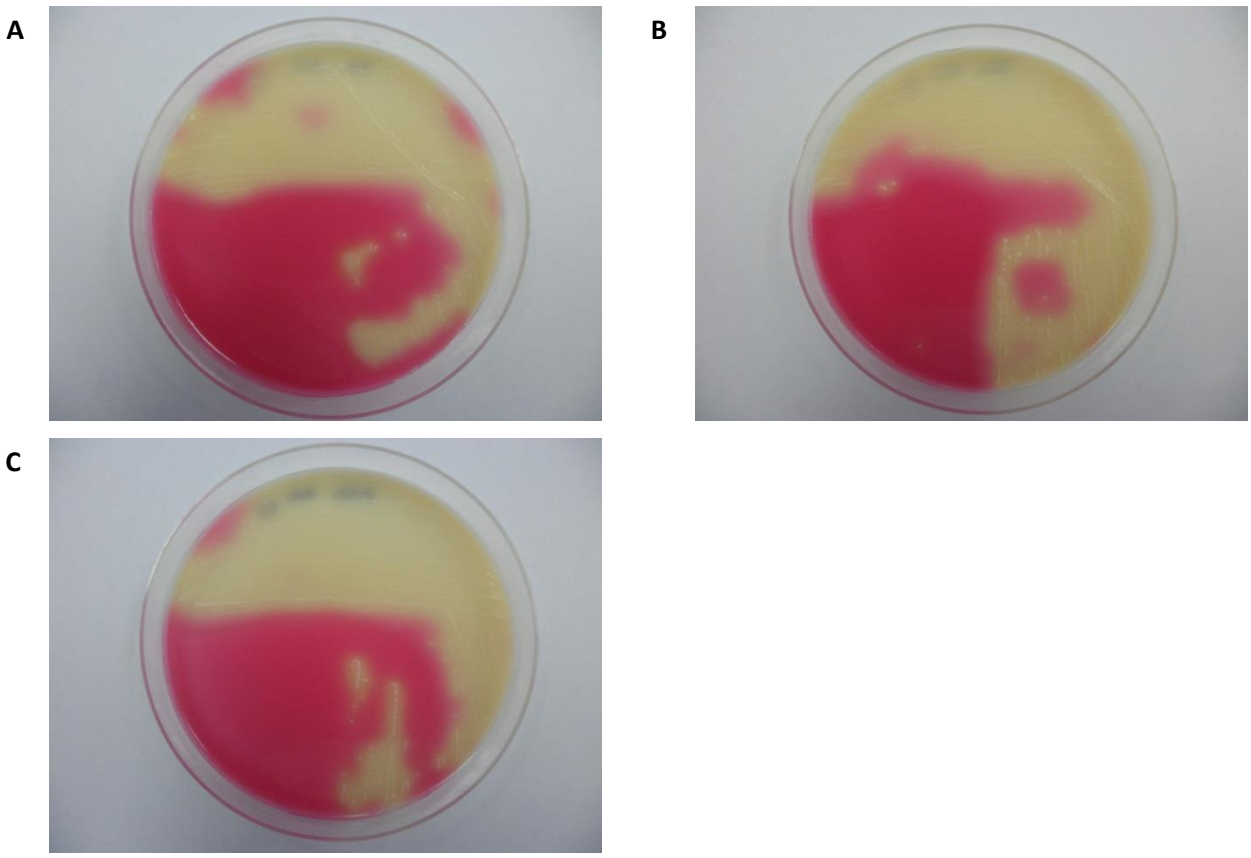


Figure 3. Growth of white colonies on ruthenium red skim milk agar indicating EPS production. **(A)** Positive control *Lb. paracasei* DPC1116; **(B)** and **(C)** test strains *Lb. rhamnosus* DPC7102 and *Lb. paracasei* DPC 7150 respectively.

3.3.7 Genome Characteristics of *Lb. rhamnosus* DPC7102 and *Lb. paracasei* DPC 7150

Whole genome sequencing and annotation was undertaken on the two strains *Lb. rhamnosus* DPC7102 and *Lb. paracasei/casei* DPC7150 in an effort to fully characterise the strains to species level and to determine if either contained antibiotic resistance, virulence or other genes associated with human pathogens. The data obtained (**Table 4**) demonstrated that both ANI and OrthoANI values were >95%, indicating that they are the same species as the reference strains used confirming that DPC7150 is in fact a *Lb. paracasei* (Rodriguez-R & Konstantinidis, 2016). ResFinder 3.2 detected no acquired antibiotic resistance genes in either draft genome. The gene *RRS* (a 16 S ribosomal RNA gene) from *Mycobacterium tuberculosis* was the only exception in *Lb. rhamnosus* 7102, but the coverage was only ≈4% (with 60% being the minimum coverage threshold cutoff) (data not shown). VirulenceFinder 2.0 detected no genes associated with virulence in either genome (data not shown). PathogenFinder 1.1 predicted that neither organism is a likely human pathogen.

	General Genomic Features of *Lactobacillus* Strains of Interest	
	***Lb. rhamnosus* DPC7102**	***Lb. paracasei* DPC7150**
BioSample Accession No.	SAMN12280510	SAMN12280511
No. of Contigs	131	280
GC Content (%)	46.61	46.17
Coverage (X)	149	44
No. of CDS*	2978	2894
ANI/OrthoANI	98.06/97.85	98.56/98.23
Probability of being Human Pathogen (%)	0.095	0.092

Table 4. General genomic features of *Lb rhamnosus* DPC7102 and *Lb. paracasei* DPC7150. *CDS, coding sequence (with protein)

3.4 Discussion

Initially, samples of 12 Irish Cheddar cheeses were subjected to SSDP followed by plating for viable NSLAB on MRS agar. Survivors under SSDP conditions, which include exposure to gastric pH conditions followed by bile acids the two primary criteria identified by the FAO for the selection of strains with probiotic potential provided an indication of potential of NSLAB to transit through the upper intestinal tract and thus, their potential to colonise the host intestines (Sumeri et al., 2012). Apart from Cheddar cheese 4, the NSLAB populations were in excess of 10^6 CFU/g prior to SSDP, which is the accepted minimum level at which probiotics should be present in food, from which a portion can be consumed daily to administer the recommended 10^8-10^9 CFU (Kechagia et al., 2013). Following exposure to SSDP, log reductions of between 0.03 and 2.84 were observed in the NSLAB populations, with >10^6 CFU/g being observed in 7 of the 12 cheeses tested. These data provide direct evidence for the first time to support the hypothesis that Cheddar cheese contains NSLAB populations of which a large proportion are capable of surviving simulated gastric digestion and thus will arrive in the small intestine in a viable state. The NSLAB in this study were extracted from the cheese prior to exposure to SSDP; however, it is expected that under normal conditions of cheese consumption the presence of the cheese matrix would provide additional protection, thus, enhancing NSLAB survival and transit to the gut (Gardiner et al., 1999). Cheddar cheese may, therefore, serve as an efficient food vehicle for delivery of acceptable numbers of viable NSLAB to the gut where they may exert health benefits. The data presented here indicate that cheese contains bacteria with probiotic traits at high levels, which can then potentially colonise the human intestines and exert further beneficial effects (Settanni & Moschetti, 2010). This is in agreement with the work of other groups who have also confirmed the feasibility of cheese as a competent microbe/probiotic delivery matrix (Gardiner et al., 1999; Gomes da Cruz et al., 2009; Karimi et al., 2011; Sharp et al., 2008; Zhang et al., 2013).

Initially, 76 isolates that were resistant to simulated digestion and deemed to be unique strains were brought forward for further subtractive screening. The majority of these comprised members of the *Lb. casei/paracasei*, *Lb. curvatus* and *Lb. plantarum* species, but also included *Lb. helveticus*, *Lb. rhamnosus*, *Lb. coryniformis* and a single *S. thermophilus* strain. These species are commonly associated with the NSLAB

component of Cheddar cheeses, although *Lb. helveticus* and *S. thermophilus* are often added intentionally as either starter LAB or adjunct cultures (Briggiler-Marcó et al., 2007; Fitzsimons et al., 2001; Peterson & Marshall, 2010; Swearingen et al., 2001). These results are in agreement with Sumeri et al. (2012) who also reported *Lb. casei/paracasei* strains being the most prominent members of the NSLAB population to survived simulated gastric transit; however, that study did not provide data on the overall proportion of the NSLAB population that survived this treatment or whether the surviving strains exhibited other probiotic characteristics (Sumeri et al., 2012). Additionally, many potential probiotics have previously been identified from the above species, although presence of beneficial probiotic traits have been cited as being heavily strain-dependant (Lebeer et al., 2008). The number and species of individual strains capable of surviving SSDP differed between Cheddar cheeses. While most cheeses were observed to contain multiple strains capable of surviving SSDP, single strains were detected in three cheeses while up to twelve strains were observed in the cheeses containing the most diverse populations. Cheese 7 contained the most variation in terms of species, including isolates of *Lb. casei/paracasei*, *Lb. curvatus*, *Lb. helveticus* and *S. thermophilus*. However, the majority of the cheeses (10 of the 12) contained strains from one or two species only. This is not unexpected as the cheeses used here had different sources, which will effect NSLAB content (Peterson & Marshall, 2010). High diversity of NSLAB can even be seen in cheeses manufactured in the same factory, as seen in a study in 2002 which determined that the number of NSLAB strains had high variability even when produced in the same milk vat during different time points, highlighting that NSLAB variability is very commonplace (Williams et al., 2002).

The ability of *Lactobacillus* to produce BSH enzymes has been documented by different groups, with high strain variability being observed (Begley et al., 2006; O'Flaherty et al., 2018). Of the NSLAB selected by the initial screen, approximately 40% produced haloes of deconjugated bile when grown on MRS agar with 0.3% cow bile. Various groups have identified beneficial *Lb. plantarum* strains with BSH activity, with evidence suggesting that expression of these enzymes aids persistence and adherence in the intestine (Kumar et al., 2011; Lambert et al., 2008; Nguyen et al., 2007; Patel et al., 2010; Yang et al., 2019). The haloes produced by all *Lb. plantarum* strains were the largest, along with the positive *Lb. reuteri* strain. Seventeen of the thirty strains that produced deconjugated bile haloes were *Lb. casei*/*paracasei* strains, although their

performance varied depending on previous incubation conditions. While less data exists of BSH positive *Lb*. *casei*/*paracasei* strains, certain groups have identified those capable of bile deconjugation such as the *Lb. casei* K17 identified in 2016 and eight *Lb. paracasei* strains in 2006 capable of at least partial hydrolysis of bile salts (Maragkoudakis et al., 2006; Xu et al., 2016). Similarly, a single *Lb. rhamnosus* and *Lb. coryniformis* strain displayed BSH activity. While the probiotic *Lb. rhamnosus* GG strain is capable of BSH production, to date, there is no published data on a *Lb. coryniformis* strain capable of BSH expression, although there is an entry on UniProt for a *Lb. coryniformis* BSH protein (https://www.uniprot.org/uniprot/A0A1P8FCL4).

Of the ten strains that failed the EFSA antibiotic susceptibility profiles, eight were resistant to Chloramphenicol. Genes primarily responsible for Chloramphenicol resistance are *cat* (chloramphenicol acetyltransferase) genes which, when present in *Lactobacillus*, have typically been located on plasmids (Gueimonde et al., 2013). This result is surprising as *Lactobacillus* are not traditionally associated with chloramphenicol resistance; however, this can vary between strains and several resistant strains have been identified previously from foods (Guo et al., 2017; Mathur & Singh, 2005). In addition, six of the eight chloramphenicol-resistant strains also showed resistance to a second antibiotic, with four also showing growth in high concentrations of Tetracycline (the remaining two being capable of growth in Kanamycin). A paper published in 2019 examined 182 *Lactobacillus* isolates selected in such a way as to represent all lactobacilli species and found that 31% were resistant to both Chloramphenicol and Tetracycline (Campedelli et al., 2019). It is interesting to observe in both studies that chloramphenicol/tetracycline resistance was observed in some of the investigated species, as seen in this study for one *Lb. casei/paracasei*, two *Lb. plantarum* and one *Lb. coryniformis* strain. Kanamycin resistance was observed in three different *Lb. casei/paracasei* strains, with two showing dual resistance with Chloramphenicol. *Lb. casei* strains with dual resistance to Kanamycin and Chloramphenicol were also observed in Campedelli et al., with 10 of the 15 strains tested exhibiting the same dual resistance (Campedelli et al., 2019). Generally, aminoglycoside antibiotics are less efficient against gram positive anaerobes due to membrane impermeability characteristics associated with this group of bacteria (Elkins & Mullis, 2004).

HT29-MTX cells produce both membrane-bound mucin proteins MUC1 and MUC3. While MUC1 is produced early on during differentiation (Day 7) at their apical borders, MUC3 levels peak later (Day 14), with levels remaining consistent in both thereafter (Lesuffleur et al., 1993; Mack et al., 2003). More importantly, Days 7-14 of differentiation leads to a marked increase in expression of the MUC5 protein which is secreted and forms the protective gel/mucus layer of the intestinal tract and the initial foothold that allows potential adhesion of ingested microbes (Kleiveland, 2015; Santini et al., 2007). As adherence varies greatly between strains, it was not surprising that the seven strains investigated here displayed adhesion levels that ranged from 20-80%. Bacterial adhesion is an important trait for a potential probiotic as high adherence allows for extended residence times in the gut, during which time any host-specific benefits can take effect. *Lb. rhamnosus* DPC 7102 had the highest adhesion at 79.2%, with the positive control *Lb. rhamnosus* GG showing 90.6% adhesion. Strain DPC 7102 was one of three strains that demonstrated high levels of adhesion that were not significantly different from that of the control strain, with the other two being *Lb. paracasei* DPC 7150 and *Lb. casei/paracasei* DPC 7087 which showed adherence values of 64.0 and 58.0, respectively. *Lb. rhamnosus* DPC 7102 and *Lb. paracasei* DPC 7150 showed the highest adherence values of the seven strains tested and were, therefore, brought forward for additional testing. The other four strains tested showed significantly different and reduced adherence values when compared to the positive control strain and, thus, were not considered for further testing.

Of the two strains tested for pathogen exclusion, *Lb. rhamnosus* DPC 7102 was more efficient at excluding *E. coli* than *Lb. paracasei* DPC 7150 (44.2% and 25.7% exclusion, respectively). These results support our observations in the adhesion assays which demonstrated that the *Lb. rhamnosus* strain was also capable of a higher level of adhesion, leaving less physical space and binding sites for pathogen adhesion once added, leading to greater exclusion of the pathogen. A member of the adhesive EPEC serotype was chosen for this assay. Once attached, they establish compact microcolonies which cause various morphological abnormalities (including destruction of intestinal microvilli and blunt enterocyte borders) and cause diarrhoea (Fagundes-Neto & Scaletsky, 2000). Therefore, the ability of the NSLAB strains to inhibit adhesion of this pathogen highlights their potential as probiotics to protect against similar infectious agents.

The ability of potential probiotic bacteria to produce EPS is desirable as EPS has associated commercial (as an emulsifying agent, improving mouthfeel and texture in dairy products) and health benefits (immunomodulation, inhibition of pathogens and cholesterol-lowering capabilities) (Liu et al., 2017). In this study, both *Lb. rhamnosus* DPC 7102 and *Lb. paracasei* DPC 7150 exhibited white colony growth when streaked onto Ruthenium Red Skim Milk agar, along with the positive control *Lb. casei/paracasei* DPC 1116. This indicates that they are EPS producers as the generation of either capsular EPS or secreted EPS will prevent staining of the cell wall by the ruthenium red agent (Mora et al., 2002).

In an effort to confirm the species information obtained by 16S rRNA gene sequence analysis and to facilitate a more in-depth analysis of the two organisms at the genetic level, with particular reference to the presence of additional antibiotic resistance genes or genes associated with virulence or human pathogens, whole genome sequencing and annotation was undertaken. This confirmed the species designations for both isolates but also further classified DPC7150 as *Lb. paracasei.* No genes associated with virulence were detected in either genome and neither organism is predicted to be a likely human pathogen. These are important observations with regard to the potential future use of either of these two strains as probiotics.

3.5 Conclusion

Of the 76 individual strains of NSLAB with gastric tolerance isolated, 30 of them were also capable of BSH activity, which has been associated with cholesterol-lowering capabilities and indicates the potential health benefits of normal Cheddar cheese microbiomes. From these 30 BSH positive isolates, two *Lactobacillus* strains, DPC7102 and DPC 7150, were further demonstrated to display promising adhesion to and pathogen exclusion from a mammalian co-culture, are capable of EPS production, are also susceptible to antibiotics to an acceptable standard as set by the European Food Safety Authority and do not harbour any genes associated with virulence or human pathogenicity. These two strains are originally from Cheddar cheeses and could potentially be used as Adjunct Cultures in future cheese making. The added protection of the cheese matrix should ensure greater survival of lactobacilli cultures through the harsh gastric environment and into the small intestine, where the bacteria can then adhere and potentially exert their beneficial effects on the host.

3.6 Supplementary Material

	Abundance of Strains in Cheddar Cheeses			
Cheese	**Strain**	**No. of Isolates (x of 20)**	**DPC No.**	**Species**
1	1.01	1	DPC7081	*Lb. plantarum*
	1.02	1	DPC7082	*Lb. casei/paracasei*
	1.03	1	DPC7083	*Lb. plantarum*
	1.04	1	DPC7084	*Lb. casei/paracasei*
	1.05	3	DPC7085	*Lb. casei/paracasei*
	1.06	3	DPC7086	*Lb. casei/paracasei*
	1.07	4	DPC7087	*Lb. casei/paracasei*
	1.08	1	DPC7088	*Lb. casei/paracasei*
	1.09	1	DPC7089	*Lb. casei/paracasei*
	1.10	1	DPC7090	*Lb. casei/paracasei*
	1.11	1	DPC7091	*Lb. casei/paracasei*
	1.12	2	DPC7092	*Lb. casei/paracasei*
2	2.01	2	DPC7140	*Lb. casei/paracasei*
	2.02	2	DPC7141	*Lb. plantarum*
	2.03	2	DPC7142	*Lb. casei/paracasei*
	2.04	1	DPC7143	*Lb. casei/paracasei*
	2.05	3	DPC7144	*Lb. casei/paracasei*
	2.06	2	DPC7145	*Lb. casei/paracasei*
	2.07	2	DPC7146	*Lb. casei/paracasei*
	2.08	1	DPC7147	*Lb. casei/paracasei*
	2.09	2	DPC7148	*Lb. casei/paracasei*
	2.10	3	DPC7149	*Lb. casei/paracasei*
3	3.01	1	DPC7098	*Lb. casei/paracasei*
	3.02	12	DPC7099	*Lb. casei/paracasei*
	3.03	7	DPC7100	*Lb. casei/paracasei*
4	4.01	11	DPC7093	*Lb. plantarum*
	4.02	9	DPC7094	*Lb. plantarum*
5	5.01	20	DPC7097	*Lb. plantarum*
6	6.01	1	DPC7101	*Lb. casei/paracasei*
	6.02	1	DPC7102	*Lb. rhamnosus*
	6.03	2	DPC7103	*Lb. casei/paracasei*

	6.04	1	DPC7104	*Lb. casei/paracasei*
	6.05	1	DPC7105	*Lb. casei/paracasei*
	6.06	4	DPC7106	*Lb. casei/paracasei*
	6.07	2	DPC7107	*Lb. casei/paracasei*
	6.08	2	DPC7108	*Lb. rhamnosus*
	6.09	1	DPC7109	*Lb. rhamnosus*
	6.10	1	DPC7110	*Lb. casei/paracasei*
	6.11	3	DPC7111	*Lb. casei/paracasei*
	6.12	1	DPC7112	*Lb. rhamnosus*
7	7.01	1	DPC7113	*Lb. helveticus*
	7.02	1	DPC7114	*Lb. curvatus*
	7.03	1	DPC7115	*Lb. casei/paracasei*
	7.04	1	DPC7116	*Lb. curvatus*
	7.05	1	DPC7117	*S. thermophilus*
	7.06	4	DPC7118	*Lb. curvatus*
	7.07	1	DPC7119	*Lb. helveticus*
	7.08	3	DPC7120	*Lb. curvatus*
	7.09	1	DPC7121	*Lb. curvatus*
	7.10	3	DPC7122	*Lb. curvatus*
	7.11	3	DPC7123	*Lb. curvatus*
8	8.01	1	DPC7124	*Lb. curvatus*
	8.02	1	DPC7125	*Lb. curvatus*
	8.03	7	DPC7126	*Lb. plantarum*
	8.04	1	DPC7127	*Lb. coryniformis*
	8.05	1	DPC7128	*Lb. curvatus*
	8.06	1	DPC7129	*Lb. curvatus*
	8.07	1	DPC7130	*Lb. curvatus*
	8.08	1	DPC7131	*Lb. coryniformis*
	8.09	2	DPC7132	*Lb. plantarum*
	8.10	1	DPC7133	*Lb. plantarum*
	8.11	2	DPC7134	*Lb. plantarum*
	8.12	1	DPC7135	*Lb. curvatus*
9	9.01	5	DPC7136	*Lb. helveticus*
	9.02	9	DPC7137	*Lb. helveticus*
	9.03	4	DPC7138	*Lb. helveticus*
	9.04	2	DPC7139	*Lb. helveticus*

10	10.01	20	DPC7096	*Lb. plantarum*
11	11.01	1	DPC7150	*Lb. casei/paracasei*
	11.02	2	DPC7151	*Lb. casei/paracasei*
	11.03	4	DPC7152	*Lb. casei/paracasei*
	11.04	2	DPC7153	*Lb. casei/paracasei*
	11.05	4	DPC7154	*Lb. casei/paracasei*
	11.06	3	DPC7155	*Lb. casei/paracasei*
	11.07	4	DPC7156	*Lb. casei/paracasei*
12	12.01	20	DPC7095	*Lb. plantarum*

Table S1. Strains of NSLAB isolated from each cheese as determined by PFGE and species identification by 16S rRNA gene sequencing.

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Chapter 4

Protection of Candidate Probiotic Lactobacilli by Cheddar Cheese Matrix during Simulated Gastrointestinal Digestion

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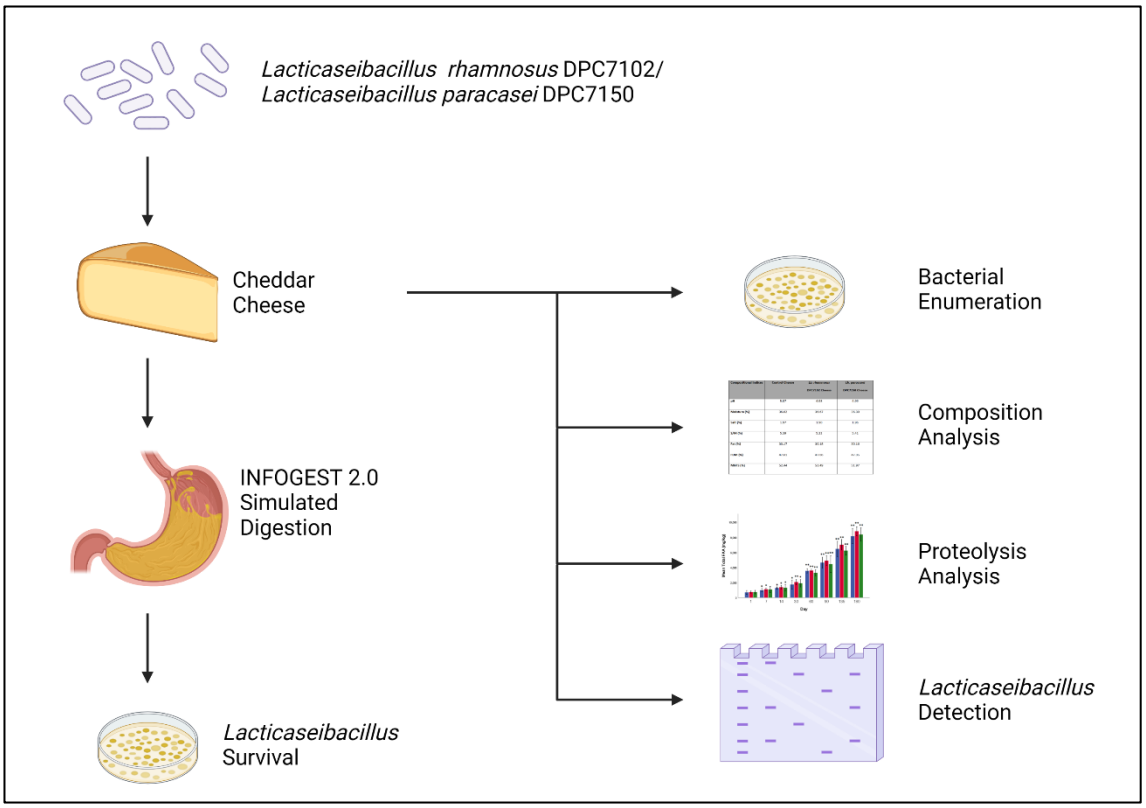
Abstract

Previously, *Lacticaseibacillus rhamnosus* DPC7102 and *Lacticaseibacillus paracasei* DPC7150 isolated from commercial Irish Cheddar cheeses were shown to exhibit probiotic traits *in vitro*. The objective of this study was to investigate how these strains survive in cheese and to explore whether a cheese or fermented milk matrix provided protection during simulated digestion using the INFOGEST 2.0 static digestion model. Manufacture of Cheddar cheese and fermented milks using these strains did not significantly impact cheese composition or ripening parameters. The Cheddar cheese matrix provided significantly more protection to non-starter lactic acid bacteria than the fermented milk. Pulsed-field gel patterns of isolates surviving simulated digestion confirmed the presence of the strains of interest. These data indicate that these two *Lacticaseibacillus* strains successfully persist in the cheese matrix during cheese ripening following which they are capable of surviving simulated digestion. This supports their potential for further development as candidate food-delivered probiotics.

Graphical Abstract



4.1 Introduction

The use of yogurt as a food matrix to deliver beneficial and probiotic microorganisms to the gut has been well documented, and health benefits have been identified by many different investigative groups (Gómez-Gallego et al., 2018). The different food matrices tested for delivery of microorganisms to the gut has expanded to include most food groups, including other dairy products, cereals, various fruits and vegetables, and some fermented meats (Kołożyn-Krajewska & Dolatowski, 2009; Rivera-Espinoza & Gallardo-Navarro, 2010; Shori, 2015, 2016). Cheese has been investigated as a probiotic carrier with varying degrees of success (Castro et al., 2015; Gardiner et al., 1998; Phillips et al., 2006). Studies have confirmed the ability of cheese to safely transport beneficial bacteria through the gastrointestinal tract (GIT) and subsequently be detected in faeces, although success of recovery from faeces is strain dependant (Lahtinen et al., 2012; Songisepp et al., 2012).

It has been suggested that cheese may be a more suitable vehicle for microbes than yogurt (Gardiner et al., 1999). Generally, cheese has a higher pH than yogurt which imparts less stress upon microbes while in the food matrix, and the high fat content offers additional protection during transit through the gastrointestinal tract (GIT) (Flach et al., 2018). An *in vitro* study indicated that the cheese matrix was more effective at protecting a probiotic *Lacticaseibacillus casei* strain at stomach pH (2.0) in comparison to yogurt (Sharp et al., 2008). A human feeding trial examined the survival and persistence of four probiotic strains (one strain each of *Propionibacterium freudenreichii* subsp. *shermanii* and *Bifidobacterium animalis* subsp. *lactis* and two strains of *Lacticaseibacillus rhamnosus*) in faeces and saliva when administered in either a yogurt, cheese or capsule matrix and found that, while the survival of the two lactobacilli was the same regardless of matrix, the *Bifidobacterium* and *Propionibacterium* strains had higher recovery numbers and persisted longer in faeces when ingested in yogurt form; however, this result may be influenced by the fact that the daily dose of the probiotic cocktail received in the cheese was only approximately a quarter to a sixth of that received in the capsule and yogurt groups, respectively (Saxelin et al., 2010).

Some cheeses have also been found to contain non-starter lactic acid bacteria (NSLAB) that have probiotic properties (Leeuwendaal et al., 2021; Settanni & Moschetti, 2010). NSLAB are the microbial populations found in cheese that are not responsible for the conversion of milk into cheese (i.e. they do not contribute to the acidification process) and are not deliberately added, usually originating from the milk itself or the plant machinery and personnel involved in the cheesemaking process (Beresford et al., 2001; Irlinger et al., 2017). Various members of the *Lactobacillaceae* family, which are common NSLAB of cheeses, have performed favourably in terms of probiotic potential, some of which were originally isolated from cheese and other dairy sources (Fijan, 2014; Maragkoudakis et al., 2006). Isolating and identifying lactobacilli from NSLAB cheese populations with probiotic potential can be advantageous as they are already known to survive in cheese during manufacture, grow during the maturation processes and can be added with the starter cultures in high numbers with little impact procedurally as they grow slowly in milk and will contribute little to acid production during cheese manufacture (Gobbetti et al., 2015).

Various organisations involved in food regulatory affairs have stated that sufficient numbers of probiotic bacteria must be present and viable in the final food product at the time of consumption to have the potential to elicit a positive health response, with the minimum requirement usually being set at between 10^6 – 10^7 colony forming units per gram of food (Leroy & De Vuyst, 2014). To satisfy this, beneficial NSLAB lactobacilli can be added during the initial stages of cheese production (along with the starter cultures) as adjunct cultures, as has been already attempted by various groups (Burns et al., 2012; Oberg et al., 2011; Oh et al., 2016). Cheese as the matrix of choice for probiotics of NSLAB origin is further enhanced by the fact that NSLAB grow in cheese during ripening, normally attaining populations in excess of 10^7 colony forming units per gram and thus, are likely to remain viable to the levels required by the regulatory agencies until the time of consumption (Leeuwendaal et al., 2021).

The aim of this study was to produce Cheddar cheeses using two NSLAB strains isolated from Irish Cheddar cheeses, *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 that have previously been found to exhibit probiotic traits when tested *in vitro* including; capacity to survive simulated gastric transit, expression of bile salts hydrolyase activity, ability to adhere to gut epithelium cells and to inhibit binding of

entropathogenic to gut epithelium cells, in addition to demonstrating antibiotic sensitivity profiles that align with the guidance of the European Food Safety Authority on assessment of bacteria of human and veterinary importance (Aquilina et al., 2012; Leeuwendaal et al., 2021), using each strain individually in high cell density as adjunct cultures. The behaviour of these strains and their impact on cheese composition and key biochemical pathways during ripening was monitored. Following a 6-month maturation period, the potential of these two NSLAB strains to survive gastric transit was investigated in the INFOGEST 2.0 static digestion method (Brodkorb et al., 2019). Fermented milk samples, using either *Lc. rhamnosus* DPC7102 or *Lc. paracasei* DPC7150, were used in parallel to test the ability of the different dairy matrices to protect the NSLAB strains during gastric transit. The INFOGEST 2.0 digestion model was developed by a network of international scientists with the objective of providing a standardised model that closely reflects the physiological conditions of the upper gastrointestinal track and thus, provide a more realistic assessment than earlier reported gastric transit models of the capacity of bacteria embedded in a food matrix to survive gastric transit.

4.2 Materials and Methods

4.2.1 Preparation of Cheese Milk

Cows' milk (1,500 L) was obtained from a local dairy company, Dairygold [Mitchelstown, Co. Cork]. The milk was obtained from commercial farms with mixed Holstein-Friesian and Friesian-Jersey cross herds. Milk was standardised to a protein to fat ratio of 0.92:1, held overnight at 4-6 °C, pasteurized at 72 °C for 15 seconds, cooled to 31 °C and 450 L was then pumped into each of three 500 L stainless steel, jacketed, cylindrical cheese vats.

4.2.2 Cultures for Cheesemaking

Defined strain starter cultures; *Lactococcus lactis* strains 223 and 303 (Chr. Hansen), were inoculated at 0.4% (v/v) into 7 L of 10% (w/v) reconstituted skim milk (RSM) powder solution (Kerry Food Ingredients) that was previously heat treated at 100 °C for 90 minutes and were grown overnight (≈16 hours) at 30 °C. Long-term stocks were stored at -80 °C and were propagated twice in 10% RSM for activation prior to experimental use.

Adjunct cultures *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 previously designated at genus level as *Lactobacillus* but following reclassification are now designated *Lacticaseibacillus*, were inoculated at 1% (v/v) into 2 L of sterile de Man, Rogosa and Sharpe (MRS) broth (containing 0.05% L-Cysteine-HCl) (BD Difco™) and grown at 37 °C for 24 hours (Leeuwendaal et al., 2021; Rogosa et al., 1951; Zheng et al., 2020). Spectrophotometric readings at wavelength 600 nm were then taken of each culture and based on previous growth data (data not shown), 4.5 x 10^{11} CFU of culture was removed, centrifuged at 2000 relative centrifugal force (RCF) for 20 minutes at 4 °C and resuspended in 0.2 L 10% RSM. Cultures were stored at 4 °C until required for cheesemaking (≈16-18 hours). Long-term stocks were stored at -80 °C and were propagated twice in MRS broth (0.05% L-Cysteine-HCl) for activation prior to experimental use.

4.2.3 Cheddar Cheese Manufacture

Cheddar cheese trials were performed in triplicate on January 22nd, January 30th, and February 12th (2019) at pilot scale (Moorepark Technology Limited, Teagasc Moorepark). Starter cultures were each inoculated at a concentration of 0.75% v/v (1.5% v/v total) into the milk in each of the three vats. One of the three vats was inoculated with the 0.2 L *Lc. rhamnosus* DPC7102 adjunct, a second vat was inoculated with the 0.2 L *Lc. paracasei* DPC7150 adjunct, and the third vat of milk served as the control with no adjunct culture added. Cheddar cheese was manufactured as previously described, with modifications (Xia et al., 2020). Milk was allowed to ripen for 35 minutes or until a pH of 6.6-6.55 was reached. CHY-MAX® Rennet (Chr. Hansen) was stirred into the milk at a rate of 0.18 mL/L milk, diluted 1:10 in autoclaved distilled water, stirring was then terminated and the milk was left stand for gelation to occur. Once the gel attained a firmness value of 30 Pa (as measured using low-amplitude strain oscillation rheometry [AR-G2 Rheometer, TA Instruments]), it was cut and cooked by increasing the temperature of the curd/whey mixture from 31 °C to 38 °C at a rate of 1°C/5 min. Curds were drained at pH 6.15, cheddared, and milled at pH 5.25, and salted at a rate of 2.7% (wt/wt). The salted curd was transferred to moulds (22.5 kg/mould), initially pre-pressed at 70 psi for 30 minutes (with the mould inverted after 15 minutes), and then transferred to the overnight press at 100 psi. Cheddar cheese blocks was removed from the moulds, cut in half, vacuum-sealed and stored at 8°C for the duration of ripening.

4.2.4 Cheese Sampling for Analysis

Cheeses were sampled for microbial, proteolysis and free amino acid (FAA) analysis at days 1, 7, 14, 30, 60, 90, 135 and 180 during the ripening period. Salt, moisture, fat and pH analysis was carried out on the day 14 samples only. Cheese samples were prepared by grating using a food processor to a particle size of ≈ 1 mm. Samples for microbial analysis were collected aseptically using a cheese trier and the resulting cheese core (≈10 g) was transferred to a sterile stomacher bag.

4.2.5 Cheese Composition

Fat content was calculated by nuclear magnetic resonance (NMR) (Fast Trac analysis system, CEM Microwave Technology [Ireland] Ltd). For pH measurements, 20 g of cheese was mixed into a slurry with 12 g of distilled water at 55 °C and pH was measured (Fenelon & Guinee, 1999). Salt analysis was performed using the potentiometric method (ISO5943, 2006). Moisture content was calculated by oven drying at 102 °C for 5 hours (O'Callaghan et al., 2017). All measurements were carried out in triplicate.

4.2.6 Primary and Secondary Proteolysis

Both primary proteolysis and secondary proteolysis, expressed as % pH 4.6 water soluble nitrogen (WSN)/g and free amino acids (FAA) respectively were analysed for all cheeses as previously described (Hickey et al., 2018). All experiments were carried out in triplicate.

4.2.7 Microbial Analysis

Microbial analysis for starter lactic acid bacteria (SLAB) and NSLAB was performed as previously described (Hou et al., 2012). The ≈10g aseptically taken core sample of cheese was diluted 1:10 in sterile 2% (w/v) trisodium citrate and macerated for 4 minutes in a stomacher (BagMixer 400 P, INTERSCIENCE). Serial dilutions of the resultant cheese slurry were prepared in sterile maximum recovery diluent. SLAB populations were enumerated on LM17 agar (M17: CM0817B, Oxoid; Lactose: S25375A, Fisher Scientific) a non-selective medium used for enumeration of lactococci from fermented dairy products following incubation at 30 °C for 3 days (Terzaghi & Sandine, 1975). NSLAB were enumerated on *Lactobacillus* Selective (LBS) agar (B11327, Fisher Scientific) a selective medium used for enumeration of lacticaseibacilli from fermented dairy products following anaerobic incubation at 30 °C for 5 days (Rogosa et al., 1951). All experiments were carried out in triplicate.

4.2.8 Fermented Milk Manufacture

Lc. rhamnosus DPC7102 and *Lc. paracasei* DPC7150 were grown in MRS broth for 24 hours and inoculated at 1% v/v into 10% (w/v) heat treated (121 °C for 5 min) RSM

(Kerry Food Ingredients) in test tubes for 18 hours at 30 °C, separately by which time the milks had coagulated confirming growth of the two cultures. The pH of the fermented milks were 5.17 ± 0.5 and 5.54 ± 0.5 the NSLAB populations were 9.03 ± 0.16 and 7.74 ± 0.12 log CFU/g for the *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 respectively.

4.2.9 *In vitro* Digestion using INFOGEST 2.0

Cheddar Cheese (at Day 180 of Ripening) and Fermented Milk (FM) samples made using either *Lc. rhamnosus* DPC7102 or *Lc. paracasei* DPC7150 were digested *in vitro* using the INFOGEST 2.0 protocol, with some modifications (Brodkorb et al., 2019). Cheese samples (5 g) were mixed at a ratio of 1:1 with simulated salivary fluid (SSF) without amylase and macerated for 4 minutes in a stomacher (BagMixer 400 P, INTERSCIENCE) to simulated mastication. The cheese/SSF mixture was then mixed at a ratio of 1:1 with simulated gastric fluid (SGF) containing lipase (60 U/mL) and pepsin (2000 U/mL). The pH of the mixture was adjusted to 3 and incubated at 37 °C for 120 minutes on a rotator (Stuart Tube Rotator SB3, Huberlab). Simulated intestinal fluid (SIF) was then added at a ratio of 1:1, containing pancreatin (100 U trypsin activity/mL) and bile (10 mM/L). The pH was adjusted to 7 and the mixture was incubated for a further 120 minutes at 37°C, with rotation. Due to the short residence time of yogurt in the mouth and the absence of mastication when ingested, the FM samples were added directly to SGF and subsequently treated identically to the cheese samples during simulated gastric and intestinal digestion. Immediately following completion of *in vitro* digestion, serial dilutions of the resultant digesta were prepared. FM digesta samples were plated on MRS agar due to only the strains of interest being present in the samples, while Cheese digesta samples were plated on the selective LBS media due to the potential persistence of *Lactococcus* starters and other adventitious microorganisms. MRS plates were incubated at 37 °C for 3 days, while LBS plates were incubated at 30 °C for 5 days, with both stored anaerobically. Simulated digestions were performed in triplicate and repeated three times. MRS and LBS, based on the work of Rogosa et al (1951) are designed to support the growth of lacticaseibacilli (Rogosa et al., 1951). The key difference between both media is that LBS contains ammonium citrate, sodium acetate, acetic acid and ferrous sulphate that act as inhibitors of lactococci and provide a low pH environment which is tolerated by

lactobacilli. Where high populations of lactococci are expected in conjunction with lacticaseibacilli, such as is the case with cheese LBS is the medium of choice.

4.2.10 Pulsed Field Gel Electrophoresis

Following INFOGEST 2.0, 18 colonies from LBS plates for each cheese were randomly selected, purified and DNA fingerprint profiles were generated using PFGE as previously described (Leeuwendaal et al., 2021). Bacterial cultures were inoculated at 1% and grown overnight in 10 mL MRS broth containing 0.02 M threonine, anaerobically. 0.5mL of each overnight culture was centrifuged at 12000 rpm for 5 minutes, the supernatant aspirated, and the pellet resuspended in 0.5 mL of Buffer 1 (0.01 M Tris-HCl, 1 M NaCl, pH 7.6). The cell suspension was re-centrifuged, the supernatant aspirated, and the pellet resuspended in 0.3 mL Buffer 1, which was then mixed with an equal volume of 2% low melting point agarose in 0.125 M EDTA (pH 7.6) and pipetted into PFGE plug moulds and allowed to solidify at room temperature. Plugs were left overnight (16-24 hours), rocking, at 37 °C in 1 mL of previously prepared EC Buffer (0.1 M EDTA, 1. 0 M NaCl, 1% [w/v] N-Lauroylsarcosine sodium salt, 0.006 M Tris-HCl, pH 7.6) with 10 mg/mL Lysozyme and 20 Units/mL Mutanolysin. Subsequently, the EC buffer was aspirated and replaced with 1 mL of Buffer 2 (0.5 M EDTA, 1% (w/v) N-Lauroylsarcosine sodium salt, pH 8.0) with 0.5 mg/mL Proteinase K, and left overnight (stationary) at 55 °C. Plugs were washed twice in TE 10/1 Buffer (0.001 M EDTA, 0.01 Tris-HCl, pH 8.0) with 0.001 M phenylmethylsulphonyl fluoride (PMSF) for 1 hour each at 37 °C (stationary), and stored in Eppendorf tubes containing 1 mL TE 10/100 Buffer (0.1 M EDTA, 0.01 Tris-HCl, pH 8.0) at 4 °C until required. Plugs were then cut into 1-2 mm slices and washed three times in 1 mL TE 10/0.1 (0.0001 M EDTA, 0.01 Tris-HCl, pH 8.0) at room temperature for 30 minutes each with gentle rocking. Slices were subsequently transferred to Eppendorf tubes containing 0.1 mL 1X Buffer Tango (Thermo Scientific) for minimum 30 minutes at 4 °C, followed by the addition of 0.4 µL of *AscI* (10 U/µL) restriction enzyme (Thermo Scientific) and incubation continued for 24 hours at 37 °C (stationary). 0.5 mL of 0.5 M EDTA was added to the slices to deactivate the enzyme, followed by loading of the slices into wells of a 200 mL 1% agarose Pulsed Field Certified™ (Bio-Rad) gel prepared with 0.5X dilution Tris-borate EDTA (TBE) buffer (55 g/L Boric Acid), 40 mL/L 0.5 M EDTA [pH 8.0], 108 g/L Tris). Gels were run using a CHEF-DR® II PFGE apparatus (Bio-Rad) in 2.3 L of

the same 0.5X TBE Buffer using the following parameters: Initial Switch Time 1 Second, Final Switch Time 20 Seconds, 6 V/cm, for 16 hours, at 14 °C. Resulting gels were stained with 10 mg/mL ethidium bromide (E1510 Merck) for 1 hour, followed by 2 destaining washes in distilled water for 40 minutes each. Gels were photographed using an Alpha Imager® 3400 (Alpha Innotech Corp). Lambda PFG Ladder (New England BioLabs® Inc.) was loaded with every gel as a reference marker. Pure strains of both the *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 were run in their respective gels as controls.

4.2.11 Statistical Analysis

Statistical analysis and graphs were generated using the IBM SPSS® software platform for Windows (Ver. 26). Analysis of Variance (ANOVA) was used to compare results between all three Cheddar Cheese types.

4.3 Results

4.3.1 Cheese Manufacture and Composition

The same manufacturing protocol was used for all three cheeses, control cheese with only SLAB, and the two test cheeses containing SLAB plus adjunct *Lc. rhamnosus* DPC7102 and SLAB plus adjunct *Lc. paracasei* DPC7150. No statistically significant ($p < 0.05$) differences in cheese composition as measured by pH, moisture, salt, salt in moisture (S/M), fat, fat in dry matter (FDM) or moisture in non-fat substances (MNFS) were observed between the different cheeses (**Table 1**).

Compositional Indices	Control Cheese	*Lc. rhamnosus* DPC7102 Cheese	*Lc. paracasei* DPC7150 Cheese
pH	5.07 ± 0.09	4.98 ± 0.15	4.99 ± 0.05
Moisture (%)	36.62 ± 1.28	36.67 ± 2.26	36.30 ± 1.20
Salt (%)	1.97 ± 0.12	1.90 ± 0.29	1.96 ± 0.12
S/M (%)	5.39 ± 0.52	5.22 ± 1.08	5.41 ± 0.23
Fat (%)	30.17 ± 0.56	30.18 ± 1.11	30.16 ± 0.64
FDM (%)	47.61 ± 0.29	47.66 ± 0.18	47.35 ± 0.23
MNFS (%)	52.44 ± 1.18	52.49 ± 1.95	51.97 ± 1.01

Table 1. Cheese Composition at Day 14 of Ripening. S/M salt in moisture, FDM fat in dry matter, MNFS moisture in non-fat substance**.** No statistically significant differences ($p < 0.05$) were observed between any of the cheese groups for any of the above parameters.

4.3.2 SLAB and NSLAB Populations during Cheese Ripening

Day 1 SLAB populations of *L. lactis* were detected in all three Cheddar cheese trials at >9 log CFU/g, only dropping below this at 90 days of ripening (**Figure 1**). The SLAB counts at 180 days ripening were 6.3, 5.2 and 5.3 log CFU/g for the Control, *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 cheeses, respectively. SLAB counts between cheeses only differed significantly at days 135 and 180 of ripening. Adjuncts *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 added to their corresponding cheeses were present at 7.6 and 7.1 log CFU/g, respectively, at the Day 1 timepoint (**Figure 2**). *Lc. rhamnosus* DPC7102 NSLAB numbers increased to eventually peak at 8.6 log CFU/g at Day 180, while the *Lc. paracasei* DPC7150 NSLAB peaked earlier at Day 90 with 8.3 log CFU/g and dropped off slightly for a final count of 7.9 log CFU/g at Day 180 (**Figure 2**). In general, NSLAB populations for both cheeses remained between 7.1 and 8.6 log CFU/g throughout ripening (**Figure 2**). In contrast, the Control cheese NSLAB population (to which no adjunct cultures were added) was only detected at Day 60 (at 2.0 log CFU/g) and reached a count of 5.8 log CFU/g at the end of the ripening process (**Figure 2**). NSLAB counts in all three cheeses differed significantly at each timepoint, which is likely due to the extremely low NSLAB counts in the Control cheese.

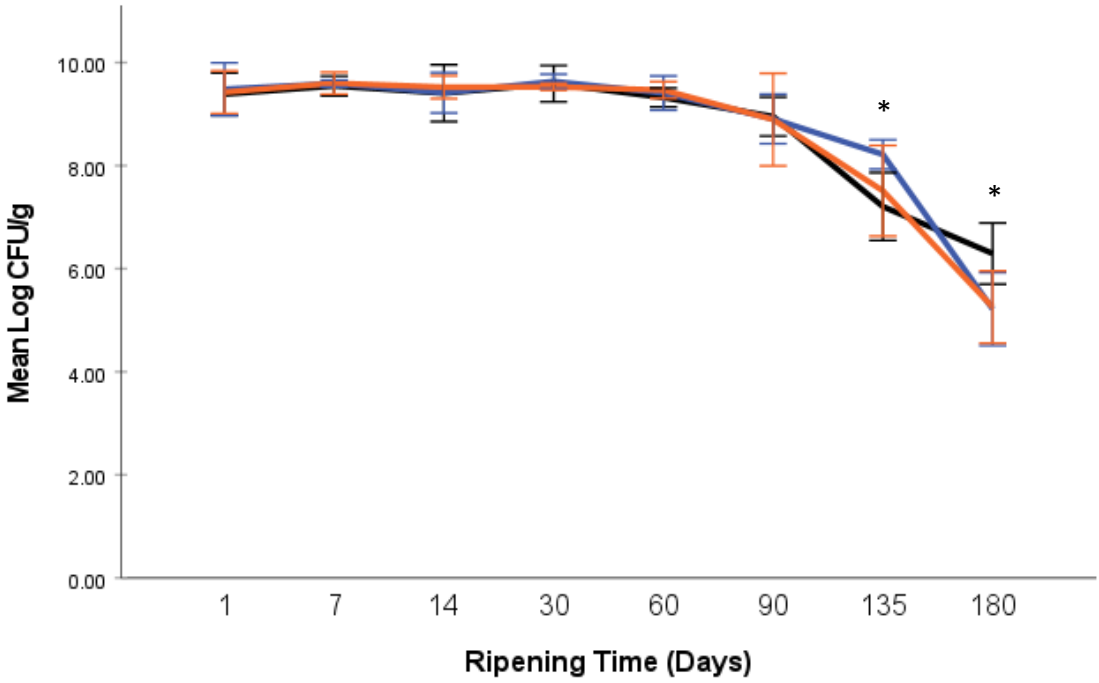


Figure 1. Quantification of *L. lactis* SLAB populations during ripening for all Cheddar cheeses, with error bars representing variation within 2 standard deviations. Significant differences were only seen in the last two timepoints. I (Black) – Control Cheddar cheese. I (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. I (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese * Denote significant differences ($p \leq 0.05$) observed between three cheeses at a single timepoint.

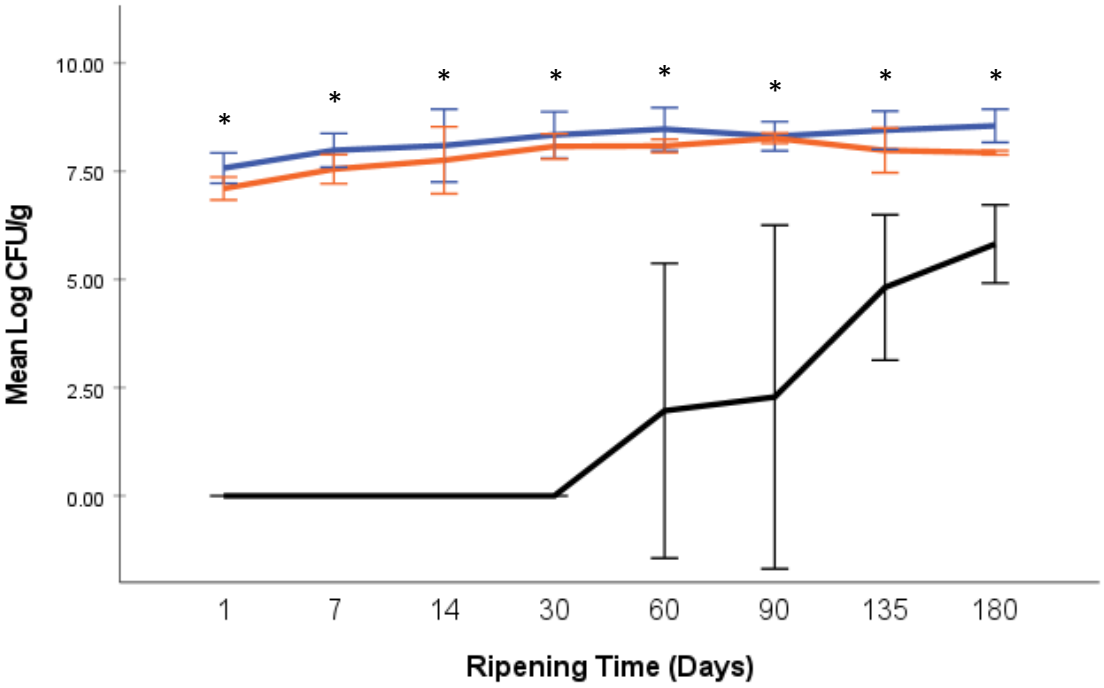


Figure 2. Quantification of NSLAB populations during ripening for all Cheddar cheeses, with error bars representing variation within 2 standard deviations. Significant differences were seen between the three cheeses at each timepoint. I (Black) – Control Cheddar cheese. I (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. I (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese * Denote significant differences ($p \leq 0.05$) observed between three cheeses at each single timepoint.

4.3.3 Proteolysis in cheese during ripening

Levels of WSN (an indicator of primary proteolysis) increased for all three cheeses as ripening time progressed. A significant increase in % WSN/g was observed in the *Lc. paracasei* DPC7150 cheese from Day 30 onward ($p \leq 0.05$), and Control and *Lc. rhamnosus* DPC7102 cheeses exhibited significantly higher % WSN/g levels from Day 60 onwards. However, no statistically significant differences were seen between cheese groups at any point during cheese ripening (**Figure 3**).

The level of FAA liberated from peptides was measured to check secondary proteolysis in each cheese. Total FAA increased significantly from Day 7 onward for the Control and *Lc. rhamnosus* DPC7102 cheeses, and from Day 14 onward for the *Lc. paracasei* DPC7150 cheese. No statistically significant differences were seen in mean levels of total FAA between cheese groups at any point during cheese ripening (**Figure 4**).

Most individual amino acid concentrations did not differ significantly between the three cheeses, although some exceptions were observed at various points during cheese ripening. Initially, no significant differences were seen between any of the groups (**Figure 5a**). At day 7, only glycine concentrations were significantly different between cheeses (**Figure S1**). At Day 14 significant differences in aspartic acid, glutamic acid, glycine and proline concentrations were observed (**Figure S2**). Day 30 showed significant differences in aspartic acid and glycine (**Figure S3**). Day 60 and day 135 only showed a significant difference in aspartic acid concentrations, while the last timepoint (day 180) showed significant differences in both aspartic acid and valine (**Figure S4, Figure S6, Figure 5b**). Aspartic acid was the most variable amino acid between the three cheeses, and differed significantly at days 14, 30, 60, 135 and 180. Glycine concentrations differed significantly at three timepoints (days 7, 14 and 30). Detection of tryptophan ceased at day 30 for all cheeses.

Additionally, day 30 showed a significant difference in the microbial metabolite γ aminobutyric acid (GABA), which was greater in cheeses produced with the adjunct cultures (**Figure S3**).

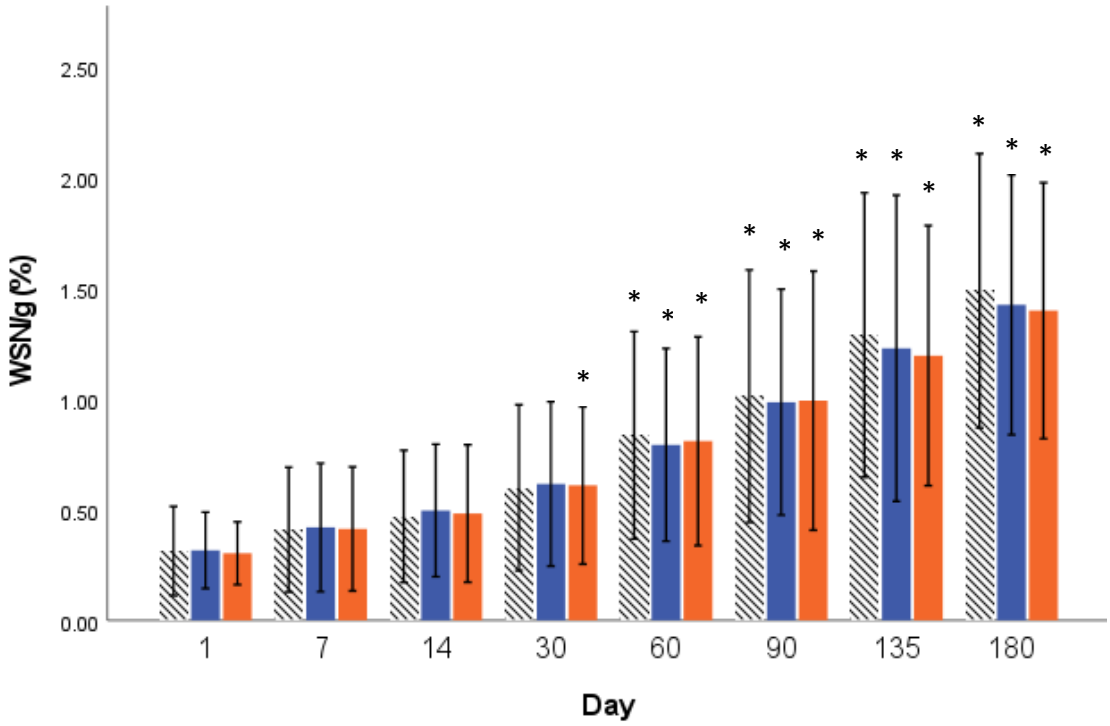


Figure 3. Primary Proteolysis as measured by % WSN/g of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen for *Lc. paracasei* DPC7150 from Day 30 onwards, and Day 60 onwards for Control and *Lc. rhamnosus* DPC7102 Cheddar cheeses. (Striped) – Control Cheddar cheese. (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences ($p \leq 0.05$) between indicated bar and 'Day 1' equivalent.

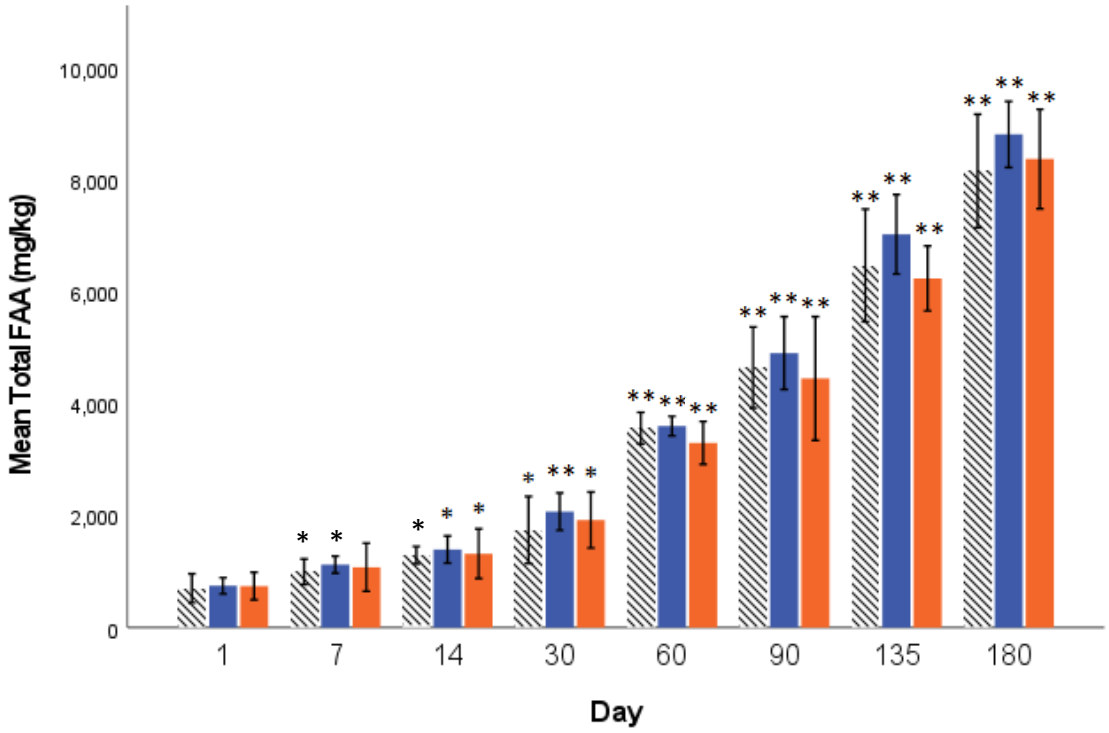


Figure 4. Mean total FAA (mg/kg) of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen for Control and *Lc. rhamnosus* DPC7102 from Day 7 onwards, and Day 14 onwards for *Lc. paracasei* DPC7150 Cheddar cheeses. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences (p≤0.05) between indicated bar and 'Day 1' equivalent. ** Denote highly significant differences (p≤0.01) between indicated bar and 'Day 1' equivalent.

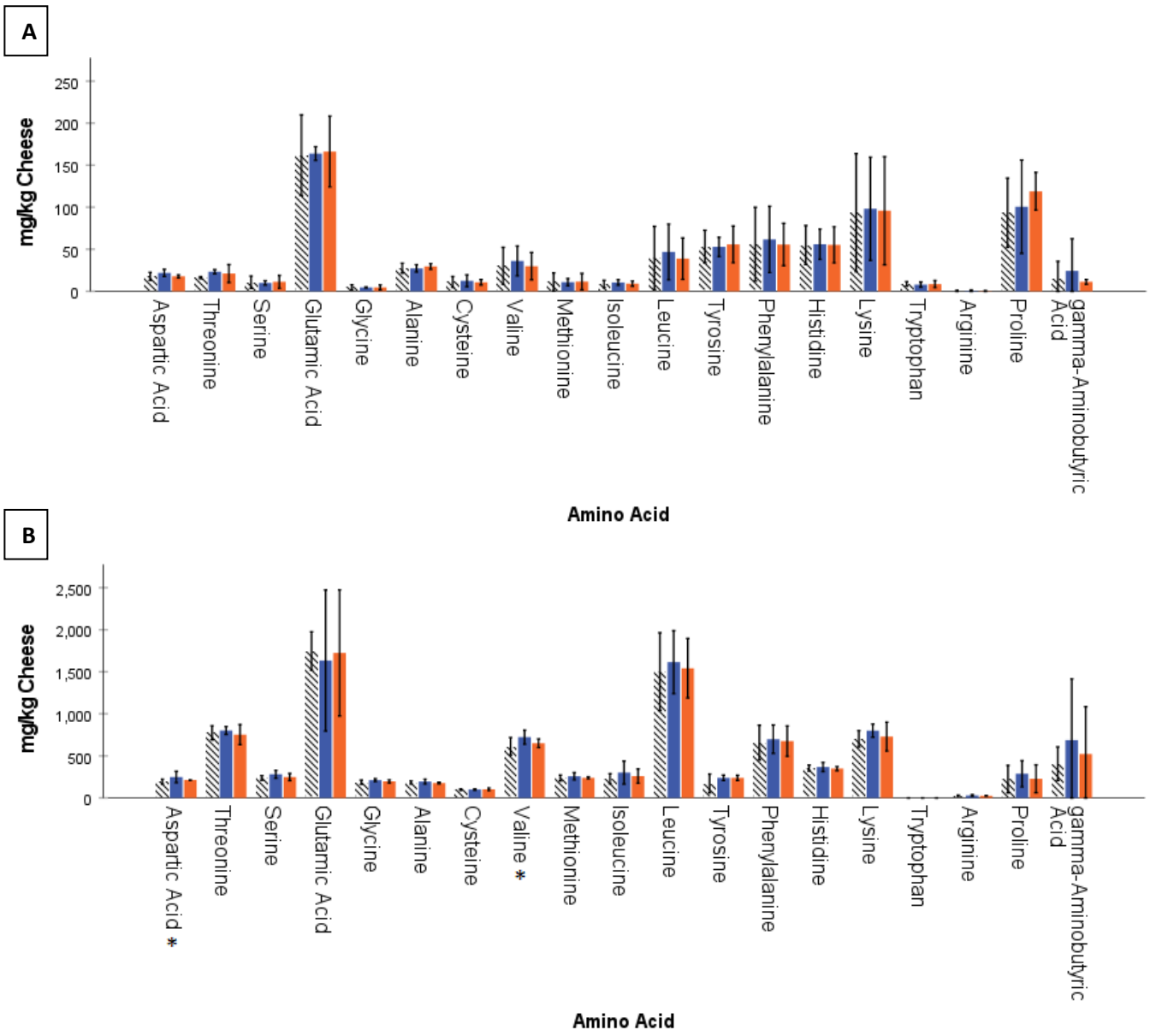


Figure 5. Individual FAA concentrations at **(A)** Day 1 and **(B)** Day 180 of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen between three Cheddar cheese groups at Day 180 **(B)** for Aspartic Acid and Valine. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences ($p \leq 0.05$) observed between three cheeses at a single timepoint.

4.3.4 Impact of food matrix on NSLAB survival during simulated gastric digestion

Cheddar cheeses and fermented milks containing *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 were exposed to the INFOGEST 2.0 protocol and the NSLAB populations of the resulting digests were investigated. In both cases, the lacticaseibacilli in the Cheddar cheese matrix exhibited a significantly higher survival rate post INFOGEST 2.0 than their fermented milk counterpart (**Table 2**). The initial populations of *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 were > 7.7 log CFU/g of cheese and fermented milk. The survival rate of *Lc. rhamnosus* DPC7102 was 10 times higher in Cheddar cheese (1.23%) than in the fermented milk (0.12%), while the survival rate of *Lc. paracasei* DPC7150 was 30 times higher when embedded in the cheese matrix (**Table 2**). Log reductions in CFU/g were also greater in the fermented milk matrix for both *Lacticaseibacillus* strains, with a 1 and 1.5 log greater reduction in the fermented milk matrix than the Cheddar matrix for the *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150, respectively (**Table 2**). In a supplementary experiment, PFGE profiles revealed the persistence of the original adjuncts in the Cheddar cheese 12 months postproduction, to varying degrees, that were also capable of surviving simulated digestion. For Cheddar cheese produced with the adjunct *Lc. rhamnosus* DPC7102, all 18 random colonies picked exhibit the same DNA profile as the original pure strain (**Figure S7A**). Four of the 18 profiles in the *Lc. paracasei* DPC7150 Cheddar matched that of the original adjunct (**Figure S7B**). The other 14 profiles seen among the *Lc. paracasei* DPC7150 Cheddar isolates appear to match the original *Lc. rhamnosus* DPC7102 strain profile, indicating potential cross contamination during cheese manufacture and subsequent dominance of DPC7102 following extended ripening (**Figure S7B**).

Strain	Matrix	Initial Log CFU/g	Final Log CFU/g	Log Reduction	% Survival
***Lc. rhamnosus* DPC7102**	Cheddar Cheese	8.46 ± 0.07	6.53 ± 0.16	1.93	1.23 ± 0.47
	Fermented Milk	9.03 ± 0.16	6.03 ± 0.13	3.00	0.12 ± 0.08
***Lc. paracasei* DPC7150**	Cheddar Cheese	7.71 ± 0.25	6.85 ± 0.09	0.86	14.33 ± 5.35
	Fermented Milk	7.74 ± 0.12	5.38 ± 0.18	2.36	0.46 ±0.18

Table 2. NSLAB Populations from 180 Day Old Cheddar Cheeses and Fermented Milks pre- and post INFOGEST 2.0.

4.4 Discussion

There is growing interest in the potential of cheese as a probiotic delivery matrix relative to other dairy food products. This interest has been strengthened as cheese has been reported to being more conducive to the growth and maintenance of probiotic species, as the acidity of cheese (pH 4.8-5.6) is less than that of fermented milks (pH 3.7-4.3), and hosts an environment capable of supporting the growth of anaerobes (bifidobacteria) and facultative anaerobes (lactobacilli) (Boylston et al., 2004). It is rich in nutrients including FAA and various metabolites produced as a result of rennet and SLAB activity as the cheese matures. In addition, its high fat content and buffering capacity afford the microbes additional protection during digestion (Boylston et al., 2004). Bacteria must be present in the final food product at levels between 10^6 – 10^7 CFU/g to potentially confer a health benefit to the host once ingested (Leroy & De Vuyst, 2014). Final counts of probiotic bacteria in cheese vary greatly depending on formulation factors (e.g. pH, oxygen availability, microbial interactions), process factors (e.g. incubation and storage temperature, heat treatment) and packaging materials and systems (for a review, see Karimi et al., 2011) (Karimi et al., 2011). This study has demonstrated that two *Lacticaseibacillus* strains with previously demonstrated probiotic potential could be added to Cheddar cheese as an adjunct culture without altering the manufacturing protocol or cheese composition, survive and persist at levels of 10^7 – 10^8 CFU/g during maturation, and could survive *in vitro* digestion at levels of > 10^6 CFU/g, significantly higher levels than when administered in fermented milk (Leeuwendaal et al., 2021).

Cheese composition was analysed at day 14 and no significant differences between cheese types were found, indicating that the addition of the adjuncts of interest had no effect on cheese composition. This is important as variation in composition, usually measured by the four parameters of pH, S/M, FDM and MNFS impacts on cheese quality and variation in environmental conditions such as pH and S/M could impact on behaviour of the adjuncts during cheese ripening and simulated gastric digestion (Gilles & Lawrence, 1973).

Microbial population shifts are important in cheese as they have the potential to influence biochemistry, in particular proteolysis and formation of FAA during cheese ripening (Smit et al., 2005). While SLAB are initially added for acidification of milk, the physical and chemical stresses experienced during cheese production and maturation impede their ability to survive (Choi et al., 2020; Ganesan et al., 2014). As pH values and lactose concentrations decrease in the aging Cheddar, SLAB populations begin to decline and are outcompeted by the more metabolically versatile NSLAB, which can reach numbers as high as 10^8 CFU/g in mature cheese (Ong et al., 2017). Bacterial enumeration completed for this study corroborated this trend, whereby initial SLAB counts were high (≈9 log CFU/g) and remained at these levels until the 90 Day time point, and then began to decrease to final counts of between 5.2 and 6.3 log CFU/g. The only significant differences seen between Cheddar types were observed at Days 135 and 180, with the Control cheese at Day 180 having ≈1 log higher population of SLAB than the cheeses containing the adjunct cultures. When monitoring levels of NSLAB in the Control cheese, quantifiable populations only appeared at Day 60, after which they continued to climb and eventually reached 5.8 log CFU/g. In contrast, the other two Cheddars had NSLAB adjuncts added to them, both of which reached numbers over 7 log CFU/g initially and remained at this level for the duration of the study. Thus, both adjunct strains were able to maintain high population levels throughout the ripening period.

Proteolysis involves the hydrolysis of cheese casein to smaller peptides and amino acids which accumulate during cheese ripening and contributes the texture and overall flavour of the finished product (Ardö et al., 2017). In addition to contributing to the organoleptic properties of the cheese, the amino acids released within the cheese matrix can be metabolised by the microbiome resulting in the formation of additional flavour and aroma active compounds (Ardö et al., 2017). In primary proteolysis, the casein molecules are broken down into smaller polypeptides, which is primarily carried out by the rennet (the coagulant, chymosin) that remains in the cheese curd postproduction (Fox et al., 1996; Soodam et al., 2015). Most of these polypeptides are soluble in water at pH 4.6 and thus nitrogen levels soluble in water at pH 4.6 are a useful indicator of primary proteolysis. In this study, we observed that the levels of % WSN/g increased as maturation progressed and no significant differences in % WSN/g between Cheddar types were observed. % WSN/g increased significantly as ripening

time progressed, with a significant increase ($p \leq 0.05$) from Day 30 onward in *Lc. paracasei* DPC7150 cheese being observed, followed by the Control and *Lc. rhamnosus* DPC7102 cheeses from Day 60 onward. While the increase in % WSN/g is typically observed in Cheddar cheeses as maturation occurs, the addition of adjunct cultures interfering with primary proteolysis appears to be strain dependant (Lane & Fox, 1996; Ong et al., 2007; Stefanovic & McAuliffe, 2018).

Secondary proteolysis occurs when proteinases and peptidases degrade the polypeptides produced in primary proteolysis into smaller peptides and free amino acids, which can be quantified in the WSN extract (Rank et al., 1985). The total concentration of FAA significantly ($p \leq 0.05$) increased in all Cheddar types, from Day 7 in the Control and *Lc. rhamnosus* DPC7102 cheeses and from Day 14 in the *Lc. paracasei* DPC7150 cheese, but no significant differences were observed in total FAA between cheese types. Tryptophan was no longer detected in any cheese from Day 30 onward, which is generally desirable as accumulation of this amino acid can lead to its catabolism by microorganisms in the cheese leading to the production of flavour defects, including flavours described as faecal, putrid and unclean (Gummalla & Broadbent, 1999; Ummadi & Weimer, 2001). Glycine levels differed significantly ($p \leq 0.05$) between cheeses at Days 7, 14 and 30, with *Lc. rhamnosus* DPC7102 cheeses tending to have higher concentrations than either the control or the *Lc. paracasei* DPC7150 cheese but were similar for the remaining timepoints. However, aspartic acid levels differed the most between cheeses at five of the eight timepoints, including the final one, with *Lc. rhamnosus* DPC7102 once again having higher levels than the other cheeses in all cases. Aspartic acid can be broken down to give a buttery flavour (diacetyl) but may also affect the chemistry of other highly volatile sulfuric compounds (Ardö, 2006). Excluding the initial timepoint, levels of GABA were always higher in the Cheddars containing the *Lacticaseibacillus* adjuncts, although this difference was only significant at day 30. GABA is a neurotransmitter and important bioactive compound with a plethora of physiological functions, and many LAB have been identified from fermented foods that are capable of generating this compound (for a review, see Cui et al., 2020)(Cui et al., 2020). It may be worth investigating the ability of both *Lacticaseibacillus* strains of interest to produce GABA due to the trend seen here, as an additional beneficial feature of these potential probiotics. Thus, the addition of the adjuncts did not have a large effect on primary or secondary proteolysis. While some

differences in individual amino acid levels were observed, the majority did not differ significantly between the three different Cheddars and no differences were seen between cheeses when total amino acid levels were examined.

In 2014, a new method for *in vitro* simulated gastric digestion was developed with the intended purpose of replacing the broad range of existing methodologies that differed between research groups and made comparison of inter laboratory studies very difficult (Minekus et al., 2014). This is referred to as the INFOGEST method, and was further improved upon to include an oral phase and lipase enzyme during the gastric phase (INFOGEST 2.0) (Brodkorb et al., 2019). In order to determine the suitability of Cheddar cheese as a microbe delivery matrix, INFOGEST 2.0 was employed on both the *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 cheeses, as well as fermented milk samples inoculated with the two *Lacticaseibacillus* strains of interest. As of now, only one previous study has attempted to digest cheese using this model and did not examine microbial survival, instead focusing on the impact of simulated digestion on peptide profiles of Prato cheese (Baptista et al., 2020). Thus, this study is the first to utilise the INFOGEST 2.0 with gastric lipase (relevant due to the high fat content of Cheddar) to determine survival of potentially beneficial microorganisms through the upper digestive system. Cheddar cheese was shown to protect lactobacilli to a significantly greater extent than the fermented milk counterparts, with >1 log CFU/g difference seen between matrices in both cases. Sharp et al. (2008) showed a reduction of 2 log CFU/g of *Lb. casei* 334e when exposed to a 30-minute acid stress test in a low-fat Cheddar matrix, while the same strain showed a much higher 6 log CFU/g reduction following the same acid test in a yogurt matrix (Sharp et al., 2008). While these log reductions vary, the overall findings presented here are in agreement with those of Sharp and colleagues (2008) concerning the potential of Cheddar cheese as a promising probiotic vehicle (Sharp et al., 2008). To determine the persistence of the two *Lacticaseibacillus* strains, an additional digestion was performed following extended ripening to 12 months on the same two Cheddars and PFGE DNA profiles of survivors were tested against the pure strain profiles of the original *Lacticaseibacillus* adjuncts. The NSLAB population of cheese with *Lc. rhamnosus* DPC7102 adjunct was found to be homogeneous and composed only of the *Lc. rhamnosus* DPC7102 strain, while the population of the cheese made with *Lc. paracasei* DPC7150 as adjunct was composed of over 20% of the added *Lc. paracasei* DPC7150 strain. The log CFU/g

counts of the potentially probiotic lactobacilli were >7.7 following 12 months of cheese maturation and >6.5 following the INFOGEST 2.0 simulated digestion, both values of which satisfy the established criteria of probiotic microbes being present at a minimum 6-7 log CFU/g in the end product, further highlighting the advantages of using Cheddar as a probiotic carrier. These data demonstrate that both strains are capable of surviving in a Cheddar matrix for at least 12 months of ripening, and that both are capable of surviving simulated gastric digestion.

4.5 Conclusion

This study demonstrated the ability of two *Lacticaseibacillus* strains previously shown to express probiotic traits to survive in a Cheddar cheese matrix during ripening when added as an adjunct and to have the ability to survive simulated gastric digestion using the INFOGEST 2.0 model (Leeuwendaal et al., 2021). Overall, the addition of *Lc. rhamnosus* DPC7102 or *Lc. paracasei* DPC7150 did not affect SLAB proliferation or cheese composition when compared to the adjunct-free Cheddar control. Additionally, neither primary nor secondary proteolysis differed significantly with the control, with the exception of a few amino acids. When comparing the survival of strains during the INFOGEST 2.0 static digestion model, Cheddar cheese proved a superior dairy matrix when compared to fermented milk grown with the same lactobacilli strains. Generally, probiotic products are expected to contain a minimum concentration of 10^6 CFU/g prior to ingestion, and that 10^8-10^9 CFU/g should be consumed on a daily basis to ensure any health benefits to take effect (Kechagia et al., 2013). Cheddar cheese produced in this study contained NSLAB with proven *in vitro* probiotic traits that were both capable of surviving at 10^7 CFU/g during cheese ripening and could also survive at levels of 10^6 CFU/g following simulated digestion. Thus, these strains can survive at sufficient levels once ingested to potentially impact on human health.

4.6 Supplementary Material

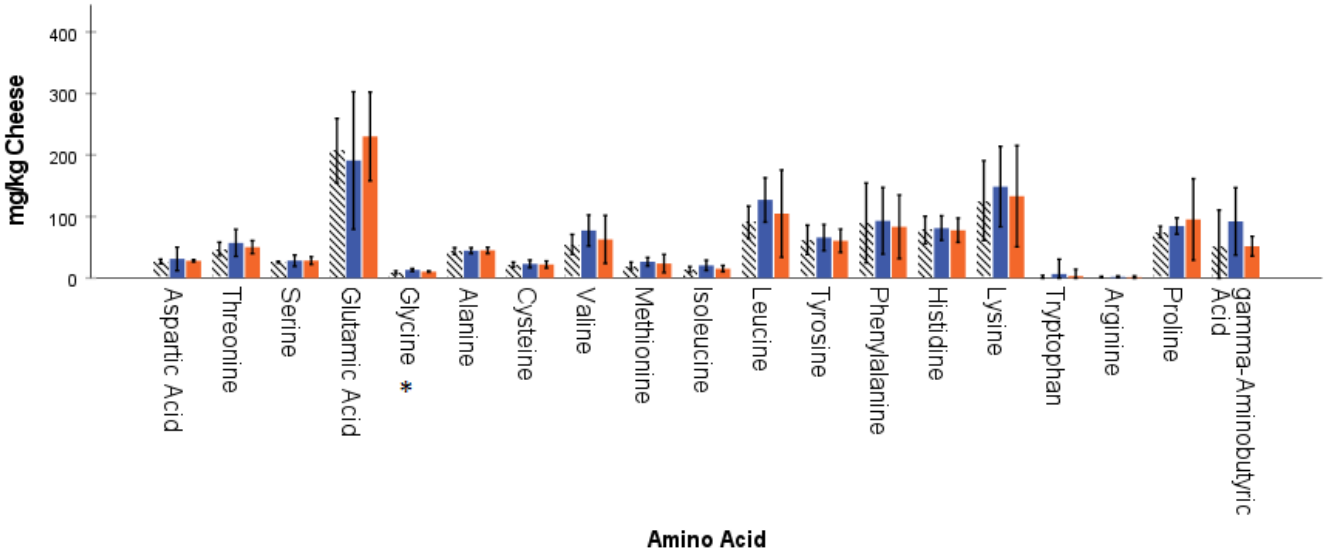


Figure S1. Individual FAA concentrations at day 7 of ripening of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen between three Cheddar cheese groups for Glycine. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences ($p \leq 0.05$) observed between three cheeses at a single timepoint.

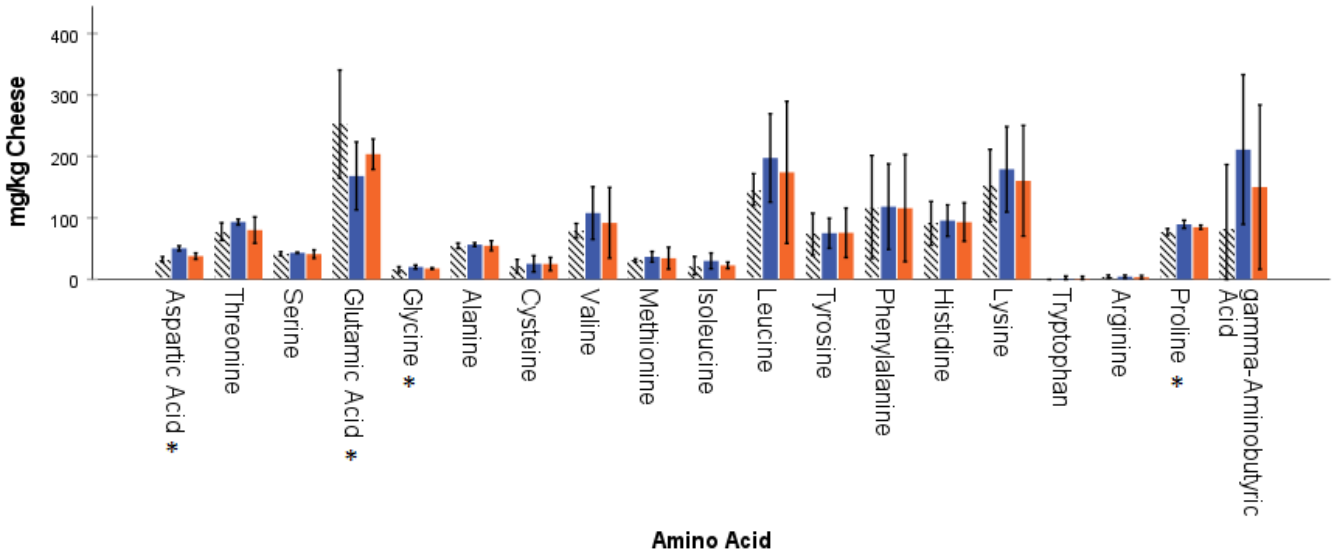


Figure S2. Individual FAA concentrations at day 14 of ripening of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen between three Cheddar cheese groups for Aspartic Acid, Glutamic Acid, Glycine, and Proline. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences ($p \leq 0.05$) observed between three cheeses at a single timepoint.

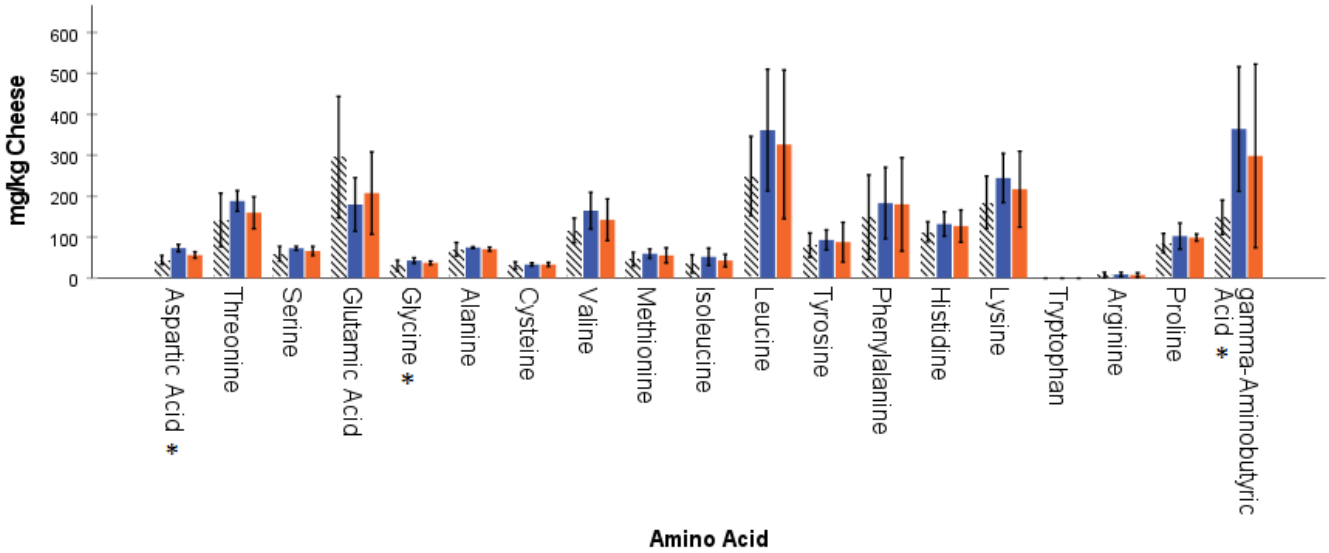


Figure S3. Individual FAA concentrations at day 30 of ripening of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen between three Cheddar cheese groups for Aspartic Acid, Glycine, and gamma-Aminobutyric Acid. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences ($p \leq 0.05$) observed between three cheeses at a single timepoint.

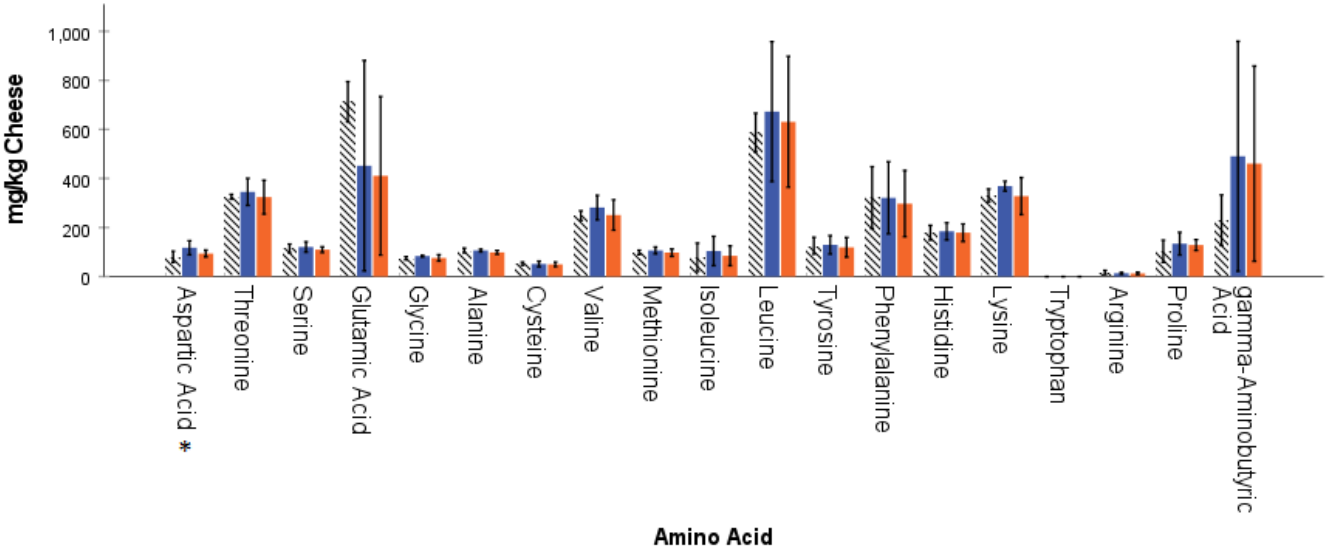


Figure S4. Individual FAA concentrations at day 60 of ripening of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen between three Cheddar cheese groups for Aspartic Acid. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences ($p \leq 0.05$) observed between three cheeses at a single timepoint.

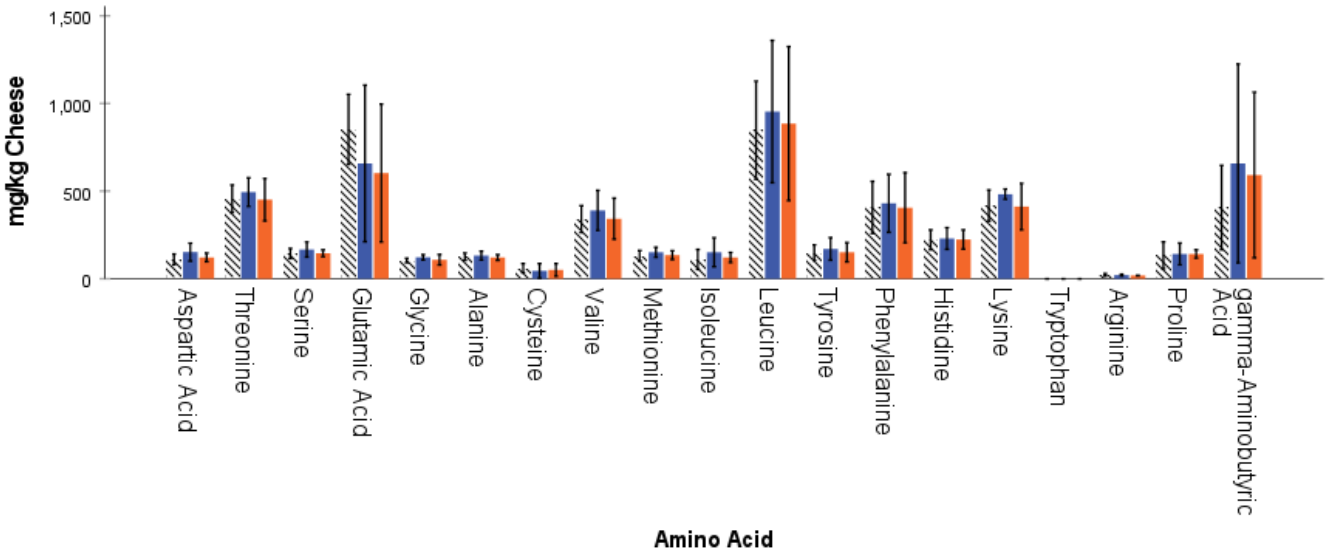


Figure S5. Individual FAA concentrations at day 90 of ripening of each Cheddar, with error bars representing variation within 2 standard deviations. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese.

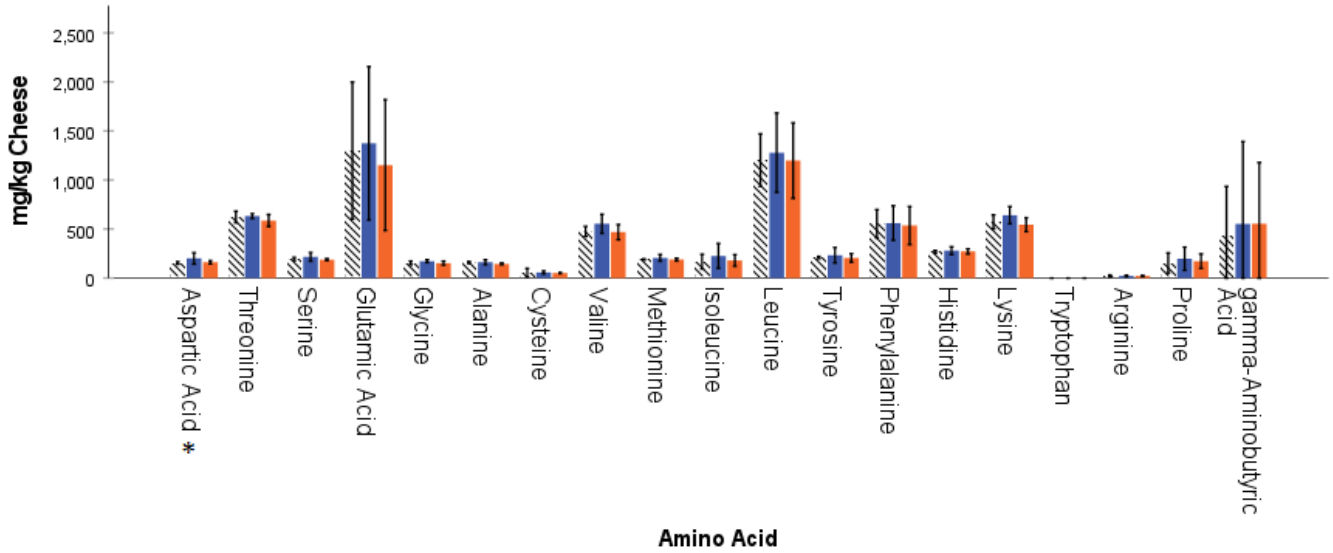


Figure S6. Individual FAA concentrations at day 135 of ripening of each Cheddar cheese, with error bars representing variation within 2 standard deviations. Significant differences were seen between three Cheddar cheese groups for Aspartic Acid. ▨ (Striped) – Control Cheddar cheese. ■ (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese. ■ (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese. * Denote significant differences ($p \leq 0.05$) observed between three cheeses at a single timepoint.

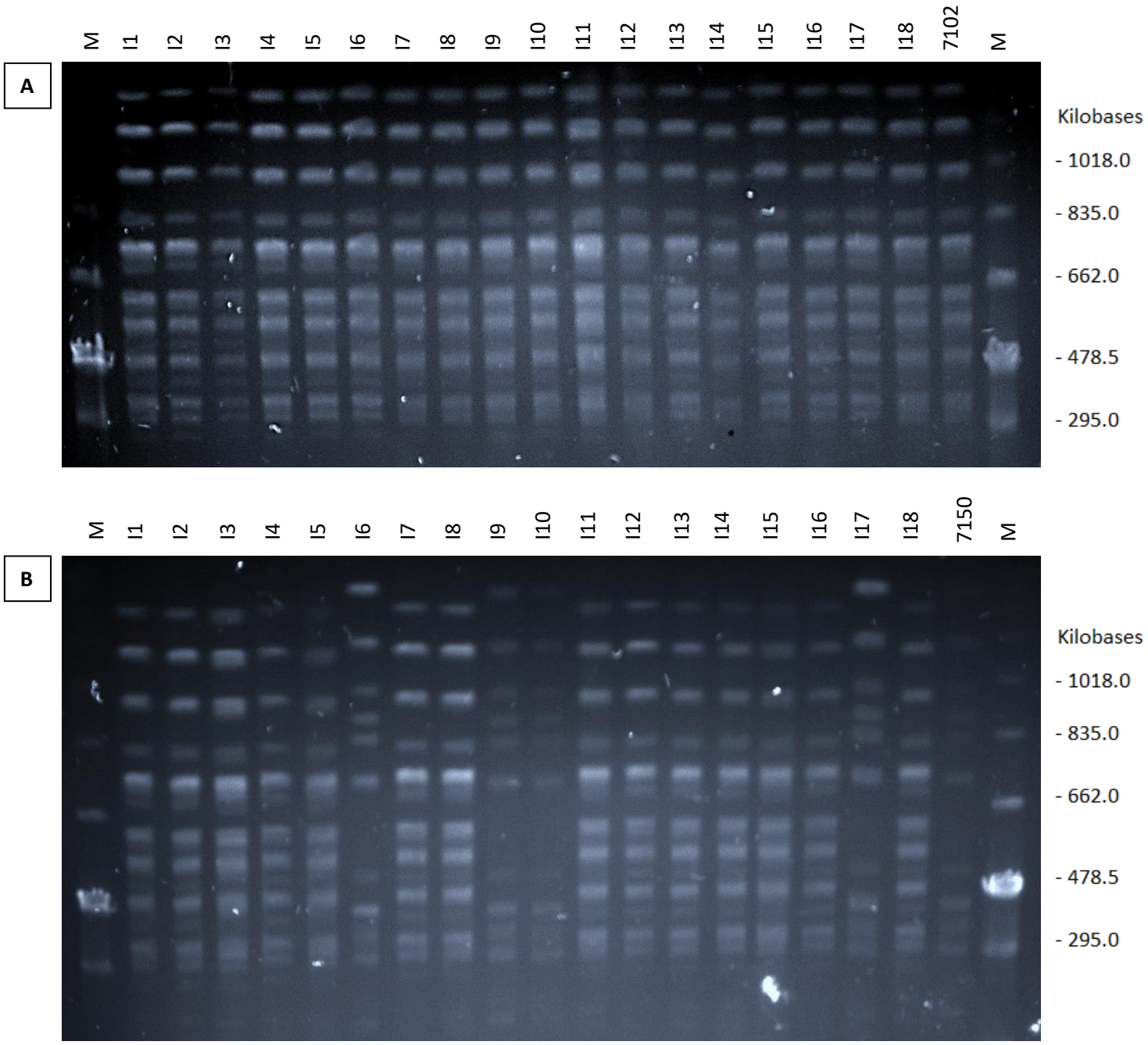


Figure S7. PFGE-generated DNA profiles of randomly selected NSLAB isolates capable of surviving INFOGEST 2.0 simulated digestion. **(A)** Profiles of Isolates from Cheddar cheese produced with the *Lc. rhamnosus* DPC7102 adjunct. **(B)** Profiles of Isolates from Cheddar cheese produced with the *Lc. paracasei* DPC7150 adjunct. M – Lambda PFG Ladder (NEB). I – Isolate. 7102 – *Lc. rhamnosus* DPC7102 control. 7150 – *Lc. paracasei* DPC7150 control.

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Chapter 5

The Effect of Cheddar Cheese containing *Lacticaseibacillus* on the Murine Microbiome during a Short-Term Feeding Trial

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Abstract

The effects of fermented foods on the gut microbiome following ingestion have been tested by various researchers. The effects of Cheese, a fermented dairy product, on the gut microbiome are largely inconsistent between groups. Similarly, cheese and dairy products and their ability to affect weight gain and other metabolic parameters show variation between studies. Cheese has also been postulated as a promising probiotic vehicle due to the protective effects of its matrix on its own microbial population against low pH and gastric fluid. The aim of this study was to determine if Cheddar cheese manufactured with either a *Lacticaseibacillus rhamnosus* or *Lc. paracasei* strain (both of which exhibited probiotic characteristics *in vitro*) was capable of affecting murine gut microbiomes in a short-term feeding study. Additionally, weight gain, organ weight, adipose tissue mass and cholesterol and triacylglycerol plasma levels were measured to determine if cheese consumption affected these important metabolic parameters. While no differences were observed between dietary groups in weight gain, organ/adipose tissue mass, or total cholesterol, mice in the Cheddar dietary groups did consume significantly less chow than their control group and had significantly higher total triacylglycerol levels. Additionally, significant differences were observed in both faecal and caecal microbiomes between treatment groups. Alpha and beta diversity were significantly different between dietary groups. The abundance of both the *Lactococcus* and *Lactobacillus* genera (the starter and adjunct/non-starter cultures of the cheeses, respectively) were significantly higher in all Cheddar groups in comparison to their Control chow diet counterparts. Thus, inclusion of Cheddar containing potentially beneficial *Lacticaseibacillus* strains did not affect weight in a murine model and was capable of causing significant shifts in microbiome composition, including within genera of interest.

5.1 Introduction

Various groups have investigated the ingestion of fermented products and their ability to modulate the gut microbiome. A recent study by Taylor and colleagues (2020) with nearly 7000 participants investigated whether ingestion of fermented plant food products altered gut microbial content, found that those who consumed the fermented diet showed a significant difference in beta diversity in comparison to the control diet group (Taylor et al., 2020).

The effects of fermented dairy products on the gut microbiome and human health have also been investigated, with varying degrees of success. In two separate studies, fermented milks were given to patients suffering irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC), and both cases yielded positive results, albeit in different manners (Veiga et al., 2014; Yılmaz et al., 2019). The IBS patients that received a fermented milk containing known cultures exhibited a decrease in the relative abundance of *Bilophila wadsworthia* (a bacteria commonly associated with inflammation/infection) and an increase in bacteria associated with butyrate production (a short chain fatty acid known to improve intestinal motility) (Veiga et al., 2014). The study of IBD patients indicated that ingestion of the fermented milk, kefir, resulted in a significantly increased quantity of lactobacilli in faeces in patients suffering from both CD and UC, and the CD patients also experienced significantly lower C-reactive protein levels and erythrocyte sedimentation rates, indicating a decrease in inflammation (Yılmaz et al., 2019). Additionally, CD patients receiving the kefir treatment reported significantly lower bloating scores and higher 'feeling good' scores (Yılmaz et al., 2019). A separate study investigated the effects of a probiotic-containing yogurt on children with *Helicobacter pylori* infection and found that it significantly increased faecal *Bifidobacterium* spp./*Escherichia coli* ratios (Yang & Sheu, 2012).

Not all studies resulted in significant disturbances to the gut microbiome. Yogurt administration to healthy subjects for 42 days did not result in any consistent, significant fluctuations within the gut microbiome, but was found to induce changes in its overall diversity and composition (Lisko et al., 2017). Similarly, Tillisch and

colleagues (2013) demonstrated that, while no significant changes in faecal microbial composition occurred following ingestion of a probiotic fermented milk product by healthy women, brain region activity associated with emotion and sensation processing was affected significantly, indicating that fermented food ingestion can have health benefits beyond affecting gut microbial composition (Tillisch et al., 2013).

The ability of cheese to modulate the gut microbiome and exert other positive effects on health has been studied to a lesser extent. One group examined Camembert consumption and its effects on the faecal microbiome of healthy subjects (Firmesse et al., 2007, 2008). Twelve patients consumed two portions of 40g of either Camembert cheese or a cheese spread containing no live bacteria every day for 4 weeks, followed by a wash out period of 2 weeks (Firmesse et al., 2007, 2008). Real time quantitative PCR analysis of stool samples revealed that native *Enterococcus faecalis* populations were significantly increased, while high levels of *Lactococcus lactis* and *Leuconostoc mesenteroides* originating from the Camembert microbiome were detected during the trial, with the *Ln. mesenteroides* persisting in stool samples 15 days post cheese consumption (Firmesse et al., 2007, 2008). Another study found that ingestion of 30 g per day of Bryndza cheese (made from sheep's milk) by female patients with a body fat percentage of 25% or more resulted in a relative increase in abundance of lactic acid bacteria, with a significant increase being observed in *Phascolarctobacterium* and *Butyricimonas* (Hric et al., 2021).

The ability of cheese to serve as a vehicle for probiotic delivery has been investigated, although the number of studies is limited. Cheese may be a more successful probiotic carrier than other fermented milk alternatives due to it being nutrient-dense and, thus, able to sustain its own microbiome for longer periods of time (Castro et al., 2015). Additionally, it has greater acid-buffering capacities than yogurt, offering greater protection to its own microorganism during transit through the gastrointestinal tract and, more importantly, the stomach (Hayes et al., 2006). Probiotics are capable of surviving at high concentrations in cheeses, although ripening times among cheese types will often vary (Dabevska-Kostoska et al., 2015; dos Santos et al., 2012). Sharp and colleagues (2008) compared the ability of yogurt and low-fat Cheddar cheese to sustain the *Lactobacillus casei* 334e probiotic and, while both were able to maintain high numbers following 3 weeks and 3 months of storage, respectively, the low-fat

cheese matrix offered greater protection to the probiotic following exposure to acid stress (Sharp et al., 2008). In yogurt, *Lb. casei* 334e populations decreased from 10^7 colony forming units (CFU)/g to lower than 10^1 CFU/g following 30 minutes of acid stress, while the counts in low-fat cheese remained at approximately 10^4 CFU/g after 120 minutes of the same treatment (Sharp et al., 2008).

Previously, two non-starter lactic acid bacteria (NSLAB) strains, *Lb. rhamnosus* DPC7102 and *Lb. paracasei* DPC7150, were isolated from commercial Irish Cheddar cheeses that exhibited *in vitro* probiotic characteristics (Leeuwendaal et al., 2021). Cheddar cheese and a fermented milk product were prepared with these two strains added as adjuncts, and *in vitro* digestion results indicated that the cheese matrix yielded higher bacterial survival rates. Additionally, the strains of interest were found to persist at 10^7 CFU/g following 12 months of ripening, and 10^6 CFU/g following synthetic digestion (Leeuwendaal et al., 2022). An *in vivo* study was now necessary to determine if these Cheddars were capable of affecting gut microbiome and beyond. Male C57BL/6J mice were chosen due to their susceptibility to diet-induced obesity, aiding in any weight-related observations that may arise when comparing diets. The ability of supplemented or non-supplemented cheese to exert benefits on a murine system has been investigated previously by several groups, with most demonstrating positive effects on different aspects of mouse health (Cordeiro et al., 2021; Kim et al., 2021; Vasconcelos et al., 2019; Yun et al., 2020). Cordeiro and colleagues (2021) demonstrated that ingestion of Minas Frescal cheese containing the probiotic *Lactococcus lactis* NCDO 2118 during colitis (induced with dextran sodium sulfate) resulted overall in a reduction in disease severity in female C57BL/6J mice (Cordeiro et al., 2021). Mice that received the probiotic cheese exhibited a lower disease activity index and histopathological score, colon length that did not statistically differ from their control counterparts and attenuated the weight loss observed in the colitis groups (including the group that received control cheese) (Cordeiro et al., 2021). Kim et al. (2021) investigated the effects of consumption of Gouda containing the bioactive component, *Allium hooker*, on male BALB/c mice and determined that, despite having a higher calorific intake, the group ingesting the bioactive-supplemented cheese showed significantly lower body weight and weight gain than their control cheese counterparts (Kim et al., 2021). The control Gouda group mice had significantly higher triacylglycerol levels than both the bioactive-supplemented cheese and control group,

and the white adipose tissue of the bioactive-supplemented cheese was significantly lower than that of the control Gouda (Kim et al., 2021). A different group investigated the effects of non-supplemented Gouda on C57BL/6N mice and found that it reduced stress and affected the gut microbiome, with an increase in the *Bacteroides*:*Firmicutes* ratio in mice that received Gouda prepared with Jersey cow milk (Yun et al., 2020).

The aim of this study was to determine whether regular ingestion of 6-month-old Cheddar cheese containing *Lacticaseibacillus* adjuncts with probiotic traits is capable of modulating the murine microbiome. Mice were fed for a period of 4 weeks, with their regular chow diet being replaced for a specified number of hours at the beginning of the dark cycle with Cheddar cheese. Microbiome perturbations were monitored via 16S sequencing of caecal and faecal samples that were collected as the study progressed and post-mortem. The ability of cheese and its microbiome to exert effects on food intake, weight gain, adipose distribution, and plasma cholesterol and triacylglycerol levels were also investigated, to determine whether other health benefits were exerted.

5.2 Materials and Methods

5.2.1 Preparation of Cheddar Cheese

Please refer to the "Materials and Methods" section of Chapter 4, sections 2.1, 2.2, 2.3, and 2.7. The three different Cheddar cheeses (Control Cheddar, *Lacticaseibacillus rhamnosus* DPC7102 Cheddar, and *Lc. paracasei* DPC7150 Cheddar) were ripened for a period of 6 months. During this time, they were analysed for macro-composition, key biochemical indices of ripening and microbial population development. The NSLAB, including the *Lacticaseibacillus* strains of interest, were tracked and their numbers in the cheese remained high (between 10^7-10^8 cfu/g).

5.2.2 Animals

This study was carried out following ethical approval from the UCC Ethics Committee and Health Products Regulatory Authority (HPRA) (AE19132/I220), and all mice were maintained according to the provisions of the European Union (Directive 2010/63/EU) and the Republic of Ireland guidelines (S.I. No. 543 of 2021). Male C57BL/6J mice were purchased at 3 weeks of age (Envigo, UK), were group housed randomly (2 per cage), and were maintained in a 12:12 h light-dark cycle. On arrival, an initial 1-week acclimatisation was given, whereby mice were all fed a low-fat (LF) diet (D12450B, Research Diets Inc., USA). Following this acclimatisation period, cages were weight matched and divided into 4 groups of 4 cages (n = 8 per diet group) each: LF Diet, Control Cheddar Cheese (CC), *Lc. rhamnosus* DPC7102 Cheddar Cheese (DPC7102), and *Lc. paracasei* DPC7150 Cheddar (DPC7150).

5.2.3 Diet

Following acclimatisation, mice were fed Cheddar cheese for 2 hours (18:00 – 20:00) for the initial 6 days of the experimental diet, and then for 4 hours (18:00 – 22:00) for the remainder of the feeding trial. Food hoppers were switched to the Cheddar diets at the beginning of the dark cycle (18:00) due to mice being nocturnal. Cheddar cheese was cut into cubes of 1 cm^3 size and distributed at 160 g/hopper for *ad libitum*

consumption by mice. Food was weighed both prior and post feeding, and test diets were replaced every 3 days to ensure the freshness of the cheese cubes. In order to account for potential moisture loss of the cheese, hoppers containing the test diet were left in empty cages and weighed in parallel with mouse-containing cages.

5.2.4 Sample Collection

The body weight of animals was recorded at the outset of the trail and once per week until the end of the study. Faecal samples were collected directly from the mice (not from the bedding/cage floor) immediately prior to allotment into dietary groups (n=10) to establish a baseline microbiome. Mice were culled following an overnight fast via an initial subcutaneous injection of 100 mg/kg Ketamidor® and 1 mg/kg Medetor® diluted in PBS, followed by decapitation once unresponsive. Faecal pellets were removed from the colon and transferred to a sterile eppendorfs and were snap-frozen on dry ice. Caecal contents were transferred to sterile eppendorfs and were snap-frozen on dry ice. Organs of interest (adipose tissue samples and those from the digestive tract) were weighed to the nearest 0.0 mg (digestive contents were removed from the organ prior to weighing) and snap frozen on dry ice. Trunk blood was collected in EDTA-lined tubes and kept on ice, and later centrifuged at 2000 rpm for 10 minutes. Plasma was then removed, aliquoted, snap-frozen on dry ice, and stored at -80 °C.

5.2.5 Plasma Analysis

Total cholesterol (MAK043, Sigma-Aldrich) and triacylglycerol (MAK266, Sigma-Aldrich) levels in collected plasma were measured using quantification kits, as per the manufacturer's specifications. Absorbance was measured and quantified using the Gen 5 Biotek Microplate Data Collection and Analysis Software.

5.2.6 DNA Extraction and 16S Amplicon Sequencing Library Preparation

DNA was extracted from caecal and faecal samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Crawley, UK) as per the manufacturer's instructions. Quantification of samples was carried out using a Qubit® dsDNA High Sensitivity Assay Kit (Fisher Scientific, Dublin, Ireland) in conjunction with a Qubit® 2.0 fluorometer (Invitrogen,

Paisley, UK). Library preparation was carried out as per an adapted protocol designed by the Teagasc Sequencing Centre (Moorepark) based on the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Illumina, Saffron Walden, UK), with some modifications to the original methodology (Illumina, 2013). During the original amplicon PCR step, all reagents were doubled in quantity from the original total of 25 µL to 50 µL to determine an adequate yield of sample DNA, with 1 µL of each primer used at a 10 µM concentration, and 30 amplification cycles were used. For the first PCR clean-up step, 36 µL of AMPure XP beads were used, and were vortexed before use. The following steps were carried out as per the original Illumina methodology (Illumina, Saffron Walden, UK), without modifications; index PCR and subsequent PCR clean-up step; library quantification, normalization, and pooling; library denaturing and MiSeq sample loading.

5.2.7 Bioinformatics Analysis

Sequences were filtered on the basis of quality (removal of low quality nucleotides at the 3' end, Q>25 by nucleotides windows at 3 nucleotides) and length (removal of sequences with <200 nucleotides) with PRINSEQ and joined using fastq-join (Aronesty, 2011; Schmieder & Edwards, 2011). Filtered sequences were denoised and clustered into unique sequences (zero-distance operational taxonomic units; zOTUs) using the UNOISE2 algorithm implemented in USEARCH (Edgar, 2016). zOTU represent unique bacterial entities and are roughly equivalent to species or strains. Contamination, which may occur when processing low-biomass samples was removed with the decontam R package (Davis et al., 2018; Salter et al., 2014). Prevalence-based identification was utilised to statistically identify and remove contaminant zOTUs. Alpha and beta-diversity was determined using phyloseq R package (McMurdie & Holmes, 2013). The results of principal coordinates analysis (PCoA) of the beta-diversity when it was calculated using distance matrices built from unweighted UniFrac distances.

5.2.8 Statistical Analysis

Statistical analysis and graphs were generated using the IBM SPSS® software platform for Windows (Ver. 26). Analysis of Variance (ANOVA) was used to compare results

between three or more diet groups while Independent Samples t-test was used for comparison of two groups.

5.3 Results

5.3.1 Body Weight Changes between Diet Groups

In order to determine whether consumption of cheese influenced weight gain, mice were weighed once per week. No significant ($p<0.05$) differences were observed in body weight between mice in different diet groups at any time point (**Figure 1**). Weight gain and relative body weight were typical of male C57BL/6J (https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000664, The Jackson Laboratory). Mice were received at 3 weeks of age, with a mean weight of 12.4 ± 1.5 g, and were culled at 7 weeks at an average mass of 23.8 ± 1.3 g.

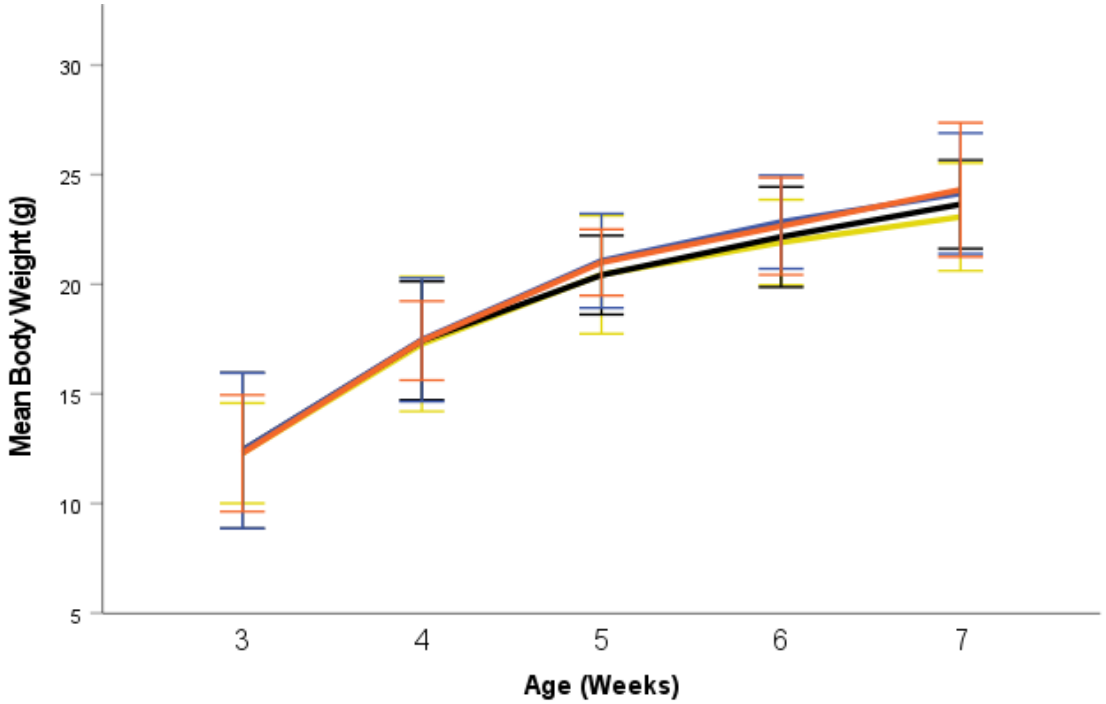

Figure 1. Mean body weight of mice with error bars representing variation within 2 standard deviations. No significant differences were observed. I (Yellow) – Control Chow Diet. I (Black) – Control Cheddar cheese Diet. I (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. I (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese Diet.

5.3.2 Total Chow Consumption between Control and Cheddar Diets

The cumulative consumption of the low-fat chow and Cheddar cheese diets are represented in **Figure 2** and **Figure 3**, respectively. The control group that received chow consumed a significantly higher amount of chow in comparison to the groups whose diet was supplemented with Cheddar cheese at every timepoint (**Figure 2**). Chow intake between diet groups given cheese did not differ significantly at any time point (**Figure 2**). Chow intake for the control group at Day 22 reached 120.7 ± 2.2 g, while the Cheddar groups peaked between 77.9 – 87.6 g. No significant differences occurred for cumulative cheese intake among the three diet groups receiving Cheddar, with intake reaching between 26.3 – 30.3 g (**Figure 3**).

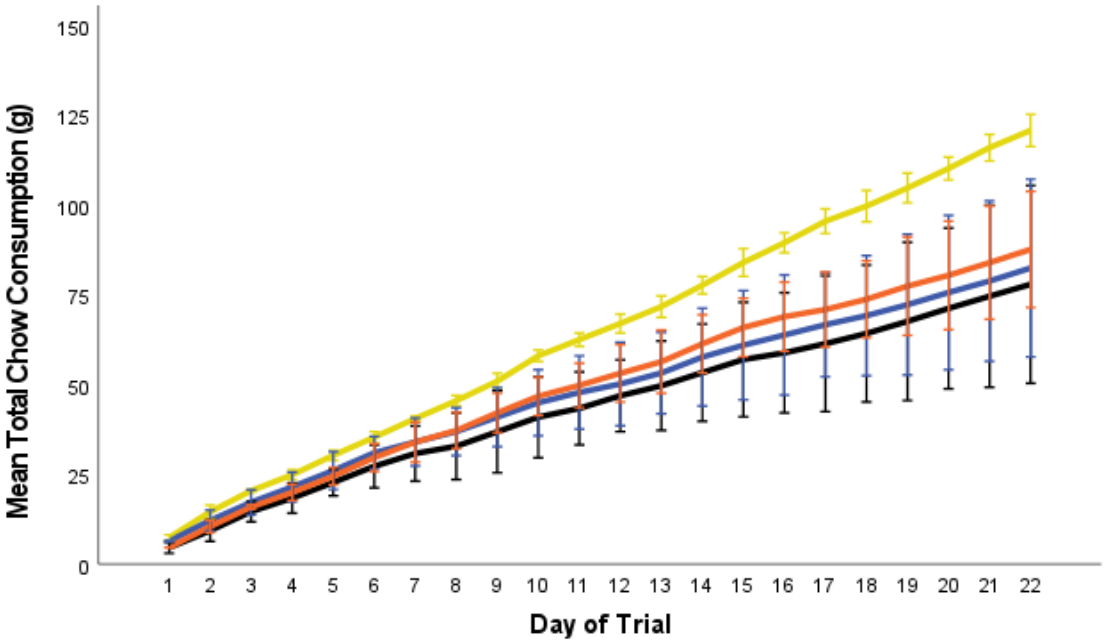

Figure 2. Mean total chow consumption of mice with error bars representing variation within 2 standard deviations. Significant differences were observed at every timepoint ($p \leq 0.01$ for Day 1, 2, 4, 5, 6, and 22, $p \leq 0.001$ for the remaining data points). I (Yellow) – Control Chow Diet. I (Black) – Control Cheddar cheese Diet. I (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. I (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese Diet.

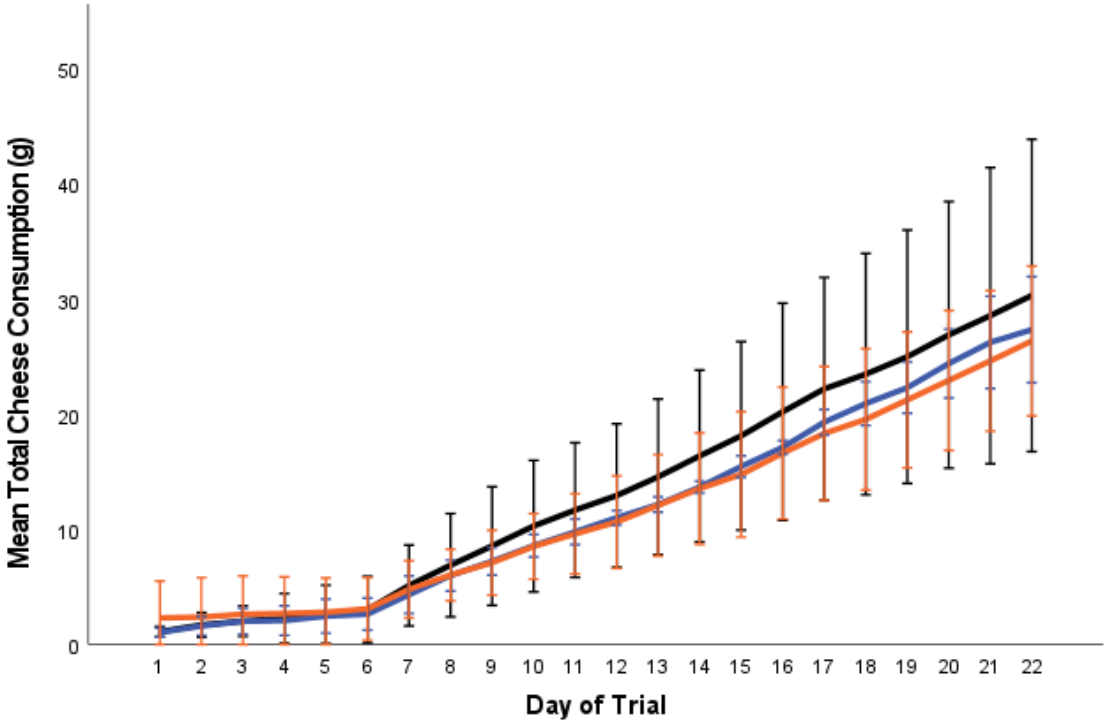

Figure 3. Mean total cheese consumption of mice with error bars representing variation within 2 standard deviations. No significant differences were observed. I (Black) – Control Cheddar cheese Diet. I (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. I (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese Diet.

5.3.3 Organ Weight between different Dietary Groups

At the time of culling, organ weight was taken to assess whether the consumption of cheese affected organ mass in the digestive tract (**Figure 4**) and selected adipose tissue (**Figure 5**). No significant differences were observed in organs between diet groups, although high variability was observed in the adipose tissue in all groups (**Figure 5**).

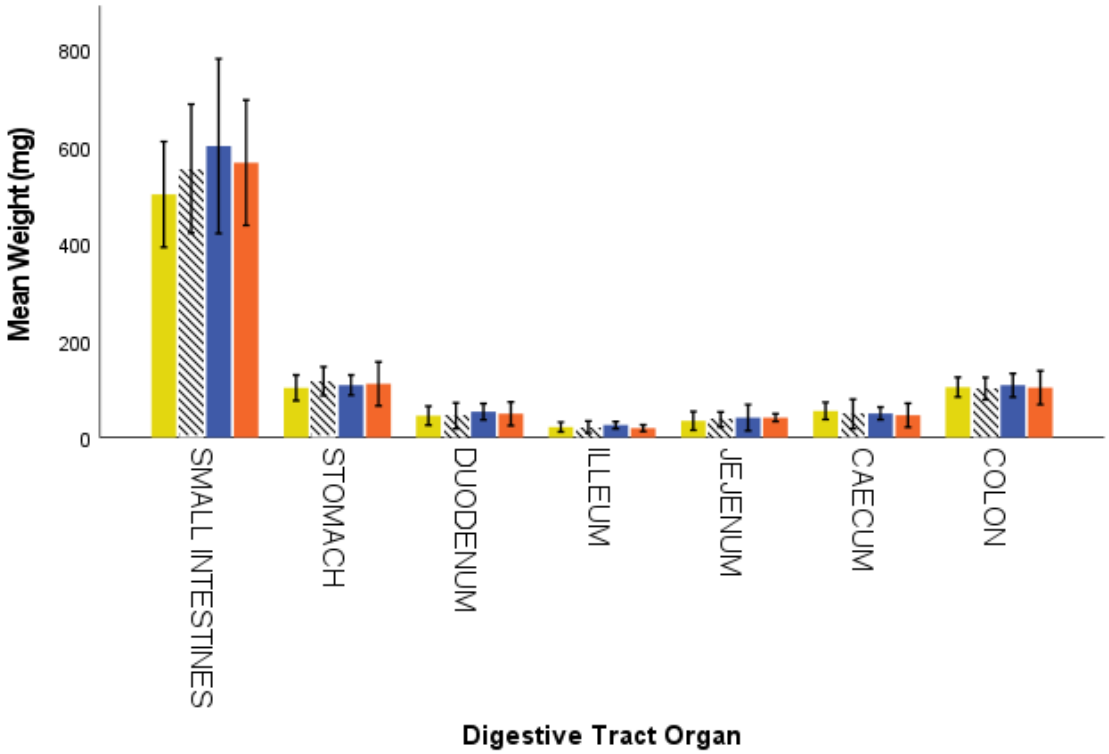

Figure 4. Mean digestive organ weight of mice with error bars representing variation within 2 standard deviations. No significant differences were observed. (Yellow) – Control Chow Diet. (Striped) – Control Cheddar cheese Diet. (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese Diet.

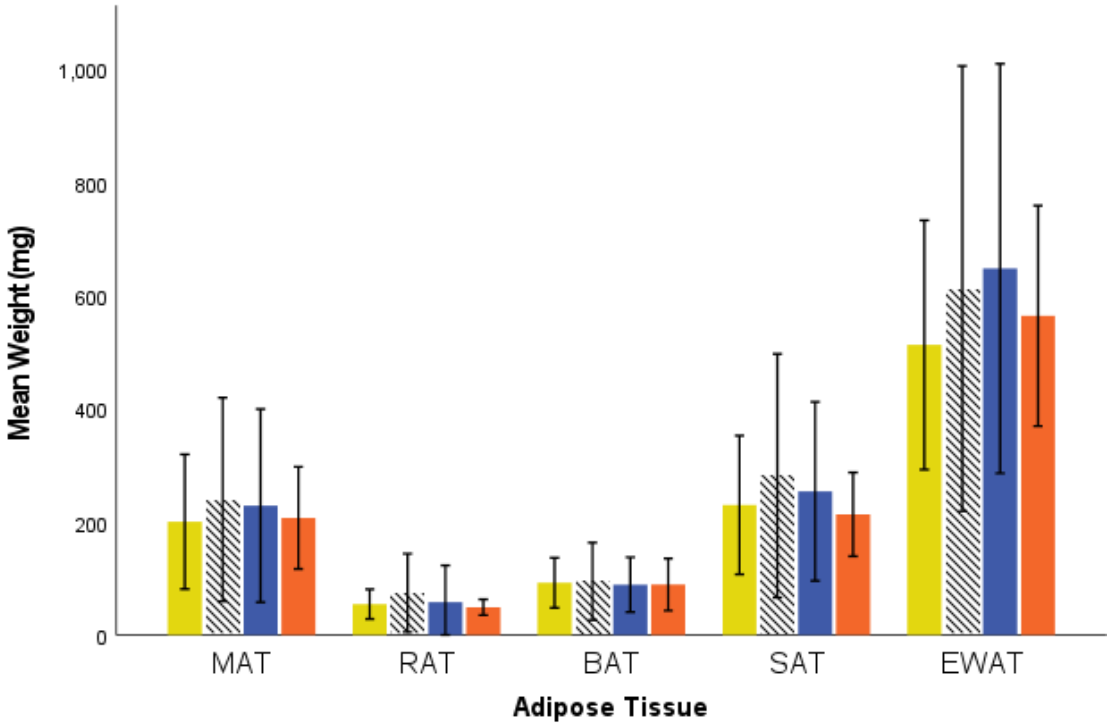

Figure 5. Mean adipose tissue weight of mice with error bars representing variation within 2 standard deviations. No significant differences were observed. (Yellow) – Control Chow Diet. (Striped) – Control Cheddar cheese Diet. (Blue) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. (Orange) – *Lc. paracasei* DPC7150 Cheddar cheese Diet. Abbreviations: MAT, mesenteric adipose tissue; RAT, retroperitoneal adipose tissue; BAT, brown adipose tissue; SAT, subcutaneous adipose tissue; EWAT, epididymal white adipose tissue.

5.3.4 Plasma Triacylglycerol Levels in different Dietary Groups

While differences in plasma cholesterol levels between dietary groups were negligible, plasma triacylglycerol levels of mice in the control group were significantly lower than those that consumed Cheddar (**Table 1**). For plasma triacylglycerols, the *Lc. rhamnosus* DPC7102 Cheddar cheese diet exhibited the highest levels at 20.97 mg/dL, while the Control Cheddar diet cause the highest plasma cholesterol levels, at 20.97 mg/dL (**Table 1**). Conversely, the lowest plasma cholesterol levels were observed in the *Lc. rhamnosus* DPC7102 Cheddar cheese diet mice (**Table 1**).

	(A) Cholesterol (mg/dL)		**(B) Triacylglycerols (mg/dL)**	
	p = 0.162		**p = 0.014 (p ≤ 0.05)**	
	Mean	*SD*	*Mean*	*SD*
Control	20.35	± 0.30	11.38	± 4.18
Control Cheddar	20.97	± 1.66	17.67	± 4.94
***Lc. rhamnosus* DPC7102 Cheddar**	19.93	± 0.44	20.97	± 5.57
***Lc. paracasei* DPC7150 Cheddar**	20.62	± 0.54	18.47	± 7.22

Table 1. Mean plasma **(A)** cholesterol and **(B)** triaclglycerol levels of mice with standard deviation values. Plasma **(A)** cholesterol levels were not statistically significant between dietary groups. Plasma **(B)** triaclglycerol levels significantly differed between dietary groups ($p \leq 0.05$).

5.3.5 Faecal and Caecal Microbiome of Mice receiving different Diets

Alpha and Beta diversity was measured to determine the microbiome diversity among single and multiple samples, respectively. Sequence analysis of both faecal and caecal samples revealed significant differences between dietary groups in both alpha and beta diversity (**Figure 6**, **Figure 7**). In terms of richness, the only dietary groups that did not significantly differ with regards taxonomic diversity were the Control Cheddar cheese and *Lc. rhamnosus* DPC7102 Cheddar cheese diet, and the Control Chow and *Lc. paracasei* DPC7150 Cheddar cheese diet (**Figure 6A**). Using the Simpson relative abundances index, the Pre-intervention diet was significantly different from all four dietary intervention groups, with the only other significant difference being between the *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 Cheddar cheese groups (**Figure 6B**). Finally, only three groups were not significantly different when measured using the Shannon richness index (**Figure 6C**). The Control Chow diet did not differ from either the Control or *Lc. paracasei* DPC7150 Cheddar cheese groups, while the Control and *Lc. rhamnosus* DPC7102 Cheddar cheese were also statistically similar (**Figure 6C**). The five dietary groups also formed distinct, significantly different clusters when beta diversity was calculated via the Bray-Curtis index (**Figure 7**).

Through sequence analysis, five phyla were identified in both faecal and caecal samples, with *Firmicutes* and *Bacteroidetes* being the most dominant at between 53-70% and 19-32% relative abundance, respectively (**Figure 8**). Significant differences between dietary groups were observed in both *Firmicutes* and *Bacteroidetes* for both faecal ($p \leq 0.01$ and $p \leq 0.05$, respectively) and caecal ($p \leq 0.001$) samples, while significant differences were observed in *Actinobacteria* and *Proteobacteria* for only the faecal ($p \leq 0.05$) and caecal ($p \leq 0.01$) samples, respectively. No differences were seen in the *Verrucomicrobia* phylum.

The dominant family identified in both sample types was *Lachnospiraceae*, which constituted between 13-26% relative abundance across all dietary groups, closely followed by *Erysipelotrichaceae,* which comprised 10-17% relative abundance (**Figure 9**). In total, twenty-eight families were identified among the faecal and caecal samples (**Figure 9**). Significant differences between dietary groups in both faecal and caecal samples were observed for the families *Lactobacillaceae*, *Porphyromonadaceae*,

Rikenellaceae, *Streptococcaceae*, *Christensenellaceae*, and *Anaeroplasmataceae*. Additionally, the relative abundances of the families *Bifidobacteriaceae*, *Staphylococcaceae*, *Corynebacteriaceae*, *Coriobacteriaceae*, *Bacillaceae*, *Aerococcaceae*, and *Carnobacteriaceae* were found to differ significantly between dietary groups in faecal samples only, while *Desulfovibrionaceae* and *Deferribacteraceae* differed significantly only in caecal samples.

Significant differences among genera between dietary groups can be seen below (**Table 2**). Of particular interest were the genera *Lactococcus* and *Lactobacillus* (referring to the old taxonomic classification system encompassing approximately 250 species), which were knowingly added to the Cheddar described previously (**Figure 10**, **Figure 11**) (N. K. Leeuwendaal et al., 2022). Dietary groups that did not involve the consumption of cheese all exhibited significantly lower levels of *Lactococcus* when compared to the dietary cheese groups*,* which comprised both the pre-intervention and control chow diets in the faecal samples and control chow diet in the caecal samples (**Figure 10A**, **Figure 11A**). Additionally, the pre-intervention and control chow dietary groups' *Lactococcus* levels were not significantly different from one another among the faecal samples group (**Figure 10A**). Conversely, the results for the *Lactobacillus* abundance levels did not show as clear a trend. In the faecal samples, the Pre-intervention group had such high variability that the abundance was only significantly different from the Control Chow group, and not to any of the Cheddar groups (**Figure 10B**). However, the Control Chow group did significantly differ from all Cheddar groups, and these results were reflected in the caecal samples (**Figure 10B**, **Figure 11B**).

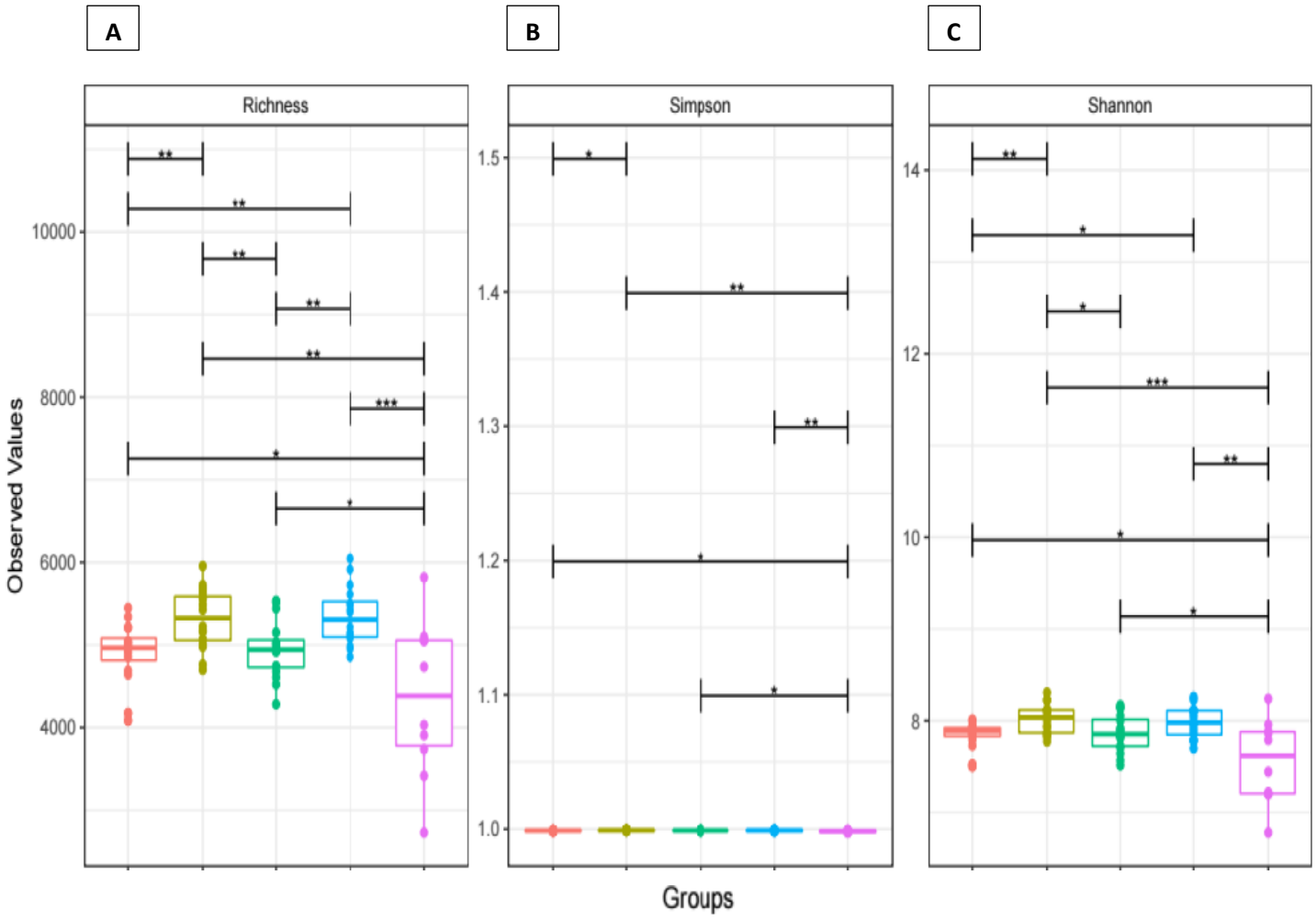

Figure 6. Microbiome Alpha Diversity. Mean alpha diversity expressed via **(A)** Richness, **(B)** Simpson, and **(C)** Shannon indices for all dietary groups. Significant differences were observed between certain groups. (Purple) – Pre-intervention Diet. (Blue) – Control Chow Diet. (Green) – Control Cheddar cheese Diet. (Orange) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. (Yellow) – *Lc. paracasei* DPC7150 Cheddar cheese Diet. * Denotes p-value ≤ 0.05. ** Denotes p-value ≤ 0.01. *** Denotes p-value ≤ 0.001.

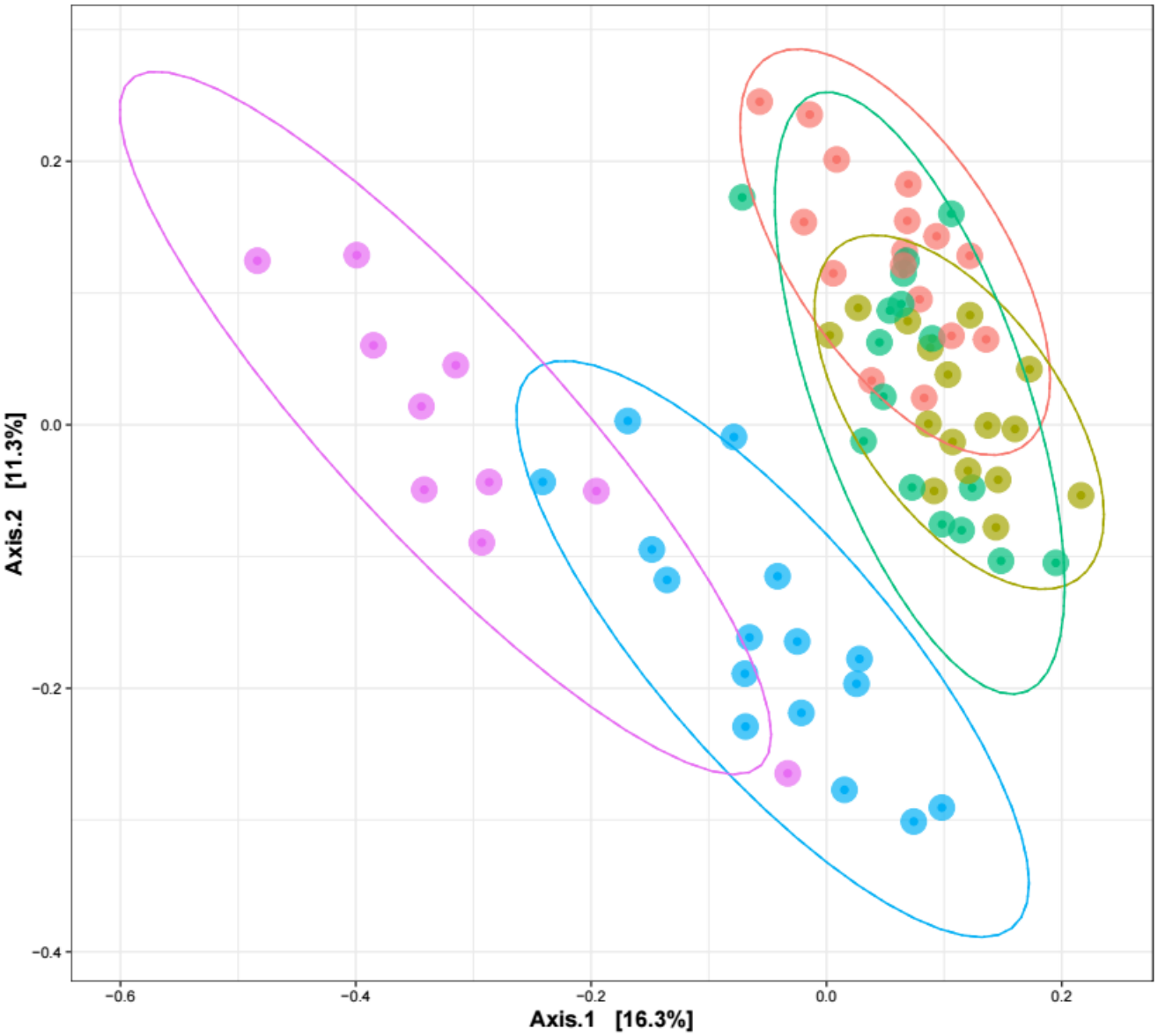

Figure 7. Microbiome Beta Diversity. Beta diversity, visualised via the Bray-Curtis index, indicates significant (p-value ≤ 0.001) differences between dietary groups. (Purple) – Pre-intervention Diet. (Blue) – Control Chow Diet. (Green) – Control Cheddar cheese Diet. (Orange) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. (Yellow) – *Lc. paracasei* DPC7150 Cheddar cheese Diet.

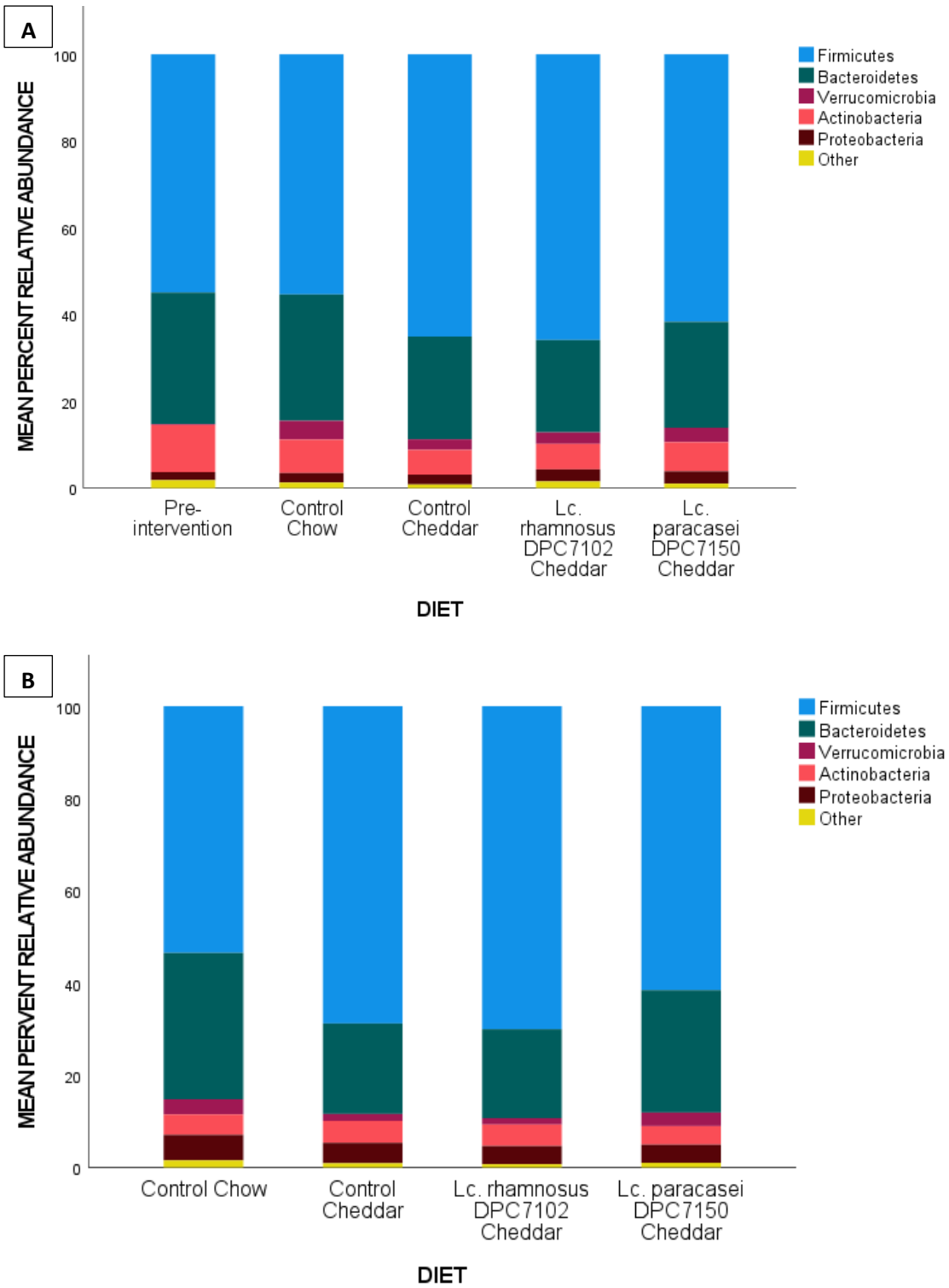

Figure 8. Mean Relative Phylum Abundance. Phylum composition in both **(A)** faecal and **(B)** caecal samples for all dietary groups.

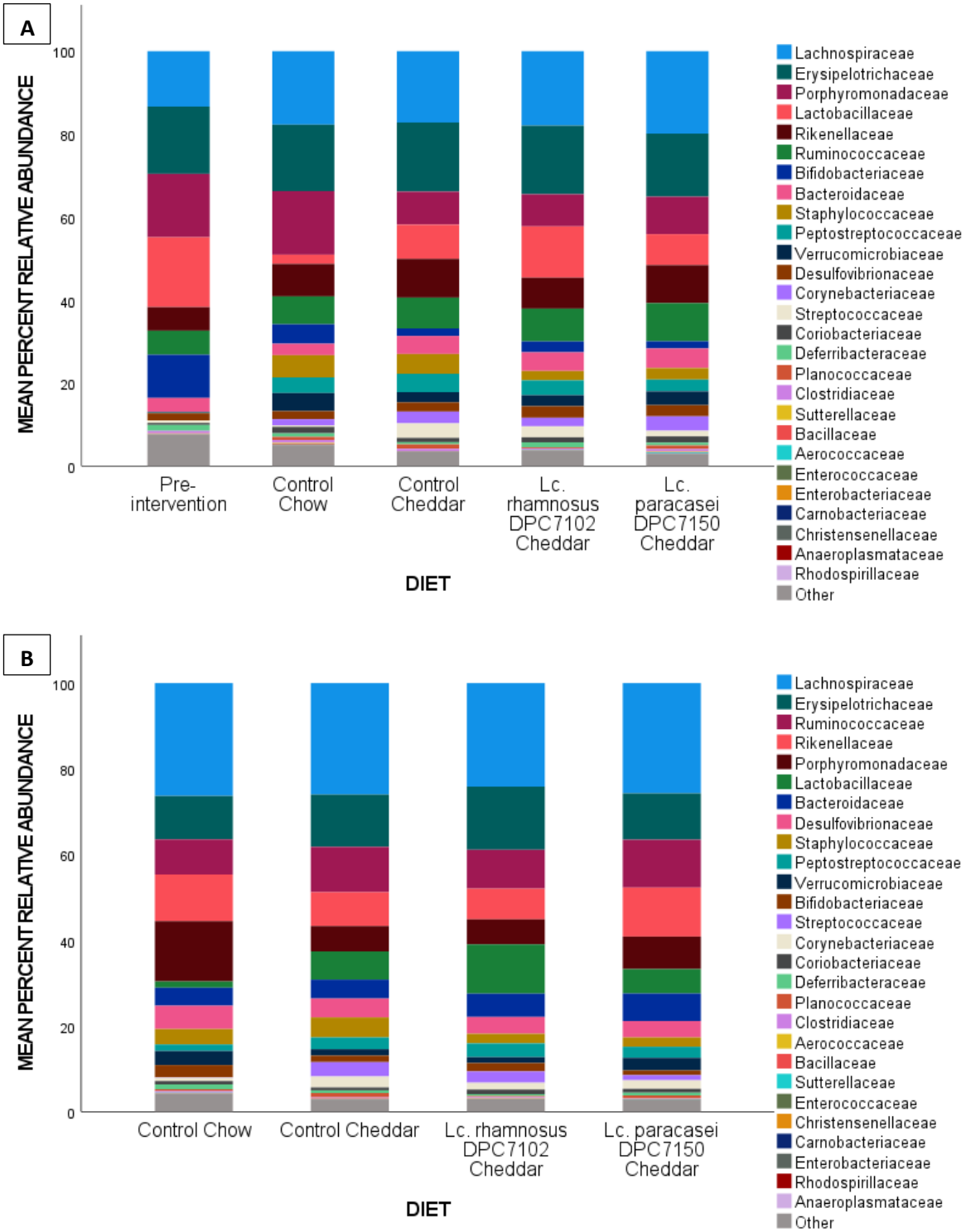

Figure 9. Mean Relative Family Abundance. Family composition in both **(A)** faecal and **(B)** caecal samples for all dietary groups.

	Significance (p-value)	
	Faecal Samples	**Caecal Samples**
Corynebacterium	**	n.s.
Bifidobacterium	***	n.s.
Enterorhabdus	***	*
Olsenella	*	n.s.
Parvibacter	n.s.	n.s.
Bacteroides	n.s.	n.s.
Barnesiella	n.s.	**
Odoribacter	***	***
Parabacteroides	n.s.	***
Alistipes	*	**
Saccharibacteria_genera_incertae_sedis	***	***
Mucispirillum	n.s.	*
Bacillus	n.s.	n.s.
Caryophanon	n.s.	n.s.
Lysinibacillus	n.s.	n.s.
Sporosarcina	n.s.	n.s.
Jeotgalicoccus	**	*
Staphylococcus	**	n.s.
Aerococcus	n.s.	n.s.
Facklamia	n.s.	*
Atopostipes	*	n.s.
Enterococcus	n.s.	n.s.
Lactobacillus	**	***
Lactococcus	***	***
Streptococcus	***	n.s.
Christensenella	***	***
Clostridium_sensu_stricto	n.s.	n.s.
Acetatifactor	n.s.	n.s.
Clostridium_XlVa	n.s.	*
Clostridium_XlVb	n.s.	***
Dorea	n.s.	n.s.
Lachnospiracea_incertae_sedis	n.s.	n.s.
Roseburia	**	n.s.
Romboutsia	n.s.	n.s.
Anaerotruncus	n.s.	*
Butyricicoccus	n.s.	n.s.
Clostridium_IV	n.s.	***
Flavonifractor	**	***
Intestinimonas	n.s.	n.s.
Oscillibacter	n.s.	n.s.
Pseudoflavonifractor	***	***
Ruminococcus	n.s.	n.s.

Allobaculum	n.s.	n.s.
Clostridium_XVIII	n.s.	n.s.
Turicibacter	***	***
Parasutterella	n.s.	n.s.
Desulfovibrio	**	n.s.
Escherichia/Shigella	n.s.	n.s.
Anaeroplasma	***	**
Akkermansia	n.s.	n.s.

Table 2. Significant Differences among Genera between Dietary Groups. Mean percent relative abundance values of the top 50 genera were compared between dietary groups to determine population differences in **(A)** faecal and **(B)** caecal samples. * Denotes p-value ≤ 0.05. ** Denotes p-value ≤ 0.01. *** Denotes p-value ≤ 0.001. n.s. Non-significant, denotes p-value > 0.05.

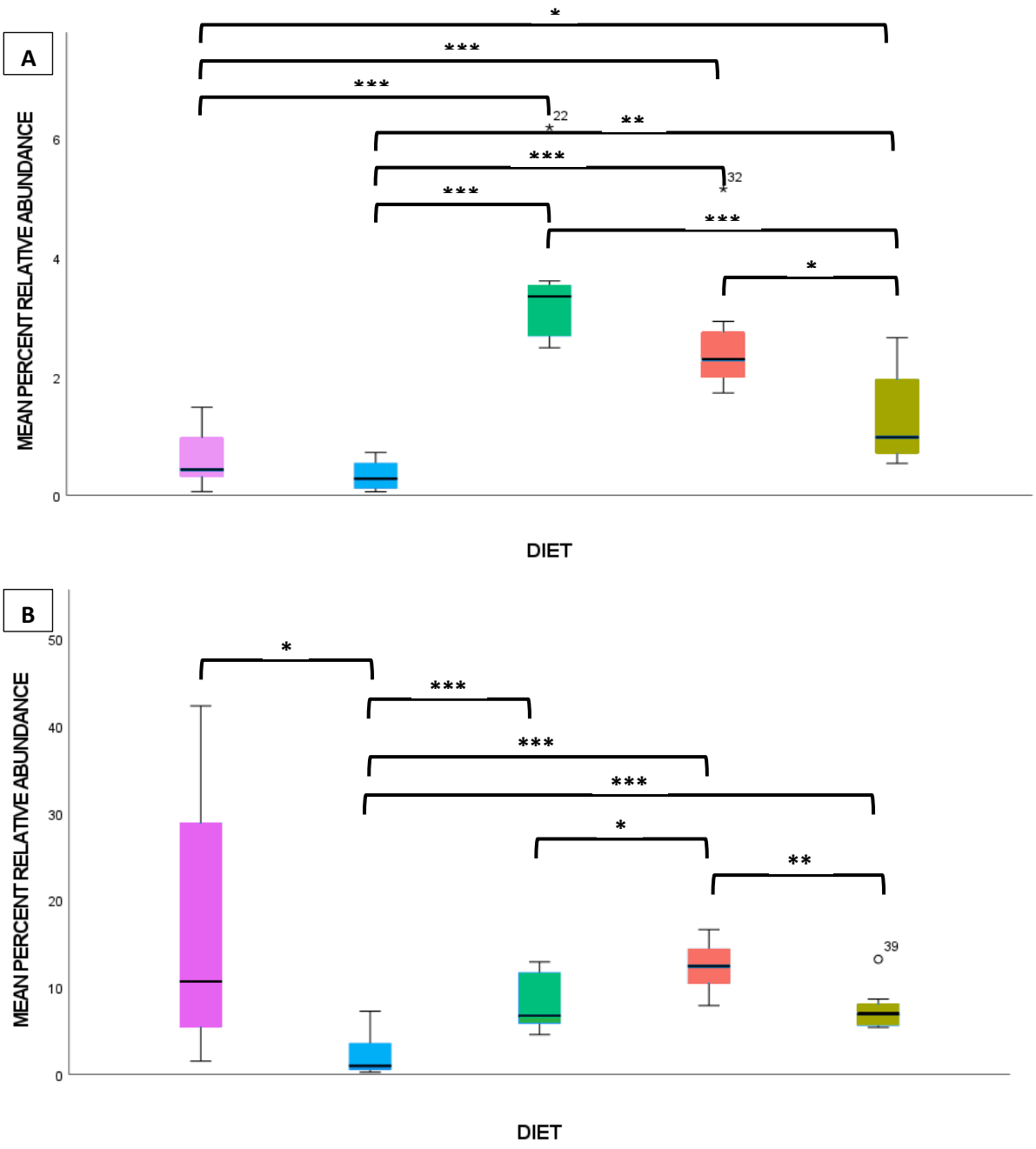

Figure 10. Mean Relative Abundance of **(A)** *Lactococcus* and **(B)** *Lactobacillus*. Abundance of **(A)** *Lactococcus* and **(B)** *Lactobacillus* in faecal samples for all dietary groups. Significant differences were observed between groups. (Purple) – Pre-intervention Diet. (Blue) – Control Chow Diet. (Green) – Control Cheddar cheese Diet. (Orange) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. (Yellow) – *Lc. paracasei* DPC7150 Cheddar cheese Diet. * Denotes p-value ≤ 0.05. ** Denotes p-value ≤ 0.01. *** Denotes p-value ≤ 0.001.

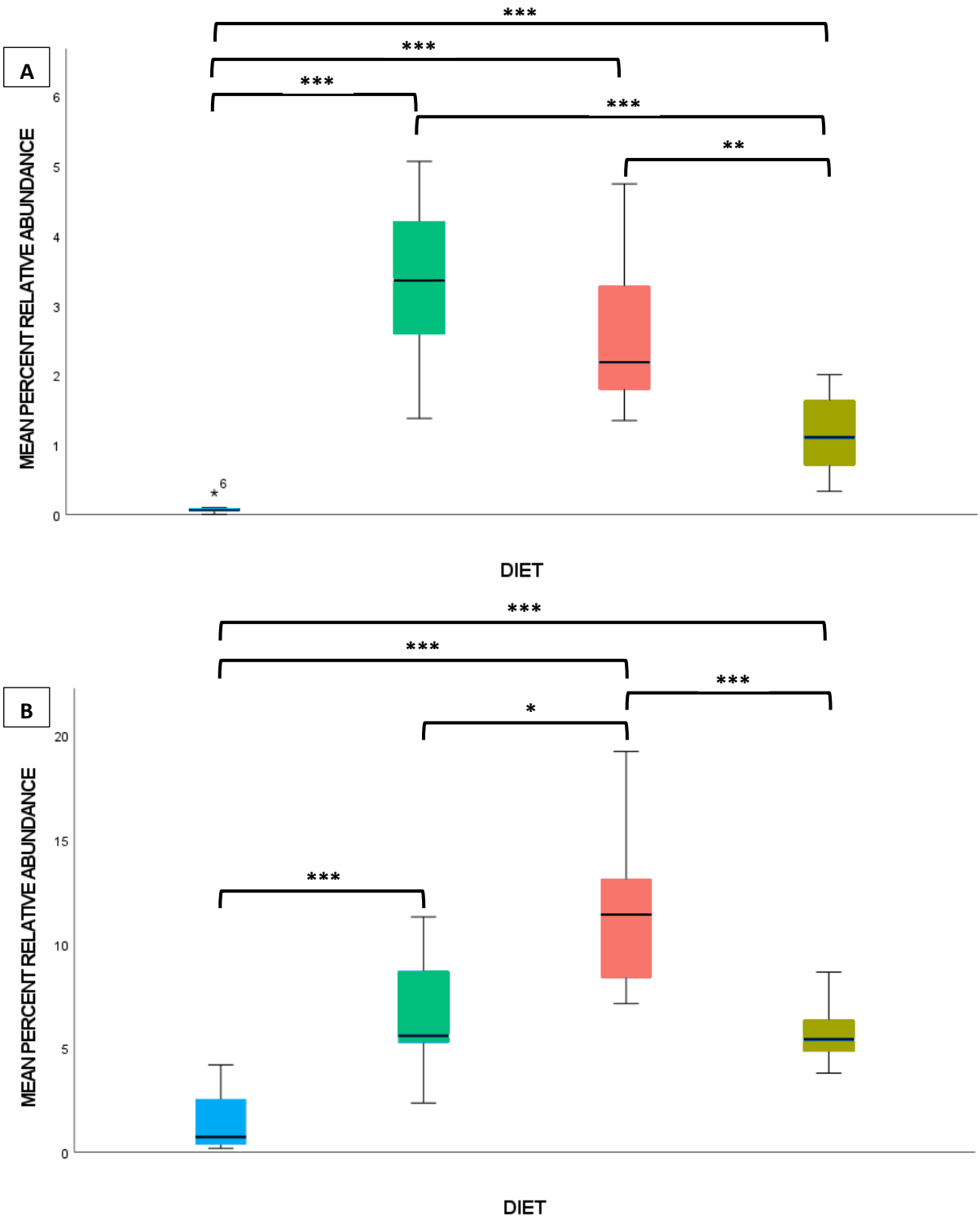

Figure 11. Mean Relative Abundance of **(A)** *Lactococcus* and **(B)** *Lactobacillus.* Abundance of **(A)** *Lactococcus* and **(B)** Lactobacillus in caecal samples for all dietary groups. Significant differences were observed between groups. (Blue) – Control Chow Diet. (Green) – Control Cheddar cheese Diet. (Orange) – *Lc. rhamnosus* DPC7102 Cheddar cheese Diet. (Yellow) – *Lc. paracasei* DPC7150 Cheddar cheese Diet. * Denotes p-value ≤ 0.05. ** Denotes p-value ≤ 0.01. *** Denotes p-value ≤ 0.001.

5.4 Discussion

The aim of this study was to determine if Cheddar cheese manufactured with *Lacticaseibacillus* adjuncts (capable of exhibiting probiotic traits *in vitro*) produced effects in a murine model during a short-term feeding trial (Leeuwendaal et al., 2021; Leeuwendaal et al., 2022). Primarily, this study aimed to determine whether this lacticaseibacilli-containing Cheddar was able to alter the gut microbiome, but other weight-related metrics were also investigated to determine if introduction if a high-fat food would have much impact on mice when compared with those ingesting a low-fat chow diet.

No significant difference was detected between the weight of mice in different dietary groups (**Figure 1**). The effects of cheese and dairy consumption on weight gain is difficult to state definitively, as various other lifestyle parameters appear to influence outcome. An older review (2011) investigating potential associations between body weight and dairy intake found that all but one of the studies fitting their inclusion parameters revealed a modest but significant inverse association, while nine of the twelve intervention studies mentioned did not show evidence of a relationship between weight loss and dairy consumption (Dougkas et al., 2011). A meta-analysis of randomized controlled clinical trials published in the following year by Abargouei and colleagues (2012) found that, overall, an increase in dairy consumption to the recommended daily intake for adults who are not on a calorie-restricted diet did not affect fat mass, lean body mass, weight or waist circumference (Abargouei et al., 2012). This study also postulated that a weight loss diet that does not exclude dairy may, in fact, accelerate weight loss, that may come about through components of the dairy matrix themselves (e.g., calcium, fatty acids) regulating appetite and metabolism (Abargouei et al., 2012). These reviews contradict evidence brought forward by Huang and colleagues (2018), who investigated over 180,000 human participants across 25 separate studies via Mendelian randomization analysis for a causal link between dairy intake and Body Mass Index (BMI) in adults (Huang et al., 2018). This was accomplished by assigning a polymorphism located upstream of the lactase gene (an allele with a previously established genetic connection to dairy intake) as an instrumental variable, and the authors demonstrated that a higher BMI was significantly associated with the genetically-associated higher dairy intake (Huang et

al., 2018). Conversely, dairy intake appeared to not have any effect on the body weight or compositions of 111 subjects with type 2 diabetes who were instructed to increase dairy consumption while not changing calorific intake (via dietary substitutions) for 24 weeks, regardless of whether the dairy products were high- or low-fat (Mitri et al., 2020). This appears to coincide with the results from our study, which reported no significant differences in weight of mice between different diet groups (**Figure 1**). A study from 2020 found similarly that, in a cohort of 54 adolescent, overweight/obese female participants, a 12-week dietary and exercise intervention whereby dairy products were increased without a significant increase in daily calorific intake did not have a significant impact on weight (Calleja et al., 2020). However, this group's focus was primarily on body composition, specifically the fat mass to lean mass ratio, as fat mass has a positive association with cardiovascular disease (Calleja et al., 2020). The study found that the group with the highest dairy intake had a significantly decreased and increased fat and lean mass, respectively, in comparison to either the low dairy intake or control group (no intervention) (Calleja et al., 2020). This observed fat and lean mass decrease and increase, respectively, was also significant between the low dairy intervention and control group, indicating that dairy products can still have positive effects on body composition that may not be reflected in weight loss alone (Calleja et al., 2020). While our study did no measure lean/fat mass specifically, all mouse digestive organs and adipose tissues that were measured posthumously were not found to differ significantly between dietary groups, indicating that the addition of Cheddar did not appear to affect body composition in that regard (**Figure 4, Figure 5**). A separate study, also using an adolescent cohort (n=700) established a negative correlation between dairy products and BMI, waist circumference and waist/height ratios, but only among its female participants, although this study assigns greater importance to milk consumption over total dairy (Yasar Firat et al., 2021).

The studies mentioned above did not differentiate between cheese and dairy products in general, making it difficult to draw conclusions about cheese specifically and its benefits/harms in dietary treatments. A cross-sectional study that came out in 2018 investigating 114,682 Dutch adults stratified dairy intake into categories (e.g., milk, yogurt, cheese, low-fat, fermented, etc.) and discovered that, while the full-fat dairy category exhibited an inverse association with the overweight BMI categories, the more specialised 'cheese and cheese snacks' category had a positive association

instead (Brouwer-Brolsma et al., 2018). This study also determined that an increase in each serving of total cheese resulted in an increase in body weight, although this was only associated with male participants despite average cheese consumption being nearly equal between sexes (Brouwer-Brolsma et al., 2018). This apparent contradiction was postulated to be due to the much higher fat content of cheese in comparison to its milk and yogurt counterparts, although high cheese intake affecting males and not females could not be explained (Brouwer-Brolsma et al., 2018). Wrotniak and colleagues (2019) also demonstrated that total dairy product intake, when consumed within a behavioural obesity treatment/intervention in 91 adolescents, resulted in a significant decrease in BMI over a period of one year prior (Wrotniak et al., 2019). However, when Wrotniak et al. (2019) investigated interactions between different varieties of dairy products with BMI after statistical adjustments were made for other physical activity and dietary factors, all dairy products, bar unflavoured milk, were found to have no significant association with decreasing BMI, including cheese (Wrotniak et al., 2019). Coinciding with these results, an exercise and nutritional education program (that lasted 16 weeks) aimed at enumerating dairy's role in weight management and body composition in 69 'sedentary' adults was carried out by Guerendiain et al. (2019). They reported that participants with a lower cheese consumption at the outset of the trial showed a significantly decreased BMI regardless of which exercise group they were assigned to (Guerendiain et al., 2019). Calorific restriction, therefore, may play a role in whether weight gain occurs due to cheese consumption. While our study did not impose calorific restrictions on the amount of Cheddar/chow the mice ingested, it is interesting to observe that, while Cheddar intake did not differ significantly between cheese-inclusive dietary groups, chow consumption was significantly different (**Figure 2, Figure 3**). All Cheddar groups consumed significantly lower chow than their chow-only counterparts, with the control Cheddar cheese group that ate a higher amount of cheese being the group that also ate the least amount of chow (**Figure 3**). Thus, the cheese appeared to substitute for the calories and satiety of the chow during the hours when the mice had access to only this food source. While it cannot be confirmed, this might be in keeping with the observations of Wrotniak et al. (2019), Mitri et al. (2020), and Calleja et al. (2020), whereby inclusion of dairy into a diet where dairy products are calorific substitutions, not additions, does not lead to weight gain (Calleja et al., 2020; Mitri et al., 2020; Wrotniak et al., 2019). Hric and colleagues (2021) focused on the ability of Bryndza

cheese (produced from ewe's milk) to impact on the health of 22 female subjects with ≥25% body fat during a 4-week weight loss intervention program that consisted of both a calorie-restricted diet and regular exercise (Hric et al., 2021). This product was of particular interest due to it being classified as a probiotic cheese, containing a microbiome rich in beneficial Lactic Acid Bacteria (LAB) (Hric et al., 2021). While both Bryndza- and control groups exhibited significant decreases in BMI, body fat and weight, a significant increase in specific LAB was only observed in the cheese-consuming group (Hric et al., 2021). Additionally, the genera *Phascolarctobacterum* and *Butyricimonas*, both of which are established short chain fatty acid producers, significantly increased in the Bryndza consumers, further indicating that the benefits of cheese consumption may be beyond simple weight loss (Hric et al., 2021).

Plasma cholesterol and triacylglycerol levels were measured and, while no differences were observed for the former, triacylglycerol levels were significantly lower for the control chow diet (**Table 1**). The murine study mentioned above by Kim and colleagues (2021) showed similar results for the triacylglycerol levels, whereby the non-supplemented Gouda group had significantly higher triacylglycerol levels than either the bioactive-containing Gouda or negative control (Kim et al., 2021). Conversely, their non-supplemented Gouda group also exhibited significantly increased total cholesterol in comparison to their negative control, which was not observed between our groups (Kim et al., 2021). Feeney and colleagues (2018) carried out a dietary intervention study examining the effects of ingesting 40 grams of dairy fat from different dairy matrices every day for 6 weeks in overweight adults of ≥50 years of age (Feeney et al., 2017). A difference was observed between the individuals who consumed dairy fat solely from a full fat Irish Cheddar and those whose source consisted of a mix of butter, calcium caseinate powder, and calcium supplements, whereby the cheese consumers exhibited significantly lower total cholesterol and LDL than the mixture group post-intervention (Feeney et al., 2017). A future study for the lacticaseibacilli in this study could include various dairy matrices to determine whether a similar matrix-dependant result for blood lipid profiles would emerge. A 3-week study exploring the ability of dairy to influence blood lipids and cholesterol in 14 young, healthy male participants determined that no significant differences were observed in total cholesterol or triacylglycerols between those with diets rich in milk, cheese or butter (Tholstrup et al., 2004). They also found no changes from blood samples taken prior to

commencement of the diet (Tholstrup et al., 2004). However, they did observe significantly low-density lipoprotein (LDL) cholesterol in the cheese-rich group in comparison to the butter-rich group, as well as a significantly higher glucose response in the cheese diet in comparison to the milk diet (Tholstrup et al., 2004).

Blood LDL cholesterol was also found to have a positive association with ricotta cheese in a study involving 802 healthy adults living in the Mediterranean area, although this was not observed in total cheese (Buscemi et al., 2021). Mitri and colleagues (2020), discussed above, also did not observe any differences in lipid parameters in their own dietary intervention study, including LDL cholesterol (Mitri et al., 2020). Future studies should include measurements for LDL cholesterol specifically to determine if similar trends are observed in mice. Interestingly, a recent intervention study on 27 hyperinsulinemic, overweight adults which observed the effects of either adequate or high dairy product ingestion on various parameters reported no difference in cholesterol or triacylglycerol levels between dietary groups but did discover that significant differences in the total levels of both existed between sexes (Khorraminezhad et al., 2021). Unfortunately, as our study only included male mice, we cannot compare as to whether a sex difference also occurs in our study.

Overall, we found that the short-term ingestion of Cheddar did cause shifts in the relative abundances of gut microbiome populations. Various alpha indices expressed significant differences between dietary groups in terms of Operational Taxonomic Units (OTU) richness and abundance, and beta analysis showed a clear clustering of Cheddar diet vs non-Cheddar groups (**Figure 6**, **Figure 7**). Significant differences between the relative abundance of different phyla and families were also observed, lending credibility to the idea that the addition of Cheddar to the diet can have consequences on gut microbiome, even following short term exposure (**Figure 8**, **Figure 9**). While many significant differences were observed among the dominant genera, of particular interest were the *Lactococcus* and *Lactobacillus* genus (**Table 2**).

The Cheddar used in this study was produced using *Lactococcus lactis* strains 223 and 303 (Chr. Hansen) as starter LAB (SLAB), while the *Lc. rhamnosus* DPC7102 and *Lc. paracasei* DPC7150 strains were added as adjuncts to their respective Cheddars (Leeuwendaal et al., 2022). These Cheddars were allowed to ripen for 180 days

(Leeuwendaal et al., 2022). The control Cheddar was produced without the addition of any cultures other than the two *Lactococcus* starters but, eventually, adventitious NSLAB did proliferate and eventually out-compete the SLAB, whereby the Control Cheddar sample NSLAB counts were found to have reached 5.8 log CFU/g at the end of the ripening period (Leeuwendaal et al., 2022). In terms of *Lactococcus* abundance, the Control Chow dietary group was always significantly lower than all Cheddar groups, in both faecal and caecal samples (**Figure 10A**, **Figure 11A**). Additionally, the Control Cheddar group mean relative abundance was higher than the *Lc. rhamnosus* DPC7102 group mean and was significantly higher than the *Lc. paracasei* DPC7150 ($p \leq 0.001$) group mean (**Figure 10A**, **Figure 11A**). Levels of *Lactococcus* were moderate in all 3 Cheddars at the end of the ripening period (between 5.2-6.3 CFU/log) but were significantly higher in the Control Cheddar ($p \leq 0.05$) after 180 days, which might explain the higher *Lactococcus* abundance in this dietary group (Leeuwendaal et al., 2022).

For the *Lactobacillus* groups, results were not as clear cut. In the faecal samples, the Pre-intervention group had such high variable relative abundance of *Lactobacillus* that no significant differences were observed between it and the Cheddar groups, although a difference was observed between it and the Control Chow group (**Figure 10B**). This is unusual as both Pre-intervention and Control Chow consumed the same diet, the only difference being that the Pre-intervention group were younger and had only been habituated for a few days. Potentially the younger mouse microbiome had not yet stabilised, or the animals were stressed and had not yet acclimatized to their new surroundings. All Cheddar cheese groups had significantly higher *Lactobacillus* abundances than the Control Chow counterpart, potentially indicating that the adjuncts and other NSLAB lactobacilli in the Cheddar can survive digestion and colonise the gut or that the digested cheese matrix itself provides metabolites that allow population increases in gut *Lactobacillus* species (**Figure 10B**). This result was mirrored in the caecal samples (**Figure 11B**). This also coincides with the results of the Hric and colleagues' study (2021) that found that microbe populations found within Bryndza cheese were higher in the gut microbiomes of female participants who ingested the cheese every day (Hric et al., 2021). A point of interest is that the *Lc. rhamnosus* DPC7102 group has highest mean *Lactobacillus* abundances, followed by the Control Cheddar, and then the *Lc. paracasei* DPC7150 group, for both faecal and caecal

samples (**Figure 10B**, **Figure 11B**). However, the Control Cheddar was shown to have a significantly lower NSLAB population than both the other Cheddars (Leeuwendaal et al., 2022). This may indicate that the NSLAB of the *Lc. paracasei* DPC7150 Cheddar do not survive gastric digestion in numbers as high as the NSLAB of the *Lc. rhamnosus* DPC7102 or Control Cheddar, or that their NSLAB populations are not as proficient at colonising the gut. It may be more likely to be the latter, as the *Lc. paracasei* DPC7150 Cheddar lactobacilli showed higher survival levels when subjected to the INFOGEST 2.0 (adult human digestion simulation) than their *Lc. rhamnosus* DPC7102 counterpart, although this inconsistency could be due to differences in the *in vitro* digestion model and digestive tracts in mice.

5.5 Conclusion

This study has shown that a short-term feeding trial involving the ingestion of Cheddar does have the capacity to influence gut microbiome composition, with significant differences seen between certain phyla, families, and genera. Additionally, the inclusion of Cheddar cheese in the murine diet did not affect overall weight or organ weight, adipose tissue mass or even total plasma cholesterol levels. Total triacylglycerol levels were significantly higher in the Cheddar cheese groups than the Control Chow diet. However, a weakness of this study is that the two adjunct *Lacticaseibacillus* strains of interest themselves could not be distinguished from the rest of the *Lactobacillus* members. It would be interesting if their specific populations could be identified in faecal samples following ingestion to investigate whether these strains in particular are capable of surviving *in vivo* and colonising the gut microbiome.

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Chapter 6

The Ability of Irish Cheddar Cheese to Modify the Gut Microbiome of Pre-diabetic Adults

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Abstract

The ability of the gut microbiome to influence host health is well established. Various groups have attempted to use dietary interventions to alter the gut microbiome in an effort to increase bacterial communities associated with positive effects on health. Fermented foods are of particular interest as they host their own diverse and unique microbiomes. Cheddar cheese is one such fermented food and contains microbes that are introduced intentionally as starter/adjunct cultures, plus an indigenous non-starter lactic acid population that eventually dominates the cheese microbiome and contributes to the organoleptic properties to the food product. Additionally, many microbes are found in both Cheddar and gut ecosystems, including members of the *Streptococcus*, *Lactococcus* and *Lacticaseibacillus* genera. This study investigates the impact of ingesting 120 g of Cheddar cheese for 6 weeks on a pre-diabetic cohort (n=10) to affect the gut microbiome and potentially produce alterations that are more typical of an anti-obesogenic microbiome. Faecal samples of patients were taken at two timepoints, Day 0 and Day 42, and sequenced via Illumina 16S amplicon sequencing. No significant differences or changes were observed over this timescale, indicating that consumption of Cheddar cheese did not result in alterations to the gut microbiome.

6.1 Introduction

The link between the gut microbiome and health has been well established, playing key roles in metabolic homeostasis, and immune and neurobehavioral aspects of human physiology (Aslam et al., 2020; Berding et al., 2021; Mota de Carvalho et al., 2018; Valdes et al., 2018). Gut microbiome composition differs between individuals and is established during early infancy, with diet being a major factor when influencing which populations will be dominant (Laursen, 2021). Perturbations of composition and diversity of the gut microbiome are implicated in certain disorders. In particular, reduced diversity is negatively associated with weight gain while increased diversity is positively associated with fibre intake, although this varied greatly across studies and cohorts (Castaner et al., 2018; Menni et al., 2017; Vallianou et al., 2019). Obese and overweight individuals currently comprise a third of the worlds' population, with levels of obesity alone being predicted to rise to 20% by the year 2030 (Chooi et al., 2019; Kelly et al., 2008; Seidell & Halberstadt, 2015). Obesity as a disease is highly complex and multi-factorial and can lead to other serious conditions, including cardiovascular disease, obstructive sleep apnoea, osteoarthritis, cancer and type 2 diabetes mellitus (T2DM) (Al-Goblan et al., 2014; Allen & Golightly, 2015; Iyengar et al., 2015; Jehan et al., 2017; Koliaki et al., 2019). Several studies have shown evidence linking obesity to systemic low grade inflammation, which can lead to insulin resistance and related metabolic disorders, including Type 2 Diabetes Mellitus (T2DM) (Esser et al., 2014).

A strategy to combat obesity and its associated metabolic side effects may be to induce positive change in the host microbiome, which has been tried in both murine and humans with varying degrees of success (Bianchi et al., 2019). Fermented foods have been investigated due to their harbouring rich microbiomes, which can be further fortified through the addition of known probiotics (Kim et al., 2017). While traditional fermented foods relied on the raw substrates' natural microbiome for fermentation, the use of defined starter cultures and strictly controlled commercial food production systems have ensured end products with consistent, reproducible microbiomes (Hill et al., 2017). This is now common practice in the manufacture of most cheeses at large commercial scale, including Cheddar cheese and this allows for greater control over the end product microbiome, supporting the growth of favourable/beneficial

microorganisms. In addition to potential benefits from its microbiome, cheese has additional nutritional benefits. These include the fatty acids conjugated linoleic acid (CLA) and phytanic acid, high calcium content, vitamin A, riboflavin and vitamin B^{12}, and can be further fortified with zinc, iron and selenium (O'Callaghan et al., 2017; Picciotti et al., 2021; Tunick & van Hekken, 2015). Cheese also contains bioactive peptides that have proven antioxidant, antihypertensive and anti-inflammatory health benefits, and are integral to a healthy diet(Khan et al., 2019; O'Callaghan et al., 2017).

Changes in the host microbiome as a result of fermented food ingestion have been recorded in previous studies and do not always reflect the microbial composition of the delivery matrix (Lisko et al., 2017; Taylor et al., 2020; Unno et al., 2015; Veiga et al., 2014; Yılmaz et al., 2019). A study by Han and colleagues (2015) investigated the effects of ingesting fresh (fermented for a very short period of time, such as a single day) and fermented kimchi on microbial populations in obese individuals and found that, while both groups experienced shifts in certain phyla, only the group consuming fermented kimchi experienced an increase in Actinobacteria that was negatively correlated with body fat (Han et al., 2015). While the LAB commonly associated with the kimchi microbiome include the genera *Leuconostoc*, *Lactobacillus* (old classification), *Pediococcus*, and *Weissella,* the fermented kimchi group exhibited an increase in the relative abundance of *Bacteroides* and *Prevotella* (Ang et al., 2022; Han et al., 2015). A high-fat diet (HFD)-induced murine model showed that mice fed milk kefir for 12 weeks resulted in a significantly lower body weight than the control group that was fed non-fermented milk (Kim et al., 2017). Additionally, mice fed kefir had a significantly lower *Firmicutes*/*Bacteroidetes* ratio, total cholesterol and low-density lipoprotein (LDL) cholesterol plasma levels, and Interleukin 6 (IL-6) plasma levels than their milk-fed counterparts (Kim et al., 2017). A study in patients with metabolic syndrome that consumed wine determined that, while no significant differences were observed when comparing dominant bacterial populations within the gut, a significant increase in beneficial bacteria (including *Bifidobacterium*, *Lactobacillus,* and butyrate-producing bacteria) was observed in faecal samples that resulted in a decrease in less desirable bacteria (Moreno-Indias et al., 2016).

The ability of cheese to alter the gut microbiome and affect human health is explored in a limited number of studies. One group determined that Camembert cheese

consumption significantly increased the proportional abundance of faecal *Enterococcus faecalis*, while *Lactococcus lactis* and *Leuconostoc mesenteroides* reached high levels during the dietary intervention (Firmesse et al., 2007, 2008). A separate group determined that Bryndza cheese, while leading to positive changes in body mass index (BMI), muscle and fat mass, also affected the relative abundances of *Erysipelotrichales*, *Lactobacillales*, *Streptococcaceae*, *Lactococcus*, and *Streptococcus* (Hric et al., 2021). Due to its high fat matrix and buffering capabilities, cheese has also been considered as a probiotic vehicle (Gomes da Cruz et al., 2009). Sharp and colleagues determined that low-fat Cheddar was potentially a viable probiotic carrier for *Lacticaseibacillus casei* 334e, with colony forming units (CFU)/g remaining at 10^4 after 120 minutes of simulated digestion (Sharp et al., 2008).

In this study we investigated the effects of mature Cheddar cheese consumption on pre-diabetic patients. This involved the ingestion of 120 g of cheese every day for 6 weeks, with faecal samples being collected immediately prior to and post dietary intervention. Weight loss as part of a lifestyle change has been implicated in preventing prediabetes from progressing to diabetes in patients for as long as 10 years (Tuso, 2014). If ingestion of a fermented dairy product could induce anti-obesogenic microbiome composition changes, then controlled Cheddar cheese consumption may be a viable strategy for tackling weight loss.

6.2 Materials and Methods

6.2.1 Ethics

This study was undertaken at the Health Research Board (HRB) Clinical Research Facility (CRFC Reference: CRF-C-NGAP 17-02) in at the Mercy University Hospital and University College Cork (UCC). The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Reference: ECM 3 (rrrrr) 07/03/18). All participants were provided with full details in order to give an informed decision, and written consent was provided by all participants prior to study commencement.

6.2.2 Visit Schedule

D-7 to -28 D0 D3(-1/+2) D20(+/-3) D42/ET D56-63

SCR*----------INIT---PFU----------SFU----------EoS*----------PI

SCR: Screening visit

INIT: Initiation of intervention

PFU: Phone Follow-up

SFU: Site Follow-up

EoS: End of study

ET: Early Termination

PI: Post Intervention

* Stool Samples were collected at these time points

6.2.3 Inclusion and Exclusion Criteria

As per the supplied supplementary material (Appendix I).

6.2.4 Feeding Schedule and Faecal Sample Collection

Participants (n=10) were asked to ingest 120 g of Cheddar cheese (provided) per day. Faecal samples were taken at Day 0 (pre-diet) and at Day 42 (6 weeks). A document entitled ‘Stool Sample Collection for CHEESE Study: Patient Instructions’ was provided

to all participants (Appendix II). Two Stool Collection Kits (Appendix III) were provided. Fresh Samples were collected by subjects and kept on ice, and immediately stored at -80 °C upon delivery to the clinician during an appointment. Samples were then transported on dry ice to Teagasc Food Research Centre (Moorepark, Fermoy, Co. Cork, Republic of Ireland, P61 C996) and immediately stored at -80 °C upon arrival.

6.2.5 DNA Extraction and 16S amplicon Sequencing Library Preparation

DNA was extracted from faecal samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Crawley, UK) as per the manufacturer's instructions. Quantification of samples was done using a Qubit® dsDNA High Sensitivity Assay Kit (Fisher Scientific, Dublin, Ireland) in conjunction with a Qubit® 2.0 fluorometer (Invitrogen, Paisley, UK). Library preparation was undertaken using an adapted protocol designed by the Teagasc Sequencing Centre (Moorepark) based on the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Illumina, Saffron Walden, UK), with some modifications to the original methodology (Illumina, 2013). During the original amplicon PCR step, all reagents were doubled in quantity from the original total of 25 µL to 50 µL (to ensure sufficient DNA was extracted), with 1 µL of each primer used at a 10 µM concentration, and 30 amplification cycles were used. For the first PCR clean-up step, 36 µL of AMPure XP beads were used, which were vortexed immediately before use. The following steps were carried out as per the original Illumina methodology (Illumina, Saffron Walden, UK), without modifications; index PCR and subsequent PCR clean-up step; library quantification, normalization, and pooling; library denaturing and MiSeq sample loading (Illumina, 2013).

6.2.6 Bioinformatics Analysis

Sequences were filtered on the basis of quality (removal of low-quality nucleotides at the 3' end, Q>25 by nucleotides windows at 3 nucleotides) and length (removal of sequences with <200 nucleotides) with PRINSEQ and joined using fastq-join (Aronesty, 2011; Schmieder & Edwards, 2011). Filtered sequences were denoised and clustered into unique sequences (zero-distance operational taxonomic units; zOTUs) using the UNOISE2 algorithm implemented in USEARCH (Edgar, 2016). zOTU represent unique bacterial entities and are roughly equivalent to species or strains. Contamination,

which may occur when processing low-biomass samples was removed with the decontam R package (Davis et al., 2018; Salter et al., 2014). Prevalence-based identification was utilised to statistically identify and remove contaminant zOTUs. Alpha and beta-diversity was determined using phyloseq R package (McMurdie & Holmes, 2013). The results of principal coordinates analysis (PCoA) of the beta-diversity when it was calculated using distance matrices was built from unweighted UniFrac distances.

6.3 Results

6.3.1 Differences in microbiomes between groups pre- and post-intervention

Illumina 16S Sequencing revealed no significant ($p > 0.05$) differences between the microbiomes of patients prior to and 6 weeks after commencement of a diet where 120 g of Cheddar cheese was consumed daily for 42 days. Alpha diversity was measured using the Richness (**Figure 1A**), Simpson (**Figure 1B**), and Shannon (**Figure 1C**) indices, while beta diversity was represented through a principal coordinates analysis (PCoA) plot on the unweighted UniFrac distance matrix (**Figure 2**). Neither alpha (**Figure 1**) nor beta (**Figure 2**) diversity was found to be significantly different ($p > 0.05$) at the two timepoints.

The majority of microbes present in faecal samples both before and after the diet belonged to the Firmicutes phylum, comprising approximately 66% relative abundance, followed by the Bacteroidetes phylum at approximately 25% relative abundance (**Figure 3**). The most abundant family present was the Lachnospiraceae at approximately 27% relative abundance, followed closely by the Ruminococcaceae family at approximately 24% relative abundance (**Figure 4**). While the most abundant genus remained *Faecalibacterium* between groups (at approximately 11% relative abundance), the second most prevalent genera were the *Prevotella* (9%) and *Bacteroides* (11%) among pre- and post-intervention participants, respectively (**Figure 5**).

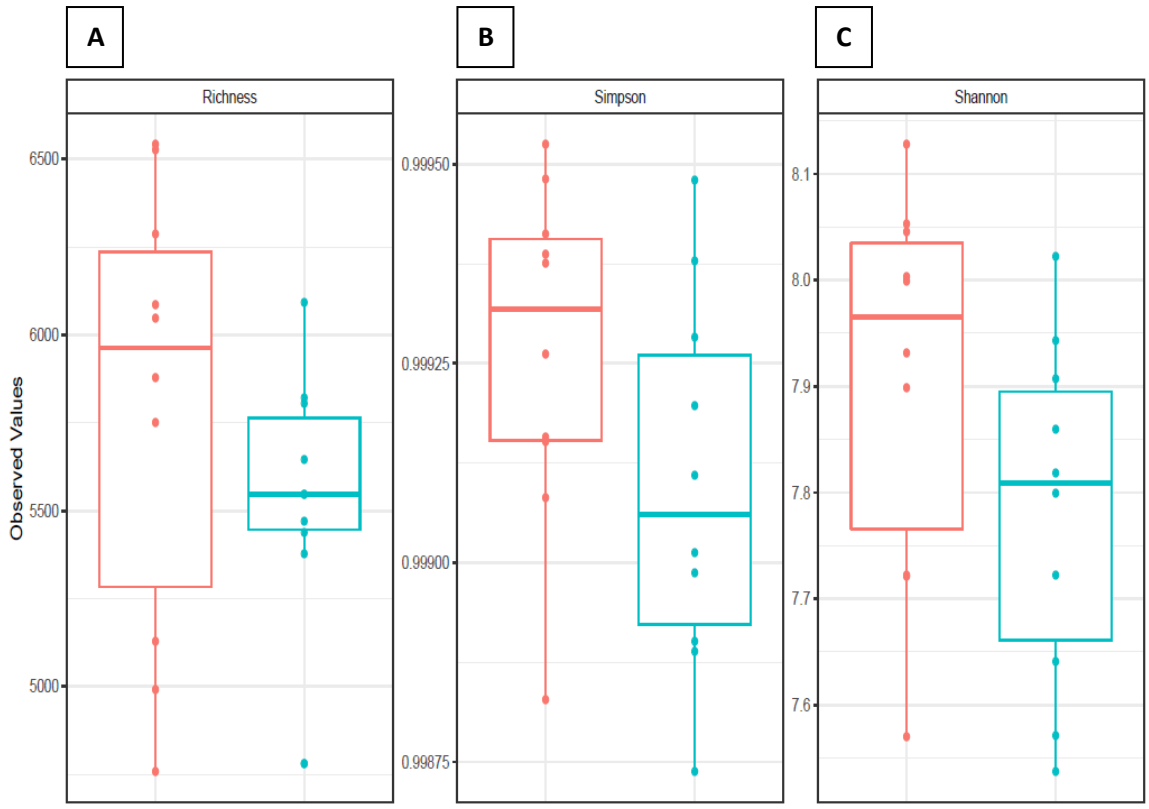

Figure 1. Alpha diversity illustrated by three indices of diversity: **(A)** Richness, **(B)** Simpson, and **(C)** Shannon. This was performed on the microbial communities present in faecal samples in pre- and post-intervention groups. No significant ($p > 0.05$) differences were observed.

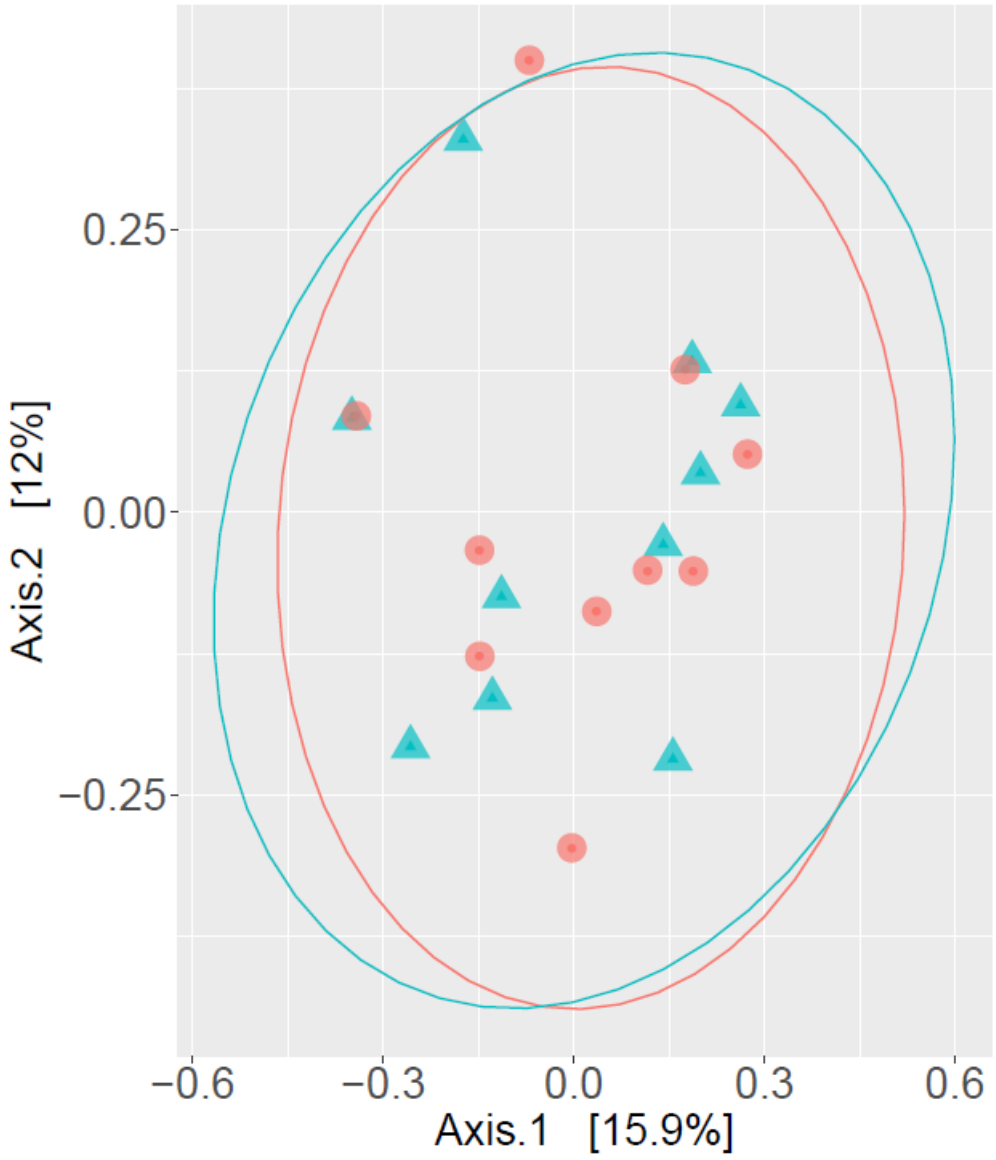

Figure 2. Beta diversity illustrated by PCoA of Unifrac distance. This was performed on the microbial communities present in faecal samples in pre- and post-intervention groups. No significant ($p > 0.05$) differences were observed.

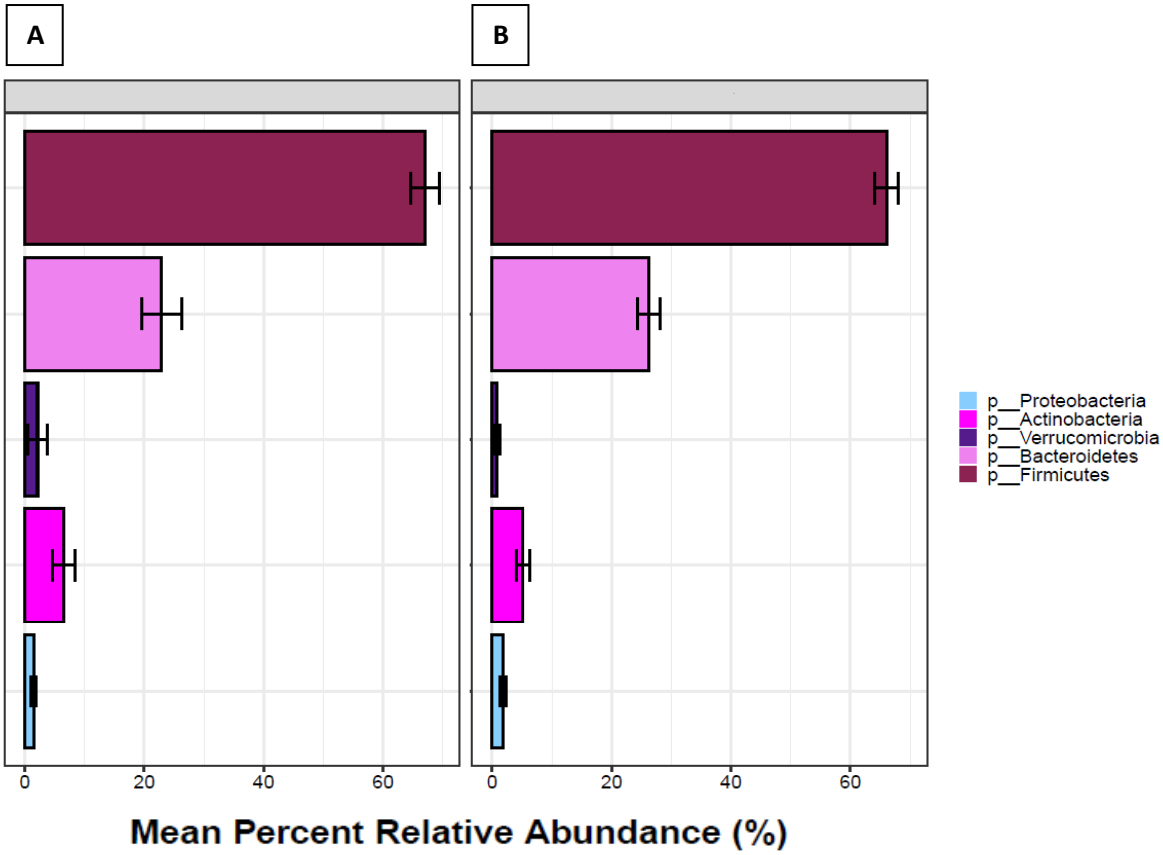

Figure 3. The mean percentage relative abundance of the major phyla present in faecal samples in **(A)** pre-intervention and **(B)** post-intervention groups. No significant ($p > 0.05$) differences were observed.

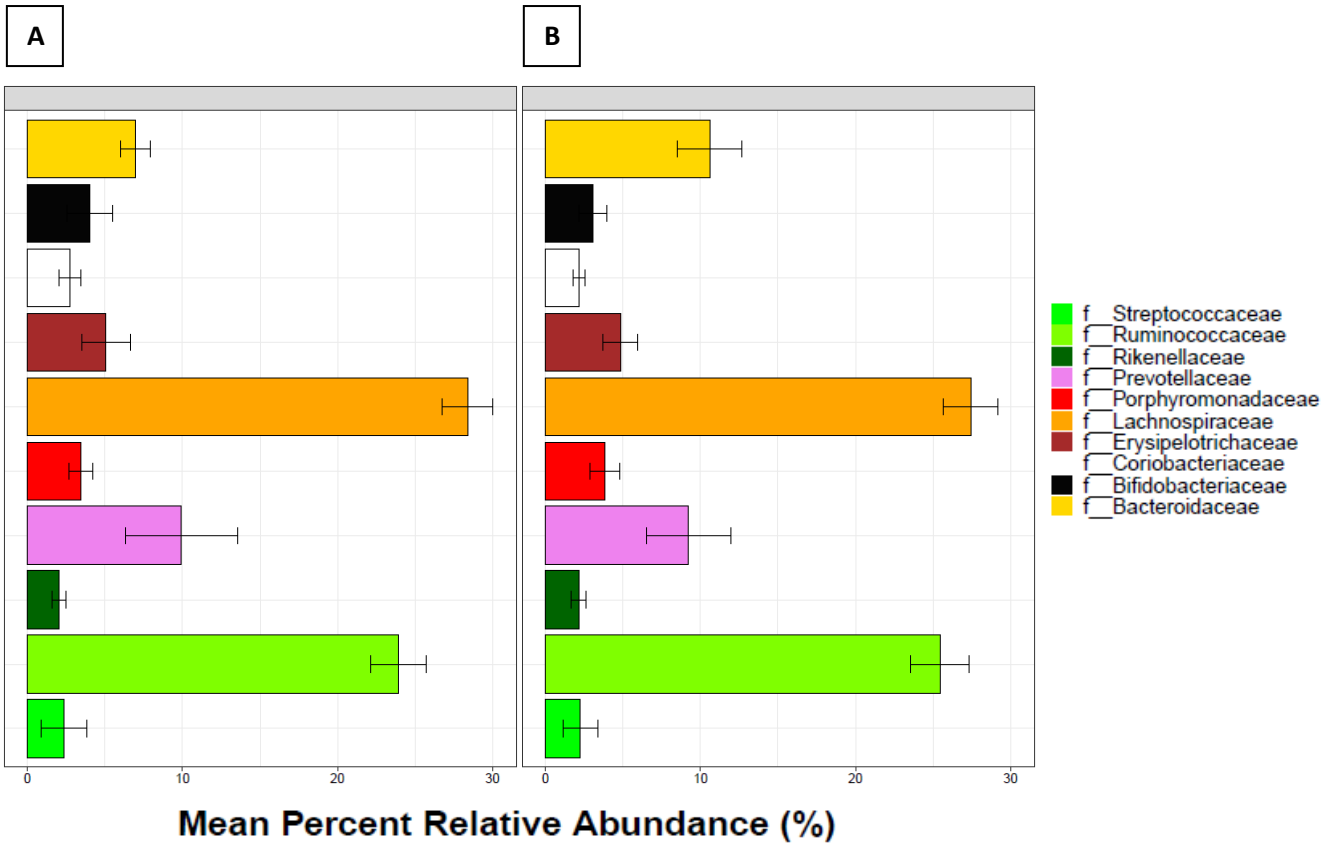

Figure 4. The mean percentage relative abundance of the major families present in faecal samples in **(A)** pre-intervention and **(B)** post-intervention groups. No significant ($p > 0.05$) differences were observed.

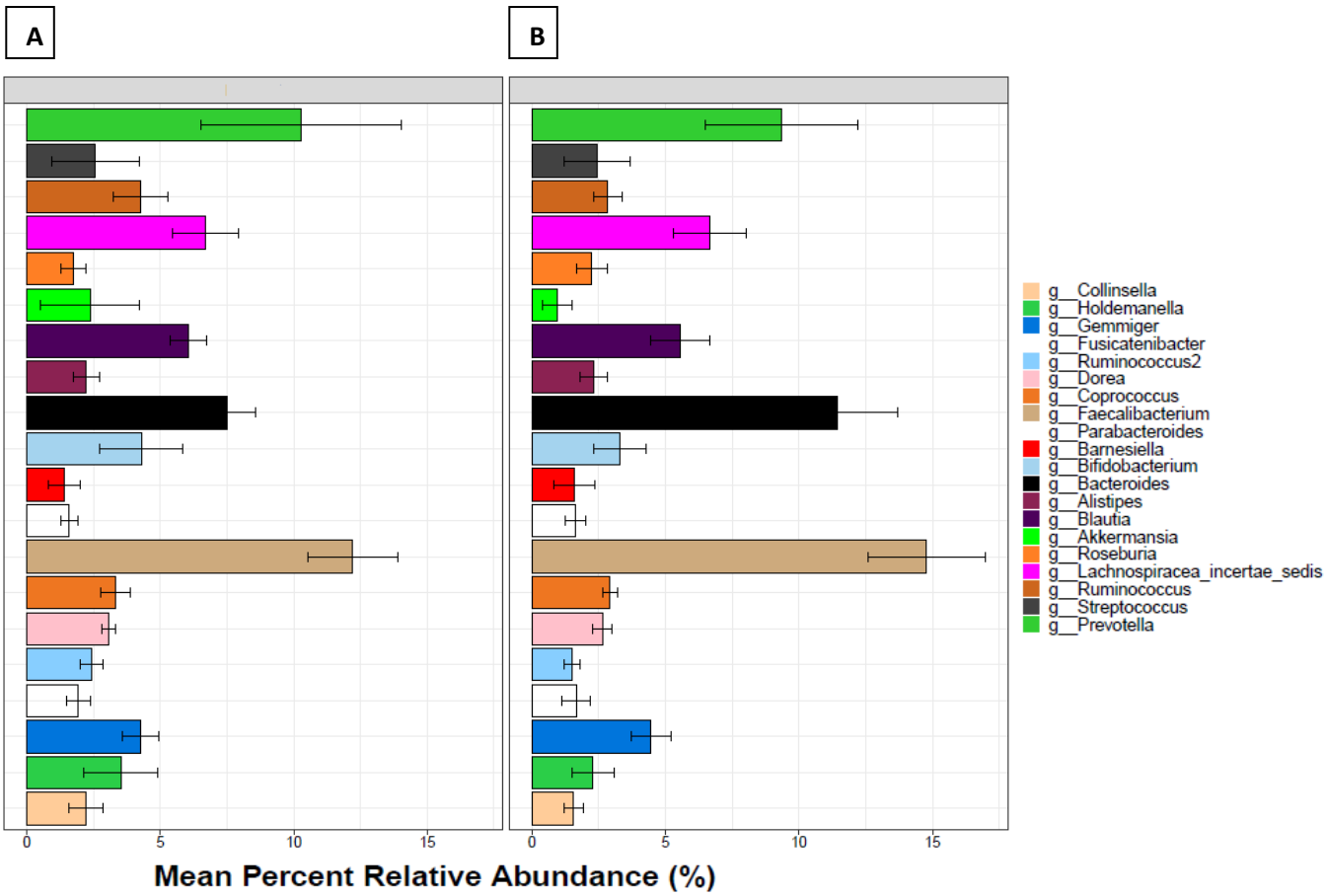

Figure 5. The mean percentage relative abundance of the major genera present in faecal samples in **(A)** pre-intervention and **(B)** post-intervention groups. No significant ($p > 0.05$) differences were observed.

6.4 Discussion

Gut microbiomes of pre-diabetic individuals were not significantly altered following a 6-week period of high Cheddar intake in this study. While no differences in microbiome composition were observed, phyla compositions were unusual. (**Figure 3**). Previously, a higher Firmicutes/Bacteroidetes ratio was associated with an obese phenotype and individuals with T2DM, although this hypothesis is not consistent through all studies (Castaner et al., 2018; Stanislawski et al., 2019). This study shows a larger Firmicutes composition, with approximately 56% relative abundance being the lowest percentage present among all 10 individuals at either timepoint (**Figure 3**). The phylum Bacteroidetes is observed at much lower levels (approximately 25% relative abundance overall), and usually encompasses one of the most abundant phyla in adults (comprising 90% of the entire gut microbiome in conjunction with Firmicutes) (Qin et al., 2010).

A study published by Zhang and colleagues (2021) investigated gut microbiome composition via Illumina 16S sequencing in 180 participants divided into three cohorts, with 60 suffering from T2DM, pre-diabetes or the control group (Zhang et al., 2021). Their pre-diabetes cohort exhibited a much more balanced Firmicutes/Bacteroidetes ratio, with approximately 41.3% and 44.9% relative abundance of Firmicutes and Bacteroidetes, respectively (Zhang et al., 2021). Our pre-diabetic participants' microbiome compositions also displayed higher Actinobacteria and Verrucomicrobia, and lower Proteobacteria relative abundances in comparison (Zhang et al., 2021). The median age of the pre-diabetes group was approximately 47 years, while the participants in our study were all 50 years of age or older, which may account for some of the discrepancies observed between Zhang et al. (2021) and the findings presented in this study (Zhang et al., 2021). Differences in microbiome between these studies will also arise due to the differing number of participants (10 vs 180) and geographical locations (Ireland vs China) for the studies (Zhang et al., 2021).

While certain dietary interventions have previously been shown to have an effect on the gut microbiome, not all interventions are successful, as appears to be the case here (de Filippis et al., 2018). The Hric and colleagues (2021) study mentioned

previously determined that a 4-week dietary intervention involving high Bryndza cheese intake in 22 female participants resulted in significant increases in relative abundance of *Erysipelotrichales*, *Lactobacillales*, *Streptococcaceae*, *Lactococcus* and *Streptococcus*, demonstrating that higher cheese intake over in a short-term period does have the potential to shift microbiome populations (Hric et al., 2021). We observed that a Cheddar cheese-heavy diet for 6-weeks did not appear to impact microbiome content of the 10 pre-diabetic patients in this study. The number of participants should be increased, and this study repeated to ensure the accuracy of these findings, and the Cheddar might also be supplemented with a known probiotic to determine if the presence of beneficial bacteria effects gut microbial populations.

6.5 Conclusion

Consumption of 120 g of Cheddar cheese per day for 6 weeks did not result in significant changes in the gut microbiomes of 10 pre-diabetic participants, aged ≥50 years. No differences were observed in either alpha or beta diversity, or between compositions at the phylum, family, or genus level. A weakness of this study is that only 10 subjects were included and should be repeated with a larger cohort to ensure the accuracy of these results.

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Chapter 7

General Discussion

The primary objective of my thesis was to investigate if consumption of Cheddar cheese could affect the gut microbiome. I hypothesised that the gut microbiome could be impacted either through provision of nutrients from the cheese or through interactions with surviving populations of the cheese microbiome. In particular, I sought to investigate if members of the Non-Starter Lactic Acid Bacteria (NSLAB), a dominant population in all long-ripened cheeses such as Cheddar, could interact with and modulate the gut microbiome. Probiotics have been isolated from many of the species that comprise NSLAB, including many members of the *Lactobacillaceae* family, and the *Bifidobacterium* and *Enterococcus* genera (Burns et al., 2012; Grujović et al., 2022). Thus, it seems plausible to theorize that some of the NSLAB strains from the Cheddar cheeses under investigation might also express probiotic traits.

Thus, to investigate the hypothesis that strains of the NSLAB population of Cheddar cheese can affect the gut microbiome I undertook the following series of experiments:

1. NSLAB were extracted from commercial Irish Cheddars using an *in vitro* simulated digestion model to test for acid and bile tolerance, as well as resistance to digestive enzymes
2. The population of *Lactobacillaceae* obtained was screened *in vitro* for a range of probiotic traits as defined by the WHO/FAO
3. Cheddar cheese was manufactured using two of the most promising *Lactobacillaceae* strains with probiotic potential as adjunct cultures
4. Survival of the NSLAB strains-of-interest during Cheddar maturation, and post *in vitro* simulated digestion was measured
5. The effects of ingesting *Lactobacillaceae*-containing Cheddar in a murine model was investigated, with particular reference to the murine gut microbiome
6. In addition, the ability of a non-related commercial Cheddar cheese to modify the gut microbiome of pre-diabetic adults was also investigated

To these ends, Cheddar samples were subjected to an *in vitro* simulated stomach duodenum passage (SSDP) digestion model, and only those NSLAB strains surviving (grown on MRS media to select for *Lactobacillaceae*) were selected for further testing. This screening process involved testing strains for the ability to express bile salt hydrolase enzymes, resistance to antibiotics at acceptable levels (as per Aquilina and

colleagues [2012]), the ability to adhere in an epithelial model, the ability to inhibit binding of enteric pathogens in the same epithelial model, and the ability to produce exopolysaccharides (Aquilina et al., 2012). Two *Lactobacillaceae* strains were identified out of the original 76 capable of gastric survival with these desirable qualities and were successfully used as adjuncts to produce Cheddar cheese. Survival of the two strains was monitored over 6-months of ripening and was found to remain dominant in the NSLAB population and to be present at 7-8 log CFU/g of cheese which conforms to the acceptable numbers for use as a functional food containing live probiotic bacteria (Leeuwendaal et al., 2022; Tripathi & Giri, 2014). Additionally, Cheddar samples were subjected to the INFOGEST 2.0 simulated digestion to determine *Lactobacillaceae* survival, and survival rates remained at acceptable levels (> 6.5 log CFU/g). The INFOGEST method has been utilised previously on a ricotta cheese matrix containing a *Lc. rhamnosus* probiotic emulsion, and only 1 of the 3 types of ricotta used maintained the bacteria of interest at > 10^6 CFU/g, levels similar to those seen in this thesis (Melchior et al., 2022). These Cheddar cheeses were then fed to male C57BL/6 mice for 4 weeks, and significant alterations to their gut microbiomes (both in faecal and caecal populations) were observed at phyla, family and genus levels between dietary groups. Very few studies have investigated the effects on cheese intake (with or without probiotic supplementation), making my study an important contribution to this area (Aljutaily et al., 2020; Yun et al., 2020). In parallel, pre-diabetic human patients were instructed to consume 120 g of Cheddar per day for 6 weeks, with faecal samples prior to and post-feeding trial revealing no significant shifts in composition of gut microbial content.

In my opinion, the most exciting finding that I have contributed to the scientific community is that I was able to confirm the hypothesis that Cheddar cheese is capable of significant microbiome modifications, at least in a murine model. To date, several studies have also examined the ability of cheese containing probiotic bacteria to influence the gut microbiome (Aljutaily et al., 2020; Hric et al., 2021). Aljutaily and colleagues (2020) examined the effects of a probiotic-enhanced cheese supplemented diet in a murine study, and significant gut microbiome changes occurred in the form of increases in relative abundances of the order *Clostridiales*, families *Ruminococcaceae* and *Lachnospiraceae* (Aljutaily et al., 2020). Yun and colleagues (2020) observed that microbial shifts in chronic, unpredictable, mildly stressed mice with a Gouda-

supplemented diet were determined by the breed of cow's milk, with the relative abundance of *Bacteroidetes* and *Firmicutes* increasing and decreasing, respectively, in only the Jersey milk Gouda group (Yun et al., 2020). Other studies have examined the effects of cheese intake on mice but, unfortunately, these studies have not included results on the microbiome (Cordeiro et al., 2021; Kim et al., 2021; Rabah et al., 2020; Vasconcelos et al., 2019). In a human cohort, Hric and colleagues (2021) also determined that probiotic containing bryndza cheese increased the relative abundance of lactic acid bacteria (*Lactobacillales*, *Streptococcaceae*, *Lactococcus* and *Streptococcus*) significantly (Hric et al., 2021). Consumption of the probiotic cheese also significantly increased the relative abundance of short-chain fatty acid producers *Phascolarctobacterium* and *Butyricimonas* (Hric et al., 2021). Thus, my study adds to the existing literature and supports the hypothesis that cheese can be used to modify gut microbiome composition.

I was also very satisfied with the level of survival of the *Lacticaseibacillus*, in terms cheese ripening and during simulated gastric digestion. Cheddar was ripened for 12 months, and NSLAB counts remained high in the NSLAB adjunct containing cheeses, at approximately 10^7 CFU/g. The minimum concentration of live probiotic within functional foods should be 10^6 CFU/g at the time of ingestion to ensure sufficient survival through digestion for exertion of health benefits (Dinkçi et al., 2019; Tripathi & Giri, 2014). Thus, the NSLAB of interest survived at levels sufficient to satisfy this stipulation following a 12-month maturation period. Survival of the adjuncts were confirmed via PFGE at 12 months of maturation, as restriction profiles of the strains of interest were confirmed. It is also interesting to note that Cheddar provided a more protective barrier to *in vitro* digestion than a fermented milk matrix.

An interesting finding from my work was the high numbers of NSLAB that survived from the initial Cheddar cheeses following simulated stomach duodenum passage (SSDP), with survival ranging from 5.83×10^7 to 1.55×10^4 colony forming units (CFU)/g (Leeuwendaal et al., 2021). Populations of NSLAB from many of the Cheddar cheeses examined experienced reductions of only ≤ 0.4 log CFU/g (Leeuwendaal et al., 2021). This is an interesting finding as it suggests that the cheese matrix is capable of protecting large NSLAB populations during SSDP (as high as 93% of the total population in some cases), thus, resulting in a larger surviving microbiome population to interact

with the gut microbiome once ingested. While the ability of cheese to protect specific probiotics from digestive stresses is frequently reported in the literature, I could locate very few articles that examined the ability of cheese to protect its microbiome in general (Firmesse et al., 2008; Martinovic et al., 2016; Ricciardi et al., 2015; Sumeri et al., 2012). In these studies, while methodologies differed greatly, survival of cheese strains during digestion was highly variable and dependent on the bacterial strains present, fat content, milk type used, but that overall survivability can be extremely high (Firmesse et al., 2008; Martinovic et al., 2016; Ricciardi et al., 2015; Sumeri et al., 2012). Thus, it is interesting to confirm that cheese can potentially contribute a high live microbial load to the gut without any additional fortification, and this in itself it worthy of further research. Exposure of cheese to SSDP prior to isolation of NSLAB enhanced the efficiency of selection of strains with the potential to survive gastric transit and thus, enriched for strains that may express desirable probiotic characteristics. In addition, as consumption of probiotics often occurs in a food matrix, exposure of cheese to SSDP as part of the selection process provides a more realistic model than investigation of survival using purified planktonic bacterial strains.

My investigations of the human cohort yielded no microbiome changes but, as only 10 patients with a pre-existing condition participated, I would strongly encourage a follow-up study with a greater number of subjects (Chapter 6). The current study would provide background data to complete a Power Calculation that would help to define the number of subjects required to result in a statistically significant study. It might also benefit to include a control group of healthy individuals for comparison. Not all dietary interventions have shown microbial perturbations, as appears to be the case here (de Filippis et al., 2018).

Future studies arising from this work should include:

- A human study examining the organoleptic properties of the Cheddar produced in Chapter 4, to determine the commercial appeal of cheese produced with the two potentially probiotic lacticaseibacilli strains. This should include additional analysis specifically on flavour compounds present in both cheeses.

- A feeding study in healthy humans involving consumption of the Cheddar produced in Chapter 4 (including the control Cheddar). This would examine weight parameters as per the murine study (Chapter 5) but should be expanded to include high-density and low-density lipid cholesterol blood levels, as well as enteroendocrine hormones responsible for satiety, such as ghrelin and leptin.

- Additionally, it would be interesting to track the two lacticaseibacilli strains specifically following ingestion, as a means of determining the duration that they are capable of remaining within the gut ecosystem.

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Appendices

Appendix I

Inclusion and Exclusion Criteria

The inclusion criteria were:

- Participants could be either gender
- Aged 50 years or older
- Provide written informed consent
- Have HbA1c levels of 42-48 mmol/mol
- Routinely ingest cheese 1-3 times per week
- Be in their usual state of health for at least 3 months prior to initiation of this study
- Have a stable diet for the 3 months prior to commencement of the study
- Be able and willing to store 21 blocks of cheese, each weighing 120 g, at their domicile, preferably in a refrigerator

The exclusion criteria were:

- Allergy to dairy products
- Use of hypoglycaemic medication or insulin in the year immediately prior to recruitment
- Planned or were believed to be at high risk for hospitalization within the next 3 months
- Were currently or had taken corticosteroids in the 6 months prior to recruitment

- Planned to begin a diet, exercise program or other major lifestyle change in the next 3 months
- Were currently on a medically mandated diet restricting cheese intake
- Planned a vacation of more than 1 week during the trial period
- Had participated in a separate trial in the last month
- Residing in a nursing home
- History of eating eating disorder, sustained nausea, vomiting or anorexia in previous 6 months
- Were unable to comply with the assigned protocol

Appendix II

Stool Sample Collection

Patient Instructions

Prepared by: Mary Daly & Nicola Ronan

Please read all instructions before beginning



A stool collection kit (as above) has been provided for you, proper use of this kit will assist the laboratory in obtaining accurate results**.**

(Stool Kit contains: disposable gloves, large collection container, lab container, spoon/scoop, zip locked bag,

paper towels, storage/transport box).

How to Collect a Stool Sample

- If possible, urinate before collecting the stool specimen to avoid contaminating the sample.

- Prepare the toilet ensuring it is flushed prior to collecting your specimen.
- Place the enclosed large container carton in the toilet bowl under the seat, toward the rear of the toilet bowl. Be careful not to get water in the container if possible
- Wash your hands before and after you collect the specimen
- Put on gloves included in the kit
- Pass the stool specimen directly into the container, try to make sure the sample does not touch the inside of the toilet if possible.

- Do not mix toilet paper, water or soap with the sample

- Using the spoon (scoop) enclosed in the kit, scoop the FULL **STOOL** specimen into the lab container.
  If you do **not** wish to transfer the stool sample from the large container, it can be returned in the collection container but making sure it is secured tightly in the container.

- Try not to spill the stool on the outside of the specimen container. If this happens clean the outside of the container with soap and water.
  .
- Place the lab container or large container in the zip locked bag provided, store the zip locked bag in a fridge or cool environment.

- Make sure anything you used to collect the stool specimen must be placed in a plastic bag, tied tightly and disposed in the waste bin.

- Wash your hands thoroughly with soap and warm water

- Bring the container back to the Clinical Research facility within 24 hours of collection if possible, and remember all specimens must be stored in the enclosed zip locked bag.

- You will need to collect two samples the first will be at the time of the Screening Visit and the second at the End of Study visit

- Give the sample to _____, who will label it with your unique study number

- Your contact telephone no: _____

Appendix III
Stool Sample Collection for the CHEESE Study
Patient Instructions

(Please read all instructions before beginning)

A stool collection kit (as above) has been provided for you, proper use of this kit will assist the laboratory in obtaining accurate results.

Stool Collection Kit contains: white lab container with integrated brown scoop, disposable gloves, large collection container, 2 small ice packs, large zip locked bag, small zip locked bag.

How to collect a stool sample at home

- Ensure that ice packs are frozen prior to collection of the stool sample.
- If possible, urinate before collecting the stool specimen to avoid contaminating the sample.
- Prepare the toilet ensuring it is flushed prior to collecting your specimen.
- Place the enclosed large container carton in the toilet bowl under the seat, toward the rear of the toilet bowl. Be careful not to get water in the container if possible.
- Wash your hands before and after you collect the specimen.
- Put on gloves included in the kit.
- Pass the stool specimen directly into the container; try to make sure the sample does not touch the inside of the toilet if possible.
- Do not mix toilet paper, water or soap with the sample.
- Using the brown scoop enclosed in the kit, scoop the FULL **STOOL** specimen into the white lab container.

If you do **not** wish to transfer the stool sample from the large container, it can be returned in the collection container but making sure it is secured tightly in the container.

- Try not to spill the stool on the outside of the specimen container. If this happens clean the outside of the container with soap and water.

- Place the white lab container or large container in the large or small zip locked bag provided with the frozen ice packs, **store the zip locked bag in a fridge or cool environment.**

- Make sure anything you used to collect the stool specimen must be placed in a plastic bag, tied tightly and disposed in the waste bin.

- Wash your hands thoroughly with soap and warm water.

- Bring the container back to the Clinical Research Facility Unit within 24 hours of collection if possible, and remember all specimens must be stored in the enclosed zip locked bag.

- Give the sample to a member of the research team, who will label it with your unique study number.

If you have any questions about the stool collection or questions about the study in general please contact the study team at: 0834148822