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Isolation and Characterisation of Antimicrobial / Antifungal Activity Produced by Bifidobacterium longum ITT 13

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Isolation and Characterisation of Antimicrobial / Antifungal Activity Produced by *Bifidobacterium longum* ITT 13

A Thesis Presented for the Award of Masters by Research by

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Abstract

Bifidobacteria are a species of GRAS organism present in the human gut flora which are claimed to confer various health benefits on the host. The antimicrobial activity of *Bifidobacterium longum* ITT 13, isolated from the neonatal gut, was evaluated in relation to both the contribution of organic acids produced by the strain and potential bacteriocin production. Bacteriocins are antimicrobial peptides produced by bacteria. This study focuses on the characterisation of the antimicrobial activity produced by *B. longum* ITT 13.

Heat-inactivated fermentate produced by ITT 13 has shown antimicrobial activity against a variety of pathogenic and food spoilage associated bacterial and fungal strains.
Physiochemical characterization of the antimicrobial activity has shown that pH stability testing has indicated activity at pH 5 and below, with activity not detectable above pH 6. Temperature stability testing has indicated stability of the antimicrobial activity at a temperature range of -20^{o}C to 100^{o}C, with maximum activity (133-213 Activity Units/ml) retained for 48 hours at 4^{o}C and room temperature (20^{o}C to 24^{o}C). Antimicrobial activity was found to be partially caused by organic acid production, as the heterofermentative *Bifidobacteria* produce several acids during growth. HPLC analysis determined between 6 mg/ml and 10 mg/ml lactic, acetic and succinic acid produced by *B. longum* ITT 13 when grown in De Man, Rogosa and Sharpe broth with and without pH control. Protease digestion by Actinase E resulted in a reduced minimum inhibitory concentration of antimicrobial activity and 80% ammonium sulphate precipitation of the fermentate resulted in the detection of antimicrobial activity in the resuspended precipitate, indicating the presence of antimicrobial activity associated with a low molecular weight proteinaceous molecule.
Concentration studies on the antimicrobial activity of the fermentate involved membrane filtration via tangential flow filtration. Use of a 10KDa and 3 kDa filter showed similar antimicrobial activity present in both the retentate and permeate, together with probable adsorption of the proteinaceous molecule to the membrane, as it is present in low concentrations.

Experimental results indicate that the antimicrobial activity associate with *B. longum* ITT 13 is due to a combination of the production of acids together with a proteinaceous component. Further studies, using higher starting concentrations of fermentate, could be used to concentrate and isolate the bacteriocin component of the detected antimicrobial activity.

Declaration

I certify that this thesis which I now submit for examination for the award of MSc by Research, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for graduate study by research of the Technological University Dublin (TU Dublin) and has not been submitted in whole or in part for another award in any other third level institution.

The work reported on in this thesis conforms to the principles and requirements of the TU Dublin's guidelines for ethics in research.

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Signature _____ Date _____

Candidate Taylor Duff B.Sc. (Hons)

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Dedicated to

My daughter

List of Abbreviations

µl	Micro-litre
µm	Micro-metre
µM	Micromolar
A_{280}	Absorbance at 280nm
AAD	Antibiotic Associated Diarrhoea
ABC	ATP-Binding Cassette
ACN	Acetonitrile
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
API	Analytical Profile Index
AS	Ammonium Sulphate
ATCC	American Type Culture Collection
ATF	Alternating Tangential Flow Filtration
ATP	Adenosine Triphosphate
AU	Arbitrary Units
AU/ml	Arbitrary Units per Milli-litre
BCA	Bicinchoninic Acid
BU/ml	Bacteriocin Unit per Millilitre
CBS	CBS-KNAW Culture Collection
CECT	Spanish Type Culture Collection
CFF	Cell Free Fermentate
CFS	Cell Free Supernatant
CFU	Colony Forming Unit

CFU/ml	Colony Forming Unit per Millilitre
Conc.	Concentration
Da	Daltons
DNA	Deoxyribonucleic Acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
FAO/WHO	Food and Agriculture Organization/World Health Organisation
g	Grams
g/L	Grams per Litre
GI	Gastrointestinal
GRAS	Generally Regarded as Safe
H_2O	Water
HCl	Hydrochloric Acid
HePS	Heteropolysaccharides
HIC	Hydrophobic Interaction Chromatography
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography with Ultraviolet Detection
HSA	Human Serum Albumin
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IgG	Immunoglobulin G
IMS	Industrial Methylated Spirit
iNKT	Invariant Natural Killer T
ITT	Institute of Technology Tallaght

IU/ml	International Unit per Millilitre
IUPAC	International Union of Pure and Applied Chemistry
kDa	Kilo Daltons
L	Litre
L.A.	Lactic Acid
LAB	Lactic Acid Bacteria
LPS	Lipopolysaccharide
M	Molarity
M/D Assay	Micro-Diffusion Assay
mCOL	Modified Columbia Medium
mg	Milligram
mg/ml	Milligrams per Millilitre
MH	Mueller Hinton
MIC	Minimum Inhibitory Concentration
MIC_{50}	Minimum Inhibitory Concentration of at least 50% growth
ml(s)	Millilitre(s)
ml/min.	Millilitre per Minute
mm	Millimetre
mM	Millimolar
MRS Agar	de Man, Rogosa & Sharp Agar
MRS Broth	de Man, Rogosa & Sharp Broth
MRSA	Methicillin Resistant *Staphylococcus aureus*
MRSc Broth	De Man, Rogosa & Sharp Broth with L-cysteine
MWCO	Molecular Weight Cut-Off

mYPD media	Modified Yeast-extract Peptone Dextrose Media
N	Normality
N/A	Not Applicable
NaAc	Sodium Acetate
NAD+/NADH	Nicotinamide Adenine Dinucleotide
NaOH	Sodium Hydroxide
NBIMCC	National Bank for Industrial Microorganisms and Cell Cultures
NCIMB	The National Collection of Industrial, Food and Marine Bacteria
nm	Nanometre
ns	Non-significant
OD	Optical Density
OD600	Optical Density at 600 nm
PBS	Phosphate-Buffered Saline
PD Agar	Potato Dextrose Agar
PD Broth	Potato Dextrose Broth
PSI	Pound Per Square Inch
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
SCFA	Short Chain Fatty Acids
SD	Standard Deviation
spp.	Species
T	Time
TFF	Tangential Flow Filtration

TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
Vol.	Volume
VRE	Vancomycin Resistant Enterococci
WHO	World Health Organisation
YPD	Yeast-extract Peptone Dextrose

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CHAPTER 1

Introduction

1.1 Gut Microflora and Probiotics

The human digestive system is a complex ecosystem of gut microflora with over 1000 known species of bacteria, which due to its anaerobic environment, comprise many obligate and facultatively anaerobic species such as *Bacteroides, Bifidobacterium, Lactobacilli, Enterococci* and *Streptococci* among many others (Nie *et al.*, 2018). The digestive system and its components are important factors in overall health, responsible for the absorption of water and nutrients, and the commensal organisms present are key players in its effectiveness due to interactions with host cells and important roles in the contribution to health and disease (Butel, 2014; Heintz-Buschart and Wilmes, 2018). The composition of the human gut flora varies between individuals due to a variety of factors such as age, geography and diet (Yatsunenko *et al.*, 2012), as well as consumption of probiotics or antimicrobials (Hugon *et al.*, 2017), yet *Bifidobacterium* appears to dominate the gut of infants, decreasing as they age (Yatsunenko *et al.*, 2012). In a study by Cooke *et al*., 2005, *Bifidobacterium* and *Lactobacilli*, were found to be more prevalent in the gut of breast fed babies and were thought to contribute to what constitutes a healthier gut based on bacterial composition.

The beneficial effect of the gut microbiota on the host illustrates the importance of maintaining a healthy digestive system and a healthy balance of the intestinal ecosystem. They produce a variety of bioactive compounds such as organic acids, fatty acids and bacteriocins which have been shown to contribute to the overall health of individuals through preventing colonisation of the gut by potentially pathogenic bacteria and promoting a healthier gut (Hladíková *et al.*, 2012; O'Shea *et al.*, 2012; Guinane *et al.*, 2016). Various enteric bacteria also play important roles in nutrition and maintenance of intestinal integrity and functions, including the production of beneficial nutrients such vitamin k and folate for the host (Quigley, 2010; Pacheco Da Silva *et al.*, 2016). The conversion of unabsorbed dietary sugars into SCFA's is also an important function of the microflora, as these fatty acids are used as an energy source by the colonic cells

for the promotion of epithelial cell growth in the intestine (Quigley, 2010). The preservation of a healthy and intact intestinal barrier is necessary to maintain overall gastrointestinal health and can aid in the prevention of diseases such as IBS and Crohn's disease (O'Flaherty and Klaenhammer, 2010).

Intestinal bacteria are evidently integral to healthy intestinal function, however they also contribute to the overall health of the host as studies have shown they have also been linked to modulating immunity in the prevention of immune-associated diseases or allergies. A study by Olszak *et al* (2012) involving mice displayed how the early colonisation of neonatal mice by commensal gut microbes prevented the accumulation of invariant natural killer T (iNKT) cells which have been associated with the immune related diseases asthma and inflammatory bowel disease (IBD). This was in comparison to germ free mice in which an increase in these cells resulted in higher expression in the intestinal and pulmonary systems of the chemokine ligand CXCL16, which is associated with these diseases. It is thus evident that the significant impact these microbes have on human health is immense.

Probiotic bacteria are defined by the World Health Organisation (WHO) as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host", and most commonly include *Bifidobacterium* and *Lactobacillus* species (FAO/WHO, 2006; Butel, 2014). The consumption of probiotic bacteria can positively influence the balance of gut microflora and increase the health of the gut through the mechanisms mentioned previously (Quigley, 2010; Olszak *et al.*, 2012; Pacheco Da Silva *et al.*, 2016). The necessary characteristics of probiotic bacteria according to the FAO/WHO (2006) can be grouped into four main criteria; the strains must be sufficiently characterised, safe for intended use, be supported by at least one positive human clinical trial carried out according to scientific standards and be alive in sufficient numbers within the probiotic product to exert an effect throughout the products shelf-life (Binda *et al.*, 2020). The FAO/Who (2006) also detail how

probiotic bacteria should display resistance to the acidic bile conditions of the gastrointestinal system and the ability to survive and proliferate during the traversal through the gut upon consumption. Many probiotic bacteria have been isolated from the human gastrointestinal tract, such as *Bifidobacteria*, that are described to offer a health benefit similar to that offered by the hosts natural gut microbiome, by helping to maintain tight junctions of protein to protect the intestinal barrier and exertion of antimicrobial activity in the gut to prevent infection (O'Flaherty and Klaenhammer, 2010; Mahmoudi *et al.*, 2013; Jomehzadeh *et al.*, 2020; Wang *et al.*, 2020). The antimicrobial activity produced by many probiotic strains is claimed to be primarily associated with organic acid and antimicrobial peptide (AMP) production (Hladíková *et al.*, 2012; O'Shea *et al.*, 2012; Guinane *et al.*, 2016). These can beneficially affect the host through the inhibition of potential pathogens in the body and maintain a healthy balance of microflora (O'Shea *et al.*, 2012). This production of antimicrobial activity aids in the prevention of infection, as well as in the prevention of enteric disease in individuals who may have compromised intestinal barriers that can lead to disorders such as Crohn's disease (O'Flaherty and Klaenhammer, 2010). *E. coli* Nissle 1917 is a strain of probiotic bacteria that has been shown in clinical trials to improve symptoms of irritable bowel diseases such as ulcerative colitis and of IBS. *E. coli* Nissle 1917 exerts beneficial effects through modulation of the immune system, production of antimicrobial peptides by the strain and subsequent antimicrobial activity against pathogenic bacteria thus preventing their invasion of intestinal epithelial cells (Altenhoefer *et al.*, 2004; Schultz, 2008; Kruis *et al.*, 2012; Parker *et al.*, 2018).

As indicated in this section, it is evident that commensal gut microbes modulate and contribute to many functions in the body, both in and outside the gut and play an important role within. Exposure to microbe colonization earlier in life appears to be the most significant time to increase health benefits and maximise contribution to future health.

1.2 Bifidobacteria

Bifidobacteria are a genus of bacteria within the phylum *Actinobacteria* and order of *Bifidobacteriaceae* (Lee and O'Sullivan, 2010) and they were originally isolated from the faeces of breast-fed babies in 1899 (Tissier, 1899). *Bifidobacteria* are Gram-positive, non-spore forming, non-motile, anaerobic bacteria with a G+C content of 55-67% and upon growth a bifid-shaped morphology can be observed, consisting of y or v-shaped branched rods (Lee and O'Sullivan, 2010; Dhanashree *et al.*, 2017). Upon the original isolation of *Bifidobacteria* from the faeces of breast-fed infants by Tissier (1899), their high presence was suggested as possibly resulting in a lower incidence of diarrhoea or gastroenteritis in infants (Tissier, 1900). Yamada *et al*., (2017) discusses the link between breast-fed neonates and higher incidences of *Bifidobacteria* species present in their gut microbiome, likely due to the presence of human milk oligosaccharides (Yamada *et al.*, 2017). Human milk oligosaccharides are complex carbohydrates present in breast milk, that are not used for energy by the infant but instead are utilised by and result in the enrichment of beneficial gut microbes such as *Bifidobacteria*, thus supporting the formation of a healthy gut microbiome (Yamada *et al.*, 2017; Thomson *et al.*, 2018). Cooke et al., (2005), also found a higher abundance of both *Bifidobacteria* and *Lactobacilli* in the guts of breast-fed neonates in comparison to formula-fed, with these bacteria linked to healthier digestive systems.

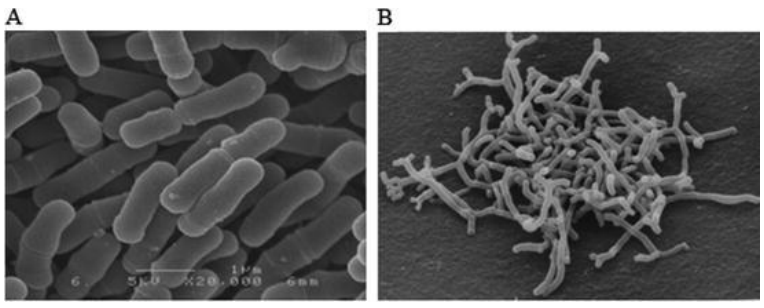

Figure 1.1: Morphology of A) *Bifidobacterium longum* subsp. *infantis* M-63 with uniform rod shape and B) *Bifidobacterium longum* BB536 with Y shaped rods (Lau *et al.*, 2015)

The understanding of the contribution of *Bifidobacteria* to a healthy human gut and their importance as probiotic organisms can be traced back to Tissier (1907). The author administered *Bifidobacteria* for the treatment of infantile diarrhoea in his research, in the first recorded instance of this application, and this strain is still widely used for probiotic treatment today. According to FAO/WHO, (2006) *Bifidobacteria* and *Lactobacilli* are primarily used as probiotic organisms, and are used in food as starter cultures due to their probiotic use and health benefits on the gastrointestinal system of the host (FAO/WHO, 2006). Probiotic bacteria must demonstrate resistance to the acidic conditions of the stomach and intestine to be capable of survival and proliferation while transiting through the gastrointestinal system (FAO/WHO, 2006), which are characteristics many Bifidobacterial strains possess. The strains *B. longum* INIA P132 and *B. infantis* P731 were assessed for their probiotic potential and applicability for food applications and it was found that both were resistant to acidic pH and bile salts, additionally *B. longum* replicated well in milk products and *B. infantis* displayed high adhesion to human intestinal lining Caco-2 cells (Llamas-Arriba *et al.*, 2019). Both strains produced heteropolysaccharides (HePS) (exopolysaccharide production has been attributed to the beneficial effect on the hosts health by probiotics (Llamas-Arriba *et al.*, 2019)), that appeared to modulate the immune system with a potential anti-inflammatory response in macrophages treated with the inflammatory lipopolysaccharide of *E. coli.* The HePS have also shown to offer protection against an *in vivo* enterocolitis model of zebrafish larvae, with a reduction in mortality when the larvae were treated with the HePS produced by both Bifidobacterial strains (Llamas-Arriba *et al.*, 2019). Another strain, *B.longum* subsp. *infantis* CECT 7210, has also displayed probiotic properties with resistance to gastric juices, bile salts and a low pH, as well as prevention of diarrhoea due to inhibition of rotavirus in both cell-cultures and in mice (Chenoll *et al.*, 2016), further indicating the beneficial properties and applicability of this genus in food and probiotic use.

Bifidobacteria are heterofermentative bacteria, producing a range of metabolites such as organic acids, upon growth, including lactic, acetic and formic acid (Makras and De Vuyst, 2006; Ruiz-Aceituno *et al.*, 2020). Makras & De Vuyst, (2006) analysed the organic acids present in a variety of *Bifidobacteria* strains and found that all strains produced varying concentrations of lactic and acetic acid, with as low as 20.9 mM acetic acid and 5.7 mM lactic acid and as high as 60.9 mM acetic acid and 39.9 mM lactic acid. Some strains also produced formic acid, however, this acid was not detected in the fermentations of *B. longum* or *B. bifidum* in this study, indicating that differing organic acids may be produced by different *Bifidobacteria* strains (Makras and De Vuyst, 2006). Ruiz-Aceituno *et al*., (2020) also detected the production of acetic, lactic and formic acid by *B. longum* NCIMB 8809 and *B. breve* UCC2003 and assessed the final concentrations of acids produced when using different carbon sources for growth including glucose, lactose, maltose and raffinose. The highest concentrations of lactic acid (29.7 mM for *B. breve* and 37.1 mM for *B.* longum) and acetic acid (66.1 mM for *B. breve* and 67.6mM for *B. longum*) were produced when using lactose as a carbon source, while the highest concentration of formic acid was 3.1 mM when using raffinose as the carbon source and was produced by *B. breve* UCC2003 alone (Ruiz-Aceituno *et al.*, 2020). The lowest concentrations of lactic acid were 1.6 mM for *B. breve* with raffinosyl-oligofructosides and 0.7 mM for *B. longum* using maltose as a carbon source, while the lowest concentrations of acetic acid were 5.6 mM with raffinosyl-oligofructosides and 2.1 mM for *B. longum* when using maltose as a growth substrate. *Bifidobacteria* also produce small concentrations of succinic acid with usually not more than a single figure millimolar concentration (Van Der Meulen *et al.*, 2006). Although it is not widely studied yet, it has been theorised that the succinic acid may be produced for NAD+ regeneration in cells, as small amounts of NAD+ coenzyme (an integral cofactor in cellular metabolic activities) are regenerated upon the conversion of phosphoenolpyruvate to succinic acid (Van Der Meulen *et*

al., 2006). Van Der Meulen *et al*. (2006) also suggested a reason for the production of such small concentrations of succinic acid may be due to the fact that the energy present in phosphoenolpyruvate dissipates and is lost from the cell when the molecule is converted to succinic acid.

Bifidobacteria do not fermentate carbohydrates using the typical metabolic glycolic pathway or the hexose monophosphate shunt pathway, as they lack the required enzymes (De Vries, Gerbrandy and Stouthamer, 1967). Instead, *Bifidobacteria* ferment carbohydrates using the "bifidum pathway" of fermentation (Figure 1.2), which uses the fructose-6-phosphate phosphoketolase enzyme (Bezkorovainy, 1989). This pathway results in acetate and lactate as the primary end products and allows *Bifidobacteria* to produce more ATP from carbohydrates than conventional pathways with 2.5 ATP produced from one mole of glucose, along with approximately 1.5 moles of acetate and 1 mole of lactate (Palframan *et al*, 2003). As varying amounts of acid are produced by different strains of *Bifidobacteria,* research has shown that the carbohydrate source used can affect the amount of acid produced, as discussed in the study by Ruiz-Aceituno *et al*., (2020). The specific rate of sugar consumption is also an important factor in the ratio of the end metabolites produced (Van Der Meulen *et al.*, 2006). A faster consumption of sugars results in higher concentrations of lactic acid, and less acetic acid, formic acid and ethanol, while slower consumption results in less lactic acid production and more acetic acid, formic acid and ethanol (Van Der Meulen *et al.*, 2006).

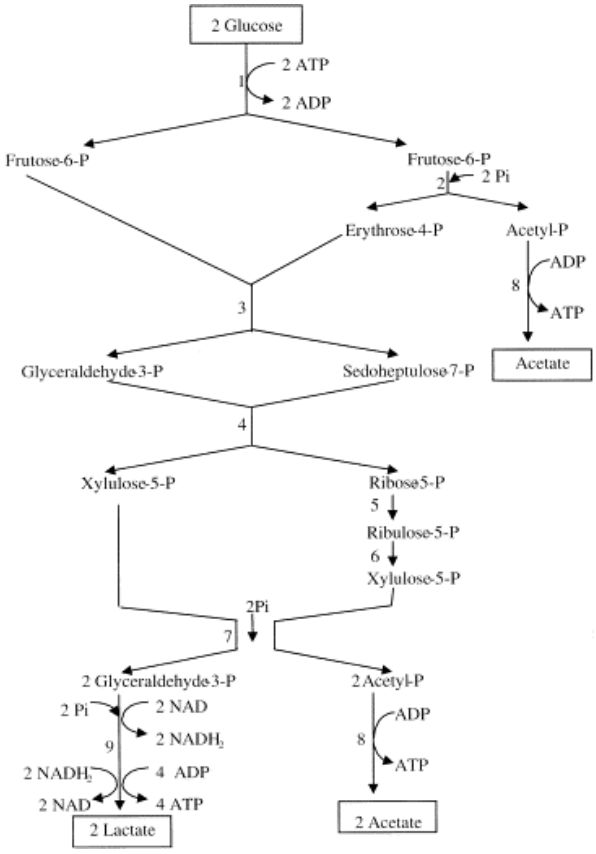

Figure 1.2: The bifidum pathway of *Bifidobacteria* resulting in the formation of lactate and acetate. The enzymes utilised in this process are numbered (1) hexokinase and fructose-6-phosphate isomerase, (2) fructose-6-phosphate phosphoketolase, (3) transaldolase, (4) transketolase, (5) ribose-5-phosphate isomerase, (6) ribulose-5-phosphate-3-epimerase, (7) xylulose-5-phosphoketolase, (8) acetate kinase, (9) enzymes as in the homofermentative pathway (Gomes and Malcata, 1999).

The organic acids produced by *Bifidobacteria* are associated with an antibacterial effect, but it is known that the antimicrobial characteristics of *Bifidobacteria* can also be associated with the production of other metabolites including antimicrobial peptides, also known as bacteriocins (Lee and O'Sullivan, 2010). The antimicrobial activity associated with the production of these organic acid and bacteriocin metabolites is an important characteristic for probiotic bacteria, as it is claimed to contribute to the maintenance of a healthy balance of gut microflora through the inhibition of colonization by pathogenic organisms (Hladíková *et al.*, 2012; O'Shea *et al.*, 2012; Guinane *et al.*, 2016).

1.3 Bacteriocins

Antimicrobial peptides (peptide chains displaying an antimicrobial effect) are produced by both prokaryotic and eukaryotic organisms (Phoenix *et al.*, 2013). They play vital roles in defence and can be found across many species (Chikindas *et al.*, 2018), with many AMP's also produced by leukocytes, now referred to as defensins of the mammalian immune system (Phoenix *et al.*, 2013). Many bacteria produce metabolites and antimicrobial peptides, also known as bacteriocins, during growth (Zacharof and Lovitt, 2012), to aid in survival and to enable them to compete with other bacterial cells for nutrients (Perumal and Venkatesan, 2017). They are ribosomally synthesised molecules (da Silva Sabo *et al.*, 2014) and display antimicrobial activity against various bacteria and in some cases fungal species (Ahn *et al.*, 2017; Aarti *et al.*, 2018; Ruggirello *et al.*, 2018).

Although bacteriocins were first discovered in 1925 (Cavera *et al.*, 2015), they are now becoming an increasingly important area of research, as an alternative to antibiotics due to antimicrobial resistance by the latter (Ventola, 2015; Biswas *et al.*, 2017; Morehead and Scarbrough, 2018) and as potential biopreservatives in the food industry (Gálvez *et al.*, 2007; Skariyachan and Govindarajan, 2019). The development of antibiotic resistance in modern times has become a major issue, with bacteria such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant Enterococci (VRE) two prominent pathogens, and some bacteriocins have reportedly shown activity against these strains (Shokri *et al.*, 2014; Ahmad *et al.*, 2017) Other applications for bacteriocins which are being actively researched, include potential use in the food industry in food preservation where they act by inhibiting foodborne pathogens and degradative microbes that result in food spoilage (Chikindas *et al.*, 2018). The bacteriocin nisin produced by *Lactococcus lactis* has been in use for decades as a preservative in several food applications in some countries (Gyawali and Ibrahim, 2014).

Bacteriocins are generally small molecules, with a molecular weight range of between 2 kDa and 300 kDa (Ahmad *et al.*, 2017). They act in both an 'offensive / defensive' role as they enable the producing bacteria to inhibit other strains in the environment to allow for an advantage in the competition for nutrients required for growth and sustainability (Singh *et al.*, 2015). According to Zacharof and Lovitt (2012), bacteriocins are synthesised during the primary phase of bacterial growth, and they undergo post translational modifications, which can involve cleavage or the addition of other molecules such as carbohydrate moieties to the bacteriocin. An example of a bacteriocin structure can be seen in Figure 1.3, with the primary structure of nisin presented.

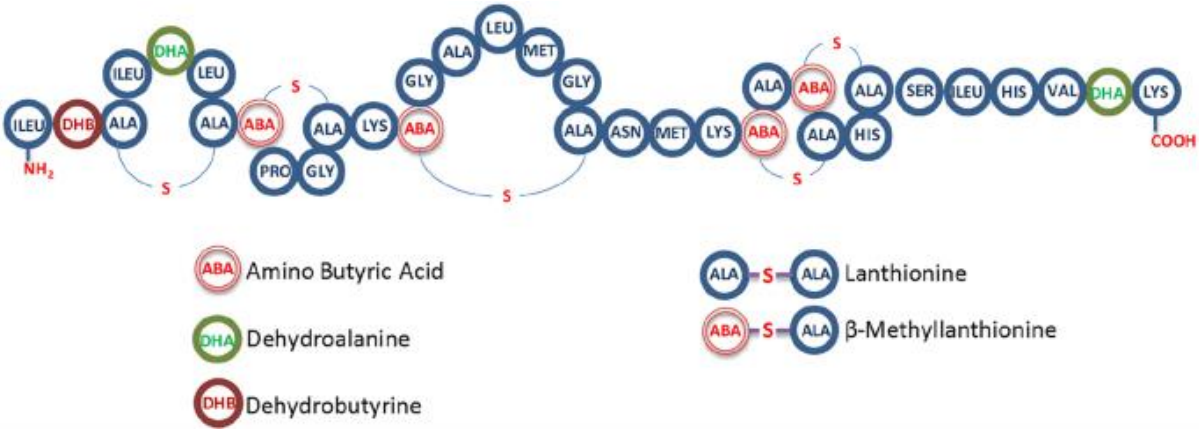

Figure 1.3: Antimicrobial Peptide Nisin (Khelissa *et al.*, 2020). This is the primary structure of the AMP nisin produced by *Lactococcus lactis*, where S denotes Sulphur found in Lanthionine and Methyllanthionine

Many bacteriocins are positively charged amphiphilic molecules (due to the presence of large amounts of the amino acids lysine and arginine in their structure) (Rodríguez *et al.*, 2003; Singh *et al.*, 2015), and they can interact with pathogen cells due to electrostatic interaction between the positively charged portion of the bacteriocin and negatively charged cell membrane. The hydrophobic portion of the polypeptide chain, which is more than 30% of the residues, enables the bacteriocin to enter within the hydrophobic environment phospholipid bilayer of the cell membrane (Singh *et al.*, 2015).

Gut microflora play an important role in the breakdown of food and nutrients in the body and thus maintaining a healthy balance of gut flora is important for a healthy digestive ssystem (O'Shea *et al.*, 2012). Many lactic acid producing bacteria in the gastro-intestinal tract have gained a reputation for notable production of bacteriocins, with many of these bacteria considered as GRAS, or generally regarded as safe, thus opening the avenue for their addition to foodstuffs (Zacharof and Lovitt, 2012). As bacteriocins are proteolytic in nature they can be degraded by the enzymes within the digestive tract and thus are considered as non-toxic with an additional benefit of reducing the development of resistance to the bacteriocin's (Zacharof and Lovitt, 2012).

1.3.1 Classification of Bacteriocins

Bacteriocins produced by lactic acid bacteria are some of the most intensely studied and have been classified into various groups on the basis of their structure and modes of action. According to Zacharof and Lovitt (2012), various classifications of bacteriocins have been proposed over the years with consistent revision of the classes by many academics. The most widely known classifications are the 4 classes of bacteriocins proposed by Klaenhammer (1993). A proposition was put forward by Cotter *et al.*, (2005) that they be divided into only the first two categories stated by Klaenhammer, with a third category separately named bacteriolysins and the fourth class disregarded. The first two classes of bacteriocins are generally mentioned academically, however the full four classifications of bacteriocins will be explored in this review.

1.3.1.1 *Class I Bacteriocins – Lantibiotics*

Class I bacteriocins are composed of small peptides of less than 5kDa that are heat stable due to the presence of rare amino acids (Deegan *et al.*, 2006). These amino acids undergo extensive levels of post translational modifications resulting in many lanthionine and methyllanthionine residues, giving rise to the name lantibiotics (Deegan *et al.*, 2006). An example of a bacteriocin in this group is Nisin which is produced by *Lactococcus lactis spp.* (Matsusaki *et al.*, 1998; Balciunas *et al.*, 2013).

The lanthionine (Figure 1.4a) and methyllanthionine (Figure 1.4b*)* amino acids are not genetically coded and are formed by post translational modifications and these residues can interact forming ring structures within the peptide (Woraprayote *et al.*, 2016). The residues are formed by the enzymatic degradation of serine and threonine, yielding dehydroalanine and dehydrobutyrine (Deegan *et al.*, 2006). Thiol groups from cysteine residues then interact with the double bond of either molecule, with dehydroalanine forming lanthionine and dehydrobutyrine forming methyllanthionine (Deegan *et al.*, 2006).

This class has also been divided into two subgroups based on their structure. *Group 1a* are elongated and have a positive charge, with a size between 2 and 4 kDa (Zacharof and Lovitt, 2012), and they act on target cells by pore formation of the cell membrane, causing cellular contents to leak out (Ahmad *et al.*, 2017). *Group 1b* are rigid and globular in shape with either a negative charge or no net charge, and their mode of action is by the inhibition of essential enzymes of the bacterial cell (Deegan *et al.*, 2006).

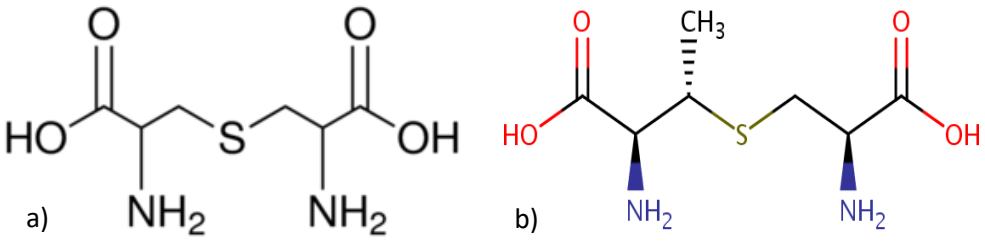

Figure 1.4: Structures of a) Lanthionine (*Lanthionine | Sigma-Aldrich*, no date) and b) Methyllanthionine (*CompTox Chemicals Dashboard*, no date)

1.3.1.2 *Class II Bacteriocins*

Class II bacteriocins are also small at less than 10 kDa and heat stable, however they do not contain lanthionine (Singh *et al.*, 2015). They exert antimicrobial activity by inserting themselves into the cell membrane of the target, causing depolarization and thus cell death (Balciunas *et al.*, 2013; Singh *et al.*, 2015). This class of bacteriocins is commonly further divided into three subclasses.

Subclass IIa are referred to as pediocin-like bacteriocins and exert their activity against *Listeria* spp. (Balciunas *et al.*, 2013). In their structure the N-terminus, or the start of the peptide chain with the amine group, contains a beta pleated sheet while the C-terminus, with the exposed carboxyl group, contains alpha helices (Fimland *et al.*, 2005).

Subclass IIb peptides need two individual peptide chains to work in synchronisation to exert maximum antimicrobial activity, but each peptide exerts low effects individually (Woraprayote *et al.*, 2016). Examples of bacteriocins within this group which show a synergistic relationship include the peptides lactococcin G and lacticin F, which act together as a bacteriocin (Woraprayote *et al.*, 2016).

Subclass IIc peptides contain covalent bonds between the N and the C terminals, thus resulting in a unique cyclical or ring structure (da Silva Sabo *et al.*, 2014; Woraprayote *et al.*, 2016).

1.3.1.3 *Class III Bacteriocins*

This class of bacteria consist of larger peptides >30 kDa, that are thermolabile, or affected significantly by heat (da Silva Sabo *et al.*, 2014) and their mechanism of antimicrobial action is through lysis of the cell wall (Balciunas *et al.*, 2013).

1.3.1.4 *Class IV Bacteriocins*

This smaller group contains bacteriocins that are modified to contain lipid or carbohydrate moieties (da Silva Sabo *et al.*, 2014; Ahmad *et al.*, 2017). This section is often disregarded and is not mentioned frequently in literature.

1.3.2 Biosynthesis of Bacteriocins

The production of bacteriocins by bacterial cells involves many different genes and mechanisms due to the many proteins involved and the extensive post translational modification process (McAuliffe *et al.*, 2001). At least four genes of known function code for; a) the production of the bacteriocin precursor, b) the production of an immunity protein for self-protection of the cell, c) the production of an ABC transporter and d) the production of an accessory protein of which the function is not known (da Silva Sabo *et al.*, 2014). The genes for bacteriocin production are usually located either on the chromosome on transposable elements (Deegan *et al.*, 2006). Transposable elements are DNA sequences that can move their location within the genome or on extrachromosomal plasmids (Deegan *et al.*, 2006).

Bacteriocins are ribosomally synthesised as biologically inactive precursors called pre-peptides, that have a leader peptide at the N terminal that renders the pre-peptide inactive (Deegan *et al.*, 2006). This is a short extension of amino acids between 18 to 30 amino acids in length (McAuliffe *et al.*, 2001; da Silva Sabo *et al.*, 2014). It has two functions; to prevent the bacteriocin becoming activated while still inside the cell as it is only active once this is cleaved upon exportation and also to function as a signal protein for the transport system across the membrane (da Silva Sabo *et al.*, 2014). The leader peptide is removed either just before, after or during exportation of the pre-peptide from the cell, forming the active bacteriocin peptide (Deegan *et al.*, 2006). According to McAuliffe *et al* (2001), for group 1a lantibiotics serine proteases are responsible for cleaving the leader sequence, whereas for group 1b lantibiotics the transporter protein cleaves the peptide simultaneously with export.

The transporter system, called the ATP-binding cassette transporter protein (ABC transporter), actively transports the bacteriocin across the membrane into the extracellular medium (McAuliffe *et al.*, 2001). The ABC transporter is composed of two sections; one which recognises the pre-peptide sequence and the other that hydrolyses the ATP molecule to generate energy for export of the peptide (Deegan *et al.*, 2006). An example of an ATP-binding cassette and its mechanism of action can be seen below in Figure 1.5, where the substrate indicated in the figure represents the bacteriocin peptide.

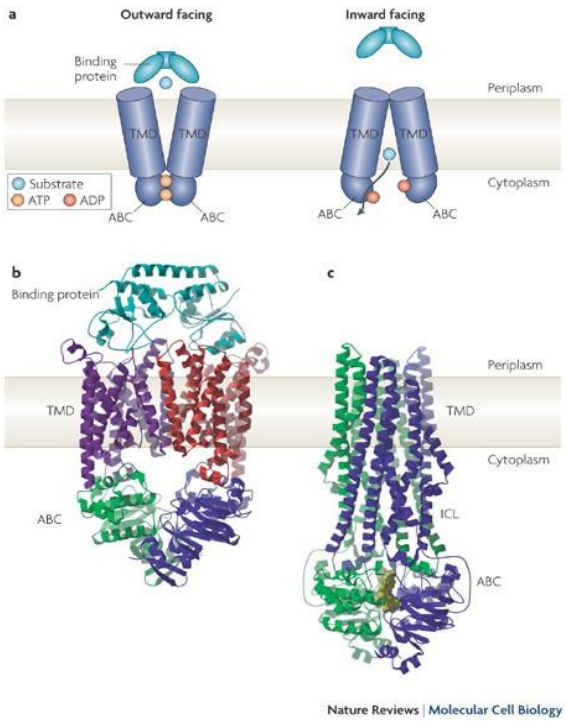

Figure 1.5 – An ATP binding cassette (ABC) Transporter (Rees *et al.*, 2009). The ABC transporter used for the exportation of bacteriocins into the extracellular medium is presented above. **a**) depicts the structure of the ABC transporter composed of two transmembrane domains (TMD) and two ATP-binding cassette domains (ABC). The substrate depicted would be representative of a bacteriocin. **b**) represents the *E. coli* vitamin B12 transporter system while **c**) represents the multidrug exporter of *S. aureus* Sav1866

There are various enzymes involved in the bacteriocin post translational modification process during their synthesis and activation and the process is reviewed in detail by McAuliffe *et al* (2001) with respect to Lantibiotics which undergo extensive levels of modifications. In terms of the lantibiotic group, LanB, LanC and LanM enzymes are responsible for the post-translational modifications, and it has been suggested that these enzymes are responsible for the reactions resulting in the production of lanthionine and methyllanthionine amino acids (McAuliffe *et al.*, 2001). Additionally, it has been suggested that LanB catalyses the dehydration of both serine and threonine, and that LanC follows this dehydration process by forming thioether molecules (McAuliffe *et al.*, 2001). The above enzymatic reactions have been discussed in further detail with respect to the formation of lanthionine and methyllanthionine residues in Section 1.3.1.

1.4 Mechanisms of Action of Inhibitory Metabolites

1.4.1 Bacteriocins

Bacteriocins have various modes of action for how they exert their antimicrobial effects on cells (Cavera *et al.*, 2015). To locate the site of action on the susceptible cell, the bacteriocin molecule recognises receptors on the surface of the cell membrane which enable the bacteriocin to interact with the membrane and exert its action (Macwana and Muriana, 2012). The cell surface molecules recognised for interacting with some bacteriocins include the mannose phosphotransferase system or lipid II molecules (Ahmad *et al.*, 2017). The mechanisms through which bacteriocins exert their antimicrobial effect vary between the different classes (Section 1.3.1), with some exerting their effect through interference with the cell membrane or through the inhibition of protein synthesis (Balciunas *et al.*, 2013). The general consensus for the mode of action of bacteriocins however, is that they primarily affect cell wall integrity by binding to precursors, or cause pores or lyse the membrane of the cell (Ahmad *et al.*, 2017).

According to Cavera *et al* (2015); several bacteriocins target the same cellular processes and systems for production of essential compounds that many antibiotics target. One of the main targets is synthesis of the cell wall, which is critical to survival of bacteria by maintaining integrity and morphology of the cell (Cavera *et al.*, 2015). The primary mode of inhibition of cell wall synthesis is through binding of the bacteriocin to the lipid II molecule, which is a membrane bound precursor of the cell wall, thus blocking its activity and inhibiting synthesis which is observed for bacteriocins such as Nisin A and Nukacin ISK-1 (Cavera *et al.*, 2015). Nisin is unusual however, in the sense that the inhibition of cell wall synthesis is not its only mode of action. Upon binding to the lipid II complex and interfering with synthesis, it also causes pore formation in the cell membrane, allowing essential molecules to leak out (Woraprayote *et al.*, 2016). The two modes of action for nisin are presented in Figure 1.6.

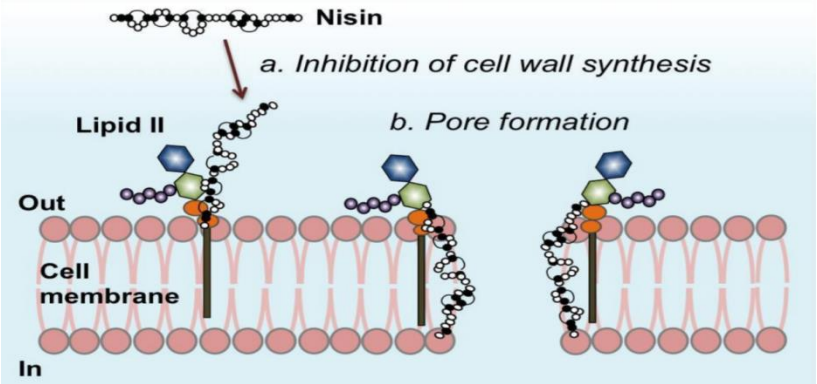

Figure 1.6 – Inhibition of cell wall synthesis and pore formation by nisin (Ibrahim, 2019). The two modes of action of the bacteriocin nisin against Gram positive bacteria are presented in the above Figure 1.6. a. indicates the binding of the nisin peptide to the lipid II molecule of the bacterial cell wall resulting in inhibition of cell wall synthesis while b. demonstrates the ability of nisin to bind to the lipid II molecule resulting in pore formation of the membrane and leakage of cellular contents.

Some bacteriocins, such as microcin B17 produced by *E. coli* and bacteriocins produced by other colicins, can interfere with the DNA structure of target cells by competitive inhibition of DNA gyrase that aids in unwinding, thus preventing two newly synthesized DNA molecules from separating (Cavera *et al.*, 2015).

Colicins have displayed several different mechanisms of activity against target cells, including colicin M which inhibits peptidoglycan synthesis by enzymatic degradation of a bond between lipid II and the lipid moiety (O'Shea *et al.*, 2012). Other colicins have been shown to cause pore formation (O'Shea *et al.*, 2012), along with many other bacteriocins such as those in class IIa, which consequently affects the proton motive force causing overconsumption of ATP and cell death (Balciunas *et al.*, 2013). Pores can be formed by the peptides attaching to the membrane at a perpendicular orientation and folding (Sengupta *et al.*, 2008). Many bacteriocins that exert their activity by the process of pore formation in the membrane, bind to lipid II or the mannose phosphotransferase system as mentioned previously in Section 1.4.1, however in the case of Lacticin Q, it can form toroidal pores by lipid flip flop – the transfer of lipids from

one membrane to the other (Cavera *et al.*, 2015). An increased peptide to lipid ratio at the membrane can cause the formation of these pores as this causes the lipid molecules on the membrane to bend inwards forming a toroidal pore (Figure 1.7), in which the peptides can then line the interior of the formed pore (Sengupta *et al.*, 2008).

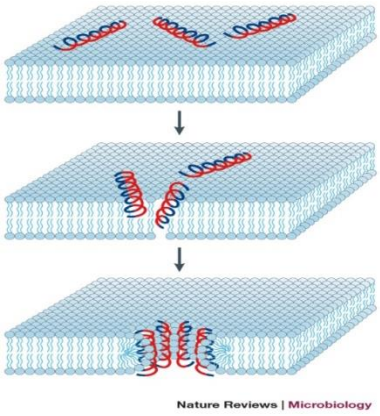

Figure 1.7 – Formation of toroidal pore (Brogden, 2005). In this figure the antimicrobial peptides aggregate causing the lipid monolayers on the membrane to bend inwards through the pore resulting in the lipid heads and peptides lining the core. The red region represents the hydrophilic portion of the peptide while the blue region represents the hydrophobic portion of the peptide.

Colicin A displays a mechanism of inhibition of protein synthesis (Ahmad *et al.*, 2017). Proteins are integral to many functions of a cell and enzymes catalyse virtually all chemical reactions in all living organisms, including bacteria (Robinson, 2015). Thus, interference with them can be detrimental to the cell. Bacteriocins have been shown to cleave the 16s portion of ribosomal RNA, inhibiting translation of a protein sequence, and some accelerate the depletion of transfer RNA in the cytoplasm, meaning protein synthesis is thus limited (Cavera *et al.*, 2015).

1.4.2 Organic Acids

Organic acids have been shown to display an antimicrobial effect that is generally attributed to causing membrane interference (Dalié *et al*., 2010) due to their ability to cross the cell membrane as a result of the lipophilic nature of the undissociated form of the acid (Dibner and Buttin, 2002; Gómez-García *et al.*, 2019). According to Dalié *et al*., (2010), the acids diffuse through the membrane of microbes in their undissociated form, thus reducing the pH of the cytoplasm with consequent inhibition of metabolic processes. One of the probable modes of action of organic acids on the membrane is neutralization of its electrochemical potential, thus increasing its permeability (Dalié *et al.*, 2010) and affecting membrane porosity resulting in interference with substrate transportation through the cell membrane of bacterial cells (Davidson, Taylor and Schmidt, 2013). The overriding effect is probably a general collapse of the proton gradients required for ATP synthesis due to the binding of free anions with the periplasmic protons pumped out by the electron transport chain, thus carrying them back across the membrane (Halstead *et al.*, 2015). However, as the pH of the inside of the cell is higher than the acid solution outside, the internalised acid will dissociate, thus causing an internal pH reduction in the cell and an increase in protons as a result of the undissociated acid ionization, and these protons must then be removed from the cell to prevent conformational changes and damage of internal structures such as proteins and DNA (Davidson *et al.*, 2013; Halstead *et al.*, 2015). Additionally, the protons cannot passively diffuse through the membrane and require high ATP usage for exportation at the expense of the cell, resulting in collapse of the cell metabolism with the consequent eventuality of cell death (Davidson, Taylor and Schmidt, 2013). Ajingi *et al.*, (2020) also mentions how organic acids can impede the oxidation of the cofactor nicotinamide adenine dinucleotide (NADH) that transports electrons to the mitochondria, preventing the electron transport chain receiving reducing agents and thus

resulting in interference with the production of metabolic energy within the cell required for cell growth.

The above suggested effects on the membrane of bacteria associated with organic acids has been observed for lactic acid activity against Gram-negative bacteria (Alakomi *et al.*, 2000). Alakomi *et al.*, (2000) demonstrated using a fluorescent-probe uptake assay how lactic acid at concentrations as low as 5 mM and pH 4, acts as a permeabilizer to the outer membrane of the tested Gram-negative strains *E. coli* O157:H7, *Pseudomonas aeruginosa* and *Salmonella enterica* serovar *typhimurium*, resulting in release of LPS and increased uptake of the fluorescent probe. The outer membrane disruption caused by lactic acid was also stronger than the effect observed by hydrochloric acid, and according to the authors the effect is likely due to the undissociated acid molecules (Alakomi *et al.*, 2000).

1.5 Applications of Probiotic Bacteria and Bacteriocins

1.5.1 Probiotics and Antibiotic Use

It is well documented in the literature that the use of antibiotics on the body can disrupt the natural balance of microbiota within the gastrointestinal tract (Burdet *et al.*, 2019; Sun *et al.*, 2019; Zhang, *et al*., 2019). Probiotic use has been shown to regulate and restore the intestinal microbiota after antibiotic use in research carried out by Engelbrektson *et al*., (2009) comparing the microbiota of individuals administered an antibiotic but no probiotic, to a group taking an antibiotic and probiotic supplement containing *Lactobacilli* and *Bifidobacteria*. Through culturing and DNA-based terminal restriction fragment length polymorphism to identify the microbial composition after treatment, in comparison to each subjects' baseline composition, a faster return to normal pre-antibiotic baseline microbiota upon consumption of probiotics was indicated.

The use of antibiotics to treat infections can result in incidence of antibiotic associated diarrhoea (AAD). This is caused by the disruption of the intestinal flora as a result of antibiotic use, or direct irritation to the GI tract, that cannot be associated with any other cause of diarrhoea (Bartlett, 2002; Giannelli, 2017). *Clostridioides difficile* is one of the most prominent and important microbes linked to causing AAD, as well as causing nearly all cases of antibiotic associated pseudomembranous colitis, which is an inflammation of the large intestine, and is the leading cause of nosocomial diarrhoeal infections in adults (Barbut and Petit, 2001; Zhou *et al.*, 2014). AAD generally results from overgrowth of pathogenic organisms within the intestinal mucosa as a result of the disruption of the healthy microflora, or it can also be effected by a response to a reduction of the healthy intestinal flora, and as a result, can cause a decrease in carbohydrate metabolism with a consequent accumulation of these solutes resulting in osmotic diarrhoea due to solute concentration causing malabsorption of water (Varughese *et al., 2013; Zhou et al., 2014)*.

Probiotic application can restore the natural balance and help to reduce the incidence of AAD (Selinger *et al.*, 2013; Hayes and Vargas, 2016; Litao *et al.*, 2018). In one collection of 23 studies observing the effects of the treatment of AAD with probiotic bacteria, patients were administered the bacteria *Bacillus* spp., *Bifidobacterium* spp., *Lactobacilli* spp., *Lactococcus* spp., *Saccharomyces* spp., *Streptococcus* spp., *Leuconostoc cremoris* or *Clostiridium butyricum*, either alone or as a combination of strains. A total of 22 of the 23 studies demonstrated improvement upon probiotic treatment based on the incidence of diarrhoea, with the incidence of AAD for those administered probiotics at 8%, as compared to occurrence of 19% in the control group who were not administered probiotics (Hayes and Vargas, 2016). Selinger *et al.*, (2013) assessed the effectiveness of the commercial probiotic treatment VSL#3, a mixture of various strains of *Lactobacilli, Bifidobacteria* and *Streptococcus* (Chapman *et al.*, 2006), on AAD incidence in hospital patients exposed to antibiotic treatment. This study found that the group administered the probiotic treatment had a significantly lower incidence of AAD, with 0% in comparison to an AAD incidence of 11.4% in the placebo treated group, with a significant p-value of 0.006 (Selinger *et al.*, 2013).

A study carried out by (Mego *et al.*, 2015) investigated the incidence of diarrhoea in patients undergoing irinotecan chemotherapy for colorectal cancer, whereby the primary metabolite of the drug (SN-28), was mainly responsible for the development of diarrhoea. The administration of 10 probiotics including *Bifidobacterium bifidum* HA-132, *Lactobacillus casei* HA-108 and *Streptococcus thermopilus* HA-110, aided in the reduction of diarrhoea induced by irinotecan chemotherapy. There was a 0% incidence of severe diarrhoea in the probiotic treated group in comparison to 17.4% in the control group with placebo treatment and in terms of overall diarrhoea occurrence, there was a 39.1% incidence within the probiotic treated group as compared to 60.9% incidence within the control group, with the results obtained indicating a notable reduction when using a probiotic formulation treatment.

An alternate way of administration of probiotic organisms and gut bacteria is through faecal microbiota transplants, which has been shown to improve symptoms of gut disease, aid in the regulation of a healthy balance of gut microbiota and can be used for hard-to-treat *C. difficile* infections (Mattner *et al.*, 2016). A study by Van Nood *et al.*, (2013) used donor faeces in the treatment of patients with recurrent *C. difficile* infections in comparison to treatment with the standard vancomycin treatment. Of the patients that received an infusion of donor faeces, 13 of 16 (81% of the group) had complete resolution of *C. difficile*-associated diarrhoea after the first infusion, and 2 of the remaining 3 had resolution after the second, in comparison to the vancomycin-treated group which only had resolution in 4 of 13 treated patients (31%). The preferred route of this therapy is generally through lower endoscopy (Mattner *et al.*, 2016), as it has provided the most favourable and effective results in more ill patients in comparison to nasogastric administration, however, nasogastric delivery has displayed similar cure-rates in more healthy patients (Gundacker *et al.*, 2017).

1.5.2 Probiotics and the Food Industry

Aside from the oral consumption of probiotic supplements, many probiotic organisms are added to food products or occur naturally in these products, such as in fermented milk products that have been consumed for their health benefits throughout history (Butel, 2014). In modern day, probiotic dairy beverages produced by the food industry, such as Yakult which contains *L. casei* and Actimel which contain *S. thermophilus, L. bulgaricus* and primarily *L. paracasei* (*Actimel,* 2019; *Yakult GB*, n.d.) are commonly consumed as a source of these beneficial microbiota. Using food products as a delivery method for probiotics has displayed benefits such as a potential synergistic effect between the nutrient rich food components and the probiotic bacterial strains has been observed and explored, potentially increasing the bacterial viability and their associated antimicrobial activity (Sireswar *et al.*, 2017). In the study by

Sireswar *et al.,* (2017), they investigated the relationship between phenolic compounds and intestinal microbiota and then assessed the effectiveness of using fortified fruit juice matrices for probiotic delivery over dairy products, and their effect on viability and activity of the selected probiotic bacteria. A juice matrix of either apple or sea buckthorn juice was fortified with either whey or malt extract and contained *Lactobacilli* spp. probiotic strains and antipathogenic activity (antimicrobial activity toward pathogenic strains) was then assessed. It was observed that the matrix and supplement influenced the antipathogenic activity and sea buckthorn juice fortified with malt was a superior matrix for the probiotic strains as it showed increased antibacterial properties against the pathogens *E. coli, Shigella dysenteriae* and S*almonella enteritidis* (Sireswar *et al.,* 2017). Another study by Dias *et al.,* (2018) also investigated the use of passionfruit juice with added *B. animalis* ssp. *lactis* BB-12 which was microencapsulated by a spray drying process with either maltodextrose or inulin as a non-dairy probiotic beverage. The results indicated 4^{o}C storage was better for viability, the use of inulin was better for *Bifidobacterium* survival and also produced more desirable physical characteristics of the juice including lower moisture and that the microencapsulation was better for stability of the juice powders (Dias *et al.*, 2018). These studies indicate the potential suitability for the incorporation of probiotic bacteria into beverages other than those that are dairy based, which may appeal to a wider range of consumers seeking food-associated health benefits.

Probiotic strains can also be employed as biocontrol agents in the food industry to prevent contamination during food production as an alternative to frequently used methods of antibiotic and disinfectant treatment, that can pose the risk of resistance or is of a high cost (Hossain, *et al.,* 2017). As many food products originate from livestock and marine life, meaning the prevention of transmission of common foodborne pathogens such as *Salmonella* spp., *E. coli, Pseudomonas* spp., *Micrococcus* and *Listeria* to humans is important, probiotics offer a natural

alternative of contamination control and can help maintain beneficial microbes within the GI system of animals consequently preventing colonization by harmful bacteria (Mountzouris *et al.*, 2009; Shim *et al.*, 2012; Hossain *et al.*, 2017). Probiotics can also be added to animal feed for supplementation, such as *B. cereus var. toyoi* which has been added to pig feed (Lodemann *et al.*, 2008) and a *Bifidobacterium lactis*-based probiotic and galacto-oligosaccharide prebiotic fed to broiler chickens, which stimulated favourable growth of *Bifidobacteria* within the intestines of the chickens (Jung *et al.*, 2008). Probiotics can also be used as an alternative to disinfection of surfaces within food-processing plants, with *Lactococcus lactis* subsp. *lactis* C-1-92 and *Enterococcus durans* 152 shown to eliminate *Listeria* from 5 of the 6 floor drains of a poultry-processing factory after 4 treatments in a week and that remained free of *Listeria* for 13 weeks after this treatment (Zhao *et al.*, 2013). Pérez Ibarreche *et al*, (2014) also investigated the ability of the probiotic strain of *Lactobacillus* spp. to form biofilms on surfaces such as stainless steel and polytetrafluoroethylene (PTFE) used in meat-processing plants, with the strong biofilm forming strain *L. sakei* CRL1862 showing potential as a biocontrol agent to prevent *Listeria* biofilm formation on these surfaces.

Aside from probiotic bacteria supplementation through capsules, these bacteria, namely *Bifidobacteria* and *Lactobacilli,* are also used as starter cultures or additives in biofunctional foods such as dairy products (Linares *et al.*, 2017; Rodríguez de Olmos *et al.*, 2017). Biofunctional foods are ordinary foodstuffs that have added components to provide health benefits (Figure 1.8), such as probiotic bacteria and their bioactive metabolites including organic acids and bacteriocins, as consumers are increasingly looking for healthy food alternatives (Linares *et al.*, 2017; Rodríguez de Olmos *et al.*, 2017). Many fermented milk products such as yoghurt, cheese and kefir are some of the main sources of probiotic delivery, and non-fermented food supplemented with probiotics include low-fat ice-cream, mousse and infant formula (Rotar *et al.*, 2007; Linares *et al.*, 2017). Lactic acid bacteria have been used for

over 4000 years in the production of fermented foods, which involves the microbial process of converting lactose to lactic acid, altering the structure and texture to produce new fermented food products (Rotar *et al.*, 2007). Probiotics are becoming more relevant and of interest as food additives due to an increasing demand for natural healthier food products by consumers (Rodríguez de Olmos *et al.*, 2017). *Bifidobacterial* species including *B. bifidum, B. infantis* and *B. longum* were incorporated into the production of Crescenza cheese to introduce probiotic *Bifidobacteria* into food products (Gobbetti *et al.*, 1998). Results indicated that the addition of *Bifidobacteria* did not inhibit or interfere with the other microflora, including the strain *S. thermophilus* that is used for the production of the cheese and sensory evaluation yielded no significant difference in comparison to the conventional cheese (Gobbetti *et al.*, 1998). Cheddar cheese was also found suitable for the incorporation of probiotic *Bifidobacteria* to generate a probiotic cheese with high *Bifidobacteria* cell counts and results indicated that the *B. longum* BB536 added to the cheese did not adversely affect the composition of the cheese and the addition of *B. lactis* Bb-12 resulted in improved flavour (Mc Brearty *et al.*, 2001). Soybean paste was also selected as another healthy food substrate for the incorporation of probiotic *Lactobacilli* and *Bifidobacteria* as a carrier for these healthy bacteria and for the production of a healthy vegetarian food with increased nutritional value (Rodríguez de Olmos *et* al., 2017).

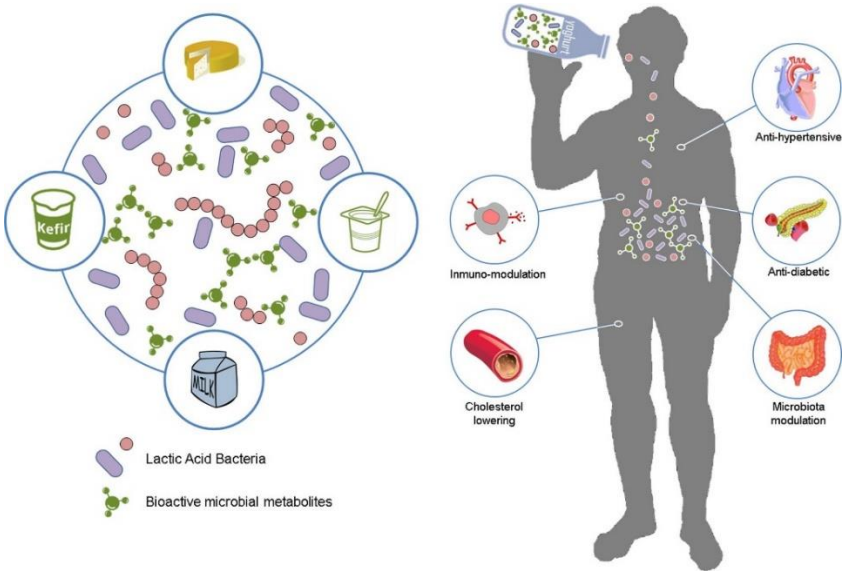

Figure 1.8: Health benefits associated with the use of probiotic bacteria in the food industry (Linares *et al.*, 2017). The beneficial effects associated with the consumption of biofunctional fermented food is associated with the production of bioactive metabolites produced by LAB. The health effects include anti-cholesterolemic, immunomodulation and microbiome modulation among others.

1.5.3 Bacteriocins and Probiotics in Food Preservation and Safety

Chemical additives have been added to processed foods at an increasing rate in recent years (da Silva Sabo *et al,* 2014), however increased awareness of the extent of additive addition and the perceived negative impact of chemicals and processed foods on health by consumers has resulted in a demand for minimally processed foods and more natural preservatives (O'Connor *et al.*, 2015). Widely used chemical preservatives include sorbic and benzoic acids, however in some cases these may have an adverse effect on the health of consumers, such as allergic reactions to benzoates resulting in uticaria and asthma (Tfouni and Toledo, 2002), and the potential formation of carcinogenic products from chemicals used in preservation, such as nitrosamines from nitrites (Johnson *et al.*, 2018). Biopreservatives, food preservatives derived from organisms and their metabolites, are becoming an increasingly popular area of research, including research into the use of antimicrobial and antifungal peptides produced by bacteria

for potential in biopreservation (Singh, 2018; Skariyachan and Govindarajan, 2019; Thery *et al.*, 2020). Lactic acid bacteria are commonly employed for biopreservation due to their generally regarded as safe (GRAS) status and their use as probiotics (Perez *et al.*, 2014; Singh, 2018). Probiotic LAB have played a role in traditional food preservation through use in fermented foods, by bacteriocin production to inhibit spoilage bacteria, as well as production of lactic acid and hydrogen peroxide to ferment the food (O'Connor *et al.*, 2015). Spoilage of food products by fungi and some bacteria can greatly reduce the shelf-life of products without the use of preservatives (Garcia *et al.*, 2019), with biopreservatives offering an appealing alternative to the use of chemical preservatives that are currently the more commonly used preservative within the food industry (Kıvanc *et al.*, 2014).

Various probiotic bacteria have been used in food for a biopreservation application, including the class of probiotic bacteria known as dairy propionibacteria, where this class of bacteria are used as starter cultures and additionally for their production of beneficial metabolites including organic acids (Rabah *et al.*, 2017). Propionibacteria have been shown to display antifungal activity against foodborne moulds and yeast as a result of organic acid production, which includes the production of propionic, acetic and lactic acids (Lind *et al.*, 2005). The most potency has been detected for propionic acid, with a concentration as low as 20 mM propionic acid at pH 5 required to inhibit fungal strains (Lind *et al.*, 2005). Other probiotic lactic acid bacteria have also been shown to offer protection against pathogens in food. One study demonstrated how a commercial preparation of lactic acid bacteria, LactiGuard^{TM}, reduced *E. coli* O157:H7 and *Salmonella* on spinach leaves during refrigerated storage (Cálix-Lara *et al.*, 2014). In this study both a bacteriocin-like inhibitory substance and lactic acid detected in the cell-free fermentates of the LAB strains were found to be associated with antimicrobial activity (Cálix-Lara *et al.*, 2014). The latter results indicated the potential and efficacy of the probiotic LAB in LactiGuard^{TM} for preservation of microbial safety in food. Various potentially

probiotic *Lactobacillus* and *Bifidobacterium* species have also displayed antimicrobial activity against food-related pathogens and spoilage organisms including the yeast *C. albicans* and bacterial strains *S. aureus, B. cereus* and *E. coli* in a study by Georgieva *et al.,* (2015). Antimicrobial activity was namely attributed to organic acid production and low pH, and upon neutralization of the supernatant it was evident that the presence of other inhibitory molecules produced by the strains were contributing towards the antimicrobial activity and may include hydrogen peroxide and/or bacteriocins, indicating their potential for use as starter cultures or bioprotective cultures in the food industry (Georgieva *et al.,* 2015). Another study demonstrated the antifungal activity of lactic acid bacteria combinations on common fungal spoilage strains including *Penicillium commune, Mucor racemosus, Galactomyces geotrichum* and *Rhodotorula mucilaginosa* when added to yoghurt, cheese and sour cream (Leyva Salas *et al.,* 2018). The combinations of *Lactobacillus plantarum* L244 and either *Lactobacillus harbinensis* L172 or *Lactobacillus rhamnosus* CIRM-BIA1113 were the most effective at reducing fungal spoilage in cheese and sour cream, delaying the growth by between 2-24 days on sour cream and between 1-6 days for cheese, thus offering prospects as bioprotective cultures (Leyva Salas *et al.,* 2018).

Although many lactic acid and probiotic bacteria offer a preservative effect due to the production of a combination of inhibitory metabolites such as organic acids, hydrogen peroxide and bacteriocins (Hladíková *et al.,* 2012; Tejero-Sariñena *et al.,* 2012; An *et al.,* 2017), the isolation of bacteriocins have attracted particular attention as natural peptide biopreservatives (Balciunas *et al.,* 2013). Bacteriocins are considered to be particularly useful due to their observed antibacterial and antifungal activity against potential foodborne pathogens and/or food spoilage organisms (Cálix-Lara *et al.*, 2014; O'Connor *et al.,* 2015; Luz *et al.,* 2017; Salazar *et al.,* 2017) with their advantages detailed in Figure 1.9. The natural presence in food and GRAS status of several LAB that produce bacteriocins opens up scope for the use of

bacteriocins in food preservation applications for human consumption (Deegan *et al.*, 2006; Messaoudi *et al.*, 2013). Adding bacteriocins to foodstuffs can be accomplished by either direct addition of a purified bacteriocin to the food product, or by addition of the bacteriocin producing starter culture to a fermentation food process (Messaoudi *et al.*, 2013). Nisin is a bacteriocin produced by *Lactococcus lactis* (Matsusaki *et al.*, 1998) that has been used since the late 1950's as a biopreservative agent in the food industry and that shows activity against Gram-positive spoilage bacteria, and has been approved for use in over 50 countries (Gyawali and Ibrahim, 2014). Nisaplin, is a commercial nisin preparation made from the fermentation of non-fat milk with nisin producing *Lactococcus lactis* (Deegan *et al.*, 2006). Nisin has been included in food applications such as pasteurized mashed potato (Thomas *et al.*, 2002) and meat products to control spoilage of bologna-type sausage meat by LAB strains including *Lactobacillus sake* and *Lactobacillus curvatus* (Davies *et al.*, 1999). It has also been established as an effective preservative in pasteurized cheese and cheese products to eliminate spores that the heat processing step does not eliminate as well as other dairy products including desserts – which cannot be fully sterilised without interfering with the food quality (Davies and Delves-Broughton, 1999). Nisin is also used in pasteurized egg products to remove the risk of spores and vegetative cells, beer and wine products without interfering with the fermentation of yeast, and many others including canned foods, yoghurt and bakery products (Davies and Delves-Broughton, 1999).

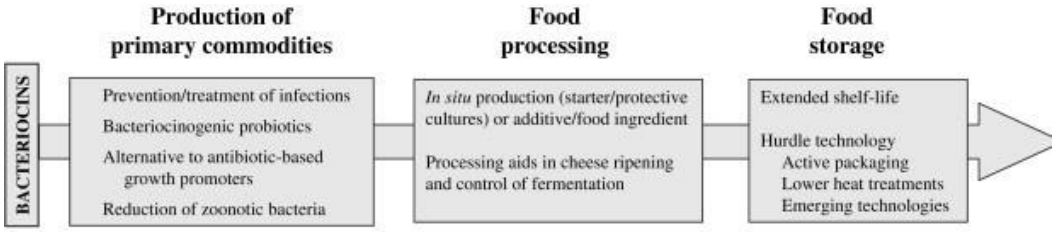

Figure 1.9: Advantages of bacteriocins/bacteriocin producing bacteria in the three primary stages of food production in the food industry (García *et al.*, 2010).

Due to the water and nutrient content of meat and meat products, the risk of microbial contamination is high, with spoilage bacteria such as *Pseudomonas* and *Enterobacter*, as well as pathogenic bacteria such as *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* species commonly present with the consequent risk of transmission into humans upon consumption (Woraprayote *et al.*, 2016). Fungal spoilage of food products including fruit and fruit juice, cereal and baked goods by strains such as *Penicillium*, *Saccharomyces*, *Candida* and *Aspergillus* is also a common issue in the food industry (Saleh and Al-Thani, 2019; Snyder *et al.*, 2019), thus reducing the shelf-life of products. As these organisms can cause spoilage of food and disease in humans, this research into extending the shelf-life and maintaining food safety is important.

There have been many studies over the years regarding bacteriocins and their applications in food as a natural preservative alternative. In one study by An *et al.*, (2017), a bacteriocin produced by *L. plantarum* M1-UVs300 that was isolated from a Chinese fermented sausage exhibited activity against both Gram-positive and Gram-negative bacteria, including the foodborne pathogens *L. monocytogenes* and *S. typhi*. The bacteriocin was also heat resistant and active over a pH range of pH 2-8 (An *et al.*, 2017). Due to the isolation of this bacteriocin from a LAB strain and its ability to withstand various elevated temperature conditions that may occur during food processing, this bacteriocin is considered to have the potential for application

as a natural bio preservative with the consequent increase in food safety. In a study by Du *et al.*, (2017), they examined the antimicrobial effectiveness of two bacteriocins, Dur 152A and enterocin L50B produced by *Enterococcus durans* 152, against the pathogen *L. monocytogenes* in meat, specifically in delicatessen ham, which due to the production process would be considered a high-risk food. Both bacteriocins were effective in preventing the growth of *L. monocytogenes* for 10 weeks at 8 °C and 30 days at 15 °C, a result showing higher efficacy than with nisin (Du *et al.*, 2017), indicating their potential applicability for use in food safety. Scannell *et al.*, (2000) carried out a study on the effect of bacteriocins lacticin 3147 and nisin on the shelf life of fresh pork sausage, with the results obtained indicating that both bacteriocins displayed similar broad-spectrum activity, and that both were more effective than the inorganic preservative sodium metabisulfite against pathogenic *Clostridium perfringens* DSM 756 and non-pathogenic *Listeria innocua* DPC 1770. The bacteriocins used in combination with organic acids also increased activity of both bacteriocins against pathogenic *C. perfringens* and *Salmonella kentucky* AT1 and non-pathogenic *L. innocua* (Scannell *et al.*, 2000). *Bacillus cereus* is a common foodborne pathogen found in a variety of foods and mejucin, a class II bacteriocin produced by *Bacillus subtilis* SN7 was found to display antibacterial activity against this pathogen, as well as against both *L. monocytogenes* and *S. aureus* (Lee and Chang, 2018). The bacteriocin was stable over a wide range of pH values (pH 3 - pH 9) and temperatures, displaying activity after treatment with temperatures as high as 120^{o}C, thus showing promising activity under various food processing conditions as a natural preservative or food biocontrol agent (Lee and Chang, 2018). The LAB *Pediococcus acidilactici* HW01, originally isolated from malt, has been shown to produce a bacteriocin, designated HW01, that was found to display antimicrobial activity against pathogenic bacteria such as *S. aureus* and *L. monocytogenes.* The HW01 bacteriocin also caused inhibition of *Pediococcus damnosus* and

P. claussenii that were isolated from spoiled beer, and thus, may have potential application for utilisation as an effective starter culture to prevent beer spoilage (Ahn *et al.*, 2017).

Bacteriocins can also display antifungal activity, as observed by Muhialdin *et al.*, (2016), where peptides produced by the strain *L. plantarum* IS10 displayed good antifungal activity against *Aspergillus flavus* MD3, *Penicillium roqueforti* MD4 and *Eurotium rubrum* MD5. In a study by (Luz *et al.*, 2017), they demonstrated how the LAB strains *L. johnsoni* CECT 289, *L. rhamnosus* CECT 278T, *L. planatrum* CECT 749 all displayed antifungal activity against *Aspergillus parasiticus* CECT 2681 and *Penicillium expansum* CECT 2268, with *L. plantarum* displaying the highest inhibitory activity that was found to be associated with the production of antifungal bioactive peptides. Overall, the research carried out to date indicates promising potential applications for antifungal peptides and their lactic acid bacterial producer strains within the food industry due to the high level of fungal associated food spoilage (Garcia *et al*, 2019).

1.5.4 Questions Still Surrounding Probiotics

Although the many potential benefits of probiotic administration have been discussed, there are still questions surrounding the true extent of their efficacy with many reviews, studies and experts doubting their safety and claimed benefits (Lerner *et al.*, 2019). According to recent studies, the ways in which probiotic bacteria influence the microbiota of the gut and dysbiosis is still not completely known (Bezirtzoglou and Stavropoulou, 2011; Laursen *et al.*, 2017; Suez *et al.*, 2018). Some studies have also shown that the intake of probiotic bacteria does not significantly affect the composition of the gut microflora (Laursen *et al.*, 2017; Singh *et al.*, 2018). Singh *et al.*, (2018) administered a probiotic preparation to 14 women for 4 weeks and monitored the composition of their faecal microbiota before and after administration of the

probiotic as well as 4 weeks after taking the probiotic. The study found that there was no significant change in the abundance or diversity of the faecal microbiota at any timepoint before and after probiotic administration with no change also observed 4 weeks after discontinuation of the probiotic. Laursen *et al.*,(2017) carried out a study on the effect of probiotic administration to children as according to the author the gut microflora of children is more responsive to external influence than the adult's gut. A probiotic supplement with *Bifidobacterium animalis* subsp. *lactis* (BB-12®) and *Lactobacillus rhamnosus* (LGG®) was administered to more than 200 Danish infants ages 8-14 months, with placebos in place, and the composition of the gut microflora was assessed before and after administration. It was found that the probiotic administration did not significantly alter the microbiota community structure or the microbial diversity within the gut of the children who had taken the probiotic as compared to the children who had taken the placebo (Laursen *et al.*, 2017).

The safety of probiotic supplementation has also been discussed, with questions surrounding the administration of probiotics to certain populations including immunocompromised individuals or patients with short bowel disease who may be more at risk of adverse effects (Doron and Snydman, 2015; Lerner *et al.*, 2019) The review by Lerner *et al.* (2019) discusses reported adverse effects following the consumption of probiotics including D-lactic acidosis, brain fog, intestinal bacterial overgrowth and bacteremia. Gargar and Divinagracia (2019) detail how three patients admitted to ICU were administered a *Bacillus clausii* probiotic that is widely used in immunocompromised patients. The use of the probiotic resulted in *B. clausii* bacteremia in the three patients after a minimum of three days on the treatment, potentially contributing to one patients death (Gargar and Divinagracia, 2019).Rao *et al.* (2018) investigated the link between probiotic use and D-lactic acidosis, as probiotics have been linked to the condition which is caused by fermentation of carbohydrates in the bowel by D-lactic producing bacteria such as *Lactobacillus* and *Bifidobacterium*. The author evaluated patients

exhibiting brain fogginess (BF) as this symptom along with elevated D-lactate in the body are characteristic of D-lactic acidosis, with the patients exhibiting other symptoms such as gas and bloating. Patients without BF were also assessed. All patients exhibiting BF consumed probiotics and it was found that D-lactic acidosis and small intestine bacterial overgrowth were more prevalent in the BF group than the non-BF group. When probiotic use was discontinued and antibiotics were administered, BF and gastrointestinal symptoms improved, indicating a potential link in the condition to probiotic use (Rao *et al.*, 2018). Another case described a child with short-bowel syndrome admitted to hospital with ataxia, neurological symptoms affecting balance, co-ordination and speech, who had been prescribed a probiotic preparation 2 weeks previously (Munakata *et al.*, 2010). The diagnosis indicated D-lactic acidosis and the probiotic was discontinued and antibiotics administered to treat the patient, while the probiotic preparation was indicated as the likely cause of the condition (Munakata *et al.*, 2010). The potential of probiotic strains to contribute to the problem of antibiotic resistance has also been discussed with the possibility of the acquisition of antibiotic resistant genes as well as the potential to pass on genes of antibiotic resistance to commensal enteric bacteria through horizontal gene transfer (van Reenen and Dicks, 2010; Lerner *et al.*, 2019). Horizontal gene transfer between probiotics has been reported for probiotic strains including *L. paracasei* and *L. rhamnosus* among others (Ceapa *et al.*, 2016; Mercanti *et al.*, 2016). Due to the extensive use of probiotic bacteria in the food industry and medically, this poses the question whether horizontal gene transfer between the strains may pose a risk for delivering hostile genetic material such as antimicrobial resistance to the hosts microbiota.

Although research into probiotics has shown various beneficial effects, there are still questions surrounding the exact benefits, efficacy and safety of probiotics in some populations including immunocompromised individuals or those with short bowel syndrome.

1.6 Conclusion

It is evident that the use of probiotic bacteria, especially *Bifidobacteria* and *Lactobacilli*, offer many benefits in areas of human health, such as probiotic supplements or for treatment as post-antibiotic therapy with future potential as an alternative to antibiotics. They are also effective agents in biopreservation within the food industry. They offer a promising alternative to current chemical preservatives, as alternatives are highly sought after due to the increase in consumer awareness of the perceived negative impacts of chemical preservatives on human health. The ability to produce antimicrobial metabolites, including organic acids and bacteriocins, are an integral characteristic of these probiotic bacteria in providing health benefits such as immunomodulation and maintenance of healthy gut microbiota, as well as inhibition of potential pathogens both within the body and within the food industry. It is evident from research that the production of these various organic acids including lactic, acetic, propionic, formic acid etc. have displayed potent antimicrobial activity, alongside the production of bacteriocins by some strains, and both contribute to the potential of these strains for use within the food industry. The antimicrobial and antifungal properties show significant opportunities to contribute to overall health, food production and for use as biopreservatives. The latter potential application to use *Bifidobacterium longum* ITT 13 strain as a biopreservative in the food industry is explored within this thesis.

CHAPTER 2

Materials and Methods

2.1 Materials

Table 2.1: Materials used throughout project and their associated supplier/manufacturer

Product	Supplier or Manufacturer
90 mm Petri Dish	Cruinn
150 mm Petri Dish	Sarstedt
Sterile Flat Bottomed 96-Well Microtitre Plate	VWR
Serological Pipette 1 ml	Cruinn
Serological Pipette 5 ml	Cruinn
Serological Pipette 10 ml	Cruinn
Sterile Pasteur Pipette	Analab
Sterile Syringe 5 ml	Carl Stuart
Sterile syringe 10 ml	Carl Stuart
0.22 µm Sterile Filter	Sarstedt
0.45 µm Sterile Filter	Sarstedt
Inoculating Loop	Cruinn
Sterile Spreader	Carl Stuart
Weigh Boat	Cruinn
Autoclave Tape	Cruinn
10 ml Polypropylene Sterile Universal Tubes	Sarstedt
50 ml Sterile Centrifuge Tubes	Lennox
0.22 µm Stericup	VWR
0.45 µm Stericup	VWR
Plastic Cuvette	Sarstedt
Quartz Cuvette	HELLMA Worldwide
AnaeroGen Sachets	Analab

Table 2.2: Reagents and chemicals used throughout project and their associated supplier/manufacturer

Product	Supplier or Manufacturer
Tryptic Soy Agar (TSA)	VWR
TSB (Tryptic Soy Broth)	VWR
De Man, Rogosa & Sharpe (MRS) Agar	Cruinn
De Man, Rogosa & Sharpe (MRS) Broth	Cruinn
Muller Hinton (MH) Broth	Analab
Potato Dextrose (PD) Broth	Analab
Yeast Peptone Dextrose (YPD) Broth	Fisher Scientific
M17 Broth	VWR
Agar	Fisher Scientific
L-cysteine	Sigma Aldrich
BCA Kit (Catalog no. 23225)	Fisher Scientific
Phosphate Buffered Saline (PBS)	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Lactic Acid	Sigma-Aldrich
Glacial Acetic Acid	Sigma-Aldrich
Hydrochloric Acid (HCl)	Sigma-Aldrich
Sodium Hydroxide (NaOH)	Fisher Scientific
Sodium Acetate	Sigma-Aldrich
Tween20	Sigma-Aldrich
Nisin (2.5%, balance sodium chloride)	Sigma-Aldrich
Natamycin	Analab
Ammonium Sulphate	VWR
Proteinase K	VWR
Pronase E (Actinase E)	Sigma-Aldrich

2.2 Bacterial Cultivation

2.2.1 Media Preparation

All media utilised was prepared according to manufacturer's instructions and autoclaved at 121^{o}C at 15 psi for 15 minutes. Sterile molten agar was stored at 55^{o}C before pouring into sterile petri dishes. Media broth was either prepared and dispensed into test tubes and autoclaved, or the media broth was batch prepared and autoclaved prior to dispensing into sterile universal tubes.

2.2.2 Growth and Cryopreservation of Bacterial Stocks

The bacterial strains utilised for this research and their growth conditions are presented in Table 2.3. All strains were quadrant streaked on Tryptic Soy agar (TSA) to ensure purity before an isolated colony was then added to 10 mls TSB and grown overnight. The cells were then centrifuged at 2722 x *g* for 5 minutes, the supernatant decanted and the pellet was then resuspended in 5 mls of TSB with 10% glycerol to concentrate cells and separated into 1 ml cryovials for storage at -80^{o}C.

2.3 Gram Stain

Gram staining was carried out as described by Bartholomew and Mittwer (1952). All bacterial strains cultured were Gram stained before storage at -80^{o}C to ensure purity of cultures.

Table 2.3: Bacterial Indicator Strains and Growth Conditions

Bacterial Indicator Strain	Strain ID	Incubation Temperature	Growth Media	Incubation Conditions
Bacillus cereus	ATCC 10876	37ºC	TSB/TSA	Aerobic
Enterococcus faecalis	ATCC 7080	37ºC	TSB/TSA	Aerobic
Kocuria rhizophilia	ATCC 9341	30ºC	TSB/TSA	Aerobic
Staphylococcus aureus	ATCC 25923	37ºC	TSB/TSA	Aerobic
Staphylococcus epidermidis	ATCC 12228	37ºC	TSB/TSA	Aerobic
Klebsiella aerogenes (previously known as *Enterobacter aerogenes*)	ATCC 13048	37ºC	TSB/TSA	Aerobic
Escherichia coli	ATCC 25922	37ºC	TSB/TSA	Aerobic
Pseudomonas aeruginosa	PA01	37ºC	TSB/TSA	Aerobic
Bacillus subtilis	ATCC 1174	37ºC	TSB/TSA	Aerobic
Streptococcus pyogenes	NCIMB 11841	37ºC	TSB/TSA	Aerobic
Staphylococcus xylosus (previously known as *Micrococcus luteus*)	ATCC 29971	37ºC	TSB/TSA	Aerobic
Salmonella typhimurium (Salmonella enterica subsp. *enterica*)	ATCC 29629	37ºC	TSB/TSA	Aerobic
Burkholderia cenocepacia	ATCC 17765 III.20	37ºC	TSB/TSA	Aerobic
L. paracasei subsp. *paracasei* (*Lactobacillus plantarum*)	NCIMB 3254	37ºC	MRS broth/agar	Anaerobic
Lactobacillus delbruickii subsp. *bulgaricus*	NCIMB 11778	37ºC	MRS broth/agar	Anaerobic
Lactococcus cremoris	M176	30ºC	M17 broth/agar	Anaerobic
Lactococcus lactis	M179	30ºC	M17 broth/agar	Anaerobic

The stocks of bacterial strains were prepared as described in section 2.2 and stocks were stored at -80ºC. The temperature and media in Table 2.3 were used in microdiffusion assay testing (section 2.6) and for preparation of the indicator organisms for MIC_{50} testing (section 2.8.4).

2.4 *B. longum* ITT 13 Growth and Storage

2.4.1 *B. longum* ITT 13 Isolation and Storage

The isolate *B. longum* ITT 13 was previously isolated from the gut flora of neonates (Costello, M, Personal Communication 2018). It was grown up from 1 ml cryovials stored at -80^{o}C and incubated anaerobically at 37^{o}C, on MRS agar supplemented with 0.05% *L*-cysteine for 3-4 days until growth was observed. The ITT 13 strain was sequentially re-streaked to purify before a single colony was inoculated into 10 tubes, each containing 10 mls of MRS broth supplemented with 0.05% *L*-cysteine and grown for 20-24 hours at 37^{o}C. The tubes were centrifuged at 2722 x *g* for 5 minutes to pellet the cells, the supernatant was decanted and the pelleted cells resuspended in 5 mls MRSc broth with 10% glycerol. The resuspended cells were aliquoted into 1 ml cryovials, yielding a total of 50 cryovials, and frozen at -80^{o}C.

2.4.2 *B. longum* ITT 13 Fermentate Production

A 0.5% aliquot from a cryovial of *B. longum* ITT 13 was inoculated from frozen stocks into MRSc broth of 10-100 ml and grown for 18-20 hours anaerobically at 37^{o}C. A 1% inoculum of the culture was then transferred to fresh MRSc broth of 100-1000 mls depending on volume of fermentate required and incubated anaerobically at 37^{o}C for a further 18-20 hours. The fermentate was heat shocked by incubating at 60^{o}C for 15 minutes. Heat shocking was required by the project sponsor as a minimally modified fermentate was required for testing with the inactivated cells present, unless a specific assay required the absence of cells i.e. testing in microtitre plates. This also meant that no purification methods were employed to purify the fermentate for testing, with testing carried out in its natural heat shocked form. Prior to use, the heat shocked fermentate was stored at 4^{o}C and used as required in individual experiments.

2.4.3 Cell Free Supernatant of *B. longum* ITT 13 Fermentate

Cell free fermentate was prepared using the heat shocked fermentate (section 2.4.2). The fermentate was centrifuged at 2722 x *g* for 5 minutes to pellet cells. Where large aliquots of the heat shocked fermentate were used, the supernatant was decanted into fresh tubes and a second centrifugation required at 2722 x *g* for 5 minutes due to large cell density. Prior to addition to tangential flow filtration (TFF) systems, the cell free fermentate was filtered through 0.45 μm stericups to remove remaining cells and cellular debris. The cell free fermentate was stored at 4^{o}C prior to use.

2.5 Nisin Preparation

Nisin was prepared according to Aplin and Barrett Nisin Reference Preparation Method. A commercial preparation of nisin (2.5%, balance sodium chloride) (SigmaAldrich, Co. Wicklow) was used with activity of 1000 IU/ml. For preparation, 0.1 g was weighed out and dissolved in 60 mls 0.02N HCl in a glass conical flask. The flask was plugged with cotton wool and boiled for 10 minutes in a 100^{o}C waterbath, before being cooled to room temperature. The solution was brought up to 100 mls with 40 mls 0.02N HCl and stored at 4^{o}C for at least 1 hour, and at maximum for 24 hours, before being frozen at -20^{o}C in 1 ml aliquots.

2.6 Microdiffusion Plate Assay

The microdiffusion assay (M/D assay), also known as the agar/well diffusion assay and for the purposes of this study was referred to as the microdiffusion or M/D assay, was utilised for antimicrobial activity testing. The assay was adapted from the method described by Tramer and Fowler (1964). TSA was prepared by weighing out 2.22 g and suspending in 60 mls of deionised water for 150 mm petri dishes or 1.11 g TSA suspended in 30 mls deionized water for 90 mm petri dishes. For *Lactobacilli* strains 3.72 g of MRS agar with 0.05% *L*-cysteine was

weighed and added to 60 mls deionized water. A 20 ml 1:1 mixture of deionised water and Tween 20 was prepared. The media and Tween 20:H_2O were then autoclaved (section 2.1) and the relevant indicator strain (5%) grown in TSB was added to the molten agar at 55^oC and 2% of the sterile Tween 20:H_2O added also. The inoculated agar mixture was poured into a 150 mm or a 90 mm petri dish and allowed to solidify before it was placed into a refrigerator prior to use. Wells of 10 mm in diameter were cut into the agar on the M/D plates using a size 6 bore dipped in 100% ethanol or IMS and flamed to sterilise. A volume of 180 µl of the required samples being tested for activity were placed into the relevant wells and plates incubated for 20-24 hours under the conditions required by the indicator strain (Table 2.1). Nisin was used as the positive control and sterile media or relevant buffer used as a negative control(s).

2.7 Biolog Identification of Strain ITT 13

The Biolog (TECHNOPATH Distribution Ltd. Ireland) was used for phentotypic identification of the ITT 13 strain. The Biolog utilises a variety of different carbon sources added to microtitre plate wells. The strain for testing is added to wells and following incubation there is a resulting pattern of growth that depends on the carbon sources that can be utilised by the test strain. Identification of the strain is based on this pattern of growth. Strain ITT 13 was grown on Biolog universal anaerobe agar in an anaerobic chamber for 48 hours. Colonies were then inoculated into the Biolog inoculating fluid at 65% Transmission (T). The turbidity was determined using a turbidometer which was calibrated using a turbidity standard. Colonies from the universal anaerobe agar plate were inoculated into the fluid until 65%T was reached. The inoculated fluid was then poured into a sterile reservoir and 100 µl of the inoculated culture added to each well on the Biolog anaerobic microplate that was then incubated in an anaerobic jar at 37^oC for 20-24 hours before the plate was removed and read using the Biolog plate reader. The growth pattern on the Biolog anaerobic microplates were read using the Biolog

Microstation^{TM} Reader and analysed in comparison to the Biolog Anaerobe database.

2.8 Determination of Antibacterial and Antifungal Spectrum of Activity for *B. longum* ITT 13

2.8.1 Antibacterial Assay Evaluation

The most suitable assay for antibacterial activity screening of the *B. longum* ITT 13 fermentate was selected from the Kirby-Bauer disk diffusion assay (Kirby *et al*, 1957, Bauer *et al*, 1966), the microdiffusion or well diffusion assay (section 2.6) and the agar spot assay (Nakano *et al*, 1968). Nisin was used as the antibacterial agent to evaluate each assay and *Kocuria rhizophilia* ATCC 9341 was used as the indicator strain for all three assays as it has provided clear zones of inhibition in previous testing. For the microdiffusion assay, serial dilutions of nisin (1000 iu/ml) from neat to 1/128 were placed into wells and zones measured following incubation of the plates. The agar spot assay is a solely qualitative assay with a small spot of clearance on the incubated agar plate indicating inhibition of the indicator strain. The agar spot assay was carried out using neat to 1/128 serial dilutions of nisin spotted onto the surface of 150 mm TSA plates inoculated with *K. rhizophilia*, in 5 µl and 10 µl volumes. The disk diffusion assay was carried out by impregnating 6 mm sterile disks with 15 µl of each nisin dilution (neat to 1/128) and then placing the disks on the surface of 150 mm TSA plates inoculated with *K. rhizophilia*. The results generated for each assay were compared for effectiveness based on interpretation of antimicrobial activity.

2.8.2 Antibacterial Spectrum of Activity Determination – Microdiffusion Assay

The microdiffusion assay as described in section 2.6, using *B. longum* ITT 13 heat inactivated fermentate (section 2.4.2) serially diluted up to 1/16 with MRSc broth, was used for the determination of the antibacterial spectrum of *B. longum* ITT 13 against a variety of Gram-

positive and Gram-negative bacteria. Bacterial indicator strains were selected based on their relevance to food spoilage and contamination, pathogenesis or their effectiveness as an indicator strain by providing clear zones of inhibition (ZOI). The microdiffusion assay was carried out using the 15 bacterial strains detailed in Table 2.2 as the indicator strains. Results were recorded as the presence or absence of a ZOI at each dilution of fermentate and the diameter of zones of inhibition were recorded. A minimum of three independent assays (n=3) with three replicates for each assay were carried out for each indicator strain.

Arbitrary Units (AU)/ml were calculated for the M/D assay to determine the level of antibacterial activity against each indicator strain by dividing the inverse of the highest dilution displaying antimicrobial activity by the volume of fermentate, multiplied by 1000 to bring up to 1 ml ($\mathrm{AU/ml} = \frac{inverse\ of\ highest\ dilution\ with\ \geq 50\%\ inhibition}{volume\ applied\ to\ well}\ x\ 1000$).

2.8.3 The Effect of pH on Antibacterial Activity of Fermentate

A volume of 10 mls of heat inactivated ITT 13 fermentate (section 2.4.2) was adjusted from pH 4.21 to 4.68 using 2 mls of 0.3M NaOH, giving a final concentration of 0.05M NaOH in the Fermentate. A minimal increase in pH was carried out so as not to eliminate observable antibacterial activity on the M./D assay as significant pH increases have resulted in a loss of activity in previous testing. The pH adjusted Fermentate as well as unadjusted Fermentate were both diluted to 1/2 and added to M/D assay plates (section 2.6). The negative control was prepared using a volume of 10 mls of MRSc broth adjusted from pH 6.43 to 4.82 with 12 mls of 60 mM Lactic Acid, giving a final concentration of 33 mM lactic acid. The microdiffusion assay was carried out using *B. subtilis, K. rhizophilia* and *P. aeruginosa* as the microbial indicator strains, and nisin (neat to 1/4 dilutions) were used as the positive controls. Negative controls of 0.05M (50 mM) NaOH and 33 mM lactic acid were also used on the microdiffusion plates. A minimum of three independent experiments (n=3) with three replicates per

experiment were carried out for each indicator strain.

2.8.4 Minimum Inhibitory Concentration Assay

The minimum inhibitory concentration causing 50% inhibition of growth (MIC_{50}) of the *B. longum* ITT 13 heat inactivated fermentate (section 2.4.2) adjusted to pH 4.55 ± 0.02 was determined used 96-well microtitre plates according to the EUCAST broth microdilution method (Eucast.org, 2020) as described below.

The bacterial indicator strains used were *S. xylosus* ATCC 29971, *B. subtilis* ATCC 1174, *E. faecalis* ATCC 7080, *P. aeruginosa* PA01, *S. typhimurium* ATCC 29629 and *E. coli* ATCC 25922 which were freshly grown to mid-log phase and diluted to $1x10^6$ CFU/ml in Mueller Hinton broth (MH broth). Mid-log phase was identified by growth curves carried out on the strains. Available growth curves are presented in Appendix A and mid-log phase for *P. aeruginosa* was informed by J. Brady – personal communication. A volume of 100 µl of the $1x10^6$ CFU/ml indicator cultures was seeded into each of the wells. In the control wells i.e. wells without cells, 100 µl of Mueller Hinton broth was added at a 1:1 dilution with the sample which was then placed into the well. The pH adjusted fermentate was generated by taking a volume of 10 mls of heat inactivated fermentate and adjusting it to pH 4.55 ± 0.02 with 1 ml of 0.3M NaOH.

A 100 µl sample of the pH adjusted fermentate was then added to wells with $1x10^6$ CFU/ml of the indicator strain, giving a final density of $5x10^5$ CFU/ml of the indicator strain and the highest concentration of the fermentate as an initial dilution of 1/2. Serial dilutions from 1/2 to a final dilution of 1/128 of fermentate were carried out in the wells. MRSc broth was also serially diluted in the wells from 1/2 to a 1/128 dilution. The MRSc controls were used to determine the OD600 of the indicator strain for that well with the specific correlating MRSc concentration in the ITT 13 fermentate, without any inhibition. The antibacterial effect of each

dilution of the pH adjusted fermentate was then calculated as a percentage reduction of growth of the indicator strain in MRSc with and without ITT 13 fermentate.

A volume of 10 mls of MRSc broth was also adjusted to pH 4.55 ± 0.02 with 8-8.5 mls of 0.1 M lactic acid and the pH adjusted MRSc was then used as a control to assess whether the antibacterial activity of the fermentate could be solely pH associated. The latter evaluation was based on the MIC_{50} of the fermentate vs. the MIC_{50} of the pH adjusted MRSc broth. A growth control of diluted MRSc broth prepared by adding 8-8.5 mls of sterile H_2O to was used to analyse the effect of the broth dilution. The pH adjusted MRSc broth and diluted MRSc broth were comparatively determined in each well. Comparative analysis meant the inhibition of the MRSc broth adjusted to pH 4.55 ± 0.02 could also be calculated as a percentage reduction of growth based on the growth compared to diluted MRSc and be directly comparable to the percentage reduction calculated of the ITT 13 fermentate.

Nisin serially diluted from 1/2 to 1/128 was used as a positive control and controls of MRSc broth at a 1:1 with Mueller Hinton broth and nisin at a 1:1 with Mueller Hinton broth were used as correction factors to adjust for absorbance due to the MRSc and MH broth mixture or the nisin and MH broth mixture.

Arbitrary Units (AU)/ml were calculated for the MIC_{50} assay to determine the level of antibacterial activity as described in Section 2.8.2. AU/ml was calculated using the $MIC_{50,}$ which was the highest dilution displaying at least 50% inhibition of the indicator strain. Generally the MIC is the lowest concentration of an antimicrobial agent that prevents visible growth of the test organism (EUCAST Definitive Document, 1998), however, in this study, the minor differences in growth of the indicator strains at each dilution of the test samples and the controls were extremely important rather than the highest dilution preventing complete growth. For example, if the test sample and the positive control both prevented visible growth of the indicator strain at the 1/4 dilution this would generally be the MIC of both, but the test sample

may prevent 70% of growth at the 1/8 dilution while the control only prevents 10% of growth. In this case, the MIC would be the same, but there is still a stronger inhibitory effect observed by the test sample. Therefore, it was decided for this study to take the MIC_{50} so a more detailed analysis could be performed and this was what was be used for AU/ml calculation. This is similar to an IC50 calculation which is the concentration of a drug that is required to inhibit 50% of a biological process and is a measure of a drugs potency and efficacy in pharmacological research (Aykul and Martinez-Hackert, 2016), but as the concentration of potential antimicrobial peptides within the fermentate is unknown this was not a suitable analysis. This method of analysis was adapted from Lim, (2016) who also calculated the bacteriocin units/ml (equivalent to AU/ml) based on the highest dilution displaying 50% inhibition.

2.8.5 Antifungal Activity

2.8.5.1 Antifungal Activity Using Soft Agar Overlay Method

A modified version of the soft-agar overlay assay was adapted from Romanens *et al.* (2019), using heat in-activated fermentate (Section 2.4.2) and was carried out as a minimum of three independent experiments (n=3) for each indicator organism. The heat inactivated *B. longum* ITT 13 fermentate was used to determine whether any antifungal activity was present against the five fungal strains *Candida albicans* ATCC2091, *Saccharomyces cerevisiae* ATCC18824, *Saccharomyces cerevisiae* CBS1171, *Rhodotorula mucilaginosa* CBS316 and *Debaryomyces hansenii* ATCC18110. The five fungal strains were grown on both Yeast Peptone Dextrose (YPD) and Potato Dextrose (PD) agar, the agar selected for the soft-agar overlay analysis was based on observation of the greatest confluency of growth on the relevant media. Soft YPD agar was used for *S. cerevisiae* and *D. hansenii*, and soft PD agar was used for *R. mucilaginosa* and *C. albicans*. The assay was carried out by adding 30 ml of TSA bottom layer agar into a

sterile petri dish allowing it to set, and then adding 10 ml of a soft top agar (PD broth or YPD broth containing 0.7% agar) inoculated with 1% v/v of the chosen fungal strain.

Natamycin in a YPD or PD broth suspension, depending on the media used as soft agar, was used at concentrations of 0.1 mg/ml on each plate, as the positive control. YPD/PD broth and MRSc broth were used as negative controls on all plates. Plates were incubated for 45 hours at 30^{o}C for *Saccharomyces cerevisiae* ATCC18824, *Saccharomyces cerevisiae* CBS1171, *Rhodotorula mucilaginosa* CBS316 and Debaryomyces hansenii ATCC18110 until confluent growth resulted. *Candida albicans* ATCC2091 was incubated for 24 hours at 30^{o}C.

2.8.5.2 Antifungal Activity Using Microtitre Plate/MIC$_{50}$ Method

The fermentate produced by *B. longum* ITT 13 was previously screened for antifungal activity using the agar-spot assay. This assay concluded that there was no observable antifungal activity displayed by the fermentate, however due to the increased sensitivity of the MIC$_{50}$ method using 96-well microtitre plates (Section 2.8.4), antifungal screening was repeated using this assay. The fungal strains *R. mucilaginosa* CBS 316, *C. albicans* ATCC 2091, *S. cerevisiae* ATCC 18824 and *S. cerevisiae* CBS 1171 were grown for 20-22 hours in either PD broth (*R. mucilaginosa* and *C.* albicans) or Yeast Peptone Dextrose broth (*S. cerevisiae* strains). The fungi were diluted 1/100 in their respective broth and 100 µl of the diluted culture plated into the wells on microtitre plates. *B. longum* ITT 13 fermentate produced in 100 mls MRSc broth in an anaerobic jar (pH 4.35) as described in Section 2.4.2 and *B. longum* ITT 13 fermentate produced in MRSc broth in a 2 L pH-controlled fermentation vessel (pH 4.8) were made cell-free using the method described in Section 2.4.3. The cell free fermentates were added to wells containing the fungal cells in 100 µl volumes and diluted serially across the wells (1/2 - 1/32 dilution). Natamycin was prepared at a concentration of 0.1 mg/ml as a suspension in YPD and PD broth as a positive control and serially diluted across wells to a 1/32 dilution equivalent to

0.0031 mg/ml. MRSc broth pH adjusted to pH 4.36 using 39.9 mM lactic acid and 60.9 mM acetic acid was also added to wells and diluted to a 1/32 dilution. A negative control of the respective fungal media used for growth of the tested fungal strain, either YPD or PD media, was used as a negative/growth control. The microtitre plates were incubated for 22-24 hours and the growth of the fungal strains determined by the optical density at 600 nm (OD600). A minimum of three independent assays (n=3) with two replicates per assay were carried out.

2.9 Physiochemical Characterisation

2.9.1 Ammonium Sulphate Precipitation of Fermentate

Ammonium sulphate (AS) precipitation was carried out using a method adapted from Wingfield (2001). Two variations of the AS precipitation were carried out on the *B. longum* ITT 13 fermentate with the aim of identifying an AS fraction that contained antimicrobial activity. The ammonium sulphate solution interferes with the proteins solubility in water causing precipitation by increasing hydrophobic interactions, while maintaining the protein conformation (Wingfield, P., 2001).

2.9.1.1 Ammonium Sulphate Precipitation with Separate Saturation

The first method involved ammonium sulphate (AS) precipitation of the fermentate at four separate AS saturations. Ammonium sulphate was added at 20%, 40%, 60% and 80% saturation to 4 separate 40 ml samples of cell free fermentate (section 2.4.2 and 2.4.3). The 4 samples were kept on ice during addition of AS and magnetic stirring rods placed into beakers on slow speed to allow dissolution of each of the AS concentrations over 1.5 hours. The samples were placed into sterile centrifuge tubes, stored overnight at 4^{o}C before centrifugation of the 40 ml samples at 4^{o}C and 13,440 x *g* for 30 minutes. The supernatant was decanted and the pellets were resuspended in 1 ml of ultra-pure H_2O, except for the 80% pellet which was

resuspended in 2 ml ultra-pure H_2O due to the size of the precipitate. Antimicrobial activity was screened for each of the supernatant fractions and resuspended pellets using the Microdiffusion assay A minimum of three independent assays (n=3) were carried out for testing of all samples plus the negative controls of AS at 20%, 40%, 60% & 80% in MRSc broth and the positive control was neat nisin (1000 iu/ml).

2.9.1.2 Sequential Ammonium Sulphate Precipitation

The second AS precipitation method involved sequential ammonium sulphate precipitation. The solutions were kept on ice during addition of AS with magnetic stirring rods in the solutions at slow speed. The AS concentration in a 100 mls fermentate sample was initially brought to a 20% saturation by the gradual addition of the AS over 30 minutes to a final weight of 10.7 grams of AS added, followed by a further 15-minute period of dissolution. The 20% AS solution was then centrifuged at 4ºC and 13,440 x *g* for 30 minutes. The supernatant from the 20% was then saturated sequentially to 40% by the addition of 11.5 g AS, 60% (12.2 g AS added) and up to 80% saturation (13.1 g AS added), with repeated centrifugation and pelleting of protein after each AS addition. Pellets from each step were resuspended in 1 ml of ultrapure H_2O. Antimicrobial activity was screened for a sample of each supernatant fraction and each resuspended pellet using the Microdiffusion assay. Nisin (1000 IU/ml) was used as a positive control and AS at the 20%, 40%, 60% & 80% saturations in MRSc broth were used as negative controls. A minimum of three independent M/D assays (n=3) were carried out.

2.9.1.3 Ammonium Sulphate Precipitation with Microtitre Plate Analysis

A *B. longum* ITT 13 heat-inactivated fermentate (pH 4.3) was prepared as described in Section 2.4.2 and made cell-free as described in Section 2.4.3. Ammonium sulphate precipitation was carried out as described in Section 2.9.1.1 with ammonium sulphate brought up to 20%, 40%,

60% and 80% saturation in 40 ml aliquots of the cell free fermentate. Precipitated pellets were resuspended in 2 ml of 50 mM pH 4.3 sodium acetate buffer. As the original fermentation was carried out in MRSc broth an equivalent ammonium sulphate precipitation fractionation was carried out with the MRSc media as a control to ensure that if any antimicrobial activity was detected, then it could not be associated with the protein fractions present in the actual media itself or the percent saturation of ammonium sulphate used.

S. xylosus cells were freshly grown to mid-log phase and diluted to $1x10^6$ CFU/ml in Mueller Hinton broth (MH broth). The cell density was known as a growth curve had been carried out (Appendix A). A volume of 100 µl of the $1x10^6$ CFU/ml cells were seeded into each of the wells. A 100 µl sample of the resuspended *B. longum* ITT 13 fermentate and MRSc broth pellets were then added to wells with $1x10^6$ CFU/ml of the indicator strain, giving a final density of $5x10^5$ CFU/ml of the indicator strain and the highest concentration of the resuspended fermentate and MRSc samples as an initial dilution of 1/2. Serial dilutions from 1/2 to a final dilution of 1/128 of fermentate were carried out in the wells. Nisin serially diluted from 1/2 to 1/128 was used as a positive control and controls of MRSc broth at a 1:1 with Mueller Hinton broth and nisin at a 1:1 with Mueller Hinton broth were used as correction factors to adjust for absorbance due to the MRSc and MH broth mixture or the nisin and MH broth mixture.

The plates were incubated on an orbital shaker at 150 rpm in a 37ºC incubator for 18-20 hours and growth in the wells measured in a microtitre plate reader at 600 nm. The growth for the *S. xylosus* cells in the presence of the resuspended *B. longum* ITT 13 fermentate was compared to the growth of the cells in the presence of the resuspended MRSc broth at each dilution and each ammonium sulphate saturation to determine if the resuspended fermentate resulted in inhibition of the cells that was not observed for the MRSc media. Three independent assays (n=3) were carried out with three replicates per assay.

2.9.2 pH Stability Testing of *B. longum* ITT 13 Fermentate with HCl and NaOH

The pH stability of the ITT 13 heat inactivated fermentate (section 2.4.2) was determined through the adjustment of neat fermentate to a variety of pH's (pH 3, 4, 6, 7 and 8) which were then screened for activity using the microdiffusion assay with *S. xylosus* as the indicator strain. Aliquots (10 mls) of ITT 13 heat inactivated fermentate were adjusted to pH 3.18, pH 4.02, pH 5.85, 6.38 and 7.96 with various volumes of 0.3M NaOH and 0.3M HCl. MRSc broth at pH's 3.00, 4.01, 6.01, 6.98 and pH 8.04 were used as the negative controls, with nisin (1000 iu/ml) as the positive control and pH unadjusted heat inactivated fermentate used a control for the expected antimicrobial activity of the fermentate sample. The pH adjusted samples were placed at room temperature (23^{o}C) for 4 hours with samples taken at 0 hours (T0), 1 hour (T1), 2 hours (T2) and 4 hours (T4) to determine if antimicrobial activity was reduced over time at any pH. Microdiffusion assay plates (90 mm) were used to screen for antimicrobial activity at each time point. All pH adjusted fermentate samples were carried out in three independent assays (n=3) and dilutions of the fermentate associated with the pH adjustment, were considered in the analysis.

2.9.3 Detailed pH Stability Assay of *B. longum* ITT 13 Fermentate with Lactic Acid and NaOH

Aliquots of *B. longum* ITT 13 heat inactivated fermentate (5 mls) (Section 2.4.2) were adjusted to pH 3, pH 4, pH 5 and pH 6 using filter sterilized 0.1 M lactic acid or 0.3 M NaOH. Volumes of 5.7 mls of 0.1 M lactic acid was used to adjust 5 mls fermentate to pH 3.08, 700 µl of 0.1 M lactic acid was used to adjust 5 mls fermentate to pH 4.00, 1.9 mls of 0.3 M NaOH was used to adjust 5 mls fermentate to pH 5.12 and 2.7 mls of 0.3 M NaOH was used to adjust 5 mls fermentate to pH 6.10. The pH of the fermentate without pH adjust was pH 4.27.

MRSc broth was also pH adjusted from pH 6.18 to pH 4.32 with 11.5 mls 0.1 M lactic acid to allow for more direct comparison to the fermentate (pH 4.27). This was filter sterilized (0.22 µm) and divided into 5 ml volumes to which the same volume of NaOH or lactic acid used to adjust the fermentate as described above were added. These were used as negative controls to assess whether any activity of the adjusted fermentate could be primarily due to acid or base. The volume of NaOH or lactic acid added to the pH 3-6 fermentate samples as described above were also added to 5 ml volumes of MRSc broth with no pH adjust. This was another control to determine the antimicrobial activity associated solely with the acid/base used to adjust the pH of the fermentate.

The analytical and control samples were added to a microdiffusion assay plate (Section 2.6) as a time 0 sample. Neat nisin (1000 iu/ml) was used as a positive control for the assay. The unadjusted fermentate was also added to plates for comparison of activity to the pH adjusted fermentate. The pH adjusted analytical samples and controls were kept at room temperature (18-20^{o}C) over the course of the assay. Samples were taken and added to microdiffusion plates at T0 (0 hours), T1 (2 hours), T3 (4 hours) and T4 (24 hours).

2.9.4 Protease Digestion of the *B. longum* ITT 13 Fermentate in Microdiffusion Plates

The ITT 13 fermentate was digested with a variety of proteases to determine whether the antimicrobial activity was partially or wholly proteinaceous in nature. Actinase E was prepared at a concentration of 10 mg/ml in sterile PBS. A volume of 100 µl of protease was incubated with 100 µl of heat inactivated fermentate (section 2.4.2) at 37^{o}C for 4 and 24 hours, resulting in a final concentration of 5 mg/ml of protease and a 1/2 dilution of the fermentate. Nisin was also digested with the proteases as a positive control for the experiment. A 1/2 dilution of the fermentate and a 1/2 dilution of nisin (500 iu/ml) diluted with sterile PBS and incubated at 37^{o}C for 4 and 24 hours were also carried out for a direct comparison of antimicrobial activity

with the protease digested samples. Following incubation, a heat inactivation step of the samples at 80^{o}C for 5 minutes was carried out to inactivate the protease before addition to plates. Neat samples of the fermentate and nisin were added to Microdiffusion plates to assess initial activity without dilution (Section 2.6). The M/D assay was carried out to screen for a loss of activity upon treatment with the proteases using *S. xylosus* as the indicator strain. Zones of inhibition of the 1/2 dilutions of the fermentate and nisin control were compared with the presence or absence of ZOI's of the protease treated fermentate and nisin control.

2.9.5 Protease Digestion of the *B. longum* ITT 13 Fermentate in Microtitre Plates

Two *B. longum* ITT 13 cell free fermentates (fermentate 1: pH 4.35 grown anaerobically in 100 mls MRSc broth, fermentate 2: pH 4.80 grown in MRSc broth anaerobically in a 2 L fermenter) were digested with both Actinase E and Proteinase K as described in Section 2.9.4 for 22 hours. Samples of both fermentate were also diluted with PBS at a 1/2 dilution and incubated at 37^{o}C for 22 hours as a direct comparison for the dilution caused by the proteases. After incubation, the samples were heat treated at 80^{o}C for 5 minutes to inactivate the protease and added to microtitre plates.

A 100 µl volume of the digested and diluted fermentate samples was added to wells containing 100 µl of 1x10^{6} CFU/ml of *S. xylosus* as the indicator strain giving a final density of 5x10^{5} CFU/ml and the highest concentration of the fermentate as an initial dilution of 1/4. As the fermentate samples were either diluted with PBS or digested with Actinase E or Proteinase K prepared in PBS, a 1:1 of MRSc broth and PBS was prepared and added to wells containing 100 µl of *S. xylosus* as a growth control to ensure the mix of PBS and MRSc broth alone did not contribute to similar inhibition of the fermentate. All samples were serially diluted across up to a 1/256 dilution.

Nisin (1000 iu/ml) was also digested and diluted with PBS in the same manner and added to

microtitre plates as described above. Instead of a 1:1 of MRSc and PBS added to wells, a growth control of a 1:1 of 0.02 N HCl and PBS was added as the former is the solvent nisin used for nisin preparation.

2.9.6 HPLC Analysis of the Organic Acid Production by *B. longum* ITT 13

In order to identify the composition and concentration of organic acids produced by *B. longum* ITT 13 during growth and fermentation, HPLC-UV analysis was carried out based on the method described by *Özcelik et al.,* (2016) with modifications, using a reverse-phase column with UV analysis. Samples of the *B. longum* ITT 13 fermentate were cultured and provided to C. Lynch who carried out the HPLC analysis using a quaternary solvent management HPLC instrument. The *B. longum* ITT 13 fermentate included two variations, the first was cultured in MRSc broth with 0.11% *L*-cysteine in 2L fermentation vessels with pH control at pH 4.8 (Section 2.9.5) and the other cultured in MRSc broth with 0.05% *L*-cysteine without pH control at pH 4.3 in lab-scale volumes of 200 ml (Section 2.4.2). The HPLC instrument was equipped with a diode array detector at 210 nm and the column used was C18 reverse phase, Phenomenex EVO-C18 (100mm x 4.76, 2.6 um particle size) column. A mobile phase composition of ortho-phosphoric acid and acetonitrile (ACN) was used, with a flow rate of 0.4 ml/min. Each chromatographic run was carried out for 15 minutes with 5-minute interval periods between runs to allow for system re-equilibration. An injection volume of 10 µl was used with the injection port kept at room temperature (20-24^{o}C). An internal standard of methylmalonic acid was used.

Stock solutions of formic acid, lactic acid, acetic acid, succinic acid, propionic acid and butyric acid were prepared at 1M concentration and diluted to 200 mM, 100 mM, 50 mM and 25 mM, for the creation of calibration curves for concentration determination of the organic acids produced by *B. longum* ITT 13. For preparation of the *B. longum* ITT 13 fermentate samples,

5 ml of the fermentates were centrifuged at 1210 x *g* for 5 minutes before the cell free fermentate (CFF) was then filtered through a Whatman syringe filter with pore size 0.45 µm to remove any remaining cellular debris. The relative centrifugal force used was different to how the cell-free fermentate was generated for previous testing as the HPLC analysis was carried out independently by an undergraduate student who designed their own method. The cell-free fermentate was diluted 1:20 with deionised water before a further 1:1 mixture was generated of the diluted CFF with the 40 mM internal standard solution – producing a *B. longum* ITT 13 CFF mixture of a 1:40 dilution. The presence of organic acids in the *B. longum* ITT 13 fermentate samples were identified based on comparison of the peaks to the retention times of standards and the concentration of the organic acids present determined using the calibration curves of the associated standards. The analysis was carried out in duplicate on two individual days.

2.9.7 Thermostability Studies of the Antimicrobial Activity Produced by *B. longum* ITT 13

The stability of the antimicrobial activity produced by *B. longum* ITT 13 was assessed from -20^{o}C to 70^{o}C to determine the effect of heat and storage at various temperatures on the stability of the antimicrobial activity displayed by the organism. The *B. longum* ITT 13 fermentate was grown in MRSc broth in a 2-litre fermentation vessel pH controlled at pH 5 for 16-18 hours. The fermentation vessel was pH controlled at pH 5 using NaOH to minimise the interference of organic acid production by *B. longum* ITT 13. The use of a fermentation vessel produced higher biomass, with 10^{11} CFU/ml of *B. longum* ITT 13 compared to 10^{9} CFU/ml when grown at laboratory scale in 100-200 ml volumes without pH control.

Cells were removed from the *B. longum* ITT 13 fermentate as described in Section 2.4.3 and the CFF was aliquoted into sterile eppendorfs in 2 ml volumes. Replicates of 24 eppendorfs

were incubated at separate temperatures of -20°C, 4°C, room temperature (20-24°C), 37°C, 55°C and 70°C. These aliquots were then stored over a 28-day period and two replicate eppendorfs removed at 1 day, 2 days, 7 days, 14 days, 21 days and 28 days incubation. The *B. longum* ITT 13 CFS was screened for activity by removing 100 µl volumes from the incubated samples and tested using the MIC procedure in microtitre plates (Section 2.8.4). Dilutions of 1/2 to 1/128 of the *B. longum* ITT 13 CFS were performed with *S. xylosus* as the indicator strain in the assay. Arbitrary units per ml (AU/ml) were calculated as described in Section 2.8.2 using the highest dilution of the CFS displaying at least 50% inhibitory activity against the indicator strain: ($\mathrm{AU/ml} = \frac{inverse\ of\ highest\ dilution\ with \geq 50\%\ inhibition}{volume\ applied\ to\ well}\ x\ 1000$).

A minimum of three independent assays (n=3) with two replicates per assay were carried out for every temperature and timepoint. Antimicrobial activity was evaluated in relation to the control of the *B. longum* ITT 13 fermentate as measured prior to incubation at any of the temperatures, with an average value of 124.4 AU/ml ± 42.1. If a result was a similar or higher value than the control it was regarded as unaffected by the temperature, however if it was reduced below the control value consecutively, it was indicative that a possible reduction in activity may have taken place after incubation at that temperature.

2.10 Concentration of Antimicrobial Activity

2.10.1 Small-scale Tangential Flow Filtration (TFF) and Concentration

Small-scale TFF concentration was carried out as described by Grzenia *et al* (2008) using a Pellicon® XL 50 Cassette and Labscale^{TM} TFF System (Millipore).

2.10.1.1 Small-scale TFF with 10 kDa Filter

A volume of 500 mls of ITT 13 CFF was produced by two sequential centrifugation steps at 2722 x *g* for 5 minutes. After centrifugation a total volume of 450 mls was added to the TFF Resevoir and was concentrated to 50 mls using a 10 kDa filter by recirculation of the retentate back into the feed vessel until 400 mls permeate was collected and 50 mls retentate remained after approximately 2 hours at room temperature (22-25^{o}C). The retentate and the permeate were assayed for antimicrobial activity using the microdiffusion assay with *S. xylosus* as the indicator strain.

2.10.1.2 Small-scale TFF with 3 kDa Filter

2.10.1.2.1 TFF with 3 kDa Filter Run 1

B. longum ITT 13 was grown anaerobically in a 2-litre fermenter in MRSc broth for 17 hours and a 1 litre sample of the culture taken. The culture was heat shocked at 60^{o}C for 20 minutes to produce a fermentate and 200 ml of the upper layer of the 1 litre fermentate with was aspirated as visually there was less cell density present. The cells were centrifuged twice at 2722 x *g* for 5 minutes and the cell free fermentate was added to a 0.22 μm 200 ml stericup filtration unit to remove any remaining cells.

The above cell free supernatant (140 mls) was stored at 4^{o}C overnight and 125 ml was used for a TFF concentration step using a 3 kDa filter. A five-fold concentration was carried out of the cell free Fermentate reducing the volume from 125 mls to 25 mls. The permeate (filtrate) was

collected in a sterile duran (Permeate 1) and a 1.5 ml sample of the retentate was kept (Retentate 1). Buffer exchange was carried out to reduce organic acids present in the fermentate and to minimise any antimicrobial activity associated with acid or pH. Three buffer exchanges were carried out, each of which involved bringing the 25ml retentate volume to 125 mls and concentrating back down to 25 mls using sterile 50 mM, pH 4.9 Sodium Acetate buffer. Permeate and Retentate samples were kept from each run at 4^{o}C until tested. After three buffer exchanges the final retentate (Retentate 4) with a 25 ml volume was retained for testing. Antimicrobial activity was screened for each permeate and retentate fraction against *S. xylosus* using the microdiffusion assay as described in section 2.6. Test samples were filtered through a 0.45 µm filter before addition to plates, with the exception of the fermentate containing cells.

2.10.1.2.2 TFF with 3 kDa Filter Run 2

TFF concentration was again carried out using a 3 kDa filter. The *B. longum* ITT 13 was grown anaerobically in 200 mls MRSc broth for 19-20 hours at 37^{o}C. The culture was heat shocked at 60^{o}C for 15 minutes to produce a Fermentate and the cells were removed from 150 mls of fermentate using centrifugation at 2722 x *g* for 5 minutes. The supernatant was then filtered using a 0.22 µm stericup filtration unit to remove any remaining cellular debris. The cell free supernatant (125 mls) was then used for the TFF concentration step using a 3 kDa filter. A five-fold concentration step was carried out on the cell free fermentate reducing the volume from 125 mls to 25 mls over. The permeate was collected in a sterile duran (Permeate 1) and approximately 1.5 ml of the retentate was kept (Retentate 1). Two buffer exchanges were carried out, each of which involved bringing the 25ml retentate volume back to 125 mls and concentrating back down to a 25 ml volume using 0.02M HCl. The latter solvent was selected as this is the solvent used for nisin preparation (section 2.5) and shows no antimicrobial effect at a pH of 1.8 on M/D assay plates. Permeate and retentate samples were kept after each

exchange at 4°C until testing. A volume of 25 mls of the retentate (Retentate 3) was collected after the two buffer exchanges.

Antimicrobial activity was screened for each permeate and retentate fraction against *S. xylosus* using the microdiffusion assay as described in Section 2.6 and samples were filtered through a 0.45 µm filter before addition to plates, with the exception of the fermentate containing cells.

2.10.1.2.3 TFF with 3 kDa Filter Run 3

The third TFF concentration was carried out using a 3 kDa filter with a bigger volume of fermentate (950 mls), with the aim to generate a more concentrated sample in order to evaluate the lack of detectable antimicrobial activity from the methods described in sections 2.9.5.1 and 2.9.5.2. *B. longum* ITT 13 was grown overnight in 100 mls of MRSc broth and 1% of the fresh overnight culture was used to inoculate two 500 ml bottles of MRSc broth which were grown anaerobically at 37°C for a further 20 hours. The 500 ml fermentations were heat shocked at 60°C for 20 minutes due to the larger volume and centrifuged twice at 2722 x *g* for 5 minutes, before addition to 0.45 µm stericup to remove any remaining cells. A 15 ml volume of the fermentate with cells and the cell free fermentate before addition to the TFF were kept for testing in M/D assay. The cell free fermentate (approximately 950 mls) was added to the TFF and concentrated to 50 mls (a 19-fold concentration). Using the TFF apparatus the concentration step took 6 days at room temperature to concentrate the sample. Two buffer exchanges were carried out on 25 mls of the retentate, by bringing the volume up to 125 mls and concentrating back down to 25 mls using 50 mM, pH 4.8 NaAc buffer. Permeate and Retentate samples were kept after each exchange at 4°C until tested. After the two buffer exchanges 25 mls of the final retentate (Retentate 3) was kept at 4°C until tested. Antimicrobial activity was screened for each permeate and retentate fraction against *S. xylosus* using the microdiffusion assay as described in Section 2.6. Samples were filtered through a 0.45 µm filter

before addition to plates, with the exception of the fermentate containing cells. The various parameters and conditions of each TFF run are displayed in Table 2.4.

Table 2.4: Details of Conditions and Parameters of all small-scale Tangential Flow Filtration (TFF) and large-scale TFF (Crossflow) Concentration Runs of the *B. longum* ITT 13 Fermentate

TFF Run No.	Initial			Centrifugation		Steri cup (Filter size)	Buffer Exchange(s)			Vol. of Ret. After Conc.
	Vol.	pH	Media	x *g*	Time (mins)		No. of exchanges	Final pH	Solvent	
1 (10 kDa)	450 mls	4.26	MRSc	2722	5	Not used	-	-	-	50 mls
2 (3 kDa)	125 mls	4.19	MRSc	2722	5	0.22 µm	3	-	NaAc pH 4.9	25 mls
3 (3 kDa)	125 mls	4.25	MRSc	2722	5	0.22 µm	2	2.27	0.02N HCl pH 1.8	25 mls
4 (3 kDa)	950 mls	4.20	MRSc	2722	5	0.45 µm	2	4.67	NaAc pH 4.8	50 mls

Vol. of Ret. after Conc. denotes the final retentate volume after concentration of the fermentate

Table 2.4 displays all the variables and various steps taken for the preparation and concentration of *B. longum* ITT 13 fermentate using small-scale TFF concentration. The initial fermentate volumes used for concentration, their starting pH and the media they were grown in are compared, as are the centrifugation steps and the use of stericups to produce cell free fermentate before application to the TFF equipment. After concentration of the fermentate and buffer exchange the number of buffer exchanges carried out, the solvent used for buffer exchange and the final pH after buffer exchange were compared. The final volume the fermentate was concentrated to was also noted. This information allows for direct comparison of all small-scale TFF assays.

2.10.2 Large-scale TFF (Crossflow) Filtration and Concentration

2.10.2.1 Crossflow Concentration with MRSc Broth

In order to overcome the issues in relation to the length of the run times on the labscale TFF system the larger-scale tangential flow filtration Sartoflow Alpha Benchtop Crossflow System (Sartorius AG, Germany) was used for the production of CFF of ITT 13 and concentration of fermentate. *B. longum* ITT 13 was grown in 100 mls MRSc broth and incubated overnight at 37^{o}C under anaerobic conditions. A 1% inoculum of the fresh fermentate was added to three 1 litre bottles of MRSc broth which were incubated anaerobically, without stirring at 37^{o}C for 19 hours. An aliquot of 15 mls was kept from each of the 3 litres of fermentate and combined for testing as the 3 litres of fermentate was combined when added to the crossflow system. Cells were removed from the combined 2.95 L using a 0.22 µm filter on the crossflow system. A total of 2.62 litres of cell free fermentate was collected and then concentrated to 330 mls (Retentate 1) with a 1 kDa filter and 2.19 L of permeate 1 was collected. The retentate (310 mls) was added back into the crossflow system. Samples of the retentate 1 and permeate 1 were kept at 4^{o}C until tested. A volume of 3 litres of 50 mM pH 4.7 NaAc buffer was gradually added to Retentate 1 until 3015 mls (3.02 L) of Permeate 2 had been collected. Only 150 mls of retentate 2 was collected due to losses in the crossflow system. Samples were filtered through a 0.45 µm filter before testing to ensure no contamination was present from the crossflow system, with the exception of the fermentate containing cells. Antimicrobial activity was screened for each permeate and retentate fraction against *S. xylosus* using the microdiffusion assay as described in Section 2.6 and the MIC_{50} assay (Section 2.8.4) however there was no pH adjustment of the fermentate as described in 2.8.4.

2.10.2.2 Crossflow Concentration with Modified YPD Broth

B. longum ITT 13 was grown in 100 mls of a modified YPD (mYPD) broth (Meat Peptone 17.5g/L, Soybean peptone 17.5g/L, Glucose 40g/L, Yeast Extract 30g/L, L-Cysteine 0.05%, - C. Whelan Personal Communication, 2019) and incubated overnight under anaerobic conditions for 18 hours. A 1% inoculum of the fresh fermentate was added to three 1 litre bottles of mYPD broth and incubated anaerobically without agitation at 37ºC for 19 hours. The 3 litres of fermentate were combined and 40 mls was retained for testing. Cells were removed from 2.96 L of the fermentation using a 0.22 µm filter on the crossflow system, with 2.65 litres of cell free fermentate collected and a sample kept at 4ºC until testing. The cell free fermentate (2.55 L) was added back into the crossflow system and initially concentrated to 350 mls (Retentate 1) with a 1 kDa filter, with 2.20 L of Permeate 1 collected. Samples of the Retentate 1 and Permeate 1 were kept at 4ºC until testing. Retentate 1 (310 mls) underwent a buffer exchange with 3 litres of 50 mM pH 4.7 Sodium Acetate buffer. The buffer was gradually added to the vessel until approximately 3 litres of permeate was collected (Permeate 2) and 290 mls of Retentate 2 remained. Samples were filtered through a 0.45 µm filter before testing, with the exception of the fermentate containing cells. Antimicrobial activity was screened for each permeate and retentate fraction against *S. xylosus* by the microdiffusion assay as described in Section 2.6 and the MIC_{50} assay as in Section 2.8.4 but without pH adjustment of the fermentate. Protein concentration of all samples was determined using the BCA assay (Section 2.9.7).

The various parameters and conditions of each Crossflow run are presented in Table 2.5.

Table 2.5: Details of Conditions and Parameters of all small-scale Tangential Flow Filtration (TFF) and large-scale TFF (Crossflow) Concentration Runs of the *B. longum* ITT 13 Fermentate

TFF Run No.	Initial			Centrifugation		Steri cup (Filter size)	Buffer Exchange(s)			Vol. of Ret. After Conc.
	Vol.	pH	Media	x *g*	Time (mins)		No. of exchanges	Final pH	Solvent	
5 (Cross flow 1kDa)	2.62 L	4.21	MRSc	N/A	N/A	N/A	1	4.46	NaAc pH 4.7	330 mls
6 (Cross flow 1kDa)	2.55 L	4.60	mYPD	N/A	N/A	N/A	1	4.67	NaAc pH 4.7	350 mls

Vol. of Ret. after Conc. denotes the final retentate volume after concentration of the fermentate

Table 2.5 displays the details of the preparation and concentration of the *B. longum* ITT 13 fermentate for large-scale TFF, also known as Crossflow, concentration. The volumes of fermentate used for concentration, their starting pH and the media they were grown in are compared. The cell-free fermentate generated on the crossflow system therefore no centrifugation or stericup steps were required. The number of buffer exchanges carried out and the solvent used for the exchange were compared, as well as the final pH of the retentate after buffer exchange and the final volume. This allows for comparison of both crossflow runs.

2.11 Bicinchoninic Acid (BCA) Assay

The BCA assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific™) was performed on fermentate from various assays and permeate and retentate samples from the TFF and crossflow runs in order to quantify the protein concentration. The BCA reaction involves the reduction of Cu^{2+} to Cu^{1+} by protein, which produces a purple colour (A562 nm) which is proportional to the concentration of protein in the sample (ThermoFisher Scientific, 2019). Bovine serum albumin standards of 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml were prepared in sterile H_2O and 25 µl volumes added to microtitre plates. Samples (25 µl) of the fermentate, permeate, retentate and broth samples were also added to the microtitre plates. The working reagent was added in 200 µl volumes to each well containing the standard or test sample, the plate was then mixed on a plate mixer for 30 seconds before incubation at 37^{o}c for 30 minutes. The A562 nm was read using a plate reader and a calibration curve was constructed for the concentration of the BSA standards versus the A562 nm. The equation of the line was used to determine the concentration of the unknown samples.

2.12 Statistical Analysis

Standard deviation was calculated for all replicates using either GraphPad Prism 8 software or where GraphPad Prism 8 could not be used an online standard deviation calculator was used, which is available at: https://www.mathsisfun.com/data/standard-deviation-calculator.html. All statistical analysis including P-value determination was carried out using GraphPad Prism 8 software.

CHAPTER 3

Spectrum of Antibacterial and Antifungal Activity of the *Bifidobacterium longum* ITT 13 Fermentate

Bifidobacteria are a genus of bacteria that have been isolated from breast-fed neonates with links to a healthier digestive system (Cooke *et al.*, 2005; Yamada *et al.*, 2017). *Bifidobacteria*, along with *Lactobacilli*, are primarily employed as probiotic organisms and are also introduced as starter cultures in food due to their probiotic use and health benefits on the gastrointestinal system of the host (FAO/WHO, 2006). *Bifidobacteria* display probiotic properties including resistance to the acidic conditions of the gastrointestinal system and resistance to bile salts, modulation of the immune system, prevention of diarrhoea and the production of bioactive metabolites such as organic acids and bacteriocins that display an antimicrobial effect to prevent pathogen colonisation of the gut (Makras and De Vuyst, 2006; Lee and O'sullivan, 2010; Chenoll *et al.*, 2016; Vlasova *et al.*, 2016; Llamas-Arriba *et al.*, 2019; Ruiz-Aceituno *et al.*, 2020).

The antimicrobial activity and mechanisms of action associated with both organic acid production and bacteriocin production by bacteria including *Bifidobacteria* have been explored in detail in Sections 1.3 and 1.4. The production of these antimicrobial metabolites are an important characteristic of probiotic strains to protect the host from pathogenic colonisation (Vlasova *et al.*, 2016), with potential applications for treatment in regulation of the gut microbiome and antibiotic associated diarrhoea (Selinger *et al.*, 2013; Hayes and Vargas, 2016; Litao *et al.*, 2018). These bacteria and their metabolites also have potential application in the food industry for use in biopreservation to increase the shelf-life of food products, as starter cultures in fermented food and in food safety applications including use as biocontrol agents to prevent contamination by pathogens during food production (Butel, 2014; Hossain *et al.*, 2017; Sireswar *et al.*, 2017; Singh, 2018; Skariyachan and Govindarajan, 2019; Thery *et al.*, 2020).

A suspected *Bifidobacteria* strain, designated ITT 13, was isolated from the gastrointestinal systems of neonates in previous research carried out within our laboratory (Cooke *et al.*, 2005).

Preliminary research work carried out within previous research projects has indicated that strain ITT 13 was most likely thought to be a *Bifidobacteria* strain, specifically *B. longum*. This strain displayed significant antimicrobial activity in preliminary research work and was thus analysed for further identification and assessment within the research carried out in this project. To further explore the spectrum of activity a combination of screening methods were used including the microdiffusion assay and MIC_{50} assay, as carried out by Wayah and Philip (2018), with both proving effective for determination. This research was also carried out to determine the nature of the antimicrobial activity produced by ITT 13 and assess its suitability for potential use as a probiotic or biopreservative by screening against food spoilage related and industrially relevant bacterial and fungal strains.

Results:

3.1 Characterisation of Strain ITT 13

3.1.1 Characterisation and Colony Morphology of *B. longum* ITT 13

The cryopreserved stock of strain ITT 13, which had been stored at -80^{o}C, was grown on MRS agar supplemented with 0.05% *L*-cysteine (MRSc) and subcultured twice on MRSc agar to ensure purity of colonies. The colony morphology was that of white, round colonies approximately 1-2 mm in diameter (Figure 3.1). Gram staining of the colonies revealed they were Gram positive rods (Figure 3.2), with distinctive bifid 'v/y' formation of rods observed (circled in Figure 3.2), that is commonly observed in *Bifidobacteria* spp (Dhanashree *et al.*, 2017). The strain grew well under strict anaerobic conditions, at 37^{o}C in MRSc broth with no pH control for 20 hours – producing approximately 10^9 CFU/ml and a pH of pH 4.2-4.3. CFU/ml were calculated by serially diluting and plating the fermentate before heat inactivation onto MRSc agar and growing overnight before counting colonies to determine CFU/ml (calculation not presented).

Figure 3.1: Colonies of Strain ITT 13 on MRS agar with 0.05% *L*-Cysteine. Figure 3.1 displays the colony morphology of the ITT 13 strain on MRSc agar after 3 subcultures from frozen cryovials stored at -80ºC. Colonies observed were white, raised and round with an approximate diameter of 1-2 mm.

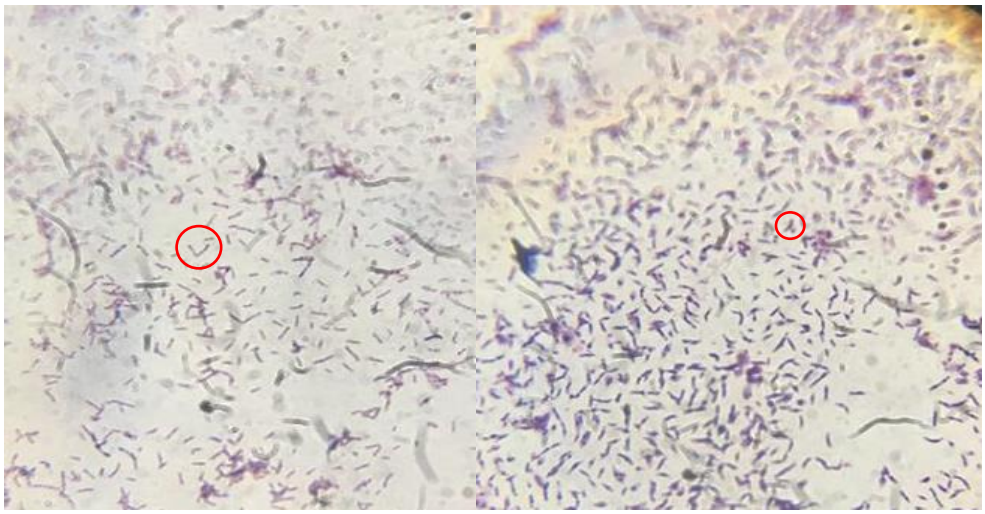

Figure 3.2: Gram Stain of Strain ITT 13 Displaying Purple Rods. The distinctive bifid formation of the ITT 13 rods that are characteristic of *Bifidobacteria* are circled in Figure 3.2 at 1000x magnification after Gram staining.

3.1.2 Phenotypic Characterisation of *B. longum* ITT 13 using the Biolog System

Biolog identification, as described in Section 2.7, was used to confirm the identity of strain ITT 13. The strain was confirmed to be a *Bifidobacterium longum*, with 0.995 probability as Biolog speciation information presented in Figure 3.3.

Date & Time of Read: Nov 12 2019 3:57 PM
Biolog ID DB: An601.kid

Result: Species ID: Bifidobacterium longum
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.995	0.866	1.934	An GP-Rod	Bifidobacterium longum
2	0.002	0.002	4.033	An GP-Rod	Bifidobacterium suis
3	0.002	0.001	4.095	An GP-Rod	Bifidobacterium choerinum
4	0.001	0.000	4.420	An GP-Rod	Clostridium clostridiiforme

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1	15	-4	-8	-4	-7	-4	-2	-6	-8	< 80
B	-5	-6	< 353	13	{ 52	3	-3	-2	2	< 102	-6	-6
C	-4	-3	-4	< 131	< 147	< 683	< 252	-2	< 518	-3	< 325	3+
D	< 540	< 385	< 317	-4	< 82	< 492	-6	-2	-1	< 391	< 582	-3
E	< 397	4	-7	-10	10	-4	-1+	4	-1	13	-7	-3
F	-2	-6	-7	25	4	0	-2	{ 37	-1	2	-1	4
G	12	-6	-7	1	-3	-5	-2	-2	2	13	0	5
H	16	{ 45	-6	-7	-5	{ 60	-6	2	-6	-9	-6	-7

Report Date: Nov 12 2019 4:05 PM

Figure 3.3: Biolog Identification of Strain ITT 13 as *Bifidobacterium longum* with 0.955 probability. Biolog identification was carried out as described in Section 2.7.

3.2 Antibacterial Activity of *B. longum* ITT 13

The spectrum of antibacterial activity produced by *B. longum* ITT 13 fermentate was determined by exposing a select number of test strains to the heat inactivated fermentate as described in Section 2.4.2 using the microdiffusion assay (Sections 2.6 and 2.8.2). The results are presented in Section 3.2.1. The precise dosage required to cause an antibacterial effect was then more accurately determined for selected strains that were shown to be inhibited by the *B. longum* ITT 13 fermentate by using the MIC_{50} assay (Section 2.8.4). The MIC_{50} results generated are presented in Section 3.2.2.

3.2.1 Antibacterial Spectrum of Activity Using Microdiffusion Plate Assay

The microdiffusion (M/D) plate assay is a commonly used method for the screening of antimicrobial activity produced by microbial sources (Miao *et al.*, 2014; Balouiri, Sadiki and Ibnsouda, 2016; Mohammadi *et al.*, 2018) and was selected for the initial screening of the *B. longum* ITT 13 heat inactivated fermentate. *B. longum* ITT 13 fermentate was serially diluted down to 1/16 with MRSc broth and screened for antibacterial activity against 15 bacterial strains. Nisin was used as a positive control from an undiluted form (neat) to a 1/16 dilution and MRSc broth was used as a negative control in the M/D assay. Results were interpreted as the presence/absence of a zone of inhibition at each dilution of fermentate, determining antibacterial activity against that strain and the diameter of any resulting zone to compare effectiveness of the antimicrobial activity. Comparative results for the antibacterial activity of the fermentate against each of the indicator strains in terms of average AU/ml are presented in Figure 3.4. AU/ml is calculated as described Section 2.8.2. The quantitative analysis of the M/D plates i.e. diameter of zone of inhibition (mm), against susceptible strains is presented in Table 3.1 (Gram positive strains) and Table 3.2 (Gram negative strains). Figures 3.5 and 3.6 display M/D plates of a Gram-positive and Gram-negative strain with zones of inhibition due to the *B. longum* ITT 13 fermentate.

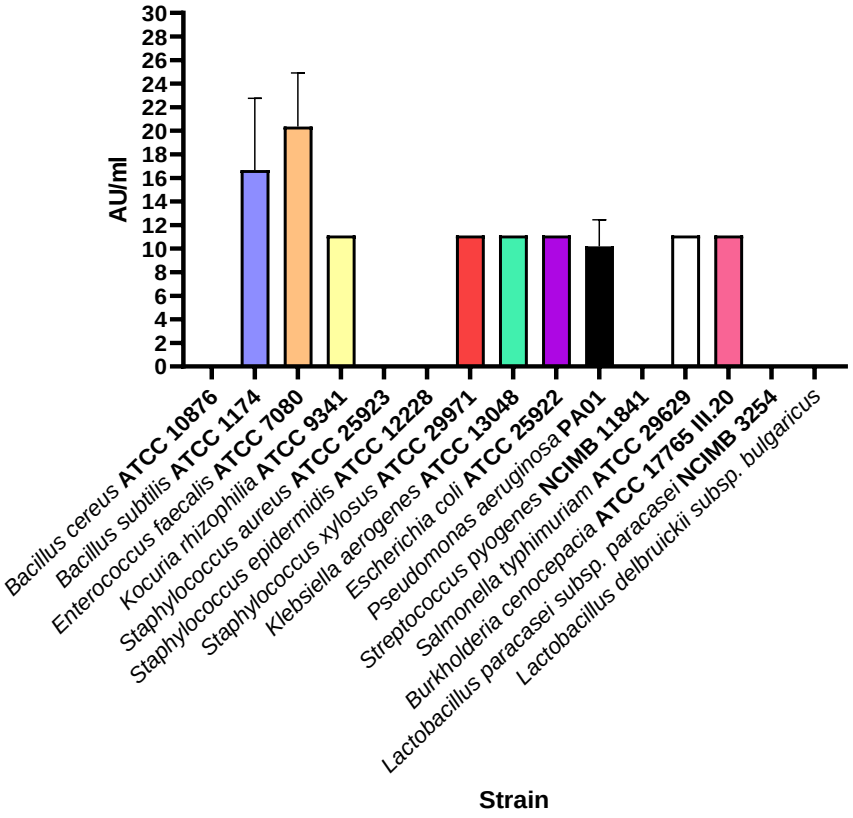

Figure 3.4: The AU/ml of the *B. longum* ITT 13 fermentate against a number of indicator Gram-positive and Gram-negative bacterial strains. The AU/ml was determined as described in Section 2.8.2, the absence of AU/ml indicates the lack of antimicrobial activity detected against that indicator strain.

In Figure 3.4, the antimicrobial activity of the *B. longum* ITT 13 fermentate against the screened bacterial strains was graphed in AU/ml (Section 2.8.2). Of the 15 strains screened for antibacterial susceptibility to the fermentate, only six strains did not display growth inhibition when exposed to the fermentate: *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* NCIMB 11841, *Lactobacillus paracasei* subsp. *paracasei* NCIMB 3254 and *Lactobacillus delbruckii* subsp. *bulgaricus* NCIMB 11778. *Bacillus subtilis* ATCC 1174 and *Enterococcus faecalis* ATCC 7080 both displayed the highest levels of susceptibility to the fermentate, giving values of 16.7 AU/ml and 20.4 AU/ml respectively. The fermentate generated zones of inhibition on the M/D assay plates giving an average of 10.2-11.1 AU/ml against *Kocuria rhizophilia* ATCC 9341, *Staphylococcus xylosus* ATCC 29971, *Klebsiella aerogenes* ATCC

13048, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PA01, *Salmonella typhimurium* ATCC 29629 and *Burkholderia cenocepacia* ATCC 17765 III.20.

Table 3.1: Antibacterial Activity of *B. longum* ITT 13 Fermentate against selected Gram-Positive Strains

Sample:	***B. subtilis***	***E. faecalis***	***K. rhizophilia***	***S. xylosus***
	Avg. Zone Size (mm) ± SD)	**Avg. Zone Size (mm) ± SD**	**Avg. Zone Size (mm) ± SD**	**Avg. Zone Size (mm) ± SD**
Fermentate Undiluted	3.1 ± 0.6	3.6 ± 0.3	3.3 ± 0.2	4.2 ± 0.2
Fermentate 1/2	1.3 ± 0.4	3.2 ± 0.4	1.7 ± 0.4	2.4 ± 0.2
Fermentate 1/4	0.3 ± 0.4	2.3 ± 0.4	0 ± 0	0 ± 0
Fermentate 1/8	0 ± 0	0 ± 0	0 ± 0	0 ± 0
AU/ml of Fermentate	17 ± 6	20 ± 4	11 ± 0	11 ± 0
Nisin Undiluted (1000 IU/ml)	2.8 ± 0.2	2.4 ± 0.2	3.5 ± 0.4	4.4 ± 0.2
Nisin 1/2 (500 IU/ml)	1.8 ± 0.3	1.9 ± 0.2	2.6 ± 0.5	3.8 ± 0.1
Nisin 1/4 (250 IU/ml)	1.0 ± 0	1.3 ± 0.3	1.8 ± 0.3	3.4 ± 0.1
Nisin 1/8 (125 IU/ml)	0.7 ± 0.2	0.8 ± 0.2	1.3 ± 0.3	3.0 ± 0.0
Nisin 1/16 (62.5 IU/ml)	<0.5 ± 0	0.5 ± 0	0.8 ± 0.2	2.5 ± 0.1
MRSc Broth	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Nisin displayed inhibition against the gram-positive strains, while, as expected, no activity was shown for nisin against the gram-negative strains. Both *B. subtilis* and *E. faecalis* displayed the highest levels of susceptibility to a 1/4 fermentate dilution (average of 16.7 AU/ml and 20.4 AU/ml respectively according to Figure 3.4), with the other strains displaying susceptibility to a 1/2 dilution (10.2-11.1 AU/ml). The largest zones of inhibition for the *B. longum* ITT 13 fermentate were observed against *S. xylosus* at 4.2 mm for the undiluted fermentate and 2.4 mm for the 1/2 dilution. The smallest zones of inhibition were observed against *B. subtilis* at 3.1 mm for the undiluted fermentate, 1.3 mm for the 1/2 dilution and 0.3 mm for the 1/4 dilution.

Table 3.2: Antibacterial Activity of *B. longum* ITT 13 Fermentate against selected Gram-Negative Strains

Sample:	*K. aerogenes*	*E. coli*	*P. aeruginosa*	*S. typhimurium*	*B. cenocepacia*
	Avg. Zone Size (mm) ± SD	Avg. Zone Size (mm) ± SD	Avg. Zone Size (mm) ± SD	Avg. Zone Size (mm) ± SD	Avg. Zone Size (mm) ± SD
Fermentate Undiluted	2.3 ± 0.5	3.6 ± 0.3	3.4 ± 0.3	2.7 ± 0.5	4.1 ± 0.3
Fermentate 1/2	1.3 ± 0.4	2.5 ± 0.4	1.5 ± 0.8	1.3 ± 0.4	2.1 ± 0.2
Fermentate 1/4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fermentate 1/8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
AU/ml of Fermentate	11 ± 0	11 ± 0	10 ± 2	11 ± 0	11 ± 0
Nisin Undiluted (1000 IU/ml)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Nisin 1/2 (500 IU/ml)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Nisin 1/4 (250 IU/ml)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Nisin 1/8 (125 IU/ml)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
MRSc Broth	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

As gram-negative strains are resistant to nisin (Matsusaki, Sonomoto and Ishizaki, 1998) inhibition of the selected strains by nisin was not expected nor experimentally determined. The MRSc broth used as the negative control also displayed no inhibition of the gram-negative strains selected for testing. Antimicrobial activity by the fermentate was detected up to a 1/2 dilution against all the gram-negative strains tested, with an average of 10-11 AU/ml determined. The largest zones of inhibition were observed against *B. cenocepacia* with zones of approximately 4 mm detected on the M/D plates for the undiluted fermentate and 2 mm for the 1/2 dilution, thus correlating with 11 AU/ml. *B. cenocepacia* is an opportunistic pathogen that can pose a significant risk for cystic fibrosis patients and is known to be naturally resistant to a variety of antibiotic classes, thus making it a potentially dangerous pathogen (Scoffone *et al.*, 2017). The ability of *B. longum* ITT 13 to inhibit this strain with the largest zones of inhibition is notable. The smallest zones of inhibition were observed against *Klebsiella*

aerogenes at 2.3 mm for the undiluted fermentate and 1.3 mm for the 1/2 dilution, which was also equivalent to 11 AU/ml of activity as the activity was detected up to a 1/2 dilution.

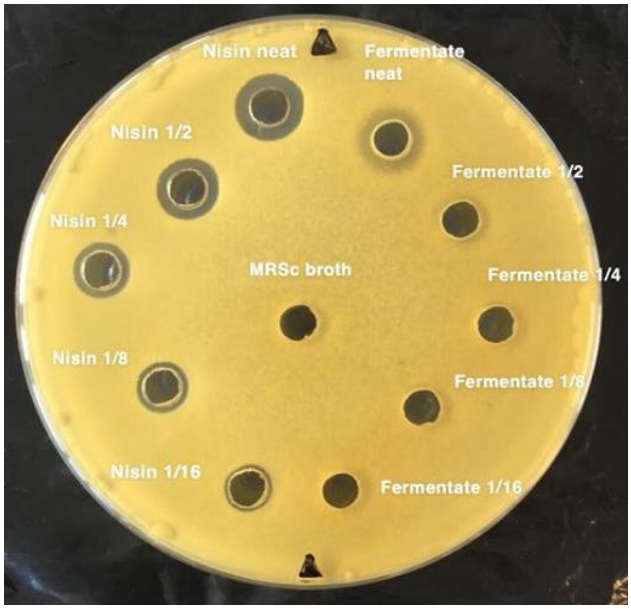

Figure 3.5: Antibacterial Activity of the *B. longum* ITT 13 Fermentate against Gram-Positive *K. rhizophilia* ATCC 9341. Nisin (62.5 ($^{1}/_{16}$) - 1000 (neat) IU/ml) was used as a positive control in the assay. The indicator strain was grown in TSA.

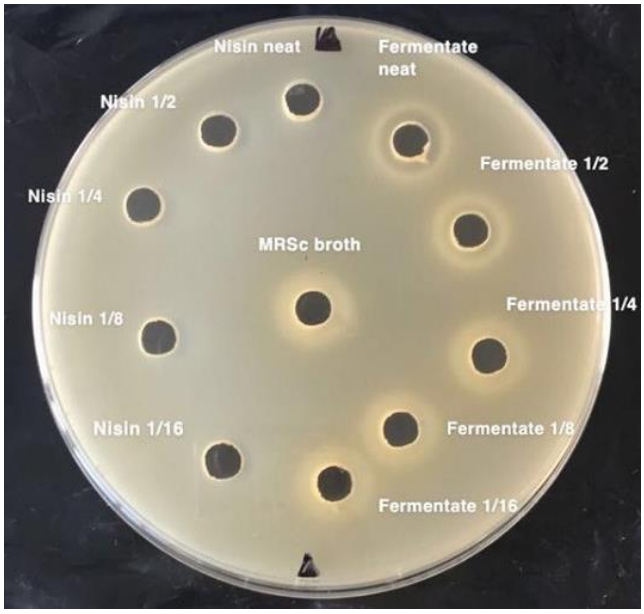

Figure 3.6: Antibacterial Activity of the *B. longum* ITT 13 Fermentate against Gram-Negative *E. coli* ATCC 25922. Nisin (62.5 - 1000 IU/ml) was used as a control in the assay with a lack of activity against the Gram-negative strain taken as validation of the assay. The opaque ring observed around the negative control and 1/4-1/16 fermentate dilutions are a result of denser growth of the *E. coli* ATCC 25922 around the well where additional nutrients have been provided by the MRSc broth as it diffuses into the agar. The indicator strain was grown in TSA.

3.2.2 MIC_{50} Testing of the *B. longum* ITT 13 Fermentate

The use of more than a single screening procedure was important to determine the antimicrobial activity of *B. longum* ITT 13, due to varying sensitivity of the assays. The microdiffusion assay had been carried out primarily for qualitative screening and select strains were then chosen for the MIC_{50} assay in microtitre plates. The MIC assay has been shown to be more sensitive, which is likely due to the removal of the agar interface, allowing the microbial cells to come into direct contact with the fermentate in a liquid medium, as observed by Inturri *et al.*, (2019) as when testing the cell free supernatants of various *B. longum* and *L. rhamnosus* strains, higher levels of antimicrobial activity were detected on average using the MIC microtitre plate procedure in comparison to the microdiffusion (well-diffusion) method.

Due to time constraints, all indicator strains could not be tested using the MIC_{50} method, therefore three gram-positive and three gram-negative strains were selected for the MIC_{50} assay. This was carried out using the method described in Section 2.8.4, with the fermentate pH adjusted from pH 4.2 to pH 4.5 to minimise any contribution of the acid produced by the *B. longum* ITT 13 strain to the antimicrobial activity. The selected indicator strains used were *B. subtilis* ATCC 1174, *E. faecalis* ATCC 7080, *S. xylosus* ATCC 29971, *E. coli* ATCC 25922, *P. aeruginosa* PA01 and *S. typhimurium* ATCC 29629. Results are presented in Figures 3.7-3.12 and AU/ml were calculated (Section 2.8.2) based on the highest dilution displaying at least 50% inhibition in comparison to the control, as calculated by Lim (2016).

For assessment of the antibacterial activity of the fermentate at pH 4.5 it was compared to the growth control of MRSc broth which should cause no inhibition of the tested strains. A separate sample of MRSc broth was also adjusted to pH 4.5 to assess the antibacterial activity of this pH and it was compared to a separate growth control of MRSc broth (10 ml volume) diluted with 8-8.5 mls of sterile H_2O. The reason for the dilution of this MRSc broth with 8-8.5 mls of sterile H_2O was due to the fact the pH 4.5 MRSc broth was adjusted with 8-8.5 mls

of 0.1M lactic acid and the dilution of nutrients in the fermentate meant a growth/negative control of MRSc broth with diluted nutrients was also required. This allowed the inhibition of the fermentate at pH 4.5 and of the MRSc broth at pH 4.5 to be directly comparable.

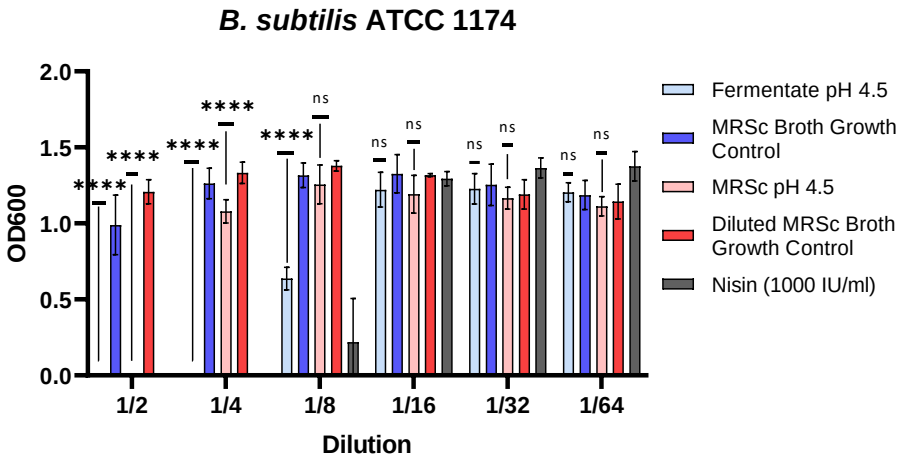

Figure 3.7: The growth (OD600) of *B. subtilis* ATCC 1174 and MIC_{50} determination of the *B. longum* ITT 13 Fermentate (pH 4.5) and MRSc broth (pH 4.5). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition of the indicator strain. Nisin undiluted was 1000 IU/ml while each successive serial dilution reduced the concentration by half. Standard deviations were determined for a minimum of three independent assays carried out on separate days (n=3) and statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison assay. The pH 4.5 fermentate was compared to the control of MRSc broth, while the pH 4.5 MRSc media was compared to the control of diluted MRSc at each dilution to assess for significant deviations in growth, as explained in Section 2.8.4. **** = p-value ≤0.0001, ns = non-significant p-value >0.05.

Antimicrobial activity for the pH 4.5 fermentate was detected up to a 1/8 dilution ($p<0.0001$), equating 80 AU/ml. Antimicrobial activity was detected for the pH 4.5 MRSc broth up to a 1/4 dilution ($p<0.0001$), however as the inhibition detected was minimal with an OD600 of 1.08 in comparison to the growth control of 1.33, and as the MIC_{50} is calculated from the dilution displaying at least 50% inhibition, the pH 4.5 MRSc broth thus displayed inhibition equivalent to 20 AU/ml as compared to the 80 AU/ml for the fermentate. Nisin as a positive control displayed antimicrobial activity also up to a 1/8 dilution which is equivalent to 80 AU/ml.

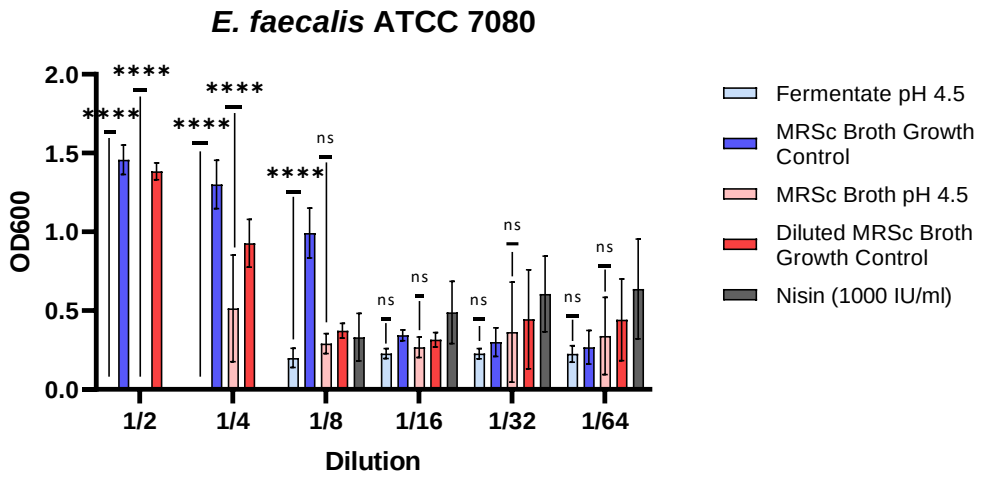

Figure 3.8: The growth (OD600) of *E. faecalis* ATCC 7080 and MIC_{50} Determination for the *B. longum* ITT 13 Fermentate (pH 4.5) and MRSc broth (pH 4.5). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition. Nisin undiluted was 1000 IU/ml while each successive serial dilution reduced the concentration by half. Standard deviations were determined for a minimum of three independent assays carried out on separate days (n=3) and statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison assay. The pH 4.5 fermentate was compared to the control of MRSc broth, while the pH 4.5 MRSc media was compared to the control of diluted MRSc at each dilution to assess for significant deviations in growth as explained in Section 2.8.4. **** = p-value ≤0.0001, ns = non-significant p-value >0.05.

Antimicrobial activity for the pH 4.5 fermentate was detected up to a 1/8 dilution ($p<0.0001$) with an OD600 of 0.20 in comparison to the OD600 of the MRSc broth growth control of 0.99. This indicates more than 50% inhibition of the *E. faecalis* at this dilution in comparison to the 100% growth of the strain in the MRSc broth growth control, therefore generating an MIC_{50} of a 1/8 dilution equating 80 AU/ml for the fermentate.

MRSc broth was adjusted to pH 4.5 for comparison to the fermentate also at pH 4.5, to determine whether the antimicrobial activity was associated with the low pH. Antimicrobial activity was detected for the pH 4.5 MRSc broth up to a 1/4 dilution ($p<0.0001$), however as the inhibition was less than 50%, with an OD600 of 0.51 in comparison to the growth control of 0.93, the 1/2 dilution was the MIC_{50} with 20 AU/ml. Therefore, the pH 4.5 MRSc broth, which was equivalent to the pH 4.5 fermentate, had 20 AU/ml in comparison to the higher

activity of 80 AU/ml generated by the pH 4.5 fermentate. The increased antimicrobial activity of the pH 4.5 fermentate thus indicates that the fermentate has significantly more antimicrobial activity and can be interpreted as indicative of more than just a pH effect. Nisin as a positive control displayed antimicrobial activity also to a 1/4 dilution with 40 AU/ml.

Growth of the *E. faecalis* ATCC 7080 reduced at higher dilutions of all samples, likely indicative of reduced growth of the strain in the minimal MH media when nutrients provided by the samples had been diluted out. The samples had likely provided some nutrients at higher concentrations as they contained MRSc media which *B. longum* ITT 13 was grown in and which was used for controls.

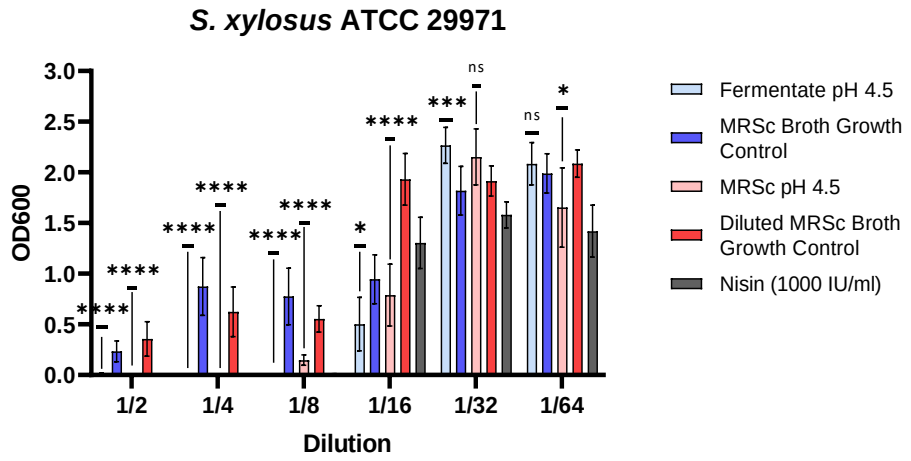

Figure 3.9: The growth (OD600) of *S. xylosus* ATCC 29971 and MIC_{50} determination for the *B. longum* ITT 13 Fermentate (pH 4.5) and MRSc broth (pH 4.5). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition. Nisin undiluted was 1000 IU/ml while each successive serial dilution reduced the concentration by half. Standard deviations were determined for a minimum of three independent assays carried out on separate days (n=3) and statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison assay. The pH 4.5 fermentate was compared to the control of MRSc broth, while the pH 4.5 MRSc media was compared to the control of diluted MRSc at each dilution to assess for significant deviations in growth as explained in Section 2.8.4. **** = p-value ≤0.0001, *** = p-value ≤0.001, * = p-value ≤0.05, ns = non-significant p-value >0.05.

For *S. xylosus* ATCC 29971, the fermentate adjusted to pH 4.5 caused 100% inhibition to the 1/8 dilution ($p<0.0001$) and 47% inhibition at the 1/16 ($p=0.0151$), therefore equating to 80 AU/ml based on at least 50% inhibition. Antimicrobial activity of at least 50% inhibition was detected for the pH 4.5 MRSc broth up to a 1/16 dilution ($p<0.0001$), with 160 AU/ml. Although the pH 4.5 fermentate visually resulted in more inhibition of the *S. xylosus* than the pH 4.5 MRSc broth at the 1/16 dilution, the AU/ml was worked out based on the % inhibition in comparison to the respective growth control. In the case of the pH 4.5 fermentate at the 1/16 dilution, it only resulted in 47% inhibition in comparison to the growth control of MRSc broth, however the pH 4.5 MRSc broth resulted in 59% inhibition in comparison to its growth control of diluted MRSc broth. Therefore, it was undetermined as to whether the antimicrobial activity caused by the fermentate in this case was primarily pH associated as it is evident that the pH

4.5 MRSc control displayed higher levels of antimicrobial activity against this strain than seen in the other strains tested. Nisin as a positive control displayed antimicrobial activity to a 1/8 dilution with 80 AU/ml.

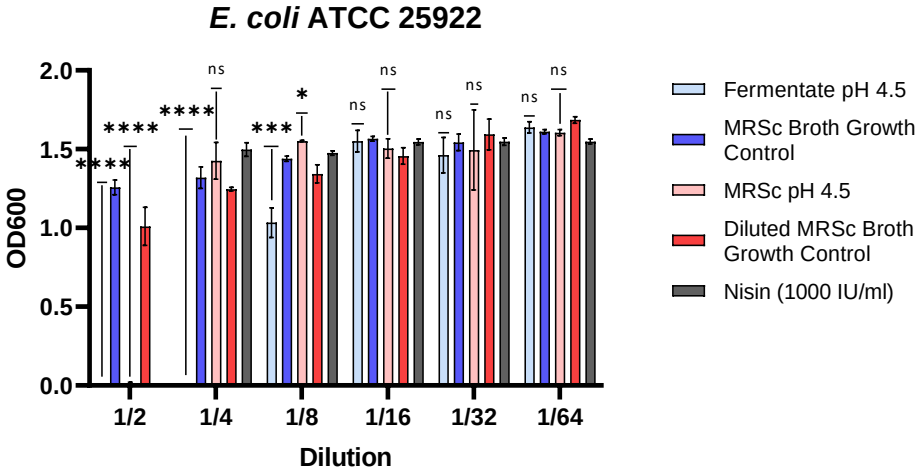

Figure 3.10: The growth (OD600) of *E. coli* ATCC 25922 and MIC_{50} determination for the *B. longum* ITT 13 Fermentate (pH 4.5) and MRSc broth (pH 4.5). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition. Nisin undiluted was 1000 IU/ml while each successive serial dilution reduced the concentration by half. Standard deviations were determined for a minimum of three independent assays carried out on separate days (n=3) and statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison assay. The pH 4.5 fermentate was compared to the control of MRSc broth, while the pH 4.5 MRSc media was compared to the control of diluted MRSc at each dilution to assess for significant deviations in growth as explained in Section 2.8.4.**** = p-value ≤0.0001, *** = p-value ≤0.001, * = p-value ≤0.05, ns = non-significant p-value >0.05.

Figure 3.10 depicts the inhibition of *E. coli* ATCC 25922 by the fermentate adjusted to pH 4.5 and the pH 4.5 MRSc broth. Antimicrobial activity for the pH 4.5 fermentate was detected up to a 1/8 dilution (p=0.006) with an OD600 of 1.03 in comparison to the MRSc broth growth control of 1.44 at the 1/8 dilution. Although there is a significant difference at the 1/8 dilution, as the inhibition was less than 50% of the growth control, the MIC_{50} result is taken from the 1/4 dilution to give a measured antimicrobial activity of 40 AU/ml. Antimicrobial activity was

detected for the pH 4.5 MRSc broth up to a 1/2 dilution with 100% inhibition ($p<0.0001$), equating 20 AU/ml in comparison to the pH 4.5 fermentate of 40 AU/ml. The higher antimicrobial activity associated with the pH 4.5 fermentate (40 AU/ml) in comparison to the equivalent pH 4.5 MRSc broth (20 AU/ml) indicates that the antimicrobial activity is likely not associated with the low pH alone. Nisin as a positive control displayed antimicrobial activity also to a 1/2 dilution to give a measured inhibitory level of 20 AU/ml.

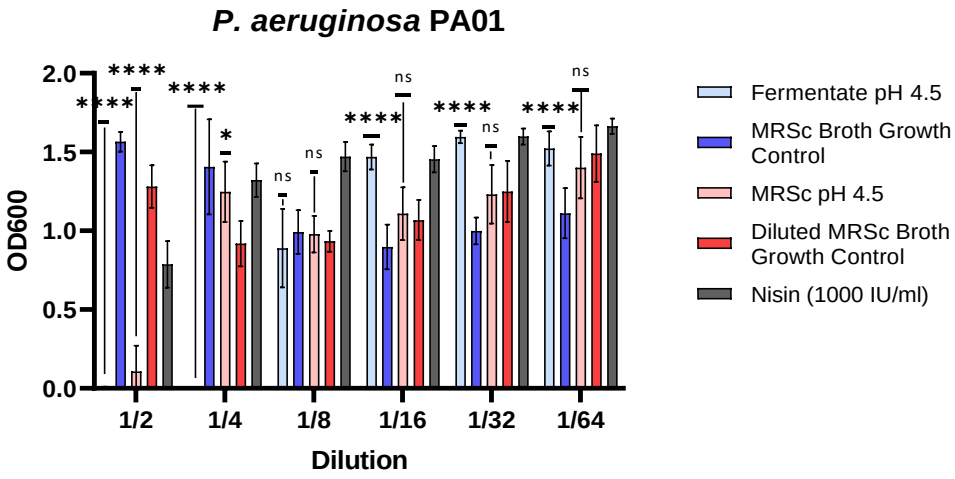

Figure 3.11: The growth (OD600) of *P. aeruginosa* PA01 and MIC_{50} determination for the *B. longum* ITT 13 Fermentate (pH 4.5) and MRSc broth (pH 4.5). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition. Nisin undiluted was 1000 IU/ml while each successive serial dilution reduced the concentration by half. Standard deviations were determined for a minimum of three independent assays carried out on separate days (n=3) and statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison assay. The pH 4.5 fermentate was compared to the control of MRSc broth, while the pH 4.5 MRSc media was compared to the control of diluted MRSc at each dilution to assess for significant deviations in growth as explained in Section 2.8.4. **** = p-value ≤0.0001, * = p-value ≤0.05, ns = non-significant p-value >0.05.

Antimicrobial activity (100%) was detected against *P. aeruginosa* PA01 by the fermentate adjusted to pH 4.5 ($p<0.0001$) with an MIC_{50} of the 1/4 dilution giving an inhibitory level of 40 AU/ml. In comparison, the equivalent pH 4.5 MRSc broth displayed antimicrobial activity only at a 1/2 dilution ($p<0.0001$), with an average OD600 of 0.11 at the 1/2 dilution in

comparison to the diluted MRSc broth growth control of 1.28. This equated to a measured inhibitory level of 20 AU/ml for the pH 4.5 MRSc broth in comparison to the 40 AU/ml of the pH 4.5 fermentate, indicating higher levels of antimicrobial activity associated with the pH 4.5 fermentate. This indicates the antimicrobial activity of the fermentate against *P. aeruginosa* is likely not associated with pH alone. Nisin as a positive control did not display complete inhibition at any dilution. Nisin is not considered to be active against gram-negative organisms and as expected, gave no inhibition of the indicator strain of *P. aeruginosa.* The MRSc broth control also resulted in decreasing growth of the indicator strain as the dilutions increased which is likely due to the diluting out of the additional nutrients provided by the MRSc broth.

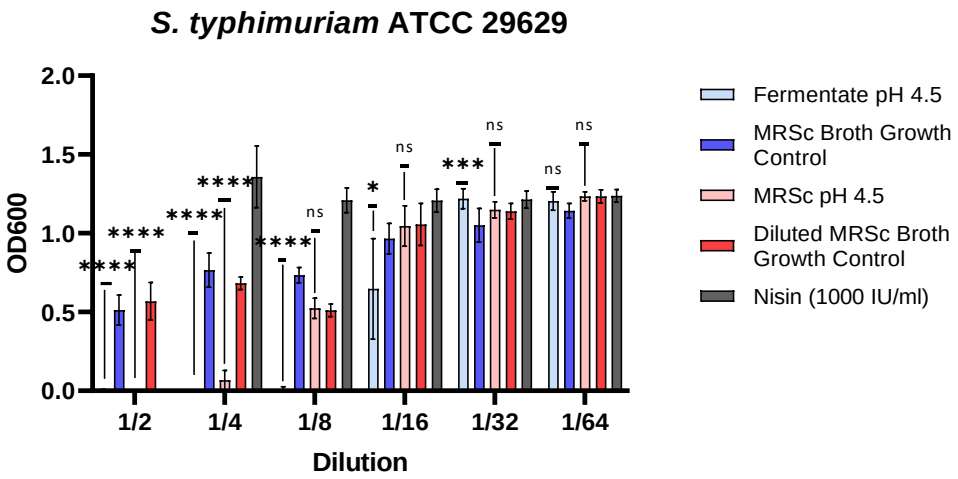

Figure 3.12: The growth (OD600) of *S. typhimurium* ATCC 29629 and MIC_{50} determination for the *B. longum* ITT 13 Fermentate (pH 4.5) and MRSc broth (pH 4.5). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition. Nisin undiluted was 1000 IU/ml while each successive serial dilution reduced the concentration by half. Standard deviations were determined for a minimum of three independent assays carried out on separate days (n=3) and statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison assay. The pH 4.5 fermentate was compared to the control of MRSc broth, while the pH 4.5 MRSc media was compared to the control of diluted MRSc at each dilution to assess for significant deviations in growth as explained in Section 2.8.4. **** = p-value ≤0.0001, *** = p-value ≤0.001, * = p-value ≤0.05, ns = non-significant p-value >0.05.

Antimicrobial activity (100% inhibition) was detected against the gram-negative strain *S. typhimurium* ATCC 29629 by the *B. longum* ITT 13 pH 4.5 fermentate to a 1/8 dilution ($p<0.0001$) to give an inhibitory measure of 80 AU/ml. The *B. longum* ITT 13 pH 4.5 fermentate also displayed inhibitory action to the *S. typhimurium* strain for the 1/16 dilution, with the significant p-value of $p = 0.0221$, but as the inhibition was less than 50% (33%) it was not used for calculation of the MIC_{50} and AU/ml.

The pH 4.5 MRSc broth displayed antimicrobial activity to a 1/4 dilution ($p<0.0001$) with an average OD600 of 0.07 in comparison to the diluted MRSc broth growth control of 0.68, thus providing an inhibitory level of 40 AU/ml. In comparison to the pH 4.5 fermentate which gave an inhibitory measure of 80 AU/ml toward the *S. typhimurium*, the equivalent pH 4.5 MRSc broth displayed less antimicrobial activity (40 AU/ml), indicating the antimicrobial activity associated with the pH 4.5 fermentate is likely not solely associated with the low pH. Nisin as a positive control displayed antimicrobial activity only at a 1/2 dilution, and as nisin would not be expected to show any significant inhibition of a gram-negative strain, this inhibition at the 1/2 dilution may be associated with sensitivity of the strain to the 0.02N HCl used for the preparation of 1000 IU/ml nisin.

Table 3.3: The MIC_{50} and AU/ml of the *B. longum* ITT 13 Fermentate (pH 4.5), the MRSc Broth (pH 4.5) and Nisin (1000 IU/ml) Against Various Bacterial Strains.

Strain:	*B. longum* ITT 13 Fermentate pH 4.5		MRSc Broth pH 4.5		Nisin (1000 IU/ml)	
	AU/ml	MIC_{50}	AU/ml	MIC_{50}	AU/ml	MIC_{50}
B. subtilis ATCC 1174	80	1/8	20	1/2	80	1/8
E. faecalis ATCC 7080	80	1/8	20	1/2	40	¼
S. xylosus ATCC 29971	80	1/8	160	1/16	80	1/8
E. coli ATCC 25922	40	1/4	20	1/2	N/A	N/A
P. aeruginosa PA01	40	1/4	20	1/2	N/A	N/A
S. typhimurium ATCC 29629	80	1/8	40	1/4	N/A	N/A

The fermentate displayed an MIC_{50} of a 1/8 dilution and 80 AU/ml against *B. subtilis* ATCC 1174, *E. faecalis* ATCC 7080, *S. xylosus* ATCC 29971 and *S. typhimurium* ATCC 29629. For both *E. coli* ATCC 25922 and *P. aeruginosa* PA01 an MIC_{50} of a 1/4 dilution and 40 AU/ml were achieved by the fermentate.

The MRSc broth (pH 4.5) displayed the highest antimicrobial activity against *S. xylosus* ATCC 29971 with 160 AU/ml and *S. typhimurium* ATCC 29626 with 40 AU/ml in comparison to 20 AU/ml for the remaining strains. With the exception of *S. xylosus* ATCC 29971, the antimicrobial activity of the MRSc broth adjusted to pH 4.5 was less than the antimicrobial activity of the fermentate displayed against each strain, indicating that pH effect is likely not the only factor responsible for activity.

The results for the positive control of nisin were as expected for the gram-positive strains. Although the IU/ml of the nisin at each dilution is known, AU/ml were calculated for direct comparison to the samples. The nisin demonstrated inhibition similar to the *B. longum* ITT 13 fermentate with 80 AU/ml for both nisin and the fermentate against *B. subtilis* ATCC 1174 and

S. xylosus ATCC 29971. In the case of *E. faecalis* ATCC 7080, the nisin displayed 40 AU/ml of antimicrobial activity in comparison to the higher 80 AU/ml displayed by the pH 4.5 fermentate, indicative of higher activity by the fermentate against this strain. The nisin showed no significant inhibition for gram-negative strains as expected (Matsusaki, *et al.*, 1998).

3.3 Antibacterial Testing with pH Adjusted Fermentate

B. longum ITT 13 is known to produce several organic acids such as lactic, acetic and succinic acid during growth (Section 4.5). Growth of the *B. longum* ITT 13 strain under conditions without pH control usually results in a fermentate that has a final value of approximately pH of 4.2-4.3. To reduce the possible antimicrobial contribution of these acids, present in the low pH *B. longum* ITT 13 fermentate, the fermentate was adjusted to pH 4.7 and screened for activity against selected strains. A pH of 4.7 was chosen as previous testing had indicated a loss of detectable activity in the microdiffusion assay above pH 4.8. The fermentate was therefore adjusted to as close to pH 4.7 as could be achieved – which resulted in a pH of pH 4.68. The MRSc broth for comparison to the pH 4.68 fermentate was adjusted to pH 4.82 instead of pH 4.7 as the addition of more acid caused a large decline in pH past pH 4.7. The pH adjusted fermentate was tested for antibacterial activity using the microdiffusion assay with *K. rhizophilia*, *B. subtilis* and *P. aeruginosa* used as the indicator strains. Only these strains were selected to provide a general overview of the effect of pH adjusting the fermentate on both Gram-positive and Gram-negative strains, instead of carrying out the assay on all previously tested strains. Fermentate with no pH adjustment was added to the M/D assay plates for comparison of activity to the pH adjusted fermentate in order to assess whether increasing the pH in some way affected the antibacterial activity detected for the fermentate.

Table 3.4: Antibacterial activity (mm) of the *B. longum* ITT 13 fermentate and pH adjusted fermentate against *K. rhizophilia, B. subtilis* and *P. aeruginosa*

Sample:	***K. rhizophilia***	***B. subtilis***	***P. aeruginosa***
	Average Zone Size (mm) ± SD	**Average Zone Size (mm) ± SD**	**Average Zone Size (mm) ± SD**
Nisin Undiluted (1000 iu/ml)	**3.8 ± 0.3**	**3.8 ± 0.3**	**0 ± 0**
Nisin 1/2 (500 iu/ml)	**3.0 ± 0**	**2.8 ± 0.3**	**0 ± 0**
Fermentate pH 4.21	**3.7 ± 0.3**	**4.2 ± 0.2**	**4.0 ± 0**
Fermentate pH 4.21 1/2	**1.8 ± 0.3**	**3.0 ± 0.9**	**2.2 ± 0.3**
Fermentate pH 4.68 Undiluted	**2.3 ± 0.3**	**4.2 ± 0.8**	**2.3 ± 0.3**
Fermentate pH 4.68 1/2	**0 ± 0**	**3.0 ± 0.9**	**0 ± 0**
MRSc broth pH 4.82	**0 ± 0**	**1.5** ± 0**	**0 ± 0**
MRSc broth (no pH adjustment)	**0 ± 0**	**0 ± 0**	**0 ± 0**
Lactic Acid 33 mM	**0 ± 0**	* ± 0**	**0 ± 0**
NaOH 50 mM	**0 ± 0**	**1.7 ± 0.3**	**0 ± 0**

*Hairline zone of inhibition **Faint zone and difficult to accurately determine.

In the M/D assay for *K. rhizophilia* neither the MRSc broth at the original pH 6.42 or adjusted to pH 4.82 generated zones of inhibition. No zones of inhibition were detectable for the 33 mM Lactic acid, which is the final concentration of acid present in the pH adjusted MRSc broth, or for the 50 mM NaOH, which is the final concentration present in the pH adjusted ITT 13 fermentate. The activity for the nisin against the *K. rhizophilia* indicator strain was comparable to the activity of the pH 4.21 ITT 13 fermentate, with zones of 3.8 mm and 3.7 mm respectively. As the antibacterial activity of the pH 4.68 fermentate was reduced in comparison to the pH 4.21 fermentate, with a zone of 2.3 mm, it was not comparable to the nisin with a zone of 1.5 mm less than the nisin. The zone of inhibition detected for the pH 4.68 fermentate was 2.3 mm in comparison to a 3.7 mm zone for the pH 4.21 fermentate without pH adjustment. This indicates the original fermentate with no pH adjustment and a lower pH retained more antibacterial activity against *K. rhizophilia* than the fermentate which had its pH raised to pH 4.68, indicating increased activity associated with a lower pH. A 1/2 dilution of the ITT 13 fermentate adjusted to pH 4.68 did not generate a zone of inhibition, while activity remained

for the original pH unadjusted fermentate (pH 4.21), which gave an average zone size of 1.8 mm.

The microdiffusion results for the *B. subtilis* strain generated faint zones of roughly 1.5 mm for the control of pH adjusted MRSc broth (pH 4.82), which was not observed for other strains and indicates a higher susceptibility of *B. subtilis* to a lower pH. A hairline zone was detectable for the 33mM Lactic acid, which is the concentration of acid used to adjust the pH 4.82 MRSc broth, further indicating its susceptibility to more acidic conditions. Incubation of the *B. subtilis* with 50 mM NaOH generated a zone of 1.7 mm, thus indicating the sensitivity of this strain to both acidic and basic solvents. Neither of the other two tested strains displayed any susceptibility to the lactic acid or the NaOH.

The activity of the nisin against *B. subtilis* gave zones on the M/D plates that indicated less antibacterial activity for the nisin than for the pH unadjusted *B. longum* ITT 13 fermentate (pH 4.21) with zones of 3.8 mm for undiluted nisin and 4.2 mm respectively for the undiluted fermentate. The pH 4.68 fermentate also had a larger zone of inhibition than the nisin, with an average zone of 4.2 mm also observed for the pH 4.68 fermentate, as for the pH 4.21 fermentate, and 3.8 mm for the nisin. The average zone of inhibition size of 4.2 mm was the same for both the original ITT 13 fermentate (pH 4.21) and for the pH adjusted ITT fermentate (pH 4.68), indicating increasing the pH did not lower the antibacterial activity observed against *B. subtilis* as it did for *K. rhizophilia*. However, it should be noted that although the zone sizes were measured as the same for both the pH 4.21 fermentate and the pH 4.68 fermentate, there was more variation for the pH 4.68 fermentate with an SD of 0.764. The zone for the 1/2 dilution of the pH 4.68 fermentate was also the same average size as the zone for the pH 4.21 fermentate (3.0 mm) with the same standard deviation observed of 0.866 observed for both. This further indicates that increasing the pH of the ITT 13 fermentate did not decrease the antibacterial activity detected against *B. subtilis* as it did for *K. rhizophilia*.

For *P. aeruginosa* no zones were detected for the controls of the MRSc broth at the original pH of pH 6.42 or for the MRSc broth adjusted to pH 4.82, which is similar to *K. rhizophilia*. No zones were present for the 33mM Lactic or for the 50 mM NaOH again similar to *K. rhizophilia* which was also not susceptible to these concentrations of solvents, indicating they have no detectable effect on these two indicator strains. The average zone of inhibition for the undiluted fermentate at pH 4.21 with no pH adjustment was 4.0 mm and for the fermentate adjusted to pH 4.68 the average zone of inhibition was 2.3 mm. Activity was lost for the 1/2 dilution of the pH 4.68 fermentate but was present for the pH 4.21 fermentate with a zone of 2.2 mm. The reduced zones of inhibition and antibacterial activity for the fermentate adjusted to pH 4.68 against the *P. aeruginosa* are similar to the reduction in zones also observed for the pH 4.68 fermentate against *K. rhizophilia* which likely indicates that an increase in the pH of the *B. longum* ITT 13 fermentate results in a reduction of antibacterial activity and maximum activity is achieved at a lower fermentation pH.

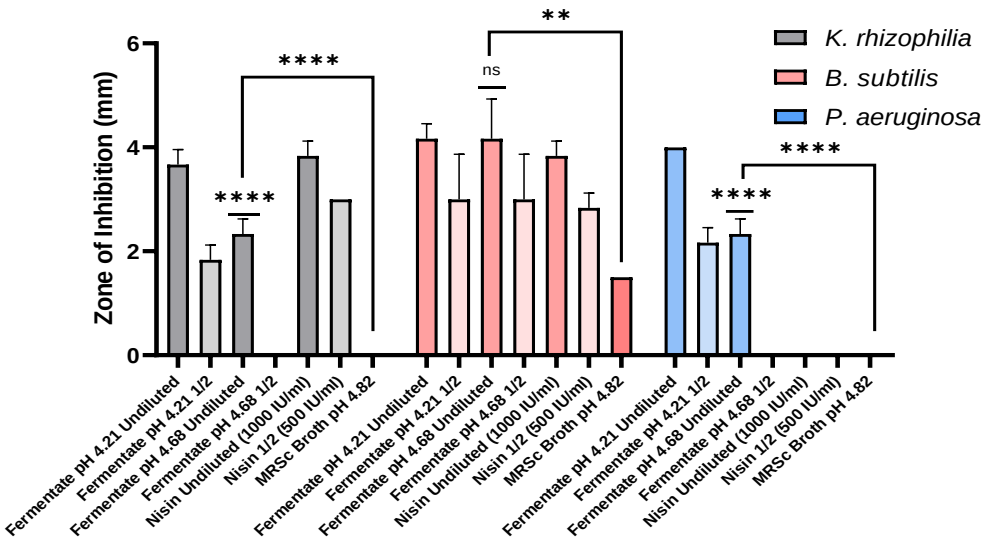

Figure 3.13: Antibacterial activity (mm) of *B. longum* ITT 13 fermentate at pH 4.21 and adjusted to pH 4.68 against selected indicator strains. The controls included a positive control of undiluted and a 1/2 dilution of nisin (1000 & 500 IU/ml) and a negative control of MRSc broth adjusted to pH 4.82. Three independent assays (n=3) were used to determine the standard deviation. Statistical analysis was carried out using a one-way ANOVA with Tukeys multiple comparison assay. The statistical comparison of the pH 4.68 fermentate to the pH 4.21 fermentate is denoted by the symbols directly above the pH 4.68 fermentate bar. The statistical comparison of the pH 4.69 fermentate to the pH 4.82 MRSc broth is denoted by the symbols above the line connecting both bars. **** = p-value <0.0001, ** = p-value of 0.0042 and ns = non-significant p-value >0.05.

In Figure 3.13 the statistical analysis was carried out in order to determine the effect of adjusting the fermentate to a higher pH value and to ascertain whether the antibacterial activity was primarily or solely attributable to the pH value of the fermentate. For all three strains, the undiluted fermentate adjusted to pH 4.68, gave a statistically significant different zone of inhibition when compared with the MRSc Broth (pH 4.83), with a p-value <0.0001 for both the *K. rhizophilia* and *P. aeruginosa* and a p-value of 0.0042 for *B. subtilis.* This indicates that the antibacterial activity of the fermentate adjusted to pH 4.68 is likely not solely associated with a pH effect.

Comparison of the zone sizes in the M/D assays (Table 3.4) for the undiluted original fermentate with no pH adjustment (pH 4.21) and the undiluted fermentate adjusted to pH 4.68, gave statistically significant p-values of <0.0001 for *K. rhizophilia* and *P. aeruginosa*, but a non-significant p-value of >0.05 for *B. subtilis* (Figure 3.13). This indicates that maximum

antibacterial activity is achieved at a lower pH with activity reduced at increasing pH's. For the *B. subtilis* however this deduction did not apply as there was no significant difference between the antibacterial activity of the original pH 4.21 fermentate and the fermentate when adjusted to pH 4.68, indicating increasing the pH of the fermentate did not reduce observed activity against this strain.

3.4 Antifungal Activity of *B. longum* ITT 13

Due to the common problem of food spoilage, the *B. longum* ITT 13 fermentate was tested for antifungal activity to assess its potential for application in biopreservation. Antifungal testing was initially carried out using the soft agar overlay assay (Section 2.8.5.1) and resulted in no antifungal activity detected against the following indicator strains: *R. mucilaginosa* CBS316, *S. cerevisiae* ATCC18824, *S. cerevisiae* CBS1171, *D. hansenii* ATCC18110 and *C. albicans* ATCC2091 (data not shown). It was decided to further test the *B. longum* ITT 13 fermentate using the more sensitive microtitre plate testing method (Section 2.8.5.2) due to increased sensitivity of the assay. Cell-free fermentate was prepared from the *B. longum* ITT 13 fermentate (Section 2.4.3) and was screened against *R. mucilaginosa* CBS316, *S. cerevisiae* ATCC18824, *S. cerevisiae* CBS1171 and *C. albicans* ATCC2091 using the MIC_{50} assay (Section 2.8.5.2). Testing for the fungal strain *D. hansenii* was not possible due to Covid restrictions and associated closing of laboratory access. Natamycin (SigmaAldrich) was used as a positive control and MRSc broth (pH 6.4) as a negative control. The broth the fungal strain was grown in was used as a growth control, either YPD or PD broth. An acid control containing 49.9 mM lactic acid and 60.9 mM acetic acid in MRSc broth was also employed as this combination of acids brought the pH of the MRSc broth to pH 4.3, which is approximately the pH of the cell-free ITT 13 fermentate used in the MIC_{50} antifungal testing. Antifungal activity was detected for the *B. longum* ITT 13 fermentate at pH 4.3 and for fermentate grown with pH control at pH 4.8, (Figures 3.14-3.17 and Table 3.5).

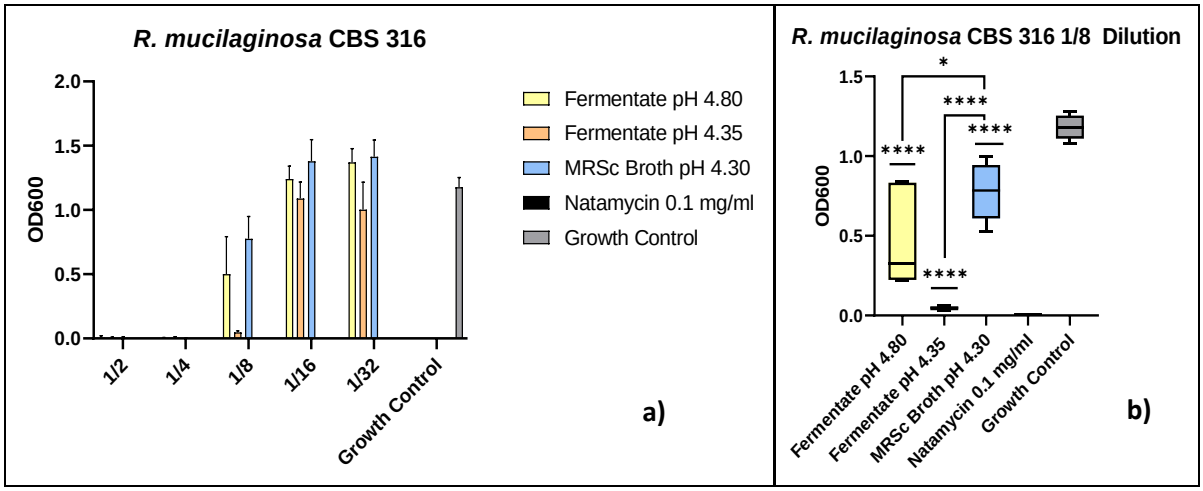

Figure 3.14: The MIC_{50} determination of the antifungal activity for the *B. longum* ITT 13 Cell-Free Fermentate against *R. mucilaginosa* CBS 316 at a) 1/2-1/32 Dilution and b) a magnification of the 1/8 dilution. The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition of growth (OD600). A growth control of PD broth (approx. pH 5.1) was used to determine maximum growth. The AU/ml was determined as described in Section 2.8.2. Standard deviations were determined for a minimum of three independent assays (n=3) and statistical analysis was carried out for Figure 3.14 (b) using one-way ANOVA with Tukey's multiple comparison assay. **** = p-value ≤0.0001, * = p-value ≤0.05.

Antimicrobial activity was detected against *R. mucilaginosa* CBS 316 for the ITT 13 cell-free fermentate grown with pH control (pH 4.80), the cell-free fermentate grown with no pH control (pH 4.35) and for the MRSc broth adjusted to pH 4.30. Both the pH 4.80 fermentate and the pH adjusted MRSc broth displayed complete inhibition for the growth of the fungal strain up to the 1/4 dilution and partial inhibition at the 1/8 dilutions was observed for all but the pH 4.35 fermentate, which displayed complete inhibition of the fungal strain at the 1/8 dilution. Natamycin gave complete inhibition of the fungal strain at every dilution tested, which was to be expected as it is a potent antifungal agent.

At the 1/8 dilution (Figure 3.14 (b)) the average OD600 for the pH 4.80 fermentate was 0.50, for the pH 4.35 fermentate was 0.05 and for the pH 4.30 MRSc broth was 0.78, which is significantly lower than for the OD600 of 1.18 measured for the growth control of PD broth, with p-values of <0.0001 recorded for all. This indicates that the pH 4.35 *B. longum* ITT 13

fermentate, the pH 4.80 fermentate and the pH 4.30 MRSc broth all displayed some level of antimicrobial activity at a 1/8 dilution, which was lost at a 1/16 dilution. There was a significant difference of $p<0.05$ when comparing the pH 4.35 and pH 4.80 fermentate samples to the MRSc broth adjusted to the same value as the original pH unadjusted fermentate i.e. pH 4.3. The latter would indicate that the fermentate has some antifungal activity present which cannot be accounted for just by the pH present in the fermentate.

The MIC_{50} was determined for both the pH 4.35 fermentate and pH 4.80 fermentate samples as well as the pH 4.30 MRSc broth based on the dilution of the sample required to cause at least 50% inhibition of the fungal strain, with the growth control taken as 100% growth. The MIC_{50} for the pH 4.80 fermentate against *R. mucilaginosa* CBS 316 was 80 AU/ml, while the value for the *B. longum* ITT 13 fermentate at pH 4.35 was 80 AU/ml and the antifungal activity detected for the MRSc pH 4.30 broth was determined as being 40AU/ml.

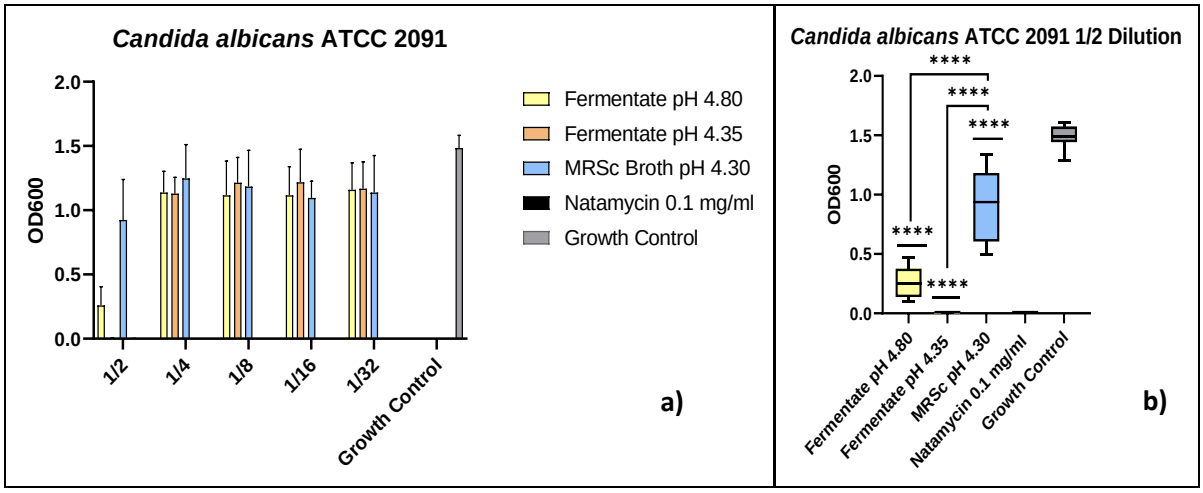

Figure 3.15: The Antifungal Activity and MIC_{50} Determination for the *B. longum* ITT 13 Fermentate against *C. albicans* ATCC 2091 at a) the 1/2-1/32 Dilution and b) the 1/2 dilution magnified. The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition of growth (OD600). A growth control of PD broth (approx. pH 5.1) was used to determine maximum growth. The AU/ml was determined as described in Section 2.8.2. Standard deviations were determined for a minimum of three independent assays (n=3) and statistical analysis was carried out on Figure 3.15 (b) using one-way ANOVA with Tukey's multiple comparison assay. **** = p-value ≤0.0001.

Antimicrobial activity was detected against *C. albicans* ATCC 2091 by the *B. longum*, ITT 13 pH 4.80 fermentate, the pH 4.35 fermentate and by the MRSc broth adjusted to pH 4.30 at the 1/2 dilution.

As antimicrobial activity was detected to varying degrees at the 1/2 dilution alone, this was magnified in Figure 3.15 (b). Only the pH 4.35 fermentate displayed complete inhibition at the 1/2 dilution, while the pH 4.80 fermentate and the pH 4.30 MRSc broth displayed partial inhibition at this dilution, with average OD600 values of 0.26 and 0.92 respectively, in comparison to the growth control of PD broth with an average OD600 of 1.48. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test was carried out on the 1/2 dilution as the three test samples (the pH 4.35 fermentate, pH 4.80 fermentate and pH 4.30 MRSc broth) displayed varying amounts of inhibition as seen in Figure 3.15 b). The statistical analysis indicates that all were found to be statistically significant in comparison to the growth control with p-values below 0.0001, which indicates that all samples displayed some level of

antimicrobial activity at the 1/2 dilution. When the pH 4.80 *B. longum* ITT 13 fermentate and the pH 4.35 *B. longum* ITT 13 fermentate were compared to the pH 4.30 MRSc broth, statistical significance was again determined with p-values of <0.0001 for both fermentates, indicating that they had also displayed higher levels of inhibition than the pH adjusted MRSc broth.

The MIC_{50} for the pH 4.80 fermentate against *C. albicans* ATCC 2091 was a 1/2 dilution with the calculated inhibition being equivalent to 20 AU/ml. For the pH 4.35 fermentate, the MIC_{50} was also determined as a 1/2 dilution equivalent to 20 AU/ml. There was no inhibition of the fungal strain of at least 50% associated with the presence of the pH 4.30 MRSc broth and therefore no AU/ml could be determined.

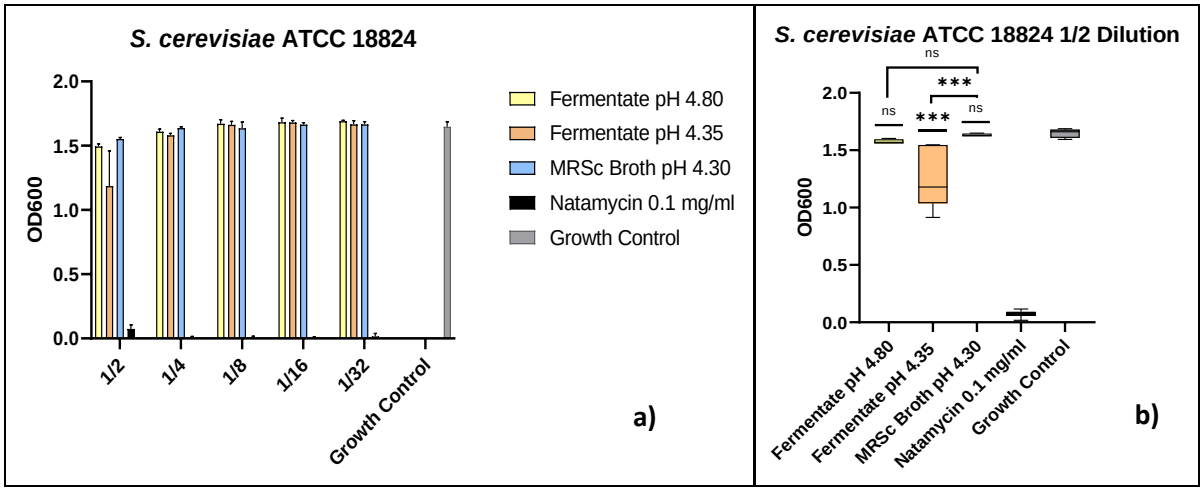

Figure 3.16: The antifungal activity and MIC_{50} determination of the *B. longum* ITT 13 Fermentate against *S. cerevisiae* ATCC 18824 a) 1/2-1/32 dilution and b) the 1/2 dilution magnified. The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition of growth (OD600). A growth control of YPD broth (approx. pH 6.5) was used to determine maximum growth. The AU/ml was determined as described in Section 2.8.2. Standard deviations were determined for a minimum of three independent assays (n=3) and statistical analysis was carried out on Figure 3.16 (b) using one-way ANOVA with Tukey's multiple comparison assay. *** = p-value ≤0.001, ns = non-significant p-value >0.05.

The pH 4.35 fermentate displayed slight inhibition at a 1/2 dilution with an average OD600 of 1.19 in comparison to the growth control of the fungal strain which was 1.65. No antimicrobial activity was detected for either the pH 4.80 fermentate or the pH 4.30 MRSc broth. As the antimicrobial activity detected for the pH 4.35 fermentate was only present at the 1/2 dilution, this dilution was magnified in Figure 3.16 (b). Statistical analysis using a one-way ANOVA with Tukey's multiple comparison test was carried out on the 1/2 dilution for the three test samples in comparison to the growth control, however only the pH 4.35 fermentate had a significant p-value of 0.0001. When the pH 4.80 fermentate and the pH 4.35 fermentate were compared to the pH 4.30 MRSc broth, statistical significance was again only determined for the pH 4.35 fermentate with a p-value of 0.0002 indicating higher antifungal activity than the pH 4.30 MRSc broth.

There was no inhibition detected of at least 50% for *S. cerevisiae* ATCC 18824 by the pH 4.35 *B. longum* ITT 13 fermentate and therefore no AU/ml could be determined. As there was also

no inhibition of the fungal strain associated with the presence of the pH 4.80 fermentate or the pH 4.30 MRSc broth, no AU/ml could be determined for these samples.

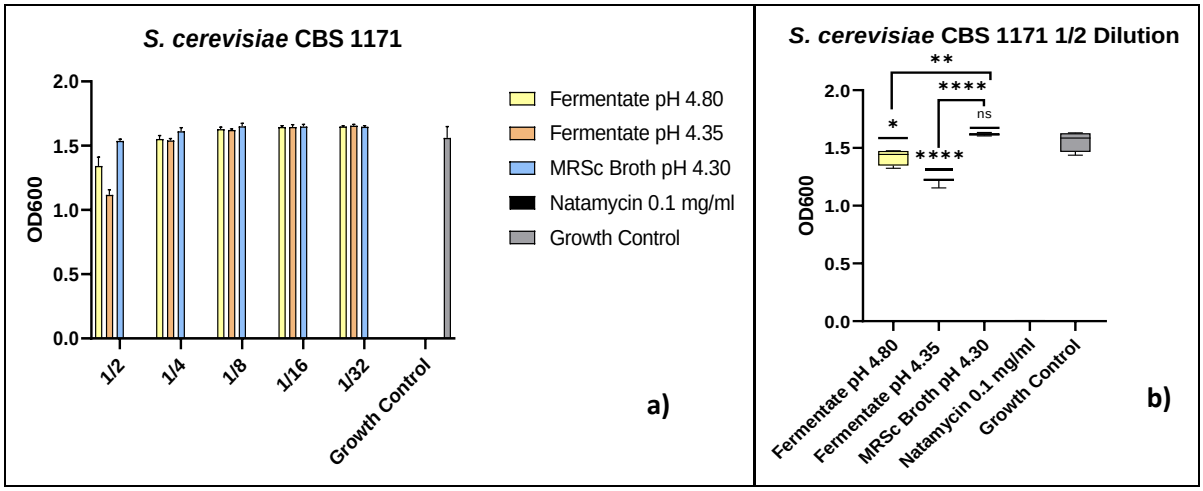

Figure 3.17: The antifungal activity and MIC_{50} determination of the *B. longum* ITT 13 Fermentate against *S. cerevisiae* CBS 1171 a) 1/2-1/32 dilution and b) the 1/2 dilution magnified. The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition of growth (OD600). A growth control of YPD broth (approx. pH 6.5) was used to determine maximum growth. The AU/ml was determined as described in Section 2.8.2. Standard deviations were determined for a minimum of three independent assays (n=3) and statistical analysis was carried out on Figure 3.17 (b) using one-way ANOVA with Tukey's multiple comparison assay. **** = p-value ≤0.0001, ** = p-value ≤0.01, * = p-value ≤0.05, ns = non-significant p-value >0.05.

The antifungal activity presented in Figure 3.17 (a) indicates that there was some antimicrobial activity detected against *S. cerevisiae* CBS 1171 by the pH 4.35 and pH 4.80 fermentate samples at the 1/2 dilution.

As both fermentates (pH 4.80 and pH 4.35) displayed slight inhibition at only the 1/2 dilution, this dilution was magnified in Figure 3.17 (b). An average OD600 of 1.34 for the pH 4.80 fermentate and 1.12 for the pH 4.35 fermentate was determined in comparison to the growth control of the fungal strain which had an OD600 of 1.56, indicating small amounts of inhibition relative to the growth control. Statistical analysis using a one-way ANOVA with Tukey's multiple comparison test was carried out on the 1/2 dilution for the three test samples in comparison to the growth control (Figure 3.17 (b)). The pH 4.80 fermentate generated a significant p-value of 0.0216, the pH 4.35 fermentate had a significant p-value of <0.0001 and the pH 4.30 MRSc broth did not generate significant results with a p-value >0.05. When the pH 4.80 fermentate and the pH 4.35 fermentate were compared to the pH 4.30 MRSc broth,

statistical significance was found for both, with p-values below 0.05, indicating both fermentates displayed higher antifungal activity than the pH 4.30 MRSc broth. Due to the low level of inhibition however it is likely not biologically significant.

There was no detectable inhibition for any of the 3 tests samples above the 1/2 dilution. As there was no antifungal activity detected for the pH 4.35 fermentate, pH 4.80 fermentate or the pH 4.30 MRSc broth that resulted in at least 50% inhibition of *S. cerevisiae* CBS 1171, no AU/ml could be determined.

Table 3.5: The MIC_{50} and AU/ml of the *B. longum* ITT 13 Fermentate (pH 4.80), the *B. longum* ITT 13 Fermentate (pH 4.35) and MRSc Broth (pH 4.30) against selected fungal strains.

Strain:	*B. longum* ITT 13 Fermentate pH 4.80			*B. longum* ITT 13 Fermentate pH 4.35			MRSc Broth pH 4.30		
	AU/ml	MIC_{50}	P-value	AU/ml	MIC_{50}	P-value	AU/ml	MIC_{50}	P-value
R. mucilaginosa CBS 316	80	1/8	<0.0001	80	1/8	<0.0001	40	1/4	<0.0001
C. albicans ATCC 2091	20	1/2	<0.0001	20	1/2	<0.0001	0	0	<0.0001
S. cerevisiae ATCC 18824	0	0	N/A	0	0	N/A	0	0	N/A
S. cerevisiae CBS 1171	0	0	N/A	0	0	N/A	0	0	N/A

No MIC_{50} was observed for either the *B. longum* ITT 13 fermentate at pH 4.8 or pH 4.35 and for the MRSc broth pH 4.3 against either of the *S. cerevisiae* strains tested and thus no AU/ml could be determined. The highest antifungal activity of 80 AU/ml with an MIC_{50} of the 1/8 dilution was observed for both the pH 4.80 fermentate and the pH 4.35 fermentate samples against *R. mucilaginosa* CBS 316. Antifungal activity was observed against *C. albicans* ATCC 2091 by both the pH 4.80 and pH 4.35 fermentate, however it was lower than that observed for *R. mucilaginosa* CBS 316 at 20 AU/ml with a corresponding MIC_{50} of the 1/2 dilution.

The pH 4.30 MRSc broth, which was equivalent in pH to the pH 4.3 fermentate, displayed less antifungal activity than both the *B. longum* ITT 13 pH 4.80 and pH 4.35 fermentate samples, with 40 AU/ml determined against *R. mucilaginosa* CBS 316 and no determined activity against the other three fungal strains. As the calculated activity (AU/ml) was lower for the pH 4.30 MRSc broth than the activity observed by both fermentate samples against *R.*

mucilaginosa CBS 316 and *C. albicans* ATCC 2091, it can be interpreted that the antifungal observed for these strains is not solely associated with a pH effect.

3.5 Discussion

3.5.1 Characterisation of Strain ITT 13

Preliminary research work carried out within previous research projects within our laboratory had indicated that strain ITT 13 was most likely thought to be a *Bifidobacterial* strain, specifically *B. longum*. The strict anaerobicity of the ITT 13 strain (Lee and O'Sullivan, 2010), along with the requirement for a nutrient rich media for growth, such as MRSc agar/broth supplemented with 0.05% L-cysteine (Liu *et al.*, 2015), both supported this suggested identification. Colony morphology and the resultant purple rods with bifid formation after Gram staining further supported this identification, as these are all characteristic of *Bifidobacteria* (Dhanashree *et al.*, 2017).

The phenotypic characterisation using the Biolog was utilised in preference to API test kits, as it runs a total of 96 assays to confirm identity and growth pattern, and is therefore considered more accurate than the API test strips which use 20 assays (Shayegani *et al.*, 1978), while also providing details of carbon sources required for nutrition (Microbial Identification – Biolog). Identification of the strain was also further validated using 16s rRNA sequencing (C. Whelan – personal communication). Both 16s rRNA sequencing and the Biolog system confirmed the identity of strain ITT 13 as a *Bifidobacterium longum* with certainty (0.995), which confirmed the use of a GRAS strain (O'Callaghan and van Sinderen, 2016) for the research and production of antimicrobial compounds and thus safe for potential applicability to the food industry for possible biopreservation or as a nutraceutical probiotic organism (O'Shea *et al.*, 2012; Liu *et al.*, 2020). *B. longum* specifically has been shown in other studies to grow faster at 37^{o}C than other *Bifidobacterium* strains (Dhanashree *et al.*, 2017), thus indicating the possibility of the potential suitability of *B. longum* ITT 13 for biomass production, which could be an important factor for the production of the strain as a probiotic organism.

Probiotics have been defined by the World Health Organisation as "Live microorganisms

which when administered in adequate amounts confer a health benefit on the host". Many probiotic bacteria are isolated from the gastrointestinal tract of humans, where they are thought to provide additional health benefits similar to those of the hosts gut microbiome such as facilitating the protection of the intestinal barrier by maintaining tight junctions of protein and modulation of antimicrobial activity within the gut to prevent infection (O'Flaherty and Klaenhammer, 2010). This production of antimicrobial activity is namely associated with organic acids and antimicrobial peptides that can be produced by *Bifidobacteria* spp. and is considered important for a healthy balance of microflora within the gut and for infection prevention (Hladíková *et al.*, 2012; O'Shea *et al.*, 2012; Guinane *et al.*, 2016).

Lactic acid bacteria such as *Lactobacillus* spp. and *Bifidobacteria* spp. are among the most common bacteria utilised as probiotic strains (Butel, 2014). *B. longum* ITT 13 has initially displayed some characteristics of probiotic organisms, such as the production of antimicrobial activity and the isolation of the strain from the human gastrointestinal system indicates the potential to be utilised as a probiotic organism that is capable of both surviving and thriving with in the gut (Cooke *et al.*, 2005). *B. longum* subsp. *infantis* CECT 7210 also exhibits probiotic properties with resistance to gastric juices, bile salts and a low pH, as well as prevention of diarrhoea due to inhibition of rotavirus (Chenoll *et al.*, 2016).

3.5.2 Antibacterial Activity of *B. longum* ITT 13

Several *Bifidobacteria* have been shown to have antimicrobial activity against various bacterial strains that has been shown to be associated with the production of antimicrobial peptides which are generally referred to as bacteriocins (Lee and O'sullivan, 2010). *Bifidobacterial* strains have produced bioactive antimicrobial peptides displaying activity against a range of bacteria including *Helicobacter pylori*, which was shown to have been inhibited by peptides produced by six different *Bifidobacterium* isolates (Collado *et al.*, 2005). The peptide bifidin I

has displayed broad-spectrum activity against bacteria including *Bacilli, Listeria* and *Salmonella* (Cheikhyoussef *et al.*, 2010). The strains *B. bifiidum 174 and Bifidobacterium* sp. 27 have produced peptides with broad-spectrum antimicrobial activity against various Gram-positive and Gram-negative strains including *Streptococcus* spp., *Clostridioides difficile, S. typhimurium, E. coli* and *Klebsiella pneumoniae* (Poltavska and Kovalenko, 2012). *Bifidobacteria* spp. also produce a range of organic acids upon growth, including formic acid, lactic acid and acetic acid (Ruiz-Aceituno *et al.*, 2020). Both lactic and acetic acid are primarily produced during growth and fermentation and are also associated with significant antimicrobial activity against some bacterial strains (Makras and De Vuyst, 2006).

3.5.2.1 Determination of the Antibacterial Spectrum of Activity using the Microdiffusion Plate Assay

The indicator strains *S. xylosus* and *K. rhizophilia* that were used in the microdiffusion assay, were chosen based on the efficacy as indicators strains as determined by providing clear zones of inhibition for the *B. longum* ITT 13 fermentate in previous work. Indicator strains were also chosen based on their relevance in food spoilage. Some of these species include *Bacillus* spp. which commonly spoil bakery products and potatoes (André *et al.*, 2017; Ajingi *et al.*, 2020), *P. aeruginosa* which can spoil meat and animal derived food (Höll *et al.*, 2016) and *Staphylococcus* spp., which are commensal bacteria commonly found on the skin and can be foodborne pathogens resulting in *Staphylococcus* food poisoning (Wang *et al.*, 2019; Maia *et al.*, 2020). The strains used for evaluation of the antimicrobial effect of the fermentate were also chosen based on food associated pathogenicity. *E. coli* and *Salmonella* spp. were selected for testing because of their pathogenicity associated with foodborne illnesses, where they have been known to contaminate meat, poultry and eggs resulting in food poisoning (Huang *et al.*, 2019; Vinayaka *et al.*, 2019). Other strains chosen for inclusion in the M/D assays were

selected because of their known presence in the gut microflora and included *Enterococcus faecalis* (Chen *et al.*, 2018) and *Klebsiella aerogenes* (Paulose *et al.*, 2019). Finally, several lactic acid bacteria which are known to be used in fermented foods were screened to determine if the *B. longum* ITT 13 fermentate affected their growth as that would have implications for co-cultivation in future possible fermented probiotic foods, e.g. lactic acid bacteria such as *Lactococcus spp.* and *Lactobacilli* spp. Of the 15 bacterial strains tested for antimicrobial susceptibility to the *B. longum* ITT 13 fermentate, it was determined that 9 strains displayed susceptibility (*B. subtilis* ATCC 1174, *E. faecalis* ATCC 7080, *K. rhizophilia* ATCC 9341, *S. xylosus* ATCC 29971, *K. aerogenes* ATCC 13048, *E. coli* ATCC 25922, *P. aeruginosa* PA01, *S. typhimurium* ATCC 26929 and *B. cenocepacia* ATCC 17765 III.20), while 6 displayed resistance (*B. cereus* ATCC 10876, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *S. pyogenes* NCIMB 11841, *L. paracasei subsp. paracasei* NCIMB 3254 and *L. delbruickii subsp. bulgaricus* NCIMB 11778.

Many bacteriocins only display inhibitory activity to bacteria of a similar ecological niche and are described as having a narrow spectrum of activity (Zou *et al.*, 2018; Vaičikauskaitė *et al.*, 2019; Pei *et al.*, 2020). However, the spectrum of activity of *B. longum* ITT 13 as determined in M/D assays (Figure 3.4, Table 3.1 & 3.2) indicate that the fermentate displays broad antimicrobial action against both Gram-positive and Gram-negative organisms from a variety of ecological environments. In this study, fifteen species were selected as indicated previously and within the fifteen strains, ten are Gram-positive and five are Gram-negative. In the M/D assays 4 of the 10 Gram-positive species displayed susceptibility to the antimicrobial activity of *B. longum* ITT 13 (Figure 3.4 and Table 3.1), while all 5 Gram-negative species tested displayed susceptibility (Figure 3.4 and Table 3.2). The susceptibility of the Gram-negative species to the fermentate was especially of significance given the resistance of many Gram-negative bacteria to antimicrobials such as antibiotics as well bacteriocins produced by some

Gram-positive strains (Martin-Visscher *et al.*, 2011; Breijyeh *et al.*, 2020). Higher levels of antimicrobial activity were observed against some Gram-positive strains in comparison to the lower-level activity observed against the Gram-negative strains, with the highest levels of antimicrobial activity detected against the Gram-positive strains *E. faecalis* ATCC 7080 (20.4 AU/ml) and *B. subtilis* ATCC 1174 (16.7 AU/ml) (Figure 3.4). The *E. faecalis* ATCC 7080 and *B. subtilis* ATCC 1174 strains were also among the most susceptible to the *B. longum* ITT 13 fermentate in the MIC_{50} assay (Section 3.2.2).

In a paper by Makras & De Vuyst (2006), a variety of *Bifidobacterium* strains, including *B. longum*, were screened for antimicrobial activity against a variety of Gram-positive and Gram-negative strains. In this paper the antimicrobial activity observed against the Gram-negative bacterial strains, which included *Salmonella* and *E. coli*, was found to be primarily due to the production of organic acids by the *Bifidobacterium* spp, in comparison to the activity against Gram-positive strains which was associated with both organic acid and bacteriocin production. This may explain the increased activity against some Gram-positive strains by *B. longum* ITT 13, as they may be susceptible to both bacteriocin and acid production resulting in higher levels of inhibition. Thus, the susceptibility of the screened Gram-negative strains to the antimicrobial activity of *B. longum* ITT 13 may be associated with sensitivity to organic acids alone, or less sensitivity to potential bacteriocins produced by ITT 13. This in turn may be indicative of why lower levels of inhibition are detected against some Gram-negative bacteria by *B. longum* ITT 13, as was observed by Makras & De Vuyst's (2006) study. Some bacteriocins produced by lactic acid bacteria also have displayed broad spectrums of antimicrobial activity against gram-negative strains. Indira *et al.* (2018) discovered a bacteriocin produced by *Enterococcus casseliflavus* MI001 that displayed antimicrobial activity against both gram-positive and gram-negative strains, including pathogenic gram-negative strains such as *P. aeruginosa, K. pneumoniae* and *E. coli*. An approximately 5.5KDa bacteriocin produced by the LAB

Lactobacillus sake C2, which was originally purified from a Chinese fermented cabbage, displayed strong antimicrobial activity against a wide range of bacteria including *S. aureus, E. coli, S. typhimurium* and other LAB including *Lactobacilli* spp. and *S. thermophilus* (Gao *et al.*, 2010). The gram-negative antimicrobial activity associated with sakacin C2 is not commonly seen in LAB and the strain also produced a large amount of lactic acid, with the authors stating that the acid production and broad spectrum of activity demonstrate the potential of *L. sake* C2 as a starter culture and bio-preservative.

A wide spectrum of antimicrobial activity was observed for *B. longum* ITT 13, with inhibition detected for the growth of both Gram-positive and Gram-negative food spoilage and pathogenic organisms.

As previously stated, many bacteriocins display antimicrobial activity against bacteria of a similar ecological niche, as observed with the inhibition of *Lactobacilli* by sakacin C2 (Gao *et al.*, 2010). *Lactobacilli* are also commonly found as part of the gut microflora (Liu *et al.*, 2020) like *Bifidobacterium* spp., however no antimicrobial activity was detected against *Lactobacillus paracasei* subsp. *paracasei* NCIMB 3254 and *Lactobacillus delbruickii* subsp. *bulgaricus* NCIMB 11778 by *B. longum* ITT 13. The lack of inhibitory activity against these bacteria by the *B. longum* ITT 13 fermentate may indicate potential suitability for co-cultivation with *B. longum* ITT 13. This would increase the suitability of *B. longum* ITT 13 as a probiotic culture or a starter culture in comparison to the aforementioned sakacin C2 described by Gao *et al.* (2010), which displayed antimicrobial activity against a variety of *Lactobacilli* and *Streptococcus thermophilus*, a common starter culture in yoghurt making (Uriot *et al.*, 2017). The production of antimicrobial compounds by probiotic bacteria to prevent colonisation of pathogenic bacteria within the gut is an important factor of probiotics, with similar attributes displayed by *B. longum* ITT 13. Antimicrobial activity by *B. longum* ITT 13 was observed against pathogenic bacteria including *E. coli* and *Salmonella*, which are

common foodborne pathogens that contaminate meat, poultry and eggs resulting in food poisoning (Huang *et al.*, 2019; Vinayaka *et al.*, 2019).

3.5.2.2 MIC_{50} Testing of the *B. longum* ITT 13 Fermentate

Increased sensitivity has been displayed using the microtitre plate method in comparison to well diffusion by Inturri *et al.*, (2019), and as discussed by Balouiri *et al.*, (2016) is suitable for quantitative analysis of antimicrobial activity.

The fermentate displayed an MIC_{50} of a 1/8 dilution and 80 AU/ml against *B. subtilis* ATCC 1174, while the pH 4.5 MRSc broth generated an MIC_{50} of 1/2 dilution and 20 AU/ml in comparison with the fermentate at the same pH (Figure 3.7 and Table 3.3). The 1/2 MIC_{50} of the MRSc broth was four-fold less than the 1/8 MIC_{50} of the fermentate, indicating that *B. subtilis* ATCC 1174 is likely not inhibited by the pH 4.5 effect alone and therefore there may be other molecules present in the fermentate contributing to antimicrobial activity. The genus *Bacillus* have displayed an ability for growth tolerance in both acidic and alkaline conditions (Logan and Vos, 2015). Some *Bacillus subtilis* strains have also been shown to be lactic and acetic acid producers and would thus possibly display a level of tolerance (Gao *et al.*, 2012; Yan *et al.*, 2013).

A two-fold difference in activity between the pH 4.5 fermentate and the MRSc broth at the same pH was the case for *E. faecalis* ATCC 7080, where the *B. longum* ITT 13 fermentate generated an MIC_{50} of a 1/8 dilution and 80 AU/ml, whereas the pH 4.5 MRSc broth generated an MIC_{50} for the 1/2 dilution and 20 AU/ml (Figure 3.8 and Table 3.3). *E. faecalis* has been shown to be acid-resistant with growth observed as low as pH 4.0 (Nakajo *et al.*, 2006). The aforementioned strains *E. faecalis* and *B. subtilis* were among the strains displaying the highest levels of susceptibility to the fermentate in the MIC_{50} assay (80 AU/ml), as well as the M/D assay (20.4 and 16.7 AU/ml respectively).

For *E. coli* ATCC 25922 (Figure 3.10), *P. aeruginosa* PA01 (Figure 3.11) and *S. typhimurium* ATCC 29629 (Figure 3.12), the *B. longum* ITT 13 fermentate generated inhibition that was a single-fold higher than that measured for the pH 4.5 MRSc which was at the same pH as for the pH adjusted fermentate. The fermentate generated an MIC_{50} of the 1/4 dilution and 40 AU/ml and the MRSc pH 4.5 broth generated an MIC_{50} of an 1/2 dilution and 20 AU/ml for both *E. coli* ATCC 25922 and *P. aeruginosa* PA01 (Figure 3.10, Figure 3.11 and Table 3.3). An MIC_{50} of a 1/8 dilution and 80 AU/ml for the fermentate and a 1/4 dilution and 40 AU/ml for the pH 4.5 MRSc broth was observed against *S. typhimurium* ATCC 29629 (Table 3.3).

In contrast to the results generated from the species previously described, *S. xylosus* ATCC 29971 displayed higher susceptibility to the pH 4.5 MRSc broth, with an MIC_{50} of a 1/16 dilution and 160 AU/ml in comparison to an MIC_{50} of a 1/8 dilution and 80 AU/ml for the fermentate (Figure 3.9 and Table 3.3). The fermentate displayed an average of 47% inhibition at the 1/16 dilution, with standard deviation of ± 26% in comparison to the average OD600 of the control of MRSc broth which was taken as 100% growth. The high standard deviation indicates fluctuation between replicates and that there was the possibility that activity of the fermentate had likely risen above 50% inhibition for some replicates. Thus some replicates had the same MIC_{50} as the pH 4.5 fermentate. The high standard deviation determined for the inhibition of *S. xylosus* by the *B. longum* ITT 13 fermentate is indicative that the results cannot be taken to be statistically significant in relation to a higher effect on the inhibitory growth of the strain for the fermentate than for the pH 4.5 MRSc broth alone. The MRSc broth at pH 4.5 displayed an average of 59% inhibition at the 1/16 dilution in comparison to the OD600 of the control of the diluted MRSc broth, which would be taken as 100% growth. However, as the MIC_{50} was based off the average inhibition and at least 50% inhibition was required to determine the MIC_{50} dilution, the *B. longum* ITT 13 pH 4.5 fermentate displayed an MIC_{50} of a 1/8 dilution (80 AU/ml) while the MRSc broth also at pH 4.5 of a 1/16 dilution (16 AU/ml).

S. xylosus has been shown to be survive at a low pH of 4 (Leroy *et al.*, 2017) with organic acid production including lactic acid resulting in beer spoilage (Yu *et al.*, 2019). *S. xylosus* has also been shown to display susceptibility to bacteriocins produced by *Pediococcus acidilactici* MCL11 (512 BU/ml i.e. Bacteriocin Unit/ml - equivalent to AU/ml) and *Enterococcus faecium* MCL13 (64 BU/ml) in a study by (Lim, 2016).

As the pH 4.5 MRSc broth displayed less activity than the fermentate in five of the six strains, this indicates there was likely other constituents other than acidic conditions and low pH contributing to antimicrobial activity. The increased antimicrobial effect of the fermentate against some strains may be caused by a combination of organic acids produced by the *B. longum* ITT 13 as *Bifidobacterium* spp. are heterofermentative species (Ruiz-Aceituno *et al.*, 2020), as only lactic acid was used to adjust the pH of the pH 4.5 MRSc broth. In the study by Makras and De Vuyst (2006) they demonstrate how a variety of *Bifidobacterium* strains exhibited antimicrobial activity associated with bacteriocin productions towards gram-positive bacterial strains, however the activity exhibited towards gram-negative strains was associated with organic acid production and the lowering of pH, namely acetic and lactic acid. This may be the case with *B. longum* ITT 13, explaining why activity was detected against all gram-negative strains tested for susceptibility using the microdiffusion assay in Section 3.2.1 (Figure 3.4). However, when using the MIC_{50} assay (Section 3.2.2) for quantitative results less activity was observed by the fermentate against the gram-negative strains (with 40 AU/ml generated for two gram-negative strains and 80 AU/ml for the other) in comparison to the gram-positive strains (80 AU/ml for all strains) (Table 3.3). This was also similar to the microdiffusion assay (Section 3.2.1) whereby increased antimicrobial activity was detected against the gram-positive strains than the gram-negative strains, with 11-20 AU/ml detected for the gram-positive strains (Table 3.1) compared to only 10-11 AU/ml for the gram-negative strains (Table 3.2).

The activity of the fermentate was also compared to the activity of the nisin positive control for effectiveness (Figures 3.7-3.12). For *B. subtilis* ATCC 1174 and *S. xylosus* ATCC 29971, the MIC_{50} of nisin was a 1/8 dilution and 80 AU/ml, which was identical to the antimicrobial activity of the *B. longum* ITT 13 fermentate (pH 4.5) against these strains. As the nisin contained 1000 IU/ml in the undiluted form, the 1/8 MIC_{50} thus equated to an MIC_{50} of 125 IU/ml for the nisin against both *B. subtilis* ATCC 1174 and *S. xylosus* ATCC 29971. As the activity had been identical to that of the pH 4.5 fermentate, this can be suggestive of the fermentate also displaying an MIC_{50} associated with 125 IU/ml, however, the reason dilutions of the fermentate are used for the calculation of the MIC_{50} of the *B. longum* ITT 13 fermentate is due to the fact there has been no determined concentration of the actual inhibitory component present within the fermentate as this has not been solely identified.

For *E. faecalis* ATCC 7080, the antimicrobial activity detectable for the fermentate was higher with the MIC_{50} value determined as being the 1/8 dilution equivalent to 80 AU/ml, in comparison to nisin with an MIC_{50} for the 1/4 dilution and 40 AU/ml, equating to 250 IU/ml (Figure 3.8). The latter results indicate the comparability of the antimicrobial activity detectable for the *B. longum* ITT 13 fermentate (pH 4.5) to nisin (1000 IU/ml undiluted), however it is noted that the nisin used as the positive control is pure, while the fermentate contains many substances, which individually or collectively could be associated with antimicrobial activity and therefore would warrant further evaluation. In the M/D assay (Section 3.2.1) the nisin (1000 IU/ml undiluted) displayed higher levels of inhibition than the fermentate for all of the gram-positive strains (Table 3.1), with approximately 88.9 AU/ml (activity detected to as low as 62.5 IU/ml) determined for all of the tested strains in comparison to the fermentate which had between 10.2 and 20.4 AU/ml against the tested strains (Figure 3.4, Table 3.1 and Table 3.2).

Although some low-level antimicrobial activity was detected for nisin at a 1/2 dilution for both

E. coli ATCC 25922 and *S. typhimurium* ATCC 26929 in Figures 3.10 & 3.12, this is likely a result of dilution of the Mueller Hinton broth nutrients, which is already a nutrient limiting media and this low-level inhibition is not observed for the MRSc broth controls as the MRSc broth provides additional nutrients. The nisin is prepared in 0.02N HCl (Section 2.5).

Nisin, produced by *Lactococcus lactis,* displays antimicrobial activity towards Gram-positive strains, with limited activity in Gram-negative strains due to the relatively impermeable outer membrane present in gram-negative cells (Masusaki *et al*., 1998). A destabilization effect of the cell membrane may take place with *B. longum* ITT 13, whereby the organic acids may weaken cell membranes thus increasing the efficacy of an antimicrobial peptide, resulting in a synergistic reaction between acid and peptide against some strains. This effect has been observed in a paper by Ajingi *et al.* (2020) where the efficacy of nisin was increased to sub-MIC concentrations against a potato spoiling strain of *B. subtilis* when used in conjunction with organic acids such as formic acid and lactic acid, providing a synergistic effect between nisin and the organic acids.

The antimicrobial activity and the spectrum of activity explored in Section 3.2.1 of the fermentate produced by *B. longum* ITT 13, and the GRAS status of *Bifidobacterium* (O'Callaghan and van Sinderen, 2016) indicates the potential suitability of *B. longum* ITT 13 as a biopreservative agent in food applications or as a potential probiotic strain. Nisin inhibits common gram-positive foodborne bacteria and has been approved for use as a food preservative in over 50 countries (Gyawali and Ibrahim, 2014). The review of *Bifidobacteria* by Cheikhyoussef *et al.* (2008) discusses the activity of antimicrobial peptides produced by various *Bifidobacterium* strains against foodborne pathogens such as *E. coli* and *Salmonella* and the potential of bacteriocins produced by this genus as biopreservatives to increase shelf-life and food safety. As *B. longum* ITT 13 has displayed activity against strains including *E. coli* and *S. typhimurium* this furthers the potential of its application in industry. *B. longum* ITT

13 was isolated from the human gut and *Bifidobacteria* for probiotic consideration are recommended to be isolated from the same species as intended use, with other requirements including low pH tolerance (Mahmoudi *et al.*, 2013), exhibited by *B. longum* ITT 13 which reduces the pH of its growth medium to pH 4 during growth.

When comparing the MIC_{50} assay to the Microdiffusion assay, the differences in sensitivity are evident. It is evident that when comparing the results determined from the M/D assay (Section 3.2.1) with those values obtained from the MIC_{50} assays (Section 3.2.2), that the antimicrobial activity in terms of AU/ml was higher when observed for the MIC_{50} assay. Thus the MIC_{50} test can be confirmed to detect higher levels of inhibition in relation to the evaluation of the antimicrobial activity generated by the fermentate produced by *B. longum* ITT 13. This conclusion is in-line with Inturri *et al.*, (2019) who detected stronger inhibition by the supernatant of *B. longum* BB536 and *L. rhamnosus* HL001 against some indicator strains when using the broth microdilution method in microtitre plates – equivalent to the MIC_{50} assay carried out in Section 3.2.2. Combinations of well-diffusion (microdiffusion) and broth microdilution to determine MIC have been used to screen antimicrobial activity produced by other bacterial strains. Salivaricin mmaye1, a bacteriocin produced by *Lactobacillus salivarius* SPW1, was screened for its antimicrobial spectrum of activity using the well-diffusion assay and microtitre plates used to determine the MIC (Wayah and Philip, 2018) both proving effective for determination.

3.5.3 Antibacterial Testing with pH Adjusted Fermentate.

Bifidobacteria are heterofermentative organisms, producing a combination of organic acids upon growth such as lactic and acetic acid (Ruiz-Aceituno *et al.*, 2020). The production of organic acids by *Bifidobacteria* has been associated with some antimicrobial activity displayed

by the species (Makras and De Vuyst, 2006) and as the *B. longum* ITT 13 fermentate had a considerably low pH of approximately pH 4.2-4.3 without pH control during growth, the impact of low-level pH increases of the fermentate, without pH neutralization, on the antimicrobial activity were assessed.

The *B. longum* ITT 13 fermentate was adjusted to pH 4.7 to assess how an increase in pH would affect the level of antimicrobial inhibition displayed against three test strains in comparison to the fermentate without any adjustment. The three indicator strains chosen included one gram-negative strain (*P. aeruginosa*) and two gram-positive strains (*B. subtilis* and to completely assess the activity. Several publications report research work involving the neutralisation of the cell-free supernatant fermentate which contains a bacteriocin and evaluating whether the lack of acidity has any effect on the overall antimicrobial activity demonstrated from the lactic acid bacteria (Yang *et al.*, 2012; Liu *et al.*, 2015; Vijayakumar and Muriana, 2015). The results of a previous research project involving ITT 13 indicated a loss of detectable antimicrobial activity above pH 5 for the *B. longum* ITT 13 fermentate in the microdiffusion assay. Therefore, it was decided to adjust the fermentate to pH 4.7 instead to keep the pH mid-range between the fermentation pH of 4.2-4.3 and the more neutral pH 5. Although activity has been lost with pH neutralization of the fermentate above pH 5 in previous work using the microdiffusion assay, it has been detected above this pH in the more sensitive MIC assay (C. Whelan – personal communication). The loss of activity at higher pH can indicate the possibility that organic acids are primarily responsible for antimicrobial activity, however some antimicrobial products retain higher activity in acidic pH conditions, as the commercially used antimicrobial peptide of nisin retains higher active at a lower pH and is prepared in 0.02 N HCl for this reason (Matsusaki *et al.*, 1998).

The results showed that the statistically significant p-values determined for all three of the strains tested indicated that the antimicrobial activity caused by the fermentate adjusted to pH

4.68 was significantly different to any antimicrobial activity caused by the pH adjusted MRSc broth (pH 4.82), meaning the activity of the pH adjusted fermentate was not solely the result of a pH effect caused by a low pH. The results presented are strongly suggesting that the antimicrobial activity of the fermentate is not solely associated with the pH values tested. *Bifidobacteria* are known to produce a number of acids including acetic, lactic and formic acids (Makras and De Vuyst, 2006; Ruiz-Aceituno *et al.*, 2020). In carrying out the pH adjustment experiment above lactic acid was used to adjust the pH and to fully evaluate the effect of this the acids content and associated pH, the contribution of the various acids needs to be evaluated further. This can be supported by evidence in the publication by Makras and De Vuyst (2006) whereby they used a variety of lactic, acetic and hydrochloric acid to adjust the pH of MRSc broth to pH 4.5 which resulted in significant antimicrobial activity, indicating a combination of acids may be more effective than just the pH.

Statistical analysis of the activity of both the undiluted and 1/2 dilutions of the fermentate without pH adjustment (pH 4.2) compared to the undiluted and 1/2 dilutions of the fermentate pH adjusted to pH 4.68 generated statistically significant p- values that were <0.0001 for both *K. rhizophilia*. This indicates that the level of antimicrobial activity displayed by the fermentate depended on the pH of the fermentate and that increasing the pH reduced the level of antimicrobial activity. The same conclusions were drawn for the 1/2 dilution of the unadjusted fermentate compared to the 1/2 dilution of the pH adjusted fermentate for both *K. rhizophilia* and *P. aeruginosa* (Figure 3.13), as the antimicrobial activity had disappeared completely at the 1/2 dilution of the pH adjusted fermentate while antimicrobial activity remained at the 1/2 dilution for the unadjusted fermentate. The dilution of the pH 4.68 fermentate caused by the addition of NaOH could be hypothesised as the actual cause for the reduction of activity as the dilution effect equated to 5-parts fermentate and 1-part NaOH, i.e. a 5/6 dilution, although a pH effect cannot be ruled out.

For *B. subtilis* there was no statistically significant differences between the undiluted or the 1/2 dilutions of the fermentate with no pH adjustment (pH 4.2) and the fermentate adjusted to pH 4.68, with p-values >0.05. This indicates that increasing the pH of the fermentate resulted in no significant change in antimicrobial activity for this strain.

3.5.4 Antifungal Activity of *B. longum* ITT 13

Fungi are the most common food spoilage organisms (Saleh and Al-Thani, 2019; Snyder, *et al.*, 2019) and can greatly reduce the shelf-life of products without the use of preservatives (Garcia, Bernardi and Copetti, 2019), with biopreservatives offering an appealing alternative to the use of chemical preservatives that are primarily used within the food industry (Kıvanc, Kıvanc and Pektas, 2014). Screening of the *B. longum* ITT 13 fermentate for antifungal activity as well as antimicrobial activity was of importance due to the widespread spoilage of food associated with fungal strains, such as the unsolved issue of fungal spoilage in bread and baked goods (Quattrini *et al.*, 2019). *B. longum* ITT 13 was screened against selected fungal strains that have been known to cause food spoilage, including yeast organisms that result in the conversion of growth substrates within the food matrices to metabolic end products that can alter the chemical and physical properties of food (Fleet, 2011).

The four fungal strains screened for activity were *R. mucilaginosa* CBS 316, *C. albicans* ATCC 2091, *S. cerevisiae* ATCC 18824 and *S. cerevisiae* CBS 1171. Fungal strains were supplied by the sponsor as strains of interest in spoilage. *R. mucilaginosa* CBS 316 was the most susceptible strain to the *B. longum* ITT 13 fermentate both at pH 4.35 (grown without pH control) and pH 4.80 (grown in fermenters with pH control) as well as the pH 4.30 MRSc broth. MIC_{50} values of 80 AU/ml for the pH 4.80 fermentate, 80 AU/ml for the pH 4.35 fermentate and 40 AU/ml for the pH 4.30 MRSc broth were observed for *R. mucilaginosa* CBS 316 (Figure 3.14). The

inhibition of *R. mucilaginosa* CBS 316 was statistically significant with all p-values below 0.05 when compared to the growth control and as the inhibition by the fermentate at both the pH 4.80 and pH 4.35 was detected up to a 1/8 dilution it was also biologically significant. LAB strains such as *Lactobacilli* have been shown to display strong antifungal activity against *R. mucilaginosa* at densities of 10^7 CFU/ml as demonstrated by Lačanin *et al.*, (2017). A combination of *Lactobacillus plantarum* L244 and *Lactobacillus harbinensis* L172 inhibited the growth of *R. mucilaginosa* for 7 days in sour cream (Leyva Salas *et al.*, 2018), thus high levels of inhibition by ITT 13 are not unusual given the similarities between *Lactobacilli* and *Bifidobacterium*.

C. albicans ATCC 2091 was also susceptible to both the pH 4.35 and pH 4.80 *B. longum* ITT 13 fermentate samples, as at a 1/2 dilution the pH 4.80 and pH 4.35 fermentates both had statistically significant p-values below 0.05 indicating that they caused inhibition of the strain in comparison to the growth control of Potato Dextrose (PD) broth (Figure 3.15). Both the pH 4.80 and pH 4.35 *B. longum* ITT 13 fermentate had an MIC_{50} of a 1/2 dilution and 20 AU/ml against this strain as both caused at least 50% inhibition at the 1/2 dilution. Although the pH 4.30 MRSc broth caused inhibition it was below 50% and therefore denoted as having 0 AU/ml – indicating a weaker inhibitory effect than the ITT 13 fermentate. Similar to *R. mucilaginosa,* LAB such as *Lactobacilli* have also displayed antifungal activity against *Candida* spp., with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 strains displaying antifungal activity against vulvovaginal candidiasis causing *C. glabrata* through both autoaggregation and coaggregation (Chew *et al.*, 2015). The neutralised supernatant of *Lactobacillus pentosus* LAP1 displayed potent antifungal activity against various *Candida* strains using the microdiffusion method which generated an inhibitory value equivalent to 150 AU/ml against *C. albicans* (Aarti *et al.*, 2018), which was significantly higher than the activity of ITT 13. For the fungal strain *S. cerevisiae* ATCC 18824, only the fermentate without pH control (pH 4.35) was found to

display antifungal activity with a significant p-value of 0.0001, while the fermentate which had been adjusted to pH 4.80 fermentate displayed no significant activity (Figure 3.16). The later results for the *S. cerevisiae* ATCC 18824 are compared with those for *S. cerevisiae* CBS 1171 (Figure 3.17) where both the pH 4.35 and pH 4.80 fermentates displayed significant antifungal acivity (p-value <0.05). Although statistically significant antifungal activity was detected by the *B. longum* ITT 13 pH 4.35 fermentate against *S. cerevisiae* ATCC 18824 and by both the pH 4.35 and pH 4.80 fermentates against *S. cerevisiae* CBS 1172, it may not be biologically significant, as the antifungal activity was below 50% inhibition for both strains at a 1/2 dilution, which in terms of the MIC_{50} calculation equates 0 AU/ml. Future work should include further concentration of the samples in order to evaluate the effectiveness of the inhibition caused by the *B. longum* ITT 13 fermentate's to a greater degree.

Bifidobacterium spp. and other lactic acid bacteria have been previously reported to display antifungal activity similar to that determined for the *B. longum* ITT 13 strain in these studies. In a study by Ghazvini *et al* (2016) *Bifidobacterium bifidum* PTCC 1644 and *Lactobacillus fermentum* PTCC 1744 were shown to reduce the mycelial mass of *Aspergillus parasiticus* PTCC 5286 by 82% and 77% respectively when the fungal strain was cultured in their presence. Both bacterial strains were also shown to reduce the aflatoxin production by the fungal strain, which is a carcinogenic mycotoxin and that can contaminate both human and animal food commodities (Ghazvini *et al.*, 2016). In another study by Fernandez *et al* (2017), various *Lactobacilli*, *Propionibacterium* and *Bifidobacterium animalis* subsp. *lactis* A026 displayed strong antifungal activity against various fungal strains including *Penicillium chrysogenum* LMA-212 and *Aspergillus versicolor* LMA-370. *Lactobacillus rhamnosus* A238, both alone and in combination with *Bifidobacterium animalis* subsp. *lactis* A026, inhibited the growth of *P. chrysogenum* for 21 days (Fernandez *et al.*, 2017). *B. longum* BB536 was also shown to display antifungal activity towards clinical and reference *Candida* strains (Inturri *et al.*, 2019).

The fungal strains were also screened for sensitivity to a low pH and organic acids in the pH adjusted MRSc, where both lactic and acetic acid were used to adjust the pH of the MRSc broth to pH 4.30. Organic acid have been shown to demonstrate antifungal activity, such as acetic acid which has been shown to be one of the most relevant antifungal compounds produced by LAB, with lactic, propionic and sorbic acids also displaying effects (Lind *et al*, 2005; Quattrini *et al.*, 2019; Debonne *et al.*, 2020). Samapundo *et al.*, (2016) demonstrated that fermentates obtained from unidentified bacteria, containing varying concentrations of propionic, lactic and acetic acids, displayed increasing antifungal activity at lower pH's against *Asperigillus* strains. The pH 4.30 MRSc broth displayed complete inhibition up to a 1/4 dilution and partial inhibition at a 1/8 dilution, with 40 AU/ml for *R. mucilaginosa* CBS 316 (Figure 3.14). The pH 4.30 MRSc broth also displayed partial inhibition of *C. albicans* ATCC 2091 at a 1/2 dilution, however the inhibition was less than 50% and based on the MIC_{50} calculation requiring at least 50% inhibition at a given dilution for MIC_{50} and AU/ml determination, the pH 4.30 MRSc broth therefore generated 0 AU/ml against *C. albicans* ATCC 2091 (Figure 3.15). Statistically significant p-values of less than 0.05 were observed for the fungal growth of both *R. mucilaginosa* CBS 316 and *C. albicans* ATCC 2091 when incubated with the pH 4.30 MRSc broth in comparison to the growth control of PD broth – indicating the pH 4.30 MRSc broth containing 39.9 mM lactic acid and 60.9 mM acetic acid displayed an antimicrobial effect. For both *S. cerevisiae* strains, the p-value of the pH 4.30 MRSc broth in comparison to the growth control was non-significant (>0.05), indicating the acids did not display a significant antimicrobial effect (Figures 3.16 & 3.17). Thus, it is possible for some fungal strains, namely *R. mucilaginosa* CBS 316 and *C. albicans* ATCC 2091, that the inhibition caused by the fermentates may be partially or completely due to the concentration of organic acids as produced by *B. longum* ITT 13.

However, it should be noted that there was higher detectable antifungal activity associated with

both the pH 4.35 and pH 4.80 *B. longum* ITT 13 fermentate samples as compared to the antifungal activity detected for pH 4.30 MRSc broth. The p-values for the antifungal activity of each fermentate in comparison to the pH adjusted MRSc broth were statistically significant (<0.05) for all fungal strains (Figures 3.14-3.17), except for the pH 4.80 fermentate with *S. cerevisiae* ATCC 18824 compared to the pH adjusted MRSc (Figure 3.16). The overall results obtained for the antifungal studies thus signify that more potent antifungal activity is associated with fermentate, even when the fermentate is at the equivalent pH as the pH adjusted pH 4.3 MRSc broth. Therefore, the MIC_{50} results for the antifungal studies on the selected strains would indicate that partial antifungal inhibition may be caused by a source other than organic acids within the fermentate and this suggestion that the antimicrobial activity associated with the *B. longum* ITT 13 is associated with more than just organic acid production is further developed in Chapter 4, with the indication of a possible proteinaceous compound with antimicrobial activity also produced by *B. longum* ITT 13 in low concentrations (Sections 4.3 and 4.4). Some proteinaceous molecules produced by lactic acid bacteria have been shown to display antifungal activity e.g. *L. plantarum* CECT 749 produced bioactive peptides displaying antifungal activity against *Aspergillus parasiticus* CECT 2681 and *Penicillium expansum* CECT 2268 (Luz *et al.,* 2017). A synergistic combination of organic acid production and proteinaceous molecules produced by LAB including *Lactobacilli,* as detailed by Ruggirello *et al.,* (2018), was responsible for some antifungal activity against various *Penicillium* and *Aspergillus* strains and may indicate this as a possibility for the antifungal activity of ITT 13 (Chapter 4).

HPLC analysis of the *B. longum* ITT 13 fermentate grown in MRSc broth both produced in 2L fermentation vessels at pH 4.80 and grown in an anaerobic chamber without pH control (pH 4.35) was carried out in a supervised undergraduate project within the laboratory, to determine the organic acid production by the strain (Section 4.5). It was found that the pH 4.80 fermentate

contained approximately 68.3 ± 10.6 mM lactic acid, 161.0 ± 13.7 mM acetic acid and 67.7 ± 6.2 mM succinic acid. This equates to approximately 34.2 mM lactic acid, 80.5 mM acetic acid and 33.9 mM succinic acid at the initial 1/2 dilution used in the microtitre plates. The pH 4.35 fermentate contained approximately 92 ± 1.5 mM lactic acid, 147.7 ± 10.3 mM acetic acid and 54.5 ± 3.0 mM succinic acid (46.0, 73.9 and 27.3 mM respectively at a 1/2 dilution in microtitre plates). The concentrations of the lactic and acetic acid of both the pH 4.35 and pH 4.80 fermentates are both significantly higher than those present in the pH 4.30 MRSc broth – which were 39.9 mM lactic acid and 60.9 mM acetic acid. The fact that the concentration of lactic and acetic acid in the *B. longum* ITT 13 fermentate is higher than in the pH 4.3 MRSc broth used as a comparison in the antifungal studies, would suggest that further studies with variable acid concentrations would be beneficial in elucidating the full effect of the organic acids present in the antifungal activity. *B. animalis* subsp. *lactis* A026 which displayed antifungal activity against various moulds, was determined to have a concentration of 70.5 ± 1.1 mM lactic acid and 91.6 ± 1.0 mM acetic acid present in the fermentate when cultured in MRSc broth and 4.9 ± 0.4 mM lactic acid and 40.9 ± 1.5 mM acetic acid when cultured in skim milk (Fernandez *et al.*, 2017). The concentrations of lactic and acetic acid produced by *B. animalis* subsp. *lactis* A026 in this study are higher in the fermentate cultured in MRSc broth than the acids in the 1/2 dilutions of the *B. longum* ITT 13 fermentate which is the first dilution on the MIC_{50} plate. However, the concentrations of the lactic and acetic acids in the skim milk fermentate produced by *B. animalis* subsp. *lactis* A026 are lower than the concentrations of the acids in the *B. longum* ITT 13 fermentates at the 1/2 dilution in the fungal MIC_{50} plates, yet the skim milk fermentate still displayed antifungal activity. This indicates that concentrations of the lactic and acetic acids lower than those present in the *B. longum* ITT 13 fermentate are capable of demonstrating detectable antifungal activity. Another study demonstrated that concentrations of acetic acid as low as 20 mM in pH 4 media displayed antifungal activity

against *Penicillium nordicum* PFE 487 (Schillinger and Villarreal, 2010). *C. albicans* SC55314 displayed increasing levels of susceptibility to acetic acid from 40-75 mM at pH 4.0-4.5 (Lourenço *et al.,* 2019), which is comparable to the concentration of acetic acid present in the ITT 13 fermentates (70-80 mM) at the 1/2 dilution, at which *C. albicans* ATCC 2091 was inhibited (Figure 3.15). *S. cerevisiae* has displayed higher tolerances to acetic acids with MIC values of 110-180 mM (Stratford *et al.*, 2013) and these results are in agreement with those observed for the *B. longum* ITT 13 pH 4.3 and pH 4.8 fermentate's at the 1/2 dilutions in the MIC_{50} assays. The concentration of acetic acid in the *B. longum* ITT 13 fermentates was significantly lower than 110-180 mM at the 1/2 dilution in the microtitre plates (73.9 mM in the pH 4.35 fermentate and 80.5 mM in the pH 4.80 fermentate at the 1/2 dilution), it offers an explanation to why there was minimal inhibition of the *S. cerevisiae* strains with no MIC_{50} detected (Figures 3.16 and 3.17).

The results generated in the MIC_{50} testing of the 4 selected indicator fungal strains display varying levels of susceptibility to both the *B. longum* ITT 13 pH 4.3 fermentate and the fermentate at pH 4.8. Significant concentrations of acids are now known to be produced by *B. longum* ITT 13 which may result in the observed antifungal activity and further studies are required to establish the potency of the acid concentrations present.

CHAPTER 4

Physicochemical Characterisation of *Bifidobacterium longum* ITT 13 Antimicrobial Activity

Characterisation of antimicrobial activity produced by bacteria is an important aspect of research to determine suitability of bacteria or their metabolites for use in food production or probiotic application. Characteristics such as thermal stability and pH stability are important for molecules that can be used in food applications with potential as biopreservatives, due to high food processing temperatures and various pH processing conditions of food, or the low pH of fermented food products (Johnson *et al.*, 2018; Bagde and Vigneshwaran, 2019; Castilho *et al.*, 2020). Bacteria for consideration as probiotics should also be tolerant of low pH for transition through the stomach and gastrointestinal system (Mahmoudi *et al.*, 2013). As *Bifidobacteria* and other LAB strains such as *Lactobacilli* and *Pediococci* have been shown to produce both organic acids and antimicrobial peptides or bacteriocins contributing to antimicrobial activity (Yildirim and Johnson, 1998; Collado *et al.*, 2005; Yang *et al.*, 2012; Liu *et al.*, 2015; Delgado *et al.*, 2019; Lourenço *et al.*, 2019; Skariyachan and Govindarajan, 2019), it is also an important factor to characterise the metabolites contributing to any antimicrobial activity produced by bacteria. *B. longum* ITT 13 has been shown to display antibacterial and antifungal activity, therefore the stability and the properties of this antimicrobial activity were investigated within this chapter through physiochemical characterisation.

Results:

4.1 Thermostability Assay of *B. longum* ITT 13 Fermentate

Food preservation is an important area of human health as improper preservation can result in human illness and disease outbreaks associated with spoilage organisms (Linscott, 2011; Singh, 2018). For consideration of the application of antimicrobial peptides in food preservation, heat stability is an important characteristic as the food industry can involve the use of high processing temperatures, as stated by Bagde and Vigneshwaran (2019). The stability of the antimicrobial activity produced by *B. longum* ITT 13 was assessed from -20^{o}C to 70^{o}C to determine the effect of heat and storage at various temperatures on the stability of the antimicrobial activity displayed by the organism.

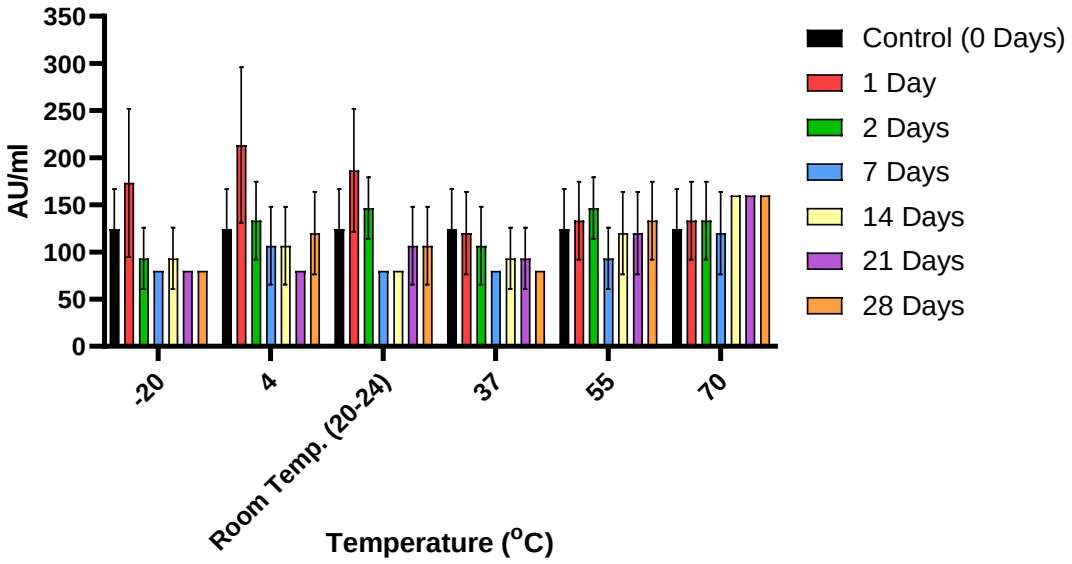

Figure 4.1: The antimicrobial activity (AU/ml) of *B. longum* ITT 13 cell free fermentate incubated at -20^{o}C to 70^{o}C over a 28-day period. The microtitre plate (MIC) procedure (Section 2.8.4) with *S. xylosus* as the indicator strain, was used for analysis. AU/ml was calculated as describe in Section 2.8.2. Standard deviations were determined for a minimum of three independent assays (n=3).

The control used for the experiment was the antimicrobial activity associated with the *B. longum* ITT 13 fermentate as measured prior to incubation at any of the temperatures and gave

an average value of 124.4 AU/ml ± 42.1.

The fermentate stored at the -20^{o}C incubation temperature had a measurement of activity with an average of 173.3 AU/ml ± 78.7 after 1 day of incubation, which is slightly higher than the value recorded from the control, however there is notable deviation between replicates due to the larger error bars for the replicate samples. The activity of the -20^{o}C incubated ITT 13 fermentate drops below the activity of the control after 2 days incubation to an average of 93.3 AU/ml ± 32.7, where it then fluctuates between 80.0 AU/ml ± 0 and 93.3 AU/ml ± 32.7 for the rest of the time period up to 28 days. The highest activity was thus retained after 1 day of incubation at -20^{o}C and there was no percent loss after the first day of incubation relative to the control. Over the 2-28 day incubations there was between 25% and 36% loss of activity determined in comparison to the control value which was taken as 100% antimicrobial activity with 124.4 AU/ml.

The *B. longum* ITT 13 fermentate incubated at 4^{o}C had an average of 213.3 AU/ml ± 82.6 after 1 day of incubation, higher than the control but again comparable as the standard deviation is large and indicates some replicates were equivalent to the control and some higher. The antimicrobial activity retained after 2 days (133.3 AU/ml ± 41.3) was also equivalent to the non-incubated control. It then dropped below the control to an average of 106.7 AU/ml ± 41.3 after 7 days incubation (14% loss), where it remained below the control and fluctuated between 80.0 and 106.7 ± 41.3 AU/ml up to 21 days, before climbing to 120.0 AU/ml ± 43.8 after 28 days. The highest antimicrobial activity was observed within the first two days of incubation at 4^{o}C, before remaining consistently below the activity of the control by 4-44 AU/ml for the remainder of the 28-day incubation equivalent to between 3-36% losses relative to the control of 100% activity. As the percent loss was equivalent to 36% after 21 days incubation at 4^{o}C, yet there was minimal loss of activity determined at 28 days with only approximately 3% loss, this indicates that with the large standard deviation taken into account for the antimicrobial

activity at some latter timepoints (i.e. day 28) the loss of activity was not consistent at the 4^{o}C incubation temperature.

The fermentate was also incubated at room temperature, which was monitored as being between 20^{o}C and 24^{o}C over the evaluation time. The fermentate incubated at room temperature had an average of 186.7 AU/ml ± 65.3 after 1 day of incubation, which although slightly higher than the control, the error bars associated with the replicates indicated no substantial reduction or change in antimicrobial activity relative to the control. After 2 days incubation the activity dropped to 146.7 AU/ml ± 32.6, which is slightly higher than the value for the control antimicrobial activity (124.4 AU/ml ± 42.2), however when the error bars are taken into consideration, there is no significant difference between the control and the samples incubated at room temperature over the two-day period. After 7 days incubation the activity dropped below that of the control (124.4 AU/ml ± 42.2) to 80 AU/ml ± 0 (36% loss of activity) where it again remained below the control between 80 AU/ml ± 0 and 106.7 AU/ml ± 41.3 for the remainder of the 28-day assay, equivalent to between 14% and 36% losses of activity. The results would indicate that there is no significant loss of activity over the first 2 days incubation at room temperature, however when incubating between 7 and 28 days there is a consistent drop in antimicrobial activity below the average of the control of between 14-36%. The 37^{o}C incubated *B. longum* ITT 13 fermentate was equivalent to the control (124.4 AU/ml ± 42.2) after 1-day incubation with an average of 120.0 AU/ml ± 43.8, which dropped to 106.7 AU/ml ± 42 after 2 days (14% loss of antimicrobial activity relative to the control) and then to 80.0 AU/ml ± 0 after 7 days (36% loss). It fluctuated between 80 AU/ml ± 0 and 93.3 AU/ml ± 32.7 between 7 and 28 days incubation and thus retained the highest activity after 1-2 days incubation, as losses of activity of between 25% and 36% were observed between 7 and 28 days incubation.

The values for antimicrobial activity retained after incubation at the 55^{o}C and 70^{o}C

temperatures were both equivalent to the control of 124.4 AU/ml ± 42.2 after 1 day of incubation with 133.3 AU/ml ± 41.3 determined for both incubation temperatures, indicating initial stability at these temperatures. Apart from the antimicrobial activity measured after 7 days incubation at 55ºC (93.3 AU/ml ± 32.7, equivalent to a 25% loss in activity), the activity of the fermentate never dropped notably below the control and began to increase in activity after 14 days incubation for both the 55ºC and 70ºC temperatures. At the 55ºC incubation temperature, the 14-day and 21-day incubations both displayed an increase in activity of 120.0 AU/ml ± 43.8 (3% loss) for both days compared to the 93.3 AU/ml ± 32.7 observed at 7-days. The antimicrobial activity increased again after 28 days to 133.3 AU/ml ± 41.3 – demonstrating a gradual increase in activity from days 7-28. The increase in antimicrobial activity was also observed for the 70ºC incubation temperature, with a slight drop to 120.0 AU/ml ± 43.8 after 7 days incubation and an increase to 160.0 AU/ml ± 0 observed for the remaining timepoints of day 14 to day 28. The lack of any standard deviation indicates no variation in the increased antimicrobial activity of 160.0 AU/ml at days 14-28 for the 70ºC incubation, relative to the control of 124.4 AU/ml. The increase in antimicrobial activity after 14 days for both incubation temperatures was the result of a concentration effect observed due to evaporation of the fermentate at these higher temperatures after multiple weeks of incubation.

There is large fluctuation in antimicrobial activity observed over the 28-day period with the highest levels of activity concentrated in the first 1-2 days for the -20ºC, 4ºC, room temperature (20-24ºC) and 37ºC, indicating a trend of the highest retention and stability of antimicrobial activity within 48 hours of incubation at a variety of temperatures. The 4ºC incubation temperature generated the least consistent reduction of antimicrobial activity in terms of percent loss relative to the control, as it displayed a 36% loss after 21-days incubation however this increased to only a 3% loss after 28-days, which was similar in activity to the control. The 3% loss at 28-days thus indicated no consistent reduction in antimicrobial activity over the 28

day storage period at 4^{o}C as this temperature generated the lowest overall losses in activity, with the exception of the 55^{o}C and 70^{o}C incubation temperatures which were not accurately comparable due to the evaporation resulting in increased antimicrobial activity.

Statistical analysis using a two-way ANOVA with Dunnet's multiple comparison assay resulted in no significant difference between the antimicrobial activity of the fermentate at any temperature or timepoint and the antimicrobial activity of the control. This absence of statistical significance for the antimicrobial activity of any timepoint/temperature likely indicates that temperature does not impact the active agents as the large standard deviation indicates there was no consistent pattern of reduction of antimicrobial activity for many samples. Although there was no statistical significance as previously mentioned, a biological trend was observed for all samples stored at -20^{o}C to 37^{o}C, whereby the maximum antimicrobial activity was retained for the first 48 hours of storage at each of these temperatures, before successively dropping to lower levels of activity, which is elaborated on in discussion Section 4.6.1.

4.2 pH Stability of *B. longum* ITT 13 Fermentate

pH stability is an important characteristic of potential biopreservatives due to the wide variety of matrices and pH processing conditions of food (Johnson *et al.*, 2018; Castilho *et al.*, 2020). The ability for biopreservation at low pH is important in foods that may contain a starter culture, such as cheese, which has been shown to decrease in pH over storage time with an increase in acid production likely as a result of starter culture bacteria or lactic acid bacteria incorporation into the foodstuff, which was observed by both Ong and Shah, (2009) and Ulpathakumbura *et al.*, (2016). Stability of activity and tolerance at a low pH is also an important characteristic of probiotic bacteria, which should be stable enough to resist passage through the gastrointestinal tract (Mahmoudi *et al.*, 2013) and metabolites such as bacteriocins should be stable enough to retain activity at a variety of food processing conditions including pH (Castilho *et al.*, 2020).

4.2.1 pH Stability with HCl and NaOH

The *B. longum* ITT 13 fermentate was assessed for stability under a variety of pH conditions. Following fermentation without pH control of the *B. longum* ITT 13 and heat inactivation to produce the fermentate (Section 2.4.2) the pH of the fermentate is approximately pH 4.2-4.3. Antimicrobial activity is detectable at this low pH (Section 3.3). The aim of this section of work was to assess the effectiveness of the antimicrobial activity at various pH values, including neutral and basic pH conditions, with the view to potential applicability for use as a biopreservative in the food industry. Preliminary screening for pH stability was carried out with HCl and NaOH, rather than with lactic acid (Section 2.9.3) to provide a general evaluation of the stability of the activity of the fermentate as preliminary work. The fermentate was adjusted to pH 3-8 using HCl (0.3 M) and NaOH (0.3 M) (described in Section 2.9.2). The results for the antimicrobial activity detected at the various pH values, where the pH was adjusted with HCl and NaOH, are presented in Table 4.1.

Table 4.1: The stability of the antimicrobial activity of the *B. longum* ITT 13 fermentate adjusted to pH 3-8 with HCl and NaOH.

Fermentate	Hours	Activity of Fermentate	Activity of MRSc (pH Adj.)
No pH Adjustment (pH 4.15)	0	++	N/A
	2	++	N/A
	24	++	N/A
pH 3.18	0	+++	+
	2	+++	+
	24	+++	+
pH 4.02	0	++	-
	2	++	-
	24	++	-
pH 5.85	0	-	-
	2	-	-
	24	-	-
pH 6.38	0	-	-
	2	-	-
	24	-	-
pH 7.96	0	-	-
	2	-	-
	24	-	-

- = No antimicrobial activity, + = 1.5-3.0 mm, ++ = 3.1-4.5 mm, +++ ≥ 4.6 mm.

In Table 4.1, antimicrobial activity was retained for the fermentate adjusted to pH 3.18 and 4.02, and lost at pH adjustment to pH 5.85, 6.38 and 7.96. The zones of inhibition measured on the M/D plates averaged at ≥ 4.6 mm for the pH 3.18 adjusted fermentate, however there were smaller zones of inhibition observed for the MRSc broth at the same pH with zones between 1.5-3.0 mm in diameter (+), indicating that at pH 3.18 there is an antimicrobial effect associated with the pH conditions. The latter pH adjustment for the fermentate required 0.07 M HCl concentration which could be contributing to the antimicrobial activity present on the M/D plates.

Antimicrobial activity was observed with zones of inhibition between 3.1-4.5 mm for the fermentate adjusted to pH 4.02. There was no activity associated with the MRSc broth at pH 4.02, indicating that the measured antimicrobial activity is only associate with something present within the fermentate and not just specifically pH effect. The zone size of the pH 4 fermentate was similar to the zone for the unadjusted fermentate with zones of inhibition

measured against the *S. xylosus* indicator strain of between 3.1-4.5 mm for the latter unadjusted fermentate on the M/D plates.

4.2.2 pH Stability with Lactic Acid and NaOH

In addition to the use of HCl to adjust the pH, it was decided to also adjust the pH of the fermentate using lactic acid, given that it is one of the acids that is produced by the heterofermentative bacterial strain (Makras and De Vuyst, 2006; Ruiz-Aceituno *et al.*, 2020). The fermentate was adjusted to pH 3, 4, 5 and 6, however as no antimicrobial activity was detected at the pH values of pH 5 and pH 6 of the pH adjusted fermentate (Figure 4.2), only the pH 3 and pH 4 results were graphed and presented with statistical analysis carried out. Subsequent work in our research lab has shown that *B. longum* ITT 13 fermentate produced following pH controlled incubation in an optimised yeast peptone glucose (YPD) produces a fermentate that is active at pH 5 and above when screened using the microtitre plate (MIC) assay (C. Whelan personal communication).

Three independent assays (n=3) for each pH value and time point were carried out using the microdiffusion assay with *S. xylosus* as the indicator strain. The volume of lactic acid or NaOH used to adjust the pH of the *B. longum* ITT 13 fermentate to either pH 3, 4, 5 or 6 was added to MRSc broth pH previously adjusted to pH 4.3 (similar starting pH to the fermentate) and to MRSc broth with no pH adjustment (initially pH 6.18), as controls to ascertain the activity of the pH adjusted fermentate screened in this assay was not solely associated with the pH adjustment (described in detail in Section 2.9.3). For example, the fermentate adjusted to pH 3 had 5.7 mls L.A. to adjust to pH 3, so this volume was also added to the MRSc broth previously adjusted to pH 4.3, as a direct control for the pH 3 fermentate. The same volume of L.A. was also added to MRSc broth that was left at its original pH (pH 6.18) to determine the activity solely associated with acid added to adjust the pH of the fermentate.

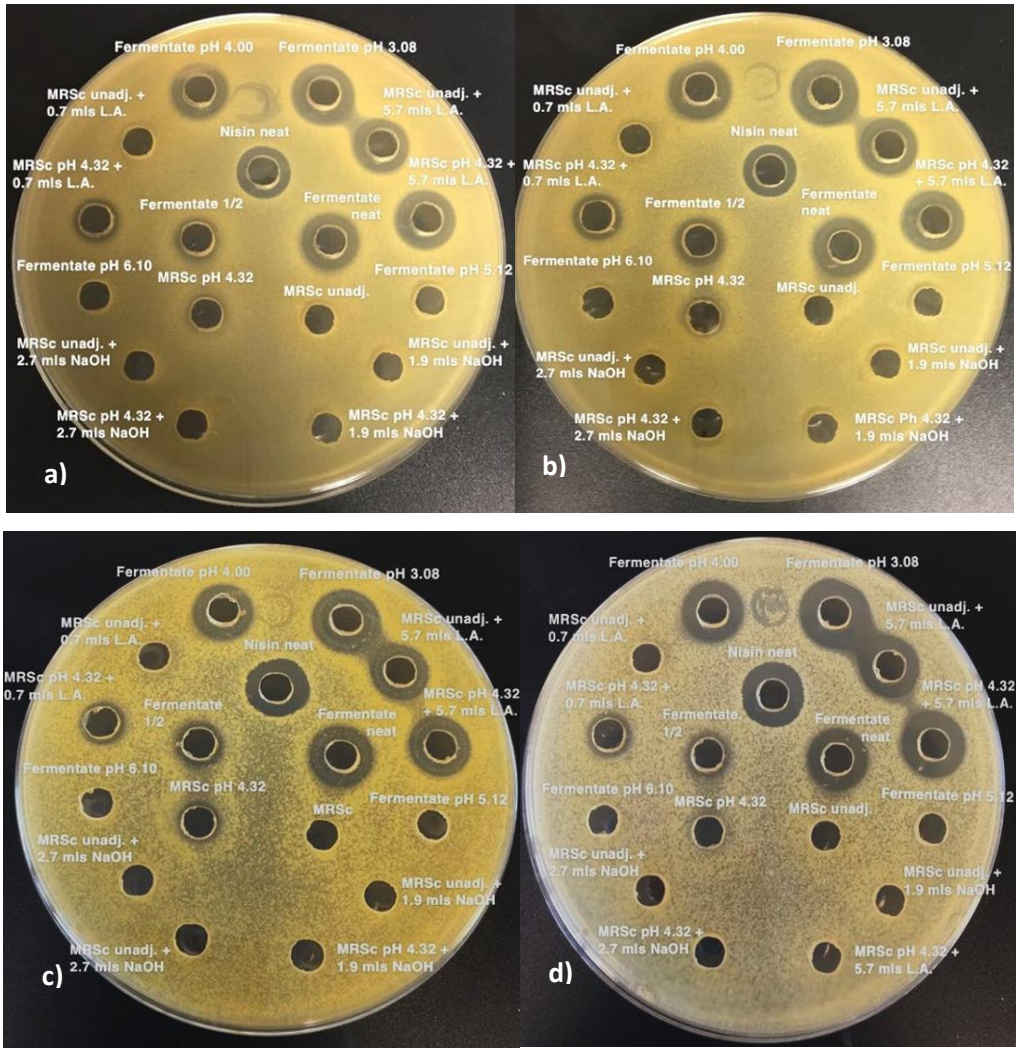

Figure 4.2: pH Stability of the *B. longum* ITT 13 fermentate at pH 3.08, 4.00, 5.20 and 6.12 at a) 0 hours, b) 2 hours, c) 4 hours and d) 24 hours. The M/D assay plates with *S. xylosus* indicator strain were carried out as three independent assays (n=3) to allow for standard deviation (SD) calculation. Zones of inhibition were measured in mm and the results are presented in Figures 4.3 and 4.4.

Zones of inhibition were detected for the *B. longum* ITT 13 fermentate after pH adjustment to pH 3 and pH 4 and incubation over the 24-hour time period, however no antimicrobial activity was detected for the fermentate adjusted to pH 5 or pH 6 at any time point. The zones of inhibition of the pH adjusted fermentate to pH 3 and pH 4 and associated controls are presented and described in Figures 4.3 and 4.4.

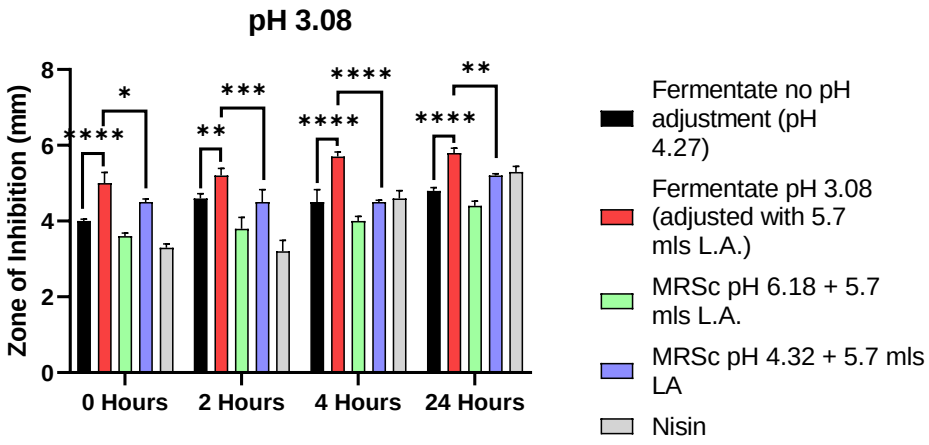

Figure 4.3: The Zones of Inhibition (mm) of the *B. longum* ITT 13 Fermentate at pH 3.08 from 0-24 Hours. The fermentate was adjusted with 5.7 mls of lactic acid (L.A.) to adjust to pH 3.08. The controls were MRSc broth pH 6.18 (original pH) with an added 5.7 mls of lactic acid, MRSc broth initially adjusted to pH 4.32 then with a further addition of 5.7 mls of lactic acid and Nisin (1000 IU/ml) as per Section 2.9.3. Three independent M/D assays (n=3) (Section 2.6) with *S. xylosus* as the indicator strain were carried out to determine standard deviation. Zones of inhibition were measured in mm. Tukeys multiple comparison assay was carried out with the pH 3.08 fermentate compared to the fermentate with no pH adjustment and the pH 4.32 MRSc + 5.7 mls L.A. * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001, **** = p-value ≤ 0.0001.

Figure 4.3 displays the activity of the *B. longum* ITT 13 fermentate at pH 3.08 from 0-24 hours in terms of zone of inhibition (mm). The zones of inhibition (mm) of the fermentate at pH 3.08 were larger (5.0-5.8 mm) than those of the pH 4.3 MRSc broth + 5.7 mls of lactic acid (4.5-5.2 mm) at each timepoint ($p < 0.05$). This is of importance, as these samples are directly comparable given that the MRSc broth was adjusted to pH 4.32 to match the pH of the unadjusted fermentate, and both the fermentate and MRSc broth were then adjusted further with 5.7 mls of lactic acid – bringing the pH of both samples to pH 3 (as described in Section 2.9.3). As the zone of inhibition of the fermentate was larger than the MRSc broth with which it is comparable, this indicates it may not just be a pH effect causing antimicrobial activity against the *S. xylosus*. The activity of the pH 3.08 fermentate is not reduced over time and appears to increase at each time point, however this is likely due to random variation on the M/D plates as the nisin zones of inhibition also increase over time.

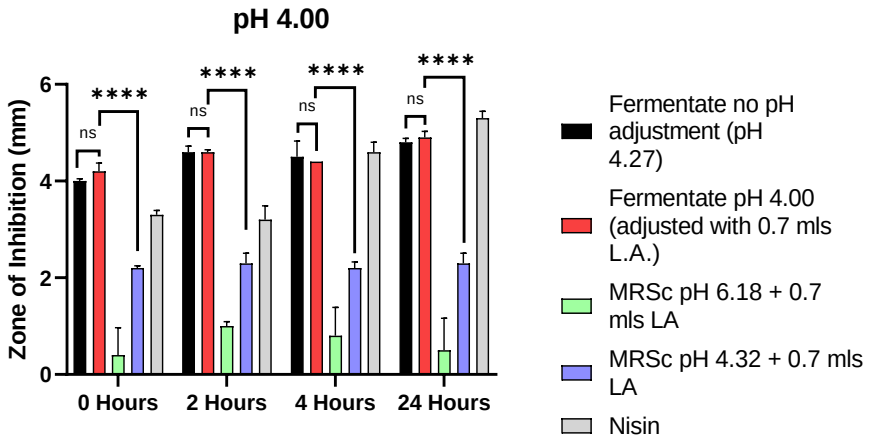

Figure 4.4: The Zones of Inhibition (mm) of the *B. longum* ITT 13 Fermentate at pH 4.00 from 0-24 Hours. The fermentate was adjusted with 0.7 mls of lactic acid (L.A.) to adjust to pH 4.27. The controls were MRSc broth pH 6.18 (original pH) with an added 0.7 mls of lactic acid, MRSc broth initially adjusted to pH 4.32 with a further addition of 0.7 mls of lactic acid and Nisin (1000 IU/ml) as per Section 2.9.3. Three independent M/D assays (n=3) (Section 2.6) with *S. xylosus* as the indicator strain were carried out to determine standard deviation. Zones of inhibition were measured in mm. Tukeys multiple comparison assay was carried out with the pH 4.00 fermentate compared to the fermentate with no pH adjustment and the pH 4.32 MRSc + 0.7 mls L.A. **** = p-value ≤ 0.0001.

The inhibition zones of the *B. longum* ITT 13 fermentate at pH 4.00 were considerably larger in size (4.2-4.9 mm) than those of the MRSc broth at pH 4.32 with 0.7 mls of lactic acid added (2.2-2.3 mm), as observed in Figure 4.4, with p-values of <0.05 providing statistical significance. Again these two samples are directly comparable as the MRSc broth was initially adjusted to pH 4.32 to match the pH of the unadjusted fermentate (pH 4.27), and both the fermentate and MRSc broth were then adjusted further with 0.7 mls of lactic acid – bringing the pH of both samples to pH 4 (as described in Section 2.9.3). The zones of inhibition for the pH 4.00 fermentate did not decrease over the time period tested and in fact appeared to increase at each timepoint (4.2 mm at 0 hours compared to 4.9 mm at 24 hours), however this is likely due to random variation on the M/D plates, as the zones of inhibition for the nisin standards also increased slightly zones over the time points, for both the pH 4.00 fermentate assay the pH 3.08 fermentate assay in Figure 4.3.

4.3 Ammonium Sulphate Precipitation of the *B. longum* ITT 13 Fermentate

Bifidobacteria have been shown in many studies to exert an antimicrobial effect through the production of compounds including organic acids and bacteriocins and the antimicrobial effect exerted by these compounds subsequently aids in a competitive advantage for the producing bacteria in relation to access and competition for nutrients and additionally maintenance of a healthy intestinal microflora through the inhibition of colonisation by pathogens (Lee and O'Sullivan, 2010). Inhibition of pathogenic bacteria in the gut by these compounds and favourable growth of the microflora is also an important characteristic of bacteria for consideration as probiotic strains, as is the safety and GRAS status of the producing strain (O'Shea *et al.*, 2012; Guinane *et al.*, 2016; Choudhary *et al.*, 2019). Bacteriocins are ribosomally synthesised peptides produced by a variety of bacteria that display broad or narrow spectrum inhibitory activity towards other bacterial and fungal strains (Lee and O'Sullivan, 2010; Indira, T. C. Venkateswarulu, *et al.*, 2018; Ruggirello *et al.*, 2018; Zou *et al.*, 2018; Vaičikauskaitė *et al.*, 2019). Bacteriocin production by probiotic bacteria could offer the potential for use in biopreservation, with the bacteriocin Nisin produced by the lactic acid bacterium *Lactococcus lactis* already approved for use as a food preservative in over 50 countries (Gyawali and Ibrahim, 2014). A method for crude purification or isolation of antimicrobial peptides present in the supernatant of producing bacteria is ammonium sulphate (AS) precipitation. This method involves interference with the proteins water solubility causing precipitation by increasing hydrophobic interactions and maintaining the protein conformation (Wingfield, 2001). This method has been widely employed to extract the antimicrobial peptides or bacteriocins produced by bacteria (Contreras *et al.*, 1997; Afshan Naz, Sheikh and Rasool, 2013; Ansari *et al.*, 2018; Ahmad *et al.*, 2019; Skariyachan and Govindarajan, 2019).

In order assess for the presence of any proteinaceous molecules that could be contributing to the antimicrobial activity produced by *B. longum* ITT 13 ammonium sulphate precipitation was

carried out as described in Section 2.9.1.3 and tested using Microtitre plates. Ammonium sulphate precipitation had previously been carried out and tested using the M/D assay as described in Sections 2.9.1.1 and 2.9.1.2 however the results were inconclusive. It was decided to further carry out the ammonium sulphate precipitation and test the *B. longum* ITT 13 fermentate using the more sensitive microtitre plate testing method due to increased sensitivity of the assay (Section 2.9.1.3). The ammonium sulphate precipitation was carried out on 40 ml aliquots of *B. longum* ITT 13 cell free fermentate (CFS) (pH 4.3). Ammonium sulphate was brought up to 20%, 40%, 60% and 80% saturation in separate aliquots of the CFS and precipitated pellets were resuspended in 50 mM pH 4.3 sodium acetate buffer. As the original fermentation was carried out in MRSc broth an equivalent ammonium sulphate precipitation fractionation was carried out with the MRSc media as a control to ensure that if any antimicrobial activity was detected, then it could not be associated with the protein fractions present in the actual media itself. No antimicrobial activity was found in the 20%, 40% and 60% ammonium sulphate fractionation of the fermentate when tested in an microtitre plate assay (Section 2.8.4) (Figure 4.5A, 4.5B, 4.5C). Testing of the 80% fraction showed some low-level antimicrobial activity present when testing the fraction against the indicator strain *S. xylosus* (Figure 4.5D). *S. xylosus* was the only indicator strain selected for the AS precipitation assay as the objective of this experiment was to ascertain whether any proteinaceous material was responsible for antimicrobial activity, and not to determine a spectrum of bacteria susceptible to any antimicrobial peptides.

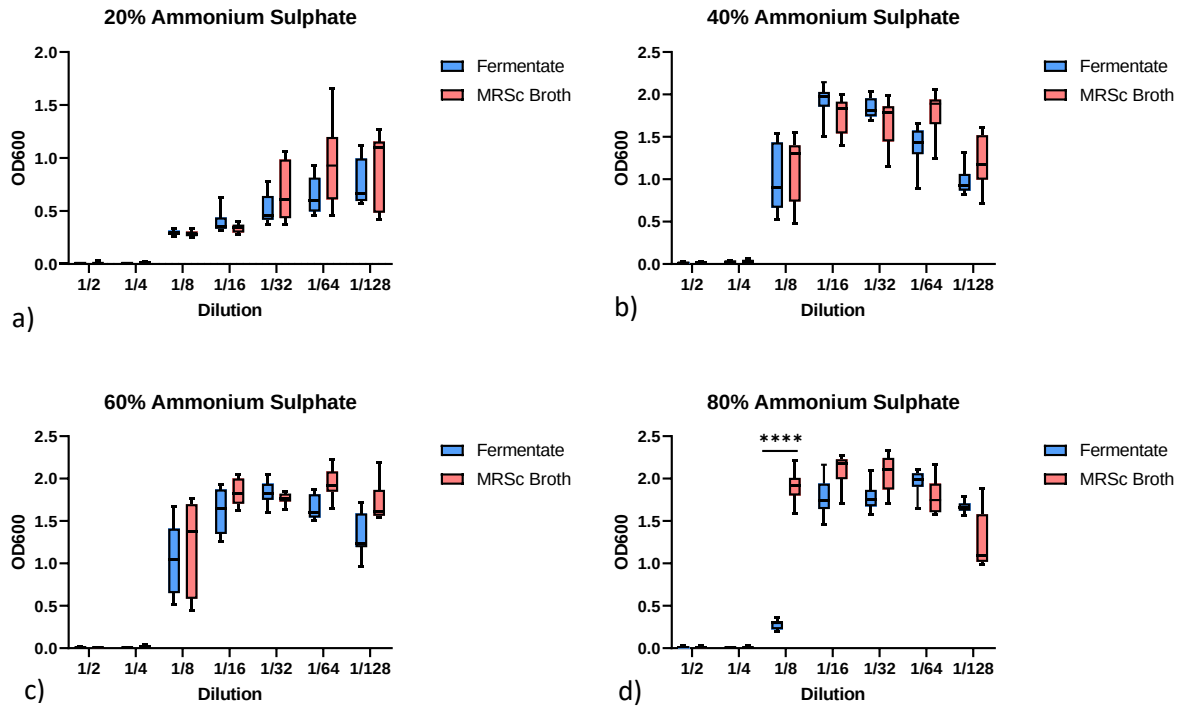

Figure 4.5: Evaluation of the supernatant precipitated with saturations of a) 20%, b) 40%, c) 60% and d) 80% ammonium sulphate. Three independent (n=3) microtitre plate (MIC) assays were carried out (Section 2.8.4) and used to determine standard deviations. The growth of the indicator strain *S. xylosus* is represented as the OD600 nm and the dilution of the resuspended pellets is observed on the x-axis. Statistical analysis was carried out using a paired t test to compare the test sample and control. **** = p-value <0.0001.

For the 20%, 40%, 60% and 80% that no growth of the indicator strain *S. xylosus* was detected for either the 1/2 or the 1/4 dilution for all 4 ammonium sulphate saturation samples for either of the fermentate or the MRSc broth samples. This is likely associated with either the dilution of nutrients in the Muller Hinton broth that the *S. xylosus* cells were seeded into the plates with or the ammonium sulphate salts used as they were not possible to remove prior to testing, hence the use of the MRSc broth saturated to the same AS percentages. Growth of the indicator strain *S. xylosus* was detected for the 1/4 dilution samples in all four AS saturation samples tested, as observed for the 20%, 40% and 60% fermentate and MRSc broth samples and the 80% MRSc broth sample. The growth pattern detected in the microtitre plates is the same for every dilution of both the fermentate and the MRSc broth samples at 20%, 40% and 60% saturation, indicating

no antimicrobial molecules were isolated from the fermentate at these AS saturations as there was no difference to the MRSc negative control. At the 1/8 dilution of the 80% saturation however, there is inhibition caused by the resuspended fermentate pellet that is not observed for the resuspended MRSc pellet, with a p-value of <0.0001. This is not observed for any dilution at any other AS saturation, whereby the inhibition and growth patterns observed for the *S. xylosus* indicator strain in the microtitre plates was the same for both the fermentate and MRSc broth at 20, 40 and 60% saturations. This inhibition observed by the fermentate for the 80% AS saturation that was not observed for the MRSc at the same saturation is likely indicative of a low molecular weight proteinaceous molecule (Wingfield, 2001) causing antimicrobial activity, and this experimental finding is further evaluated in Section 4.4 which describes the use of protease digestion in determining whether some or all of the antimicrobial activity present in the fermentate is associated with proteinaceous molecule(s).

4.4 Protease Digestion

Protease digestion, which uses various enzymes such as Proteinase K, trypsin and Actinase E, to cleave and inactivate protein structures, is commonly employed to validate the presence and assess the stability of bacteriocin production by various bacteria (Gautam and Sharma, 2015; Pilasombut *et al.*, 2015; Biswas *et al.*, 2017; Lv *et al.*, 2018; Ahmad *et al.*, 2019). Nisin is a bacteriocin that is currently used in food preservation (Gyawali and Ibrahim, 2014), and is digested by the protease enzymes Proteinase K and Actinase E (Matsusaki *et al.*, 1998). Proteases inactivate proteins through the cleavage of peptide bonds; the site of cleavage by the Proteinase K enzyme is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups (*Proteinase K | Sigma-Aldrich*; Saenger, 2013), while Actinase E, (also known as pronase E) exerts hydrolysis on the carboxyl side of glutamic or aspartic acid (*Pronase E | Sigma-Aldrich*). Both Proteinase K and Actinase E are stable from pH 4 above (*Proteinase K | Sigma-Aldrich*, no date; Sigma-Aldrich, no date; Saenger, 2013).

Protease digestion was carried out on the *B. longum* ITT 13 fermentate as described in Section 2.9.5 to further validate that some of the antimicrobial activity produced by the strain was proteinaceous in nature. A positive result for the presence of antimicrobial peptides would cause either partial or complete loss of antimicrobial activity. Proteinase K and Actinase E (each at 5 mg/ml) were the proteases selected due to their capability for digesting nisin, which was used as the positive control in the assay (Matsusaki *et al*, 1998). The microtitre plate (MIC) procedure (Section 2.8.4) was used to evaluate the antimicrobial activity of the digested fermentate (1:1 sample:protease) in comparison with fermentate diluted 1/2 with PBS (the buffer for protease preparation), with the dilutions carried out so that the concentrations in the samples of digested and undigested were equivalent in relation to each other thus allowing for direct comparison.

4.4.1 Proteinase K Digestion

The results for the proteinase K digestion as described in Section 4.4 are presented in Figures 4.6 (nisin), 4.7 (*B. longum* ITT 13 fermentate pH 4.35) and 4.8 (*B. longum* ITT 13 fermentate pH 4.80).

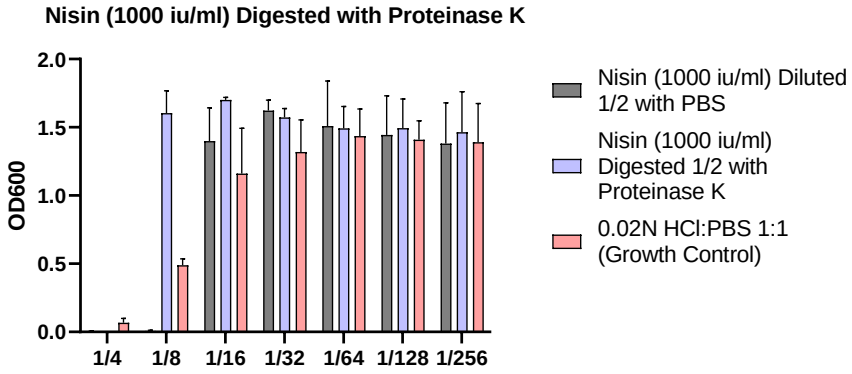

Figure 4.6: The antimicrobial activity detected for undigested and proteinase K digested nisin (1000 IU/ml) against *S. xylosus*. The assay was carried out using the microtitre plate procedure (Section 2.8.4) with *S. xylosus* as the indicator strain. Standard deviations (SD) were determined for three independent assays (n=3). The initial dilution is 1/4 as the samples for testing had already been diluted 1/2 with PBS/Proteinase K. The growth control consisted of a 1:1 ratio of 0.02N HCl:PBS as this is equivalent to the solvent combination present in the 1:1 of nisin:Proteinase K therefore allowing for accurate dilution of the nutrients in the MH broth used for the *S. xylosus* indicator strain in both the growth control and test samples.

It was observed that undigested nisin displays antimicrobial activity up to a 1/16 dilution, while the digested nisin showed reduced growth inhibition of the *S. xylosus* and resulted in inhibition up to a 1/8 dilution.

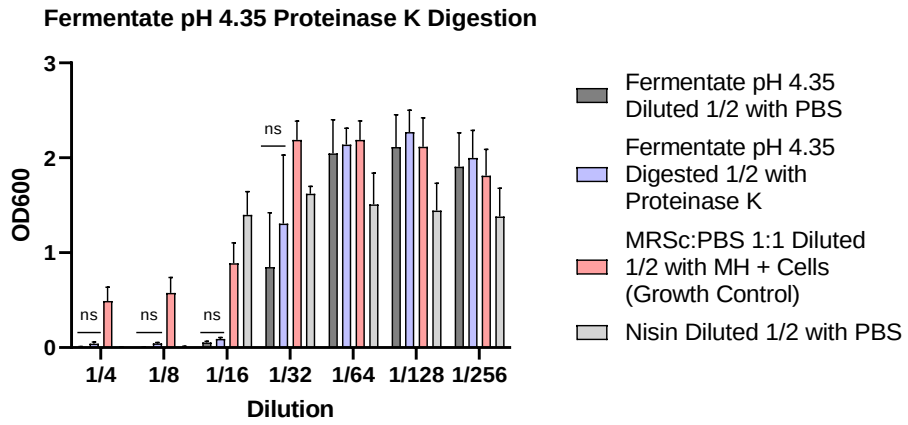

Figure 4.7: The antimicrobial activity detected for undigested and proteinase K digested *B. longum* ITT 13 fermentate at pH 4.35 and controls. The initial dilution is 1/4 as the samples for testing had already been diluted 1/2 with PBS/Proteinase K. Standard deviations were determined for a minimum of three independent assays (n=3). ns = non-significant p-value >0.05. The growth control consisted of a 1:1 ratio of MRSc:PBS as this is equivalent to the combination present in the 1:1 of fermentate:Proteinase K therefore allowing for accurate dilution of the nutrients in the MH broth used for the *S. xylosus* indicator strain in both the growth control and test samples. Nisin (1000 iu/ml) diluted 1/2 with PBS, which is equivalent to the digested and diluted fermentate samples, was used as a positive control for antimicrobial activity.

Both the undigested and digested pH 4.35 fermentate displayed a similar pattern of inhibition of the *S. xylosus*, with antimicrobial activity observed up to the 1/16-1/32 dilutions for both samples and no inhibition detectable on the plate for the 1/64 dilution. P-values for the activity of the undigested fermentate in comparison to the digested fermentate at the 1/4-1/32 dilutions were $p > 0.05$, which indicated that there was no reduction in activity as a result of protease digestion with the Proteinase K of the pH 4.35 fermentate. The p-values were only determined for these dilutions as they displayed the largest difference in the growth of the *S. xylosus* indicator strain between the undigested and digested fermentate.

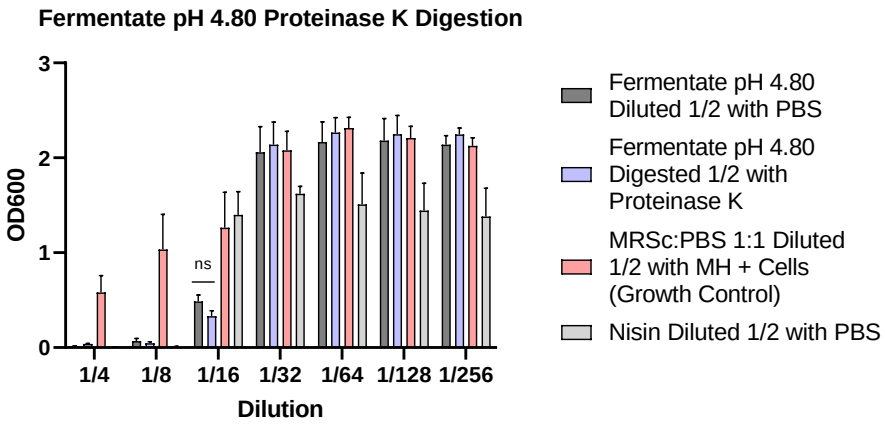

Figure 4.8: The antimicrobial activity detected for the undigested and proteinase K digested *B. longum* ITT 13 fermentate at pH 4.80 and controls. The initial dilution is 1/4 as the samples for testing had already been diluted 1/2 with PBS/Proteinase K. Standard deviations were determined for a minimum of three independent assays (n=3). ns = non-significant p-value >0.05. The growth control consisted of a 1:1 ratio of MRSc:PBS as this is equivalent to the combination present in the 1:1 of fermentate:Proteinase K therefore allowing for accurate dilution of the nutrients in the MH broth used for the *S. xylosus* indicator strain in both the growth control and test samples. Nisin (1000 iu/ml) diluted 1/2 with PBS, which is equivalent to the digested and diluted fermentate samples, was used as a positive control for antimicrobial activity.

It is observed in Figure 4.8 that the antimicrobial activity of the undigested fermentate begins to reduce at approximately a 1/8-1/16 dilution and inhibition is lost completely between a 1/16-1/32 dilution. This pattern of antimicrobial activity is also observed for the digested fermentate indicating the Proteinase K digestion did not reduce or eliminate antimicrobial activity. It is also evident that all antimicrobial activity of nisin is lost at a 1/16 dilution. Statistical analysis was carried out using a one-way ANOVA with Tukey's multiple comparison test and no significant difference in activity between the undigested and digested fermentate was detected ($p > 0.05$), further validating that the incubation with the Proteinase K did not result in any loss of antimicrobial activity of the fermentate.

4.4.2 Actinase E Digestion

The Actinase E digestion as described in Section 4.4 is presented in Figures 4.9 (nisin), 4.10 (*B. longum* ITT 13 fermentate pH 4.35) and 4.11 (*B. longum* ITT 13 fermentate pH 4.80).

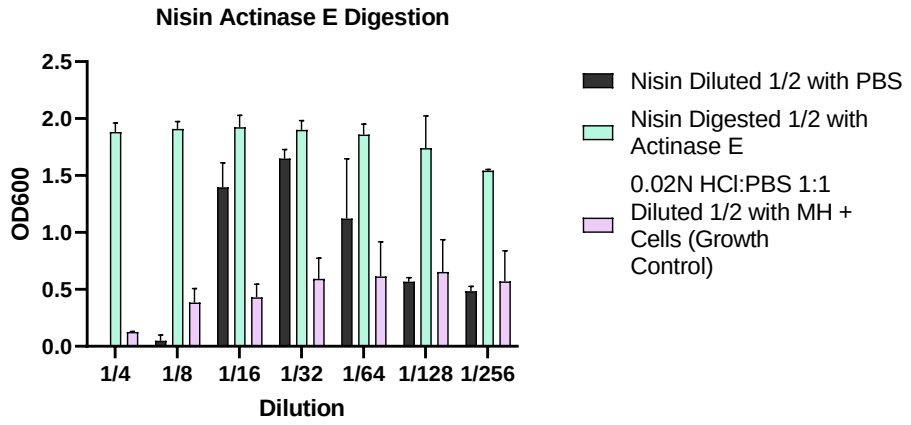

Figure 4.9: The antimicrobial activity of undigested and actinase E digested nisin. The initial dilution is 1/4 as the samples for testing had already been diluted 1/2 with PBS/Actinase E. Three independent assays (n=3) were used to determine standard deviation. The growth control consisted of a 1:1 ratio of 0.02N HCl:PBS as this is equivalent to the solvent combination present in the 1:1 of nisin:Actinase E therefore allowing for accurate dilution of the nutrients in the MH broth used for the *S. xylosus* indicator strain in both the growth control and test samples.

The nisin digested with Actinase E resulted in good growth of the *S. xylosus* indicator strain with an OD600 between 1.5-2.0, which tapered off slightly at the higher dilutions of 1/128-1/256. The activity of the nisin digested with Actinase E showed the bacteriocin to be completely inactivated for the 1/4 and 1/8 dilutions, with an MIC_{50} for the 1/8 dilution. In comparison, the undigested nisin displayed inhibition at both the 1/4 and 1/8 dilutions, with growth for the indicator strain returning for the 1/16 dilution with an average OD600 of 1.40. The growth also tapered off at higher dilutions of the undigested nisin before reaching an average OD600 of 0.48 at the 1/256 dilution. The Actinase E enzyme was therefore active as growth of the indicator strain *S. xylosus* was not inhibited for the nisin digested by the Actinase E enzyme in comparison with the undigested nisin. The latter result indicates that the nisin, as

the positive control for the enzymatic reaction, was digested by the Actinase E enzyme with the concomitant loss of antimicrobial activity.

Higher levels of growth are noted for all dilutions for the *S. xylosus* in figure 4.9 when it was grown in the presence of samples containing the undigested nisin in comparison to the growth control, which only contains PBS. The higher level of growth may be associated with additional nutrient being supplied into the minimal Muller Hinton growth media that the nisin provides when either inactivated by the protease or by higher dilutions. This may also account for why the growth of the *S. xylosus* indicator strain tapers at the highest dilutions of both the digested and undigested nisin, as the nisin is diluted out.

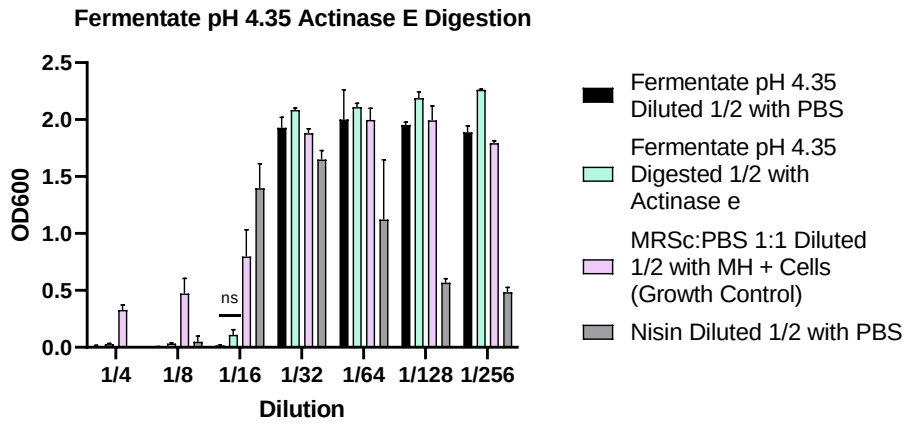

Figure 4.10a: The Antimicrobial Activity of Undigested and Actinase E digested *B. longum* ITT 13 pH 4.35 Fermentate. The initial dilution is 1/4 as the samples for testing had already been diluted 1/2 with PBS/Actinase E. Standard deviations were determined for three independent assays (n=3). ns = non-significant p-value >0.05. The growth control consisted of a 1:1 ratio of MRSc:PBS as this is equivalent to the combination present in the 1:1 of fermentate:Actinase E therefore allowing for accurate dilution of the nutrients in the MH broth used for the *S. xylosus* indicator strain in both the growth control and test samples. Nisin (1000 iu/ml) diluted 1/2 with PBS, which is equivalent to the digested and diluted fermentate samples, was used as a positive control for antimicrobial activity.

Nisin inhibited growth at the 1/4 and 1/8 dilutions and the growth of the indicator strain at higher nisin dilutions was reduced slightly and as described previously for figure 4.9, may be indicative of less nutrients being added from the inactivated/diluted nisin solution into the Muller Hinton media. The growth of the indicator strain was inhibited by both the undigested and the digested pH 4.35 fermentate up to a 1/16 dilution, with inhibition lost completely by both samples at the 1/32 dilution. However, although the undigested pH 4.35 fermentate completely inhibited the *S. xylosus* at the 1/16 dilution, the digested pH 4.35 fermentate did not completely inhibit the growth at the same dilution and minor growth of the *S. xylosus* was observed with an average OD600 value of 0.11. This indicates a small reduction in the antimicrobial activity for the Actinase E digested pH 4.35 fermentate at the 1/16 dilution, in comparison to the undigested pH 4.35 fermentate. The results for the Actinase E digested and undigested samples at the aforementioned 1/16 dilution, together with the controls, are further evaluated in the box plot presented in Figure 4.10b below.

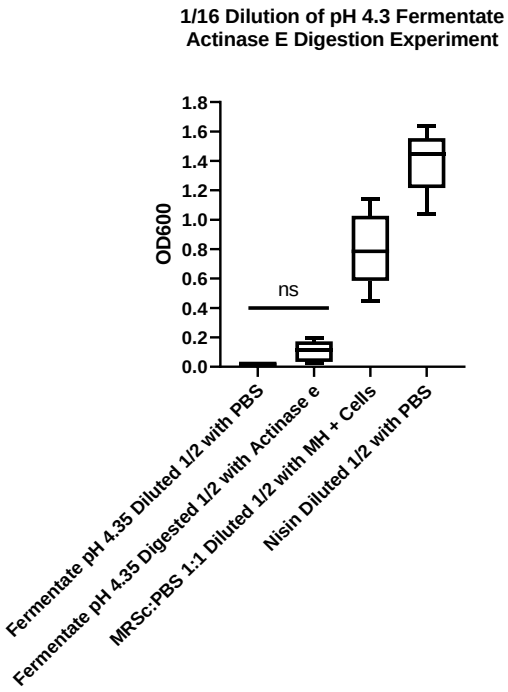

Figure 4.10b: A box Plot Displaying the Enlargement of the 1/16 dilution from Figure 4.10a of the *B. longum* ITT 13 pH 4.35 Fermentate Digested with Actinase E. Standard deviations were determined for three independent assays (n=3). ns = non-significant p-value >0.05.

As observed in Figure 4.10a, the digested *B. longum* ITT 13 pH 4.35 fermentate did not completely inhibit the growth of the indicator at the 1/16 dilution and minor growth was observed with an average OD600 value of 0.11 in comparison to an average OD600 of 0.02 for the undigested pH 4.35 fermentate. Although minor growth was observed for the *S. xylosus* with the Actinase E digested pH 4.35 fermentate, it did not significantly reduce antimicrobial activity in comparison to the undigested pH 4.35 fermentate, with a non-statistically significant p-value of 0.6973.

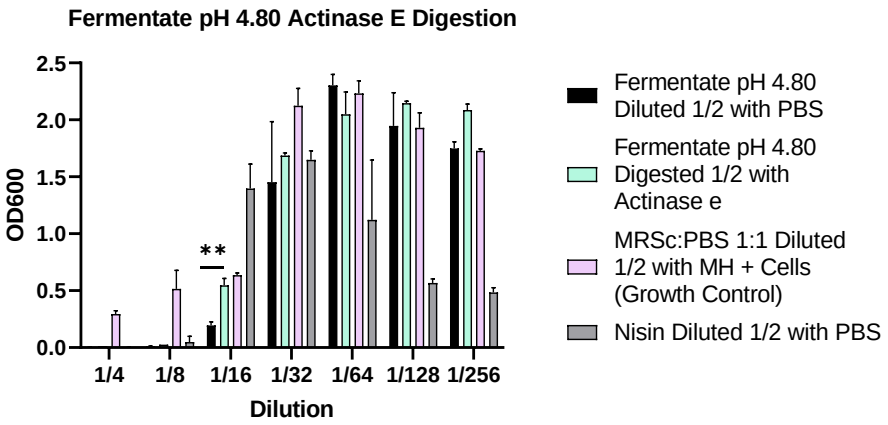

Figure 4.11a: The Antimicrobial Activity of Undigested and Actinase E Digested *B. longum* ITT 13 pH 4.80 Fermentate. The initial dilution is 1/4 as the samples for testing had already been diluted 1/2 with PBS/Actinase E. Standard deviations were determined for three independent assays (n=3). ** indicates p-value of 0.0023. The growth control consisted of a 1:1 ratio of MRSc:PBS as this is equivalent to the combination present in the 1:1 of fermentate:Actinase E therefore allowing for accurate dilution of the nutrients in the MH broth used for the *S. xylosus* indicator strain in both the growth control and test samples. Nisin (1000 iu/ml) diluted 1/2 with PBS, which is equivalent to the digested and diluted fermentate samples, was used as a positive control for antimicrobial activity.

In figure 4.11 nisin inhibited growth at the 1/4 and 1/8 dilutions and the growth of the *S. xylosus* indicator strain at higher nisin dilutions of 1/64-1/256 again progressively reduced as observed and discussed in Figures 4.9 and 4.10.

The digested and undigested pH 4.80 fermentate also inhibited *S. xylosus* at both the 1/4 and 1/8 dilutions.

Both the undigested and digested pH 4.80 fermentate samples were compared to the growth control at each dilution to determine whether they had displayed antimicrobial activity. Complete inhibition of the *S. xylosus* indicator strain was observed by both the undigested pH 4.80 fermentate and the Actinase E digested pH 4.80 fermentate at the 1/4 and 1/8 dilutions. At the 1/16 dilution, no antimicrobial activity was detected for the Actinase E digested pH 4.80 fermentate in comparison to the MRSc growth control, however the undigested pH 4.80 fermentate displayed partial inhibition that was not observed by the digested fermentate

(p=0.0023). The results for the Actinase E digested and undigested samples at the aforementioned 1/16 dilution, together with the controls, are further evaluated in the box plot presented in Figure 4.11b below. This 1/16 dilution is enlarged in Figure 4.11b.

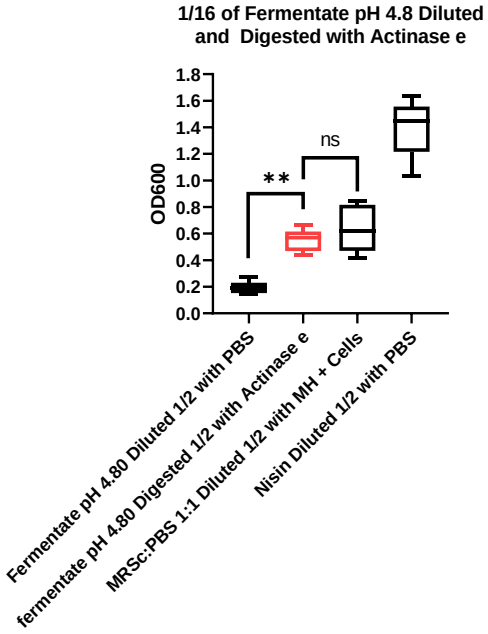

Figure 4.11b: A box Plot Displaying the Enlargement of the 1/16 dilution of Figure 4.11a, of the *B. longum* ITT 13 pH 4.35 Fermentate Digested with Actinase E. The assay was carried out using microtitre plates as described in Section 2.8.4 with *S. xylosus* as the indicator strain. Standard deviations were determined for three independent assays (n=3). (** indicates p-value of 0.0023, ns = p-value >0.05).

The 1/16 dilution of undigested pH 4.80 fermentate inhibited the *S. xylosus* in comparison to the growth control, with average OD600 values of 0.20 observed for the undigested pH 4.80 fermentate and 0.64 for the growth control (p = 0.004). This result clearly demonstrates inhibition of the *S. xylosus* by the undigested pH 4.80 fermentate.

In comparison, the 1/16 dilution of the Actinase E digested pH 4.80 fermentate resulted in an average OD600 of 0.55 as compared with the growth control OD600 of 0.64, with statistical evaluation generating a p-value >0.05, thus indicating no significant difference between the growth control and the protease digested pH 4.80 fermentate. The latter result suggests

complete elimination of the antimicrobial activity of the Actinase E digested pH 4.80 fermentate at the 1/16 dilution in comparison to the undigested fermentate, meaning the antimicrobial activity observed at the 1/16 dilution of the pH 4.80 fermentate may be proteinaceous in nature. This is likely only observed at the 1/16 dilution as the organic acids present may have contributed to higher levels of antimicrobial activity at the lower dilutions thus only allowing the effect of the digested peptide to be seen when the acids were diluted past the point of causing any antimicrobial activity. As the pH 4.80 fermentate was generated in a 2L fermenter with a higher cell density (10^{11} CFU/ml) compared to the pH 4.35 fermentate produced in laboratory scale conditions (10^{9} CFU/ml) there were likely higher levels of produced proteins, as well as higher stability of the actinase E at a higher pH resulting in potentially higher specific activity (*Pronase E | Sigma-Aldrich*).

Further validating this result is the significant difference in growth of the *S. xylosus* indicator strain between the undigested pH 4.80 fermentate and the Actinase E digested fermentate, which generated a significant p-value of 0.0023. The latter result indicates that there was significantly higher growth of the *S. xylosus* in the presence of the digested pH 4.80 fermentate, which displayed a lack of antimicrobial activity in comparison to the undigested pH 4.80 fermentate that resulted in inhibition. These latter results are indicative of proteinaceous associated antimicrobial activity caused by the fermentate at a 1/16 dilution, similar to the hypothesis for the pH 4.35 fermentate digested with Actinase E in Figure 4.10.

4.5 HPLC Analysis of Organic Acid Production by *B. longum* ITT 13

Lactic and acetic acids produced by *Bifidobacteria* have been shown to display antimicrobial activity against a variety of bacterial strains (Makras and De Vuyst, 2006). Organic acid concentrations of between 20.9 mM ± 0.3 to 60.9 mM ± 4.2 acetic acid, 5.7 mM ± 0.2 to 39.9 mM ± 3.1 lactic acid and 3.6 mM ± 0.7 to 10.8 mM ± 2.1 mM formic acid were produced by various *Bifidobacterial* strains and shown to exhibit antimicrobial activity against both Gram-positive and Gram-negative bacterial strains in a study by Makras and De Vuyst (2006). A study by Tejero-Sariñena *et al.*, (2012) also demonstrated the organic acid production of a variety of LAB, including *Lactobacilli, Lactococci* and *Bifidobacterium* strains, with 44-180 mM lactic acid and 45-99 mM acetic acid detected over 24 hours of growth of these LAB. In this study by Tejero-Sariñena *et al.* (2012), antimicrobial activity was correlated with organic acid production and pH, as the lower the pH the larger the zones of inhibition observed and through HPLC analysis it was determined that lactic and acetic acid were responsible for pH reduction and thus antimicrobial activity.

Some LAB are homofermentative, whereby through the glycolysis pathway they produce only lactic acid as an organic acid metabolite, in comparison, other strains including *Bifidobacteria* produce a combination of organic acids including ethanol, lactic acid and acetic acid upon the metabolization of sugars (Basso *et al.*, 2014; Nguyen *et al.*, 2019). Many other LAB also produce organic acids such as lactic and formic acid and analysis of these organic acid concentrations produced by bacteria can be performed using HPLC methods (Özcelik, Kuley and Özogul, 2016; Nuryana *et al.*, 2019). HPLC analysis was carried out as described in Section 2.9.6. on samples of *B. longum* ITT 13 fermentate, generated from an MRSc pH-controlled fermentation at pH 4.8 and an MRSc fermentation grown without pH control to give a terminal pH of 4.3. Further work in our laboratory has shown that a modified YPD media had been optimised to maximise cell growth and antimicrobial activity production by *B. longum* ITT 13

(C. Whelan, personal communication) and was also assessed for the acid production profiles using the HPLC analysis. Both qualitative and quantitative measurement of organic acids were performed to determine the composition of acids produced by *B. longum* ITT 13, as well as the concentration of these acids in both the pH 4.80 fermentate and the pH 4.35 fermentate grown in MRSc and a pH 5.0 fermentate grown in the mYPD, so as to evaluate if the determined acid composition and concentrations would have a contributory effect to the antimicrobial activity determined for the strain.

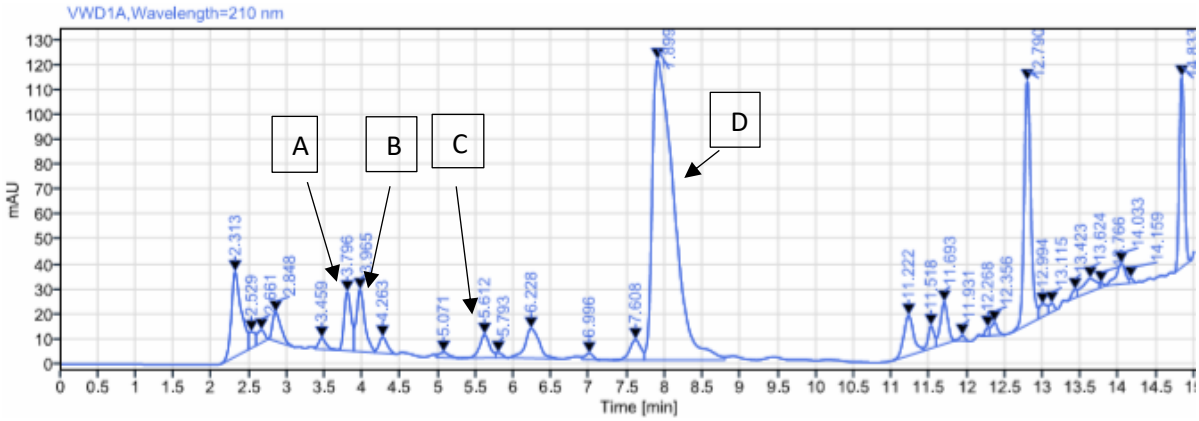

Figure 4.12: Chromatogram of the *B. longum* ITT 13 pH 4.3 fermentate (MRSc) HPLC analysis (Run 1). The pH 4.3 *B. longum* ITT 13 fermentate was analysed in duplicate using the analytical method described in section 2.9.6. with o-phosphoric acid and acetonitrile as the mobile phase using a Phenomenex C18-EVO (100 x 4.6, Particle Size 2.6 um) column. The peaks for selected acids are indicated as follows - Lactic Acid (A), Acetic Acid (B), Succinic Acid (C), and Internal Standard of methylmalonic acid (D).

In Figure 4.12, retention times of 3.796 for lactic acid, 3.965 for acetic acid, 6.228 for succinic acid and 7.899 for the internal standard were detected. The media used for the fermentation (MRS broth + 0.05% *L*-cysteine) was compared as a blank, to eliminate peaks associated with the media, leaving the three peaks associated with the lactic, acetic and succinic acids identifiable in comparison to the retention times of the organic acid standards ran previously (Appendix B). The results thus determined in the HPLC analysis would indicate the presence

of lactic acid, acetic acid and succinic acid in the *B. longum* ITT 13 pH 4.3 fermentate as produced by the strain during growth.

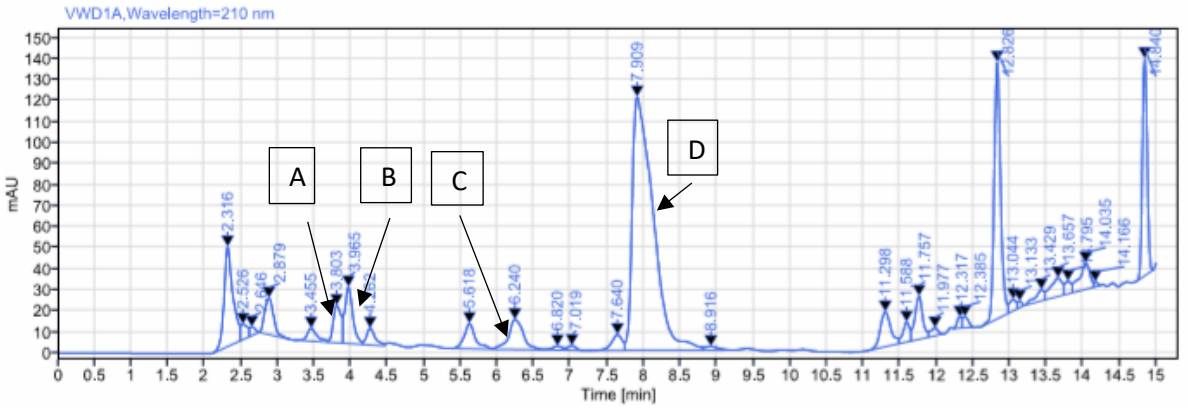

Figure 4.13: Chromatogram of the *B. longum* ITT 13 pH 4.8 fermentate (MRSc) HPLC analysis (Run 1). The pH 4.8 *B. longum* ITT 13 fermentate was analysed in duplicate using the analytical method described in section 2.9.6. with o-phosphoric acid and acetonitrile as the mobile phase using a Phenomenex C18-EVO (100 x 4.6, Particle Size 2.6 um) column. The peaks for selected acids are indicated as follows - Lactic Acid (A), Acetic Acid (B), Succinic Acid (C), and Internal Standard of methylmalonic acid (D).

In Figure 4.13, the retention times observed were 3.803 corresponding to lactic acid, 3.965 corresponding to acetic acid, 6.240 for succinic acid and 7.909 for the internal standard. The broth used for the fermentation (MRS broth + 0.11% L-cysteine) was compared as a blank to eliminate peaks associated with the media, thus resulting in the peaks associated with the lactic, acetic and succinic acids identifiable as present in the pH 4.80 Fermentate sample but not the media. The same peaks were observed and labelled for lactic acid, acetic acid and succinic acid, as well as the peak for the internal standard, for the pH 4.8 *B. longum* ITT 13 fermentate with pH control grown in a 2-litre fermentation vessel, as for the pH 4.3 fermentate.

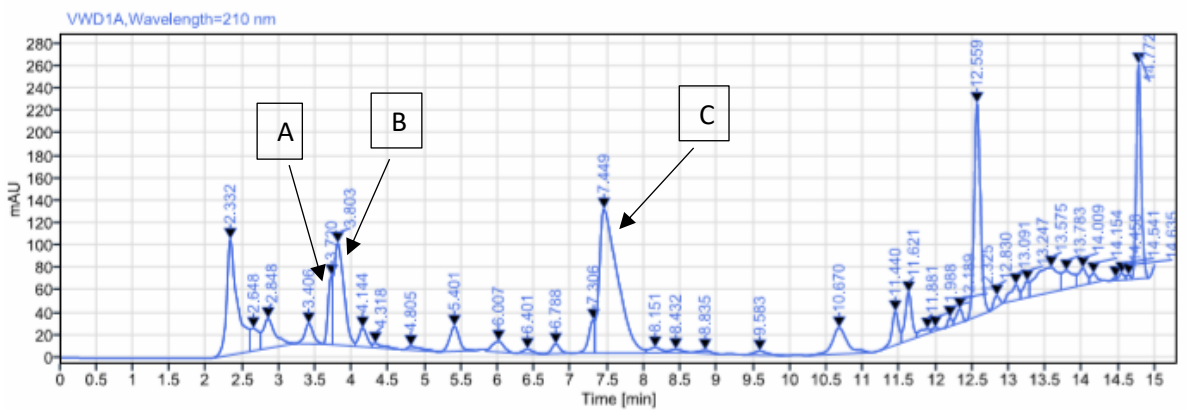

Figure 4.14: Chromatogram of the *B. longum* ITT 13 pH 5 (mYPD) fermentate HPLC analysis. The pH 5 *B. longum* ITT 13 fermentate, grown in mYPD media, was analysed using the analytical method described in section 2.9.6. with o-phosphoric acid and acetonitrile as the mobile phase using a Phenomenex C18-EVO (100 x 4.6, Particle Size 2.6 um) column. It should be noted that this analysis was carried out as one run with no replicates. The peaks for selected acids are indicated as follows - Lactic Acid (A), Acetic Acid (B) and Internal Standard of methylmalonic acid (C).

The use of modified YPD media, a media that had been optimised to maximise cell growth and antimicrobial activity production by *B. longum* ITT 13 (C. Whelan, personal communication), resulted in the acid production profiles presented in Figure 4.14. Both lactic acid and acetic acid were detected in the analysis with a retention time of 3.720 corresponding to lactic acid and a retention time of 4.144 corresponding to acetic acid. There was no determined peak for succinic acid in this fermentation, which was identified in both the pH 4.3 fermentate and pH 4.8 fermentate grown in MRSc media. This indicates that the succinic acid was not produced in this specific media composition of an mYPD broth and that media composition can thus influence the organic acid combination produced. A retention time of 7.499 was determined for the internal standard. The peaks associated with the lactic and acetic acids were identifiable as present in the pH 4.80 Fermentate as the broth used for the fermentation (mYPD – C. Whelan, personal communication) was compared as a blank to eliminate peaks associated with this media.

Table 4.2: The Concentrations of Organic Acids present in the *B. longum* ITT 13 pH 4.3 and pH 4.8 Fermentates Grown in MRSc Media and the pH 5 Fermentate Grown in mYPD media.

The concentration of organic acids present in the fermentate were determined using the peak area of the associated acids detected using HPLC analysis. Duplicate experiments were carried out for average (Avg.) concentration and standard deviation (SD) determination of the acids.

Organic Acid	**pH 4.3 Fermentate (MRSc media)**		**pH 4.8 Fermentate (MRSc media)**		**pH 5.0 Fermentate (mYPD media)**	
	Avg. mM ± SD	Avg. mg/ml ± SD	Avg. mM ± SD	Avg. mg/ml ± SD	Avg. mM ± SD	Avg. mg/ml ± SD
Lactic Acid	92.22 ± 1.53	8.27 ± 0.17	68.32 ± 10.62	6.15 ± 0.95	154.98	13.94
Acetic Acid	147.72 ± 10.34	8.86 ± 0.62	160.95 ± 13.72	9.66 ± 0.82	650.68	39.04
Succinic Acid	54.52 ± 3.00	6.43 ± 0.35	67.69 ± 6.14	7.99 ± 0.73	0	0

The concentration of lactic acid was highest in the pH 5.0 *B. longum* ITT 13 fermentate (mYPD) at 154.98 mM, as compared to the pH 4.3 *B. longum* ITT 13 fermentate (MRSc) at 92.22 mM ± 1.53 and the pH 4.8 fermentate (MRSc), which contained 68.32 mM ± 10.62. The concentration of acetic acid was also the highest in the pH 5.0 fermentate (YPD) at 650.68 mM in comparison to the pH 4.8 fermentate (MRSc) with 160.95 mM ± 13.72 and the pH 4.3 fermentate (MRSc) with 147.72 mM ± 10.34. Of the fermentates produced in MRSc broth, the pH 4.3 fermentate had a higher concentration of lactic acid than the pH 4.8 fermentate, while the latter fermentate had a higher concentration of acetic acid than the pH 4.3 fermentate. The media composition used for growth of the *B. longum* ITT 13 strain thus has a direct influence on the concentration of organic acids and metabolites produced during growth.

The succinic acid was only detected in the two fermentates cultured in MRSc broth. The concentration of succinic acid was higher in the pH 4.8 fermentate at 67.69 mM ± 6.14 than in

the pH 4.3 fermentate at 54.52 mM ± 3.00. The lack of succinic acid present in the fermentate cultured in the mYPD broth indicates the media used for growth also affects the composition of metabolites produced. A total of 23.56 mg/ml ± 1.14 organic acid was present in the pH 4.3 fermentate (MRSc) and a total of 23.80 ± 2.50 mg/ml of organic acid was detected in the pH 4.8 fermentate (MRSc). Therefore, the two fermentates produced using MRSc media contained similar total acid concentrations considering the large difference in final fermentation pH. The pH 4.8 fermentate only contained 0.24 mg/ml more acid than the pH 4.3 fermentate considering the lower pH of the latter sample. In comparison, a total of 52.98 mg/ml organic acid was present in the pH 5.0 fermentate (mYPD), which, considering the lack of succinic acid production, is significantly higher than the total concentration of organic acid produced by the fermentates produced using MRSc as a growth medium.

4.6 Discussion:

4.6.1 Thermostability of *B. longum* ITT 13 Antimicrobial Activity

The stability of the antimicrobial activity produced by *B. longum* ITT 13 at a variety of temperatures was an important component for analysis in relation to consideration of the applicability of the fermentate as a biopreservative in the food industry. The food industry involves the use of high processing temperatures (Bagde and Vigneshwaran, 2019), thus indicating the necessary relative stability of potential biopreservatives.

For the -20^{o}C temperature, the activity was higher than any successive day after 1 day of incubation with an average of 173.3 AU/ml, after which it dropped below the control (124.4 AU/ml) to between 80 and 93.3 AU/ml over the successive 2-28 day incubation period, a 25-36% reduction relative to the control. The latter results were not statistically significant however and indicate no significant reduction in activity relative to the control. Freeze-thaw effect has sometimes been described in literature when bacteriocins are subjected to freezing temperatures, such as the study by Ponce *et al.*, (2008) which investigated the antimicrobial activity associated with four lactic acid bacteria (LAB), *Enterococcus faecium*, *Lactococcus lactis, Enterococcus hirae and Enterococcus canis*. The cell free supernatants of these strains were subjected to three freeze-thaw cycles over 24 hours to determine the stability, with activity detected against the indicator strains *Lactobacillus plantarum* CRL 691, *Pediococcus pentosaceus* ATCC 10791, GIIA 30, *Listeria monocytogenes*, and *E. coli*. Although the antimicrobial activity of all the LAB against the indicator strains generally remained stable relative to the control after the freeze-thaw cycles, the activity produced by *E. faecium* against *L. plantarum* CRL 691, decreased by approximately 42-43% in comparison to the control after the first freeze-thaw cycle and remained at that same level of reduced activity for the subsequent cycles. This study indicates that the antimicrobial activity produced by many LAB can generally remain stable upon freezing, but for some strains as little as one freeze-thaw

action may reduce the antimicrobial activity produced. As there was no significant loss of activity by ITT 13 upon freezing the antimicrobial activity detected may display stability upon freezing.

For the 4^{o}C temperature, the average antimicrobial activity was highest after 1 day of incubation (213.3 AU/ml) and then dropped slightly to between 133.3 AU/ml after 2 days incubation and 80 AU/ml after 21 days (36% loss). This indicated a minor loss of activity in relation to the control. However, the loss of activity was reduced at the 28-day timepoint as only a 3% loss was observed with 120.0 AU/ml determined. The increase in activity after 28 days and the largest loss of activity observed for only day 21 indicates that the overall losses were inconsistent with the 4^{o}C temperature and that the reduction in activity did not remain consistently below the control over the 28-day period as observed with the -20^{o}C, room temperature and 37^{o}C temperatures, which may be indicative of the most stability at this temperature. No statistically significant loss of activity was detected further indicating stability at this temperature.

The highest retention of antimicrobial activity after 2 days of incubation that was observed for the 4^{o}C incubation temperature was also observed at room temperature (20-24^{o}C), whereby the activity was highest after 2 days incubation (average of 186.7 AU/ml after 1 day and 146.7 AU/ml after 2 days) before it dropped below the control value of 124.4 AU/ml from 7 days onwards with losses of activity between 14% and 36%. This indicates maximum stability of the antimicrobial activity of the fermentate at room temperature for 2 days in relation to the control, although again no significant losses of activity were detected indicating relative stability.

For the fermentate incubated at 37^{o}C the average activity was slightly below the control result of 124.4 AU/ml after the 1 day of incubation period with 120.0 AU/ml (3% loss) and continued to decrease after 2 days incubation at this temperature to an average of 106.7 AU/ml (14%

loss), before dropping again and fluctuating between 80.0 and 93.3 AU/ml for day 7-28 (25-36% loss of activity). Although the antimicrobial activity was the highest (86-97% activity retained) for the first two days of incubation before dropping again and fluctuating between two values (64-75% activity retained) for the remaining days of incubation, like previous samples there was no significant loss of activity relative to the control at any timepoint.

The average activity observed for 55^{o}C and 70^{o}C was irregular with high levels of fluctuation (75-128% activity retained relative to the control) and increasing activity at later time points, with activity increasing from 75% to 107% from day 7-28 at the 55^{o}C incubation temperature and from 97% to 129% from day 7-28 for the 70^{o}C incubation temperature. This increase is likely an artefact of the experiments due to evaporation in the samples with the reduction in volume been associated with a perceived increase in activity.

The activity fluctuated over the time period of the 28-day study for the samples, with a fluctuation from as low 80 AU/ml (64% activity) to as high as 213 AU/ml (172% activity), with some AU/ml values increasing rather than decreasing between subsequent timepoints. The evaluation of the effect of temperature on the antimicrobial activity would possibly indicate that the antimicrobial activity present seems to retain the highest consistent levels at 4°C and room temperature storage for up to 48 hours, with some loss (14-36%) over the 28-day period tested. The latter retention of maximum activity up to the 48 hours was also observed in a bacteriocin produced by *L. plantarum* KL-1 described by Pilasombut *et al.* (2015), as when thermal stability analysis was carried out, the maximum antimicrobial activity was retained for 2 days at 4^{o}C against the indicator strain *L. sakei* subsp. *sakei* JCM1157, before dropping in the days thereafter.

With this being said however, the overall result indicated that there was no significant loss of activity at any timepoint or temperature indicating the temperature likely did not impact the fermentate. Dunnett's multiple comparison assay was carried out to assess the antimicrobial

activity determined in the assay at each of the temperatures tested (-20°C to 70°C) over the time period of 0 to 28 days and compared to the control of the freshly produced fermentate before incubation at each temperature. No statistically significant difference was determined ($p>0.05$), thus the antimicrobial activity detected over the time period at the various temperatures indicate that the antimicrobial activity produced by *B. longum* ITT 13 is relatively stable under all of the incubation conditions tested. Antimicrobial activity previously associated with bacteriocins produced by *Bifidobacterium* spp. can be quite thermostable in nature, with activity often retained after heat treatment above 100°C. The latter thermostability was observed by Yildirim and Johnson, (1998), whereby the bacteriocin bifidocin B, produced by *Bifidobacterium bifidum* NCFB 1454, displayed resistance to heating up to 90°C for 60 minutes and 121°C for 15 minutes, while also retaining biological activity after storage at -20°C for 3 months. Cheikhyoussef *et al.*, (2009) investigated the stability of a partially purified bacteriocin-like inhibitory substance (BLIS) produced by *B. infantis* BCRC 14602 that displayed complete retention of activity upon heat treatment, with 1600 AU/ml activity retained following heat treatment at 50-90°C for 60 minutes, 100°C for 20 minutes and 121°C for 15 minutes. A study by Liu *et al.*, (2015) explored the characteristics of the bacteriocin bifidocin A, produced by *Bifidobacterium animals* BB04, that, like *B. longum* ITT 13 was isolated from the gut flora, but in this case from centenarians rather than the infant gut flora. The bifidocin A peptide was stable at heating to heating of 100°C for 30 minutes and 121°C for 15 minutes, which was in agreement with the above discussed studies.

It is possible that antimicrobial activity detected for *B. longum* ITT 13 could be associated with a proteinaceous component i.e. bacteriocin and/or organic acid production. The results indicating no significant drop in antimicrobial activity over the temperature and time period of the thermostability studies for the *B. longum* ITT 13 fermentate, may indicate that organic acid production by *B. longum* ITT 13 likely contributes to some of the antimicrobial effect observed

by the strain (Makras and De Vuyst, 2006). This organic acid production was explored further in Sections 4.4 and 4.5. It also does not rule out the contribution of thermostable bacteriocins to activity as the above previous literature has stated that bacteriocins produced by *Bifidobacteria spp.* are relatively thermostable.

Higher stability of the antimicrobial activity was observed for 1-day incubation compared to subsequent days, as evident for -20^{o}C to 37^{o}C in Figure 4.1, with averages of 120-213 AU/ml observed for the activity at day 1 of these temperatures, before dropping to as low as 80 AU/ml on subsequent days. The results generated for each of the temperatures tested over the 28 day time period, well not generating statistically significant differences from the original control sample, indicate the trends observed may be biologically significant as it was observed that for the majority of temperatures (-20^{o}C, 4^{o}C, room temperature and 37^{o}C) that shorter storage times of 1-2 days seem to be associated with the highest levels of antimicrobial activity. Thus, the biological trend observed was that the most antimicrobial activity retained up to 1-2 days incubation for -20^{o}C to 37^{o}C, with decreased activity thereafter. This trend of higher activity in the first 2 days of incubation and the fact that there was no complete loss of activity, thus indicates the relative thermostability of the molecule at a wide variety of temperatures. If the antimicrobial activity detected for the *B. longum* ITT 13 fermentate has a proteinaceous component i.e. a bacteriocin, then the results on thermostability would indicate that this bacteriocin could be either a class I class II bacteriocin which are known to be heat-stable peptides (Deegan *et al.*, 2006; Singh *et al.*, 2015). As the results have shown thermostability of the fermentate and previous literature stated has indicated thermostability of many bacteriocins, it does not rule out the possibility that there may be antimicrobial peptide/s present in the fermentate.

4.6.2 pH stability of *B. longum* ITT 13 fermentate

Like thermostability, pH stability of potential biopreservatives is another important factor for consideration, due to the wide variety of matrices and pH processing conditions used within the food industry (Johnson *et al.*, 2018; Castilho *et al.*, 2020) and thus potential biopreservatives should be able to withstand these processing conditions. Many foodstuffs containing a starter bacterial culture, i.e. fermented foods, have a low pH including dairy products such as cheese, which has been shown to decrease in pH over storage time due to acid production by the starter bacteria (Ong and Shah, 2009; Ulpathakumbura *et al.*, 2016). Stability at a low pH is also important for probiotic bacteria when passing through the stomach and gastrointestinal tract (Mahmoudi *et al.*, 2013). The pH stability assay of the *B. longum* ITT 13 fermentate was initially carried out using HCl (0.3M) and NaOH (0.3M) at a pH range from pH 3-8 to provide preliminary studies of the stability of the antimicrobial activity over this pH range. The initial pH studies on the stability of the antimicrobial activity was carried out using HCl (Section 4.2.1), with antimicrobial activity found to only be detected after adjustment to pH 3 and 4. It was then decided to repeat the pH studies but using 0.1M lactic acid (L.A.) to adjust the pH values of the fermentate rather than HCl (Section 4.2.2). The decision to use L.A. was made on the basis that L.A. (together with other acids) is already produced by *Bifidobacteria*, it would be more appropriate to adjust the pH using the L.A. rather than using HCl (Makras and De Vuyst, 2006; Lee and O'sullivan, 2010; Delgado *et al.*, 2019).

The fermentate adjusted to pH 3.08 (Figure 4.3) displayed higher levels of antimicrobial activity than the unadjusted fermentate ($p<0.05$), with averages of 5.0-5.8 mm of inhibition observed for the pH 3.08 fermentate and 4.0-4.8 mm for the unadjusted fermentate. The additional antimicrobial activity observed for the pH 3.08 fermentate in comparison to the unadjusted fermentate could most likely be attributed to the addition of the 5.7 mls of L.A. used to lower the pH of the fermentate. The concentration of lactic acid as then present in the

latter pH adjusted media, generated antimicrobial activity with zones of inhibition between 3.6 mm and 4.4 mm of activity on average. The antimicrobial activity of the pH 3.08 fermentate was compared to the control of the pH 4.32 MRSc broth + 5.7 mls of L.A., as these were directly comparable as previously discussed i.e. comparing the pH adjusted fermentate with the pH adjusted MRSc broth using the same volumes of acids, using ANOVA with Tukey's multiple comparison assay. The latter comparative analysis showed that although activity was caused by the acidic pH, there was more activity associated with the fermentate in comparison to the pH control at each time point of 0-24 hours ($p<0.05$). The significance i.e. p-value <0.05 in the difference between the pH 3.08 fermentate and the pH adjusted MRSc broth would indicate that the antimicrobial activity is not associated with the pH value alone. *Bifidobacterial* spp. are heterofermentative and produce several acids including acetic and lactic acid (Nguyen *et al.*, 2019), however only lactic acid was used to adjust the pH of the fermentate and controls. The fermentate was also diluted by more than 1/2 with the addition of 5.7 mls lactic acid to adjust the pH, which should consequentially reduce the antimicrobial activity associated solely with the fermentate, meaning the activity observed at pH 3.08 for the fermentate may be primarily associated with the addition of acid.

The results for the fermentate adjusted to pH 4.02 (Figure 4.4) generated antimicrobial activity values similar to those for the original undiluted pH 4.35 fermentate when the 2 sets of results are compared, indicating no significant difference of the activity between the two samples. Very little dilution of the fermentate was also carried out when adjusted to pH 4.02, with 0.7 mls L.A. added to 5 mls fermentate, as evidenced by the similarity of the original pH 4.35 fermentate and the adjusted pH 4.02 fermentate.

Comparison of the activity between the pH 4.02 fermentate and the equivalent pH 4.3 MRSc broth with an additional 0.7 mls L.A. was assessed using ANOVA with Tukey's multiple comparison assay. The activity of the pH 4.02 fermentate was significantly higher than the

MRSc broth with p-values <0.001 at each time point. The p-value of <0.001 is indicative of the presence of significant antimicrobial activity for the pH 4.02 fermentate, which cannot be solely attributed to the pH of the media itself.

Antimicrobial activity of the *B. longum* ITT 13 fermentate was not detected when the fermentate was pH adjusted to pH 5 and 6 and was in agreement with the results generated by Yi *et al.*, (2020), whereby the antimicrobial activity of the bacteriocin produced by *Lactobacillus pentosus* DZ35, began to reduce in antimicrobial activity above pH 4. However, later work where the fermentation was carried out in a pH controlled MRSc media fermentation and separately in an optimised YPD medium and tested using the MIC assay, has shown that antimicrobial activity is present to pH 5.5 (C. Whelan, personal communication).

The pH activity studies measuring the antimicrobial activity for *B. longum* ITT 13 fermentate suggest that the antimicrobial activity is more stable at lower pH values. The results presented in Section 4.2 indicate that the antimicrobial activity is more stable at pH values of 4 and up to pH 5, however further fermentation studies were carried out by one of my laboratory colleagues (C. Whelan, personal communication) which would suggest that in pH controlled fermentations the antimicrobial activity can be stably detected at values around pH 5 in the more sensitive microtitre plate MIC assays. The media composition may also be a factor in the stability of the antimicrobial activity at higher pH's in the pH controlled fermentations as a modified YPD medium was utilised for growth of the ITT 13 strain in the pH controlled fermentations carried out in fermentation vessels. Throughout Sections 4.1 and 4.4, a pH 4.8 *B. longum* ITT 13 fermentate was used that had been grown with pH control in fermentation vessels using MRSc media as the growth medium, which also displayed significant antimicrobial activity. Therefore, the pH stability of the antimicrobial activity at pH 5 is likely correlated to both the medium used for growth of the *B. longum* ITT 13 in conjunction with the use of fermentation vessels for growth as opposed to static growth in laboratory conditions at

smaller volumes, as the use of fermentation vessels produces cell densities of up to 10^{11} CFU/ml.

The bacteriocin nisin has also been shown to be more stable in the acidic pH range of pH 5 and below with the highest stability of the bacteriocin in the lower pH range at around pH 3 (Matsusaki *et al.*, 1998). Increasing the pH to neutral or alkaline pH ranges reduces the antimicrobial activity associated with nisin (Rollema *et al.*, 1995; Matsusaki *et al.*, 1998) with a sharp decrease in stability at pH 7-8 (Rollema *et al.*, 1995). The results in this research project indicate that the *B. longum* ITT 13 antimicrobial activity, like nisin, is primarily active within the acidic pH range with antimicrobial activity detected to between pH 5-6 in microtitre plates (C. Whelan, personal communication) dependent on the YPD or MRSc media used, with maximum activity around pH 4 which is similar to the stability pattern of nisin. Many bacteriocins have displayed similar pH stability activity pattern to the *B. longum* ITT 13 fermentate, with maximum antimicrobial activity in acidic and neutral pH ranges, with activity decreasing or lost in the alkaline pH range (Yue *et al.*, 2013; Bagde and Vigneshwaran, 2019; Pei *et al.*, 2020; Yi *et al.*, 2020). In the study by Yue *at al.*, (2013) a bacteriocin produced by *Lactobacillus rhamnosus* CICC 20975 remained stable at pH values 3-7 when adjusted to pH's 2-10 using HCL and NaOH. Bagde and Vigneshwaran (2019) examined the stability of a bacteriocin from *E. faecium* LMG 11423(T) at pH 2-12 and found that antimicrobial activity decreased at alkaline pH, with >4 mm zones of inhibition at pH's 2, 4 and 6, and <4 mm at pH 8 and 10, with <2 mm zones of inhibition at pH 12 when tested on a microdiffusion plate. Pei *et al.* (2020) examined the pH stability of bacteriocin SLG10 produced by *L. plantarum* SLG10 at pH 2-10 and found that at pH 3-7 there was approximately 200 AU/ml, but the antimicrobial activity began to reduce at pH 8 and was close to 0 AU/ml for pH 9 and 10. The reduction and loss of activity at high alkaline pH's may be associated with intramolecular electrostatic interactions of the peptides as the electrostatic repulsion of intermolecular and hydrogen bonds

break, as altering the pH alters the charge on the amino acids within the protein due to their zwitterionic nature which can have major effect on the electrostatic interactions within the protein (Yi *et al.*, 2016).

The antimicrobial activity produced by *B. longum* ITT 13 has been shown to be stable at pH 5 when grown in a modified YPD media with pH control in 2-litre fermentation vessels and assessed using an MIC microtitre plate assay (C. Whelan personal communication). As all of the antimicrobial detection for the 2L fermentations were carried out using the more sensitive MIC microtitre plate assay, there is a possibility that the sensitivity of the microdiffusion assay versus the MIC assay may have limited detection of activity at the higher pH values of pH 5 or pH 6, with the research work carried out on the pH stability within this project using the microdiffusion assay. Additionally, the lack of detection of activity at the higher pH (pH 5-6) could also have something to do with the specific media and fermentation conditions used for growing the *B. longum* ITT 13 in laboratory conditions as opposed to the optimised fermentation vessel conditions. A lower sensitivity of the microdiffusion assay in comparison to the microtitre plate MIC assay was observed by Inturri *et al.*, (2019) and this coupled with the likelihood more antimicrobial peptides would be produced when grown in fermentation vessels due to a higher cell density of 10^{11} CFU/ml (C. Whelan personal communication) in comparison to 10^{9} CFU/ml when grown in static laboratory conditions, may contribute to activity detectable at higher pH levels when cultured in the fermentation vessels. The antimicrobial activity observed by *B. longum* ITT 13 may be from multiple sources as the pH alone doesn't contribute fully to the antimicrobial activity as discussed in the results for the fermentate adjusted to pH 3 and 4. Although the combination of acids produced by the heterofermentative *B. longum* ITT 13 cannot be ruled out as causing the majority of antimicrobial activity, it is also possible that a proteinaceous component may be contributing to antimicrobial activity as ammonium sulphate precipitation (Section 4.3) and protease

digestion (Section 4.4) indicated the presence of low-level antimicrobial activity that is proteinaceous in nature produced by *B. longum* ITT 13.

4.6.3 Ammonium Sulphate Precipitation of the *B. longum* ITT 13 Fermentate

Ammonium sulphate precipitation is a method of partial protein purification and concentration that has been widely used as a method to extract antimicrobial peptides produced by bacteria from cell free supernatant (Contreras *et al.*, 1997; Afshan Naz, Sheikh and Rasool, 2013; Ansari *et al.*, 2018). The ammonium sulphate solution interferes with the proteins solubility in water causing precipitation by increasing hydrophobic interactions, while maintaining the protein conformation, allowing the peptide to be extracted without denaturation and preserve activity (Wingfield, 2001).

As the structure of any potential proteinaceous compounds associated with antimicrobial activity e.g. bacteriocins produced by *B. longum* ITT 13 are unknown, preliminary saturation studies were carried out on a range of ammonium sulphate concentrations as it was not obvious from work within the laboratory as to what the potential size of a potential antimicrobial peptide/protein would be. Saturations of 20%, 40%, 60% and 80% AS were added to the *B. longum* ITT 13 cell free fermentate to determine which saturation would precipitate the suspected bacteriocin(s). The AS protein pellets were resuspended in 2 ml volumes of 50 mM sodium acetate buffer – resulting in a 20-fold concentration.

It is evident that at the 1/2 and 1/4 dilutions of the 20%, 40%, 60% and 80% AS saturations, there is inhibition caused by both the resuspended pellet of the fermentate and by the resuspended MRSc broth – which is just media. This may be indicative of the ammonium sulphate contributing to antimicrobial activity at these initial dilutions before it is diluted out at higher dilutions. It was not possible to remove the ammonium sulphate prior to testing due to the initial low levels of detection for antimicrobial activity i.e. even at the 1/2 dilutions it is

sometimes difficult to detect any antimicrobial activity for the fermentate. It is also possibly due to a result of dilution of the Mueller Hinton broth nutrients, which is already a nutrient limiting media, as the NaAc buffer was used for resuspension of the fermentate and MRSc media pellets.

It can be seen in Figure 4.5 a, b and c, that the growth pattern of the indicator strain (*S. xylosus*) in the microtitre plates, measured as OD600 nm, was similar at the 20%, 40% and 60% AS saturations, for both the fermentate precipitate resuspended in 50 mM sodium acetate pH 4.3 and the resuspended MRSc broth precipitate. This growth pattern of the *S. xylosus* indicates there was no inhibition detectable for the resuspended pellet of the fermentate at these AS saturations (20%-60%) in comparison to the resuspended MRSc and thus the results would indicate that the resuspended precipitates do not contain any antimicrobial activity or possibly the antimicrobial activity is below the detectable level. In contrast, at the 80% AS saturation, there is a significant difference (p-value <0.0001) for the OD600 which is significantly lower for the fermentate pellet (0.277) in comparison to the MRSc broth pellet (1.902) at the 1/8 dilution, indicating a reduction of growth of the indicator strain in the presence of the resuspended 80% fermentate pellet. The detection of antimicrobial activity causing decreased growth of the indicator strain when exposed to the resuspended fermentate pellet, would suggest that some of the antimicrobial activity is proteinaceous in nature. The negative control of the 80% MRSc broth resuspended pellet did not display similar inhibition at this 1/8 dilution, possibly eliminating the 80% AS or the 50mM sodium acetate buffer at pH 4.3 causing this inhibition. The significant difference in the inhibition of the *S. xylosus* indicator strain when incubated with the 80% pH 4.3 MRSc resuspended pellet versus the 80% resuspended *B. longum* ITT 13 pH 4.3 fermentate pellet, would indicate a strong probability that there is some low molecular weight antimicrobial proteinaceous material precipitated at the 80% AS concentration and that the antimicrobial activity is stable when the precipitate is resuspended.

Makras and De Vuyst (2006) used 40% and 70% AS saturations to extract the bacteriocins of many *Bifidobacterium* strains and found that the higher saturation of 70% resulted in the precipitation of the antimicrobial peptides in comparison to the 40% AS. The use of the high 80% AS concentration to generate a precipitate from the *B. longum* ITT 13 fermentate that exerts antimicrobial activity is thus in agreement with the studies of Makras & De Vust (2006). The requirement of a high AS concentration for precipitation may be common for many bacteriocins produced by *Bifidobacterium* spp. as this further suggested by Lievin *et al.*, (2000) who used a concentration of 60% AS saturation to precipitate the spent culture supernatants of *Bifidobacterial* strains CA1 and F9, determining that activity was associated with the production of proteinaceous material.

Fujiwara *et al.*, (1997) assessed the production of antimicrobial activity by *B. longum* SBT 2928, used ammonium sulphate as a salting out method by bringing the saturation to 85%, which resulted in binding-inhibitory activity i.e. inhibition of the binding activity of various bacteria, of this fraction that was comparable to the activity of the supernatant before AS precipitation. The precipitate reduced the binding activity of *E. coli*, *P. aeruginosa* and *B. cepacia* to gangliotetraosylceramide (GA1), a sphingoglycolipid that is present in the intestinal brush border membrane of the mouse and the use of AS to isolate the activity indicated the likely proteinaceous nature of the binding inhibitor.

The concentration of ammonium sulphate required to precipitate target proteins depend on the physiochemistry of the protein, as low molecular weight proteins require higher salt concentrations for precipitation and as the AS decreases solubility of the protein, highly soluble proteins would also require higher salt concentrations for precipitation (Wingfield, 2001). As the 80% AS saturation was required to precipitate the proteinaceous material causing antimicrobial activity in the *B. longum* ITT 13 fermentate, this indicates it is likely of a low molecular weight and highly soluble. Both class I and class II bacteriocins are of a low

molecular weight, at less than 10 kDa (Deegan *et al.*, 2006; Singh *et al.*, 2015). Many bacteriocins in various studies have been shown to be of a low molecular weight in studies, such as for the bacteriocin HW01, produced by *P. acidilactici* HW01, which had a molecular weight of 6 kDa (Ahn *et al*, 2017) and *E. faecium* MMT21 produced two bacteriocins of 4.8 kDa (enterocin A) and 5.5 kDa (enterocin B). The commercially used bacteriocin nisin also is a low molecular weight peptide of approximately 3.4 kDa (Williams and Delves-Broughton, 2003), which is used as a positive control in many of the experiments carried out in this research. Two bacteriocins produced also by a *Bifidobacterial* strains (CA1 and F9), were found to have molecular weights estimated at lower than 3.5 kDa, indicating low-molecular weight peptides, which may be in agreement with the possible antimicrobial peptide produced by *B. longum* ITT 13.

The antimicrobial activity of the 80% AS precipitated fermentate was compared to the activity of nisin, as the antimicrobial activity associated with organic acid production in the fermentate was eliminated by resuspension of the AS precipitate in an NaAc buffer. Nisin displayed antimicrobial activity against *S. xylosus* ATCC 29971 to a 1/8 dilution, equating 125 IU/ml, with growth of the *S. xylosus* indicator strain returning at the 1/16 nisin dilution in the microtitre assay plates (Section 3.2.2). The antimicrobial activity associated with the nisin thus equated to 125 IU/ml or 80 AU/ml, which was also the same antimicrobial activity in AU/ml associated with the precipitated protein of the *B. longum* ITT 13 fermentate with 80% AS saturation. The dilution is used to calculate the MIC_{50} values of the *B. longum* ITT 13 fermentate as there has been no determined concentration of the actual inhibitory component present within the fermentate. Further studies would be required on a purified bacteriocin to elicit more information in relation to the nature and activity of the bacteriocin and its comparability to nisin.

The MIC_{50} results of 80 AU/ml observed against *S. xylosus* for the precipitated peptide

produced by *B. longum* ITT 13 are comparable to a peptide produced by *Enterococcus faecium* MCL13, which displayed 64 BU/ml (bacteriocin units/ml - equivalent to AU/ml) against *S. xylosus* in a study by Lim, (2016). The activity of the precipitated *B. longum* ITT 13 fermentate is comparable to the antimicrobial activity produced by *E. faecium* MCL13 in this study, as it was also crudely purified using ammonium sulphate precipitation and tested using microtitre plates, with determination of the BU/ml for *E. faecium* MCL13 calculated similarly to the AU/ml calculation of the fermentate in Section 2.8.2.

As a gradual reduction in antimicrobial activity with an increasing pH was observed for the antimicrobial activity produced by *B. longum* ITT 13 in Section 4.2, indicative of antimicrobial activity caused by low pH, it may suggest the possibility of a synergistic effect between the detected proteinaceous component causing antimicrobial activity and the lactic and acetic acid produced by ITT 13. This synergistic effect was described by Pasteris *et al.* (2014) and would account for the absence/reduction of activity with an increase in pH observed for the antimicrobial activity produced by *B. longum* ITT 13. The study by Pasteris *et al.* (2014) described how *Lactococcus lactis* CRL 1584 produced antimicrobial activity found to be proteinaceous in nature, that was indicative of a bacteriocin which inhibited the growth of *Listeria monocytogenes* through synergistic activity with organic acids, as well as hydrogen peroxide. The cell free supernatant was tested for antimicrobial activity using the microdiffusion assay and the synergism was determined as the zone of inhibition decreased upon neutralization of the supernatants and additionally when catalase was added, which inactivated the hydrogen peroxide and thus overall were indicative of organic acids and hydrogen peroxide as causing antibacterial activity. The antibacterial effect observed was then completely eliminated by further treatment with α-chymotrypsin, indicating a proteinaceous nature of some inhibitory molecules, and suggesting a combination of all three molecules caused the observed antimicrobial activity. Therefore, upon the evident antimicrobial activity

caused by a proteinaceous component of the *B. longum* ITT 13 fermentate observed with the ammonium sulphate precipitation at 80% saturation, couples with the antimicrobial activity associated with the organic acids produced by the strain, synergism is a possibility.

4.6.4 Protease Digestion

Many LAB and probiotic bacteria demonstrate antimicrobial activity through the production of both organic acids and antimicrobial peptides i.e. bacteriocins, which aim to prevent colonisation of the host by pathogenic bacteria (Vlasova *et al.*, 2016). The antimicrobial nature of various strains is often proteinaceous in nature, with various *Bifidobacteria* in a study by Makras and De Vuyst, (2006) shown to produce antimicrobial activity both proteinaceous and partially or wholly associated with organic acid production. Bacteriocin production is an important factor for probiotic bacteria (O'Shea *et al.*, 2012; Choudhary *et al.*, 2019) and would further indicate the suitability for the use of *B. longum* ITT 13 as a probiotic strain, or for biopreservation similar to the use of the bacteriocin nisin. Enzymatic digestion with proteases is carried out to confirm the proteinaceous nature of molecules and commonly employed to determine the nature of antimicrobial activity associated with potential bacteriocins (Miao *et al.*, 2014; Du *et al.*, 2017; Lv, Ma, *et al.*, 2018) and was carried out to determine the nature of the antimicrobial activity produced by *B. longum* ITT 13. Proteinase K and Actinase E (Pronase E) were chosen as the proteases for digestion of the *B. longum* ITT fermentate as they have been shown in previous studies to digest nisin which is also involved in food preservation (Matsusaki *et al*, 1998).

The inhibition of *S. xylosus* by nisin was detected in the assays at a 1/4 and 1/8 dilution (250 and 125 IU/ml of nisin), with no inhibition detected at the 1/16 dilution (62.5 IU/ml) (Figures 4.6-4.11). Incubation of nisin with Proteinase K caused a reduction in nisins ability to inhibit

the growth of *S. xylosus*, with inhibition only detectable at the 1/4 dilution (250 IU/ml) of the nisin, rather than at the 1/8 of the undigested nisin (Figure 4.6). Incubation of nisin with Actinase E caused a complete loss of ability of the bacteriocin to inhibit the growth of *S. xylosus*, with no inhibition detected at any dilution in the microtitre plate (Figure 4.9). This reduction or loss of antimicrobial activity of the nisin control when incubated in the presence of proteases confirms the enzymatic activity of the Proteinase K and Actinase E.

The results generated for the statistical analysis of the Proteinase K incubated pH 4.35 fermentate versus the undigested pH 4.35 fermentate generated non-significant results with the P-value above 0.05, indicating the Proteinase K did not reduce or eliminate the antibacterial activity of the fermentate.

For the pH 4.80 fermentate (Figure 4.8) the statistical analysis was carried out only on the 1/16 dilution, as this was the only dilution displaying a visual difference between the growth of the indicator strain for the undigested and the digested fermentate samples. There was no significant difference detected between the growth of the *S. xylosus* with either the pH 4.80 fermentate samples incubated with or without Proteinase K at the 1/16 dilution, with the generated p-value > 0.05. The comparison of the Proteinase K digested pH 4.80 fermentate to the *S. xylosus* growth control generated p-values of <0.0001, indicating significant inhibition was observed by the Proteinase K digested pH 4.80 fermentate, and that incubation with the Proteinase K did not reduce or eliminate the antimicrobial activity produced by *B. longum* ITT 13.

The fact that the antimicrobial activity is not lost for the pH 4.35 or pH 4.8 fermentate's following incubation with the Proteinase K is not indicative that there is no proteinaceous component contributing to the antimicrobial activity, as it is possible that whatever potential proteinaceous molecule present in the fermentate's may be resistant to Proteinase K digestion. Bacteriocin sensitivity to various proteases appears to be variable, with many susceptible to a

wide range of proteases and others resistant to various proteases. BAC-IB17, a bacteriocin produced by *Bacillus subtilis* KIBGE-IB1 and described by Ansari *et al.*, (2018), was found to be completely resistant to various proteases including Proteinase K and Protease V8 after a 2 hour incubation and only a 50% reduction in antimicrobial activity was detectable after treatment with the proteases for 24 hours. Aureocin A53, a class II bacteriocin, displayed high resistance to Trypsin with 90-95% of the peptide intact after an 18-hour incubation with the protease (Netz *et al.*, 2002), indicating not all proteinaceous material with antibacterial activity is reduced or eliminated with protease treatment. Some bacteriocins have also displayed resistance to Proteinase K including BM1157, produced by *Lactobacillus crustorum* MN047, which only had a 6% reduction in activity when treated with the enzyme (Yi *et al.*, 2018).

For the Actinase E digestion, the pH 4.35 fermentate incubated with the enzyme showed no significant difference in antimicrobial activity ($p>0.05$) when compared with the undigested fermentate (Figure 4.10). However, it was observed that there was low-level growth of the *S. xylosus* at the 1/16 dilution (average OD 600 = 0.11) for the digested pH 4.35 fermentate as compared to the undigested fermentate which had no growth of the *S. xylosus* with an average OD 600 of 0.02 (Figures 4.10a and 4.10b). The difference in the OD 600 measurements for the digested versus undigested pH 4.35 fermentate was not statistically significant with a p-value >0.05, but there is a possibility that the difference observed indicates potential biological significance that may warrant further investigation around the 1/16 dilution as a small amount reduction in antimicrobial activity may be indicative of proteinaceous material at a very low concentration that is contributing to the antimicrobial activity.

The growth observed for the pH 4.80 fermentate digested with Actinase E (Figure 4.11) was similar to that of the *S. xylosus* growth control ($p >0.05$), indicating no significant antimicrobial activity was observed for the digested pH 4.80 fermentate and that the Actinase E had inactivated possible proteinaceous material contributing to the antimicrobial activity. There

was a significant difference between the antimicrobial activity of the original pH 4.80 fermentate that was not incubated with Actinase E and the Actinase E digested pH 4.80 fermentate ($p = 0.0023$). This indicates that the fermentate with no Actinase E digestion still displayed antimicrobial activity, but that the antimicrobial activity was then reduced after digestion with the Actinase E enzyme. It is likely the digestion of the pH 4.80 fermentate and reduction of activity were only observed at the 1/16 dilution as it is known that *B. longum* ITT 13 produces a combination of organic acids resulting in some antimicrobial activity. As these acids are more concentrated at the lower dilutions the antimicrobial activity of the organic acids is likely stronger than that associated with the proteinaceous material, with the activity of the latter not detected until the acids have been diluted. As the pH 4.80 fermentate was generated in a 2L fermenter with a higher cell density (10^{11} CFU/ml) compared to the pH 4.35 fermentate produced in laboratory scale conditions (10^{9} CFU/ml) there were likely higher levels of produced proteins which may offer explanation to why there was significant reduction in antimicrobial activity only for the pH 4.80 fermentate upon digestion in comparison to the pH 4.35 fermentate. Another possibility is that as the pH of the pH 4.80 fermentate is higher, and that actinase E displays higher levels of stability of the at higher pH (pH 5-8) (*Pronase E | Sigma-Aldrich*) resulting in potentially higher specific activity and increased digestion of the pH 4.80 fermentate. The statistically significant partial reduction in antimicrobial activity when the *B. longum* ITT 13 pH 4.80 fermentate is incubated with the Actinase E is indicative that the antimicrobial activity observed for the strain is partially associated with an antimicrobial proteinaceous molecule. As the MIC_{50} dilution is calculated on the basis of at least 50% inhibition of the indicator strain (see Section 4.1), it can be deduced that the MIC_{50} of the pH 4.80 fermentate was a 1/16 dilution and the MIC_{50} of the same fermentate after digestion with Actinase E was the 1/8 dilution. Actinase E is an enzyme that has been shown to digest bacteriocins produced by many bacteria. A bacteriocin produced by the LAB *Pediococcus*

acidilactici HW01, was inactivated by various proteases including Actinase E (stated as Pronase E in the study), when the cell free supernatant was incubated with the enzymes and tested using the well diffusion assay (Ahn *et al.*, 2017). The supernatant of the strain *Enterococcus faecium* MMT21 that had displayed antimicrobial activity was digested with Trypsin, Proteinase K and Actinase E (Protease E in the study) and tested for activity using the well diffusion (microdiffusion) assay with *Listeria ivanovii* BUG 496 as the indicator strain, with all activity lost after digestion (Ghrairi *et al.*, 2008). In another study by Biswas *et al.*, (2017) various LAB including *Lactobacillus, Pediococcus* and *Enterococcus*, were shown to display antimicrobial activity that was inactivated after digestion with various proteases including Pronase E (Actinase E). Many of the species indicated in the above studies are found in the intestinal flora – similar to *B. longum* ITT 13 which was isolated from the neonatal gut flora in a previous research project carried out in our laboratory (Cooke *et al.*, 2005) and may display similarities due to being found within the same ecological niche.

The results observed at the 1/16 dilution for the pH 4.35 fermentate, although non-significant, suggest a trend that is in agreement with the Actinase E digestion effect on the pH 4.80 fermentate and it is possible that as the amount of antimicrobial proteinaceous molecule is at a very low concentration in the pH 4.35 fermentate, that the effect of the digestion by the Actinase E may just be below the limit of detection and it is suggested that further studies be carried out on a concentrated pH 4.35 fermentate. The pH 4.80 fermentate may have displayed a larger decrease in antimicrobial activity when digested with Actinase E due to the fact that this fermentate would be expected to have a larger concentration of antimicrobial peptides, as it was cultured in a 2L fermenter leading to higher levels of growth ($1x10^{11}$ CFU/ml). This is in comparison to the pH 4.35 fermentate, which was grown at a smaller scale (100 mls) in an anaerobic chamber and had less cell growth ($1x10^{9}$ CFU/ml) and in turn would be expected to have less bacteriocin production.

Antimicrobial activity was observed at the 1/4 and 1/8 dilutions for the Actinase E digested pH 4.80 fermentate in Figure 4.11a, even though the enzyme had successfully digested antimicrobial peptides and reduced activity at the 1/16 dilution. The observance of antimicrobial activity at the lower dilutions after the enzymatic digestion may be indicative of antimicrobial activity most likely associated with organic acid production and this acid may only be diluted out by the 1/16 dilution, at which point the effect of the incubation with the Actinase E was observable in relation to the difference in activity between the digested and undigested pH 4.80 fermentate. The latter effect can be taken as indicative of the contribution of a proteinaceous molecule to the antimicrobial effect of the *B. longum* ITT 13 fermentate. It was observed that the antimicrobial activity of the *B. longum* ITT 13 fermentate was reduced by the Actinase E, however there was no observable statistically significant decrease in antimicrobial activity when the incubated with Proteinase K. In a study by Collado *et al.*, (2005), the antimicrobial activity of bacteriocins produced by *Bifidobacterium* strains were reduced when treated with multiple proteases including Protease A, a component of Pronase E (Actinase E). However, in the case of Proteinase K, the enzyme did not inhibit the antimicrobial activity and instead displayed a synergistic effect by increasing the antimicrobial activity observed against *Helicobacter pylori* (Collado *et al.*, 2005). This finding is comparable to the activity of ITT 13, as it is also a *Bifidobacterium* strain with resistance to Proteinase K and susceptibility to Pronase E (Actinase E). Although synergism has not been observed with the Proteinase K digested fermentate of *B. longum* ITT 13, antimicrobial peptides may be present in such a low concentration the effect may not be observed, and this is something that would warrant further investigation with a concentrated fermentate.

The fact that the Actinase E was demonstrated to show the presence of a proteinaceous component contributing to the antimicrobial activity is a statistically and biologically significant result and thus is in agreement with the detection of antimicrobial activity for the

resuspended 80% ammonium sulphate fraction. It should be noted that the 80% AS fraction was resuspended in a 50 mM NaAc pH 4.3 buffer and thus the antimicrobial activity would not be considered to be associated with the presence of organic acids. As the Actinase E enzymatic digestion resulted in inactivation of the proteinaceous material contributing to the antimicrobial activity, this could indicate the possible presence of aspartic and glutamic acids in the peptide, as some of the possible sites of action of Actinase E, which is a mix of enzymes with multiple potentials sites, are the carboxyl side of glutamic and aspartic acid (*Sigma-Aldrich*, 2020). In studies glutamic acid and aspartic acid have been shown to increase the solubility of proteins due to their negative charge, whereby negative charge is associated with increased solubility (Trevino, *et al*., 2007; Kramer *et al.*, 2012), indicating the possibility the antimicrobial peptide may be a soluble protein. If this is the site of action in the peptide, the agreement between the protease digestion and ammonium sulphate precipitation results indicates the possibility of a low molecular weight, potentially soluble, likely negatively charged peptide.

4.6.5 HPLC Analysis of the *B. longum* ITT 13 Fermentate

Organic acids including lactic and acetic acid are widely produced by *Bifidobacteria* (Lee and O'Sullivan, 2010; Delgado *et al.*, 2019; Nguyen *et al.*, 2019) and organic acid production by bacteria has been associated with antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as anti-fungal activity against both mould and yeast fungal strains (Schillinger and Villarreal, 2010; Yang *et al.*, 2012; Lourenço *et al.*, 2019; Ajingi *et al.*, 2020; Chen *et al.*, 2020). HPLC analysis has been used to identify and quantify the organic acid production of many LAB (Özcelik *et al.*, 2016; Nuryana *et al.*, 2019) and in this study was carried out on the fermentate produced by *B. longum* ITT 13, both without pH control at pH 4.3 and pH controlled during growth in a 2-litre fermentation vessel with a final pH in the fermentate of pH 4.8.

Lactic, acetic and succinic acids were determined as the organic acids produced by *B. longum* ITT 13 during growth and fermentation in the MRSc broth medium for both the fermentation with and without pH control (Figures 4.12 and 4.13). In comparison, only lactic and acetic acid were specifically determined as the organic acids produced by *B. longum* ITT 13 when cultured in the mYPD media (C. Whelan – personal communication).

For the fermentates produced using MRSc media, the pH 4.8 fermentate, grown with pH control, was found to produce higher levels of acetic acid and succinic acid, with concentrations of 160.95 mM ± 13.72 and 67.69 mM ± 6.14 respectively, compared to 147.72 ± 10.34 acetic acid and 54.52 ± 3.00 succinic acid for the pH 4.3 fermentate. In comparison, the lactic acid concentration was higher for the pH 4.3 fermentate at 92.22 mM ± 1.53 compared to 68.32 mM ± 10.62 for the pH 4.8 fermentate.

The pH 5.0 fermentate produced in the mYPD media in our laboratory (C. Whelan – personal communication) and grown with pH control contained higher levels of lactic acid and substantially higher levels of acetic acid than the fermentates grown in MRSc, with 154.98 mM and 650.68 mM respectively. Both the pH 4.8 fermentate grown in MRS media + 0.11% *L*-cysteine and the pH 5.0 fermentate grown in mYPD in 2L fermentation vessels with pH control generated a high cell count of 10^{11} CFU/ml, which would likely correspond to the higher levels of production of organic acids by the *B. longum* ITT 13 in both fermentates. In comparison, the pH 4.3 fermentate grown as static low volume cultures (100-200 mls) in MRSc without pH control grew at a lower density of approximately 10^{9} CFU/ml and also has lower production of organic acids except lactic acid.

The media used for growth also demonstrates an effect on the composition and concentration of organic acids produced due to the significantly higher concentrations of both lactic and acetic acid produced by the pH 5.0 fermentate grown in an mYPD and the lack of succinic acid produced in the same fermentation.

The concentrations of lactic and acetic acid produced by various LAB including *Bifidobacteria* were analysed in a study by Tejero-Sariñena *et al.*, (2012), with 180 mM lactic acid and <50 mM acetic acid produced by *B. longum* PXN 30 and 99 mM acetic acid and <50 mM lactic acid produced by *B. infantis* PXN 27. In comparison, *B. longum* ITT 13 cultured in MRSc broth produced less lactic acid, but more acetic acid than *B. longum* PXN 30 and higher concentrations of both lactic and acetic acid than *B. infantis* PXN 27 (Table 4.3). These results indicate the variability in acid production between different *Bifidobacterial* strains, as all were cultured using the same MRS media.

The organic acid production profile of two *B. longum* strains, CA1 (grown in MRSc) and F9 (grown in modified Columbia medium (mCOL)), was also assessed in a study by Makras and De Vuyst, (2006). Strain CA1 was found to produce 60.9 mM ± 4.2 acetic acid and 39.9 mM ± 3.1 lactic acid with a final pH of 4.34, and strain F9 was found to produce much lower concentrations of 28.0 mM ± 2.4 acetic acid and 17.8 mM ± 1.2 lactic acid (Table 4.3), with a final pH of 4.14. This further demonstrates how different *B. longum* strains have the ability to produce vastly different concentrations of acid, as the *B. longum* ITT 13 fermentate with no pH control had a pH of 4.30-4.35 comparable to the pH of *B. longum* CA1 at pH 4.34, yet much higher concentrations of acid were detected in the fermentate of ITT 13 even though they were grown in the same media. It is also evident that the concentration of these acids does not directly correlate to the pH as F9 contained much lower acetic and lactic acid concentrations than both CA1 and ITT 13, yet had the lowest pH profile of all three strains. The media used for growth also appears to affect the concentration and composition of acids produced as *B. longum* F9 cultured in mCOL had the lowest concentrations of lactic and acetic acid produced out of all assessed strains, while *B. longum* ITT 13 cultured in the mYPD media (C. Whelan – personal communication) had the highest lactic and acetic concentrations in comparison to ITT 13 cultured in MRSc (Table 4.3).

Table 4.3: Lactic and Acetic Acid Concentrations Produced by *Bifidobacteria*

Strain + media	Lactic acid (mM)	Acetic acid (mM)
[(1)] *B. longum* PXN 30 (MRSc)	180	<50
[(1)] *B. infantis* PXN 27 (MRSc)	<50	99
[(2)] *B. longum* CA1 (MRSc)	40	61
[(2)] *B. longum* F9 (mCOL)	18	28
B. longum ITT 13 (pH 4.3 fermentate) (MRSc)	92	147.72
B. longum ITT 13 (pH 4.8 fermentate) (MRSc)	68	161
B. longum ITT 13 (pH 5 fermentate) (mYPD)	155	651

The acid concentrations (mM) produced are as described by [(1)] Tejero-Sariñena *et al.*, (2012), [(2)] Makras and De Vuyst, (2006) and by *B. longum* ITT 13. It is evident upon comparison that the *B. longum* ITT 13 fermentations both pH controlled at pH 4.8 and cultured without pH control at pH 4.3 produced higher concentrations of acetic acid than the various other *Bifidobacterial* strains cultured in MRSc broth. *B. longum* PXN 30 produced the highest concentrations of lactic acid at 180 mM in comparison to the other strains, while *B. longum* ITT 13 cultured in mYPD produced significantly higher levels of acetic acid than all strains.

Although the acid concentrations may differ between *B. longum* strains, the profile of acids produced may be similar within the various *Bifidobacterial* species, as in the study by Makras and De Vuyst, (2006), where formic acid was detected in supernatants produced by multiple strains of *B. animalis* and *B. bifidum*, yet not by either of the *B. longum* strains CA1 or F9, and was also not detected in this study by *B. longum* ITT 13. This may indicate that formic acid is not produced by *B. longum* strains or is produced below detectable levels compared to other *Bifidobacteria* spp.. Succinic acid is not mentioned in either paper for comparison to *B. longum* ITT 13.

Antimicrobial activity associated with the organic acid production of *Bifidobacteria* has been documented (Lee and O'Sullivan, 2010). In a study by Georgieva *et al.* (2015), the acidic and neutralized cell-free supernatant of various *Lactobacilli* and *Bifidobacterium* strains were

tested for activity and it was found that the *Bifidobacteria* generally lost their antimicrobial activity once neutralized to pH 7, except for *B. animalis* subsp. *lactis* L3, when tested in the microdiffusion assay against the indicator strain *S. aureus* NBIMCC 3703. The results indicated in the aforementioned study indicate that the organic acid production contributes to antimicrobial activity alongside the low molecular weight proteinaceous material exhibiting activity (Section 4.3 and 4.4). Makras and De Vuyst, (2006) determined that 39.9 mM lactic acid and 60.9 mM acetic acid was produced by *B. longum* CA1. When these concentrations of acid were tested for antimicrobial activity by the authors, they were found to display antibacterial activity through inhibition of the growth of *Salmonella enterica* ser. *typhimurium* SL1344 and a decrease in viability of *E. coli* C1845 cells in time-kill assays, which was the same as the antimicrobial activity observed by the *B. longum* CA1 cell-free fermentate indicating the activity was associated with the organic acids present in the fermentate. As the acid concentrations produced by *B. longum* ITT 13 are higher than the concentrations displaying activity produced by CA1, it is probable that the concentration of the acids are contributing to the antimicrobial activity and further indicate the likelihood of synergism between acid and peptide resulting in activity, in agreement with the studies observed by Pasteris *et al.*, (2014).

A destabilisation effect of the cell membrane may also take place with *B. longum* ITT 13, whereby the organic acids may weaken cell membranes thus increasing the efficacy of the antimicrobial peptide, resulting in a synergistic reaction between acid and peptide. This effect has been observed in a study by Ajingi *et al.* (2020) where the activity of nisin was increased to sub-MIC concentrations against a potato spoiling strain of *B. subtilis*, as well as other bacteria including *S. aureus* and *E. coli*, when used in conjunction with organic acids such as formic acid and lactic acid. This increase in the antibacterial activity of nisin indicated a synergistic effect between nisin and the organic acids. For example, in this study by Ajingi *et*

al. (2020) the MIC concentrations of 0.25% lactic acid and 0.016% nisin were observed against *B. subtilis,* with the MIC decreasing to 0.025% lactic acid and 0.0016% nisin when both were used in conjunction, which was indicative of synergism. This synergistic effect was also observed for nisin in combination with many organic acids including formic, citric and malic acid, against both Gram-positive and Gram-negative indicator strains (Ajingi *et al.,* 2020). A probable mode of action of organic acids could be interference with subtrate transportation through the cell membrane of bacterial cells by interference with the porosity of the membrane (Davidson *et al.*, 2013).

Due to the organic acid production by *B. longum* ITT 13 of lactic, acetic and succinic acid and the production of potentially low-molecular weight, antimicrobial proteinaceous material, a synergistic effect is a potential scenario in this case. This is another possibility for the antimicrobial activity observed against all tested Gram-negative species susceptible to the *B. longum* ITT 13 fermentate which are more susceptible to inhibition by organic acid production (Makras and De Vuyst, 2006). Therefore, the organic acid produced by *B. longum* ITT 13 may cause interference with the cell membrane as described above, thus making the cells highly susceptible to the antimicrobial effects of the antimicrobial peptide produced at low concentrations. Further concentration and purification of the antimicrobial activity produced by *B. longum* ITT 13 may concentrate and isolate these produced antimicrobial molecules allowing future testing and insight into the specific modes of action of the antimicrobial peptide production and possible synergistic activities between the proteinaceous material and the organic acids produced by the strain.

CHAPTER 5

Partial Purification and Concentration Studies of the *Bifidobacterium longum* ITT 13 Fermentate

The production of antimicrobial metabolites are an important characteristic of probiotic strains to protect the host from pathogenic colonisation (Vlasova *et al.*, 2016). Bacteriocins are of a particular interest as these are ribosomally synthesised peptides that have demonstrated an antimicrobial effect towards various bacteria and fungi, including those associated with food spoilage and pathogenesis (Cheikhyoussef *et al.*, 2010; Poltavska and Kovalenko, 2012; Lim, 2016; Luz *et al.*, 2017; Aarti *et al.*, 2018). The results indicated within Chapter 4 suggest that *B. longum* ITT 13 produces antimicrobial activity associated with an antimicrobial peptide produced at low concentrations, as well as antimicrobial activity associated with the production of organic acids including lactic acid, acetic acid and succinic acid. In order to increase the antimicrobial activity produced by *B. longum* ITT 13 for analysis, methods of concentration of the fermentate were evaluated.

Cross flow filtration has been reported by the International Union of Pure and Applied Chemistry (IUPAC) recommendation as "flow through a membrane module in which the fluid on the upstream side of the membrane moves parallel to the membrane surface and the fluid on the downstream side of the membrane moves away from the membrane in the direction normal to the membrane surface" (Koros *et al.,* 1996). It is a method of filtration whereby the sample flows tangentially to the surface of the filter, consisting of a semi-permeable membrane with a particular pore size and a pump system is used to promote the feed flow throughout the system to the membrane (Musumeci *et al.*, 2018). Therefore, it can be used to separate molecules above a certain size from those small enough to pass through the membrane, separating the filtered fluid into a retentate which cannot pass through the filter, and a filtrate or permeate which passes through and is collected, allowing for separation of two samples or a concentration effect of the retentate (Musumeci *et al.,* 2018) (Figure 5.1). It is becoming increasingly used as a key step in the biotechnology, pharmaceutical and food industries (Holland *et al.,* 2012; Kelly *et al.*, 2014; Musumeci *et al.*, 2018; Pires and Palmer, 2021; Tan *et al.*, 2021).

To enable further detailed studies of the component(s) contributing to the antimicrobial activity observed for *B. longum* ITT 13 fermentate it is necessary to purify them. This chapter looks at methods evaluated to concentrate the antimicrobial activity together with preliminary purification studies. One of the experimental methods evaluated in concentration of the antimicrobial activity was crossflow filtration as this method has been used previously as a step in purification schemes for antimicrobial activity produced by bacteria (Izquierdo *et al.*, 2009; Lv, Ma, *et al.*, 2018). To concentrate the antimicrobial activity produced by *B. longum* ITT 13 in the fermentate, the filtration method of tangential flow filtration was employed by using both a Pellicon® XL 50 Cassette and Labscale^{TM} TFF System (Millipore) and larger-scale tangential flow filtration Sartoflow Alpha Benchtop Crossflow System (Sartorius AG, Germany).

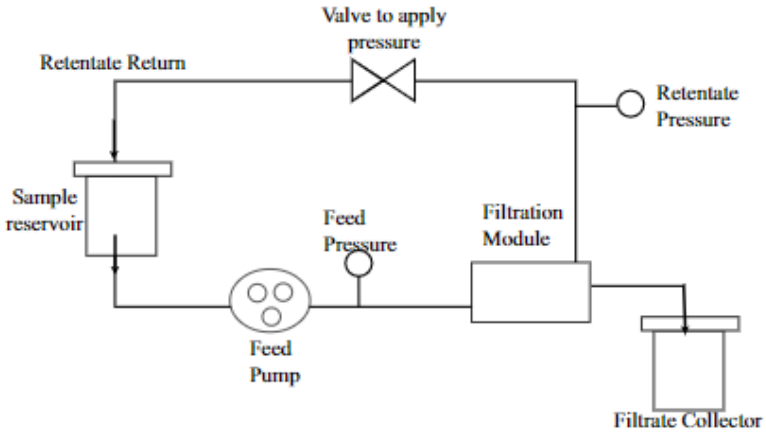

Figure 5.1: A diagram representation of a Tangential Flow Filtration system (Musumeci *et al.*, 2018). In the schematic representation of a TFF system, the samples for concentration are applied to the sample reservoir where they travel through the system propelled by the feed pump to the filtration module, which consists of a membrane of specified pore size. Samples that pass through the filter are collected as the filtrate/permeate, while the retained molecules are fed back toward the sample reservoir. Repeated circulation is carried out until the retentate is of the required, concentrated volume.

Results

5.1 Small-scale TFF Concentration Experimental Results

For the small-scale TFF concentration of the *B. longum* ITT 13 fermentate, a Pellicon® XL 50 Cassette and a Labscale^{TM} TFF System (Millipore) benchtop laboratory system were used, with various experiments carried out using filter sizes between 3 kDa and 10 kDa. The filtration of the fermentate was carried out as describe in Section 2.10.1.

Table 5.1: Details of Conditions and Parameters of all small-scale Tangential Flow Filtration (TFF) Runs of the *B. longum* ITT 13 Fermentate

TFF Run + Filter Size	**Initial Information**			**Centrifugation**			**Buffer Exchange(s)**			**Vol. of Ret. After Conc.**	**Final Conc. (x-fold)**	**BCA Test (Y/N)**
	Vol.	pH	Media	x *g*	Time (mins)	Steri cup (µm)	No.	Final pH	Solvent			
1. TFF 10 kDa	450 mls	4.26	MRSc	2722	5	No	-	-	-	50 mls	9-fold	Yes
2. TFF 3 kDa (1)	125 mls	4.19	MRSc	2722	5	0.22	3		NaAc pH 4.9	25 mls	5-fold	No
3. TFF 3 kDa (2)	125 mls	4.25	MRSc	2722	5	0.22	2	2.27	0.02N HCl pH 1.8	25 mls	5-fold	No
4. TFF 3 kDa (3)	950 mls	4.20	MRSc	2722	5	0.45	2	4.67	NaAc pH 4.8	50 mls	19-fold	Yes

A total of 4 TFF runs were carried out, with either no use of a stericup or stericups of 0.22 or 0.45 µm used and either no buffer exchange or a buffer exchange in either 50 mM pH 4.9 NaAc buffer, 50 mM pH 4.8 NaAc buffer or 0.02N HCl. A BCA protein assay test was performed on TFF runs 1 and 4.

5.1.1 Small-scale TFF Run 1 (10KDa)

The first TFF run carried out on the *B. longum* ITT 13 fermentate involved the use of a 10 kDa membrane.

Table 5.2: The Antimicrobial Activity of each Fraction of the *B. longum* ITT 13 Fermentate After TFF Concentration (Run 1)

The results were only noted up to a 1/4 dilution as this is the highest dilution at which antimicrobial activity was detected for the samples. AU/ml was not calculated for the positive control of nisin and it was not relevant for this experiment.

	Sample	Avg. Zone (mm) ± SD			Average AU/ml
		Undiluted	1/2	1/4	
TFF (10 kDa)	Permeate (pH 4.28)	4.0 ± 0	1.7 ± 0.2	0 ± 0	11 ± 0
	Retentate (pH 4.26)	4.0 ± 0	2.0 ± 0	0 ± 0	11 ± 0
	Nisin (1000 IU/ml undiluted)	2.3 ± 0.2	1.2 ± 0.3	0.7 ± 0.2	N/A
	MRSc broth	0 ± 0	N/A	N/A	N/A

Both the permeate and retentate samples gave equivalent levels of antimicrobial activity, which could not be statistically differentiated. For both, the antimicrobial activity was detected up to a maximum of a 1/2 dilution with activity equivalent to 11 ± 0 AU/ml.

A BCA assay was carried out to determine the protein content of the fractions (Table 5.3). As no BCA assay or microdiffusion assay was carried out on the fermentate before concentration using TFF, the protein concentration and activity could not be determined for the original fermentate. The entire volume of fermentate was used for TFF concentration with no fermentate kept for activity or BCA testing before concentration.

Table 5.3: Activity table for TFF concentration (Run 1) of the *B. longum* ITT 13 Fermentate against *S. xylosus*

Sample	Total Volume	Protein Conc. (mg/ml)	Protein Content (Total mg)	Activity (AU/ml)	Total Activity (AU/ml x total vol.)	Specific Activity (AU/mg)	% Volume Recovery	
Fermentate (pH 4.26)	450 mls	N/A	N/A	N/A	N/A	N/A	N/A	
Permeate (pH 4.28)	400 mls	12.30	4,920	11	4,400	0.9	89%	100%
Retentate (pH 4.26)	50 mls	23.63	1182	11	550	0.5	11%	

No sample of the initial fermentate was available for testing using the BCA method for protein concentration determination. Both the permeate and retentate had 11 AU/ml of antimicrobial activity, even though the retentate had almost double the protein concentration (23.63 mg/ml) in comparison to the permeate (12.30 mg/ml). The lower protein concentration of 12.3 mg/ml and antimicrobial activity of 11 AU/ml for the permeate generated a specific activity of 0.9 AU/mg, which is almost double that of the retentate which had a specific activity of 0.5 AU/mg. The results presented in Table 5.3 show that although there was a ninefold concentration effect from 450 ml to 50 ml, and the retentate contained nearly double the protein concentration of the permeate, the specific activity decreased by almost 50% and most of the antimicrobial activity is still present in the permeate. This indicates that although a concentration effect was observed for the retentate as expected, the antimicrobial activity observed was not reflective of this as the AU/ml remained the same for both samples. The pH of the permeate and retentate was equivalent at pH 4.28 and pH 4.26 respectively, indicating that this low pH and the contribution of organic acids in the *B. longum* ITT 13 fermentate (see Section 4.5) may have masked any activity associated with the protein concentration within the retentate.

5.1.2 Small-scale TFF Run 2 (3KDa filter using 50 mM NaAc exchange buffer at pH 4.9)

The second TFF run carried out on the *B. longum* ITT 13 fermentate involved the use of a smaller 3 kDa membrane in place of the 10 kDa membrane to concentrate the activity within the retentate.

Table 5.4: Antimicrobial activity of *B. longum* ITT 13 fractions following TFF concentration using a 3 kDa filter and pH 4.9, 50 mM NaAc buffer exchange (Run 2).

The results were noted up to a 1/4 dilution, as this is the highest dilution antimicrobial activity was detected. Zones detected for the 1/4 dilution were faint in appearance. The fermentate containing cells as well as the cell free fermentate after cell removal were both tested for activity. AU/ml was not calculated for the positive control of nisin and it was not relevant for this experiment.

	Sample	Avg. Zone (mm) ± SD			Average AU/ml
		Undiluted	1/2	1/4	
TFF (3 kDa)	Fermentate (pH 4.19)	4.2 ± 0.2	2.2 ± 0.2	1.7 ± 0.2	22 ± 0
	Cell Free Fermentate	3.8 ± 0.2	2.2 ± 0.2	1.5 ± 0.4	22 ± 0
	Permeate 1 (no buffer exchange)	3.7 ± 0.6	2.0 ± 0	1.3 ± 0.9	18 ± 5
	Permeate 2 (1 buffer exchange)	0 ± 0	0 ± 0	0 ± 0	0
	Permeate 3 (2 buffer exchanges)	0 ± 0	0 ± 0	0 ± 0	0
	Permeate 4 (3 buffer exchanges)	0 ± 0	0 ± 0	0 ± 0	0
	Retentate 1 (no buffer exchange)	3.3 ± 0.5	N/A	N/A	N/A
	Retentate 2 (1 buffer exchange)	1.2 ± 0.2	N/A	N/A	N/A
	Retentate 3 (2 buffer exchanges)	0 ± 0	N/A	N/A	0
	Retentate 4 (3 buffer exchanges)	0 ± 0	0 ± 0	0 ± 0	0
	Nisin (1000 IU/ml undiluted)	2.5 ± 0.1	2.0 ± 0.2	1.4 ± 0.2	N/A
	MRSc broth	0 ± 0	N/A	N/A	N/A
	NaAc buffer pH 4.9	0 ± 0	N/A	N/A	N/A

No BCA assay was carried out on these samples to determine protein concentration and distribution after TFF concentration. The pH of the samples was also not monitored throughout the buffer exchange. The fermentate before concentration displayed antimicrobial activity to a

maximum of a 1/4 dilution with 22 ± 0 AU/ml and the cell free fermentate displayed equivalent antimicrobial activity i.e. the 1/4 dilution gave an activity value of 22 ± 0 AU/ml.

Permeate 1 from TFF run 2, obtained following filtration with a 3kDa filter but with no buffer exchange, showed only slightly lower antimicrobial activity at 18+ 5 AU/ml as compared to the original fermentate (22+ 0 AU/ml). As this was the initial permeate and did not involve a media exchange, any constituents of the fermentate smaller than the 3kDa filter size would be excluded in this permeate, therefore, permeate 1 was the only permeate diluted to 1/16 for AU/ml determination.

Retentate 1, generated before buffer exchange, would contain particles small enough to be retained by the filter and should thus remain in the retentate throughout the TFF process. Therefore, only the final retentate after the buffer exchange was diluted to a 1/16 dilution for AU/ml determination. Retentate 1 was plated in its undiluted form using the microdiffusion assay to determine whether antimicrobial activity was present, and generated a zone of inhibition of 3.3 mm ± 0.5, approximately 0.4 mm less than permeate 1 in the undiluted form which had a zone of 3.7 mm ± 0.6, however both values were very similar when standard deviation is taken into account.

Microdiffusion assay results for Permeate 2 and Retentate 2 showed that while there is no detectable activity present in the permeate, the zone size for the retentate had decreased to 1.2 mm ± 0.2 relative to the original cell-free fermentate (3.8 mm ± 0.2). As Retentate 2 was screened for activity qualitatively in the undiluted form, no AU/ml could be determined. The results indicate that there was antimicrobial activity detectable in the retentate after the buffer exchange which was either absent or below the limit of detection in the more dilute permeate. Both permeates 3 and 4 and retentates 3 and 4 generated after subsequent buffer exchanges retained no antimicrobial activity even in the undiluted form.

5.1.3 Small-scale TFF Run 3 (3KDa filter using pH 1.8, 0.02N HCl as exchange buffer)

The third TFF run carried out on the *B. longum* ITT 13 fermentate was repeated with the use of a 3 kDa membrane to concentrate the antimicrobial activity with buffer exchanges carried out using 0.02N, pH 1.8 HCl, which is the same solvent used for preparation of the bacteriocin nisin (Section 2.5) and does not display antimicrobial activity despite its low pH.

Table 5.5: The Antimicrobial Activity of each Fraction of the *B. longum* ITT 13 Fermentate after TFF concentration using a 3 kDa filter and buffer exchange with pH 1.8, 0.02N HCl (Run 3).

The results were only noted up to a 1/4 dilution as this is the highest dilution activity was present at. The fermentate before cells were removed as well as the cell free fermentate after cell removal were both tested for activity. AU/ml was not calculated for the positive control of nisin and it was not relevant for this experiment.

	Sample	Avg. Zone (mm) ± SD			Average AU/ml
		Undiluted	1/2	1/4	
TFF (3 kDa)	Fermentate (pH 4.25)	4.5 ± 0	2.5 ± 0	1.0 ± 0.7*	18 ± 5
	Cell Free Fermentate	4.3 ± 0.2	2.2 ± 0.2	0.7 ± 0.9*	15 ± 5
	Permeate 1 (no buffer exchange)	3.7 ± 0.2	0 ± 0	0 ± 0	6 ± 0
	Permeate 2 (1 buffer exchange)	1.8 ± 0.3	N/A	N/A	N/A
	Permeate 3 (2 buffer exchanges)	0 ± 0	N/A	N/A	N/A
	Retentate 1 (no buffer exchange)	3.8 ± 0.2	N/A	N/A	N/A
	Retentate 2 (1 buffer exchange)	1.8 ± 0.2	N/A	N/A	N/A
	Retentate 3 (2 buffer exchanges) (pH 2.27)	0 ± 0	0 ± 0	0 ± 0	0
	Nisin (1000 IU/ml undiluted)	4.9 ± 0.3	4.5 ± 0.4	3.9 ± 0.3	N/A
	MRSc broth	0 ± 0	N/A	N/A	N/A
	0.02N HCl (negative control)	0 ± 0	N/A	N/A	N/A

*indicates the zones of inhibition were hazy in comparison to other zones on the microdiffusion plates.

Both the original fermentate (with cells) and the cell free fermentate displayed similar antimicrobial activity that was detected up to a maximum of the 1/4 dilution, with 18 ± 5 AU/ml associated with the fermentate with cells and 15 ± 5 AU/ml associated with the cell free

fermentate. Although both the fermentate samples displayed activity to the same dilution, the cell free fermentate displayed zones of inhibition 0.2-0.3 mm smaller than those produced by the fermentate with cells at every dilution tested using the micro-diffusion assay. Antimicrobial activity was only detected for Permeate 1, with no buffer exchange, in the neat form (6 ± 0 AU/ml), indicating a loss of antimicrobial activity in comparison to the fermentate and cell free fermentate before TFF concentration. Retentate 1 was screened for the presence of antimicrobial activity in its undiluted form, as described previously for Table 5.4, and displayed antimicrobial activity with an average zone of inhibition of 3.8 mm ± 0.2, similar to permeate 1 which generated an average zone of 3.7 mm ± 0.2. Antimicrobial activity testing for Permeate 2 and Retentate 2, following a single buffer exchange with pH 1.8, 0.02N HCl (in comparison to NaAc buffer used in Run 2), generated zone sizes averaging 1.8 mm for both samples. Both permeate 2 and retentate 2 were only screened for antimicrobial activity qualitatively in the undiluted form as described previously in Table 5.4, therefore no AU/ml calculation was determined. Antimicrobial activity testing of the Permeate 3 and Retentate 3 fractions that had undergone 2 buffer exchanges showed no detectable antimicrobial activity on the microdiffusion assay.

5.1.4 Small-scale TFF Run 4 (3KDa filter using 50 mM NaAc exchange buffer at pH 4.8)

The fourth TFF concentration run carried out on the *B. longum* ITT 13 fermentate was carried out with the use of the 3 kDa membrane to concentrate the antimicrobial activity detected within the fermentate and two subsequent buffer exchanges with 50 mM, pH 4.8 NaAc buffer.

Table 5.6: The Antimicrobial Activity of each Fraction of the *B. longum* ITT 13 Fermentate After TFF Concentration and pH 4.8, 50 mM NaAc buffer exchange (Run 4).

The results were only noted up to a 1/4 dilution as this is the highest dilution at which antimicrobial activity was detected for the samples. The fermentate before cells were removed as well as the cell free fermentate after cell removal were both tested for activity. AU/ml was not calculated for the positive control of nisin and it was not relevant for this experiment.

	Sample	**Avg. Zone (mm) ± SD**			**Average AU/ml**
		Undiluted	**1/2**	**1/4**	
4. TFF (3 kDa)	Fermentate (pH 4.20)	4.7 ± 0.5	2.7 ± 0.5	0.8 ± 0.6	18 ± 5
	Cell Free Fermentate (pH 4.18)	4.8 ± 0.2	2.8 ± 0.2	1.7 ± 0.5*	22 ± 0
	Permeate 1 (no buffer exchange) (pH 4.20)	4.3 ± 0.2	2.5 ± 0	0.5 ± 0.7*	15 ± 5
	Permeate 2 (1 buffer exchange)	1.2 ± 0.2	N/A	N/A	N/A
	Permeate 3 (2 buffer exchanges)	0 ± 0	0 ± 0	0 ± 0	0
	Retentate 1 (no buffer exchange) (pH 4.22)	4.5 ± 0	2.5 ± 0	0.3 ± 0.5*	15 ± 5
	Retentate 2 (1 buffer exchange) (pH 4.43)	1.3 ± 0.3	N/A	N/A	N/A
	Retentate 3 (2 buffer exchanges) (pH 4.67)	0 ± 0	0 ± 0	0 ± 0	0
	Nisin (1000 IU/ml undiluted)	4.7 ± 0.3	3.9 ± 0.3	3.3 ± 0.2	N/A
	MRSc broth	0 ± 0	N/A	N/A	N/A
	pH 4.8 50 mM NaAc Buffer	0 ± 0	N/A	N/A	N/A

* indicates the zones of inhibition were hazy in comparison to other zones on the microdiffusion plates.

Both the fermentate (with cells) and the cell free fermentate which had cells and cellular debris removed via centrifugation and the use of a 0.45 µm Stericup, displayed antimicrobial activity to a 1/4 dilution with 18 ± 5 AU/ml and 22 ± 0 AU/ml respectively.

Retentate 1, permeate 1 and the fermentate samples all generated similar detectable

antimicrobial activity to the 1/4 dilution. The undiluted zones of inhibition for both fermentate samples, permeate 1 and retentate 1 were similar in size and between 4.3 and 4.8 mm and the 1/2 diluted samples were also similar with a diameter between 2.5 and 2.8 mm. At the 1/4 dilution, the fermentate with cells, permeate 1 and retentate 1 displayed average zone measurements between 0.3-0.8 mm and the cell free fermentate displayed a larger average zone size of 1.7 mm, however it should be noted that there was higher fluctuation in zone sizes across the replicates at this dilution and the zones were hazy in comparison to the undiluted and 1/2 dilutions. Both the Retentate 1 and Permeate 1 fractions demonstrated antimicrobial activity of 15 ± 5 AU/ml, which was less than the activity determined for the fermentate samples (18 ± 5 AU/ml and 22 ± 0 AU/ml).

Permeate 2 and retentate 2 which had undergone one buffer exchange were screened for antimicrobial activity using the microdiffusion assay and were tested in their undiluted form, with similar zones of inhibition of 1.2 mm ± 0.2 detected for permeate 2 and 1.3 mm ± 0.3 detected for retentate 2.

No antimicrobial activity was detected using the microdiffusion assay for both permeate 3 and retentate 3, which had undergone two buffer exchanges. An M/D assay plate displaying the analysis of permeate 2 and 3 and retentate 2 and 3 can be seen in Figure 5.3.

A BCA assay was carried out on all samples to assess the protein concentration and distribution of each sample from each step of the TFF run and is displayed in Table 5.7.

Table 5.7: Activity table for TFF concentration (Run 4) of the *B. longum* ITT 13 Fermentate with TFF Concentration

Sample	Total Volume	Protein Conc. (mg/ml)	Protein Content (Total mg)	Activity (Avg. AU/ml)	Total Activity (AU/ml x total vol.)	Specific Activity (AU/mg)	% Volume Recovery		% Protein Recovery	
Fermentate (pH 4.20)	1000 mls	18.16	18,160	18	18,000	1.0	N/A		N/A	
Cell Free Fermentate	950 mls	16.97	16,122	22	20,900	1.3	N/A		N/A	
Permeate 1 (pH 4.20)	900 mls	14.52	13,068	15	13,500	1.0	95%	100%	81%	89%
Retentate 1 (pH 4.22)	50 mls	24.43	1,222	15	750	0.6	5%		8%	
Permeate 2	100 mls	0.52	52	N/A	N/A	N/A	80%	100%	9%	25%
Retentate 2 (pH 4.43)	25 mls	3.79	95	N/A	N/A	N/A	20%		16%	
Permeate 3	100 mls	0	0	0	0	0	80%	100%	0%	0%
Retentate 3 (pH 4.67)	25 mls	0	0	0	0	0	20%		0%	

From the results for TFF (Run 4) presented in table 5.7 it is evident that after the first TFF concentration of the fermentate, 100% of the initial volume was recovered in both the permeate 1 and retentate 1, however only 89% of the initial protein concentration was recovered, indicating possible losses in the filtration concentration protocol.

Of the total protein concentration in Retentate 1 (24.43 mg/ml), which was then used for the second TFF concentration with a buffer exchange, only a total of 25% of the protein concentration was recovered, even though 100% volume was recovered, indicating potential losses through absorbance on the filter. Although the protein concentration of Retentate 2 was 3.79 mg/ml prior to the final TFF filtration and the second buffer exchange, no detectable protein or antimicrobial activity was detected in either Retentate 3 or Permeate 3, indicating a complete loss of protein.

Although there were evident protein losses with repeated TFF cycles, for both Retentate 1 and Permeate 1 together with Retentate and Permeate 2, the concentration effect was somewhat effective, as the protein concentration in the retentates was higher than for the permeates. Retentate 1 had 24.43 mg/ml protein, while permeate 1 contained 14.52 mg/ml and retentate 2 had 3.79 mg/ml while permeate 2 had 0.52 mg/ml protein.

Activity values were calculated for the *B. longum* ITT 13 fermentate and cell free fermentate, Permeate 1, Retentate 1, Permeate 3 and Retentate 3. However, the activity values were not calculated for Permeate 2 and Retentate 2, as these samples were only evaluated for the presence or absence of antimicrobial activity on the micro-diffusion plates and thus only plated in the undiluted form. The fermentate had an average of 18 AU/ml of antimicrobial activity, while the cell free fermentate was slightly higher at 22 AU/ml. As there was less protein in the cell free fermentate yet higher activity, this resulted in a higher specific activity of 1.3 AU/mg of protein for the cell-free fermentate in comparison to the fermentate with cells which had 1.0 AU/mg. The activity was slightly reduced as compared to the original fermentate after the first TFF cycle to 15 AU/ml for both the permeate 1 and retentate 1. The higher concentration of protein in Retentate 1 (24.43 mg/ml) generated a specific activity of 0.6 AU/mg as compared to a protein concentration of 14.52mg/ml for Permeate 1, which thus generated a slightly higher specific activity of 1.0 AU/mg as the activity was equivalent to the retentate yet there was less protein present. Antimicrobial activity was detected at low level for the undiluted samples of Permeate 2 & Retentate 2 and then was completely undetectable following the final buffer exchange via TFF in Permeate 3 and Retentate 3. Following the reduction of protein concentration from Retentate 1 and Permeate 1 to Retentate 2 and Permeate 2, there was a complete loss of detectable protein in Retentate 3 and Permeate 3.

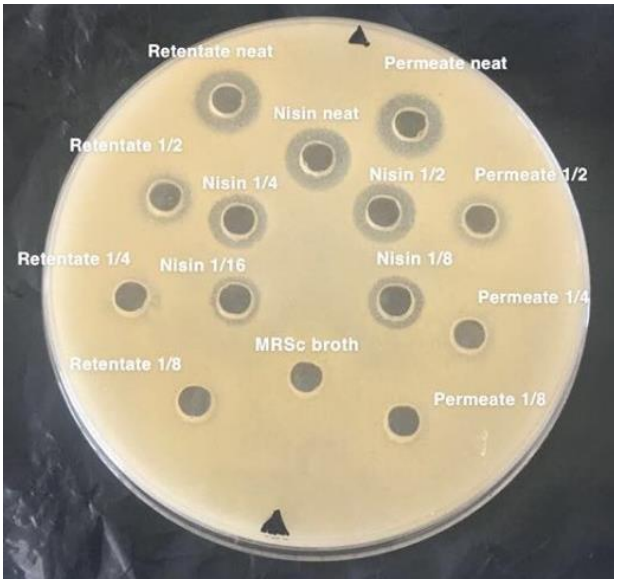

Figure 5.2: The antimicrobial activity of Permeate 1 and Retentate 1 (no buffer exchange) obtained from the TFF run 4. The samples in Figure 5.2 were produced using TFF concentration using a 3 kDa filter. The analysis of antimicrobial activity was carried out using the M/D assay (Section 2.6), with *S. xylosus* as the indicator strain and serial dilutions of nisin from undiluted (neat) to a 1/16 dilution (1000-62.5 IU/ml) as a positive control.

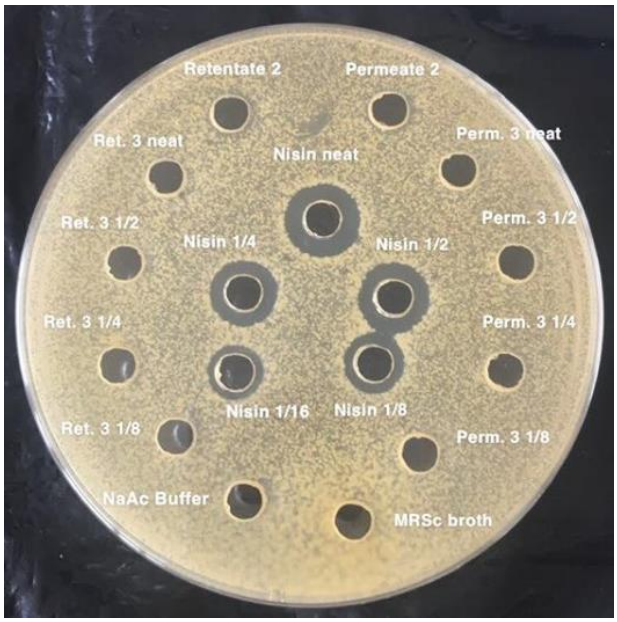

Figure 5.3: The antimicrobial activity of permeates 2 and 3, and retentates 2 and 3 with buffer exchange(s) from TFF run 4. The samples in Figure 5.3 were produced using TFF concentration with a 3 kDa filter. The analysis of antimicrobial activity was carried out using the M/D assay (Section 2.6) with *S. xylosus* as the indicator strain and serial dilutions of nisin from undiluted (neat) to a 1/16 dilution (1000-62.5 IU/ml) as a positive control.

5.2 Large-scale TFF (Crossflow) Concentration Experimental Results

To increase the volume of fermentate utilised for concentration and minimise run time, thus maximising efficiency and increasing potential concentrated yield of antimicrobial molecules, the larger-scale TFF Sartoflow Alpha Benchtop Crossflow System (Sartorius AG, Germany) was utilised. Crossflow filtration experimentation was carried out, initially on a *B. longum* ITT 13 fermentation produced using MRSc broth as the growth medium for the strain and secondly using a modified YPD media which had been previously optimised in our laboratory to maximise growth of the *B. longum* ITT 13 strain (C. Whelan – personal communication).

Table 5.8: Conditions and Parameters for Crossflow Concentration Runs of the *B. longum* ITT 13 Fermentate

Crossflow Run + Filter Size	**Initial Information**			**Buffer Exchange(s)**			**Vol. of Ret. After Conc.**	**BCA Test**
	Vol. (L)	pH	Media	No.	Final pH	Solvent		
TFF 1. Cross flow 1 kDa	2.62	4.21	MRSc	1	4.46	NaAc pH 4.7	330 mls	Yes
TFF 2. Cross flow 1 kDa	2.55	4.60	mYPD	1	4.67	NaAc pH 4.7	350 mls	Yes

The initial fermentate volumes used for the Crossflow concentrations, the starting pH of the fermentates and the media they were grown in were compared in Table 5.8. As the fermentate with cells could be applied directly to the Crossflow system to generate cell free fermentate (Section 2.10.2), there was no requirement for centrifugation steps or the use of a Stericup to remove cells and cellular debris prior to the crossflow concentration. After concentration of the fermentate and buffer exchange, the number of buffer exchanges carried out, the solvent used for buffer exchange and the final pH after buffer exchange were compared. The final volume the fermentate was concentrated to was also noted.

5.2.1 Crossflow Run 1

The initial run using the larger-scale TFF Crossflow system was carried out on the *B. longum* ITT 13 fermentate cultured in MRSc broth. The cell free fermentate was generated through the use of a 0.22 µm filter in the system before a 1 kDa membrane was used for concentration of the fermentate. The antimicrobial activity of the fractions generated from the first crossflow run with a single buffer exchange using 50 mM NaAc buffer at pH 4.7 were determined using both the microdiffusion and MIC_{50} assays and are presented below in in Table 5.9. The graphed results of the MIC_{50} plate are presented in Appendix C.

Table 5.9: Antimicrobial activity for the *B. longum* ITT 13 fermentate (MRSc broth) fractions following Crossflow concentration and buffer exchange with 50 mM, pH 4.7 NaAc buffer (run 1)

Antimicrobial activity was detected using both the M/D assay (Section 2.6) and the MIC_{50} assay (Sections 2.8.4 and 2.10.2.1) with *S. xylosus* as the indicator strain. Three independent assays (n=3) were used to determine the standard deviation (SD) Antimicrobial activity was only detected as far as the 1/4 dilution and therefore the negative results for higher dilutions factors were not recorded. AU/ml was not calculated for the positive control of nisin and it was not relevant for this experiment.

	Sample	Avg. Zone (mm) ± SD			AU/ml M/D assay	AU/ml MIC_{50} Assay
		Undiluted	1/2	1/4		
Run 1. Crossflow 1 kDa MRSc	Fermentate (pH 4.21)	3.8 ± 0.2	2.7 ± 0.2	2.0 ± 0	22 ± 0	N/A
	Permeate 1 (no buffer exchange) (pH 4.22)	3.8 ± 0.2	2.7 ± 0.5	1.3 ± 0.9	18 ± 5	160
	Permeate 2 (buffer exchange) (pH 4.42)	0 ± 0	0 ± 0	0 ± 0	0	40
	Retentate 1 (no buffer exchange) (pH 4.21)	4.0 ± 0	2.7 ± 0.2	2.5 ± 0.4	22 ± 0	160
	Retentate 2 (buffer exchange) (pH 4.46)	0 ± 0	0 ± 0	0 ± 0	0	20
	Nisin (1000 IU/ml undiluted)	3.6 ± 0.3	2.9 ± 0.5	2.6 ± 0.5	N/A	N/A
	MRSc Broth	0 ± 0	N/A	N/A	N/A	N/A
	50 mM pH 4.7 NaAc buffer	0 ± 0	N/A	N/A	N/A	N/A

Antimicrobial activity was detected up to a 1/4 dilution, with 22 AU/ml for the initial fermentate before the crossflow concentration. Permeate 1 also displayed antimicrobial activity up to a 1/4 dilution equivalent to 18 ± 5 AU/ml and similar average zones of inhibition to the fermentate, with average zone diameters of 3.8 mm and 2.7 mm for the undiluted (neat) and 1/2 dilutions, for both the fermentate and permeate 1. The 1/4 dilution of Permeate 1 generated a smaller zone size of 1.3mm + 0.9 as compared to the fermentate, that had an average zone of 2.0 mm ± 0, however the standard deviation was significantly larger for permeate 1 and may account for the smaller average. Retentate 1 also displayed antimicrobial activity up to the 1/4 dilution, with a value of 22 ± 0 AU/ml, generating the same activity values as the fermentate. Although retentate 1 had similar average zones of inhibition to the fermentate and permeate, with 4.0 mm and 2.7 mm for the undiluted and 1/2 dilution samples, the 1/4 dilution displayed a slightly larger zone of inhibition than the original fermentate at 2.5 mm ± 0.4. This may suggest that the smaller zone of the 1/4 dilution of permeate 1 in comparison to the original fermentate and the larger zone of the 1/4 dilution of retentate 1 may be due to a slight concentration effect. The antimicrobial activity generated for the permeate 1 and retentate 1 samples on the M/D assay can be seen in Figure 5.4.

No antimicrobial activity was detected after the buffer exchange for either permeate 2 or retentate 2 using the microdiffusion assay, however the MIC_{50} assay (graphically presented in Appendix C) detected low-level antimicrobial activity of 40 AU/ml for permeate 2, with antimicrobial activity detected to the 1/4 dilution, and 20 AU/ml for retentate 2 i.e. antimicrobial activity was detected up to the 1/2 dilution. The activity for permeate two and retentate two, generated after a buffer exchange, were significantly lower than the values obtained for the permeate 1 and retentate 1 samples prior to buffer exchange which had an average MIC_{50} value of 160 AU/ml on the MIC_{50} assay in microtitre plates.

Upon determination of the antimicrobial activity of the samples generated from the crossflow

concentration and buffer exchange, a BCA assay was performed to assess the protein concentration in each of the fractions generated and is presented in Table 5.10.

Table 5.10: Activity results for Crossflow concentration (Run 1) of *B. longum* ITT 13 Fermentate cultured in MRSc media

The antimicrobial activity results as determined using the M/D assay (Table 5.9) were used to determine the activity relative to the protein concentration within Table 5.10 below

Sample	Total Volume	Protein Conc. (mg/ml)	Protein Content	Activity (Avg. AU/ml)	Total Activity (AU/ml x total vol.)	Specific Activity (AU/mg)	% Volume Recovery		% Protein Recovery	
Fermentate (pH 4.21)	2620 mls	20.59	53,946	22	57,640	1.07	N/A		N/A	
Permeate 1 (pH 4.22)	2290 mls	17.89	40,968	18	41,220	1.01	87%	100%	76%	90%
Retentate 1 (pH 4.21)	330 mls	22.54	7,438	22	7,260	0.98	13%		14%	
Permeate 2 (pH 4.42)	3015 mls	1.81	5,457	0	0	0	91%	96%	78%	79%
Retentate 2 (pH 4.46)	150 mls	0.47	71	0	0	0	5%		1%	

The activity was calculated using the antimicrobial activity results of the M/D assay (Table 5.9) rather than the MIC_{50} assay for comparability with the TFF results in Section 5.1. During the initial concentration of the *B. longum* ITT 13 fermentate, 100% of the volume was recovered between the Permeate 1 and Retentate 1, along with 90% of the protein present in the original fermentate used for the Crossflow concentration. Of the 310 mls of Retentate 1 used for the buffer exchange to produce Permeate 2 and Retentate 2, 96% of the total volume was recovered from the crossflow system and 79% of protein. Only one buffer exchange was carried out using the crossflow system as larger volumes (up to 3L) of buffer could be used in a single run, which also minimised protein losses on the membrane caused by subsequent runs as previously observed with the TFF results presented in Table 5.7.

After separation of the initial fermentate into Permeate 1 and Retentate 1, it was evident that a concentration effect was observed as Retentate 1 had a higher concentration of protein with

22.54 mg/ml in comparison to Permeate 1 which had 17.89 mg/ml. This concentration effect of protein in the retentate was only partially reflected in the activity, as Permeate 1 showed an average antimicrobial activity of 18 AU/ml, while Retentate 1 had an average of 22 AU/ml of antimicrobial activity, which was the same as for the antimicrobial activity of the fermentate at 22 AU/ml. A similarity in the generated specific activity values for both Permeate 1 and Retentate 1 were observed, with 1.01 AU/mg and 0.98 AU/mg respectively, despite the difference in protein concentration levels between the two fractions.

The Retentate 1 was used for the Crossflow buffer exchange, resulting in the production of Permeate 2 and Retentate 2. It was evident that the concentration effect of the protein was not observed as the Retentate 2 had a lower concentration of protein at 0.47 mg/ml as compared to Permeate 2 which had 1.81 mg/ml. There was a significant reduction in antimicrobial activity following the buffer exchange as antimicrobial activity at a low level was detectable in the more sensitive MIC_{50} assay but not with the M/D assay. The M/D assay for Permeate 1 and Retentate 1 is presented in Figure 5.4.

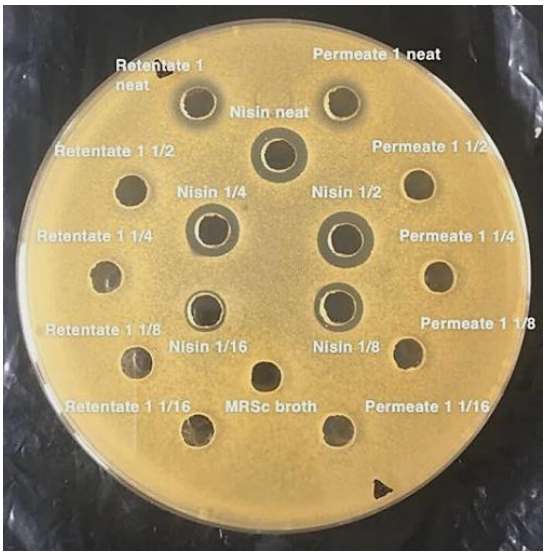

Figure 5.4: The antimicrobial activity of permeate 1 and retentate 1 with no buffer exchange from crossflow concentration experiment 1. Samples analysed in Figure 5.4 were produced using crossflow concentration with a 1 kDa filter. The analysis of antimicrobial activity was carried out using the M/D assay (Section 2.6) with *S. xylosus* as the indicator strain and nisin (undiluted (neat) to 1/16 dilution) as a positive control.

5.2.2 Crossflow Run 2

The second concentration experiment using the Crossflow system was carried out with the *B. longum* ITT 13 fermentate produced in a mYPD media. A 0.22 µm filter within the Crossflow system was used to generate the cell free fermentate before a 1 kDa membrane was used for concentration. The antimicrobial activity of the fractions generated from the second crossflow run with a single buffer exchange using 50 mM NaAc buffer at pH 4.7 were determined using both the microdiffusion and MIC_{50} assays and are presented below in in Table 5.11. The graphed results of the MIC_{50} plate are presented in Appendix C.

Table 5.11: Antimicrobial activity for the *B. longum* ITT 13 fermentate (mYPD broth) fractions following Crossflow concentration and buffer exchange with 50 mM, pH 4.7 NaAc buffer (run 2).

Antimicrobial activity was detected using both the M/D assay (Section 2.6) and the MIC_{50} assay (Sections 2.8.4 and 2.10.2.1) with *S. xylosus* as the indicator strain. Three independent assays (n=3) were used to determine the standard deviation (SD). The results were only noted up to a 1/4 dilution as this is the highest dilution activity was present at. AU/ml was not calculated for the positive control of nisin and it was not relevant for this experiment.

	Sample	**Avg. Zone (mm) ± SD**			**AU/ml M/D Assay**	**AU/ml MIC_{50} Assay**
		Undiluted	**1/2**	**1/4**		
2. Crossflow 1 kDa mYPD	Fermentate (pH 4.57)	4.8 ± 0.2	2.3 ± 0.2	0 ± 0	11 ± 0	N/A
	Cell Free Fermentate (pH 4.64)	4.8 ± 0.2	1.5 ± 1.1	0 ± 0	9 ± 2	160
	Permeate 1 (no buffer exchange) (pH 4.60)	4.7 ± 0.2	0.7 ± 0.9	0 ± 0	8 ± 2	160
	Permeate 2 (buffer exchange) (pH 4.67)	0 ± 0	0 ± 0	0 ± 0	0	40
	Retentate 1 (no buffer exchange) (pH 4.62)	4.8 ± 0.2	0.7 ± 0.9	0 ± 0	8 ± 2	160
	Retentate 2 (buffer exchange) (pH 4.67)	0 ± 0	0 ± 0	0 ± 0	0	20
	Nisin (1000 IU/ml undiluted)	4.1 ± 0.1	3.3 ± 0	2.9 ± 0.1	N/A	N/A
	MRSc broth	0 ± 0	N/A	N/A	N/A	N/A
	mYPD Broth	3.7 ± 0.5*	0 ± 0	0 ± 0	6 ± 0	40

* indicates the zones of inhibition that were less clear/defined than other zones.

Table 5.11 displays the antimicrobial activity of all the fractions produced during the second crossflow run of the *B. longum* ITT 13 fermentate produced in modified YPD (mYPD) broth, which was detected using both the microdiffusion assay (Section 2.6) and MIC_{50} assay (Section 2.8.4). Both the M/D and MIC_{50} methods of antimicrobial evaluation were employed for analytical purposes, with the latter presented visually in Appendix C. The results of the MIC_{50} assay are presented visually in Appendix C. The mYPD broth used as the growth medium for the *B. longum* ITT 13 was also tested for antimicrobial activity as a negative control. The mYPD broth itself displayed antimicrobial activity on the microdiffusion assay against *S. xylosus*. Zones of inhibition smaller than those of the fermentate, permeate or retentate samples were determined, at an average of 3.7 ± 0.5 mm and equivalent to 6 AU/ml attributed to the media. No antimicrobial activity was detected on this assay beyond the undiluted form of the mYPD broth and the zones of inhibition had less clarity than those for the undiluted fermentate, permeate and retentate samples.

Antimicrobial activity was detected up to a 1/2 dilution for both the fermentate and cell free fermentate in the M/D assay, with 11 ± 0 AU/ml and 9 ± 2 AU/ml respectively. Although the average zones of inhibition for both the undiluted fermentate and cell free fermentate samples were equivalent at 4.8 mm ± 0.2, the diameter of the 1/2 dilutions were variable, with 2.3 mm ± 0.2 for the fermentate and 1.5 mm ± 1.1 for the cell free fermentate and the standard deviation is notably larger for the cell free fermentate and thus the results are not significantly different. The cell free fermentate could be screened using the MIC_{50} assay however the fermentate with cells could not, as only cell-free samples can be screened using this method. Antimicrobial activity equivalent to 160 AU/ml was thus determined for the cell-free fermentate using the MIC_{50} assay.

Permeate 1 and Retentate 1 after crossflow concentration displayed similar antimicrobial activity, with activity detected to a 1/2 dilution and equivalent to 8 ± 2 AU/ml for both in the

M/D assay. The zones of inhibition on the M/D assay were also equivalent with 4.7 mm ± 0.2 and 0.7 mm ± 0.9 for the undiluted and 1/2 dilutions of Permeate 1, and 4.8 mm ± 0.2 and 0.7 mm ± 0.9 for the undiluted and 1/2 dilutions of Retentate 1. Using the MIC_{50} assay, both Permeate 1 and Retentate 1 were also found to have equivalent antimicrobial activity of 160 AU/ml. These similarities in antimicrobial activity, using both assays, indicate that there was likely no concentration effect on the antimicrobial activity observed for Retentate 1 using the 1kDa filter in the Crossflow.

For Permeate 2 and Retentate 2, which had both undergone a buffer exchange with 50 mM NaAc buffer, pH 4.7, there was no detectable antimicrobial activity using the microdiffusion assay, however similar to the previous Crossflow run (Run 1), 40 AU/ml was detected for Permeate 2 and 20 AU/ml was detected for Retentate 2 when using the MIC_{50} assay. Thus, there was noticeably reduced antimicrobial activity present following buffer exchange with values of 160 AU/ml determined for the cell free fermentate, Permeate 1 and Retentate 1.

Upon determination of the antimicrobial activity of the samples generated from the crossflow concentration and buffer exchange, a BCA assay was performed to assess the protein concentration in each of the fractions generated and is presented in Table 5.12.

Table 5.12: Activity results for Crossflow concentration (Run 2) of *B. longum* ITT 13 Fermentate cultured in mYPD media

Sample	Total Volume	Protein Conc. (mg/ml)	Protein Content	Activity (AU/ml)	Total Activity (AU/ml x total vol.)	Specific Activity (AU/mg)	% Volume Recovery		% Protein Recovery	
Fermentate (pH 4.57)	2960 mls	39.49	116,890	11	32,560	0.28	N/A		N/A	
Cell Free fermentate (pH 4.64)	2550 mls	36.44	92,922	9	22,950	0.25	N/A		N/A	
Permeate 1 (pH 4.60)	2200 mls	32.19	70,818	8	17,600	0.25	86%	100%	76%	89%
Retentate 1 (pH 4.62)	350 mls	33.84	11,844	8	2,800	0.24	14%		13%	
Permeate 2 (pH 4.67)	3010 mls	3.03	9,120	0	0	0	91%	100%	87%	93%
Retentate 2 (pH 4.67)	290 mls	2.08	603	0	0	0	9%		6%	

The activity was calculated using the antimicrobial activity results of the M/D assay (Table 5.11) rather than the MIC_{50} assay for comparability with the TFF results in Section 5.1. Of the 2.55 L of the cell free fermentate used for the crossflow concentration, 100% of the volume was recovered between both Permeate 1 and Retentate 1. Of the protein present within the fermentate 89% of the total protein content was recovered between Permeate 1 and Retentate 1 – equating to an 11% protein loss. Of the 310 mls of Retentate 1 used for the buffer exchange with 3 L of 50 mM, pH 4.7 NaAc buffer, close to 100% of the total volume of the Retentate 1 and buffer solution was recovered between Permeate 2 and Retentate 2, along with 93% of the protein that had been present in the solution – equating to a 7% protein loss from the protein content present in the initial Retentate 1 and buffer solution. A single buffer exchange was carried out on the crossflow system, as a much larger volume of buffer could be used in one run, which, while allowing for considerable buffer exchange, minimise multiple pass requirements through the membrane and minimised protein fouling on the membrane which can occur in these systems (Kujundzic *et al.*, 2011).

After separation of the cell free fermentate into Permeate 1 and Retentate 1, it was evident that there was only a minor concentration effect observed with 33.84 mg/ml protein in the Retentate 1, as opposed to 32.19 mg/ml in Permeate 1, which constitutes only a minimal concentration difference of 1.65 mg/ml. As observed for Crossflow concentration experiment (Run 1), there was no concentration effect but rather a significant protein loss after the buffer exchange on Retentate 1 which was used to generate Permeate 2 and Retentate 2. Permeate 2 contained 3.03 mg/ml protein, in comparison to 2.08 mg/ml determined for Retentate 2. The results obtained for the buffer exchange in Crossflow (Run 2) thus indicate that there was no successful protein concentration effected in this method following buffer exchange.

In comparison to the results for the Crossflow concentration experiment (Run 1) (Table 5.10), the calculated specific activity results for Run 2 were lower than expected which may be a result of the higher protein concentrations present in the samples, as compared to the detectable antimicrobial activity. The calculated specific activity was similar across all samples tested – between 0.28 AU/mg and 0.24 AU/mg for the Fermentate (with cells), the cell free Fermentate, Permeate 1 and Retentate 1. Permeate 2 and Retentate 2 displayed no antimicrobial activity and therefore had a specific activity of 0 AU/mg. The overall results obtained for the crossflow experiment (Run 2) suggest that this protocol is not suitable for concentration of the antimicrobial activity detected in the original fermentate because there was no increased activity associated with the retentate fraction as would be expected. The similar antimicrobial activity observed for both the retentate and permeate fractions may be associated with organic acid production by *B. longum* ITT 13 (Section 4.5), which is masking the low-level antimicrobial activity associated with the potential proteinaceous molecule as determined in Sections 4.3 and 4.4 that would likely be concentrated within the retentate. This protocol is thus not suitable as the buffer exchange used to remove the media and these organic acids contributing to activity results in a complete elimination of detectable antimicrobial activity,

possibly as a result of adsorption of the low-molecular weight peptide to the TFF filter at the protein concentrations used in the experiment, which can be known to occur in these systems (Kujundzic *et al.*, 2011).

5.3 Discussion

Partial purification and concentration studies were carried out on the *B. longum* ITT 13 Fermentate through the use of tangential flow filtration (TFF) methods, including bench top TFF and Crossflow filtration systems. These TFF methods have previously been used in various studies for the concentration of metabolites produced by various organisms, including the purification of proteins which is widely reported in literature (Musumeci *et al.*, 2018). Pires and Palmer, (2021) used TFF to purify complexed human serum albumin using an anti-HSA polyclonal immunoglobulin G (IgG) from a haemoglobin mixture, while Aspelund and Glatz, (2010) employed the ultrafiltration technique of TFF to evaluate its effectiveness for the purification of recombinant proteins from aqueous corn endosperm and germ extracts, proving effective for the separation and purification of proteins above 100 kDa with 89-99% purity. TFF has been utilised as a purification method within the food industry to reduce microbial load of raw wines (Umiker *et al.*, 2013) and for beer clarification and pasteurization (Fillaudeau and Carrère, 2002). TFF has also been used within the pharmaceutical industry for the manufacturing and purification of nanoparticles used in nanomedicine by removing impurities and carrying out buffer exchange (Musumeci *et al.*, 2018; Roces *et al.*, 2020; Webb *et al.*, 2020). In terms of bacteriocin production, TFF has been widely utilised as a step within purification and/or concentration protocols to purify and isolate bacteriocins from the supernatants of producing strains, enabling further characterization studies of these peptides (Izquierdo *et al.*, 2009; Amado *et al.*, 2016; Kumar and Tiwari, 2017; Lv, Ma, *et al.*, 2018).

The *B. longum* ITT 13 fermentate was initially concentrated through the use of a Pellicon® XL 50 Cassette and Labscale^{TM} TFF System (Millipore) benchtop laboratory system for small-scale concentration using volumes of 125-950 mls of fermentate. A variety of filter sizes of 3 kDa to 10 kDa were used for these TFF concentration experiments and the details of the four TFF runs can be found in Table 5.1, including filter size, volumes of fermentate used and

whether a buffer exchange was carried out on the fermentate after concentration. Small-scale TFF systems have been previously used for the purification of antimicrobial peptides, such as in the study by Nanjundan *et al.*, (2019) whereby a miniature TFF module from PALL Lifesciences was employed for the purification of the surfactin lipoprotein produced by *Bacillus amyloliquefaciens* SR1 that had displayed antimicrobial activity against fungal plant pathogens.

The first TFF experimental run involved the concentration of 450 mls of the *B. longum* ITT 13 fermentate to a final volume of 50 mls using a 10 kDa filter and no buffer exchange carried out in order to solely assess the concentration effect on the fermentate (Table 5.1 & Table 5.2). Both the Permeate and Retentate were screened for antimicrobial activity using the microdiffusion assay (Section 2.6), with antimicrobial activity detected up to a 1/2 dilution equivalent to 11 AU/ml for both the Retentate and Permeate (Table 5.2). A BCA assay was carried out to assess the protein distribution between the Retentate and Permeate in order to assess the concentration effect on the total protein content in addition to assessing the antimicrobial activity (Table 5.3). The Permeate was determined as having 12.30 mg/ml of protein, whereas the Retentate had nearly double this concentration with 23.63 mg/ml protein. Therefore, although a concentration effect for the protein in the Fermentate was evident for the Retentate fraction, this did not correlate with higher levels of antimicrobial activity for the Retentate fraction as compared to the Permeate fraction. This indicates that a pH or acid effect was likely the overriding cause of antimicrobial activity here as there was no increase in activity associated with concentrated protein.

The second lab scale TFF run involved the use of a smaller filter size of 3 kDa as opposed to the 10 kDa used in run 1, to concentrate 125 mls of the *B. longum* ITT 13 fermentate to 25 mls – equivalent to a five-fold concentration (Table 5.1). A total of 3 buffer exchanges were also carried out on the Retentate after the initial concentration to 25 mls using pH 4.9 NaAc buffer

(Table 5.1 & Table 5.4). The buffer exchanges were carried out to remove the organic acids present in the Fermentate that may mask the antimicrobial activity associated with the concentration of proteinaceous compounds (Makras and De Vuyst, 2006; Tejero-Sariñena *et al.*, 2012; Georgieva *et al.*, 2015). It should be noted that the retentate was not tested for the presence of organic acids to determine whether residual or lower concentrations of acid remained, though due to large volumes of buffer used for the exchange it was assumed they would have been diluted below the point of causing antimicrobial activity. Both the *B. longum* ITT 13 fermentate with cells and the cell free fermentate had 22 AU/ml of antimicrobial activity before concentration and after the initial concentration prior to the buffer exchange Permeate 1 displayed activity equivalent to 18 AU/ml ± 5. The initial Retentate before the buffer exchange (Retentate 1) was not diluted on the M/D plates for an AU/ml determination and only screened for antimicrobial activity in the undiluted form. It displayed antimicrobial activity with zones of inhibition of 3.3 ± 0.5 mm which was similar to the zones produced for the undiluted Permeate 1 of 3.7 ± 0.6 mm, indicating no obvious concentration of antimicrobial activity for the Retentate fraction. A total of 3 buffer exchanges were carried out generating Permeates 2, 3 and 4 and Retentates 2, 3 and 4, however no antimicrobial activity was detected for any of these Permeate samples indicating that there was no detectable activity present following the buffer exchanges. No antimicrobial activity was detected for Retentate 3 and 4 however, Retentate 2 displayed antimicrobial activity when tested in the undiluted form with an average zone of inhibition of 1.2 ± 0.2 mm. The lack of detectable antimicrobial for Permeate 2 after the first buffer exchange, in comparison to the antimicrobial detected for Retentate 2 indicates that either a source other than organic acid may be responsible for the antimicrobial activity and that a concentration effect was observed within the Retentate.

Izquierdo *et al.*, (2009) used TFF as the second step within the purification process of an antimicrobial peptide produced by *E. faecalis* WHE 96, whereby the use of a 10 kDa filter

resulted in the peptide detected within the Permeate and the use of a 5 kDa filter resulted in retention of the peptide. This indicated that the size of the peptide was between 5 kDa and 10 kDa. The use of a 3kDa filter during TFF filtration run 2 (Section 5.1.2) as opposed to the use of the 10 kDa filter run 1 (Section 5.1.1) of the *B. longum* ITT 13 Fermentate, resulted in higher antimicrobial activity detected in the Retentate as opposed to the Permeate, with the result suggestive that the proteinaceous component of the antimicrobial activity may be between 3kDa and 10 kDa. Another study involving the concentration of an antimicrobial peptide DY4-2 produced by *L. plantarum* DY4-2, using TFF with 10 kDa, 5 kDa and 1 kDa determined the presence of the peptide in the Permeate after the use of the 10 kDa and 5 kDa filters, however the 1 kDa filter retained the peptide indicating a size of between 1-5 kDa (Lv, Ma, *et al.*, 2018). Many antimicrobial peptides are small, with both Class I and Class II bacteriocins less than 10 kDa (Deegan *et al.*, 2006; Singh *et al.*, 2015). Thus, there is the likelihood that the potential antimicrobial peptide produced by *B. longum* ITT 13 is of a low molecular weight – as previously discussed in Chapter 4 (Section 4.6). The lack of detection of antimicrobial activity in the Retentate 3 and 4 fractions could potentially be reflective of significant losses associated with adsorption to the TFF filter at the protein concentrations used in the experiment that can occur in these systems (Kujundzic *et al.*, 2011) due to the repeated circulation throughout the instrument for each buffer exchange. Although this is likely a potential reason for the lack of activity in Retentates 3 and 4 this could not be definitively proven as transmembrane pressure was not monitored during the run.

A third TFF run was carried out with the same volumes (125 mls concentrated to 25 mls) and conditions as for the previous TFF run 2, however instead of a buffer exchange with a 50 mM NaAc pH 4.9 buffer, buffer exchanges were instead carried out using 0.02 N HCl (pH 1.8) (Table 5.1), which is an acidic solvent used for nisin preparation (Section 2.5). The reason this solvent was chosen was on the basis that some antimicrobial peptides, e.g. nisin, display

maximum activity in the lower pH range below pH 5 (Rollema *et al.*, 1995; Matsusaki *et al.*, 1998) and as the fermentate had retained maximum detectable antimicrobial activity in the microdiffusion assay below pH 5 in Section 4.2, an acidic solvent with no antimicrobial effect (Table 5.5) was chosen for the buffer exchange. The *B. longum* ITT 13 fermentate with cells and the cell free fermentate before TFF concentration were diluted to a 1/16 dilution and displayed antimicrobial activity up to the 1/4 dilutions equivalent to antimicrobial activity values of 18 ± 5 and 15 ± 5 AU/ml respectively on the M/D assay plates (Table 5.5).

Permeate 1 was also diluted to a 1/16 dilution but only displayed antimicrobial activity in the undiluted form with 6 ± 0 AU/ml, indicating a loss of activity in the Permeate in comparison to the original fermentate (Table 5.5). Retentate 1 (no buffer exchange) was only screened for activity in the undiluted form to determine qualitatively whether antimicrobial activity was absent or present. Retentate 2, Permeate 2 and Permeate 3 were also only screened for antimicrobial activity in their undiluted form to determine whether any antimicrobial activity was retained after buffer exchange. The final retentate after the two buffer exchanges (Retentate 3) was diluted to a 1/16 dilution and antimicrobial activity determined quantitatively with an AU/ml calculation, however no activity was detected (Table 5.5). The zone of inhibition for the undiluted Retentate 1 was 3.8 ± 0.2 mm, which was comparable to the zone of the undiluted Permeate 1 at 3.7 ± 0.2 mm thus indicating similar activity for the undiluted forms of both. Permeate 2 and Retentate 2, which had undergone a single buffer exchange with 100 mls of 0.02 N HCl, also displayed comparable antimicrobial activity on the microdiffusion plates, with a zone of inhibition of 1.8 ± 0.3 mm detected for Permeate 2 and 1.8 ± 0.2 mm detected for Retentate 2 (Table 5.5). The above results are in contrast to the antimicrobial activity detected for the previous TFF Run 2 with a pH 4.9 NaAc buffer exchange, whereby after the single buffer exchange, Retentate 2 retained some antimicrobial activity, however no activity was detected for Permeate 2 (Table 5.4). The results generated in TFF run 3, which

give similar results for the permeate and retentate despite the differences in volume, do not indicate any appreciable concentration of the antimicrobial activity. Following the second buffer exchange step with 0.02N HCl, no antimicrobial activity was detectable on an M/D assay for either the Retentate or the Permeate. The buffer exchange using the acidic 0.02N HCl in the TFF step therefore did not generate antimicrobial activity results that would be suggestive of a successful concentration step. This result was not anticipated as the antimicrobial activity produced by *B. longum* ITT 13 had previously displayed antimicrobial activity at a more acidic pH range (Section 4.2) and antimicrobial peptides produced by LAB have often displayed stability at a lower pH, including nisin which retains maximum antimicrobial activity in the lower pH range below pH 5 (Rollema *et al.*, 1995; Matsusaki *et al.*, 1998). A bacteriocin designated bovicin HC5, produced by *Streptococcus bovis* HC5, demonstrated a greater ability to cause pore formation in the cellular membrane of the target cells of *S. bovis* JB1 at a more acidic pH of pH 5.5 as compared to the peptide at pH 7, causing a greater measurable loss of potassium from the cells (Houlihan *et al.*, 2004). Pei *et al.* (2020) also examined the pH stability of bacteriocin produced by *L. plantarum* SLG10 at a pH range of pH 2-10, determining that at the more acidic pH range of pH 3-7 there was increased antimicrobial activity of approximately 200 AU/ml, however the antimicrobial activity began to decline at pH 8 and was close to 0 AU/ml for pH 9 and 10.

As the pH 1.8 HCl did not have any discernible beneficial effect for the buffer exchange, the buffer for the fourth TFF concentration run was reverted back to 50 mM NaAc buffer at pH 4.8. The pH of the buffer was reduced from pH 4.9 in TFF Run 2 to pH 4.8 to try preserve antimicrobial activity after the buffer exchange which may only be active at a lower pH, as the pH of the original fermentate was pH 4.2-4.3. The volume size of the fermentate to be concentrated was also increased to 950 mls concentrated to 50 mls to increase the concentration of any potential antimicrobial compounds from 5-fold in TFF Run 2 to a 20-fold concentration

in TFF Run 4 (Table 5.1). The *B. longum* ITT 13 fermentate with cells displayed antimicrobial activity at an average of 18 ± 5 AU/ml, while the cell free fermentate displayed an average of 22 ± 0 AU/ml (Table 5.6). Upon the initial TFF separation of the cell free fermentate, the activity was reduced to an average of 15 ± 5 AU/ml for both the Permeate (Permeate 1) and the Retentate (Retentate 1) (Table 5.6). This pattern of the antimicrobial activity decreasing slightly in the Permeate and Retentate after the initial separation, but before buffer exchange, was also observed in the previous TFF runs and may be indicative of binding of some antimicrobial constituents in the Fermentate to the 3 kDa filter.

A total of two buffer exchanges were carried out, with reduced antimicrobial activity detected for both the Permeate 2 and the Retentate 2 generated after the first buffer exchange in comparison to Permeate 1 and Retentate 1, with the zone of inhibition for the undiluted fractions decreasing from an average of 4.3 ± 0.2 mm for Permeate 1 to 1.2 ± 0.2 mm for Permeate 2 and from 4.5 ± 0.0 for Retentate 1 to 1.3 ± 0.3 for Retentate 2 (Table 5.6). The pattern observed previously in Runs 2 and 3 whereby only the retentate retained antimicrobial activity after the buffer exchange was not observed on this run. It is possible that the buffer exchange reduced the antimicrobial activity due to a combination of the removal of the organic acids produced by *B. Longum* ITT 13 e.g. acetic acid, lactic acid and formic acid, together with a probable loss of antimicrobial activity through absorption on the filter (Makras and De Vuyst, 2006; Kujundzic *et al.*, 2011; Tejero-Sariñena *et al.*, 2012; Delgado *et al.*, 2019).

No antimicrobial activity was detected for any dilution of Permeate 3 and Retentate 3 after the second buffer exchange in TFF run 4 (Table 5.6). The unfortunate observed complete loss of antimicrobial activity following more than a single buffer exchange for TFF runs 2-4 could be due to the overall TFF concentration process. TFF involves repeated circulation of the retentate through the equipment, which should enable retention of any fermentate components larger than 3kDa within the instrument e.g. antimicrobial proteinaceous molecules, but the multiple

passes through the filter would increase the risk for adsorption of proteinaceous molecules to the filter (Kujundzic *et al.*, 2011), especially as the filter was not depolarised during runs.

Grund *et al.* (1992) discussed how issues may arise at the filter interface when concentrating soluble proteins on crossflow/TFF membranes. High flow rates during concentration of proteins can be impeded through the concentration of soluble proteins, due to rejection of these proteins at the surface of the membrane causing the formation of a concentration-polarization layer. The formation of this layer then often increases the build-up of soluble protein at the filter, which in turn mediates protein adsorption onto the surface of the membrane. The latter adsorption, combined with proteins that enter the pores causing blockage, can also cause resistance to the flow rates of the permeate. Thus, liquid permeation of the membrane/filter is affected by the protein adsorption onto the membrane through protein membrane interactions and adsorption of protein on the membrane surface due to soluble protein mediated protein interactions (Grund *et al*, 1992).

Another possibility is that the production of antimicrobial molecules, such as bacteriocins, may be at a very low concentration and therefore much larger volumes of multiple litres may be required to generate significant detectable antimicrobial activity when concentrating and carrying out buffer exchanges. Antimicrobial peptides are generally produced at a low concentration unless a complex, specific media is used for growth, therefore purification and concentration methods are commonly employed to increase yield, as discussed in the review by Garsa *et al.*, (2014). Garsa *et al.*, (2014) details the many small and large-scale purification processes often employed in the food and pharmaceutical industries to increase bacteriocin yield, including various chromatographic methods (gel-filtration, hydrophobic interaction chromatography, cation exchange), ultrafiltration and precipitation methods (ammonium sulphate, ethanol etc.). Previous bacteriocin purification work carried out in our laboratory has also indicated low bacteriocin purification levels. The latter consideration was the reason for

the decision to carry out concentration using the larger scale Crossflow concentration process. A BCA assay was carried out on all the samples from the fourth TFF run (Table 5.7). This determined that the cell free fermentate used for the TFF concentration contained approximately 16.97 mg/ml of protein, which was equivalent to a total of 16,122 mg protein in the 950 ml volume of fermentate. The initial separation into Permeate 1 and Retentate 1 would be expected to show an increase in concentration of protein for the Retentate and a decrease for the Permeate in comparison to the Fermentate used for the separation and that was what was observed with a decreased average of 14.52 mg/ml protein determined for Permeate 1 and an increase to 24.43 mg/ml observed for the Retentate 1. This larger concentration of protein within the Retentate 1 was not reflective of an increase in antimicrobial activity however, as observed in Table 5.6, with the same average activity determined for both Retentate 1 and Permeate 1 at 15 ± 5 AU/ml. However, the antimicrobial activity associated with the organic acid production by *B. longum* ITT 13 may have masked any small differences in antimicrobial activity associated with the concentration of potential antimicrobial peptides. The overall recovery of protein was 89% in comparison to the cell free Fermentate, indicating binding is likely taking place within the TFF instrument incurring losses which may be associated with the observed protein loss.

With the first buffer exchange, this likely binding of the protein to the filter or within the tubing of the instrument, became even more evident as only 25% of the total protein from Retentate 1 that was used for the buffer exchange was recovered. The distribution of protein between Permeate 2 and Retentate 2 indicated that a concentration effect was still taking place, however the overall concentration in the recovered samples protein were lower than for the original Retentate 1 sample applied for the buffer exchange step, with only 0.52 mg/ml present in Permeate 2 and 3.79 mg/ml present within Retentate 2.

The second buffer exchange resulted in a complete loss of any detectable protein, meaning the

recirculation of the samples throughout the instrument during the buffer exchanges resulted in complete protein loss and the latter protein loss possibly accounts for the complete loss of antimicrobial activity detected for each TFF run when any more than a single buffer exchange was carried out. The Pierce™ BCA Protein Assay Kit (Thermofisher) procedure used detects protein concentration as low as 20 µg/mL indicating high sensitivity of the assay and sufficient sensitivity for the analysis of the samples generated through TFF as they are measured in mg/ml (*Pierce^{TM} BCA Protein Assay Kit*).

The use of TFF to purify an antimicrobial peptide was utilised by Izquierdo *et al.*, (2009), who generated an activity table through AU/ml calculated using the spot-on-lawn assay and A_{280} readings for protein concentration. The author found that after purification using TFF concentration with a 5 kDa filter, the AU/ml increased from 100 AU/ml for the culture extract to 1600 AU/ml upon purification using TFF, with a total of 70% of activity present in the 220 mls of the retentate generated from the 5000 mls of the culture extract that contained 100% of activity. In the reported case, the TFF concentration of the peptide was efficient in partial purification and concentration of the antimicrobial activity. This was not the case for the concentration of the *B. longum* ITT 13 fermentate as in the TFF experiments Runs 1-4 carried out, it was observed that there was no significant concentration of the *B. longum* ITT 13 antimicrobial activity, as the AU/ml was not increased for the Retentate in comparison to the original cell-free Fermentate (Tables 5.4-5.6).

Due to the limitations of the labscale TFF runs including repeated recirculation causing protein losses, the volume limitations of the equipment allowing only 450-500 mls of fermentate at a time and the length of the run times to efficiently concentrate the fermentate, a larger scale Crossflow filtration system was utilised allowing for the concentration of larger Fermentate volumes of up to approximately 3L in a shorter timeframe. A total of two crossflow runs were carried out, with the initial run involved the growth of the *B. longum* ITT 13 Fermentate in an

MRSc media and the second run involved the production of Fermentate using a modified YPD (mYPD) media (C. Whelan – personal communication). The fractions produced from each crossflow run i.e. the Permeates and Retentates, were tested for antimicrobial activity using both the microdiffusion assay (Section 2.6) and the more sensitive microtitre plate MIC_{50} assay (Section 2.8.4). Nakamura *et al.*, (2013) produced a concentrated fraction of Gassericin A, a circular bacteriocin produced by *Lactobacillus gasseri* LA39, from the culture supernatant using crossflow filtration with a 30 kDa membrane, by concentrating 9L of supernatant to a 600 ml retentate resulting in a 15-fold concentration effect. This method of concentration resulted in a concentrated fraction with antimicrobial activity 16-times higher (3940 AU/ml) than that of the culture supernatant (246 AU/ml) and was used for further characterization studies including temperature and storage stability testing.

The first crossflow concentration run involved cultivation of the *B. longum* ITT 13 in MRSc broth and after the initial concentration step, a single buffer exchange was carried out on the retentate using pH 4.7 NaAc buffer. The microdiffusion assay yielded antimicrobial activity equivalent to 22 ± 0 AU/ml for the original *B. longum* ITT 13 Fermentate produced in MRSc broth, 18 ± 5 AU/ml for the Permeate 1 and 22 ± 0 AU/ml for the Retentate 1 with both samples produced prior to buffer exchange (Table 5.9). The antimicrobial activity measurements for the Permeate 1 and Retentate 1 are only marginally different as the standard deviation results do not indicate any significance in the difference. Buffer exchange of Retentate 1 (310 ml) with 3 L of 50 mM pH 4.7 NaAc buffer was used to generate Permeate 2 and Retentate 2. The buffer exchange resulted in no detectable antimicrobial activity when samples were tested in the microdiffusion assay with 0 AU/ml calculated for both Permeate 2 and Retentate 2.

Samples were additionally tested on the more sensitive MIC_{50} assay and these results, together with the M/D assay results, are presented Table 5.9. The MIC_{50} was determined as the dilution inhibiting at least 50% of the growth of the *S. xylosus* indicator strain. Both Permeate 1 and

Retentate 1 generated antimicrobial activity with an MIC_{50} for the 1/16 dilution, equivalent to 160 AU/ml (Table 5.9), which indicates that both samples were similar in activity. The latter equivalent results for both Permeate 1 and Retentate 1 in the MIC_{50} assay thus indicates that the lower detected activity of 18 ± 5 AU/ml for Permeate 1 in the microdiffusion plates in comparison to 22 ± 0 AU/ml for the Retentate 1 was likely due to variation in the M/D assay replicate results for Permeate 1. Small amounts of antimicrobial activity were detected for both the Permeate 2 with an MIC_{50} of a 1/4 dilution, equivalent to 40 AU/ml (Table 5.9) and Retentate 2, with an MIC_{50} of a 1/2 dilution, equivalent to 20 AU/ml (Table 5.9). The results generated were disappointing in that the expected results would be where the retentate should likely contain everything retained by the filter and thus display more antimicrobial activity due to the presence of concentrated antimicrobial molecules such as bacteriocins. Nanjundan *et al.*, (2019) used ultrafiltration to successfully purify surfactin lipopeptides produced by *Bacillus amyloliquefaciens* with antifungal activity against a variety of plant pathogens, using a miniature TFF instrument with 1 kDa and 3 kDa membranes to purify the peptides. Golneshin *et al.*, (2020) also carried out purification and concentration steps of a bacteriocin, plantacyclin B21AG produced by *Lactiplantibacillus plantarum* B21, using ultrafiltration techniques with a 10 kDa membrane, which resulted in 16-fold concentration of the bacteriocin increasing the antimicrobial activity from 800 AU/ml to 12,800 AU/ml. Amado *et al.*, (2016), also employed TFF as a partial purification method of a bacteriocin produced by *Pediococcus acidilactici* NRRL B-5627, however the author retained the bacteriocin of 2.7 kDa in the permeate through use of a 10 kDa filter, to exclude and reduce higher molecular weight molecules present in the culture media. The latter idea of reducing the high molecular weight compounds in the fermentate could be used in partial purification of the *B. longum* ITT 13 fermentate though it is noted that this would not concentrate activity. Further membranes with a smaller molecular weight cut off could be used for concentration of the permeate containing the antimicrobial

peptide produced by ITT 13 after the use of the 10 kDa membrane to remove larger molecular weight molecules, which in turn would likely reduce protein adsorption to the 3 kDa membrane and minimise fouling.

A BCA assay was carried out to determine the protein distribution to assess whether a concentration effect had taken place. The results for the protein concentrations obtained from the BCA assay as presented in Table 5.10 indicate that following the initial concentration step but prior to the buffer exchange, the Retentate 1 contained a slightly higher concentration of protein at 22.54 mg/ml in comparison to Permeate 1 which had 17.89 mg/ml. The results are suggestive that a low-level concentration had taken place with slightly higher concentration of protein present in the Retentate. When the buffer exchange was carried out however, the concentration of protein was greatly reduced for both the Permeate 2 and the Retentate 2, whereby Retentate 2 had a lower protein concentration at 0.47 mg/ml in comparison to the Permeate 2 value of 1.81 mg/ml. The higher concentrations of protein in the permeate was not expected as the original sample applied for buffer exchange had already been run through the 1 kDa filter. The lower protein concentrations determined in the BCA assay for Retentate 2 in comparison to Permeate 2 could be taken to account for the lower-than-expected antimicrobial activity detected for Retentate 2 in comparison to Permeate 2 in the MIC_{50} assay (Appendix C). The significant protein losses experienced previously using the TFF apparatus were not experienced with the Crossflow system, as upon the initial concentration of the fermentate producing Permeate 1 and Retentate 1, 90% of the protein was recovered and when carrying out the buffer exchange step using 310 mls of the Retentate 1, 79% of the protein was recovered (Table 5.10). Therefore, although losses of 10-21% protein were observed for the crossflow system, they were not comparable to losses of up to 100% experienced using the smaller TFF system with significantly more cycles through the filter to enable concentration. Considering the high protein recovery yet lack of concentrated antimicrobial activity, it may be plausible in

future work to utilise much larger volumes of the *B. longum* ITT 13 fermentate, as work by Nakamura *et al.*, (2013) demonstrated that a 15-fold concentration of 9 litres of a cell-free supernatant to a 600 ml retentate, resulted in a 16-fold increase of antimicrobial activity by the bacteriocin Gassericin A, produced by *Lactobacillus gasseri* LA39, against *Lb. delbrueckii* subsp. *bulgaricus* JCM 1002T.

The second crossflow concentration took place with a *B. longum* ITT 13 Fermentate grown in a modified YPD (mYPD) media that had been modified to yield greater levels of antimicrobial activity (C. Whelan – personal communication). As with crossflow Run 1, buffer exchange was carried out using 50 mM pH 4.7 NaAc buffer, with the aim of removing the contribution of any acids produced by *B. longum* ITT 13 to the detected antimicrobial activity. The Permeates and Retentates generated were tested for antimicrobial activity using both the microdiffusion assay (Section 2.6) and the microtitre plate MIC_{50} assay (Section 2.84). The *B. longum* ITT 13 Fermentate with cells grown in the mYPD media displayed antimicrobial activity in the microdiffusion assay plates equivalent to 11 ± 0 AU/ml, whereas the cell-free fermentate gave a slightly lower antimicrobial activity value of 9 ± 2 AU/ml, and both the Permeate 1 and Retentate 1, before the buffer exchange, displayed the same activity equivalent to 8 ± 2 AU/ml (Table 5.11). As with the results obtained for the crossflow run 1 fractions, no antimicrobial activity was detected for the Permeate 2 and Retentate 2 fractions that had undergone a buffer exchange with 3L of 50 mM, pH 4.7 NaAc buffer (Table 5.11).

In contrast to the media used in the previous crossflow run 1, it was noted that the mYPD media by itself generated small zones of partial inhibition in the M/D assay with zone sizes of 3.7± 0.5 mm, equivalent to 6 ± 0 AU/ml as compared to the larger clear zones of 4.7-4.8 mm generated by the undiluted Fermentate, Permeate 1 and Retentate 1 (Table 5.11). The source of inhibition observed for sterile mYPD media alone was not ascertained. However, a possible suggestion for the inhibition observed in the M/D assay for the mYPD media may be a result

of the high solute and sugar concentration within the media which had a glucose concentration of 40 g/L. Sugars have been used as preservatives in industry, with high sugar contents, including glucose, shown to inhibit the growth of various microbes to varying degrees (White, 2018; Mizzi *et al.*, 2020).

Results for the microtitre plate MIC_{50} assay (Table 5.11), showed an MIC_{50} for the 1/16 dilution equivalent to 160 AU/ml detected for the cell free Fermentate, Permeate 1 and Retentate 1, indicating similar levels of activity across all three samples. Some of the antimicrobial activity may be primarily due to organic acid production as discussed previously in Sections 4.6.4 and 4.6.5. The mYPD media also displayed antimicrobial activity to a 1/4 dilution - equivalent to 40 AU/ml, however it never completely inhibited the growth of the *S. xylosus* ATCC 29971 indicator strain when the media was diluted further as the other samples did. After the buffer exchange, reduced antimicrobial activity was detected for Permeate 2 and Retentate 2, with levels of activity similar to those detected for the previous Crossflow run 1 and an MIC_{50} recorded for the 1/4 dilution equivalent to 40 AU/ml for Permeate 2 and a slightly lower MIC_{50} value of the 1/2 dilution and 20 AU/ml for Retentate 2 (Table 5.11). The lower antimicrobial activity detected in the Retentate as opposed to the Permeate was not the expected result, as the Retentate should retain all the molecules unable to pass through the filter, as they had already been passed once through the 1 kDa filter and thus it would be expected that the antimicrobial activity should be retained within the Retentate.

The protein concentrations of the fractions were determined in the BCA assay (Table 5.12), the cell-free fermentate had 36.44 mg/ml of protein and this concentration of protein was not effectively further concentrated in Retentate 1 which gave similar values of 33.84 mg/ml protein, while 32.19mg/ml was measured in Permeate 1. As also observed in the previous crossflow Run 1, the Permeate 2 had a slightly higher concentration of protein at 3.03 mg/ml compared to 2.08 mg/ml for Retentate 2 after the buffer exchange (Table 5.12). The higher

concentration of protein in the permeate was not expected as the original retentate sample applied for buffer exchange had already been run through the 1 kDa filter and the buffer exchange involved only a change of media.

In the activity tables generated for TFF run 4 and crossflow runs 1 and 2, it is observed that the specific activity was not increased for the Retentate as would be expected with a successful partial purification and concentration effect, with the original fermentate or cell free fermentate retaining the highest specific activity in comparison to the generated Retentate or Permeate. For the activity table produced for TFF run 4 (Table 5.7), the fermentate with cells displayed specific activity of 1.0 and the cell free Fermentate generated calculated specific activity of 1.3 AU/mg, while Permeate 1 and Retentate 1 had specific activity values of 1.0 AU/mg and 0.6 AU/mg respectively. For the first crossflow run using MRSc broth as the media for growth, the Fermentate had displayed a specific activity of 1.07 AU/mg, which was reduced to 1.01 AU/mg for Permeate 1 and 0.98 AU/mg for Retentate 1. The specific activity for the Fermentate with cells of the second crossflow experiment using mYPD as the growth medium was 0.28 AU/mg, for the cell free Fermentate and Permeate 1 the specific activity was 0.25 AU/mg and for Retentate 1 was 0.24 AU/mg. These specific activity values were much lower as the media which the bacteria were grown in has significant protein content and this would have a significant effect on the measured specific activity and total protein present. The results obtained above indicate that the specific activity of the retentate was never increased, as would be expected with a successful concentration of the Fermentate. The concentration effect upon the use of tangential flow filtration methods has been observed in other studies, such as the study by Lv *et al.*, (2018) where the supernatant of *L. plantarum* DY4-2 was concentrated using the TFF ultrafiltration method with a 1 kDa filter, similar to the 1 kDa filter used for both crossflow concentration runs of the *B. longum* ITT 13 fermentate. This use of ultrafiltration by Lv *et al.*, (2018) resulted in a specific activity of 507 AU/mg for their Retentate in comparison

to the initial cell free supernatant which had a specific activity value of 51 AU/mg, the results indicating a ten-fold increase of antimicrobial activity with the use of TFF. In another study, the class II bacteriocin Enterocin 96 was subjected to multiple purification steps which included TFF with a 10 kDa filter as the first step (Izquierdo *et al.*, 2009). The specific activity as determined by Izquierdo *et al.*, (2009) was calculated as AU/ml divided by the A_{280} protein readings and the specific activity increased from 7.13 $AU.ml^{-1}/A_{280}$ for the crude culture extract to 17.85 $AU.ml^{-1}/A_{280}$ for the Retentate after TFF and there was no detection of the peptide in the permeate. These are the expected results when using TFF to efficiently concentrate and partially purify crude supernatant and it is evident this was not the case for the antimicrobial activity produced by *B. longum* ITT 13, with future work required to efficiently develop the method.

5.4 Future Work/Potential Studies

TFF systems have been used widely to concentrate and partially purify protein molecules including bacteriocins (Li *et al.*, 2003; Freiherr Von Roman *et al.*, 2014; Amado *et al.*, 2016; Golneshin *et al.*, 2020). In theory, the concentration of the antimicrobial activity through the retention and concentration of the antimicrobial peptides present in the fermentate would have been the desired outcome for the experiments described in Sections 5.1 and 5.2. Subsequent buffer exchanges would have been expected to remove the acidic medium surrounding the retained and concentrated peptides allowing for accurate determination of the antimicrobial activity associated solely with these molecules. Use of the benchtop TFF system and the larger Crossflow filtration system, as discussed in Section 5.3, was associated with loss of both protein from the retentate, as well as the antimicrobial activity that was present in the original samples. The latter findings would suggest that the techniques tested to date would need significant optimisation in order to be useful in a concentration and partial purification procedure for the antimicrobial activity observed for the *B. longum* ITT 13 fermentate. As discussed previously (Section 5.3), protein fouling due to soluble-protein–adsorbed-protein interactions and protein-membrane interactions, may have been the cause of a loss of protein (Grund *et al.*, 1992) when samples were being recycled through the various filters. Given that TFF technology has been successfully applied in the purification of antimicrobial peptides, it is suggested that the technology should be useful, but requires significant optimisation for ITT 13 (Izquierdo *et al.*, 2009; Amado *et al.*, 2016; Kumar and Tiwari, 2017; Lv, Ma, *et al.*, 2018).

Antimicrobial activity associated with bacteriocins tends to be produced at relatively low concentrations and thus it would be preferable to work with larger scale equipment to increase larger initial volumes of fermentate in the concentration steps. The production of low concentrations of these peptides increases the need for concentration of the activity in industry (Garsa *et al.*, 2014), thus it would be preferable to work with larger volumes of the *B. longum*

ITT 13 fermentate. Nakamura *et al.,* (2013) used crossflow filtration as a method to successfully concentrate the antimicrobial activity associated with Gassericin A, produced by *Lactobacillus gasseri* LA39, against *Lb. delbrueckii* subsp. *bulgaricus* JCM 1002T, by concentrating a large volume of 9 litres of the culture supernatant to a 600 ml concentrate (retentate), that resulted in a 16-fold increase of antimicrobial activity. The volumes of the *B. longum* ITT 13 fermentate used in this thesis research were up to 2.6L, and suggest a requirement to work with larger volumes on the Crossflow apparatus to generate a successfully concentrated fraction. Coronel *et al*. (2019) described the use of a single-use bioreactor used with a TFF system to produce the influenza A virus. In this study, a large-scale 12L bioreactor connected to a TFF system was utilised for the production of a high cell concentration of influenza A. This type of bioreactor coupled with a TFF system could be explored as a future possibility for the production and concentration of high volumes of the *B. longum* ITT 13 fermentate. The use of a bioreactor similar to the one described by Coronel *et al.,* (2019) would enable efficient and rapid TFF concentration upon growth of the strain in the bioreactor at high concentrations, resulting in potentially higher concentrations of antimicrobial peptide production and minimising the risk of loss of protein activity due to storage. Bhugaloo-Vial *et al.,* (1997) used a bioreactor coupled with a crossflow filtration system for continuous bacteriocin production. The author described the use of the system with a membrane of a large molecular weight cut-off of 5 x 10^5 Da. However, even with the use of a large membrane a higher bacteriocin concentration was retained within the retentate with l0^8 AU/ml as compared to l0^3 AU/ within the permeate. Fouling of the membrane occurred after 75 hours and 102 hours growth (Bhugaloo-Vial *et al.,* 1997), however this could likely be combatted with batch production in place of continuous, as *B. longum* ITT 13 had been shown to display optimum antimicrobial activity between 16-18 hours growth in fermentation vessels (C. Whelan – personal communication).

A larger scale TFF system with a filter of a larger surface area could also be used for future fermentate concentration, as described by Cai *et al.* (2015). In the latter study, microbes in water samples were concentrated and separated from water samples through the use of TFF with a) a smaller scale filtration system of 50 cm^2 that could filter several litres of water, similar to the crossflow system used for *B. longum* ITT 13, and b) a large scale system with a filter of 0.5 m^2, which could filter hundreds of litres of water. A TFF system with a filtration membrane of a large surface area, similar to the latter large-scale system, would be a viable option for the filtration of the *B. longum* ITT 13 fermentate, thus enabling the concentration of much larger volumes while potentially minimising membrane fouling due to the large surface area of the membrane.

Another form of TFF exists called alternating tangential flow filtration (ATF), whereby reverse flow is used once per cycle to minimise membrane fouling (Kelly *et al.*, 2014), which could be promising for application in the concentration of the *B. longum* ITT 13 fermentate. Tan *et al.* (2021) discussed the use of this ATF method in terms of continuous buffer exchange in downstream processing of bioproducts using a single piece of equipment. As the retentate flows through a channel between two membranes, one which removes permeate and the other which supplies a buffer, the use of an alternating flow direction of diafiltration buffer through the membranes reduces the incidence of concentration polarization at the membrane (Tan *et al.*, 2021). Concentration polarization has already been discussed as a major factor and mediator in the fouling and interaction of soluble proteins at the membrane interface (Grund *et al.*, 1992). By employing the ATF method described above, the fouling of membranes within the TFF and crossflow systems may be reduced with consequent improvement in the flow rates and minimisation of protein losses, as observed during the successive buffer exchanges carried out with *B. longum* ITT 13 Fermentate.

A study by Pires & Palmer (2021) describes the use of TFF to purify and isolate proteins, however instead of concentrating the proteins in the Retentate they were kept in the Permeate through the use of target-protein binding molecules and a filter with a molecular weight cut-off (MWCO) below the target protein. This allowed impurities below the MWCO to be extracted in the Retentate, while the target protein remained in the Permeate. A target-protein binding molecule was then added to form protein-protein complexes with the target protein, which was the only complex larger than the MWCO thus enabling this complex to be selectively separated from the other protein components in the sample. A buffer exchange was carried out allowing for dissociation of the target protein from the binding molecule and separation using a membrane with a MWCO between the molecular weight of the protein and the binding molecule. An issue arose with low protein-protein complex recovery from the TFF system, however based on mathematical models this could be combatted and recovery increased with a series of three filters of the same MWCO in sequence, whereby the Permeate of the first system is fed into the second and the second is fed into the third filter system (Pires and Palmer, 2021). This study could be indicative of a potential option for future TFF concentration and purification of the *B. longum* ITT 13 fermentate, although the fermentate would likely need partial purification in advance to remove larger proteins that would also be above the MWCO of the filter alongside the antimicrobial peptide-binding molecule complex. The isolation of the peptide in the Permeate would potentially minimise protein interactions with the membrane when collecting the Retentate for concentration. Using the above method, a suitable specific target-protein binding molecule would also be required to isolate the antimicrobial peptide produced by *B. longum* ITT 13, meaning the biochemistry of the specific ITT 13 peptide molecule would need to be understood and as the antimicrobial peptide produced by ITT 13 is likely of low molecular weight indicated by the TFF work, this process may not be suitable.

Another option for future concentration and purification studies would be to consider other methods of protein concentration including techniques such as ammonium sulphate precipitation. The latter method, which is widely employed in current literature to extract and concentrate antimicrobial peptides (Afshan Naz, Sheikh and Rasool, 2013; Ansari *et al.*, 2018; Lei *et al.*, 2020), was utilised in Chapter 4, Section 4.3 and 4.6.3 to extract antimicrobial proteinaceous compounds. As evidenced in Sections 4.3 and 4.6.3, antimicrobial activity was isolated in the 80% ammonium sulphate saturation that was validated as associated specifically with the proteins produced within the *B. longum* ITT 13 fermentate. Due to limitations with apparatus, only small-scale volumes of 40 ml were used for the ammonium sulphate precipitation, however as this method was successful in the isolation of antimicrobial peptides, it could potentially be employed on a large-scale basis in future work. To minimise repeated passes through the TFF and crossflow membrane systems with buffer exchanges, ammonium sulphate precipitation could be carried out on large volumes of the already concentrated retentate post TFF concentration, subsequently removing the protein from the acidic medium without the requirement for a buffer exchange. This could be a suitable method for scale up studies, as the ammonium sulphate solution interferes with the solubility of protein in water by increasing hydrophobic interactions resulting in precipitation that maintains protein conformation and preventing denaturation (Wingfield, 2001). Therdtatha *et al*. (2016) used ammonium sulphate precipitation as the first of three purification steps for a bacteriocin produced by *Lactobacillus salivarius* KL-D4, whereby a 20% ammonium sulphate saturation was utilised. A large volume of 1 litre of the *L. salivarius* KL-D4 cell-free supernatant solution was employed and left stirring in the ammonium sulphate solution for 18 hours to facilitate full saturation of the ammonium sulphate in the solution. Once the protein pellets were isolated following centrifugation, they were then resuspended in another 1 litre volume of a buffer solution to dilute the salt concentration of the ammonium sulphate (Therdtatha *et al*., 2016).

Volumes of this size would be ideal to increase the concentration of antimicrobial peptide present and thus concentrate the antimicrobial activity produced by *B. longum* ITT 13. In addition, instead of resuspending the extracted protein in the same volume, it could be resuspended in a reduced volume, as it was done in Section 4.3 for the *B. longum* ITT 13 ammonium sulphate precipitation previously carried out. This would both concentrate and partially purify the activity on a larger scale. A potential issue with the resuspension of the AS pellets in a minimal volume would likely result in high salt concentration present within the sample which would require removal to minimise interference. To combat this issue, the use of hydrophobic interaction chromatography (HIC) could be administered as the next step in a purification protocol, as the high salt concentration would facilitate binding on the hydrophobic resin (Galeotti *et al.*, 2020; Muca *et al.*, 2020). HIC is a form of chromatography often used extensively in the biotechnology and pharmaceutical industries for the downstream processing of proteins and is based on the differences in hydrophobic properties between the target proteins for purification and the impurities within the sample (Muca *et al.*, 2020). Bacteriocins are generally hydrophobic in nature with large hydrophobic portions present within the peptide chain to facilitate interaction and penetration of the phospholipid bilayer of target cell membranes (Singh *et al.*, 2015; Daba and Elkhateeb, 2020). In HIC, a salt or salt mixture is often utilised within the protein sample to promote adsorption to the HIC media, including salts used in protein precipitation such as ammonium sulphate (Galeotti *et al.*, 2020; Muca *et al.*, 2020) and thus HIC is additionally suitable for a suggested next step in a potential purification process. Ray Mohapatra and Jeevaratnam, (2019) used HIC as a purification step for isolation and characterization of a bacteriocin produced by *Lactobacillus plantarum* subsp. *argentoratensis* SJ33. The supernatant suspended in 0.1 N NaCl was partially purified using gel permeation chromatography, which generated an active fraction that was passed through HIC (Sep-Pak C18 cartridge) and eluted with 20%, 30% and 50% of isopropanol to obtain

various fractions with different polarity. The active fraction containing the bacteriocin was further purified using reverse phase HPLC, before antimicrobial and characterization studies were carried out on the peptide (Ray Mohapatra and Jeevaratnam, 2019). Deraz *et al.*, (2005) also used HIC as part of a purification process of a bacteriocin produced by *Lactobacillus acidophilus* DSM 20079, designated acidocin D20079, following ammonium sulphate precipitation and cation exchange chromatography, generating a purified peptide suitable for characterization including partial sequencing and molecular weight determination.

Experiments carried out by Lv *et al.* (2018) and Izquierdo *et al.* (2009) using TFF as a single step in a purification process, provide another option in place of carrying out TFF with a subsequent buffer exchange on the same system. Lv *et al.* (2018) first carried out an ethyl acetate extraction on 1 litre of the cell free supernatant of *Lactobacillus plantarum* DY4-2 that contained the bacteriocin, resulting in a crude extraction from the culture media, with a specific activity value of 493 AU/mg in a 200 ml volume. This was then subjected to tangential flow filtration at 10 kDa, 5 kDa and 1 kDa to further purify and concentrate to an 80 ml volume fraction with 507 AU/mg, of which 2 ml was then subjected to further purification by size-exclusion chromatography on a Sephadex G25 column with bacteriocin-containing fractions pooled, freeze-dried and resuspended in 6 ml of ultrapure water, resulting in a sample which had a specific activity of 1064 AU/mg. The final purification step for the concentrated bacteriocin fraction involved the use of a HPLC method, with the eluted fraction containing the peptide collected and concentrated to 1 ml by freeze-drying, with a final specific activity of 3560 AU/mg (Lv, Ma, *et al.*, 2018). The overall method therefore did not involve repeated passages through a TFF filter to facilitate buffer exchanges. The TFF in combination with other purification methods thus successfully concentrated and increased the antimicrobial activity of the bacteriocin produced by *L. plantarum* DY4-2.

A similar purification process to that described in the previous paragraph was carried out by

Izquierdo *et al.*, (2009), whereby TFF was employed as a single step in the purification process. A 5-litre volume of the cell free supernatant of *E. faecalis* WHE 96 grown in MRS broth was first ultrafiltered using a TFF system with a 10 kDa filter and the Permeate was concentrated to 220 mls using a 5 kDa filter resulting in specific activity of 17.85 $AU.ml^{-1}/A_{280}$ (70% activity yield). Instead of carrying out a buffer exchange, the retentate was pH adjusted to pH 5.5 using NaOH before the next purification step of cation exchange chromatography, which resulted in specific activity of 1,564 $AU.ml^{-1}/A_{280}$ (16% activity yield), followed by the application of the active fractions to reverse-phase HPLC generating a concentrated bacteriocin fraction with specific activity of 206,451 $AU.ml^{-1}/A_{280}$ (10% activity yield). The active fractions containing the concentrated bacteriocin, detected at 280 nm, were then vacuum dried and resuspended in deionized water for testing (Izquierdo *et al.*, 2009). The specific activity was calculated as the bacteriocin activity (AU/ml) divided by the A_{280}. The above method resulted in significant concentration of the bacteriocin and its associated antimicrobial activity without repeated TFF circulation and eliminated a buffer exchange, which is where the largest concentrations of protein in the *B. longum* ITT 13 fermentate were lost in the practical approach described in this thesis (Section 5.1). Therefore, future work could likely entail the design of a series of purification steps that incorporates TFF concentration without the subsequent use of a buffer exchange. The overall aim with *B. longum* ITT 13 was concentration of the antimicrobial activity within the fermentate rather than purification, in order to generate a more concentrated sample for physiochemical characterization work including protease digestion and temperature stability. As discussed through Sections 5.3 and 5.4, successful concentration of the antimicrobial activity within the *B. longum* ITT 13 fermentate was not achieved with the practical approach of TFF described, thus the methods described above in Section 5.4 may be explored for future concentration of the activity.

Aside from purification and concentration, characterisation of the proteinaceous material displaying antimicrobial activity produced by *B. longum* ITT 13 is an important area of research and could be carried out using commonly used methods of protein analysis such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 2D gel electrophoresis or matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

SDS-PAGE is used to determine the molecular weight of proteins in a mixture through denaturation of the proteins using SDS and separation of the proteins on a polyacrylamide gel using electrophoresis that separates the proteins based on their molecular mass (Nowakowski *et al.*, 2014). Yamamoto *et al.*, (2003) utilised SDS-PAGE to determine the molecular weight of a bacteriocin produced by *E. faecalis* RJ-11 and determined the bacteriocin to be of approximately 5 kDa. Lv *et al.*, (2018) also used SDS-PAGE to determine the molecular weight of plantaricin JY22, which is a bacteriocin produced by Lactobacillus plantarum JY22 that displays activity against *Bacillus cereus.* The author determined the approximate molecular weight of the bacteriocin as 4.1 kDa (Lv *et al.*, 2018). Both authors separated the gel into two after the electrophoresis was ran, with half of the gel stained for molecular weight determination and the other half of the gel used for antimicrobial testing to determine any bands on the gel displaying antibacterial activity which would indicate bacteriocins (Yamamoto *et al.*, 2003; Lv *et al.*, 2018). This was carried out by overlaying the gels with a soft agar inoculated with an indicator bacteria and incubation to grow the bacteria within the soft agar before examining the growth for any areas of inhibition associated with protein bands on the gel (Yamamoto *et al.*, 2003; Lv *et al.*, 2018). This latter method is a useful method for determining if any separated protein bands on the gel display antimicrobial activity, thus validating that there are antimicrobial peptides present as well as determining which bands to use for molecular weight determination. This method would be useful for future testing of the

B. longum ITT 13 fermentate to further validate the presence of suspected antimicrobial peptides thus allowing for molecular weight determination.

Mulyawati *et al.*, (2019) also partially purified and characterised bacteriocins produced by *Lactobacillus plantarum* SB7 and *Bacillus amyloliquefaciens* BC9 using SDS-PAGE. The bacteriocins were partially purified from cell-free supernatant by precipitation using the ammonium sulphate precipitation method at 80% saturation followed by dialysis using a semi-permeable membrane with a 5 kDa cutoff. SDS-PAGE was then employed to determine the molecular weight of the bacteriocins. The samples were all ran in duplicate on separated wells of the same gel which was cut in half following the electrophoresis as also carried out in the above studies. Half of the gel was stained with Coomassie blue R-250 and the other half used for the soft agar overlay assay inoculated with indicator bacteria to confirm antibacterial activity. Potential bacteriocin bands produced by both *L. plantarum* SB7 and *B. amyloliquefaciens* BC9 were found to be approximately 48 kDa in size based off staining and antimicrobial activity associated with both *L. plantarum* SB7 on the gel in the soft agar overlay assay indicated activity associated with bands of 48 and 17 kDa in size. The authors noted that the antimicrobial activity associated with *B. amyloliquefaciens* BC9 had disappeared when the SDS-PAGE gel was added to the soft agar overlay assay and hypothesised this was to do with denaturation of the protein by the SDS, advising that MALDI-TOF mass spectrometry would be a more suitable method of molecular weight determination. It would also be possible to carry out a PAGE gel without SDS denaturation on the *B. longum* ITT 13 proteins to prevent denaturation and loss of activity of samples.

MALDI-TOF involves ionisation of a sample with a laser beam, with the ions accelerated causing them to separate based on their mass to charge ratio (m/z), which can be measured based on the length of time they take to pass down the flight tube and reach the TOF analyser (Singhal *et al.*, 2015). MALDI-TOF was the first mass spectrometry technique employed to

detect bacteriocins within the cell free supernatant (Rose *et al.*, 1999). In the study by Rose *et al.*, (1999) they determined that spotting the sample onto the MALDI-TOF probe, allowing it to dry and a simple wash of the probe with milli-Q water for 30 seconds was effective for removing contaminants from the sample. The author tested the supernatant of *L. lactis* ATCC 11454 which should contain nisin and detected a bacteriocin in the samples ranging between 3.355 and 3.359 kDa, likely indicating nisin which has a molecular mass of 3.354 kDa. Rose *et al.*, (1999) also successfully detected the bacteriocins enterocin A and B, brochocin A and B and pediocin from the cell free supernatants of the producing cultures. Goh and Philip (2015) also used MALDI-TOF mass spectrometry to determine the molecular weight of a bacteriocin produced by *Weissella confusa* A3, which was found to produce a bacteriocin of approximately 2.7 kDa that displayed antibacterial activity towards the pathogens *E. coli, P. aeruginosa* and *B. cereus* among others. This method of MALDI-TOF seems to suggest a potential method of analysis of any bacteriocins produced by *B. longum* ITT 13, requiring minimal modification due to the detection of bacteriocins in the cell-free supernatants with only a water wash of the sample to remove contaminants as described by Rose *et al.*, (1999).

As described there are many potential avenues of characterisation and purification of potential antimicrobial peptides produced by *B. longum* ITT 13 that can be explored in future work. As only some characterisation of the organic acids produced using HPLC was carried out, this could also be explored further to determine the full antimicrobial effect of the acids. Further HPLC analysis with more replicates could be carried out to ensure accuracy in the determination of the concentration of acids produced by *B. longum* ITT 13. The organic acids could also be quantified at various timepoints during the growth of ITT 13 to determine the time point that contains the least concentration of organic acids within the fermentate yet still retains antimicrobial activity. This time point analysis would also be useful to monitor how the antimicrobial activity increases proportionally to acid production during growth of *B. longum*

ITT 13. From the quantification of the concentrations of lactic, acetic and succinic acids produced during growth, the antimicrobial activity of these concentrations alone and in combination should be screened against all bacterial and fungal indicator strains tested within the thesis to determine the levels on inhibition associated solely with the acids. The study by Makras and De Vuyst (2006) involved the quantification of the organic acids produced by HPLC, with concentrations of 60.9 mM acetic acid and 39.9 mM lactic acid found to be produced by *B. longum* CA1. This combination of acids at these concentrations was then used as a control for comparison to the antimicrobial activity of the cell-free supernatant generated by *B. longum* CA1 to determine whether the activity associated with the supernatant was solely the result of organic acid production. When tested against *S. typhimurium* it was found that the antibacterial activity observed was associated with the concentration of organic acids present. Similar antibacterial results were also found for the supernatant produced by *B. longum* BB536 against *E. coli* and for the MRSc control with the same amount of organic acids as present within the supernatant. These results coupled with results from testing the supernatants and controls against Gram-positive strains led the authors to determine the organic acids were responsible for antibacterial activity against Gram-negative strains while bacteriocins were responsible for activity against Gram-positive strains. Therefore, this sort of detailed analysis involving a control of the concentration of organic acids within the fermentate should be carried out for *B. longum* ITT 13 against all tested bacteria and fungi to aid in determining the source of antimicrobial activity against all tested microbes.

There are several concentration and purification methods for bacteriocins detailed in the literature which could provide several different approaches to future experimental work on the concentration and purification of the antimicrobial activity identified to be produced in the *B. longum* ITT 13 fermentate. It is evident throughout Chapter 4, from the ammonium sulphate precipitation, protease digestion and organic acid analysis work, that the detectable

antimicrobial activity produced by *B. longum* ITT 13 is most probably associated with a low molecular weight proteinaceous molecule produced in low concentrations. Additional to the antimicrobial activity associated with the proteinaceous molecule some of the antimicrobial activity is associated with the production of the organic acids by *B. longum* ITT 13. To enable full physicochemical characterisation of the antimicrobial activity associated with the proteinaceous compound it will be necessary to purify out the bacteriocin and remove any interference from the produced organic acids. Approaches to the concentration and purification of the activity produced by *B. longum* ITT 13 would incorporate methods already utilised for bacteriocin studies such as ammonium sulphate precipitation, tangential flow filtration systems and various chromatographic approaches. Overall, the results presented in this thesis showing that the antimicrobial activity is active against a number of different organisms, warrants further research in the purification and characterisation of the antimicrobial activity produced by the GRAS organism *B. longum* ITT 13.

CHAPTER 6

Summary

Probiotics are defined by the World Health Organisation (WHO) as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host", and most commonly include *Bifidobacterium* and *Lactobacillus* species (FAO/WHO, 2006; Butel, 2014). Probiotic bacteria are said to contribute to the maintenance of a healthy gastrointestinal system by offering health benefits similar to the hosts natural gut microflora. These benefits include the maintenance of tight junctions of protein to protect the intestinal barrier and exertion of antimicrobial activity in the gut to prevent infection and colonisation by pathogenic bacteria (O'Flaherty and Klaenhammer, 2010; Mahmoudi *et al.*, 2013; Jomehzadeh *et al.*, 2020; Wang *et al.*, 2020). The production of antimicrobial activity by probiotic bacteria is primarily associated with organic acid and antimicrobial peptides, also known as bacteriocins (Hladíková *et al.*, 2012; O'Shea *et al.*, 2012; Guinane *et al.*, 2016). Bacteriocins are ribosomally synthesised proteinaceous molecules produced by bacteria that display antimicrobial activity against other bacterial and in some cases fungal species (Ahn *et al.*, 2017; Aarti *et al.*, 2018; Ruggirello *et al.*, 2018).

Bifidobacteria are Gram-positive, non-spore forming, non-motile, anaerobic bacteria that are commonly utilised as probiotic strains (Lee and O'sullivan, 2010; Dhanashree *et al.*, 2017). They are heterofermentative bacteria and produce metabolites such as organic acids including lactic and acetic acid upon growth (Makras and De Vuyst, 2006; Ruiz-Aceituno *et al.*, 2020). *Bifidobacteria* have also been associated with the production of other metabolites including bacteriocins (Lee and O'Sullivan, 2010). Probiotic bacteria have been used to restore the balance of gut flora following antibiotic treatment (Engelbrektson *et al.*, 2009) and help to treat antibiotic associated diarrhoea (Selinger *et al.*, 2013; Litao *et al.*, 2018), and many are used for the production of fermented or biofunctional food products such as fermented dairy products including yoghurt, cheese and infant formula (Rotar *et al.*, 2007; Butel, 2014; Linares *et al.*, 2017). It is noted however, that there are still questions surrounding the exact benefits, efficacy

and safety of probiotics in some populations including immunocompromised individuals or those with short bowel syndrome (Munakata *et al.*, 2010; Rao *et al.*, 2018; Gargar and Divinagracia, 2019), as well as some experts doubting their claimed benefits (Lerner *et al.*, 2019). .

Bacteriocins have also been used in the food industry as potential biopreservatives in place of the use of chemical preservatives, which have an increasingly perceived negative impact on health (Paula M O'Connor *et al.*, 2015; Skariyachan and Govindarajan, 2019). Bacteriocins are considered to be particularly useful in the food industry due to their observed antibacterial and antifungal activity against potential foodborne pathogens and/or food spoilage organisms (Cálix-Lara *et al.*, 2014; O'Connor *et al.*, 2015; Luz *et al.*, 2017; Salazar *et al.*, 2017). This study aimed to characterise the antibacterial and antifungal activity produced by *Bifidobacterium longum* ITT 13 and determine whether the nature of the activity was associated with either organic acid or bacteriocin production, or a combination of both.

Various methods were implemented to characterise the antimicrobial activity produced by *B. longum* ITT 13. The bacteria was grown anaerobically in MRSc broth that was heat-inactivated by incubation at 60^{o}C for 15 minutes without removal of the cells to generate a fermentate. A heat inactivated fermentate was required at the behest of the project sponsor instead of using cell-free supernatant, as a minimally modified media with the ITT 13 cells present was of interest. Heat inactivated fermentate was screened for antimicrobial activity against a variety of bacterial and fungal strains using a combination of the microdiffusion assay, also known as the well diffusion assay, and a microtire plate MIC_{50} assay. The strains were selected primarily based on their pathogenicity and role in food spoilage or food poisoning. Various Gram-positive and Gram-negative bacteria were screened for susceptibility including *Bacilli, Staphylococci, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* and *Enterococcus faecalis.* Screened fungal species include *Saccharomyces cerevisiae, Candida*

albicans Rhodotorula mucilaginosa and *Debaryomyces hansenii*.

Physiochemical characterisation was carried out to determine the temperature and pH stability of the *B. longum* ITT 13 fermentate by incubation of the fermentate at various temperature or pH conditions, followed by antimicrobial screening to observe any losses or reduction in activity. The nature of the antimicrobial activity was determined through protease digestion, ammonium sulphate precipitation and HPLC analysis. Concentration studies on the antimicrobial activity of the fermentate involved membrane filtration via small-scale TFF and large-scale TFF on a crossflow system with the use of 10 kDa, 3 kDa and 1 kDa membranes. This was carried out to concentrate the antimicrobial activity of the fermentate without the use of a purification protocol.

B. longum ITT 13 was identified through Gram-staining, cell morphology and Biolog identification using the Biolog system (TECHNOPATH Distribution Ltd. Ireland). 16s ribosomal RNA sequencing was also carried out to verify the identity of the strain (C. Whelan – personal communication). The antimicrobial activity of the strain was then assessed as previous work had indicated antibacterial properties. The microdiffusion assay was carried out to screen 15 bacterial strains for susceptibility to the *B. longum* ITT 13 fermentate, of which 9 displayed growth inhibition when exposed to the fermentate. These were *Bacillus subtilis* ATCC 1174, *Enterococcus faecalis* ATCC 7080, *Kocuria rhizophilia* ATCC 9341, *Staphylococcus xylosus* ATCC 29971, *Klebsiella aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PA01, *Salmonella typhimurium* ATCC 29629 and *Burkholderia cenocepacia* ATCC 17765 III.20.

The fermentate adjusted to pH 4.5 and a control of MRSc broth at pH 4.5 were added to microtitre plates for an MIC_{50} assay as a more detailed analysis of the antibacterial activity and pH effect of the fermentate. This assay was screened against 6 selected bacterial strains and although conventional MIC assays assess dilutions of the analyte causing inhibition of visible

growth of the indicator strain, this MIC_{50} assay involved calculation of arbitrary units (AU)/ml of activity using the highest dilution displaying at least 50% activity for detailed analysis of inhibition compared to controls. The pH 4.5 fermentate had 80 AU/ml against *B. subtilis* and *E. faecalis* while the pH 4.5 MRSc broth had 20 AU/ml against both, the pH 4.5 fermentate had 40 AU/ml against *E. coli* and *P. aeruginosa* while the pH 4.5 MRSc broth had 20 AU/ml against both strains, the pH 4.5 fermentate had 80 AU/ml against *S. typhimurium* while the pH 4.5 MRSc broth had 40 AU/ml and the pH 4.5 fermentate had 80 AU/ml against *S. xylosus* while the pH 4.5 fermentate had 160 AU/ml. Although the latter result indicated more activity associated with the pH 4.5 MRSc broth indicating its sensitivity to acid, it should be noted that the pH 4.5 fermentate eliminated complete growth at the 1/8 dilution while the pH 4.5 MRSc broth had partial growth at this dilution. These results indicate that there may be more than a pH effect contributing to antibacterial activity as there was more activity associated with the fermentate than the control.

To assess how raising the pH of the fermentate affected the associated antibacterial activity the pH was increased to pH 4.68 and compared to a control of MRSc broth adjusted to pH 4.82 for comparison. This was tested against *K. rhizophilia, B. subtilis* and *P. aeruginosa* and the inhibition associated with the fermentate was significantly higher than the pH 4.82 MRSc broth control for all three strains ($p<0.05$) further indicating that antimicrobial activity is likely associated with more than a pH effect.

The fermentate was also screened against the fungal strains *R. mucilaginosa* CBS316, *S. cerevisiae* ATCC18824, *S. cerevisiae* CBS1171 and *C. albicans* ATCC2091 using the microtitre plate MIC_{50} assay to screen for antifungal activity. The fermentate at pH 4.35 and pH 4.80 was screened for antifungal activity, along with a control of MRSc broth at pH 4.30. Both the pH 4.80 and pH 4.35 fermentates displayed antifungal activity towards *R. mucilaginosa* and *C. albicans* while no notable antifungal activity was detected against either

S. cerevisiae strain. *S. cerevisiae* are relatively resistant to organic acids (Stratford *et al.*, 2013), therefore it is possible that the antifungal activity is primarily pH associated.

Physiochemical characterisation was carried out to assess the stability of the fermentate in a variety of temperature and pH conditions due to high food processing temperatures and various pH processing conditions within the food industry (Johnson *et al.*, 2018; Bagde and Vigneshwaran, 2019; Castilho *et al.*, 2020) if the strain or its fermentate were to be considered for this application. A thermostability assay was carried out by incubating 2 ml samples at temperatures of -20^{o}C, 4^{o}C, room temperature (20-24^{o}C), 37^{o}C, 55^{o}C and 70^{o}C over a 28-day period with multiple time-points taken. The results indicated no significant reductions in antibacterial activity over the course of the 28-day period, indicating the fermentate as a whole is relatively thermostable, however as any potential bacteriocins were not isolated the stability of these alone could not be determined. For the pH stability assay the fermentate was adjusted to pH 3, 4, 5 and 6 using lactic acid and NaOH and incubated for up to 24 hours. No antibacterial activity was detected when the fermentate was adjusted to pH 5 or 6, thought it should be noted that this assay was carried out using the less sensitive microdiffusion assay and the more sensitive MIC assay has detected activity at pH 5. The fermentate was stable for 24 hours at pH 3 and pH 4 with no significant reduction in activity over the entire 24-hour period.

The nature of the antimicrobial activity was investigated using ammonium sulphate precipitation, protease digestion and HPLC analysis. Saturations of 20%, 40%, 60% and 80 % ammonium sulphate were used to precipitate the proteins present within the fermentate. There was no significant antibacterial activity associated with the fermentate precipitated at 20%, 40% or 60% saturations in comparison to the control, however the 80% saturation resulted in the precipitation of proteinaceous material displaying an antibacterial effect in comparison to the control. This indicates the presence of a low-molecular weight proteinaceous molecule.

Protease digestion was carried out with pH 4.80 and pH 4.35 fermentate. Digestion of the fermentate at both pH's with Proteinase K resulted in no reduction of antibacterial activity indicating there was no successful digestion of any antimicrobial peptides. Digestion of the pH 4.35 fermentate with Actinase E also resulted in no reduction in activity, however digestion of the pH 4.80 fermentate resulted in a reduction of activity relative to the fermentate without Actinase E digestion at the 1/16 dilution. This indicates that a proteinaceous element of the pH 4.80 fermentate was contributing to the antimicrobial activity observed. It is likely that the pH of the pH 4.35 fermentate may have been too low for maximal activity of the Actinase E enzyme.

HPLC using a reverse-phase column with UV analysis was carried out to quantify the organic acids present within the fermentate. Fermentate at pH 4.35 generated in MRSc broth in lab-scale conditions in an anaerobic chamber, pH 4.80 fermentate also generated in MRSc broth in a 2 litre pH-controlled fermentation vessel and pH 5.0 fermentate generated in a modified YPD (mYPD) broth in a 2 litre pH-controlled fermentation vessel were analysed. The pH 4.35 fermentate had 147.72 mM ± 10.34 acetic acid, 92.22 mM ± 1.53 lactic acid and 54.52 mM ± 3.00, the pH 4.80 fermentate had 160.95 mM ± 13.72 acetic acid, 68.32 mM ± 10.62 lactic acid and 67.69 mM ± 6.14 succinic acid, while the pH 5.0 fermentate had 650.68 mM acetic acid, 154.97 mM lactic acid and no succinic acid detected.

Concentration studies involved concentrating volumes of between 125-2620 mls of fermentate using membrane filtration via small-scale TFF and large-scale TFF (crossflow). Use of a 10KDa and 3 kDa filter showed similar antimicrobial activity present in both the retentate and permeate of the fermentate after small-scale TFF concentration. Use of a 1 kDa filter also showed similar activity in both the retentate and permeate after concentration using the large-scale TFF crossflow system. Buffer exchanges were carried out to remove organic acids to assess activity associated solely with other molecules within the fermentate, however no

concentrated activity was detectable after buffer exchanges. This could indicate possible adsorption of the proteinaceous molecule to the membrane as it is present in low concentrations and a BCA assay showed losses of protein within the samples with each successive buffer exchange.

Experimental results indicate that the antimicrobial activity associate with *B. longum* ITT 13 is due to a combination of organic acid production together with a low molecular weight, proteinaceous component. Further studies, using higher starting concentrations of fermentate, could be used to concentrate and isolate the bacteriocin component of the detected antimicrobial activity when concentrating using TFF methods. Other methods to further validate the presence of an antimicrobial peptide could include larger scale ammonium sulphate precipitation, the use of chromatographic methods such as hydrophobic interaction chromatography to isolate and purify the peptide and methods including SDS-PAGE and MALDI-TOF mass spectrometry.

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APPENDIX

Appendix A

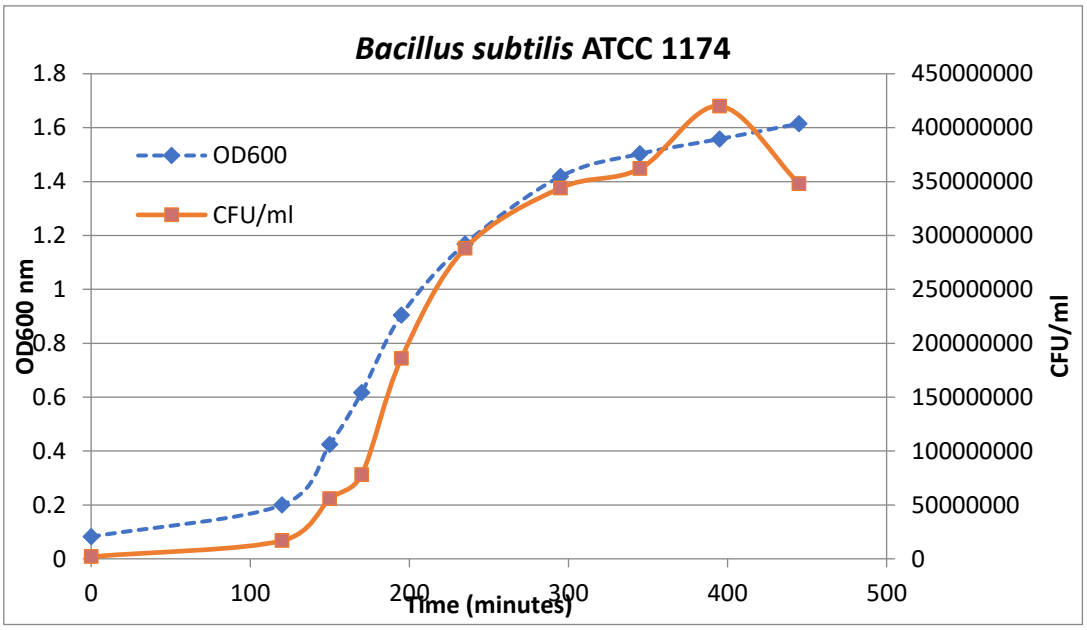

Figure 7.1: Growth curve of *Bacillus subtilis* ATCC 1174 in TSB showing OD600 and CFU/ml over time

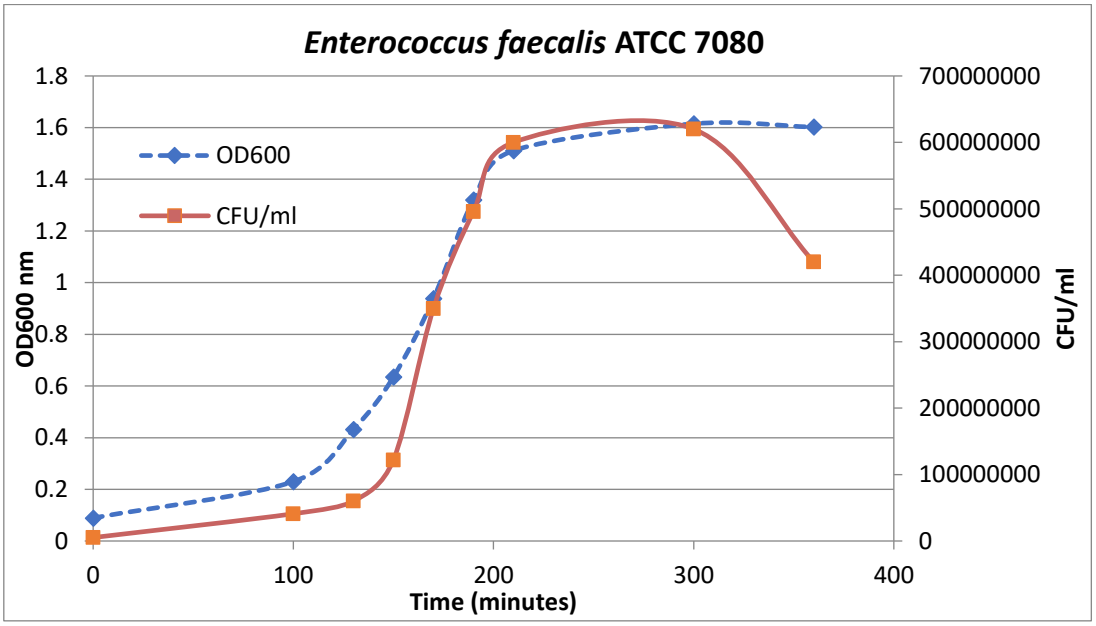

Figure 7.2: Growth curve of *Enterococcus faecalis* ATCC 7080 in TSB showing OD600 and CFU/ml over time

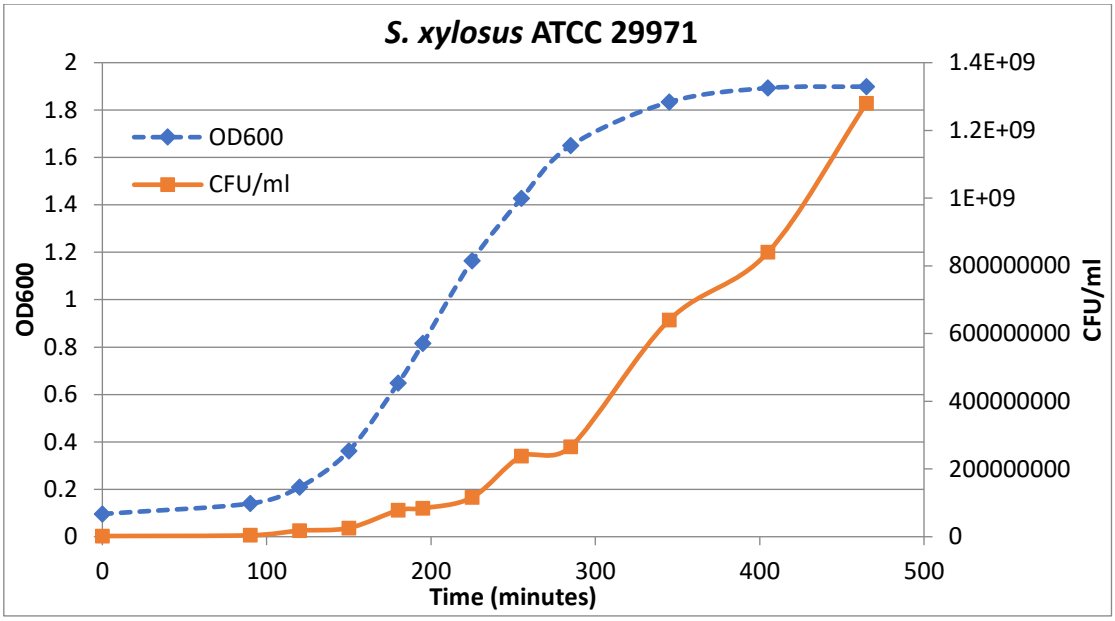

Figure 7.3: Growth curve of *Staphylococcus xylosus* ATCC 29971 in TSB showing OD600 and CFU/ml over time

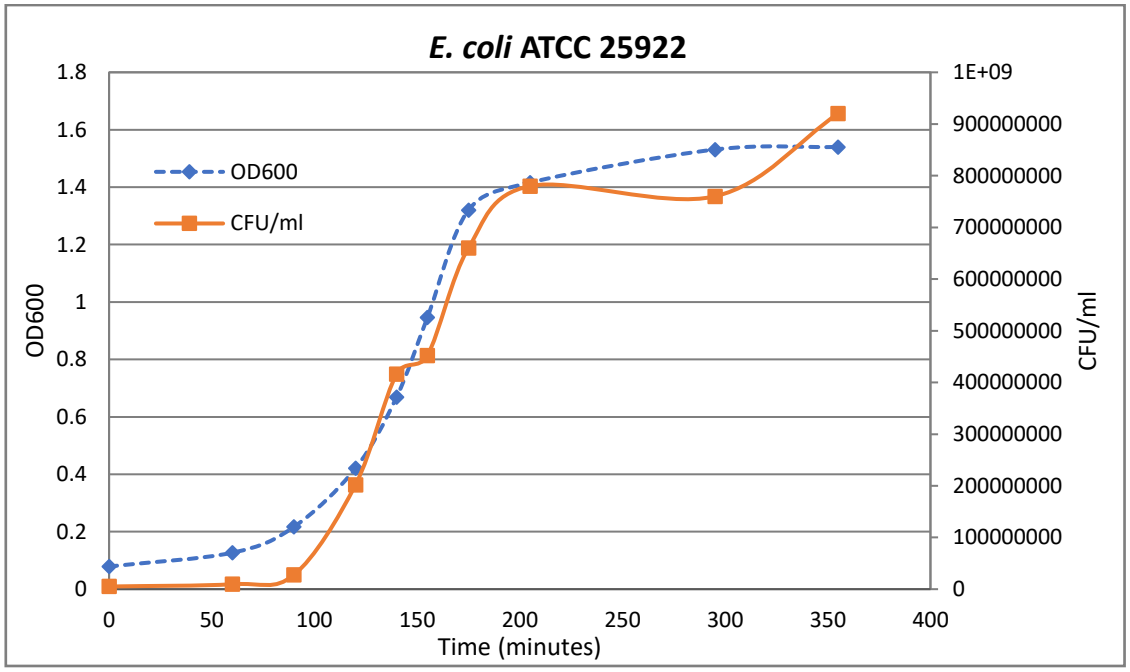

Figure 7.4: Growth curve of *Escherichia coli* ATCC 25922 in TSB showing OD600 and CFU/ml over time

Appendix B

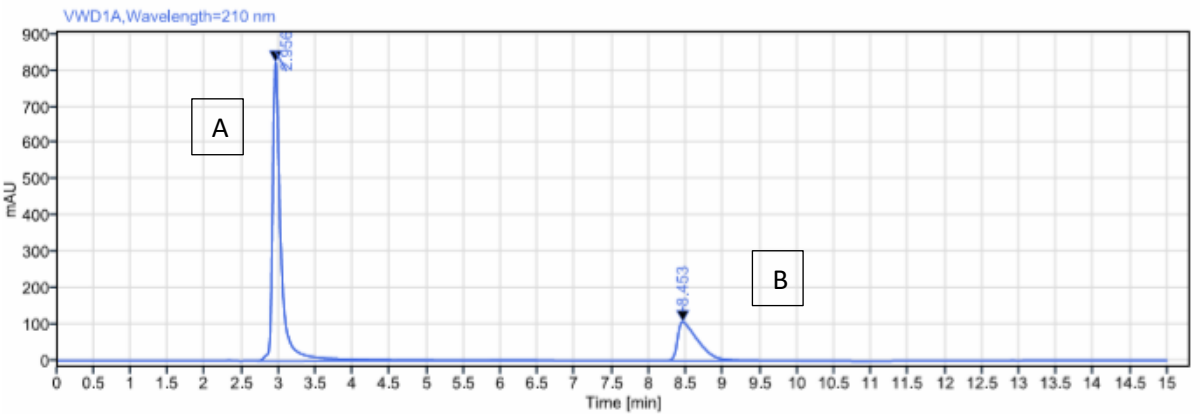

Figure 7.5: Chromatogram of 100 mM standard of formic acid standard. Formic acid (A) and 10 mM IS Methylmalonic Acid (B). Chromatogram is representative of one of n=3.

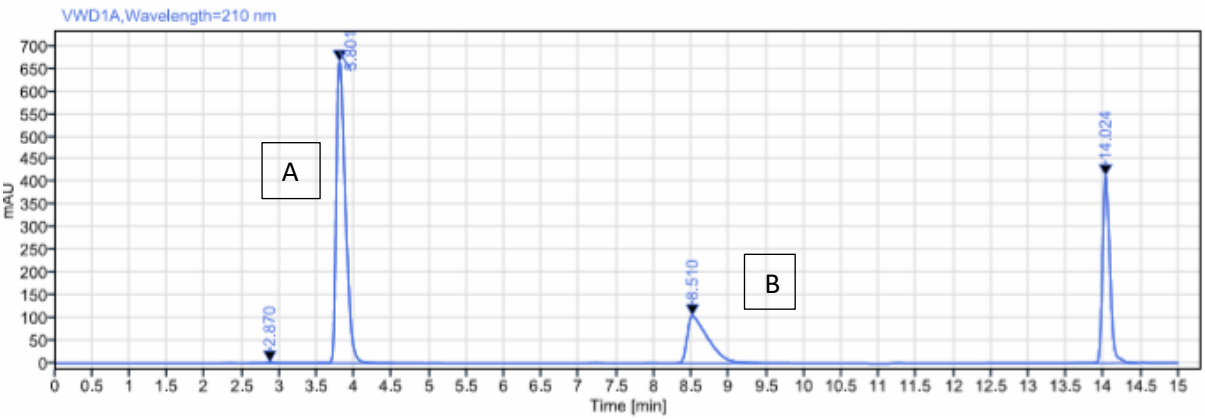

Figure 7.6: Chromatogram of 100 mM standard of lactic acid standard. Lactic acid (A) and 10 mM IS Methylmalonic Acid (B). Chromatogram is representative of one of n=3.

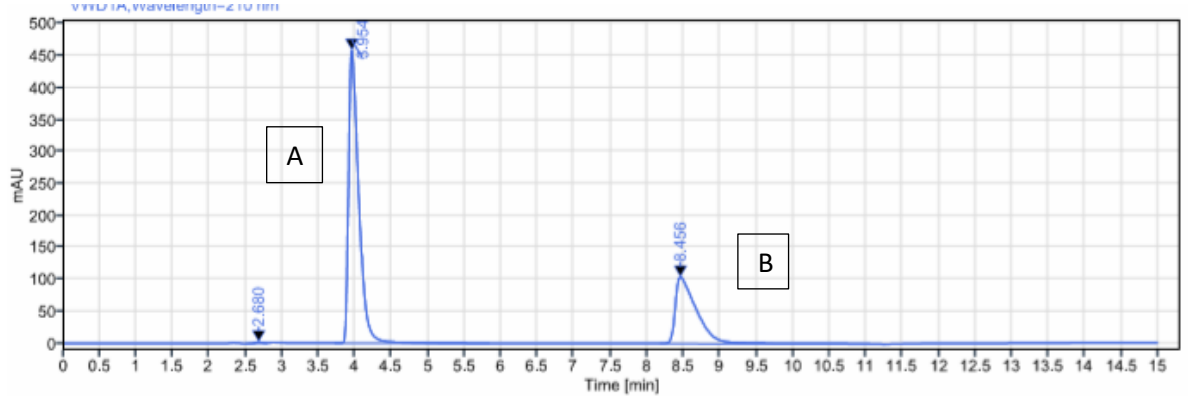

Figure 7.7: Chromatogram of 100 mM standard of acetic acid standard. Acetic acid (A) and 10 mM IS Methylmalonic Acid (B). Chromatogram is representative of one of n=3.

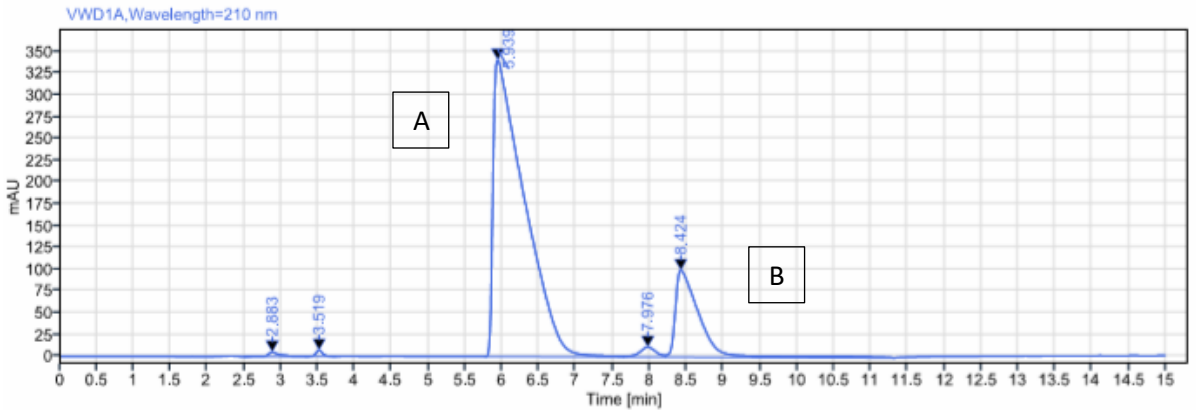

Figure 7.8: Chromatogram of 100 mM standard of succinic acid standard. Succinic acid (A) and 10 mM IS Methylmalonic Acid (B). Chromatogram is representative of one of n=3.

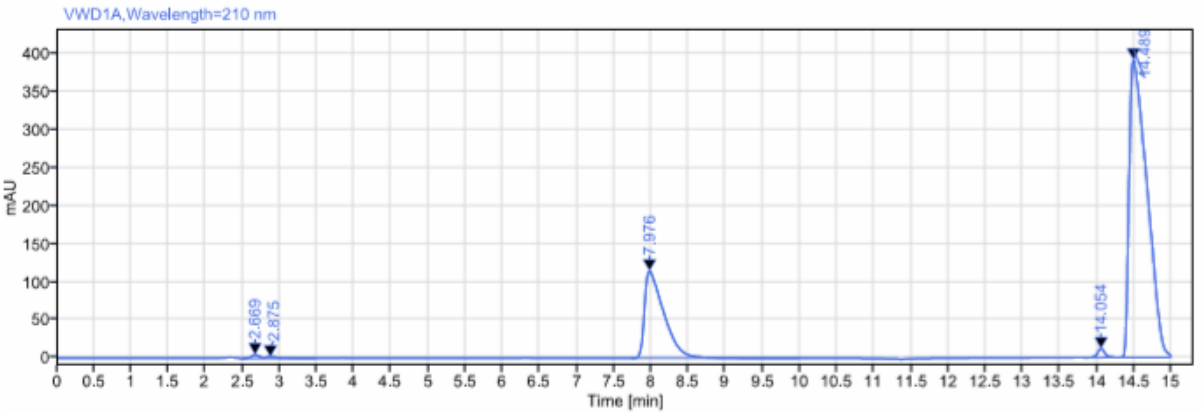

Figure 7.9: Chromatogram of 100 mM standard of butyric acid standard. Butyric acid (A) and 10 mM IS Methylmalonic Acid (B). Chromatogram is representative of one of n=3.

All tested organic acids were used to spike the fermentate samples. An increase in peak size of any peaks already present indicated that the associated organic acid was present within the fermentate and produced by *B. longum* ITT 13. An introduction of a new peak to the chromatogram indicated that the associated acid was not produced by *B. longum* ITT 13.

Appendix C

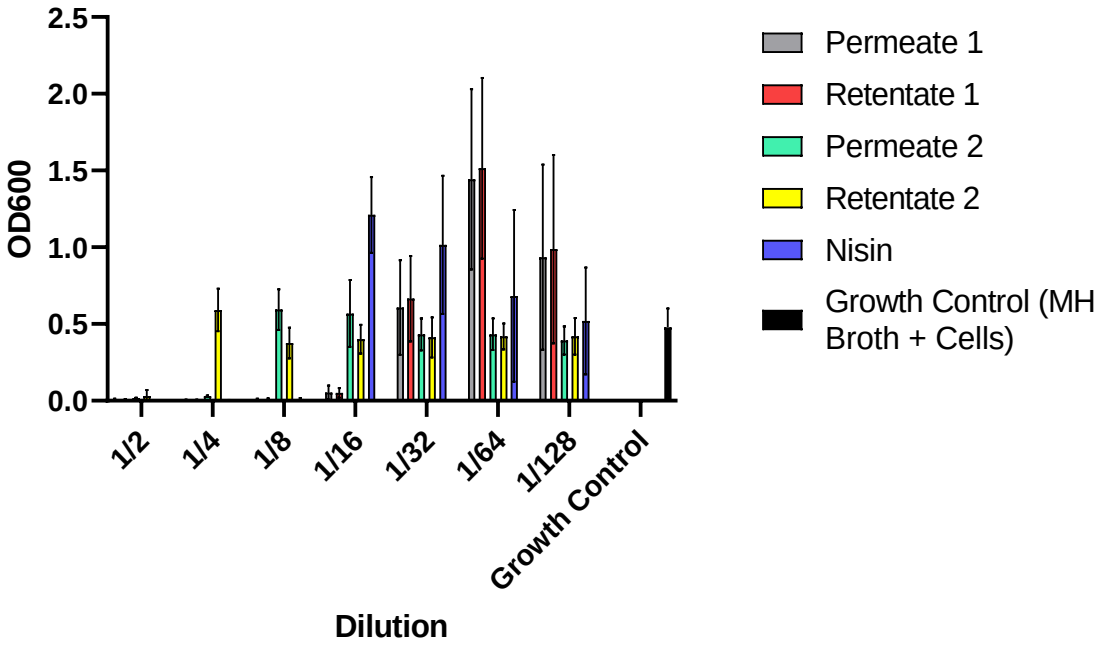

Figure 7.10: The growth (OD600) of *S. xylosus* ATCC 29971 and MIC_{50} determination of the *B. longum* ITT 13 permeates and retentates grown in MRSc broth after Crossflow concentration (Run 1). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition in comparison to the growth control. The AU/ml was determined as described in Section 2.8.2. Standard deviations and averages were determined for three independent assays (n=3).

Figure 7.10 details the growth of *S. xylosus* ATCC 29971 in the presence of the various samples from the Crossflow run 1 of the *B. longum* ITT 13 fermentate generated in MRSc broth. The growth control consisting of *S. xylosus* cells in MH broth had an average OD600 value of 0.48, indicating 100% growth in this medium and anything with an OD600 of less than 50% of this growth control was taken as the MIC_{50} value. Permeate 1 and retentate 1 generated from the first pass through the Crossflow were tested for inhibitory activity, along with permeate 2 and retentate 2 that were generated from the buffer exchange using 50 mM NaAc buffer at pH 4.7. It is evident from the graph in Figure 7.10 that the MIC_{50} for permeate 1 and retentate 1 was a 1/16 dilution with antimicrobial activity of 160 AU/ml. For Permeate 2, the MIC_{50} value was

generated from the 1/4 dilution, with the equivalent antimicrobial activity of 40 AU/ml, while for Retentate 2, it was lower with an MIC_{50} value of a 1/2 dilution equivalent to 20 AU/ml. Nisin as a positive control displayed complete inhibition to a 1/8 dilution, with no antimicrobial activity then observed beyond this dilution. The Permeate 1 and Retentate 1 samples displayed the highest amount of growth by *S. xylosus* beyond their MIC_{50} values in comparison to the Permeate 2, Retentate 2 and nisin samples, which is likely attributed to the presence of nutrients in the original MRSc fermentation broth, as the MRSc broth has been exchanged for the 50 mM NaAc buffer pH 4.7 in Permeate 2 and Retentate 2.

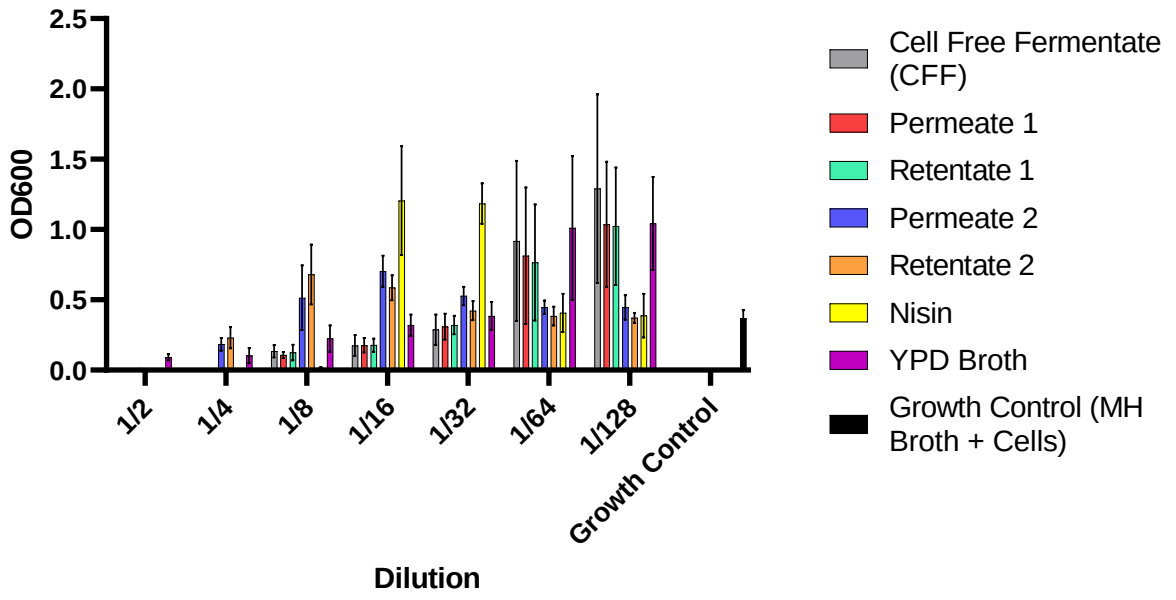

Figure 7.11: The growth (OD600) of *S. xylosus* ATCC 29971 and MIC_{50} determination of the *B. longum* ITT 13 permeates and retentates grown in modified YPD broth after Crossflow concentration (Run 2). The MIC_{50} was determined as the highest dilution displaying at least 50% inhibition in comparison to the growth control. The AU/ml value was determined as described in Section 2.8.2. Standard deviations were determined for three independent assays (n=3).

The growth control of MH broth and *S. xylosus* cells for this assay had an average OD600 of 0.37 and the MIC_{50} for all samples was determined as the dilution with at least 50% inhibition

in comparison to the growth control. The mYPD media was also diluted to 1/128 and assessed using this assay for any inhibition, as it had resulted in small potential zones of inhibition on the microdiffusion assay. Based on the average OD600 values, the fermentate had an MIC_{50} of a 1/16 dilution with antimicrobial activity of 160 AU/ml, Permeate 1 also had an MIC_{50} value of a 1/16 dilution and 160 AU/ml, as did Retentate 1. Similar to the previous Crossflow concentration with MRSc broth, the Permeate 2 had an MIC_{50} value of a 1/4 dilution and 40 AU/ml, while Retentate 2 displayed an MIC_{50} for the 1/2 dilution and 20 AU/ml. The mYPD broth displayed an MIC_{50} of a 1/4 dilution and antimicrobial activity of 40 AU/ml, however although it did have an MIC_{50} value, the broth did not completely inhibit the growth of *S. xylosus* ATCC 29971 at any dilution, unlike the Fermentate, Permeate and Retentate samples which all generated complete inhibition at both the 1/2 and/or 1/4 dilutions. In agreement with previous results, nisin displayed complete inhibition to a 1/8 dilution, which was the MIC_{50} value and indicated 80 AU/ml, with complete growth detected with dilutions beyond the 1/8 dilution. The growth of *S. xylosus* in the presence of nisin after the 1/8 dilution decreases with each subsequent dilution, however this pattern has been observed in all other graphs containing nisin diluted to this extent.