Ollscoil na hÉireann, Corcaigh

National University of Ireland, Cork

LAB as probiotics and silage inoculants for enteric methane inhibition in ruminants

Thesis presented by

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for the degree of

Doctor of Philosophy

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2022

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Acknowledgements

This work would not have been possible without the financial support from the sponsors of the MethLAB project, FACCE-ERAgas. I want to thank my supervisors, Catherine Stanton, Paul Ross, and Colin Hill, for the opportunity to do the PhD and for their guidance throughout the years. I value all the academic and technical advice you provided me with and sincerely appreciate your mentorship.

Much appreciation for my family, particularly my mom, for her unwavering confidence in me and to my siblings for their endless love, optimism and support throughout the years, especially Sonto. Your inexhaustible faith in me has enabled me to achieve much. My husband, Mark, the last few months have been emotionally, intellectually and physically challenging, but your support made all the difference. Much appreciation to friends and extended family for their encouragement and continued interest in my study progress. Moreover, to Martin and Margaret for proofreading my chapters.

I want to extend my gratitude to the past and present members of APC 2 and Lab 64 especially Natasha for their willingness to help and share knowledge. My sincere thanks to Dr Paula O'Connor for peptide purification-related work, Dr Kiera Healy for helping me with the bioinformatics when I had no idea where to begin, and Dr Ghjuvan Grimaud for assistance with statistics for metabolomics and methane analysis work. Special thanks to the farm staff for helping us with sample collection for our silage and animal trials.

And to everybody else whom I may have missed here but who has contributed significantly in my life and in completing this PhD, your assistance is gratefully acknowledged. Most importantly, my sincerest gratitude to my God, the creator of this world, who bestowed resources, strength, and knowledge to enable me to achieve this milestone in my life.

Declaration

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

Contributions

Chapter 3.

Ms Natasha Doyle carried out part of the LAB isolation.

Dr Paula O' Connor performed mass spectrometry.

Chapter 4.

Mass spectrometry and peptide purification were performed by Dr Paula O' Connor.

Mr Gareth Gillen performed GC methane analysis.

Chapter 5

Dr Michael Egan and Dr Pat Dillon were responsible for the silage inoculation.

Ms Helen Slattery analysed lactate using HPLC.

Chapter 6.

Mr Micheal O'Donovan and Dr Michael Egan were responsible for conducting the animal trial, and methane measurements from the ruminants.

Dr Deidre Hennessy and Mr Michael Kennedy were responsible for rumen fluid collection.

Ms. Fatma Koc ran the GC for VFA analysis.

Dr Kiera Healy performed part of the bioinformatics analysis of 16S rRNA sequencing data and generated corresponding graphs.

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Signed: _____

List of Publications

Book Chapters

Mbandlwa, P., Doyle, N., Hill, C., Stanton, C., and Ross, R. P. (2022). Bacteriocins: Novel Applications in Food, and Human and Animal Health. In *Encyclopedia of Dairy Sciences* (pp. 46-54). https://doi.org/10.1016/b978-0-08-100596-5.23030-8

Doyle, N*., **Mbandlwa, P***., Leahy, S., Attwood, G., Kelly, B., Hill, C., Ross, R. P., and Stanton, C. (2021). The use of feed supplements to reduce livestock greenhouse gas emissions: direct-fed microbials. In Reducing greenhouse gas emissions from livestock production (pp. 261-286). https://doi.org/10.19103/as.2020.0077.14

Reviews

Doyle, N*., **Mbandlwa, P***., Kelly, W. J., Attwood, G., Li, Y., Ross, R. P., Stanton, C., and Leahy, S. (2019). Use of Lactic Acid Bacteria to Reduce Methane Production in Ruminants, a Critical Review. *Frontiers in Microbiology*, 10, 2207. https://doi.org/10.3389/fmicb.2019.02207.

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Abbreviations and Acronyms

AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSH	Bile salt hydrolase
BLAST	Basic local alignment search tool
CFU	Colony forming unit
cDNA	Complimentary deoxyribonucleic acid
CH_4	Methane
CO_2	Carbon dioxide
CoA	Coenzyme A
CoB	Coenzyme B
CoM	Coenzyme M
CP	Crude protein
CT	Cycle threshold
DFM	Direct-Fed Microbial
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
EFSA	European food safety authority
EPS	Exopolysaccharide
Fdx	Ferredoxin
Ftr	Formylmethanofuran
FID	Flame ionisation detector
GIT	Gastrointestinal tract
GWP	Global warming potential
GC	Gas chromatography
GHG	Green house gases
HPLC	High presuure liquid chromatography
H_4MPT	Tetrahydromethanopterin
H_2	Hydrogen

IPCC	Intergovernmental Panel on Climate Change
LAB	Lactic acid bacteria
MFR	Methanofuran
MCR	Methyl coenzyme M reductase
NCBI	National Center for Biotechnology Information
NADH	Nucleotide adenine diphosphate hydrogenase
NH3-N	Ammonia
PBS	Phosphate buffered saline
p-value	probability value
PCR	Polymerase chain reaction
PCoA	Principal coordinates analysis
qPCR	Quantitative polymerase chain reaction
RPM	Revolutions per minute
RF	Rumen fluid
SF_6	Sulphur hexafluoride
OD	Optical Density
OM	Organic Matter
VFAs	Volatile fatty acids
WGS	Whole Genome Sequencing

Thesis Abstract

This thesis describes how *Lactobacillus kimchicus*, isolated from the faeces of the great tit bird (*Parus major*) was assessed as a potential probiotic for birds, using various *in vitro* and *in silico* tests. Secondly, a biobank of lactic acid bacteria (LAB) were isolated from the ruminant environment, characterised and tested for bacteriocin production and potential to reduce enteric methane production *in vitro*. Commercial bacteriocin-producing LAB strains were used to create silage as a delivery mechanism to reduce enteric methane production in ruminants *in vivo*. Overall, the results show that the ruminant environment is a good source of LAB, which produce novel antimicrobial agents, some with methane inhibition properties. It also demonstrated that using bacteriocin-producing LAB as co-inoculants can produce silage of superior quality with potential to reduce methane production in dairy cows.

Chapter one describes LAB probiotics, their functionality and exclusion criteria. It details their application as direct-fed microbials (DFMs) and methane inhibitors. The role of LAB as silage inoculants and the role they play in different stages of fermentation is described. Methane is a notorious green house gas (GHG) which is primarily produced as a result of enteric fermentation by ruminants. Methanogens the sole producers of methane have different orders and have a synergistic relationship with other microbes in the rumen. Understanding this relationship between different microbes and metabolites in the rumen can help in designing strategies that reduce methane emissions.

Chapter two describes the isolation of a novel *Lactobacillus* and assessment of the identified strain for its probiotic potential using *in vitro* and *in silico* methods. The novel *L. kimchicus* 5.1 was characterised as a homofermentative, non-EPS producing LAB and subsequently tested for its ability to grow in conditions simulating the gastrointestinal tract (GIT) of birds. Whole-genome sequencing (WGS) was used to examine the antimicrobial resistance profile, screen for bacteriocins genes, and investigate the presence of fermentation machinery in this strain. WGS confirmed the phenotypic antimicrobial resistance results, and *L. kimchicus* 5.1 showed borderline resistance to streptomycin. However, there were no genes found conferring resistance to this antibiotic. *In silico* screening for potential bacteriocin genes was also performed, which found that a bacteriocin-encoding locus for leuococin A was present. Its amenability to freeze-drying and stability under different temperatures are essential characteristics for a potential probiotic. This strain maintained stability (~1×10^{10} CFU/g) at 20°C and 4°C for six weeks, comparable to *L. rhamnosus* GG (APC 2947). Indigenous origin

probiotic strains may have an advantage over general non-indigenous strains. These can better tolerate conditions posed by GIT, such as acidic and high bile salts levels, possibly because they are derived directly from the host's microbiota.

Chapter three outlines the screening for LAB and the characterisation of the produced antimicrobial compounds. Lactic minimal media (LMM) was used to isolate different LAB strains. Bacteriocin characterisation methods involved using cell-free supernatants (CFS) to inhibit *Lactobacillus bulgaricus delbrueckii* ssp. *bulgaricus* LMG 6901 in well diffusion assays. The CFS were assayed under various conditions, such as investigating their antimicrobial activity under different temperatures (37-121°C), inhibiting indicator pathogens, and tested for protease sensitivity. The majority of LAB isolated were *Lactobacillus* spp. The LAB produced bacteriocins of different sizes, with MS spectrometry showing peptides ranging from 1101.36 Da produced by *L. plantarum* (L6) to 6310 Da produced by *Enterococcus thailandicus* (L16). *Lactobacillus paracasei* (D1) exhibited strong inhibition against Gram-negative bacteria (*Escherichia coli and Enterobacter cloacae*), while *Pediococcus acidilactici* (D12) inhibited Gram-positive and Gram-negative bacteria. *E. thailandicus* (L16), an isolate that showed consistently better activity units compared to the other isolates, was subjected to *in silico* bacteriocin screening, and this was found to have genes coding for enterocin and uberolysin.

In Chapter 4, bacteriocins, in addition to LAB isolates from Chapter three and other bacteria from the APC culture collection and commercially available strains, were tested for their ability to reduce methane *in vitro*. Fluorescence microscopy was used to confirm the growth and purity of *Methanobrevibacter sp*. and *Methanosarcina barkeri* based on the presence of coenzyme F420, which is a diffusible enzyme that fluoresces when in its excitation state at 420 nm. CFS from LAB strains and purified bacteriocins (nukacin 2922 and lacticin 3147) were tested against actively growing pure methanogen cultures (*M. ruminantium*, *M. boviskoreani* and *M. gottschalkii*). Headspace gas chromatography showed that *Lactobacillus plantarum* (LP58) could significantly inhibit methane production in *M. gottschalkii* and not in *M. ruminantium.* Purified bacteriocins nukacin 2922 and lacticin 3147 did not significantly reduce methane in the methanogens tested. The results confirmed that CFS from LAB reduces methane production *in vitro*.

Chapter five expands on bacteriocin-producing *L. plantarum* (LP58) and *Lactococcus lactis* (SL242) as silage inoculants and quality assessment of the resulting silage. Two silage production trials were performed. The first was a lab-scale silage trial where *L. plantarum* (LP58) and *L. lactis* (SL242) were used separately. Results from this trial were used to inform decisions on creating the subsequent large-scale silage with *L. plantarum* (LP58) and *L. lactis* (SL242) as co-inoculants. The fermentative and nutritive properties of silage were tested on both occasions. The results of the lab-scale trial revealed the benefits of using *L. plantarum* (LP58) in silage, such as better reduction of silage pH as shown by lower final pH, while *L. lactis* (SL242) remained viable longer and exhibited higher colony counts after three months of fermentation. Using these organisms as co-inoculants improved the fermentation of the baled grass, as shown by the sustained proliferation of LAB throughout the fermentation period and significantly higher levels of lactic acid (P= 0.0034) compared to the untreated control silage.

Chapter six outlines an animal feeding trial using the LAB inoculated silage on lactating dairy cows to modify rumen function and reduce enteric methane production. An animal trial of 30 dairy cows (n=15) fed LAB-treated silage, and untreated control silage was conducted over seven weeks. Based on the Dry matter intake (DMI) of 15-18 kg per cow, each cow ingested 10^{11}-10^{12} CFU of LAB per day. Six cannulated cows (n=3) were tested for rumen modification by examining changes in rumen pH, lactic acid concentration, volatile fatty acids (VFAs), and microbiome analysis using 16S rRNA sequencing and methanogen quantification via qPCR. Individual methane production was measured using Greenfeed on the remaining 24 cows (n=12). After four weeks, cows fed LAB-treated silage produced significantly less methane than those on the control diet ($P < 0.05$). LAB did not cause significant changes to the methanogen population copy numbers as *Methanobrevibacter* DNA copy numbers ranged from 10^5- 10^7 in the control and 10^5- 10^8 per ml of ruminal fluid in the treatment group. Although LAB addition did not significantly impact total SCFA production between groups, there was a significant increase in the amount of acetate and butyrate produced in week seven compared to week one in the treatment group. Acetate increased from 25.01 in week one to 28.62 µmol/ml in week seven, whereas butyrate increased from 8.9 to 10.73 µmol/ml ($P<0.05$). This study provides insight into the efficiency of LAB as a potential mitigator of enteric methane. Overall, this thesis expands on the current knowledge of using LAB probiotics for animal use; and demonstrates the effectiveness of bacteriocin-producing LAB at inhibiting pathogens, creating good quality silage, and reducing methane production *in vitro* and *in vivo*.

Chapter 1.

Part 1: LAB as animal probiotics and silage inoculants.

Abstract

It has become increasingly clear that the GIT of birds and ruminants plays an important role in host health and nutrition as it harbours a variety of microbes. Probiotics have been used as alternatives to antibiotics as growth promoters and to facilitate good health through pathogen inhibition accomplished by organic acids or bacteriocins. Bacteriocins have been preferred over other antimicrobials because of minimal risk of microbial resistance. The most commonly used probiotics belong to the LAB. The exclusion criteria for probiotics safeguards which species can be included to this group based on their genetic content and safety. The many applications of LAB probiotics range from methane inhibition to animal feed and silage inoculants. Silage inoculation is a potential route of administration of LAB bacteriocins in ruminant production systems and can result in the reduction of enteric methane emissions.

The Gastrointestinal tract

The gastrointestinal (GI) tract has the most extensively exposed surface in the body (Yegani and Korver, 2008), and its development and health are crucial to the productivity of animals and birds (Gauthier, 2002). The GI tract harbours a complex and diverse community of microorganisms with various mechanisms influencing host functionality. The microbial communities within the GIT of different animals have co-evolved with their host to form a mutualistic relationship (Apajalahti, 2005; Hillman et al., 2017). The gut microbiota extract energy from feed and, in turn, synthesize vitamins such as the vitamin B group (folate, thiamine, biotin, pyridoxine, cobalamin, riboflavin, nicotinic acid, and pantothenic acid), vitamin K, ion absorption (iron, calcium and magnesium), and amino acids (Rowland et al., 2018). The bacteria also contribute to the production of VFAs, organic acids and antimicrobial compounds such as bacteriocins, which provide nutrition and protection to the host. In ruminants, the GIT is key to nutrient absorption, metabolism, and immune system development. Therefore, the state of the GIT influences growth and milk production (Zhang et al., 2021). In birds, the GIT also functions in nutrient production, and the gut microbiome inhibits the growth and establishment of pathogens by attaching to the epithelial cell walls and forming a protective barrier (Yegani and Korver, 2008) and is involved in host immune maturation (Aruwa et al., 2021). These factors can determine productivity, impacting meat and egg quality (Celi et al., 2017). The central role of gut microbiota in metabolism and health has motivated research into identifying specific microbes involved in different symbiotic host interactions. Traditional culture-based methods that are selective for cultivable microbes have typically been used to study the gut microbiome. Such methods are highly selective and represent only a portion of the gut microbiome (Shang et al., 2018). However, advances in next-generation DNA sequencing techniques have enabled for better understanding of the composition of the microbial populations inhabiting the GIT.

Probiotics

In animal nutrition, the DFMs and probiotics are used interchangeably, but their applications may differ (McAllister et al., 2011). The FDA stipulates that DFMs should be feed products that contain only one source of live naturally existing microorganisms (Brashears et al., 2005), while probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host,” according to the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO/WHO, 2001). Probiotics

are used in pharmaceuticals, livestock production and food. The European Union has in "Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition", banned the use of antibiotics growth promoters in animal production since 2006 (Markowiak and Slizewska, 2018). The ban was in response to the challenge of antibiotic resistance in both animals and consumers of animal products. Due to the ban on growth-promoting usage of antibiotics, DFMs have been considered antimicrobial alternatives for use in the farming and livestock industries (Ban and Guan, 2021). Probiotic use has been proposed as an alternative to antibiotic growth promoters in the livestock and poultry industries. Available probiotics include various microorganisms, mainly of bacterial origin or yeast. These provide a safe and healthy approach to improving animal feed, increasing livestock productivity, and improving health and general well-being (Lambo et al., 2021). This literature review focuses on bacterial strains as animal probiotics.

Health benefits conferred by probiotics may be local in the GIT or exerted systemically throughout the host's body (Figure 1.1). Locally, probiotics may benefit the host by increasing the abundance of beneficial microorganisms in the intestine and inhibiting the proliferation of pathogens and their associated toxins. They may enhance the production of enzymes that contribute to the digestion and absorption of nutrients in the intestinal epithelial cells (IEC) and refine the morphology of IEC (Ding et al., 2021).

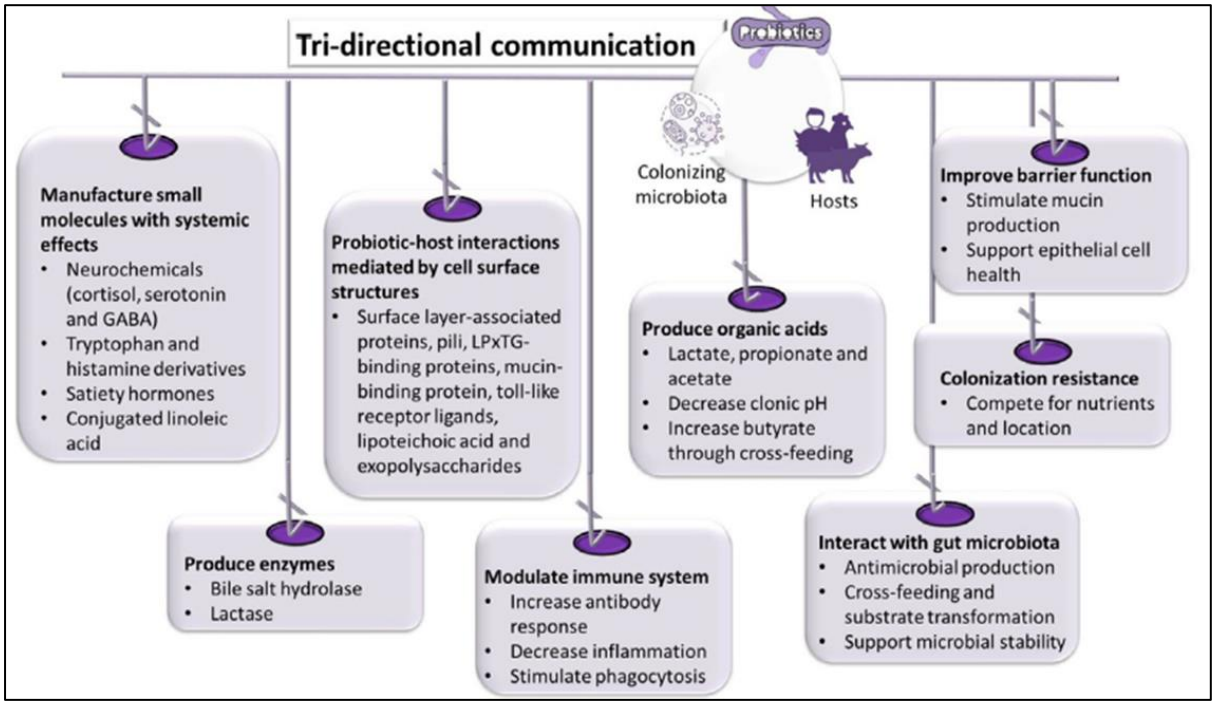

Figure 1.1: Benefits conferred by probiotics to host (Abd El-Hack et al., 2020).

Multistrain probiotics contain more than one strain of the same species, and multispecies probiotics refer to probiotic preparations comprising strains that belong to one or more genera (Kwoji et al., 2021). Probiotic products may contain more than one selected microbial strain based on the assumption that the functionality of a multistrain or multispecies probiotic could be more effective and efficient compared to a single strain probiotic (Timmerman et al., 2004). The benefits exhibited by probiotics are usually strain-specific and host-specific; using a combination of probiotics with different mechanisms of action could amplify their efficacy due to synergistic or additive effects between the strains (Musa et al., 2009). A meta-analysis by Signorini et al. (2012), which analysed the impact of LAB probiotic administration on the health and faecal microbiota of young calves, found that a protective probiotic effect was achieved in trials that used multistrain inocula compared to a single strain in livestock. Similarly, a systemic review by Leão et al. (2021) showed that there was an increase in feed intake by broiler chickens when multiple bacterial probiotic LAB strains were used (SMD = 0.67, 95% CI = 0.22–1.12; P = 0.03) and a decrease when a single strain was used (SMD = − 0.62, 95% CI = − 1.18 to − 0.06; P = 0.03).

Benefits of probiotics in animals

- Increase in the growth rate of the host.
- Protection against pathogens.
- Better immune response.
- Enhance feed intake and weight gain.

Fesseha et al. (2021) investigated the effects of probiotic strain, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* on the growth performance of 120-day-old Sasso dual-purpose chickens. This study revealed that the chickens supplemented with *Lactobacillus* probiotics during the first week exhibited higher body weight than the control ($p < 0.05$). The feed intake of week one of birds fed a diet supplemented with 4 g probiotics and birds fed a diet supplemented with 2 g probiotics were significantly higher ($p< 0.05$) than the control diet with no probiotics. Overall, the results from this study suggest that *L. paracasei* and *L. rhamnosus* positively affect the growth performance of the chickens. Similarly, Timmerman et al. (2004) studied the impact on health and growth upon feeding veal calves with milk replacers supplemented with multispecies probiotics of human origin and calf-specific probiotics or milk

replacement with no probiotic. Probiotic administration increased the growth rate during the first two weeks of the trial. It was concluded from this study that probiotic supply reduced the need for antibiotic treatments for digestive and respiratory diseases.

Although the exact mechanisms by which bacterial probiotics exert their benefits are yet to be fully understood, probiotics confer benefits through three primary pathways (Lambo et al., 2021). These are (i) Competitive exclusion: The probiotic strain competes with pathogens for nutrition sources or adhesion sites. A probiotic strain that establishes itself after ingestion may accomplish the barrier effect, a condition in the intestinal tract that protects the host from colonisation by external microbes. Furthermore, adhesion to epithelial sites would enhance colonisation and reduce the need for daily probiotic feeding. (ii) Bacterial antagonism: Primarily achieved by producing antimicrobial agents such as bacteriocins, organic acids, hydrogen peroxide, and other low-molecular-weight metabolites (Brashears et al., 2005). (iii) Immune system stimulation by activating macrophages, antigen-specific cytotoxic T-lymphocytes, natural killer (NK) cells, and releasing several cytokines in a strain-specific and dose-dependent manner (Ashraf and Shah, 2014).

A selection criterion is employed before an isolated strain is characterised as a probiotic (Stanton et al., 1998). When selecting a potential probiotic strain, special requirements include the ability of the strain to survive passage through the GIT, as well as processing, and having the capacity to survive and grow during storage and delivery to the host. Probiotics should be safe for use in animals, be genetically stable, produce antimicrobial substances such as bacteriocins, and be antagonistic against pathogenic or carcinogenic organisms (Yi et al., 2020). Probiotics should not carry any transferable antibiotic resistance genes. Criteria categorised under safety, functionality and technological amenability are summarised below.

Safety

- Preferably, it should be of animal origin if it is to be used as an animal probiotic (Sornplag and Piyadeatsoontorn, 2016).
- The individual from which it is isolated should be healthy.
- Antibiotic resistance genes in transposable elements should be absent.
- It should be accurately characterised phenotypically and genetically.

- There should be no evidence of adverse effects or association with infectious disease and it should have an established history of biosafety and health benefits.

Functionality

- Should be able to inhabit the GIT competitively.
- Capable of survival and maintaining metabolic activity in the target site.
- It should be resistant to bile salts and stomach enzymes and withstand acidic pH.

Technological amenability

- High biomass productivity (10^6- 10^8 CFU per gram), generally an increase in concentration, results in a more effective probiotic.
- It should be genetically stable and maintain the desired properties during manufacture and freeze-drying.
- It should survive under different conditions as the finished product and be resistant to bacteriophages.
- The potential probiotic should have acceptable sensory properties.

Applications of probiotics

Methane inhibition in ruminants

Since DFMs are already exploited for their ability to enhance the productivity and health of ruminant livestock, they are promising candidates that are being explored as a possible option to reduce enteric methane production. It is proposed that probiotics may reduce methane production by increasing butyrate or propionate production as these similarly to methanogenesis, are H_2 utilising pathways (Kataria, 2016). There is a shift in research, which is focusing on selecting LAB probiotic strains specifically for methane-reducing ability in addition to improving ruminant health and productivity. This topic is covered further in-depth in our publication Doyle et al. (2019) in supplementary material.

Avian probiotics

Bird diversity is a central component of biodiversity, which plays a significant role in ecological balance. Birds (Aves) have received attention because they have a critical role in

ecology as they control pests and act as pollinators; however, they are also viewed as potential vectors of diseases that could be transmitted to humans (Benskin et al., 2009). Human-induced pressures have resulted in a global decline in bird populations (Liu et al., 2019). Gut microbiomes are central to the host's health because they are essential in many biochemical and physiological functions such as maturation of the gut immune system, vitamin synthesis and nutrient metabolism (Liu et al., 2019). The complex microbial communities in bird guts enable birds to sustain their ecological and biological roles. Gut bacteria facilitate the detoxification of food items permitting bird hosts to feed on otherwise toxic diets. In flesh-eating wild birds such as owls, the gut microbiome protects the host from microbial pathogens in decomposing flesh (Bodawatta et al., 2021). Benskin et al. (2009) summarised the various enteropathogens prevalent in various birds, including *Salmonella*, *Campylobacter*, *Klebsiella*, *Yersinia* and *Listeria spp*. A large proportion of research in avian probiotics has been conducted in poultry, with very few studies focusing on wild birds, and as such, dietary functions of specific bacterial symbionts are fundamentally unknown (Heidenreich, 2012). For a probiotic to be effective, it should withstand the different conditions in the GIT of birds, as shown in Table 1.1. LAB probiotics stimulate the proliferation of intestinal epithelium and regulate the mucosal barrier formed by mucin in the small intestine of birds (Abd El-Hack et al., 2020). Probiotic administration to birds has also been associated with increased villus height, increased nutrient absorption, and growth performance. Increased knowledge about the composition of avian gut microbiomes can help develop targeted probiotics for different bird species.

Table 1.1: The pH and mean duration of the transit time of feed in the different compartments of broiler gut (Gauthier, 2002).

GIT Compartment	**Duration/ Transit time (min)**	**pH**
Crop	50	5.5
Proventriculus and gizzard	90	2.5 - 3.5
Duodenum	5-8	5 - 6
Jejunum	20-30	6.5 - 7
Ileum	50-70	7-7.5
Rectum	25	8

Lactic acid bacteria (LAB)

LAB are so named because they can ferment carbohydrates into lactic acid. They are a group of Gram-positive, non-sporulating, microaerophilic, physiologically related rods and cocci that are ubiquitous and occur naturally in the environment (Ma et al., 2019). These bacteria have been commonly isolated from grass silage, dairy food products and sourdough, among many ecological niches. LAB have traditionally been used as starter cultures in food fermentation, flavour and texture development and as pathogen inhibitors in food and silage (McAuliffe et al., 2001). Many have gained wide acceptance because of their generally regarded as safe (GRAS) status in the USA and have the Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) and the effects they impart as probiotics. Other LAB are exploited because they produce various secondary beneficial metabolites, many of which have antimicrobial activity. Such metabolites include organic acids, specifically lactic acid, hydrogen peroxide, diacetyl, bacteriocins and other low molecular weight compounds, which they may release into their surrounding environment (Henning et al., 2015).

Due to LAB possessing various beneficial properties, it is imperative that these organisms are identified accurately for their correct use in their many applications, whether industrial, agricultural, or medical (Novik et al., 2017). Taxonomically, LAB are found in two phyla, namely Actinobacteria and Firmicutes (Giraffa, 2012). The Actinobacteria group comprises *Atopobium* and *Bifidobacterium* genera with a GC content of 36–46% and 58–61%, respectively (Witkin and Linhares, 2017). Firmicutes comprise genera such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, *Weisella*, *Aerococcus*, and *Alloiococcus*. Firmicutes are the low GC group (Giraffa, 2012). *Lactobacillus* is the most widely studied and used LAB genera (Vieco-Saiz et al., 2019). Generally, LAB are classified based on their phenotypic characteristics. However, results from phenotypic classification may be limited in that they may pose ambiguity, poor reproducibility and may be time-consuming if many LAB are identified (Novik et al., 2017). Therefore, genotypic methods may be preferred over phenotypic characterisation (identification based on cell morphology, utilisation of different substrates, fermentation products and their ability to produce specific enzymes) as these rely on the provision of the appropriate growth conditions and cultivation media. There have been advancements in developing molecular typing for identification and classification of bacteria, with the most powerful being genetic-based molecular methods (Amor et al., 2007).

Genotyping methods also exhibit various levels of differentiation, which can be achieved down to strain or serovar level. The most commonly used tool for molecular identification is 16S rRNA sequencing. The main advantage of molecular-based methods is that they are highly accurate (Donelli et al., 2013). WGS technology is quickly replacing traditional microbial typing and characterisation methods (Sharma et al., 2020). WGS is a powerful tool that provides insight into bacteria at the genomic level, accurately revealing bacterial structure and function (Buron-Moles et al., 2019). WGS helps provide information on the antimicrobial resistance of the LAB strains. Understanding specific genotype-phenotype associations can bring better insights into the function of certain proteins and elucidate the potential roles of particular genes in carbohydrate metabolism. This would permit better exploration and application of technologically useful LAB.

LAB produce adenosine 5'-triphosphate (ATP) primarily through the fermentation of sugars (Abedi et al., 2020). The absence of cytochromes and porphyrins means that these organisms do not use oxygen, although they can grow in aerobic conditions due to the protection conferred by peroxidases against oxygen by-products such as peroxides (Novik et al., 2017). LAB are classified as either homo- or hetero-fermentative based on their final fermentation products. Heterofermentative LAB are further divided into facultatively and obligately fermentative species. Obligately heterofermentative bacteria degrade hexoses using the phosphogluconate pathway resulting in lactate, ethanol or acetic acid and carbon dioxide. LAB, such as *Leuconostoc* and some *Lactobacillus*, degrade hexoses to lactic acid by the Embden–Meyerhof–Parnas (EMP) pathway, in addition to degrading pentoses and often gluconate as they possess both aldolase and phosphoketolases (Felis and Dellaglio, 2015). Obligate homofermentative LAB ferment only hexoses almost completely to lactic acid using glycolysis or EMP pathway. This results in two moles of lactate per mole of glucose. Examples of obligate homofermentative LAB are selected *Lactobacillus*, *Pediococcus*, *Streptococcu*s, and *Lactococcus* (Buron-Moles et al., 2019).

Antibiotic resistance (AR) is a safety issue and becomes a challenge when there is a risk of transfer to other bacteria. Resistance is acquired by gene transfer systems such as conjugative or nonconjugative plasmids or transposons (Gueimonde et al., 2013). The presence of mobile resistance genes means that AR could be transferred through the food chain between food, humans, and the environment. The LAB genus most commonly associated with antibiotic

resistance is *Enterococcus*, especially *Enterococcus faecalis* and *E. faecium* (Brashears et al., 2005). It is vital to carefully assess antibiotic resistance in new strains before including them in products as probiotics. There are several antibiotic resistance gene databases, such as the Comprehensive Antibiotic Resistance Database (CARD), a bioinformatics database of resistance genes, their products and associated phenotypes; and Resfinder, an online resource for identifying antimicrobial resistance genes in whole genome datasets (Florensa et al., 2022).

Bacteriocins

Bacteriocins are proteins or ribosomally synthesised peptides produced by bacteria that kill or inhibit the growth of microorganisms (Gálvez et al., 2010) but are ineffective against the species by which they are produced (Joerger 2001). These peptides can be broad-spectrum, where they possess antimicrobial activity against a diverse range of microorganisms, or narrow-spectrum, where only closely related microorganisms are targeted (Lee et al., 2021). Generally, activity is directed against low-GC Gram-positive species. Mbandlwa et al. (2021) describes in-depth the applications of bacteriocins in food, human and animal health. Bacteriocins from LAB have several properties that make them desirable agents with numerous applications in different disciplines (Kayalvizhi, 2016). Bacteriocins have applications in various industries such as agriculture, pharmaceuticals (de Arauz et al., 2009), veterinary, and aquaculture (Galvez et al., 2010). They have been proposed as alternatives to antibiotic feeding in animals, as additives in cheese ripening (Silva et al., 2018), and as inhibitors of spoilage organisms and pathogens during food production, processing, and distribution (biopreservatives) (Soltani et al., 2021). Bacteriocin-producing organisms have been routinely used in animal health applications to protect animals such as swine and other livestock from gastrointestinal infections (Hernández-González et al., 2021).

The characteristics of bacteriocin peptides include:

i. These peptides are safe since they are not active against the host cell and are non-hazardous, particularly to eukaryotic cells.
ii. They are inactivated by digestive enzymes, thereby causing minimal disturbance of the intestinal microbiota.
iii. Tolerant to industrially relevant conditions such as heat treatment, NaCl and pH change.
iv. Many have broad-spectrum antimicrobial activity against several spoilage microbes and foodborne pathogens (O'Connor et al., 2015).

v. Their genetic determinants are generally encoded by a plasmid, facilitating easier genetic manipulation (Gálvez et al., 2007).

Bacteriocins are abundant and are structurally diverse (Anastasiadou et al., 2008; Mathur et al., 2017; Ryan et al., 1996). Bacteriocins are classified into three major classes according to Cotter et al. (2005). Class 1 are the Lantibiotics, which are the Lanthionine-containing small post-translationally modified peptides. This group comprises small peptides ranging between 19 - 38 amino acids in length and mainly produced by Gram-positive bacteria. Lantibiotics are further subdivided based on their mode of action and structure. Class 2 are the non-lanthionine-containing, unmodified bacteriocins. Class 3 comprises larger, thermolabile peptides, which are greater than 10 kDa. The limitations to the widespread use of bacteriocins are the high manufacturing costs associated with their large-scale production and that they may be susceptible to proteolysis (Jha et al., 2020).

Nukacin

Nukacin is a 27- residue peptide, which belongs to the lacticin 481 group of bacteriocins (Fujinami et al., 2018). There is a linear N-terminal and a globular C-terminal region, that function co-operatively to confer antimicrobial activity and these regions are completely inactive separately (Islam et al., 2009). It is produced by *Staphylococcus warneri* and was initially described as bacteriostatic bacteriocin by Asaduzzaman and colleagues (2009), however, this lantibiotic has more recently been described as possessing both bactericidal and bacteriostatic mechanisms of action (Roy et al., 2014). Nukacin has been investigated in applications against antibiotic resistant bacteria and against causative pathogens of bovine mastitis (Zheng et al., 2018, Ceotto et al., 2010).

Silage

Silaging is the process used to store and preserve green fodder in an enclosed pit or silo and is dependent on the production of adequate amounts of lactic acid to prevent the growth of spoilage microorganisms under anaerobic conditions when sugar is converted to lactic acid (Yang et al., 2016). Ensiling is a low-cost method used to preserve and improve fermentative metabolites in crops. It prohibits the introduction of oxygen, which would encourage the growth of moulds and yeasts (which are a source of mycotoxins) (Acosta 2012), as well as spore-forming and sometimes pathogenic microorganisms including clostridia and Bacillus

(Kharazian et al., 2017). *Escherichia coli* are the main competitors for sugar and sometimes produce the Shiga toxin. *Firmicutes* and *Proteobacteria* are the most abundant epiphytic microbes in silage. LAB represent between 0.1 and 1.0% of the normal epiphytic microbiota and have long been known to result in spontaneous silage fermentation (Kharazian et al., 2017). LAB are commonly used in making silage for animal consumption. Silage inoculants often contain one or more strains of LAB. The most widely used silage inoculants include traditional homofermenters, such as *Lactobacillus plantarum*, *Pediococcus* species, and *Enterococcus faecium*, as well as the heterofermentative bacterium *Lactobacillus buchneri* (Contreras-Govea and Muck, 2006).

Inoculants such as LAB positively influence silage by improving fermentation and dry matter (DM), maximising fermentation, enhancing nutrient recovery, and extending aerobic stability. In the ensiling process, the rapid production of acids and the accompanying decrease in pH brought about by lactic acid fermentation are essential. Bacterial silage inoculants are usually heterofermentative or homofermentative depending on the dominant microbial species and the substrates fermented (Borreani et al., 2018). Homofermentative LAB ferment glucose only lactate only; therefore, minimal DM loss occurs, whereas heterofermentative LAB (*L. buchneri*) ferment one mole of glucose to one mole of carbon dioxide resulting in approximately 24% DM loss. The acidity levels of an ensiled sample are determined by measuring its pH. Lactic acid is the primary acid in silage and is responsible for decreasing pH during fermentation. Other acids in silage include acetic acid, propionic acid and butyric acid (Table 1.3). Having insight into lactate metabolism is important when ruminants are fed silage treated with lactate-producing bacterial inoculants.

The silage-making process is typically divided into six stages (Seglar, 2003) (Table 1.2). The initial short-lived aerobic phase occurs immediately after harvest. This step is followed by the more extended fermentation phase that lasts between seven and 21 days. During this phase, the dominant microorganisms are acid-tolerant LAB, which produces lactic acid and other acids, reducing the pH to a range of 3.8-5.0. The LAB that dominate the early stages of fermentation are lactococci *(Lactococcus lactis*) and enterococci (*E. faecalis*), and some lactobacilli (*L. plantarum* and *L. cellobiosis*) (Ohmomo et al., 2002; Tanaka et al., 2014). The stable storage phase in the silo is a phase of biological inactivity when air is prevented from re-entering the silo, and the final step is the feed-out phase when the silo is opened, and the organic material is exposed to air (Borreani et al., 2018).

Table 1.2: The different phases of silage production (Seglar, 2003).

Phase	Age of silage	PH	Temperature	Dominant microbes	Silage conditions
1. Aerobic	Lasts a few hrs to a day after harvesting.	6-6.5	Ambient temperature	Aerobic spoilage organisms: Yeasts and Bacillus occur in a poorly sealed silo.	There is the depletion of oxygen and water-soluble carbohydrates.
2. Lag phase	1-2 days.	~5	32-28	*Enterobacter*.	Anaerobic condition. Heterofermenters produce lactic acid and acetic acid.
3. Transitional phase	3- 4 days.	5-4	28	Homofermentative lactic acid bacteria.	Silage pH declines below 5.7, a level at which LAB can grow.
4. Stable phase	4-21 days.	4.5-4	28	Homo-fermentative bacteria. Acid-tolerant microbes are in almost an inactive state. Clostridia and Bacilli produce spores.	Conversion of water-soluble carbohydrates into lactic acid by homofermenters.
5. Storage	21 days onward.	4.2-3.8	28	Good quality silage would be heavily colonised by LAB. Sour silage is indicated by the growth of clostridia, which produces butyric acid.	Longer storage times result in quicker degradation of starches in the rumen.
6. Feed out	3 months post-production.	7-4	Ambient	LAB, if silage is well preserved. The presence of yeast and mould indicates spoiled silage.	Aerobic decomposition upon reintroduction of oxygen. If this phase is neglected as a part of fermentation, it can result in a loss of dry matter since oxygen is reintroduced.

Table 1.3: Typical concentrations of common fermentation end products in grass silage (Kung et al., 2018).

End product	**Grass silage**
pH	4.3– 4.7
Lactic acid (%)	6–10
Acetic acid (%)	1–3
Propionic acid (%)	<0.1
Butyric acid (%)	<0.5–1.0
Ethanol (%)	0.5–1.0
NH_3-N (% of total N)	8–12
LAB (CFU/g)	10- 10^6
Spoilage organisms: Yeast and clostridia (CFU/g)	10^3- 10^5
	10^2-10^3

Conclusion

Probiotics such as LAB are useful tools that can modify the animal's gut microbiome and enhance its function, thereby preventing potential disease by inhibiting pathogens by producing metabolites such as bacteriocins. LAB probiotics are effective in several applications, including enhancing weight gain in poultry and livestock, where they are used as DFMs or silage inoculants. New isolates undergo various genomic and phenotypic tests to characterise and qualify them as probiotics. Literature has shown that these organisms can be used in livestock or even birds such as poultry to offer protection from pathogens and increase their growth rate, reduce mortality and enhance productivity. This group of organisms has been widely used in silage production to facilitate fermentation. Silage fermentation is a potential route of administration of LAB bacteriocins in ruminant production systems and can result in the reduction of enteric methane emissions. LAB have facilitated the production of good quality grass-based silages through anaerobic fermentation. Better quality silage means better hygiene and consequently improved animal welfare.

Although studies have demonstrated the advantages of probiotics, in some cases, the exact mechanism by which probiotics confer benefits are yet to be elucidated. Future studies should focus on finding the mechanisms of action and determining the optimal dosage for their efficacy. Their use can also be limited by antimicrobial resistance, which is prevalent in *Enterococcus*; therefore, it is essential to test for such characteristics before use in animal nutrition applications.

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Chapter 1.

Part 2: Methane as a by-product of enteric fermentation.

Abstract

GHGs such as methane contribute to the greenhouse effect by absorbing and re-emitting long-wave radiation back to the earth's surface. The largest source of anthropogenic methane is agriculture, with ruminant livestock producing approximately 3.3 Gt CO_2 equivalents of enteric methane annually. Enteric methane is a by-product of feed fermentation, where methanogens and other rumen microbes work synergistically to stabilise hydrogen pressure in the rumen, after which the ruminant releases the methane into the atmosphere through eructation or flatulence. The nature of the feed determines fermentation products. When rumen microorganisms degrade nutritional components, these are converted into microbial cells, VFAs, and gases as by-products of the fermentation. Various models have been employed to measure or to inhibit methane *in vitro* and *in vivo*, as methane production results in loss of metabolizable energy for the ruminant and contributes to climate change. Understanding the function of the rumen microbes and their interactions with the host could be key to reducing methane emissions from livestock and is instrumental in the attempts to mitigate the climate change crisis.

Methane as a greenhouse Gas

GHGs are atmospheric gases that contribute to the greenhouse effect by absorbing and re-emitting long-wave radiation back to the earth's surface (Kebreab et al., 2006). Since preindustrial times, the planet earth has warmed up by 1.2°C due to the anthropogenic emission of GHGs (Chen et al., 2020), such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and other ozone-depleting substances (Montzka et al., 2011). Climate change is a global threat with negative consequences such as environmental degradation, changes in water quality, negative impact on human health and well-being, and a risk to food security. The introduction of taxes and new regulations suggests an urgent need to reduce greenhouse gas emissions (Stanton et al., 2020). Therefore, considerable research is being conducted to find methods that reduce GHG emissions while supporting sustainable and efficient food production.

Methane (CH_4) is a colourless, odourless gas emitted by natural and anthropogenic sources (Chand et al., 2017). The major sources of atmospheric methane include the combustion of fossil fuels, landfills, oil and natural gas systems, coal mining, natural wetlands, paddy rice fields, and livestock production systems emissions (including intrinsic fermentation and animal waste) (Heilig, 1994). Methane is a highly reactive chemical and more efficient in absorbing infrared radiation than carbon dioxide. Enteric methane is referred to as a Short-Lived Climate Pollutant (SLCP) because of its inability to persist in the atmosphere for an extended time frame (Liu et al., 2021). It has a relatively short atmospheric half-life of 12-15 years compared to the lifetime of CO_2, which is 100 years (Hook et al., 2010). The effect of this higher energy absorption combined with a shorter half-life is reflected in its global warming potential (GWP). It is approximately 21 times more potent than carbon dioxide in terms of its GWP over one century and heats the atmosphere 86 times more efficiently over 20 years (Liu et al., 2021).

Methane contributes to global warming by reacting with hydroxyl radicals in the atmosphere during oxidation, which decreases the amount of hydroxyl radicals available to eliminate other types of air contaminants (Li et al., 2018). Hydroxyl radicals play an important part in the atmosphere's self-cleansing as they naturally react with most air pollutants, convert them into water-soluble compounds, and subsequently remove them. Therefore, they are known as the detergent molecules of the atmosphere since they are cleansing agents (Reidal and Lassey, 2008). Secondly, as methane exits the atmosphere through oxidisation, it forms water vapour and carbon dioxide. Hence, methane indirectly contributes to global warming by releasing

carbon dioxide (Poschl and Shiraiwa, 2015). Methane is emitted directly by livestock and from their manure. The amount of methane emitted by livestock is primarily influenced by the number of ruminants, the type of digestive system, and the type and quantity of feed consumed. Globally, ruminant livestock produce about 3.3 Gt CO_2 equivalents of enteric methane annually (FAO, 2022). In Irish agriculture, one of the significant drivers of the increased emissions in 2020 was the 3.7% increase in national dairy herd size (Buckley and Donnellan, 2021). Lanigan et al. (2019) projected that in the absence of mitigation strategies, emissions from Irish agriculture will increase by 9% in 2030. An increase in dairy herd will be the main driver of this increase. Because enteric methane emissions contribute a significant proportion to Ireland's total GHG, mitigating enteric methane emissions could substantially reduce the national GHG emissions.

Methane production is the terminal step in the anaerobic degradation of organic carbon. Through the concerted efforts of various rumen microbes, enteric methanogens produce methane as an end product of feed fermentation (Danielsson et al., 2017), along with CO_2 and H_2. Methane production by ruminants is influenced by various factors such as the physical and chemical characteristics of the feed, the feeding schedule and the feed additives. Because methane is derived from ingested feed, diet composition and intake can be used to manipulate fermentation by altering the microbial interactions through feed additives. This would also have the potential to impact animal production positively. Manipulating the host's diet may reduce methane emissions by shifting the site of fermentation of organic matter from the rumen to the intestine, consequently diverting the hydrogen away from methane production (McGinn et al., 2004). However, there is a challenge to maintaining a balance between productivity, household food security, and environmental preservation (Wright et al., 2007).

The main electron donor for methanogenesis is hydrogen (H_2), and its availability affects gene expression and regulates key steps in the methanogenesis pathway. Enteric methanogenesis also depends on the availability of ATP derived from the enteric fermentation of rumen anaerobic bacteria. ATP production occurs primarily through hydrogen in the stepwise reduction of carbon dioxide (Mathison et al., 1998). It is a unique and complex form of anaerobic respiration which requires the biosynthesis of several unusual coenzymes, a long multistep pathway for methane and several unique membrane-bound enzyme complexes for coupling to the proton motive force (Hedderich and Whitman, 2006). The three enzymatic methanogenesis pathways (hydrogenotrophic, methylotrophic, and acetoclastic) share three

common steps (Figure 1.2). These steps are the transfer of the methyl group to coenzyme M (CoM-SH), the reduction of methyl-coenzyme M with coenzyme B (CoB-SH), and the recycling of the heterodisulfide CoM-S-S-CoB (Lyu et al., 2018). The chemical reactions for the three methanogenesis pathways are as follows.

Hydrogenotrophic: $4H_2 + CO_2 \rightarrow CH_4 + H_2O$

Acetoclastic: $CH_3COOH \rightarrow CH_4 + CO_2$

Methylotrophic: $CH_3OH + H_2 \rightarrow CH_4 + H_2O$

$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$

$CH_3NH_2 + H_2 \rightarrow CH_4 + NH_3$

Hydrogenotrophic methanogenesis is also referred to as CO_2/H_2 reduction or H_2-dependent methanogenesis (Megonigal et al., 2004). The hydrogenotrophic pathway is the most significant methane-producing pathway within the rumen, with approximately 82% of methane synthesis in the rumen derived from the reduction of CO_2 with H_2 (Yan and Lang, 2019). It is a chemoautotrophic process, and the H_2 is both the source of energy and electrons, while CO_2 is frequently both an electron sink and the source of cellular carbon. It has been suggested that hydrogenotrophic methanogenesis is the ancestral form of methane production (Vanwonterghem et al., 2016). It occurs in multiple environments, from the intestinal tracts of animals extending to anaerobic sediments and hot springs. Hydrogenotrophic methanogens, alongside syntrophic bacteria, degrade acetate more efficiently at high temperatures than acetoclastic methanogens in a shared environment (Goyal et al., 2016). Hydrogenotrophic methanogenesis occurs in *Methanobacteriales*, *Methanosarcinales*, *Methanococcal*e, *Methanomicrobiales* and *Methanopyrales* (Bapteste et al., 2005).

Acetoclastic methanogenesis is a minor methanogenic pathway in the rumen (Gaci et al., 2014). Methanogens that perform this process exclusively are in the order of *Methanosarcinales* (*Methanosarcina* and *Methanosaeta*). These methanogens split acetate to produce carboxyl groups further oxidised to CO_2 (Welte, 2018). Thereafter, the acetoclastic methanogens activate acetate into acetyl-coenzyme A (acetyl-CoA), from which a methyl group is then transferred into the central methanogenic pathway (Fournier and Gogarten, 2008) to produce methane. In methylotrophic methanogenesis, the oxidation of additional methyl-groups to CO_2 provides the electrons required for the reduction of the methyl groups to methane, which proceeds stepwise as the reverse of hydrogenotrophic methanogenesis (Liu and Whitman, 2008).

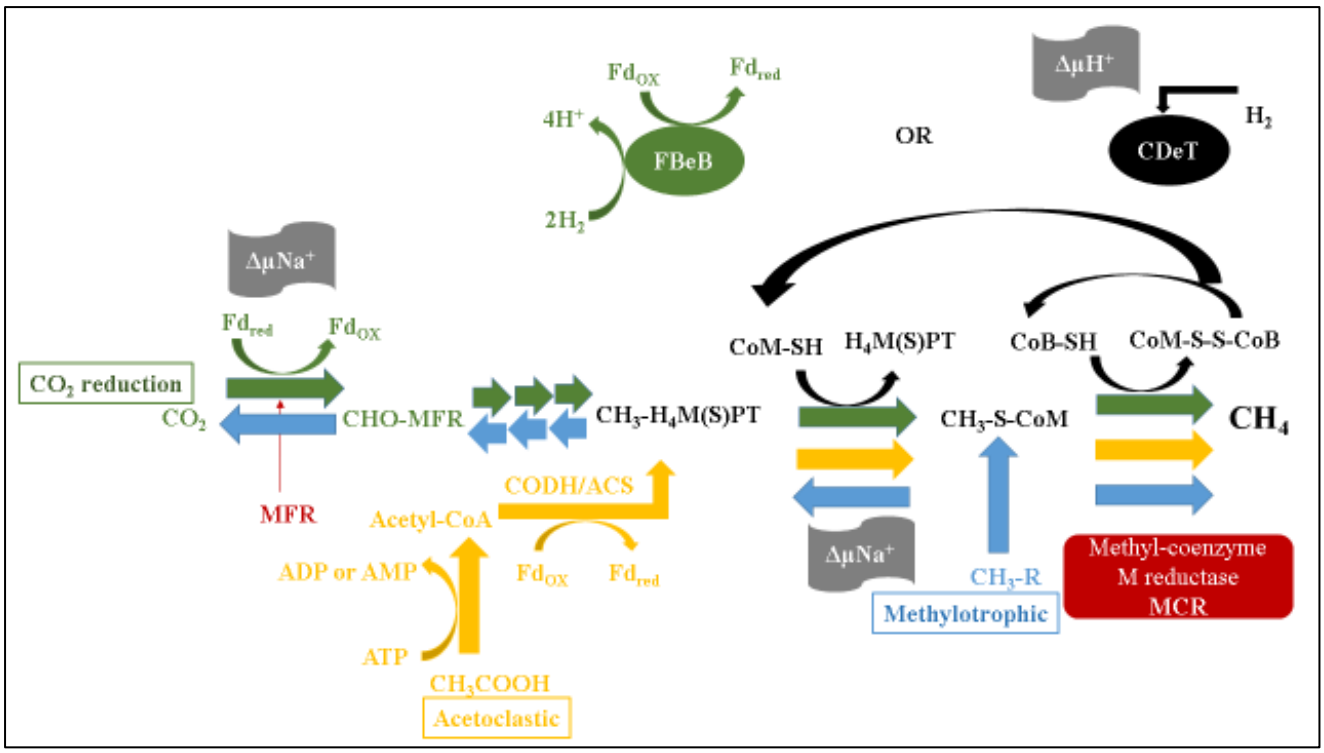

Figure 1.2: The three enzymatic pathways of methanogenesis (Lyu et al., 2018). Reactions in green correspond to the hydrogenotrophic pathway, and reactions in orange represent the acetoclastic pathway. The methylotrophic pathway is shown in blue.

Abbreviations:

MFR: methanofuran, $Fdr_{ed:}$ reduced ferredoxin, Fd_{ox}: oxidized ferredoxin, CHO-MFR: formylmethanofuran, $H_4M(S)PT$: tetrahydrosarcinapterin, CoM-SH: Coenzyme M, H_4MPT: tetrahydromethanopterin, CH_3-$H_4M(S)PT$: Cyclohydrolase; CH_3-R: methyl-containing compounds such as methanol and methanethiol, FBeB: flavin-based electron bifurcation, CDeT: cytochrome-dependent electron transfer, CoB-SH: Coenzyme B, CoM-S-S-COM: heterodisulfide, CODH/ACS: carbon monoxide dehydrogenase/acetyl-CoA synthase complex, $\Delta\mu Na^+$: electrochemical sodium ion gradient, $\Delta\mu H^+$: electrochemical hydrogen potential.

Measurement of Enteric Methane Production

Numerous *in vivo* and *in vitro* methods are used for measuring methane production and emissions. Several *in vitro* methods are presently being used to measure enteric methane production depending on the condition of application. The *in vitro* gas production technique (IVGPT) simulates ruminal fermentation of feed and feedstuffs (Storm et al., 2012). Indigenous rumen microbes ferment the feed or feedstuff while incubated at 39°C for a predetermined

period. The fermentation vessel is supplemented with buffers and minerals in conjunction with ruminal fluid. In IVGTP, the total gas produced is measured, and gas composition is analysed for *in vitro* methane production, which is then reported as methane per gram of dry matter or methane per gram of dry matter degraded (Storm et al., 2012). There are various IVGTP methods, such as closed vessel batch fermentations, the rumen simulation technique (RUSITEC) (Wetzels et al., 2018) and the gas production technique, which involves using graduated syringes (Navarro-Villa et al., 2011).

Methane determination in pure cultures is not as common as IVGTP. Only a few pure culture studies have been reported (Patel et al., 1991; Prins et al., 1972). Some methods include using pure cultures in Balch tubes, according to Balch and Wolfe (1976), which is a method developed for rapid and efficient handling of large numbers of cultures of methanogenic bacteria. In this instance, micromoles of methane produced per tube are calculated from its partial pressure and headspace volume (Salvador et al., 2017).

Measuring and monitoring enteric methane emissions is essential to developing reputable inventories and assessing the efficacy of mitigative strategies (Zhao et al., 2019). Because ruminant production systems are important contributors to anthropogenic methane emissions, it is imperative to have accurate and robust methane measurement techniques in place. Various indirect practical methods to detect methane emissions, such as handheld laser methane detectors, laser technology, and enclosure chambers, are used. These vary in their accuracy. *In vivo* methane measurements are compulsory to refine reduction efforts and pursue global mitigation strategies. The most widely used methods for *in vivo* experiments for cattle are the sulphur hexafluoride (SF_6) tracer technique, respiration chambers (regarded as the gold standard in terms of accuracy but with limited applicability) and the automated head-chamber system Greenfeed (Hristov et al., 2018; Huhtanen et al., 2019).

The Greenfeed emissions monitoring system is an automated head chamber system that measures the production of methane, carbon dioxide, and hydrogen gas mass fluxes from the breath and eructation gas of ruminant animals (Patra, 2016). This is considered a reliable technique for measuring enteric methane emissions. Using the Greenfeed, methane is measured in short increments of time, typically over 3-7 mins, while the animal is receiving food enticement as a reward to stay in the chamber as methane is measured (Zimmerman and

Zimmerman, 2016). Electronically controlled restrictions are imposed on over-enthusiastic ruminant visitors to limit pellet drops to once every four hrs per animal. Typically, cattle visit the Greenfeed system three times or less. Many short-term methane emission values from each animal are measured at different times of the day for a set number of days, and these are aggregated to estimate an average daily methane emission from the animal. As the animal visits the Greenfeed, a fan system attracts air past the animal's muzzle into an intake manifold and through an air collection pipe where continuous airflow rates are measured. Absolute daily production methane measurements are reported in grams per day (Garnett, 2012; Patra, 2016).

When the ruminant visits the Greenfeed unit, it is identified (usually by an ear tag) using a radio frequency identification (RFID) reader, while it receives some pellet concentrate (Figure 1.3) (Hammond et al., 2015). The gas expelled by each animal is extracted into the system. A rotating cup dispenser controlled by a computer is used to dispense the concentrate pellets from above the Greenfeed to encourage the animal to keep a suitable head position for measurements to be correctly and accurately recorded. This system simultaneously measures airflow rate, gas concentration, and the animal's head position. The data is transmitted to a server where it is subsequently processed, enabling the user to access a summarised report of calculated fluxes.

There are several factors to consider when measuring methane using the Greenfeed unit, according to Hristov et al. (2015).

i. The ruminant has to be adapted to the receiving bait for it to approach the system.
ii. The head should be inserted correctly for accurate methane measurement data.
iii. Trained personnel should operate the system, ensuring proper calibration before use.
iv. Sufficient time between animals to collect background methane and carbon dioxide data should be allowed.
v. Enough data should be collected per animal per 24-hr sampling cycle.

One of the advantages of using Greenfeed to the other methane measurement methods is that it is non-invasive and does not cause pain or discomfort to the animal being tested (Hristov et al., 2015). The system can be used in various environments, such as grazing conditions. Additionally, the animal can move freely and is not restricted to the chamber. Furthermore, the analytical equipment is simpler and less expensive than the SF_6 tracer method (Huhtanen et al., 2019). However, the Greenfeed system is also more costly than conventional respiratory

chambers. The drawbacks of using Greenfeed are that they may be cases of unrepresentative sampling, especially in grazing systems where the animals visit the unit voluntarily (Hristov et al., 2015). Enticement feed, typically a pellet composed of grass, vegetable oil, alfalfa, grain concentrates, and molasses, could represent up to 5% of the animal's dry matter intake during a gas measurement event. Greenfeed does not measure methane from the hindgut, which contributes approximately 3% of the total methane emitted. Because the Greenfeed system is a fairly novel invention, more studies are needed to fully understand and appreciate its on-farm implementation (Garnett, 2012). In terms of reliability, when comparing methane measurements from Greenfeed and those from the SF_6 tracer technique, (Hammond et al., 2015) found that generally, methane emissions measured using SF_6 were higher ($P < 0.001$) than Greenfeed (186 *vs* 164 g/d), but significant ($P < 0.01$) concordance was observed between the two methods (0.6017).

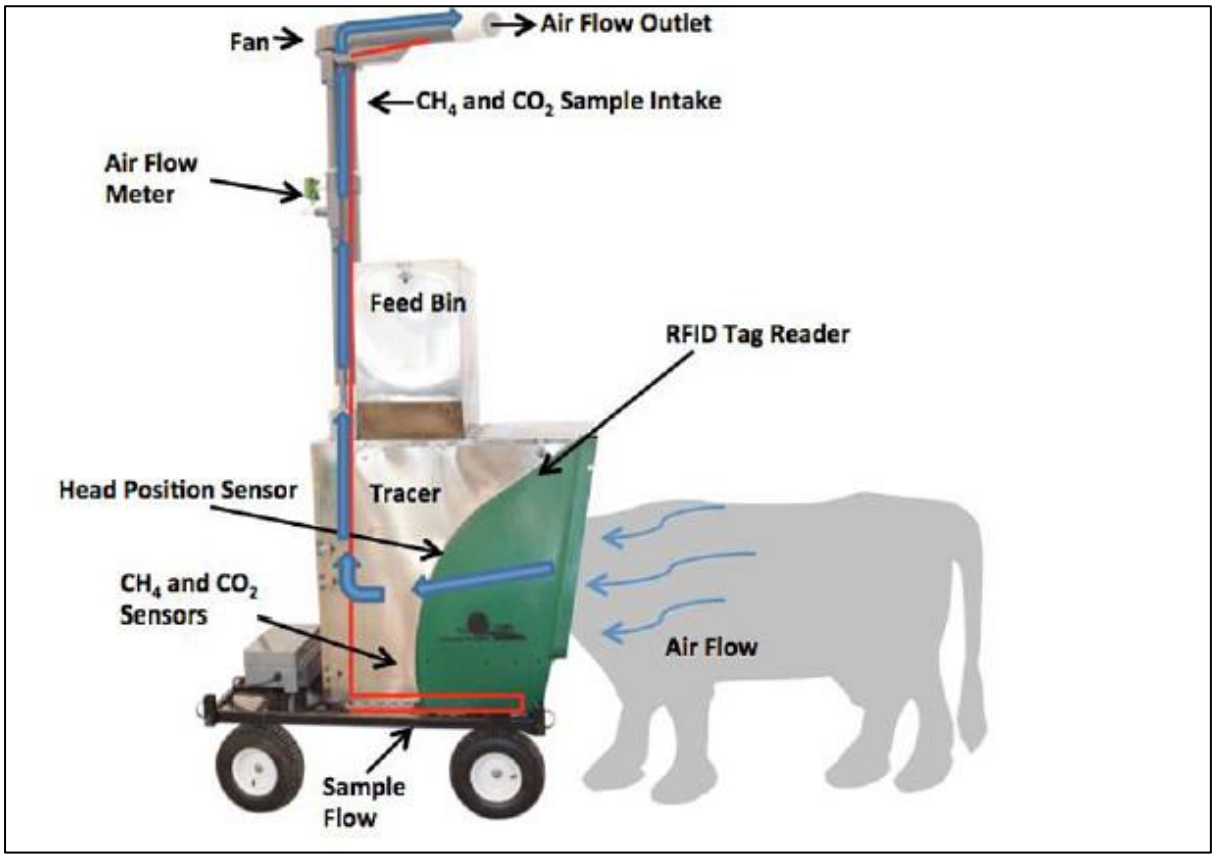

Figure 1.3: Greenfeed components for measuring methane production in ruminant animals (Hristov et al., 2015).

The Rumen

The rumen is the biggest organ of the digestive tract of the ruminant animal. It is an open anaerobic fermenter with discontinuous solid (feed) and liquid (saliva and drinking water) inputs and multiple fractions with different turnover rates. The rumen environment is a product of microbial and host interactions (Amanzougarene and Fondevila, 2020), containing many diverse microbial species. A millilitre of rumen content consists of 10–50 billion bacteria, 1 million protozoa and variable numbers of yeasts and fungi (8%), as well as archaea (0.3-4%) (Chellapandi et al., 2018) and phage (Gilbert et al., 2017). The physiological pH range for an active and healthy rumen is 5.5–7 and is controlled by the large amounts of bicarbonate and phosphate entering the rumen in saliva (Ness and Steiner, 2017). The diverse and complex community of microorganisms in the digestive tract of ruminant livestock profoundly influences the conversion of plant biomass polymers from feed into end products, allowing the host access to otherwise inaccessible nutrients (Cammack et al., 2018). The digestive physiology of ruminants is unique, and ruminants and rumen microbes have co-evolved to form a symbiotic relationship. Cellulose, for example, cannot be digested by enzymes of the ruminant but is fermented by the microbial community. The host provides a habitat for the growth of microorganisms and offers microbial protein and fermentation acids (Stanton et al., 2020). Approximately 65% of digestion occurs in the rumen via the concerted effort of many anaerobes. The rumen microbiome is essential for maintaining digestive and metabolic functions in the host (Morgavi et al., 2010).

Each microbial species within the rumen possesses a unique combination of characteristics (such as amylases, cellulases, proteases, and lipases) that contribute to digestion, energy harvesting and ultimately methanogenesis. These microbes collectively create an environment that is suitable for methanogen survival or create substrates required by the methanogens (Morgavi et al., 2010). Although there have been considerable technological advancements during the last decade, the function of the rumen bacteria and their interactions with other rumen microbiome members is still not well understood (Huws et al., 2018). It is important to understand the rumen microbial community structure of animals that are considered high methane producers versus those that are low methane producers. Herein could lie the answer to which microbial groups play a crucial role in controlling methane emissions in the host (Kittelmann et al., 2014).

Rumen microbes can be free-living organisms or associated with protozoa or fungi (Lan and Yang, 2019). A small portion of rumen methanogens co-exists with protozoa as ectosymbionts and endosymbionts (Teklebrhan et al., 2018). Methanogens can only convert a limited number of substrates to gain their energy for growth. Methanogenic archaea are at the terminal position of the trophic chain of decomposers degrading organic matter and have a mutually beneficial syntrophic relationship with other members of the rumen. This is achieved through interspecies H_2 transfer, i.e., the unilateral supply of H_2 by one microbial species and the subsequent capturing of this substrate, enabling syntrophic metabolic association between the interacting microbial species (Hamilton et al., 2015) (Figure 1.4). Interspecies electron transfer from primary fermenting organisms produces H_2 and VFAs (Leng, 2014). Methanogens contain hydrogenases which enable them to utilise the H_2 to generate ATP via methane generation. The interspecies transfer is advantageous for methanogens as it provides them with a direct supply of hydrogen (Lan and Yang, 2019).

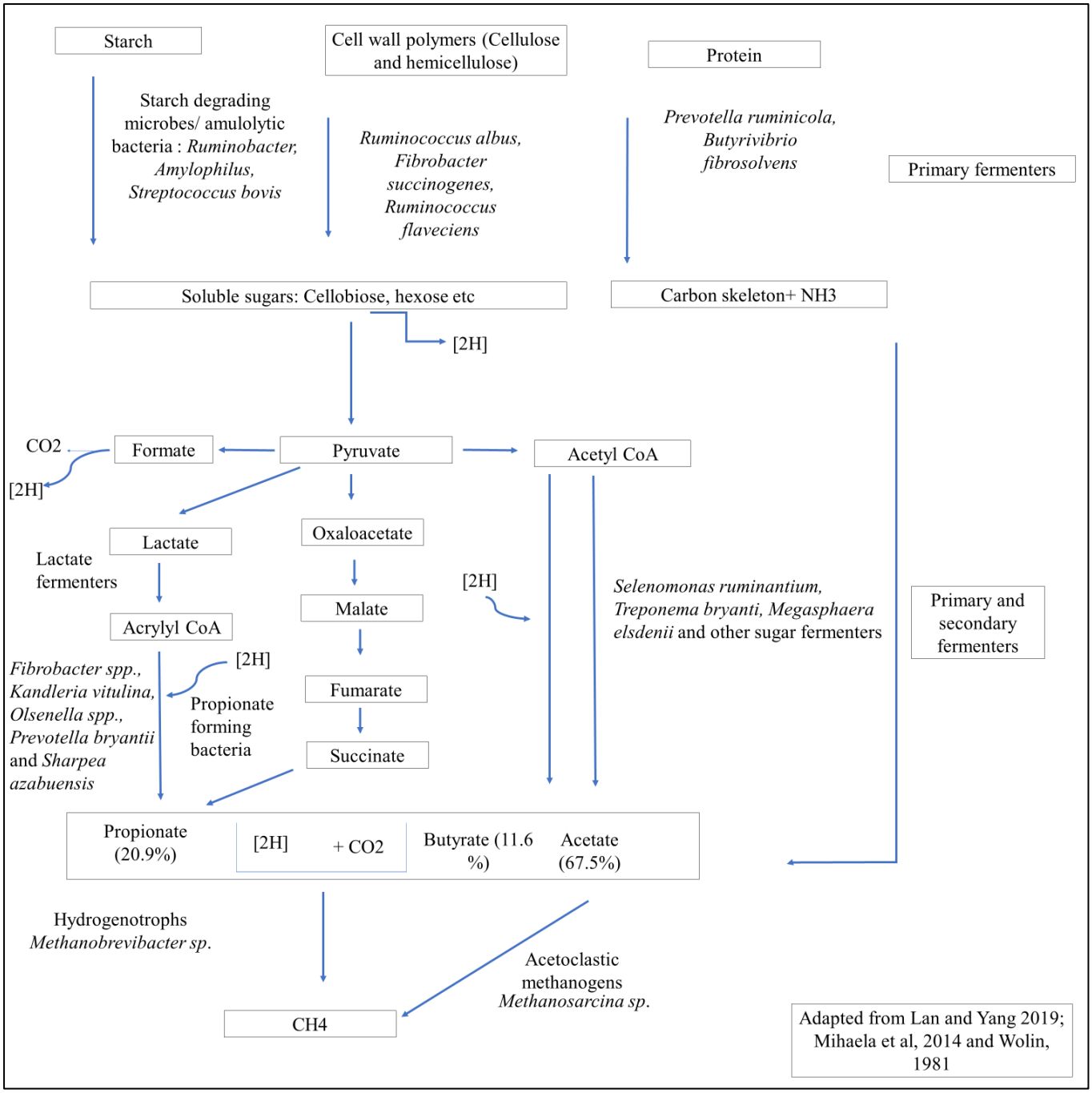

Figure 1.4: Feed fermentation and interspecies transfer in the rumen. Adapted from Lan and Yang, 2019; Mihaela et al., 2014; Wolin, 1981.

High levels of molecular hydrogen in the rumen act as a feedback inhibitor and may impair fibre digestion and fermentation by impeding the normal function of microbial enzymes such as NADH dehydrogenase, which is responsible for electron transfer reactions (Ungerfeld, 2020). Ruminal bacteria are the most diverse group within the rumen. The Gram-positive bacteria in the rumen are responsible for lactic acid and hydrogen production as a result of feed

fermentation (Melchior et al., 2018). Fibrolytic bacteria, such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*, are well-studied H_2 producers (Tapio et al., 2017). The fermentation of structural carbohydrates (such as fibre) results in the formation of butyrate and acetate, producing H_2. The released H_2 is a methanogen substrate to reduce CO_2 and produce methane (Ellis et al., 2008). To maintain the low hydrogen levels within the rumen, hydrogen-utilising methanogens use H_2 and carbon substrates, mainly CO_2, acetate, or methanol, to generate methane gas and reduce hydrogen pressure within that habitat, increasing the efficiency of the system (Danielsson et al., 2017). The efficient removal of H_2 leads to a nutritionally more favourable formation of VFAs (Janssen and Kirs, 2008). An accumulation in NADH ultimately reduces rumen fermentation (Morgavi et al., 2010). The methane generated and eructed into the atmosphere represents a loss of energy from animal feed (Miller and Wollin, 2001).

Anaerobic bacteria digest complex polysaccharides from plant material (cellulose, hemicellulose, starch and pectin), proteins, and lipids from feed, to produce short-chain fatty acids, namely propionate, acetate, butyrate and succinate (Chellapandi et al., 2018), which the host uses for maintenance, growth, and performance. As a result, H_2, CO_2 and formic acid are also produced in varying amounts as fermentation products (Tapio et al., 2017). Acetic, propionic, and butyric acids normally make up 95% of total VFA in the rumen, of which propionic acid ranges between 25% and 44% (Yao et al., 2017). Methane production can be calculated from the stoichiometry of the main volatile fatty acids formed during fermentation (Moss et al., 2000). Acetate and butyrate production results in more H_2 being produced and may aid in methane production, whereas, during propionate and valerate production, H_2 is utilised and may serve as a competitive pathway to methane production (Ungerfeld, 2020). Therefore, stimulating propionate and valerate fermentation in ruminants may lower methane emissions (Chen et al., 2020). Theoretically, methanogenesis can be reduced by shifting the VFA patterns toward enhancing propionate formation (Asanuma et al., 1999). Since propionate is the main glucose precursor and produces most of the energy required for mammary lactose production and live weight gain, this redirection of H_2 could enhance animal productivity while decreasing its environmental impact (Wang et al., 2018). Furthermore, propionate is gluconeogenic and thus can increase the availability of energy to the mammary gland, thereby increasing milk production in ruminants (Chen et al., 2020). Changes in the acetate to propionate ratio due to treatment have been associated with modifications in methane production both *in vitro* and *in vivo*. A study performed by Holtshausen et al. (2009) found that

a decrease in the acetate: propionate ratio as a response to treatment of *in vitro* fermentation with saponin-containing extracts was coupled with a reduction in total gas and methane production. Similarly, supplementation with extruded linseed lipids combined with starch in the feedlot diet decreased enteric methane emissions and lowered the acetate to propionate ratio of ruminal VFAs in bulls (Eugène et al., 2011).

Methanogens

Methanogens belong to the Euryacheota phylum and share similar metabolism and energy conservation systems (Enzmann et al., 2018; Liu, 2010). The unifying features of methanogens are that they are all obligate methane producers and strictly limit their growth to extremely anaerobic environments. Regarding their morphology, methanogens display a variety of shapes and sizes, including rods, regular and irregular cocci, long-chained rods, spirilla, sarcina and irregular, unusual, flattened plates. Some may be motile, and some species can aggregate into clusters or exist as free-living organisms (Garcia et al., 2000).Methanogens were first detected in the early 1930s by Stephenson and Stickland (1933). Balch et al. (1979) established the original classification system of methanogens in which three orders consisted of four families, seven genera and 13 species (Koga et al., 1998). Since then, many more methanogens have been isolated and named or discovered through bio molecular techniques. To date, 155 different species have been isolated that can be further grouped into 29 different genera, 14 families, six taxonomic orders, and four classes (Patra et al., 2017). The current seven orders are the M*ethanococcales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanopyrales*, *Methanocellales* and *Methanomassiliicoccales.* The last two were added later to the phyla between 2008 and 2012 (Enzmann et al., 2018). Some classifications, however, group methanogens based on their presence or absence of cytochromes and according to these classifications, there are only two classes of methanogens, those which possess cytochromes (hydrogenotrophic) and those that lack cytochromes (*Methanosarcinales*). Cytochrome-lacking methanogens are less efficient in producing methane when compared to those that contain cytochromes (Thauer, 2019).

The cell wall of methanogens contains pseudomurein also known as pseudopeptidoglycan which is a structural analogue of peptidoglycan found in bacteria (Subedi et al., 2021). The lipids in their cell walls are unique and not present in other life forms. The cell wall lipids of methanogens distinguish them from bacteria and other microbes in that the membrane lipids in

methanogens in that the bonds which link alkyl chains to the glycerol are phytane or biphytane, contrary to ester-linked fatty acyl glycerol derivatives in bacteria (Zhou et al., 2011). Furthermore, 31 proteins are uniquely found in various methanogenic archaea (Gao and Gupta, 2007). The cell wall characteristics are diverse and vary significantly among methanogens. These can range from protein subunits to eubacterial murein and pseudomurein (Sirohi et al., 2010). Methanogens, like bacteria, differ in their cell wall components (Balch et al., 1979). For example, the peptidoglycan is replaced by pseudomurein in *Methanobrevibacter* and *Methanobacterium* (Steenbakkers et al., 2006), heteropolysaccharide in *Methanosarcina*, and protein in *Methanomicrobium* (Hook et al., 2010). Although the structure and appearance of pseudomurein and murein are similar, they differ in their biosynthesis, bindings, and amino acids (Lange and Ahring 2001). The main differences between pseudopeptidoglycan and peptidoglycan are the occurrence of talosaminuronic acid in place of muramic acid, the linkages in the glycan components of methanogens is a beta (1→3) linkage, contrasting to beta (1→4) linkage found in bacteria (Gottlieb et al., 2016). Because of their difference in cell walls, methanogens are insensitive to antibiotics that inhibit cell wall synthesis, such as beta-lactams (Megonigal et al., 2004). Lysozyme and some proteases have no or only minimal effect on the cell walls of methanogens (Lange and Ahring, 2001).

Archaea have a self-assembling proteinaceous surface (S-) layer as their cell envelopes' primary and outermost boundary. This layer also maintains structural integrity. Although little is known about the proteinaceous archaeal S-layer, it is postulated to be a primitive component of the cell envelope structure and has additional essential biological functions (Arbing et al., 2012), such as cell surface recognition and may act as molecular sieves. S-layer proteins are frequently glycosylated, and it has been hypothesised that this plays a role in maintaining stability in high temperature environments (Rodrigues-Oliveira et al., 2017).

Conclusion

Enteric methane production is one of the main targets for GHG mitigation in agriculture (Yang et al., 2016). There is an urgent need to reduce global methane emissions, as this gas does not only pose an environmental threat due to its global warming potential but enteric methane production results in a high energetic cost to ruminants. Rumen methanogens, the sole producers of methane, utilise various unique enzymes to create methane via a series of methanogenic pathways. Because methanogens have pseudomurein, a structural analogue of peptidoglycan, they are insensitive to antimicrobial agents that inhibit cell wall synthesis. Methane production is influenced by feed type and is closely associated with the production of volatile fatty acids, some of which aid in methane creation, such as acetate and butyrate, while others (propionate) compete with methanogenesis for hydrogen, which is an important and limiting substrate in the rumen. The gold standard method of measuring methane from ruminants has always been the SF_6 tracer technique; however, Greenfeed offers several benefits such as ease of use and reliability of results. Because methanogenesis is an important hydrogen sink in the rumen, its inhibition needs consideration of alternate pathways to dispose off rumen hydrogen and maintain homeostatic feed fermentation. For this to occur, various methane mitigation strategies have been explored empirically. Understanding how rumen modification impacts the microbiome, especially methanogens, is vital to mitigating enteric methane production and is foundational in solving the climate change crisis.

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Chapter 2.

Isolation and characterisation of *Lactobacillus kimchicus* for use as a potential probiotic.

Abstract

This study aimed to screen for *Lactobacillus* species with probiotic potential for birds. *Lactobacillus*-specific media (LBS) was used to isolate *L. kimchicus* 5.1 from faecal droppings of the great tit bird (*Parus major)*. This isolate was identified using phenotypic testing and 16S rRNA sequencing. A potential probiotic needs to survive the adverse conditions of the GIT, therefore, *in vitro* tests that simulated the GIT were conducted. *L. rhamnosus* GG was used as a reference strain. The characterisation tests included bile salt and acidity tolerance testing, antimicrobial agent production, and pathogen inhibition, amongst others. The study found that this organism is a homofermentative LAB that produces D- lactic acid. Very slight inhibitory activity was observed against *S. simulans* and *E. coli*, using spot and well diffusion assays. After 5 hrs of exposure to bile salts (0.5%), 95.2% of *L. kimchicus* 5.1 cells survived. The lowest pH that this strain could tolerate was 2.5 since cell viability at this pH was maintained at 10^7 CFU/ml for 6 hrs. The susceptibility of *L. kimchicus* 5.1 to various antibiotics was tested according to EFSA guidelines. *L. kimchicus* 5.1 was sensitive to ampicillin, chloramphenicol, erythromycin, clindamycin, tetracycline, gentamycin, kanamycin, and vancomycin but was found to be slightly resistant to streptomycin. The complete genome sequence of *L. kimchicus* 5.1 consists of 2,535,859 bp with no plasmids or transposable elements. The majority of the genes identified were associated with carbohydrate metabolism (19.6%), amino acid (18.7%), and protein metabolism (14.3%). This study contributes to the body of knowledge about *Lactobacillus* and helps improve understanding of novel *Lactobacillus* isolated from bird faeces, which may have potential probiotics characteristics to enhance avian and animal health and nutrition.

Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host," according to the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO/WHO, 2001). Some of the most commonly used probiotics belong to the LAB group (Campana et al., 2017). The beneficial effects of LAB as probiotics are well known and well established, hence, different species of LAB have been used for the improvement of intestinal health and animal growth performance (Smith, 2014). Probiotic use has been suggested as an attractive method to manage dysbiosis in endangered species and mitigate disease in wildlife (McKenzie et al., 2018). Birds carry various microbes potentially transmissible to humans (Abulreesh et al., 2007). The primary bacterial pathogens associated with birds are enteropathogens, including *Salmonella sp*, *Klebsiella sp*, *Enterobacter sp*, *Escherichia coli*, and *Pseudomonas sp* (Benskin et al., 2009). Avian probiotics can and have been used to prevent disease associated with the ingestion of pathogenic organisms and to decrease the shedding of pathogenic microorganisms in birds (Smith, 2014). Feed and indeed the probiotic contained within feed spends a set mean duration time in the different compartments of birds' gastrointestinal tract (GIT), which is characterised by different conditions and pH. To colonise the intestinal tract, a probiotic organism for birds should survive passage through the acidic environment of the proventriculus and gizzard and persist in the presence of bile in the intestine (Corcoran et al., 2005).

Pursuing beneficial probiotic cultures is a complex process requiring multidisciplinary methods. For an organism to be classified as a probiotic that will benefit its host's health, it must fulfil several criteria, such as the ability to tolerate acid and bile salts and grow in the lower intestinal tract (Hameed et al., 2022). Once administered, the microorganisms should not be killed by the host's defence mechanisms (Dowarah et al., 2017). *In vitro* or *in vivo* tests should ascertain whether the strain can remain viable during commercial preparation and maintain its desirable characteristics during storage. Furthermore, viable cells of the microorganism should be present in adequate numbers to confer a health benefit (Kailasapathy and Chin, 2000).

Lactobacillus are part of the LAB group, and the genus *Lactobacillus* comprises numerous Gram-positive facultative anaerobic or microaerophilic, non-spore-forming, rod-shaped or coccobacilli bacteria (Fijan, 2014). *Lactobacillus* ferment carbohydrates to produce lactic acid

and are divided into three groups based on their products resulting from carbohydrate fermentation (Bernardeau et al., 2006). The three groups are i) Obligately homofermentative lactobacilli, ii) Facultatively heterofermentative lactobacilli, and iii) Obligately heterofermentative (Vandamme et al., 1996). *Lactobacillus* are the largest genus in the LAB group and encompass some of the most important strains in food microbiology, biotechnology, and nutrition (Claesson et al., 2007). Most research and development of probiotic products has focused on *Lactobacillus* due to their health-promoting properties and because these species are generally better suited to handle the stress associated with processing and delivery. Lactobacilli are diverse at the genetic level, and their G+C content ranges from 32-54% (Goldstein et al., 2015). An update to the *Lactobacillus* nomenclature in 2020 has specified 261 species categorised based on their genetic likeness and phylogeny (Zheng et al., 2020).

In humans and animals, *Lactobacillus* naturally inhabit the GIT, the oral cavity and the vagina of mammals (Walter, 2008). Members of this species are found in the presence of carbohydrate-rich substances, and the nutritional requirements of these organisms are quite complex. *Lactobacillus* species may control pathogens by producing antimicrobial compounds such as hydrogen peroxide (H_2O_2), organic acids, and bacteriocins or bacteriocin-like substances (possibly biosurfactants) that are inhibitory against pathogens (Giraffa, 2012). Such compounds are proposed to reduce the viable cell number of pathogens and affect bacterial toxin production in addition to disabling the metabolic systems of pathogens (Rolfe, 2000). *Lactobacillus spp.* probiotics are widely used as growth promoters in poultry (Fesseha et al., 2021). The treatment of broiler chickens with *Lactobacillus* strains has been associated with increased weight gain and a reduction in *Enterobacteriaceae* (Angelakis and Raoult, 2010). Some probiotic strains exhibit poor persistence after administration is discontinued due to being competitively displaced by the indigenous gut bacteria (Walter, 2008). Therefore, it is reasonable to consider autochthonous strains for development as probiotics for a particular host. Such strains may be better suited as probiotics, assuming they already have a symbiotic relationship with the host and could colonise its GIT better (De Cesare et al., 2020).

Lactobacillus kimchicus was first isolated from South Korean kimchi by Liang et al. (2011). It was described as a rod-shaped, Gram-positive, non-spore-forming, non-motile, β-glucosidase-producing bacterium. The organism has been found to exhibit a 16S rRNA gene sequence most similar to *Lactobacillus paracollinoides* AB 74 (96.9%). Regarding metabolism, *L. kimchicus*

oxidises glucose to D-lactic acid and does not produce L-lactic acid. According to available literature, there is limited research conducted on this strain. The only available studies to date are by Kim et al. (2019) where the strain was developed alongside gold nanoparticles for the delivery of ginsenoside compound K. Similarly, Markus et al. (2016) used this strain for the intracellular synthesis of gold nanoparticles with antioxidant activity.

In this study, we aimed to isolate *Lactobacillus* from faeces of the great tit (*Parus major*) to develop a probiotic for the same bird species. Faecal droppings were screened for *Lactobacillus* using selective media (LBS, Difco). An isolate was identified via biochemical and phenotypic tests, and its identity was confirmed as *L. kimchicus* using genomic sequencing. The ability of *Lactobacillu*s to survive in simulated GIT conditions could contribute to potential probiotic characteristics. Therefore, we examined the tolerance of the isolate to acid and bile solutions *in vitro* and checked for bacteriocin production and antibiotic resistance using *in vitro* and *in silico* methods. Stress tolerance was investigated by checking for EPS production and exposure to different temperatures and determining the loss of viable cells. A stability test of freeze-dried probiotic powder was conducted over six weeks under different storage temperatures.

Materials and Methods

Isolation of *Lactobacillus*

Bird droppings were obtained from Duke Wood, from a nestling of the great tit (*Parus major*) and were inoculated into BD Difco *Lactobacillus* broth de Man, Rogosa, and Sharpe (MRS; Difco Laboratories, Detroit, MI). This was incubated anaerobically at 37°C overnight and serially diluted using phosphate-buffered saline (PBS), then spread onto *Lactobacillus* Selective (LBS) agar (Difco Laboratories, Detroit, MI) plates and incubated under different conditions, i.e., anaerobically at 30°C and 37°C, and aerobically at 30°C and 37°C for two days. Colonies with different morphologies were streaked and re-streaked on LBS to obtain pure cultures. The pure LAB cultures were subsequently kept in LBS broth supplemented with 35% (v/v) glycerol and frozen at −80°C until further analysis.

Identification of putative *Lactobacillus* isolates

Preliminary identification consisted of phenotypic methods where the colony characteristics in terms of colour, size, shape, and texture were recorded after examining 1-2 day old cultures on LBS agar. Subsequently, catalase testing was performed by placing a drop of 3% (v/v) hydrogen peroxide solution on a pure, single colony and examined on a glass slide. Immediate gas formation (bubbles) was considered a positive result. Microscopic analysis was carried out on 2-day-old pure colonies that were Gram-stained and viewed under a 1000× magnification on an Olympus light microscope. Only the Gram-positive, catalase-negative, rod-shaped bacteria were taken forward for analysis. For comparison, *Lactobacillus rhamnosus* GG (APC 2740) was obtained from the APC/ DPC culture collection (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) and treated similarly to the isolate.

Genomic DNA extraction for 16S rRNA sequencing

Genomic DNA was extracted (strains grown anaerobically overnight in 10 ml MRS broth at 37°C) using the GenElute bacterial genomic DNA kit (Sigma). The extracted DNA was quantified using the Qubit 2.0 fluorometer (ThermoFisher Scientific, 168 Third Avenue Waltham, MA, USA 02451). Polymerase Chain Reaction (PCR) was performed using Biomix red (Meridian Bioscience, Dublin, Ireland). For amplification of the 16S rRNA gene, universal primers B27F (5-AGAGTTTGATCCTGGCTCAG-3) and U1492R (5-GGTTACCTTGTTACGACTT-3) (Sigma-Aldrich) were used with the following run conditions: initial denaturation: 94°C × 5 mins, cycling conditions: 30 cycles of 94°C × 40 s,

55°C × 30 s, 72°C × 1 min, final extension: 72°C × 10 mins. PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma) and sent for sequencing to Genewiz (Hope End, Takeley, Essex, CM22 6TA, United Kingdom). The resulting sequences were compared to existing genomic data using the Basic local alignment search tool (BLAST) on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The identity of the isolates was determined based on the highest scores (≥ 98%).

Whole Genome sequencing (WGS)

To prepare for WGS, *L. kimchicus* 5.1 was streaked on LBS plates from frozen stocks and incubated anaerobically at 37°C overnight. A single colony was suspended in 200 µl of sterile PBS buffer. From this, 100 µl was used to inoculate LBS broth, while the remaining culture suspension was streaked out again to ascertain the purity of the culture. The broth with culture was incubated overnight and harvested at the exponential phase as indicated by absorbance of 0.5 at OD_{600}. Cells were harvested by centrifugation at 4000 × g for 10 mins at 4°C. The pellet was washed with 1 ml sterile PBS and centrifuged again at 12000 × g for 3 mins. Finally, the concentrated pellet was suspended in 0.5 ml of 1× DNA/RNA shield buffer and sent for sequencing at MicrobesNG (Microbes NG, 97 Vincent Drive, Birmingham, United Kingdom).

Production of antimicrobial compounds

Screening for bacteriocins

The isolated colonies were overlaid with an indicator organism to check for the production of antimicrobial agents using spot assays. The indicator culture had been grown anaerobically at 37°C overnight. Briefly, the isolate was grown overnight in MRS broth. Thereafter, 10 µl was spotted onto LBS agar, incubated overnight, and overlaid with 0.75% sloppy MRS agar, seeded with the appropriate indicator. The indicator that the isolate was tested against was *Lactobacillus bulgaricus delbrueckii* ssp. *bulgaricus* LMG 6901, which is an acid-resistant strain.

Antipathogen spectra

Seven bacterial strains were used to investigate the antagonistic activity of *L. kimchicus* 5.1 against pathogens, using spot assays as detailed above. The chosen pathogens were based on immediate availability in the lab. These strains were *Staphylococcus epidermis, Escherichia coli* (K12), *Staphylococcus simulans, Streptococcus lutetiensis, Enterobacter cloacae,*

Enterococcus faecium, and *Streptococcus agalactiae*. The growth conditions of the various pathogens are listed in Table 2.1. Cell-free supernatant (CFS) from *L. kimchicus* 5.1 was obtained through centrifugation (Sorvall Legend RT) at 4000 × g for 20 mins at 4°C, repeated twice. To eliminate inhibition from acid production, the CFS was neutralised to a pH of 6.9 – 7.2 using 1 M Sodium Hydroxide (NaOH). Tempered sloppy media (0.5% agar) was seeded with 0.25% (v/v) of the appropriate indicator pathogen inoculum. Wells (7 mm) were bored into the sloppy agar plates and 50µl of the neutralised and filter sterilised supernatant was added to the wells, and the plates incubated accordingly. A zone of inhibition was suggestive of antimicrobial activity against that particular pathogen.

Table 2.1: Growth conditions for the different target organisms

Target organism	Media	Growth conditions
S. epidermis	Brain Heart Infusion (BHI)	Aerobic 37°C
E. coli	Luria Broth (LB)	Aerobic 37°C
S. simulans	BHI	Aerobic 37°C
S. lutetiensis	Tryptic Soy Agar (TSA)	Aerobic 37°C
E. cloacae	LB	Anaerobic 37°C
E. faecium	McConkey broth	Anaerobic 37°C
S. agalactiae	TSA	Aerobic 37°C

Fermentation characteristics

A standard growth curve was generated with measurements based on optical density (OD_{600}). Overnight culture (1 ml) was inoculated into 9 ml LBS and incubated anaerobically at 37°C. OD_{600} was measured over time intervals for 48 hrs, and absorbance was recorded in triplicate. This was also done for the reference strain *L. rhamnosus* GG. Growth and acid production were determined using a homofermentative-heterofermentative differential (HHD) agar medium, according to McDonald et al. (1987). The concentration of reagents required for the formulation of HHD agar plates is detailed in Table 2.2. The bromocresol green stock solution was prepared by dissolving 0.1 g bromocresol green powder in a 30 ml solution of 0.01N NaOH. When all the reagents in Table 2.2 had been combined, the solution was adjusted to pH 7, using NaOH (0.01N), and autoclaved (121°C, 15 mins). The inoculum for the assay on HHD media was prepared by growing bacterial cells in LBS under the appropriate conditions until

they reached the early stationary phase. The cells were harvested by centrifugation and washed twice with sterile molecular grade water. The cells were then washed in PBS, streaked onto the HHD agar plates, and incubated at 37°C under anaerobic and aerobic conditions. The cultures were also inoculated into liquid HHD media. For comparison, a known heterofermentative LAB (*L. rhamnosus* GG) and a known homofermentative LAB (*Lactococcus lactis*) were used because they produce colonies with distinct morphologies.

Table 2.2: Composition of HHD media for the differentiation of homofermentative and heterofermentative LAB.

Component	**Amount per litre**	**Supplier**
Trypticase peptone	10 g	Becton, Dublin, Ireland
Casamino acids	3 g	Merck, Darmstadt, Germany
Fructose	2.5 g	Sigma Aldrich, Wicklow, Ireland
Phytone peptone	1.5 g	Thermo Fisher Scientific, Dublin, Ireland
KH_2PO4	2.5 g	Sigma Aldrich, Wicklow, Ireland
Tween 80	1 ml	Sigma Aldrich, Wicklow, Ireland
Yeast extract	1 g	VWR International Limited, Dublin, Ireland
Bromocresol green	20 ml	Sigma Aldrich, Wicklow, Ireland
Agar	20 g	Sigma Aldrich, Wicklow, Ireland

Lactic acid configuration

The configuration and relative amounts of L (+) and D (-) lactic acid isomers produced and released into broth by the isolate were determined using the D-lactic and L-lactic acid assay kit (Megazyme inc., Bray, Ireland), following the protocol as outlined by the supplier. Two-day-old cultures (1 ml) were centrifuged to remove the pellet at 14,000 × g for 5 mins. Thereafter, the assay procedure was performed according to the manufacturer's instructions with a 2.24 ml reaction volume. Absorbance was read at 340 nm using a spectrophotometer. The standard in the kit was D/L Lactic acid supplied at a concentration of 0.15 mg/ml. LBS media (broth) was used as the control while molecular grade water was the blank.

Characterisation of *L. kimchicus* 5.1 as probiotic

Antibiotic susceptibility

The minimum inhibitory concentration (MIC) was determined using Epsilometer-test (E-test) strips (bioMérieux; Marcy-l'Étoile, France) of ampicillin, streptomycin, chloramphenicol, erythromycin, clindamycin, tetracycline, gentamycin, kanamycin, and vancomycin (0.016 to 256 µg/ml). For MIC assays, the isolate *L. kimchicus* 5.1 and the reference strain *L. rhamnosus* GG (APC 2740) were grown overnight to densities corresponding to a McFarland standard of 0.5 or a spectrophotometric equivalent (3×10^8 CFU/ml). *Lactobacillus* sensitivity media (de Jong et al.), supplemented with L-cysteine-HCl, was prepared according to Klare et al. (2006) by combining Iso-Sensitest broth (90%), MRS broth (10%), agar (15 g/l), and L-cysteine–HCl (0.3 g/l). LSM plates (approximately 4 mm thick) were swabbed with culture, rotating at 60° angles to ensure even distribution of the inoculum, using a sterile swab. Excess moisture on the surface of the plate was allowed to be absorbed for approximately 15 mins. Thereafter, the E-test strips were applied, MIC scale facing upward. The plates were incubated anaerobically at 37°C for 48 hrs. The MIC was defined by the intersection of the growth eclipsing the margin of the E-test strip.

Stress tolerance

Exopolysaccharide (EPS) production

The loop touch test (Ruas-Madiedo and de los Reyes-Gavila´n, 2005) was used to determine if *L. kimchicus* 5.1 could produce exopolysaccharides (EPS) by spotting 5µl of overnight culture on MRS agar plates supplemented with cysteine-HCl ((0.05%) w/v) and glucose ((5%) w/v). EPS production was also tested in MRS broth. Agar plates and broths were incubated anaerobically (anaerobic jars with Anaerocult® A gas packs (Merck, Darmstadt, Germany) for three days at 37°C. In this case, known EPS producer *Lactobacillus mucosae* (DPC 6426) (London et al., 2014) was used as a positive control.

Heat tolerance

Thermotolerance of the isolate was determined by incubating 1 ml aliquots of the overnight culture in different temperatures, i.e., 37, 40, 45, 50, and 60°C for 30 mins. After thermal treatment, surviving microorganisms were serially diluted tenfold using PBS and subsequently plated out and incubated anaerobically at 37°C for two days.

Acid tolerance

Tolerance to low pH was assessed as described by Kobierecka et al. (2017), with minor modifications. Briefly, 1 ml of overnight culture, diluted to OD_{600} of 1.0, was inoculated into 9 ml of *Lactobacillus* broth (Difco MRS) that had been pH adjusted with 1M HCl, which was filter sterilised using a 200 µM membrane filter. The pH conditions tested were pH 2, 2.5, 3, 4, 5, 6 and untreated control broth at 6.67. Enumerations were performed every 2 hrs until 6 hrs. Cultures were serially diluted tenfold at each enumeration point, plated on LBS agar, and incubated under the appropriate conditions. Plating was performed in duplicates.

Bile salt tolerance

The bile tolerance assay was carried out according to Shokryazdan et al. (2014), with modifications. Resistance to bile salts was assessed in triplicates in terms of viable colony counts and enumerated after anaerobic incubation at 37°C at 0 and 5 hrs, in 0.3% and 0.5% Oxgall (Sigma Aldrich, Wicklow, Ireland). The control was MRS broth without bile. Tenfold serial dilutions were prepared using PBS, then spread plated on LBS agar plates without bile. The control samples were incubated in MRS broth without adding bile for 0 and 5 hrs, then plated. Bile tolerance was calculated by comparing viable cell counts of the cells incubated in broth with bile salts and those without bile salt.

Deconjugation of bile salts

Bile salt hydrolase activity testing was based on the method of Ahn et al. (2003). Difco *Lactobacillus* MRS (55 g/l) was supplemented with L-cysteine-HCl (0.5 g/l) and calcium chloride (0.37 g/l). This was mixed well while heating and boiled for a min to allow reagents to get into the solution. Bacteriological agar (12.5 g/l) was then added. Thereafter, it was autoclaved for 15 mins at 121°C. A membrane filter (0.20 µM) was used to sterilise conjugated bile salts (taurodeoxycholic acid (TDCA) and taurocholic acid (TCA). To prepare the BSH assay plates, 0.25 g of each salt was dissolved in 2 ml of deionised water. Thereafter, 2 ml was added per 40 ml of tampered agar to achieve a final concentration of 0.3%. The plates with no bile served as control plates. When plates were dry, 10 µl of culture was spotted onto the plate and left to dry. Subsequent cultivation of the bacterial strains was carried out at 37°C for 72 hrs under an anaerobic atmosphere. A visible halo around the colony was used to indicate positive BSH activity of the strains. Lack of a halo was considered a negative result for BSH activity.

Viability as a freeze-dried powder

L. kimchicus 5.1 was grown from frozen stock in Difco MRS media under the appropriate cultivation conditions. It was subcultured 2-3 times before the freeze-drying process. The culture was routinely streaked out to check for purity. Using a 1% (v/v) inoculation, *L. kimchicus* 5.1 was grown in 800 ml of modified MRS broth (Difco) supplemented with 0.05% L-cysteine HCl (Sigma) and incubated anaerobically at 37°C for 24 hrs. The culture was centrifuged (Sorvall Lynx 600 centrifuge) at 4000 × g for 20 mins at 4°C, with slow deceleration. The supernatant was removed by pouring it off gently, ensuring that the pellet stayed intact. The remaining pellet was washed by resuspending it in PBS and then centrifuged using the same conditions stated above. The washing step was repeated once again. The bacterial cells were concentrated (×20) by finally suspending the pellet in 10% (w/v) trehalose (Sigma Aldrich, Wicklow, Ireland). The suspension (1 ml) was transferred to FD vials, placing the cap loosely. These were loaded to a decontaminated (with 70% ethanol) freeze-dryer and allowed to freeze-dry for ~24 hrs. Thereafter, the dry weight of the freeze-dried bacterial powder was measured. Subsequently, the powder was suspended in PBS, serially diluted, and plated onto LBS plates to determine the viable cells (CFU/g) of *L. kimchicus* 5.1 post-freeze-drying. Viable cell counts were determined before freezing and after freeze-drying.

To determine if freeze-dried *L. kimchicus* 5.1 could maintain viability long-term, a 6-week stability trial was performed under different conditions, i.e., at room temperature, at 4°C and -20°C. Approximately 50 g of powder from one freeze-drying batch was split into three and stored under the above conditions. The powders were stored in sealed sterile plastic bags. During each sampling episode, one gram of powder was suspended into 9 ml PBS and serially diluted accordingly. The dilutions were spread onto LBS agar plates and incubated anaerobically at 37°C. Duplicate plates were used for each dilution. Colony-forming units (CFUs) were calculated and presented as means when colonies had fully grown. For comparison, *L. rhamnosus* GG (APC 2740) was included in the stability trial and treated the same as *L. kimchicus* 5.1.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism version 9.1.0 (GraphPad Software, La Jolla, California, USA). The results were expressed as means ± standard deviations of at least two replicates. An unpaired T-test was used to identify any statistically significant differences in the experiments, and differences were considered statistically significant at $P<0.05$.

Results

Strain isolation and characterisation

A faecal sample received from the wild nestling great tits (*Parus major*) in Duke Wood, Cork, Ireland, was inoculated into *Lactobacillus* MRS broth which supports the cultivation of *Lactobacillus* species and incubated overnight. After this enrichment, the inoculated broth was serially diluted and plated on LBS plates in different incubation conditions (anaerobic 37°C, aerobic 37°C, anaerobic 30°C, and aerobic 30°C). The plates were checked intermittently after 24 and after 48 hrs of incubation. Colonies of different morphologies and colours were streaked for purity and incubated again under the conditions previously described. There were five phenotypically different colonies initially isolated. The best growth of colonies was observed when the plates were incubated anaerobically at 37°C for 48 hrs. The colonies of the isolated bacteria were small to medium, cream, and white coloured, and smooth in texture. When the Gram-stained colonies were viewed under the light microscope (×1000), the cells were Gram-positive, short rods that occurred single or in pairs (Figure 2.1). Only one isolate tested catalase positive, which was immediately excluded from the study.

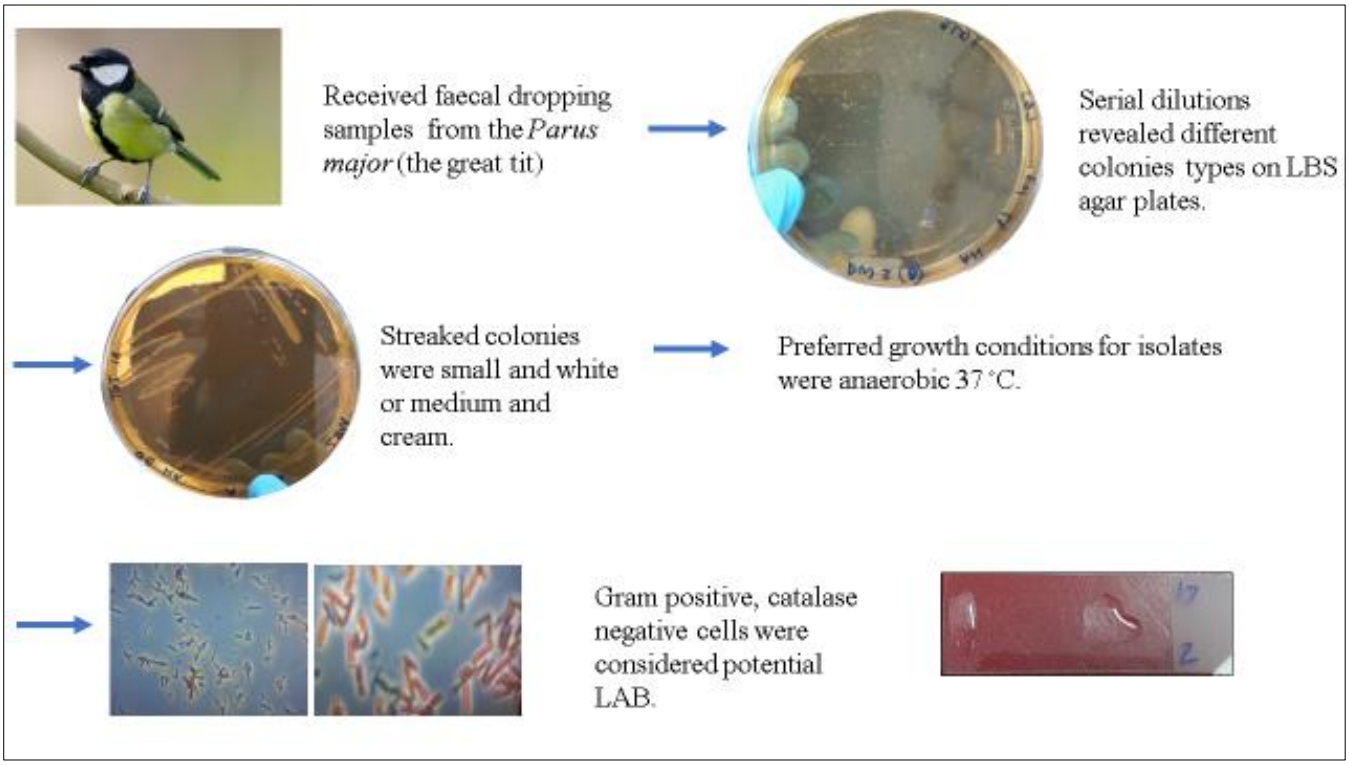

Figure 2.1: Isolation and preliminary identification of *L. kimchicus.*

Genomic identification

16S rRNA sequencing

The isolates were further identified based on 16S rRNA sequencing. The sequences obtained from the four colonies were compared to an existing NCBI genomes database and found to have 98 to 98.86% similarity to *Lactobacillus kimchicus,* confirming the species identity. Because all the isolates had exhibited genotypic similarity, only one of the original five isolates was taken forward for analysis. This isolate was thus designated *as L. kimchicus* 5.1. This isolate had small to medium, round, cream-white, and convex colonies.

Whole Genome sequencing (WGS)

To expand on the knowledge base of this *Lactobacillus* species, WGS was conducted on *L. kimchicus* 5.1. The annotation (Table 2.3) was performed with RAST (version 2.0) (Aziz et al., 2008), and assembly was carried out using tools available on the public server at http://cgview.ca. The complete genome of *L. kimchicus* 5.1 consists of 2,535,859 bp and has no plasmids or transposable elements (Figure 2.2(A)). The G+C content of this strain is 46.4 mol%. Using RAST software, 2730 coding sequences were found, including 71 RNAs and 955 protein-coding open reading frames (ORFs) divided into 27 subsystem groups. The majority of the genes identified were associated with carbohydrate metabolism, amino acid and protein metabolism (Figure 2.2(B)). Other notable regions were protein-encoding genes for ldhD (D-lactate dehydrogenase) and the acid-resistant locus fragment (Arl7), which is 759 bases long. A coding region for the general stress protein family Gls24 was found in contig 5. The 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MetE) gene was also present. Heat shock-related protein sequences that were found include GroES and GroEL, which are heat shock protein family chaperones and are 10 kDa and 60 kDa, respectively. The S4 paralogue, a ribosome-associated heat shock protein implicated in the recycling of the 50S subunit, was found between positions 9664 and 9936. A coding sequence for leucocin A was found in the genome. The coding sequence between nucleotide 83594 and 85531 was for tetracycline resistance-related proteins.

Table 2.3: General features of *L. kimchicus* 5.1.

Feature	**Size**
Genome length (bp)	2,535,859
Number of contigs with Protein Encoding Genes (PEGs)	39
G+C content (%)	46.4
Number of coding sequences (CDs)	2730
Number of plasmids	0
Number of Subsystems	237
Number of RNAs	71

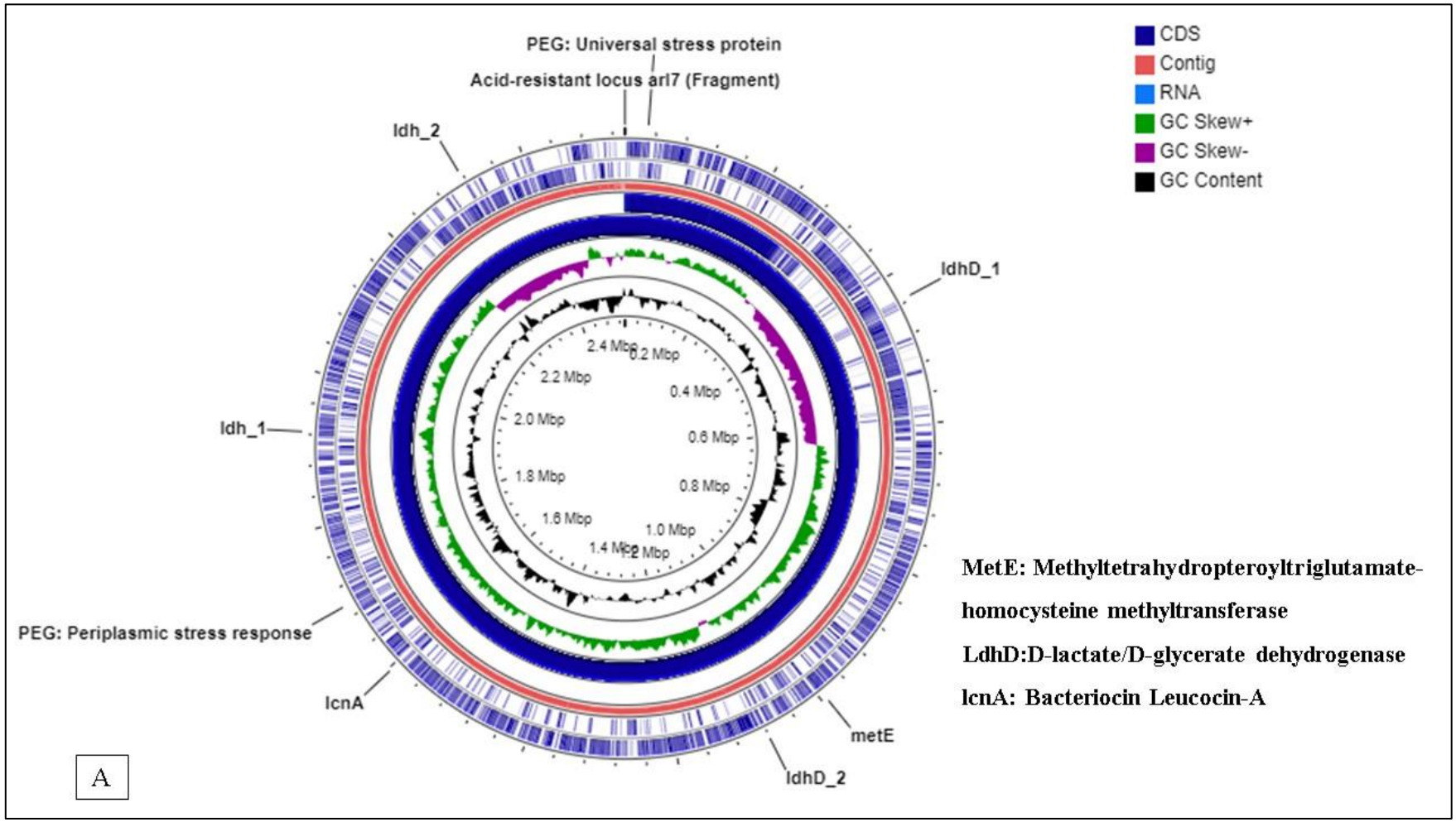

Figure 2.2(A): Circular genome of *L. kimchicus* 5.1 with a subset of representative genes highlighted.

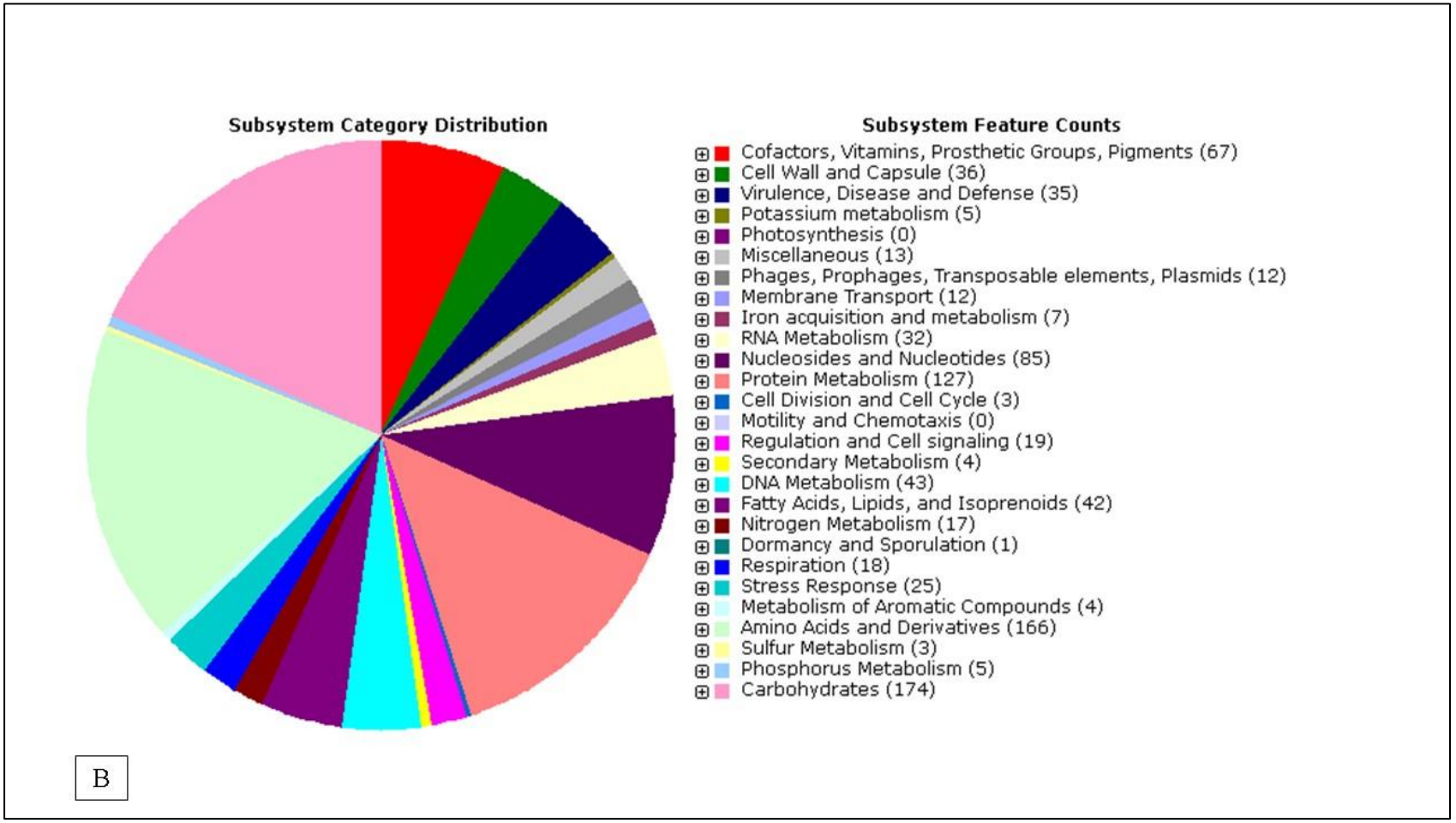

Figure 2.2(B): Pie graph of the gene subsystems for different functions in *L. kimchicus* 5.1.

In silico screening for bacteriocins

Screening the entire genome of *L. kimchicus* 5.1 revealed that the bacteriocin-encoding locus (*LcnA* locus) occurred between positions 1898 and 2291. RAST showed this as encoding for the bacteriocin Leucocin A. Bagel was used to identify bacteriocin encoding gene clusters and showed coding regions for leucocin C and plantaricin 423 (Figure 2.3). The results of the antiSMASH analysis showed a ribosomally synthesised and post-translationally modified peptide (Ripp) like region and no other secondary metabolites in the genome.

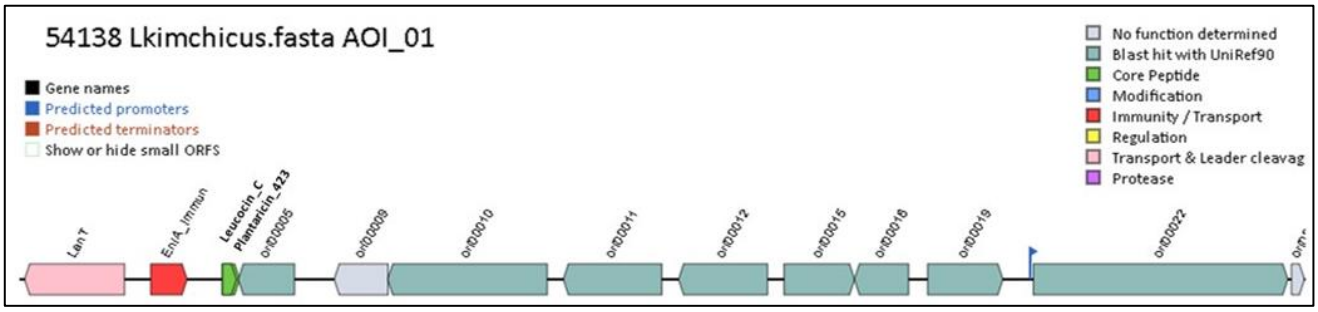

Figure 2.3: Potential bacteriocin operon in *L. kimchicus* 5.1 as shown by Bagel4.

In silico screening for antibiotic resistance genes

The genome sequence of *L. kimchicus* 5.1 was analysed using an online resource for identifying antibiotic resistance genes known as Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/). When antibiotic resistance was initially investigated using Resfinder, no genes known to be involved in antibiotic resistance were detected. Therefore, the Comprehensive Antibiotic resistance database (CARD; http://arpcard.mcmaster.ca) was used to verify these results. No antibiotic resistance genes were detected in the genome using CARD when the resistance gene identifier was set at selection criteria to find only "Perfect and strict complete genes". An antibiotic resistance mechanism with a 31.9% match to a set of genes for the antibiotic efflux pump that confers tetracycline resistance was found when the resistance gene identifier criteria were set to "loose."

In vitro screening for bacteriocins

Antimicrobial activity is important for LAB to colonise the intestinal mucosa effectively, enabling them to defend against potential pathogens (Vijayabaskar and Somasundaram, 2008). This study examined the antimicrobial activity of LAB against common pathogens using the

spot assay and the well diffusion method. When tested against *L. delbrueckii* subsp. *bulgaricus* LMG 6901, *L. kimchicus* 5.1, did not demonstrate any bacteriocin production. Of all the indicators used (Table 2.1), *L. kimchicus* 5.1 exhibited a slight inhibition against *S. simulans* (Figure 2.4(A)) and a slightly more potent inhibition against *E. coli* (Fig 6B). A subsequent well diffusion assay using neutralised CFS demonstrated continued inhibitory activity against *E. coli* (Figure 2.4(C)); however, against *S. simulans*, inhibitory activity was lost.

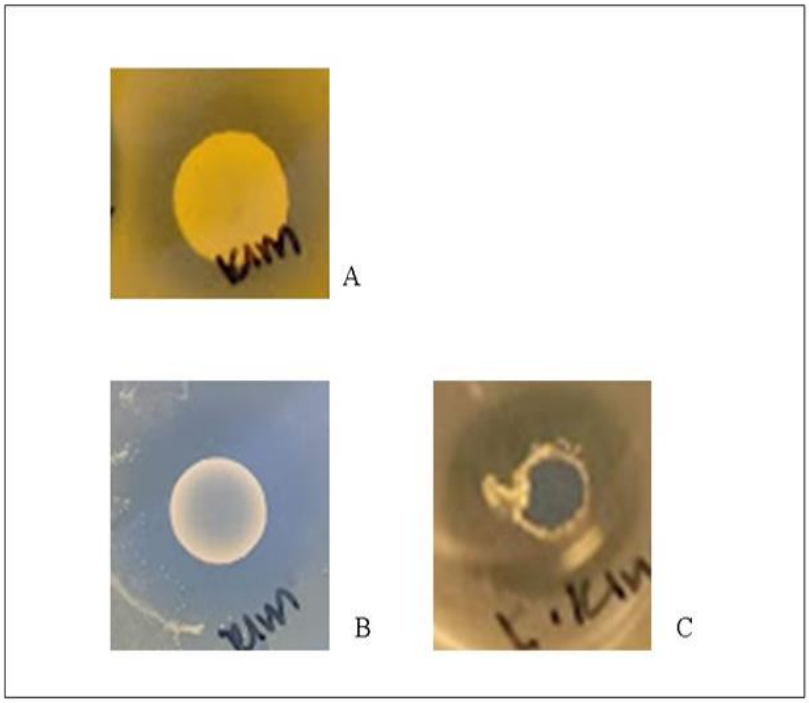

Figure 2.4: Antimicrobial activity using a spot assay against *S. simulans* (A) and *E. coli* (B) and well diffusion against *E. coli* (C).

Growth and Fermentation pattern determination

To establish the growth rate of the newly isolated *L. kimchicus* 5.1 strain, a pure colony was inoculated into 10 ml Difco MRS broth and incubated in anaerobic 37°C, and turbidity (OD_{600}) was regarded as an indicator of growth. This was also performed for *L. rhamnosus* GG (APC 2740). Growth was measured at various time intervals using a spectrophotometer (Ultraspec 10 Cell density meter) (Biochrom, Cambridge, United Kingdom) for 48 hrs. A growth curve was generated for both *L. kimchicus* 5.1 and *L. rhamnosus* GG (Figure 2.5). *L. kimchicus* 5.1

displayed a lag period of 8 hrs and a doubling time of 4.65 hrs, and *L. rhamnosus* showed a doubling time of 3.64 hrs. The formula (1) stated below was used to calculate doubling time.

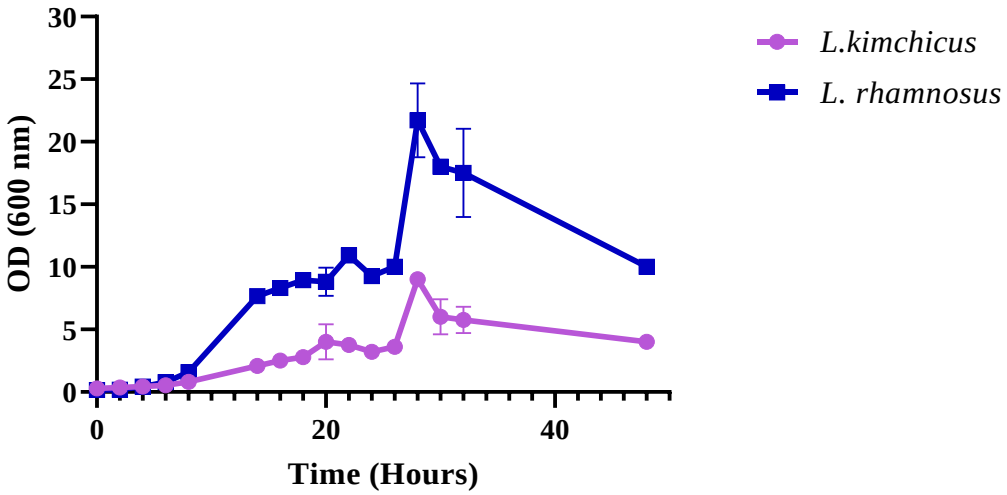

Figure 2.5: Representative growth curves using optical density.

$$(1)\ Y = Y0 * exp(k * x)$$

Y0 = Starting OD

K= Rate constant (*L. kimchicus* 5.1: 0.071; *L. rhamnosus* GG: 0.060)

Y= Population

X= Time (hrs)

The *L. kimchicus* 5.1 strain was tentatively identified based on phenotypic characteristics by determining growth and acid production on HHD agar medium. After two days of incubation, *L. rhamnosus* GG, a known heterofermentative organism, produced white colonies (Figure 2.6(A)), while the known homofermentative *L. lactis* produced blue/green colonies (not shown). *L. kimchicus* also produced blue colonies (Figure 2.6(B)) on HHD media, suggesting that the organism is a homofermentative LAB.

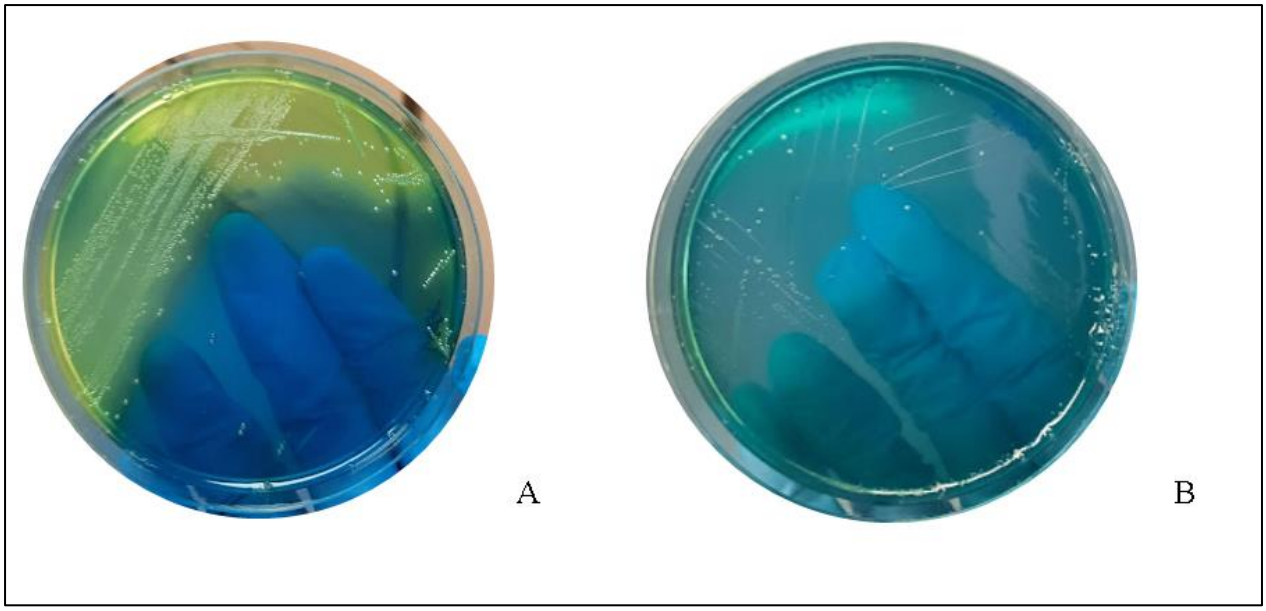

Figure 2.6: Growth and acidification on HHD agar medium of *L. rhamnosus* GG (A) and *L. kimchicus* 5.1 (B).

Lactic acid isomers

A rapid assay kit (Megazyme, Bray, Ireland) was used to calculate the ratio of each of the optically active forms of lactic acid produced by *L. kimchicus* 5.1 (Table 2.4). Each sample was analysed in duplicate, and the assay was performed twice. The accuracy of the assay was confirmed by using *L. rhamnosus* GG as a positive control and comparing the results to the supplied lactic acid standard. Only the D-lactic acid isomer was detected at 0.82 g/l when *L. kimchicus* 5.1 was grown in LBS.

Table 2.4: The optically active isomer of lactic acid produced by LAB.

Organism	**Lactic acid isomer (g/l)**	
	L-lactic acid	**D-lactic acid**
L. kimchicus 5.1	-0.002 ± 0.001	0.82 ± 0.0009
L. rhamnosus GG	0.199 ± 0.001	0.612 ± 0.0003

Characterisation of *L. kimchicus* 5.1 as a probiotic

Antibiotic susceptibility

Table 2.5 shows the antibiotic susceptibility of the two LAB against eight antibiotics using LSM plates. In this study, *L. kimchicus* 5.1 was sensitive to all the necessary antibiotics as specified by the European Food Safety Authority (EFSA) except streptomycin, where the cut-off was 16 µg/ml, and the bacteria was inhibited at 32 µg/ml. *L. rhamnosus* GG was used as the positive control and was susceptible to all the specified antibiotics. Figure 2.7 shows an example of the results obtained using the E-test strips.

Table 2.5: MIC (µg/ml) testing using E-test strips on *Lactobacillus* Sensitivity Media (LSM).

Antibiotic	***L. rhamnosus GG***		***L. kimchicus* 5.1**	
	MIC (µg/ml)	Result	**MIC** (µg/ml)	Result
Chloramphenicol	0.19	**S**	CI	**S**
Erythromycin	0.125	**S**	0.125	**S**
Gentamycin	1.5	**S**	0.38	**S**
Clindamycin	1	**S**	1	**S**
Kanamycin	24	**S**	32	**S**
Streptomycin	6	**S**	32	**R**
Ampicillin	1.9	**S**	0.125	**S**
Tetracycline	0.5	**S**	4	**S**

Key: CI: Complete inhibition; S: Susceptible; R: Resistant.

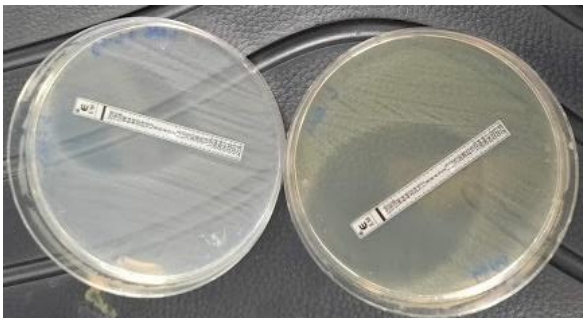

Figure 2.7: An example of MIC testing of *L. kimchicus* 5.1 using E-Test strips.

Stress tolerance

Exopolysaccharide (EPS) production

EPS production is identified by the ropiness or mucoid nature of bacterial colonies when lifted with an inoculation loop. As expected, EPS production was observed in the positive control strain *L. mucosae* (DPC 6426), which was used as a reference strain as this organism is a well-documented EPS producer (Ryan et al., 2019). No EPS production was observed for *L. kimchicus* 5.1 when inoculated with 5% (w/v) glucose.

Heat tolerance

Tolerance to heat was evaluated by incubating the cultures at increasing temperatures from 40°C to 60°C for 30 mins (Figure 2.8). Incubation at 37°C was considered the control, as it had already been established that this strain grows well when incubated at this temperature. When *L. kimchicus* 5.1 culture was incubated at 40 to 45°C, viable cell counts remained at 10^7 CFU/ml, similar to the growth observed at 37°C. At 50°C, there was a log reduction in viable cells compared to 37°C. However, upon incubation at 60°C, there was a dramatic 5 log reduction in viable cells, from 10^7 to 10^2 CFU/ml, compared to the 37°C control temperature. *L. rhamnosus* GG tolerated incubation at 50°C better than *L. kimchicus* 5.1, as viable cell counts were 5.1 $\times 10^6$ and 1.8 $\times 10^6$ CFU/ml for *L. rhamnosus* GG and *L. kimchicus* 5.1 respectively. The tolerance of *L. rhamnosus* GG to 60°C was significantly better than the test isolate *L. kimchicus* 5.1 (P= 0.0198).

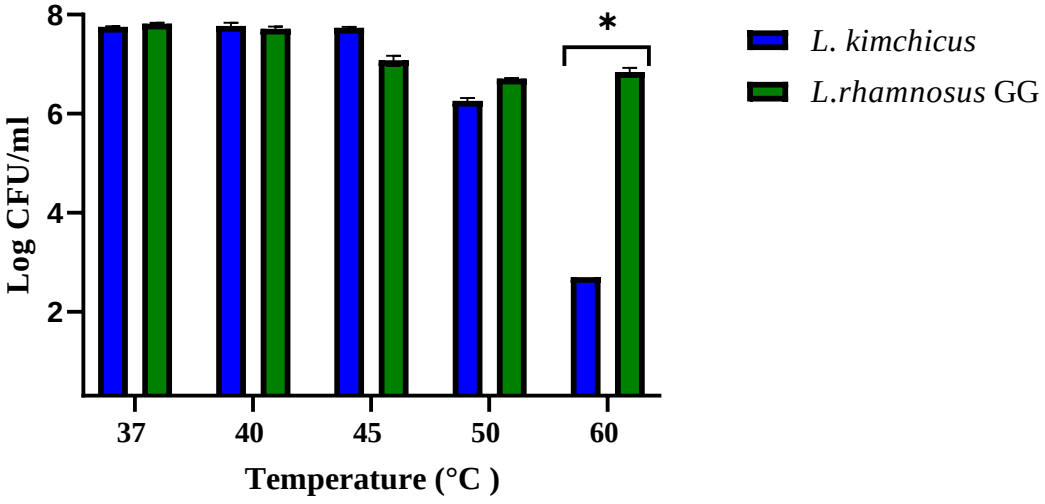

Figure 2.8: Cell counts (log CFU/ml) of *L. kimchicus* 5.1 and *L. rhamnosus* GG after heat treatment challenge at 37°C to 60°C incubation for 30 mins.

Tolerance to acidic conditions

The *L. kimchicus* 5.1 isolate was tested for its ability to survive under different acidic conditions, at pH ranging from 2-6, and control media (pH 6.7) was included. After 4 hrs of exposure to pH 2, *L. kimchicus* 5.1 viable cells were 1×10^3 CFU/ml, while at T_0, the number of bacteria was at 10^7 CFU/ ml for all the pH values (Figure 2.9). Therefore pH 2 appears to be the cut-off for *L. kimchicus* 5.1 since growth at pH 2.5 was maintained at 1×10^7 CFU/ml for 6 hrs. For the reference strain used in this study, *L. rhamnosus* GG counts were maintained at 10^7 CFU/ml for 6 hrs for pH values between 3-6. There was a 3-log reduction of *L. rhamnosus* GG viable cells at pH 2 after 2 hrs of incubation.

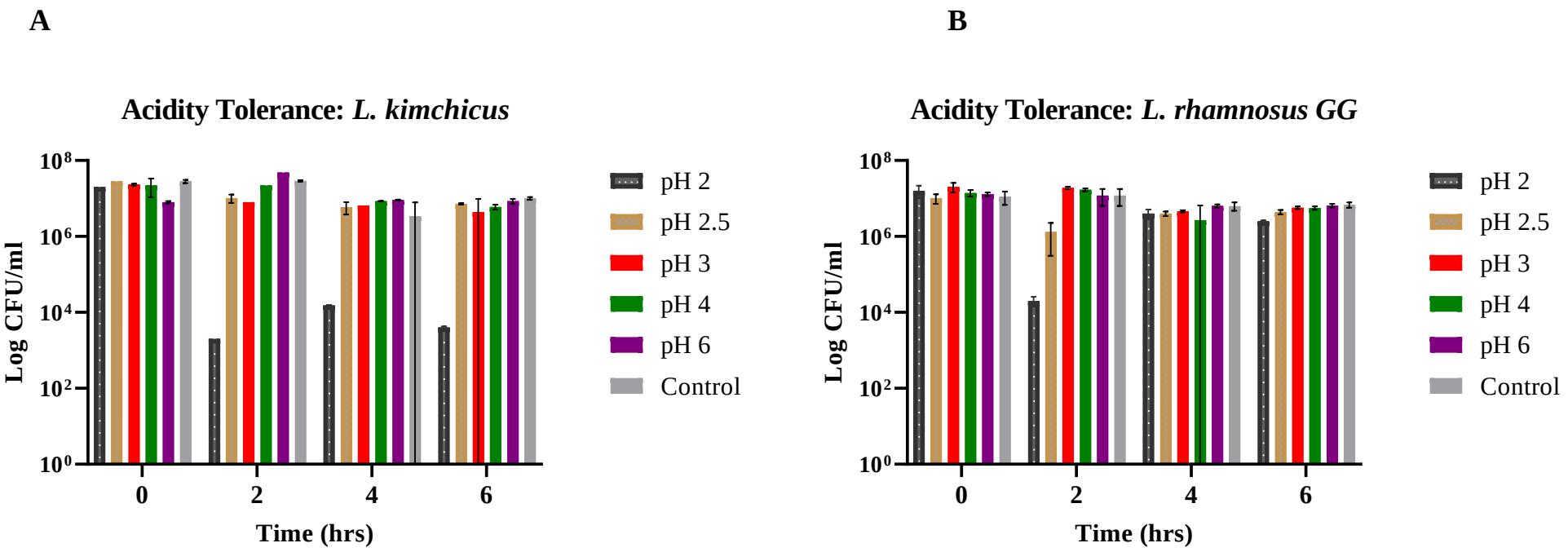

Figure 2.9. Acidity tolerance testing on isolate *L. kimchicus* 5.1 (A) and reference strain *L. rhamnosus* GG (B).

Error bars represent standard deviation.

Bile salt tolerance

After incubation in 0.3% and 0.5% (v/v) bile (oxgall), viability of bacterial cells was assessed by colony counts (CFU/ml) on plates incubated at 37°C (Figure 2.10). Bile tolerance was calculated by comparing viable cell counts in MRS with and without bile. Survival ratios were calculated using Formula 2. *L. kimchicus* 5.1 survived up to 5 hrs in 0.3% and 0.5% bile salt. After 5 hrs of exposure, 98.5% of cells exposed to 0.3% bile salts had survived, while 95.2% of those exposed to 0.5% bile salt remained. The reference strain *L. rhamnosus* GG was unaffected by the presence of 0.3% bile salts, as indicated by 100% recovery after 5 hrs of exposure.

$$\text{Survival ratio} = (\text{Log CFU}_{\text{MRS}} - \text{Log CFU}_{\text{Test bile}}) / \text{Log CFU}_{\text{MRS}}$$

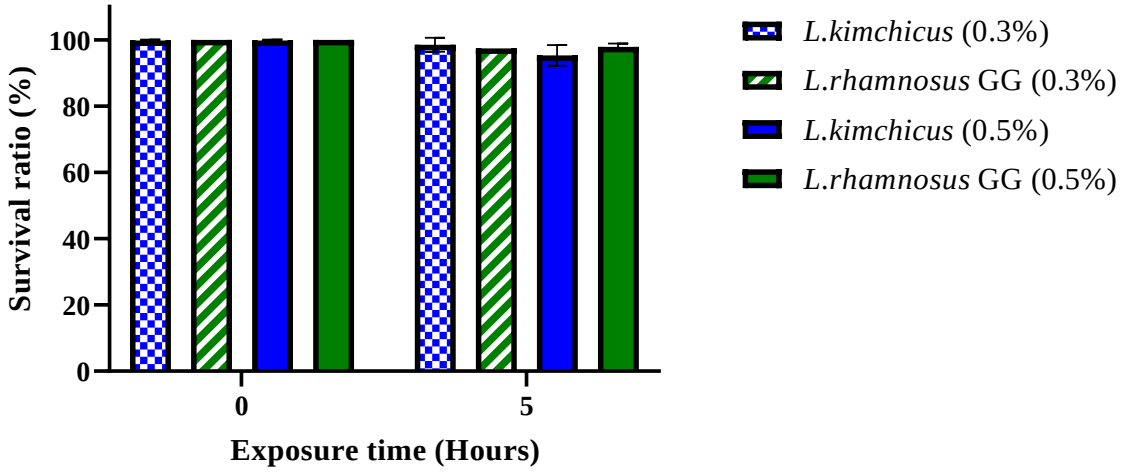

Figure 2.10: Percentage survival ratios of *L. kimchicus* 5.1 and *L. rhamnosus* GG in 0.3 and 0.5% bile salts. The error bars represent the standard deviation.

Bile salt hydrolase activity

The direct plate assay method was used to assess the BSH activity of *L. kimchicus* 5.1 qualitatively. Overnight cultures were spotted (10 µl) in triplicate on LBS agar that had been supplemented with conjugated bile salts: taurodeoxycholic acid (TDCA) and taurocholic acid (TCA). No halos were observed in *L. kimchicus* 5.1, which indicates an absence of bile salt hydrolase activity (Figure 2.11).

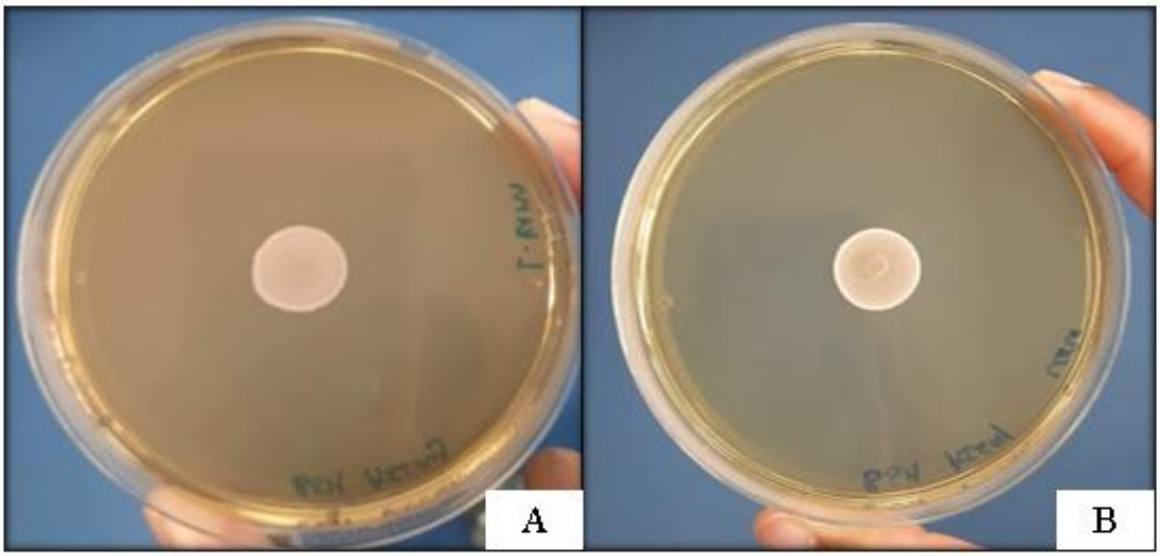

Figure 2.11: *L. kimchicus* 5.1 spotted on BSH assay plates on (A) TDCA and (B) TCA supplemented MRS plates.

Stability of freeze-dried *L. kimchicus* 5.1 at different temperatures

Freeze-dried powders obtained from *L. kimchicus* 5.1 and *L. rhamnosus* GG were stored at different conditions for six weeks, i.e., at room temperature (RT), 4°C, and -20°. Enumerations were performed on the first day of the stability trial, after 1, 4, and 6 weeks. At each time point, the number of viable *Lactobacillus* cells was determined by the plate count method and presented as CFU/ g. The initial viable colonies at T_0 were 1.1×10^{11} and 3.52×10^{11} CFU/ g for *L. kimchicus* and *L. rhamnosus* GG, respectively. After six weeks, the overall decrease in the viability of *L. rhamnosus* GG and *L. kimchicus* 5.1 was less than 1 log CFU/g when the freeze-dried powder was stored at 4°C and -20°C (Figure 2.12 (B and C)). When *L. kimchicus* 5.1 was kept at room temperature (Figure 2.12(A)), a decrease of up to 3 log CFU/g was observed after four weeks of storage. A further sharp decline in cell viability of 2 log CFU/g was observed between week four and week six. A similar trend was observed for *L. rhamnosus* GG.

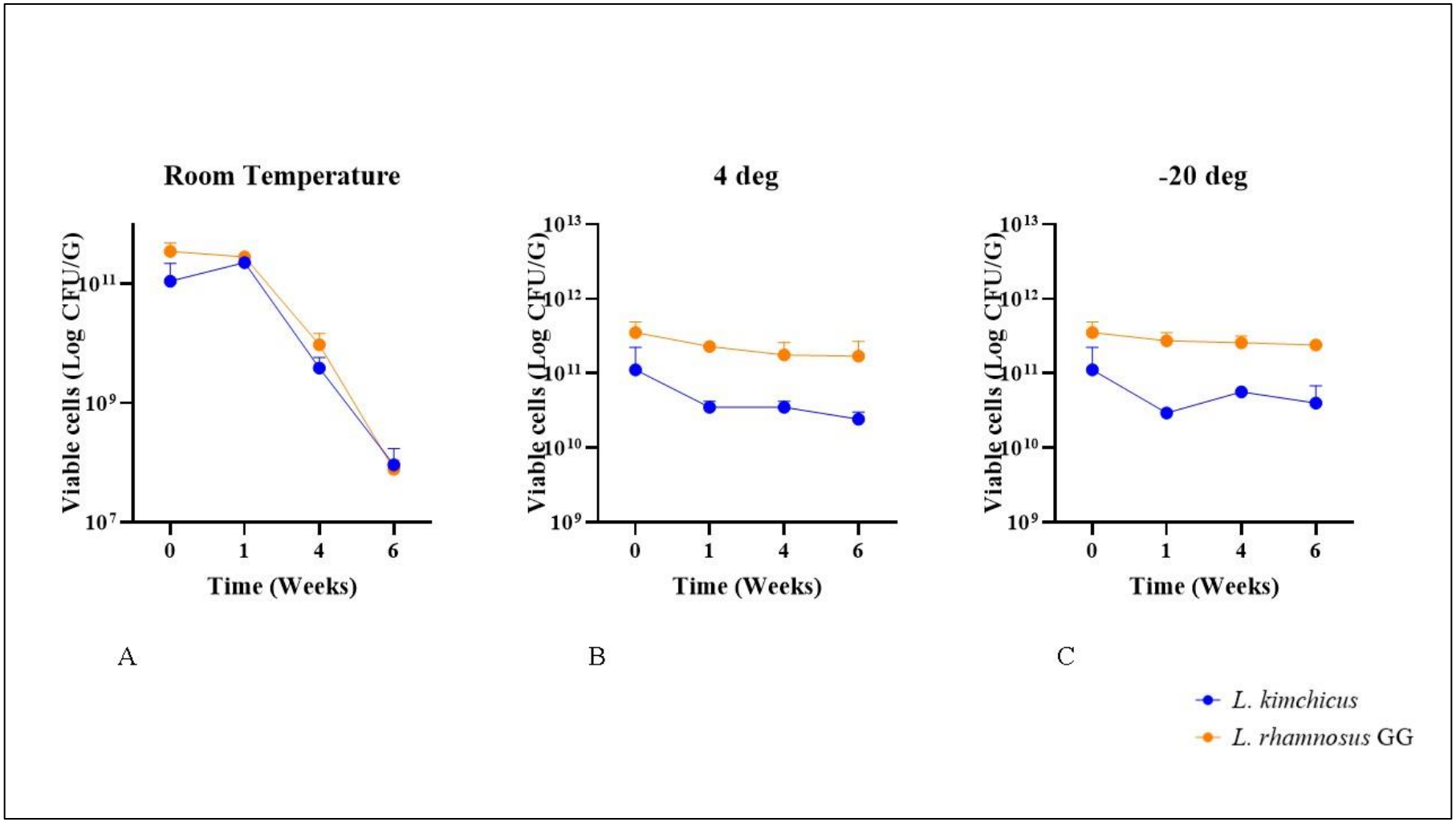

Figure 2.12: Cell viability of *L. kimchicus* 5.1 and control strain (*L. rhamnosus* GG) stored at room temperature (A), 4°C (B), and -20°C (C) for six weeks.

Discussion

Lactobacillus colonies were isolated from bird faeces and characterised for probiotic potential using various *in vitro* tests and *in silico* screening approaches. The first step involved the isolation and identity confirmation of the *Lactobacillus* sp., and the second step was an investigation of the probiotic characteristics of the isolated strain. *Lactobacillus* sp. are the most widely used probiotic group and have an essential role in gut microbiome restoration and maintaining balance after antibiotic treatment of a host (Das et al., 2020).

The isolated colonies grew best under anaerobic conditions, at 37°C, which is characteristic of *Lactobacillu*s, as their optimal growth temperature range is 40°C (Slizewska and Chlebicz-Wojcik, 2020). Initially, five different colonies were identified, but upon testing, one isolate was excluded due to testing catalase positive. Only Gram-positive and catalase-negative isolates were taken forward for analysis. Physiological and biochemical tests confirmed that the isolate was indeed a LAB and 16S rRNA sequencing revealed that the organism is *Lactobacillus kimchicus*. Although there was some slight phenotypic variation in the colour of the colonies, genotypic identification showed that the four colonies sequenced were all *L. kimchicus*. As such, one colony designated *L. kimchicus* 5.1 was carried forward, and all analysis was performed on this strain. *L. kimchicus* is a novel organism that was first isolated from Korean kimchi. The biochemical identification results were similar to those of Liang et al. (2011). The isolate was found to have a long lag phase of 8 hrs at 37°C and a doubling time of 4.65 hrs (Figure 2.5). Evaluating the growth patterns of this organism was important to enable ease of use during processes such as freeze-drying and ensuring that the organism is given sufficient time to grow for other *in vitro* tests.

To gain a thorough insight into the genetic composition of this isolate, WGS was performed on *L. kimchicus* 5.1. There were 2730 coding sequences found, including 71 RNAs and 955 protein-coding open reading frames (ORFs) divided into 27 subsystem groups. The largest subsystem of genes (174 of 955) in the *L. kimchicus* 5.1 genome is dedicated to carbohydrates (Figure 2.2(B)). The nutritional requirements of Lactobacilli could be due to their various habitats, which are typically rich in carbohydrates and proteins (Mayo et al., 2008). A preferred trait when selecting a probiotic strain is the demonstrated ability to produce bacteriocins to positively influence the host's health (Dobson et al., 2012). *Lactobacillus* species primarily exert their essential role in GI tract function by producing bacteriocins that generally have

potent activity against intestinal pathogens (Dempsey and Corr, 2022). Environmental factors such as temperature, pH, culture incubation time, and media composition affect the extent to which bacteriocins are produced (Bibalan et al., 2017). Challenges related to the conventional methods of bacteriocin screening can be overcome by using *in silico* screening (Bibalan et al., 2017). In this study, the draft genome was analysed using BAGEL4 to search for potential antimicrobial-encoding operons. Gene clusters were identified containing putative genes for the production of leucocin C and plantaricin 423 (Figure 2.3). One drawback of *in silico* screening methods is that they cannot be used in isolation to confirm bacteriocin production conclusively (Walsh et al., 2015). Although no bacteriocin activity was detected *in vitro*, there was slight inhibition against *S. simulans* and *E. coli* (Figure 2.4). This inhibitory activity against *E. coli* could not be attributed to lactic acid production as it was maintained despite CFS being neutralised. However, activity was lost against *S. simulans* upon neutralising the CFS (Figure 2.4). This could mean that inhibition was due to the production of another antimicrobial agent such as H_2O_2 (Reis et al., 2012). Antimicrobial agent production could enable the strain to competitively exclude potential pathogens in the GIT when used as a probiotic.

HHD media was developed to enable differential enumeration of heterofermentative and homofermentative LAB based on media and colony colour (Soro-Yao et al., 2014). Fructose was used in this media as the sole carbohydrate source. Heterofermentative organisms reduce fructose to mannitol while producing lactic acid, CO_2, and acetic acid, whereas homofermentative bacteria produce two moles of lactic acid from fructose. This study used known heterofermentative organisms, *L. rhamnosus* GG (APC 2947), and homofermentative *L. lactis* (SL242) (Golomb and Marco, 2015), as the controls for comparison. *L. rhamnosus* produced the expected white colonies, while the media surrounding the colonies turned from blue to light green. The test organism exhibited homofermentative characteristics as blue colonies grew and the media remained blue (Figure 2.6). The D/L lactate assay confirmed the finding of Liang et al. (2011) that this species only produces D-lactate. LAB metabolises sugars via lactate dehydrogenase, which reduces pyruvic acid to lactate. Both the *ldh1* and *LdhD* genes were present in the genome of *L. kimchicus* 5.1. The *ldh1* gene codes for L-Ldh, which synthesises L-lactate in bacteria (Rico et al., 2008). *LdhD*, the D-lactate dehydrogenase gene, is involved in D-lactate catabolism. Similarly, *Lactobacillus helveticus* possesses both L and D-lactate dehydrogenases (Kochhar et al., 1992). *L. helveticus*, a homofermentative LAB, can produce large amounts of lactic acid. However, the expression of ldhD and ldhL genes does

not always correlate to changes in the amounts of D and L- lactate produced (Nikkila et al., 2000).

The emergence and transfer of antibiotic resistance genes is a major concern. The potential to transfer resistance factors to pathogenic bacteria in the gut is a real threat if antibiotic-resistant strains are used as probiotics (Gueimonde et al., 2013). It was therefore, necessary to test the strain *L. kimchicus* 5.1 for antibiotic susceptibility, according to EFSA guidelines. The susceptibility of *L. kimchicus* 5.1 to the various antibiotics as stipulated by EFSA guidelines was evaluated by phenotypic testing. This test revealed that although the organism was susceptible to all the antibiotics tested (ampicillin, chloramphenicol, erythromycin, clindamycin, tetracycline, gentamycin, kanamycin, and vancomycin); it exhibited a slight resistance toward streptomycin. Some *Lactobacillus* strains have a naturally high resistance to streptomycin (Campedelli et al., 2019). This natural resistance of *Lactobacillus* to streptomycin was also documented by Danielsen and Wind (2003). Antibiotic resistance is conferred by the presence of antibiotic resistance genes (Zhang and Zhang, 2019). Antibiotic resistance was investigated using *in silico* methods such as Resfinder and the Comprehensive Antibiotic resistance (CARD) database because it was critical to evaluate whether this putative probiotic has any transferable resistance genes. Although no genes encoding antibiotic resistance were initially found when the selection criteria were set to "perfect and strict hits only" on the resistance gene identifier (RGI) on CARD, RGI found 31.9% similarity to genes encoding for tetracycline resistance when RGI was set to "loose" parameters. This was in agreement with the findings from RAST, which revealed a match to a set of genes encoding for the antibiotic efflux pump that confers tetracycline resistance. No transposable or mobile elements were detected in the *L. kimchicus* 5.1 genome, meaning that any resistance genes present could not be transferred horizontally.

Low pH environments can impair the metabolism and inhibit the growth and viability of lactobacilli (Corcoran et al., 2005). The persistence of a potential LAB in acidic conditions is crucial for it to survive passage through the GIT. A 4 log decrease in *L. kimchicus* 5.1 viable cells was observed after incubation at pH 2 for 4 hrs. At pH 2.5, although there was a decrease in the viable cell numbers, this difference was less than 1 log indicating that this organism is tolerant to this pH value. These results are in agreement with those obtained from a study by Faye et al. (2012), where the viability of *Lactobacillus* strains *(L. rhamnosus* GG and *L. paracasei* INF-448) decreased from log 7 to about log 4 CFU/ml after 3 hrs of exposure at pH

2. The acid-resistant locus (Arl7) located between positions 35676 and 36425 of the *L. kimchicus* 5.1 genome was also found to be part of unique genes belonging to *Streptococcus thermophilus* ACA-DC 2, which is a strain that was isolated from traditional Greek yoghurt by Alexandraki et al. (2017). It can be hypothesised that Arl7 contributes to *L. kimchicus* being able to survive under acidic conditions. LAB utilise fermentable carbohydrates to produce lactic acid. Therefore, acid resistance in LAB is vital for their growth, fermentation, and use as probiotics. LAB employ various mechanisms to regulate acid resistance and survive in low pH environments. These include changes in cell membrane composition, DNA and protein repair (Wang et al., 2018). The protein-encoding gene (PEG) for (Gls24), which is defined as a "general stress protein," was identified in the genome of *L. kimchicus* 5.1. This could have contributed to the varying degrees of tolerance to acidic conditions of this strain when exposed to pH 2- 6 for 6 hrs (Figure 2.9(A)).

Another criterion for assessing potentially probiotic bacteria is the ability to survive the hostile environments in the GIT. In particular, they must be bile tolerant to survive in the small intestine. The stress caused by incubation in 0.3% bile salt resulted in the death of less than 3% of the *L. kimchicus* 5.1 cells (Figure 2.10). This concentration of bile is considered physiologically relevant and is often regarded as critical for evaluating strain resistance to bile (Gharbi et al., 2018). After 5 hrs of incubation at 0.5% bile salts, greater injury to the cells was observed as a 4.8% reduction in viable cells occurred. Bile salt hydrolase, also known as cholylglycine hydrolase, plays a role in deconjugating bile salts (Chand et al., 2017). BSH has various essential functions in colonising the host and is involved in host physiological processes (Grimm et al., 2014). *Lactobacillus* strains with this ability have the added advantage of colonising the small intestine where the enterohepatic cycle occurs (Kumar et al., 2011). Other functions include a nutritional role, where the amino acids released from bile salt deconjugation can be used as nitrogen and carbon for bacterial survival and growth. BSH can also facilitate incorporating cholesterol or bile into bacterial membranes, increasing the membrane tensile strength of bacteria and enabling gastrointestinal persistence (Begley et al., 2006; Chand et al., 2017; Tanaka et al., 1999). BSH activity confers a benefit to the host by reducing serum cholesterol levels in the body (Sedláčková et al., 2014*),* and BSH activity is perceived as a beneficial probiotic trait (Begley et al., 2006). For bacteria to be useful as a potential probiotic strain, the organism should survive in the presence of bile salt in the GIT. Because we could not find the *bsh* gene in the genome of *L. kimchicus* 5.1, this strain was not expected to survive well under conditions with bile. There are some reports of studies that do

not correlate the bile tolerance of strains with BSH activity (Moser and Savage, 2001, Usman and Hosono, 1999). For example, using tauroconjugated bile acids instead of glycoconjugate bile acids may yield differing results (Begley et al., 2006). Although no BSH activity was observed for *L. kimchicus* in the qualitative plate assay, it is important to note that the presence and genetic organisation of *bsh* genes in lactobacilli are quite variable. Furthermore, in some *Lactobacillus* strains, enzyme activity may depend on substrate specificity, temperature and pH range (Fang et al., 2009).

Temperature variation is probably the most common stress bacteria encounter in the natural environment. Some LAB can survive at high temperatures between 45 and 80°C (Mbye et al., 2020). *L. kimchicus* 5.1 cells showed a moderate level of tolerance to high temperatures. For *L. kimchicus* 5.1 cells, exposure to 50°C for 30 mins reduced viable cell numbers by less than 1 log compared to incubation at the control temperature (37°C). At 60°C, there was a log reduction in viable cells, suggesting that the highest temperature that *L. kimchicus* 5.1 can tolerate is 50°C.

Lyophilisation is a commonly used method for preservation of biological material such as viable bacteria to safeguard proteins and liposomes, using sugars such as trehalose and sucrose as the main protectants (Zhang et al., 2017). Freeze-drying can result in cell damage and loss of viability. When *L. kimchicus* 5.1 was freeze-dried using trehalose as a cryoprotectant, the resulting powder consistently harboured *L. kimchicus* 5.1 at levels between log 10 and log 11 CFU per gram. Trehalose was useful in providing the necessary protection for *L. kimchicus* 5.1. Trehalose was chosen because it had been reported by Li et al. (2011) to protect *Lactobacillus reuteri* during freeze-drying sufficiently. Another important characteristic of probiotic bacteria is maintaining viability during the product life cycle (Muller et al., 2013). The therapeutic potential of these bacteria in the GIT depends on their survival during manufacture and storage (Kailasapathy and Chin, 2000). Determining the appropriate conditions for storage of a probiotic powder increases the survival of LAB and, consequently, its effectiveness in the host. There was a 3-log loss of viability of *L. kimchicus* 5.1 during six weeks of powder storage at room temperature. After six weeks of storage at room temperature, the number of viable cells remained above the desired minimum of 10^6 CFU per gram viable cells. However, storage at 4°C resulted in higher viability (log 10) CFU over six weeks (Figure 2.12). This indicates that *L. kimchicus* 5.1 can be stored as a probiotic powder for six weeks at refridgerated temperature.

Conclusion

In this study, small to medium, cream and white coloured, and smooth colonies, which grew best when incubated anaerobically at 37°C, were successfully isolated from the great tit bird faeces using LBS. Genomic sequencing was used to identify the four isolates as *L. kimchicus.* A representative isolate, *L. kimchicus* 5.1, was found to have a doubling time of 4.65 hrs and was characterised as a D-lactate producing homofermentative strain. Although the isolate exhibited slight resistance to streptomycin, *in silico* screening for antibacterial genes using CARD found an antibiotic resistance mechanism that had a 31.9% match to a set of genes for the antibiotic efflux pump that confers tetracycline resistance. WGS also showed that *L. kimchicus* 5.1 harbours no transposable elements or plasmids. The strain was capable of survival under acidic conditions down to pH 2.5 as cell viability was maintained at $1x10^7$ CFU/ml after 6 hrs of exposure. Furthermore, tolerance to *in vitro* digestive conditions was demonstrated in that the strain survived at 98.5% in the presence of 0.3% bile salts for 5 hrs. *L. kimchicus* 5.1 did not exhibit an ability to deconjugate bile salts TCA and TDCA, which was in agreement with WGS results where there were no detectable bile salt hydrolase genes. *L. kimchicus* demonstrated noticeable tolerances to high temperatures. When incubated at 40-45°C for 30 mins, viable cell counts remained at 10^7, similar to the growth observed at 37°C. As a freeze-dried powder, the strain maintained high viability when stored at 4°C for six weeks. Although more research is needed for product development technology, evaluation of extended shelf-life properties, and the actual health benefits conferred to the host, this study lays the foundation for the use of *L. kimchicus* 5.1 as a potential probiotic strain.

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Chapter 3.

Isolation and characterisation of bacteriocin-producing LAB from ruminants' environment.

Abstract

LAB are physiologically and metabolically related organisms with a traditional and important role in food, agriculture, and clinical applications. Bacteriocins produced by some LAB are structurally and functionally diverse heat-stable cationic proteins, possessing broad or narrow spectrum antimicrobial activity. This study aimed to isolate and identify LAB from the rumen environment and to screen and characterise any bacteriocins that these LAB produce. Lactic minimal media (LMM) was used to screen for LAB specifically, and *L. delbrueckii* subsp. *bulgaricus* LMG 6901 was used as an indicator during bacteriocin screening via spot and well diffusion assays. Using LMM, the most frequently isolated genus was *Lactobacillus* (62.5%), followed by *Enterococcus* (31.25%), and there was an equal distribution of *Streptococcus* and *Pediococcus*, each constituting 6.25% of the total isolates. The antimicrobial compound containing CFS were neutralised and characterised in terms of proteinase K sensitivity and thermal stability (incubation at 37, 60, 70, 80, 90, 100, and 121°C for 10 mins). The inhibition spectra against common pathogens was evaluated using CFS from LAB. Colony mass spectrometry was used to confirm the peptide sizes of putative bacteriocins. Of the 16 isolates, which exhibited antimicrobial activity, 1 CFS from *Lactobacillus mucosae* (D11) was insensitive to proteinase K treatment. A general trend showed a decrease in bacteriocin activity associated with increased temperature for most of the bacteriocins assessed. Mass spectrometry showed inhibitory compounds of various sizes from 1100-6000 Da. The whole-genome sequence of *Enterococcus thailandicus* (L16) was subjected to *in silico* screening to identify potential bacteriocins using Bagel4, and two potential bacteriocins were found, i.e., Subtilosin A and Enterocin. This study shows that the agricultural environment is a source of a variety of LAB, many of which are bacteriocin-producing. These bacteriocin-producing LAB may have applications in food and in animal health.

Introduction

LAB occur naturally in the environment and have been commonly isolated from grass silage, dairy food products and sourdough, among other settings (Ma et al., 2019). LAB have traditionally been used as starter cultures in food fermentation, flavour and texture development and in various applications requiring pathogen inhibition (McAuliffe et al., 2001). Many LAB have gained acceptance because of their GRAS status and the effects they impart as probiotics, while others are exploited because they produce various secondary beneficial metabolites, some of which have antimicrobial activity. Such metabolites include organic acids, specifically lactic acid, hydrogen peroxide and bacteriocins which they may release into their surrounding environment (Henning et al., 2015). The LAB group comprises genera such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Streptococcus*, in addition to *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella*, with *Lactobacillus* being the most widely studied and used genus from this group (Vieco-Saiz et al., 2019). A recent update to the *Lactobacillus* nomenclature has specified 261 species belonging to this genus categorised based on their genetic similarity and phylogeny (Zheng et al., 2020).

One of the most exploited properties of LAB is their ability to produce bacteriocins against spoilage and pathogenic microorganisms. Bacteriocins are structurally and functionally diverse peptides that are ribosomally synthesised (O'Connor et al., 2015). They are antimicrobial compounds produced by LAB that may be bacteriocidal or bacteriostatic toward other bacteria (Galvez et al., 2010) but are ineffective against the species by which they are produced (Joerger, 2003). Bacteriocin producers protect themselves against their own compounds by immunity which they express concomitantly with the bacteriocin production (Settanni and Corsetti, 2008). The classification of bacteriocins has been revised recently (Soltani et al., 2021). Bacteriocins are classified into two major classes: Class 1 lantibiotics, and Class 2 non-modified bacteriocins or non-lantibiotics (Alvarez-Sieiro et al., 2016). There is a smaller, lesser-known third class known as bacteriolysins, consisting of heat-labile proteins (Darbandi et al., 2022).

The most promising bacteriocins for food safety and human and animal health applications are those produced by LAB(Daba and Elkhateeb, 2020). Bacteriocins are different from most antimicrobial agents because they are proteinaceous and are degraded in the digestive system by various proteases. Because bacteriocins are non-persistent in the environment or human

body, there is a reduced chance of unintended interaction with bacteria, reducing the likelihood of bacteriocin resistance (Parada et al., 2007). This property allows for the development of many applications in food preservation and human and animal health. Literature on bacteriocin use has highlighted applications in diverse therapeutic purposes such as treatment of peptic ulcers, anticancerous agents, spermicides, veterinary use, skin and oral care, and plant growth promotion in agriculture (Negash and Tsehai, 2020; Norouzi et al., 2018). They have also been investigated for their potential in enteric methane mitigation (Callaway et al., 1997).

Based on their distinctive mechanisms of action, bacteriocins are categorised as bacteriocidal or bacteriostatic (Silva et al., 2018). Bacteriocins attack their target bacteria by forming an interaction with the bacterial cell surface and cell wall. The primary mechanisms of action are cell permeabilisation and pore formation (Simons et al., 2020). The most extensively studied and commercially used bacteriocin is nisin, a heat-stable bacteriocin produced naturally by *Lactococcus lactis* subsp. *lactis* and some strains of *Streptococcus* (Jozala et al., 2015).

The use of *in silico* screening methods has increased over the past decade due to its ability to process larger projects in less time and is more cost-effective than culture-based methods (Egan et al., 2018). Such methods prevent potential challenges relating to finding the susceptible indicator strains or identifying bacteriocins from difficult to culture strains (Papagianni et al., 2006). To further exploit the probiotic properites of LAB, the study described in this chapter aimed to isolate LAB from the ruminant environment, screen these for antimicrobial activity such as bacteriocin production, identify the producing strain, and characterise the antimicrobial agent produced. Characterisation included checking for inhibition against various common pathogens, identifying the peptide size using mass spectrometry (MS), evaluating thermal stability of the CFS, and determining if the compounds produced are proteinaceous, thereby permitting them to be classified as bacteriocins. *In silico* bacteriocin screening was used to identify the putative genes present in one isolate (L16 identified as *E. thailandicus*).

Materials and Methods

Isolation of Lactic Acid Bacteria

Environmental samples were screened for the presence of LAB. Sources included cow patties, grass, clover, and rumen fluid fractions obtained from rumen-cannulated cows at Teagasc Moorepark Research Centre, Ireland. Phosphate buffer saline (PBS) was used to serially dilute samples (10-10^9). After spread-plating on lactic minimal azide media (LMM), plates were incubated anaerobically at 30°C and 37°C for 24 hrs. LMM agar plates were prepared according to Lawson and tanner (2014) using glucose (10 g/l), yeast extract (10 g/l), $CaCO_3$ (2 g/l), KH_2PO_4 (1 g/l), purified agar (15 g/l), and sterilised at 121°C for 15 mins. Thereafter, filter sterilised LMM vitamins (10 ml) (Table 3.1), 10 ml mineral solution (Table 3.2), 1 ml trace metals (Table 3.3) and 2 ml of sodium azide (5%) (w/v) were added to 1 litre of molten agar with LMM components. Single phenotypically different colonies were selected and restreaked onto De Man, Rogosa, and Sharpe, MRS plates (BD™ Difco™ Trafalgar Scientific Ltd, Leicester, United Kingdom) and brain heart infusion (BHI) plates (Oxoid) for routine cultivation. The colonies were restreaked several times thereafter to ensure purity. The pure cultures were subsequently kept in MRS broth supplemented with glycerol (final concentration of 35% w/v) and stored frozen at −80°C until further analysis.

Table 3.1: Vitamin solution for LMM.

Component	Milligrams per litre
Pyridoxine HCl	10
Thiamine HCl	5
Riboflavin	5
Calcium pantothenate	5
Thioctic acid	5
p- aminobenzoic acid	5
Nocitinic acid	5
Vitamin B12	5
Biotin	2
Folic acid	2
Mercaptoethanesulfonic acid	10

Table 3.2: Mineral solution for LMM.

Component	Grams per litre
NaCl	80
NH_4Cl	100
KCl	10
KH_2PO_4	10
$MgSO_47H_20$	20
$CaCl_22H_20$	4

Table 3.3: Trace metal solution for LMM

Component	Grams per litre
Nitriloacetic acid (Adjust pH 6 with KOH)	2
$MnSO_4H_2O$	1
$Fe(NH_4)_2(SO_4)_26H2O$	0.8
$CoCl_26H_20$	0.2
$ZnSO_47H_2O$	0.2
$CuCl_2H_2O$	0.02
$NiCl_26H_2O$	0.02
$Na_2MoO_42H_2O$	0.02
Na_2SeO_4	0.02
Na_2WO_4	0.02

Biochemical characteristics of isolates

Preliminary identification of strains was carried out using phenotypic methods, namely Gram staining and assessment of the morphology of cells by light microscopy (1000× magnification) and catalase testing. Cells were grown on MRS media under the appropriate conditions and Gram-stained according to standard protocol. Catalase testing was performed by placing a drop of 3% (v/v) hydrogen peroxide solution on a single pure colony. Immediate gas formation (bubbling) was considered positive. Isolates that were Gram-positive and catalase-negative were further analysed as described below.

Molecular identification

Genomic DNA extraction

Genomic DNA was extracted from 16 antimicrobial-producing strains (grown anaerobically overnight in 10 ml MRS broth at 37°C) using the Repeated Bead Beating and Column (RBB+C) method according to Yu and Morrison (2004). Sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm) were combined with 0.25 ml of overnight bacterial culture. Cells were lysed using a mini bead beater for 3 mins at maximum speed in the presence of a lysis buffer which comprised of 4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, and 50 mM EDTA. This was followed by sample incubation at 70°C for 15 mins, with gentle mixing by hand, at 5 min intervals. To remove impurities and SDS, samples were centrifuged at 16,000 × g for 5 min at 4°C. Subsequently, 260 µl of 10M ammonium acetate was added to each lysate tube, homogenised thoroughly, and incubated in the fridge for 5 mins. This was centrifuged at 16,000 × g for 10 mins at 4°C, then the supernatant was split into two 1.5-ml Eppendorf tubes, and one volume of isopropanol was added and incubated at 4°C overnight. Centrifugation at 16,000 × g for 15 mins at 4°C was followed by removing the supernatant, washing the nucleic acid pellet with 70% ethanol, and air drying in the laminar flow hood. The nucleic acid pellet was dissolved in 100 µl of TE (Tris-EDTA) buffer. Thereafter, the 2 aliquots were pooled together. Genomic DNA was purified via sequential digestions with RNase and proteinase K. Briefly, 2 µl of DNase-free RNase (10 mg/ml) was added to the sample and incubated at 37°C for 15 mins. Proteinase K (15 µl) and 200 µl of Buffer AL (from the QIAamp DNA Stool Mini Kit) were added, vortexed to mix, and incubated at 70°C for 10 mins. Afterwards, 200 µl of ethanol was added and mixed well, then transferred to a QIAamp column and centrifuged at 16,000 × g for 1 min at room temperature. After discarding the eluate, 500 µl of Buffer AW1 (Qiagen) was added and centrifuged for a further 1 min at room temperature. Subsequently, 500 µl of Buffer AW2 (Qiagen) was added, and the solution was centrifuged for 1 min at room temperature. The column was centrifuged for 1 min at room temperature to dry it, and 200 µl of Buffer AE (Qiagen) was added. Finally, the samples were incubated at room temperature for 2 mins and centrifuged for 1 min to elute the DNA.

DNA quality was checked by analysing 2 µl of sample in 1% SYBR Safe gel electrophoresis, operated at 100 volts for 30 min. The gels were visualised using the Odysseyâ FC imaging system. The extracted DNA was quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, 168 Third Avenue Waltham, MA, USA 02451).

16S rRNA sequencing

Polymerase Chain Reaction (PCR) was performed using Biomix red PCR mix (Meridian Bioscience, Dublin, Ireland). For amplification of the 16S rRNA gene, universal primers F27 (5-AGAGTTTGATCCTGGCTCAG-3) and U1492 (5-GGTTACCTTGTTACGACTT-3) (Sigma- Aldrich) were used with the following run conditions: initial denaturation: 94°C × 5 mins, cycling conditions: 30 cycles of 94°C × 40 s, 55°C × 30 s, 72°C × 1 min, final extension: 72°C × 10 mins. The PCR products were confirmed via gel electrophoresis and purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, to clean up the PCR product, 5 volumes of the binding solution were added to 1 volume of the PCR reaction solution and mixed. The solution was transferred to a binding column and centrifuged at 15,000 × g for 1 min at room temperature. The eluate was discarded then 500 µl of wash solution was added and centrifuged again at 15,000 × g for 1 min. The empty column was centrifuged at 15,000 × g for 2 mins to remove residual eluate. The column was transferred to a new collection tube, and 50 µl of elution solution was added to the centre of the column and incubated for 5 mins at room temperature. The column was centrifuged at 15,000 × g for 1 min to recover DNA and sent for sequencing at Genewiz (Hope End, Takeley, Essex, CM22 6TA, United Kingdom). The resulting sequences were compared to existing genomic data using the Basic local alignment search tool (BLAST) on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The identity of the isolates was determined based on the highest scores (≥ 98%).

Detection of antimicrobial agent production

Deferred antagonism assay

The pure isolated colonies of different morphologies and colours were tested for bacteriocin production against *L. delbrueckii* subsp. *bulgaricus* LMG 6901. Briefly, the isolates were grown overnight in MRS broth, then 10 µl was spotted onto MRS agar, incubated overnight, and overlaid with 0.75% (w/v) sloppy agar, and seeded with the appropriate indicator. The indicator culture had been grown anaerobically at 37°C overnight. Anaerobic conditions were achieved by incubating in an anaerobic chamber (Baker Ruskinn Concept 400, Fannin Ltd., Dublin, Ireland). Colonies from spot assays that displayed zones of inhibition, identified by a clear zone around each colony, were then inoculated into MRS broth, grown overnight at 37°C, and cryopreserved in 35% (vol/vol) glycerol at −80°C while awaiting further characterisation.

Characterisation of antimicrobial peptides produced

Well diffusion assays (WDAs)

Well diffusion assays were performed according to Ryan et al. (1996). Strains that had produced a zone of inhibition in the spot assay were grown overnight in 10 ml MRS broth. The supernatant was centrifuged at 4000 × g for 20 mins at 4°C (Sorvall, Legend RT). CFS were neutralised to pH 6.9-7.2 using a 2 M filter-sterilised NaOH to eliminate the possibility of inhibition due to acid. Sloppy media (0.5% (w/v) agar) was seeded with 0.25% (v/v) of the appropriate indicator inoculum, which had been grown to the log phase. Wells (7 mm) were bored on the plates, and 50 µl of CFS was added. The plates were incubated in the appropriate conditions and observed for inhibition zones surrounding the wells. Strains that maintained activity by displaying inhibition zones were selected for further study.

Heat stability and protease sensitivity

To determine the effect of heat, treatment of the antimicrobial agents was conducted by incubating 1 ml of CFS at 37, 60, 70, 80, 90, 100, and 121°C on a hot plate (Fischer Scientific) for 10 mins, then 50 ul was used in WDAs against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 as described previously. To investigate protease susceptibility of the antimicrobials produced, CFS were subjected to proteinase K treatment (Sigma- Aldrich) by incubating 1 ml of CFS with 1 ml of proteinase K at a final concentration of 20 mg/ml at 37°C for 3 hrs and then at 100°C for 10 mins to inactivate the enzyme. Antimicrobial activity was determined against *L. delbrueckii subsp. bulgaricus* LMG 6901 indicator by assaying 50 µl of CFS into wells as described above.

Spectrum of inhibition

WDAs were carried out as described above with the strains in the appropriate medium and growth conditions according to Table 3.4. Indicators included Gram-positive (*Lactobacillus fermentum, Blautia schinkii, Enterococcus faecium, Staphylococcus epidermis, Staphylococcus simulans*, *Streptococcus agalactiae*, and *Streptococcus lutetiensis* previously termed *Streptococcus bovis* type ii) and Gram-negative bacteria (*Escherichia sp.* and *Enterobacter cloacae*). Inhibition assays were performed following the protocol by O'Sullivan et al. (2019). All indicator strains were adjusted to the same optical density. (OD_{600} nm of 0.8), using a Biochrom Libra S2 Colorimeter (Biochrom Ltd., Cambourne Business Park, Cambridge, United Kingdom) before seeding the agar.

Table 3.4: Growth conditions for the different target organisms.

Target organism	**Media**	**Growth conditions**
S. epidermis	Brain Heart Infusion (BHI)	Aerobic 37°C
Escherichia sp.	Luria Broth (LB)	Aerobic 37°C
S. simulans	BHI	Aerobic 37°C
S. lutetiensis	Tryptic Soy Agar (TSA)	Aerobic 37°C
E. cloacae	LB	Anaerobic 37°C
E. faecium	McConkey broth	Anaerobic 37°C
S. agalactiae	TSA	Aerobic 37°C
L. fermentum	MRS	Aerobic 37°C
B. schinkii	Anaerobic basal broth	Anaerobic 37°C

Cross immunity

To investigate if the bacteriocin produced by the strains were related to each other, cross-immunity tests were performed. Overnight cultures of producing strains were used as target strains, and neutralised cell-free supernatant from another producer was assayed against it using the WDA method described previously.

Activity units

For the different antimicrobial-producing strains identified, bacteriocin activity units (AU) were calculated as described by Zhang et al. (2018), in which one bacteriocin unit is arbitrarily defined as the reciprocal of the highest dilution that showed a clear inhibition zone and was expressed as activity units (AU) per ml. Using the WDA, 100 µl of CFS was diluted 2-fold each time, and 50 µl of the dilutions were dispensed into wells of plates seeded with *L. delbrueckii* subsp. *bulgaricus* LMG 6901.

Molecular sizes of the antimicrobial agents

Colonies of the antimicrobial agent producers were streaked on the appropriate media, grown overnight, and assayed by MALDI-TOF (Matrix Supported Laser Desorption/Ionization Flight Time) mass spectrometry to determine the molecular mass of antimicrobial compounds (Axima

TOF2 MALDI-TOF mass spectrometer; Shimadzu Biotech, Manchester, UK). Positive-ion linear or reflectron mode was used to detect peptide masses.

In silico screening for bacteriocin production

Whole Genome Sequencing (WGS)

Isolate L16 (*E. thailandicus*) was prepared for WGS by extracting DNA using the RBB+C method. Thereafter, DNA was quantified using a Qubit 2.0 fluorometer and submitted for sequencing (Genewiz, UK). Annotated genomes were analysed for predicted bacteriocins using the Bagel4 webserver (http://bagel4.molgenrug.nl/) according to van Heel et al. (2018). The resulting protein sequences were compared to an existing protein database, Blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (Camacho et al., 2009) to check for homology.

Results

Isolation and identification of LAB

Based on colony morphology, 16 phenotypically different strains were isolated from environmental samples using LMM plates. As a general approach, cultures were identified as LAB based on their Gram stain and catalase test reaction and their physiological characteristics such as growth temperature and sensitivity to oxygen. The isolates were mainly Gram-positive, and a negative Gram stain was used as an exclusion criterion to exclude isolates. Selected isolates also showed negative catalase results, as is characteristic of LAB. Putative LAB strains were identified using 16S rRNA sequencing. The 16 LAB strains were classified into four genera, with the dominating genus being *Lactobacillus* (56.25%), followed by *Enterococcus* (31.25%), and there was an equal distribution of *Streptococcu*s and *Pediococcus*, each constituting 6.25% of the total isolates identified (Table 3.5). At strain level, three *Lactobacillus paracasei*, two *Lactobacillus mucosae*, a *Lactobacillus zeae* and an *Enterococcus thailandicus* were isolated.

Table 3.5: Identification via 16S rRNA sequencing of LAB isolates.

Code	Strain identity
D1	*Lactobacillus paracasei*
L1	*Lactobacillus zeae strain RIA*
L14	*Lactobacillus plantarum*
Pat	*Lactobacillus mucosae*
D14	*Lactobacillus paracasei*
D12	*Pediococcus acidilactici*
D11	*Lactobacillus mucosae*
D7	*Lactobacillus coryniformis* subs *torquens*
L18	*Enterococcus sangunicola*
L16	*Enterococcus thailandicus*
L6	*Lactobacillus zeae strain* RIA 412
D10*	*Streptococcus cristatus* ATCC 51100
D7*	*Enterococcus faecium*
L2	*Entercoccus thailandicus*
L20	*Lactobacillus paracasei subs tolerans*
D17	*Enterococcus hirae* ATCC 9790

Screening for antimicrobial production

Using the spot assay, the isolated strains were evaluated for bacteriocin production by screening for antimicrobial activity against *L. delbrueckii* subsp. *bulgaricus* LMG 6901. The inhibition zones produced were small to medium in size and were sharp, clear zones (Figure 3.1).

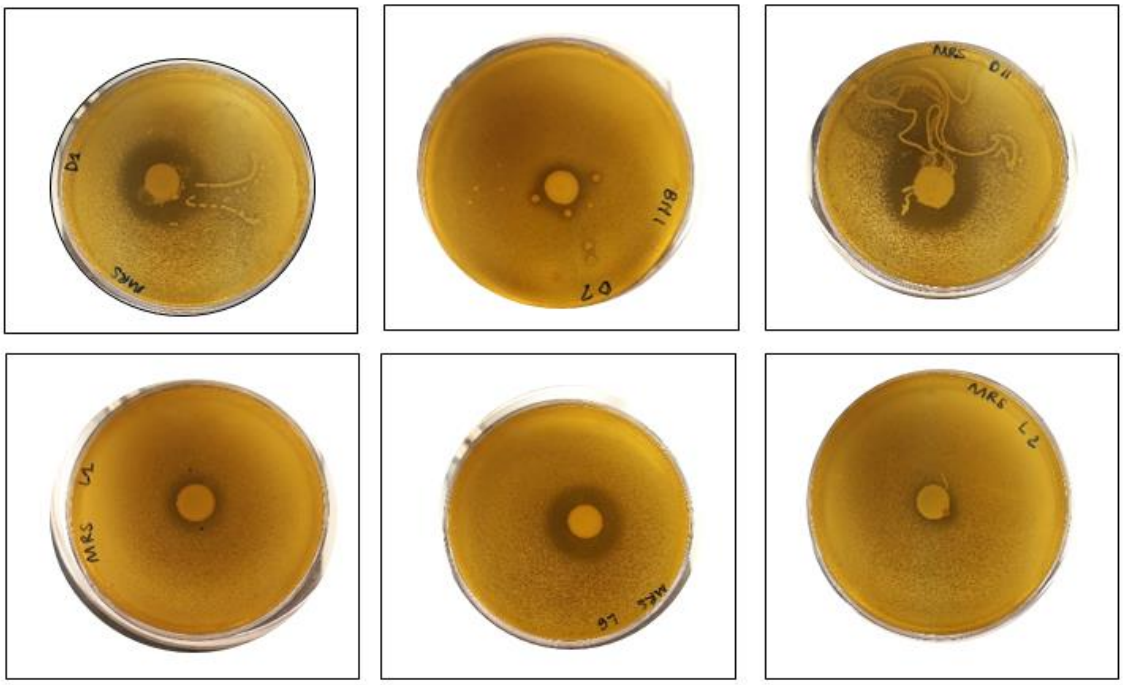


Figure 3.1: Initial screening of antimicrobial-producing colonies. Spot assays of a selection of isolates overlayed with *L. delbrueckii* subsp. *bulgaricus* LMG 6901.

Characterisation of antimicrobial compounds

CFS from the 16 isolates that exhibited antimicrobial activity were used to characterise the antimicrobial compounds produced. The secondary screening, which involved the well diffusion method, was conducted using neutralised CFS collected from the isolates. These were also tested against *L. delbrueckii* subsp. *bulgaricus* LMG 6901. Figure 3.2 shows that when the neutralised (pH 6.9 to 7.2) CFS from LAB were dispensed into agar wells, antimicrobial activity was reduced in CFS from *L. zeae* (L6 and L1), *L. plantarum* (L14), and *E. faecium* (D7*). In some instances, antimicrobial activity was completely lost, as indicated by the absence of inhibition zones in *E. sanguinicola* (L18) and *L. paracasei* (D14). CFS from six isolates were unaffected by changes in pH as inhibition zones remained similar in size and

intensity, pre and post-neutralisation. These CFS were from *P. acidilactici* (D12), *L. mucosae* (D11), *L. paracasei* (D1 and L20), *E. thailandicus* (L16), and *E. hirae* (D17).

Proteinase K treatment was performed on the neutralised CFS to determine susceptibility to protease activity. Antimicrobial activity was eliminated in 93% of the isolates, indicating that the antimicrobials produced were proteinaceous, except for *L. mucosae* (D11) supernatant, which remained inhibitory post proteinase K treatment.

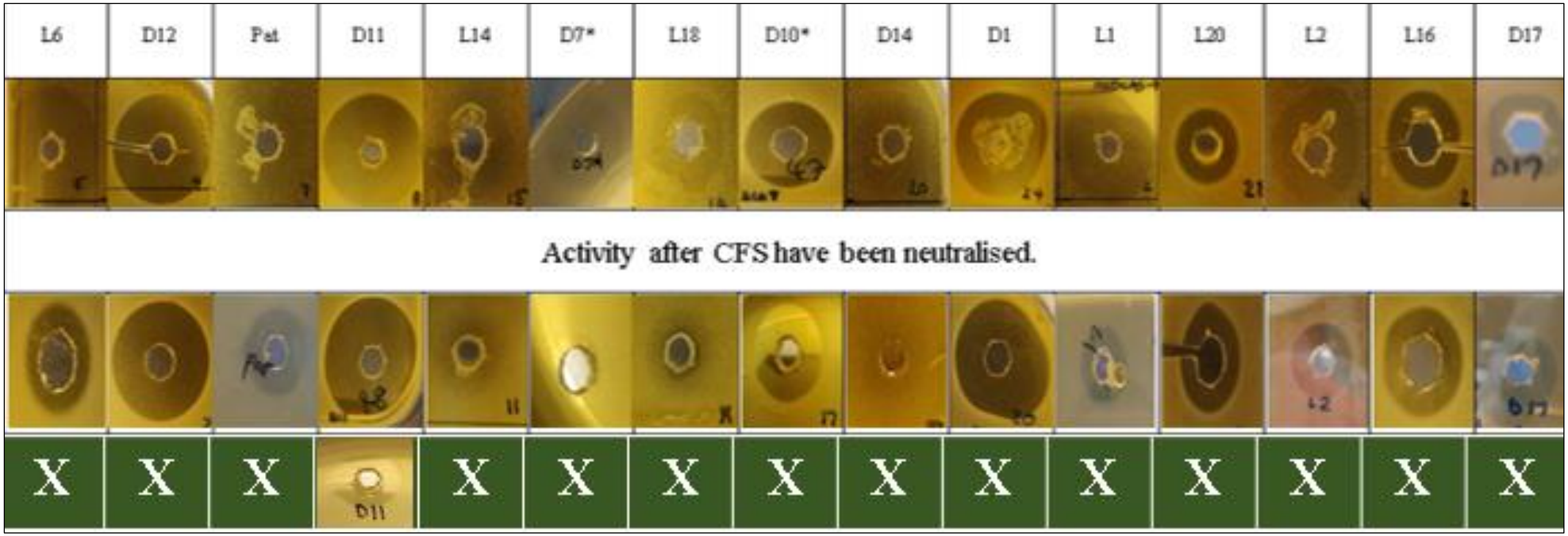


Figure 3.2: Well diffusion assays were performed using neutralized cell free supernatants (CFS) against acid-tolerant *L. delbrueckii* subsp. *bulgaricus* LMG 6901.

Key: **X** = Activity lost **+** = Activity maintained.

Thermal stability of bacteriocins produced by LAB strains

Heat stability tests were conducted to classify further the antimicrobial compounds produced by the various strains. When the CFS from the LAB strains were heated at different temperatures, i.e., from 37°C to 121°C, activity was lost at varying degrees. The following strains produced antimicrobial compounds that were only active at 37°C: *L. plantarum* (L14), *L. zeae strain* RIA 412 (L6), *L. mucosae* (Patty and D11), *L. coryniformis* subs torquens (D7), and *S. cristatus* (D10 *). Three of the isolates, i.e., *L. paracasei* (D1), *E. faecium* (D7*) and *E. thailandicus (*L2), produced CFS that were active at 90°C. At the same time, CFS from *P. acidilactici* D12 did not lose activity at 121°C, indicating that the compound(s) responsible for antimicrobial activity may not be heat sensitive.

Activity units

Most of the supernatants exhibited low activity units, ranging from 20 to 160 AU/ml (Figure 3.3 (A)). Isolates that produced bacteriocins with low activity units of 20 AU/ml were *L. zeae* (L1), *L. plantarum* (L14), *L. mucosae* (Pat) and *E. hirae* (D17). Moderate activity of 160 AU/ml was observed in both strains of *E. thailandicus* (L16 and L2) and *L. mucosae* (D11). The antimicrobial agent produced by *P. acidilactici* (D12) (Figure 3.3(B)) exhibited potent inhibitory activity against *L. delbrueckii* subsp. *bulgaricus* LMG 6901, with activity units exceeding 1280 AU/ml.

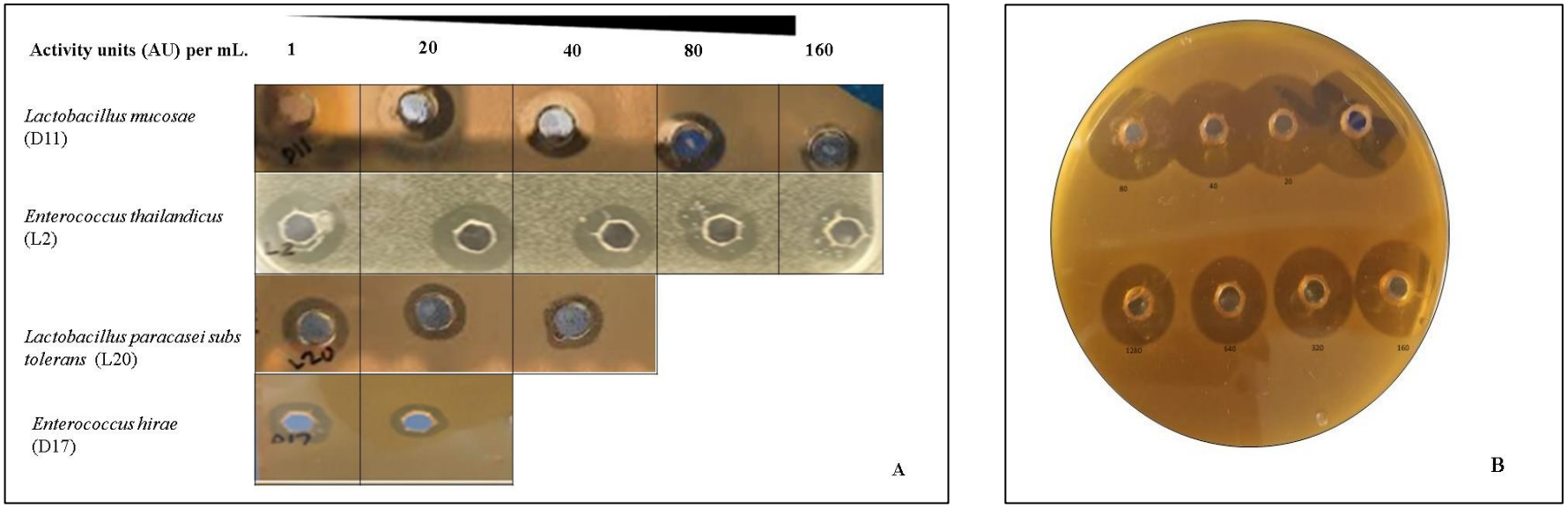


Figure 3.3: Antimicrobial activity (Active units) of a selection of neutralised CFS from isolated LAB (A) and *P. acidilactici* (D12) (B) using well diffusion assays.

Cross immunity and determining the spectra of inhibition

Cross-immunity assays were performed using the active CFS from all the antimicrobial-producing strains tested against themselves and each other (data not shown). For the isolates, the antimicrobial producers were immune to their own produced antimicrobial compound, as anticipated. The supernatants were tested against six common pathogens, as well as against a rumen anaerobe *B. schinkii* and LAB *L. fermentum*. Table 3.6 shows the inhibition spectra of antimicrobial producers against the eight indicators. The values presented are the mean of two independent experiments. Inhibition zones were measured (mm) as the radius from the start to the end of the zone. These were represented as No inhibition = -, 1-2 mm= +; 3-5mm= ++; 5-10 mm = +++. None of the isolates tested displayed strong antimicrobial activity against all the pathogens used. Notably, D1, which is *L. paracasei,* exhibited strong activity against gram-negative organisms *E. cloacae* and *Escherichia sp*, and slight inhibition was observed against Gram-positive *S. epidermis and S. simulans*. The strongest antimicrobial activity was exhibited by *P. acidilactici* (D12) toward *S. epidermis, S. lutetiensis, E. faecium,* and *L. fermentum*.

Table 3.6: Spectrum of inhibition of potential bacteriocin-producing LAB isolates against indicator strains using agar well diffusion assay.

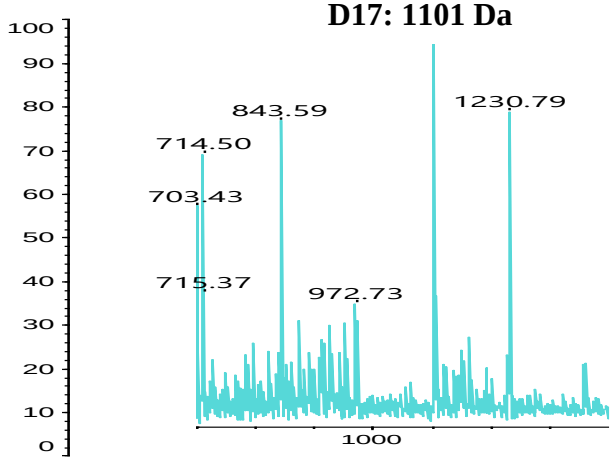
Supernatant	**D11**	**D17**	**D12**	**D1**	**L1**	**L14**	**PAT**	**L2**	**D7***	**L16**
Indicator										
S. epidermis	-	-	++	+	-	+	-	-	-	-
Escherichia sp	-	+	-	+++		-	-	+	++	+
S. simulans	-	-	-	+	-	-	-	+	+	-
S. lutetiensis	-	-	++	-	-	-	-	-	+	-
E. cloacae	-	-	-	++	-	+	-	+	+	+
E. faecium	+	-	+	-	+	+	+	-	-	-
S. agalactiae	-	-	-	-	-	-	-	-	-	-
L. fermentum	-	-	++	-	-	-	-	-	-	-
B. schinkii	+	-	-	-	-	-	+	+	-	-

Molecular sizes of the antimicrobial agents

Colony mass spectrometry (MS) was used to identify the antimicrobial agents produced (Figure 3.4 (A-L)). MS showed that the isolates had peptides with masses ranging from 1101.36 Da, from an isolate identified as *L. plantarum* (L6) to 6310 Da produced by *E. thailandicus* (L16). *P. acidilactici* (D12) produced a CFS with a peptide of mass 3354.39 Da. *S. cristatus* (D10*) and *L. zeae* strain RIA (L1) had multiple peptides, indicating that these strains produce several metabolites. In *S. cristatus* (D10*), the peaks were 3443, 4372, 5381 and 5794 Da.

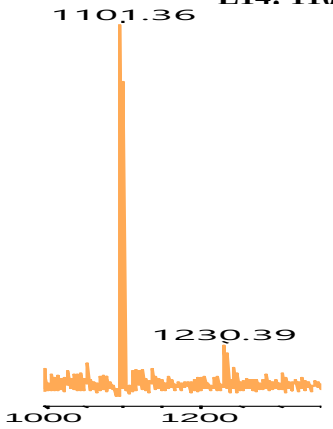
(A)

D17: 1101 Da



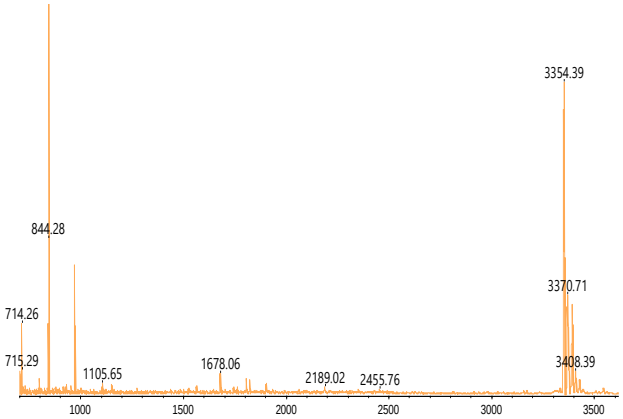
(B)

L14: 1101.36 Da



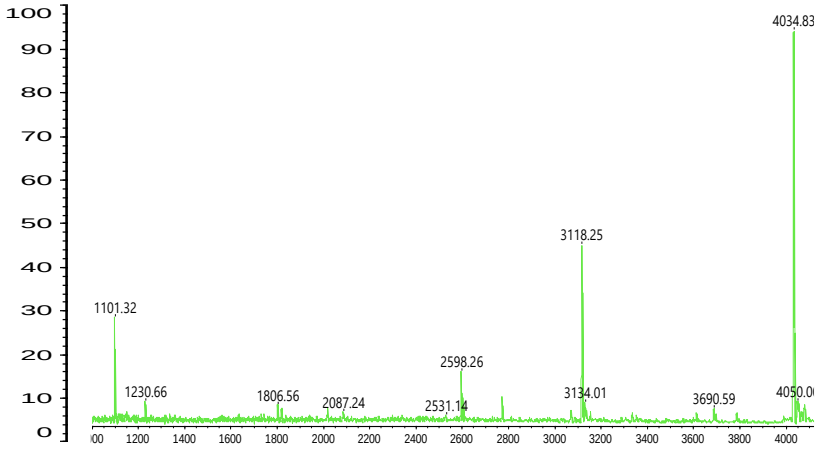
(C)

D12: 3354.39 Da



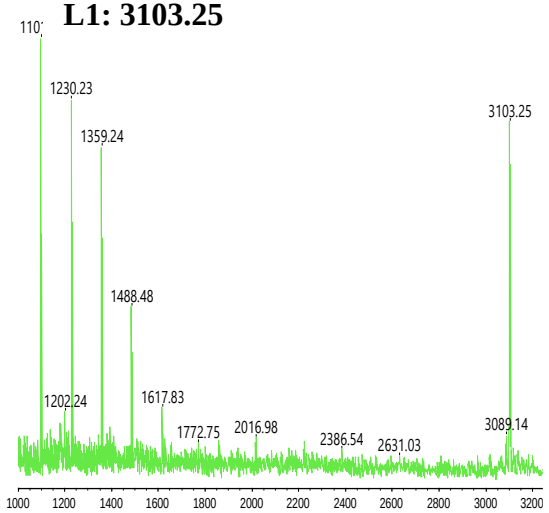
(D)

L6: 4034.86 Da



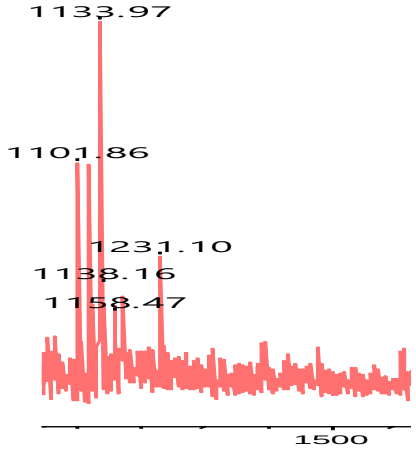
(E)

L1: 3103.25



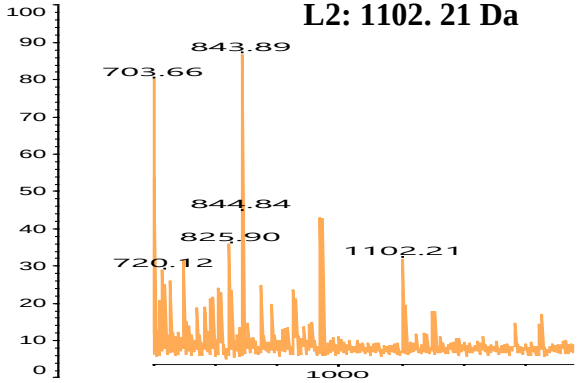
(F)

D1: 1133.97 Da



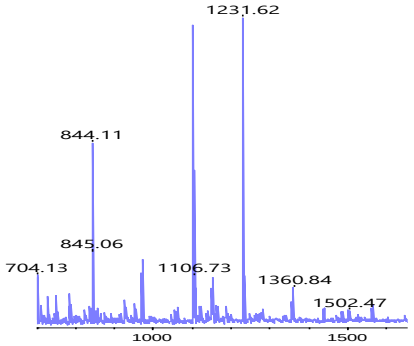
(G)

L2: 1102. 21 Da



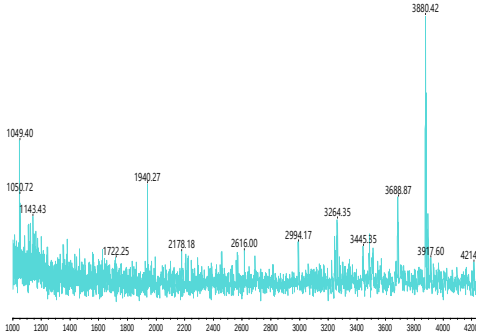
(H)

D7: 1231.62 Da



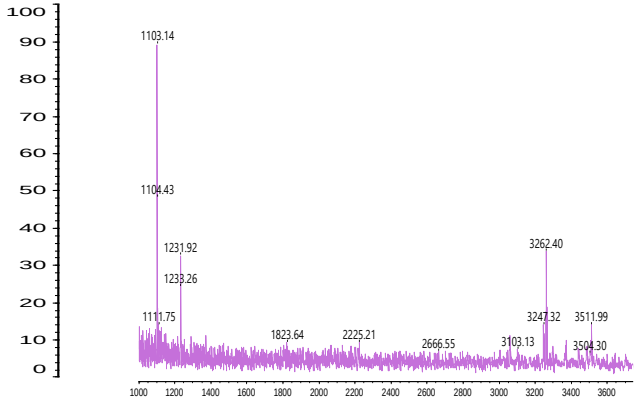
(I)

Pat: 3889.42 Da



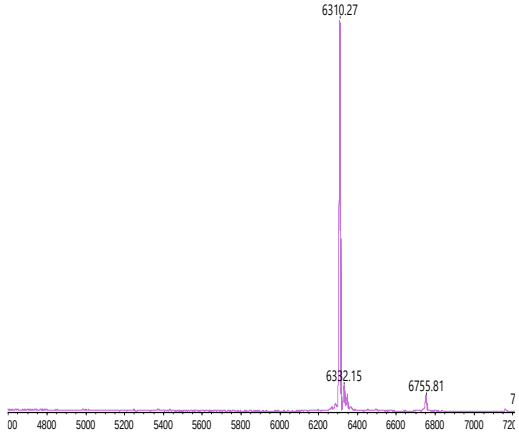
(J)

L18: 1103.14 Da and smaller peak of 3262.40



(K)

L16: 6310.27



(L)

D10*: Multiple peaks of 3443, 4372, 5381, and 5794 Da

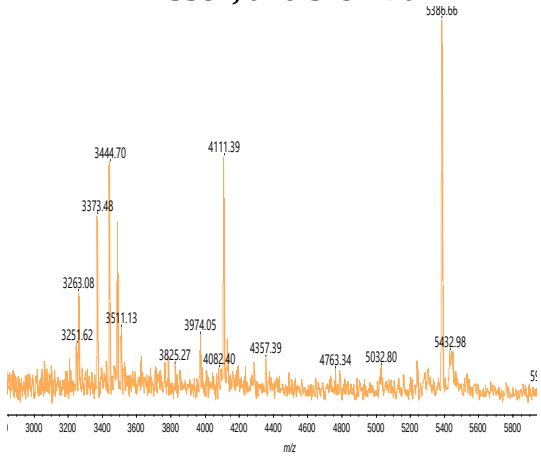
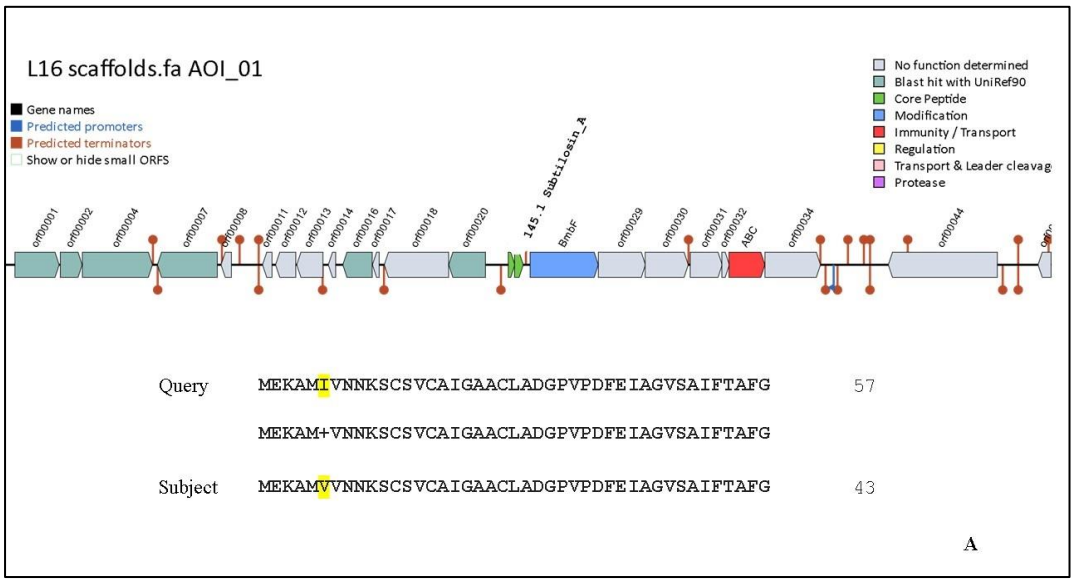


Figure 3.4 (A-L): A selection of the potential bacteriocins sizes produced by isolated colonies on a plate.

In silico bacteriocin screening

The genome of *E. thailandicus* (L16) was analysed using BAGEL4 for potential bacteriocins, and two areas of interest (AOI) were returned. The proteins within the area of interest were blasted on NCBI blastp to find the closest relative. There was 98% similarity to subtilosin A (Figure 3.5(A)) with a single amino acid change at position five (isoleucine instead of valine). This bacteriocin was consistent in Bagel4 and Blastp. The second protein had a 95% similarity to uberolysin, according to Blastp, although Bagel4 showed enterocin Nkr- 5-3B (Figure 3.5(B)). There were three amino acid changes in this protein compared to uberolysin.



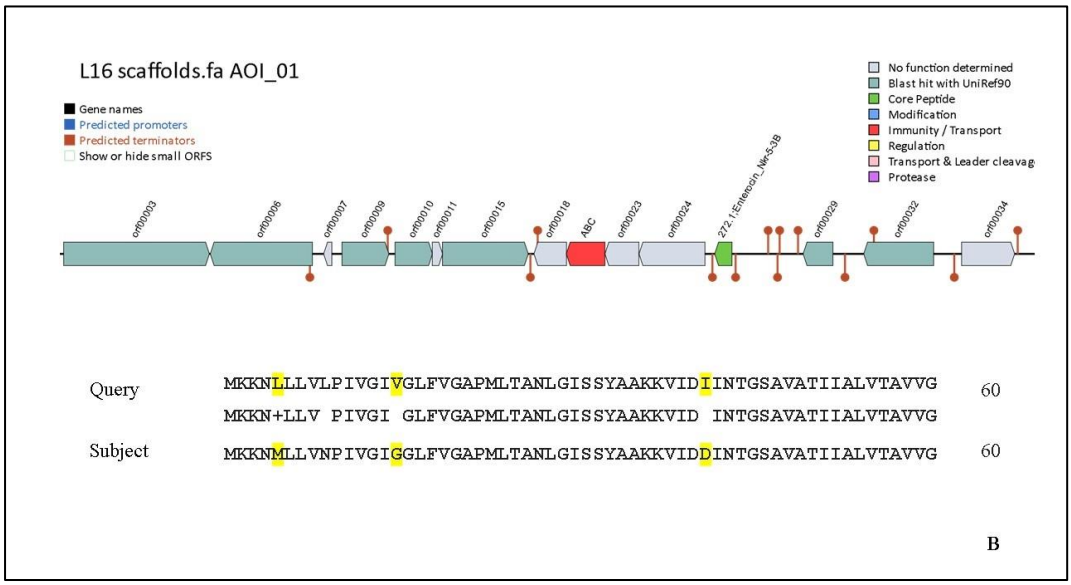


Figure 3.5: Potential bacteriocins in *E. thailandicus* (L16). Residues that did not match are highlighted.

Discussion

The agricultural environment harbours a variety of LAB, both on the animal itself and the surrounding environment. The aim of this study was to use a culture-based approach to isolate LAB present in ruminant environments and to screen and characterise bacteriocins produced by these LAB. Tentative bacteriocins from the isolates were characterised using *in vitro* techniques. *In silico* screening was used to screen for a potential bacteriocin from *E. thailandicus* (L16). The indicators used are organisms found in the rumen, and the pathogens screened against represented organisms that cause illnesses in humans and animals.

LAB are ubiquitous and can be found in many environments, extending from food and plants to the gastrointestinal tracts (GIT) of humans and animals (Adams, 1999). In this study, novel bacteriocin-producing LAB were isolated and characterised. The initial screening was performed on LMM plates composed of azide, a cytochrome *bd* oxidase inhibitor used to grow anaerobic organisms selectively. Sodium azide helps isolate LAB from samples containing numerous indigenous microbes by binding to heme and inactivating cytochrome oxidase to inhibit aerobic respiration (Bennet et al., 1996), thereby facilitating the growth of only anaerobes. In total, 16 presumptive LAB were isolated, first identified as LAB based on their physiological and biochemical characteristics; thereafter, strain identity was confirmed by Sanger sequencing of the 16S rRNA gene. The most frequently isolated genus was *Lactobacillus* (Table 3.5), which is not surprising as *Lactobacillus* is the most common genus in the LAB group (Goldstein et al., 2015). The most commonly isolated LAB in this study was *L. paracasei*, which is a normal inhabitant of the human GIT, but it has also been isolated from natural habitats consisting of plant materials such as pickles, silage and kimchi (Toh et al., 2013). Dos Santos et al. (2019) observed a variation in LAB diversity in corn silage, where 47% of the strains isolated were *L. paracasei* (reclassified to *Lacticaseibacillus paracasei* subsp. *paracasei).* In our study, three isolated organisms were identified as *Enterococcus* sp. Although *Enterococcus* sp. are LAB, they are also classified as pathogenic and commensal microorganisms (Hanchi et al., 2018). Currently, the genus *Enterococcus* has not yet acquired GRAS status. However, some strains are used as probiotics and feed additives to increase animal growth (Zommiti et al., 2022). In our study, *Streptococcus* and *Pediococcus* were found in similar amounts. Unexpectedly, no *Lactococcus* was isolated in our study. Because the isolation environment was agricultural and characteristically rich in carbohydrates,

the isolates found in this study were expected, except for *S. cristatus* (D10*), which is typically associated with the oral environment (Hanel et al., 2020).

In this study, bacteriocin production was tested against *L. delbrueckii* subsp. *bulgaricus* LMG 6901, first through deferred antagonism by spot assays and subsequently by well diffusion assays. Isolates produced antimicrobial agents that were inhibitory to this indicator organism to varying degrees. The possibility that other inhibitors such as organic acids common to LAB may give inhibitory reactions appearing as bacteriocin activity was eliminated by neutralising the CFS before conducting WDAs. Although the inhibition zones produced on spot assays were larger when the CFS were tested, these were reduced for some isolates when the supernatants were neutralised (Figure 3.2). Antimicrobial activity was eliminated post neutralisation in *E. sanguinicola* (L18) and *L. paracasei* (D14). This indicates that inhibition may have been due to acid in the media. Organic acids, especially lactic acid, could be the antagonistic substance that inhibited the indicator strain. The finding that *E. sanguinicola* lost activity is contrary to the findings of Chahad et al. (2012), who confirmed bacteriocin activity in *E. sanguinicola* UPAA57 isolated from seabass. The dissimilarities in isolation sources may explain the differences in antimicrobial activity. Furthermore, they may not be the same strain, just members of the same species.

Bacteriocins are proteinaceous (Simons et al., 2020), and in this study, protease sensitivity was evaluated using proteinase K. It was found that the compounds produced from all the isolates tested lost activity after proteinase K treatment except D11 (*L. mucosae*) (Figure 3.2). According to Bactibase, a database for bacteriocins (Hammami et al., 2010), *L. mucosae* does not produce any known bacteriocin; therefore, this antimicrobial agent was not a bacteriocin. However, Azevedo et al. (2015) reported *L. mucosae* AGR63 to have an operon for class 3 bacteriocin synthesis. Further analysis is necessary to determine the nature of the antimicrobial agent produced by this isolate. The sensitivity of bacteriocins to proteinases means that bacteria are unlikely to develop resistance toward them since they do not linger in the environment, as they are quickly digested compared to other antimicrobial agents such as antibiotics. On the other hand, this limits some of their application, for example, in the GIT, since they may be quickly degraded by digestive proteases before reacting with the target pathogens (Dicks et al., 2011).

A fundamental characteristic of bacteriocins is that this group of peptides are heat stable (Perez et al., 2014). Temperature affects bacteriocin activity in varying degrees, and this was tested by comparing the zone of inhibition of the indicator organisms before and after exposing bacteriocin–containing CFS to different temperatures. Similar to Hassan et al. (2020), there was a general trend showing a decrease in bacteriocin activity associated with an increase in temperature for most of the bacteriocins assessed. The results of the heat stability assays confirmed that the bacteriocin produced by D12 (*P. acidilactici*) was fully heated stable as there was no reduction in antimicrobial activity even after exposing it to 120°C for 10 mins. However, other bacteriocins lost activity at a lower temperature, namely, CFS from *L. paracasei* (D1), *E. faecium* (D7*), and *E. thailandicus* (L2) maintained activity at 90°C. This sustained antimicrobial activity at such temperatures permits use in various commercial processes, such as in the food processing industry (Zangeneh et al., 2020). In contrast, *L. zeae* (L6) only maintained activity at 37°C, demonstrating that the bacteriocin produced is heat sensitive. Although uncommon, there have been reports of heat-sensitive bacteriocins. For example, Kato et al. (2014) identified enterocin E1B from *E. faecium*, whose molecular mass was more than 300 kDa, and inactivated by heat.

Bacteriocins can be broad-spectrum or narrow-spectrum (Ryan et al., 1996). Because bacteriocins have an entirely different mode of action from conventional antibiotics, they are often pursued as alternative methods for controlling microbial pathogens (Dicks et al., 2018). Gram-positive (*S. epidermis, S, simulans, S. lutetiensis*, *E. faecium*, and *S. agalactiae*), Gram-negative *(Escherichia sp*., and *E. cloacae*) pathogenic bacteria as well as non-pathogenic *L. fermentum* and *B. schinkii* were used to investigate the inhibitory spectrum of the various antimicrobial agents produced by the isolates. *E. hirae* (D17) produced an antimicrobial compound that is active only against *Escherichia sp.* Bacteriocins from several isolates *E. hirae* ATCC 9790 (D17), *P. acidilactici* (D12), *L. paracasei* (D1), *L. plantarum* (L14), *E. thailandicus* (L2), and *E. faecium* (D7*) displayed broad-spectrum inhibitory activity against both Gram-positive and Gram-negative organisms (Table 3.4). A well-known example of a LAB that produces a broad spectrum bacteriocin is *L. lactis* which produces lacticin 3147 (Ryan et al., 1996). *Lactobacillus zeae* strain RIA (L1) produced an antimicrobial agent inhibitory only against *E. faecium*. Although not overtly attributed to bacteriocin production, Yin et al. (2014) observed that *L. zeae* reduced *Salmonella* colonies in pig feed.

None of the strains in this study were sensitive to the bacteriocin, which they produced. The mechanism of cell self-protection (immunity) is an essential yet poorly understood aspect of bacteriocin biology (Diep et al., 2006). Some bacteriocins confer immunity to the producing strain by directly binding to bacteriocin receptors in the producer's cell membrane, thereby inhibiting pore formation (Kjos et al., 2010). The genetic determinants proposed or confirmed to give immunity are located downstream of the bacteriocin structural gene(s) in the bacteriocin operon (Gajic et al., 2003).

Mass spectrometry enables the accurate determination of peptide mass and has been routinely used to characterise bacteriocins (Hindre et al., 2003). The substances responsible for the antimicrobial activity of some of the LAB strains selected were confirmed in this study through colony MS and loss of activity when neutralised. In addition to bacteriocins, LAB can produce several compounds, such as hydrogen peroxide and lactic acid, which are inhibitory towards bacteria and may be misinterpreted as bacteriocins (Kormin et al., 2001). Isolates L14, identified as *L. plantarum*, was found to produce an antimicrobial compound of 1101.36 Da. Usually, *L. lantarum* produces the bacteriocin plantaricin, which is around 4347.8 Da (Wang et al., 2018). However, similar to our study, Giani et al. (2019) found a low molecular weight bacteriocin-like compound from *L. plantarum*, which was 1053.05 Da. The MS profile obtained from *S. cristatus* revealed multiple peaks indicating the possibility of this strain producing multiple bacteriocins (Figure 3.4). Some LAB are known to be capable of producinge multiple bacteriocins, for e.g. *Lactobacillus gasseri* LM19 produces several bacteriocins (Garcia-Gutierrez et al., 2020). *E. thailandicus* (L16) produced an antimicrobial agent that was 6310 Da, similar to data fromAl- Madboly et al. (2020), who also found a similarly sized bacteriocin of 6797 Da from *E. thailandicus*.

In vitro screening for bacteriocins is typically time-consuming and requires some form of trial and error to successfully isolate bacteriocin-producing LAB and optimise growth conditions that favour maximum bacteriocin production and purification (Egan et al., 2018). Bacteriocin-producing *Enterococcus* are widespread in nature, and they have been isolated from various environments. This study used BAGEL4 and NCBI blastp to perform an *in silico* screen of *E. thailandicus* (L16) to identify the bacteriocin operon present in its genome. This was one of the isolates that consistently demonstrated good antimicrobial activity. The *in silico* screen resulted in the identification of two putative bacteriocins. Bagel4 predicted the bacteriocins to be subtilosin A and enterocin NKR-5-3B. The amino acid sequence of the putative subtilosin

from our study had isoleucine in position 5, while Subtilosin A has a valine in that position (Figure 3.5(A)). In the enterocin NKR-5-3B sequence, there were 3 amino acid substitutions between the online match uberolysin and the query sequence (Figure 3.5(B)). There was a 98 and 95% similarity to subtilosin A and uberolysin, respectively, according to NCBI blastp. In our study, the BAGEL4 result seemed more acceptable as it was in agreement with the literature. This result from Blastp is somewhat contradictory to expectation because, according to literature, *Enterococcus* species produce bacteriocins that are encoded by similar structural genes for enterocin A, enterocin nmr10B, and enterocin mr10A (Henning et al., 2015; Shastry et al., 2020). The exception is a study by Al-Madboly et al. (2020), who observed a similar result to our study. When the enterocin sequence from *E. thailandicus* was subjected to blastp for homology, it revealed 94.7% identity similarity to uberolysin. *In silico* bacteriocin screening results from the present study are in agreement with the findings of Perez and colleagues, who proposed that the orientation of the bacteriocin enterocin NKR-5-3B gene locus resembles that of uberolysin. Subtilosin A is a 35-amino-acid bacteriocin first isolated from *Bacillus subtilis* by Babasaki et al. (1985). Initially, subtilosin A was classified as a circular bacteriocin; however, due to it being significantly smaller than other circular bacteriocins, having intramolecular disulfide bridges and being negatively charged, it is currently classified as a sactibiotic (Perez et al., 2018). To our knowledge, this bacteriocin has only been isolated from *Bacillus* sp. and not from LAB. Enterocin NKR-5-3B is a circular, broad-spectrum bacteriocin produced by *E. faecium* (Himeno et al., 2015), whereas uberolysin is also a cyclic bacteriocin (Ahmad et al., 2017). The bacteriocins from *E. thailandicus* (L16) identified in our study using *in silico* methods seem to match the profile of circular bacteriocins. Future work should focus on elucidating the structure of the bacteriocins produced and determining whether the second bacteriocin more closely resembles uberolysin or enterocin NKR-5-3B.

Conclusion

The use of LAB selective media resulted in isolation of a range of diverse bacteria. Most LAB strains isolated were *Lactobacillu*s (56.25%), and *Enterococcus* was the second most abundant (31.25%). Neutralising the CFS from the isolates caused a loss of antimicrobial activity in two isolates, specifically, *E. sanguinicola* (L18) and *L. paracasei* (D14), while activity was reduced in *L. zeae* (L6 and L1), L. *plantatum* (L14), and *E. faecium* (D7*). Proteinase K treatment caused a loss of inhibition in all isolates except *L. mucosae* (D11), indicating that the majority were proteinaceous in nature. Colony mass spectrometry showed that the peptides produced by the isolates ranged from 1101 to 6300 Da. Upon determining the antipathogenic spectra, *P. acidilactici* (D12) exhibited the most potent inhibition against the four indicators used in this study. *In silico* and *in vitro* screening showed the isolate *E. thailandicus* (L16) produces a bacteriocin as indicated by inhibition of various indicators when the well diffusion tests were performed, and colony mass spectrometry revealed a peptide of 6310.27 Da. This is in agreement with the general size of enterocins, which generally have an estimated size of ≤6 kDa (Fugaban et al., 2021). *In silico* results revealed that *E. thailandicus* possesses bacteriocins genes for subtilosin A and enterocin NKR-5-3B. This study showed that the ruminant environment is a source of a range of LAB, some of which have bacteriocin-producing abilities.

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Chapter 4.

Methanogen cultivation and *in vitro* inhibition of methane using bacteriocins and cell-free supernatants.

Abstract

Methane is a potent greenhouse gas with a global warming potential that is 21 times higher than carbon dioxide. Agriculture is the largest source of anthropogenic methane emissions. Most methane produced by ruminants through enteric feed fermentation is released into the atmosphere through eructation. A network of rumen microbes functions collaboratively during methanogenesis, but methanogens are responsible for methane production. Methanogens remove the excess hydrogen in the rumen; however, methane production represents a 6-12% energetic cost to the ruminant. Mitigation of methane production by ruminants has economic and environmental benefits. LAB are generally regarded as safe (GRAS) and have a long history of use in on-farm technologies and food production. They produce a variety of inhibitory compounds such as bacteriocins, which could potentially reduce methane by targeting the bionetwork for methanogenesis. This study aimed to investigate the potential of nukacin and lacticin 3147 and CFS from bacteriocin-producing LAB and other bacteria to reduce methane emissions in pure methanogen cultures. Rumen methanogens were cultivated for *in vitro* methane inhibition trials. *M. ruminantium*, *M. gottschalkii*, and *M. boviskoreani* were all grown in the standard DSMZ medium 1523. Spectrophotometry was used to investigate their growth rates, and fluorescence microscopy was used to verify methanogen growth, visualise the methanogen cells, and confirm purity. *Methanosarcina barkeri* was grown for comparison. Fluorescence microscopy revealed bright green, fluorescent cells characterised by the coenzyme Cofactor F420. The actively growing *Methanobrevibacter* were treated with CFS from bacteriocin-producing LAB, and the methane produced was measured over two weeks. Gas chromatography was used to analyse headspace methane for the LAB-treated and untreated methanogen samples. *L. plantarum* (LP58) significantly inhibited methane production in *M. gottschalkii*. Methane inhibition by nukacin and lacticin was comparable to nisin in *M. ruminantium*. *S. equinus* (HUN037) was the most effective ($P<0.05$) at inhibiting methane from *M. boviskoreani*, with a 64.8% methane inhibition.

Introduction

Methane (CH_4) is a potent contributor to the GHG effect. Methane is a highly reactive chemical and more efficient at absorbing infrared radiation than carbon dioxide; this makes it approximately 21 times more potent than carbon dioxide in terms of its global warming potential (GWP). It has a relatively short atmospheric half-life of 12-15 years compared to the lifetime of CO_2, which is 100 years (Hook et al., 2010). A prominent source of anthropogenic methane production is enteric fermentation, particularly by domesticated ruminants (McAllister et al., 1996). Concerted efforts of various rumen microbes produce methane as an end-product of feed fermentation (Danielsson et al., 2017), along with CO_2 and H_2. The methane production pathway (methanogenesis) acts as a hydrogen sink in the rumen; otherwise, rumen function is impaired (Barabasz et al., 2017). However, enteric methane mitigation strategies are being sought as its production negatively impacts the formation of volatile fatty acids and results in 6-12% metabolisable energy loss for the ruminant. Furthermore, methane exacerbates the problem of climate change (Ellis et al., 2008; Johnson and Johnson, 2014).

Methanogens are a diverse group of archaea that belong to the phylum Euryacheota (Liu, 2010) and share similar metabolism and energy conservation systems (Enzmann et al., 2018). The unifying features of methanogens are that they are all obligate methane producers and strictly limit their growth to extremely anaerobic environments. It has been proposed that methanogens possess unique enzymes, such as Methyl Coenzyme M reductase (MCR), Factor 420 and methanopterin, which contribute to methanogenesis (Gao and Gupta, 2007) and are not found in other organisms. The cell characteristics vary significantly among methanogens. Regarding their morphology, methanogens display a variety of shapes and sizes. Some may be motile, and some species can aggregate into clusters or exist as free-living organisms (Garcia et al., 2000). *Methanobrevibacter* is the predominant genus in the rumen, and these hydrogenotrophs account for two-thirds of the rumen methanogens (Danielsson et al., 2017).

LAB are physiologically, and metabolically related organisms traditionally used in food. LAB are generally regarded as safe and have a long history of use in on-farm technologies. They produce a variety of inhibitory compounds such as bacteriocins, which could potentially reduce methane *in vitro* by targeting the bionetwork of methanogenesis. Bacteriocins have been proposed to inhibit methane production (Callaway et al., 1997; Lee et al., 2002; Patra et al.,

2017; Sar et al., 2004). These bacterially-produced, heat-stable, antimicrobial, cationic peptides exhibit broad or narrow inhibition spectra (Collins et al., 2017). They are generally non-toxic to eukaryotic cells (Soltani et al., 2021). The bacteriocin nisin, produced by *Lactococcus lactis*, has been successfully used in the food industry for food safety and preservation purposes. This is a 34 amino acid peptide comprising of five lanthionine rings. An *in vivo* study in sheep showed that nisin decreased methane production by up to 10% (Santoso et al., 2004). Therefore, it is imperative to investigate the role of bacteriocins in modulating methanogenesis. Lacticin 3147 is a two-peptide, broad-spectrum bacteriocin produced *by Lactococcus lactis* subsp. *lactis* DPC3147 (McAuliffe et al., 1998). It is heat-stable and active at physiological pH and has an inhibition spectrum similar to nisin; however, it is genetically distinct (Draper et al., 2013). The two lacticin 3147 peptides, Ltnα and Ltnβ, work synergistically in a 1:1 ratio. *Staphylococcus warneri* produces various bacteriocins such as warnericin RB4 (Minamikawa et al., 2005), nukacin SK1 (Sashihara et al., 2000) and warnerin (Prema et al., 2006). Bacteriocins from *S. warneri* have not previously been investigated for methane inhibition characteristics. Based on the antimethanogenic effect of nisin and other bacteriocins, it is worth exploring lacticin and nukacin for this property.

There are limited studies investigating the effect of LAB isolated from the previous chapter (Chapter 3), or bacteriocins on methane emission in pure rumen methanogen cultures. The aim of this study was to explore the potential of CFS from LAB, *Staphylococcus* sp., and pure bacteriocins nukacin and lacticin 3147 when used to inhibit methane production in pure cultures of *Methanobrevibacter* species, i.e., *M. ruminantium*, *M. boviskoreani* and *M. gottschalkii* and to compare the effectiveness of synthesised nukacin peptide from *S. warneri* (APC 2922) and CFS from *S. warneri* as methane inhibitors in *M. ruminantium*. The potential-methane inhibitors evaluated in this study can be grouped into three catagories: CFS from bacteriocin-producing LAB, CFS from bacteriocin-producing *Staphylococcus* sp., and synthesised bacteriocins lacticin 3147 and nukacin 2922.

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Materials and Methods

Methanogen cultivation

Methanobrevibacter ruminantium (DSM 1093), *Methanobrebivacter gottchalkii* (DSM 119) and *Methanobrevibacter boviskoreani* (DSM 25824) (JH1) were purchased actively growing from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strains were grown anaerobically on complex medium 1523 according to DSMZ guidelines. The trace element solution was prepared separately and then added. The composition of the trace element solution SL10 is provided in Table 4.1. The medium vessel was then connected to a nitrogen gas line, and the headspace was flushed for approximately 20 mins to remove oxygen from the headspace while the medium was boiled over a flame (Figure 4.1). Sodium bicarbonate was added. The nitrogen line was then replaced with H_2/CO_2 (80/20, v/v) while cooling.

The medium was then dispensed with glass pipettes into serum bottles or anaerobic Balch tubes (Bellco Glass Inc, New Jersey, USA). The serum bottles or tubes were flushed with H_2/CO_2 (80/20) before and during the addition of medium and sealed with butyl rubber septa and aluminium crimp caps. The medium was autoclaved at 121°C for 15 mins. This method of preparation ensures that the medium remains reduced as indicated by the resazurin colour change (Miller and Wolin, 1973).

Before inoculation, filter-sterilised vitamins prepared according to DSMZ medium 1523 were injected. Thereafter, the mineral medium was reduced with 0.8 mM sodium sulfide ($Na_2S{\cdot}7H_2O$). The headspace of serum bottles or Balch tubes was flushed with a mixture of H_2: CO2 or nitrogen via a gassing cannula to ensure anaerobic conditions. All the inoculations and transfers were performed aseptically using sterile syringes and 25 g needles needles (Sigma Aldrich, Wicklow, Ireland) at 10% v/v. Cultures were incubated statically at 37°C in the dark. Turbidity was taken as an indicator of culture growth. Cultures were grown in smaller (12") Hungate tubes with screw cap tubes for absorbance readings. A sterile mixture of 80% H_2 and 20% CO_2 gas was added at 0.5 - 1 bar overpressure to maintain the growth of cultures. The decrease in headspace gas pressure was periodically compensated for by adding H_2: CO2 (80:20, v/v).

Table 4.1: Trace element solution SL10.

Reagent	**Quantity**
HCl (25%; 7.7 M)	10 ml
*$FeCl_2$ x 4 H_2O	1.5 g
$CoCl_2$ x 2 H_2O	190 mg
$MnCl_2x4H_2O$	100 mg
$ZnCl_2$	70 mg
H_3BO_3	6 mg
$NiCl_2x6H_2O$	24 mg
$Na_2MoO_4x2H_2O$	36 mg
$CuCl_2x2H_2O$	2 mg
Sterile distilled water	990 ml

***** $FeCl_2$ was first dissolved in HCl, diluted in water, then added the remaining salts and finally topped up to 1000.0 ml. The trace element solution was made in pre-autoclaved bottles and sterilised using 400 µM membrane filters.

Table 4.2: Salt solution A.

Reagent	**Quantity**
NaCl	15 g
KH_2PO4	7.5 g
$(NH4)_2SO_4$	3.75 g
$CaCl_2.2H_2O$	1.98 g
$MgSO_4.7H_20$	3.0 g
Make up to 2.5 litres with distilled water and aliquot.	

Table 4.3: Salt Solution B.

Reagent	**Quantity**
KH_2PO_4 x $3H_2O$ or	19.65 g
K_2HPO_4	15 g
Make up to 2.5 litres with distilled water and aliquot.	

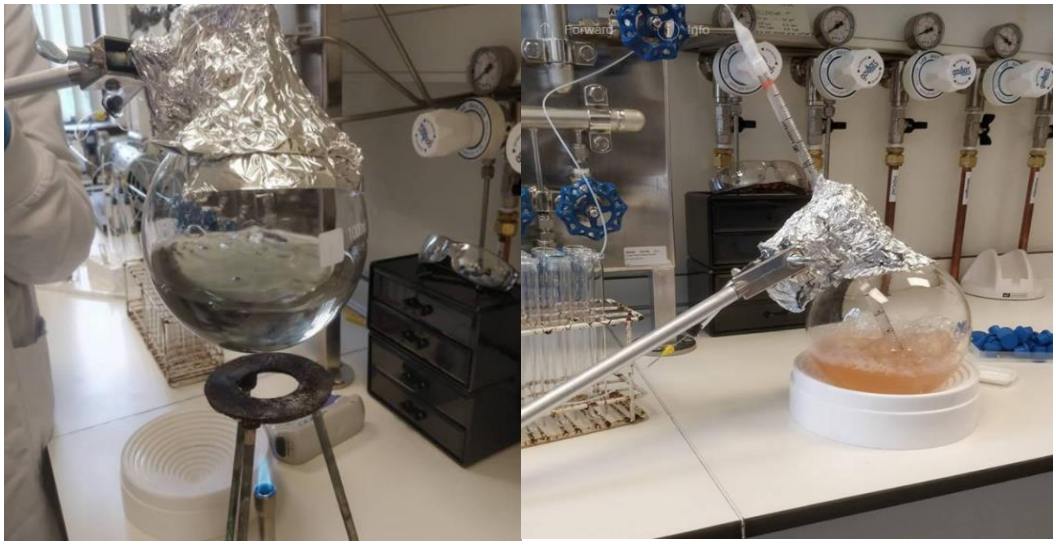

Figure 4.1: Images of boiling and reducing basal medium for methanogen cultivation.

Methanosarcina barkeri was purchased from DSMZ. *M. barkeri* (DSM 1538) was cultivated using DSMZ medium 120 using the same method described above for *Methanobrevibacter* sp. Methanol (50% v/v stock solution) and the reducing agents were prepared separately as side components under N_2 gas (100%) as concentrated solutions in tightly closed Balch tubes. Vitamins were prepared under N_2 gas (100%) and sterilised by filtration. Appropriate volumes of the side components were injected into the sterile medium with hypodermic syringes, before culture inoculation at 10% v/v. The culture was incubated statically at 37°C in the dark. Turbidity was taken as an indicator of culture growth. A sterile mixture of 80% H_2 and 20% CO_2 (Air products, 950 Knockmitten Cl, Western Industrial Estate, Dublin 12, Ireland) gas was added at 0.5-1 bar weekly.

Measuring Growth using a spectrophotometer and fluorescence microscopy

Growth was monitored by measuring the absorbance of the cultures. To obtain optical density (OD_{600}) readings, methanogens were cultivated in smaller Hungate tubes (16 × 125 mm) that can fit into the spectrophotometer (Figure 4.2). Anaerobic conditions were maintained by sealing the tubes with a grey butyl stopper after inoculation. The growth of methanogens was measured (OD_{600} nm) using a Biochrom Libra S2 Colorimeter (Biochrom Ltd., Cambourne Business Park, Cambridge, United Kingdom), and a growth curve was generated. The grey butyl stoppers for Hungate tubes cannot maintain high pressure for extended periods; therefore, the experiments could not be prolonged for longer than two weeks.

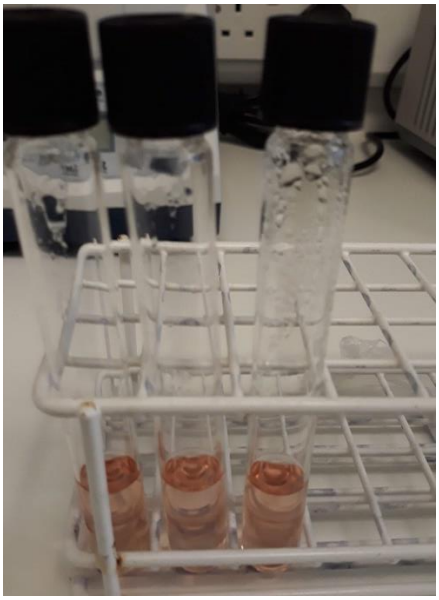

Figure 4.2: Hungate tubes for cultivating methanogens and measuring OD.

Visualising methanogens using fluorescence microscopy

Methanogenic archaea can be identified by their inherent fluorescent cofactor F420. Fluorescence microscopy was used to view the various methanogens. Before the microscopic examination, to concentrate the methanogens, 30 ml of methanogen-containing medium was centrifuged at 4000 × g for 20 mins at 4°C. Most of the supernatant was discarded, leaving approximately 1 ml of supernatant with the pellet. Samples were kept on ice to slow down oxidative degradation of cofactor F420. The methanogen slurry was suspended in PBS buffer (735 µl). Thereafter, 40 µl of absolute ethanol was added. Sybr green 1 (20 µl) (Sigma Aldrich, Wicklow, Ireland) was used to stain the cells for 3 hrs to differentiate the methanogens from their background. Autofluorescence was visualised with a 395–440 nm excitation filter, a 475–495 nm emission filter, and a 460 nm beam splitter (Carl Zeiss). SYBR Green I fluorescence was visualised with a 475–495 nm excitation filter, a 515–565 nm emission filter and a 510 nm beam splitter, according to Lambrecht et al. (2017).

Methanogen maintenace

For long-term maintenance, methanogen cultures were stored in glycerol at a final concentration of 10% (v/v). An anaerobic glycerol solution (500 ml) was prepared by combining salt solution A (85 ml), salt solution B (85 ml), distilled water (130 ml), glycerol (200 ml), $NaCO_3$ (2.5g), L- cysteine HCl (250 mg) and two drops of 0.1% (w/v) resazurin. All components were mixed in a cannulated round bottom flask and brought to boil while bubbling with oxygen-free nitrogen, except $NaCO_3$ and L-cysteine HCl. The solution was then cooled in an ice bath while still bubbling with nitrogen; $NACO_3$ was added, followed by L-cysteine HCl. The cold solution was dispensed into nitrogen sparged serum bottles and autoclaved for 20 min at 121°C, at 15 psi. Cultures were added to achieve a final concentration of 10%. Bottles were kept at -20°C until frozen and then transferred to -80°C for long-term storage. To recover the cultures, the cultures were thawed, and a 10% inoculum was transferred to new tubes of fresh media.

Methane sampling

Leakproof 10 ml graduated syringes (Medguard, Unit 6/7, Block 4, Ashbourne Business Park, Ashbourne, Co. Meath, A84 VK52, Ireland) fitted with 25 g Luer lock needles (Sigma Aldrich, Wicklow, Ireland) were used to sample headspace methane gas. The syringe was flushed with nitrogen gas, thereafter, 10 ml of gas was suctioned through the stopper of the Balch tubes containing the growing methanogen. The gas sample was immediately transferred to 7 ml screw-cap glass GC vials (Sigma Aldrich, Wicklow, Ireland) which had been air-evacuated using a vacuum pump (Figure 4.3). Methane concentration was measured using gas chromatography (GC) with a flame ionisation detector (FID) fitted with a 3.6 mm column, and helium was used as the carrier gas as described by Rira et al. (2015).

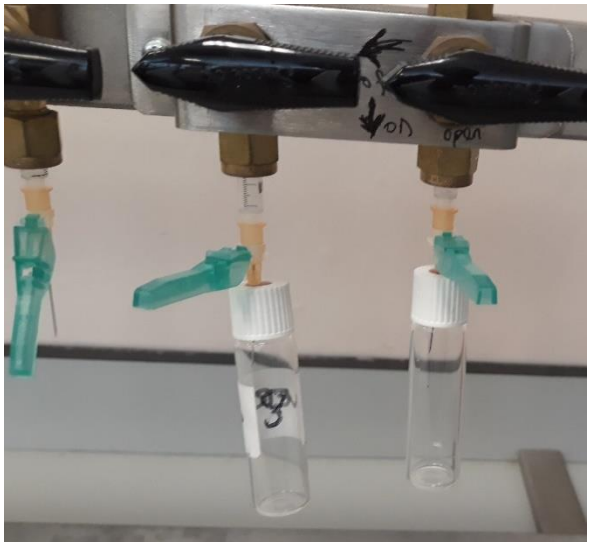

Figure 4.3: Evacuation of stopper-sealed GC vials in preparation for injecting with headspace gas for methane analysis.

Methane inhibition using Cell-Free supernatants (CFS) and bacteriocins

Selection of LAB, Streptococcus equinus and Staphylococcus species

There were various LAB chosen for *in vitro* methane inhibition (Table 4.4). Commercial strains *Lactobacillus plantarum* (LP58), *Pediococcus acidilactici* (PA09) and *Lactococcus lactis* (SL242 and SL147) were obtained from SACCO (Sacco Srl, Via Manzoni 29/A, 22071 Cadorago (CO), Italy). These are well-documented bacteriocin producers of plantaricin, pediocin and nisin, respectively (Papagianni and Anastasiadou, 2009; Shen et al., 2017; Wang et al., 2018). The remaining LAB strains (*Lactobacillus plantarum* (L14), *Lactobacillus coryniformis* subsp. *torquens* (D3), and *Pediococcus acidilactici* (D12)) were isolated from the various agricultural sources in Teagasc, Moorepark, Ireland, as detailed in Chapter three. These bacteriocin producers were investigated for their ability to reduce methane *in vitro*. *Streptococcus equinus* strains (HUN035 and HUN037) and *Lactococcus lactis* (KF245) were obtained from AgResearch (AgResearch Limited, Grasslands Research Centre, Palmerston North, New Zealand). *S equinus*, formerly known as *Streptococcus bovis*, has been reported in the literature to reduce methane production *in vitro* (Doyle et al., 2019, Lee et al., 2002). *Staphylococcus capitis* (APC 2918) and *Staphylococcus warneri* (APC 2922) were obtained from the APC culture collection (Cork, Ireland), and *Staphylococcus* species attracted attention for this study because *S. warneri* CFS had been found to directly inhibit *M. boviskoreani* growth in a separate preliminary study conducted in AgResearch (data not shown). CFS from *S. warneri* were subjected to proteinase K treatment (Sigma-Aldrich) by incubating 50% (v/v)

CFS with proteinase K at a final concentration of 20 mg/ml at 37°C for 3 hrs. Following incubation, enzymes were denatured by heating to 100°C for 10 mins. Residual antimicrobial activity, which was not affected by the enzymatic treatment, was determined through a WDA using *L. delbrueckii* subsp. *bulgaricus* LMG 6901 as an indicator, as described previously in Chapter three.

Verification of bacteriocin presence or other inhibitors in CFS

Bacteria were grown according to the conditions listed in Table 4.4 in liquid media. The cultures were centrifuged at 8000 × g for 20 mins at 4°C, the different supernatants were saved, and the pellets were discarded. Each supernatant was neutralised and then tested for inhibition against *L. delbrueckii* subsp. *bulgaricus* LMG 6901. A zone of inhibition was taken as an indication of antimicrobial activity, and such supernatants were subsequently carried forward for the methane inhibition assay.

Purification of nukacin.

Cryopreserved stocks of *S. warneri* (APC 2922) were grown in BHI broth aerobically at 37°C. These were subcultured three times, and activity was checked by performing WDAs, using *L. delbrueckii* subsp. *bulgaricus* LMG 6901 as an indicator. Once antimicrobial activity was confirmed, overnight cultures of *S. warneri* (APC 2922) were grown in 1 litre of BHI broth. The cultures were centrifuged at 8000 × g for 20 mins at 10°C, and the supernatant was retained for purification. The cell-free supernatants were passed through Econo columns containing 30 g of Amberlite XAD16N. The columns were washed with 250 ml 35% (v/v) ethanol and antimicrobial activity eluted with 250 ml 70% (v/v) isopropanol containing 0.1% (w/v) TFA. Eluents were assayed against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 indicator to ascertain that activity was maintained during column flow-through and that the peptide of interest was eluted in the IPA eluent. Peptide size was confirmed using mass spectrometry and subsequently purified using High-Pressure Liquid Chromatography (HPLC). The most active fractions were pooled and assessed on the analytical HPLC column. Thereafter, the fractions were lyophilised to produce nukacin-containing powder, referred to as nukacin 2922.

Table 4.4: LAB and *Streptococcus* growth conditions.

Strain identity	**Media**	**Growth conditions**	**Potential bacteriocin**
L. delbrueckii subsp. bulgaricus LMG 6901	de Man, Rogosa, and Sharpe (MRS)	Anaerobic 37°C, 24 hrs	No known inhibitor
L. plantarum (LP58)	*Lactobacillus* selective broth (LBS)	Aerobic 30°C, 48 hrs	Plantaricin N
L. lactis (SL242)	GM17	Anaerobic 30°C, 24 hrs	Nisin A (García Méndez et al., 2021)
L. lactis ssp. *lactis* (DPC 3147)	GM17	Anaerobic 30°C, 24 hrs	Lacticin 3147
L. plantarum (L14)	GM17	Anaerobic 37°C, 24 hrs	Plantaricin
L. coryniformis (D3)	Brain Heart Infusion (BHI)	Anaerobic 37°C, 24 hrs	Lactocin MXJ 32A (Lü et al., 2014)
P. acidilactici (D12)	MRS	Anaerobic 37°C, 24 hrs	Pediocin
S. warneri (APC 2922)	BHI	Aerobic 37°C, 24 hrs	Nukacin (O'Sullivan et al., 2019)
L. lactis (SL147)	MRS	Anaerobic 37°C, 24 hrs	Lacticin
S. capitis (APC 2918)	BHI	Aerobic 37°C, 24 hrs	Capidermicin (Lynch et al., 2019)
S. equinus (HUN037)	BHI	Anaerobic 37°C, 24 hrs	Nisin U, SalivaricinA Nukacin A (Nukacin ISK1)
S. equinus (HUN035)	BHI	Anaerobic 37°C, 24 hrs	Nisin U, Nukacin A (Nukacin ISK1)
L. lactis subs *lactis* (KF245)	MRS	Anaerobic 37°C, 24 hrs	Enterolysin A, Lactococcin B (LCN-B), and Nisin Z
P. acidilactici (PA09)	MRS	Anaerobic 37°C, 24 hrs	Pediocin

Preparing bacteriocin stocks

Nisin (Sigma Aldrich, Wicklow, Ireland) stocks of 100 µM were prepared by suspending 3.35 mg of nisin powder in sterile water (10 ml), which had been supplemented with 0.05% acetic acid (Sigma Aldrich, Wicklow, Ireland). It was filter-sterilised, then transferred into a sterile serum bottle, and a gas exchange system was performed. Nisin was used as the positive control for this study. The bacteriocin lacticin 3147 was obtained from Teagasc Moorepark (Cork, Ireland). Lacticin 3147, a two-peptide bacteriocin, was prepared by combining Ltnα and Ltnβ in a 1:1 ratio. Two milligrams of each two-peptide bacteriocin were dissolved in 5 ml sterile water to produce a 0.4 mg/ml stock. The solution was filter sterilised and made anaerobic using nitrogen, as mentioned previously. The purified nukacin 2922 was prepared at 5 mg/ml. Both nukacin 2922 and lacticin 3147 were prepared anaerobically similar to nisin.

In vitro Methane inhibition assays: The addition of various inhibitors to actively growing methanogens

Pure methanogen cultures were cultivated as described earlier in this chapter. LAB capable of producing inhibitory compounds were grown under the conditions outlined in Table 4.4. At the beginning of the log phase of methanogen growth (on day 3), CFS (30% v/v) were added anaerobically into the actively growing methanogens (*M. ruminantium*, *M. gotschalkii* and *M. boviskoreani*). Before addition to the methanogens, the supernatants were centrifuged at 4000 x g for 20 mins at 4°C to remove live cells. Each CFS was neutralised and transferred into a stopper-sealed serum bottle. A gas exchange system was created by sparging the bottle with nitrogen while allowing gas to escape and inhibiting oxygen re-entry. Sparging is a degassing method in which a gas, in this instance nitrogen, is bubbled through a liquid medium or supernatant to remove dissolved oxygen. Sparging was performed for 30 mins per 40 ml of CFS. The negative control was CFS from *L. delbrueckii subsp. bulgaricus* LMG 6901 with no known inhibitory compound production. The bacteriocin stocks were treated similarly to the CFS, as illustrated in Figure 4.4.

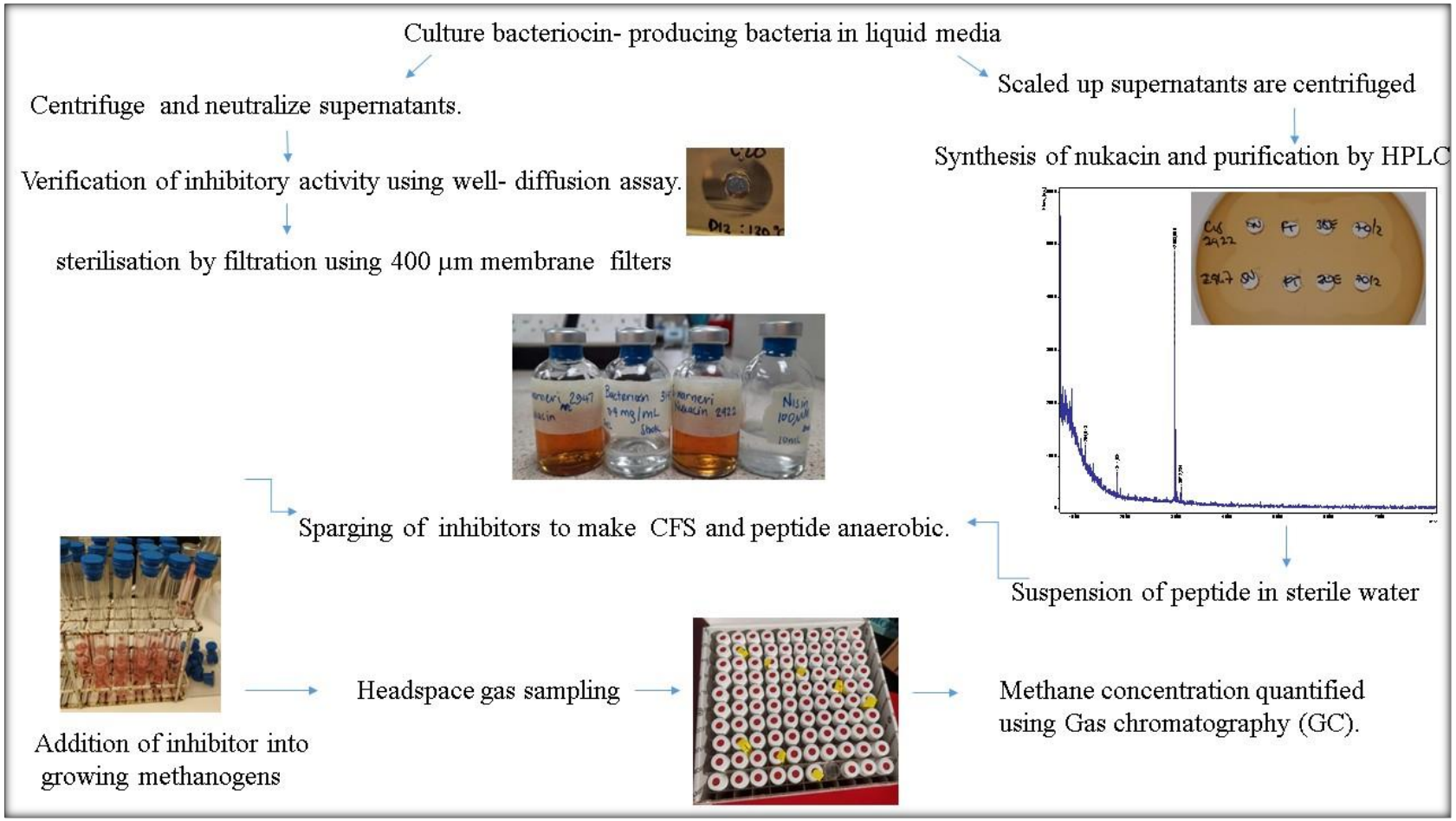

Figure 4.4: The steps involved in the *in vitro* methane inhibition assay.

Statistical *analysis*

Statistical analyses were performed using the software package GraphPad Prism version 9.10 for Windows (GraphPad Software, La Jolla, California, USA). Statistical difference between groups was assessed using a one-way analysis of variance (ANOVA). P-values ≤ 0.05 were considered statistically significant.

Results

Methanogen cultivation

Growth indicated by turbidity

Figure 4.5 shows the growth of *M. ruminantium, M. gottschalkii, and M. boviskoreani* (JH1) grown in complex media with hydrogen at 37°C. The negative controls remained sterile in all the experiments, and all the methanogens grew as expected in the recommended standard DSMZ culture medium. The growth phases distinguished were the lag, exponential and stationary phase in *M. gottschalkii*, while a death phase was observed in *M. boviskoreani* and *M. ruminantium*. The OD_{600} was used to calculate the doubling times (with a 95% confidence interval) based on the observed trend. For *M. ruminantium*, the doubling time was predicted to be 5.4 days, *M. gotschalkii* doubled every 10 days, and *M. boviskoreani* was found to have a doubling time of six days. A lag phase of two days was identified for *M. gottschalkii* and JHI, while *M. ruminantium* had a lag phase of three days. The highest growth was observed on day 10 at an OD_{600} of 0.2- 0.22 for all thre methanogens. *M. gottschalkii* remained at the stationary phase until day 15. The death phase occurred after 10 days for *M. boviskoreani* and *M. ruminantium.*

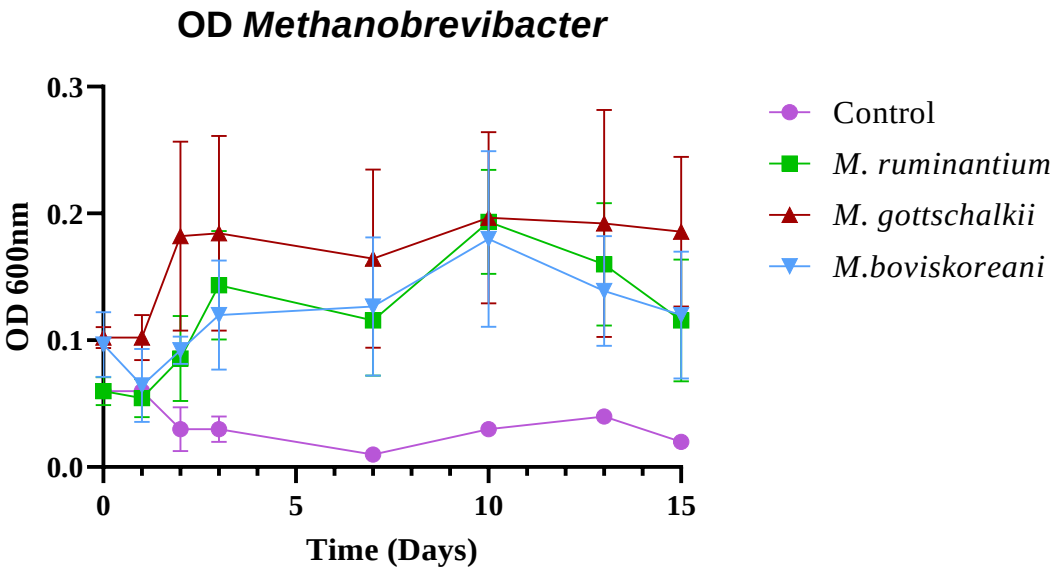

Figure 4.5: Methanogen growth curve of *Methanobrevibacter sp*. as indicated by optical density (OD_{600} nm). Error bars indicate the standard deviations and results are a mean of three replicates.

In vitro methane production

Headspace methane analysis was performed for the *Methanobrevibacter* species. Methane production over time for the methanogens is shown in Figure 4.6. Of the organisms studied, *M. boviskoreani* produced the most methane, with the maximum methane of 90 g/l produced on day 10. *M. gottschalkii* and *M. ruminantium* peaked for methane production on day 10 with 30 g/l of methane produced. Thereafter, methane production declined. On day 15, *M. boviskoreani* still produced 70 g/l of methane, while *M. ruminantium* and *M. gottschalkii* produced 20 g/l of methane.

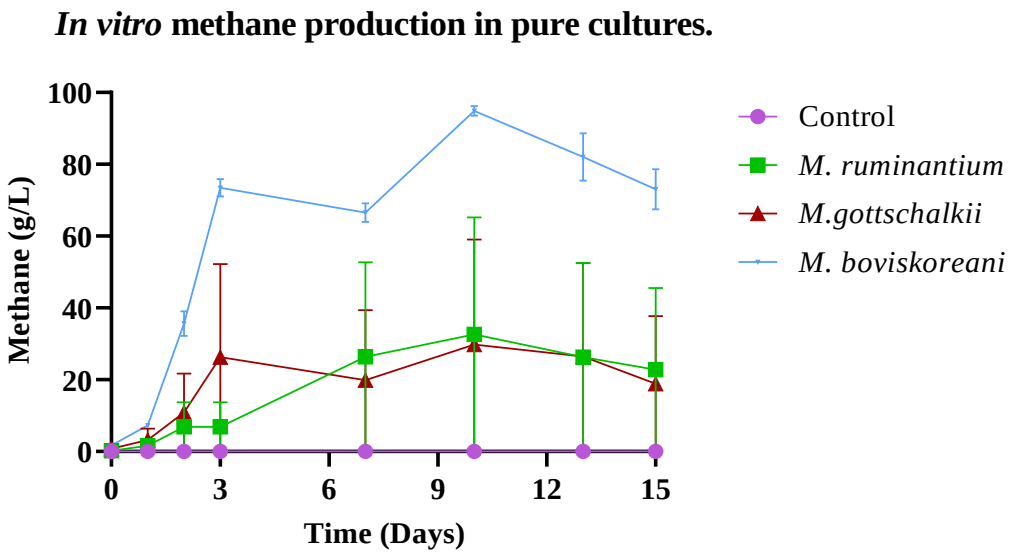

Figure 4.6: Methane production by pure cultures of methanogens. Each point represents the mean of three replicate tubes.

Visualising methanogens with fluorescence microscopy

In order to gain more insight into the appearance of the methanogens, fluorescence microscopy was used to view their cells (Figure 4.7). The methanogenic cells showed fluorescence at 420 nm excitation that was bright green with a tinge of yellow. The *Methanobrevibacter* cells all looked similar. They were small, short-rod shaped and mainly occurred as single cells. *M. gottschalkii* (C) displayed some cells that occurred in pairs.

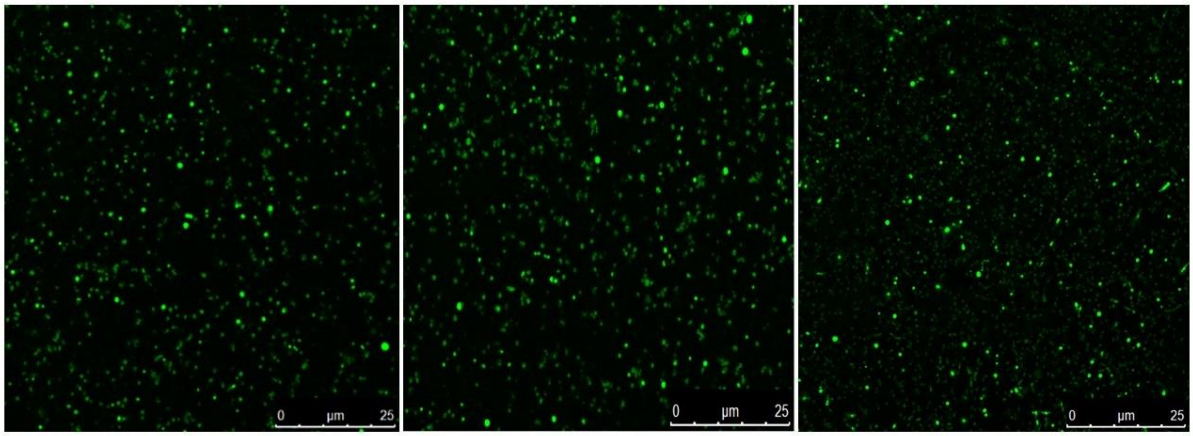

Figure 4.7: Auto fluorescent photomicrograph *of M. boviskoreani* (A), *M. ruminantium* (B), and *M. gottschalkii* (C) at 420 nm.

Figure 4.8 shows fluorescence images illustrating cell aggregates of *M. barkeri* cell suspensions exposed to the SYBR green I stain. Some cells seemed to be clustered in tetrads. These were distinctly different from the smaller *Methanobrevibacter* species. No flagella or pili were observed on the *Methanobrevibacter* species or *Methanosarcina*.

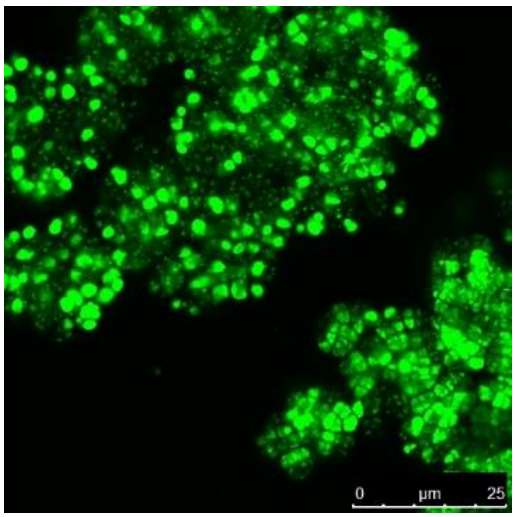

Figure 4.8: Auto fluorescent photomicrograph of *M. barkeri* at 420 nm.

Nukacin purification

The bacteriocin was purified from *S. warneri* (APC 2922) CFS using an Econo column containing 30 g of Amberlite XAD16N beads (C18 SPE; reversed-phase HPLC) and MALDI-TOF MS. Active peaks were detected at a mass of 2959 ± 1 Da (Figure 4.9 (A)). The chromatogram showed these peaks to be eluting from 30 to 50 mins. The antimicrobial activity of HPLC bioactive fractions was determined using a well-diffusion assay using *L. delbrueckii subsp. bulgaricus* LMG 6901 as the indicator strain. The fractions with the most active peaks were pooled together for lyophilisation (Figure 4.9 (B)), and the purity of the peptides was confirmed by MALDI-TOF MS. Consequently, 13.01 mg of a nukacin-containing powder was produced, which was used for *in vitro* methane inhibition assays.

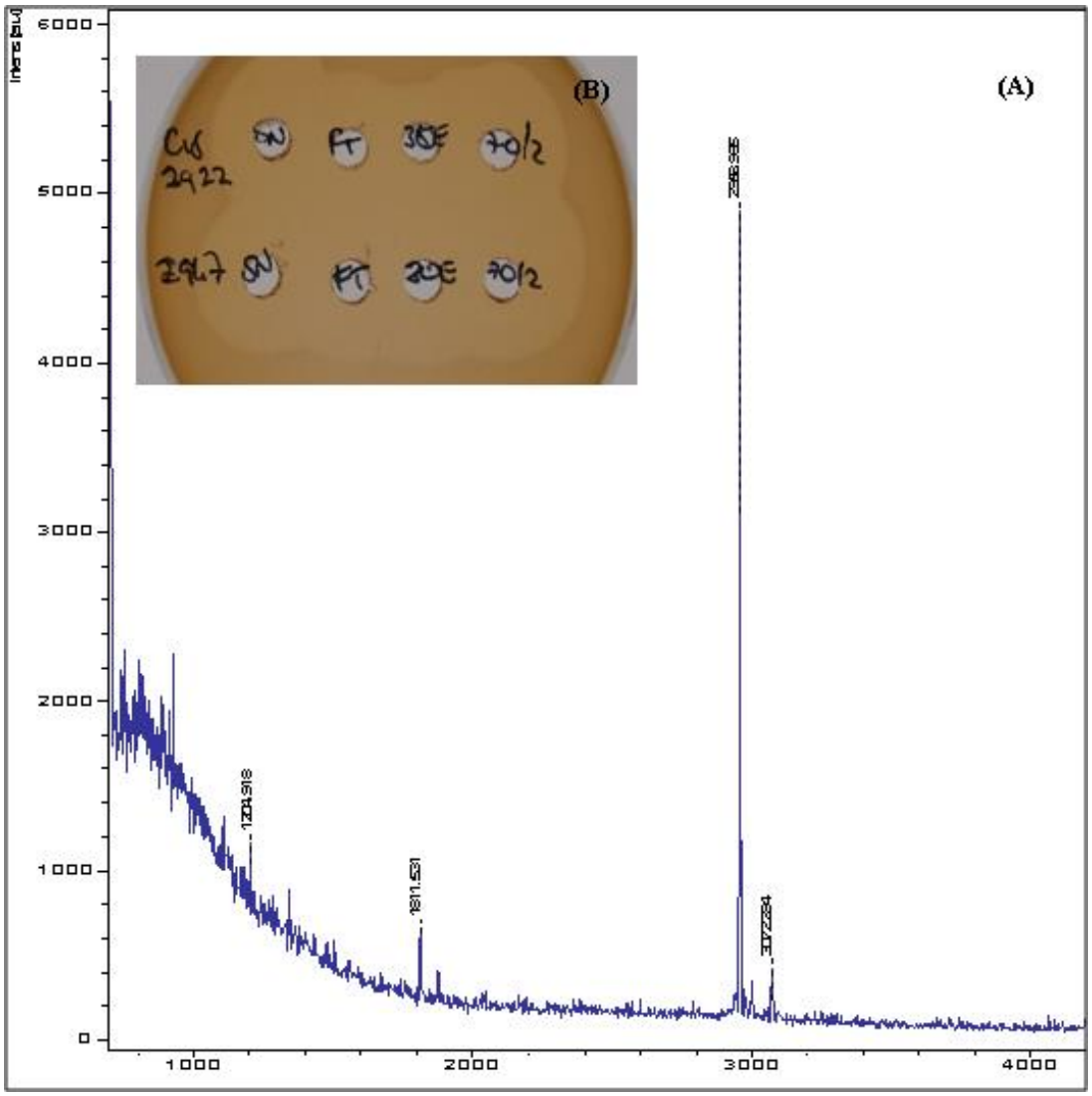

Figure 4.9: Detection of nukacin at 2959 ± 1 Da by MALDI-TOF MS from cell-free supernatant of *S. warneri* (APC 2922).

In vitro Methane inhibition assays

Inhibition of methane by various inhibitors

Headspace methane gas was measured using GC equipped with an FID column. CFS from different bacteria, capable of producing various bacteriocins, were used as inhibitors of methane production (Table 4.4). Different inhibitors divided into separate groups were tested against *M. ruminantium*, *M. gottschalkii* and *M. boviskoreani* (JH1) for methane inhibition. These inhibitors were added on day three of methanogen growth. The inhibitors were CFS from various LAB and staphylococci. These were *L. plantarum* (LP58), *L. lactis* (SL242), *L. lactis* ssp. *lactis* (DPC 3147), *L. coryniformis* (D3) and *L. lactis* subs *lactis* (KF245), *S. equinus* (HUN035 and HUN037), *S. warneri* (APC 2922), as well as peptides that were purified from *S. warneri* (nukacin 2922) and *L. lactis* ssp. *lactis* (lacticin 3147). Pure bacteriocins lacticin 3147 and nukacin 2922 were added to *M. ruminantium.* The bacteriocins were added at a final concentration of 0.04 mg/ml for lacticin 3147 and 0.2 mg/ml for nukacin 2922. The concentration of pure bacteriocins used in the assays was determined based on the available amount of peptide at the time. Commercially available nisin (Sigma Aldrich, Wicklow, Ireland) was used as a positive control, while the supernatant from *L. delbrueckii* subsp. *bulgaricus* LMG 6901 was used as a negative control. Headspace methane samples (10 ml) were taken at intervals from day three to nine. Methanogen cultures were pressurised to 1 bar with H_2/CO_2 after sampling.

The focus of this study was to investigate the effects of the chosen inhibitors on methane production compared to the untreated control or negative control. The inhibitory effect during methanogen exposure to CFS or pure bacteriocins was determined by assessing methane production by three rumen *Methanobrevibacter* species. Methane inhibition percentages were calculated via the following formulation: “Methane inhibition = 100 * (1- (sample/control)”.

Figure 4.10 (A) shows methane production in *M. gottschalkii* supplemented with 30% CFS (v/v) from commercial LAB strains *L. plantarum* (LP58) and *L. lactis* (SL242). Adding *L. plantarum* (LP58) CFS resulted in significant (27%) inhibition of methane compared to the *M. gottschalkii* culture to which the negative control was added. *L. lactis* (SL242) caused 8.25% methane inhibition in *M. gottschalkii*. In Figure 4.10 (B), *L. mucosae* (Pat) inhibited methane by 51%, similar to inhibition levels achieved by the positive control nisin (53%). Figure 4.10 (C) shows methane production when *M. gottschalkii* was supplemented with CFS from

different staphylococci, i.e., *S. capitis* (APC 2918), *S. warneri* (APC 2922), and proteinase K treated CFS from *S. warneri* (APC 2922). From the staphylococci CFS, the best methane inhibition of 53% was obtained from *S. capitis* (APC 2918) treated *M. gottschalkii*, although this result was not statistically significant. Adding the proteinase K-treated CFS did not significantly impact methane inhibition compared with non-treated CFS.

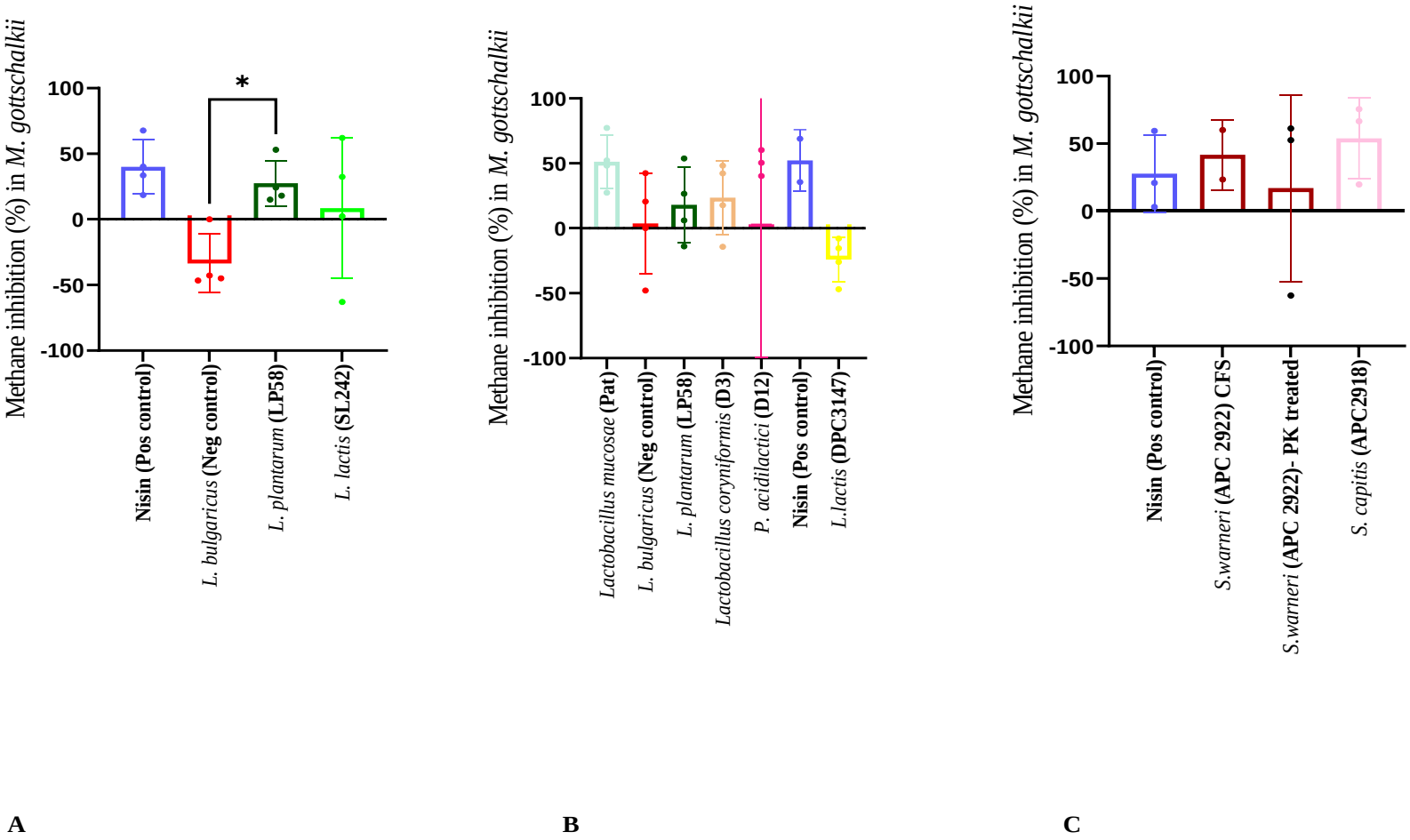

Figure 4.10: Methane inhibition percentages in the actively growing culture of *M. gottschalkii* supplemented with CFS from (A) commercially supplied SACCO (LAB) strains, (B) Bacteriocin- producing LAB strains, and (C) *S. warneri* and *S. capitis* from the culture collection. The different inhibitors were all added on day three of *M. gottschalkii* growth. *P-value <0.05.

Figure 4.11 shows the effect of different bacterial CFS and bacteriocins on methane inhibition in *M. ruminantium*. The addition of supernatant from *L. plantarum* (LP58) did not reduce methane production in *M. ruminantium*; instead, an increase in methane was observed (Figure 4.11 (A) and (B)). Similarly, methane inhibition was not observed after the addition of CFS from *P. acidilactici* (D12) and *L. lactis* (DPC 3147) (Figure 4.11 (B)). *L. lactis* (SL242) reduced methane by 7.5%, whereas *L. mucosae* (Pat) and *L. coryniformis* (D3) inhibited methane production by 26.9 and 49.4%, respectively. The effect of CFS from *Staphylococcus* species on methane production by *M. ruminantium* was investigated, as shown in Figure 4.11 (C). *S. capitis* (APC 2918) inhibited methane production by 68% compared to the untreated control. The purified bacteriocins lacticin 3147 and nukacin 2922 caused methane inhibition of 34.4 and 36.4%, respectively, similar to that of the positive control, nisin, which inhibited methane production by 34.1% *M. ruminantium* (Figure 4.11 (D)).

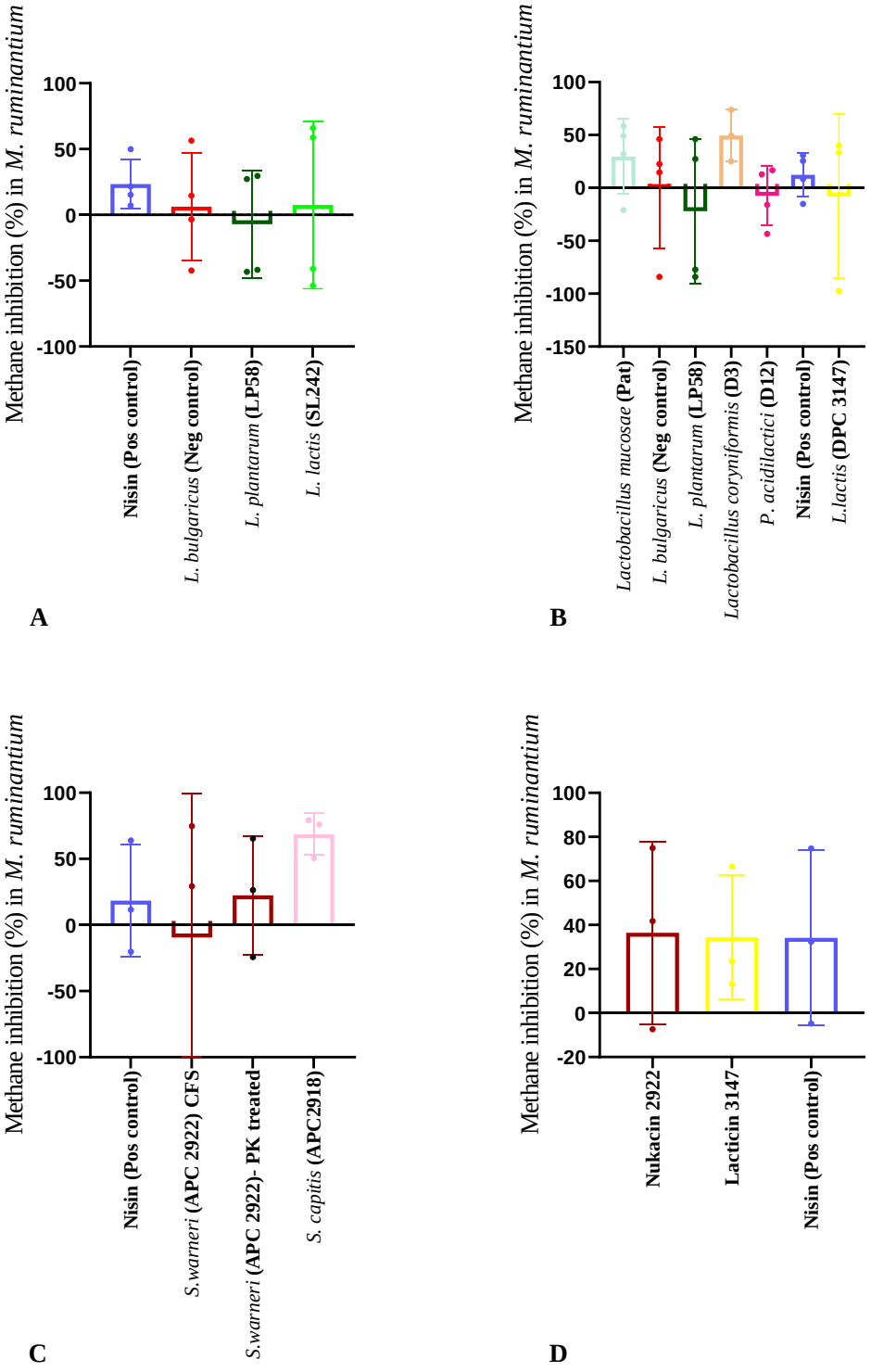

Figure 4.11: Methane inhibition percentages in the actively growing culture of *M. ruminantium* supplemented with CFS from (A) commercially supplied SACCO (LAB) strains, (B) isolates from the agricultural environment, (C) *S. warneri* and *S. capitis* from the culture collection, and (D) Purified bacteriocins lacticin 3147 and nukacin 2922. The different inhibitors were all added on day three of *M. ruminantium* growth.

Supernatants from *L. lactis* strains (SL242, SL147, and KF245), *S. equinus* (HUN035 and HUN037), and *P. acidilactici* (PA09) were tested for methane inhibition against *M. boviskoreani* (Figure 4.12 (A)). *S. equinus* (HUN037) was the most effective ($P<0.05$) at inhibiting methane from *M. boviskoreani*, with a 64.8% methane inhibition, and *L. lactis* (SL147) was the least effective, with none of the other *L. lactis* strains tested performing significantly better. *L. plantarum* (LP58) achieved 15% methane inhibition in *M. boviskoreani* (Figure 12 (B)). *P. acidilactici* strains (D12 and PA09) did not result in methane inhibition; instead, the methane produced was higher than the untreated *M. boviskoreani* control.

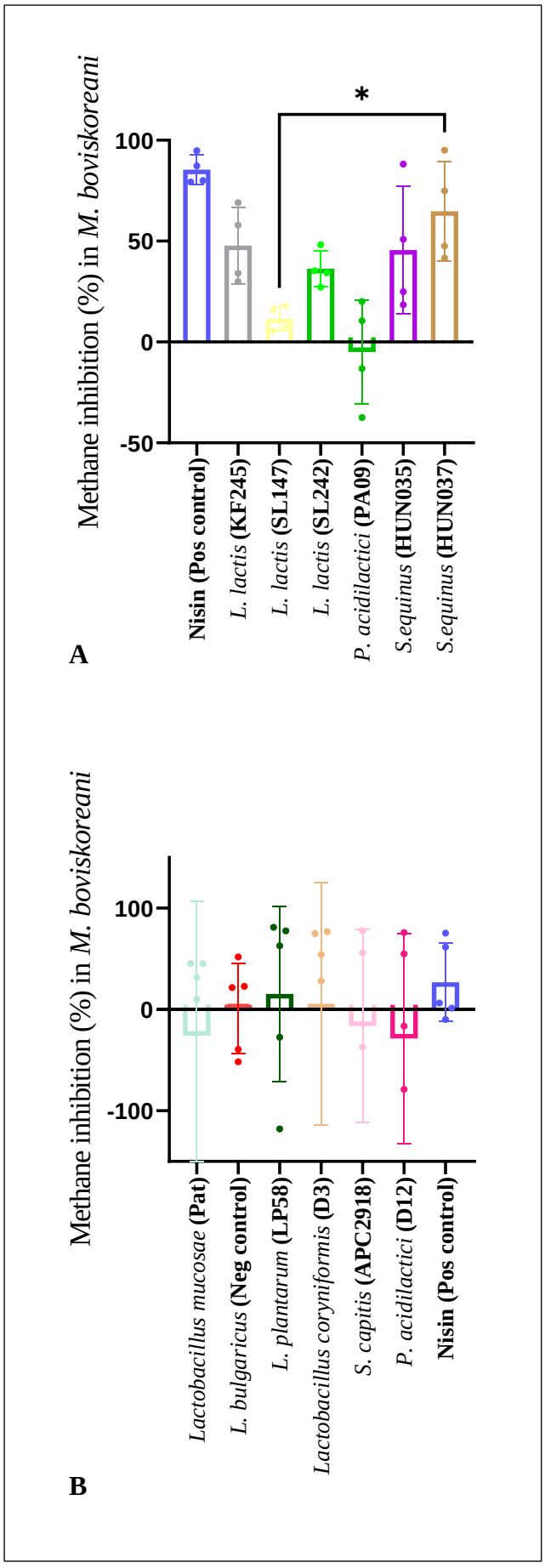

Figure 4.12: Methane inhibition percentages in the actively growing culture of *M. boviskoreani* supplemented with CFS from (A) commercial and AgResearch supplied LAB strains and (B) isolates from the agricultural environment and APC culture collection.

Discussion

This study investigated the potential of bacteriocins lacticin 3147 and nukacin and CFS from LAB and staphylococci to inhibit methane production *in vitro* in pure cultures of the rumen methanogens (*M. ruminantium, M. boviskoreani* and *M. gottschalkii*). Pure cultures of *Methanobrevibacter* spp. were used to limit confounding factors such as interactions between feed, minerals, and microorganisms that may occur *in vivo* or with rumen fluid *in vitro*.

Hydrogenotrophic methanogenesis is the most common methanogenic pathway observed in rumen methanogens and is utilised by the predominant *Methanobrevibacter* spp (Leahy et al., 2013). In the rumen, hydrogenotrophic microorganisms are important for stability in the whole methanogenesis pathway. They metabolise hydrogen and reduce its concentrations to levels suitable for the activity of syntrophic acetogens, which are sensitive to high concentrations of hydrogen (Zabranska and Pokorna, 2018). Many hydrogenotrophic methanogens are autotrophic, needing only CO_2, H_2, and inorganic salts to produce energy through methanogenesis and synthesise biomass through CO_2 fixation (Goldman et al., 2009). Therefore, cultivating this group of methanogens for methane was imperative in our *in vitro* studies.

Methanobrevibacter are considered extremely oxygen-sensitive, fastidious organisms, and their culturing requires specifically controlled conditions. *M. ruminantium* showed a lag phase of three days (Figure 4.5). Taylor et al. (1974) found that *M. ruminantium* had a lag phase of around 42 hrs when grown without mercaptoethanesulfonic acid (Co-enzyme M). For the *M. gottschalkii* studied, the death phase was not observed during the 15 days of the study, indicating that the organism could take longer to reach the death phase. A decline in OD_{600} was observed after 10 days for *M. ruminantium* and *M. boviskoreani*. Once these organisms reached the peak, the death phase, as indicated by a decline in $OD_{600,}$ followed. The dead methanogen cells could have pelleted and settled to the bottom of the Hungate tube. This could have caused the spectrophotometric measurement (dependent on light absorption) to be reduced and seen as a decline in OD_{600} (Lewis et al., 2014).

In the methanogen cultures cultivated, there was a correlation between OD (Figure 4.5) and methane production (Figure 4.6). In general, an increase in methane production corresponded with an increase in methanogen biomass. *M. gottschalkii* displayed the highest biomass

production as indicated by absorbance, while *M. boviskoreani* displayed the highest methane production rate. The availability of hydrogen is one of the main drivers of methane production; therefore, this was kept constant by adding H_2/CO_2 (80%/ 20%) (v/v) regularly to ensure that methanogenesis occurred at a constant rate throughout the experiment.

Coenzyme F420 is a fluorescent, cytoplasmic cofactor synonymous with methanogenesis (Greening et al., 2019). It functions as an electron carrier in anabolic and catabolic reactions. Along with methnopterin derivatives, it gives methanogens their characteristic blue-green fluorescence when illuminated at 420 nm (Lambrecht et al., 2017). Autofluorescence was observed in the three *Methanobrevibacter* sp (Figure 4.7) tested and *M. barkeri* (Figure 4.8). SYBR Green I staining facilitated the discrimination of the methanogen community from the ubiquitous particle noise. *M. boviskoreani* and *M. ruminantium* displayed single coccobacilli, while some *M. gottschalkii* cells appeared in pairs. Microscopic observations in our study revealed the expected morphology according to the literature. Cells of *Methanobrevibacter* are characteristically short, non-motile, oval or lancet-shaped rods or cocci and are usually found in pairs or chains (Oren, 2014). *M. barkeri* (Figure 4.8) showed a micrograph of large, clustered cells similar to Lambie et al. (2015). The microscopic analysis served as a verification step for the growth and purity of the methanogen cultures.

The results of this study demonstrate the sensitivity of three *Methanobrevibacter* species to several supernatants from bacteriocin-producing bacteria. In each instance, the inhibitors were added on day three of methanogen growth. Samples were taken on days 0, 1, 3, 5, 7, 9 and 11. However, the analysis only includes samples from days 3-9 because the methane production curve showed methane to be actively produced at this time (Figure 4.6). *L. plantarum* (LP58) CFS demonstrated significant methane inhibition against *M. gottschalkii* (Figure 4.11 (A)). *L. plantarum* produces the bacteriocin plantaricin (Todorov, 2009). The use of *L. plantarum* CFS to reduce methane production was reported by Nollet et al. (1998). In that study, researchers used LP80 to supplement ruminal samples during short-term (one day) batch experiments and observed a 5-15% decrease in methane production. Asa et al. (2010) also tested *L. plantarum* TUA1490L for the ability to reduce methane in a mixed ruminal sample and found that methane was significantly reduced compared to other treatments and the untreated control. Most studies investigated methane inhibition in mixed ruminal samples (Asa et al., 2010; Jeyanathan et al., 2016; Lee et al., 2002; Nollet et al., 1998; Puniya et al., 2013) whereas this study targeted a single methanogen at a time under highly defined media conditions. Studies with mixed

cultures and undefined media do not demonstrate the effect of inhibitors on individual methanogens present in the rumen (Patel et al., 1978).

L. mucosae was originally isolated from piglets' intestines but has also been found in cattle rumen (London et al., 2014). In our study, *L. mucosae* (Pat) was isolated from cattle faeces and was able to inhibit methane production in *M. gottschalkii* by 51%, although not significantly. (P=0.5). Soriano et al. (2014) investigated the effect of *L. mucosae* on *in vitro* rumen fermentation and reported that inoculation with this LAB increases methane in *vitro. L. mucosae* produces CO_2 and H_2 during its growth. These are substrates for methanogenesis (Ferguson and Ma, 1983) and could therefore explain the differences in the results in our study and that of Soriano et al. (2015). Furthermore, the *L. mucosae* used in our study was verified to produce an antimicrobial agent, which could potentially be antimethanogenic, and it was only the supernatant that was used and not a live strain, which could have produced substrates for methanogenesis. Supernatants from *S. capitis* (APC 2918) were inhibitory against two of the three methanogens tested, i.e., 53% methane inhibition was observed in *M. gottschalkii*, and 68% was observed against *M. ruminantium*. Lynch et al. (2019) discovered that *S. capitis* produces capidermicin, a novel bacteriocin with a mass of 5,464 Da. While antimicrobial production by *S. capitis* strains has been shown by agar-based studies against organisms such as *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Staphylococcus intermedius* and *Micrococcus luteus* (Janek et al., 2016; Lynch et al., 2019; O'Sullivan et al., 2019), its antimethanogenic properties have not yet been investigated.

In this study, it was observed that the different *L. lactis* strains (SL242, DPC 3147, and KF245) (Figure 4.12(A)) had varying results on methane inhibition in *M. boviskoreani*. The best methane inhibition in *M. boviskoreani* was achieved by adding *L. lactis* KF245 CFS, which inhibited methane production by 47.7%. Species of *L. lactis* are known to produce a variety of bacteriocins that inhibit bacteria using different mechanisms (Holo et al., 1991; Morena et al., 2000). Most bacteriocins from *L. lactis* are lantibiotics, which generally disrupt the integrity of the cytoplasmic membrane of the target cells through pore formation. This group uses lipid 2 as a specific docking site to interact with target cells (Barbour et al., 2016). Some bacteriocins from *L. lactis* are Class 2a bacteriocins (Ennahar et al., 2000). Class 2a bacteriocins kill target cells by forming pores and disrupting the integrity of target cell membranes, causing dissipation of proton motive force, depletion of intracellular ATP and leakage of amino acids and ions (McAuliffe et al., 2001). The addition of CFS from *L. lactis* (SL242), a nisin producer

(DeVuyst and Vandamme, 1992), did not significantly impact methane production in *M. ruminantium* in our study. Nisin was found by Callaway et al. (1997) to reduce methane production by 36% in ruminal fermentation when added to concentrations that are above 1 µM. This result was accompanied by a decrease in starch fermenting ruminal bacteria, indicating that nisin could potentially reduce methane by disrupting precursors in the methane formation pathway rather than targeting methanogens and directly inhibiting methanogenesis. Several strains used in this study are known nisin producers. These are *S. equinus* (HUN035 and HUN037) (Shin et al., 2016). It would be interesting to see if nisin can use its conventional inhibition mechanism of pore formation and cause cell contents to leak, especially since methanogens have pseudomurein instead of peptidoglycan in their cell wall. Nisin uses two mechanisms to form pores in the cytoplasm. Firstly, it inhibits bacterial growth by forming pores in the cell membrane and interferes with cell-wall biosynthesis through specific lipid II interaction. Secondly, if lipid II occurs in low concentrations or is absent, nisin disrupts microbial cell membranes by interacting with membrane phospholipids and can displace or release enzymes, resulting in lysis of the cell wall (Paiva et al., 2011). The positively charged C-terminal domain of nisin binds the negatively charged phospholipids of the membrane. The methanogens *Methanobrevibacter* and *Methanobacteria* contain psudomurien (Klaus and Koning 2016). A study by Hammes and Colleagues (1979) found nisin to be inhibitory against pseudomurein-containing *Methanobacterim* strains (*Methanobacterium arbophilicum*, *Methanobacterium* strain M.o.H.G, *Methanobacterium* strain M.o.H, and *Methanobacterium formicicum*). In contrast, the pseudomurein lacking strains (*Methanospirillum hungatei* and *M. barkeri*) were not sensitive to nisin. Therefore, it can be suggested that nisin may inhibit methane production by interfering with the biosynthesis of pseudomurein in methanogens (Varnava et al., 2017). A more recent study by Shen et al. (2017) used qPCR to show that nisin added at 5 µM significantly reduced ($P < 0.05$) the total population of methanogens compared with the negative control in rumen *in vitro* studies. One drawback to using this bacteriocin is that nisin could lose its inhibitory activity through protease degradation in the rumen (Russsel and Mantovani, 2004). This phenomenon was not observed in our study because the methanogens were cultivated in the media most suitable for their growth, which did not truly represent rumen conditions.

Streptococcus equinus, also referred to as *Streptococcus bovis*, produces bovicin HC5, warnericin, nukacin and warnerin, apart from nisin (Azevedo et al., 2015; Minamikawa et al., 2005; O'Sullivan et al., 2019; Prema et al., 2006) According to Bagel4 (de Jong et al., 2006),

these rumen streptococci contain an 11-gene cluster for producing a bacteriocin belonging to the gallidermin/nisin family. Adding CFS from *S. equinus* (HUN035 and HUN037) strains inhibited methane production in *M. boviskoreani* by 45.6 and 64.79%, respectively (Figure 4.12 (B)). Bacteriocins from *S. bovis* have been investigated for the potential to reduce methane. Bovicin H5 was found to inhibit ruminal methane by 50% *in vitro* (Lee et al., 2002). Proteinase treatment of the CFS from *S. warneri* did not affect methane production. Bacteriocins administered orally to ruminants may be susceptible to proteolytic degradation (Brock et al., 1982), reducing their efficacy *in vivo* (Benitez-Chao et al., 2021). This can be circumvented by engineering bacteriocin derivatives that demonstrate resistance to ruminal protease while retaining antimicrobial activity (Soltani et al., 2021).

Purified bacteriocins lacticin 3147 and nukacin 2922 did not significantly reduce methane production in *M. ruminantium,* and the methane inhibition achieved by these bacteriocins was similar to the positive control (nisin) (Figure 4.11 (D)). Nukacin is a 27-amino-acid bacteriostatic lantibiotic (Asaduzzaman et al., 2009) and exerts antimicrobial activity through binding to lipid II (Fujinami et al., 2018). The cell wall lipids of methanogens are distinct from that of bacteria in that the bonds which link alkyl chains to the glycerol are phytane or biphytane in methanogens, contrary to the ester-linked fatty acyl glycerol derivatives in bacteria (Zhou et al., 2011). Lacticin 3147 is a potent broad-spectrum bacteriocin that is inhibitory against *L. lactis, Listeria monocytogenes, Bacillus subtilis* (McAuliffe et al., 1998) and various mastitis pathogens (*Streptococcus agalactiae, Streptococcus uberis, S. aureus* and *Streptococcus dysgalactiae*) (Ryan et al., 1998). Jayanegara et al. (2014) investigated the ability of lacticin 3147 to reduce methane emission under *in vitro* rumen environment and found that its addition at 50 µM did not inhibit methane production. Similar to our study, Jeyanagara and colleagues suggested that future antimethanogenesis testing should be carried out using lacticin in higher concentrations.

Conclusion

The addition of LAB supernatants had varying results on the different methanogen species. The production of methane by pure, growing cultures of *M. gottschalkii* was inhibited by the addition of CFS from *L. plantarum* (LP58), whereas *L. plantarum* (LP58) did not significantly impact methane production in *M. ruminantium.* From the staphylococci strains, the best methane inhibition of 53% and 68% was obtained when *M. gottschalkii and M. ruminantium* were respectively treated with CFS from *S. capitis* (APC 2918). *S. equinus* (HUN037) was effective ($P < 0.05$) at inhibiting methane from *M. boviskoreani* by 64.8%. The purified bacteriocins lacticin 3147 and nukacin 2922 resulted in methane inhibition of 34.4 and 36.4%, respectively, in *M. ruminantium*, which was comparable to nisin. Whether the effects of the bacteriocin-containing CFS on methane production could be ascribed to the membrane interaction with the structurally different ether lipids of methanogens or an additional intracellular mode of action has to be further studied using biophysical methods. These could be live-dead staining, electron microscopy and molecular biology approaches. The key strengths of this study are its long duration and its examination of the effect of bacterial CFS and pure bacteriocins on individual methanogen cultures. The main limitation of the assays in this study is that methane concentration was quantified after the assays were complete. This meant that a decrease or increase was observed retrospectively and not in real-time. The drawback of this was that even when the methanogens in the Hungate tubes were not actively growing, we continued to sample them, which made drawing conclusions about certain inhibitors extremely difficult. For future studies, it would be advisable to measure OD_{600} concurrent with methane production measurements to get a sense of the growth of the methanogens. Additionally, it would be preferable to measure methane production as the study progresses to enhance the efficiency and reliability of results. Future work may also involve viewing the structure (via scanning electron microscopy) of the methanogens susceptible to the inhibitors to examine the cell wall for structural changes. This could give insight into the mechanism by which some bacteriocins can reduce methane production in *Methanobrevibacter*. There is a limited number of available studies on methane production in pure cultures of methanogens. This study may be the key to understanding how LAB, other bacteria and bacteriocins can be used to inhibit methane and how such results may be translated to intervention studies *in vivo*.

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Chapter 5.

Evaluation of *Lactobacillus plantarum* (LP58) and *Lactococcus lactis* (SL242) as silage inoculants.

Abstract

Silage is any crop that is harvested and preserved under anaerobic conditions. Ensilaging depends on LAB, which use water-soluble carbohydrates and produce organic acids such as lactic acid and moderate amounts of acetic acid, thereby preventing silage degradation by oxidation or spoilage microbes. This study aimed to determine the effect of *Lactococcus lactis* (SL242) and *Lactobacillus plantarum* (LP58) separately in lab-scale silage and large-scale silage fermentation trials as co-inoculants. Three groups of mini silages of 200g each were created, i.e., the untreated controlled group and the two treatment groups each inoculated with *L. plantarum* (LP58) or *L. lactis* (SL242), both at 0.25 $\times 10^6$ CFU/g. The mini silages were packaged in polyethene vacuum-sealed bags and stored in the dark at room temperature. The nutritional composition and microbiological quality of the silage were monitored for three months after silage manufacture. Spoilage microbes (yeast and clostridia) were assessed using plating methods. The results of the lab-scale trial revealed the benefits of using *L. plantarum* LP58 in silage, such as better reduction of silage pH as shown by lower final pH of 5.3, and *L. lactis* SL242 as it remained viable longer and exhibited higher colony counts (8.6 log CFU/g) after three months of fermentation. None of the treatments showed significant inhibition of spoilage organisms such as yeast and clostridia in the lab-scale silage. Lactate and ammonia levels were similar in the control and treated samples. The large-scale silage was made into bales of 800 kg each. *L. plantarum* (LP58) and *L. lactis* (SL242) were co-inoculants applied at 1.76×10^7 CFU/g and 8.64×10^6 CFU/g, respectively. Silage fermentation proceeded for three months, and samples were taken regularly. The proliferation of the inoculants was assessed using culture-based methods and quantitative PCR (qPCR). Lactic and acetic acid concentrations were measured using enzyme assay kits, and an ammonia assay was performed. The inoculants could thrive and replicate within the silage as indicated by colony counts of 8 log CFU/g and confirmed via qPCR. *L. lactis* (SL242) viable counts and DNA copy numbers were statistically higher in the LAB-treated silage than in the control (P= 0.026). LAB silage co-inoculation resulted in a rapid decrease in silage pH. LAB inoculated silage had significantly higher L- and D- lactate levels than the control (P= 0.0034). Acetic acid gradually increased from 0.28 mg/g to 4.2 mg/g for the LAB-treated silage and from 0.21 mg/g to 2.9 mg/g for the control silage over 90 days. LAB did not affect ammonia concentration or the inhibition of yeast and clostridia in the large-scale silage. The use of *L. plantarum* (LP58) and *L. lactis* (SL242) as silage co-inoculants improved the nutritional quality of silages through sufficient production of lactic acid and reduced silage pH.

Introduction

Silage is a fermented crop, forage or agricultural byproduct consisting of a high-moisture content (greater than 50%) which is used as feed for ruminants and is primarily used for ensuring an adequate supply of animal feed throughout the year (Bolsen et al., 1996; Oliveira et al., 2017). To be ensiled, the crop must meet specific criteria, i.e., dry matter should be above 20%, water-soluble carbohydrates (generally glucose, fructose and sucrose) should be above 3%, and the crop must have good buffering capacity (resistance to acidification) (Bolsen et al., 1996). There are three main factors at work within the silo: plants undergo physical changes, microbial interactions, and various chemical reactions (Huo et al., 2021). Silage has traditionally been used as an essential feed source for ruminants worldwide (Ramos, 2016). Its quality affects the feed intake of animals in addition to the resultant meat and milk flavour and quality (Acosta, 2012). The main goal of silage making is to conserve the crop with minimal loss of nutritional value by fermentation of soluble carbohydrates, guaranteeing high nutritional value when fed to ruminants. An important objective in ensiling is to avoid extensive proteolysis, which decreases the nutritional quality and intake of the silage. Ensiling is a multifaceted biochemical and microbiological process that progresses from aerobic to anaerobic conditions (Li et al., 2018). Silage creation is a preservation technique that depends on the spontaneous fermentation by LAB and occurs naturally under anaerobic conditions (Adesoki et al., 2010).

There are different types of silage additives: a) nutrients and adsorbents, b) fermentation stimulants and fermentation inhibitors, and c) inhibitors to aerobic deterioration (Muck et al., 2018). An ideal inoculant must also promote anaerobic stability by inhibiting the growth of aerobic spoilage organisms. The effect of the inoculant on the efficiency of the animal must also be noted to ensure that the consumed silage does not negatively impact the host's microbiome upon ingestion.

LAB have a central role as inoculants for silage preservation and fermentation due to their long history of use in agriculture and food production because of their numerous beneficial properties (Xu et al., 2017). LAB produce compounds used as feed-grade enzymes, veterinary medicines and bio preservatives (Kim et al., 2021). This metabolically related group produce bacteriocins, which enable them to colonise silage effectively, and they can withstand an acidic environment, which allows them to competitively exclude many spoilage and pathogenic

microbes (Choi et al., 2018; Mathur et al., 2017). Many LAB inoculants such as *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Pediococcus acidilactici* and *Enterococcus faecium* have been commercially developed to make silage (Ni et al., 2015). LAB are known for their positive effects during ensiling, such as, enhancing fermentation, improving dry matter (DM) and nutrient recovery and prolonging aerobic silage stability (Queiroz et al., 2018). Silage making is divided into six phases. The initial four stages are fermentation phases and include an aerobic phase, which occurs immediately after harvest, followed by the lag, transition, and the stable phase. The final two are the storage and feed-out phase (Beglar, 2006, Borreani et al., 2018). Understanding the microbial ecology of silage is vital for identifying the type of microorganisms needed for optimal silage production and circumvent pathogen growth, which can potentially compromise animal safety (Romero et al., 2017). An inoculation rate of $10^5 – 10^6$ viable cells per gram of crop is often sufficient for the inoculant LAB to overwhelm the epiphytic microorganisms and become the predominant population in the silage (Oliveira et al., 2017). The most commonly used measurements to evaluate silage fermentation include pH, quantifying the microbial population using culture-dependent and independent methods, assessing the concentrations of organic acids, primarily lactic acid and acetic acid, and determining the rate of proteolysis indicated by ammonia concentration (Kung et al., 2018).

The aim of this study was to evaluate the effectiveness of LAB as silage inoculants and determine if fermentation and nutritional benefits were achieved using one strain of LAB or a combination of inoculants. *L. plantarum* is a well-known, homofermentative silage inoculant (Muthusamy et al., 2020) (Xie et al., 2020) (Yang et al., 2020), however, it has not been tested in combination with *L. lactis* as silage inoculants. Furthermore, *L lactis* (SL242) and *L. plantarum* (LP580 showed potential as methane inhibitors *in vitro* (Chapter 4). This study was performed in two parts. The first trial involved the creation of lab-scale silages (200 g each), which were inoculated with *L. plantarum* (LP58) and *L. lactis* (SL242) separately. Phosphate buffered saline (PBS) was used for the control silage. The fermentation characteristics of silage were monitored and compared. The second trial involved using *L. plantarum* (LP58) and *L. lactis* (SL242) as co-inoculants for large-scale baled silage of 800 kg each, wrapped in plastic and stored in the fields. Nutritional and fermentative properties were monitored for three months. The silage was then fed to dairy cows (Chapter six), with feed acceptability assessed via dry matter intake and feed refusal percentages. Comparative analysis was performed on the untreated control silage versus LAB treated silage, where nutritional and fermentation characteristics were examined.

Materials and Methods

Trial 1: Preparation of Lab-scale silage

In order to understand the microbial ecology within silage and test the viability of LAB strains in an animal trial (Chapter six), it had to be established whether the cultures could be effectively incorporated into animal feed by initially creating mini silage. Commercially available strains of *Lactococcus lactis* (SL242) supplied at a concentration of 5.4 $\times 10^{11}$ CFU/g and *Lactobacillus plantarum* (LP58) at 11×10^{11} CFU/g were obtained from SACCO (Sacco Srl, Via Manzoni 29/A, 22071 Cadorago (CO), Italy). These were diluted using phosphate-buffered saline (PBS) to a final concentration of 0.25×10^{9} CFU/g. PBS was used to suspend the culture and subsequently spread onto 4 kg wilted (for 24 hrs) grass from Teagasc Moorepark. The final concentration of the inoculants was 0.25×10^{6} CFU/g on the fresh matter, which is 5.39 log CFU per gram. The ensiled grass was mixed thoroughly to distribute each inoculant evenly. Then, 200 g of grass was weighed onto sterile polythene bags. The bags were vacuum-sealed for 25 s, then enveloped with a second bag and labelled accordingly. There were 2two treatments (*L. lactis* (SL242) and *L. plantarum* (LP58)) and a control group (C) without inoculant. The bags were kept in the dark at room temperature for 90 days to mimic normal silage-making conditions. During the ensiling period, fermentation properties were monitored. Mini silages were opened and analysed on days 0, 30, 60 and 90.

Microbial enumeration

To evaluate the microbial composition of silage over time, silage samples (2 g) were mixed with 18 ml PBS, then mechanically pulverised using a stomacher (BagMixer lab blenders, Interscience, France) for 3 mins at the highest setting. Serial (ten-fold) dilutions were performed using PBS, and microbial suspensions were plated on media specific to certain microorganisms. De Man, Rogosa, and Sharpe (MRS) (Difco™ Trafalgar Scientific Ltd, Leicester, United Kingdom) media was used for total LAB counts and plates were incubated for 48 hrs under anaerobic conditions at 30°C. *Lactobacillus* selective agar (LBS) (Difco™ Trafalgar Scientific Ltd, Leicester, United Kingdom) was used to enumerate *L. plantarum* (LP58), and plates were incubated aerobically at 30°C for 48 hrs. LM17 (Merck, Darmstadt, Germany) agar was used to select for *L. lactis* (SL242), and plates were incubated anaerobically at 30°C for 48 hrs. Reconstituted clostridia media (RCM) (Merck, Darmstadt, Germany) was used to detect the presence of any clostridia species, and incubation was for 48 hrs anaerobically at 37°C. Yeast extract agar (Oxoid c/o Fannin Healthcare, Dublin, Ireland) was

used for yeast enumeration, and the plates were incubated for 24 hrs under aerobic conditions at 30°C. The YEA media was supplemented with 0.1% (w/v) chloramphenicol (Sigma Aldrich, Wicklow, Ireland). Whenever possible, plates comprising 30-300 colonies were counted. An average result for the triplicate plates was calculated and corrected for dilution. The number of microorganisms present was expressed as colony forming units (CFU) per gram of silage.

Silage fermentation

Silage pH was measured on day zero, then at monthly intervals until day 90. Silage (2 g) was placed in a stomacher bag, and 8 ml of PBS was added. Silage was mechanically homogenised in a stomacher for 3 mins at the highest speed. The extract was removed, and pH was measured with a glass electrode pH meter (pH FE28, Mettler-Toledo Instruments Co., Ireland).

Silage extractions were prepared using a mechanical homogeniser as stated above. The method of Van Winssen et al. (2000) was modified to prepare silage samples for D- lactate and L-lactate analysis using high-pressure liquid chromatography (HPLC) with a Chirex 3126 (D)-penicillamine 150 mm column. Briefly, silage extract samples were centrifuged at 2000 × g at 4°C for 10 mins. The pellet was discarded, and the supernatant was centrifuged again at 18,500 × g for 10 mins at 4°C. The supernatant was filtered through a 0.2 µM nylon filter (Thermofischer scientific Currabinny, Carrigaline, Co Cork, Ireland) and stored at -20°C until further analysis. Samples were mixed for 1 min after thawing and centrifuged again at 18,500 × g at 4°C for 10 mins. For HPLC analysis (Water 2487λ dual absorbance detect, Shimadzu, Biotech, Manchester, United Kingdom), filtered $CuSO_4$ (0.002 M) was used as a buffer, and the standards were D and L-lactate prepared as 1 mg/ml stocks. Silage extract samples (20 µl) were injected into the HPLC vials, and analysis was performed in duplicate with the operating pressure set at 100 bar.

Dry matter analysis

Dry matter was determined as described by Romero et al. (2017), where 100 g of silage was incubated at 60°C for 72 hrs with a beaker of water to prevent the possibility of incineration. The samples were weighed until constant weight indicated by a difference of less than 1 gram from the previous weight. This weight was then represented in percentage value.

Ammonia analysis

Ammonia concentrations were determined by using the method developed by Van Laar (2013). Silage was added to PBS in a 1:10 ratio and mixed vigorously using a stomacher for 3 mins at the highest setting. The mixture was centrifuged at 2500 × g for 10 mins at 4°C using 50 ml Falcon tubes (Sigma, Adrich). The supernatant (0.75 ml) was then transferred into an Eppendorf tube (Accuscience Ireland Ltd, M7 Business Park, Unit C3, Newhall, Naas, Co. Kildare, Ireland) and 0.75 ml of 10% (w/v) trichloroacetic acid (TCA) (Sigma, Aldrich) was added and vortexed to mix. This mixture was centrifuged at 14000 × g for 10 mins at 4°C. The supernatant was then transferred into a 15 ml Falcon tube, and 2 ml of TCA (10% w/v) was added and vortex mixed. The suspension (0.25 ml) was then transferred into a second tube containing 2.5 ml phenol solution. Before vortexing, 2.5 ml hypochlorite buffer solution was added and incubated in a 37°C water bath for 30 min while the tube was covered with parafilm. After removing the samples from the water bath, the solution was left to cool to room temperature for 30 mins before quantifying ammonia in silage samples. In parallel, different concentrations of ammonia sulphate (5-30 ppm) were treated the same as the samples (as described above) and used to generate a standard curve, with R^2 = 0.98. Ammonia concentration from the samples and the standard solutions was measured using a spectrophotometer at 623 nm against water as a blank.

Bacteriocin production from silage sample

The cell acidification method of Yang et al. (1992) was modified to extract bacteriocins from silage. Ten grams of silage was added into a 50 ml Falcon tube. Thereafter, 35 ml of PBS (pH 7), was added and stirred for 1 hr in an agitating incubator (150 RPM) for 90 mins at room temperature. In order to inactivate the bacterial cells in the sample, the silage was incubated at 70°C for 45 mins. To collect the pellet, samples were centrifuged at 2600 × g at 4°C for 15 mins. The supernatant was separated from the grass and centrifuged again at 7000 × g, at 4°C for 15 mins. The pellet was resuspended in 25 ml of NaCl and adjusted to pH 2, using 1M HCl. The bacteriocin fraction was collected by incubating the extract at 4°C for 4 hrs, with gentle agitation (100 RPM). This solution was centrifuged at 4000 × g at 4°C for 15 mins, and the supernatant was finally adjusted to pH 6.8 using 1M NaOH. The pH-adjusted supernatant was sterilised using 0.22 µM membrane filters (Thermofischer scientific Currabinny, Carrigaline, Co Cork, Ireland). A well-diffusion assay (as described in Chapter three) was carried out

against *L. bulgaricus* LMG 6901 as the indicator strain, with 50 µl of the bacteriocin-containing silage extract dispensed into the wells.

Trial 2: Large-scale silage production

This study aimed to investigate the effects of LAB on the nutritive quality and the aerobic stability of silage when used as co-inoculants. A freeze-dried commercial preparation of *Lactobacillus plantarum* (LP58) and *Lactococcus lactis* (SL242) was used as grass silage co-inoculants. Before being used as co-inoculants, the CFU of each strain was determined. The CFU of the different LAB strains in the dry powder was confirmed by suspending the inoculants in PBS and plating serial dilutions onto the MRS, LBS, and LM17 agar. The freeze-dried preparations were diluted with chlorine-free water and spread onto the grass before bailing the silage. The inoculants were applied onto the grass wilted to ~25% DM using a Hardi sprayer (Hardi International, Herthadalvej 10, 840 Nørre Alslev, Denmark) mounted onto a 250L liquid tank holder on a tractor. The inoculant was sprayed directly onto the 1.2 m swath immediately before ensiling, and a flow rate of 6.08 l/min with a forward driving speed of 8 KPH to achieve an application rate of 0.12 kg additive/1000 kg DM of grass. The final CFU of the freeze-dried inoculant was calculated to be 1.76×10^7 CFU/g for *L. plantarum* (LP58) and 8.64×10^6 CFU/g for *L. lactis* (SL242). The preparation concentrations were based on probiotic powder availability and recommendations from the manufacturer. The control silage was prepared similarly to the treatment but without adding inoculant. Grass silage was made on July 11, 2020, at Teagasc Moorepark. Two batches of grass silage were prepared for the control and inoculated treatments. Each bale contained approximately 800 kg of grass. Then fermentation was allowed to proceed for three months.

For analysis, silage cores were taken from four different bales, combined, and split into two. One sample was analysed immediately, while the remaining sample was stored at -20°C for processing at a later stage. The samples were analysed for fermentation and nutritional characteristics. In order to avoid possible cross-contamination, untreated silages were sampled first, followed by the LAB-treated grass. The sampling corer was rinsed with ethanol (70%) and then rinsed with water between sampling. Different tests were carried out on every sampling occasion (Figure 5.1). Silage samples were diluted in PBS or water and then homogenised for 3 mins using a stomacher at the highest setting to obtain silage extracts.

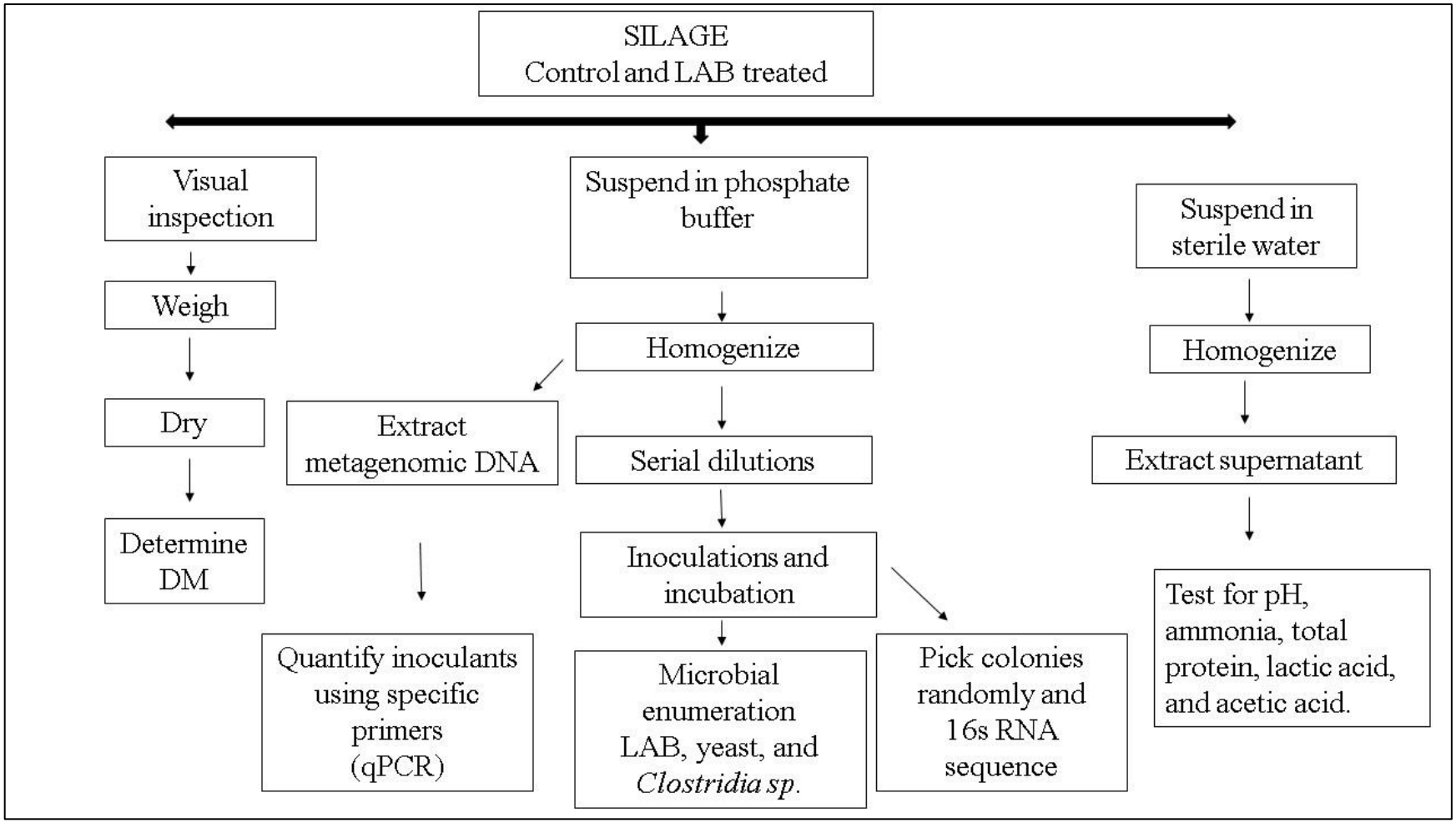

Figure 5.1: Flow diagram for silage analysis

Microbiological analysis and preliminary phenotypical characterisation of isolates

To evaluate the microbial progression of silage over time, silage samples (2 g) were mixed with 18 ml PBS, as mentioned previously. Solid selective media was used to cultivate the inoculants *L. plantarum* (LP58) and *L. lactis* (SL242) and spoilage microbes, namely clostridia and yeast. For CFU/g, colonies phenotypically characteristic of the silage inoculants were counted. *L. plantarum* (LP58) forms large yellow colonies on LBS, whereas *L. lactis* (SL242) forms small white colonies on LM17. Furthermore, colonies that grew on MRS were randomly picked for genomic analysis via 16S rRNA sequencing. Genomic DNA was extracted using the GenElute kit (Sigma Aldrich, Wicklow, Ireland), following the manufacturer's instructions for DNA isolation from Gram-positive colonies. Polymerase Chain Reaction (PCR) was performed using Biomix red (Meridian Bioscience, Dublin, Ireland). For amplification of the 16S rRNA gene, universal primers F27 (5-AGAGTTTGATCCTGGCTCAG-3) and U1492 (5-GGTTACCTTGTTACGACTT-3) (Sigma, Aldrich) were used with the following run conditions: initial denaturation: 94°C × 5 mins., cycle conditions: 30 cycles of 94°C × 40 s, 55°C × 30 s, 72°C × 1 min, final extension: 72°C × 10 mins. The PCR products were confirmed via gel electrophoresis. The resulting sequences were compared to existing genomic data using the Basic local alignment search tool (BLAST) on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The identity of the isolates was determined based on the highest scores (≥ 98%).

Species-specific identification using qPCR

DNA extraction on silage samples was carried out using the Qiagen Powersoil DNA isolation kit (Qiagen Ltd, Manchester, England), according to the manufacturer's instructions, with minor modifications. Briefly, 0.5 g of grass material was removed from the silages into a sterile stomacher bag, and subsequently diluted with 4.5 ml of sterile PBS to create a 1:10 dilution. The mixture was blended at the highest speed for 3 mins and subsequently centrifuged at 4000 × g for 10 mins at 4°C to pellet the bacterial cells. DNA was extracted from a 1 ml overnight culture of the inoculants at 10^{10} CFU for the positive control. DNA yield and quality were checked using a Nanodrop1000 spectrophotometer (ThermoFisher Scientific, Ireland).

Primer specificity

For *L. plantarum* (LP58), the PCR-specific primers LbP11 (5'-AATTGAGGCAGCTGGCCA-3') and LbP12 (5'-GATTACGGGAGTCCAAGC-3') (Sigma, Aldrich) were used, according to Quere et al. (1997). To amplify the 16S rRNA gene, each sample was subjected to a primary denaturation cycle at 95°C for 5 mins and at 94°C for 30 s, 36°C for 1 min and 72°C for 1 min for 35 cycles. The reaction mixture consisted of 2 µl of each primer (10 µM), 25 µl of Biomix red buffer, 20 µl PCR grade molecular water and 1 µl DNA. For *L. lactis* SL242, primers specific for the Lactococcin A, B, and M genes were used. These were LactBM-F (5'-GAAGAGGCAATCAGTAGAG-3') and LactA-R (5'- GTGTTCTATTTATAGCTAATG-3') (Sigma, Aldrich). The PCR conditions were as follows: initial denaturation at 94°C for 2 mins, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 mins. The PCR reaction was the same as that for *L. plantarum* (LP58). To determine the size of amplified DNA, 8µl of DNA was examined using 1% agarose gel stained with SYBR fast and viewed under the Odysseyâ FC imaging system. Amplicon sizes were further verified *in-silico* using primer blast (Ye et al., 2012). The qPCR analysis was performed to investigate the copy numbers of the genes mentioned above. The quantification of DNA for each bacterial species in silage was performed in a Roche 96 light cycler using the Fast SYBR green master mix (Roche Diagnostics Ltd). The standards and samples were assayed in triplicate in a 10 µl reaction mixture containing 5 µl Fast SYBR Green Master Mix, 0.2 µl of each primer (10 µM), 3.6 µl nuclease-free water, and 1 µl DNA template. All DNA was standardised by adjusting concentration to 5 ng/µl. The Roche 96 light cycler system automatically generated the standard curve.

Large-scale silage fermentation

Silage pH was determined from the filtrate solution using a pH electrode. PH testing of the large-scale silage extracts was performed similarly to the lab-scale silage, as mentioned previously, using water instead of PBS. Sampling was performed as needed. Detection of lactic acid was carried out according to Kobierecka et al. (2017), with minor modifications. Briefly, silage was added into PBS in a 1:5 ratio and mixed vigorously for 5 mins using a stomacher at the highest setting. The extract was centrifuged (Sorvall, Legend RT) at 2500 × g for 10 mins at 4°C. The CFSs were analysed for D and L-lactate using an enzymatic method where a commercial assay kit (D-/L-lactic acid rapid assay; Megazyme, Wicklow, Ireland) was used according to the manufacturer's instructions.

The acetic acid concentration was determined using the commercial rapid assay kit (Acetic Acid Assay Kit; Megazyme, Ireland), following the protocol as set out by the manufacturer. Briefly, the optical density of silage extracts was measured at 340 nm. Analysis of each sample was performed twice. Water was used as the blank, whereas the manufacturer-supplied standard of acetic acid (0.10 mg/ml) was used as a positive control.

Total protein in the silage was determined using the Pierce bicinchoninic acid (BCA) assay (ThermoFisher Scientific), and absorbance was measured at 562 nm. Protein concentrations were calculated from the standard curve by 20 mg/ml of bovine serum albumin (BSA). Assays for ammonia testing and determining DM percentage were performed similarly to the lab-scale silage.

Assessment of the physical quality of silage

Silage was physically assessed in terms of its colour, texture, and odour throughout the fermentation process. Grass silage was expected to change from green to gold/green as fermentation proceeded. It was also anticipated that the silage would exhibit a sweet fruity smell. Dark brown acidic or rancid silage was undesirable because it would have indicated poorly preserved silage and would not be suitable as feed for ruminants.

Dry Matter Intake (DMI) and feed refusals

One of the indicators of silage quality is its acceptability as feed by the ruminants (Kalio et al., 2012). The prepared silage was offered to 30 late lactation dairy cows (Chapter six). The quantity of silage fed daily was recorded using the Keenan InTouch feeding system (Clonygoose, Borris, Co. Carlow, Ireland). For silage refusal, excess silage was collected and weighed to estimate silage consumed per treatment group twice per week. Individual DMI was calculated using the n-alkane technique (Mayes et al., 1986) as modified by Dillon and Stakelum (1989). All cows were dosed twice daily, before milking, for twelve consecutive days with a paper bullet (Carl Roth GmbH, Karlesruhe, Germany) containing 500 mg of dotriacontane (C32 – alkane). Feed refusal was calculated by subtracting the leftover feed from the total weight of feed offered to each group per week. The average DMI and feed refusals were reported for each group of cows per week (Debarry et al., 2007).

Statistical analysis

Microbial populations of samples were determined as CFU/g of fresh matter and were log-transformed before statistical analysis. Results are reported as the mean of two or three replicate groups. An assumption made for the interpretation of the quantitative PCR is that the efficiency of DNA extraction was similar for all samples. The one-way ANOVA was used to compare differences in three groups in the lab-scale silage, and the Mann–Whitney T-test was used to analyse differences between two groups in the large-scale silage. *P*-values < 0.05 were considered statistically significant.

Results

Trial 1: Lab-scale silage

This experiment aimed to select the most suitable microorganism(s) from the biobank collection of cultures, to be used as inoculants for large-scale silage production. The selection of strains was based on their performance as inhibitors of *Methanobrevibacter boviskoreani* (JH1) (data not shown) and methane inhibition *in vitro* (Chapter four). A comparative analysis was performed in mini silage to determine which strain was superior between *Lactobacillus plantarum* (LP58) and *Lactococcus lactis* (SL242) for silage creation. The mini silages were all made into 200 g each. The sample was discarded after completing the analysis.

Silage microbiota evaluation

MRS was used for the enumeration of the total LAB in silage. LAB was inoculated at 5.39 log CFU/ gram for each inoculant. The total LAB count on the silage, which had been inoculated with *L. lactis* (SL242), ranged from 6.12 log CFU/ g on day 30 and increased to 8.6 log CFU/ g on day 90 (Figure 5.2). Mini silages inoculated with *L. plantarum* (LP58) exhibited an increase of almost 3 logs to 7.9 log CFU/ g on day 30; however, after three months, LAB were at 8.3 log CFU/ g for this treatment. *L. lactis* (SL242) treatment was the more effective than *L. plantarum* (LP58) at silage colonisation (P =0.049).

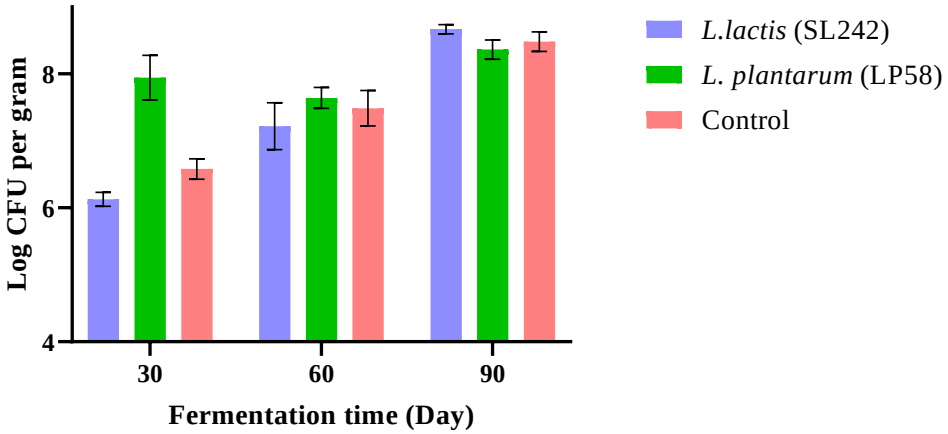

Figure 5.2: Total LAB growth isolated from silage inoculated with *L. lactis* (SL242), *L. plantarum* (LP58), or untreated control. All bacterial counts are expressed as the log of viable bacteria (CFU) per gram of silage.

Table 5.1 shows changes in clostridia CFU counts in the LAB-treated silage. Silage treated with *L. lactis* (SL242) had the lowest clostridia counts until day 60 of silage fermentation. On day 90, the control and treated silage had similar clostridia counts at log 5 CFU/g. Yeast counts (Table 5.2) ranged from log 5.34 to 7.81 ± 0.021 CFU/g in the *L. lactis* (SL242) treated silage and remained around 5 log CFU/g in the *L. plantarum* (LP58) treated silage for the duration of the fermentation which was 90 days. Yeast counts in the untreated control decreased from 6.19 log CFU/g on day 30 to 4.67 log CFU/g on day 90. These yeast counts were higher than the threshold 5 log CFU/g typically associated with silage spoilage and did not differ significantly among treatments.

Table 5.1: Mean CFU clostridia counts in LAB-treated silage.

Treatment	***L. lactis* (SL242)**	***L. plantarum* (LP58)**	**Control**
Day			
30	4.38	4.27	6.07
60	3.60	4.57 ±0 .13	7.70
90	5.81±0.02	5.36 ± 0.015	5.43 ± 0.06

Table 5.2: Mean CFU yeast counts in LAB-treated silage.

Treatment	***L. lactis* (SL242)**	***L. plantarum* (LP58)**	**Control**
Day			
30	5.34 ± 0.19	5.47 ± 2.38	6.19 ± 0.03
60	6.38 ± 1.28	5.89 ± 2.62	7.15 ± 0.72
90	7.81 ± 0.02	5.3 ± 2.74	4.67 ± 3.02

Fermentation characteristics of LAB-treated silage

Silage pH was measured from the silage extract, and on day zero, the pH for grass ensiled with *L. lactis* (SL242) was 5.58, while it was 5.08 in the *L. plantarum* (LP58) treated silage and 5.77 in the control silage (Figure 5.3). On day 60, the pH was similar in the three silage groups, at pH 6.0. After 90 days of silage fermentation, the pH had further increased for *L. lactis* (SL242)

and the control, to 8.15 and 7.5, respectively, while *L. plantarum* (LP58) treated silage had decreased to pH 5.53.

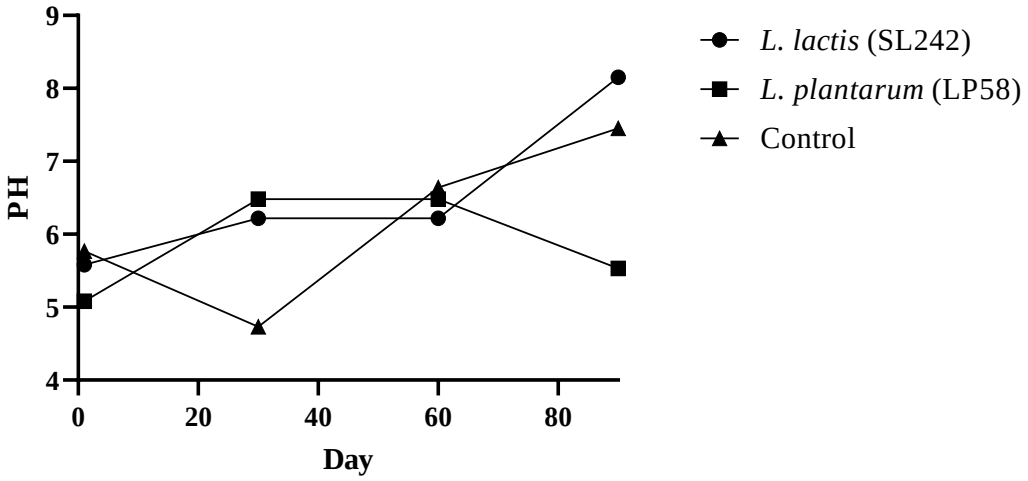

Figure 5.3: Changes in pH during fermentation of grass in mini silages (200 g), inoculated with *L. lactis* (SL242), *L. plantarum* (LP58) or untreated control.

D and L-lactate concentrations in grass silage treated with LAB and untreated control are shown in Table 5.3. The highest lactic acid concentration for the two LAB-treated silages and the control was observed in the first 30 days. The untreated control exhibited the highest L- lactate (58.45 mg/g) and D-lactate (52.42 mg/g) production during this period. No further increase was observed between days 30 and 90 for all treatments. *L. lactis* (SL242) treatment resulted in the lowest levels of lactate production throughout the study, with D-lactate at 35.83 mg/g and L- lactate at 38.41 mg/g on day 30 and subsequently declining to 0.3 mg/g (D-lactate) and 1.4 mg/g (L-lactate) on day 90.

Figure 5.4 shows the calibration curve used to determine ammonia concentration. Table 5.4 presents ammonia concentration in mg/kg of silage extract. The untreated control grass silage consistently had the highest levels of ammonia. After three months of silage fermentation, *L. lactis* (SL242) inoculated silage had ammonia levels of 110.8 mg/kg.

Table 5.3: Mean D and L-lactate (mg/g) concentration during fermentation.

Day	Treatment					
	***L. lactis* (SL242)**		***L. plantarum* (LP58)**		**Control**	
	D- lactate	**L- lactate**	**D- lactate**	**L- lactate**	**D- lactate**	**L- lactate**
30	35.83 ± 0.33	38.41 ± 0.09	51.51 ± 0.26	38.56 ± 0.16	52.42 ± 0.04	58.45 ± 0.58
60	Not determined	ND	1.46 ± 0.03	2.25	7.23 ± 0.15	4.32 ± 0.03
90	0.30 ± 0.11	1.40 ± 0.27	2.49 ± 0.05	3.32 ± 0.07	1.06 ± 0.18	1.03 ± 0.52

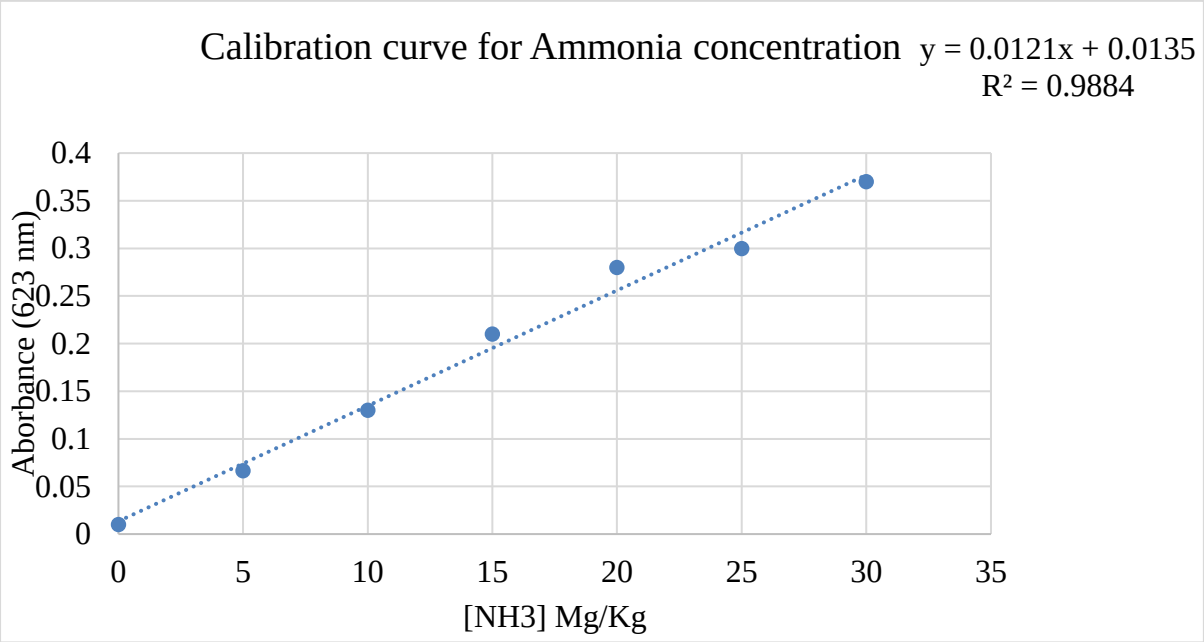

Figure 5.4: Calibration curve used to determine ammonia concentration.

Table 5.4: Ammonia concentration (mg/kg) of silage extract.

Time (Days)	30	60	90
L. lactis (SL242)	105.2	44	110.8
L. plantarum (LP58)	77.4	94	444
Control	110.8	110.8	227.4

Ambient temperature and DM are the main factors affecting the growth rate of LAB in silage (Chen et al., 2022; Li et al., 2019). There was no difference in DM when comparing untreated silage to that inoculated with *L. plantarum* (LP58) or *L. lactis* (SL242). At the end of 90 days, the DM (%) content was 20.24, 19.69, and 20.02% for *L. lactis* (SL242), *L. plantarum* (LP58) and the control, respectively.

Bacteriocin Production from silage sample

Well-diffusion assays were performed using extracts from silage, which had been treated according to Yang et al. (1992) to test if bacteriocins produced by *L. plantarum* (LP58) and *L. lactis* (SL242) were present and active in the silage. When silage extract was tested against *L. delbrueckii* subsp. *bulgaricus* LMG 6901, no inhibition was observed in the samples. Because the inoculants had been confirmed as bacteriocin producers via well-diffusion assays using CFS, it can be postulated that the concentration of bacteriocins in the silage extract were below detectable levels.

Summary of results of the lab-scale silage trial

The fermentation quality of silages was generally good in all treatments. When comparing the effect of the two treatments, *L. lactis* (SL242) and *L. plantarum* (LP58), rapid establishment of anaerobiosis could not be achieved due to the small size of the mini silages. However, valuable information was obtained from this study to inform the creation of large-scale silage. The two inoculants provided differing benefits for the fermentation and aerobic stability of ensiled grass reflected as lower terminal pH in the *L. plantarum* (LP58) inoculated silage while viable colony counts indicated that *L. lactis* flourished better in silage. For such reasons, it was decided that a combination of these inoculants would be used to create large-scale silage.

Trial 2: Large-scale silage production

The enumeration of LAB inoculants in silage

MRS media, suitable for general LAB growth, was used to simultaneously enumerate both *L. lactis* and *L. plantarum* (Figure 5.5). The inoculants quickly dominated the fermentation as they were already at 9 log CFU/g on day 3. This was maintained until day 30. Soon after, LAB counts dropped to 8 log CFU/g by day 35 and declined by less than 1 log until day 90, where counts remained stable. At the end of fermentation, LAB counts were similar in both the treated and untreated silage and were at 8 log CFU/g.

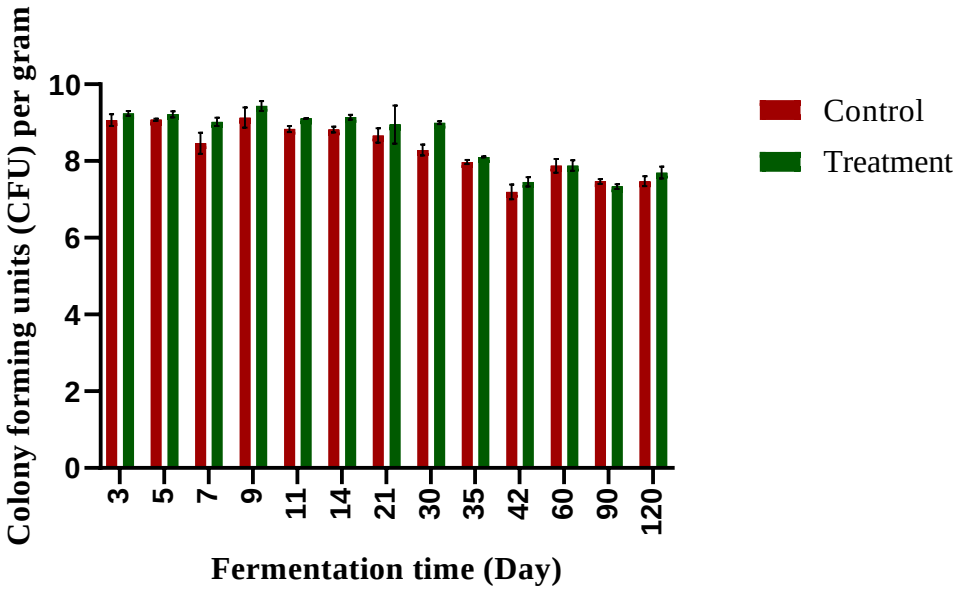

Figure 5.5: Changes in LAB counts during ensilage for 120 days. P= 0.2428.

A random selection of 56 putative LAB isolates from MRS plates was performed throughout the sampling period. There were 26 colonies isolated from the control silage and 30 from the LAB-treated silage. The isolates were then characterised using the identification procedures outlined in Chapter three for genomic identification of bacteria. The identities of a random selection of LAB colonies isolated from the ensiled grass are presented in Figure 5.6, revealing that the control silage contained a small LAB population at time zero, which was before inoculant addition and ensiling. Results from the 16S RNA identification showed *Leuconostoc kimchi* and *Pediococccus pentosaceus* as the predominant organisms in the grass before ensiling. The identity of strains isolated in the first week mainly was *L. plantarum* from the

LAB treated silage. In the first month, the dominating isolates were *L. plantarum*, where 15% of the isolates were from the treatment group, and 7% were from the control group. Other than the inoculants, *P. pentosaceus* were the second-highest group isolated and identified, most of which came from the untreated control silage (11%). Month 2-3 fermentation was again dominated *by L. plantarum* and *Lactobacillus parapalantarum*. Although *P. pentosaceus* was consistently isolated as fermentation progressed, this organism was more prevalent in the control group than in the LAB treatment group. *L. lactis* was not identified in any of the randomly isolated colonies, despite being one of the inoculants.

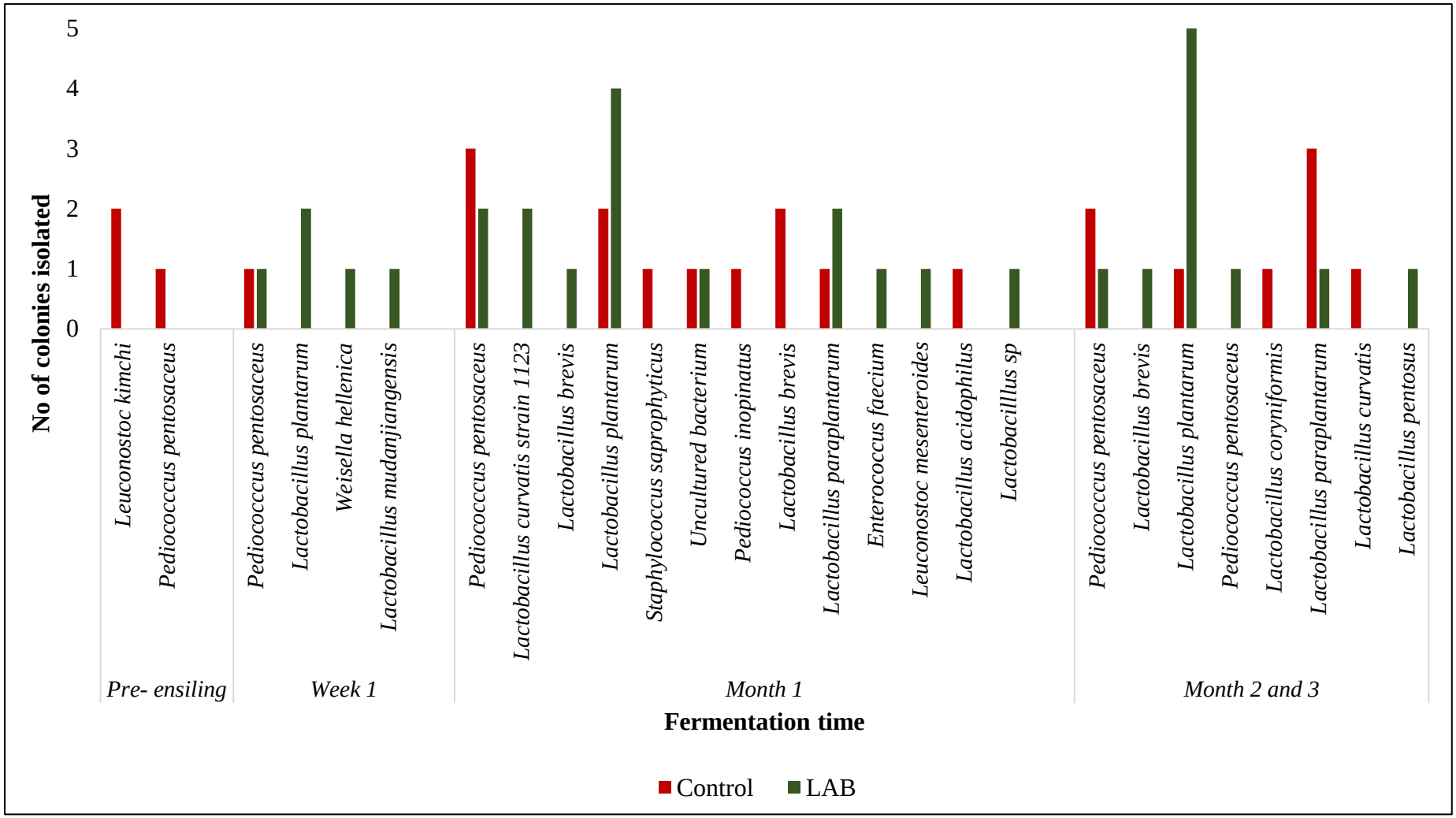

Figure 5.6: The identification via 16S rRNA sequencing of a random selection of LAB colonies isolated during fermentation.

Species-specific primers were used for qPCR to confirm the identity of the colonies in the silage as *L. lactis* (SL242) and *L. plantarum* (LP58). The cDNA template concentrations were 32.5 ng/µl and 30.7 ng/µl for LP58 and SL242, respectively. QPCR was used to determine the DNA copy number per millilitre of silage extract according to formula (1) developed by Li et al. (2009).

DNA (Number of molecules) =

$$\frac{[6.02 \times 10^{23} (\frac{\text{molecules}}{\text{mol}}) \times \text{DNA amount (g)}]}{[\text{DNA length (bp)} \times 660 (\frac{\text{g}}{\text{mol}}/\text{bp}]}$$

The extracted microbial DNA from silage ranged from 0.7 ng/µl to 10.4 ng/µl and was generally of good quality as indicated by the 260/280 ratios, except for day 30 samples (Appendix 5.1). To enumerate *L. lactis* (SL242) microbial counts, LM17 media, which is selective for the cultivation of *Lactococcus* species (Cavanagh et al., 2015), was used to cultivate colonies, and these were counted based on phenotypic similarity to *L. lactis* colonies grown from stock. To verify the continued presence of the inoculants in the silage, *Lactococcus lactis*-specific primers were used for qPCR to quantify its copy numbers. Changes in viable *L. lactis* counts are represented in log CFU/g, and target copy numbers of *L. lactis* during ensilage are illustrated in figure 5.7(A). Plate counts showed a higher prevalence of *L. lactis* presumptive viable colonies in the LAB treated silage compared to the control throughout fermentation. The highest cell counts of 9 log CFU/g were observed from day seven until day 30. The presence of *L. lactis* in terms of the number of colonies and DNA copy number in the treated silage was significantly higher (P-value for SL242= 0.0268) than those from the untreated silage. LBS was used to grow *L. plantarum* (LP58). Changes in *L. plantarum* counts in the silage are represented in log CFU/g and target copy numbers of 16S rRNA. *L. plantarum* gene counts during ensilage are shown in Figure 5.7(B). *L. plantarum* presumptive colonies were at 8 log CFU/g on day three and increased to 10 log CFU/g on day 7. The proliferation of this organism decreased to 9 log CFU/g from day 14 to day 30 and declined even further to 7 log CFU/g on day 35, after which the CFU remained stable for the remainder of the experiment. The log copy numbers for *L. plantarum* remained stable at 5 log DNA copies/g throughout the experiment for both LAB treated and untreated control groups.

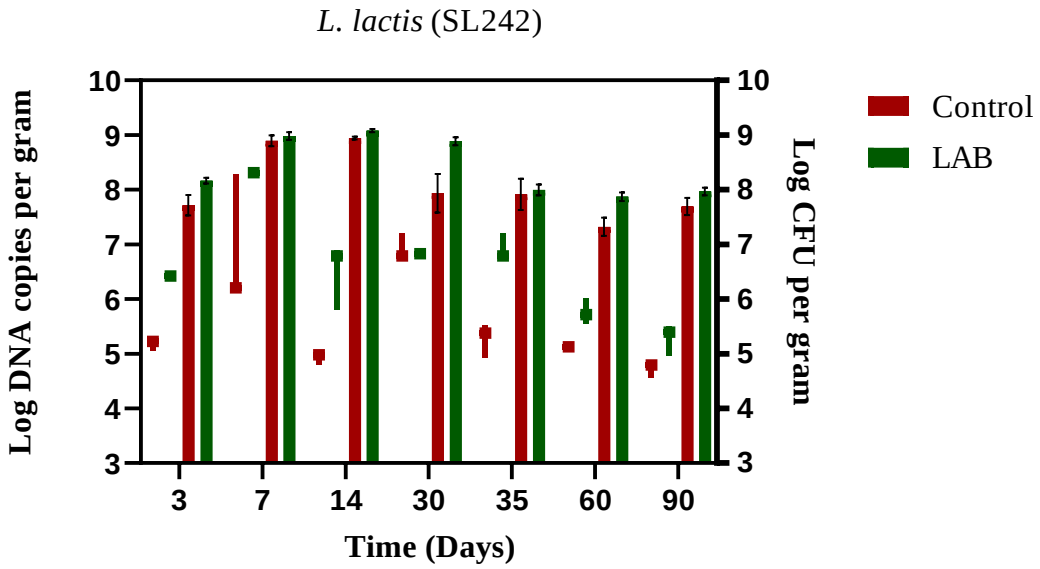

Figure 5.7(A): Changes in *L. lactis* (SL242) counts represented in log CFU/g and target copy numbers of 16S rRNA *L. lactis* gene during ensilage (P= 0.0268)

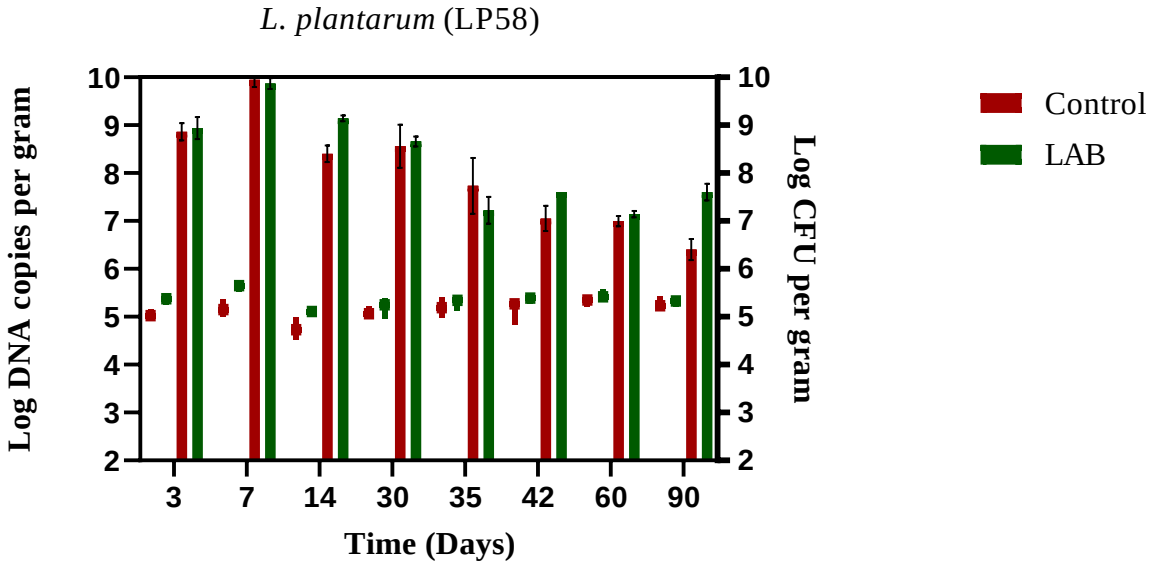

Figure 5.7(B): Changes in *L. plantarum* (LP58) counts represented in log CFU/g and target copy numbers of 16S rRNA *L. plantarum* gene during ensilage (P= 0.35).

Spoilage microbes

When analysing the presence of spoilage microorganisms, the initial clostridia counts were higher in the LAB treatment group on day one of fermentation (Table 5.5). However, as time progressed, the enumerated counts of clostridia decreased from 5.78 log CFU/g to 4.71 log CFU/g on day 60. No significant differences for clostridia CFU were observed between the control and treatment samples. Yeast levels within the silage were 6.42 log CFU/g for the treatment silage and 6.04 log CFU/g for the control silage at the end of 90 days which was not statistically significant.

Table 5.5: Mean log CFU/g of clostridia in silage.

Treatment	**Control**	**LAB**
Time (Days)		
1	5.60 ± 0.24	5.78 ± 0.33
30	5.0	0
60	5.06 ± 0.026	4.71 ± 0.33
90	Not Determined	Not Determined

Silage fermentation

The most rapid pH decline occurred in the first nine days in the treated and control silage, as shown in Figure 5.8. Silage pH stabilised in both groups around day 20, with only minor fluctuations occurring until the end of the experiment. Although silages inoculated with or without LAB met the standard for good fermentation quality silage, as shown by a decline in pH, the LAB treatment improved silage quality by lowering pH to a greater extent, from 6.3 to 3.8. The difference in pH between the control and treatment was not statistically significant.

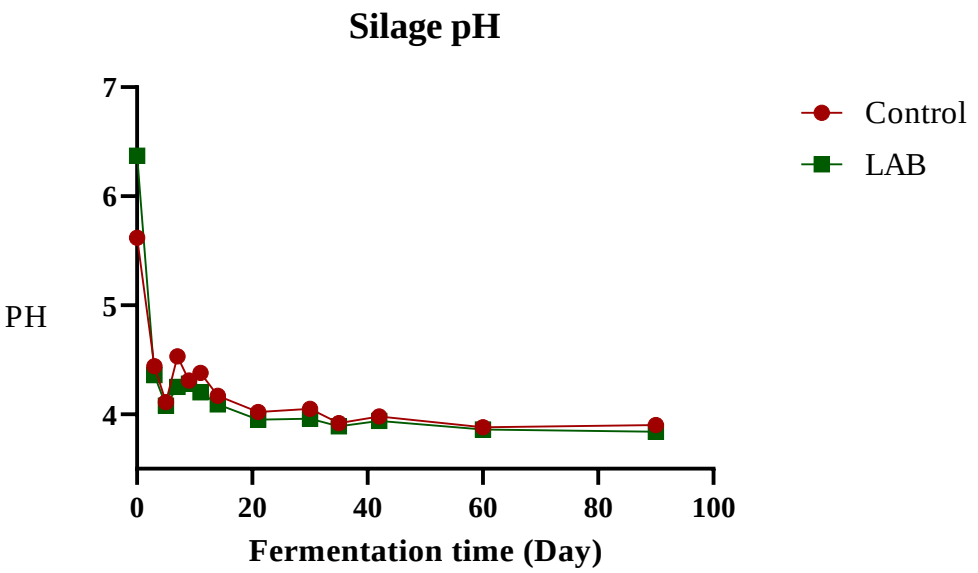

Figure 5.8: Changes in pH of the grass inoculated with a combination of LAB strains (*L. lactis* (SL242) and *L. plantarum* (LP58)) and untreated control (P=0.41).

Figure 5.9 shows D and L-lactate proportions in fermented grass silage over the fermentation time of 90 days. The pre-ensiled grass on day 0 contained 0.98 mg/ g of L-lactate. On day one, only L-lactate was observed in the treatment group in very low concentrations of 0.52 mg/g. L-lactate increased in both groups from day seven, contributing to a higher proportion of the lactate produced. The addition of silage inoculants, *L. lactis* and *L. plantarum* significantly impacted lactate production during silage fermentation (P=0.0034). L-lactate was the predominant isomer in the treatment group throughout the fermentation.

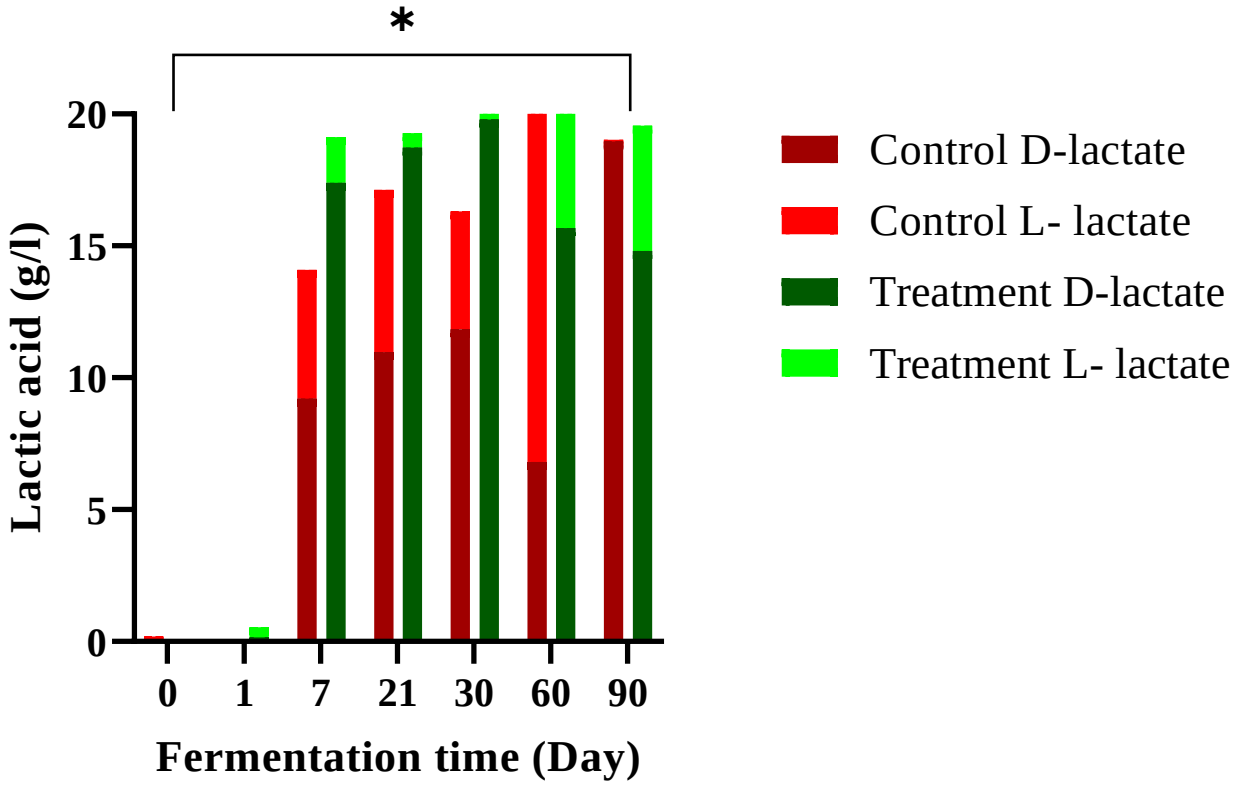

Figure 5.9: Proportions of D and L- lactate at different times during fermentation of grass co-inoculated with *L. plantarum* (LP58) and *L. lactis* (SL242) and in the untreated control silage (P= 0.0034).

Low (0.069 mg/g) levels of acetic acid were observed at the beginning of the trial (Figure 5.10). Acetic acid levels increased throughout the fermentation, with the LAB-treated silage showing higher levels of acetic acid, which was trending toward being statistically significant. After one week of fermentation, the control silage had 1.57 mg/g of acetic acid, whereas the LAB-treated silage had 2.5 mg/g of acetic acid. The acetic acid levels continued to increase in both groups, with the highest levels of acetic acid observed on day 90 of the fermentation trial, which was 4.2 mg/g for the treatment and 2.9 mg/g for the control.

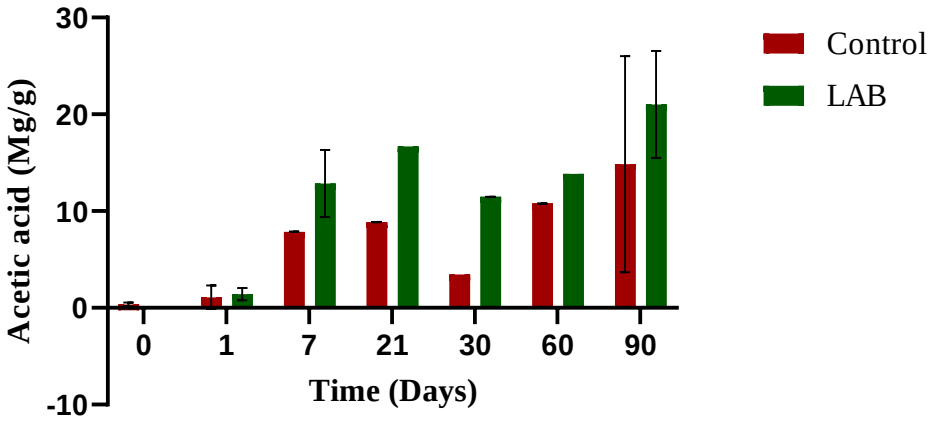

Figure 5.10: Acetic acid levels in grass silage during 90 days of fermentation (P= 0.073).

The total protein levels are shown in Figure 5.11. A decline in protein levels was seen on day one. Soon after, protein levels remained around 2200 µg/ml of silage extract throughout the silage fermentation process. Levels of crude protein were very similar in quantities in both the LAB-treated and untreated control groups (P=0.94).

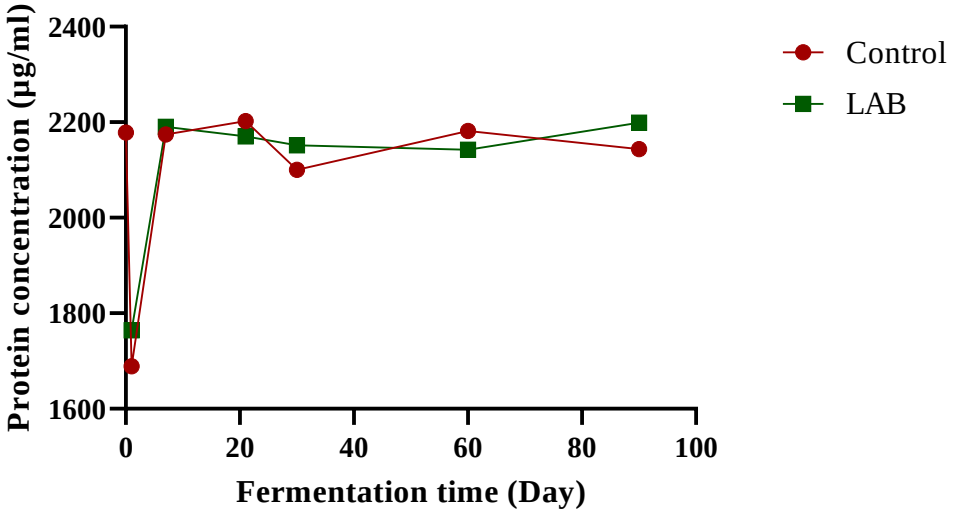

Figure 5.11: The total protein levels in silage during fermentation.

Figure 5.12 represents the ammonia levels observed during the 90 days of silage fermentation. The control and LAB-treated silage had an average of 18 and 12.1 mg/kg of ammonia, respectively, throughout fermentation. LAB inoculation did not negatively impact silage fermentation as the ammonia levels did not significantly differ between the two groups.

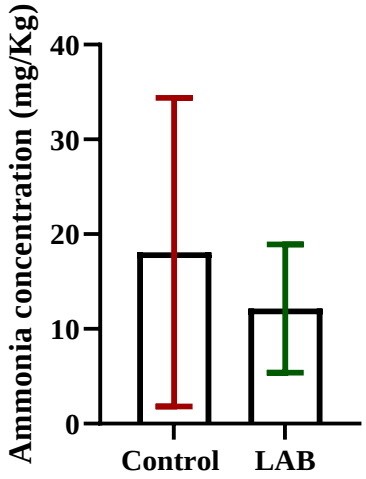

Figure 5.12: The effect of different LAB inoculation on ammonia concentration in silage (P=0.32).

The grass was wilted to ~25% DM before ensiling into bags. As the silage fermentation progressed, DM (%) was monitored throughout the experiment. For the different sampling points throughout the three months of silage fermentation, the DM percentage of the untreated silage ranged from 27- 25%, while the LAB treated silage had DM ranging from 28 to 23%.

Organoleptic properties such as the colour and odour of silage were monitored throughout the fermentation period. The colour of grass in both the control and treated silage progressed from green to olive-green to greenish-yellow, although the progression from green to olive-green and golden occurred more rapidly in the LAB-treated silage. It took nine days for the odour to change from a fresh grass to a mild sweet odour. The LAB-treated grass started to develop the characteristic silage odour on day 21. For the control, the fermented silage odour occurred much later, around day 30.

Dry matter intake (DMI) and feed refusals

Poor silage quality may lead to low feed intake and can be inversely correlated with dry matter intake. The average DMI per group was calculated to determine if there was a preference for the LAB-treated silage over the control silage. DMI and feed refusals are presented in Figure 5.13. It was observed that the dairy cattle consumed approximately 15-18 kg of DM per day on average (Chapter six). DMI was marginally higher for the LAB-treated silage. The total DMI was 13.89% and 14.63% in week one for the control and treatment cattle, respectively. By week seven, total DMI had increased to 14.2% for the control group and 15.2% for the LAB treatment group. Feed refusal percentage considers the amount of silage offered per group of animals. The remaining silage is subtracted from the total feed that has been offered and subsequently converted to a percentage. Feed refusals were only higher during week three for the treatment group (4.8%), and the control had 2.78% of feed refusals during this week. LAB-treated silage was preferred over control silage for the remaining six weeks, although the difference was not statistically significant.

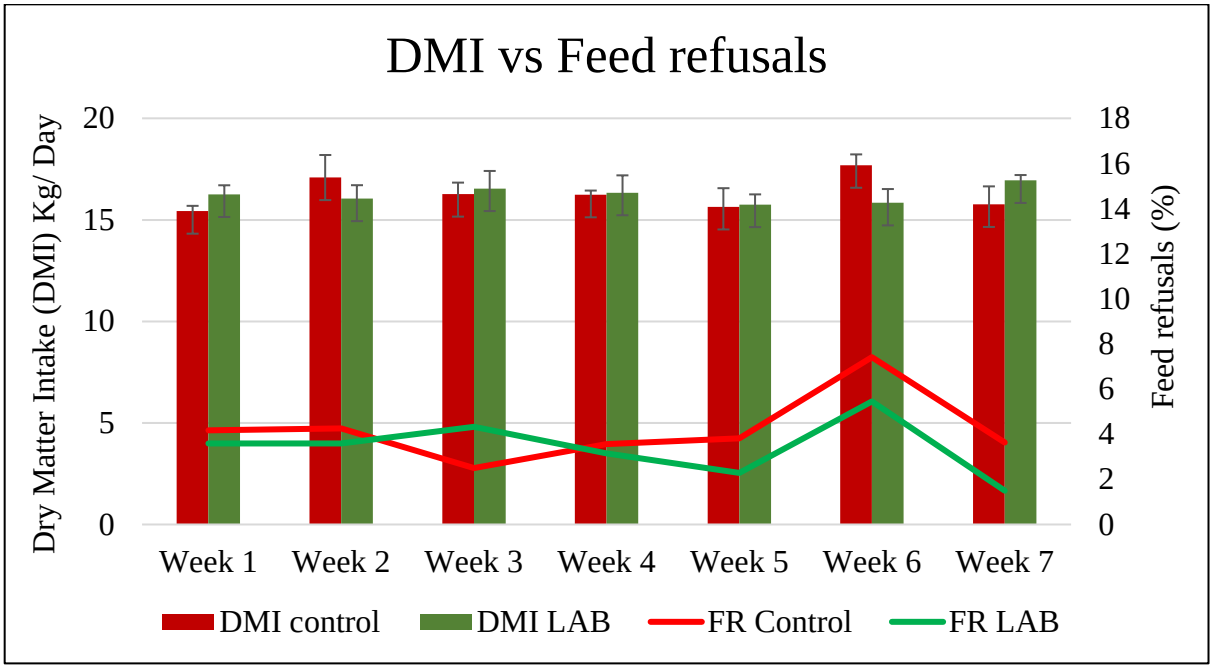

Figure 5.13: Average DMI of silage per cow per week over seven weeks (P = 0.87) and the average percentage of feed refusals per group per week (P =0.9).

Discussion

This study aimed to identify the most suitable large-scale silage inoculant between two widely used LAB strains, *L. lactis* (SL242) and *L. plantarum* (LP58). For this purpose, lab-scale mini silages of 200 g each were created using these inoculants separately. Results from the preliminary study were used to inform the design of the large-scale silage trial (bales of 800 kg each), which would be offered to bovine ruminants in Teagasc Moorepark, Ireland (Chapter six). Silage fermentation and nutritional properties were monitored for three months in both instances.

LAB are responsible for silage fermentation and influence silage quality. Growth of LAB is required for the stabilisation and preservation of silage. The most suitable silage inoculants are based on the GRAS status, their tolerance to various temperatures and low pH, their ability to utilise the carbohydrates found in silage and their efficiency in reducing silage spoilage microbes through the production of organic acids or antimicrobial agents such as bacteriocins (Adesoki et al., 2010). Bacterial inoculants encounter a variety of physical conditions in silage. A good silage inoculant needs the capacity to outcompete other microbes by growing fast and colonising the silage (Agarussi et al., 2019). In the smaller, lab-scale trial, *L. lactis* (SL242) treated silage had the highest CFU counts at the end of three months (Figure 5.2). *L. lactis* is generally associated with food production, but the resilience of this organism is profound because it has evolved an acid tolerance response (ATR) (Alemayehu et al., 2000). This characteristic makes it a good silage inoculant candidate. It is a mesophile with an optimum growth temperature of 30°C. During the storage phase, the temperature of well-fermented silage ranges between 21 and 32°C. *L lactis* is well able to survive in these conditions. *L. lactis* is also a well-documented bacteriocin producer, enabling it to inhibit competitive microbes within the silage (Ryan et al., 1996). *L. plantarum* is a well-known silage inoculant, popular as a facultative homofermentative LAB that produces various antimicrobial agents and sufficient lactic acid production, promoting a decrease in pH (Muthusamy et al., 2019, Xie et al., 2020, Yang et al., 2020). Furthermore, this species is commonly isolated from silage (De Giani et al., 2019; Guo et al., 2013). As shown in Figure 5.2, the difference in the LAB counts of treated and untreated lab-scale silage was not statistically significant. This is not surprising because the total counts include epiphytic LAB that may already be present in the silage, which may not be phenotypically distinguishable from the inoculants. Epiphytic LAB constitutes between

0.1-1% of natural silage microflora. Furthermore, *L. plantarum* is widespread in anaerobic plant matter (Lorenzo et al., 2018).

Combining two or more LAB species as silage inoculants can be more advantageous than using one species due to the positive interaction that may occur, and the differences in inoculant growth patterns may produce better quality silage (Jatkauskas et al., 2013). In the large-scale silage, where *L. plantarum* (LP58) and *L. lactis* (SL242) were used as co-inoculants (Figure 5.5), the LAB viable counts were similar at 8 log CFU/g at the end of fermentation for both the LAB treated and untreated control silage. A high number of indigenous LAB means that these could function co-operatively with the inoculants to ferment silage. Low LAB levels may allow more significant loss of nutrients or could be outcompeted by other microbes to dominate fermentation (Ellis et al., 2016). The results of this study indicate that the tested LAB can survive in silage. This was very important in this study since the inoculants needed to be alive to impact the ruminants fed the silage (Chapter six). The enumeration of live LAB in the silage is a valuable technique that can be used to interpret some of the chemical and compositional changes in the silage. The identification of randomly picked colonies from MRS agar showed that *L. plantarum* was consistent in both the control and treated silage throughout the fermentation period (Figure 5.6). *L. paraplantarum* was also commonly isolated from the treated silage. *L. plantarum* and *L. paraplantarum* are genotypically closely related and exhibit similar phenotypes. RecA gene-derived primers show amino acid substitutions between these two microorganisms (Saito et al., 2011; Torriani et al., 2001).

Quantitative PCR (qPCR) can be used to enumerate specific bacteria of interest. The growth and survival of the inoculants can then be accurately differentiated from their epiphytic counterparts, resulting in a better understanding of how the inoculated bacteria proliferate and favourably alter the ensiling process (McAllister et al., 2018). Compared to culture-dependent techniques, the benefits of qPCR include reproducibility, rapidness, sensitivity, and quantitative ability. The drawback of using qPCR as most DNA-based techniques is that it requires specific primers appropriate for the organism of interest. Furthermore, this method quantifies both viable and non-viable cells (Singh et al., 2014). The qPCR conducted on samples collected in different time intervals revealed that the control silage contained a population of *L. lactis* (SL242), which steadily increased in copy number to 7 log DNA copies per gram until day 30, then declined again to 5 log copies/g and remained at these levels until day 90 (Figure 5.7(B)). *L. plantarum* (LP58) copy numbers in the treated silage remained stable

as fermentation progressed. However, *L. plantarum* was also evident in the control silage. *L. plantarum* is an indigenous LAB in silage (Ennahar et al., 2002). Our results are similar to a study by Stevenson and colleagues (2006), who conducted real-time PCR (RT-PCR) reactions on corn stover and alfalfa silage and found that the control silage contained a small population of LAB that had traces of *L. plantarum* amongst other LAB, at time zero of silage creation. For greater specificity to improve the qPCR, primers that are strain-specific for *L. plantarum* LP58 and *L. lactis* SL242 could be used in future.

Clostridia and yeast growth are expected to be inhibited when silage is inoculated with LAB. Yeasts and moulds are undesirable because they hydrolyse and metabolise nutrients. Furthermore, some moulds produce mycotoxins, which harm animals and humans (Mauro et al., 2013). The high presence of yeast in the mini silages (Table 5.2) could be attributed more to the ensiling technique rather than the performance of the bacteria used as silage inoculants. A household sealer was used to close the bags of silage, which could have allowed air to seep into the silage causing spoilage. Going forward, this problem could be avoided in large-scale silage since proper sealing mechanisms were used. *L. plantarum* has been found to reduce yeast and fungal populations in silage (Agarussi et al., 2019, Muthusamy et al., 2019). Poorly preserved silage has a proliferation of clostridia species. No significant differences were observed in clostridia prevalence between the control and treatment samples. Clostridia endospores are expected to range from 100- 1000 CFU per gram of crop before ensiling (Muck, 2010). The prevalence of unwanted microorganisms in silage results in inefficient fermentation and poses significant disease risks if consumed by ruminants.

Silage pH is the primary indicator of silage quality and fermentation efficiency (Kung et al., 2018). Adding silage inoculants ensures a rapid decrease in pH to facilitate fermentation. The desired pH in grass silage is 3.8- 4.2, indicating well-preserved silage (Denek et al., 2011). The lab-scale silage (Figure 5.3) inoculated with the *L. lactis* (SL242) showed that pH increased with time, which is uncharacteristic of this LAB species as an inoculant. In the lab-scale silage, high pH correlated with high yeast counts. Several factors can be responsible for silages presenting higher than normal pH. The high silage pH may be caused by aerobic contamination, which would have been a likely occurrence in small 200 g silage, as these would not have been compact enough to prevent air seepage (Kung et al., 2018). This aerobic deterioration process is typically initiated by yeasts, utilising the silage's sugars and lactic acid. When this occurs, the pH increases and the less acid-tolerant microbes such as moulds and yeast dominate the

silage (Pahlow et al., 2015). The higher pH observed between days 60 and 80 of ensiling was above the optimum for LAB and may have encouraged the growth of microbes not customarily associated with silage making. Such an occurrence could further promote the proliferation of coliform bacteria associated with deaminative activity (Lorenzo et al., 2018). The metabolism of lactic to butyric acid by clostridia may also explain why grass silages exhibited higher than normal pH. In the large-scale silage where a combination of LAB inoculants was used (Figure 5.8), pH rapidly declined in the first seven days and remained relatively stable and low for the rest of the fermentation trial of 90 days. A similar effect of a rapid drop in pH was also observed by Nkosi et al. (2009), where maise crop was ensiled with Bonsilage Mais Flussig (BMF), a commercial bacterial silage inoculant consisting of *L. plantarum*, *P. pentosaceus*, and *L. buchneri* at 2.5×10^{11} CFU per gram of BMF powder. The organic acid primarily responsible for the decrease in silage pH is lactic acid, which is more potent than the other acids found in silage (Santos et al., 2013). This is important because a lower pH is inhibitory toward spoilage microbes and stabilises the silage fermentation (Kung et al., 2018). Saarisalo et al. (2007) described a strain of *L. plantarum* capable of reducing the pH of grass silage with low proteolytic activity, which makes it effective in decreasing deamination in silage.

One of the main objectives of applying an inoculant to grass to be ensiled is to increase the number of LAB in the crop to guarantee high lactic acid production, facilitating efficient silage fermentation. In the lab-scale silage, lower pH was observed in *L. plantarum* treated silage. Applying *L. plantarum* to the grass silages resulted in a lower silage pH because homolactic acid bacteria often produce more lactic acid than other LAB. High lactic acid production decreases pH, limiting acetic acid production, resulting in a more homolactic silage fermentation. There was a temporary increase in lactic acid until day 30, observed in the lab-scale silage, although this was accompanied by an increase in pH in the same sample. Insufficient lactic acid production during fermentation may fail to reduce silage pH and inhibit clostridia resulting in poor quality silage. This is evident in Table 5.1, where clostridia CFUs in the treatment and control groups were high.

In the first week of silage fermentation, only L-lactate was detected in both groups of our large-scale silage. Similarly, Zhang et al. (2000) observed that L-Lactic acid was produced predominantly in the first three days of ensiling guinea-grass silage with *L. plantarum* and glucose. The concentration of D and L-lactate increased in both groups of silage until day 60,

then slightly declined on day 90 (Figure 5.9). However, L-lactate was the dominant isomer continuously throughout the fermentation trial. This finding was consistent with that of Cai and Sumai (1993), who found that L-lactate was the predominant isomer in LAB (*Lactobacillus casei* and *L. lactis*) inoculated silages. In contrast, Schaadt and Johnson (1968) reported that lactate production in silage largely involves the D-isomer.

Acetic acid is the second most abundant metabolite in silage after lactic acid and is produced mainly by heterofermentative LAB (Kung et al., 2018). It imparts the characteristic slight vinegar smell on silage, and high concentrations of this acid may be associated with low dry matter intake (Ranjit et al., 2002). Conversely, it has fungicidal effects and can mitigate silage deterioration, thus improving stability. A gradual increase in acetic acid (0.3-21 mg/g) was observed in the large-scale LAB-treated silage (Figure 5.10). The desirable levels of acetic acid are 10-30 mg/g of silage (Gerlach et al., 2021). Because of this, it can be postulated that this silage demonstrated improved stability during the feed-out stage since acetic acid increases silage stability under aerobic conditions (Danner et al., 2003). When the acetic acid present in silage is consumed by the ruminant, it is absorbed from the rumen and can be utilised for energy or be integrated into milk or body fat (Kung et al., 2018).

A decline in the total protein content during the fermentation process indicates plant and microbial proteolysis in the ensiled material, which is an undesirable process. Although a high ammonia concentration may indicate the aerobic deterioration of silages, proteolysis commonly occurs even in silage harvested and stored under adequate management (Grant and Ferraretto, 2018). There is an intricate relationship between total protein, ammonia concentration, and silage DMI. An inverse relationship exists between ammonia concentration and DMI. In the lab-scale silage experiment (Table 5.4), the control had the highest ammonia levels throughout, while less than 200 mg of ammonia per kg was observed in silage inoculated with *L. lactis* or *L. plantarum* until the second month of fermentation. LAB inoculants are used in ensiling to enhance the preservation efficiency of silage and increase the desirable properties of silage, such as reduced ammonia nitrogen content. In the large-scale silage, ammonia concentrations were extremely low, averaging below 50 mg/kg throughout the trial for both groups (Figure 5.12), while crude protein levels remained constant (Figure 5.11). *Lactococcus lactis* because of its nisin-producing capabilities, has been found to reduce ammonia production indirectly by inhibiting clostridia which degrade amino acids and produce ammonia (Jatkauskas et al., 2013).

Organoleptic observations refer to the use of senses to evaluate silage quality (Kung et al., 2018). These factors contribute to the acceptability of the silage feed by the ruminant. Organoleptic observations note the colour, odour and texture of silage which could all be influenced by the inoculants used (Baroon and Razzaque, 2013). These properties were monitored throughout the large-scale silage fermentation experiment. In this study, the colour development from green to the characteristic golden straw coloured occurred simultaneously for both the treatment and control. This colour development took one week from ensiling. Odour changed from freshly cut grass to bittersweet in nine days and subsequently to the characteristic fermented silage odour around day 21 for the treated silage. This change is due to lactic and acetic acid production and accumulation. For the untreated control, the fermented silage odour occurred much later, around day 30. None of the silage exhibited any signs of spoiled silage, such as a slimy texture or mould. The LAB-treated silage was more acceptable to ruminants, as demonstrated by higher dry matter intake and lower feed refusals (Figure 5.13).

Testing for bacteriocin activity in the lab-scale silage, where silage extracts were used to inhibit *L. delbrueckii* subsp. *bulgaricus* LMG 6901 showed a lack of antimicrobial activity from the *L. plantarum* (LP58) and *L. lactis* (SL242) treated silage. Antibacterial activity may be reduced or lost after long periods of ensiling. Ogunade et al. (2016) discovered a loss of activity of silage extracts (inoculated with *L. plantarum* and *L. buchneri*) at day 100. This inactivity was attributed to the loss of metabolic activity of the LAB. The lack of antimicrobial activity could also mean that the concentration of bacteriocins was below detectable levels.

Conclusion

Although the quality of the lab-scale silage was less than optimal, performing this preliminary experiment enabled us to better understand the characteristics of the inoculants *L. plantarum* and *L. lactis* and refine our silage-making technique. The large-scale silage treated with the LAB co-inoculants had significantly higher CFU counts and copy numbers *of L. lactis*. The co-inoculants were able to survive in the silage for three months and a various epiphytic LAB were identified. LAB improved silage quality indicated by higher lactate and acetate levels in the treated silage. According to Weinberg and Muck (1996), there are certain instances where LAB silage inoculants may be classified as not genuinely successful in improving silage quality if they did not completely dominate fermentation. In the case of this experiment, the inoculants were prolific during the fermentation in the treated silages. However, the control silages exhibited characteristics of similar good quality overall. Therefore, minimal beneficial characteristics of the silage can be attributed purely to LAB inoculation. The practical implication of this study is that it was demonstrated that bacteriocin-producing LAB can be used as silage co-inoculants to produce good quality silage which is acceptable for consumption by ruminants.

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Appendix

Appendix 5.1: Purity and concentration of DNA extracted from silage and used for qPCR

Silage samples	**Elapsed silage fermentation time (Days)**	**DNA (ng/ul)**	**260/280 ratio**	**260/230 ratio**
Fresh pre-ensiled grass	0	1.8	1.3	0.45
A1	1	1.6	2.81	0.25
B1	1	0.7	0.85	0.22
A2	3	0.9	0.81	0.12
B2	3	3	1.81	0.37
A7	14	1.8	1.77	0.65
B7	14	1.5	1.86	0.92
A9	30	1.9	6.64	0.38
B9	30	1.5	5.86	0.71
A10	35	0.7	0.77	0.23
B10	35	1	0.97	0.38
A11	42	1.5	1.2	0.55
B11	42	2.8	1.01	0.5
A12	60	2	0.93	0.57
B12	60	2.1	1.12	0.6
T0A	90	2.7	1.97	0.12
T0B	90	10.4	1.49	0.7

A= Control, B=LAB-treated silage.

Chapter 6.

Investigating the potential of LAB to modify rumen function and inhibit enteric methane production.

Abstract

LAB silage co-inoculants *Lactococcus lactis* (SL242) and *Lactobacillus plantarum* (LP58) were investigated for their potential to reduce enteric methane and their impact on rumen fermentation was tested. These LAB strains were confirmed to have methane reduction effects *in vitro* through GC and spectrophotometry methods (Chapter four). Thirty dairy cows (24 non-cannulated and six rumen-cannulated) housed indoors were on a silage feeding trial for seven weeks. Treatment and control silage was fed *ad libitum* (Chapter five). The total bacterial CFU consumption of each inoculant strain, based on the DMI for each cow, was determined to be ~10^{11} CFU per day. The Greenfeed system was used to measure methane and hydrogen emissions for seven weeks from the 24 non-cannulated cows (n=12). Rumen fluid from six cannulated cows (n=3) was collected for analysis on weeks 1, 4 and 7. Ruminal pH was measured immediately upon collection. VFA concentrations were measured using GC, and lactic acid was determined using a commercial lactate kit. Genomic DNA was extracted from ruminal fluid, and methanogen copy numbers were determined using quantitative PCR (qPCR). Changes in the rumen microbiome, as a result of the intervention, were assessed using 16S rRNA gene amplicon sequencing. Non-cannulated cows fed LAB-treated silage produced significantly lower methane levels between weeks 4-7 ($P < 0.001$) than those fed untreated control silage. LAB had no effect on the hydrogen expelled. Rumen pH ranged between 5.96-6.6 for the control group and 5.96-6.8 for the treatment group. Different diets did not influence the total VFAs or lactic acid concentration; however, an increase in acetate and butyrate was observed in LAB-treated ruminants ($P<0.05$). The LAB-treated silage diet did not affect the overall methanogen population or *Methanobrevibacter* sp, the primary methane producers in the rumen. The Shannon diversity index showed a significant difference in the alpha diversity of rumen microbes in the control and treatment groups. The propionate-producing *Prevotella* was most abundant at the genus level in both groups and increased by 3% in the LAB treatment group. LAB demonstrated the ability to inhibit the intricate methanogenesis process and modify rumen function by increasing acetate and butyrate production while significantly decreasing Proteobacteria and Cyanobacteria. This study shows that ingested LAB can be used to modify rumen function and reduce methane emissions from ruminants.

Introduction

The largest source of global anthropogenic methane is livestock production, with enteric fermentation in ruminants reportedly responsible for 18% of the methane produced (Chang et al., 2019). Ruminants are essential to humans, especially domesticated livestock, as they convert inedible biomass such as plant cellulose into high-quality protein (milk and meat) and fibre products such as leather (Doyle et al., 2019). However, as a result of enteric fermentation, depending on diet, dairy cows can produce up to 260 g of methane per day (Lassey et al., 1997). Enteric methane production poses a dietary inefficiency, as approximately 6-12% of an animal's gross energy intake is lost as methane (Eckard et al., 2010). Methane is also a potent greenhouse gas. Livestock farming productivity is projected to increase in the next ten years, meaning that without mitigation, methane levels are set to increase to 60% by 2030 (Grainger and Beauchemin, 2011).

Rumen microbiota have co-evolved with ruminants, and rumen microbes are essential for maintaining digestive and metabolic functions in the host (Morgavi et al., 2010). Approximately 65% of digestion occurs in the rumen via the concerted effort of many anaerobes. Anaerobic bacteria digest complex polysaccharides, proteins, and lipids from feed, to produce VFAs, namely propionate, acetate, butyrate and succinate, which are used by the host for maintenance, growth, and performance (Chellapandi et al., 2018). As a result, H_2, CO_2 and formic acid are also produced in varying amounts as products of fermentation (Tapio et al., 2017). To maintain low hydrogen levels within the rumen, hydrogen-utilising methanogens use the H_2 and carbon substrates, mainly CO_2, acetate, or methanol, to generate methane. Alleviating rumen pressure increases the efficiency of the rumen system (Duin et al., 2016). Methanogenesis primarily occurs in the rumen as the terminal step during the anaerobic conversion of organic matter to methane. Methanogens, the organisms solely responsible for methane production, are abundant in the rumen and lower segments of the intestines of ruminants (Patra et al., 2017). They are estimated to range between 10^7 to 10^9 CFU per gram of rumen contents in concentrate-fed ruminants and 10^9 to 10^{10} CFU per gram of rumen in pasture-fed ruminants (Attwood et al., 2011). Culture-independent techniques used to study microbial genes from ruminants have revealed that *Methanobrevibacter* species dominate the rumen, regardless of ruminant diet or geographical location. Although there is a core microbial community (a set of microbial taxa or genes that are shared by all or most ruminants) (Risely, 2020) in the rumen, studies have reported that the composition of the rumen microbiome can

be influenced by factors such as dietary source, feeding system and age (Guo et al., 2020). Therefore, manipulating the ruminal microbial community is likely the key to lowering enteric methane production (Ellis et al., 2016). Theoretically, methane mitigation can be achieved using one or a combination of the following strategies: i. Direct inhibition of rumen methanogens by adding certain feed additives such as probiotics, which are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001); or DFMs which are feed additives containing live naturally existing microbes that can benefit animal health and production performance (Ban and Guan, 2021; Leng, 2014). ii. Redirecting hydrogen in the rumen to pathways that could act as alternative hydrogen sinks, such as propionate production, nitrate and nitrite reduction, and reductive acetogenesis (Kobayashi, 2010). iii. Dietary manipulation targeting microbes in the rumen, which provide substrates for methanogenesis (Morgavi et al., 2010). iv. Inhibiting the methanogenesis pathway using enzyme blockers or structural analogues for methane-specific enzymes (Huws et al., 2018).

LAB occur naturally in the GIT of animals such as ruminants and have a long history of use as silage inoculants. Although generally more prevalent in young ruminants, these organisms have been isolated from the adult rumen (Doyle et al., 2019). LAB have been used as additives to improve fermentation quality and nutrient digestibility (Guo et al., 2020). They have also been investigated for their potential to reduce enteric methane since LAB produce various end products such as bacteriocins and may activate microbial changes within the rumen, thereby affecting enteric methane production (Ellis et al., 2016). LAB provide a safe, practical, and natural way to influence the rumen microbial community for methane mitigation. The use of LAB as rumen modifiers has the potential to provide a sustainable, economically viable, and environmentally conscious food production system. In order to implement strategies that will result in the consistent long-term reduction of enteric methane, it is vital to understand the factors that influence microbial community composition. Currently, the rumen microbial community profile that confers low methane production is not well understood (Lyons et al., 2018).

The aim of this study was to assess the potential of LAB strains *L. plantarum* (LP58) and *L. lactis* (SL242) delivered as silage co-inoculants, created in chapter 5, to inhibit enteric methane production in dairy cows. We also sought to determine the metabolic and microbial changes in the rumen of LAB-treated cattle compared to those fed control silage.

Materials and methods

Ethical approval

The handling and care of the animals followed an experimental protocol approved by the Teagasc Animal Welfare Body (AWB) and Animal Ethics Committee, project authorization license AE/I9132/I052. The Health Products Regulatory Authority (HPRA) provided project authorisation for the animal trial. HPRA is the competent authority in Ireland responsible for the implementation of European Union legislation (Directive 2010/63/EU) for the protection of animals used for scientific research.

Experimental design and sample collection

The animal trial was undertaken at Moorepark Research Farm, Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork. The silage was made in July 2020 (Chapter five), and the animal feeding experiment was conducted from late October to mid-December 2020. Extraneous variables were controlled for by housing the dairy cows indoors in a purpose-built cubicle shed for the duration of the experiment. There were two treatments, grass silage and grass silage plus LAB inoculants. The LAB inoculants used were *Lactococcus lactis* (SL242) and *Lactobacillus plantarum* (LP58) obtained from SACCO System, Italy and applied onto the grass at a concentration of 8.64×10^6 CFU/g and 1.76×10^7 CFU/g respectively as described in Chapter five.

Thirty late lactation dairy cows were blocked and assigned to 1 of two treatments (n=15). Of these, 24 were for methane, and hydrogen analysis (n=12 per group) and six were for studying rumen modification (n=3 per group). Existing rumen cannulated cows at Moorepark were used to study rumen modifications. Cow blocking was based on breed, parity, calving date, pre-experimental milk yield and milk solids yield, body weight, body conditioning score (BCS) and methane emissions during the two weeks before the commencement of the trial. Cows were housed separately in cubicles according to their treatment group. Adequate feeding space was permitted per cow, and water was freely accessible to the ruminants *ad libitum*. Cows consumed approximately 13 kg DM of grass silage (+/-inoculant depending on treatment) and 2 kg of concentrate per day for the duration of the study. Silage was offered once daily (am) using a Keenan diet feeder and silage fed was recorded using the Keenan InTouch feeding system. Based on DMI, the total bacterial CFU consumption of LAB strains *L. plantarum* (LP58), and *L. lactis* (SL242) was determined to be ~10^{11} CFU per day. Rumen fluid samples were taken in the morning and the evenings of two consecutive days on weeks one, four, and seven.

Methane and hydrogen emissions

Methane (CH_4) and hydrogen (H_2) emission measurements were taken across seven weeks using a Greenfeed emissions monitoring system (Greenfeed; C-Lock Inc., Rapid City, USA). The Greenfeed gas flux quantification system measures methane, carbon dioxide, and hydrogen production from animals. For this study, only methane and hydrogen values were reported. The Greenfeed was made available to cows throughout the day, and access was voluntary (Figure 6.1). Various short-term methane and hydrogen emission values from an individual animal were measured at different times throughout the day and aggregated to estimate each cow's average daily methane and hydrogen emissions. Emission rates were calculated using volumetric airflow rate adjusted to standard temperature and pressure (STP) and corrected for capture rate, according to Patra et al. (2016). Cows received a maximum of 320 g per day of concentrate composed of barley, corn, rapeseed, soya, and molaprem used as an enticement in the Greenfeed.

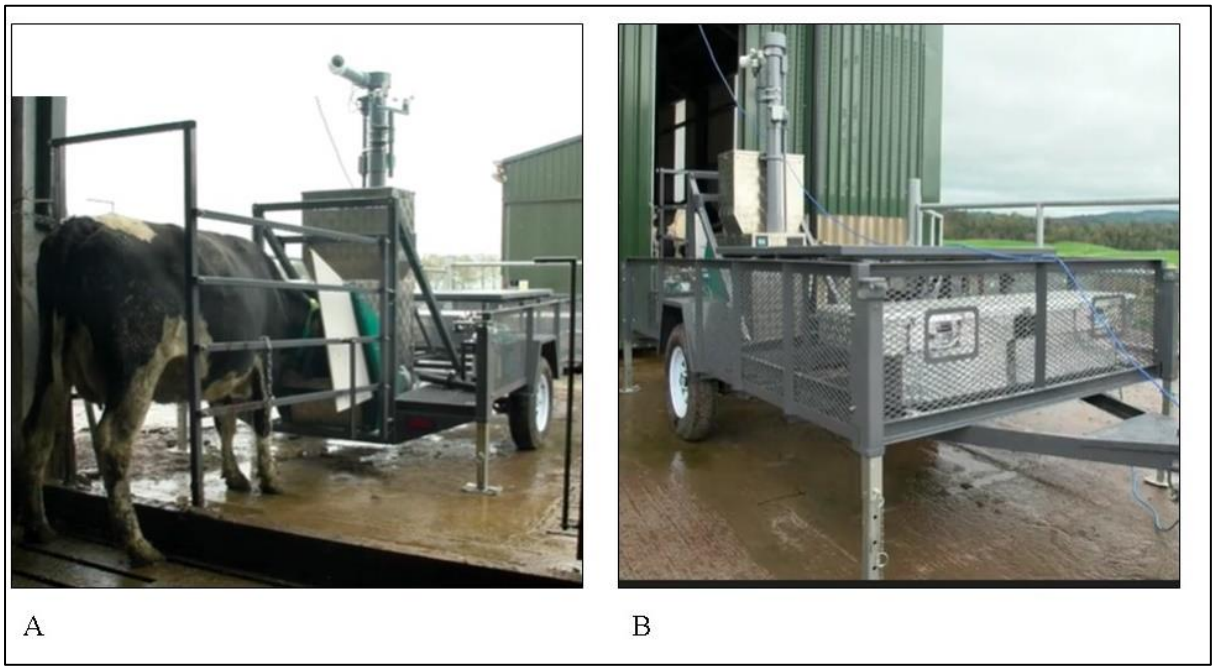

Figure 6.1: Back view of Greenfeed (A) and front view of Greenfeed (B) for measuring methane emissions from dairy cows.

Rumen sampling and processing

Six rumen-cannulated (101.6 mm internal diameter, Bar Diamond Inc., Parma, USA) cows that were already available at Teagasc, Moorepark, were selected. Cows were blocked on calving date, lactation number and their previous two weeks' milk yields; and assigned to treatments (n= 3 per group). Cows remained on their allocated treatment for the duration of the experiment. Rumen samples were taken from each cannulated cow at two timepoints; 07.30 am and 3.00 pm on two consecutive days on weeks 1, 4, and 7. Solid and liquid rumen contents were obtained from four representative locations of the rumen of each cow and strained through three layers of synthetic cheese cloth to separate the solid and liquid fractions (O'Callaghan et al., 2018). The liquid fraction was used for analysing VFAs, pH and lactic acid. For rumen microbiome analyses, two whole (containing solid and liquid fractions) samples were snap-frozen in liquid nitrogen and frozen at -80°C until analysis. Two additional snap-frozen samples were stored at -20°C.

Rumen pH and total lactic acid concentration

Ruminal fluid pH was measured immediately following collection using a glass electrode pH meter (pH FE28, Mettler-Toledo Instruments Co., Ltd., Ireland). The total lactic acid concentration in the liquid fraction was determined using a commercial kit (Megazyme, Arklow) according to the manufacturer's instructions.

Volatile fatty acids (VFAs)

Complete fractions of rumen fluid were analysed for VFAs using gas chromatography with a flame ionisation detector with a chrompack glass column using the method of Tao et al. (2016). Briefly, 0.5 ml acidified water (supplemented with 50µl of HCl (25% (v/v)) was added to 0.5 ml rumen fluid samples and vortexed for 5 mins. The tubes were centrifuged at 15,000 × g for 10 mins at 4°C and subsequently filtered through a 200 µm membrane filter. The sample was split into two, each containing 270 µl to which 30 µl internal standard (IS) was added. After being vortexed vigorously, each sample was centrifuged at 15,000 × g for 10 mins at 4°C; thereafter, 280 µl of each sample was transferred into a glass amber vial and frozen until analysis. Quantification of total VFAs and individual VFAs (propionic acid, butyric acid, acetic acid, and valeric acid) was performed using GC.

Microbiome analysis

DNA extraction

Total genomic DNA was extracted from rumen samples collected throughout the trial. DNA extraction was performed using the Qiagen QIAamp DNA Stool Kit (Qiagen Ltd, Manchester, England) protocol, with some modifications to accommodate rumen fluid and maximise DNA yield, according to Zeale et al. (2011). The starting material was 200 µl, added into a tube containing tungsten carbide beads and 1.4 ml buffer ASL and shaken on a Qiagen TissueLyser for 5 mins at 24 Hz. Proteinase k (supplied in the kit at 20 mg/ml) was added to the suspension, homogenised, and then incubated for 30 min at 70°C. The protocol was followed unmodified, except for the final DNA step, where 50 µl of Buffer EB (Sigma) was used to elute the DNA. Genomic DNA concentration was determined using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Ireland).

Quantifying rumen methanogens through qPCR

Genomic DNA from *M. ruminantium* and *Methanosarcina barkeri* was isolated from two week- old culture grown on DSMZ media 1523 and media 119, respectively, using the repeated bead beating plus column (RBBC) method as developed by Yu and Morrison (2004). Quantitative PCR (qPCR) was used to monitor the changes in ruminal methanogen populations. The quantified groups were general methanogens, *Methanobrevibacter*, and *Methanosarcina* species, using the primer sets and conditions outlined in Table 6.1.

Table 6.1: PCR primers targeted at methanogens.

Primer set	**Primer sequence**	**Target group/ Primer specificity**
1. Meth 86F and Met1340 (Wright et al., 2004)	GCTCAGTAACACGTGG CGGTGTGTGCAAGGAG	Methanogen-specific forward and reverse primers.
2. Mbb-g-1-f and Arch R1386 (Skillman et al., 2004)	GCTAATACYGGATAGATRAT GCGGTG TGTGCAAGGAGC	*Methanobrevibacter* species-specific primers.
3. MB1b and SAR835R (Narihiro and Sekiguchi, 2011)	CGGTTTGGTCAGTCCTCCGG AGACACGGTCGCGCCATGCCT	*Methanosarcina* species-specific primer.

PCR was performed according to Banning et al. (2005) with minor modifications to verify the specificity of the different primers. The PCR reactions were performed in a total volume of 25 µl, which was composed of 0.5 µl methanogen DNA template, 12.5 µl Biomix Red (Bioline, Memphis, USA), 1 µl each of forward and reverse primers (10 µM), and 10 µl PCR grade water. Amplification conditions were as follows: denaturing step of 94°C for 4 mins; 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 45 s, extension at 72°C for 60 s; and post extension at 72°C for 10 mins. DNA was checked by analysing a 2 µl amplicon sample in 1% SYBR Safe gel at 100 volts for 30 mins to determine the DNA size and quality. The gel was visualised using the Odysseyâ FC imaging system. The amplified PCR product was purified using a GenElute bacterial DNA purification kit (Sigma-Aldrich, Wicklow, Ireland) according to the manufacturer's instructions. The DNA concentration of the PCR product was determined using a nanodrop 1000 spectrophotometer.

qPCR conditions

Methanogen changes in the rumen were quantified using qPCR. The methanogen copy number for each primer set was calculated using the formula by Li et al. (2009). To prepare standards, each DNA methanogen PCR template per primer was adjusted to 1×10^{10} copies/ µl by diluting the DNA with PCR grade water. Tenfold dilutions were prepared to create standard curves ranging from 1×10^{5} molecules to 1×10^{10} molecules of DNA per microliter (Figure 6.2). The qPCR mix was carried out in 10 µl volume containing 5 µl of 2 × Brilliant Sybr Green ® qPCR Master Mix (Roche Diagnostics Ltd), 1 µl DNA (adjusted to 5 ng/µl), 0.2 µl methanogen species-specific (forward and reverse) primers (10 µM), and 3.6 µl PCR grade water. All PCR reactions were performed in triplicate, and standards were treated the same as samples. The Roche LightCycler® 480 System was used to perform qPCR. The qPCR conditions are listed in Appendix 6.1.

DNA (Number of molecules)

$$= \frac{[6.02x^{23}\left(\frac{\text{molecules}}{\text{mol}}\right)\text{x DNA amount (g)}]}{[\text{DNA length (bp) x 660 (g/mol/bp)}]}$$

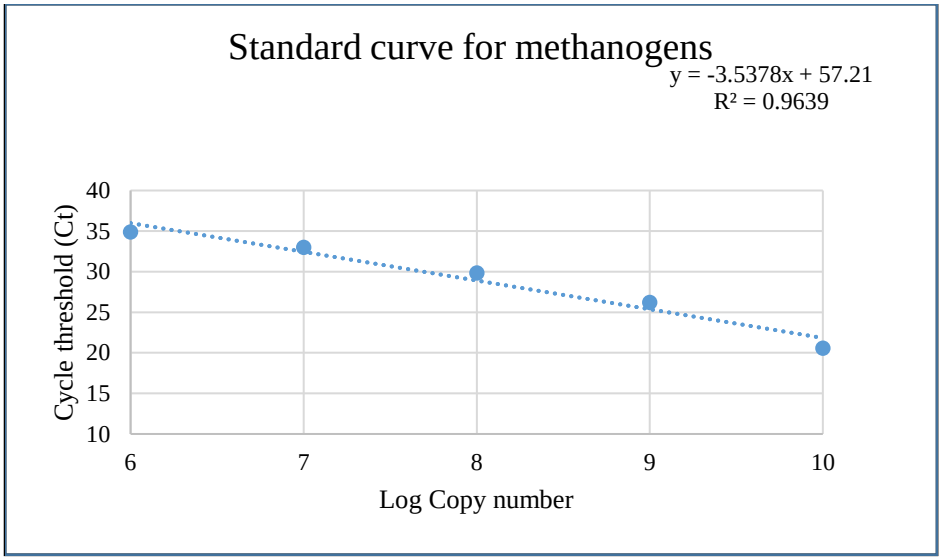

Figure 6.2: The standard curve used for methanogen qPCR.

16S rRNA MiSeq sequencing

DNA libraries were prepared according to the Illumina MiSeq protocol. The V3-V4 hypervariable regions of the 16S rRNA gene were amplified using Forward primer (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCWGCAG 3’) and reverse primer (5 ′GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC 3’). All PCR reactions were carried out in 25 µl volume, consisting of 12.5 µl KAPA HiFi Hotstart ready mix (2×) (Roche, Dublin, Ireland), 5 µl of the forward and reverse primers (2 µM), and 2.5 µl microbial DNA. Microbial DNA was first diluted to 5 ng/ µl using 10 mM Tris HCl (pH 8.5). The PCR conditions were as follows: 95°C for 3 mins, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 mins. PCR products were visualised using gel electrophoresis (1× TAE buffer, 1.5% agarose gel, 100 v for 30 mins). The remaining PCR products were purified using AMPure XP magnetic beads (Labplan, Dublin) according to the Illumina MiSeq protocol. Next, amplicons were bound with two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) per sample. Each 50 µL reaction contained 25 µl Kapa HiFi HotStart ReadyMix (2X), 10 µl PCR grade water, 5 µl index 1 primers (N7xx), 5 µl index two primers (S5xx) and 5 µl template DNA. The PCR conditions were kept the same as mentioned in the first stage, with samples undergoing 12 cycles instead of 35. PCR products were cleaned using the AMPure XP magnetic bead-based purification (Labplan, Dublin) method and validated via gel electrophoresis described above. DNA concentration was determined using

the Qubit 2.0 fluorometer (Invitrogen) with a high sensitivity DNA quantification assay kit (BioSciences, Dublin, Ireland). Samples were pooled to an equimolar concentration. Paired end 2 × 300 bp sequencing was carried out on a MiSeq platform at the Teagasc Sequencing Centre, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork.

Bioinformatics analysis

All sequences were processed in R (version 4.0.2) using Divisive Amplicon Denoising Algorithm 2 (DADA2), version 1.16 (Callahan et al., 2017). Standard filtering parameters were used with forward reads truncated at position 280 bp and reverse reads at position 210 bp. Primer sequences were removed using the trimLeft function. Forward and reverse reads were merged to obtain the full denoised sequences. An amplicon sequence variant (ASV) table was generated, and chimeric sequences were removed. Taxonomic assignment of ASVs was performed using the IDTAXA algorithm (Murali et al., 2018) and the SILVA database (version 138). Bootstrapping was applied at a threshold of 80. Microbiome Analyst was used to analyse the ASV table for alpha diversity, beta diversity and differential abundance measures (Chong et al., 2020). Data were filtered for abundance for those ASVs with a minimum total count of four, or less than 20% total abundance in all samples. The ASVs in the lowest 5% for variance based on the inner-quartile range were removed. Finally, a centred log-ratio (CLR) transformation was performed. Abundance profiles were visualised at phylum, family and genus levels. The Bray-Curtis dissimilarity matrix was calculated and displayed using Principal Coordinate Analysis (PCoA). Alpha-diversity analysis was performed on unfiltered data using Shannon, Simpson, Observed and Chao diversity measures.

Statistical analysis

The animal feeding trial was conducted over seven weeks. Each group consisted of 12 cows (excluding cannulated cows) from which methane and hydrogen were measured weekly from weeks 1-7. The first two weeks were for adaptation to the Greenfeed sampling system. Statistical differences between control and LAB groups were assessed using the Wilcoxon test. SCFA analyses were performed in duplicate, and values are reported as means ± standard deviation. Statistical significance was assessed using the Wilcoxon test for metabolomics analysis (n=3). For microbiome data, statistical analysis of beta diversity was examined with PERMANOVA. Differential abundance analyses were used to assess potential differentially abundant taxa between control and test using DESeq2 on untransformed data (Love et al., 2014). DESeq2 uses a negative binomial generalised linear model with a variance stabilising transformation on abundances. P-values ≤ 0.05 were considered statistically significant.

Results

Methane and hydrogen production

Methane and hydrogen measurements from non-cannulated cows (n=12) were determined using a GreenFeed system from weeks 1-7 of the animal trial. Figure 6.3 shows the effect of feeding dairy cows with LAB-inoculated silage or control silage on the average methane emitted (in grams per day) for seven weeks. Cows in the LAB-inoculated silage group consumed approximately 2.56 x10^{11} CFU of *L. plantarum* (LP58) and 1.26 x 10^{11} CFU of *L. lactis* (SL242) per day, based on the average DMI of silage. After three weeks, cows fed the LAB-treated silage produced significantly lower levels of methane ($P < 0.05$). During weeks 3-7, cows on the control silage diet produced an average of 381.96 g of methane per day compared to 377.54 g/d produced by ruminants on LAB-treated silage. From weeks 4-7, the decrease in methane produced by the LAB-treated cows was even more evident ($P< 0.001$). The trend for methane production seemed to decrease with time for the treatment group, with week seven having the lowest methane produced (350 g/d). The most significant reduction in methane emissions from the LAB-treated cattle was observed in week five, with 7.74% less methane emissions compared to the control group during the same period.

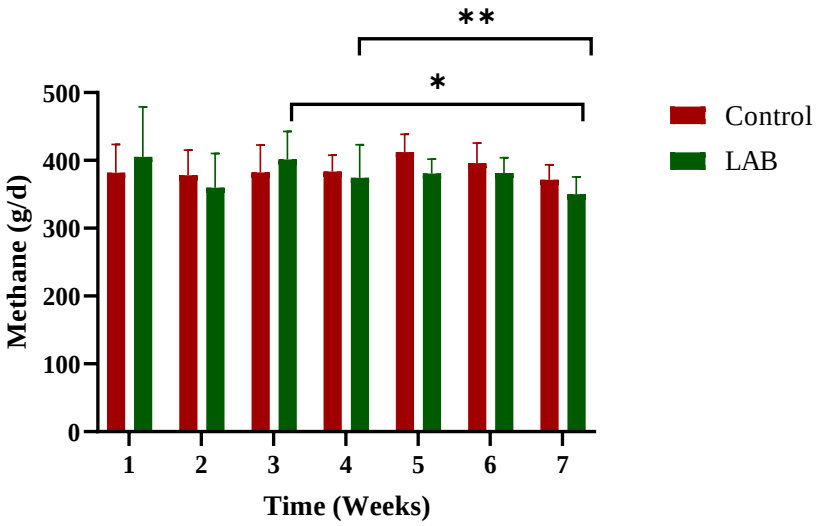

Figure 6.3: Mean (n=12) methane emissions (grams/day) from cows fed control silage or LAB-inoculated silage. *P value <0.05, ** P value <0.001

Between weeks 1-3, cows in the LAB-inoculated silage group emitted more hydrogen (H_2) than the control group (Figure 6.4). On week four, both groups expelled 1 gram of hydrogen per day on average. From weeks 5-6, the control group expelled more hydrogen, although not significantly. The lowest level of hydrogen emitted was observed on week seven, which was 0.8 g/d for both groups.

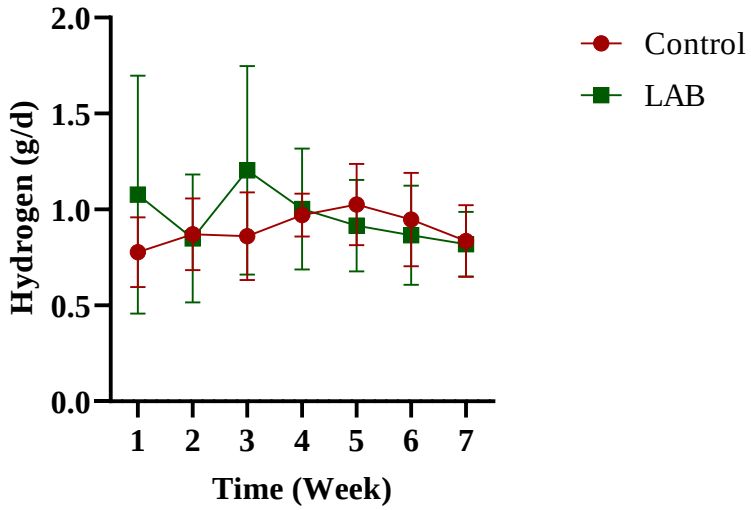

Figure 6.4: Mean (n=12) hydrogen emissions (Grams/day) in cows fed control silage or LAB-inoculated silage.

Rumen fermentation parameters

Rumen pH and lactic acid concentration

The results of ruminal fluid pH analysis are presented in Figure 6.5. In general, rumen pH was lower for the evening samples for the treatment and control groups. When comparing morning and evening samples, the evening samples had an average pH of 5.93-6.4 for the control group, while the average evening pH ranged between 5.98 and 6.1 in the LAB-treated ruminants. In the morning control samples, the average pH ranged between 6.3-6.5; and 6.4-6.6 in the treatment group. A fluctuation in pH was observed throughout the animal trial for both groups, and the differences observed were not statistically significant.

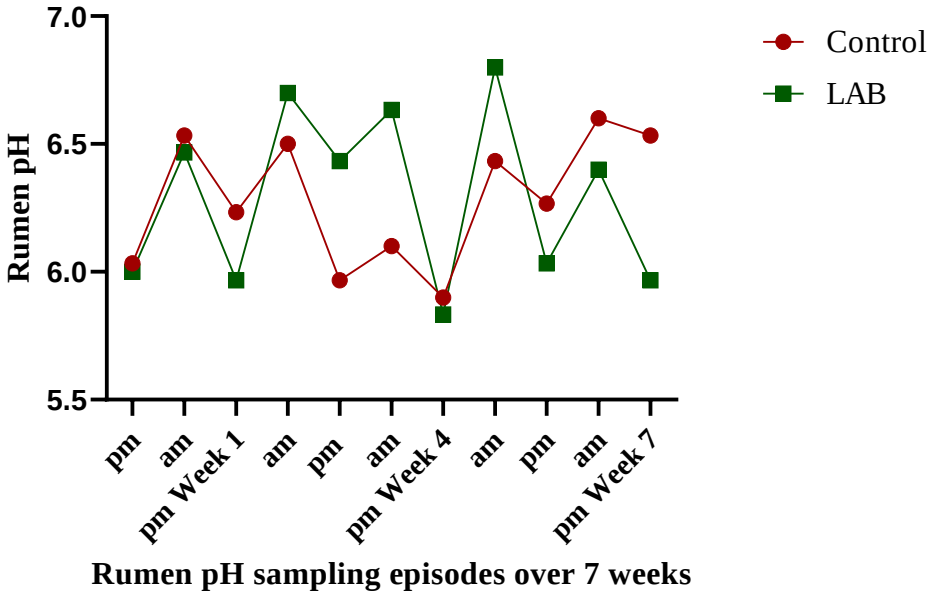

Figure 6.5: Changes in ruminal fluid pH (n= 3).

Accumulated lactic acid was low throughout the study for cows fed the treatment or control silage. The lactic acid concentration was higher in the treatment group from week one to week four, although the difference was not statistically significant. Lactic acid concentration ranged from 5 mmol/l on week 1 to 7 mmol/l on week four. At the end of seven weeks, both groups had an average of 4 mmol/l of rumen lactic acid.

Volatile fatty acids (VFAs)

GC was used for the determination of VFAs. Figure 6.6 shows the effect of feed on ruminal VFAs. Total VFAs consists of the sum of acetate, propionate, butyrate, valerate and branched-chain fatty acids (isovalerate, and isobutyrate). No significant difference was observed in the total VFAs concentrations between the treatment and control groups, although marginally higher concentrations were observed in the LAB treatment group on week seven.

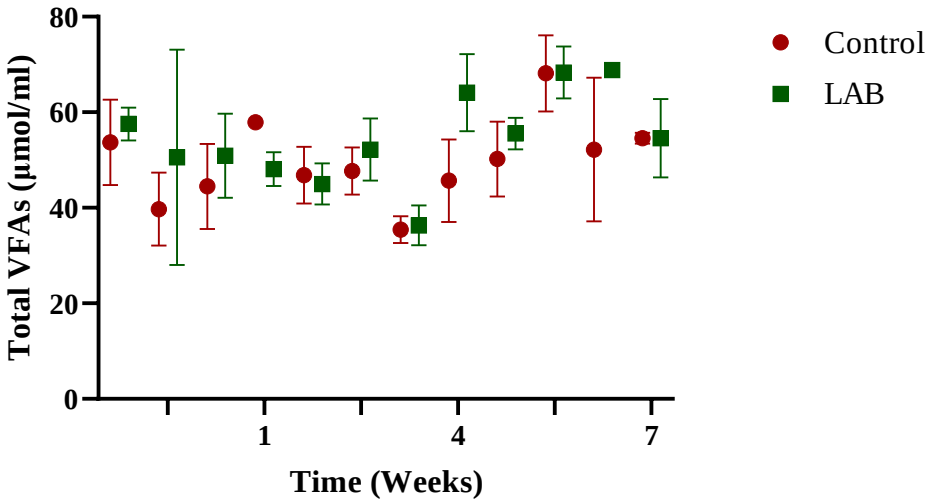

Figure 6.6: The effect of feed on total ruminal VFAs including branched chain fatty acids.

The two diets did not influence the individual VFAs proportions (Figure 6.7 (A-D)). The average acetate concentration was 22.88 and 26.85 µmol/ml for the control and treatment groups, respectively, in week one. Acetate concentration curves exhibited a similar pattern throughout the study for both groups. Acetate was produced in the highest proportions of all the tested VFAs, followed by propionate, then butyrate. The levels of propionate ranged between 9.6 µmol/ml and 20 µmol/ml for both groups throughout the study. The VFAs produced in the lowest molar concentration was valerate, with a maximum concentration of 1.5 µmol/ml displayed on week seven. LAB-treated silage did contribute to an increase in acetate and butyrate production in the treatment group ($p < 0.05$) (Figure 6.8). Acetate increased from 25.01 in week one to 28.62 µmol/ml in week seven, whereas butyrate increased from 8.9 to 10.73 µmol/ml.

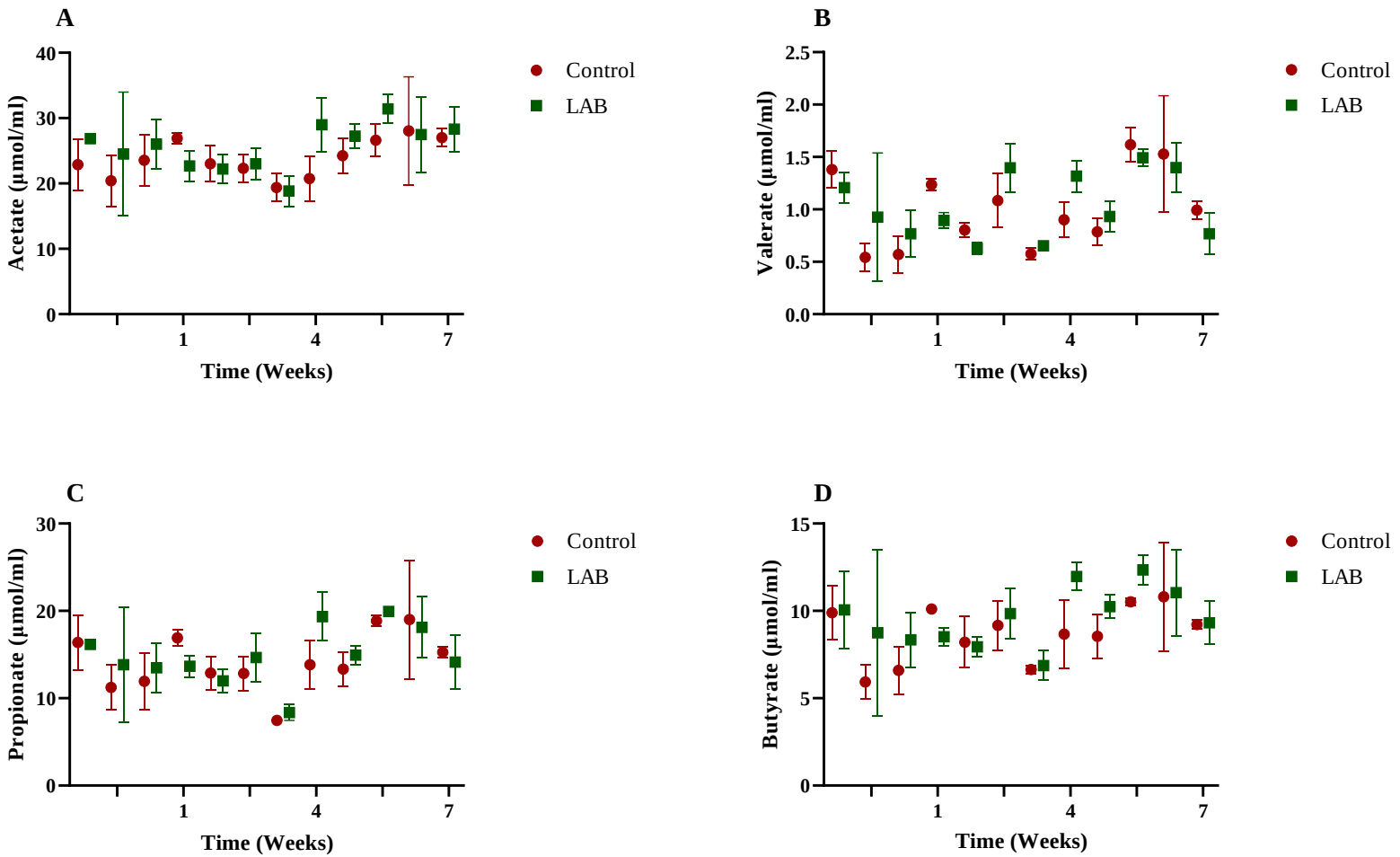

Figure 6.7: Changes in acetate (A), valerate (B), propionate (C), and butyrate (D) concentration in rumen samples from cows fed control silage or LAB inoculated silage for seven weeks.

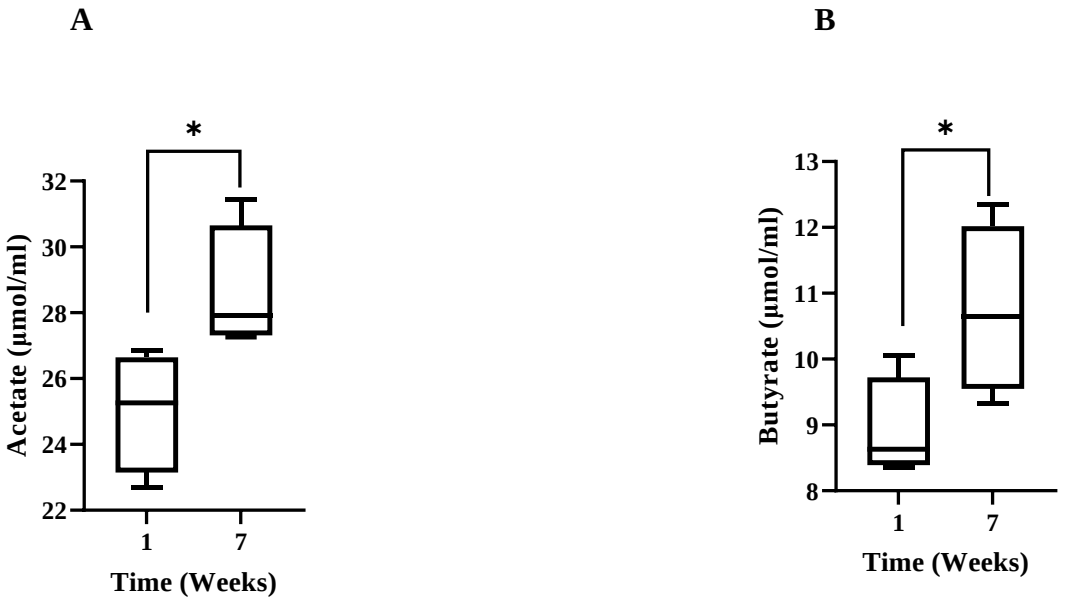

Figure 6.8: Changes in acetate (A) and butyrate (B) comparing weeks one and seven in the LAB treatment group.

Microbiome changes

Methanogen quantification

Because methanogens are the primary producers of enteric methane, it is logical to assume that their abundance could be correlated with methane emissions; hence, the total and specific methanogens were quantified via their 16S rRNA copy numbers using specific primers by qPCR. Figure 6.9 shows the abundance of general methanogens found in the rumen and two distinct genera based on their copy numbers of *Methanobrevibacter sp.* and *Methanosarcina sp.* The administered LAB silage inoculants did not significantly affect the total methanogens, *Methanobrevibacter* or *Methanosarcina sp* in the rumen. *Methanosarcina* was consistently lower in abundance compared to *Methanobrevibacter* in both groups. In week four, a 3-log decrease in *Methanobrevibacter* was detected in the treatment group, whereas a 2-log decrease was observed in the control group, compared to week one. However, at week seven, *Methanobrevibacter* was restored to values similar to week one. *Methanosarcina* was higher in the treatment group during week one and week seven, although the differences were not statistically significant.

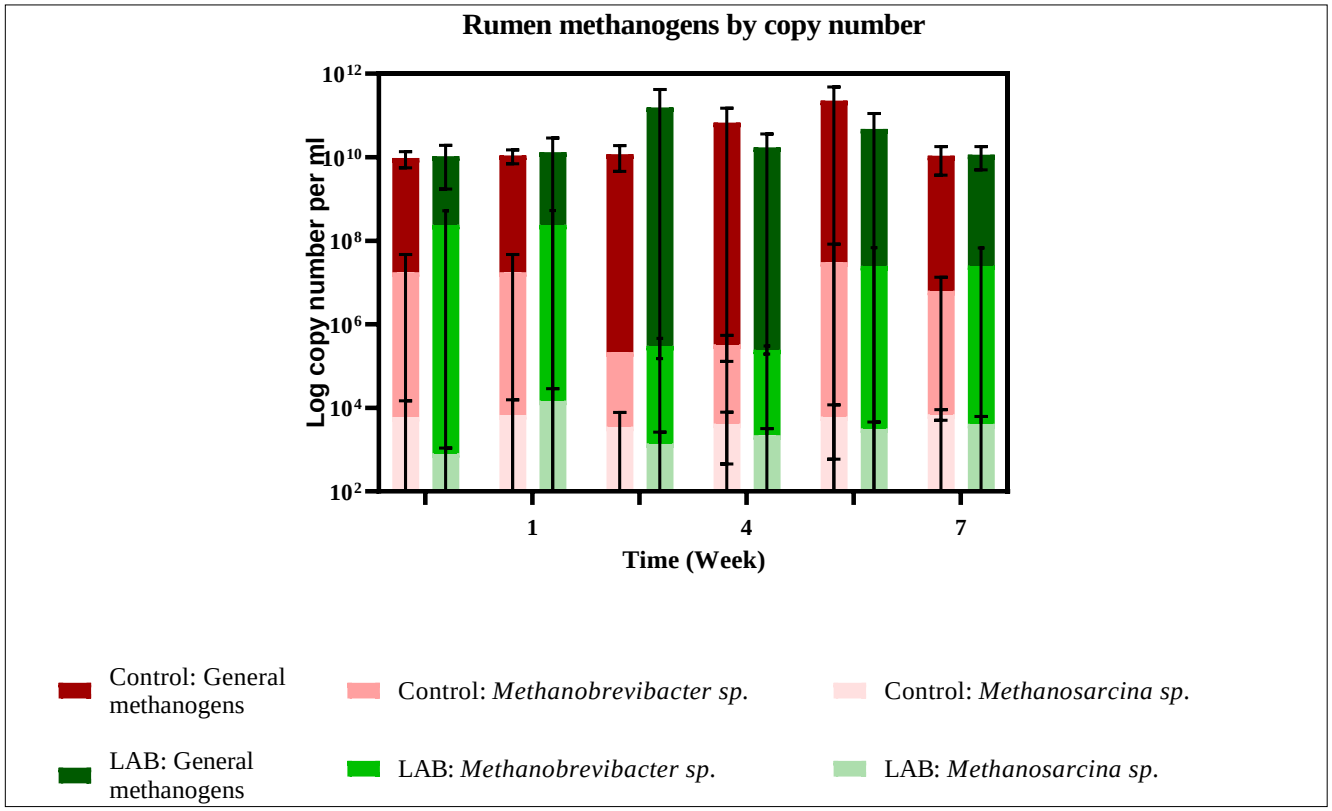

Figure 6.9: Bar representation of mean log copy number (with standard deviation) of target methanogen DNA per ml of rumen fluid.

16S rRNA MiSeq sequencing

In total, 11,266,981 paired end reads with an average of 165,691 reads per sample were generated. After trimming and filtering, 3,588,022 high-quality reads per sample remained for further processing. Following abundance and variance filtering, 1971 ASVs were obtained from the reads in rumen samples. The Shannon diversity index shown in Figure 6.10 accounts for both abundance and evenness of taxa present, and it presents a significant difference in alpha diversity between the control and treatment groups. Other measures of alpha diversity such as the Simpson, Chao1 and observed ASVs indices showed a trend toward a significant difference between the two groups.

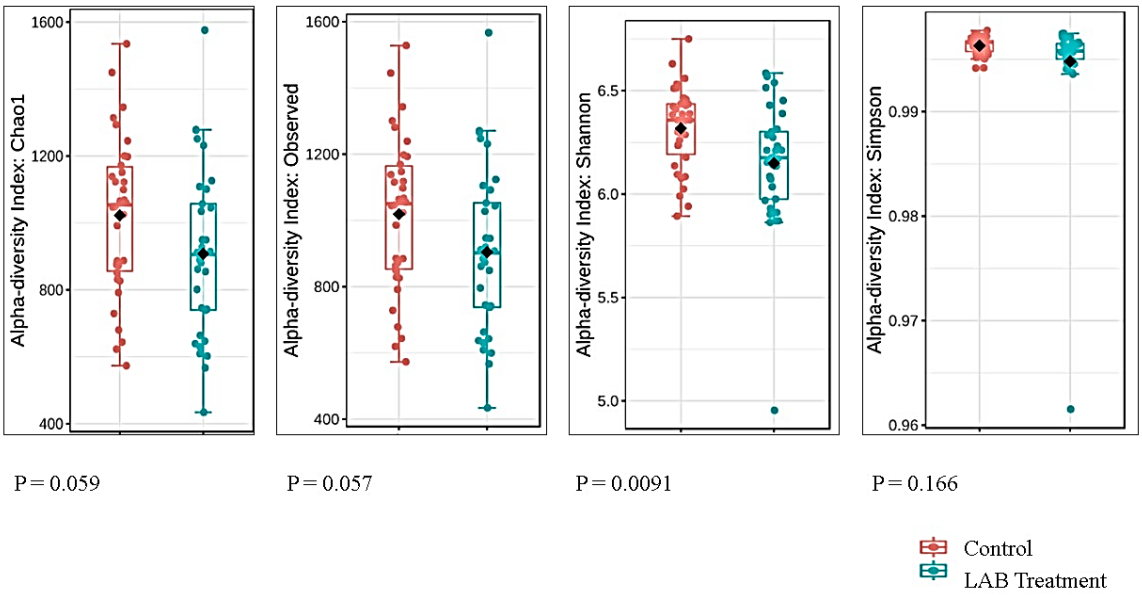

Figure 6.10: Comparison of richness and alpha diversity of microbial communities. Boxplots showing variations in alpha diversity in the rumen liquid represented in the control and LAB treatment groups.

A comparison of the rumen bacterial community structure between the control and the LAB treatment was estimated using PCoA based on Bray-Curtis distance (Figure 6.11). PERMANOVA analysis revealed a significant (P = 0.049) difference in the overall rumen prokaryotic microbiota profiles between the two groups. There is more variability in the treatment group compared to the control due to a larger inter-sample distance.

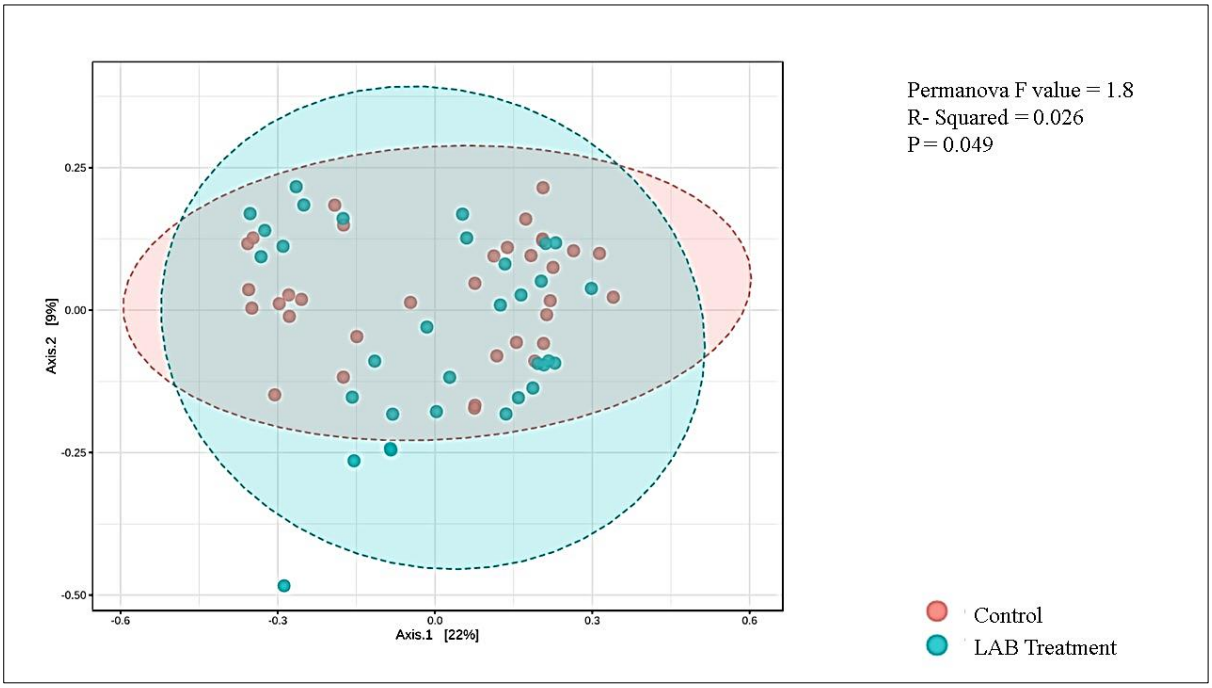

Figure 6.11: Principal coordinate analysis (PCoA) using Bray-Curtis distance of the control and LAB treatment groups.

The differences in microbiome were also reflected in the relative abundance of the major phyla (Figure 6.12 (A) and 6.12 (B)), families, and genera. A total of 19 phyla (Figure 6.12 (A)) were identified within ruminal microbiota. Relative abundances of Bacteroidetes (41.09% in control and 40.40% in the LAB treatment group) and Firmicutes (35.36% in control and 35.76 % in the LAB treatment group) dominated the phylum dataset within each group. The unassigned taxa accounted for approximately 8% of the reads in both groups. The Euryarchaeota, which are archaeal in nature, accounted for 1.8% in the control and 3% of the total sequences in the treatment group. Cyanobacteria, Planctomycetota, and Proteobacteria appeared to be low in relative abundance. When these phyla are combined, they contribute 1.4 and 1.5% for the control and treatment groups, respectively. However, upon analysing the differences in abundance, the control group had a statistically significantly higher relative abundance of Cyanobacteria ($P = 0.007$) and Proteobacteria ($P = 0.006$), whereas Planctomycetota occurred in higher abundance in the LAB treatment group ($P = 0.015$) (Figure 6.12(B)).

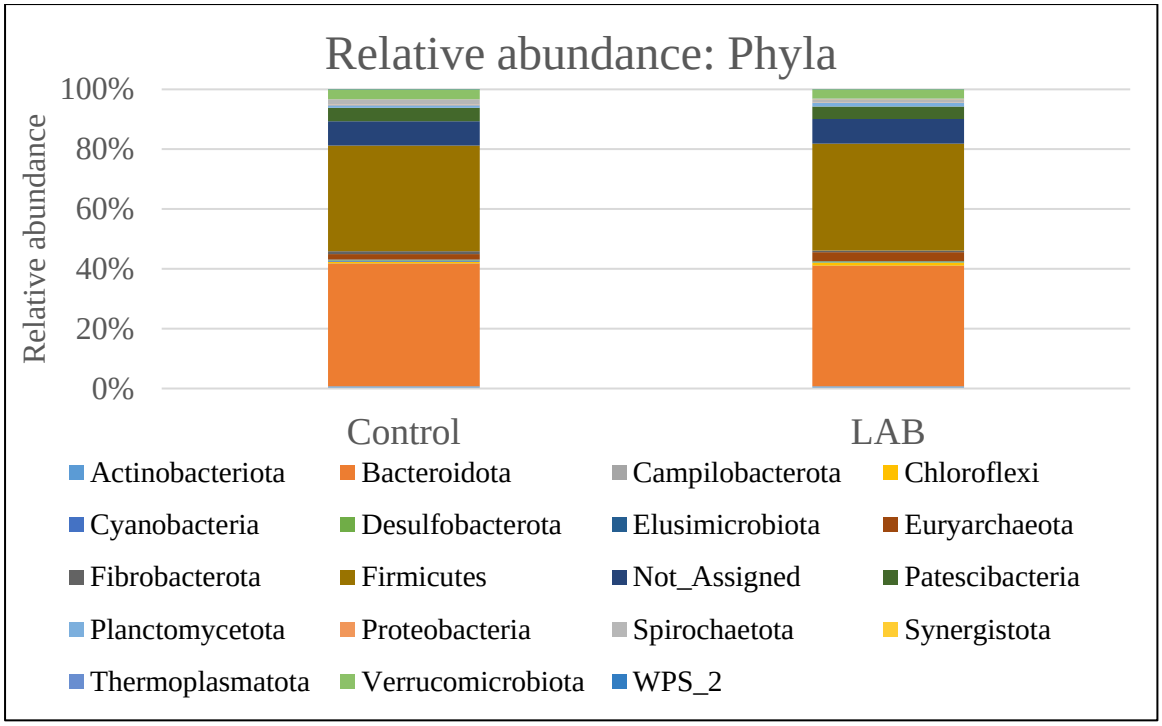

Figure 6. 12(A): The taxonomic profiles for the relative phylum abundance of each group are classified by percentage representation of the total sequences.

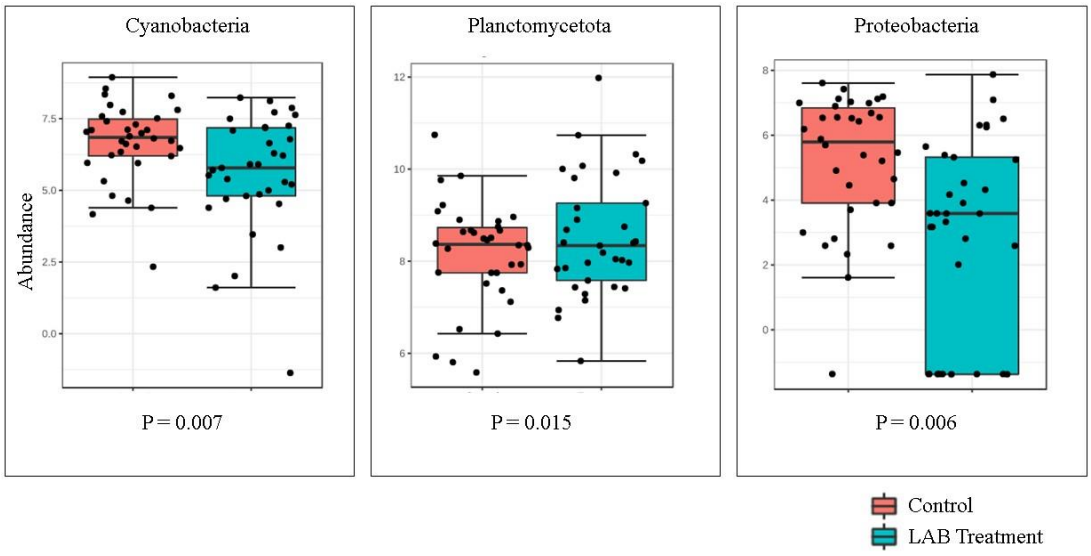

Figure 6.12(B): Boxplots depicting the phyla (Cyanobacteria, Planctomycetota, and Proteobacteria) that were significantly different between the control and treatment groups in the rumen.

The average relative abundance of each microbial family in the rumen fluid of cows fed untreated control silage and LAB inoculated silage is shown in Figure 6.13(A). Prevotellaceae was the greatest in relative abundance at the family level, representing 26% of the microbes in both groups. This was followed by Rikenellaceae, which was 7.8% in the control group and 8.3% in the treatment group. A high percentage of ASVs belonged to unclassified bacteria in both groups (17 %) at the family level. Lactobacillaceae constituted less than 0.1% of the total sequences, but the control group had 0.01% of Lactobacillaceae while the treatment group had 0.03% (P= 0.24). Surprisingly, the family of Methanobacteriaceae was significantly higher in relative abundance in the treatment group (P = 0.001) than in the control group. Other families for which significant differences were observed were the Pirellulaceae (P = 0.0018) and the Bacteroidales BS11 gut group (P = 0.0002) (Figure 6.13(B)).

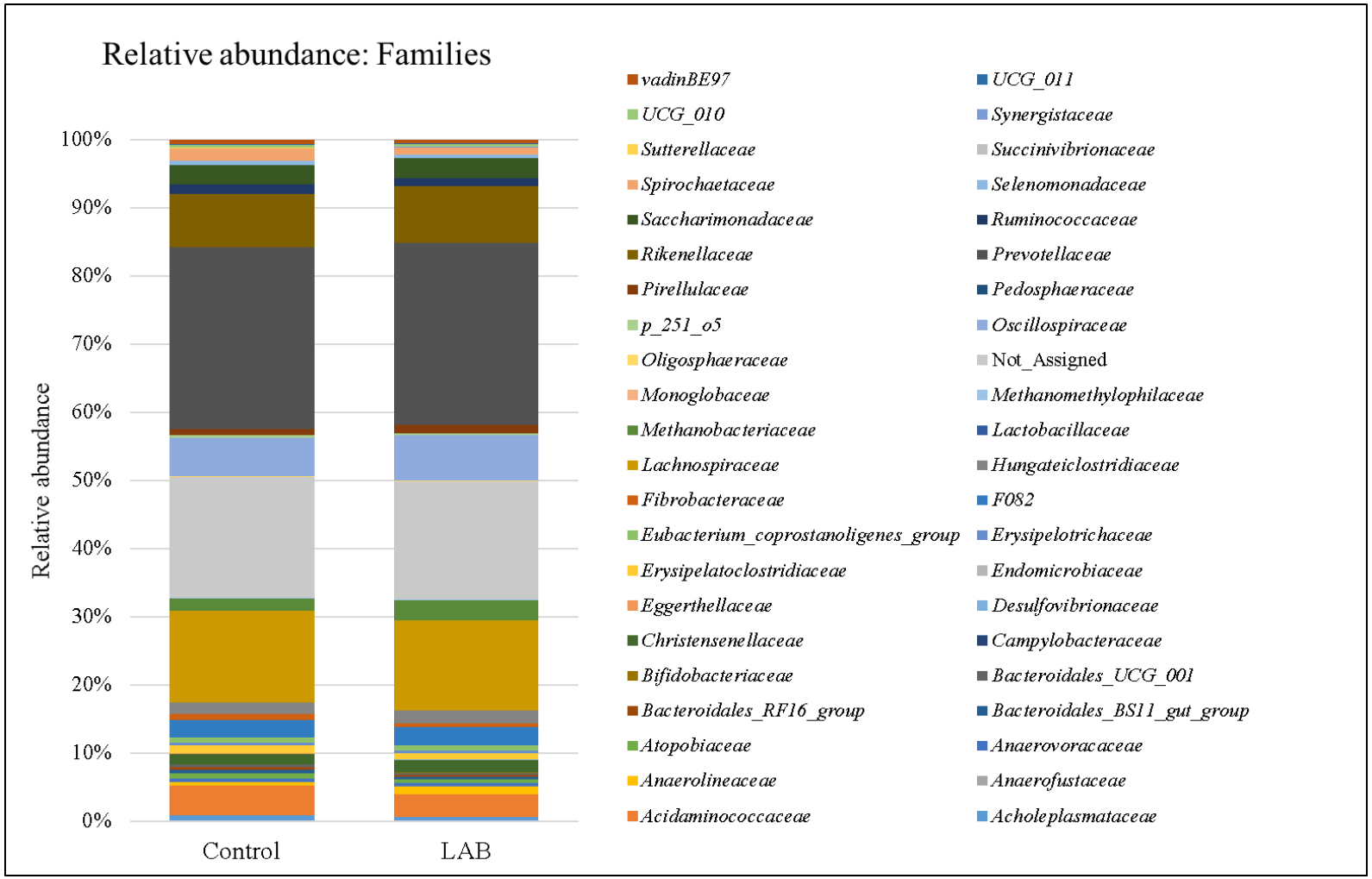

Figure 6.13(A): The taxonomic profiles for the relative family abundance of each group are classified by percentage representation of the total sequences.

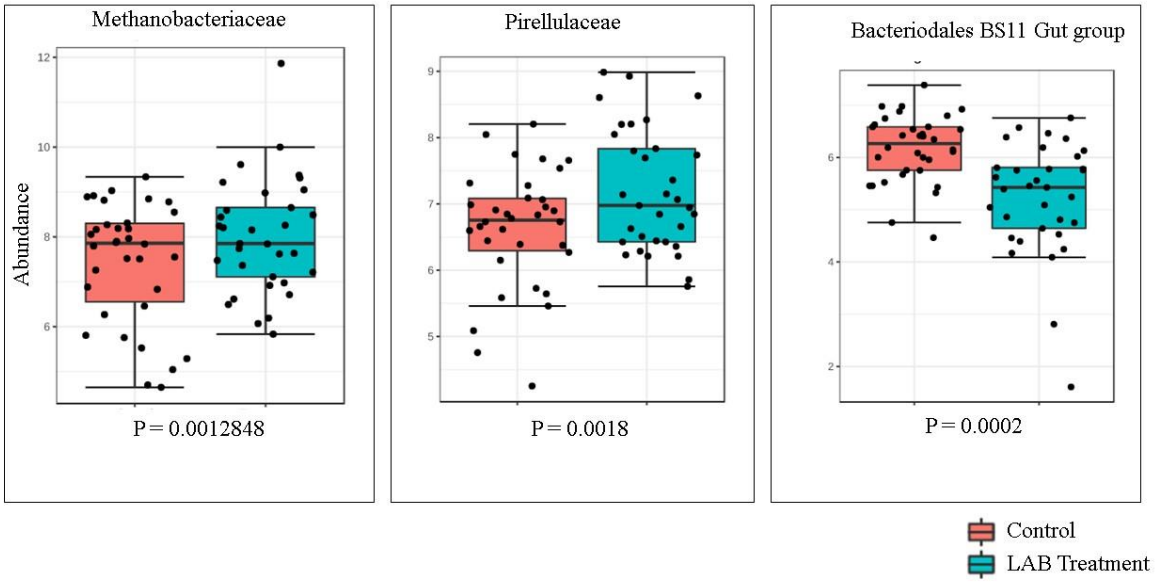

Figure 6.13(B): Boxplots depicting the families (based on the representative 16S rRNA gene) which were significantly different between the control and treatment groups in the rumen.

This study demonstrated high bacterial diversity in the rumen of animals on both the control and LAB-treated silage. Up to 97 genera were detected, including an unassigned group (Figure 6.14 (A and B)). Relative abundances of each genus were compared between the control and treatment groups for weeks 1, 4 and 7, excluding the "Not assigned" group (averaging at 34.7% in the control group and 33.5% in the treatment group). The most abundant genus overall was *Prevotella.* The relative abundance of this genus increased from 16.48% in week one to 18.78% in week seven in the control group. Similarly, in the treatment group, an increase in *Prevotella* from 16.3% to 19.4% in week seven was observed. Other genera that consistently had a relative abundance >1% in the community were *Candidatus saccharimonas, Lachnospira* group, *Methanobrevibacter*, NK4A214_group, *Succiniclasticum*, *Christensenellaceae* R_7_group, and *Treponema*. *Methanosphaera* levels increased per week. *Lactobacillus*, a genus of interest in this experiment, was relatively low (<0.1%) in both groups, although an increase was observed in the treatment group, with the highest relative abundance of this genus displayed in week 7. Conversely, a decrease in the abundance of *Lactobacillus* was seen in the control group. The treatment group had significantly higher abundance in the Cpla 4 termite group, UCG 009 and *Candidatus soleaferrea* (Figure 6.14(C)).

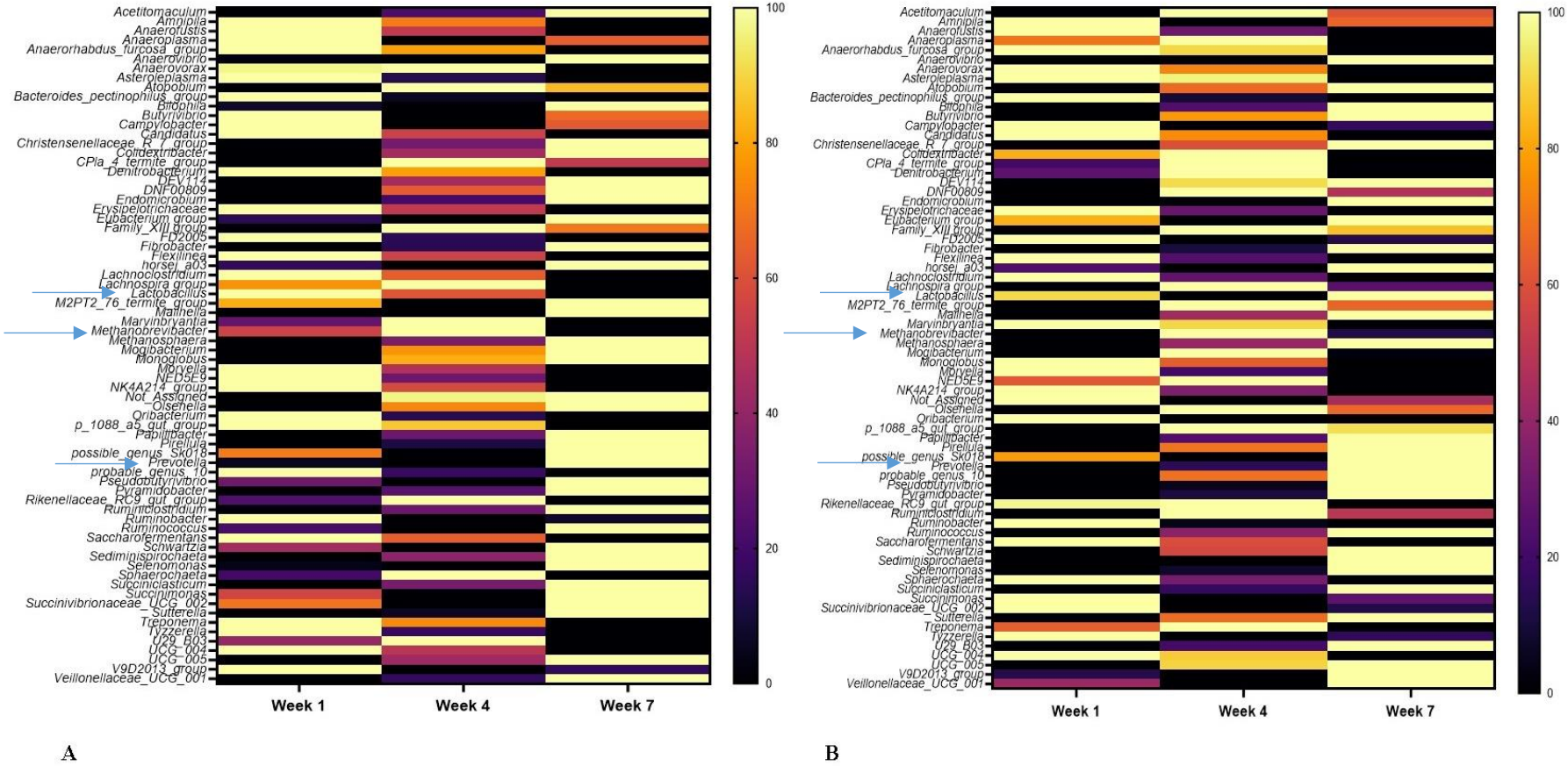

Figure 6.14: Heat maps of relative abundance (genera) in the control group (A) and treatment group (B).

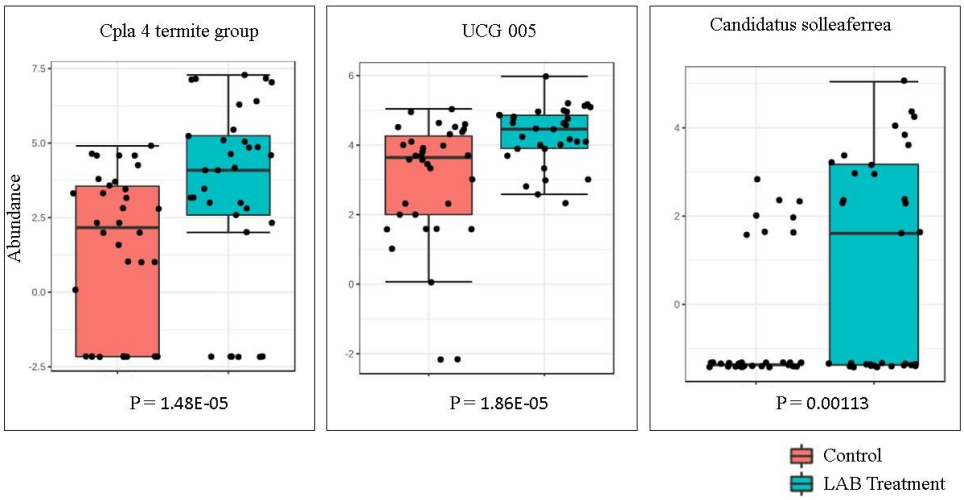

Figure 6.14(C): Boxplots depicting the genera (based on the representative 16S rRNA gene) that showed statistically significant differences between the control and LAB-treated rumen (Cpla 4 termite group, UCG 005 and *Candidatus solleafera*).

Discussion

This study aimed to investigate the effect of ingestion of LAB-silage inoculants *Lactococcus lactis* (SL242) and *Lactobacillus plantarum* (LP58) on enteric methane production and the subsequent impact on rumen fermentation in dairy cows, concerning changes in metabolites such as VFAs and lactic acid production, rumen methanogens and other rumen microbes. Methane production was reported for 24 non- cannulated dairy cattle (n=12 per group), and rumen modification results were reported for six cannulated dairy cows (n=3 per group). The LAB tested in this animal trial were chosen based on their anti-methanogenic properties, discovered through *in vitro* GC methods (Chapter four).

Enteric methane is produced as a result of microbial fermentation of feed components (Boadi et al., 2004). Modification of the rumen is often achieved via dietary manipulation and is the most commonly used approach for enteric methane mitigation (Haque, 2018). Dry matter intake, especially the type and rate of fermentation of carbohydrates (Moss et al., 2000), is a significant determinant for enteric methane production (Ellis et al., 2008). In this experiment, cows fed LAB-treated silage produced less methane, although differences were statistically significant only between week three and week seven of the animal trial. There are limited studies on the effects of LAB on methane production in dairy cattle. Jeyanathan and colleagues (2016) tested the antimethanogenic potential of LAB strains (*Propionibacterium freudenreichii, L. pentosus* and *L. bulgaricus*) on 12 rumen-cannulated Texel wethers sheep. That study found that methane production was reduced by 13% ($P < 0.05$) with *L. pentosus* after two weeks of administering the DFM, and the effect was maintained throughout the 4-week treatment period and two weeks after treatment had been discontinued. Conversely, *Propionibacterium* treatment increased methane production by 16% ($P < 0.05$) after four weeks of DFM administration, and *L. bulgaricus* had no effect. McAllister and Newbold (2008) state that although methane production can be temporarily inhibited for short periods, the rumen ecosystem has several adaptive mechanisms that enable it to revert to its initial levels of methane production. Thus, in our study, it would have been informative to test whether lower methane levels were maintained in the LAB-treated cows over a longer period of dietary supplementation and after the feeding trial had ended. An *in vivo* trial by Berger et al. (2014) found that administering *Lactobacillus plantarum* strain 115 to four lactating Holstein cows for a month resulted in approximately 20% less methane than in the control cows.

Molecular hydrogen is a product of carbohydrate fermentation and is utilised by methanogens to produce methane, thereby reducing partial pressure in the rumen (Ma et al., 2019). Hydrogen accumulation in the rumen is undesirable as it acts as a feedback inhibitor and may impair fibre digestion and fermentation by impeding the normal function of microbial enzymes such as NADH dehydrogenase (Li et al., 2021; Ungerfeld, 2015). Our study found no significant difference in the hydrogen (H_2) expelled from the cows fed LAB-inoculated silage compared to the control group (Figure 6.4). At the beginning of the experiment (week 1-3), the LAB treatment group expelled more H_2 than the control group. However, an equal amount of hydrogen (0.8 g/d) was expelled from both groups at seven weeks. There was a decrease in H_2 expelled which correlated with a decrease in methane emitted from week four onwards. This outcome is contrary to that of Martinez-Fernandez et al. (2016) who observed an inverse relationship between methane decrease and H_2 loss, when chloroform was used to inhibit methanogenesis in rumen fistulated Brahman steers (n= 8/group).

Ruminal pH values in this study were within the normal range (6.0–7.0) (Nur Atikah et al., 2018); however, a fluctuation in pH was observed in both groups; when comparing morning and evening samples, rumen pH was lower in the evenings. A variation in pH is influenced by the type of diet and feeding frequencies (Franzolin and Dehority, 2010). Maintaining a stable rumen pH is vital for the persistence of the gut microbiota and the overall health of the animal. Rumen pH is also an important indicator of rumen fermentation function. Changes in pH can cause a shift that favours the growth of certain bacterial species that may impact fermentation and influence methane production. For example, a reduction in pH may affect cellulolytic bacteria in the rumen that have a symbiotic relationship with methanogens by providing precursors for methanogenesis, which may indirectly influence methane production (Russel, 2002). Lower rumen pH can be caused by excessive production of VFAs (Huws et al., 2019). Furthermore, it has been reported that low rumen pH is not conducive to methanogenesis because the microbiota present are altered (Van Kessel and Russel, 1996).

The rumen of healthy adult ruminants has a lactic acid concentration between 1-20 mmol/l (Møller et al., 1997). This is because, in a fully functional rumen system, accumulation is prevented through removal by lactic acid utilising bacteria. The rumen consists of lactic acid-producing and lactic acid utilising microbes (*Megasphaera, Propionibacteria*, *Prevotella*, and *Bacillus*), which ferment lactic acid to short chain fatty acids (SCFAs), which keeps lactic acid concentration in the rumen low (Mills et al., 2014; Seo et al., 2010). In this study, it was

observed that the amount of accumulated lactic acid was consistently low throughout seven weeks for cows fed the treatment or control silage, with the highest lactate concentrations being 7 mmol/l observed in week four of the animal trial. Lactate has a pKa of 3.9, making it a ten-fold stronger acid than VFAs, which have a pKa of 4.9. Therefore, lactate accumulation can result in a faster decrease in ruminal pH (Russel, 2002). Lactate can accumulate if the rate of carbohydrate fermentation is rapid, and the ruminant has not yet adapted to the diet. The functioning of rumen bacteria also stabilises rumen pH, thereby reducing ruminal acidosis.

VFAs are normal end-products of ruminal fermentation (Ungerfeld, 2020). SCFAs are VFAs produced by the gut microbiota (Rios-Covian et al., 2016). The main VFAs produced during fermentation in the rumen are acetate, butyrate, valerate and propionate and serve different functions for the host (Moss et al., 2000). For example, they provide approximately 50-85% of the ruminant's energy supply; and are of paramount importance. The VFA profile has an influence on the rate and efficiency at which energy is utilised, as well as on the composition of animal products such as milk components. The molar percentage of VFAs influences the production of methane. Proportions of VFAs determine the volume of gases such as CO_2 and methane that are released into the atmosphere by the ruminant (Jatkauskas et al., 2013). Acetate and butyrate production results in more H_2 being produced and may aid in methane production. During propionate and valerate production, H_2 is utilised and may serve as a competitive pathway for methane production (Ungerfeld, 2020).

When comparing the concentration of the total VFAs, no significant difference was observed between the LAB treatment and control groups; however, acetate and butyrate had significantly increased in the treatment group when comparing week one and week seven. Overall, it was found that the average levels of propionate were marginally higher in the LAB treatment group than in the control. It is hypothesised that LAB stimulate the growth of lactic acid-utilising bacteria by providing lactate as a substrate, thereby improving propionate production with a subsequent reduction in methane production (Jeyanathan et al., 2016). Propionate production is a competitive pathway to methanogenesis. This could explain the decrease in methane observed in the LAB treatment group (Figure 6.3). If propionate production is both energetically enhanced and proportionately increased, then it is likely that the energy available to the cattle will also increase, thereby improving the overall wellbeing of the animal (Elam et al., 2003).

The method of qPCR is a useful technique to enumerate difficult-to-culture microbes such as the methanogen community, and it was utilised in this study to monitor the changes in ruminal methanogen populations. Initially, methanogen-specific primers were used; however, to increase specificity, primer sets targeting *Methanobrevibacter* and *Methanosarcina* were added. *Methanobrevibacter* species are generally predominant ruminal methanogens (Leahy et al., 2010) and *Methanosarcina sp.* are metabolically versatile, slow-growing methanogens that produce methane from methanol, methylamine substrates, and hydrogen (Lackner et al., 2018). The method used in this study demonstrated that total methanogens were present at log 10^{10}-10^{11} copies/ml for both the treatment and control groups (Figure 6.9). Denman et al. (2007) found methanogen populations were 10^{9} copies/ml in the rumen control and 10^{8} copies/ml in the treatment (bromochloromethane) group. The researchers used primer sets ME1 and ME2, which have been found to reduce diversity coverage of the methanogenic lineages, which could explain the log difference in methanogen copy numbers between the two studies.

The order Methanobacteriales*,* which encompasses the family Methanobacteriaceae, are reportedly the most abundant methanogens in the ruminal environment (Skillman et al., 2004). Methanobacteriaceae constitutes the genera *Methanobrevibacter, Methanobacterium,* and *Methanosphaera*. This family was present at 1.8% in the control group and 2.9% in the treatment group in terms of their relative abundance (Figure 6.13(B)). This order consists of mesophilic or moderately thermophilic and neutrophilic methanogens (Oren, 2014). Generally, *Methanobacteriaceae* reduce CO_2 with H_2 to produce methane. Enteric methanogenesis is primarily hydrogenotrophic. Some members of the *Methanobacteriaceae* family utilise formate to produce methane. In this study, the only genera detected from this family were *Methanobrevibacter,* which are hydrogenotrophs and *Methanosphaera,* which are methylotrophic. *Methanobacterium* were either less abundant or were not present at detectable levels in the LAB treatment group.

The diverse and complex community of microorganisms in the digestive tracts of ruminant livestock allow the host access to otherwise inaccessible nutrients (Lan and Yang, 2019). Cyanobacteria, which was significantly more abundant in the control group (Figure 6.11), has a recent designation as a new candidate class known as *Melainabacteria* (Soo et al., 2014). This group has a highly conserved 16S rRNA gene and is phylogenetically different from the photosynthetic cyanobacteria. Gut Cyanobacteria have a role in nitrate reduction (Flores et al., 2005), carbohydrate fermentation and converting sugars into acetate and butyrate (Neves et al.,

2017). Our result was unexpected as Prasanna et al. (2002) reported on the ability of Cyanobacteria to inhibit methane production in soil samples from rice fields. Nevertheless, more research is needed to verify the extent of its impact on carbohydrate fermentation in the rumen. Planctomycetota was significantly more abundant in the rumen cattle fed the LAB-inoculated silage. Planctomycetota have many carbohydrate-active enzymes in their genomes, suggesting that their high metabolic potential contributes to the functioning of the rumen (Gharechahi et al., 2022). The relative abundance of Proteobacteria, which include a wide variety of pathogenic genera (Auffret et al., 2017), was significantly lower in the LAB treatment group than in the control group (Figure 6.12(B)). The Bacteroidales BS11 gut group were significantly higher in the treatment group. This group activates acetate production in the rumen (Liu et al., 2019). Furthermore, an increase in *Prevotella* from 16.3% to 19.4% was observed in the treatment group. This genus incorporates H_2 in the succinate (randomising) or acrylate (non-randomising) pathway to produce propionate (Ahmed et al., 2020) which was found to be marginally increased in the treatment group in our study. *Lactobacillus* was relatively low (<0.1%) in both groups in the current study; however, a non-significant increase was observed in its relative abundance in the LAB treatment group as the feeding trial progressed, with the highest abundance found in week seven. This could be attributed to the consumption of *L. plantarum* (LP58) in the silage fed to the cattle and could indicate that this organism persisted within the rumen, which is an important factor to consider when choosing silage inoculants for rumen modification.

Conclusion

Methane production is the inevitable result of enteric fermentation, and it helps stabilise rumen pressure by reducing available hydrogen. This pathway cannot be altered extensively without causing an adverse reaction to enteric fermentation and, ultimately, the ruminant. The current study suggests that feeding dairy cows with silage that has been co-inoculated with *L. plantarum* (LP58) and *L. lactis* (SL242) can reduce methane emissions. The LAB supplementation did not affect rumen pH and lactate production. The total VFAs remained unchanged compared to the control group, although acetate and butyrate increased significantly between week one and week seven for the treatment group only. The rumen microbiome showed differences in relative abundances at genus, family and phylum levels between the LAB treatment and control groups, suggesting that the ingested LAB changed the abundance of specific microbes in the rumen. At the phylum level, the control group had significantly higher Cyanobacteria and Proteobacteria, while Planctomycetota was significantly higher in the LAB treatment group. This study demonstrated that feeding *L. lactis* (SL242) and *L. plantarum* (LP58) as silage co-inoculants to lactating cows resulted in reduced methane and does not negatively impact the rumen microbiome. Because research using LAB to reduce methane production *in vivo* is limited, more experimental evidence is needed to investigate if methane inhibition is sustained over longer periods.

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Appendix

Appendix 6.1: qPCR conditions for methanogens

Denaturation			
Cycles	1	Analysis mode	None
Target °C	Acquisition mode	Hold	Ramp Rate (°C/s)
95	None	3 min	4.4
Amplification			
Cycles	40	Analysis mode	Quantification
Target °C	Acquisition mode	Hold	Ramp Rate (°C/s)
95	None	10 sec	4.4
60	None	20 sec	2.2
72	Single	1 sec	4.4
Melting			
Cycles	1	Analysis mode	Melting curve
Target °C	Acquisition mode	Hold	Ramp Rate (°C/s)
95	None	5 sec	4.4
60	None	1 min	2.2
97	Continuous 5 *acquisitions (per °C)	1 sec	4.4
Cooling			
Cycles	1	Analysis mode	None
Target °C	Acquisition mode	Hold	Ramp Rate (°C/s)
40	None	20 sec	2.2

Chapter 7.

Discussion.

The enteric microbial ecosystem is important because it performs significant biochemical functions such as nutrient management and it ultimately influences host fitness, function and response to disease. In ruminants and birds, selective enrichment for beneficial microorganisms can be achieved through probiotics such as LAB. The great tit (*Parus major*) provides a powerful natural system for addressing microbiome-related effects on host phenotypes. This species allows for research explaining how indigenous strains of bacteria beneficial to the host can be developed and used as probiotics. The overall aim of this thesis was first to identify the *Lactobacillus* strain isolated from the great tit bird and evaluate its probiotic potential and safety. Secondly, to screen for and characterise bacteriocins from LAB, which were used as silage inoculants to reduce methane production in dairy cattle, following *in vitro* confirmation of efficacy. Ruminants produce enteric methane as a by-product of microbial feed fermentation. As a GHG, it poses an environmental threat, and as a non-nutritionally beneficial compound, its reduction saves metabolisable energy for the host. This can be achieved by employing several dietary strategies, such as using LAB as silage inoculants or DFMs. Therefore, an investigation of LAB as rumen modifiers and their effect on the host's microbiome and metabolome was conducted.

Chapter two aimed to isolate *Lactobacillus* strains from faecal samples of the great tit bird (*Parus major*) and to characterise the isolate using *in vitro* and *in silico* techniques as a potential probiotic for great tits in the wild. The gut microbiota of birds include many bacterial species, such as *Lactobacillus,* which are generally considered non-pathogenic and impart many benefits to the host, such as improving intestinal health (Li et al., 2018), pathogen inhibition, immune modulation and increasing growth rates (Alizadeh et al., 2021). Our study isolated a Gram-positive, homofermentative, D- lactic acid-producing rod-shaped bacterium that grew best at 37°C under aerobic and anaerobic conditions. The strain *L. kimchicus* exhibited acid tolerance since cell viability was maintained at 1×10^7 CFU/ml for 6 hrs at pH 2.5, while incubation at pH 2 resulted in a 3-log reduction in viable cells. The acid-resistant locus (Arl7) was found in the genome of this strain, and it is hypothesised that this locus could be for genes that confer acid resistance. It is expected that potential probiotic strains are able to withstand the acidic environment in order to effectively colonise the GIT of their host (Reuben et al., 2019). Fortunately, the alimentary tract of birds is shorter compared to other animals, and bird feed journeys through the entire alimentary tract in a short time of 2.5 hrs (Jin et al., 1998), therefore a probiotic needs to only tolerate acidity for that short amount of time. One of the

main obstacles to the viability of probiotics is the presence of bile salts that probiotics encounter in the GIT when ingested. Bile salts damage the bacterial cell membrane causing cell death. Under physiological bile concentrations (0.3%), *L. kimchicus* maintained more than 97% of viable cells after 5 hrs of exposure to bile salt. Bile salt hydrolase (BSH) activity contributes to colonisation (Joyce et al., 2014), and no *bsh* genes conferring BSH activity were found in the genome of *L. kimchicus* (Bi et al., 2016; McAuliffe et al., 2005), which was validated by the BSH assay where BSH activity was not detected using a qualitative plate assay. A high concentration of bile salts and low pH as stressors in the GIT interact and affect the probiotic strain synergistically (Pinto et al., 2006). Therefore, a future experiment of viability testing, which would evaluate a combination of low pH and high bile salt concentration, is recommended. This would better represent the bird GIT rather than testing the effect of each condition in separate *in vitro* experiments.

The phenotypic and genotypic antibiotic resistance profile and bacteriocin production capabilities of *L. kimchicus* were investigated. No transferrable antibiotic resistance genes were detected in the genome of *L. kimchicus* 5.1, although the isolate was resistant to streptomycin. The emergence and transfer of antibiotic resistance genes are a significant concern. Antibiotic resistance can be developed intrinsically through natural recombination and integration into the bacterial genome, or acquired through horizontal gene transfer (Martinez and Baquero, 2014). Harbouring antibiotic resistance genes excludes a LAB strain as a probiotic. Therefore, the FAO/WHO (2006) recommends screening for antibiotic resistance genes in potential probiotic strains. *In silico* screening methods amplify our ability to identify and study antibiotic resistance. The European Food Safety Authority (EFSA) considers WGS-based information suitable for the risk assessment of microorganisms (EFSA, 2021). *In silico* screening showed that *L. kimchicus* possessed the genetic machinery for bacteriocin production. However, the isolate did not demonstrate bacteriocin production when tested against the acid-resistant *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901, and only weak antimicrobial activity was observed against gram-negative *E. coli* and Gram-positive *S. simulans*. This suggests that the inhibitory activity observed could be potentially due to the production of other antimicrobial agents known to be produced by LAB, such as hydrogen peroxide or 1,2-propanediol (Vieco-Saiz et al., 2019).

Further *in vitro* testing using microassays can be conducted to investigate the presence of the aforementioned antimicrobial agents (Demmano et al., 1996; Ju et al., 2020). Future experiments may be necessary to establish the inhibition spectrum of the isolate against a larger panel of pathogens because bacteriocin screening can be limited by the indicators used. Pathogens to include are those common to birds, such as *Campylobacter jejuni* and *Salmonella enterica* (Chung et al., 2018).

Freeze-drying is an important technique that enables long-term probiotic storage while maintaining cell viability. The six-week stability test showed that *L. kimchicus* 5.1 is best stored at refrigerated temperatures as storage at room temperature resulted in a 3-log loss in cell viability. Probiotic bacteria are often packaged in foil sachets, liquid suspensions or marketed as capsules. Various studies have consistently shown low viability of probiotics in commercial preparations (Blandino et al., 2016; Masco et al., 2005; Shah, 2000; Ullah et al., 2019) therefore, packaging *L. kimchicus* in the appropriate medium, such as in sachets, could help it maintain viability at room temperature, facilitating its application as a probiotic in the wild.

This study has shown that the gut of the great tit bird is a source of *L. kimchicus*, which possesses fundamental properties that make it a potentially good candidate for probiotics. *L. kimchicus* grows in both anaerobic and aerobic conditions and is inhibitory against *E. coli* and *S. simulans*. Therefore, it could be used in the field to optimise the performance of the great tit bird and control pathogens, which would minimise pathogen transmission to humans. Moreover, using this strain as a probiotic would contribute to the preservation of the great tit population, a model species for ecological and evolutionary research that has been intensively studied for several decades, particularly in Europe (Groothuis and Carere, 2005). This species is used for addressing questions related to microbiome-related effects on host phenotypes (Davidson et al., 2020)

Chapter three focused on isolating potential bacteriocin-producing LAB strains from the ruminant environment, i.e., grass, cow faeces, clover and rumen fluid fractions. It also concentrated on the characterisation of potential bacteriocins produced by these LAB. Due to the ban on using antibiotics as animal supplements, DFMs that are LAB have increased in popularity. LAB have traditionally received attention for their probiotic properties, use in food, and promotion of animal growth and productivity (Raeth-Knight et al., 2007), but more recently, they have been investigated for their antimethanogen potential (Cao et al., 2011; Vyas

et al., 2014). Several researchers have isolated LAB from the agricultural environment using various conventional methods (Bui et al., 2017; Lin et al., 2020; Miller and Wolin, 2001; Peng et al., 2021). The 16S rRNA gene analysis provided a reliable, highly discriminatory method for identifying bacteria at the species level. Of the 16 isolates, nine (56.25%) belonged to the *Lactobacillus* genus. This was expected as LAB are ubiquitous, naturally diverse, and the largest most common genus in this group is *Lactobacillus* (Gueimonde et al., 2013). Due to the many applications in food and biotechnology, Lactobacilli are of economic importance and have been the subject of intense empirical research. In this study, the LAB species most frequently isolated was *L. paracasei*. This organism is indigenous to the human intestinal tract and has been commonly isolated from plant materials such as silage, pickles and kimchi (Toh et al., 2013). Similar results were discovered by Plessas and colleagues (2020), who found that the dominating LAB isolate was *L. paracasei* in Kefir grains. Pediococci and streptococci were isolated in equal amounts. Pediococci are commonly used in fermented meats, while the streptococci genus comprises various pathogens (Barnett et al., 2015; Seleshe and Kang, 2021). Our study highlighted the agricultural environment as a source of diverse LAB strains. This variation in the diversity of LAB isolates is comparable to that of Santos et al. (2019), where 11 strains of *L. paracasei,* four of *P. acidilactici*, and two of *L. zeae* were isolated from corn silage.

Owing to their benefits over other antimicrobials, bacteriocins have been explored for several applications. Notably, using bacteriocins and bacteriocin-producing LAB is an excellent method to target and inhibit pathogens that could harm livestock and poultry. Such pathogens include *E. coli* and *S. simulans,* which are regularly implicated in bovine mastitis (Andrade et al., 2021). In this study, when *L. delbrueckii* subsp. *bulgaricus* LMG 6901 was used directly as a primary indicator strain to check for bacteriocin production activity from isolates, it was found that neutralisation of the CFS resulted in the reduction of inhibition zone sizes, with CFS from six isolates unaffected by changes in pH, and two isolates completely losing inhibitory activity post neutralisation. However, activity was maintained in some isolates, such as *P. acidilactici* (D12). Broad-spectrum inhibitory activity against both Gram-positive and Gram-negative organisms was observed in this organism. *P. acidilactici* has attracted interest for its use in food as protective cultures or bio-preservatives due to its production of bacteriocins (Papagianni et al., 2006). *Pediococcus sp.* are known to produce pediocins, which are class 2 bacteriocins. A well-known bacteriocin from this group is pediocin PA-l, which is best recognised for its antimicrobial activity against *Listeria monocytogenes* (Kuniyoshi et al.,

2022). In our study, anitlisterial activity was not assessed, but *P. acidilactici* (D12) displayed inhibitory activity against *S. epidermis, S. lutetiensis, E. faecium,* and *L. fermentum*. Future research will have to focus on the additional characterisation of the bacteriocin produced by *P. acidilactici* (D12).

For bacteriocins from LAB to be commercially viable, it is preferred that they are amenable to large-scale production and manufacture in addition to maintaining activity after consumption through the GIT. After determining the antimicrobial spectra, we characterised the produced antimicrobial agents in terms of their protease sensitivity and heat tolerance. Protease sensitivity is a defining characteristic of bacteriocins and is vital because it reduces the persistence of bacteriocins in nature and the organic environment (Hols et al., 2019). This minimises the likelihood of unintended interaction with non-target bacteria, thereby reducing the emergence of bacteriocin resistance (Parada et al., 2007) and extending their applications. An exception is salivaricin D, which is naturally resistant to proteases such as trypsin (Birri et al., 2012). More recently, through partial characterisation, a bacteriocin from *L. plantarum* was found to be resistant to proteolytic enzymes by Zangeneh et al. (2020). The bacteriocins in our study were only tested for proteinase K sensitivity. Further characterisation of these bacteriocins should include testing for susceptibility to other proteases such as pepsin, trypsin, and chymotrypsin. Mass spectrometry analysis is a convenient way to identify known bacteriocins and discover novel bacteriocins by accurately determining the mass of the antimicrobial peptide (Zendo et al., 2008). This study showed that the isolates had peptides with masses ranging from 1101.36 Da to 4034.83 Da, except for the biomass of *E. thailandicus* (L16), which had a mass of 6310 Da. Low molecular weight bacteriocins (<5 kDa) such as those identified in this study typically belong to Class 1 bacteriocins, also named lantibiotics and are generally produced by Gram-positive bacteria. It is hypothesised that lantibiotics aid in the survival of the producing bacteria by inhibiting the growth and survival of potential competitive and sometimes pathogenic bacteria in a particular ecological niche (Barbour et al., 2016). The smaller bacteriocins (>5 kDa) are typically heat-resistant due to the presence of dehydrated amino acids, lanthionine and 3-methyllanthionine, which undergo post-translational modifications to generate the mature, structurally stable peptides (Meade et al., 2020; Simons et al., 2020). Prominent examples of lantibiotics include nisin, lacticin 481, lacticin 3147, and staphylococcin C55 (Chatterjee et al., 2005; McAuliffe et al., 2001). The isolation of bacteriocin-producing LAB adds to the arsenal of LAB that can be used against antimicrobial-resistant pathogens. The mechanisms by which lantibiotics exert their

antimicrobial activities are complex. The mode of action is to target lipid II (a cell wall precursor), inhibit cell wall biosynthesis and form pores in the target membrane (Cotter et al., 2005). It has been highlighted that bacteriocin production by LAB is greatly dependent on culture conditions (temperature, pH, growth phase, inoculum age, and size) and media composition (organic versus inorganic nitrogen and carbon sources) (Fahim et al., 2017) (Jawan et al., 2020; Yang et al., 2018). These factors can make bacteriocin screening tedious and time-consuming. *In silico* bacteriocin screening of one isolate (*E. thailandicus* (L16)), chosen based on its high activity units and superior antipathogen spectra, showed that this organism possesses the genetic machinery with a 98 and 95% similarity to that of subtilosin A and Enterocin, respectively according to Bagel4. *Enterococcus* spp. are well-known producers of enterocin, whereas subtilosin is a 35-amino-acid bacteriocin initially isolated from *Bacillus subtilis* by Babasaki et al. (1985). Future studies should focus on fully characterising the bacteriocin produced by *E. thailandicus* (L16).

Sixteen LAB strains were isolated from the ruminant environments. Of these, nine were *Lactobacillus,* five were *Enterococcus,* and one of each belonged to the *Pediococcus* and *Streptococcus* genera. CFS from six (*P. acidilactici* (D12), *L. mucosae* (D11), *L. paracasei* (D1 and L20), *E. thailandicus* (L16), and *E. hirae* (D17) isolates were unaffected by changes in pH as inhibition zones remained similar in size and intensity, pre and post-neutralisation indicating the inhibitory activity was not due to acid production. The bacteriocins produced by the LAB exhibited strong antimicrobial activity against several pathogens and were resistant to high temperatures. This study indicates that bacteriocin-producing LAB from the agricultural environment have the potential to be used as DFMs and for application in pathogen control. Bacteriocins and their producer LAB strains can be a safe alternative to antibiotics in animal feed. Further research is needed to purify these bacteriocins and study their characteristics in detail before their application as ruminant feed additives with antimethanogen potential.

Chapter four involved using some of the isolated and characterised strains in Chapter three, along with LAB from the APC culture collection (Ireland), commercial LAB strains from Sacco (Italy) and LAB received from AgResearch (New Zealand). This research chapter was initiated from the necessity to inspect the effect of bacteriocin-producing LAB on *Methanobrevibacter*, the primary producers of methane in the rumen. Because LAB already have many applications in the food and animal husbandry industry, their addition to the

ruminants' diets would be safe with minimal disturbance to the animal. *In vitro* studies are an important tool for preliminary screening of potential antimethanogenic compounds to determine suitability for evaluation *in vivo*. Very few studies are similar to our study, which have researched the effect of methane inhibitors on pure methanogen cultures. This may be due to the fastidious nature of methanogens and their strict anaerobic requirements. Hence, it can be challenging to prepare a specific culture medium for each methanogen species (Khelaifia et al., 2013). Methane production levels were analysed only from day three to day nine because the methane production curve showed methane to be actively produced at this time. Variations in response to different inhibitors were detected in the *Methanobrevibacter* species tested. CFS from *L. plantarum* (LP58) demonstrated outstanding inhibitory activity against *M. gottschalkii* and merits further studies to investigate the mechanism with which methane production is inhibited and to maximise the efficacy of the CFS against pure methanogens. However, adding supernatant from *L. plantarum* (LP58) did not significantly reduce methane production in *M. ruminantium*. Although these methanogens are classified under the same genus, *M. ruminantium* and *M. gottchalkii* belong to different clades (Kelly et al., 2016). Clade-specific sensitivity to different inhibitors has been observed in bacteria and viruses (Dick et al., 2022; Palucci et al., 2019). Analysing the whole genome can help determine the cause for differences in susceptibility in *M. ruminantium* and *M. gottschalkii*. *L. plantarum* (LP58) achieved 15% methane inhibition in *M. boviskoreani*. *M. boviskoreani* is a newly identified methanogen species (Lee et al., 2013). It was observed that the different *L. lactis* strains used in this study had varying results on methane inhibition in *M. boviskoreani*. This methanogen was most susceptible to *L. lactis* (KF245) CFS, where 47.7% methane inhibition was observed. *L. lactis* generally produces class 2a bacteriocins (Ouzari et al., 2008). Some LAB strains are known to produce multiple bacteriocins (Ennahar et al., 2003). This could explain the difference in methane inhibition as it could have been brought about by the different bacteriocins produced by the *L. lactis* strains. The addition of CFS from *S. equinus* strains inhibited methane production in *M. boviskoreani* and showed a trend toward being statistically significant. These strains were from the Hungate1000 collection and had been isolated from the rumen of young animals (Kelly and Waghorn, 2000). The absence of a statistically significant difference between treatments could be due to the small sample size. Although not characterised in this study, *S. equinus*, sometimes referred to as *S. bovis*, is known to produce the bacteriocin bovicin. Bovicin is a class 2 lantibiotic (Garsa et al., 2019), and its mechanism of action is the interaction with lipid II molecule, resulting in the inhibition of the bacterial cell wall synthesis

and, ultimately, pore-formation (Paiva et al., 2013). The role of bovicin in suppressing methane production under *in vitro* conditions has been investigated (Lee et al., 2002).

The purified peptide Lacticin 3147 and nukacin did not demonstrate significant methane inhibition in *M. ruminantium*. For this experiment, bacteriocins were added at a final concentration of 0.04 mg/ ml for lacticin 3147 and 0.2 mg/ml for nukacin 2922. The successful inhibition of methane *in vitro* using bacteriocins is dose-dependent (Callaway et al., 1997). It is therefore recommended that higher concentrations of nukacin and lacticin 3147 be tested in future studies. The two-component bacteriocin, lacticin 3147, produced by *L. lacti*s (DPC 3147), has been routinely shown to inhibit Gram-positive microorganisms, including mastitis-causing pathogens and *E. coli* (Kitching et al., 2019; Ryan et al., 1998; Ryan et al., 1999). However, there is only one known study where lacticin 3147 was investigated as a methane inhibitor (Jayanegara et al., 2014). As far as we know, our study is the first to test nukacin for its ability to reduce methane production in a pure culture of methanogens. The nukacin used in this study was produced by *S. warneri* (APC 2922), which was isolated from the skin of Irish volunteers (O'Sullivan et al., 2019). Bacteriocins can be used with other antimicrobial agents and physical methods known as the hurdle effect to enhance targeted microbial inhibition (Galvez et al., 2010). Using a combination of bacteriocins or using them in tandem with other antimicrobials to increase their inhibitory effects may also reduce the chances of developing resistance to the bacteriocins (Mathur et al., 2017). This property can be tested in future *in vitro* studies where nukacin and lacticin 3147 can be combined with other food-grade antimicrobials such as nisin to suppress methane production.

An important factor for *in vitro* methanogen inhibition assays is adequate microbial activity to support fermentation, which can be determined by measuring the absorbance of the culture. The growth rate of *Methanobrevibacter in vitro* was slow and produced low turbidity at $OD_{600,}$ with the highest turbidity being 0.2 observed in *M. gottschalkii*. The growth of these methanogens was likely limited by some nutrient or growth factor. Many researchers supplement growth media with rumen fluid to promote methanogen growth during *in vitro* studies (Altermann et al., 2018; Chen and Wolin, 1979; Leahy et al., 2010). Rumen fluid is undefined, and its composition may vary between animals (Mizrahi and Jami, 2018). Although it may have been beneficial in improving methanogen growth, rumen fluid was not used in this study to circumvent methane production fluctuation, which could reduce the reliability of the methane inhibition assays.

A limitation of this study is that methanogen growth was not measured in conjunction with the methane inhibition assays. This could have clarified whether the CFS inhibited methanogen growth or inhibited methanogenesis in actively growing strains. Furthermore, the cultivation conditions were optimal for the growth of *Methanobrevibacter* species, and these conditions did not necessarily mimic the physiological conditions of the rumen. Therefore, it cannot be stated with certainty if the efficacy of the peptides or CFS will be maintained if tested in the rumen environment. In a practical sense, the main limitation to the broader application of this assay and using bacteriocin peptides as enteric methane inhibitors is that a large quantity of bacteriocin powder may be required to have an effect on rumen, which is a large, complex and dynamic organ (Wright et al., 2004). Digestive proteases can inactivate bacteriocins. In some instances, LAB can reduce methane production *in vitro,* but this does not always translate to success *in vivo* trials, as observed by Jeyanathan et al. (2019; 2016) hence it is imperative to develop an efficient and economical method to inhibit enteric methanogenesis *in vivo*.

This study only focused on three hydrogenotrophic methanogen species, which do not fully represent all the rumen methanogens. Future studies should include methylotrophic and acetoclastic methanogens, which make up one-third of rumen methanogens. The general hypothesis on the mechanism by which bacteriocins or bacteriocin-producing LAB reduce methane is by targeting and inhibiting bacteria that are the hydrogen producers in the rumen. In this study, there was a direct inhibition of methane. Consequently, it would be advisable to use microscopy to investigate the mechanism by which methane production is suppressed. In the future, conducting these assays in an environment that better represents the rumen would be ideal. Because methanogens are quite challenging to culture due to their strict requirements for anaerobic conditions and long doubling time, *in silico* studies may be better suited for exploring methane inhibition in rumen methanogens. This study involved screening different bacterial groups for their ability to reduce methane and combined the cultivation of extreme anaerobes, peptide synthesis and GC use. Such knowledge and techniques can supplement the methods researchers currently use to study *in vitro* methane mitigation.

In the *in vitro* trial outlined in Chapter four, the methane inhibition efficacy of *L. plantarum* (LP58) was confirmed in *M. gottschalkii* but not in *M. ruminantium*. Purified bacteriocins did not impact methane production in *M. ruminantium*. According to the European Council, developed countries have to decrease GHG emissions by 80% by 2050 (Huyen et al., 2020). Finding methods to reduce methane *in vitro,* such as using CFS from *L. plantarum,* could help

decrease methane emissions' intensity (methane produced per unit of meat or milk) when applied to animal models. This, in turn, would create an environmentally sustainable and efficient food production system in Irish agriculture.

Upon silage creation, it is important to calculate the concentration of viable LAB to be applied, the efficacy of strains to be used and decide whether to use homofermentative or heterofermentative species for improved aerobic stability. In Chapter five, two LAB strains, *L. plantarum* (LP58) and *L. lactis* (SL242), supplied by the commercial partners (Sacco, Italy), were compared as silage inoculants in a lab-scale study to identify the extent of their influence on nutritional and fermentative properties. Information obtained from the preliminary lab-scale study was used to inform the design of the large-scale silage trial. LAB as silage inoculants offers the potential for control over the silage fermentation process. Silage inoculation is an excellent way to deliver LAB to ruminants. The large-scale silage involved the use of *L. plantarum* (LP58) and *L. lactis* (SL242) as co-inoculants, and this silage was fed to dairy cows in Chapter six.

In both our trials, the inoculants could survive in the silage for the duration of the fermentation period, as indicated by the viable colony counts on specific solid media. LAB viability is vital in silage because live LAB can be ingested as potential probiotics for ruminants. QPCR showed a gradual increase in *L. lactis* (SL242) DNA, whereas *L. plantarum* (LP58) remained stable throughout the fermentation and was present in the untreated silage samples. Strains of *L. plantarum* have been isolated from silage (You et al., 2021). Furthermore, this microorganism is an EFSA-approved technological additive intended to optimise the ensiling process (EFSA, 2017); therefore, it can be incorporated into Irish silage without hindrance. *L. plantarum* (LP58) is already commonly used as a silage inoculant, meaning its behaviour in silage is well documented and understood (Alhaag et al., 2019; Contreras-Govea et al., 2013; Yang et al., 2020). Similar to our large-scale silage trial, Xu et al. (2017) observed that the pH value of the silage co-inoculated with heterofermentative and homofermentative LAB reduced more drastically. A rapid drop in silage pH is desirable and generally correlates with lactic acid production. Incidentally, in our study, the lactic acid concentration was significantly higher in the LAB co-inoculant-treated silage than in the control silage, facilitating efficient silage fermentation. Furthermore, LAB-treated silage had higher acetic acid contents compared to untreated silage. Generally, for silage inoculation, homofermentative LAB strains are

preferable to heterofermentative LAB strains (Kim et al., 2021). However, in this study, strains were chosen for their preservation properties and capacity to produce bacteriocins. Low pH values in silage stimulate the release of nisin from *L. lactis* (SL242), which exhibits strong antibacterial activity against spoilage bacteria. The bacteriocin-producing inoculants did not negatively affect the epiphytic LAB communities involved in silage fermentation, as shown in Figure 4.6. This was an encouraging result because epiphytic microbial populations affect the results of silage inoculation trials and the subsequent animal trials (Filya et al., 2007).

Silage is an essential part of the cattle diet, especially in dairy cows in milk and meat-producing countries such as Ireland (Puntillo et al., 2020). Grass silage is commonly used as feedstuff in Irish farms and accounts for 20-25% of total annual feed in dairy farms. As discussed in the first chapter, various environmental factors influence the ensilaging process, including crop respiration, aerobic microbial and plant enzyme activity (Muck, 1988). Results from the two trials showed that LAB have a crucial role in deterring unwanted microbes such as yeast and Clostridia sp. Agarussi et al. (2019) confirmed that LAB inoculation decreased undesirable yeast growth in treated alfalfa silage. Several studies have found that some *L. plantarum* strains produce phenyllactic acid, which has been linked to antifungal activity (Deepthi et al., 2016; Russo et al., 2017). This could explain why the *L. plantarum* (LP58) inoculated silage had the lowest yeast counts. Grass for the treatment and control silage was wilted to ~25% DM before ensiling. One function of wilting is to reduce water activity and thereby minimise clostridial development (Pahlow et al., 2015). This could be the reason for the similarity in clostridia levels in the large-scale silage. Tight compaction is part of good silage management practices as it ensures that anaerobic conditions are achieved and prevents the proliferation of spoilage organisms. Anaerobic conditions were easier to accomplish in the large-scale silage and contributed to the overall quality of the silage.

One of the strengths of this study is that LAB were used, and these organisms are easy to apply silage, already known to be safe for animal and human consumption; therefore, there would be no issues with alteration of milk taste and quality from cows which consumed the LAB-treated silage. Additionally, this study's small-scale trial was used to inform methods to produce good quality silage. In some studies, only the final parameters such as pH, lactic, and acetic acid values after ensiling were used to assess the effect of LAB inoculants in improving the quality of silage fermentation (Huo et al., 2021; Jatkauskas et al., 2013; Kleinmans et al., 2011). Such

an approach does not account for the dynamic changes initiated by inoculants during the silage fermentation process.

LAB has been used for many decades as inoculants for the preservation of silages (Soundharrajan et al., 2021) and has consistently been relied upon to produce feed supply for ruminants. This means that introducing the use of *L. plantarum* (LP58) and *L. lactis* (SL242) as co-inoculants may be easily acceptable to Irish farmers and highly likely to be implemented. The economic benefit of using LAB as silage inoculants is that LAB minimise dry matter losses as they facilitate more efficient silage fermentation, which reduces feed cost for the farmer (Borreani et al., 2018). Furthermore, LAB are considered natural feed additives with GRAS status (Brashears et al., 2005), which will further contribute to the favourable endorsement of their use by farmers and consumers. Due to their probiotic effect, LAB silage inoculation may provide a cost-effective method for farmers as the ingestion of LAB- inoculated silage may improve animal health and productivity (Oliveira et al., 2017).

Chapter six describes how the silage created in Chapter five was subsequently used as a strategy to deliver LAB into the rumen of dairy cows to reduce methane production in the treatment group. We hypothesised that feeding ruminants with LAB-treated silage confers benefits to the animal by changing the levels of VFAs in the rumen, modifying the microbiome and rumen function, which subsequently results in a decrease in enteric methane production. The agricultural industry faces pressure to increase animal production, safety, and health while reducing its negative environmental impact. The European Green Deal has identified excessive methane emissions as an important and urgent issue requiring a swift reaction (Annual review and outlook for agriculture, food and the marine, 2021).

Methane production was significantly lower in cows on the LAB-treated silage diet between weeks three and seven of the study. Methane associated with ruminant livestock production makes up 68% of Irish agricultural GHG emissions (Teagasc, 2017). While the results of our study seem small, they can have a considerable impact when translated into real-life figures. A lactating dairy cow produces 263 ±10 grams of methane daily (Lassey et al., 1997). A reduction of 19.4%, as observed in this study between weeks 4-7, and if sustained, would have an impact on GHG emissions in Irish livestock farming. Methane is the second most significant contributor to GHG in Ireland and this is due to the large population of cattle. Therefore, the

use of *L. lactis* (SL242) and *L. plantarum* (LP58) to reduce enteric methane could help reach the Irish agricultural sector target of a 10% reduction in enteric methane emissions by 2030 (Lanigan et al., 2019). LAB as silage inoculants can be used with other methane mitigation strategies such as dietary additives, vaccinations and selective breeding of low methane-producing ruminants to reduce methane from agriculture.

Hydrogen unifies microbial fermentation and methanogenesis in the rumen. Our study found that the decrease in methane was coupled with a reduction in hydrogen expelled. Hydrogenotrophic methanogenesis is the largest hydrogen sink in the rumen. Therefore, an alternate hydrogen sink is needed to redirect hydrogen, preferably into an energy-yielding pathway when methanogenesis is inhibited. This could theoretically improve animal productivity (Martinez-Fernandez et al., 2016). Greenfeed was used to measure methane and hydrogen emissions from individual cows. Although the SF_6 tracer technique is regarded as the gold standard, Hammond et al. (2015) found that the accuracy of the Greenfeed is comparable to the SF_6 tracer technique and holds additional benefits such as being non-invasive and not causing pain or discomfort to the animal being tested (Hristov et al., 2015).

The digestion of polysaccharides into monosaccharides and, ultimately, VFAs is accomplished by various interactions amongst specialised rumen microbes and several chemical reactions. The degradation of microbial feed results in various VFAs, mainly acetate, propionate, valerate, butyrate, and hydrogen (Bach Knudsen, 2015). A relationship exists between certain groups of bacteria, VFAs and methane production. For example, in this study, *Prevotella*, associated with propionate production in literature (Chen et al., 2017; Poeker et al., 2018; Ren et al., 2021), increased by 3% in the treatment group. This correlated with increased propionate (a competitive pathway to methanogenesis) and decreased methane production. Propionate production likely functioned as a hydrogen sink in this study. Although lactate was not detected in high concentrations in the rumen fluid of the cows in both groups, it can be argued that the addition of lactate-producing LAB increased this secondary metabolite, which can be converted to propionate. This could have further contributed to the decrease in enteric methane production observed in the treatment group.

Research has revealed that *Methanorevibacter* dominate the rumen, regardless of ruminant diet or geographical location. Therefore, they are potential targets, which could be inhibited to

reduce methane production in the rumen. Even though it was not clear why, it was unexpected to observe a higher prevalence of Methanobacteriaceae in the treatment group. This outcome is contrary to Oskoueian et al. (2021), who found that the LAB treatments decreased methanogens. Because rumen microbes exist in a symbiotic relationship with each other and the host ruminant, a future study could include shotgun metagenomic profiling as this technique could reveal the links between microbial genes and methane emissions as described by Roehe et al. (2016).

The main strength of this study is that methane reduction in ruminants was achieved using LAB strains, which are already well-documented silage inoculants with probiotic characteristics. The effective reduction of enteric methane emissions from livestock has societal benefits in that it may help ease consumers' concerns about the environmental impact of animal production and may increase consumer acceptance of animal-based products (Vijn et al., 2020). Dairy production in Ireland is economically sustainable (Dillon et al., 2018). Therefore, instead of reducing the dairy herd as suggested by Emmet-Booth et al. (2019), it would be advisable to incorporate LAB as silage inoculants for enteric methane reduction, as demonstrated by our study. Successful implementation of climate change mitigation measures largely depends on farmers' acceptance and adoption (Kreft et al., 2021). The use of LAB to reduce methane as an option that contributes to GHG emissions reduction while increasing farm productivity due to probiotic properties is more likely to be adopted than practices which would negatively impact the farmer's income (Smith et al., 2008). Emissions in Irish agriculture are projected to continue increasing by 9% in 2030 due to expanding dairy herd if no mitigation strategies are employed (Lanigan et al., 2019; Läpple et al., 2021). Because methanogenesis is not nutritionally beneficial to the ruminant, its reduction could enhance animal prodctivity, which would offset national methane emissions. Furthermore, methane has a short half-life of 12 years; therefore, even moderate decreases in the rate of emission of methane would have a noticeable impact on Ireland's contribution to climate change.

In summary, the bird GIT and agricultural environment are a source of diverse LAB strains dominated by *Lactobacillus* species. LAB was used to reduce methane production *in vitro* and create good quality silage, which decreased methane emissions when fed to lactating dairy cows. This study is in keeping with Ireland's Food vision commitment to reduce methane by at least 10% by 2030 (Smyth, 2021).

Conclusion

This thesis covers a broad range of topics centralised on LAB and the animal GIT. It describes how *L. kimchicus* was isolated, identified, and characterised as a potential bird probiotic using various *in vitro* and *in silico* methods. There is a constant need for antimicrobial agents and producers that can be used in animal feed and other applications in the agricultural industry. This need justifies the research on novel antimicrobial alternatives such as bacteriocins. Data showed the agricultural environment as a source of LAB, dominated by *Lactobacillus* species with bacteriocin-producing abilities. Subsequently, an investigation on using CFS and bacteriocins from LAB to reduce methane production in *Methanobrevibacter* found that *L. plantarum* (LP58) was most effective in reducing methane from *M. gottschalkii* while individual strains of *L. lactis* affected methane production in *Methanobrevibacter* differently. Purified bacteriocins nukacin and lacticin 3147 did not influence *in vitro* methane production. The nutritive and fermentative properties of *L. plantarum* and *L. lactis* were detailed as silage co-inoculants and these inoculants enhanced the beneficial microbes in the silage and resulted in silage with significantly higher lactic acid concentration. Finally, an animal feeding trial showed significant methane reduction in the dairy cattle fed LAB-treated silage. Gaining insight into the different ruminotypes can enable more targeted approaches for methane mitigation. An investigation of the role of LAB in modifying the rumen microbiome and metabolome showed that LAB positively impacts the rumen by significantly increasing acetate and butyrate production while significantly decreasing Proteobacteria. Methanogen levels remained unaffected.

Livestock farming is a significant sector in the Irish economy as it creates jobs, enables export, and is an intricate part of Irish culture. Although it will be challenging for Irish agriculture to meet its climate targets, concerted efforts, such as using LAB as enteric methane mitigators, can enable Ireland to reach its target of reducing atleast 10% of its GHG emissions by 2030. If overall methane emissions are lowered, the national dairy herd can be maintained or increased, and jobs can be created while continuing to supply meat and milk to the growing world population. Future research will focus on finding even more potent methane reducing LAB and characterisation and elucidating the mechanism of methane inhibition *in vitro* using LAB CFS.

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Supplementary information/ Publications.