New antimicrobials to target gut and food pathogens

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ABSTRACT

There is a pressing need for the discovery of new antimicrobials to fight antibiotic resistant bacteria. The aim of this thesis was the discovery and characterisation of new bacteriocins from two sources, fermented foods and human faeces, testing the hypothesis that bacteria from the same niche will produce antimicrobials uniquely suited to act in this niche. Isolates from culture collections and new isolates from food and faecal samples were screened against a panel of pathogens responsible for food spoilage and human disease. Promising candidates were selected for genome sequencing, antimicrobial characterisation and biological study. The genome of *Lactobacillus gasseri* LM19 showed the presence of antimicrobial genes encoding, among others, a new bacteriocin, gassericin M. *L. gasseri* LM19 could survive and express its bacteriocin genes under colonic conditions. Its administration modulated the effects of *Clostridium perfringens* on the gut microbiota composition. *Streptococcus agalactiae* DPC7040 was previously shown to produce the natural variant nisin P. MALDI-ToF analysis confirmed that nisin P is three amino acids shorter than nisin A and that two lanthionine rings were absent in 50% of molecules. This structure impacted on its antimicrobial activity, which was weaker than that of nisin A and nisin H in faecal fermentations. *Staphylococcus epidermidis* strains isolated from faecal samples were compared with skin isolates with respect to genomic and phenotypic traits. It was concluded that *S. epidermidis* has no specific genomic features to colonise different body sites but is likely to adapt its metabolism to the different conditions. Potential novel antimicrobials were found in *Lactobacillus amylovorus* and *Lactobacillus crispatus* isolates, which showed probiotic properties and interesting phenotypic differences between strains. Together, this work further demonstrates that fermented food and gut environments are valuable sources of new isolates, the study of which can yield new antimicrobials and give insights into bacterial ecology and evolution.

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Outputs of this Project

Publications

• Garcia-Gutierrez, E., Walsh, C.J., Sayavedra, L., Diaz-Calvo, T., Thapa, D., Ruas-Madiedo, P., Mayer, M.J., Cotter, P.D., Narbad, A. Genotypic and phenotypic characterization of fecal *Staphylococcus* epidermidis isolates suggests plasticity to adapt to different human body sites. Under review.

• Garcia-Gutierrez, E., O'Connor P.M., Saalbach, G., Walsh, C.J., Hegarty, J.W., Guinane, C.M., Mayer, M.J., Narbad, A., Cotter, P.D. First evidence of production of the lantibiotic nisin P. Under review.

• Garcia-Gutierrez, E., O'Connor P.M., Colquhoun, I.J., Miguel-Vior, N., Rodriguez, J.M., Mayer M. J., Cotter P. D., Narbad A. *Lactobacillus gasseri* LM19, isolated from breast milk, exhibits antagonist activity against enteropathogens. Under review.

• Garcia-Gutierrez, E., Mayer M. J., Cotter P. D., Narbad A. (2018). Gut microbiota as a source of novel antimicrobials, Gut Microbes, 10:1, 1-21, DOI: 10.1080/19490976.2018.1455790

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• Garcia Gutierrez E., Saalbach G., O'Connor P.M., Hegarty J. W., Mayer M. J., Narbad A., Cotter P. D. (2019) Nisin P structure International Meeting of Antimicrobial Peptides IMAP 2019. Utrecht, Netherlands. Poster presentation.

• Garcia Gutierrez E., O'Connor P.M., Mayer M. J., Cotter P. D., Narbad A. (2019) Bacteriocin production and other probiotic traits of food and gut isolates 13th International scientific conference on probiotics, prebiotics, gut microbiota and health IPC2019. Prague, Czech Republic. Poster presentation.

• Garcia-Gutierrez E., Mayer M. J., Cotter P. D., Narbad A., Ruas-Madiedo P. (2018) Anti-biofilm features of bacteriocin-producing bacteria isolated from the human gut. 6th International Symposium on Antimicrobial Peptides 2018. Poitiers, France. Poster presentation.

• Garcia-Gutierrez E., O' Conor P., Mayer M. J., Cotter P. D., Narbad A. (2017) Bacteria strike back: a coevolution tale in the quest for antimicrobials. International Scientific Conference on Probiotics and Prebiotics IPC2017. Budapest, Hungary. Poster presentation.

Abbreviations

ACN	Acetonitrile
BHI	Brain heart infusion
BSA	Bovine serum albumin
BSH	Bile salt hydrolase
C-terminal	Carboxy-terminal
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DTT	Dithiothreitol
EB	Elution buffer
EMA	Ethidium bromide monoazide
FPLC	Fast protein liquid chormatography
gDNA	Genomic DNA
GIT	Gastrointestinal tract
HPLC	High pressure liquid chromatography
HPMC	Hydroxypropil methylcelulose
IAA	Iodoacetamide
IPA	70% propan-2-ol, 0.1% TFA
kb	Kilobase
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography-mass spectrometry
MRS	De Man-Rogosa-Sharpe
MS	Mass spectrometry
NaCl	Sodium chloride
NMR	Nuclear Magnetic Resonance
NMR	Nuclear magnetic resonance
N-terminal	Amino-terminal
o/n	Overnight
OD	Optical density
OFF	Open reading frame
PCR	Polymerase chain reaction

rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RT	Room temperature
SCFA	Short chain fatty acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TEAB	Triethylammonium bicarbonate buffer
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TFA	Trifluoroacetic acid
UPH_2O	Ultra-pure sterile water
UV	Ultraviolet
WGS	Whole genome sequencing

Symbols

%	percentage
^{o}C	degrees Celsius
±	plus/minus
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
bp	base pairs
d	days
g	gram
h	hours
l	litre
min	minutes
M	molar
mg	milligrams
ml	millilitre
mM	millimolar
mm	millimetre
ng	nanograms
nm	nanometre
nM	nanomolar
pH	potential hydrogen
ppm	parts per million
rpm	revolutions per minute
s	seconds
v/v	volume per volume
w/v	mass per volume
x g	times gravity

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CHAPTER I

General Introduction

1.1. The antimicrobial resistance problem

Antimicrobials are substances that have the ability to kill or inhibit the growth of microorganisms. They have been used as preservatives and as a way of managing infections over centuries. The presence of tetracyclines has been reported in histological studies in ancient bones in Egypt and traditional knowledge relating to the use of antimicrobials has been transmitted over generations in Chinese medicine, Greco-Roman and Hindu cultures [1]. However, in the pre-antibiotic era, events involving a microbial infection had considerable potential to have fatal consequences and it was not until the discovery of penicillin, the first natural antibiotic in 1928, that humankind experienced a significant antimicrobial-mediated improvement in life quality, reflected in an increase in life expectancy and the absence or minimisation of sequelae after infectious processes. Unfortunately, prescription policies are not always adhered to and there are many examples of antibiotic misuse by humans [2, 3]. Some antibiotics can be used as prophylactics, and they were provided to animals in farms in order to avoid epidemics and accelerate growth until 2006. [3]. Agricultural production is another big market for antibiotic use [3]. All of these factors have increased the appearance of antibiotic resistance. Resistance is manifested by an antibiotic not being effective against a certain pathogen at a certain dose. This is brought about by the fact that bacteria have developed different ways to avoid the effect of antibiotics, including modifications that decrease the ability of the antibiotic to penetrate through the cell envelope, efflux pumps that take the antibiotic out of the cell, targeted modification and enzymatic inactivation of the antibiotic, among others [4]. These resistance traits can be products of an innate resistance or occur by acquisition of resistance. This acquisition might be by spontaneous mutation (especially in membrane receptors and targets) or by incorporation of foreign DNA (plasmid exchange, transformation or transposons). Pathogens frequently exchange genetic material in order to gain ecological fitness, but these resistance genes do not necessarily come from other pathogenic bacteria, they can also be in a reservoir of commensal cells, forming what is known as the ‘resistome’ [5, 6]. The use of broad-spectrum antibiotics instead of more target specific antibiotics is one of the causes of the antibiotic resistance problem [7, 8]. Multidrug resistant (MDR) [9], extensively drug resistant (XDR), and pandrug-resistant (PDR) organisms [10, 11] are a product of this situation. Some of them are also known as ‘ESKAPE’ organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,

Acinetobacter baumannii, *Pseudomonas aeruginosa*, and *Enterobacter* species). Right now, the treatment approach in these situations is the use of a combination of different drugs, but effectiveness of this solution is also becoming more limited. Unfortunately, the dearth of new antibiotics is further exacerbating the situation. Since the 1980's, very few classes of new antibiotics have been discovered and, as a general rule, the new antimicrobials on the market are modifications of the pre-existing ones [12]. Challenges with the development of new antibiotics include the need of sourcing new compounds, identifying novel antibiotic targets (ribosome, cell wall synthesis and DNA gyrase or topoisomerase are the current ones) and the challenge of breaking through the bacterial envelope, especially in Gram-negatives [9]. Persisters are also another important challenge. Antibiotics can only work in the presence of active targets, but persisters are dormant cells produced stochastically during bacterial growth that are not active, and therefore antibiotics cannot be effective on these cells. All pathogens studied have shown the ability to form persisters and the pathways for their formation are highly redundant [13, 14]. Similarly, spore formation is another challenge since spores are more resistant to antibiotics than the corresponding vegetative cells. Some spore-forming organisms are pathogenic and also cause food spoilage [15]. Biofilms can be considered another challenge in antibiotic resistance. It is more common to find bacteria in biofilm communities rather than as isolated cells. These biofilms provide advantages to bacteria because cells share molecules like enzymes, siderophores and extracellular polymers. Biofilms offer bacteria advantages against dehydration, predators and antibiotics, and are a major problem in healthcare, contributing to persistent infections [16, 17].

It is necessary to develop platforms that allow a systematic strategy to have an efficient search for new antibiotics. The combination of old and new strategies and the incorporation of new tools could be the key to identify and develop prodrugs, biologically inactive compounds that require metabolisation to activate, and/or species-specific compounds and overcome previous challenges, like the 'great plate count anomaly', that states that only 1% of microorganisms present in natural samples are able to be cultured, limiting antibiotic discovery. Screening of natural sources in combination with genomics and transcriptomics could help with silent operons and could enable drug discovery from microorganisms that have previously gone uncultured [4, 12, 18].

Antibiotic resistance is a serious problem. The World Health Organisation (WHO) has predicted a huge rise in deaths by infections due to resistant microorganisms, reaching 10 million deaths per year by 2050 [3]. Given the time necessary to develop an effective drug, including the associated clinical trials, considerable efforts in novel antibiotic discovery/development is needed in the coming years.

1.2. Microbial ecology

Most of the antibiotic compounds found during the 'golden era' of antibiotics discovery (1940-1960) came from natural sources, specifically, soil-derived actinomycetes [9]. Natural environments continue to be reliable sources for new compounds of interest [9, 12]. Natural environments can be considered as balanced ecosystems, and within them, living organisms establish different relationships that can be positive, negative or neutral. An example of positive relationship is 'mutualism', where both parties are benefiting from the interaction. The opposite end is 'competition' and usually happens when two parties share the same resources in the same niche, experiencing a decrease in their ecological fitness because the access to resources is limited. Competition can be interspecies (between different species) and intraspecies (between individuals of the same species). Among microorganisms, competition for resources can very intense and antimicrobials producers can gain a considerable advantage. Other hypotheses contend that antimicrobials might have an additional social purpose, serving as signals or even promoting biodiversity [19, 20]. Like many compounds, the production of antimicrobials is likely to be linked to a phenomenon known as 'quorum sensing', related to population density. Bacteria are very likely to live in communities and this quorum sensing is how they regulate their gene expression in response to biotic or abiotic stress [21, 22].

1.3. Bioactive potential of the human gastrointestinal tract (GIT)

The human body hosts a rich microbiome consisting of many different microbial ecosystems. Trillions of microorganisms have co-evolved with us over time and the richest microbial communities are found in the gastrointestinal tract, skin and urogenitalia, although microbial communities are also present in lungs, placenta, breast milk and eyes [23]. Additionally, some of these communities may be related. For example, the transference that occurs between the oral and gut microbiota has been linked to the development of a number of conditions [24, 25]. The presence of an entero-mammary pathway has also been suggested [26], the existence of which could be critical for the early

colonisation of the human gut and for future systemic development [27]. Because of this microbial vastness, there remains much to be learned about the human microbiome. However, it is clearly a potentially significant source of metabolites, including broad and highly specific antimicrobials [28]. At the moment, this metabolite variety includes production of lipids and glycolipids, oligosaccharides, terpenoids, polyketides, amino acids, nonribosomally synthesised peptides and ribosomally synthesised post-translationally modified peptides (RiPPs). Although relatively unexplored, the studied compounds showed different functions, such as immunomodulation, cytotoxicity, antioxidant activity and, of course, antimicrobial capacity [29]. For these reasons, the human microbiome, more specifically, the human GIT, constitutes a promising source of new antimicrobials.

1.3.1. Conditions and microbiota composition in the human GIT

The diversity of microbial species in the human GIT can be linked to the variety of environmental conditions present therein. Several factors influence the abundance and diversity of microorganisms in the gut:

1.3.1.1. Geography. The GIT is not uniform. There is a small and a large intestine with different functions and special structures. The small intestine is composed of the duodenum, jejunum and ileum and its main function is absorption of nutrients and chemical digestion. The large intestine is mainly represented by the colon, but has other structures i.e. the caecum, appendix and rectum. Its functions include the final absorption of water and salts and the fermentation and breakdown of the previously undigested products. The caecum and appendix have been described as reservoirs of microorganisms that might have an immunomodulatory function. The interior of the GIT is also varied, the surface of the epithelium presents crypts in the small intestine and the colon, where microorganisms are more protected than in the lumen from the flow of nutrients [30]. Both have a mucus layer composed of mucins, glycoproteins of high molecular mass, that obstruct the access of the microbial cells to the host cells, but also can be a nutrient source to certain bacteria.

1.3.1.2. Temperature. Temperature is one of the most important factors regulating gut microbial composition and growth. It affects important aspects like pH, water activity, ion activity, viscosity, hydration and aggregation of macromolecules, toxic action of metabolites and solubility of gases (especially oxygen and carbon dioxide). The small and large human intestine have an average temperature of 37°C. This is essential for the

optimal performance of enzymes and the breakdown of indigestible fibres. This temperature will select mesothermic microorganisms to live within the gut.

1.3.1.3. Gas composition. The gas mixture in the GIT has a microbial origin, with the exception of N_2 and O_2 that originate from swallowed air. This swallowed air has an influence on the upper regions of the intestinal tract. There is a decreasing gradient of oxygenation from the proximal to the distal part of the small intestine that ultimately will determine the distribution of aerobic and anaerobic bacteria. Oxygen tolerant bacteria and facultative anaerobes will be present in small intestine [30]. In the colon, fermentation processes make it a hypoxic environment and the gases such as H_2, CO_2, H_2S and CH_4 are present, as a result of the different processes carried out by fermentative bacteria and hydrogenotrophs, their symbiotic counterparts. Archaea are responsible for methane production [31].

1.3.1.4. pH. pH is not constant across the gastrointestinal tract. The proximal part of the small intestine is more acidic due to its proximity to the stomach and the presence of bile acids, while the pH increases progressively through the rest of the GIT, being highest at the descending part of the colon and the rectum, where it reaches over 6.5. In regions of the gut where the presence of microorganisms is higher, products of microbial fermentation also influence the pH.

1.3.1.5. Osmotic effects. Osmolarity depends on the type of food eaten and the region of the gut. Osmotic pressure is one of the main factors that control emptying of the stomach and the outcome is a mixture with the isotonic gastric secretions [32].

1.3.1.6. Surface tension. Surface tension is one of the parameters that affect the physiology of bacteria [32] and is variable across the different regions in the gut, mainly because of the effects of fat digestion and bile secretions at the proximal end of the small intestine. It has an influence over other parameters, like the redox potential. Food and additives also play a major role over this parameter.

1.3.1.7. Nutrients. Food ingested will be the main source of nutrients for the microorganisms that inhabit the gut [33]. Nutrients that arrive to the gut might be digestible or indigestible, and the proportions of which will influence the composition of the microbiota. Primary ingested foods will be used by certain species to obtain nutrients, and the products generated by this metabolism will serve as substrates for other species.

Bacteria also synthesise compounds that are beneficial for the host, in the case of humans for example, B complex vitamins.

1.3.1.8. Liquid flow. Flow rates are not constant across the gut, being controlled by the chemical and physical nature of the food, drink, secretions in the gut and absorption that removes products. Liquid flow influences the degree to which certain microbes are washed out of the gut. Organisms that are better able to adhere to the epithelium and mucus layer will be more protected and more likely to be successful in the colonisation of the small intestine [30, 32].

As a result of the variation of all of these parameters, the gut microbiota is heterogeneous across the GIT, but it is also heterogeneous through time. From the moment of delivery, the GIT begins to be colonised and changes until it stabilises at the third year of life [34, 35]. After this, the composition remains constant and tends to recover after acute changes, like use of antibiotics [30] but the influence of long-term habits, such as consumption of a specific type of diet, are more difficult to overcome if trying to modulate microbe composition and proportion [34]. Because of the considerable variability in microbial composition across individuals, defining what exactly constitutes a healthy GIT microbiome is a difficult challenge [23]. However, a functional approach has provided more promising results in this area. This is rooted in the ability of many components of the microbiota to present redundant functionality beyond the specifics of species composition [35]. Regardless, both approaches, i.e., determining taxonomies and identification of functional patterns, continue to undergo further development [36]. Cultivability problems might improve by culturomics approach, pointed as a solution to fill gaps left by traditional cultures and culture-independent techniques [37-39]. There are other strategies that do not rely on microbial cultures, such as metagenomic profiling [35, 40, 41], that allow an accurate compositional characterisation from stool samples and can help to identify microbial groups and functions related to healthy and dysbiotic metagenomic profiles and specific diseases and syndromes. However, non-abundant groups can be under represented [39] suggesting that a combination of techniques might be the best approach for a reliable characterisation of the human microbiota. Also, most of the studies carried out regarding microbiological composition of the gut have been performed using stool samples, and that might imply a misrepresentation of gut mucosal microbiota [30, 42].

In terms of composition, the most abundant bacterial phyla in the gut are Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia [30]. There are also Archaea and Eukarya representatives and viruses [36]. In general, colonisation of the small intestine is led by groups of facultative anaerobes able to grow fast and tolerate the effects of bile acids, like Firmicutes (Lactobacillaceae) and Proteobacteria (Enterobacteriaceae), favoured by lower pH and higher oxygen levels and antimicrobial compounds (both of host and bacterial origin). Once in the colon, when pH increases, and oxygen levels and antimicrobials decrease, the transit time is slower and with the presence of easily digestible nutrients there is a proliferation of fermentative organisms able to degrade more complex compounds, like polysaccharides and fibre. Bacteroidetes group (Bacteroidaceae, Prevotellaceae and Rikenellaceae) in the lumen and Firmicutes (Lachnospiraceae and Ruminococcaceae) in the inter-fold regions are the most common organisms.

1.3.2. Antimicrobial activity in the gut

Microbes in the gut employ a range of different strategies to compete for the resources in the environment. Some of them are direct, such as competitive removal of essential substrates, accumulation of D-amino acids, and lowering of the oxidation-reduction potential. Other strategies involve the production of metabolites with the ability to limit the growth of surrounding bacteria. This activity can be classified as specific, such as the case for some bacteriocins, or non-specific, if the regulatory agent does not address a specific target. In the latter case, activity might have other impacts, beneficial or undesirable, in the host. Some of the compounds that possess non-specific antimicrobial properties are summarised below.

1.3.2.1. Unspecific antimicrobial activity

1.3.2.1.1. Hydrogen peroxide

Hydrogen peroxide consists of two atoms of hydrogen and two atoms of oxygen. It is an example of a ‘reactive oxygen species’, and although these are by-products of oxidative cell metabolism, it has been suggested that its production might have a biological purpose itself [43]. Hydrogen peroxide can be produced by pathogens and opportunistic species, species with probiotic properties and commensal microbiota. Usually, microbial species that accumulate hydrogen peroxide are able to adapt their energy metabolism to anaerobic environments, but in the presence of molecular oxygen, hydrogen peroxide and other

partially reduced oxygen species can be produced, when this molecular oxygen captures electrons from the enzymes involved in electron-transfer, mainly flavoenzymes [44]. Hydrogen peroxide is the result of central carbon and energy metabolism, oxidases are a pivotal part of its production: pyruvate oxidase (Pox), lactate oxidase (Lox) and NADH oxidases (Nox), but depending on the producing species, the importance of each specific oxidase in the production will vary [43]. However, specific reactions and enzymes that catalyse hydrogen peroxide formation are still being characterised [43, 44]. The absence of enzymes that scavenge hydrogen peroxide, such as catalase and NADH peroxidase, is also an important disseminated characteristic of these producers.

Traditionally, hydrogen peroxide is well known for its antimicrobial properties. This activity is based on the oxidising effects that hydrogen peroxide has over the bacterial cells and their molecular structures. Therefore, DNA and protein repair system are used by cells to minimise its effect. Those systems were reviewed by Imlay [44]. Hydrogen peroxide regulation is not very well characterised in gut environments and it has been suggested that it contributes to maintaining a homeostatic microbiota. It is known, though, that some lactobacilli, streptococci and enterococci can produce relative high amounts of hydrogen peroxide and that these amounts can be similar to those released by phagocytic cells [45]. Production of hydrogen peroxide can be an asset in certain environments, but that does not mean that it is beneficial at all times as its activity is not only toxic for bacteria, but also for human cells [46]. Strus *et al* [45] reported differences in gut microbiota composition and abundance in chronic inflammatory lesions in colons of people affected by Crohn's disease and ulcerative colitis, with aerobic streptococci and lactobacilli, respectively, being more abundant. These might lead to an increase of hydrogen peroxide concentration, exacerbating harm of the epithelium [45]. Despite the fact that the gut is mainly an anaerobic environment, the presence of oxygen gradients close to the mucosal surface has been proven [47], which contributes to harm the epithelium.

It has also been noted that the antimicrobial activity of hydrogen peroxide seems to work synergistically with other compounds produced by bacteria available in the environment, such as lactic acid. This may be explained by an ability of lactic acid to induce sensitivity or permeabilise bacterial membranes, thus facilitating hydrogen peroxide diffusion [48].

1.3.2.1.2. Organic acids

An organic acid has carboxyl groups, sulfonyl groups and sometimes alcohols that promote their acidic properties. Short-chain fatty acids (SCFAs) are saturated aliphatic organic acids with less than six carbon atoms, such as formate, acetate, propionate, lactic acid and butyrate. Their activity is highly influenced by the conditions in the environment that surrounds them. One of the factors is pH, since they are mainly weak acids and both dissociated and undissociated forms are common. Their antimicrobial mode of action is not fully understood, but it is believed that the undissociated form of the organic acid crosses the lipid membrane with dissociation occurring once inside in the neutral pH, producing ions that affect the homeostasis of the cell, causing stress. Another level of action for organic acids is at the cell membrane, where they interfere in the regulation of transport at the membrane and cell wall function. These activities of organic acids have resulted in their use in food conservation against foodborne pathogens [49].

SCFA origin in the gut is through fermentation of resistant carbohydrates that have not been digested in the small intestine. This fermentation takes place mainly in the colon using substrates such as starches, dietary fibre, sugars and alcohols among others [50], including protein and amino acid fermentation. The type of starch is very likely to have an influence in the production and proportion of SCFAs [51]. This process provides extra energy to the host. SCFAs are produced in millimolar concentrations in anaerobic conditions. It has been reported that acetate production is common among gut bacteria, but propionate and butyrate production is not as extended.

SCFAs are considered to be very important as regulators of different functions in the human gut. They impact inflammatory processes and are an important part of glucose homeostasis. Immune processes, along with lipid metabolism and appetite regulation, are also influenced by SCFAs [52]. Lactic acid is produced by lactic acid bacteria and it is one of the most important products of their carbohydrate metabolism, either following a homofermentative or heterofermentative pathway. Lactic acid bacteria include a variety of groups: lactobacilli, carnobacteria, streptococci, lactococci, enterococci, leuconostoc, pediococci, aerococci and tetragenococci. Lactic acid has an inhibitory activity against a wide range of bacteria, including Gram-positive and Gram-negative targets. Recent studies [53, 54] proved that low concentrations, 0.5% (v/v), were enough to have an inhibitory effect on *Salmonella*, *E. coli* and *L. monocytogenes* over a two hour exposure period. SDS-

PAGE confirmed that there was a leakage of proteins from the target cells, meaning that cell membrane damage is the main physiological effect.

1.3.2.1.3. Other non-specific inhibitory compounds

Many of these inhibitory compounds have a fermentative origin and are products of the degradation of other compounds, like lactic acid or ethanol, that ultimately become SCFAs, CO_2 and H_2. Carbon dioxide exhibits antimicrobial activity by lowering intra- and extracellular pH and its disrupting effect on the cell membrane, and by replacing the molecular oxygen leading to a transformation of the surrounding atmosphere into an anaerobic environment.

Another fermentation product is diacetyl or 2, 3-butadione. It is produced by some species of *Lactobacillus* and *Pediococcus*, among others. The antimicrobial ability of diacetyl has been described previously [55]. Gram-negative bacteria and fungi seem to be the most affected by diacetyl activity, followed by yeast and Gram-positive non-lactic acid bacteria. Lactic acid bacteria have shown the less susceptibility to its action, at any pH. This effect over Gram-negative organisms might be explained because diacetyl interferes with arginine-binding proteins of Gram-negative bacteria. The activity of diacetyl is influenced by pH, requiring different optimums to act against different organisms [55]. Its use in combination with other compounds, such as reuterin, makes it act synergistically [56]. Diacetyl has been studied mostly as a food preservative.

Protein and amino acid fermentation can lead to the production of ammonia and amines. Ammonia can be formed by oxidative or reductive deamination of amino acids while amines are the result of decarboxylation and in an acidic environment can form nitrogen (N-nitroso) compounds. There are amino acids that are of special interest, like cysteine and methionine, whose degradation derives in the generation of H_2S. This compound is also generated by sulphate-reducing bacteria (SRB) using lactate, ethanol, succinate and H_2. Aromatic amino acids also produce phenols and indoles.

1.3.2.2. Specific antimicrobial activity

1.3.2.2.1. Peptidic antimicrobial activity

Bacteria can produce target-specific antimicrobial peptides. Antimicrobial peptides are classified based on their biosynthesis, which can be ribosomal or non-ribosomal. Typically, antimicrobial peptides are 10–50 amino acids in length and their ability to kill bacteria

depends on their interaction with bacterial membranes and cell walls, and so does their selectivity. The identification and direct isolation of those compounds is difficult. Recently, a bioinformatics tool, ClusterFinder, has been able to identify thousands of biosynthetic gene clusters from human metagenomic samples encoding antimicrobial peptides, among other compounds. This highlights the potentially underexploited therapeutics that can be sourced from within the human microbiome.

1.3.2.2.1.1. Non-ribosomal peptides (NRPs)

NRPs are synthesized by multienzyme complexes of multifunctional peptide synthetases and constitute secondary metabolites. NRPs are a large class of bacterial products and they are present in several environments. Their study has provided many of the antibacterial compounds currently in use, such as vancomycin or polymyxin, that are considered as peptide derived. However, there are very few NRPs characterised from the human microbiota. The peptides cereulide and zwittermicin, both produced by pathogenic *Bacillus cereus*, and tilivalline, produced by pathogenic *Klebsiella oxytoca*, were isolated initially from bacteria isolated from the human gut, although they are not common human gut microbiome representatives [28]. Their activity has been described as cytotoxic [28]. Zwittermicin activity has been reported as antimicrobial but resistance genes for it have also been identified [28]. Therefore, the use of NRPs as therapeutics would be limited.

1.3.2.2.1.2. Ribosomally synthesised peptides (bacteriocins)

Bacteriocins are ribosomally synthesised antimicrobial peptides. Although they are not referred to as traditional antibiotics, they do have antibiotic activity and they have been proven to provide an ecological advantage to the producing strains in order to survive in highly competitive environments [57]. Bacteriocins are important in bacterial population dynamics. They are found to be very effective and specific against competitors from the same environment as their producers and many of them have been discovered by screening a variety of natural environments [58-66].

1.4. Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria. They constitute a heterogeneous group that include many different subgroups. The first bacteriocin was discovered by Andre Gratia in 1925 and was named a ‘colicin’ because they were active against *Escherichia coli*. Perhaps the best known bacteriocin is nisin, produced

by *Lactococcus lactis* and is most frequently used as a food preservative [67]. Bacteriocins have low toxicity and that makes them suitable for being used as food and beverage preservatives, since traditional antibiotics are prohibited for use in foods. These low toxicity effects have been proven for a number of bacteriocins in different studies [68, 69]. However, some bacteriocins have shown some cytotoxic activity [70]. Bacteriocins can exhibit broad-spectrum antimicrobial activity, giving them the possibility of being used as a traditional antibiotic, and some exhibit narrow-spectrum, which makes them suitable for targeting specific harmful microorganisms without negatively impacting on other microbes. Bacteriocins activity has been shown *in vitro* and *in vivo* [70, 71]. As a general rule, bacteriocins produced by Gram-positive bacteria show better activity against Gram-positive pathogens and bacteriocins produced by Gram-negative bacteria work more effectively against Gram-negative pathogens. However, the use of bioengineered derivatives has made it possible to cross this barrier somewhat [72]. As bacteriocins have a ribosomal origin, they offer the possibility of being bioengineered, either by gene manipulation or partial/complete chemical synthesis, enhancing their features [73, 74]. Additionally, bacteriocins can have synergistic activity when combined with other substances, such as lactic acid, that allows the permeabilization of the bacterial membrane. Bioactivity is in a nano- to micromolar range and the absence of taste, colour or odour, makes them also suitable for industrial and clinical purposes [75].

Bacteriocins are produced by bacteria in natural environments, and this property can be exploited in order to produce the antimicrobial *in situ* [76]. Probiotic strains could be considered for this purpose and there are several studies that report beneficial outcomes of different strains controlling pathogens or infection-associated microorganisms [76, 77]. A very important advantage of bacteriocins over traditional antibiotics is that resistance is not easily developed against them.

1.4.1. Classification

Because bacteriocins are a heterogeneous group, different criteria have been used for their classification, such as primary structure, molecular weight, mode of action, heat stability or genetic properties. Currently the most accepted classification is based on their structure. Here, we follow the classification proposed by Cotter *et al*, 2013 [70].

1.4.1.1. Class I

This class contains different groups of small heat-stable peptides (<10 KDa), all having in common the presence of modified amino acids that are introduced through enzymatic modification during their biosynthesis. These modifications influence their function through the formation of characteristic amino acids and structures. Representatives of lantibiotics, sactibiotics, microcins, linear azole or azoline-containing peptides or lasso peptides have been described in strains of gut origin and are being increasingly studied (Table 1.1). Other class I groups that do not yet have representatives isolated from gut bacteria are linaridins, proteusins, patellamide-like cyanobactins, anacyclamide-like cyanobactins, thiopeptides and bottromycins [70].

Table 1.1. Class I bacteriocins produced by bacteria originally isolated from the human gut.

Class I (modified)					
Group	Distinctive feature	Bacteriocin from human gut isolate	Gut Producer first isolated from human	Activity against	Reference
Microcins		Microcin L	*E. coli* LR05	*Shigella sonnei* *E. coli* *Pseudomonas aeruginosa*	[150]
		Microcin M	*E. coli* Nissle 1917	*E. coli* *Salmonella*	[151]
		Microcin V (ColicinV)	*E. coli*	*E. coli*	[49]
		Microcin H47 (MccH47)	*E. coli* H47	*E. coli, Salmonella, Enterobacter, Shigella, Klebsiella*, *Proteus* spp.	[152]
		Microcin E492	*Klebsiella pneumoniae* RYC492	*Escherichia, Klebsiella, Salmonella*, *Citrobacter*, *Enterobacter*	[153]
Lasso peptide	Lasso knot structure	Microcin J25	*E. coli* AY25	*E. coli* *Salmonella* sp, *Shigella flexneri*	[96]
Sactibiotics	Sulphur-α-carbon linkages	Thuricin CD	*Bacillus thuringiensis* DPC 6431	*Clostridium difficile, Bacillus cereus, Bifidobacterium firmus, Listeria monocytogenes*	[154]

Lantibiotics	Lanthionine bridges	Ruminococcin A	*Ruminococcus gnavus*	*Bacteroides* sp, *Clostridium* sp, *Bifidobacterium* sp, *B. cereus*	[116]
		Nisin O	*Blautia obeum* A2-162	*Clostridium perfringens, C. difficile, Lactococcus lactis*	[118]
		Cytolysin ClyLl and ClyLs	*Enterococcus faecalis*	Gram-positive bacteria	[114]
		BLD 1648	*Bifidobacterium longum* (strain DJO10A)		[117]
Linear azole- or azoline-containing peptides	Heterocycles but no other modifications	Microcin B17	*E. coli* Nissle 1917	*E. coli, Salmonella, Shigella*	[151]

1.4.1.1.1. Lantibiotics

Lantibiotics are the most studied group of bacteriocins. The 'lantibiotic' term comes from 'lan-thionine containing antibiotic'. Lantibiotics are part of the group of lanthipeptides. However, not all exhibit antimicrobial activity, and the ones that do are referred to as lantibiotics. They are small peptides (<5 kDa) that include modified amino acids known as dehydroalanine (Dha) and dehydrobutyrine (Dhb) that are the result of the dehydration of Ser and Thr residues respectively, forming 2,3-didehydroalanine and (Z)2,3-didehydrobutyrine. There is a stereospecific intramolecular addition of a cysteine residue to form a lanthionine (Lan) or methyllanthionine (MeLan) bridges [78]. There are described up to 17 uncommon amino acids [79]. The function of these amino acids in unknown, but it is suspected to be related to the interaction with free sulfhydryl groups on cell envelopes of target organisms.

Lantibiotic genes are organised in highly conserved clusters that usually encode proteins with similar functions. A typical lantibiotic cluster presents genes for a precursor peptide (LanA), modification enzymes (LanB and LanC or LanM), usually a protease to remove the leader peptide (LanP); LanT encodes a family of transport proteins, ABC, that translocate the peptide and sometimes encodes the protease activity too; LanR and LanK constitute a two-component regulatory system, and LanI and LanFEG are responsible for self-immunity (Figure 1.1).

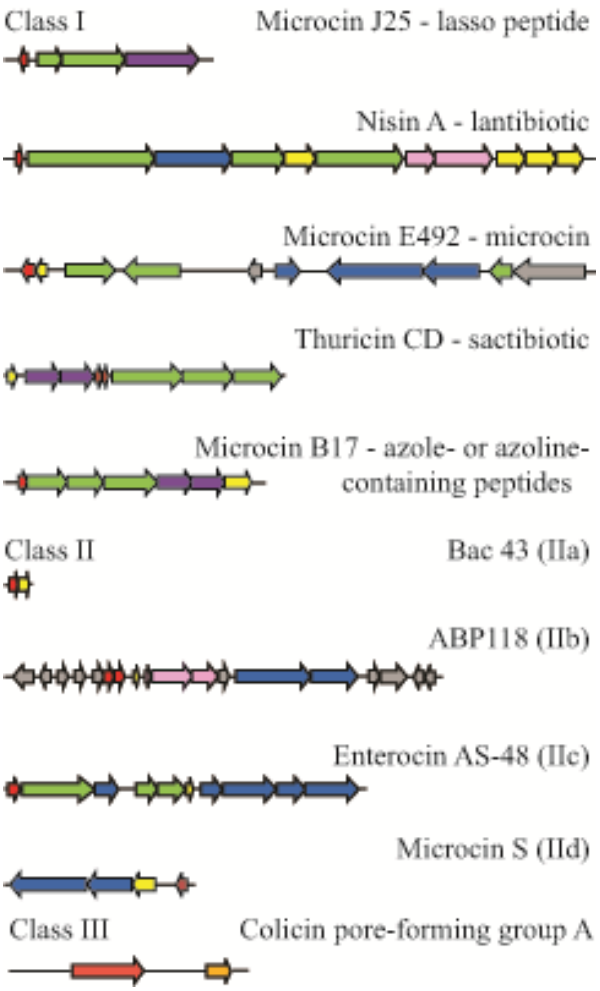


Figure 1.1. Examples of bacteriocin cluster organisation among the different bacteriocin classes. Genes are coloured according to the function of their products: red, precursor peptides; green, post-translational modifications; blue, export; yellow, immunity; pink, regulation; purple, export and immunity; orange, lysis; grey, unknown function. Class I: microcin J25 [80]; nisin A [81]; microcin E-492 [80]; thuricin CD [82]; microcin B17 [80]; class IIa bacteriocin 43 [83]; class IIb ABP-118 [84]; class IIc AS-48 [85]; class IId microcin S [86]; class III colicin [87].

There are differentiated steps that have been described in the production and maturation of lantibiotics: synthesis of the prelantibiotic, dehydration and linkage reactions, removal of the leader sequence and secretion. There are four groups that are differentiated based on the enzymes present during the maturation process: class I, LanBC-modified, class II, LanM-modified. Class III uses LanKC and class IV uses LanL, but they do not show antimicrobial activity. Lipid II binding is the most common mechanism of action of lantibiotics, a target in the cell-wall formation process. There are two ways of disrupting the cell wall, either by inhibiting its synthesis or by pore formation. Nisin A, first isolated

from *Lactococcus lactis* and the most studied lantibiotic (Figure 1.2), has both functions, but not all lantibiotics do. However, this activity on the cell wall is the reason for lantibiotics to not be very effective against Gram negative bacteria, since they are protected by a lipopolysaccharide membrane that shields the cytoplasmic membrane [88]. It has been a common belief that lantibiotics are only produced by Gram positive bacteria. Despite this, the identification of pinensins, a lantibiotic type bacteriocin produced by Gram-negative bacteria that exhibits antifungal activity [89], refutes this idea and highlights the vastness of metabolic diversity. Currently, the transference of the *in vitro* efficacy to the *in vivo* tests is one of the main focuses in lantibiotic research [90]. There are lantibiotics tested *in vivo* for a variety of health applications, especially targeting bacteria of clinical relevance, and currently in pre-clinical development.

Other lantibiotics include ruminococcin A, produced by *Ruminococcus gnavus*, isolated from human faeces. It was the first characterised bacteriocin from a strict anaerobe isolated from a human faecal sample and its activity was shown to be induced by trypsin [91]. Genomic analysis of *B. longum* DJO10A identified the presence of a novel lantibiotic, BLD1648. This is important because bifidobacteria are classified as beneficial commensal bacteria, but the isolation and characterisation of antimicrobial compounds of ribosomal origin has been very difficult. Activity against bifidobacteria suggests competition functionality. Another lantibiotic recently described from human gut origin is nisin O, produced by *Blautia obeum* A2-162 [92, 93]. Butyrivibriocin [94], Butyrivibriocin OR79 [95] and Butyrivibriocin AR10 [94] were bacteriocins produced by bacteria isolated from gastrointestinal tracts (rumens) of animals. Nisin H has also been identified produced by a porcine gut isolate, *Streptococcus hyointestinalis* DPC6484 [61].

Human pathogens can synthesise lantibiotics too. The two-component cytolysin, isolated from *E. faecalis* is an example, contributing causes to bacterial virulence and antibiotic resistant infections and being highly efficient against other Gram-positive bacteria. Cylolysin has two subnunits, large (L) and small (S) whose post-translational modification is controlled by the gene *cylM*. They are transported outside the cell by the ABC transport system and activated by *cylA*. Its activity is regulated by quorum-sensing autoinduction. The process starts with two genes, *cylR1* and *cylR2*, whose products prevent the transcription of the bacteriocin components. The small subunit of the cytolysin induces the

expression and when it reaches a certain concentration, derepression from the two inhibitory genes takes place [96].

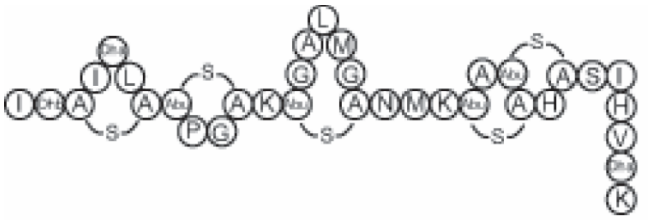


Figure 1.2. Nisin A structure [97].

1.4.1.1.2. Microcins

Microcins are peptides of less than 10 kDa produced by Enterobacteriaceae that are differentiated from colicins, also produced by Enterobacteriaceae and are bigger than 20 kDa, by their molecular mass. Microcin clusters typically involve a precursor, secretion proteins and elements for self-immunity. There are two groups of microcins, a class I of peptides smaller than 5 kDa with post-translational modifications, and a class II (between 5-10 kDa) that can be divided into class IIa, encoded on plasmids, and class IIb, encoded on the chromosome. They are described as hydrophobic and heat-, pH- and protease-stable [80]. Microcins have a precursor peptide that is cleaved at the transport stage [98]. Although 16 microcins have been identified, only eight are structurally characterised [98]. Microcins are believed to mimic essential nutrients to act like a 'Trojan horse', like iron-siderophore complexes, that the target bacterial cell detects and incorporates and once inside the cell, microcins act on the enzymes or inner membrane [99]. Microcins are involved in *in vivo* interspecies and intraspecies competition among Enterobacteriaceae in the gut during inflammation and they are able to inhibit cell growth [100, 101]. The structure of microcin E-492 is shown in Figure 1.3.

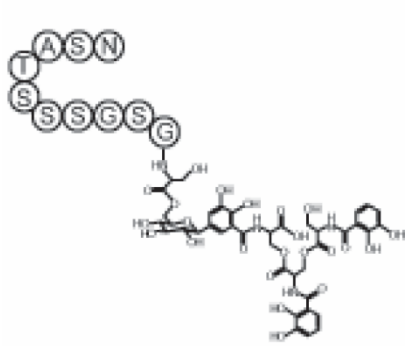


Figure 1.3. Microcin E-492 structure [102].

1.4.1.1.3. Lasso peptides

Lasso peptides have bacterial origin and named by their distinctive structure as a knot, including a macrolactam ring crossed through by the C-terminal tail (Figure 1.4). The structure is stable and sustained by steric interactions and disulphide bridges. Three classes of lasso peptides are described at the moment, based on the number of disulphide bridges. Class I has two disulphide bonds, class II does not have any and class III possesses just one disulphide bond [103, 104]. They have enzymes with inhibitory activity, receptor antagonistic behaviour and some of them also exhibit antimicrobial activity. Genome mining has increased the discovery of biosynthetic gene clusters encoding lasso peptides. In addition to activity-based discovery, 38 lasso peptides were known in 2015 [105]. Most of the described lasso peptides have been reported from soil, sludge and water bacteria, with the exception of zucinodin, isolated from an intracellular bacterium of erythroleukemia cell line origin, and microcin J25, from a bacterium of human origin. Their stability has also made them attractive to be used as new chemical scaffolds.

Microcin J25 is considered the paradigm of lasso peptide and has been extensively studied. It is made up of 21 amino acids and produced by *E. coli* AY25, isolated from the faeces of a newborn. Typically, it forms a ring by a lactam linkage between the first glycocin and the eighth glutamate [106]. The tail is formed by the thirteen residues left, with the phenylalanine in position 19 and the tyrosine in position 20 acting as plugs [107]. There are four genes in the biosynthetic cluster: a precursor (*mcjA*), cleavage of leader peptide (*mcjB*), a macrolactam ring formation gene (*mcjC*) and a gene that encodes the ATP-binding cassette transporter (*mcjD*) (Figure 1.1). The biosynthesis of lasso peptides is not fully understood at the moment [108]. The microcin J25 antimicrobial target is the Gram-negative RNA polymerase, which it inhibits, but also the depolarization of cell membranes in *Salmonella* and *E. coli* species [105].

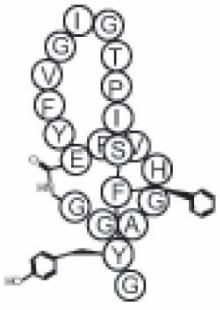


Figure 1.4. Microcin J25 structure [109].

1.4.1.1.4. Sactibiotics

Sactipeptides are a general denomination for peptides that are characterised by having an intramolecular bridge between a cysteine sulphur and an α-carbon (Figure 1.5). Sactipeptides do not always exhibit inhibitory activity, and those who do are known as sactibiotics. They have been mainly identified by genome mining [110]. Currently there are four sactibiotics described, although not all of them have been deeply characterised: Subtilosin A, Propionicin F, Thuricin H and Thuricin CD [111]. Thuricin CD is one of the most studied. It was isolated from *Bacillus thurigiensis* DPC6431, a representative of the human gut. It is particularly important because it has been shown to have very specific activity against important pathogens: *Clostridium difficile, Bacillus cereus, Bacillus firmus* and *Listeria monocytogenes* [112]. It consists of two subunits, Trnα and Trnβ (Figure 1.4). Its genomic cluster presents structural genes, transport, immunity and post translational modification genes (Figure 1.1). The presence of genes encoding for the radical S-adenosylmethionine (SAM) is also characteristic. Research on thuricin CD mode of action is still ongoing. Both subunits act on the cell membrane, collapsing the membrane potential irreversibly. More specifically, results suggest that the peptides get inserted in the membrane of the cell, forming a pore that leaks ions and ultimately lyses the cell [113]. Thuricin CD effects were similar to the ones exhibited by vancomycin and metronidazole, both used to treat *C. difficile* infections.

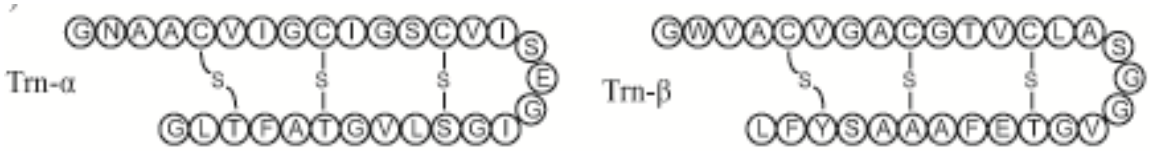


Figure 1.5. Structure of thuricin CD subunits [114].

1.4.1.1.5. Linear azole-or azoline-containing peptides (LAPs)

These peptides are also known as LAPs and their main feature is that they present different combinations of heterocyclic rings of thi-azole and (methyl)oxazole, produced by a cyclodehydration of cysteine, serine and threonine, and a dehydrogenation depending on flavin mononucleotide. Their antimicrobial activity is not fully understood yet. Microcin B17 is a LAP produced by *E. coli* isolated from baby faeces [115] (Figure 1.6). Its antimicrobial activity targets the gyrase [116] and it is extensively studied to connect the effect between its structure and its function, in order to obtain information to design new antimicrobials

that mimic its function [117], especially since the inhibition of topoisomerases and gyrases constitute new targets for designing antimicrobials [118, 119]. Its applications have been studied by conducting *in vivo* studies to address its ability to control infections in infants [120] and cattle [121], proving its beneficial action.

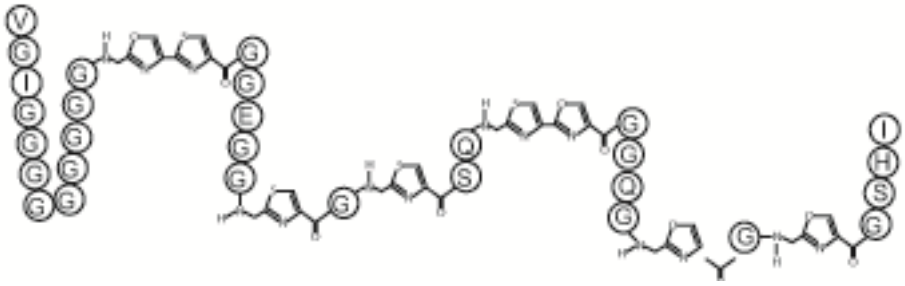


Figure 1.6. Microcin B17 structure [122].

1.4.1.2. Bacteriocins class II

Class II is the largest group of bacteriocins. They are unmodified small heat stable peptides (<10 kDa). There are four subgroups, according to Cotter *et al*, based on their structure-function relationships [123].

Table 1.2. Class II bacteriocins produced by bacteria originally isolated from the human gut.

Class II (unmodified or cyclic)					
Group	Distinctive feature	Bacteriocin from human gut isolate	Gut Producer first isolated from human	Activity against	Reference
IIa peptides (pediocin PA-1-like bacteriocins)	Conserved YGNGV motif (N represents any amino acid)	Bac43	*Enterococcus faecium* VRE82	*E. faecalis*, *E. faecium*, *Enterococcus hirae*, *Enterococcus durans*, and *L. monocytogenes*	[122]
		Bacteriocin RC714	*E. faecium strain* RC714	*E. faecalis, E. faecium, L. monocytogenes, Listeria innocua, Listeria murrayi, Listeria grayi, Lactobacillus paracasei, Lactobacillus plantarum, Leuconostoc sp., and Pediococcus pentosaceus*	[124]
		Bacteriocin 31	*E. faecalis* YI717	*E. hirae 9790, E. faecium, and L. monocytogenes*	[155]
IIb peptides	Two unmodified peptides	ABP-118	*Lactobacillus salivarius* UCC-118	*L. monocytogenes*	[127]

	are required for activity	Acidocin J1132	*Lactobacillus acidophilus* JCM1132	*Lactobacillus* sp	[126]
		Gassericin T	*Lactobacillus gasseri* SBT 2055	*Lactobacillus delbrueckii subsp. bulgaricus*	[130]
		Lactacin F	*Lactobacillus johnsonii*	*Lactobacillus sp, E. faecalis*	[156]
IIc peptides	Cyclic peptides	Gassericin A	*L. gasseri* LA39	*Bacillus, Clostridium, Lactobacillus spp, Lactococcus lactis, Leuconostoc mesenteroides, Listeria spp, Pediococcus cerevisiae, S. aureus, Streptococcus agalactiae*	[157]
		Reutericin 6	*Lactobacillus reuteri* LA 6	*L. acidophilus, L. delbrueckii subsp. bulgaricus and L. delbrueckii subsp. lactis*	[158]
		AS-48	*E. faecalis* AS-48	Gram-positive and Gram-negative	[159]
IId peptides	Unmodified, linear, non-pediocin-like, single-peptide bacteriocins	Microcin S	*E. coli* G3/10	*E. coli* G3/10	[136]
		Rhamnosin A	*Lactobacillus rhamnosus* strain 68	*Micrococcus lysodeikticus* ATCC 4698	[138]
		Bac 32	vancomycin-resistant *E. faecium* (VRE) 200	*E. faecium, E. hirae*, and *E. durans*	[139]

1.4.1.2.1. Class IIa

Also known as pediocin-like bacteriocins, they are characterised by a highly conserved N-terminal consensus sequence YGNGVXaaCXaaK/NXaaXaaC, including the 'pediocin box' (YGNGV). They also have two cysteines in the conserved region forming a disulfide bond, required for the antimicrobial activity (Figure 1.7). Their specificity is based in the C-terminus and they act by permeabilising the cell wall. The target receptor is the sugar transporter mannose phosphotransferase system (Man-PTS), present in Gram-positive and Gram-negative bacteria. They are known for having strong activity against *Listeria* spp. Typically, biosynthesis requires at least four genes, a structural gene encoding a precursor, and three others encoding an immunity protein, ATP-binding cassette transporter and a protein for extracellular translocation (Figure 1.1). The cluster is regulated by quorum sensing by a three component system: an inducer peptide, a membrane associated histidine protein kinase, and a cytoplasmic response regulator [124] .

A typical class IIa bacteriocins of human gut origin is bacteriocin 43, isolated and characterised from a clinical isolate vancomycin-resistant *E. faecium* (VRE). Its sequence showed high homology with bacteriocin 31 from *E. faecalis* YI717 and bacteriocin RC714 from a similar origin [125, 126].

Figure 1.7. Structure of bacteriocin class IIa enterocin NKR-5-3C [127].

1.4.1.2.2. Class IIb

Bacteriocins that belong to this subclass require two peptides acting synergistically to exhibit their antimicrobial activity (Figure 1.8). Usually, the structural genes are in the same operon and are expressed simultaneously. The killing mechanism involves membrane permeabilization, that leads to the leakage of small cytoplasmic molecules and monovalent cations (Na^+, K^+, Li^+, Cs^+, Rb^+ and choline), but not divalent cations or anions. Some also include H^+. The peptides that constitute the bacteriocin are typically of 30 to 50 amino acids in length and have similar characteristics to one peptide bacteriocins, being usually cationic, hydrophobic or amphiphilic. There is a typical double-glycine leader type sequence in the N terminus that it is cleaved off during transport. It is commonly accepted that individual peptides do not have individual antimicrobial activity by themselves, but there are exceptions [128].

Examples of this class of bacteriocins produced by human gut representatives include acidocin J1132, isolated from *L. acidophilus* JMC 1132 [129]. It was found that its production is limited by pH of the medium not being above 7.0, having its maximum production at pH 5.0. Its mode of action is bactericidal instead of bacteriolytic [129]. ABP-118 is produced by the probiotic *L. salivarius* UCC118 [84]. *L. salivarius* UCC118 produces ABP-118 *in vivo* and it has able to control *L. monocytogenes* infection in mice [130]. It can act also as a gut microbiota regulator, increasing Bacteroidetes and Proteobacteria and decreasing Actinobacteria [131]. Gassericin T and Lactacin F also belong to this class. Gassericin T is produced by a *L. gasseri* isolated from human faeces and it is part of the Lactacin F family, sharing a 60% similarity [132]. Lactacin F requires two hydrophobic peptides LafA and LafX for its antimicrobial activity and it has been shown to act synergistically in combination with nisin [125].

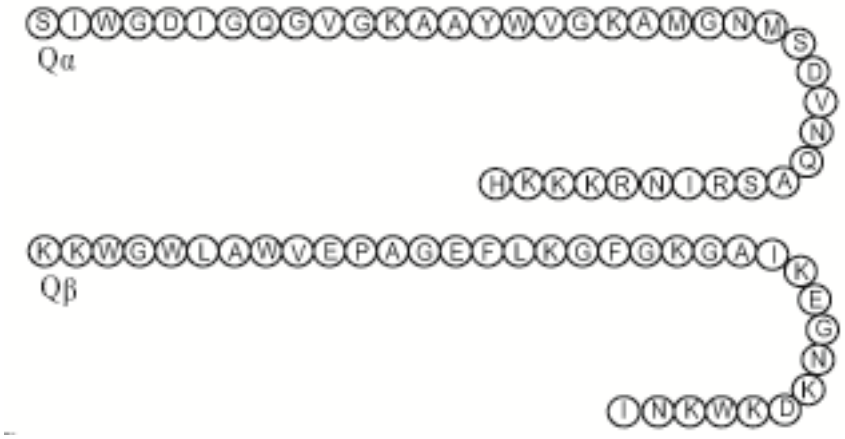


Figure 1.8. Structure of bacteriocin class IIb lactococcin Q [127].

1.4.1.2.3. Class IIc

Class IIc bacteriocins are generally cationic peptides that present a covalent bond between C and N termini and are cyclic peptides (Figure 1.9). They have some degree of hydrophobicity. Traditionally, their mode of action was attributed to disruption and permeation of the bacterial cell membrane to small molecules, leading to cell death. However, new modes of action have been identified, such as inhibition of nucleic acid, protein and cell wall synthesis and enzyme activity [133]. Their three-dimensional structure is considered to have a role in their stability, making them more resistant to proteolysis [134].

The most studied circular bacteriocin is AS-48, an α-helical and cationic peptide, first isolated from *E. faecalis* but produced by different *Enterococcus* species isolated from clinical and food samples. It is very broadly distributed among *Enterococcus*, and also presents different derivatives, both engineered and natural (e.g. AS-48J, isolated from goat cheese [135]). It has shown stability and solubility through a broad range of pH and temperature, and the most attractive feature is its spectrum of action, having activity over Gram-positive and Gram-negative bacteria. Its applications include being a well-proven food preservative, but with new strategies like lipome-encapsulation, its functionality might be increased. The successful treatment against *S. aureus* from cow mastitis opens the possibility to veterinarian applications. Other applications are being investigated at the moment, like its use as a leishmanicidal agent [136], for *Propionibacterium acnes* control [137] or against bacterial biofilms [138-141]. Gassericin A is another example of a class IIc bacteriocin produced by the human gut isolate *L. gasseri* LA39, presenting the same primary amino acid sequence as reutericin 6 produced by *L. reuteri* La6, both isolated from

faeces of the same baby with a difference of two months' time between sample collection [133]. However, they differ in one D-alanine residue that confers on gassericin A a broader spectrum of activity. In fact, gassericin A can inhibit the growth of *L. reuteri* La6, but not the other way around.

Figure 1.9. Structure of bacteriocin class IIc lactocyclicin Q [127].

1.4.1.2.4. Class IId

This group has been proposed as a miscellanea group for different bacteriocins of class II that did not fit elsewhere. It comprises unmodified, linear, non-pediocin like, single bacteriocins (Figure 1.10). Some examples of this class are microcin S, a bacteriocin produced by an *E.coli* strain that is part of the probiotic Symbioflor, constituted of six different *E. coli* genotypes [142]; rhamnosin A, bac 43 and bac 32 were also assigned to this subclass of bacteriocins. Microcin S is capable of inhibiting the adhesion of the enteropathogenic *E. coli* E238/6 to intestinal epithelial cells. Bac 43 and bac 32 have a clinical origin. There are other class II bacteriocins produced from bacteria isolated from the gastrointestinal tract of animals: bovicin 255 [143], coagulin A [144], enterocin 1071 [145], lacticin Z [146] and lichenin [147].

Figure 1.10. Structure of bacteriocin class IId lacticin Q [127].

1.4.1.3. Bacteriocins class III

Class III are large, heat-labile proteins (>30 kDa). Their mode of action and structure differ from other bacteriocins and it is common that they present different domains. In general, bacteriocins from class III are not very well characterised. Their mode of action consists of promoting lysis of the cell wall by a conserved endopeptidase-like domain in the N-terminal region. The C-terminal region is responsible for recognition of the target (Figure 1.11). They

are classified in two groups, class IIIa, formerly known as bacteriolysins, an example being helveticin J [148]. Class IIIb disrupt membrane potential but are known for being non-lytic bacteriocins.

Colicin V was the first colicin described, originally isolated from *E. coli* and initially it was classified as a microcin. Since then, many colicins have been isolated from other enteric bacteria. They are described as a narrow spectrum bacteriocins and their antimicrobial activity is conducted in different ways, such as inhibiting macromolecular synthesis, causing DNA breakdown and stopping protein synthesis [87]. Colicins can be divided into two subgroups. The criteria is based on their encoding plasmids: group A are on small plasmids and excreted and group B are encoded by large plasmids and are not excreted, although some of them have mixed characteristics. Typically, colicin operons are formed by a structural gene, an immunity gene and a lysis protein gene (Figure 1.1). Other bacteriocins are bacteriocin 28b, from *Serratia marescens* clinical isolates and helveticin J, identified from the genome of *L. acidophilus* NCFM, a probiotic strain that is widely commercialized and helveticin-M from *L. crispatus* isolated from chicken gut [149]. Other class III bacteriocins with animal gastrointestinal tract origin are alveicins A and B [150], albusin B [151], bacteriocin 28b linocin M18 [152].

Figure 1.11. Structure of bacteriocin class III colicin A [87].

Table 3. Class III bacteriocins produced by bacteria originally isolated from the human gut.

Class III					
Group	Distinctive feature	Bacteriocin from human gut isolate	Gut Producer first isolated from human	Activity against	Reference
Bacteriolysins		Colicins	*E. coli*	Enterobacteria	[49]
		Bacteriocin 28b	*Serratia marcescens*	*E. coli*	[141]
Non-lytic bacteriocins		Bacteriocin helveticin J	*L. acidophilus NCFM*		[142]

1.4.1.4. Class IV

Although some researchers do not consider class IV to be bacteriocins and they are not listed in the classification of Cotter *et al* [70], they will be briefly mentioned. They are complex bacteriocins that require carbohydrate or lipid moieties to be functional. Leucocin S is an example of a bacteriocin with a glucidic moiety and lactostrepcins produced by *L. lactis* [153] are examples of bacteriocins with lipidic moieties. Lactocin 27 needs both groups [154].

1.4.2. Regulation of bacteriocin biosynthesis

The production and excretion of bacteriocins is subject to different kinds of regulation. Some require quorum sensing which, as stated previously, is a cell-density-dependent mechanism of regulation [155]. Production of antimicrobial peptides usually happens at the end of the bacterial growth phase and the beginning of the stationary phase. There is a characteristic signal molecule production during growth and at a certain concentration it triggers rapid production of the antimicrobial peptide. The objective is very likely to kill the targets without giving them time to defend themselves. This is very common in lantibiotics and their own products can act as triggers in order to autoregulate their own synthesis, for example, nisin and subtilin and their two-component regulatory systems (RK) [156]. Mersacidin is another lantibiotic produced by *Bacillus sp*., in stationary phase, and is regulated by itself in an exponentially growing culture [157].

Some bacteriocins do not require a specific regulator and their promoters respond to pH at the transcriptional level. Promoters P1 and P3 in lacticin 481 produced by *L. lactis* [158] depend on the intra and extracellular acidification. This acidic sensitivity might have an ecological response related to quorum sensing, since lactic acid is released in media during fermentation, meaning that a very acidic environment is due to a dense population and the secretion of the bacteriocin is necessary for survival. This pH regulatory system has also been observed in lactocin S and nisin production [159].

Trypsin can also act as a regulator in production of bacteriocins. Ruminococcin A is an example of such, a lantibiotic produced by *Ruminococcus gnavus* E1. Trypsin is responsible for processing an inactive precursor peptide that, once turned into an active form and over a certain threshold, induces biosynthesis pathway. This proteolytic interaction is exclusive for trypsin, with other proteases not being able to generate the same effect [160].

There are also examples of general regulators involved in bacteriocin production, like diacyl-glycerol kinase in mutacin II or the galactose inducing nisin promoter [161].

1.4.3. Identification, production and characterisation of bacteriocins

Traditionally, bacteriocins have been identified by antagonistic activity using bioassays such as overlays, cross-streaks, drop test, filter discs or well-diffusion (Figure 1.12).

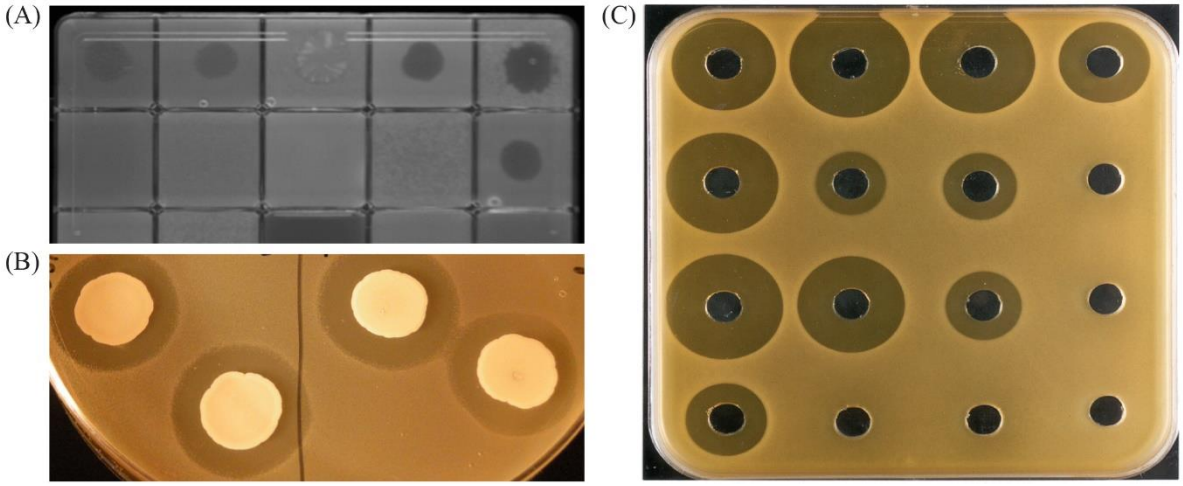


Figure 1.12. Examples of assays performed to identify antimicrobial activity. (A) spot test; (B) overlay; (C) well diffusion.

This is a very limited strategy as the first challenge that presents is known as the 'plate count anomaly', where just a small percentage of the bacterial species are able to grow in culture media. Among the ones that grow, they do not necessarily find the appropriate conditions to induce the production of bacteriocins, as all bacterial products depend also on the environmental conditions, including diet [126, 162, 163]. Some of the bacteriocin-like compounds identified required the presence of a specific trigger to release its activity, like for example, presence of trypsin [58], proven to be effective as an antimicrobial inductor in human gut isolates. This reinforces the idea of the important role that environment plays in the production of these compounds. Other problems may arise, like false positives due to other antimicrobial compounds, like organic acids or hydrogen peroxide activity. Bacteriophages are another source of false positives. To test that, zones of clearing are excised and tested by plaque assay [164].

Overcoming these limitations requires the understanding of the factors and interactions that take place in the environment of the producing bacteria, and the development and implementation of strategies to mimic these interactions. Current limited knowledge on microbial ecology makes this task highly difficult. However, there are new strategies, like

peptidogenomics, that attempt to integrate genomic and phenotypic information with a mass spectral molecular networking of microbes grown in a plate [165]. Other approaches are increasing the success in the identification of new bacteriocins too. Currently, the development and implementation of molecular tools with genomic information has allowed the identification of putative bacteriocin clusters that might be not expressed in genomes, both in culturable and unculturable species [166]. There are public software and databases to identify putative bacteriocin clusters in sequenced genomes and access to bacteriocin peptide sequences. BAGEL with 482 sequences [167] and BACTIBASE with 345 sequences [168] are the most commonly used. Other databases are available to identify potential new bacteriocin clusters in the gut, like the Human Microbiome Project's reference genome database [169]. Furthermore, other genome mining strategies, like Hidden Markov Model, have been proved useful for bacteriocin identification. Database combinations are available too, like BUR ('Bacteriocins of the URMITE database'), where BAGEL, BACTIBASE and NCBI databases for bacteriocin published sequences are combined. The next challenging stage is the bacteriocin purification. In general, since bacteriocins are a heterogeneous group, it is difficult to identify a standardised protocol [170]. Different methods for purification can be performed depending on biochemical structures. Some of the commonly used ones consist of different phases, such as ammonium sulphate precipitation, ion exchange, hydrophobic interaction, gel filtration and reversed-phase high pressure liquid chromatography. Another one extensively used is a protocol with three steps: ammonium sulphate precipitation, chloroform/methanol extraction/precipitation and reversed-phase high pressure liquid chromatography. Mass spectroscopy and nuclear magnetic resonance (NMR) spectroscopy are other techniques used to characterised bacteriocins further [77].

Regarding bacteriocins, use of these –omics tools and bioinformatics can help to understand some of the problems that their research presents, for example instability in their production, or loss of activity, either because they are encoded by plasmids or because they have complex regulatory mechanisms [126, 171].

1.4.4. Bioengineering of bacteriocins

Rational design might be the key to have more potent bacteriocins, based on the structure-function relationship [171]. The objective is to achieve a higher stability for application in industry. For example, four analogues of lactocin S have been generated using solid-phase

peptide chemistry to enhance the oxidative stability to atmospheric oxygen by removing sulphur atoms [172]. Mersacidin has undergone modifications that have enhanced its activity against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) [73]. Nisin has been one of the most modified bacteriocins in order to enhance its ability to have more pathogen targets and treat biofilms [17, 74], but it has not been the only one [173]. The generation of nisin derivatives can be achieved by different strategies, the non-targeted approach, using random mutagenesis to create derivatives that are analysed further or site-directed mutagenesis and mutagenesis by saturation. The enhanced activity is tested against a wide range of targets, both Gram-positives and Gram-negatives, and food settings in complex matrices. [174]. The analysis of these mutants has contributed to elucidating the mechanisms that control activity and regulation, by knowing which residues are key for activity and which residues modify activity in different bacteriocins, allowing the possibility of further improving the desired features.

1.4.5. Prospective applications of bacteriocins

1.4.5.1. Industrial applications

Bacteriocin could be considered as a serious solution for food industry and veterinary purposes [175]. However, the use of bacteriocin-producing strains is still questioned due to their doubtful functionality under industrial food processing conditions, and the development of bacteriocin preparations for commercial use is not fully developed due to the difficulties in bacteriocin production and in the food use approval. Nonetheless, this situation is starting to change, and countries like Japan are expanding bacteriocin use as preservatives in food industry and veterinary [127].

Despite these problems, bacteriocins have proved to be able to solve preservative problems in bakery industry, alcoholic beverages, to avoid spoilage bacteria, meat products, brined shrimp industry, protection of fresh fruits, vegetables and sauces, baby foods [176]. One of the most extended uses of bacteriocins is in dairy industry, especially with the use of nisin, that has been used as a preservative in different types of cheese to control *Bacillus* sp spore germination, and also has been tested against bacterial contamination in chilled desserts, flavoured mil and canned evaporated milks [177]. Nisin is licensed as a food preservative (known as E324) and is considered as safe by the WHO. Other bacteriocins used in dairy industry are class IIa bacteriocins that go under the

commercial name Alta 2341™ of Microgard™, able to control *L. monocytogenes*, *S. aureus*, *Pseudomonas* and *E. coli* [177]. Lacticins and enterocins, especially AS-48 and KP are other bacteriocins that have been proved to be useful in dairy industry for food preservation [177].

Bacteriocins applications in food industry not only involved the addition of the bacteriocin or the production *in situ* by the responsible bacteria. Thus, another application is the production of edible films or coatings with bacteriocins for food preservation in raw food or food without further cooking, that has proved effective to reduced pathogens such as *L. monocytogenes* and *S. aureus* [177].

Other industrial applications involve their use as disinfectants or their use in aquaculture, especially as probiotics and encapsulated bacteriocins, livestock and in agriculture [176].

Potential applications involve the pharmaceutical industry, since they have been suggested as potential anti-tumoral drug candidates [178]. This is the case of pore-forming colicin A and E1, able to inhibit the growth of human tumor-cell lines. Colicins D, E2, E3 and A were also capable of inhibiting murine leukaemia cells [178]. However, these applications still need to be further developed.

1.4.5.2. Clinical applications

The regulatory properties attributed to natural antimicrobials, and especially the ones produced by human GIT bacteria and the ones produced by bacteria with probiotic characteristics from fermented foods, are considered desirable traits towards controlling potential gut dysbiosis, and therefore, are considered for clinical applications. Research conducted to establish microbiota alterations in certain pathologies has shown dysbiotic relationships in the gut microbiota in certain pathologies. Dysbiosis in the gut has been associated not only with metabolic syndrome, type 2 diabetes, colorectal cancer or IBD, but also with psychological problems, like anxiety and depression, via the gut-brain axis [179]. Rational application of bacteriocins, either directly or produced by bacteria *in situ* as probiotics, have been started to be studied as a method to modulate the structure and function of gut microbiota in these acute and chronic host etiologies [180-184] . In general terms, the analysis of potential activity inferred from structure and amino acid composition of bacteriocins [185] suggests two strategies. One is the production of many of low activity and the other, the production of highly effective specific-target bacteriocins. The aim would be maintaining homeostasis with a functional focus and developing defensive strategies

against elements that could cause dysbiosis. Despite the increasing research in this area, the complexity of GIT microbial ecology makes it a difficult task at the moment, with further understanding required. For example, despite the fact that administration of probiotics is taken often as a beneficial approach, it has been suggested that the abrupt interruption of a probiotic administration can cause gut dysbiosis by increasing pathogen susceptibility and other alterations in the intestinal epithelium and metabolites, according to research conducted in a tilapia model [186]. Further research is required to elucidate whether these results can be extended to humans or to all the probiotic bacteria.

The lack of a stablished a paradigm of a healthy gut microbiota makes the identification of specific targets difficult [34]. However, general features can be highlighted, like the ratio of Firmicutes / Bacteroidetes. A correlation between this altered ratio and the development of metabolic syndrome and diabetes type 2 has been established and reviewed previously [33, 34, 187]. Besides that, some species have been identified as enriched in mice models with induced metabolic syndrome, like *Enterobacter cloacae B29* and *Clostridium ramnosum*. Bacteriocin producer *L. salivarius* UCC118 was tested in obese mice showing temporary reductions in weight gain. The most promising effect was the modification of this ratio, by the increase of Bacteriodetes and Proteobacteria and the lower number of Actinobacteria.

Another example of the regulatory potential of bacteriocins in gut microbial populations is bactofencin A, produced from a *L. salivarius* isolated from porcine intestine. Despite the fact that it did not have a direct inhibition activity against *Clostridium*, *Bacteroides*, *Bifidobacterium* and *Fusobacterium*, when tested in a distal colon model, populations showed modifications in a positive way [188]. More recently, in an experiment where probiotic bacteriocin producers were supplemented to water given to mice, initial metataxonomic data suggested that gut microbiota populations were not affected. However, a deeper analysis at lower taxonomic levels showed that potentially problematic bacteria, like *Clostridium* or *Staphylococcus*, were inhibited [189]. Analysing these results show us that, although we cannot expect big changes in *in vivo* experiments at higher taxonomy level in microbiota, there are subtle changes that are favourable for the host without major disturbances in the gut. In addition, these studies highlight the importance of the analysis scale.

Bacteriocin production *in situ* by a probiotic is one of the desirable, yet challenging tasks for developing treatments. To be effective, the probiotic cells need to survive and colonise the human gut. Additionally, the antimicrobial needs to be produced and be active in the gut environment. Thuricin CD is an example of this challenge. Despite its efficacy against *C. difficile*, when delivering *B. thuringiensis* DPC6431 through the intestinal tract of mice, results showed the inefficiency of this approach. However, alternative supplementation routes were found to be effective, like rectal administration [190]. Also, other possibilities can be explored, like synbiotics and delivery systems to help and protect the producer strain or the compound. Tablets of nisin with a pectin / HPMC polymer mixture are an example of this delivery strategy that is gaining interest [191].

But not only target-specific bacteriocin producers help to control infections in the gut. As mentioned before, the acetate-producing bifidobacteria protects epithelial cells in mice infected with *E. coli* O157 from damage [192]. A common trend that colorectal cancer presents with T2D is the lower levels of butyrate producing microorganisms and SCFA have been highlighted for inducing transcriptional changes in *C. albicans*, being the butyric acid responsible for the inhibition of yeast-hyphal transition [193]. Despite that, nonspecific action against pathogens can lead to undesirable and uncontrolled effects. Thus, acetate has been suggested as a promoter of metabolic syndrome and obesity [194].

1.4.6. Challenges and future developments in bacteriocin research

Antibiotic resistance has transformed the search for new antimicrobials into one of the most important tasks in the following decades. By understanding the natural environment where problematic bacteria grow, we may be able to find solutions to address this problem. Bacteriocins are antimicrobials that can develop an important role against antibiotic resistance. There are limitations in the identification and isolation of bacteriocins that may be overcome by new techniques like genomic and bioinformatics tools, genome mining and metagenomics. However, ultimately, this genomic information has to be translated into the laboratory, and then to achieve efficacy *in vivo*. The combination with other disciplines and the bioengineering process are offering the possibility to improve the technology necessary to make bacteriocins a serious and efficient alternative to antibiotics, including production improvement and the scale-up process. Besides the bioengineering opportunities, there is also improvement in delivery. Nanotechnology offers opportunities to get bacteriocins to the location where they have to exert their action, avoiding possible modifications due to

environment and conditions that might reduce or destroy their function. For example, the use of liposomal nanovesicles containing bacteriocins has been reported against *Listeria monocytogenes* in goat milk [195]. There is more research to conduct in the food area in order to assess any impact on taste, flavour or sensorial analysis regarding nanovesicles. Another possibility that is being explored is the combination of bacteriocins and traditional antibiotics. Bacteriocins and antibiotics have different mechanisms of action and resistance, therefore a synergistic effect would be desirable, improving and enhancing the effects of both of them individually [17, 196]. Bacteriocins delivery by probiotics to be produced at the site of infection [70, 76] also offer huge possibilities. Complex health alterations, like IBD, colon cancer, anxiety, depression and irritable bowel syndrome have been linked to gut microbiota [197-200]. Ultimately, further understanding of the microbial relationships and ecology will help to the development of more effective treatments, both for targeted intervention and to maintain gut and host homeostasis.

1.5. Aims and objectives of the thesis

This project aims to identify new antimicrobials produced by isolates from gut and food origin, with a special focus on the identification of bacteriocins, ribosomally synthesised peptides and proteins that will be effective against gut pathogens and food spoilage bacteria. Our hypothesis is **that mining bacteria from the same niche as the pathogen will identify novel antimicrobials which are uniquely suited to act in this niche**.

This thesis has four main objectives.

Objective 1: *Identify bacterial isolates with antimicrobial activity against gut and food pathogens*

Strains from the culture collections of both QIB and Teagasc were tested. Those isolates had previously shown antimicrobial activity but not characterised. The use of neutralising agents, heat- and protease-resistance tests helped to identify which of those strains were likely to be bacteriocin producers rather than producers of organic acids. The selected strains were phylogenetically identified by 16S rDNA sequencing and screened against a selected panel of gut and food pathogens to test the host range of the bacteriocins. Isolates showing good antimicrobial activity against relevant pathogens were further characterised.

Objective 2: *Purify and sequence bacteriocins by MS and identify biosynthetic gene clusters*

Antimicrobials of proteinaceous origin were purified with the most suitable method and MS protocols and NMR methods were used for structure characterisation. The relevant biosynthetic gene clusters were identified by combining genomic information obtained in the previous objective.

Objective 3: *Examine mechanisms of synthesis, biological activity and host range*

The purified peptides were used to determine the full relevant host range of the antimicrobials and the calculations of their minimum inhibitory concentrations (MICs). Gene expression was tested using RT-qPCR as response to changes in environmental conditions to obtain information on how the antimicrobials are produced in the native environment and how their production might be controlled or improved. Regulatory activity was also examined.

Objective 4: *Test stability and activity of selected bacteriocins in complex environments (colon model and Micro-Matrix) and assess their effects on the native microbiota*

The stability and activity of the peptides was tested within complex environments *in vitro* in a human colon model and a Micro-Matrix fermenter, with a mixed microbiota, both

spiked or not with the pathogen of interest. Effects on pathogens and the native microbiota was assessed by next generation sequencing.

CHAPTER II

General Materials and Methods

2.1. Microbiology

This compilation of materials and methods were used throughout the PhD project. Chemicals and consumables were purchased from Sigma Aldrich/Merck (UK) unless stated otherwise.

2.1.1. Culture media

Table 2.1 summarises the media used for microbiology work. For solid media preparation 15 g/l agar (Difco, UK) were added before autoclaving. For overlay assays, sloppy agar containing 7 g/l was prepared.

Table 2.1. List of media used in this study.

Media	Details	State
MRS medium (de Man-Rogosa-Sharpe) (Oxoid, UK)	Prepared from ready-prepared commercial powder.	Liquid, solid
MRS in-house preparation	10 g/l trypticase peptone, 2.5 g/l yeast extract, 3 g/l trypticase peptone, 3 g/l K_2HPO_4, 3 g/l KH_2PO_4, 2 g/l tri-ammonium citrate, 0.2 g/l pyruvic acid, 0.3 g/l cysteine · HCl, 0.575 g/l $MgSO_4 \cdot 7H_2O$, 0.12 g/l $MnSO_4 \cdot 7H_2O$, 0.034 g/l $FeSO_4 \cdot 7H_2O$ and 1 ml Tween 80, supplemented with 2% glucose after autoclaving	Liquid, solid
BHI (brain heart infusion)	Prepared from ready prepared commercial powder.	Liquid, solid
BHI + complements media	BHI media (Oxoid), 5 g/l yeast extract, 0.1 g cysteine, 0.02 g/l haemin and 200 µl of 10 mM vitamin K1 solution	Liquid media
Robertson's cooked meat medium	Purchased from Southern Group Laboratories Ltd, UK.	Liquid
M17 (Oxoid)	Prepared from ready prepared commercial powder	Liquid media
GM17	5 g/l glucose to M17 media added to after autoclaving	Liquid, solid

Batch model and Micro-matrix media	2g/l peptone water, 2g/l yeast extract, 0.1 g/l NaCl, 0.04 g/l K_2HPO_4, 0.04 g/l KH_2PO_4, 0.01 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $CaCl_2 \cdot 2H_2O$, 2 g/l $NaHCO_3$, 0.5 g/l cysteine · HCl, 0.5 g/l bile salts, 2 ml Tween 80, 0.02 g/l hemin (pre-dissolved in 1M NaOH) and 10 µl vitamin K1	Liquid
Carbon Free Basal Medium CFBM	2 g/l peptone water, 2 g/l yeast extract, 0.1 g/l NaCl, 0.04 g/l KH_2P0_4, 0.04 g/l K_2HP0_4, 0.01 g/l $MgS0_4 \cdot 7H_20$, 0.01 g/l $CaCl_2 \cdot 6H20$, 2 g/l $NaHC0_3$, 2 ml Tween-80, 0.05 g/l haemin solution, 10 µl Vitamin K1, 0.5 g/L L-cysteine HCl and 0.5 g/l bile salts.	Liquid
Minimal media M9	64 g/l Na_2HPO_4 x $7H_2O$, 15 g/l KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 20% v/v of M9 salts, 0.2% v/v of 1M $MgSO_4$, 2% v/v of 20% glucose, 0.001% v/v of 1M $CaCl_2$	Liquid
LB (Luria Bertani) medium	Prepared from ready prepared commercial powder.	Liquid, solid
L medium	10 g/l tryptone (Difco, UK), 5 g/l yeast extract (Difco, UK), 5 g/l NaCl and 1 g/l D-glucose	Liquid, solid
ROGOSA (Oxoid)	Prepared from ready prepared commercial powder	Solid

2.1.2. Bacterial strains and their growth conditions

Typically, strains were stored at -80 ^oC in 1 ml working aliquots of multiple use in 40% glycerol and grown in conditions listed in tables 2.2 and 2.3. Antibiotics used during the project are listed in table 2.4. Stocks of *Clostridium* species were made using Robertson's cooked meat medium (Southern Group Laboratory, UK). Microbial cultures were set up by adding 100 µl of the stock to the appropriate media. Anaerobic conditions were set up by pre-reducing media overnight in anaerobic chamber (Don Whitley, UK) in an atmosphere of 5% CO_2, 10% H_2 and 85% N_2 or, alternatively, by using Anaerocult ®A (Merck, UK) to maintain anaerobic conditions in an anaerobic jar.

Table 2.2. List of bacterial strains used in this project.

Strain name	Isolate code	Media	Growth conditions	Source
INDICATOR STRAINS				
S. enterica serovar Typhimurium	LT2 (QIB)	LB or L	37 °C, 250 rpm aerobic or anaerobic	QIB culture collection
E. coli	ATCC 25922	LB or L	37 °C, 250 rpm Aerobic or anaerobic	QIB culture collection American Type Culture Collection
E. coli	LMG 2092	LB or L	37 °C, static, aerobic or anaerobic	Instituto de Productos Lacteos (IPLA)
Cronobacter sakazakii	DSMZ 4485 NCTC 11467	BHI	37 °C, static, aerobic	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
C. sakazakii	LMG 5740	BHI	37 °C, static, aerobic	IPLA
Clostridium perfringens	NCTC 3110	BHI + complements	37 °C, static, anaerobic	QIB culture collection
Clostridium difficile	NCTC 11209	BHI + complements	37 °C, static, anaerobic	QIB culture collection
Listeria innocua	NCTC 11288	BHI	37 °C, static, aerobic or anaerobic	QIB culture collection
Campylobacter jejuni	NCTC 11168	Brucella agar	37 °C, static, microaerophilic, 5% O_2	QIB culture collection
Micrococcus luteus	MC8166	MRS	37 °C, static, aerobic	QIB culture collection
Lactobacillus bulgaricus	5538	MRS	37 °C, static, anaerobic	Teagasc culture collection
L. bulgaricus	LMG 6901	MRS	37 °C, static, anaerobic	Teagasc culture collection
QIB ISOLATES[a]				
Lactobacillus amylovorus	Lac 20	MRS	37 °C, static, anaerobic	QIB culture collection

Lactobacillus gallinarum	Lac 24	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus mucosae	Lac 25	MRS	37 °C, static, anaerobic	QIB culture collection
L. mucosae	Lac 28	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus saerimneri	Lac 29	MRS	37 °C, static, anaerobic	QIB culture collection
L. mucosae	Lac 30	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus crispatus	Lac 32	MRS	37 °C, static, anaerobic	QIB culture collection
L. crispatus	Lac 34	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus taiwanensis	Lac 37	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus reuteri	Lac 40	MRS	37 °C, static, anaerobic	QIB culture collection
L. gallinarum	Lac 43	MRS	37 °C, static, anaerobic	QIB culture collection
L. crispatus	Lac 44	MRS	37 °C, static, anaerobic	QIB culture collection
L. crispatus	Lac 45	MRS	37 °C, static, anaerobic	QIB culture collection
L. reuteri	Lac 46	MRS	37 °C, static, anaerobic	QIB culture collection
L. gallinarum	Lac 47	MRS	37 °C, static, anaerobic	QIB culture collection
L. crispatus	Lac 50	MRS	37 °C, static, anaerobic	QIB culture collection
L. crispatus	Lac 51	MRS	37 °C, static, anaerobic	QIB culture collection
L. crispatus	Lac 52	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus frumenti	Lac 53	MRS	37 °C, static, anaerobic	QIB culture collection
L. reuteri	Lac 54	MRS	37 °C, static, anaerobic	QIB culture collection

L. reuteri	Lac 55	MRS	37 °C, static, anaerobic	QIB culture collection
L. reuteri	Lac 56	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus paracasei	R1	MRS	37 °C, static, anaerobic	QIB culture collection
L. paracasei	R2	MRS	37 °C, static, anaerobic	QIB culture collection
L. paracasei	R3	MRS	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium sp	FI10480	BHI	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium sp	FI10481	BHI	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium longum	FI10917[a]	BHI	37 °C, static, anaerobic	QIB culture collection
Enterococcus faecalis	LM1	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	LM4	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	LM14a	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	LM18	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	LM31	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM4	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM5	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	M12	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	M22	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	SC1	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	SC8	MRS	37 °C, static, anaerobic	QIB culture collection

E. faecalis	VM6	MRS	37 °C, static, anaerobic	QIB culture collection
L. gasseri	PM9	MRS	37 °C, static, anaerobic	QIB culture collection
L. gasseri	PM19	MRS	37 °C, static, anaerobic	QIB culture collection
Staphylococcus epidermis	LM10	BHI	37 °C, static, anaerobic	QIB culture collection
Klebsiella milletis	LM11	MRS	37 °C, static, anaerobic	QIB culture collection
Klebsiella pneumoniae	LM12	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobadillus gasseri	LM19	MRS	37 °C, static, anaerobic	QIB culture collection
L. gasseri	LM21	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	LM24	MRS	37 °C, static, anaerobic	QIB culture collection
K. milletis	LM26	MRS	37 °C, static, anaerobic	QIB culture collection
L. gasseri	HM8	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM3	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM6	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM7	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM9	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM10	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM11	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM12	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM13	MRS	37 °C, static, anaerobic	QIB culture collection

K. milletis	HLM14	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM15	MRS	37 °C, static, anaerobic	QIB culture collection
L. gasseri	HLM17	MRS	37 °C, static, anaerobic	QIB culture collection
E. faecalis	HLM20	MRS	37 °C, static, anaerobic	QIB culture collection
K. milletis	HLM21	MRS	37 °C, static, anaerobic	QIB culture collection
L. gasseri	HLM23	MRS	37 °C, static, anaerobic	QIB culture collection
L. gasseri	HLM24	MRS	37 °C, static, anaerobic	QIB culture collection
L. paracasei	Lac1	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus plantarum	Lac2	MRS	37 °C, static, anaerobic	QIB culture collection
L. plantarum	Lac3	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus pentosus	Lac4	MRS	37 °C, static, anaerobic	QIB culture collection
L. pentosus	Lac5	MRS	37 °C, static, anaerobic	QIB culture collection
L. plantarum	Lac6	MRS	37 °C, static, anaerobic	QIB culture collection
Lactobacillus rhamnosus	Lac17	MRS	37 °C, static, anaerobic	QIB culture collection
L. plantarum	Lac18	MRS	37 °C, static, anaerobic	QIB culture collection
L. plantarum	Lac20	MRS	37 °C, static, anaerobic	QIB culture collection
L. rhamnosus	Lac21	MRS	37 °C, static, anaerobic	QIB culture collection
L. rhamnosus	Lac22	MRS	37 °C, static, anaerobic	QIB culture collection
L. rhamnosus	Lac23	MRS	37 °C, static, anaerobic	QIB culture collection

L. rhamnosus	Lac24	MRS	37 °C, static, anaerobic	QIB culture collection
L. rhamnosus	Lac25	MRS	37 °C, static, anaerobic	QIB culture collection
L. rhamnosus	Lac26	MRS	37 °C, static, anaerobic	QIB culture collection
L. rhamnosus	Lac27	MRS	37 °C, static, anaerobic	QIB culture collection
L. rhamnosus	Lac28	MRS	37 °C, static, anaerobic	QIB culture collection
L. plantarum	Lac29	MRS	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium adolescentis	Bif1	BHI	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium animalis	Bif3	BHI	37 °C, static, anaerobic	QIB culture collection
B. animalis	Bif4	BHI	37 °C, static, anaerobic	QIB culture collection
B. animalis	Bif5	BHI	37 °C, static, anaerobic	QIB culture collection
B. animalis	Bif8	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif9	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif10	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif12	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif14	BHI	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium catenulatum	Bif15	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif16	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif17	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif18	BHI	37 °C, static, anaerobic	QIB culture collection

B. adolescentis	Bif19	BHI	37 °C, static, anaerobic	QIB culture collection
B. adolescentis	Bif20	BHI	37 °C, static, anaerobic	QIB culture collection
B. longum	Bif21	BHI	37 °C, static, anaerobic	QIB culture collection
B. catenulatum	Bif22	BHI	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium sp	Bif25	BHI	37 °C, static, anaerobic	QIB culture collection
B. longum	Bif27	BHI	37 °C, static, anaerobic	QIB culture collection
Bifidobacterium sp	Bif30	BHI	37 °C, static, anaerobic	QIB culture collection
TEAGASC ISOLATES				
Pediococcus acidilactici	Li3	BHI	37 °C, static, anaerobic	Teagasc culture collection
Enterococcus spp	133	BHI	37 °C, static, anaerobic	Teagasc culture collection
Streptococcus salivarius	107	BHI	37 °C, static, anaerobic	Teagasc culture collection
Streptococcus mutans	130	BHI	37 °C, static, anaerobic	Teagasc culture collection
Enterococcus spp	106	BHI	37 °C, static, anaerobic	Teagasc culture collection
Streptococcus spp	134	BHI	37 °C, static, anaerobic	Teagasc culture collection
Streptococcus agalactiae	Sagal	BHI	37 °C, static, anaerobic	Teagasc culture collection
S. salivarius	130.3	BHI	37 °C, static, anaerobic	Teagasc culture collection
Enterococcus faecium	111 Li	BHI	37 °C, static, anaerobic	Teagasc culture collection
S. mutans	110	BHI	37 °C, static, anaerobic	Teagasc culture collection
S. salivarius	DPC 6988	BHI	37 °C, static, anaerobic	Teagasc culture collection

E. faecalis	102	BHI	37 °C, static, anaerobic	Teagasc culture collection
S. epidermidis	104 Lb	BHI	37 °C, static, anaerobic	Teagasc culture collection
Bacteroides intestinalis	DSM 17393	BHI	37 °C, static, anaerobic	Teagasc culture collection
B. fragilis	LMG10263	BHI	37 °C, static, anaerobic	Teagasc culture collection
Bacteroides vulgatus	LMG 11767	BHI	37 °C, static, anaerobic	Teagasc culture collection
Bacteroides thetaiotaomicran	LMG 11262	BHI	37 °C, static, anaerobic	Teagasc culture collection
Bacteroides dorei	DSM 17855	BHI	37 °C, static, anaerobic	Teagasc culture collection
Bacteroides spp	9 -1-42FAA	BHI	37 °C, static, anaerobic	Teagasc culture collection
Bacteroides uniformis	DSM 6597	BHI	37 °C, static, anaerobic	Teagasc culture collection
Coprobacillus sp	29_1/D6	BHI + complements	37 °C, static, anaerobic	Teagasc culture collection
FAECAL AND FOOD ISOLATES FROM THIS STUDY				
--	Strain 12c	LB	37 °C, static, anaerobic	This work
S. epidermidis	9[c]	BHI + complements	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI01	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI02	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI03	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI04	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI06	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI07	MRS	37 °C, static, anaerobic	This work

L. plantarum /L. paraplantarum	GI08	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI09	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI11	MRS	37 °C, static, anaerobic	This work
L. plantarum /L. paraplantarum	GI13	MRS	37 °C, static, anaerobic	This work
S. salivarius	GI14	GM17	37 °C, static, anaerobic	This work
S. salivarius	GI16	GM17	37 °C, static, anaerobic	This work
S. epidermidis	YI03	BHI	37 °C, static, anaerobic	This work
S. epidermidis	YI04	BHI	37 °C, static, anaerobic	This work
S. epidermidis	99	BHI	37 °C, static, anaerobic	This work
Blautia sp.	F505G	GM17	37 °C, static, anaerobic	This work
Blautia sp.	F501B	BHI	37 °C, static, anaerobic	This work
Blautia sp.	F505B	BHI	37 °C, static, anaerobic	This work
Blautia sp.	F508B	BHI	37 °C, static, anaerobic	This work
Blautia sp.	F514B	BHI	37 °C, static, anaerobic	This work
Roseburia sp.	F516B	BHI	37 °C, static, anaerobic	This work
Blautia sp.	F521B	BHI	37 °C, static, anaerobic	This work
Ruminococcus sp. K-1	F522B	BHI	37 °C, static, anaerobic	This work
Blautia sp.	F523B	BHI	37 °C, static, anaerobic	This work
Ruminococcus sp. K-1	F524B	BHI	37 °C, static, anaerobic	This work

Blautia sp.	F525B	BHI	37 °C, static, anaerobic	This work
S. epidermidis	F530B	BHI	37 °C, static, anaerobic	This work
B. bifidum	F501M	M17	37 °C, static, anaerobic	This work
B. bifidum	F503M	M17	37 °C, static, anaerobic	This work
B. bifidum	F505M	M17	37 °C, static, anaerobic	This work
B. bifidum	F510M	M17	37 °C, static, anaerobic	This work
B. bifidum	F511M	M17	37 °C, static, anaerobic	This work
B. bifidum	F512M	M17	37 °C, static, anaerobic	This work
B. bifidum	F513M	M17	37 °C, static, anaerobic	This work
B. bifidum	F515M	M17	37 °C, static, anaerobic	This work
B. bifidum	F516M	M17	37 °C, static, anaerobic	This work
B. bifidum	F519M	M17	37 °C, static, anaerobic	This work
B. adolescentis	F520M	M17	37 °C, static, anaerobic	This work
B. bifidum	F523M	M17	37 °C, static, anaerobic	This work
B. bifidum	F524M	M17	37 °C, static, anaerobic	This work
B. bifidum	F525M	M17	37 °C, static, anaerobic	This work
B. bifidum	F526M	M17	37 °C, static, anaerobic	This work
B. bifidum	F527M	M17	37 °C, static, anaerobic	This work
B. bifidum	F528M	M17	37 °C, static, anaerobic	This work

CONTROL STRAINS				
S. epidermidis	DSM 20042	BHI	37 °C, static, anaerobic	DSMZ
S. epidermidis	DSM 28764	BHI	37 °C, static, anaerobic	DSMZ
S. epidermidis	DPC 6293	BHI	37 °C, agitation, anaerobic	Teagasc culture collection
S. epidermidis	DPC 6010	BHI	37 °C, agitation, anaerobic	Teagasc culture collection
L. rhamnosus	GG ATCC 53103	MRS	37 °C, static, anaerobic	IPLA
VECTOR STRAINS				
E. coli pUK200	FI10474	L + Cm	37 °C, static, aerobic	QIB culture collection

- No match in Ribosomal Database project. [a], strains from the QIB culture collection were previously isolated from human faecal material by Carmen Nueno Palop, Daniela Musolino, Valeria Rizello and Diane Hatziioanou.

Table 2.3. List of genetically modified organisms made and growth conditions used in this project

Strain name	Plasmid	Media	Growth conditions	Antibiotic resistance
L. gasseri LM19	pUK200[b]	MRS	37°C, static, anaerobic	Chloramphenicol (Cm)
L. amylovorus Lac 20	pUK200	MRS	37°C, static, anaerobic	Cm
L. crispatus Lac 50	pUK200	MRS	37°C, static, anaerobic	Cm
L. crispatus Lac 51	pUK200	MRS	37°C, static, anaerobic	Cm
E. coli ATCC 25922	pUK200	MRS	37°C, static, anaerobic	Cm

[b] [201]

Table 2.4. Antibiotics used for general genetic modifications and selection studies.

Antibiotic	Solvent used for preparation	Final concentration
Chloramphenicol	Ethanol	7.5 µg/ml
Erythromycin	Ethanol	400 ng/ml
Gentamycin	dH_2O	10 and 50 µg/ml
Streptomycin	dH_2O	50 µg/ml
Penicillin	dH_2O	50 I.U./ml
Amphotericin	DMSO	1.25 µg/ml
Kanamycin	dH_2O	50 µg/ml

2.1.3. Measurement of cell growth

Bacterial cell growth was calculated in broth and plates using different methods. For growth measurements in broth growth, Jenway spectrophotometer (UK) and Synergy HT Microplate reader (Biotek, UK) were used measuring optical density at 600 nm (OD_{600}). Additionally, growth curves were measured using Bioscreen C (Growth Curves USA, USA). Omnilog ®Phenotype Microarray™ (Biolog Inc, USA) was used for phenotypic characterisation based on bacterial growth.

Bacterial enumeration in colony forming units (CFU) was conducted by plating 10 µl in triplicates for each 10-fold dilution on the appropriate solid media.

2.1.4. Microscopy

2.1.4.1. Gram stain and light microscopy

Typically, Gram staining was conducted using a kit from Invitrogen (UK) by transferring a loopful of liquid culture to a clean glass slide, this was spread and dried by flaming briefly using a Bunsen burner on the underneath of the slide. The slide was flooded with crystal violet solution for one min, washed off briefly with tap water to remove stain and drained. The slide was flooded again with Gram's Iodine solution for one min and washed off with tap water and drained again carefully before flooding again using 95% alcohol for 10 s for decolorization and washed with tap water. The slide was drained again and flooded with safranin solution for 30 s before washing off again using tap water and dried with blotting paper. For examination under light microscope (Leica, UK), immersion oil was used.

2.1.4.2. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

Bacterial strains were grown overnight following specific growth conditions. 1 ml of culture was centrifuged at 4000 g for 5 min at RT. SEM and TEM was performed by Kathryn Cross and Catherine Booth (QIB) following protocols described previously [202].

2.1.5. Bioassays to measure antimicrobial activity

2.1.5.1. Antimicrobial overlay assay

5 µl aliquots of an overnight culture of the test strain were spotted onto agar plates (containing 2% v/v of $NaHCO_3$ if lactic acid production was expected) and incubated for 1-2 d. Grown bacterial colonies were exposed to UV light (302 nm) for 15 minutes and then covered with 5 ml of soft agar cooled to <50^oC inoculated with 100 µl of the indicator strain overnight culture (with the exception of *E. coli* and *S. enterica*, where the inoculum was 200 µl). Overlay plates were incubated overnight in aerobic or anaerobic conditions depending on the indicator strain growth requirements. Antimicrobial activity was considered positive if an inhibition zone in the growth of the indicator strain was observed [203].

2.1.5.2. Cross streak assay

The test strain was streaked onto an agar plate (containing 2% v/v of $NaHCO_3$ if lactic acid production was expected) and incubated to allow growth. These streaks were exposed to UV light for 15 minutes and cross-streaked with different indicator strains. Plates were incubated overnight in different conditions depending on the indicator strains. Antimicrobial activity was considered positive if an inhibition zone in the growth of the indicator strain was seen [203].

2.1.5.3. Drop test

Indicator strains were cultured overnight depending on their growth conditions and diluted 1:100 in phosphate buffered saline (PBS). 100 µl was plated onto agar plates using an L-shaped loop to produce a lawn. 10 µl of cell free supernatants (centrifuged at 3220 g for 10-15 min and filtered through a 0.22 µm filter (Millipore) to remove bacteria), from overnight cultures of the test strain, were spotted onto the lawn. Plates were incubated as appropriate and antimicrobial activity was considered positive if an inhibition zone in the growth of the indicator strain was seen.

2.1.5.4. Filter disc

The method was followed as above (drop test) but supernatants were spotted onto a 5 mm filter disc (3 mm, Whatman) that was placed onto the bacterial lawn and plates were kept at 4ºC for 2 hours to allow diffusion through the agar and then incubated at different conditions depending on the indicator strain. Antimicrobial activity was considered positive if an inhibition zone in the growth of the indicator strain was seen [203].

2.1.5.5. Well-diffusion assays

The aim of this method is to assess the antimicrobial activity of a liquid supernatant, placed in a well in the agar, by the inhibition in the growth of an indicator strain inoculated in that agar. For this purpose, *L. bulgaricus* 5583 was used as an indicator strain and cultured overnight in MRS broth, anaerobically at 37ºC. 1 mL of this culture was inoculated in 200 mL of molten MRS agar (normal strength) and poured in plates where wells were performed. To obtain the cell-free supernatants, 1.5 mL of culture of the strain to be tested were centrifuged at 16,000 g for 2 min. After this, 50 µL were placed inside the corresponding well and the plate was left for overnight anaerobic incubation at 37ºC. The expected result is the presence of an inhibition zone of growth of the indicator strain around the well. For a positive control *L. salivarius* 6502 was used, and for negative control, MRS broth without inoculation was used.

2.1.5.6. *Campylobacter* assays

Skirrow plates (Oxoid) were inoculated with 50 µl of a 'one-shot' *C. jejuni* glycerol stock and incubated overnight at 37ºC in a microaerobic cabinet. The following day, cells grown on the Skirrow plate were resuspended in 2 ml of PBS and the OD_{600} was measured. A dilution of final A600 = 1 was prepared in 1 ml of PBS. 5 ml of Brucella/agar mix (1.5 g agar in 100 ml of Brucella broth with 0.01% of triphenyl tetrazolium chloride (TTC)) were added and quickly mixed and poured into a fresh Brucella plate. Filter discs were placed onto the agar and spotted with 10 µl of the previously filtered supernatant of the cultured strain of interest. A positive control of 10% hydrogen peroxide was used. Plates were placed faced up in a bag and incubated overnight in microaerobic conditions (85% N_2, 5% O_2, 10% CO_2) in a MACS-MG-1000 controlled atmosphere cabinet (Don Whitley Scientific, UK). Presence of antimicrobial activity was considered positive if an inhibition zone in the growth of *C. jejuni* was seen.

2.1.5.7. Modification of growth conditions and inducers

In order to induce antimicrobial production, growth conditions of antimicrobial-producing bacteria were modified. They were grown in 20 ml of different media (GM17, L, BHI, BHI+C, M17, MRS without glucose, MRS + 0.05M cysteine HCl + 0.5M sucrose, M17 Broth + 0.5 M sucrose, M17 + 2.5% glycine or M9 minimal media), with different period of incubation (24, 48 and 72 h), and under aerobic and anaerobic conditions and tested for antimicrobial activity by drop test method.

2.1.5.8. Co-culture assays

In order to induce the release of antimicrobials in the media, strains that showed antimicrobial activity were co-cultured in different combinations, by inoculating first the strain of interest and at 24 h the pathogen or by inoculating both of them at the same time. After that, drop tests were performed as described previously.

2.1.5.9. Minimum inhibitory concentration (MIC) assays

96-well plates (Sarstedt) were pre-treated with bovine serum albumin (BSA). For this purpose, 0.5 g of BSA (Sigma) was mixed with 2.5 ml of 20X PBS. Water was added to the mix for a total volume of 50 mL. 200 µL were added to each well and the plate was incubated for 30 to 45 mins at 37^{o}C. After this time, BSA was removed and the wells washed with 1X PBS and air dried in a sterile hood. This pre-treatment must be prepared the day of assay. For MICs, peptide concentrations of 4X the highest test concentration were prepared in 350 µL of the growth media required for the target strain to grow, and 100 µL of this mix was added to the first well of each row. 100 µL of the same media were added to each well and 2-fold serial dilution was performed alongside the row. After this, 100 µL of culture inoculated with the target strain was added to each well. To prepare this inoculum, an overnight culture was sub-cultured the day of the assay and incubated until an OD_{600} of 0.5 was reached. The culture was diluted 1:10 and 20 µL of this added to 980 µL of media. 150 µL of this new mix was added to 14.85 mL of growth media, from where the 100 µL per well were added. The objective is to standardise the final inoculum to 10^5 cfu/mL in 200 µL. The plate was incubated under the requirements of the target strain, and MIC determined as the lowest peptide concentration where there was no growth of the target strain.

2.1.6. Assessment of characteristics of probiotic activity

2.1.6.1. Autoaggregation

Autoaggregation assays were carried out following the protocol described by Tuo *et al* [204], with some modifications. Bacterial cultures were grown with the requirements of each strain for 24 h and their OD_{600} was corrected to 1.2 ± 0.05. 1 ml of each corrected culture was measured after 2, 4 and 24 h for autoaggregation using a Ultrospec 10 spectrophotometer (Amersham Biosciences). Incubation was performed on the bench at room temperature. Percentage of autoaggregation was calculated by $(A_t / A_o\text{-}1) \times 100$ where A_t is absorbance at time = 24 hours and A_o represents absorbance at time = 0 h.

2.1.6.2. Antioxidant activity

In order to evaluate the antioxidant activity of the tested strains, a protocol described by Gil-Rodriguez *et al* was used [205]. This method was based on measuring the percentage of reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Bacterial cells were harvested by centrifuging 1 ml of a 24 h cell culture, at 17000 x g during 5 min. The pellet was washed twice with 0.9% NaCl sterile solution and resuspended in 1 mL of the same saline solution to maintain the viability of the cells. 800 µL were transferred to a new tube and 1 mL of the DPPH solution prepared at a concentration of 0.2 mM in methanol was added, vortexed and incubated in darkness for 30 min at room temperature. After that, tubes were centrifuged at 17000 g for 5 min, and 300 µL transferred to a 96 well plate. Absorbance was measured at 517 nm in a Synergy HT plate reader from BioTek and Gen5 software version 2.00.17. Percentage of reduction of DPPH was calculated by $(1\text{-}A_{517\ sample} / A_{517\ blank}) * 100$.

2.1.6.3. Bile salt hydrolase (BSH) activity

To test BSH activity, an adaptation of Sedlackova *et al* was followed [206]. Strains were grown in their appropriate conditions for 24 h. After that, 5 µL of the culture were spotted onto a soft agar (0.7%) plate containing calcium chloride 0.375 g/L and 0.3% Sodium taurodeoxycholate hydrate. Plates were incubated for 3 d. BSH activity was characterised by the presence of a bright white precipitate.

2.1.7. Preparation of a faecal inoculum for faecal fermentations

The samples of healthy donors, with no antibiotic treatment during the last six months, were collected and maintained at 4ºC during 1-2 h before processing. 200 g of faeces in

total were placed in a Circulator 400 stomacher bag (Seward, UK) alongside an equal volume of 50 mM phosphate buffer with 0.05% (w/v) cysteine hydrochloride. After filtering and homogenising in a Stomacher 400 circulator (Seward), the faecal slurry was centrifuged (4000 x g) and resuspended in 50 mM phosphate buffer.

2.1.8. Biofilm formation

2.1.8.1. Biofilm formation on abiotic surfaces

To measure biofilm formation of the strains of interest, three abiotic surfaces were tested: gold (16-well E-plates), glass and polystyrene (PE). Bacterial cultures were standardised for a couple of days. To do that, a colony of each bacteria was selected and cultured in 10 ml of the appropriate liquid medium. After 24 h, it was subcultured in 10 ml of fresh medium at 1% and another subculture was performed after another 24 h. Bacterial counts were performed at this point at -6 to -8 dilutions. An appropriate volume of bacterial culture was added to each well of the 16-well E-plate (ACEA Biosciences Inc), 96-well PE plate (200 μl in each case) and 12-well plate (BD Falcon) (1ml) where a sterilised glass cover was added previously. Bacterial adhesion was monitored by xCelligence RTCA-DP (ACEA Biosciences Inc., USA) for 24 h and biofilm formation was confirmed by crystal violet staining assay.

2.1.8.2. Crystal violet staining

Supernatants were removed from the wells where the biofilms were grown, and these were washed with PBS in equivalent volumes to the previously removed from the wells. One volume of 0.1% crystal violet solution equivalent to the volume added to each well was added and incubated at room temperature for 15 min. Crystal violet was removed from the wells and an equivalent volume of 33% acetic acid was added. The mixture was transferred immediately to a 96-well plate and OD read at λ=595 nm, used to measure crystal violet.

2.1.8.3. Adhesion on biotic surface

The HT29 cell line (human colon adenocarcinoma, ECACC 91072201, European Collection of Cell Cultures, Salisbury, UK) was used as a model to test adhesion of the tested strain to a biotic surface [207]. Cells were grown until confluency in a 12-well plate (BD Falcon). Bacterial cultures were standardised for two d in 10 ml of the appropriate medium and an overnight culture was centrifuged (17,000 x g, 10 min) and washed twice with PBS. The pellet was resuspended in 10 ml of PBS and 2.5 ml were taken, pelleted and resuspended

in 2.5 ml of McCoy's medium (MM) without antibiotics. Bacterial counts were performed at this stage in dilutions -6 to -8. HT29 cells were washed twice with 1 ml of Dubelco's PBS. 1 ml of the bacterial suspensions were added to each well. The plate was incubated for 1 h at 37°C in microaerophilic conditions and after that wells were washed twice with 1 ml Dubelco's PBS. 1 ml of Trypsin-EDTA solution 0.25% was added to each well and incubated for 5-10 min at 37°C in microaerophilic conditions. The reaction was stopped by adding 1 ml of the HT29 culture medium MM, and the suspensions were homogenised and serial dilutions (-3 to -7) performed in PBS. *L. rhamnosus* GG that showed previously adhesion levels of 1-5% was used as a positive control. Percentage of adhered bacteria was calculated by (adhered bacteria/added bacteria) x 100.

2.1.8.4. Exclusion of pathogens

2.1.8.4.1. Exclusion of pathogens using live strains

To identify possible ability of the Lactobacilli and *S. epidermidis* isolates to prevent and/or impede pathogen attachment to a biotic surface such as the HT29 cell line, a fluorescence dye (SYTO-9) that targets DNA was used. The HT29 cell line was grown to confluence in a 48 well plate. Tested bacteria and pathogenic bacteria were grown separately overnight after being standardised over 2 d. 1.5 ml of those overnight cultures were washed with PBS and OD_{600} readings were standardised to approximately $1x10^9$ cfu/ml. The pellets were resuspended in 1.5 ml McCoy's medium with antibiotics (gentamycin, streptomycin, penicillin and amphotericin)[207]. In the pathogen suspensions, 4.5 μl of SYTO9 (Invitrogen) were added, to a final concentration of 15 μM and the mix was incubated in darkness for 2 h at room temperature. The unbound dye was removed by centrifugation at 3,200 x g for 10 minutes and the pellet was resuspended in 1.5 ml of MM with antibiotics, and the suspension kept in darkness. 100 μl of labelled pathogen and 100 μl of probiotic suspension to test were mixed and added to the HT29 cell plate. Tested lactobacilli bacteria were combined in equal proportion in 100 μl and added to 100 μl of each pathogen. Before the addition of the 200 μl, HT29 cell wells were washed at least once with PBS. A control of labelled pathogen was set, consisting of 100 μl of labelled pathogen and 100 μl MM with antibiotics. The plate was incubated in darkness for an hour at 37°C in microaerophilic conditions. The supernatant of each well was removed and the well washed with PBS. 100 μl trypsin-EDTA solution was added to each well and incubated for 5-10 min at 37°C in

darkness and microaerophilic conditions. To stop the reaction, 100 μl MM with antibiotics were added and the 200 μl of each well transferred to an appropriate fluorometer-validated 96-well plate. Fluorescence was measured in a Cary Eclipse Fluorescence Spectrophotometer (Agilent) at excitation wavelength of 470 nm and emission wavelength of 512 nm. Percentage of adhesion was measured by (fluorescence emitted by bacteria adhered / fluorescence emitted by bacteria add) x 100.

2.1.8.4.2. Exclusion of pathogens using dead strains

To test if the possible influence of the tested bacteria on the adhesion of the pathogens to the HT29 cell line required the viability of the tested strains, a treatment to supress their viability was added. HT29 cells of approximately 150 passages were grown in a 48-well plate and the tested bacteria were standardised for 2 d and grown overnight. After this, tested bacteria were harvested by centrifugation, washed with PBS and recentrifuged at 3000 x g for 10 min, and resuspended in PBS. Suspensions were treated with UV light in a UV chamber (15 W, Selecta, Spain) in three cycles of 30 min each, resuspending for a few minutes after each one. 100 μl of each tested bacteria was plated in the appropriate agar media to confirm that no viable cells were present in those suspensions. Non-viable bacterial suspensions were stored at 4°C. 1.5 ml of those suspensions were washed with PBS and resuspended in 1.5 ml McCoy's medium with antibiotics. Pathogens were grown overnight after being standardised for two days and 4.5 μl of SYTO9 were added to the viable pathogen suspensions to a final concentration of 15 μM. The mix was incubated in darkness for 2 h at room temperature and the unbound dye removed by centrifuging at 3200 g for 10 min. The pellet was resuspended in 1.5 ml of MM with antibiotics, and the suspension kept in darkness. The protocol used for live bacteria (2.3.1) was then followed.

2.1.9. Phenotypic studies using BIOLOG system

BIOLOG studies were conducted using an OmniLog® Phenotype MicroArray™ following manufacturer's instructions for panels PM1, PM2, PM3, PM11, PM12, PM13, PM16, PM18 and PM19.

2.2. Molecular biology

Protocols were performed following methods described in Sambrook *et al*. [208]. Kits and conditions were followed according to manufacturer's instructions.

2.2.1. PCR primers

Primers used during this project are summarised in Table 2.5.

Table 2.5. List of primers used in this project.

Name	Target	Sequence	Product (bp)	Reference
AMP_F	16S	5'-GAG AGT TTG ATY CTG GCT CAG	1500	[209]
AMP_R	16S	5'-AAG GAG GTG ATC CAR CCG CA		[209]
Bif_F	Bifid 16S	5'-GAA CGG GTG AGT AAT GCG TG	700	Designed by C. Nueno-Palop
Bif_R	Bifid 16S	5'-TGT TCT TCC CGA TAT CTA CAC		
plnC_F	Plantaricin C	5'-AGC AGA TGA AAT TCG GCA G	109	[210]
plnC_R	Plantaricin C	5'-ATA ATC CAA CGG TGC AAT CC		[210]
plnG_F	Plantaricin G	5'-TGC GGT TAT CAG TAT GTC AAA G	454	[210]
plnG_R	Plantaricin G	5'-CCT CGA AAC AAT TTC CCC C		[210]
CO1	16S Teagasc	5'-AGT TTG ATC CTG GCT CAG	1500	[211]
CO2	16S Teagasc	5'-TAC CTT GTT ACG ACT		[211]
P181	pUK200 comprobation	5'-GCG AAG ATA ACA GTG ACT CTA	140	[212]
P54	pUK200 comprobation	5'-CGG CTC TGA TTA AAT TCT GAA G		
LGgyrAF	Gyrase A *L. gasseri* LM19	5'-TTG ATT GCC TTA ACC CTT CG	136	This work
LGgyrAR		5'-TTC CCAT TGA ACG AAC ATCA		
Cluster 1.1F	LM19 C.1.1	5'-TAT TGG TGC ATG GAG AGG TG	124	This work
Cluster1.1R		5'-CCA GCC CAC ACA TTG TAC TG		
Cluster1.2F	LM19 C.1.2	5'-TTG GGG TAG TGT TGC AGG AT	97	This work
Cluster1.2R		5'-TGA TGT TGC AGC TCC GTT AG		
Cluster2F	Helveticin J-like	5'-CTT GGG TAC AAA GCG GAG AA	176	This work
Cluster2R		5'-GCC TGC TCG GTT AAG ATA AG		
Cluster3.1F	GamA (=Gassericin T)	5'-CTG GAT GGG CTC TTG GAA AT	112	This work

Cluster3.1R		5’-TTT CCG AAT CCA CCA GTA GC		
Cluster3.2F	GamX (=Gassericin X)	5’-TGG GGG AAT GCT GTA ATA GG	100	This work
Cluster3.2R		5’-CTC CTA AGC CAC AGG CAG TC		
GamYF	Gassericin Y	5’-ACT CAA ATC GTA GGA GGA AAA GG	150	This work
GamY R		5’-AAA GCA TGC ACC TGA ACCA		
GasM F	Gassericin M	5’-AGC AGG AGG AGC ATT TTC AA	90	This work
GasM R		5’-CCT GCT GCA CCA CCT AAA AT		
Cluster 3.3 F	Immunity gene gamI3	5’-CAG ATG AAG AAG CAT TAC TTG AAA A	102	This work
Cluster 3.3 R		5’-TTC CAG GCC AAG TAT TAG TTG TA		
s-Clper-F	*C. perfringens*	5’-GGG GGT TTC AAC ACC TCC	170	[213]
ClPER-R		5’-GCA AGG GAT GTC AAG TGT		
515F	Region V4	5’-GTG CCA GCM GCC GCG GTA A	292	[214]
806R		5’-GGA CTA CHV GGG TWT CTA AT		

2.2.2. General PCR methods

Thermal cycling was typically performed using a Veriti 96 well Thermal Cycler (Applied Biosystems, USA) and PCR Sprint (Thermo Scientific, UK). Primers were designed with annealing temperature of 55-60^{o}C and were purchased from Sigma Genosys. GoTaq (Promega) and Platinum PCR SuperMix (Thermo Fisher) were used for general PCR testing and Phusion polymerase (Finnzymes) was used for cloning purposes because of its high fidelity. dNTPs were purchased from Bioline.

Table 2.6. PCR reaction mix composition for the three polymerases used during the project.

GoTaq polymerase		Phusion polymerase		Platinum PCR SuperMix	
Component	Quantity	Component	Quantity	Component	Quantity
DNA template	1 µl	DNA template	1 µl (~ 5 or 50 ng)	DNA template	1 µl
5 x GoTaq Reaction Buffer	10 µl	5 x Phusion Reaction Buffer	10 µl	Reaction buffer	45 µl
dNTPs (0.2 mM of each one)	0.4 µl	dNTPs (0.2 mM of each one)	0.4 µl	dNTPs (0.2 mM of each one)	Included in buffer
Forward primer 20 µM	1 µl	Forward primer 20 µM	1.25 µl	Forward primer 20 µM	2 µl
Reverse primer 20 µM	1 µl	Reverse primer 20 µM	1.25 µl	Reverse primer 20 µM	2 µl
UPH_2O	36.35µl	UPH_2O	35.7 µl	UPH_2O	Included in buffer
1.25 U/µl GoTaq DNA polymerase	0.25 µl	1 U/µl Phusion Polymerase	0.4 µl	22 U/ml Taq Polymerase	Included in buffer
Total volume	50 µl	Total volume	50 µl	Total volume	50 µl

Table 2.7. PCR conditions for the three polymerases used during the project.

GoTaq polymerase			Phusion polymerase			Platinum PCR SuperMix		
Temperature and time		Cycles	Temperature and time		Cycles	Temperature and time		Cycles
95°C	2 min	x 1	98°C	30 s	x 1	94°C	30 s	x 1
95°C	30 s	}	98°C	10 s	}	94°C	30 s	}
55-60°C	30 s	}x 25-30	TA°C	30 s	}x 25-30	55°C	30 s	}x 25-35
72°C	1 min/kb	}	72°C	15-30 s/kb	}	72°C	1 min/kb	}
72°C	5 min	x 1	72°C	5 min	x 1	72°C	5 min	x 1

2.2.2.1. PCR from liquid cultures

150 µl of overnight bacterial culture were centrifuged at 13,000 x g for 1 min. Supernatant was removed and 150 µl of UPH_2O added. In the case of lactic acid bacteria, colony wash buffer (100 mM NaCl, 10 mM Tris pH 7 and 1 mM EDTA) was used instead of UPH_2O. The pellet was resuspended by vortexing then the suspension was centrifuged again,

supernatant was removed, and the pellet was resuspended in 15 µl of UPH_2O. The sample was boiled at 95 ^oC for 5 min and 1 µl was used as a DNA template for PCR reaction.

2.2.2.2. PCR from bacterial colonies

A colony was sampled using a toothpick and resuspended in 10 µl of UPH_2O. The sample was boiled at 95^oC for 5 min and 1 µl was used as a DNA template for PCR reaction.

2.2.2.3. 16S identification

Identification of strains was determined using PCR amplification of the 16S ribosomal DNA gene following primers and conditions summarised in tables 2.6 and 2.7. PCR cycles were limited to 25 to avoid errors. If a high G+C content was expected, 5% DMSO was added.

2.2.3. Agarose gel electrophoresis

Agarose gels were produced by adding agarose powder at 1% w/v (2% for small fragments, 0.7% for genomic (g)DNA to 0.5 x TBE buffer (Fisher Scientific). 1 kb Hyperladder (Bioline) was used as a DNA size marker. 5 µl of DNA marker and a mix of 9 µl of DNA sample plus 1 µl of loading buffer were typically loaded in the gel wells. 0.5 x TBE buffer was used to conduct electrophoresis. To visualise DNA, gels were stained for 30 min in ethidium bromide 1 µg/ml and washed in water afterwards. DNA bands were visualised with UV using an AlphaImager (Alpha Innotech).

2.2.4. DNA purification

PCR products were purified (to remove buffers, dNTPs, short DNA fragments and enzymes) using Sure Clean (Bioline), following manufacturer's instructions with small variations. Briefly, 5 µl of a pink co-precipitant were added to the PCR mix. An equal volume of Sure Clean was added, vortexed and left to incubate for 30 min at room temperature. After this, the mixture was centrifuged at 16,000 x g for 30 min and the supernatant was removed. The pink pellet was washed in a volume of 70% ethanol of twice the original sample volume and centrifuged again for 5 min. All the ethanol was removed, and the sample was left to air dry on the bench. Once dried, sample was resuspended in 20 µl of EB buffer (10 mM Tris-HCl pH 8.5).

2.2.5. 16S rDNA sequencing

DNA concentration was measured using a Nanodrop 2000 (Thermo Scientific, UK) and sequencing was performed by Eurofins Genomics (Germany). 300 ng of DNA were required

in a volume of 15 µl. Additionally, 1 µl of a primer at a concentration of 20 µM and UPH_2O to a final volume of 17 µl. Data provided by Eurofins was analysed using Finch TV sequence viewer software and SeqMan and Seqmatch II (DNAStar, Lasergene). Species were identified using the Ribosomal Database Project [215].

2.2.6. Extraction of genomic DNA (gDNA)

2.2.6.1. From single bacterial culture

Genomic DNA extraction was carried out using the GenElute ^{TM} Bacterial Genomic DNA Kit from Sigma Aldrich. The protocol provided in the kit was followed and the concentration of gDNA was measured by Qubit 3 (Invitrogen, UK) and Nanodrop 2000 (Thermo Scientific, UK), with agarose gel electrophoresis to confirm the integrity of the gDNA. The gDNA was eluted in EB buffer (10 mM Tris-HCl pH 8.5).

2.2.6.2. From faecal fermentation samples

Genomic DNA extraction was carried out using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) and and AllPrep DNA/RNA Mini Kit (Qiagen, UK), following manufacturer's guidelines.

2.2.7. RNA work

2.2.7.1. RNA isolation

Each fermentation sample was mixed with two volumes of RNAlater (Sigma-Aldrich, UK) and centrifuged for 10 min at 18,000 x g and 4^{o}C. Supernatant was discarded, and pellets stored at -80 ^{o}C until extraction which was performed using the Qiagen RNeasy extraction kit with minor modifications. Pellets were resuspended in 1 ml RLT buffer provided in the kit, complemented with 10 µl of β-mercaptoethanol (Millipore) and transferred to lysing matrix E tubes (MP biomedicals LLC, France). Samples were lysed in a FastPrep-24 homogeniser (MP biomedicals) by applying 2 pulses of 30 s and intensity 6.0 with an interval of 1 minute on ice between each pulse. After this, samples were centrifuged for 10 min at 17,000 x g and the supernatant transferred to clean 15 ml tubes and mixed with an equal volume of 70% ethanol. The mixture, including any precipitate, was transferred to spin tubes and centrifuged at 8,000 x g for 1 min. The flow-through was discarded and 700 µl buffer RW1 added to the RNeasy spin column and centrifuged again at 8,000 x g. 500 µl buffer RPE were added to the spin column and centrifuged for 1 min at 8,000 x g and the flow- through discarded. The last step was repeated, but this time centrifuged for 2 min.

The RNA was eluted in 100 µl RNase-free water and quantified by Nanodrop 2000 (Thermo Scientific, UK).

2.2.7.2. RNA cleaning

DNase treatment was performed using the Turbo DNA-free™ kit (Invitrogen, UK). Isolated RNA was adjusted to a concentration of at most 100 ng/µl. 87 µl of the RNA sample was mixed with 10 µl 10X Turbo DNase buffer and 3 µl of Turbo™ DNase (2 U / µl). The reaction was incubated at 37 °C for 30 min. After the incubation, 10 µl of resuspended DNase Inactivation Reagent were added, mixed and incubated at room temperature for 5 min. Samples were centrifuged at 17,000 g for 2 min, the supernatant transferred to a RNase free tube, and concentration and RNA quality measured by Nanodrop.

2.2.7.3. cDNA synthesis

cDNA synthesis was carried out using the QuantiTect® Reverse Transcription Kit (Qiagen) using 100 ng RNA per reaction in 12 µl RNAse-free water. 2 µl of gDNA Wipeout buffer 7x were added to a total reaction volume of 14 µl and incubated for 2 min at 42°C before being placed on ice. Reverse-transcription master mix was prepared according to manufacturer's recommendations and a control reaction replacing the reverse transcriptase with water was set up at the same time. The mixture was incubated for 15 min at 42°C then 3 min at 95°C to inactivate the transcriptase. cDNA was stored at -20°C.

2.2.8. qPCR and RT-qPCR

2.2.8.1. qPCR primer design

qPCR primers were designed using Primer 3 (v. 0.4.0) free software. Primer size were designed for a maximum of 20 bases and the product size between 50-200 bp, with preference around 100 bp. Melting temperature was established between 60-62°C for all of them. The presence of secondary structures and dimers were tested with Netprimer (Premier Biosoft) and primers were tested on 10-fold dilutions of gDNA of test strains to confirm their specificity and their efficiency (> 90%, slope -3,1 and Rsq >0.985). Oligos were also tested to check that they do not form primer dimers by a control test where the DNA template was substituted by UPH_2O.

2.2.8.2. qPCR

Aliquots were taken from fermenters and DNA extraction was performed using QIAmp Fast DNA Stool Mini Kit (Qiagen, UK), following the manufacturer's instructions. Absolute

quantification by qPCR was performed using the Roche LightCycler 480 II platform (Roche, UK). To quantify 16S bacterial counts, a standard curve was created using 1000 to 100 copies of 16S rDNA/µl and KAPA Lightcycler 480 mix (KAPA Biosystems Ltd., UK) according to manufacturer's instructions summarised in table 2.8. All samples were run in triplicate and controls for no DNA template were set up in each run. qPCR conditions are summarised in table 2.9.

2.2.8.3. RT-qPCR

qPCR was performed using 384-well plates (4titude) in the ViiA™ 7 System (Applied Biosystems) and the SensiFAST™ SYBR® No-ROX Kit (Bioline, USA). Reaction mix composition and conditions are summarised in tables 2.8 and 2.9. Reactions were set up in duplicate and controls for primers and no transcriptase samples were set in each run.

Table 2.8. Reaction mix composition for the two polymerase mixes used during the project for qPCR and RT-qPCR.

SensiFAST™ SYBR® No-ROX		KAPA SYBR® FAST	
Component	Quantity	Component	Quantity
DNA template	0.6 µl	DNA template	1 µl
SensiFAST™ master mix	3 µl	KAPA master mix	5 µl
Forward primer 20 µM	0.24 µl	Forward primer 20 µM	0.5 µl
Reverse primer 20 µM	0.24 µl	Reverse primer 20 µM	0.5 µl
UPH_2O	1.92 µl	UPH_2O	3 µl
Total volume	6 µl	Total volume	9 µl

Table 2.9. PCR conditions for the two polymerases used for qPCR and RT-qPCR.

	SensiFAST™ SYBR® No-ROX			KAPA SYBR® FAST		
	Temperature and time		Cycles	Temperature and time		Cycles
Preincubation	95°C	5 min	x 1	95°C	5 min	x 1
Amplification	95°C	20 s	}	95°C	20 s	}
	60°C	1 s	}x 40	55°C	20 s	}x 45
	-	-		72°C	20 s	}
Melting curve	95°C	15 s	x 1	95°C	5 s	x 1
	55°C	10 s	x 1	47°C	1 min	x 1
	-	-		97°C	1 min	x 1
Cooling	40°C	10 min	x 1	40°C	5 min	x 1

2.2.9. Bioinformatics analysis

2.2.9.1. Whole genome sequencing (WGS), trimming, assembly and annotation by MicrobesNG

Microbes NG performed the WGS. Briefly, DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Eppendorf AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250 bp paired end protocol. The reads were trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [216] and the quality was assessed using in-house scripts combined with the software Samtools [217], BedTools [218] and bwa-mem [219]. All the sequenced genomes had a minimum coverage of 30x. De novo assembly was performed on samples using SPAdes version 3.7 [2], and contigs were annotated using Prokka 1.11.

2.2.9.2. Genome mining for bacteriocin genes

Genomes were analysed with different software to identify putative bacteriocin clusters. BAGEL 3 and BAGEL 4 [220] to target bacteriocin clusters and antiSMASH to target secondary metabolites [221]. The assembly was also annotated with RAST (Rapid Annotation using Subsystem Technology) and visualised with SEED [222]. Genome data was visualised using Artemis [223] and Integrative Genomics Viewer (IGV) version 2.4 [224]. DNA and amino acid sequences identified as putative bacteriocin genes and proteins were analysed using BLAST [225] to assess their relationships with other peptides using default parameters. Genedoc was used for sequence alignments and Clone manager (Sci-Ed software) for cluster construction.

2.2.9.3. 16S-based metataxonomics analysis

Total gDNA concentration was measured by Qubit 3 (Invitrogen, UK) and normalised. The V4 region of the 16S rRNA gene was used for high throughput sequencing using the Illumina MiSeq platform. Data analysis was conducted using the Quantitative Insights into Microbial Ecology (QIIME2 version 2018.11) [226].

2.2.9.4. Identification of biogenic amines

Presence of genes related to biogenic amines metabolism was assessed by screening manually the RAST annotation of the sequenced genomes.

2.2.10. Transformation of Lactobacilli

2.2.10.1. Plasmid preparation

10 ml of an o/n culture of the *E. coli* strain containing the plasmid of interest were centrifuged for 10 min at 13,000 x g and 4ºC. Plasmids were extracted using the E.Z.N.A. Plasmid DNA Mini Kit (VWR, UK) following manufacturer's instructions.

2.2.10.2. Preparation of competent lactobacilli

The method used for the generation of electro-competent lactobacilli was based on the use of glycine [227]. Three subcultures were done at 37°C: first day, inoculating 10 ml of medium (MRS [Oxoid], 0.5 M sucrose, 3% glycine). Second day, 5 ml of that culture were used to inoculate 50 ml of pre-warmed medium, and the third day, 25 ml of the culture were used to inoculate 200 ml of prewarmed medium and this was incubated until OD_{600} 0.2-0.3. After that, cells were harvested by centrifuging 10 min at 5000 x g. A first wash of 225 ml of ice cold 10% glycerol/0.5 M sucrose was performed. The second wash was done using 55 ml of 10% glycerol/ 0.5 M sucrose/ 50 mM EDTA and incubated on ice for 15 min. The third wash was with 55 ml of 10% glycerol/0.5 M sucrose. Cells were resuspended in 2.25 ml of 10% glycerol/ 0.5 M sucrose, aliquoted in volumes of 40 µL and flash frozen on dry ice.

2.2.10.3. Electroporation of electrocompetent Lactobacilli cells

Plasmid pUK200 [228] was added to 40 µL of electro-competent cells. The mixture was incubated for 1 min on ice and transferred to a pre-chilled electroporation cuvette (Geneflow Limited Paul Fisher House). A pulse of 1500 V, 800 Ω and 25 µF was applied using a BioRad electroporator. 450 µL of pre-chilled MRS+glucose/ 20 mM $MgCl_2$/ 2mM $CaCl_2$ were added to the cuvette and the mixture transferred to a chilled 2ml screwcap tube and incubated for 2 h at 37ºC. 100 µL of the cells were plated in MRS plates with chloramphenicol 7.5 µg/ml and incubated overnight at 37ºC. 6 colonies of each were selected and grown in MRS medium with chloramphenicol 2.42 µg/ml and the inserted plasmid confirmed by PCR using primers p181 and p54 [229] using GoTaq DNA Polymerase according to manufacturer's instructions (Promega). Transformation efficiency was

calculated using "Bacteria Transformation Efficiency Calculator" (https://www.sciencegateway.org/tools/transform.htm).

2.3. Protein work

2.3.1. Assays to assess the nature of the antimicrobial

2.3.1.1. Heat stability test

30 µl of the cell-free supernatant from the test strain was heated to 60˚C for 10 min, 30 min and 1 h, at 90˚C for 10 min and 30 min, and 121˚C for 15 min. Samples were cooled on ice before being tested by drop test or filter disc test.

2.3.1.2. Protease resistance test

Cell-free supernatant from the test strain was incubated with 1 and 0.1 mg/ml of different proteases, trypsin (Sigma T1426), proteinase K (Melford MB2005), pancreatin (Sigma P3292) and protease (Sigma P6911), using sodium phosphate buffer 20 mM, pH 6, for 1 hour at 37˚C. Samples were cooled on ice and tested for antimicrobial activity by drop test or filter disc test.

2.3.2. Antimicrobial analysis of supernatant proteins separated by SDS-Polyacrylamide gel electrophoresis (PAGE)

To prepare the samples to be electrophoresed to test the activity of the separated peptides, cultures of the strains of interest were grown overnight according to their specific requirements. Cultures were centrifuged at 8,000 x g for 20 min and up to 12 ml of the supernatant were transferred to a Millipore concentration column of 3,000 or 10,000 MW cut-offs (or others as appropriate), that was centrifuged again at 1700 g for 20 min. The resulting fractions, both the eluted and the concentrated ones, were tested by well-diffusion assay. The following day, after identifying the fractions with activity, 75 µL of the sample and 25 µL of LDS 4x sample buffer (Sigma Aldrich, Co. Wicklow, Ireland) were mixed and heated at 70^{o}C for 10 minutes then cooled on ice for 2 min.

TruPage ^{TM} Precast gels of 4-20% SDS for peptides (Sigma Aldrich, UK) were used. The gel was rinsed with RNAse free water after being removed from the packaging and placed into the gel unit with wells facing inwards. 1X MOPS SDS buffer (Sigma) diluted from 20x stock with Milli Q water was added to the chamber. After washing the wells with buffer, 10 µL of protein marker and 25 µL of sample were added to the wells using a Hamilton syringe. BioRad Precision Plus protein Dual Extra Standards was used as a reference for protein

mass. Gels were run at 30 Amps until the dye left the gel, removed from the case and washed for 2-3 minutes with water first and left shaking at room temperature for 45 minutes in 1% tween 80. Gels were run in duplicate. The gel was stained by being washed with water once and added to Coomassie stain (Sigma), with shaking for an hour. After that, it was washed with water 2-3 times for 10 min and photographed. The duplicate gel was washed with water three times, overlaid with the indicator strain in molten agar (*L. bulgaricus* 5583) and incubated at 37^{o}C overnight.

2.3.3. Protein extraction by bead beating

Bacterial cells were pelleted by centrifuging the culture at 17,000 x g for 5 min. 500 µl of extraction buffer (TN 20mM Tris-HCl pH8, 50mM NaCl or NP 20 mM sodium phosphate pH6) was added to the cell pellet. It was vortexed to resuspend the pellet and transferred to a 2 ml screw cap tube with 100 µl of sterile acid washed glass beads (0.1 mm) and maintained on ice. Bead beating was performed using a FastPrep FR120 cell disrupter (MP Biomedical, UK) by applying 4 pulses of 30 s at speed 6 with 5-10 minutes on ice in between pulses. Samples were centrifuged at 13,000 x g for 20-30 min at 4^{o}C. Supernatant and cell pellets were assayed for antimicrobial activity by drop test.

2.3.4. Soluble and insoluble cell protein extracts by sonication

Bacterial cells were pelleted by centrifuging the culture at 17,000 x g for 5 min. Cells were resuspended in 500 µl of extraction buffer (TN 20mM Tris-HCl pH8, 50mM NaCl or NP 20 mM sodium phosphate pH6) by vortexing. Samples were sonicated using a Soniprep 150 (MSE (UK) Ltd, UK) machine by applying 7 pulses of 15 s and cooling the samples during 30 s on ice in between pulses. Debris was pelleted by centrifuging 25 min at 13,000 x g and 4^{o}C. Supernatant was transferred to a new tube and pellet resuspended in 500 µl of extraction buffer.

2.3.5. Ethanol precipitation of proteins

100 ml of culture supernatant and an equal volume of 100% ethanol previously cooled at -80^{o}C o/n were placed in a 250 ml centrifuge tube. The mixture was incubated for 5 min shaking continuously and centrifuged at 10,000 x g for 30 min at 4^{o}C. After this, it was air dried for 15 min and then resuspended in the appropriate buffer into 1/1000 of the original volume.

2.3.6. Acid treatment for protein extraction

1 ml of o/n cultures were treated by adding 35 µl of 50% HCl before being centrifuged to pellet cells at 13,000 x g for 2 min. pH was restored with NaOH 1M and supernatants were tested by well diffusion assay using negative controls of acidified and non-acidified media.

2.3.7. Colony mass spectrometry

Strains were grown in normal media plates and after that, a few colonies were picked to be analysed. The process consisted of spinning down the cells and mixing them with 50 ul 70% propan-2-ol, 0.1% TFA, vortexed a few times and centrifuged down again. Samples were analysed using mass spectrometry (MS) by Paula O'Connor (Teagasc).

2.3.8. Ammonium sulphate precipitation

Ammonium sulphate was used to precipitate and concentrate peptides and proteins in culture. Ammonium sulphate was added to the culture in fractions of 10% w/v and left stirring for 2 hours at 4^{o}C. After 2 hours, volumes were centrifuged at 10,000 x g and pellets were resuspended in 50 mM sodium acetate buffer at pH 6 and assayed by well-diffusion assay against indicator strain as stated before.

2.3.9. Protein and peptide partial purification

In order to purify the bacteriocins, as a general rule, tested isolates were grown in 500 ml of media. Cells were separated from the supernatant by centrifugation (8000 g, 20 min) and cells and supernatant were assayed independently. The cell pellet was resuspended in 100 ml of 70% propan-2-ol, 0.1% TFA using a stirrer for 3 or 4 h at room temperature, centrifuged again and the supernatant retained to test its activity. The solvent stability of the antimicrobial was characterised by mixing of 300 µl with 700 µL of water, acetonitrile, 0.1% TFA or acetonitrile with 0.1% TFA, incubating at room temperature for 4-5 h and plating on the indicator. Peptides were characterised by being stable at low pH and in the presence of acetonitrile. After that, a run in the mass spectrometer was conducted. The next step is the ability of the antimicrobial to bind to reversed phase and cation exchange columns. For reversed phase, 3 ml of the supernatant was incorporated on a 3ml, 200 mg Strata-E C18 SPE column (Phenomenex, Chesire, UK) pre-equilibrated with methanol and water (100%). After washes with 3 ml of 30% ethanol and 3 ml of 70% 2-propanol 0.1% TFA. The fractions needed to be tested for antimicrobial activity to check if the activity has been lost and were then analysed through MS. For the cation exchange column, 10 ml of

supernatant were added to an Econo column that contained 2 ml of SP sepharose (GE Healthcare, UK). The column was washed with 15 ml of 10 mM potassium phosphate, 25% acetonitrile pH 2.5 and 10 ml of 20 mM potassium phosphate, 25% acetonitrile pH 2.5 containing 1M KCl. Different buffers were tested in order to find the most suitable one for the compound (generally sodium phosphate at different concentrations and salts). After eluting and identifying the different active fractions and analysis by MS, they were further analysed by analytical reversed phase HPLC column Proteo Jupiter C18 RP-HPLC (Phenomenex, UK) running a 35%-65% acetonitrile, 0.1% TFA gradient and buffer B is 90% acetonitrile 0.1% TFA. Each sample was aliquoted at 250 µl, monitored at 214 nm and fractions collected at intervals of 1 minute. Fractions were further analysed against the indicator strain and the mass spectrometer. Heat and protease tests were also performed.

2.3.10. Preparation of gel slices for trypsin digestion

To identify antimicrobial peptides in culture, bands of interest (inhibition of indicator strain previously shown) were cut from the protein gel and destained with 30% ethanol for 30 min at 65°C, before being washed with triethylammonium bicarbonate buffer (TEAB) /50% acetonitrile (ACN). After that, it was incubated with 10 mM dithiothreitol (DTT), 30 min, 55°C. DTT was removed and iodoacetamide (IAA) 30 mM in 50 mM TEAB was added and incubated for 30 min at room temperature in the dark. The IAA solution was removed and the sample washed with TEAB/50% ACN for 15 minutes at room temperature and washed with 50 mM TEAB afterwards. Buffer was removed, and the slices cut in 1x1mm pieces before being transferred to a low bind tube (Eppendorf) and being washed with TEAB/50% ACN for 15 minutes and 100% acetonitrile for another 15 minutes. After this, the slices were put in a speed vac and dried for 30 min followed by trypsin digestion and MS.

2.3.11. Trypsinisation and MS

Treated gel slices were given to Dr. Gerhard Saalbach (JIC) and he performed trypsinisation on them followed by MS [230]. Results were visualised using proteome software Scaffold ^{TM} version 4.8.4.

2.3.12. Identification of peptides of interest

To identify candidates for antimicrobial peptide, the proteins and peptides obtained by MS were analysed manually to look for cleavage sites and calculate the different size options for each one. The peptide sequences were correlated with their genes and Artemis [231]

was used for visualising the gene neighbourhood and identify genes that could be linked to the presence of a bacteriocin.

2.4. General chemistry

2.4.1. Organic acid quantification

2.4.1.1. High Pressure Liquid Chromatography (HPLC)

Fermented samples were defrosted and filter sterilised using 0.2 µm-pore-size syringe filters (Whatman International Ltd, UK) and 300 µl injected into Chromacol vials (Fisher Scientific, UK). Concentrations of formic, acetic, lactic, propionic, butyric acid and γ-aminobutyric acid (GABA) were quantified by HPLC using a Perkin-Elmer Flexar HPLC system coupled with refractive index and photo diode array detectors for 210, 280 and 325 nm wavelengths (PerkinElmer Inc, USA). Analyses were conducted using an Aminex HPX-87H organic acid analysis column (Bio-Rad Laboratories Ltd, UK) and Chromera Manager version 3.0, 2011 (PerkinElmer Inc, USA). Chromatographic conditions were as described previously [232]. A standard curve was constructed using reference concentrations at 100, 60, 40, 20, 10, 5 and 1 mM of formic acid (eluted at 14.3 min), acetic acid (15.3 min), lactic acid (13.1 min), propionic acid (17.6 min), butyric acid (18.9 min) and GABA (22.0 min) (all from Sigma Aldrich). Standards were run at the beginning and the end of each set of samples.

2.4.1.2. Nuclear Magnetic Resonance (^{1}H-NMR)

SCFA were measured using Nuclear Magnetic Resonance (^{1}H-NMR) by Dr Ian Colquhoun (QIB). NMR buffer was mixed with cell-free sample supernatant in a proportion 1:10. NMR buffer composition per 100 ml: D_2O: 0.26 g NaH_2PO_4, 1.44 g K_2HPO_4, 17.1 mg sodium 3-(Trimethylsilyl)-propionate-d4 (TSP), 56 mg NaN_3. 500 µl were collected in 5 mm NMR Tubes (GPE Scientific Ltd, UK). High resolution ^{1}H NMR spectra were recorded on a 600 MHz Bruker Avance III HD spectrometer fitted with a 5 mm TCI cryoprobe and a 60 slot autosampler (Bruker, Rheinstetten, Germany). Sample temperature was controlled at 300^0K and the D_2O signal was used as lock. Each spectrum consisted of 512 scans of TD = 65,536 data points with a spectral width of 20.49 ppm (acquisition time 2.67 s). The *noesygppr1d* presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 3 s) and mixing time (D8 = 0.01 s). A 90^0 pulse length of approximately 8.8 µs was used, with the exact pulse length determined automatically for each sample. Spectra were

transformed with 0.3 Hz line broadening and zero filling and were automatically phased and referenced (to TSP) using the TOPSPIN 3.2 software. The resulting Bruker 1r files were converted to Chenomx (.cnx) format using the 'Batch Import' tool in the Processor module of Chenomx NMR Suite v8.12 with the TSP concentration set to 0.1 mM. Concentrations were obtained using the Chenomx Profiler module (Chenomx Inc, Canada).

2.5. Statistical analysis

Significant differences between groups were established using a paired *t*-test, assuming normal distribution, equal variances. Both sides of the distribution were considered. Significance was considered when *P* value was <0.05. Calculations were performed using Excel 365.

CHAPTER III

Faecal and Food Screening for Antimicrobial Activity

3.1. Introduction

There is a need for new antimicrobials to fight the global rise of antimicrobial resistance. Since the golden era of antibiotic discovery, new antibiotics have most frequently been derived through chemical modifications of pre-existing structures. Previously, antimicrobials originated from natural sources, and recently it has been proposed that researchers should turn again to that strategy for new antimicrobial discovery. This is partially rooted in the premise that the production of antimicrobials represents an expression of antagonistic ecological relationships among bacteria. Therefore, there are microbiology techniques to uncover these traits, like direct and deferred antagonism procedures. The main difference between these approaches is that for the direct procedure, test and indicator isolates are grown at the same time, while in the deferred procedure the test organism is grown and killed before the application of the indicator strain. Deferred antagonism has been established to be the more sensitive assay, and therefore is considered the better option for bacteriocin discovery, where it has been used extensively. It should be noted that, in some cases, antimicrobials may not be expressed in the absence of competitor strains, which may result in antimicrobial producers being overlooked if only this assay is used [233].

More recently, classical approaches, such as the aforementioned deferred antagonism assay, can be coupled with new advanced techniques to provide a more comprehensive picture of the potential of each isolate. Thus, MS has been used to identify the presence of potential products of interest in bacterial cultures [234]. In the case of detecting antimicrobial peptides, MS is able to show the presence of products with masses within the range 3000-8000 Da associated with antimicrobial activity, narrowing the range of potential bacterial isolates to work with. Genomes can also be screened for antimicrobial-encoding clusters using software designed for this purpose, such as BAGEL 3 (currently version 4) [220] and antiSMASH [221]. This is notable as the drop in sequencing costs has allowed a rapid increase in genomic analysis data to complement other data or to provide primary information directly. In summary, a combination of different strategies can increase the efficiency of the search for bioactive compounds and the identification of potential probiotic bacterial isolates.

The aim of the work in this chapter was the identification of new antimicrobials active against pathogenic bacteria in the human GIT and food. For this reason, five faecal samples

and one sample of yoghurt, kefir, gherkins in brine and miso were screened to isolate bacteria that exhibited antimicrobial activity. 208 out of 1736 (11.98 %) isolates showed inhibitory activity by different bioassays. In addition, 130 faecal isolates from culture collections at QIB and Teagasc were tested for the same purpose. From these 338 strains, 39 isolates that showed interesting antimicrobial activity were investigated further for additional probiotic activity and MS was performed on their cultures. From these, the genomes of 11 representative promising isolates were sequenced and analysed. For purification purposes, we were particularly interested in those isolates whose antimicrobial activity was present in the culture supernatant, and different attempts were conducted to release cell-associated activity. Only 4 of the QIB isolates (*L. gasseri* LM19*, L. amylovorus* Lac 20, *L. crispatus* Lac 50 and *L. crispatus* Lac 51) and 12 from the Teagasc isolates showed non-manipulated inhibitory activity in the supernatant. Some of the antimicrobials produced by these isolates are studied further in the following chapters.

3.2 Methods

3.2.1. Food and faecal screening

Faecal inoculum was prepared as explained in section 2.1.7. Ten-fold dilutions of samples were prepared in 450 µl of pre-reduced PBS. 100 µl of dilutions 10^{-4}, 10^{-5} and 10^{-6} were plated on pre-reduced and non-pre-reduced MRS, LB, ROGOSA and GM17 using a L-shaped loop. Plates were incubated in aerobic and anaerobic conditions at 37°C for 3-4 d in order to allow the growth of different microorganisms. Up to 50 colonies grown in each media were selected and streaked onto fresh media plates for testing antimicrobial activity by overlay assay as described in section 2.1.5.1. A master plate for each media was prepared for further stock preparation. Faecal samples were provided by different donors, from a study approved by the QIB Human Research Governance committee (IFR01/2015) registered at http://www.clinicaltrials.gov (NCT02653001). Food samples were processed in the same way as the faecal samples. Kefir samples were provided by Nourish Kefir (UK), yogurt by The Coconut Collaborative Ltd (UK), gherkins in brine by Morglel Fine and Organic Foods Ltd (UK) and organic miso by Source Foods (UK).

3.2.2. Isolates obtained from culture collections

3.2.2.2. QIB culture collection

Strains previously isolated from faecal samples and breast milk, and placed in the QIB collection, were screened. A total of 105 isolates showed antimicrobial activity. 27 of them were isolated by Carmen Nueno-Palop and Daniela Musolino from human faecal material. Strain FI10917 was isolated by Diane Hatziioanou from a human faecal sample. 37 breast milk isolates were provided by Complutense University of Madrid. Valeria Rizzello, from Messina University, provided 18 *Lactobacillus* sp. isolates and 20 *Bifidobacterium* sp.

3.2.2.3. Teagasc isolates

Strains previously isolated by James Hegarty and Calum J. Walsh from faecal samples were assayed for antimicrobial activity by overlay, cross-streak and well-diffusion assay.

3.3. Results

3.3.1. Isolation and screening

3.3.1.1. Screening of faecal samples

Screening of faecal samples from five different donors was conducted and a total of 900 strains isolated using anaerobic conditions and 480 from aerobic conditions were analysed for antimicrobial activity. 32 strains (3.6%) showed antimicrobial activity to at least one indicator strain, when tested against *C. perfringens, L. innocua*, *C. sakazakii*, *E. coli* and *S. enterica sv Typhimurium* (Table 3.1). One isolate (strain 12c) lost the activity previously shown against *S. enterica sv Typhimurium* as soon as it was cultured in liquid media after the initial isolation. 16S rRNA sequencing was used to identify the bacterial isolates.

3.3.1.2. Screening of food samples

Kefir products were screened on two occasions after which 180 isolated strains were tested but none of them showed any antimicrobial activity. Screening of bacteria isolated from organic miso, gherkins in brine and yogurt was also performed and 176 colonies showed antimicrobial activity (Table 3.1). However, only 47 could be subcultured.

Table 3.1. Spectrum of action of cultured isolates from faecal and fermented food screenings.

Isolate	Identification by 16S rDNA	% similarity	Activity against				
			C. p.	*L. i.*	*C. s.*	*S. e.*	*E. c.*
Strain 12c	nt	nt					
GI01	*Lactobacillus plantarum-Lactobacillus paraplantarum*	100					
GI02	nt	nt					
GI03	*L. plantarum - L. paraplantarum*	100					
GI04	*L. plantarum - L. paraplantarum*	100					
GI06	*L. plantarum - L. paraplantarum*	100					
GI07	*L. plantarum - L. paraplantarum*	100					
GI08	*L. plantarum - L. paraplantarum*	100					
GI09	*L. plantarum - L. paraplantarum*	100					
GI11	*L. plantarum - L. paraplantarum*	100					
GI12	nt	nt					
GI13	*L. plantarum - L. paraplantarum*	98.9					
GI14	*Streptococcus salivarius*	100					
GI16	*S. salivarius*	100					
YI03	*S. epidermidis*	100					
YI04	*S. epidermidis*	100					
99	*S. epidermidis*	100					
F505G	*Blautia sp.*	92.7					
F501B	*Blautia sp.*	94.9					
F505B	*Blautia sp.*	100					
F508B	*Blautia sp.*	100					

F512B	nt	nt					
F514B	*Blautia sp.*	97.2					
F516B	*Roseburia sp.*	93.4					
F521B	*Blautia sp.*	95.6					
F522B	*Ruminococcus sp. K-1*	98.3					
F523B	*Blautia sp.*	98.7					
F524B	*Ruminococcus sp. K-1*	95.5					
F525B	*Blautia sp.*	100					
F529B	nt	nt					
F530B	*S. epidermidis*	100					
F501M	*Bifidobacterium bifidum*	99.7					
F503M	*B. bifidum*	93.9					
F505M	*B. bifidum*	99.0					
F510M	*B. bifidum*	93.5					
F511M	*B. bifidum*	100					
F512M	*B. bifidum*	100					
F513M	*B. bifidum*	96.5					
F515M	*B. bifidum*	99.8					
F516M	*B. bifidum*	99.4					
F519M	*B. bifidum*	100					
F520M	*B. adolescentis*	93.5					
F523M	*B. bifidum*	100					
F524M	*B. bifidum*	99.5					
F525M	*B. bifidum*	98.3					
F526M	*B. bifidum*	98.0					
F527M	*B. bifidum*	83.3					
F528M	*B. bifidum*	99.5					

Green showed antimicrobial activity in overlay assays; red showed no antimicrobial activity. *C. p. C. perfringens* NCTC 3110*; L. i., L. innocua* NCTC 11288*; C. s., C. sakazakii* NCTC 11467*; S. e., S. enterica sv Typhimurium* LT2; *E. c., E. coli* ATCC 25922, nt, not tested.

3.3.2. Isolate testing

3.3.2.1. QIB culture collection

A total of 105 isolates from different culture collections in QIB were tested against a panel of 8 indicator strains (*C. perfringens*, *S. enterica sv Typhimurium*, *E. coli*, *L. innocua*, *C. sakazakii*, *M. luteus*, *C. jejuni* and *L. bulgaricus*) and showed varying patterns of antimicrobial activity. Results are summarised in Table 3.2. In general, test strains showed stronger activity when they were directly in contact with the indicator strain, such as overlay and cross streak assays. *Bifidobacterium* spp and *Lactobacillus* spp isolated from faecal samples showed the strongest activity. Drop test and filter disc assays were all negative except against *C. jejuni*. 52 isolates were able to inhibit *C. jejuni* growth. However, these plates did not have sodium carbonate in their composition, therefore, this inhibitory activity might be non-specific and due to the effect of the lactic acid, instead to the production of a novel antimicrobial. One interesting result of a cross streak assay showed that two strains, *E. faecalis* LM1 and *E. faecalis* LM4, exhibited antimicrobial activity against *C. sakazakii* only in the region between the two test producer streaks, indicating the antimicrobial production might be regulated by quorum sensing (Fig. 3.1). Well-diffusion assays that were carried out using *L. bulgaricus* DPC5583 as indicator strain identified four culture supernatants that displayed inhibitory activity: from *L. gasseri* LM19, *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51.

Figure 3.1. Inhibition zone in combination of *E. faecalis* LM1 and LM4 against *C. sakazakii* indicating potential quorum sensing regulation.

3.3.2.2. Teagasc isolates

The most interesting Teagasc isolate was a *S. agalactiae* isolate, that was able to produce nisin P and was further investigated within this thesis (Chapter V). *Coprobacillus* sp. strain 29_1/D6 was an isolate that had a gene for a new lantibiotic in its genome. However, it was not further studied due to the difficulties in detecting its antimicrobial activity.

Table 3.2. Summary of activity of QIB and Teagasc isolates against different indicator strains.

			Cj	*Cp*		*Se*		*Ec*		*Li*		*Ml*		*Cs*		*Lb*
16S rDNA match	Isolate code	(%) similarity	F	O	C	O	C	O	C	O	C	O	C	O	C	W
QIB ISOLATES																
L. amylovorus	Lac 20	85.4	++	-	+++	-	-	-	-	+	+++	-	++	+++	-	++
L. gallinarum	Lac 24	100	-	-	+	-	+	-	+	+	+	-	++	+++	-	-
L. mucosae	Lac 25	100	-	-	+	-	+	-	++	-	+++	-	-	+	-	-
L. mucosae	Lac 28	90.1	++	-	-	-	+++	-	+++	+	++	-	-	+	-	-
L. saerimneri	Lac 29	92.7	++	-	+++	-	++	-	++	+	+++	-	++	+++	-	-
L. mucosae	Lac 30	100	+++	-	+	-	+	-	+	++	+	-	+	+++	-	-
L. crispatus	Lac 32	100	-	-	+++	-	+++	-	+++	-	++	-	++	+++	+++	-
L. crispatus	Lac 34	100	+++	-	-	-	++	-	++	++	++	-	++	+++	-	-
L. taiwanensis	Lac 37	96.9	-	-	+	-	+	-	+	++	-	-	-	+++	-	-
L. reuteri	Lac 40	97.1	-	-	-	-	+	-	+	-	-	-	-	+++	-	-
L.gallinarum	Lac 43	99.6	++	-	+	-	+	-	+	-	+	-	+	+++	+++	-
L. crispatus	Lac 44	100	-	-	-	-	+++	-	+++		++	-	-	+++	+++	-
L. crispatus	Lac 45	98.0	-	-	-	-	+	-	+	+	+	-	+	+++	-	-
L. reuteri	Lac 46	93.4	++	-	+	-	+	-	+	++	+	-	+	+++	+	-
L. gallinarum	Lac 47	100	++	-	-	-	+++	-	+++	+	+++	-	++	+++	+++	-
L. crispatus	Lac 50	100	+	-	-	-	++	-	++	-	++	-	++	+++	+++	+++
L. crispatus	Lac 51	99.3	+++	-	+	-	+	-	+	-	-	-	-	+++	-	+++
L. crispatus	Lac 52	96.7	-	-	+	-	+	-	+	-	-	-	-	+++	+++	-
L. frumenti	Lac 53	91.2	++	-	+++	-	+++	-	+++		+++	-	+	+++	-	-
L. reuteri	Lac 54	84.6	+++	-	+++	-	++	-	++	++	++	-	++	+++	-	-
L. reuteri	Lac 55	83.7	-	-	-	-	+++	-	+++	+	+++	-	-	+++	-	-
L. reuteri	Lac 56	83.4	+	-	-	-	+++	-	+++	+	+++	-	-	+++	-	-
L. paracasei	R1	100	+	-	+++	-	+++	-	+++	+	+++	-	-	+++	-	-
L. paracasei	R2	100	-	-	+++	-	+++	-	+++	+	+++	-	-	+++	+++	-
L. paracasei	R3	100	-	-	+++	-	+++	-	+++	+	+++	-	++	+++	-	-
Bifidobacterium sp	FI10480	100	+++	-	++	-	+	-	+	+	++	-	+	-	-	-
Bifidobacterium sp	FI10481	-	+	-	++	-	++	-	+	+	++	-	+	-	+++	-
B. longum	FI10917[a]	100	+	-	+++	-	+++	-	+++	-	+	-	++	-	+++	-
E. faecalis	LM1	100	-	-	-	-	-	-	-	-	-	-	++	-	+++	-
E. faecalis	LM4	100	+	-	-	-	-	-	-	-	-	-	+	-	+++	-
E. faecalis	LM14a	100	+	-	-	-	-	-	-	-	+	-		-	-	nt
E. faecalis	LM18	100	-	-	-	-	-	-	-	-	-	-	-	-	-	nt
E. faecalis	LM31	100	-	-	-	-	-	-	-	-	++	-	++	-	++	nt
E. faecalis	HLM4	100	-	-	-	-	-	-	-	-	++	-	+	-	++	nt
E. faecalis	HLM5	100	+	-	-	-	-	-	-	-	-	-	+	-	+	nt
E. faecalis	M12	100	-	-	-	-	-	-	-	-	-	-	+	-	+	nt
E. faecalis	M22	100	-	-	-	-	-	-	-	-	++	-	+	-	-	nt

E. faecalis	SC1	100	+	-	-	-	+	-	-	-	-	-	+	-	-	nt
E. faecalis	SC8	100	+	-	-	-	-	-	-	-	-	-	+	-	-	nt
E. faecalis	VM6	100	+	-	-	-	-	-	+	-	-	-	+	-	-	nt
L. gasseri	PM9	100	-	-	-	-	-	-	+	-	++	-	+	-	-	-
L. gasseri	PM19	100	-	-	-	-	-	-	+	-	-	-	-	-	-	-
S. epidermis	LM10	100	+	-	-	-	-	-	-	-	+	-	+	-	-	-
K. milletis	LM11	99.0	+	-	-	-	-	-	-	-	+	-	+	-	-	-
K. pneumoniae	LM12	100	-	+	+	-	+	-	-	-	+	-	+	-	+	-
L. gasseri	LM19	100	+	+	+	-	-	-	-	-	++	-	++	-	++	+++
L. gasseri	LM21	100	+	-	-	-	-	-	-	-	++	-	++	-	++	-
E. faecalis	LM24	100	-	-	-	-	-	-	-	-	+	-		-	-	-
K. milletis	LM26	99.0	-	-	-	-	-	-	+	-	-	-	-	-	+	-
L. gasseri	HM8	100	-	-	-	-	-	-	-	-	+	-	+	-	-	-
E. faecalis	HLM3	100	-	-	-	-	-	-	-	-	-	-	+	-	+	-
E. faecalis	HLM6	100	+	-	-	-	-	-	-	-	-	-	+	-	++	-
E. faecalis	HLM7	100	+	-	-	-	-	-	-	-	-	-	+	-	++	-
E. faecalis	HLM9	100	+	-	-	-	+	-	-	-	-	-	+	-	-	-
E. faecalis	HLM10	100	-	-	-	-	-	-	-	-	-	-	+	-	-	-
E. faecalis	HLM11	100	-	-	-	-	-	-	-	-	-	-	+	-	-	-
E. faecalis	HLM12	100	-	-	-	-	+	-	-	-	-	-	++	-	-	-
E. faecalis	HLM13	100	+	-	-	-	+	-	+	-	-	-	++	-	-	-
K. milletis	HLM14	99.0	+	-	-	-	+	-	+	-	-	-	+	-	-	-
E. faecalis	HLM15	100	-	-	-	-	+	-	-	-	-	-	++	-	-	-
L. gasseri	HLM17	100	-	-	-	-	-	-	-	-	-	-	+	-	-	-
E. faecalis	HLM20	100	+	-	-	-	-	-	-	-	-	-	-	-	++	-
K. milletis	HLM21	99.0	-	-	-	-	-	-	-	-	-	-	-	-	++	-
L. gasseri	HLM23	100	+	-	-	-	-	-	-	-	-	-	+	-	-	-
L. gasseri	HLM24	100	-	-	-	-	-	-	-	-	-	-	+	-	-	-
L. paracasei	Lac1	100	++	-	-	++	+++	++	+++	++	-	-	-	+++	+++	-
L. plantarum	Lac2	100	++	-	-	++	+++	++	+++	++	-	-	-	+++	+++	-
L. plantarum	Lac3	100	++	-	-	++	+++	++	+++	++	-	-	++	+++	-	-
L. pentosus	Lac4	100	++	-	-	++	+++	++	+++	++	+++	-	++	+++	-	-
L. pentosus	Lac5	100	++	-	-	++	+++	++	+++	++	+++	-	++	+++	++	-
L.plantarum	Lac6	100	++	-	-	++	+++	++	+++	++	-	-	++	+++	+++	-
L. rhamnosus	Lac17	100	++	-	-	++	+++	+++	+++	++	-	-	++	+++	+++	-
L. plantarum	Lac18	100	++	-	-	++	+++	++	+++	++	+++	-	++	+++	-	-
L. plantarum	Lac20	100	++	-	-	++	+++	++	+++	++	+++	-	++	+++	+++	-
L. rhamnosus	Lac21	100	+	-	-	++	+++	++	+++	+	-	-	++	+++	+++	-
L. rhamnosus	Lac22	100	++	-	+++	++	+++	++	+++	++	+++	-	++	+++	+++	-
L. rhamnosus	Lac23	100	+	-	-	++	+++	++	+++		-	-	++	+++	+++	-
L. rhamnosus	Lac24	100	+	-	-	++	+++	++	+++	++	+++	-	++	+++	+++	-
L. rhamnosus	Lac25	100	+	-	-	++	+++	++	+++	++	+++	-	++	+++	+	-
L. rhamnosus	Lac26	100	+	-	++	++	+++	++	+++	+	-	-	++	+++	+++	-
L. rhamnosus	Lac27	100	+	-	-	++	+++	++	+++	++	+++	-	++	+++	+++	-
L. rhamnosus	Lac28	100	+	-	-	++	+++	++	+++	++	-	-	++	+++	+	-
L. plantarum	Lac29	100	++	-	++	++	+++	++	+++	++	+++	-	++	+++	+++	-

B. adolescentis	Bif1	100	-	-	-	-	+++	++	+++	-	+++	-	+	++	-	-
B. animalis	Bif3	100	-	++	-	-	-	-	-	+++	-	-	+	++	++	-
B. animalis	Bif4	100	-	++	-	-	-	-	-	+++	-	-	-	++	-	-
B. animalis	Bif5	100	-	+	-	-	-	-	-	+++	-	-	-	++	++	nt
B. animalis	Bif8	83.5	-	++	-	-	-	-	-	+++	-	-	-	++	-	nt
B. adolescentis	Bif9	100	-	-	-	-	-	-	-	-	-	-	-	-	-	nt
B. adolescentis	Bif10	100	-	-	-	+	+++	+++	+++	+++	+++	-	-	++	+++	-
B. adolescentis	Bif12	100	-	-	-	-	-	-	-	+++	+++	-	-	++	-	-
B. adolescentis	Bif14	100	-	-	-	+	+++	+++	+++	+++	+++	-	+	+	+++	-
B. catenulatum	Bif15	100	-	+	-	+	+++	+++	+++	+++	+++	-	+	+	+++	-
B. adolescentis	Bif16	100	-	-	-	+	-	-	+++	+++	+++	-	-	+	+++	-
B. adolescentis	Bif17	100	-	-	-	+	-	-	-	+++	-	-	-	++	-	-
B. adolescentis	Bif18	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. adolescentis	Bif19	100	-	-	-	+	-	-	-	+++	-	-	-	-	-	-
B. adolescentis	Bif20	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. longum	Bif21	100	-	++	-	+	+++	+++	+++	+++	+++	-	+	++	++	-
B. catenulatum	Bif22	100	-	+	-	+	+++	-	+++	+++	+++	-	+	++	++	-
-	Bif25	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-
B. longum	Bif27	100	-	++	-	+	+++	-	+++	+++	+++	-	-	++	++	-
-	Bif30	-	-	-	-	+	+++	-	+++	+++	+++	-	+	++	++	-
S. epidermidis	Strain 9[c]	94.8		-	+	-	+++	-	+++	+	+++			-	+++	-
TEAGASC ISOLATES																
P. acidilactici	Li3	nt	-	-	-	-	-	-	-		-	-	-	-	-	+
Enterococcus sp	133	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S. salivarius	107	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S. mutans	130	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Enterococcus sp	106	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Streptococcus sp	134	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S. agalactiae	Sagal	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S. salivarius	130.3	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. faecium	111 Li	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. mutans	110	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. salivarius DPC 6988	E-999	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. faecalis	102	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. epidermidis	104 Lb	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacteroides intestinalis	DSM 17393	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. fragilis	LMG 10263	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Bacteroides vulgatus	LMG 11767	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Bacteroides thetaiotaomicran	LMG 11262	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacteroides dorei	DSM 17855	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Bacteroides sp	9-1-42FAA	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacteroides uniformis	DSM 6597	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coprobacillus sp	strain 29_1/D6	nt	-	-	-	+	-	-	-	-	-	-	-	-	-	+

C j- C. jejuni NCTC 11168*; C p – C. perfringens* NCTC 3110*; L i- L. innocua* NCTC 11288*; S e - S. enterica* LT2*; E c-E. coli* ATCC 25922*; M l – M. luteus* MC8166*; C s-C. sakazakii* DSMZ 4485 *; L b – L. bulgaricus* DPC5538; F, filter disc assay; O, overlay assay; C, cross streak assay; W, well diffusion assay; nt- not tested.

-, No activity; +, 1mm radius inhibition zone; ++, 1-5mm radius inhibition zone; +++, >5mm inhibition zone.

3.3.3. Attempts to release antimicrobial activity in the supernatant

As antimicrobial activity was more evident from direct contact assays, it was hypothesised that antimicrobials were remaining attached to the producer cells. Different methods were used in order to improve the release of possible antimicrobial peptides and proteins into the supernatant, such as through the use of acidic treatments with HCl or with isopropanol, but inhibition of the indicator strain with these supernatants was not increased relative to the negative controls. A second possibility was that the presence of a competing strain would induce or improve antimicrobial production. However, no antimicrobial activity was detected after test strains and indicator strains were co-cultured in media recommended for their growth or in minimal medium M9. Lastly, a range of culture conditions and potential inducers were used. Despite the multiple combinations of different conditions, no consistent inhibitory activity was apparent. The inhibitory activity that was observed sporadically was not clear and may have been due to the pH of the media. Attempted induction with trypsin, bile salts and Tween-80 did not produce any noticeable antimicrobial activity. Acetone precipitation also did not show any effect on the antimicrobial activity of the treated supernatants.

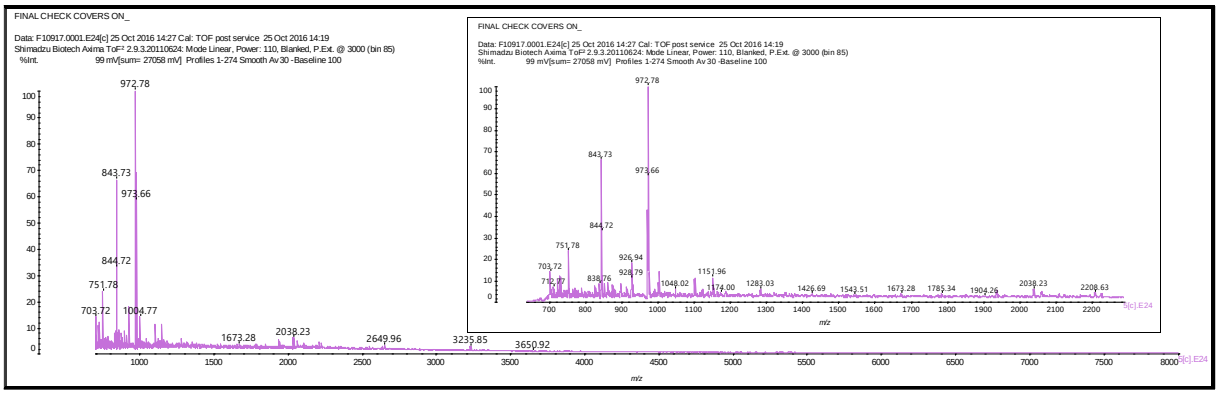
3.3.4. Chemical production and characterisation

3.3.4.1. Colony Mass Spectrometry (MS)

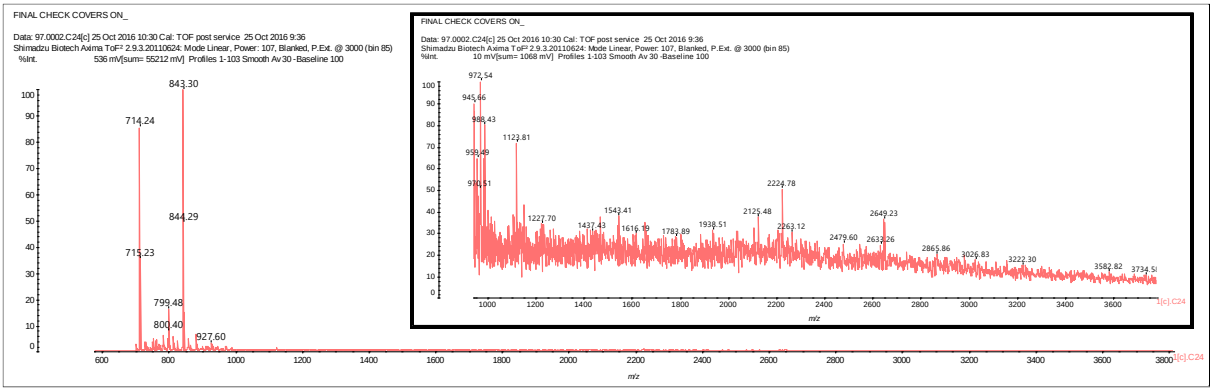
This technique allowed a primary screening of the compounds produced by colonies of the isolates grown on agar. Isolates of *S. epidermidis* or the bifidobacteria did not present noticeable peaks. Chromatograms of *E. faecalis* isolates also did not show any remarkable

peaks. Some of the lactobacilli, i.e., *L. gasseri* LM19 and *L. crispatus* Lac 50 and Lac 51, whose antimicrobial activity in the cell-free supernatant was tested, presented peaks that, after further study, corresponded to bacteriocins (Chapters 4 and 7). However, *L. amylovorus* Lac 20, that also presented activity in the supernatant, did not produce a chromatogram with any possible masses, suggesting that its antimicrobial might be secreted, and it does not remain attach to the cell in a manner that would facilitate easy detection. Figure 3.2 shows the chromatograms resulting from the colony MS analysis of the sequenced isolates.

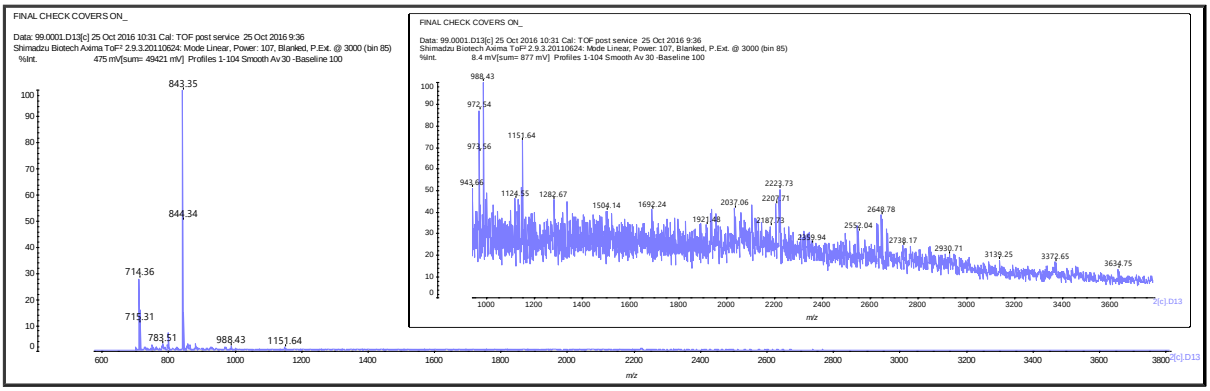
B. longum FI10917



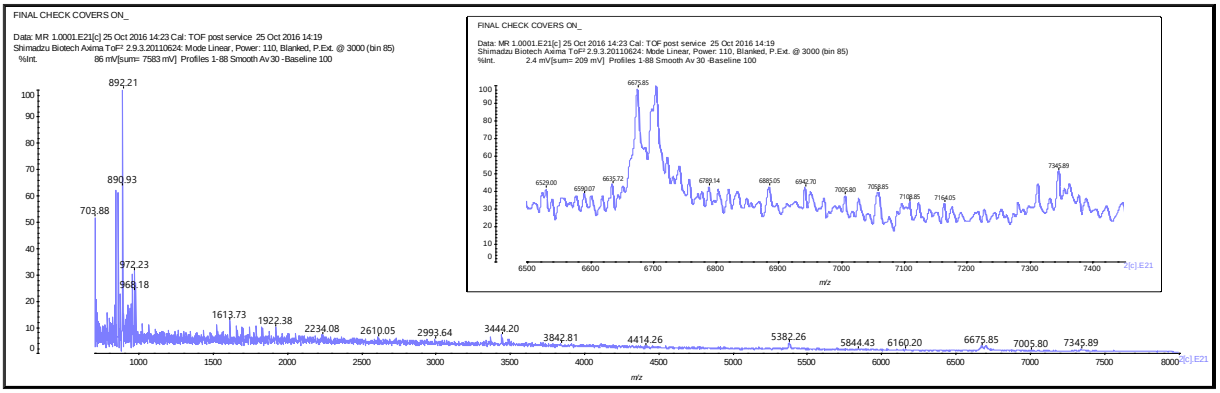
S. epidermidis 9^{C}



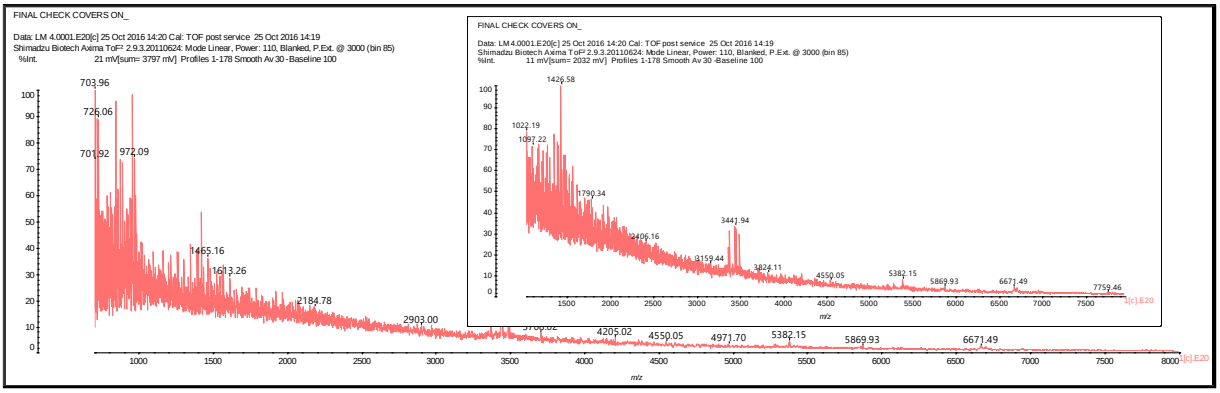
S. epidermidis F530B



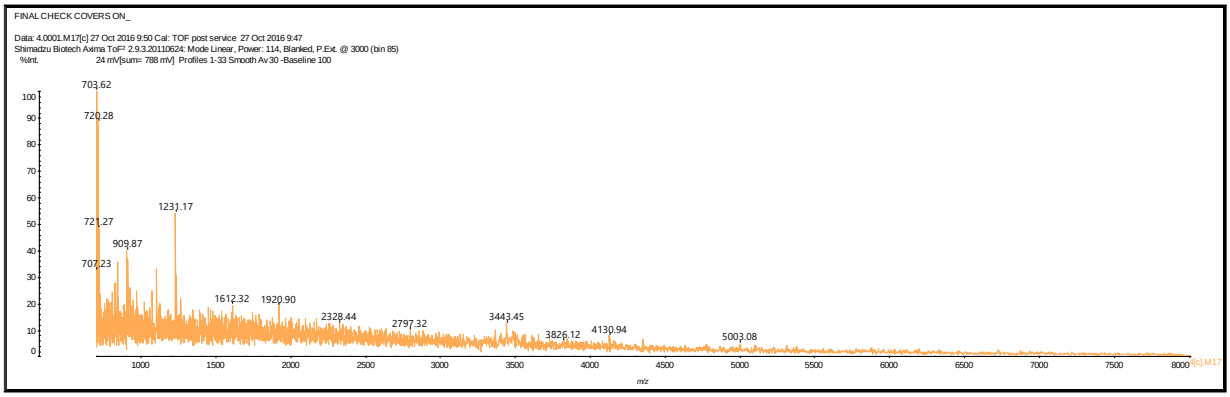
E. faecalis LM1



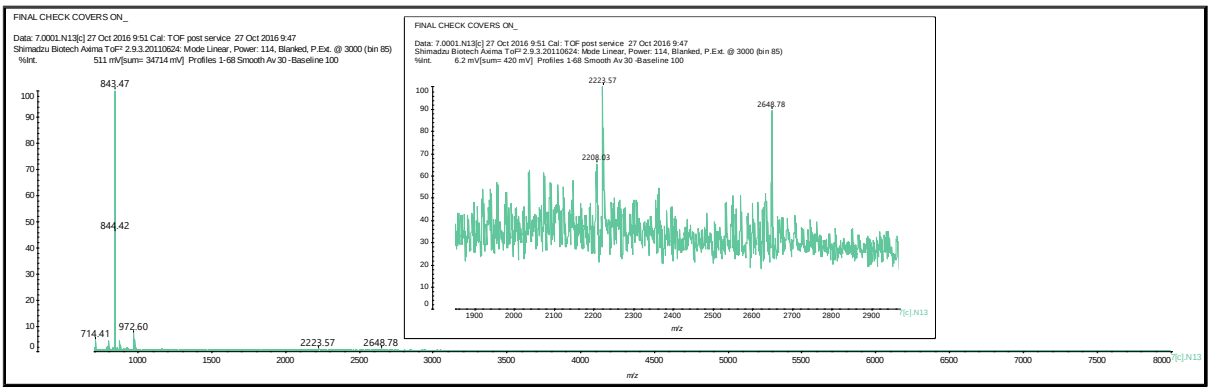
E. faecalis LM4



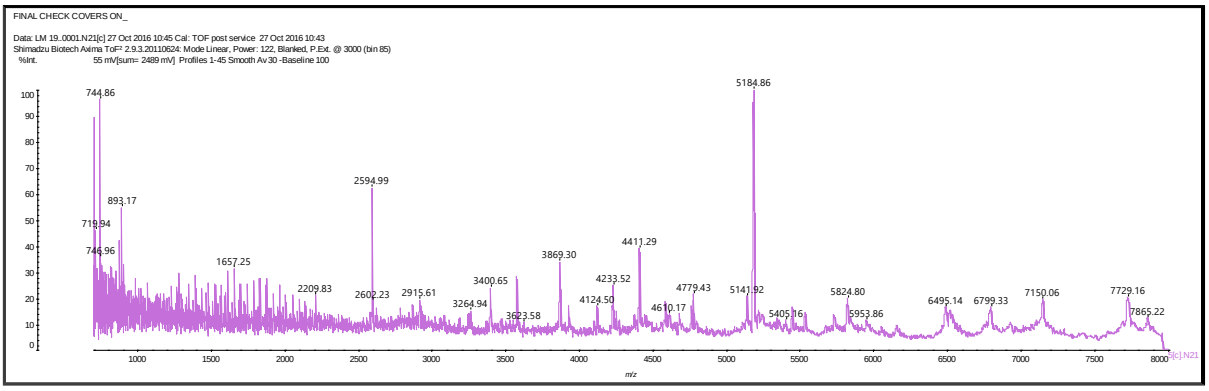
L. plantarum GI14



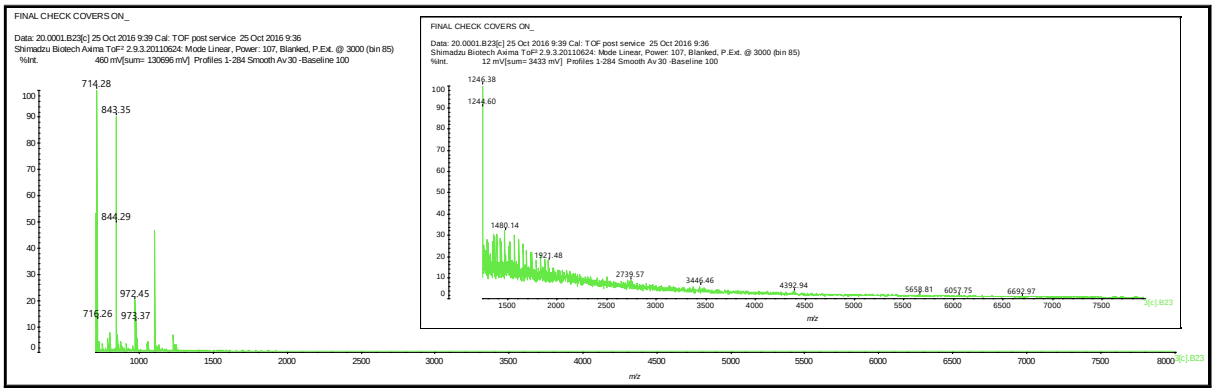
S. epidermidis 99



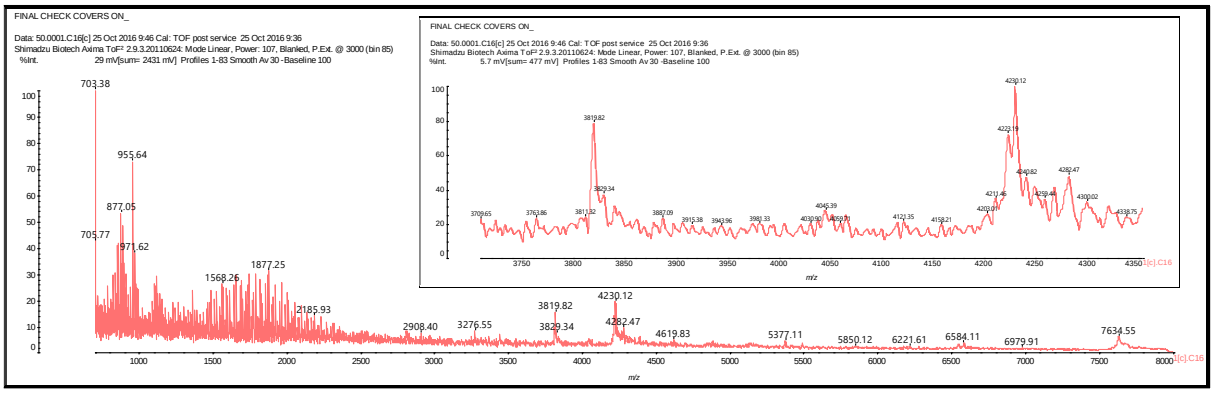
L. gasseri LM19



L. amylovorus Lac 20



L. crispatus Lac 50



L. crispatus Lac 51

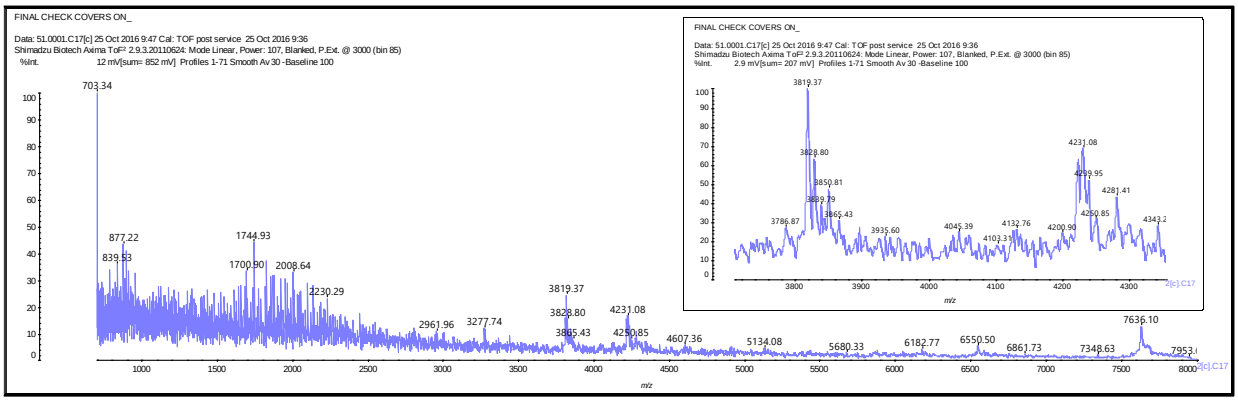


Figure 3.2. MS chromatograms of colonies from selected isolates

3.3.4.2. Characterisation of antimicrobial peptides and proteins: temperature and protease tests

Protease tests on active cell-free supernatants showed that the antimicrobials were proteinaceous in nature, i.e., antimicrobial activity was not detectable after protease treatment (Table 3.3). In the case of temperature tests, cell-free supernatants retained activity in the majority of the cases, suggesting the antimicrobials were peptides that were stabilised by modification, i.e. disulphide bridges or more considerable modifications. However, *L. crispatus* Lac 50 and Lac 51 cell-free supernatants lost their activity after incubation at/or above 90 ºC, indicating that the antimicrobial activity could be due to a protein or unmodified peptide that, when denatured, loses the activity.

Table 3.3. Summary of antimicrobial activity shown by different supernatants after heat and protease treatment.

	Heat treatment						Protease treatment			
	60 °C 10 min	60 °C 30 min	60°C 1 h	90 °C 10 min	90 °C 30 min	121 °C 15 min	Proteinase K	Protease	Trypsin	Pancreatin
L. gasseri LM19	++	++	++	++	++	++	-	-	-	-
L. amylovorus Lac 20	++	++	++	++	++	++	-	-	-	-
L. crispatus Lac 50	++	++	++	-	-	-	-	-	-	-
L. crispatus Lac 51	++	++	++	-	-	-	-	-	-	-
P. acidilactici Li3	+	+	+	+	+	+	-	-	-	-
Enterococcus sp 133	+	+	+	+	+	+	-	-	-	-
S. salivarius 107	+	+	+	+	+	+	-	-	-	-
S. mutans 130	+	+	+	+	+	+	-	-	-	-
Enterococcus sp 106	+	+	+	+	+	+	-	-	-	-
Streptococcus sp 134	+	+	+	+	+	+	-	-	-	-
S. agalactiae Sagal	++	++	++	++	++	++	-	-	-	-
B. fragilis LMG 10263	+	+	+	+	+	+	-	-	-	-
Bacteroides vulgatus LMG 11767	+	+	+	+	+	+	-	-	-	-

-No activity; + :1mm radius inhibition zone; ++: 1-5mm radius inhibition zone; +++: >5mm inhibition zone.

3.3.5. Identification of antimicrobial genomic traits

The presence of potential antimicrobial and metabolic gene clusters in the genomes of antimicrobial-producing strains was investigated using specialist software tools antiSMASH and BAGEL3 and manually using searches following a general annotation (RAST) of the genome. AntiSMASH did not identify as many regions of interest as BAGEL3, which specifically targets regions similarity to bacteriocin-encoding clusters (Figure 3.3). More specifically 24 putative bacteriocin-encoding clusters were identified using BAGEL 3, while only two were identified by antiSMASH. AntiSMASH identified a number of microcins that BAGEL3 did not (Table 3.4). Further analysis showed that it was the same microcin structural gene and no additional genes for transport, immunity or regulatory functions were identified by the program. Thus, these microcins were not followed up. Also, antiSMASH identified a number of terpenoids and other products of secondary metabolism

that were also not followed up, due to our main interest in antimicrobial peptides. Manual identification of bacteriocins based on RAST results did not provide any additional information.

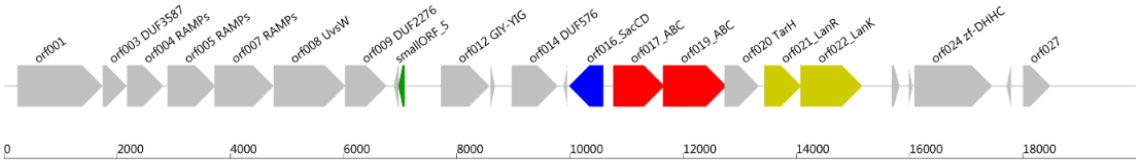
Table 3.4. Potential bacteriocin hits using BAGEL 3 and antiSMASH.

Sequenced isolate	BAGEL 3	antiSMASH
B. longum FI0917	-	-
S. epidermidis 9C	Delta lysin Sactipeptide	Microcin (*Parvimonas micra*)
S. epidermidis F530B	Delta lysin Sactipeptide	Microcin (*P. micra*)
E. faecalis LM1	Cytolysin ClyLl and ClyLs Glycocin	Cytolysin ClyLl and ClyLs
E. faecalis LM4	Cytolysin ClyLl and ClyLs Glycocin	Cytolysin ClyLl and ClyLs
L. plantarum GI14	Bovicin 255 peptide Plantaricins N and K	Microcin (*P.micra*)
S. epidermidis 99	Delta lysin	Microcin (*P.micra*)
L. gasseri LM19	LS2 chains a and b Helveticin J Acidocin LF221B (Gassericin K7B)Pediocin Gassericin T	-
L. amylovorus Lac 20	-	-
L. crispatus Lac 50	Enterolysin A (2) Helveticin J (2) Lactococcin 972	-
L. crispatus Lac 51	Enterolysin A (2) Helveticin J (2) Putative bacteriocin	-

(A) ***S. epidermidis* F530B and 9[C]**

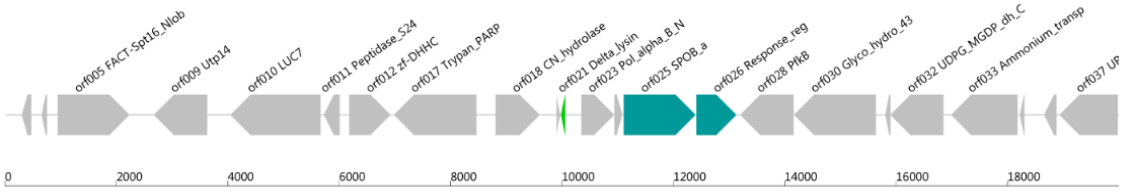
- **Sactipeptide**

MSSRPLLCGVVIEFRCAGVGLDSFNLFSSPFRRGG



- **Delta lysin**

MAADIISTIGDLVKWIIDTVNKFKK

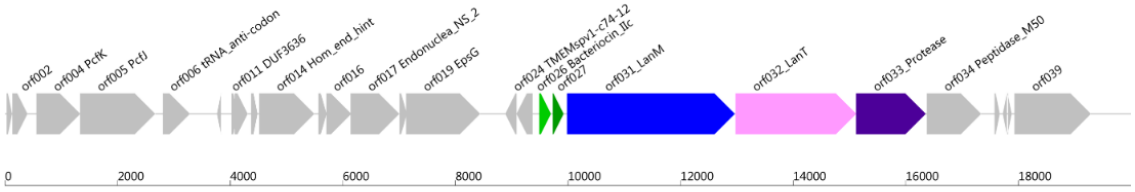


(B) ***E. faecalis* LM1**

- **Cytolysins ClyLl and ClyLs**

MENLSVVPSFEELSVEEMEAIQGSGDVQAETTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC

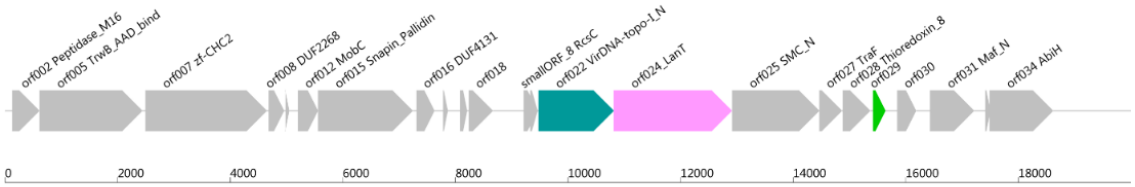
VLNKENQENYYSNKLELVGPSFEELSLEEMEAIQGSGDVQAETTPACFTIGLGVGALFSAKF**C**



- **Glycocin**

MLNKKLLENGVVNAVTIDELDAQFGGMSKRDCNLMKACCAGQAVTYAIHSLLNRLGGDSSDPAGCNDIVRKY

CK

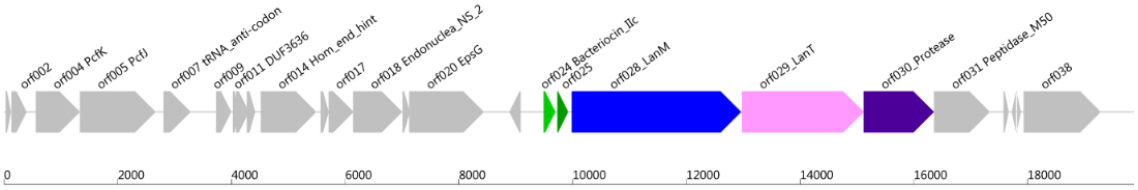


(C) ***E. faecalis* LM4**

- **Cytolysins ClyLl and ClyLs**

MENLSVVPSFEELSVEEMEAIQGSGDVQAETTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC

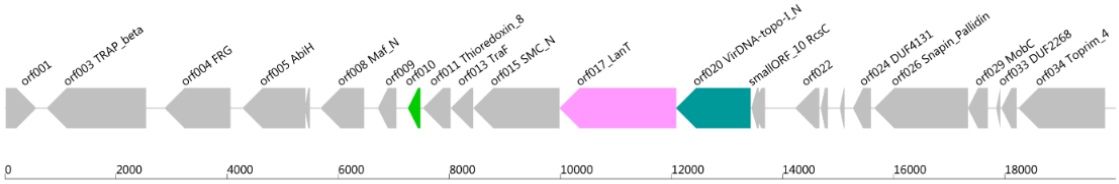
VLNKENQENYYSNKLELVGPSFEELSLEEMEAIQGSGDVQAETTPACFTIGLGVGALFSAKFC



- **Glycocin**

MLNKKLLENGVVNAVTIDELDAQFGGMSKRDCNLMKACCAGQAVTYAIHSLLNRLGGDSSDPAGCNDIVRKY

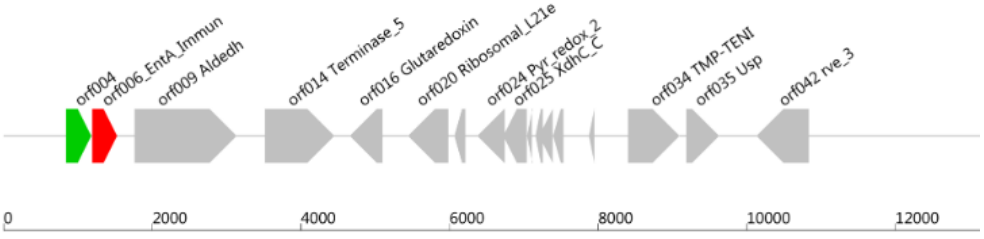
CK



(D) ***L. plantarum* GI14**

- **Bovicin 255 peptide**.

MILTIPANSGLTTQQIQEAQRIVDQFNEQAINNNVILDGESNPQSSITSPKFGLMLAKQYRYYTANGYEYRDKKG
HWHYVVTKSPFEAAFGVALHGWEGALGGSWKSGHEK



- **Plantaricins N and K**

MKIKLTVLNEFEELTADAEKNISGGRRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK

MKSLDKIAGLGIEMAEKDLTTVEGGKNYSKTWWYKSLTLLGKVAEGTSSAWHGLG

LPQKKLAKISGGFNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR

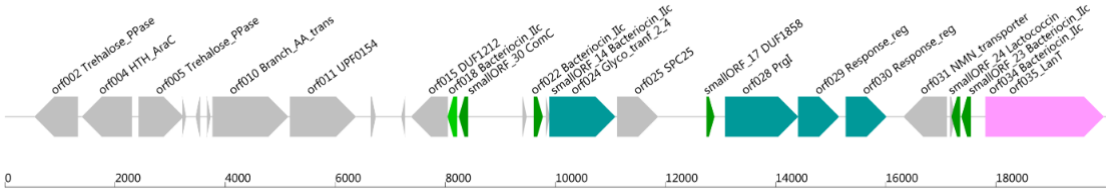
MAEKDLTTVEGGKNYSKTWWYKSLTLLGKVAEGTSSAWHGLG

MKIQIKGMKQLSNKEMQKIVGGKSSAYSLQMGATAIKQVKKLFKKWGW

MLQFEKLQYSRLPQKKLAKISGGFNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR

MKKFLVLRDRELNAISGGVFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG

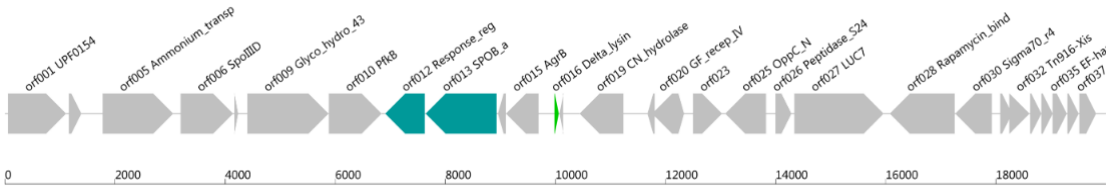
MTVNKMIKDLDVVDAFAPISNNKLNGVVGGGAWKNFWSSLRKGFYDGEAGRAIRR



(E) ***S. epidermidis* 99**

- **Delta lysin.**

  MAADIISTIGDLVKWIIDTVNKFKK



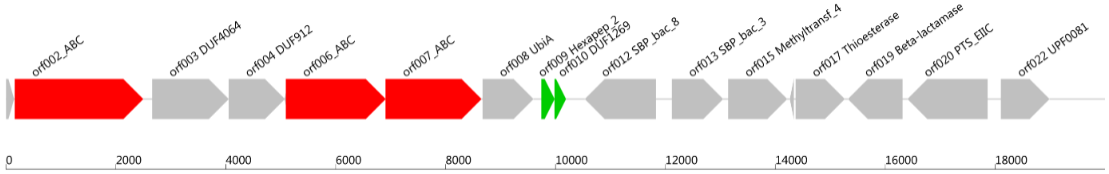
(F) ***L. gasseri* LM19**

- **LS2 chains a and b**

MKVLNECQLQTVVGGKNWSVAKCGGTIGTNIAIGAWRGARAGSFFGQPVSVGAGALIGASAGAIGGSVQCV

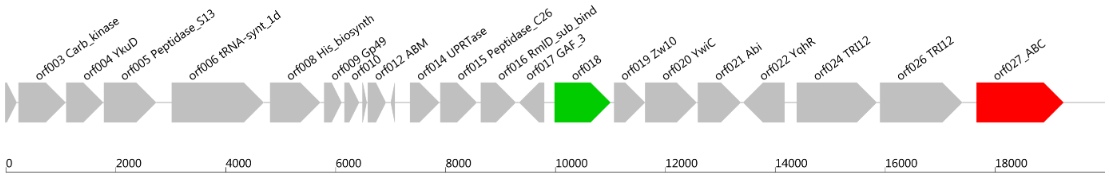
GWLAGGGR

MIEKVSKNELSRIYGGNNVNWGSVAGSCGKGAVMGIYFGNPILGCANGAATSLVLQTASGIYKNYQKKR



- **Helveticin J**

MKKGFVQIMIGKETQLCLVNKLENIHHVVVQASAIDGNNVFALQLLHKQSDVIVYQTPNDSETVTFNEDRPILYL
KGPNSAGTAGGHTQTWVQSGENNKWFVGTKPKRHGNTYWTTQIARVAVSGYQTQTFTNNTELPRLSYLNRA
GSGYGDGSVAYPGKDLVRVEAAVSPNRQYFLIASIDINHTGHFAVYNLDEVNKKLDEAEEKAEDVNIQNLNCLG
AFNVPHFNDQKIISIQGYGIDDNKNIYISSQPSPHTTFLGFPKQGKPREIVKIPWGISDPSKWSVVNLDNSLKLDA
LNFCTEFEGIQVTSDCLYLTVAYHQRNSDLTTLMNRIYQVEKF

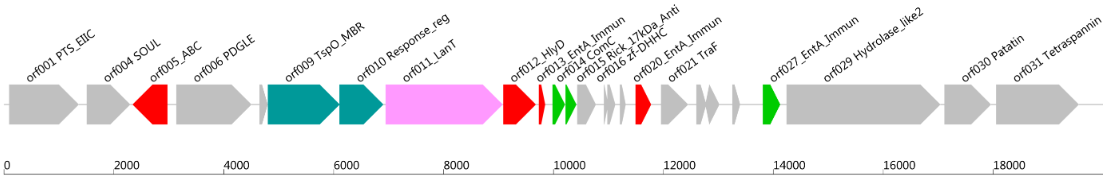


- **Acidocin LF221B (Gassericin K7B), Pediocin and Gassericin T**

MKNFNTLSFETLANIVGGRNNWAANIGGVGGATVAGWALGNAVCGPACGFVGAHYVPIAWAGVTAATGGF
GKIRK

MALKTLEKHELRNVMGGNKWGNAVIGAATGATRGVSWCRGFGPWGMTACGLGGAAIGGYLGYKSN

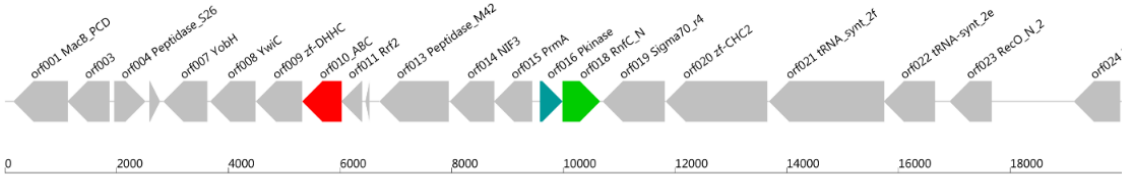
MKNELKQVLNDFISLAKTHYDRKGNEYLLEQLEVALDKLEHNVQDEVDEARATYQNINTICFANHLHLETDEEAL
LEKIKEFSMSKGWLGGLNSWNTTNTWPGR



(G) ***L. crispatus*** **Lac 50**

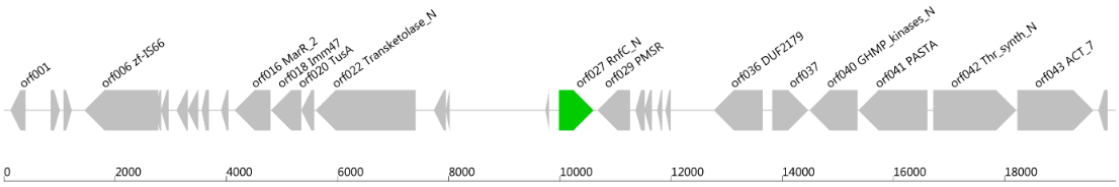
- **Enterolysin A**

MKKTESKFALLAALIAILAFASIPLWQNNLNSLRPQTHTVKKKKTAKKKKVVHVTWGYPFKKLYEKKIKFKSGQKF
GETDVIRRVYPSKSYFHDGYDFGFSEVGHSPVYAVHAGTVHRVKYAPGLGLYIWIISDDGYVEVYQEGFLSITDIY
VKKGQKVKLGQKIGKLTGSHIHLGVTKTDKDYIDKKHDNTPCKYYWKDNGTWLNPMKIIEDNLRAAGKDPVQ



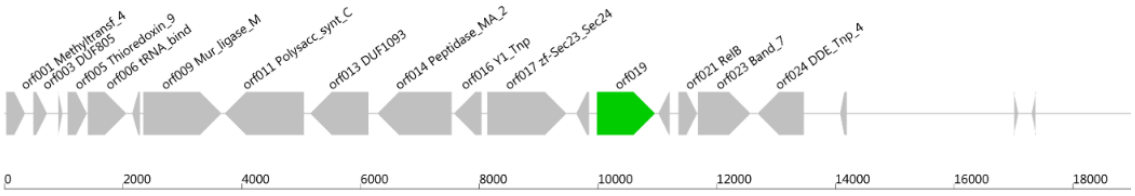
- **Enterolysin A**

MKFRKLIISLLGTALLTSSVGLSTTTASADTLDDSQNTTEVQPKNLKWAYPFKANKKNGVRPMYNAQTFGITNY
MRSTTPPSYFHDGWDFGFSEVGHSNVYAIHQGTVKKVAYGNGLGWFIWVISPDNYVEVYQEGFNKKKDIYVK
TGQKIKLGQKIGKLTGSHLHLGVTQTNKDYINKYGFPCKNWNVNNGTWLNPIEVIKSNLKK



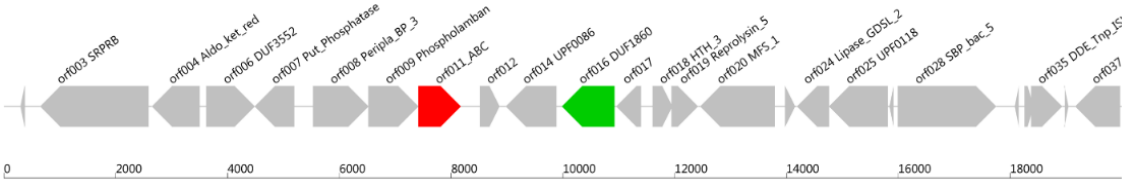
- **Helveticin J**

MVKSITPHLVYRLNGMHHVVAQVGVVNGDHVFALQLLHSAHDVLVYRKHEGLTKNIDYTDPHLVMMGFGHT
QTWVAANDKDEYFVGAKPNSGNWTTQIARVKYPRLLPERYTSNTQLPRLSHLNHVTDVPYDGHDHLHRVEAS
VSPNGKYFMIASIWDDGSGHFGLLDLNEVNQKLNENGTKNTPITDLHCLSAFHIDNFDNPSVAPDEEMPQMID
SVQGYAIDDDKNIYISNQLSPKINHETGEVTTWSRKIVKFPWGETNSDNWQVAMVDGIDLPDRYSEMESIHVN
AANDIYLTVAYHQKYIKGGEYKLRTLENQIFHITDL



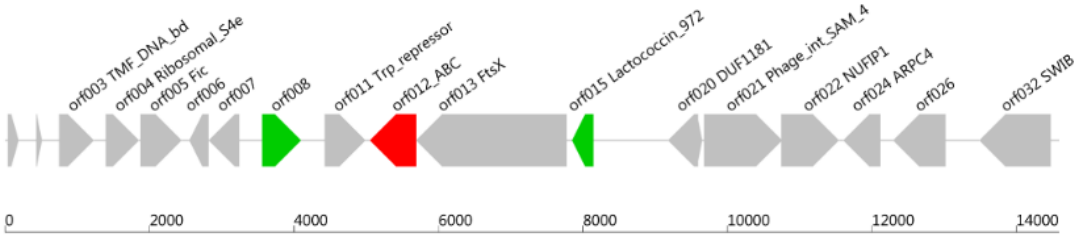
- **Helveticin J**

MVDISLKSRLYAPFARVVQKANIGHTYTYVLQMYKNNTYVSRAKNGASISSPTPGLTLVGRTGAEIKAGKNKYAA
GGHTQTWEYAGPTGDGSWFIGTKPNDDRWTTQIARVKYNSGRVSNNTQMARISNLVEITNGDWHGKHIKR
VEAAVSPNYKYLMIATVWTDNSGHFGLYELPKVNALLNGNPGGNVTVSELKQCQAGEVIDIDNFVGRIGSIQG
YDIDDDLNVYVSSQYDPTHADSNKRKIVKFSWEQPGALNTLDLTGDTRLNQNFAGYPTELEGIQVIGNNDLYLT
VAYHNKNGVGTIGNQIFRVKW



- **Lactococcin 972**

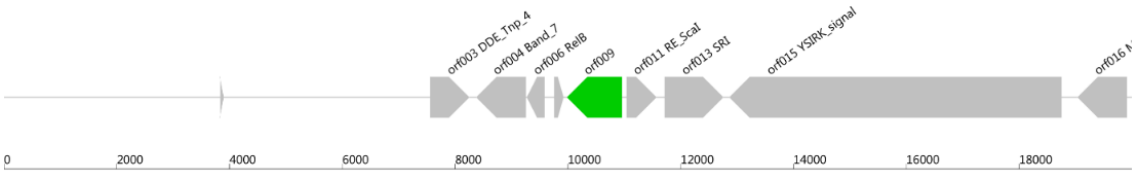
MKKKNFTKAFTIAMVSASLLAVGPASAVLAKNVGGGDWNYGYSWNYGWSKYYHGTRKHSATVAYNSDEHR
DTRARGYTAYAEYHKIPPTGLSYWWNVY



(H) ***L. crispatus* Lac 51**

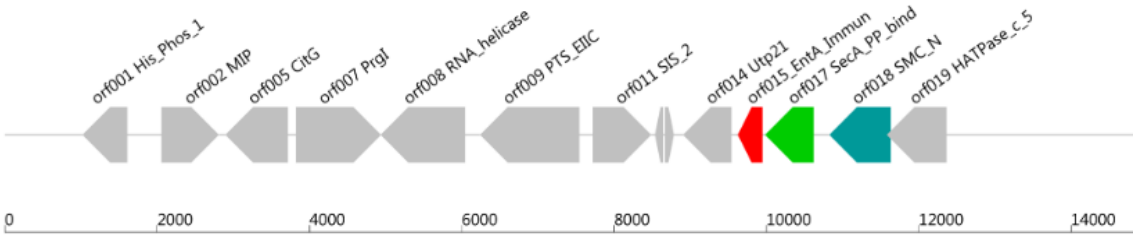
- **Helveticin J**

MVKNITPELVYRLNGMHHVVAQVGAVIDKHVFALQLLHSAHDVLVYRKHEGLTKNVDYTEPHLVMTGFGHTQ
TWVPANDNDEYFVGVKPNSGNWTTQIARVKYPKLLPENYTSNTQLPRLSHLNRATDVPYDGHNHLHRVEASV
SPNGKYFMIAAIWDDDSGHFALYDLNEVNQKLDENGTTNTPITDLHCLSAFHIDNFDHPSVAPSEEAPQMIDSV
QGYAIDDDKNIYISNQLSPKINHETGEVTTWSRKIIKFPWGETNSDNWQVAMIDGIDLPDRYSEMESIHVKAPD
DIYLTVAYHQKYIKGGEYKLRTLENQIFHITDL



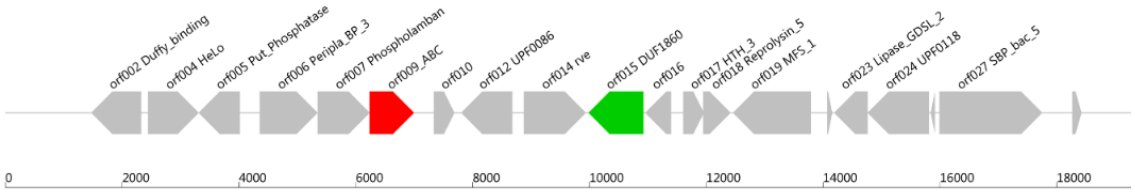
- **Putative bacteriocin**

MRKKKMKIFNKKNLLLSSAVVAGMLLVSDVNSATFTVYAAEQQSSVGQASQAVKAGNSLTQEQIQQANKYIKI
ENNQYMLDTSNKSCTLSPETIHKIQEVLTLFNKTVRENNVVLLSNEDVENNNLDQEVSPFITFVKGKKRKHRKSH
GYIVGYGSNGYCYKDSKGNFHYYVTKSPLQTVLDVNRQGLESALGGGWVQAPENAHWHGFEYHR



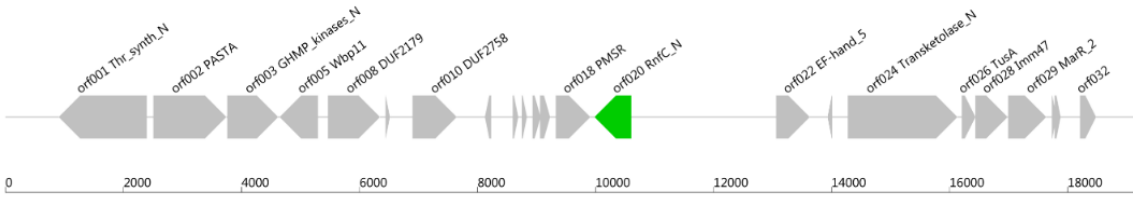
- **Helveticin J**

MVDISLKSRLYAPFARVVQKANIGHTYTYVLQMYKNNTYVSRAKNGASISSPTPGLTLVGRTGAEIKAGKNKYAA
GGHTQTWEYAGPTGDGSWFIGTKPNDDRWTTQIARVKYNSGRVSNNTQMARISNLVEITNGDWHGKHIKR
VEAAVSPNYKYLMIATVWTDNSGHFGLYELPKVNALLNGNPGGNVTVSELKQCQAGEVIDIDNFVGRIGSIQG
YDIDDDLNVYVSSQYDPTHADSNKRKIVKFSWEQPGALNTLDLTGDTRLNQNFAGYPTELEGIQVIGNNDLYLT
VAYHNKNGVGTIGNQIFRVKW



- **Enterolysin A**

MKFRKLIISLLGTALLTSSVGLSTTTASADTLDDSQNTTEVQPKNLKWAYPFKANKKNGVRPMYNAQTFGITNY
MRSTTPPSYFHDGWDFGFSEVGHSNVYAIHQGTVKKVAYGNGLGWFIWVISPDNYVEVYQEGFNKKKDIYVK
TGQKIKLGQKIGKLTGSHLHLGVTQTNKDYINKYGFPCKNWNVNNGTWLNPIEVIKSNLKK



- **Enterolysin A**

MKKTESKFALLAALIAILAFASIPLWQNNLNSLRPQTHTVKKKKTAKKKKVVHVTWGYPFKKLYEKKIKFKSGQKF
GETDVIRRVYPSKSYFHDGYDFGFSEVGHSPVYAVHAGTVHRVKYAPGLGLYIWIISDDGYVEVYQEGFLSITDIY
VKKGQKVKLGQKIGKLTGSHIHLGVTKTDKDYIDKKHDNTPCKYYWKDNGTWLNPMKIIEDNLRAAGKDPVQ

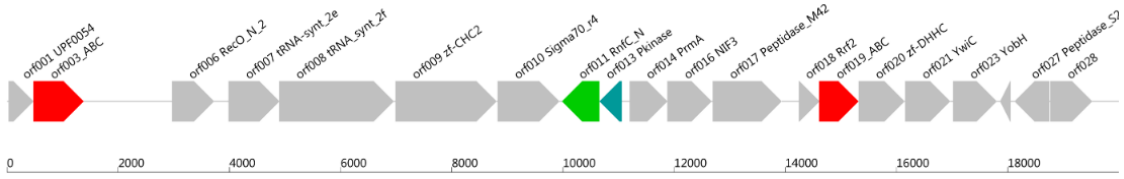


Figure 3.3. Bacteriocin cluster prediction from BAGEL3 in studied genomes with the sequence of the predicted bacteriocin operons.

Table 3.5 summarises the results of the BLAST analysis of the putative peptide sequences identified by BAGEL3. *L. gasseri* LM19 contained a number of regions of potential interest,

but a more detailed analysis of the homologous sequences identified by BLAST, both DNA (Blastn) and amino acid (Blastp), showed that those of greatest interest were associated with production of the bacteriocin gassericin T. The presence of sequences with similarity to helveticin J in some genomes (*L. gasseri* LM19, *L. crispatus* Lac 50 and *L. crispatus* Lac 51) was studied, and these analyses showed that the match was to a hypothetical protein that has been sequenced in another organism but does not have an associated function. This was also the case with glycocins identified in *E. faecalis*. *L. plantarum* GI14 was predicted to encode a bovicin 255-like protein where similarity is based on a partial match of the amino acid sequence. Lactococcin 972 from *L. crispatus* Lac 50 and the putative bacteriocin of *L. crispatus* Lac 51 present a similar situation. However, there was no evidence of production of these peptides.

Table 3.5. Summary of Blastn and Blastp analysis of putative bacteriocins identified by BAGEL3

Isolate	Bacteriocin	Percentage identity nucleotide	E-value	Top match	Percentage identity amino acid	E-value	Top match
S. epidermidis 9C and *S. epidermidis* F530B	Delta lysin	100	2e-10	Delta hemolysin	100	2.6e-21	Delta hemolysin
	Sactipeptide	100	6.6e-29	*S. epidermidis* CRISPR2 repeat region	58	2e-10	2-hydroxyacid dehydrogenase
E. faecalis LM1	Cytolysin Cly Ll	100	2e-06	Cytolysin *E. faecalis*	100	4e-45	Cytolysin *E. faecalis*
	Cytolysin Cly Ls	100	1e-42	Cytolysin *E. faecalis*	100	4e-15	Cytolysin *E. faecalis*
	Glycocin	94	9.9e-05	*E. faecalis plasmid*	No hits	No hits	No hits
E. faecalis LM4	Cytolysin Cly Ll	100	2e-06	Cytolysin *E. faecalis*	100	4e-45	Cytolysin *E. faecalis*
	Cytolysin Cly Ls	100	1e-42	Cytolysin *E. faecalis*	100	4e-15	Cytolysin *E. faecalis*
	Glycocin	100	3.2e-8	*E. faecalis* complete genome	100	3e-52	Hypothetical protein *E. faecalis*
L. plantarum GI-14	Bovicin 255 peptide	No hits	No hits	No hits	76	9e-11	Hypothetical protein *L. plantarum*
	Plantaricin N	100	3e-35	Plantaricin N	100	4.7e-05	Plantaricin N
	Plantaricin K	100	2e-37	Plantaricin K	100	2.5e-05	Plantaricin K
S. epidermidis 99	Delta lysin	100	2e-10	Delta hemolysin	100	3.2e-21	Delta hemolysin

L. gasseri LM19	LS2 chain a	99	1e-117	Gassericin K7A	100	1e-45	Hypothetical protein, Bacteriocin leader domain
	LS2 chain b	100	1e-102	Gassericin K7A Gassericin A	100	9e-42	Hypothetical protein, Bacteriocin leader domain
	Helveticin J	97	0	*L. gasseri* complete genome	98	0	Hypothetical protein Helveticin J
	Acidocin LF221B (Gassericin K7B)	100	1e-112	Gassericin T cluster	100	2e-44	Gassericin T
	Pediocin	100	4e-96	Gassericin T cluster	100	2e-38	Gassericin T
	Gassericin T	100	4e-133	Gassericin T	100	2e-69	Gassericin T
L. crispatus Lac 50	Enterolysin A	99	0	*L. crispatus* ST1	100	0	Peptidase M23
	Enterolysin A	99	0	*L. crispatus* ST1	100	0	Peptidase M23
	Helveticin J	99	0	*L. helveticus*	99	0	Hypothetical protein Helveticin J
	Helveticin J	No hits	No hits	No hits	100	7e-19	Hypothetical protein Helveticin J
	Lactococcin 972	No hits	No hits	No hits	46	0	Lactococcin A
L. crispatus Lac 51	Helveticin J	89	0	*L. crispatus* ST1	92	0	Hypothetical protein Helveticin J
	Putative bacteriocin	99	0	*L. crispatus* ST1	99	7e-151	Hypothetical protein *L. crispatus*
	Helveticin J	No hits	No hits	No hits	100	6e-163	Hypothetical protein Helveticin J
	Enterolysin A	99	0	*L. crispatus* ST1	100	0	Peptidase M23
	Enterolysin A	99	0	*L. crispatus* ST1	100	3e-156	Peptidase M23

3.3.6. Screening for probiotic traits

Some typical probiotic traits were screened in order to have a deeper background of the potential of the different isolates, for future applications.

3.3.6.1. Auto-aggregation

In general, with the exception of *L. plantarum* Lac 3, *L. paracasei* R2 and *L. reuteri* Lac 46, tested strains showed some level of auto-aggregation capability. *L. paracasei* Lac 1, *L.*

reuteri Lac 54, *L. gallinarum* Lac 43 and *L. rhamnosus* Lac 26, only showed auto-aggregation in aerobic conditions (Figure 3.4). *L. pentosus* Lac 4 and *L. frumenti* Lac 53 showed different levels of auto-aggregation but only under anaerobic conditions. Many of the tested isolates showed a high variability. The isolates that showed more consistent activity, with less variation and high percentage aggregation, were *L. crispatus* Lac 50 and Lac 51, *L. rhamnosus* Lac 22*, L. mucosae* Lac 30 and the Bifidobacteria FI10480 and FI10481. All these strains exhibited similar levels of auto-aggregation under both aerobic and anaerobic conditions.

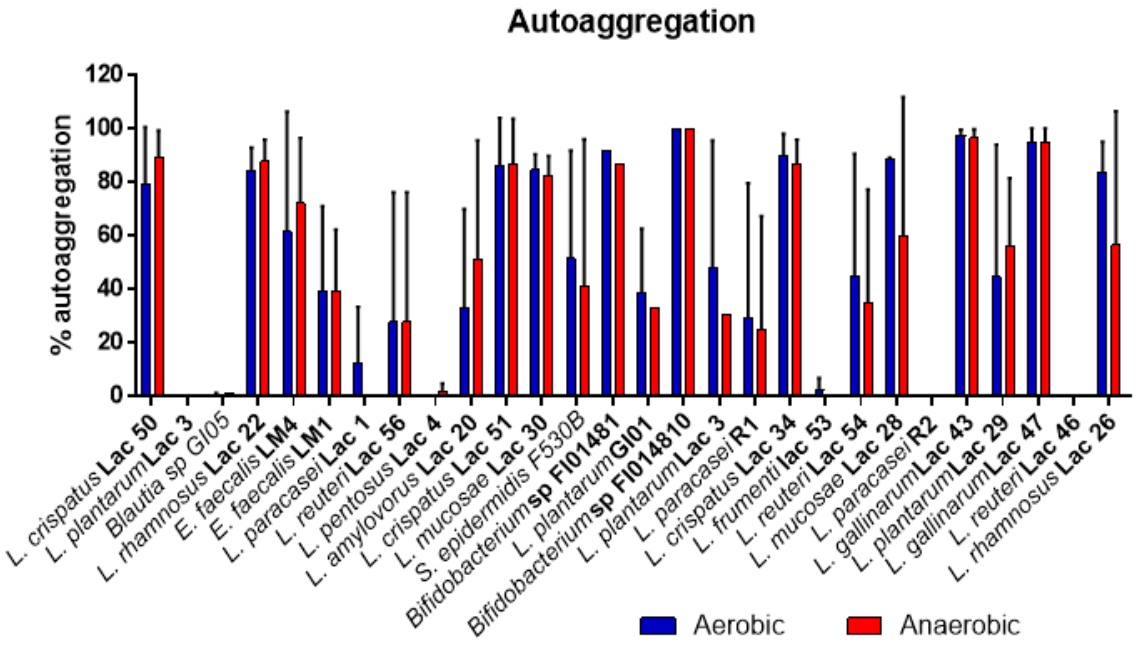


Figure 3.4. Percentage of auto-aggregation capability of different food and faecal isolates when grown in aerobic and anaerobic conditions. Results are the mean of triplicate measurements +/- standard deviation.

3.3.6.2. Antioxidant activity

The antioxidant activity of tested isolates was, in general, low relative to that of other strains considered as probiotics, that usually present levels of DPPH reduction around 80%. The highest levels observed were associated with *B. longum* FI10917, which showed levels of around 70% DPPH reduction. However, none of the other bifidobacteria or lactobacilli showed similarly high levels of antioxidant activity. Indeed, of the strains tested, only *S. epidermidis* F530B displayed comparatively high levels of anti-oxidant activity.

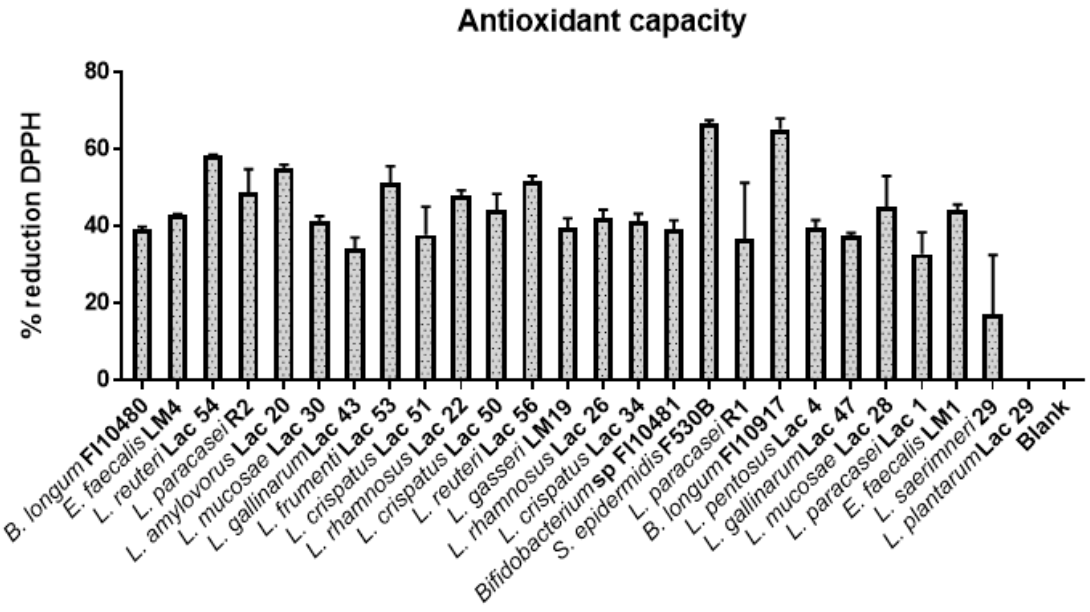


Figure 3.5. Percentage of reduction of DPPH as a measurement of antioxidant capacity of different food and faecal isolates. Results are the mean of triplicate measurements +/- standard deviation.

3.3.6.3. Bile salt hydrolase (BSH) activity

BSH activity was also measured for some of the isolates. The activity was characterised by the presence of a bright white precipitate from the colonies growth on the plates supplemented with bile salts. Table 3.6 summarises the isolates that presented this BSH activity.

Table 3.6. BSH activity of different isolates.

Isolate	BSH activity	Isolate	BSH activity
L. mucosae Lac 30	√	*Bifidobacterium* sp FI10480	√
L. gasseri LM19	√	*L. amylovorus* Lac 20	√
L. reuteri Lac 56	√	*L. plantarum* GI13	√
L. crispatus Lac 50	√	*L. crispatus* Lac 51	√
L. mucosae Lac 28	√	*L. paracasei* R1	x
L. gallinarum Lac 43	√	*L. paracasei* R2	x
L. frumenti Lac 53	√	*E. faecalis* LM4	x
L. reuteri Lac 54	√	*L. rhamnosus* Lac 26	x
L. crispatus Lac 34	√	*L. rhamnosus* Lac 22	x
L. reuteri Lac 46	√	*S. epidermidis* 99	x
L. gallinarum Lac 47	√	*L. pentosus* Lac 4	x

S. salivarius GI16	√	*E. faecalis* LM1	x
L. plantarum GI01	√		

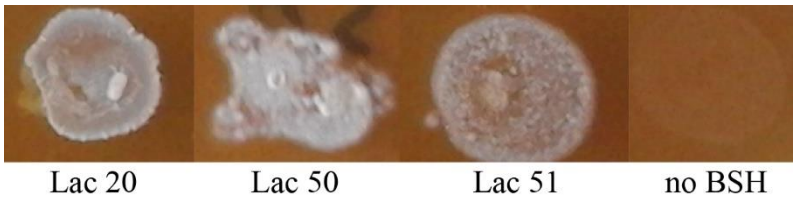


Figure 3.6. White precipitate as product of the BSH activity of *L. amylovorus* Lac 20 *and L. crispatus* Lac 50 and Lac 51. Absence of white precipitate in negative control.

3.4. Discussion

The screening for antimicrobial activity from among 1803 strains from two new sources (fermented foods and faeces) in addition to existing strains in the QIB and Teagasc culture collections yielded a total of 313 isolates that showed antimicrobial activity against at least one indicator strain (c. 17 %). 32 isolates of faecal origin exhibited antimicrobial activity initially, but the majority of them were not able to be further subcultured. Kefir samples did not provide any isolates with antimicrobial activity based on the methods used, but screening of strains from other fermented foods such as organic miso, yogurt and gherkins, yielded 176 colonies that showed antimicrobial activity. 16S rRNA analysis established that the isolates from these sources were not very diverse.

There are challenges in the implementation of culture-based techniques with a view to isolating antimicrobial-producing strains. The first relates to the culture media itself, since it is estimated that only a small percentage of bacteria are able to grow in conventional media [235]. In the case of fermented foods, the presence of a predominant species is common, especially if these fermented foods are commercially available and not naturally fermented [236]. In the case of screening from faecal samples, it is thought that growth factors are frequently retained on the agar surface during the initial screening, permitting initial growth but that, when subcultured, these factors are diluted or no longer present, and bacteria are not able to grow again or antimicrobial production can no longer be induced.

The outcome of the analysis of QIB and Teagasc isolates was different to that for freshly isolated bacteria, most likely as these were bacteria that had previously shown both antimicrobial activity and the ability to be subcultured. The main difficulty with these

isolates was, in some instances, the loss of the antimicrobial-producing phenotype. With the exception of three isolates, these strains inhibited at least one indicator strain on the basis of at least one bioassay. It is important to note that not all bioassays indicated the presence of antimicrobial activity. The clearest differences was between the bioassays that required contact with the indicator strain, such as the overlay and cross-streak assays, and those that used cell-free supernatant, like the drop test, filter disc and well-diffusion assay. As presented in table 3.2, it is clear that the antimicrobial activity exhibited is most frequently observed in the contact bioassays. In fact, only four isolates from QIB and 12 from Teagasc exhibited antimicrobial activity when assessed by well-diffusion assay. In these instances activity was against *L. bulgaricus* DPC 5583, a strain that has been typically used for antimicrobial discovery as a consequence of its extreme sensitivity to bacteriocin [61]. Similarly, *C. jejuni* was also very sensitive to the supernatants, but the lack of sodium carbonate in the plates composition suggests that this activity might be due to the lactic acid. It can be culture supernatants that showed antimicrobial activity by well-diffusion assay, did not show the same activity by drop test or filter disc. This situation can be due to a concentration factor or to the fact that drop tests and filter discs get dry at a relatively early stage during the incubation period of the assay, whereas the well-diffusion assay allows a longer exposure to a bigger volume of cell-free supernatant containing the potential antimicrobial compounds. Overall, this highlights the importance of using different bioassays when screening for new antimicrobials, since their potential activity might vary depending on its origin or mode of action.

Despite the fact that there was evidence of the presence of antimicrobial activity in the studied isolates, the absence of this antimicrobial activity in the supernatant of most strains was a challenge when it came to the further characterisation of the antimicrobial compounds [62, 237, 238]. The use of a co-culture-based approach did not yield a qualitative increase in the production of antimicrobial in the supernatant when assessed using the drop test bioassay, despite the use of the minimal medium M9 to induce higher competition. Treatments performed to release the antimicrobial compound did not produce active supernatants. Methods for crude protein extraction and ethanol and acetone precipitation were performed to attempt the release and concentrate the activity in the supernatant. However, none of the strategies were successful on a regular basis. One

explanation might be that the compounds were not produced in sufficient amounts for concentration to provide a solution.

Mass spectrometry (MS) has proved to be useful for identifying the presence of antimicrobial compounds of the cell surface of bacteria [239]. In particular, colony MS can be useful as a screening method if one is interested in identifying the presence of compounds within the range of bacteriocin masses. In this situation, although colony MS was useful to identify potential masses of interest, the information provided was not always definitive. For example, despite the fact that colony MS of *L. gasseri* LM19 and the two *L. crispatus* Lac 50 and Lac 51 revealed peaks consistent with the putative antimicrobial peptides encoded by these strains, *L. amylovorus* Lac 20, that displayed inhibitory activity in well-diffusion assays, did not. However, in the case of *L. amylovorus* Lac 20 it could be that the compound is released in the media rather than be attached to cell surface. It should be noted that the absence of peaks corresponding to putative antimicrobials encoded does not mean that these antimicrobials cannot be produced, but rather it can reflect the level of production, the nature of the compound, the degree to which it is attached to the cell surface or the absence of the factors required to trigger its production. We sequenced isolates that showed interesting features (such as *E. faecalis* LM1 and LM4 that showed the inhibition zone in combination, indicating a potential quorum sensing regulation [240]) or that in previous work showed antimicrobial activity (such as *B. longum* FI10917). Additionally, we sequenced the QIB isolates that showed inhibitory activity in cell-free supernatant-based assays. Of the eleven sequenced isolates, only two, *B. longum* FI10917 and *L. amylovorus* Lac 20, did not contain predicted bacteriocin clusters using the BAGEL 3 software. *B. longum* FI10917 did not yield detectable antimicrobial activity, but *L. amylovorus* Lac 20 did, as described in Chapter VII. A total of 30 potential antimicrobials in 24 operons were predicted by BAGEL 3. This included a number that were described previously (plantaricins N and K [241], three delta-lysins, gassericin T and four enterolysins A that were not the subject of further analysis. However, there were some potentially novel antimicrobials that had exhibited only a low percentage similarity to other annotated bacteriocins. Thus, two sactipeptides, two glycocins, one bovicin-like, five helveticin J-like and one lactococcin 972-like clusters had the potential to yield new antimicrobials. Further study of the *L. gasseri* LM19 genome showed that one of those clusters also apparently encoded new bacteriocins (Chapter IV).

One of the possible applications of this antimicrobial activity can be to the production of these bacteriocins *in situ* if their bacterial producers showed further probiotic activities. For this reason, some probiotic traits were also examined. Previously, isolates from the QIB culture collection were studied for probiotic activity [242], so additional information could improve our knowledge and the applicability of these isolates. Autoaggregation, antioxidant capability and BSH activity were measured. It has been noted in the literature that isolates from the human gut are very likely to have these feature [243]. However, one third of the tested isolates did not show a white precipitation. This is not indicative of absence of BSH activity, but only that these isolates were not able to precipitate sodium taurodeoxycholate hydrate in visible form. There are different BS [244] and different bacteria present different BSH [245]. Our work showed that although BSH activity and autoaggregation were relatively common our bacteria did not show notable antioxidant activity.

Based on the different results summarised in this chapter, it was decided to further study *L. gasseri* LM19 for its multibacteriocinogenic characteristics and the presence of two novel bacteriocins in its genome. Additionally, nisin P was further studied because its activity was not reported previously, and it was only described by genome mining. *L. amylovorus* Lac 20 was further investigated because the heat and protease tests suggested that its antimicrobial activity could have a peptidic origin, despite the absence of results using the predictive bioinformatics tools. *L. crispatus* 50 and *L. crispatus* 51 were studied for the same reason as *L. amylovorus* Lac 20. Finally, *S. epidermidis* 9^{c}, F530B and 99, although not directly related to the thesis scope, were studied due to the nature of their isolation, since no human gut isolates of this species were reported in databases to date.

CHAPTER IV

Discovery of Gassericin M Produced by *L. gasseri* LM19

4.1. Introduction

Lactobacillus gasseri is one of six closely related species which are collectively referred to as the *Lactobacillus acidophilus* complex [246, 247]. These species are considered ecologically and commercially important and have been extensively studied, revealing antimicrobial and other probiotic properties [248-252]. *L. gasseri* has been divided in two subgroups using the average nucleotide identity (ANI) [253]. Strains from this species have been isolated from the GI tract of animals and humans [254], the vaginal tract [253, 255], human milk [256] and oral cavity [257] and used for their probiotic properties, including antimicrobial and bacteriocin production [252].

Many of the bacteriocins produced by *L. gasseri*, known as gassericins because of their origin, fall into different bacteriocin subgroups. Gassericin A is a cyclic peptide produced by *L. gasseri* LA39 isolated and purified from a human infant faecal sample [258-260]. Gassericins B1, B2, B3 and B4 were isolated from the vaginal isolate *L. gasseri* JCM 2124, with B1 and B3 being found to be identical to the α and β peptides of the two-component acidocin J1132 from *L. acidophilus* and B2 and B4 potentially representing modified forms of B3 [261]. Another bacteriocin, gassericin T, was found to be produced by *L. gasseri* SBT 2055, an adult human faecal isolate [132]. Gassericin T is notable because its amino acid sequence showed high similarity to one of the peptides (LafA) of the two-component lactacin F family produced by *L. johnsonii* VPI11088 [132]. Along with the LafA peptide, *L. johnsonii* produces another hydrophobic peptide, LafX, which was highly similar to lactobin A and the putative product of *gatX* found in the operon of *L. gasseri* SBT 2055 [132]. Despite establishing the presence and activity of gassericin T, mature GatX could not be detected in the supernatant of SBT2055 during the characterisation process and the synergy of a possible two-peptide gassericin T could not be established. GatX was detected later by Mavric *et al*, in a different strain, *L. gasseri* K7 [262]. Acidocins LF221A and LF221B were isolated from the infant faecal isolate of *L. acidophilus* LF221, later renamed *L. gasseri* LF221, [263] but their classification as members of the two-peptide bacteriocin group has not been established experimentally [264]. Furthermore, the genome of *L. gasseri* K7, isolated from the faeces of a breast-fed baby, contains two putative two-peptide bacteriocin-encoding operons [265] that share a high level of homology to acidocins LF221A and LF221B and gassericin T [262]. More recently, the isolation and purification of

gassericin E from *L. gasseri* EV1461, isolated from the vagina of a healthy woman, has been reported [264]. Gassericin E exhibits high similarity to gassericin T, differing only by one amino acid residue in its mature form [264]. Interestingly, the gassericin E operon also contains a putative bacteriocin-like peptide-encoding gene, *gaeX*, the predicted product of which shares 100% identity to GatX of *L. gasseri* SBT 2055 and gassericin K7 B of *L. gasseri* K7 [264]. Finally, peptides encoding gassericin T (GatA and GatX) and novel gassericin S with similarity to acidocin LF221A (GasA and GasX) were all found in the genome of *L. gasseri* LA327, isolated from human large intestine tissue [266]. Kasuga *et al* demonstrated the synergistic activity between the two components of gassericin T, and those of gassericin S [266]. However, they could not demonstrate synergistic activity between gassericin S and gassericin T when they mixed the four peptides together.

Here it is reported a new *L. gasseri* strain, LM19, isolated from breast milk, which possesses three bacteriocin clusters in its genome, including one encoding a novel gassericin, gassericin M. These studies reveal how different carbon sources influence the expression of the different bacteriocin clusters and the production of SCFA. Notably, LM19 survives, expresses bacteriocin genes and produces SCFA in detectable amounts in a complex faecal environment mimicking colonic conditions.

4.2. Methods

4.2.1. Isolation and analysis of *L. gasseri* LM19

L. gasseri LM19 was originally isolated from breast milk on MRS agar at 37^{o}C by Professor Juan Miguel Rodriguez from University Complutense of Madrid. Bioinformatic analysis was performed as described previously in sections 2.2.9.1. and 2.2.9.2.; alignments were performed using ClustalW [267] and visualised using Genedoc [268].

4.2.2. Detection and purification of antimicrobial peptides

This work was performed by Paula O'Connor at Teagasc Moorepark (Ireland). *L. gasseri* LM19 was grown anaerobically at 37^{o}C in 2 l MRS broth for 24-48 h. The culture was centrifuged (8,000 x g, 20 min, 10^{o}C) to separate cells from supernatant, and both samples were analysed independently. The cell pellet was resuspended in 400 ml 70% propan-2-ol, 0.1% trifluoroacetic acid (TFA – 'IPA') using a stirrer for 3-4 h at room temperature, centrifuged again and the supernatant retained for further purification and activity testing by drop test using *Lactobacillus bulgaricus* LMG 6901 as an indicator strain. IPA was

removed from this extract by rotary evaporation until the sample volume was 120 ml, and it was applied to a 2 g 12 ml Strata® C_{18}-E solid-phase extraction (C18-SPE) column (Phenomenex, UK), pre-equilibrated with methanol and water following manufacturer's instructions. The column was washed with 20 ml 30% ethanol, 20 ml of 30% acetonitrile and the active fraction eluted with 30 ml of IPA. The IPA was removed from the C18 SPE IPA eluate and 4 ml aliquots of sample applied to a semi preparative Jupiter C5 Reversed Phase HPLC column (10 x 250 mm, 10 µm, 300Å) (Phenomenex, Cheshire, UK) (HPLC run I) running a 30-70% acetonitrile 0.1% formic acid (FA) gradient over 95 minutes where buffer A is 0.1% FA and buffer B is 100% acetonitrile 0.1% FA. Flow rate was 2.5 ml/min and fractions were collected at 1 min intervals. The fractions were further analysed by matrix assisted laser deionisation -time of flight-mass spectrometry (MALDI-TOF-MS; Axima TOF^2 MALDI-TOF mass spectrometer in positive-ion reflectron mode, Shimadzu Biotech, UK) to determine the masses of the potential peptides. For purification from the cell-free supernatant, the supernatant was applied to an Econo-column (BioRad, UK) containing 60 g Amberlite XAD 16N. The column was washed with 400 ml 35% ethanol followed by 400 ml 30% acetonitrile and antimicrobial activity eluted with 450 ml IPA. The IPA was removed from the XAD IPA eluate by rotary evaporation until the sample volume was 145 ml and it was then applied to a 5 g 20 ml C18-SPE column pre-equilibrated with methanol and water following manufacturer's instructions. The column was washed with 30 ml 30% ethanol followed by 30 ml 30% acetonitrile and antimicrobial activity eluted with 30 ml IPA and fractionated by semi-preparative reversed phase HPLC as before. To increase purity, some HPLC fractions were reapplied to the C5 semi prep column, running shallower gradients. Specifically, 30-40% acetonitrile 0.1% FA gradient over 95 min for GamX and Bact_2, 30-45% gradient for GamA, 35-65% gradient for Bact_1, GamM and GamY. Additionally, the six peptides were synthesised using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a Liberty Blue microwave peptide synthesizer (CEM Corporation, USA). GamA and GamM were synthesised on a H-Lys(BOC)-HMPB)-ChemMatrix® resin, GamX was synthesised on H-Asn(Trt)-HMPB-ChemMatrix® resin, Bact_1 and Bact_2 on H-Arg(PBF)-HMPB-ChemMatrix® resin and GamY on Fmoc-Phe-Wang (Novobiochem®, Germany) resin. Crude peptide was purified using RP-HPLC on a Semi Preparative Vydac C4 (10 x 250 mm, 5 µ, 300 Å) column (Grace, USA) running acetonitrile-0.1% TFA gradients specific to the peptide of interest. Fractions containing the desired molecular mass were

identified using MALDI-TOF-MS on an Axima TOF2 MALDI TOF mass spectrometer and were pooled and lyophilised on a Genevac HT 4X lyophiliser (Genevac Ltd., UK). All peptides, from HPLC runs and synthetic ones, were assayed by well-diffusion assay using *L. bulgaricus* DPC6091 to compare activity and to assess synergistic activity among them.

4.2.3. Transformation of *L. gasseri*

Generation of electro-competent cells of *L. gasseri* LM19 was performed as described in sections 2.2.10.2. and 2.2.10.3.

4.2.4. Fermentation studies

L. gasseri was inoculated at 1% in 20 ml of MRS without glucose prepared in-house with different supplementation (Table 4.1) and incubated in anaerobic conditions at 37^{o}C over 48 h. 3 ml of each treatment were harvested at 24 and 48 h for further analysis of gene expression. Lower concentrations of glucose (0, 5, 1, 1.5%) were also tested in the same way. In addition to MRS, Batch model media was also prepared as described in section 2.1.1. The pH was adjusted to 6.8 in both media and filter sterilised carbohydrate source supplementation (glucose, lactose, galactose, inulin, starch or pectin (Sigma, UK)) was added at 2% (w/v) after autoclaving. Fermentations were conducted in triplicate and 2 ml of each sample were collected at 24 h and 48 h. 1 ml was used for enumeration by plate count, pH measurement using a pH-000-85282 probe (Unisense, Denmark) and, once filter sterilized, antimicrobial activity using a well diffusion assay; the other was centrifuged at 16,000 x g and the cell-free supernatant stored at -20^{o}C for further analysis.

Table 4.1. Summary of supplements to MRS culture media

Treatment	Supplement 2% (w/v)
1	-
2	Glucose
3	Lactose
4	Sucrose
5	Inulin
6	Starch
7	Pectin
8	2% Glucose + 1% *L. bulgaricus* DPC 5383
9	2% Glucose + 1% *E. coli* MC1022
10	2% Glucose + 1% *S. enterica* LT2

4.2.5. *In vitro* colon model

Fermentations to simulate human colon conditions were performed, in 150 ml volumes, as described previously [269, 270]. Faecal inoculum was prepared as explained in section 2.1.7. The temperature was maintained at 37 ^{o}C by a circulating water bath. Batch culture media was prepared as stated before, and 1% glucose was added as a carbohydrate source. Cultures were stirred, anaerobic conditions were maintained with oxygen-free nitrogen and pH maintained between 6.6 -7.0 by adding 1M NaOH or 1M HCl with automated pH controllers (Electrolab Ltd, UK) [269]. Overnight cultures of *L. gasseri* LM19 pUK200 and *C. perfringens* NCTC 3110 were added to the vessels at 1% (v/v). 6 ml samples were taken at 4 h, 8 h, 24 h and 48 h for DNA and RNA extractions, SCFA analysis and enumeration of *L. gasseri* LM19 pUK200 by plate count on MRS supplemented with 7.5 µg/ml chloramphenicol. Experiments were carried out in triplicate using three different faecal donors.

4.2.5.1. Detection of expressed bacteriocin genes from *L. gasseri* LM19 in faecal samples

To test *L. gasseri* LM19 bacteriocin gene expression in faecal samples, 3 ml of each aliquot was treated for RNA extraction and cDNA synthesis as previously described in section 2.2.7. The presence or absence of *L. gasseri* LM19 bacteriocin genes was confirmed by PCR [271]. Thermal cycling was performed using a Verity 96 well Thermal Cycler (Applied Biosystems) using GoTaq G2 DNA polymerase (Promega) according to manufacturer´s instructions.

Primers used were summarized in table 2.5 and cycle conditions were the same as for RT-qPCR.

4.2.5.2. Quantification of *C. perfringens* NCTC 3110

A standard curve for *C. perfringens* NCTC 3110 was constructed by extracting gDNA as described previously [272] at different concentrations (colony forming units (cfu)/ml) of *C. perfringens* NCTC 3110. Each DNA concentration was measured using qPCR to determine the cycle signal associated with each cell density. Colon model treatments were analysed by RT-qPCR and total cfu calculated for each treatment.

4.2.5.3. DNA extraction, 16S sequencing and metagenomics analysis

For DNA extraction, 16S rRNA amplification and sequencing and 16S-based metataxonomic analysis, 3 ml of each aliquot at different time points was treated for DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) following manufacturer's guidelines. Total DNA concentration was measured by Qubit 3 (Invitrogen, UK) and normalized. The V4 region of the 16S rRNA gene was used for high throughput sequencing using the Illumina Miseq platform with primers 515F and 806R [273]. Data analysis were conducted using the Quantitative Insights into Microbial Ecology (QIIME2 version 2018.11) [226].

4.2.5.4. Quantification of SCFA

SCFA were measured using ^{1}H-NMR as described in section 2.4.1.2.

4.3. Results

4.3.1. Antimicrobial activity

L. gasseri LM19 showed antimicrobial activity against both Gram-positive and Gram-negative pathogens (Table 4.2). The assay method affected the outcome, and it was noted that fewer strains were sensitive to the cell free supernatant than to cells grown on a plate.

Table 4.2. Summary of inhibitory activity of *L. gasseri* LM19 against different indicator strains. Different methods were tested.

	Overlay	Cross-streak	Drop test	Filter disc	Well-diffusion
S. enterica LT2	-	-	-	-	-
E. coli ATCC 25922	-	-	-	-	-
C. sakazakii NCTC 11467	-	++	-	-	-
C. perfringens NCTC 3110	+	+	-	-	-
L. innocua NCTC 11288	-	++	-	-	-
L. bulgaricus 5583	+++	+++	-	++	+++
C. jejuni NCTC 11168	np	np	np	+	np
M. luteus FI10640	-	++	-	+	-

-, No activity; +, 1 mm radius inhibition zone; ++, 1-5 mm radius inhibition zone; +++, >5 mm inhibition zone; np, not performed

4.3.2. Identification of bacteriocin gene clusters

The sequenced genome of *L. gasseri* LM19 was assembled into contigs and analysed to identify putative bacteriocin clusters. The sequence has been submitted to the NCBI under accession number SHO00000000. RAST failed to reveal any clusters of interest; antiSMASH 3.0 recognised a single Microcin M-like cluster, while BAGEL 4, which specifically targets regions with bacteriocin similarities, found three clusters predicted to encode a number of potential bacteriocins. Manual investigation confirmed the presence of two clusters, whose putative structural peptides showed a high similarity to previously identified antimicrobial peptides from Class IIb bacteriocins (clusters 1 and 3), and a helveticin-like protein. The latter contained no other bacteriocin-associated genes on the basis of Blastp analysis, consisting only of a single gene, the product of which showed 31.9 % identity and 43.1% amino acid consensus to helveticin J, which was originally characterised in *Lactobacillus helveticus* following heterologous expression [274]. Table 4.3 summarises current known gassericins.

Cluster 1 (939 bp) is highly similar to the class IIb gassericin K7A cluster (EF392861) with 99% nucleotide identity. The cluster was predicted to encode two short peptides with leader sequences (bact_1 and bact_2) and a putative immunity protein (Figure 4.1A). *bact_1* and *bact_2* show 100% similarity with the gassericin S structural genes *gasA* and *gasX* respectively [266], while the surrounding genes do not resemble any other genes

known to be associated with bacteriocin production. The putative immunity protein showed 97% amino acid homology to that of the acidocin LF221A cluster [275].

Cluster 3 is 9736 bp in length and the associated open reading frames (orfs) 1-8 show a high nucleotide homology to the gassericin T cluster from *L. gasseri* LA158 (AB710328, 99% over 100% coverage) and the gassericin E cluster from *L. gasseri* EV1461 (KR08485, 99% over 95% coverage) (Figure 4.1B). There are two structural peptide-encoding genes, *gamA* and *gamX*, that are preceded by homologues of the gassericin E cluster as described previously [264]. It is likely that they perform the same functions, i.e., *gamP*, *gamK*, *gamR* for regulation, *gamT* and *gamC* for transport and, after the structural peptides, *gamI* for immunity, although a homologue to *gaeX* is missing. The predicted GamA peptide has the same sequence as GasT, Gas K7B cf and acidocin LF221B cf and has a single amino acid difference (W-L) from GasE (4.1). The second putative peptide, GamX, has the same sequence as GatX and GaeX, all of which differ by a single amino acid (G-A) from Gas K7 B and acidocin LF221B (Figure 4.1B).

In cluster 3, there are two additional putative structural genes, designated as *gamM* and *gamY*, which appear to encode a two-component bacteriocin. These putative peptides also show some similarity to other two-peptide component gassericins, but to a lesser extent than those previously reported (Figure 4.1B). GamY shows similarity to GamM, with 25.4% identity and 47.6% consensus, and they both have similarity to K7 A cf (27.5% identity and 38.8% consensus; 25.3% identity, 44.3% consensus, respectively) and to GamA (18.7% identity, 33.3% consensus with GamM). Surrounding *gamY* and *gamM* are genes encoding a short hypothetical protein with no matches (*orf9*), followed by a gene encoding a putative immunity protein, GamI2, with homology to an enterocin A immunity domain (pfam 08951, $2.8e^{-7}$) and a putative transport accessory protein, GamC2, with some similarity to TIGR01295 bacteriocin transport accessory protein ($1.18e^{-9}$), but also to a thioredoxin superfamily cd02947 ($5.21e^{-7}$). After *gamM*, there is a further orf14 with no matches, followed by a third putative immunity gene with similarity again to pfam 08951 ($1.1e^{-6}$). The genes on either side of the cluster resemble transporters involved in solute or cation transport, and so are not predicted to be part of the cluster.

Table 4.3. Bacteriocins described in *L. gasseri*.

Gassericin	Amino acid sequence	Molecular mass (Da)	Reference
A	**MIEKVSKNELSRIYGG**NNVNWGSVAGSCGKGAVMGIYFGNPILGCANGAATSLVLQ TASGIYKNYQKKR	5652	[258, 259]
B1	(N-terminal) NPKVAHCASQIGRSTAWGAVSGAATGTAVGQAVGA-X	6217	[261]
B2	(N-terminal) MISKPEKNTLRL-X	4400	[261]
B3	(N-terminal) GNPKVAHCASQIGRSTAW-X	6273	[261]
B4	(N-terminal) NPKVAHCASQIGRSTAW-X	5829	[261]
GatX	**MALKTLEKHELRNVMGG**NKWGNAVIGAATGATRGVSWCRGFGPWGMTACGLG GAAIGGYLGYKSN	4763	[262]
Acidocin LF221A α	**MIEKVSKNELSRIYGG**NNVNWGSVAGSCGKGAVMEIYFGNPILGCANGAATSLVLQ TASGIYKNYQKKR	3393	[263]
Acidocin LF221A β(cf)	**MKVLNECQLQTVVGG**KNWSVAKCGGTIGTNIAIGAWRGARAGSFFGQPVSVGTG ALIGASAGAIGGSVQCVGWLAGGGR	5523	[263]
Acidocin LF221B α	**MALKTLEKHELRNVMGG**NKWGNAVIGAATGATRGVSWCRGFGPWGMTACALG GAAIGGYLGYKSN	3393	[263]
Acidocin LF221B β	**MKNFNTLSFETLANIVGG**RNNWAANIGGVGGATVAGWALGNAVCGPACGFVGA HYVPIAWAGVTAATGGFGKIRK	5542	[263]
K7 A	**MIEKVSKNELSRIYGG**NNVNWGSVAGSCGKGAVMEIYFGNPILGCANGAATSLVLQ TASGIYKNYQKKR	5523	[265]
K7 A (cf)	**MKVLNECQLQTVVGG**KNWSVAKCGGTIGTNIAIGAWRGARAGSFFGQPVSVGTG ALIGASAGAIGGSVQCVGWLAGGGR	3393	[265]
K7 B	**MALKTLEKHELRNVMGG**NKWGNAVIGAATGATRGVSWCRGFGPWGMTACALG GAAIGGYLGYKSN	4777	[265]
K7 B (cf)	**MKNFNTLSFETLANIVGG**RNNWAANIGGAGGATVAGWALGNAVCGPACGFVGA HYVPIAWAGVTAATGGFGKIRK	5542	[265]
GasE	**MKNFNTLSFETLANIVGG**RNNLAANIGGVGGATVAGWALGNAVCGPACGFVGAH YVPIAWAGVTAATGGFGKIRK	5469	[264]
GamA (=GasT)	**MKNFNTLSFETLANIVGG**RNNWAANIGGVGGATVAGWALGNAVCGPACGFVGA HYVPIAWAGVTAATGGFGKIRK	5542	This work, [132]
GamX (=GaeX)	**MALKTLEKHELRNVMGG**NKWGNAVIGAATGATRGVSWCRGFGPWGMTACGLG GAAIGGYLGYKSN	4763	This work
Bact_1 (=GasS)	**MKVLNECQLQTVVGG**KNWSVAKCGGTIGTNIAIGAWRGARAGSFFGQPVSVGAG ALIGASAGAIGGSVQCVGWLAGGGR	6060	This work, [266]
Bact_2 (=GasX)	**MIEKVSKNELSRIYGG**NNVNWGSVAGSCGKGAVMGIYFGNPILGCANGAATSLVLQ TASGIYKNYQKKR	5451	This work, [266]
GamY	**MKTLNEQELTQIVGG**KGNKGINWANVRCASAVTIGALGGGLAGPGGMVGGFLLGS GACF	4105	This work
GamM	**MRKINKEELVEITGG**FNAAKCAVGTAGGAFSIARGSAAFGVPGMVIGGILGGAAGAL ASCK	4124	This work

X, sequence not available; cf, complemental factor; leader sequences are marked in bold where known.

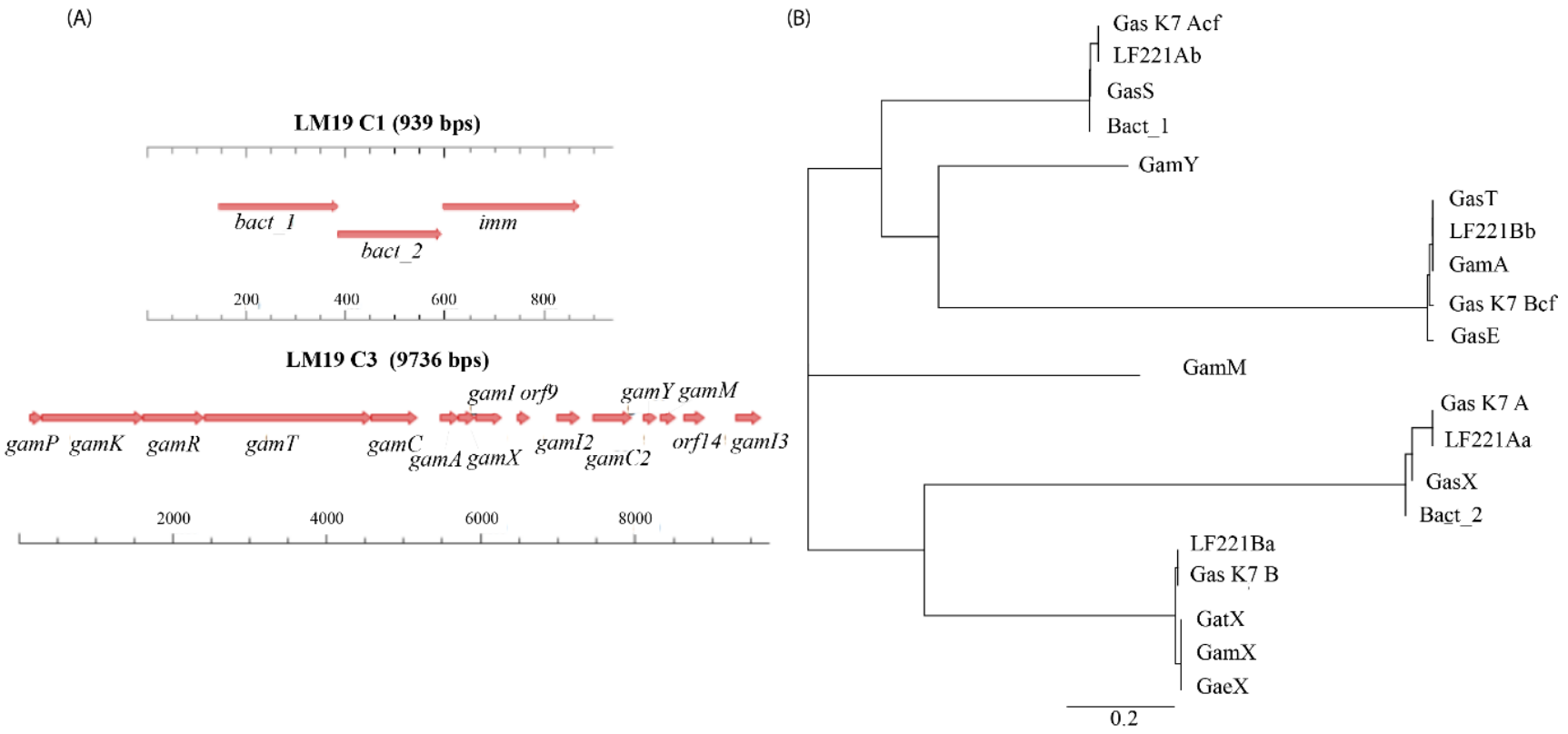
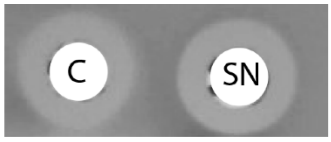


Figure 4.1 (A) Cluster LM19 C1, encoding Bact_1 and Bact_2, and Cluster LM19 C3 encoding GamA, GamX, GamY and GamM. (B) Phylogenetic tree of the amino acid sequences of putative bacteriocins identified in *L. gasseri* LM19 in context with the other class IIb gassericins.

4.3.3. Identification of antimicrobial peptides in culture

This work was performed by Paula O'Connor at Teagasc Moorepark (Ireland). Cell and supernatant extracts from *L. gasseri* LM19 cultures were fractionated by HPLC and analysed by MS and their antimicrobial activity was assessed using *L. bulgaricus* 6901 as an indicator (Figures 4.2 and 4.3). Antimicrobial activity was present in both cells and supernatant (Figure 4.2A), with fractions containing the highest activity eluting in 50% and 75% IPA. MS shows that all peptide masses of interest are present.

(A)



(B)

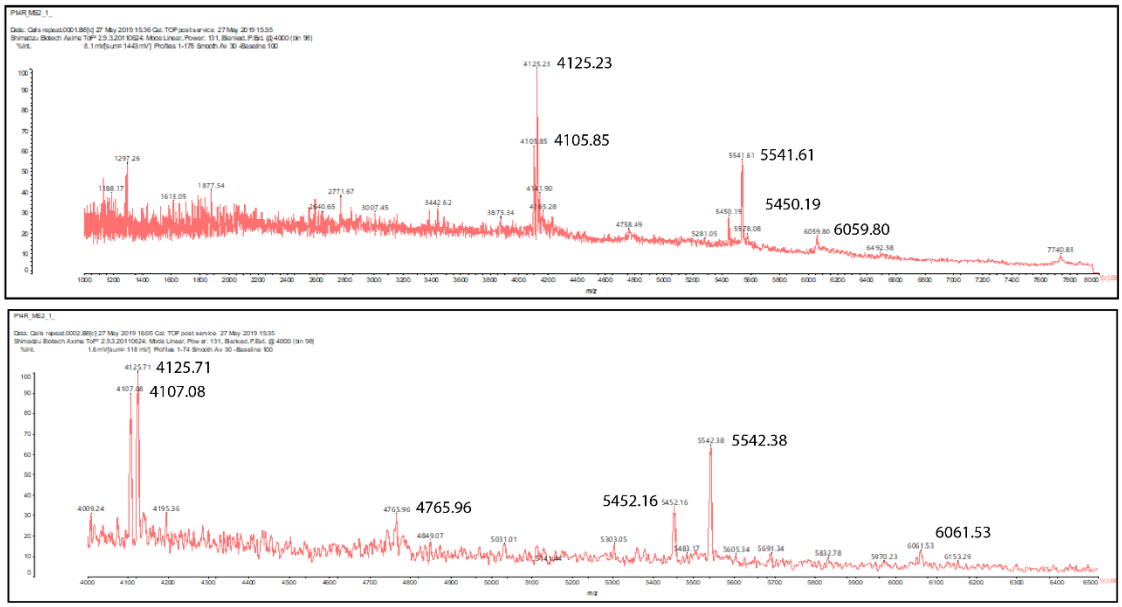


Figure 4.2 (A) Test for antimicrobial activity of cell (C) and supernatant (SN) fractions of *L. gasseri* LM19 culture against *L. bulgaricus* 6901. (B) MS chromatograms showing peptide masses of interest in the cell extracts.

4.3.3.1. Purification of antimicrobials from cells

MS analysis of HPLC fractions showed that many of them contained one or more peptide masses that were consistent with those predicted by in silico analysis of the genome (Table 4.3, Figures 4.2 and 4.3). mV response was very low (40 mV compared to around 1000 mV from a good producer), suggesting that production and consequently yield was very low. Mass corresponding to GamX (4763 Da) was detected in fractions 32-36 (Figure 4.3A). Fractions 37-39 showed mass corresponding to GamA, 5541 Da (Figure 4.3B). Masses

corresponding to GamY and GamM co-eluted in fraction 49-55, equating with a 10 mV peak on the chromatogram suggesting extremely low yield (Figure 4.3C). Fractions 57-60 showed the expected mass from Bact_1, 6060 Da (Figure 4.3D). Fractions 85-97 showed putative GamM mass, 4126 Da (Figure 4.3E).

4.3.3.2. Purification of antimicrobials from supernatant

MS analysis of HPLC fractions 20-97 showed masses corresponding to Bact_1 and GamM. Masses corresponding to putative Bact_1 eluted in fractions 59-65, while putative GamM mass was detected in fractions 76-97. Masses corresponding to Bact_2 and GamA eluted also in the first HPLC fractionation and were fractionated again. GamX eluted in fractions 86-90 (Figure 4.3E), and also in fractions 66-68, co-eluting with GamA. Putative Bact_1 eluted in fractions 58-60. GamY eluted in fractions 69-72, and GamM appeared in fractions 86-91.

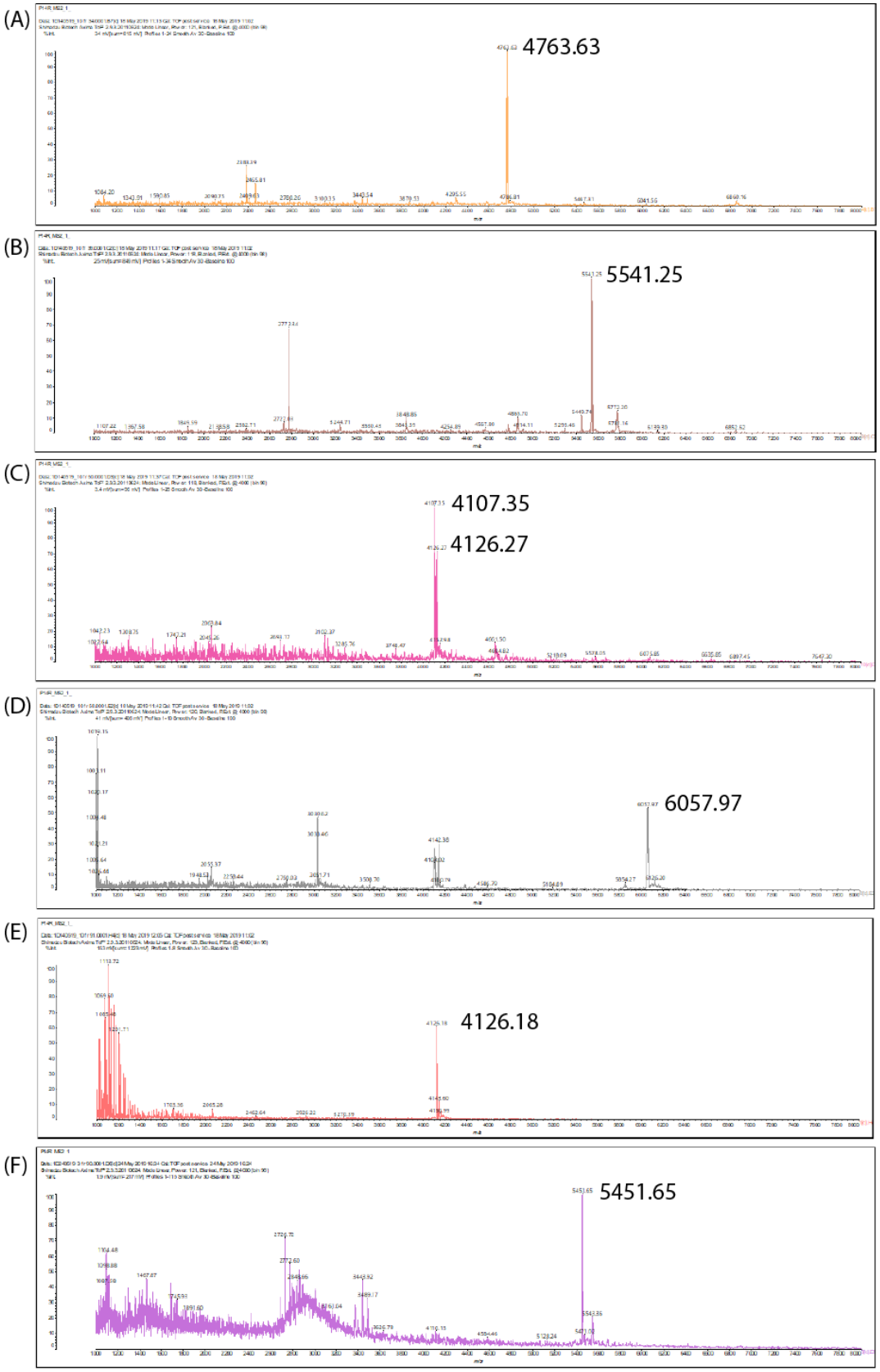


Figure 4.3. MS chromatograms of fractions showing putative masses for A, GamX, 4763 Da; B, GamA, 5541 Da; C, GamM, 4126 Da and GamY, 4107 Da; D, Bact_1, 6057 Da; E, GamM, 4126 Da; F, Bact_2, 5451 Da.

4.3.3.3. Synergy between peptides

Three sets of fractions were compared, fractions from HPLC run I, fractions from from HPLC II run and synthetic peptides resuspended in milli Q water at 1 mg/ml (Figure 4.4A). All synthetic peptides except GamY showed antimicrobial activity, with the highest activity coming from GamA and Bact_2. Figures 4.4B and C show synergy assays between synthetic peptides. We observed clear synergy between Bact_1 and Bact_2 and a possible synergy between Bact_1 and GamA. No synergy was observed between GamM and GamY or between GamA and GamX.

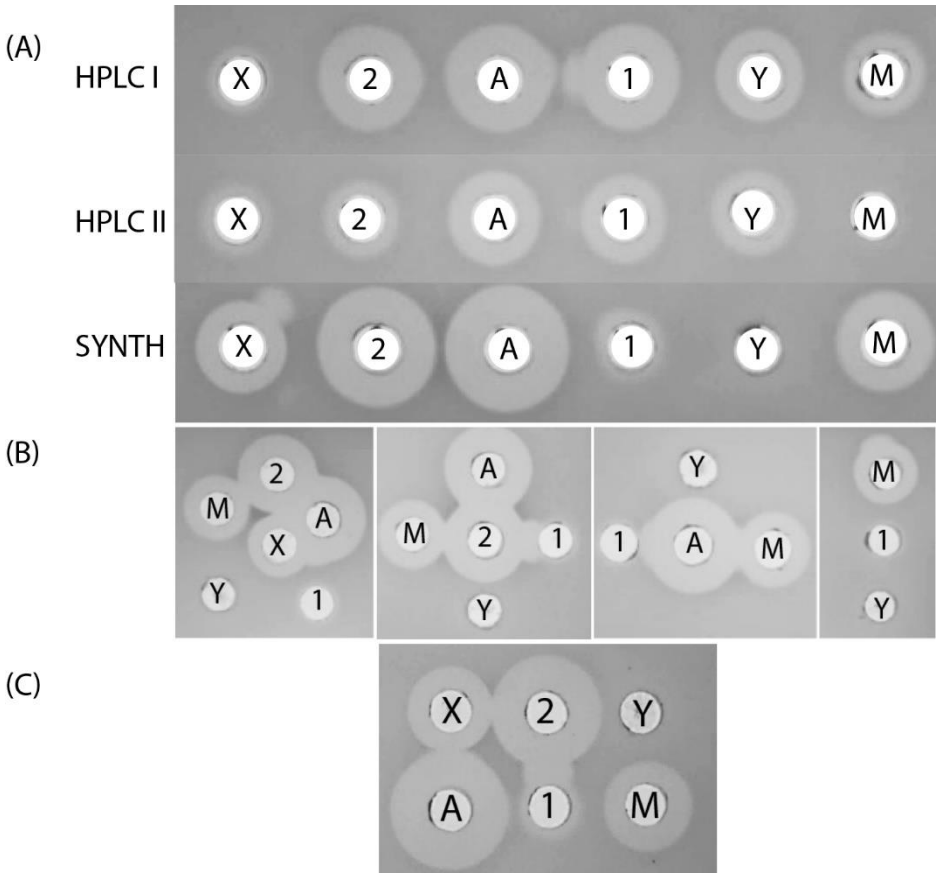
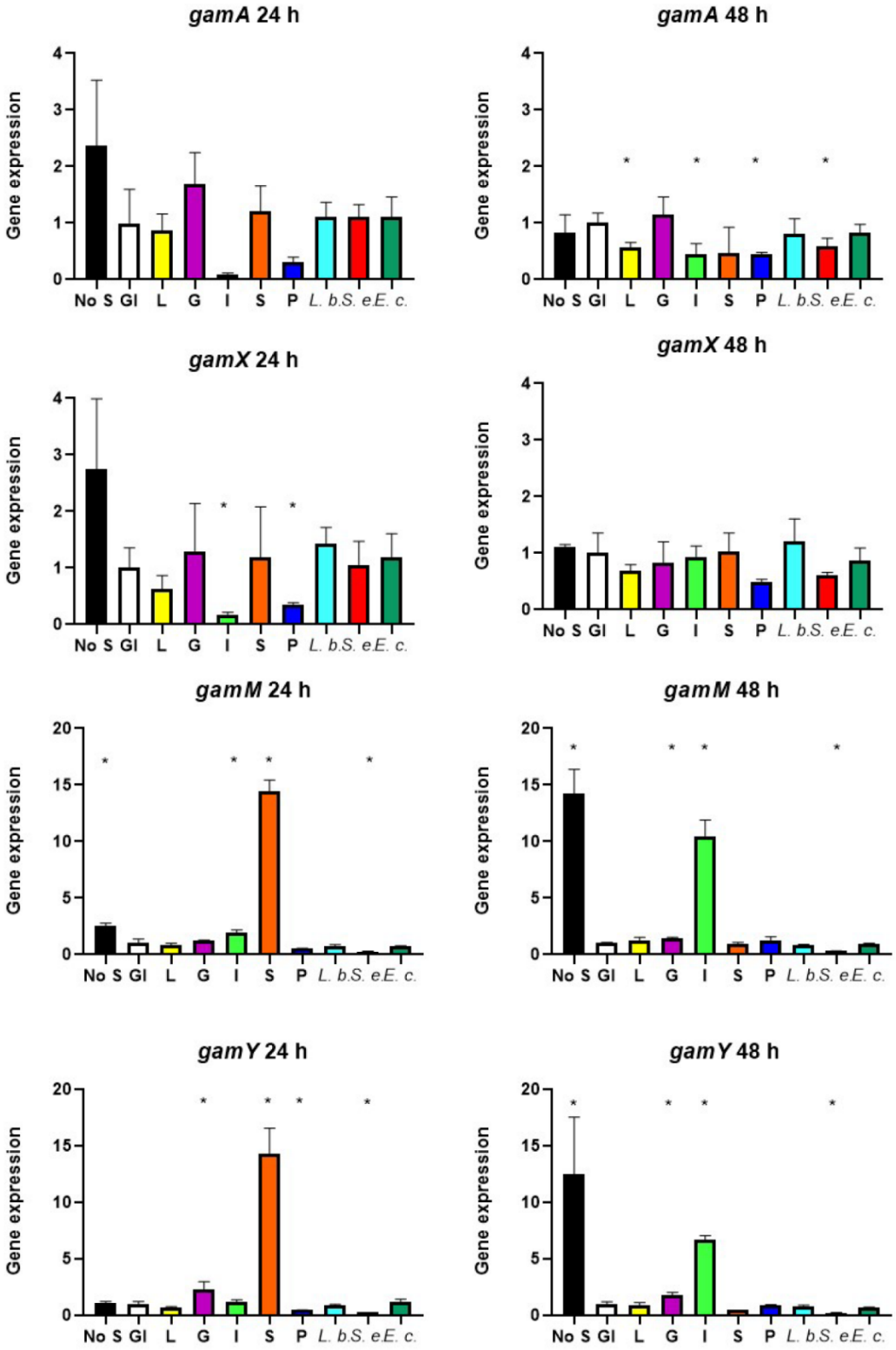


Figure 4.4 (A) Activity of GamX, (X); Bact_2, (2); GamA, (A); Bact_1, (1); GamY (Y); GamM, (M) from synthetic peptides and selected fractions from HPLC runs I and II containing similar masses; (B) Synergy between the different peptides; (C) Synergy between pairs of peptides.

4.3.4. Fermentations

4.3.4.1. Carbon supplementation in media influences the expression levels of bacteriocin genes in *L. gasseri* LM19

RT-qPCR was used to compare the expression of LM19 genes encoding bacteriocin structural peptides/proteins during growth with different carbon sources at 2% (v/v) or in the presence of competing microbes over 2 d, corrected on the basis of expression of the housekeeping gene encoding gyrase A. Ultimately, it was apparent that each bacteriocin gene was expressed at different levels depending on the carbon source that was used (Figure 4.5). *Bact_1* and *bact_2* exhibited a similar pattern across each treatment and time. There was very little expression during growth without a carbon supplement or with starch, and a tendency for higher expression of *bact_1* and *bact_2* with simple sugars rather than inulin or pectin. All genes were expressed during co-culture with other bacteria, but no significant changes were seen when compared to glucose alone. In contrast, the helveticin J-like gene showed increased expression in the absence of a carbon supplement and with the complex carbohydrates inulin, starch and pectin and, after 48 h, disaccharide lactose in comparison with the glucose control. Expression was not increased in co-culture. Indeed, the presence of *E. coli* caused a slight repression. The genes encoding GamA and GamX also showed similar behaviour to each other in that, although expression at 24 h without a carbon supplement was higher than that with glucose, this effect was lost at 48 h. Expression was lower with inulin and pectin at 24 h and at 48 h. Co-culture did not have a large effect, but expression after 48 h growth with *S. enterica* was slightly lower than in the absence of the pathogen. Genes encoding GamM and GamY differed from other putative bacteriocin-encoding genes in that an upregulation of their expression was observed at 48 h in the absence of carbon supplementation. The same behaviour was observed with inulin supplementation, while starch expression was high at 24 h but lower at 48 h. As with *gamA* and *gamX*, 48 h co-culture with *S. enterica* LT2 lowered their expression levels.


gamA 24 h
gamA 48 h
gamX 24 h
gamX 48 h
gamM 24 h
gamM 48 h
gamY 24 h
gamY 48 h
Gene expression
No S Gl L G I S P L. b S. e E. c.

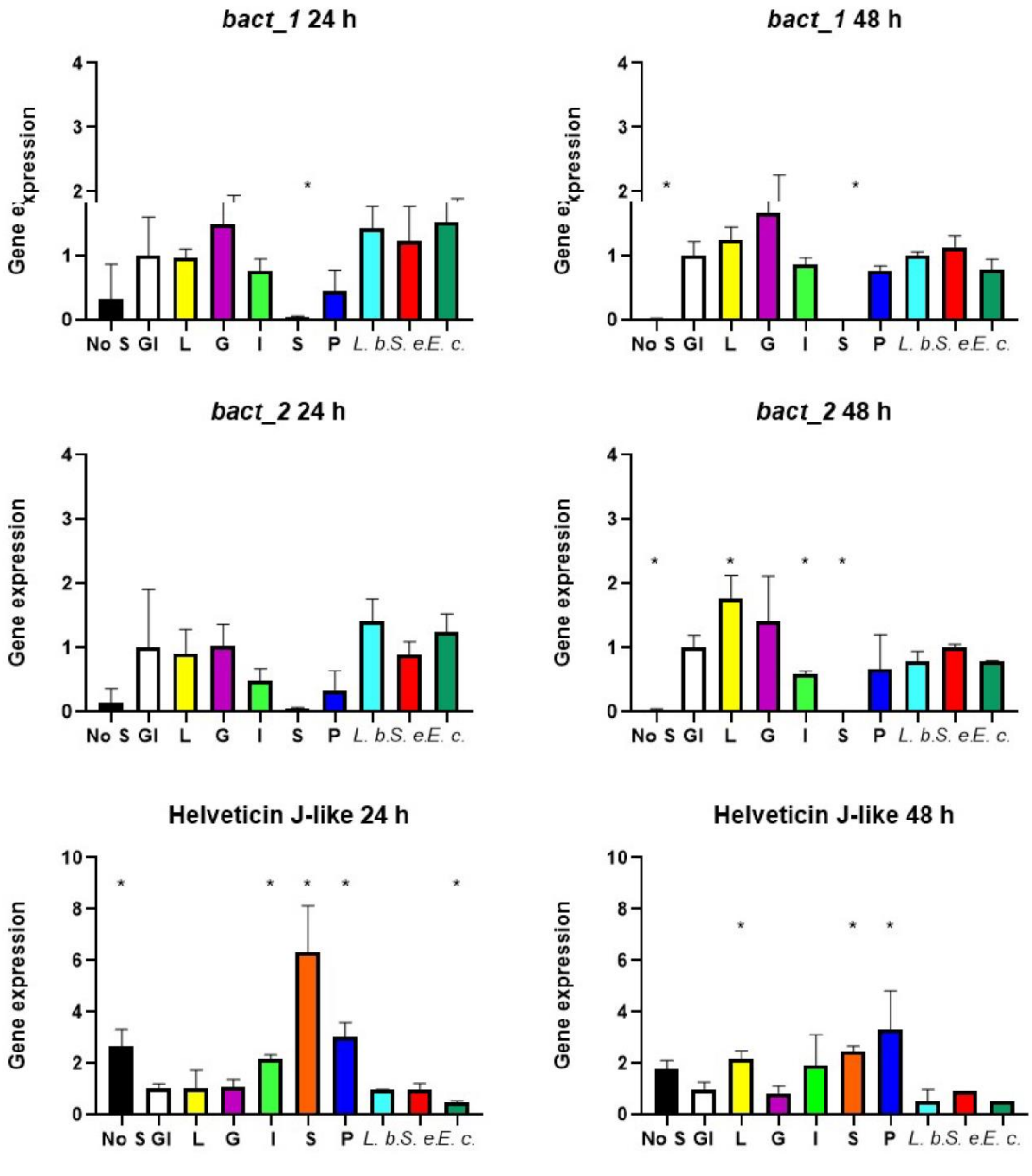


Figure 4.5. Gene expression levels of bacteriocin genes present in *L. gasseri* LM19 genome when grown in home-made MRS supplemented with different carbohydrate sources. (N, no carbohydrate; Gl, glucose; L, lactose; G, galactose; I, inulin; S, starch; P, pectin; L.b. *L. bulgaricus*; S. e., *S. enterica* and E. c. *E. coli*). *, significant difference to glucose, $p<0.05$.

4.3.4.2. Different levels of available glucose affect the expression of bacteriocins in *L. gasseri* LM 19 in different ways

Given the differences in the expression levels of the bacteriocin genes in *L. gasseri* LM19 in the absence of a carbon source supplement, the impact of growing in lower percentages of glucose was tested. The results confirmed previous observations, i.e., expression of *bact_1* and *bact_2* genes was increased in line with increases in glucose availability, while expression of the helveticin J-like gene showed the opposite pattern. Expression levels of genes encoding GamA, GamX, GamM and GamY were increased in the absence of carbon supplementation at 48 h (Figure 4.6).

4.3.4.3. Complex polymers increase viability of *L. gasseri* LM19

L. gasseri LM19 was grown in colon model medium, simulating gut conditions, or home-made MRS - alone or supplemented with simple sugars (glucose, lactose and galactose) or complex polymers (inulin, starch and pectin). In general, more viable cells were recovered from MRS; growth on simple sugars was highest at 24 h but, at 48 h, complex carbohydrates gave higher counts (Figure 4.7A). Interestingly, growth on no carbon source at 48 h was similar to that with simple sugars. On batch model medium, cell counts with glucose were lower than with all other treatments, and starch and pectin improved growth in at 48 h. Antimicrobial activity from batch model medium with glucose was almost as high as that from MRS despite a c. 3 log difference in cfu (Figure 4.7B). Glucose and galactose supplementation showed the highest antimicrobial activity at 24 h, however, after 48 h, complex carbohydrates produced the highest activity. At 48 h, higher levels of antimicrobial activity correlate with lower levels of pH and higher levels of cfu, suggesting that activity is related to bacterial numbers. The changes in activity with carbon supplementation over time suggest control of antimicrobial production in different nutritional environments.

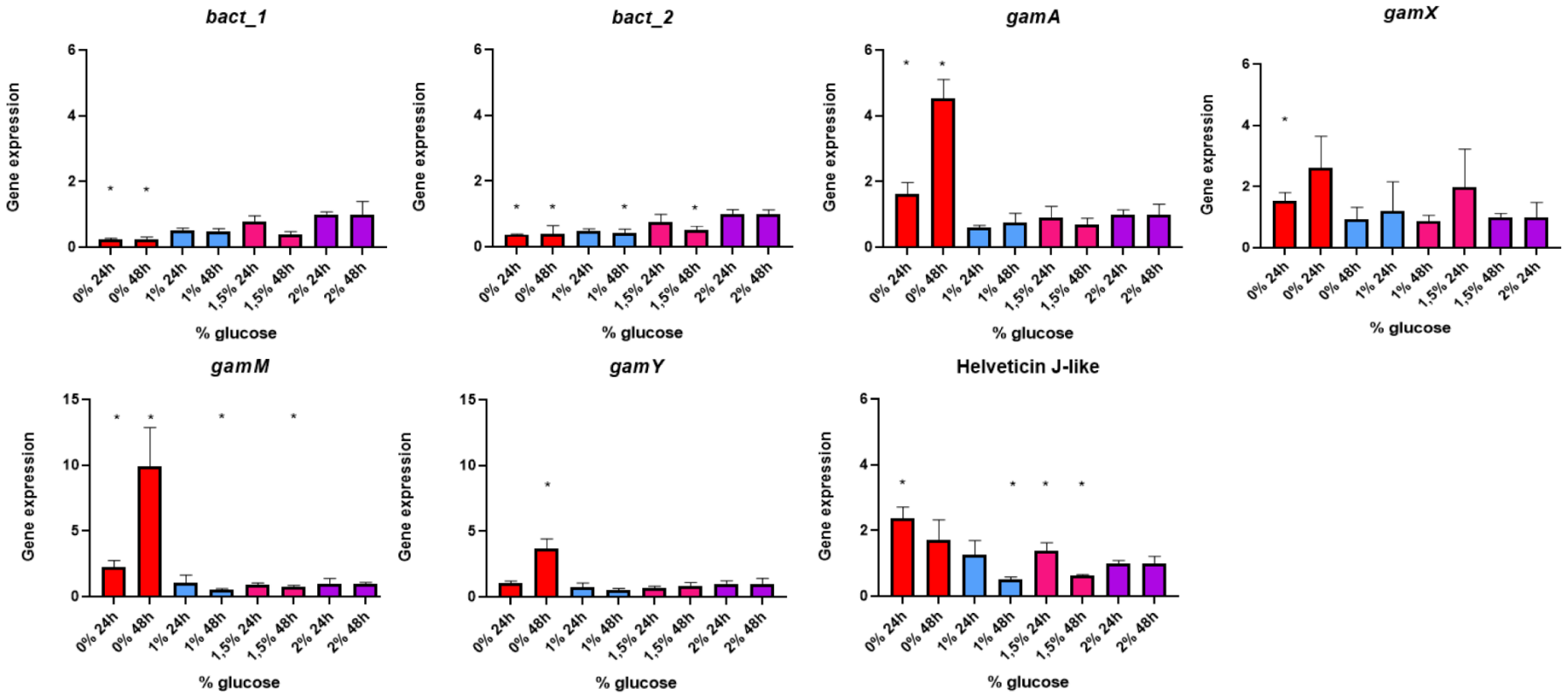


Figure 4.6. Gene expression levels of bacteriocin genes present in *L. gasseri* LM19 genome when grown in home-made MRS supplemented with different glucose percentages. *, significant difference to glucose 2%, p<0.05.

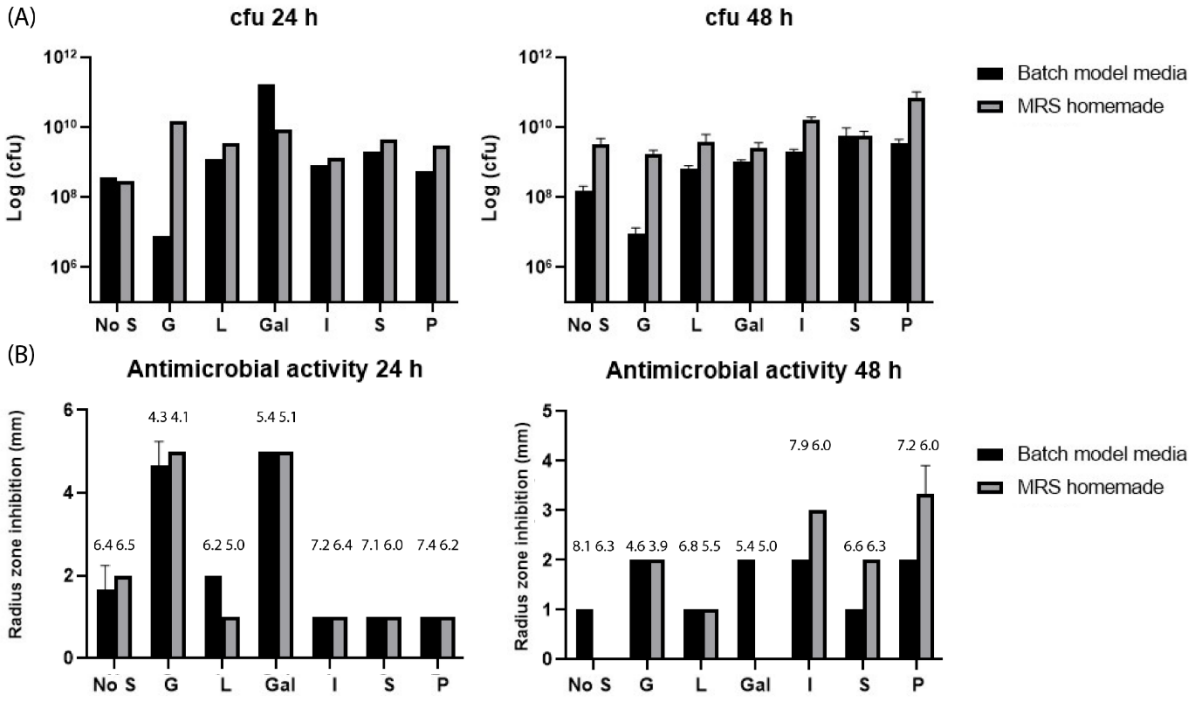


Figure 4.7 (A) Cfu counts of *L. gasseri* LM19 recovered when grown in batch model media or home-made MRS supplemented with different carbon sources. (B) Antimicrobial activity of cultures in (A) measured by well diffusion assay (Figures above bars indicate mean pH) No S, no supplementation; G, glucose; L, lactose; Gal, galactose; I, inulin; S, starch and P, pectin.

4.3.5. Colon model

4.3.5.1. Survival of *L. gasseri* LM19 and *C. perfringens* in an *in vitro* colon model

L. gasseri LM19 was transformed with a plasmid expressing chloramphenicol resistance to allow selection and enumeration of this strain from within a mixed microbial community. Transformation of electrocompetent cells gave an efficiency of 1.07×10^2 transformants/ng of DNA. Fermentations with three different faecal donors were performed with four vessels per fermentation inoculated with *L. gasseri* LM19-pUK200, *C. perfringens* NCTC 3110, *L. gasseri* with *C. perfringens*, or a media control. *L. gasseri* numbers recovered increased from 5.3, 5.22 and 5.22 Log_{10} cfu/ml in donors 1, 2 and 3, respectively at 4 h, to 6.12, 6.39 and 6.36 Log_{10} cfu/ml at 8 h and 7.30, 7.31 and 7.47 Log_{10} cfu/ml at 24 h. However, after 48 h, levels of recovery dropped to 3.66, 4 and 3.72 Log_{10} cfu/ml.

C. perfringens levels were measured by qPCR, which detects DNA from both live and dead cells. Addition of *L. gasseri* LM19 did not have a negative effect on the *C. perfringens*

population in the fermentation with faecal sample from donor 1; there was a tendency to lower *C. perfringens* counts in co-culture at 24 h with donors 2 and 3, but the changes were not significant (Figure 4.8).

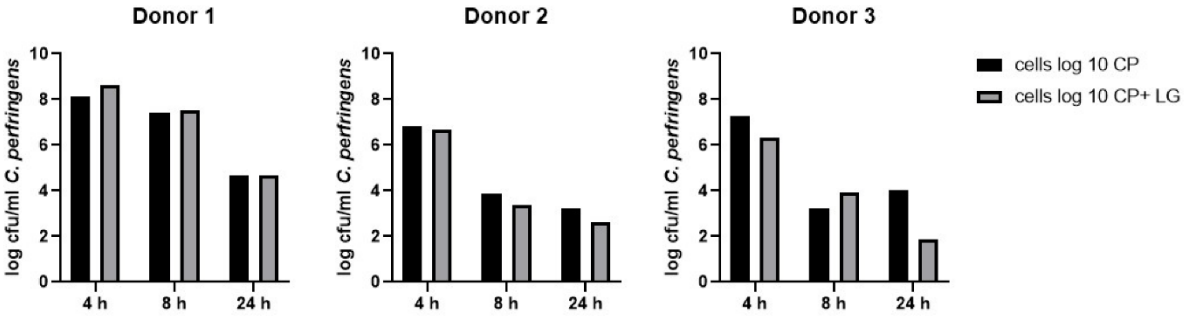


Figure 4.8. *C. perfringens* NCTC 3110 population in the presence of *L. gasseri* LM19 in three different faecal fermentations measured by RT-qPCR.

4.3.5.2. Bacteriocin gene expression

PCR amplification of cDNA obtained from colon model samples using primers to detect the bacteriocin genes *bact_1, bact_2, helveticin-J like*, *gamA*, *gamX*, *gamM* and *gamY*, showed detectable levels of bacteriocin gene expression at 24 h (Figure 4.9). At 48 h, expression of only helveticin-J like, *gamM* and *gamY* was detected (data not shown).

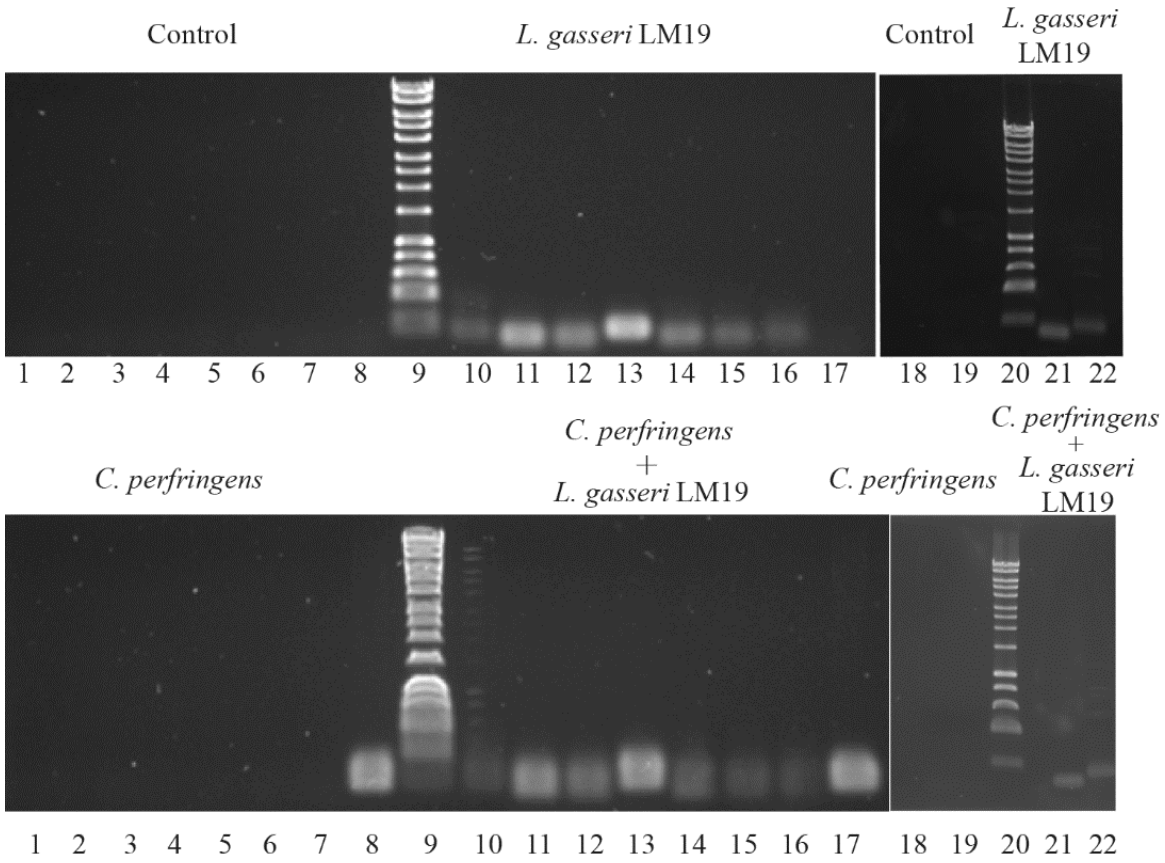


Figure 4.9. Expression of bacteriocin genes in colon model at 24 h with donor 1. Lane 1, negative control; lane 2, *bact_1*; lane 3, *bact_2*; lane 4, *gamA*; lane 5, *gamX*; lane 6, *helvJ*; lane 7, *gyrA*; lane 8, *cper*; lane 9, molecular marker; lane 10, *bact_1*; lane 11, *bact_2*; lane 12, *gamA*; lane 13, *gamX*; lane 14, *helvJ*; lane 15, *gyrA*; lane 16, *gam*I3; lane 17, *cper*; lane 18, *gamM*; lane 19, *gamY*; lane 20, molecular marker; lane 21 *gamM*; lane 22, *gamY*.

4.3.5.3. Impact of *L. gasseri* LM19 on gut microbiota composition

Analysis of relative abundance at order, family and genus level was conducted using 16S rRNA metagenomics. The initial bacterial composition was, as expected, different between the three donors (Figure 4.10). Bacterial populations from donor 1 remained relatively stable over 24 h. The addition of *L. gasseri* LM19, *C. perfringens* or both did not result in a significant increase in proportions of Lactobacillales or Clostridiales, and all three treatments resulted in similar increases in Bifidobacteriales and Coriobacteriales relative to the control, with the *L. gasseri* LM19 with *C. perfringens* more similar to the *L. gasseri* LM19 alone.

The initial population from donor 2 was constituted mainly of Clostridiales, with some Bacteroidales, Coriobacteriales and Bifidobacteriales. A change can be observed at 24 h in both the control and the samples where *L. gasseri* LM19 or *L. gasseri* LM19 with *C.*

perfringens were added, with an increase in Lactobacillales along with a small increase in Enterobacteriales. The decrease in relative abundance of Bifidobacteriales and Coriobacteriales in the control, *L. gasseri* and *C. perfringens* + *L. gasseri* treatments was statistically significant ($p<0.05$) and not observed in the *C. perfringens* sample. *C. perfringens* alone appeared to prevent the overgrowth of Lactobacillales, while Clostridiales were decreased, being replaced by Enterobacteriales, Bacteroidales, Coriobacteriales and Bifidobacteriales. It was noted that addition of *L. gasseri* LM19 with *C. perfringens* gave a profile with more similarity to the control or *L. gasseri* LM19 samples. In *L. gasseri* LM19 treatment of donor 3 samples, Bifidobacteriales and Enterobacteriales increased over time in a similar way to the control, but Clostridiales were almost completely replaced by Lactobacillales. This rise was not as large when the *L. gasseri* LM19 was co-inoculated with *C. perfringens*, while addition of *C. perfringens* alone did not manage to maintain levels of Clostridales, with increases seen in Enterobacteriales, Bifidobacteriales and Lactobacillales. In this case, the *L. gasseri* LM19 + *C. perfringens* treatment at 24 h was more similar to the control, with the exception of the presence of Lactobacillales, suggesting that the *C. perfringens* effect on the microbial composition was changed by the inoculation with *L. gasseri*.

4.3.5.4. SCFA analysis

Increases in the production of formic, acetic, propionic and butyric acids were observed in the three faecal fermentations in colon model conditions inoculated with *L. gasseri* LM9. However, there was a high variability in SCFA production between the three donors (Figure 4.11). In donor 1, production of SCFA, ethanol, succinate and, at 8 h only, lactate was increased compared to the control. In donor 2 there were notable increases in lactic acid from 4 h. Given the similar relative abundance of Lactobacillales (Figure 4.10) in control and *L. gasseri* treatment, this suggests an influence of *L. gasseri* LM19 on the native microbiota

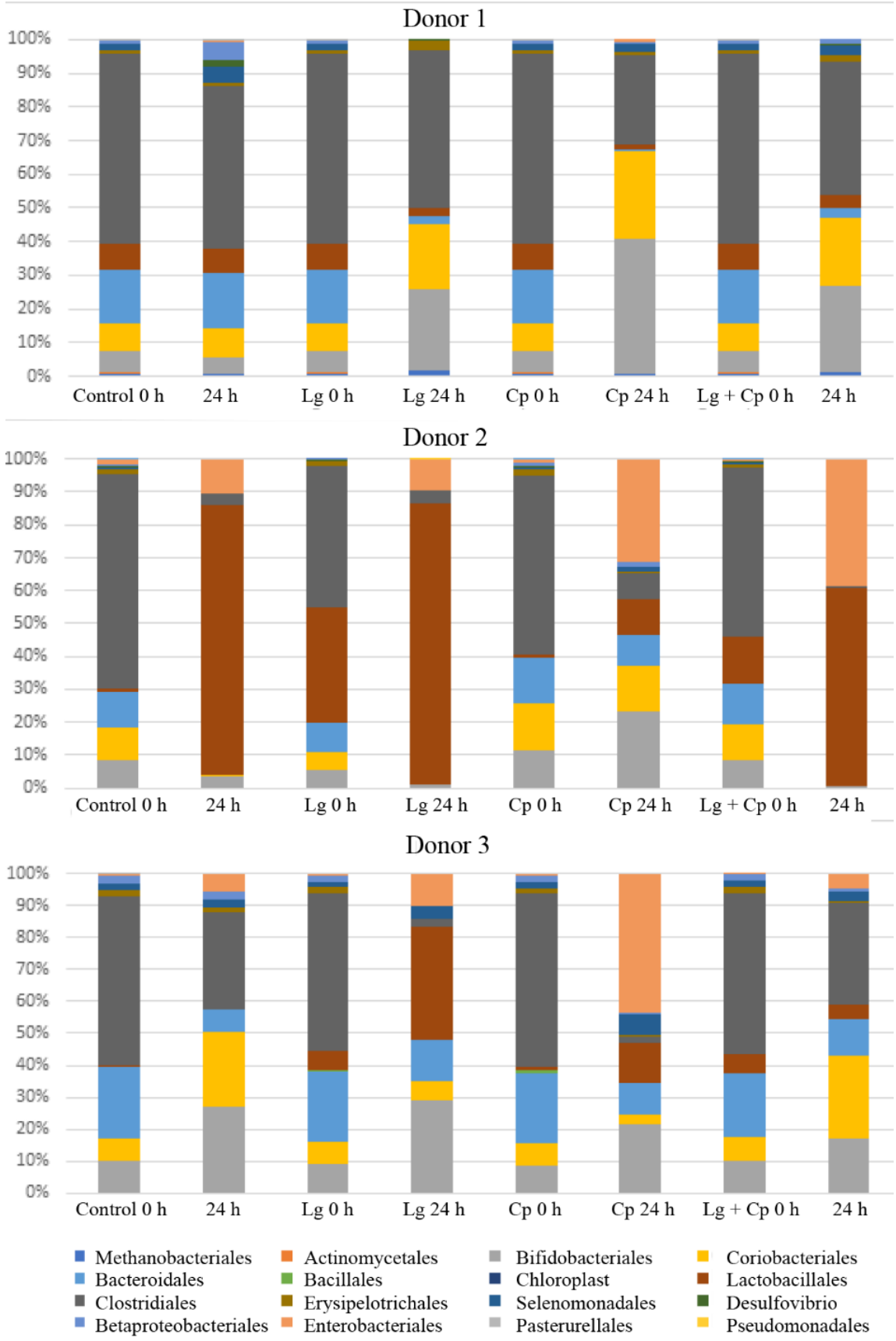


Figure 4.10. Representation of relative abundance at order level in the faecal batch model fermentation for the three donors.

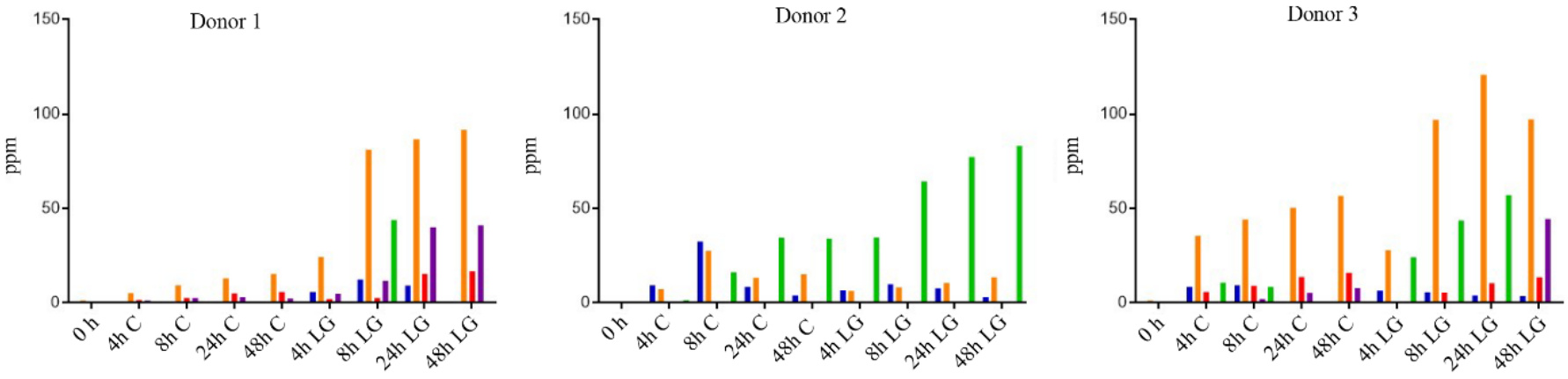


Figure 4.11. Production of SCFA in batch model faecal fermentation using inoculum from three different donors: blue, formate; orange, acetate; red, propionate; purple, butyrate; green, lactate.

4.4. Discussion

This work reports the ability of *L. gasseri* LM19 isolated from human breast milk microbiome to exhibit antagonistic activity against different enteropathogens via the production of previously identified bacteriocins as well as one novel gassericin M. From these investigations, it became apparent that different media and carbon sources influence the expression of these bacteriocin genes. Furthermore, we have confirmed the ability of *L. gasseri* LM19 to survive and express these antimicrobial genes in a complex faecal environment under simulated colon conditions. Additionally, other characteristics that are considered desirable, such as the increase in SCFA production in a faecal fermentation, were studied.

The presence of bacteria in human breast milk has been reported previously and the existence of a bacterial entero-mammary pathway has recently been proposed. [276]. These bacteria might have a gut origin and that could explain their ability to survive in GI tract conditions and exhibit antagonistic traits against other gut bacteria such as enteropathogens that might share the same environment. Gassericins are antimicrobial peptides that have been identified originally in *L. gasseri*. Several gassericins have been identified in sets of four, both two-peptide class II bacteriocins. K7 bacteriocins are a variant of acidocins LF221 and share similar sequences to GasT and its complementary peptide GatX, respectively, while GasE could be considered a variant of GasT. These two-component bacteriocins also show similarities with other two-component bacteriocins isolated from species previously grouped with *L. gasseri* [129]. *L. gasseri* LM19 also presents two clusters of bacteriocins that show homology to acidocin LF221A and Gas K7A on one hand and acidocin LF221B and Gas K7B on the other hand. But additionally, it can be observed the presence of structural genes corresponding to a new two-component bacteriocin that show a greater variation in sequence to previously described gassericins.

Partial purification of the products of these structural genes was conducted and it was observed the presence of masses matching the expected size in eluted fractions that exhibited antimicrobial activity. Particularly, masses predicted to match those of the new potential peptides GamM (4124 Da) and GamY (4105 Da) were associated with antimicrobial activity. Synthetic peptides confirmed the activity of GamM but GamY did not show activity or synergy with GamM. Synergistic activity was reported previously between

GasT and GatX and between the two components of gassericin S (here Bact_1 and Bact_2) respectively [266]. While Bact_1 and Bact_2 showed synergistic activity our GasT and GatX homologues, GamA and GamX, did not. However, it was observed a possible synergistic activity between GamA and Bact_1.

It was demonstrated that *L. gasseri* LM19 is able to survive in simulated colon conditions within a complex faecal microbiota. Moreover, it is capable of expressing the bacteriocin genes in this context. In previous work it was demonstrated that another probiotic *L. gasseri*, strain K7, able to produce both two-component bacteriocins K7 A, K7 A (cf), K7 B and K7 B (cf), was able to survive in faecal samples. Its bacteriocins were also targeted by using conventional PCR and RT-qPCR [271]. The authors noted that bacteriocin genes were detected by PCR from other LAB species. However, in these controls and treatments where *L. gasseri* LM19 was not present, no PCR products were detected.

L. gasseri LM19 showed mixed effects on a population of *C. perfringens* added to faecal fermentations of three different donors, causing a slight decrease in *C. perfringens* in only two out of three fermentations. This might indicate that the surrounding microbiota might play a synergistic or antagonistic role on the effect of *L. gasseri* LM19. However, it should be noted that in antimicrobial assays *C. perfringens* was only inhibited by *L. gasseri* LM19 cells, not cell-free supernatant, which might suggest that they should be in close proximity for an antimicrobial effect. However, co-inoculation of *L. gasseri* LM19 with *C. perfringens* did seem to alter the effect of *C. perfringens* on the background microbiota in all three donors, the profiles seen after addition of *L. gasseri* LM19 with *C. perfringens* were more similar to *L. gasseri* alone or control samples than to samples containing only *C. perfringens*.

Colon model fermentations also allowed to quantify production of formic, acetic, propionic and butyric acids using NMR. It was observed an increase in SCFA production in the faecal fermentation of the three donors. However, as with the microbial composition, the amount of each SCFA produced was very different from one donor to another, which might be related to production by other members of the microbiota that varied between the three donors. In a previous study of consumption of a beverage prepared with *L. gasseri* CP2305, the stools of the participants presented an increased level of SCFA too, while the microbiota experienced some alterations, including an increase in the presence of bacteria from Clostridium cluster IV, known for producing higher amounts of SCFA [277]. They could not conclude if the increase of SCFA was due to the effect of *L. gasseri* or due to the

proliferation of bacteria that produced more SCFA. SCFA production will also depend on diet and availability of nutrients in the gastrointestinal tract as well as the resident microbiota [278, 279].

This work shows the ability of *L. gasseri* LM19, a multi-bacteriocin breast milk isolate, to survive in colon conditions. Its ability to express different bacteriocin genes, including a novel gassericin M, under these conditions, makes it a candidate for further application studies.

CHAPTER V

Activity and Structural Characterisation of Nisin P

5.1. Introduction

Nisin is a small peptide with antimicrobial activity against a wide range of pathogenic bacteria. It was identified originally from a *Lactococcus lactis* subsp. *lactis* isolated from a dairy product [280] and classified as a class I bacteriocin because it is ribosomally synthesised and post-translationally modified [70]. Nisin has been studied extensively and its use has been extended to a wide range of applications in the food industry, biomedicine, veterinary and research fields [281-284]. It is approved as a food preservative by the Food and Agriculture Organisation and is Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration [282].

Structurally, nisin is considered a lantibiotic because it contains lanthionine (Lan), an unusual amino acid formed by two alanine residues linked by a sulphur atom through their β-carbon [282]. Other unusual amino acids present in nisin are dehydroalanine (Dha), dehydrobutyrine (Dhb) and β-methyl-lanthionine [282]. Nisin activity and stability are closely related to its structure and it can be altered by pH changes. When the pH rises, the activity decreases as a consequence of alterations in structure, and therefore, nisin is more stable at lower pHs. Nisin is heat-stable and also exhibits high stability at low temperatures, which makes it suitable for freeze-storage [285].

Nisin has nine natural variants reported at the moment (Table 5.1, Figure 5.1). When nisin A was purified, four forms were isolated, named A, B, C and D [286], with A the most abundant. Another nisin form, named nisin E, was isolated some time later [287]. Nisin Z was described as the first natural variant of nisin A. They differ in the presence of an asparagine amino acid in position 27 instead of a histidine residue [288]. This variation has, in comparison, very little effect on most of the properties of the two molecules, such as antimicrobial activity, thermal and pH stability, but affects the solubility of the molecule, with nisin Z being more soluble at neutral pH [289] . A study of its distribution also revealed that the nisin Z variant was more widespread than the A variant [289]. Nisin Q was another variant that was identified in an environmental *L. lactis* and it was different from the original nisin A in the presence of a valine, leucine, asparagine and another valine in positions instead of alanine, methionine, histidine and isoleucine respectively [290]. Nisin F, produced by *L. lactis* isolated from fish gut, also has asparagine and valine in positions 21 and 30 [291]. Nisin U and U2 were two new variants reported by Wirawan *et al* produced for the first time by a different species than *L. lactis* [292]; *Streptococcus uberis* strain 42

was isolated from bovine mammary secretions [293] and the nisin U and U2 variants that are produced were different to nisin A by nine and ten amino acids respectively. Nisin H, produced by *S. hyointestinalis*, was the first nisin variant isolated from a representative of a mammalian gastrointestinal (GI) tract (porcine) [61] and has five different amino acids in positions 1, 6, 18, 21 and 31. Recently, the nisin O operon has been reported, present in a *Blautia obeum* isolated from human gastrointestinal (GI) tract [92]. Unusually this operon has 4 structural genes, the first 3 being identical in the core peptide and have similarity to nisin U, and the fourth shows the highest variation compared to nisin A. Nisin P is predicted to be three amino acids shorter than nisin A in the C-terminus and differs to nisin A in ten amino acids (Figure 5.1). The Nisin P operon has been identified in the genomes of two *Streptococcus* species [294, 295] but its activity had not been reported before.

Streptococcus agalactiae DPC 7040 was isolated and identified from human faeces by Dr. James Hegarty at Teagasc Moorepark. Its genome was sequenced and assembled by Dr. Catriona Guinane and using BAGEL3 identified a nisin P cluster, although it was not curated. Following their work, I curated the nisin P cluster and, after purification performed by Paula O'Connor at Teagasc Moorepark, I performed an *in vitro* characterisation of its activity, in comparison with the activities of nisin A and nisin H. The first approach was related to its inhibitory activity towards other nisin producers (cross-immunity assays) and testing its spectrum of action. After that, I performed minimum inhibitory concentration assays (MIC) to determine its potency as an antimicrobial in comparison with nisin A, and a faecal fermentation experiment was conducted to identify their effect on the faecal microbiota numbers. Additionally, a brief regulation experiment was carried out to determine its ability to induce the nisin A operon.

Table 5.1. Summary of nisin variants reported in the literature

Nisin variant	First reported in	Reference
A	*L. lactis* subsp. *lactis*	[280]
Z	*L. lactis* strain NIZO 22186 (dairy product)	[288]
Q	*L. lactis* 61-14 (Japanese river water)	[290]
F	*L. lactis* F10 (freshwater catfish)	[291]
U and U2	*S. uberis* strain 42 (bovine mammary secretions)	[292]
P	*Streptococcus gallolyticus subsp. pasteurianus*	[294]
H	*S. hyointestinalis DPC 6484* (porcine intestine)	[61]
O	*Blautia obeum A2-162* (human GI tract)	[92]

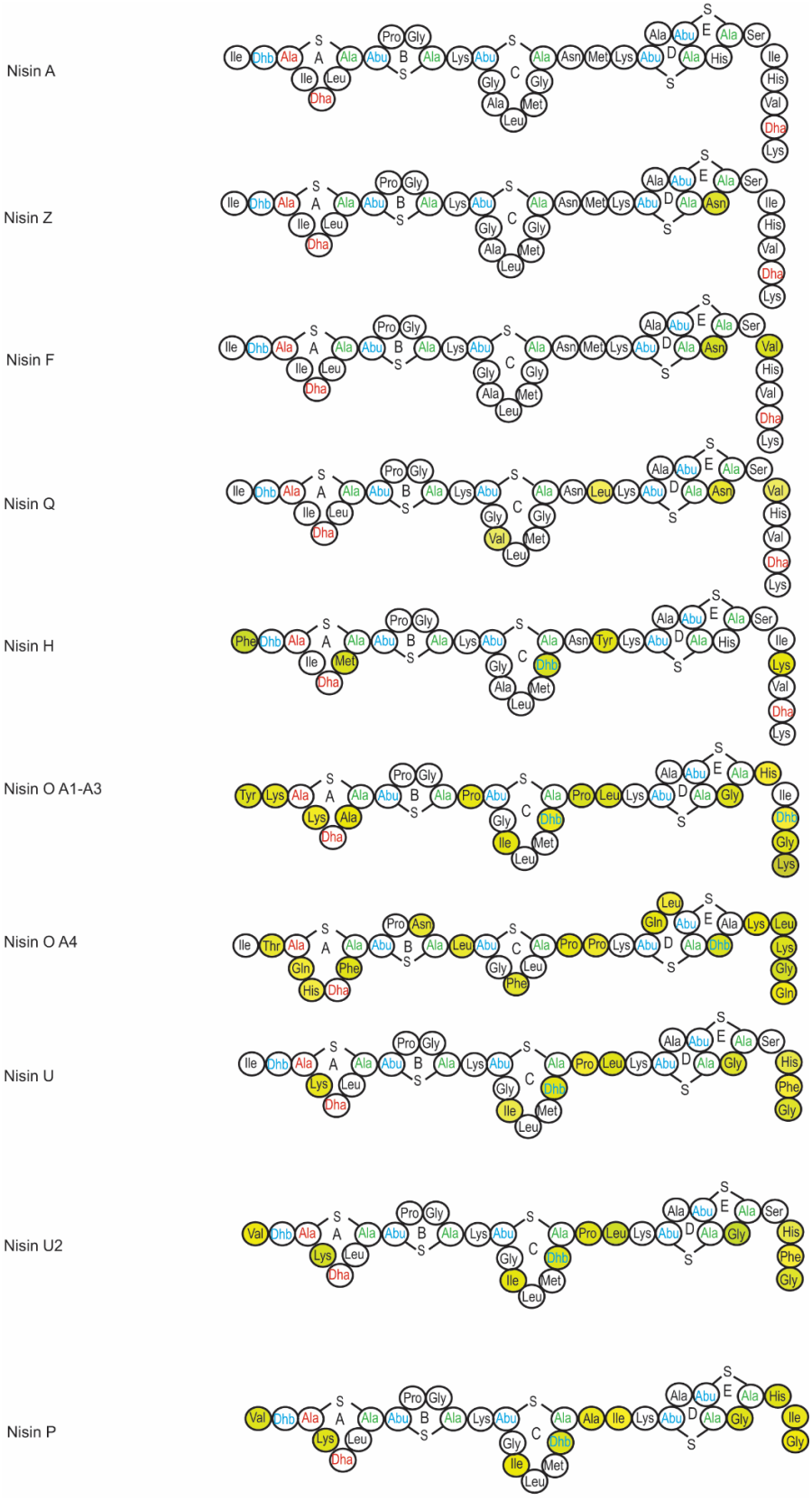


Figure 5.1. Predicted ring structures of nisin natural variants. Residues highlighted in yellow depict amino acid changes when compared to nisin A.

5.2. Methods

5.2.1. Optimisation of growth conditions for nisin P production

S. agalactiae DPC 7040 was grown under different conditions and the activity of the supernatant was tested for antimicrobial activity. It was grown for 24, 48 and 72 h in BHI, MRS and TSB, both in aerobic and anaerobic conditions and in presence of 500 ng/ml of nisin A for induction purposes. 1 ml of each culture was tested each day by well-diffusion assay using *L. bulgaricus* 5583 as an indicator strain. Antimicrobial activity was measured in activity units per ml ($AU*ml^{-1}$) [296, 297]. For this purpose, a serial 1:2 dilution was performed, and each fraction tested as stated.

5.2.2. Extraction and purification of nisin A, H and P

Nisin P, A and H were extracted and purified by Paula M. O'Connor at Teagasc Moorepark (Ireland). Nisin P production required initial induction with 500 ng/ml of nisin A which was included in all *S. agalactiae* DPC 7040 subcultures. Nisin P was purified from 2 litres of *S. agalactiae* DPC 7040 grown anaerobically in BHI for 48 h, (2.0×10^8 cfu/ml). Cell-free supernatant was obtained by centrifuging the culture at 8,000 x g, 10 min. Culture supernatant was applied to a 30 ml SP Sepharose column pre-equilibrated with 20 mM sodium acetate pH 4.4. The column was washed with 100 ml of 20 mM sodium acetate pH 4.4 and then eluted with 150 ml of 20 mM sodium phosphate buffer pH 7 containing 1M NaCl. The NaCl-containing eluate was applied to a 60 ml, 10 mg Strata–E C18 SPE column (Phenomenex, UK) pre-equilibrated with methanol and water. The column was washed with 60 ml 30% ethanol and antimicrobial activity eluted with 70% 2-propan-ol 0.1% TFA (IPA). The IPA sample was applied to a semi preparative Proteo Jupiter (10 x 250 mm, 90Å, 4µ) RP-HPLC column (Phenomenex, Cheshire, UK) running a 30-37% acetonitrile 0.1% TFA gradient where buffer A was 0.1% TFA and buffer B was 100% acetonitrile, 0.1% TFA. The eluate was monitored at 214 nm and fractions were collected at intervals. Nisin P containing fractions deemed pure by MALDI TOF mass spectrometry were lyophilised using a a Genevac lyophiliser (Suffolk, UK). Nisin H was purified from 2 l of culture (1.2×10^8 cfu/ml) grown overnight at 37 °C in TSB without induction and purified as per nisin P except a 25-40% acetonitrile 0.1%TFA gradient was used for HPLC purification. Nisin A was purified from nisin A supplied by Handary SA (Belgium) by reversed phase HPLC using a 25-45% acetonitrile 0.1% TFA gradient as described above.

Nisin P, H and A HPLC fractions containing antimicrobial activity against *L. bulgaricus* were analysed via MALDI-TOF MS to determine the molecular mass of the peptides and to assess their purity. HPLC fractions deemed pure by MALDI-TOF MS were combined and were lyophilised in a Genevac lyophiliser. MALDI-TOF mass spectrometry was performed with an Axima TOF2 MALDI-TOF mass spectrometer in positive-ion reflectron mode (Shimadzu Biotech, UK).

5.2.3. Nisin P structure

Purified nisin P was analysed for mass and structure confirmation by Dr. Gerhard Saalbach at the John Innes Centre (JIC) in Norwich. The intact peptide was analysed by LC-MS on a Synapt G2-Si mass spectrometer coupled to an Acquity UPLC system (Waters, UK). Aliquots of the sample were injected onto an Acquity UPLC® BEH C18 column, 1.7 µm, 1x100 mm (Waters, UK) and eluted with a gradient of 1-50% acetonitrile in 0.1% formic acid in 9 min and then ramped to 100% acetonitrile in 1 min at a flow rate of 0.08 ml min^{-1} with a column temperature of 45°C.

The LCMS was operated in positive MS-ToF resolution mode and with a capillary voltage of 3 kV and a cone voltage of 40 V in the range of m/z 100–2000. Leu-enkephalin peptide (1 ng ml−1, Waters) was infused at 10 µl min^{-1} as a lock mass and measured every 20 s. Masslynx 4.1 software (Waters, UK) was used to generate the spectra by combining several scans. The peaks were centred, and the mass calculated from the monoisotopic peak.

For structure confirmation, purified nisin P was digested with trypsin and analysed using nanoLC-MS/MS on an Orbitrap Fusion™ Tribrid™ Mass Spectrometer coupled to an UltiMate® 3000 RSLCnano LC system (Thermo Scientific, UK). The samples were loaded and trapped using a pre-column which was then switched in-line to the analytical column for separation. Peptides were separated on a nanoEase *m/z* column (HSS C18 T3, 100 Å, 1.8 µm; Waters, UK) using a gradient of acetonitrile at a flow rate of 0.25 µl min^{-1} with the following solvent steps A (water, 0.1% formic acid) and B (80% acetonitrile, 0.15 formic acid): 0-4 min 3% B (trap only); 4-15 min increase to 13% B; 15-77 min increase to 38% B;77-92 min increase to 55% B; followed by a ramp to 99% B and re-equilibration to 3% B.

Data dependent analysis was performed using parallel CID and HCD fragmentation with the following parameters: positive ion mode, orbitrap MS resolution = 60k, mass range (quadrupole) = 300-1800 m/z, MS2 top20 in ion trap, threshold 1.9e4, isolation window 1.6 Da, charge states 2-5, AGC target 1.9e4, max inject time 35 ms, dynamic exclusion 1 count,

15 s exclusion, exclusion mass window ±5 ppm. MS scans were saved in profile mode and MS2 scans were saved in centroid mode.

MaxQuant 1.6.2.3 was used to generate recalibrated peak lists, and the database search was performed with the merged HCD and CID peak lists using Mascot 2.4.1 (Matrixscience, UK). The search was performed, with a precursor tolerance of 6 ppm and a fragment tolerance of 0.6 Da, on a *S. agalactiae* protein sequence database, which included the nisin P gene cluster (Uniprot, January 2018, 2,123 sequences) to which the nisin P peptide sequence had been added. The enzyme was set to trypsin/P with a maximum of 2 allowed missed cleavages. Dehydration (−18 Da) of serine and threonine, oxidation of methionine and carbamido-methylation (CAM) of cysteine were set as variable modifications. The Mascot search results were imported into Scaffold 4.4.1.1 (www.proteomsoftware.com) using identification probabilities of 99% for proteins and 95% or 0% for peptides, as discussed in the results.

5.2.4. pH stability of nisin P compared to nisin H and A

Nisin P, A and H were resuspended at 1 µg/ml of distilled water. Duplicate samples at pH 3, 5, 6, 7 and 8 were prepared for each nisin. Antimicrobial activity was tested at 24, 48 and 72 h via well-diffusion assay by adding 50 µl of each solution to a well previously made in the agar containing *L. bulgaricus* 5583 as indicator and incubating the plates overnight at 37 ^{o}C anaerobically.

5.2.5. Cross- immunity assays

Cross immunity assays were conducted by well diffusion assay to test the activity of the purified nisin P and nisin A against natural nisin A, P, H and U producers. For that purpose, 100 µl of an overnight inoculum of each indicator strain was added to 20 ml of melted agar media. 50 µl of pure peptides at 0.20 mg/ml were added to a well pre-formed in the agar and incubated overnight according to the growth requirements of the different nisin producers used as indicator strains. Growth of the indicator strain in the presence of peptide was taken as confirmation of immunity. Blastp analysis was performed on the proteins responsible of the immunity system, NisF, functioning as an ATPase, and NisE and NisG, the membrane spanning domain of the transporter, of natural producers of nisin A, P, H and U.

5.2.6. Spectrum of inhibitory activity

Well diffusion assays were performed to determine the inhibitory targets of nisin P in comparison with nisin A. 50 µl of the purified peptides at a concentration of 0.2 mg/ml were added to wells preformed in agar inoculated with the different indicator strains. Inoculation consisted of adding 100 µl of an overnight culture of the indicator strain to 20 ml of melted agar and incubating overnight under the requirements of the indicator strain. Inhibitory activity was determined by the absence of growth around the well.

5.2.7. Activity induction

Experiments were performed using *L. lactis* NZ9000 pNZ8150 gfp+, where gfp acts as a reporter of expression from a nisin inducible promoter [298]. An overnight culture of this strain was diluted 1:100 into fresh media and incubated until the OD600 reached 0.5. Purified nisin P, A and H were added to 1 ml of GM17 at a concentration of 1 mg/ml, 10 µg/ml, 1 µg/ml, 500, 100, 20, 10, 5, 2 and 1 ng/ml and a further 20 µl of the L. lactis NZ9000 pNZ8150 gfp+ were added to the mix. 200 µL were transferred to black 96-well plates (Nunc^{TM}, ThermoFischer, UK) and GFP was detected using a Synergy HT Microplate reader (BioTek, UK). The excitation filter was set at 485 nm and the emission filter at 528 nm.

5.2.8. MicroMatrix fermentations

5.2.8.1. Preparation of a faecal standard

A faecal standard for fermentation in a micro-Matrix was prepared following methodology described by O'Donnell *et al* [299]. The samples of six healthy donors with no antibiotic treatment in the previous six months were collected and maintained at 4^{o}C for 1-2 hours before being transferred to an anaerobic chamber (5% CO_2, 10% H_2 and 85% N_2) for processing. 200 g of faeces in total were placed in a stomacher bag with 200 ml of 50 mM phosphate buffer containing 0.05% (w/v) cysteine hydrochloride. After filtering and homogenizing, the faecal slurry was centrifuged (4,000 x g, 25 min, Sorvall 3000 centrifuge) and re-suspended in 50 mM phosphate buffer. Sterile glycerol was added to the mix to a final concentration of 25% and aliquots were stored at -80^{o}C until required.

5.2.8.2. MicroMatrix fermentation

Faecal standards were defrosted at 37^{o}C before use and prepared at 10% concentration in faecal medium as described by Fooks and Gibson [300]. 2 ml of the mix were added to each well of a MicroMatrix 24-well cassette (Applikon ® Biotechnology, Netherlands). Purified

nisin A, P and H were added to the wells to a final concentration of 15 and 50 µM. Fermentations took place for 24 h at 37 °C, with pH of the medium adjusted and maintained to 6.8 in a MicroMatrix fermenter (Applikon ® Biotechnology). At time point 8 h, half of the wells received an additional dose of nisin A, P and H in the same concentration as at time 0 h. 500 µl aliquots were withdrawn from each well at time points 0, 8, 24 h (T0, T8, T24) and kept frozen for further analysis.

5.2.8.3. qPCR

200 µL of aliquots from each Micro-Matrix fermentation were taken for DNA extraction using QIAmp Fast DNA Stool Mini Kit (Qiagen, Crawley, UK), following the manufacturer's instructions. To compare total cell numbers with live cells, ethidium bromide monoazide (EMA) treatment was performed by adding EMA to the sample at a concentration of 10 µg/ml, incubating the samples in darkness for 5 min and exposing them to 70-Watt HQI light for 10 min at 20 cm while keeping the samples on ice [301, 302]. Samples were then centrifuged at 12,000 g for 5 min and the supernatant discarded before proceeding with the standard DNA extraction with QIAmp Fast DNA Stool Mini Kit. Absolute quantification by qPCR was performed to determine bacterial numbers, using the Roche LightCycler 480 II platform. To quantify 16S bacterial counts, a standard curve was created using 10^{10} to 100 copies of 16S rDNA/µL. For amplification, 16S rRNA primers used were CO1 forward primer (5'-AGTTTGATCCTGGCTCAG-3'), and CO2 reverse primer (5'-TACCTTGTTACGACT-3') [211] and KAPA Lightcycler 480 mix (KAPA Biosystems Ltd., UK) according to manufacturer's instructions. All samples were run in triplicate. qPCR conditions are summarised in section 2.2.8.3.

5.3. Results

5.3.1. Characterisation of the nisin P cluster from *S. agalactiae* DPC 7040

The prototypical nisin A gene cluster consists of the structural gene *nisA*, followed by *nisBCIPRKFEG* [303]. In contrast the nisin P gene cluster in *S. agalactiae* DPC7040 has a similar gene order but *nipPRKFEG* genes have translocated to the front of the *nipA* structural, a phenomenon which also occurs in nisin U [229] (Figure 5.2). This was observed when the nisin P gene cluster was first reported [295].

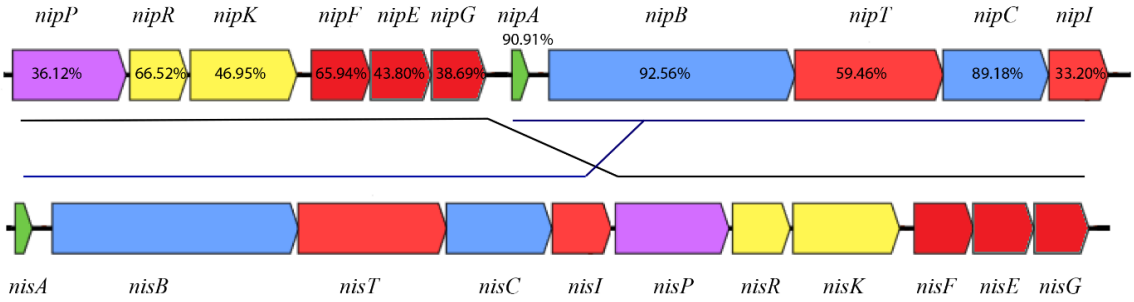


Figure 5.2. Representation of the bacteriocin-encoding nisin P gene cluster compared to the nisin A operon. Figures indicate percentage of similarity with nisin A homologues.

5.3.2. Extraction and purification of nisin P

Nisin P and nisin H are produced by Streptococci that require complex media (BHI and MRS respectively) for growth and bacteriocin production. Nisin P production by *S. agalactiae* DPC7040 also required initial induction by nisin A, taking advantage of the inducing activity of nisin peptides [304]. The highest antimicrobial activity was achieved when *S. agalactiae* DPC7040 was cultured in BHI broth under anaerobic conditions for 48 h and induced with 500 ng/ml of nisin A (Figure 5.3). Purification was achieved by a four-step process using Amberlite XAD16N, SP Sepharose, C18 SPE and Reversed phase HPLC. Overall yield was very low, i.e., 0.2-0.35 mg/l for nisin H and 0.1-0.2 mg/l for nisin P (Figure 5.4), while the yield for nisin A production was 3-4 mg/l. The lower yield obtained for nisin H and P could be partially due to less efficient purification, but it is most likely due to lower production of bacteriocin due to lower cell numbers, typically in the range of 10^8 for *S. hyointestinalis* and *S. agalactiae* compared to 10^9 cfu/ml for the nisin A producer, *L. lactis* NZ9000.

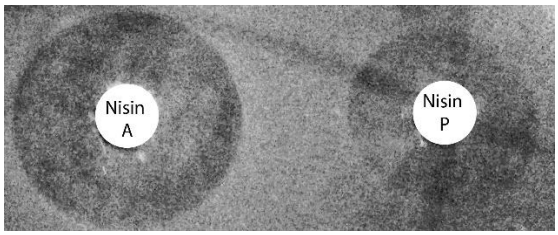


Figure 5.3. Inhibitory areas of nisin A and nisin P against indicator strain *L. bulgaricus* DPC5583.

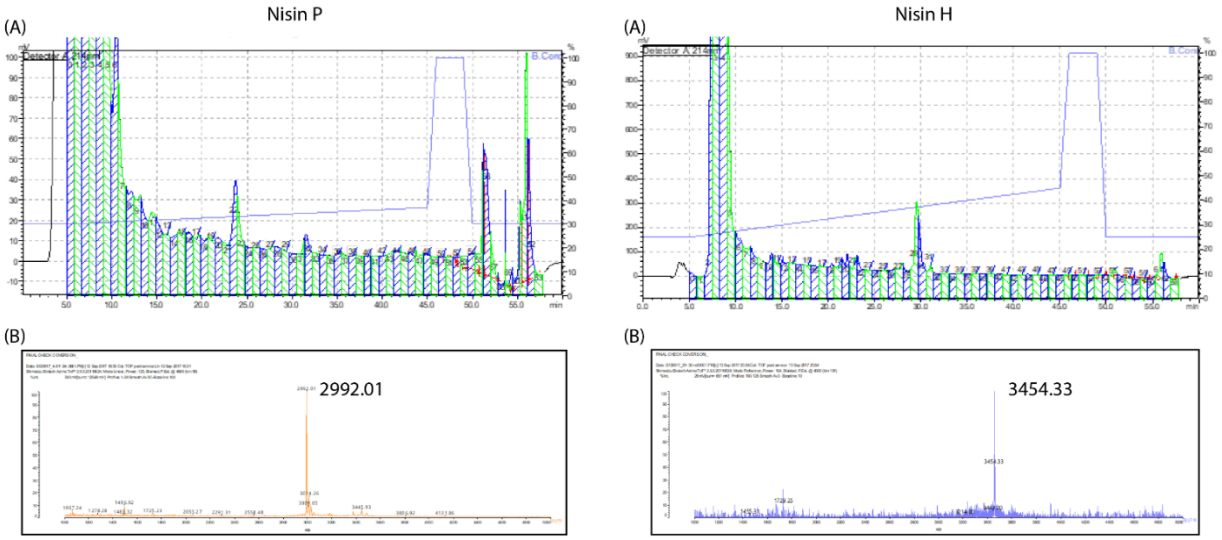


Figure 5.4. Purification of nisin P from *S. agalactiae* DPC7040 and nisin H from *S. hyointestinalis* DPC6484 cultures. For nisin P: (A) RP-HPLC chromatogram and (B) MALDI TOF MS of the active fraction number 24. Expected mass for nisin P is 2991 Da. For nisin H: (A) Fractionation of cell samples eluted from the Jupiter Proteo column; (B) MS detail of fraction 30 showing mass corresponding to the expected nisin H mass of 3453 Da.

5.3.3. Prediction of nisin P structure

5.3.3.1. Intact peptide

The purified nisin P peptide was tested by Dr. Gerhard Salbaach (John Innes Centre) to confirm that the expected peptide was intact (Figure 5.5A). The extracted-ion chromatogram (XIC) shows the recovered nisin P at 9.56 min (Figure 5.5B). The base peak intensity chromatogram shows that some impurities were found (12.55 min), but presence of intact nisin P was confirmed (Figure 5.5C).

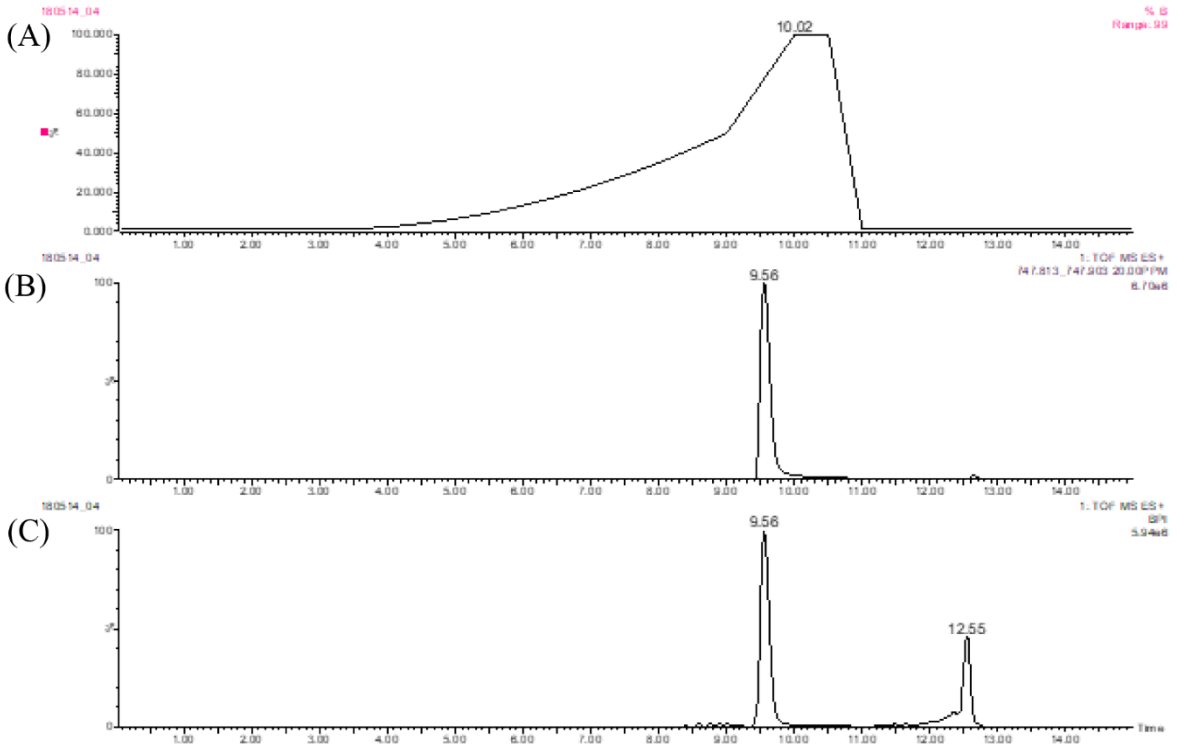
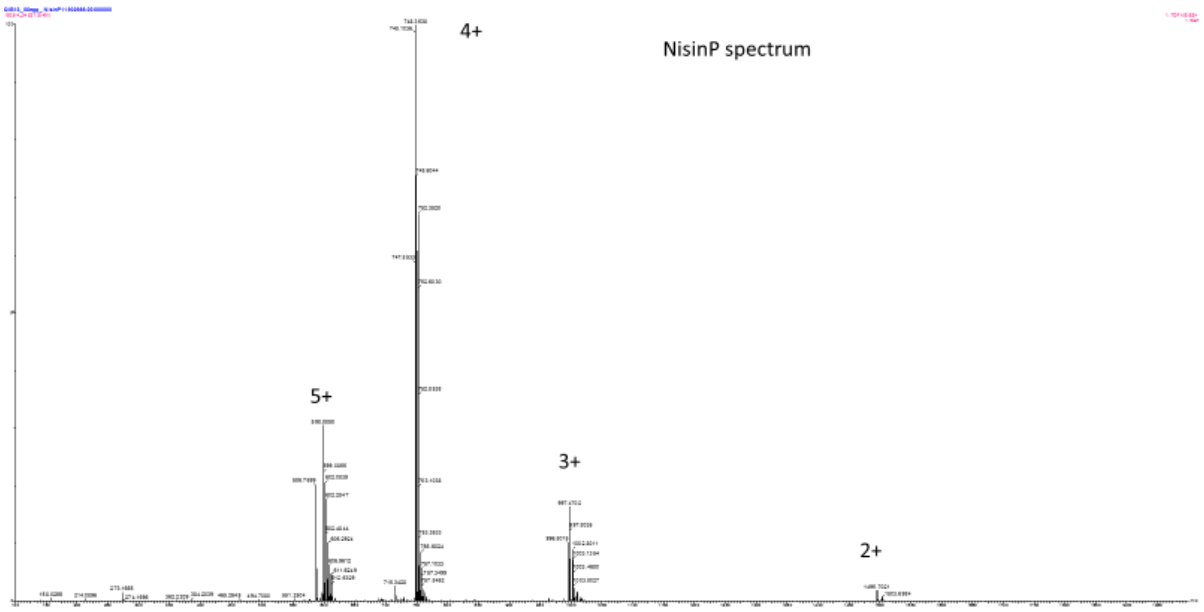


Figure 5.5. Spectra originated from LC-MS analysis of purified nisin P. (A) Elution gradient of 1-50% acetonitrile in 0.1% formic acid in 9 min and to 100% acetonitrile in 1 min (B) Extracted-ion chromatogram (XIC) of nisin P. (C) Base peak intensity chromatogram (BPI) for nisin P.

Possible modifications were further assessed by the analysis of the eluted samples, that showed a series of charge states ranging from 5+ to 2+ (Figure 5.6A). The most abundant isotope was the one showing a charge state of 4+. A more detailed analysis of this isotope pattern showed a *m/z* value of 2987.3841, that matched the expected dehydrated mass of nisin P (*m/z* 2987.3922) (Figure 5.6B). The mass calculation is performed by multiplying per 4 the 747.8533 ion mass and removing four charges of protons (747.8533*4) - (4*1.007276) = 2987.3841. Expected mass of dehydrated (-8 H_2O) is 2987.3922 Da. Additional peaks have an addition of 4 m/z which is 16 Da = oxidation. Addition of 18 Da (H_2O) (or multiples) cannot be seen, meaning that the major part of nisin P is completely dehydrated.

(A)



(B)

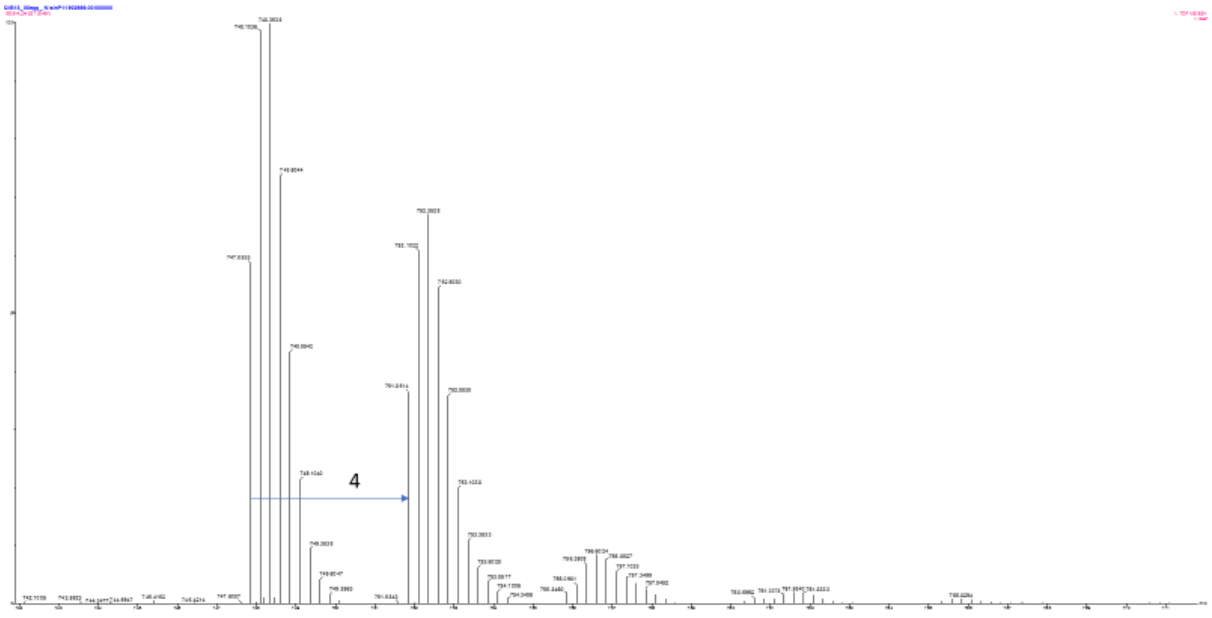


Figure 5.6 (A) Raw spectrum of series of charge states from 5+ to 2+. (B) Spectrum detail of charge state 4+, confirming expected mass of purified nisin P. The mass calculation is performed by multiplying per 4 the 747.8533 ion mass and removing four charges of protons (747.8533*4) - (4*1.007276) = 2987.3841.

5.3.3.2. Presence of lanthionine rings and final confirmation of structure

After trypsin digestion, 7 exclusive unique peptides with 30 exclusive unique spectra, matching the sequence of Nisin P with 100% probability were detected (Table 5.2). Those spectra cover 31 of the 31 amino acid residues of Nisin P corresponding to 100% sequence coverage. The presence or absence of rings can be determined by the suppression of the MS2 fragmentation within the regions that correspond to those rings. Two types of modifications were observed: dehydrations and carbamido-methylation. Dehydrations (-18) were present in S and T residues (Fig. 5.7). Dehydrations on the T residue have been reported only in some nisin variants [61, 93]. Carbamido-methylations (CAM, C+57) were observed in modified cysteine residues and are an indicator of the absence of a lanthionine ring, while unmodified cysteines indicate participation in ring formation. The N-terminal peptide VTSKSLCTPGCK and the C-terminal peptides TGILMTCAIKTATCGCHFG and TATCGCHF showed CAM modifications in approximately 50% of the 342, 144 and 91 peptide spectra matches (PSMs) obtained by trypsin digestion with a probability of 100%. Another peptide, VTSKSLCTPGCKTGILMTCAIK and the full peptide, showed two modifications, with a low probability, in the same residues as VTSKSLCTPGCK. This indicates the absence of ring B in approximately 50% of the peptides. The presence of CAM modifications in the C-terminus in peptides TGILMTCAIKTATCGCHFG and TATCGCHFG indicates the absence of ring E in approximately 50% of the peptides. Based on this observation, the structure of nisin P was confirmed (Figure 5.8).

Table 5.2. List of peptides obtained by digestion with trypsin and LC-MS/MS analysis.

Domain	Tryptic peptide	m/z (1+)	MC	PSMs	CAM	Highest probability (%)
N-term	VTSKSLCTPGCK	1,207.58	1	342	145	100
N-term	VTSKSLCTPGCKTGILMTCAIK	2,162.09	2	6	2	7
Core	TGILMTCAIK	1,029.54	0	37	0	-
Core	SLSLCTPGCKTGILMTCAIK	1,820.91	1	4	0	-
Full	VTSKSLCTPGCKTGILMTCAIKTATCGCHFG	3,003.39	3	17	1	23
C-term	TGILMTCAIKTATCGCHFG	1,984.88	1	144	70	100
C-term	TATCGCHFG	916.3329	0	91	52	100

MC: Missed cleavage. PSMs: Peptide Spectra Match.

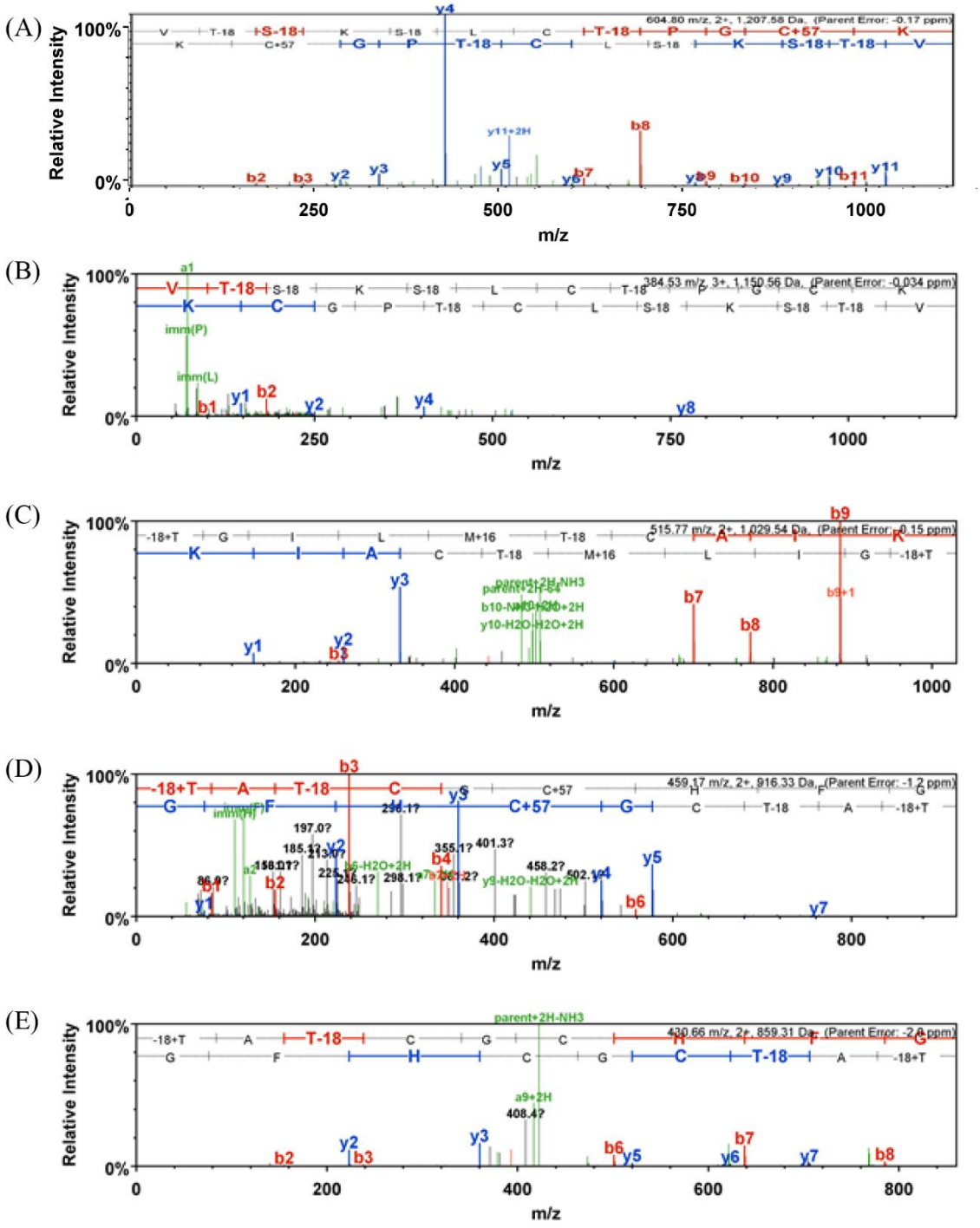


Figure 5.7. MS/MS spectra for nisin P peptides obtained by trypsinisation and generated in Scaffold. b- ions are represented in red and y- ions in blue. (A) Spectrum of peptide showing presence of CAM modification (C+57) on one C residue indicating ring B is not present but ring A is. (B) Spectrum showing no CAM modification in ring B region. (C) detail of presence of ring C and hinge region, AIK (D) Presence of ring D and CAM modification indicating the absence of ring E. (E) Unmodified cysteine indicating ring E is present.

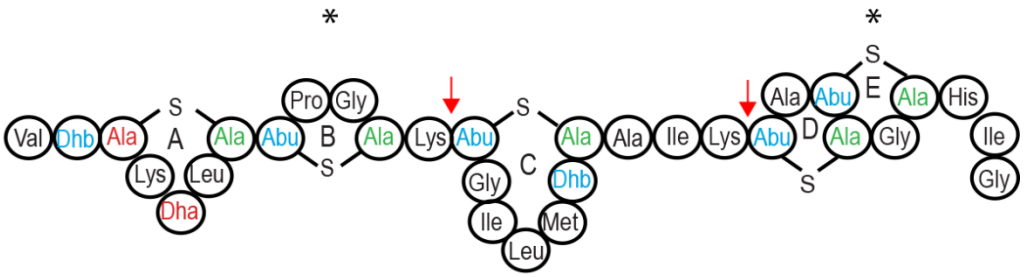


Figure 5.8. Location of the sites in nisin P cleaved by trypsin to form the different peptides summarised in table 5.2. * indicates that the ring is not always formed.

5.3.4. Stability

The antimicrobial activities of nisin A, P and H stored at pH 3, 5, 6, 7, and 8 for 24, 48 and 72 h were compared by well-diffusion assay against *L. bulgaricus* 5583 (Figure 5.9). Overall, nisin A antimicrobial activity was more stable than both P and H to 72 h and at acidic pH. Nisin P was the least stable as it lost most inhibitory activity against *L. bulgaricus* 5583 after 24 h and was inactive at 72 h at acidic pH though it retained some activity at pHs 7 and 8. While Nisin H activity was less stable than nisin A it was more stable than nisin P as it retained more activity at 24 h, showing an optimum at pH 5, also at 48 and 72 h, and it showed some activity at 72 h at pHs 5, 6, 7 and 8.

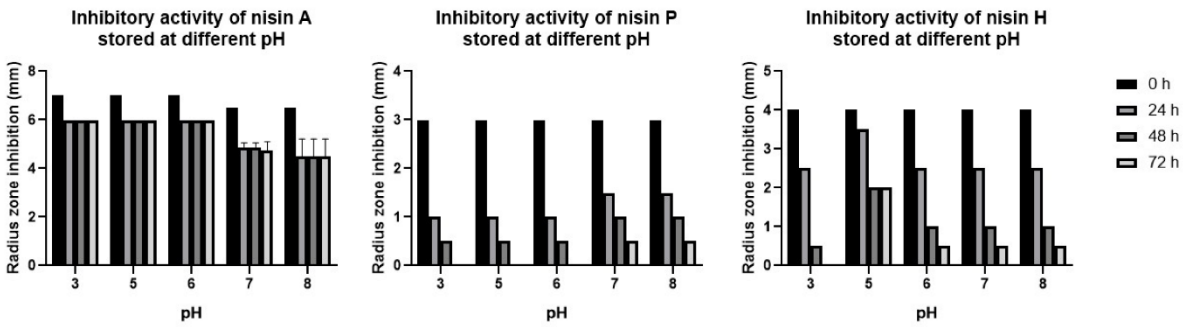


Figure 5.9. Stability of nisin A, P and H stored at different pH for 24, 48 and 72 h measured by radius (mm) of inhibitory activity against *L. bulgaricus* 5583 using well-diffusion assay. Results are the mean of triplicate measurements +/- standard deviation.

5.3.5. Cross- immunity assays

Purified nisin P at 0.20 mg/ml had inhibitory activity against natural nisin A and U but not against *S. hyointestinalis* (nisin H producer, Table 5.3). *S. agalactiae* DPC 7040 required a lower concentration of nisin A to be inhibited (0.022 µM) than *S. uberis* strain 42 (0.4 µM) and *L. lactis* NZ9800 (26µM). Nisin P inhibited *L. lactis* NZ9800 growth at 8.5 µM and *S. uberis* strain 42 at 4.25 µM. Nisin P inhibited nisin P producer *S. agalactiae* DPC7040 at 17

µM. Nisin clusters encoding nisin A, U and P have two systems involved in immunity, one through the immunity protein nisI and the other one through the ABC transporter system nisFEG. However, the cluster for nisin H does not have a *nisI* gene [61]. For this reason, a Blastp analysis was performed on the other system nisFEG. The analysis showed that, despite that these proteins are similar in nisin A, P and U, nisF and nisG from the nisin H cluster have very low percentage of similarity to them. Thus, nisF from nisin A, P and U only had 13.8%, 14.1% and 15.4% similarity to nisF from nisin H. NisG from nisin A, P and U had 9%, 8.9% and 9.8% similarity to nisG from nisin H. NshE and NisE showed 48.3% and 43.8% identity to NipE, while NsuE shared 79.8% identity.

Table 5.3. Summary of inhibitory activity of nisin P and A against different natural nisin producers

Strain	Activity nisin P	Activity nisin A	MIC nisin P	MIC nisin A
L. lactis NZ9800 (nisin A)	++	+	8.5 µM	26 µM
S. uberis strain 42 (nisin U)	++	+++	4.25 µM	0.4 µM
S. hyointestinalis DPC6484 (nisin H)	-	-	-	-
S. agalactiae DPC 7040 (nisin P)	+	+++	17 µM	0.022 µM

5.3.6. Spectrum of inhibitory activity and MIC

In general, purified nisin A was more potent and had wider spectrum of inhibition than nisin P (Table 5.4). Nisin P inhibited all lactobacilli and staphylococci strains assayed but inhibition was weaker compared to nisin A, which was able also to inhibit the growth of *Listeria* and enterococci. This is consistent with the spectrum of action of other nisins, that have targeted lactobacilli and staphylococci and in general Gram positive [305].

Table 5.4. Summary of inhibitory activity of nisin P and A

Strain	Activity nisin P	Activity nisin A	MICs nisin P	MICs nisin A
S. enterica LT2	-	-	-	-
E. coli ATCC 25922	-	-	-	-
C. sakazakii NCTC 11467	-	-	-	-
C. perfringens NCTC 3110[a]	-	-	-	-
L. innocua NCTC 11288	-	-	-	-
L. bulgaricus 5583	+++	+++	0.01 µM	0.0005 µM
L. monocytogenes (3564)	-	++	-	-
E. faecium (4955)	++	+++	0.265 µM	0.1 µM
E. faecalis (LM1)	-	++	-	6.5 µM
E. faecalis (LM4)	-	++	-	6.5 µM
P. aeruginosa (2064 APC)	-	-	-	-
S. aureus (7016) mastitis	++	+++	0.531 µM	0.05 µM
S. capitis (20G)	++	+++	8.5 µM	0.812 µM
S. simulans (2B10)	++	+++	0.531 µM	0.325 µM
S. epidermidis (99)	+	+++	34 µM	0.812 µM
L. gallinarum (43)	+	++	8.5 µM	0.812 µM
L. frumenti (53)	+++	+++	1.06 µM	0.27 µM
L. reuteri (54)	+	++	8.5 µM	0.812 µM
L. paracasei (R1)	++	+++	17 µM	1.62 µM
L. plantarum (Lac 29)	+++	+++	1.06 µM	0.1 µM
L. mucosae (28)	+++	+++	1.06 µM	0.54 µM
L. reuteri (56)	+	++	69 µM	3.25 µM

[a] *C. perfringens* NCTC 3110 has usually been reported to be sensitive to nisin A

5.3.7. Activity induction

Nisin P, A and H, have different concentration ranges for activating the nisin promotor (Table 5.5). The promoter was more sensitive to nisin A (1 ng/ml – 1 µg/ml) than nisin H (10 ng/ml – 1 µg/ml) and nisin P (100 ng/ml – 10 µg/ml). Higher concentrations of nisin P were required to activate the promotor, but its ability to induce *gfp*+ also worked at higher concentrations (10 µg/ml) than nisin A and H. The maximum concentration that nisin A and H were capable of inducing *gfp*+ was 1 µg/ml. The highest fluorescence using nisin A was

detected at a concentration of 500 ng/ml, after increasing with the concentration. However, at 1 µg/ml the fluorescence was lower than at 100 ng/ml. This weakening in fluorescence at high levels of concentration was not shown by nisin P nor nisin H induction.

Table 5.5. Fluorescence levels of nisin induction of *L. lactis* NZ9000 pNZ8150 *gfp*+ by nisin P, A and H.

	1 mg/ml	10 ug/ml	1 ug/ml	500 ng/ml	100 ng/ml	50 ng/ml	20 ng/ml	10 ng/ml	5 ng/ml	2 ng/ml	1 ng/ml
Nisin P	-	1758.33 ± 41.66	658.33 ± 8.33	316.66 ± 33.33	75 ± 8.333	-	-	-	-	-	-
Nisin A	-	-	1408.33 ± 25	1916.5 ± 83.5	1458.33 ± 25	1050 ± 0	549.99 ± 16.66	358.33 ± 8.33	224.99± 8.33	116.66 ± 16.66	83.33± 16.66
Nisin H	-	-	1566.66 ± 33.33	1133.33 ± 66.66	608.33 ± 41.66	358.33 ± 8.33	208.33 ± 8.33	108.33 ± 25	-	-	-

5.3.8. Nisin activity in faecal fermentation

The analysis of total dead and live bacteria in faecal samples after fermentation in the presence of two concentrations (15 µM and 50 µM) of the different nisins showed different profiles depending on nisin type and treatment at 8 and 24 h (Fig 5.10). There were significant differences in the total numbers of DNA from live cells between the control and the treatments with nisin A, P and H both at 8 and 24 h. Nisin A treatments had the biggest effect on bacterial numbers. The effect of the 15 µM and 50 µM concentrations of nisin A, both with and without reinforcement dose, was also significant at 8 h and at 24 h. At 24 h, nisin A treatments that had received a reinforcement dose at 8 h showed the least amount of DNA copies of live cells. The nisin H treatment showed less inhibitory activity than nisin A, and there were significant differences between 15 µM and 50 µM concentrations at 8 h, but not at 24 h. All treatments showed cell numbers between Log_{10} 6-7. Nisin P showed the least difference between total DNA and DNA from live cells and also showed the highest total DNA numbers, with all the treatments with cell numbers between Log_{10} 7-8. The effect of the 15 µM and 50 µM concentrations of nisin P was not significant at 8 h nor at 24 h, and neither with the reinforcement doses. The 50 µM concentration showed significant higher numbers of DNA than the 15 µM concentration in nisin A treatment at 8 h and 24 h with the reinforcement dose, and in nisin H treatment at 8 h. It is worth noting that the no nisin controls showed lower numbers of rDNA copies and a larger difference between live and dead cells than nisin P and H treatments.

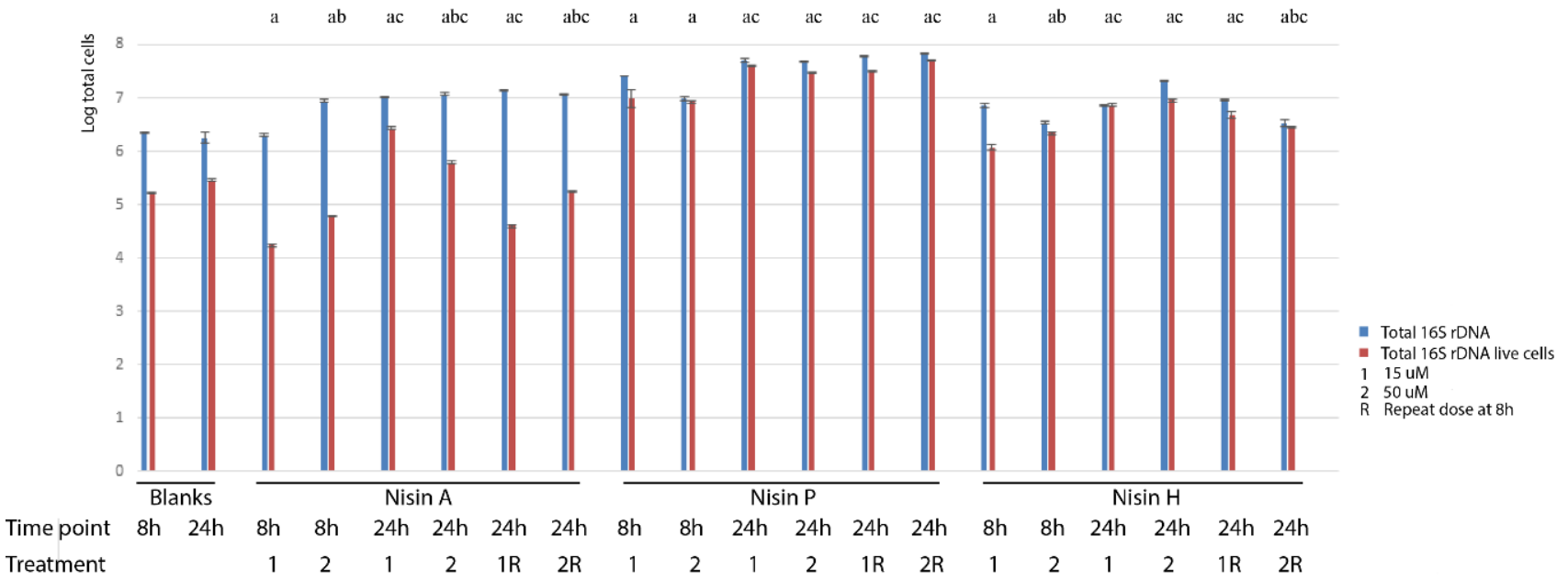


Figure 5.10. Total bacteria after nisin A, P and H treatments measured by 16S rDNA qPCR after faecal fermentation at 8 and 24 h. Two concentrations of peptide were tested, 15 µM and 50 µM. Half of the wells received a reinforcement dose of the same concentration at 8 h. Results are the mean of duplicate measurements +/- standard deviation. Statistics was conducted with live cells data: a, indicates significant difference between the treatment and the blank at 8 or 24 h; b, indicates significant difference between 15 µM and 50 µM concentrations; c, indicates significant difference between 8 and 24 h within the same nisin treatment.

5.4. Discussion

This work is the first report of production and purification of nisin P, a natural variant of nisin, originally identified in the genomes of other two Streptococci species by genome mining [294, 295]. Genes related to nisin P were identified previously by phylogenetical categorization of LanB and LanC enzymes in *Streptococcus pasteurianus* and posterior analysis of its *lanA* gene suggested the ability of this strain to produce an analogue of nisin U that was designated nisin P [294]. The gene cluster was later reported on *Streptococcus suis* [295]. The faecal isolate *S. agalactiae* DPC 7040 produced nisin P after initial induction with nisin A.

Nisin A has been considered the lantibiotic archetype for decades. It is the best known bacteriocin and it has been used extensively as a food preservative for more than half century. Its positive attributes and efficacy make it suitable scaffold for further antimicrobial development. Indeed, bioengineering has enabled the modification of the nisin A molecule to increase its bioactivity, specificity, stability and / or its range of action by targeted amino acid modifications for over 25 years [74, 90, 174, 285, 298, 306-309]. However, nature developed its own nisin variation system and new nisin variants are continuously being discovered. These variants have been found in a number of representatives from different environments, from dairy products (nisin A) to human (nisin O, P) animal (nisin H) and fish (nisin F) intestines and river water (nisin Q). We could hypothesize that the wide dissemination of the clusters encoding this molecule could be linked to its ability to provide a competitive advantage and increased fitness to the organisms that possess them, even among the different nisin variants.

Cross-immunity assays indicated that nisin P was able to inhibit the growth of the nisin A and nisin U producers, suggesting that their immunity systems are different and cannot protect them from the effect of the nisin P. *S. agalactiae* DPC7040 was also very sensitive to nisin A effect. Nisin P and nisin A inhibited the growth of the nisin U producer, *S. uberis* strain 42. Although this has been reported previously [61], initially *S. uberis* strain 42 was reported as cross-resistant to nisin A effect, and it was hypothesised that this effect was due to the effect of the different nisin immunity systems, NisI and NisFEG [292]. Nisin A and nisin P were not able to inhibit the growth of *S. hyalointestinalis* DPC6484, the nisin H producer, something that was observed previously with nisin A [61]. *S. hyalointestinalis* DPC6484 does not have one of the two systems involved in lantibiotic immunity, nisI. NisI

has been reported to have a more crucial role in immunity than the NisFEG, the ABC transporter system, which was considered to provide around 20% of the wildtype immunity [310-312]. However, another study highlighted that *nisI* expression provided a very low level of immunity (1-4%) [313]. The low levels of similarity in NisFEG of *S. hyalointestinalis* DPC6484 to the ones from the nisin A, P and U producers, suggest that this system might have a more important role in lantibiotic immunity than previously suspected.

When nisin P activity was compared to other nisin variants activities, A and H, a lower inhibitory effect was observed. Nisin P showed a higher MIC against a panel of food and gut isolates. It was expected that a bacteriocin produced by a human gut isolate would be more effective against other gut isolates due to potential environmental specificity. However, in this case, nisin A exhibited higher inhibitory activity than nisin P against these tested species. This was observed in the faecal fermentations too, where samples treated with nisin P showed a rise in total cell numbers and higher numbers of viable cells, while samples treated with nisin A and H showed lower numbers of live cells, with nisin A being the treatment that showed the lowest numbers of live cells.

These differences in bioactivity might be explained by the structural differences between molecules. Thus, the nisin A molecule is characterised for having five rings containing lanthionine or methyl-lanthionine residues, in addition to three dehydro amino acid residues, Dhb2, Dha5 and Dha33. During the large number of studies conducted to assess the relationship between structure and activity, it was highlighted that some rings play a key role in the inhibitory activity of nisin A. Some examples include the observation that the hydrolytic cleavage of Dha33 had a very little biological effect, but the cleavage of Dha5 led to a substantial loss of activity [314, 315]. However, it was not clear if the loss of activity was due to the loss of Dha5 or due to the conformational change of losing the ring A. For this reason, Dha5 was replaced with an alanine, resulting in no effect in inhibitory activity but substantially decreased its inhibitory activity of spore outgrowth [315]. Cleavage or removal of ring C abolished fully nisin activity, indicating its importance. However, the hydrolytic cleavage of the last five amino acids in the C-terminal part of the molecule only showed a 10 fold drop in growth inhibition activity against *L. lactis*, mainly attributed to the Val residue [314]. Nisin P is three amino acids shorter than nisin A in the C-term, which might me linked to a certain decrease in its antimicrobial activity. Further removal of amino acids in the C-terminal region, this time affecting rings D and E, showed a decrease of up to

100 fold of its inhibitory activity against *L. lactis* and 50 fold against *M. luteus*, suggesting that different residues or conformations might have a different outcome against different bacteria [314]. It was possible to establish a N-terminal part of the nisin A molecule, that includes rings A, B and C, responsible for the binding of nisin to the membrane surface and / or its oligomerization. A more cationic C-terminal was thought initially to be involved in pore formation, but mode of action studies helped to rule out this idea, pointing that is was likely to work only by the interference with lipid II and cell wall biosynthesis [306, 316]. Ring B is known for being very sensitive in substitution studies and the replacement with large amino acids was highly detrimental for the activity, probably due to the inability of the cysteine thiol at position 11 to cyclize onto the Dhb present in position 8 [316]. In summary, the removal of the ring B constituted a loss of activity in the nisin mutants [316].

Another important region involved in bioactivity in lantibiotics, and therefore, the nisin molecule, is the hinge region, in positions 21, 22 and 23 [317, 318]. The hinge region is constituted by the three amino acids (NMK in case of nisin A) that separate the N-terminal and C-terminal domains and are responsible of the reorientation after the binding with lipid II to penetrate into the target cell membrane [319]. The hinge region has also been linked to lantibiotic immunity and resistance. Thus, modifications to extend the hinge region showed that immunity and resistance proteins were not able to recognize it. However, the insertion into the cell membrane and its ability to form pores was malfunctioning [319]. Its importance in nisin antimicrobial activity has also been previously established through bioengineering studies [318-321]. These studies suggested a preference for small, chiral amino acids in that region [320]. Nisin H has one change, an aromatic polar tyrosine (Y) instead of the non-polar methionine in the second position. Nisin P has two changes in the hinge region, in comparison with nisin A, in positions 21 and 22. In position 1 there is an exchange of a polar asparagine (N) for a non-polar alanine, with a small methyl side chain, and in position 22 there is an exchange of methinonine with an isoleucine (I), both non-polar amino acids. The third position remains conserved in all known nisin variants, including the most recent nisin O, that along with nisin U, U2 and P, are the four natural nisin variants that have two substitutions in the hinge region, in positions 21 and 22. However, the introduction of serine (S) in position 21 and the naturally conserved position 23 of the hinge region, has been proved to enhanced bioactivity [321]. In the same study, directed amino acid changes in positions 21 and 22 showed that presence of alanine in

position 21 reduced bioactivity against Methicillin resistant *Staphylococcus aureus*, ST528, *S. aureus* DPC5245 and *S. agalactiae* ATCC13813 to half of the reference bioactivity from nisin A. However, molecules carrying an isoleucine in position 21 showed an enhanced bioactivity against the three microorganisms in comparison with nisin A natural hinge region [321]. Despite this, no studies with positions 21 and 22 of the hinge region carrying alanine and isoleucine residues were performed previously. Hence, although small peptide substitution, like alanine, would appear to benefit bioactivity, and the presence of isoleucine in the second position was reported to increase bioactivity, the combination of both modifications does not seem to present an enhanced bioactivity, in comparison with nisin A, against our indicator strains.

In the case of the structure of nisin P, analysis of trypsin digested and carbamidomethylated peptides established that, while the ring C was always present, rings B and E were only present partially, around 50 % in each case. This could explain the lower activity when it is compared with nisin A. On the other hand, nisin P might receive a benefit from the presence of a lysine residue in position 4 instead an isoleucine. Previous studies have shown that the presence of an additional positive charge in the ring A has a positive effect of its activity [316]. Further studies are required to determine if there are indeed instances where nisin P benefits by virtue of these changes.

In addition to a lower activity, nisin A promoter seems to require higher concentrations of nisin P in comparison to nisin H and A to induce its production. However, this might be due to certain level of specificity of the molecule or the receptor. Given the structural differences between the two branches of nisin variants, Lactococci and Streptococci origin, it could be interesting to investigate if using nisin P or U as inducers and using cognate receptors would give the same levels of induction requirements.

The effect of the three different nisin variants (P, A and H) over the microbiota in a fermentation of faecal material was studied using rDNA copy numbers of the 16S gene. Although bacteria can have multiple copies of rDNA [322], we have used the amplification of this gene as a reference to measure the effect of the different treatments. It is possible to observe a trend where the number of rDNA copies is bigger in nisin P than in nisin H treatments, and bigger in nisin H than in nisin A treatments, suggesting that the potency of each one individually correlates with the potency for general treatments in the faecal fermentation.

The study of both bioengineered and natural variants of nisin aids the understanding of structure-activity relationships of this molecule and that knowledge can be applied to other lantibiotics and to the further development of more efficient antimicrobials. Future investigation of nisin P modification and production in mixed communities might help to establish further knowledge on whether it would be a good candidate as antimicrobial agent in certain environments.

CHAPTER VI

Characterisation of *S. epidermidis* of Gut Origin

6.1. Introduction

S. epidermidis is a member of the coagulase-negative staphylococci (CoNS) and is considered a commensal, being frequently found as a component of the human microbiome. *S. epidermidis* has also been identified as a nosocomial pathogen responsible for antibiotic resistant infections, and has been isolated from catheters, bloodstream, prosthetic joints, ocular and mastitis infections [323]. This bacterium would appear to be highly adaptive, having also been associated with other hosts, including sheep, rodents and plants [324, 325]. Despite this, there is a pointed lack of comparative and evolutionary studies of ecologically diverse *S. epidermidis* isolates [324]. Within the human body, the GI tract is one of the primary locations where *S. epidermidis* can be found [323]. Furthermore, a recent study has identified a variety of gut pathogens, including *S. epidermidis*, in bloodstream infections, suggesting the GI tract as a source of those infections [326]. This highlights the importance of identifying traits that could reveal the origin *S. epidermidis* infection for accurate tracking and treatment of an infection, as well as potentially revealing opportunities for disease prevention. However, public databases do not contain complete genomes of *S. epidermidis* isolates of gut / stool origin and there is relatively little known about distinct features that *S. epidermidis* of this origin may possess.

There are many factors which influence the composition and stability of microbes in the gut including pH, anaerobiosis, water activity, nutrients, and the presence and concentration of compounds such as bile acids (BA). It has been noted that bacteria adapted to live in the mammalian gut have developed strategies to overcome the antimicrobial activity of BAs, such as efflux systems, bile salt hydrolase (BSH) activity or remodelling of the cell wall [12]. Another important feature is the ability to resist other antimicrobial compounds of host and bacterial origin [327]. These compounds can have non-specific activity, like hydrogen peroxide or short chain fatty acids (SCFA), or target specific microbes in the ecosystem, such as some antimicrobial peptides (AMPs) [328]. In addition to needing to survive exposure to such antimicrobials, many gut microbes are producers of these compounds in order to gain a competitive advantage with respect to niche colonisation. Indeed, *S. epidermidis* is able to produce a delta lysin and an antimicrobial $^{\delta}$-toxin called phenol-soluble modulin-γ [329].

Some lineages of *S. epidermidis* can cause infections, a phenomenon which has been associated with an increased density of antimicrobial resistance and virulence-related genes in the genomes of those disease-causing lineages [330]. Such *S. epidermidis* have been shown to cause pathological changes in the kidney and liver of rats and mice, among other organs [323]. Other features associated with the colonising ability of *S. epidermidis* include lipase activity, as this species is found mainly in lipid-rich regions (gut, skin, milk, etc) and biofilm formation. Indeed, biofilm formation is considered one of the most important traits in *S. epidermidis* colonisation, with a number of genes involved in the molecular mechanisms of the different stages of biofilm formation, such as adhesins involved in initial attachment, polysaccharide intercellular adhesin (PIA) matrix components involved in accumulation, teichoic acids in maturation and proteases and modulins in detachment [331, 332]. Taken together, there is evidence of traits that could reflect the ability of different strains of *S. epidermidis* to colonise different parts of the human body and suggest that pan-genome studies, such as that which highlighted formate dehydrogenase as a potential clinical biomarker of pathogenesis in *S. epidermidis* [333], have the potential to be of great value.

Here it is described the study of the pan-genome of *S. epidermidis*, including three new isolates of gastrointestinal origin, with a view to identifying niche specific traits. The existence of traits associated with lifestyle adaptation to the human gut, such as growth in anaerobic conditions and the presence of bile acids (BA), antimicrobial relationships with representatives of the enterobacteria, and potential pathogenic traits, like biofilm formation, were also investigated.

6.2. Methods

6.2.1. Genomic analyses

6.2.1.1. Genome Data Collection

All available *S. epidermidis* genomes were downloaded from the RefSeq genome database on 04/07/18 and metadata was downloaded from PATRIC database on 05/02/2019. To have a consistent annotation, these genomes were annotated using Prokka (v. 1.11) and pan-genome-wide associations were performed using Roary [334] and Scoary [335] with default parameters. Probability was calculated using Benjamini and Hochberg with default parameters. The tree was built using PhyloPhlAn (v. 0.99) [336] and visualised using GraPhlAn [337]. PlasmidFinder 2.0 [338] was used to identify potential plasmids.

6.2.1.2. Genome-Phenotype association

A pan-genome of the four strains for which both genome sequence and OmniLog® growth patterns were available was created using Roary and all identified genes were associated with growth on individual substrates using Scoary. An area under the curve (AUC) cutoff of 500 was chosen to denote growth on a particular substrate. If the AUC in that well was less than 500, it was considered that the strain did not grow on that substrate. Only substrates on which at least one of the strains could grow are plotted. The list of remaining substrates was filtered to only those where there were differential growth patterns between strains (i.e., substrates on which either, none or all strains could grow were deemed uninformative and removed). MetaCyc was used for further confirmation of the results obtained [339].

6.2.2. Bile acids

6.2.2.1. Sample preparation

Skin isolates of *S. epidermidis* DSM 20042, DSM 28764 and faecal isolates 9^c and F530B (Chapter III) were grown in 20 ml of suitable media supplemented with porcine bile (Sigma, UK) up to a final concentration of 0.3% v/v. Cultures were set in triplicate. 1 ml of each culture was collected at 24 and 48 h and kept at -20^{o}C for further analysis. Solid phase extraction (SPE) clean-up was performed in the samples using Waters Oasis Prime HLB 1 30 mg SPE cartridges in a SPE vacuum system and washed with 1 ml of 5% methanol. Elution was performed with 500 µl 100% methanol using the same procedure and 25 µl of internal standards for each bile acid (Steraloids, USA) were added. The final volume was transferred to low volume autosampler tubes for LC-MS analysis.

6.2.2.2. LC-MS

Clean extracts were analysed using HPLC – mass spectrometry operated in multiple reaction monitoring (MRM) mode by Dr. Mark Philo (QIB). Each sample (5 µl) was analysed using an Agilent 1260 binary HPLC coupled to an AB Sciex 4000 QTrap triple quadrupole mass spectrometer. HPLC was achieved using a binary gradient of solvent A (water + 5mM ammonium acetate + 0.012% formic acid) and solvent B (methanol + 5mM ammonium acetate + 0.012% formic acid) at a constant flow rate of 600 µl/min. Separation was made using a Supelco Ascentis Express C18 150 x 4.6, 2.7 µm column maintained at 40°C. Injection was performed at 50% B and held for 2 min, ramped to 95% B at 20 min and held until 24 min. The column equilibrated to initial conditions for 5 min. The mass spectrometer was

operated in electrospray negative mode with capillary voltage of -4500 V at 550°C. Instrument specific gas flow rates were 25 ml/min curtain gas, GS1: 40 ml/min and GS2: 50 ml/min. Quantification was applied using Analyst 1.6.2 software to integrate detected peak areas relative to the deuterated internal standards.

6.2.2.3. Growth curves

Growth behaviour of *S. epidermidis* DSM 20042, DSM 28764, 9^c and F530B was monitored aerobically and anaerobically. 20 ml of BHI was inoculated with 1% of each strain with or without 0.3% porcine bile and triplicate 300 µl aliquots were transferred to a honeycomb® sterile plate (Thermo Fisher Scientific) for Bioscreen C (Labsystems Oy) for measuring aerobic growth and to a 96 well plate for Infinite F50 (Tecan Group, Switzerland) for anaerobic conditions, using Bioscreener and Gen5 Data Analysis as software respectively. Growth rate (µ) was calculated using the formula $\mu = 2.303 (\log OD_2 - \log OD_1) / t_2 - t_1$ [340] where OD_2 is the value that doubles OD_1, and t_2 and t_1 the time at those two measurements.

6.2.4. Phenotypic analyses

6.2.4.1. Antimicrobial activity

Antimicrobial activity was measured as described in section 2.1.5.

6.2.4.2. Adhesion and biofilm measurements

Adhesion and biofilm formation abilities were conducted as described in section 2.2.

6.2.4.3. Biolog analyses

BIOLOG assays were performed as described in section 2.1.9. Additional strains used were bovine mastitis isolate *S. epidermidis* DPC6010 and cheese isolate DPC6293.

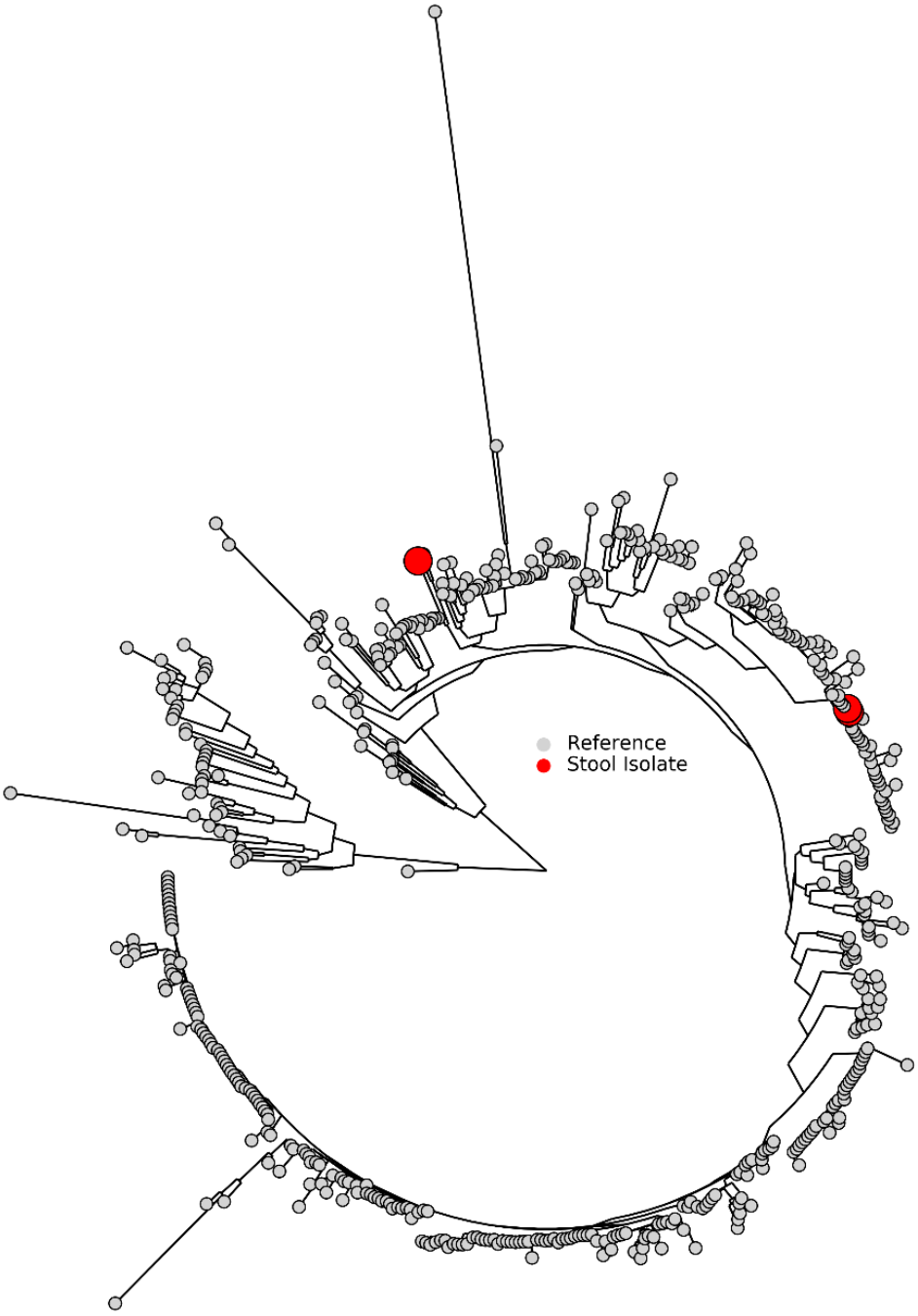
6.3. Results

6.3.1. *S. epidermidis* pan-genome

The *S. epidermidis* pan-genome was constructed by Dr. Calum Walsh (Teagasc) and Dr. Lizbeth Sayavedra (QIB) and contained 16,399 total genes across 581 genomes: 1,036 core genes present in ≥ 99% of genomes, 419 soft core genes present in 95-99% of genomes, 1,419 shell genes present in 15-95% of genomes, and 13,525 cloud genes present in less than 15% of genomes. To visualise results, two phylogenetic trees were constructed (Figure 6.1 and B). The three stool isolates did not cluster together (Figure 6.1A). In the same way, cattle isolates did not cluster. However, rice and murine isolates did cluster (Figure 6.1B).

The genomes from the different body sites isolates did not cluster either, suggesting no specialisation.

(A)



(B)

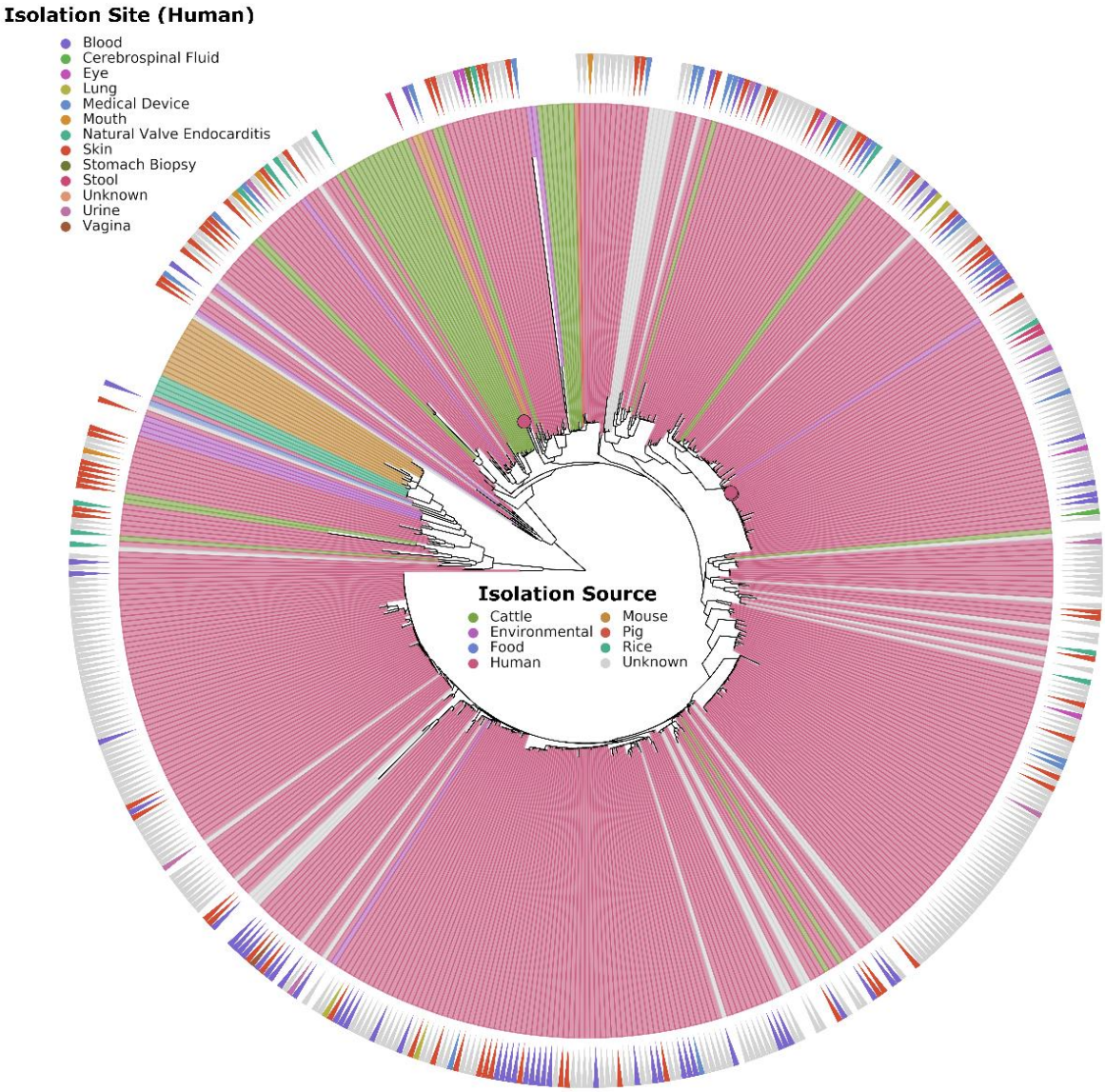


Figure 6.1(A) Presence of *S. epidermidis* isolated from stool in the pan-genome. (B) Different origins of *S. epidermidis* in the pan-genome. Coloured inner circle shows different isolation sources. Coloured outer circle shows different isolation sites within the human body.

Scoary identified 44 genes whose presence was significantly associated with being isolated from human stool (Table 6.1). 28 of these 44 ORFs (coloured in Table 6.1) were located on a 30 kb contiguous sequence which was identified as a probable plasmid by PlasmidFinder2.0, suggesting the likely acquisition of a horizontally transmitted genetic

element conferring the ability to survive in the human GI tract. Although not significantly associated, all three stool isolates contain a type V ACME gene cluster, a type of arginine catabolic module element (ACME).

Table 6.1. Genes significantly correlated with gut-associated *S. epidermidis* isolates

Gene	Significance*	Top Blastp match	Blastp Identity
group_464	0.01788	ISSag7, transposase OrfA [*S.epidermidis* VCU045]	100.00%
sdrG	0.04113	MSCRAMM family cell wall-anchored protein SesJ [*S. epidermidis*]	93.19%
group_4391	0.01151	lipopolysaccharide biosynthesis protein [*S. epidermidis*]	100.00%
group_12034	0.01506	MULTISPECIES: hypothetical protein [*Staphylococcus*]	100.00%
hsdR_1	0.01788	MULTISPECIES: type I restriction endonuclease subunit R [*Staphylococcus*]	99.80%
group_9501	0.01151	MULTISPECIES: restriction endonuclease subunit S [*Staphylococcus*]	100.00%
hsdM_1	0.01643	MULTISPECIES: SAM-dependent DNA methyltransferase [Staphylococcus]	100.00%
group_10501	0.01329	hypothetical protein SEVCU037_0543 [*S. epidermidis* VCU037]	100.00%
group_12033	0.01506	DUF1643 domain-containing protein [*S. epidermidis*]	100.00%
group_317	0.01643	tandem lipoprotein [*S. epidermidis* VCU045]	100.00%
group_6301	0.04732	MULTISPECIES: type III-A CRISPR-associated RAMP protein Csm5 [*Staphylococcus*]	100.00%
cas6	0.04732	MULTISPECIES: CRISPR-associated endoribonuclease Cas6 [*Staphylococcus*]	100.00%
group_10720	0.01329	metal-dependent transcriptional regulator [*S. epidermidis*]	100.00%
fecE_2	0.01329	MULTISPECIES: metal ABC transporter ATP-binding protein [*Staphylococcus*]	99.59%
mntB_2	0.01329	MULTISPECIES: metal ABC transporter permease [*Staphylococcus*]	100.00%
mntA_2	0.01329	MULTISPECIES: zinc ABC transporter substrate-binding protein [*Staphylococcus*]	100.00%
group_12447	0.01329	MULTISPECIES: recombinase family protein [*Staphylococcus*]	100.00%
group_13561	0.00155	MULTISPECIES: hypothetical protein [*Staphylococcus*]	99.85%
group_13560	0.00155	MULTISPECIES: hypothetical protein [*Staphylococcus*]	100.00%
group_13559	0.00110	MULTISPECIES: NERD domain-containing protein [*Staphylococcus*]	100.00%
group_13558	0.00110	hypothetical protein HMPREF2913_01140 [*Staphylococcus* sp. HMSC065A08]	94.74%
group_13154	0.01329	MULTISPECIES: poly(glycerol-phosphate) alpha-glucosyltransferase [*Staphylococcus*]	99.44%
sdrI_1	0.04732	YSIRK signal domain/LPXTG anchor domain surface protein [*S. epidermidis*]	97.94%
sdrI_2	9.49E-05	YSIRK-type signal peptide-containing protein [*S. epidermidis*]	87.93%
group_9826	0.01000	helix-turn-helix domain-containing protein [*S. aureus*]	85.71%
group_16636	9.49E-05	hypothetical protein HMPREF2566_04185 [*Staphylococcus* sp. HMSC070A07]	100.00%
group_16637	9.49E-05	No significant similarity found	
group_16638	9.49E-05	MULTISPECIES: hypothetical protein [*Staphylococcus*]	100.00%

group_16639	9.49E-05	MULTISPECIES: peptidase domain-containing ABC transporter [*Staphylococcus*]	100.00%
group_16640	9.49E-05	MULTISPECIES: DsbA family protein [*Staphylococcus*]	100.00%
group_10569	0.01643	hypothetical protein [*S. haemolyticus*]	97.92%
group_503	0.02693	MULTISPECIES: replication initiator protein A [*Staphylococcus*]	100.00%
racA	9.49E-05	MULTISPECIES: DUF536 domain-containing protein [*Staphylococcus*]	100.00%
group_588	0.00155	RepB family plasmid replication initiator protein [*Staphylococcus* sp. HMSC070A07]	100.00%
fas6	0.02047	MULTISPECIES: TIGR00730 family Rossman fold protein [*Staphylococcus*]	100.00%
group_11017	0.00110	MULTISPECIES: hypothetical protein [*Staphylococcus*]	100.00%
group_11121	0.00033	hypothetical protein [*Staphylococcus* sp. HMSC068G11]	100.00%
group_7970	0.00232	hypothetical protein [*Staphylococcus* sp. HMSC070A07]	99.33%
group_2880	0.00812	MULTISPECIES: DUF3139 domain-containing protein [Bacteria]	100.00%
group_4374	0.04113	MULTISPECIES: hypothetical protein [*Staphylococcus*]	100.00%
group_481	0.01788	MULTISPECIES: recombinase [*Staphylococcus*]	99.75%
group_9397	0.00110	MULTISPECIES: hypothetical protein [Bacilli]	100.00%
group_8755	0.00812	hypothetical protein B467_01449 [*S. epidermidis* M0881]	99.59%
group_12141	0.04732	MULTISPECIES: hypothetical protein [Bacilli]	100.00%

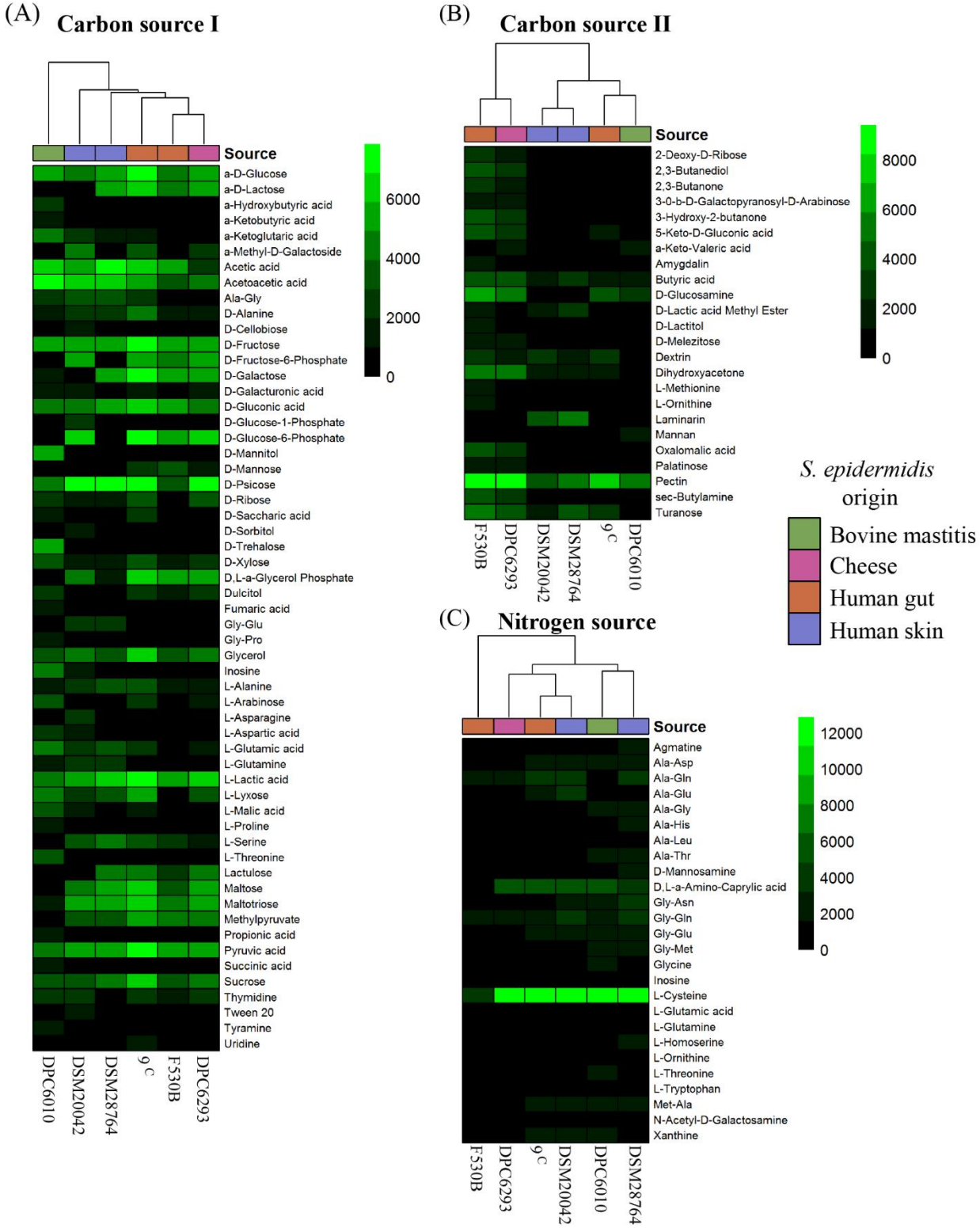
* Benjamini and Hochberg

6.3.2. Phenotype analysis using BIOLOG

Phenotype experiments conducted using BIOLOG technology showed differences in the utilisation of carbon and nitrogen substrates and antibiotic sensitivity between strains (Figure 6.2). The mastitis isolate *S. epidermidis* DPC6010 showed an ability to grow in a broader range of carbon sources than the other isolates. All isolates were able to grow, to differing extents, on α-D-glucose, acetic acid, acetoacetic acid, D-alanine, D-fructose, D-gluconic acid, D-psicose, D- xylose, glycerol, L-alanine, L-lactic acid, maltotriose, pyruvic acid, sucrose, butyric acid, pectin, Gly-Gln, and L-cysteine. The mastitis isolate was the only strain able to grow on α-hydroxybutyric acid, α-ketobutyric acid, D-mannitol, D-trehalose, fumaric acid, gly-pro, L-proline, L-threonine, tyramine, propionic acid, succinic acid and mannan. Skin isolates *S. epidermidis* DSM 20042 and DSM 28764 were the only isolates able to grow on gly-glu, and laminarin, and were the only strains not able to grow on dulcitol and D-glucosamine. The human stool isolates F530B and 9^c did not show a distinctive substrate growth pattern from the other isolates. Looking at nitrogen sources,

no isolates were able to grow in ala-leu, inosine, L-glutamic acid, L-glutamine, L- ornithine, L- tryptophan nor N-acetyl-D-galactosamine.

Antibiotic sensitivity only showed one differential pattern between skin and stool isolates in that the growth of the stool isolates was inhibited by lincomycin, but it did not fully inhibit the growth of skin isolates. The skin isolate DSM 28764 showed growth in the presence of all antibiotics tested.



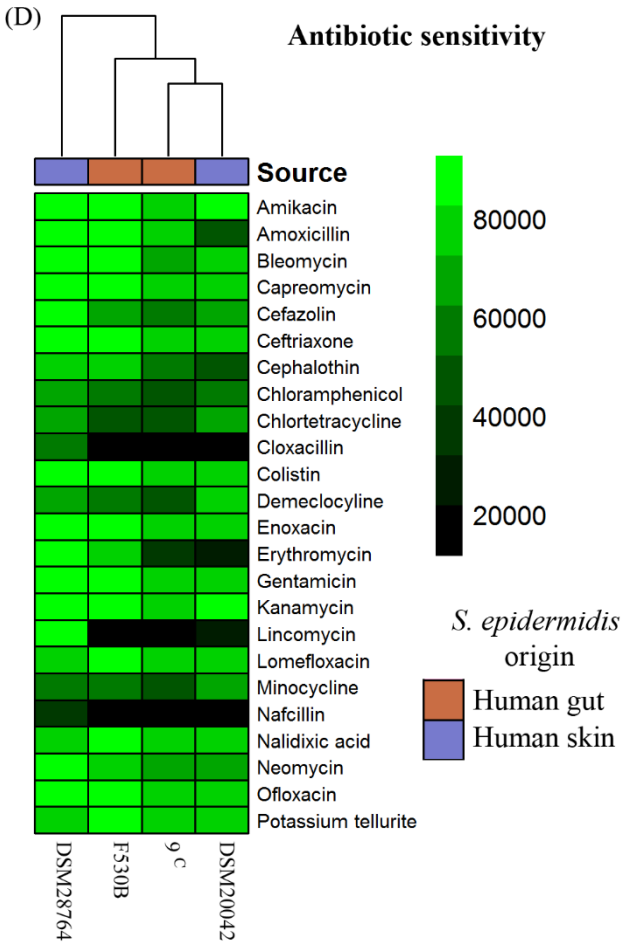


Figure 6.2. Omnilog results for growth of *S. epidermidis* stool (9^{c} and F530B), skin (DSM 20042 and DSM 28764), cheese DPC6293 and mastitis DPC 6010. (A) Carbon sources (I) PM1; (B) Carbon sources (II) PM2A; (C) Nitrogen sources PM3B; (D) Antibiotic PM11C. Colour represent modified Omnilog units after the cut-off.

6.3.3. Phenotype-genotype association

A genome-wide association study to link genomes of *S. epidermidis* 9^{c}, F530B, DSM 20042 and DSM 28764 with the results obtained from BIOLOG-based phenotype analysis provided a list of 7697 genes related to the 78 substrates that yielded different growth patterns between the strains. Table 6.2 shortlists the genes significantly associated with stool isolates from table 6.1 that were involved in the metabolism of the BIOLOG substrates. It can be observed that several genes are linked to the metabolism of the same substrates, such as *hsdR_1*, *hsdM_1*, *cas6*, *fecE_2*, *mntB_2*, *mntA_2*, *racA* and *fas6*, which have all been associated with the metabolism of dulcitol, oxalomalic acid, 2,3-butanone, 2-deoxy-D-ribose, 5-keto-D-Gluconic_acid, D-glucosamine and D-mannose. ISSag7 and the recombinase from group_481 have been linked with metabolism of the substrates

adenosine, D-melibiose, phenylethylamine, tyramine and uridine, but given the nature of these genes, it is likely that any such association is indirect. MetaCyc was used to establish a potential connection of the genes listed in table 6.3 with metabolic pathways, but none were found.

Table 6.2. Genes significantly different in stool isolates of *S. epidermidis* and their connection to the metabolism of BIOLOG substrates

Gene	Name	BIOLOG substrates
Group_464	ISSag7, transposase OrfA	Adenosine, D-melibiose, phenylethylamine, tyramine, uridine.
sdrG	Fibrinogen-binding protein	D-Glucose-6-Phosphate, D-Fructose-6-Phosphate
hsdR_1	Type-1 restriction endonuclease subunit R	Dulcitol, Oxalomalic acid, 2,3-Butanone, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Mannose
hsdM_1	Type I restriction enzyme EcoKI M protein	Dulcitol, Oxalomalic acid, 2,3-Butanone, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Glucosamine, D-Mannose
cas6	CRISPR-associated endoribonuclease Cas6	Dulcitol, Oxalomalic acid, 2,3-Butanone, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Glucosamine, D-Mannose
fecE_2	Metal ABC transporter ATP-binding protein	Dulcitol, Oxalomalic acid, 2,3-Butanone, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Glucosamine, D-Mannose
mntB_2	Metal ABC transporter permease	Dulcitol, Oxalomalic acid, 2,3-Butanone, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Glucosamine, D-Mannose
mntA_2	Zinc ABC transporter substrate-binding protein	Dulcitol, Oxalomalic acid, 2,3-Butanone, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Glucosamine, D-Mannose
racA	Chromosome-anchoring protein RacA	2,3-Butanone, Agmatine, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Cellobiose, D-Glucosamine, D-Glucose-1-Phosphate, D-Glucuronic acid, D-Mannose, D-Sorbitol, Dulcitol, Histamine, L-Alanine_PM03, L-Asparagine_PM01, L-Aspartic_acid_PM01, L-Ornithine_PM03, L-Threonine_PM01, L-Tryptophan,

		Mannan, Oxalomalic_acid, Propionic_acid, Uracil, Uric acid
fas6	Cytokinin riboside 5'-monophosphate phosphoribohydrolase	Dulcitol, Oxalomalic acid, 2,3-Butanone, 2-Deoxy-D-Ribose, 5-Keto-D-Gluconic_acid, D-Glucosamine, D-Mannose
Group_481	Recombinase	Adenosine, D-melibiose, phenylethylamine, tyramine, uridine.

6.3.4. Antimicrobial activity

6.3.4.1. Putative antimicrobial and phage-related traits

In addition to the antimicrobial-associated genomic traits identified by BAGEL3 in the stool isolates 9^c, F530B and 99 (section 3.3.5), further genome analysis identified other elements associated with potential antimicrobial activity. Several antibacterial proteins, i.e., phenol soluble modulins, were identified in the genomes of *S. epidermidis* 9^c, F530B, DSM 20042 and DSM 28764. Additionally, different phage elements were identified in both stool and skin isolates. The elements identified by BAGEL3 in stool isolates, i.e., sactipeptide and delta-lysin, were also identified in the skin isolates (Table 6.3).

Table 6.3. Putative antimicrobial traits in the genome of S*. epidermidis* isolates

		9^c	F530B	DSM 20042	DSM 28764
Phenol soluble modulin					
group_110	antibacterial protein (phenol soluble modulin)	√	√	√	√
group_2324	antibacterial protein (phenol soluble modulin)	√	√	√	√
group_2524	antibacterial protein (phenol soluble modulin)	√	√	√	√
group_2453	antibacterial protein (phenol soluble modulin)	√	√		√
group_1491	antibacterial protein (phenol soluble modulin)			√	
group_1492	antibacterial protein (phenol soluble modulin)			√	
group_2454	antibacterial protein (phenol soluble modulin)	√			
BAGEL 4 results					
	Delta-lysin	√	√	√	√
	Sactipeptide	√	√	√	√
Other potential antimicrobials in the genomes					
	Prophage phiRv2 integrase	√			√
	Phage portal protein SPP1 Gp6-like	√			√
	Phage Mu protein F like protein	√	√		
	Phage gp6-like head-tail connector protein	√		√	

	Phage head-tail joining protein	√			√
	Phage infection protein	√	√	√	√
	Phage integrase	√	√	√	√
	Phage protein		√	√	√
	Phage repressor				√
	Phage tail length tape-measure protein T	√			√
	Phage tail fiber protein	√			√
	Phage holin				√
	Phage N-acetylmuramoyl L-alanine amidase	√	√	√	√
	Phage lysin, glycosyl hydrolase, family 25	√	√	√	√
	Phage capsid and scaffold	√	√	√	√
	Phage terminase	√			√
	Prophage Clp protease-like protein	√			
	Phage Rha protein	√			
	DNA helicase phage-associated	√			
	Single-stranded_DNA-specific exonuclease RecJ Bacteriophage SPBc2-type				√

6.3.4.2. Antimicrobial activity

Antimicrobial assays showed different results for the tested isolates (Table 6.4). No inhibition zones were obtained from drop test, filter disc and well-diffusion assay for any isolate. However, overlay and cross streak assays, both involving direct cell contact between the indicator and the tested strain, showed different degrees of inhibitory activity. None of the strains inhibited *C. perfringens* NCTC 3110 or *L. bulgaricus* DPC 5583. On the other hand, all *S. epidermidis* strains inhibited the growth of *E. coli* ATCC 25922 by cross streak. *S. enterica* LT2 was inhibited, by cross streak assay, by all except isolate 99. According to BAGEL3 results in section 3.3.5 9^c, F530B, DSM 20042 and DSM 28764 contained a sactipeptide-like cluster that was absent from isolate 99, which may be responsible for this phenotypic difference. *M. luteus* was inhibited by all strains except by DSM 28764. *L. innocua* NCTC 112888 was inhibited by stool isolates 9^c and F530B, using the cross streak technique, but only by 9^c using the overlay assay. Only 9^c and F530B inhibited *M. luteus*, as determined by overlay assays, while F530B was the only strain to inhibit *C. sakazakii* NCTC 11467, as determined by cross streak.

Table 6.4. Summary of inhibitory activity of *S. epidermidis* using different techniques

	C. perfringens NCTC 3110		*S. enterica* LT2		*E. coli* ATCC 25922		*L. innocua* NCTC 112888		*M. luteus* MC8166		*C. sakazakii* NCTC 11467		*L. bulgaricus* DPC 5583	
Isolate	O	C	O	C	O	C	O	C	O	C	O	C	O	C
9^c	-	-	-	+	-	+	+	+	+	++	-	-	-	-
F530B	-	-	-	++	-	++	-	+	+	+	-	+	-	-
99	-	-	-	-	-	+	-	-	-	+	-	-	-	-
DSM 20042	-	-	-	++	-	+	-	-	-	+	-	-	-	-
DSM 28764	-	-	-	+	-	+	-	-	-	-	-	-	-	-

- -, No activity; +, 1 mm radius inhibition zone; ++, 1-5 mm radius inhibition zone; O, overlay assay; C, cross streak

6.3.5. *S. epidermidis* behaviour in presence of bile

6.3.5.1. Growth curves

Growth curves of stool and skin isolates were performed in the presence and absence of porcine bile and under aerobic and anaerobic conditions. When grown under aerobic conditions, F530B, 9^c DSM 28764 and DSM 20042 behaved similarly and exhibit similar exponential growth rates (Figure 6.3). The four isolates showed the ability to grow in the presence of bile and their growth rate was not affected by it. Thus, the exponential growth rates of stool isolates 9^c and F530B were 0.62 ± 0.01 and 0.59 ± 0.01 with BS and 0.62 ± 0.01 and 0.60 ± 0.01 without BS respectively, while skin isolates DSM 20042 and DSM 28764 presented 0.51 and 0.48 in presence of BS and 0.52 and 0.51 in absence of BS (Figure 6.3A). However, differences were observed when the four isolates were grown in the presence of BS under anaerobic conditions (Figure 6.3B). Differences in exponential growth rate were observed between stool and skin representatives: 9^c and F530B showed 0.33 ± 0.01 and 0.32 ± 0.01 in the presence of BS and 0.31 and 0.36 in absence of BS while DSM 20042 and DSM 28764 showed 0.15 ± 0.02 and 0.15 ± 0.06 with BS and 0.18 and 0.15 without BS in the media. The maximum exponential growth of 9^c and F530B is reached at 9 h, with an OD_{600} of 1.0-1.1 and 1.2 respectively, and the OD_{600} starts to decrease progressively at 10 h, arriving to 0.4-0.5 by 48 h. Both strains grow slightly better in the presence of BS. The skin isolates, DSM 28764 and DSM 20042 showed different growth behaviour: DSM 20042 displayed slightly faster growth in the absence of BS in anaerobic conditions than in presence of BS. However, at 15 h this growth reaches its maximum, with an OD_{600} of 0.95 nm, remaining at this level until 48 h. On the other hand, the DSM 20042 grown with BS in the medium reached a maximum OD_{600} of 1.18 at 25 h and remained at that level for the rest of the experiment. DSM 28764 growth was also slightly better in the absence of BS

until 22 h, when the DSM 28764 grown in the presence of BS improves and reaches 1.18 OD_{600} by 48 h, while the DSM 28764 grown in absence of BS only reached an OD_{600} of 0.9 OD. It is important to note that, in anaerobic conditions, while the stool isolates exhibit a fast growth at the beginning, this growth gets quickly exhausted and there is a depletion in OD, probably due to settling or lysis. On the other hand, although the skin isolates need more time to reach their maximum growth, they eventually achieved the same ODs as the stool isolates.

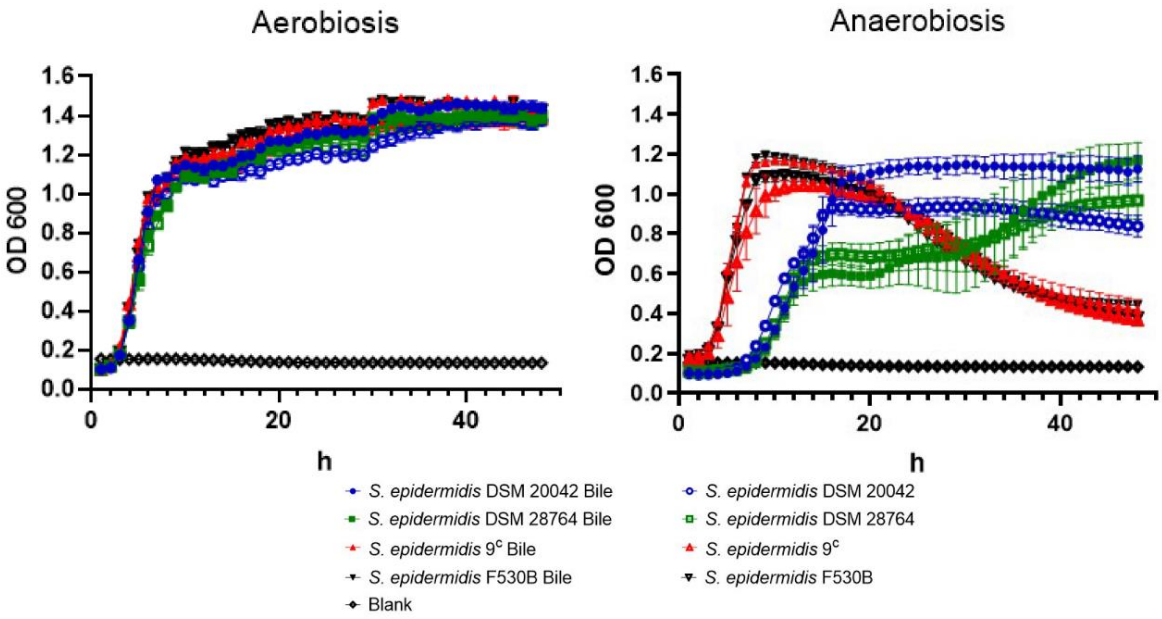


Figure 6.3. A. Growth of skin and stool isolates in aerobic conditions and in presence or absence of bile. B. Growth of skin and stool isolates in anaerobic conditions and in presence or absence of bile. Results are the mean of three replicates ± standard deviation.

6.3.5.2. Bile acids quantification

The degradation of cholesterol yields the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) that are later transformed by intestinal bacteria into secondary bile acids by removing a hydroxyl group from the C7 of the primary bile acid molecule, giving deoxycholic acid (DCA) from CA and lithocholic acid (LCA) from CDCA. Bile also contains bile acids conjugated with glycine and taurine. Bile acids are commonly very stable and incubations in the absence of no bacteria had no effect on composition. However, when stool and skin isolates of *S. epidermidis* were grown in the presence of porcine bile, we detected a progressive increase of primary bile acids, CA and CDCA with time, as a result of the cholesterol degradation. However, we observed that concentrations of secondary bile acids, DCA and LCA, rose too. The concentrations of DCA in the culture of

skin isolates were very variable, while the much lower levels of LCA varied widely in all isolates. It is worth noticing the absence of LCA in the culture of DSM 28764 until 48 h (Figure 6.4). There is a decrease of almost 100% at 24 h of glycine conjugated primary and secondary bile acids glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA) and glycolithocholic acid (GLCA), which somewhat mirrored the rise in deconjugated acids for CDCA and DCA. The glycocholic acid (GCA) levels did not show as sharp decrease as the others. The levels of the taurine conjugated bile acids remained constant over time in *S. epidermidis* cultures of both skin and stool origins.

6.3.6. Ability of *S. epidermidis* isolates to form biofilms

6.3.6.1. Genomic comparison

The comparison of the genes involved in biofilms development, previously described as virulence factors, in *S. epidermidis* 9^c, F530B, DSM 20042 and DSM 28764 showed no major differences. Only two genes of potential relevance, *sdrC_2*, Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrC and *clfB*, clumping factor B, were in the genomes of the stool isolates and absent in the genomes of skin isolates (Table 6.5). Both genes are related to the accumulation stage of biofilms, involved in cell-cell aggregation and matrix production. The SdrC protein has been highlighted as being a determinant of staphylococcal biofilm formation [341]. *Ica* genes, characteristic *Staphylococcus* genes involved in biofilm formation, were in the genome of all four isolates. Other genes involved in the biofilm maturation stage and related to thickness, such as teichoic acids, were also identified in the genomes of all the isolates.

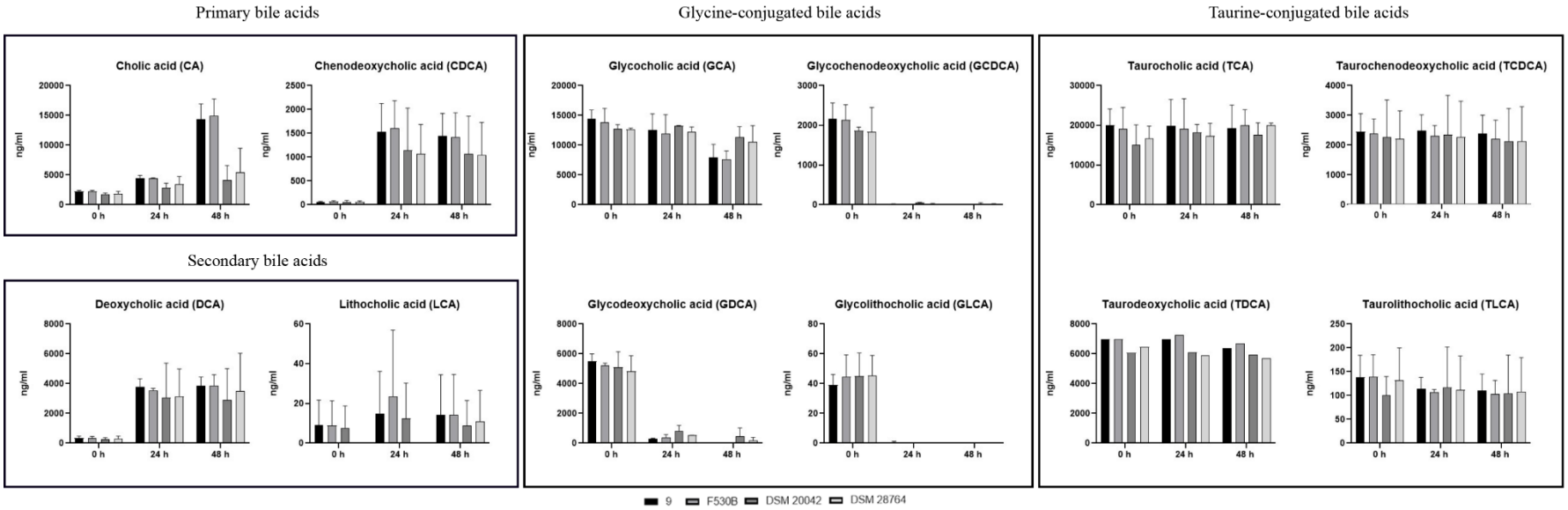


Figure 6.4. Primary, secondary and conjugated bile acids in the media after fermentations with stool isolates 9^{c} and F530B and skin DSM 20042 and DSM 28764 isolates. Results (ng/ml) are the mean of duplicate measurements +/- standard deviation.

Table 6.5. Presence of genes involved in biofilm formation in *S. epidermidis* stool isolates and skin type strains.

Biofilm function Gene annotation		9[c]	F530B	DSM 20042	DSM 28764
1. Initial attachment. Adhesion to material surface.					
atl	bifunctional N-acetylmuramoyl-L-alanine amidase/endo-beta-N-acetylglucosaminidase, Atl	√	√	√	√
atl_2	bifunctional N-acetylmuramoyl-L-alanine amidase/endo-beta-N-acetylglucosaminidase, Atl	√			
2. Accumulation. Cell-cell aggregation, matrix production.					
icaD	intercellular adhesion protein IcaD	√	√	√	√
icaC	intercellular adhesion protein IcaC	√	√	√	√
icaB	intercellular adhesion deacetylase IcaB	√	√	√	√
icaA	intercellular adhesion protein IcaA	√	√	√	√
group_28	extracellular matrix binding protein		√	√	√
group_29	extracellular matrix binding protein			√	
group_30	extracellular matrix binding protein			√	
group_31	extracellular matrix binding protein	√			
sdrD	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrD	√	√	√	√
sdrC	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrC	√	√	√	
sdrE_3	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE_1	√		√	√
sdrH	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrH	√	√	√	
sdrD_1	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE		√	√	√
sdrE_1	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE_1		√		√
group_20	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE	√		√	
group_61	cell-wall-anchored protein SasC	√		√	
sdrC_2	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrC	√	√		
clfB	clumping factor B	√	√		
group_16	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE_1	√			
group_1853	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrH				√
group_21	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE			√	
group_22	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE				√
group_23	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE	√			
sdrE_2	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE_1			√	
3. Maturation. Thickness increase, dependence of structural features.					
agrA	autoinducer sensor protein response regulator protein	√	√	√	√
agrB	accessory gene regulator B	√	√	√	
agrD	staphylococcal accessory gene regulator protein D	√	√	√	
agrB_1	accessory gene regulator B	√			√
wecG	teichoic acid biosynthesis protein	√	√	√	√

tagG	teichoic acid ABC superfamily ATP binding cassette transporter, membrane protein	√	√	√	√
group_2824	D-Ala-teichoic acid biosynthesis protein	√	√	√	√
tagH	Teichoic acid export ATP-binding protein, TagH	√	√	√	√
tagB	teichoic acid biosynthesis protein B	√	√	√	√
tagX	teichoic acid biosynthesis protein X	√	√	√	√
group_3224	teichoic acid ABC superfamily ATP binding cassette transporter, membrane protein	√	√	√	√
dltD	D-alanine lipoteichoic acid and wall teichoic acid esterification secreted protein	√	√	√	√
4. Regulation					
rsbU	putative sigma factor sigB regulation protein	√	√	v	√
sarA	staphylococcal accessory regulator family protein	√	√	√	√
rsbW	anti-sigma-B factor, serine-protein kinase	√	√	√	√
sigB	RNA polymerase sigma factor SigB	√	√	√	√
rsbV	anti-sigma-B factor, antagonist	√	√	√	√
msa	modulator of SarA, Msa	√	√		√
sigB_2	RNA polymerase sigma factor SigB	√			
5. Other genes involved in biofilm formation					
group_2411	putative polysaccharide biosynthesis protein	√	√	√	√
group_2526	lipopolysaccharide biosynthesis-related pr-like protein	√	√	√	√
kdtB	lipopolysaccharide core biosynthesis protein KdtB	√	√	√	√
paaD	putative metal-sulphur cluster biosynthetic enzyme, PaaD	√	√	√	√
fmhB	peptidoglycan pentaglycine interpeptide biosynthetic protein FmhB	√	√	√	√
group_3157	SCP/PR1-like extracellular protein	√	√	√	√
fmhA	FemAB family peptidoglycan biosynthesis protein	√	√	√	√
fmhB_2	peptidoglycan pentaglycine interpeptide biosynthetic protein FmhB	√			

6.3.6.2. Biofilm formation

S. epidermidis strains F530B, 9^{c}, DSM 20042 and DSM 28764 were equally able to form biofilms on abiotic surfaces, such PE and glass, at 1:100 and 1:1000 dilution (Table 6.6). *S. epidermidis* F530B was studied further to establish the ability of the cells to adhere to an abiotic gold electrode (RTCA) (Figure 6.5) and a biotic surface, the HT29 cell line**.** It showed good abiotic adhesion but a low percentage of adhesion to HT29 cells of 1.6 ± 0.4% (Figure 6.6), however, a positive control, *L. rhamnosus* GG, only showed adhesion of 1.5 ± 0.5%.

S. epidermidis F530B was also tested in competition with enteropathogens to assess if it could influence the adhesion levels of those pathogens to the biotic surface of the HT29

cell line, using both live and dead cells (Figure 6.7). Adhesion levels of *E. coli* LMG 2092 to HT29 were reduced significantly using both live and dead cells, while adhesion levels of *S. enterica* LT2 were reduced significantly only in the presence of live *S. epidermidis* F530B.

Table 6.6. Biofilm formation measured by crystal violet staining on glass and PE

	Glass		PE	
Dilution	1:100	1:1000	1:100	1:1000
S. epidermidis F530B	0.531 ± 0.027	0.026 ± 0.016	0.712 ± 0.027	0.032 ± 0.007
S. epidermidis 9[c]	0.483 ± 0.104	0.011 ± 0. 009	0.698 ± 0.113	0.045 ± 011
S. epidermidis DSM 20042	0.512 ± 0.085	0.012 ± 0.006	0.675 ± 0.051	0.012 ± 0.003
S. epidermidis DSM 28764	0.611 ± 0.077	0.082 ± 0.032	0.669 ± 0.095	0.033 ± 0.008
Media control	0.063 ± 0.001	0.057 ± 0.002	0.054 ± 0.002	0.059 ± 0.002

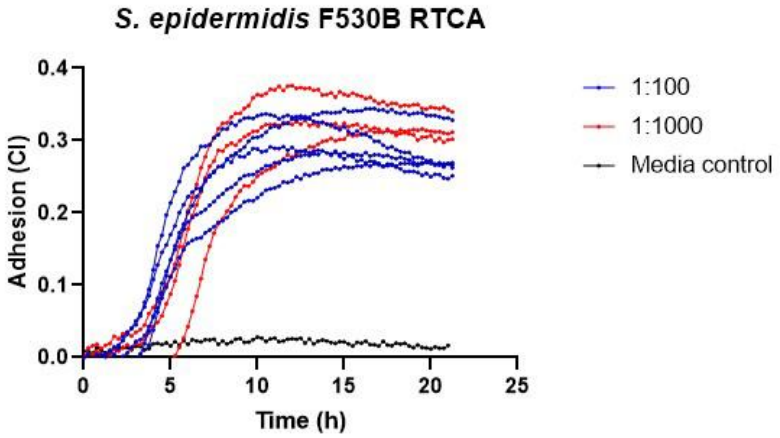


Figure 6.5. Adhesion of *S. epidermidis* F530B to E-plates measured by RTCA.

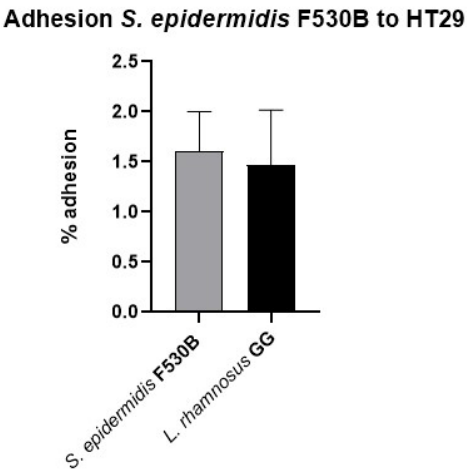


Figure 6.6. Percentage of adhesion of *S. epidermidis* F530B to HT29.

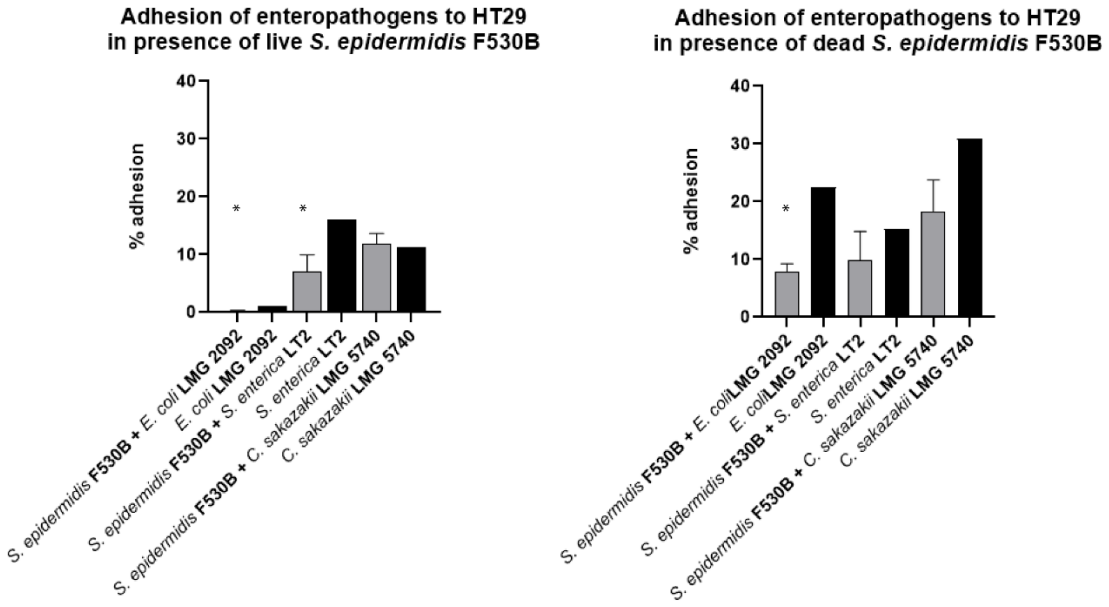


Figure 6.7. Percentage of adhesion of *E. coli* LMG 2092, *S. enterica* LT2 and *C. sakazakii* LMG 5740 to HT29 with and without the presence of live or dead cells of *S. epidermidis* F530B.

6.3.7. Microscopy

TEM images show clustered cocci cells. It is possible to observe fibres that could be related to exopolysaccharides involved in biofilm matrix (Figure 6.8).

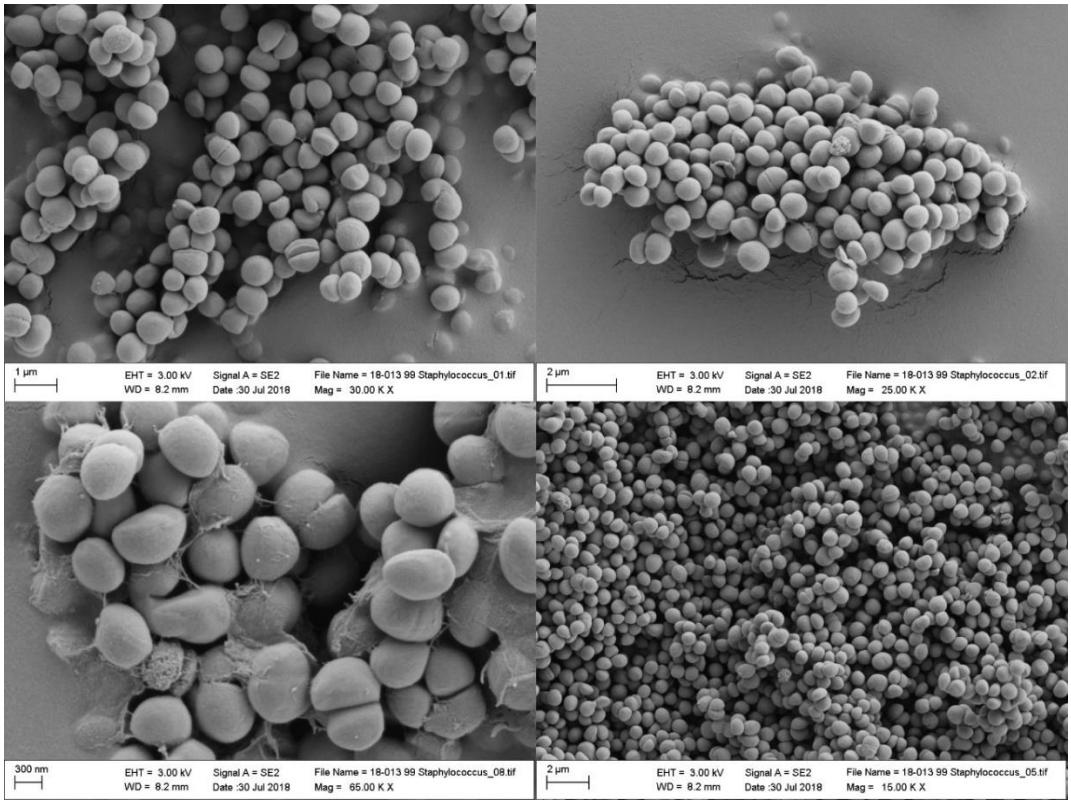


Figure 6.8. TEM images from *S. epidermidis* 99 at different scales.

6.4. Discussion

This work has investigated three *S. epidermidis* strains isolated from stool, a source which, to our knowledge, is not represented in online databases. These strains were placed in an updated *S. epidermidis* phylogenetic tree and further established genomic traits that differentiate these from *S. epidermidis* isolated from other sources, such as human body sites and other environmental niches. There were established phenotypic differences between these strains and other *S. epidermidis* isolated from human skin, cases of bovine mastitis and cheese, specifically in terms of utilisation of carbon and nitrogen sources and antibiotic sensitivity, and these differences with genomic information in strains of human origin were associated. Finally, the behaviour of these strains was compared in terms of traits that could influence their lifestyles, such as growth in the presence of bile and virulence traits such as biofilm formation.

While there was no phylogenetic clustering of the three stool isolates of *S. epidermidis,* other strains with a common origin exhibited clustering, such as those isolated from rice and mice. However, the fact that these isolates were sourced from the same study may be significant [324]. *S. epidermidis* isolated from different sites in and on the human body appeared to be more heterogeneous than their non-human counterparts, a phenomenon that has been previously reported for *S. epidermidis* [342]. One pan-genome analysis conducted in 2012 examining skin commensal and hospital infection-associated *S. epidermidis* showed high levels of intra-site diversity and two clear phylogenetic groups which differentiated the commensal and the nosocomial isolates [333]. A second whole-genome comparison study reported that rice endophytic strains are more related to rodent isolates than the majority of human isolates, an observation which is also reported using our approach [324]. These two studies were conducted on 99 and 93 genomes respectively, while this pan-genome approach used 581 genomes.

While the three stool isolates described here do not form a distinct phylogenetic cluster, our pan-genome-wide association approach was able to identify 44 genes, in four clusters, significantly associated with stool isolates, including a predicted 30 kb plasmid. In addition, it is reported that all three stool isolates contain a type V ACME gene cluster, a recently described type of arginine catabolic module element (ACME) characterised by the presence of the arginine deaminase pathway-encoding *arc* operon, the oligopeptide permease ABC transporter-encoding *opp*3 operon, and the potassium transport system-encoding *kdp*

operon [343]. These ACME gene clusters play a role in colonisation of the host and evasion of the immune system [344, 345], have been previously described in *S. epidermidis* isolated from the human skin and oral cavity [343, 345], and are significantly enriched in infected subgingival oral implants compared to non-infected counterparts [346].

These results led to study possible phenotypic differences between stool and skin *S. epidermidis* isolates. Observations made from the simplified phylogenetic tree led to select strains isolated from bovine mastitis and cheese in addition to two of our stool isolates. It is shown that there were pronounced differences in the use of carbon sources between *S. epidermidis* of different origin, with the bovine mastitis-associated strain being the clear outlier. The cheese isolate showed a closer relationship with one of the stool isolates, F530B, while isolate 9^{c}'s behaviour seemed to be closer to that of the skin isolates. There were identified common traits for both skin and stool isolates. Stool isolates showed significantly associated genes such as *hsdR_1*, *hsdM_1*, *cas6*, *fecE_2*, *mntB_2*, *mntA_2*, *racA* and *fas6*. These genes seemed to be involved in the ability to grow in dulcitol, oxalomalic acid, 2,3-butanone, 2-deoxy-D-ribose, 5-keto-D-gluconic acid, D-glucosamine and D-mannose. However, further analysis indicated that this was a wrong association, highlighting the need of a careful analysis of bioinformatics data. Despite a small sample size, these carbon sources can be pointed to as an area for further research as a potential means for identifying the source of *S. epidermidis* isolates and designing better treatments for nosocomial infections. The nitrogen usage for the different *S. epidermidis* did not show major differences that could be associated to the isolate origin. However, many studies have been made on the nitrogen usage of *S. epidermis* [347, 348]. *Staphylococcus* spp have been proved extensively as being able to be trained to use different nitrogen sources by reorganising its metabolism to the extent of being able to use ammonia as a nitrogen source instead of amino acids [349, 350]. Despite the highly conserved core genome of *Staphylococcus* spp [351], they can show strain-specific metabolic adaptations [351-353].

No known BSH genes were identified in the genomes of stool and skin isolates. However, it was observed that there was a change in the concentrations of bile acids in the culture media of both stool and skin isolates. With both stool and skin isolates, the glycine bond to the steroid nucleus of the bile was removed (deconjugation) in GCDCA, GDCA and GLCA, a process that increases bile tolerance and survival in the gut and is the pre-requisite for biotransformations in human colon, where DCA and LCA are the predominant BS in human

faeces [245]. When glycine and taurine are separated from the BS, they are used as sources of C and N [354]. The deconjugation is catalysed by the BSH and, despite the fact that no BSH were identified in the genomes, it is likely that these *S. epidermidis* genomes encode enzymes able to display that function when necessary. These results, in combination with the growth curves observed in anaerobic conditions, suggest that skin isolates are able to behave similarly to stool isolates but need some time to reorganise their metabolism in order to adapt to the surrounding environment. Interestingly, the growth displayed by the skin isolates in the presence of BS suggests that they used these BS as source of nutrients that help them increase their growth once their metabolism was adapted to the anaerobic conditions. The presence of BS not only did not cause growth inhibition, as it is often reported, but appeared beneficial for the growth of the *S. epidermidis* strains. However, this does not necessarily mean that they are using BS, as they could be using other compounds which are costly from a metabolic point of view, that led them to grow slowly anaerobically. The drop in glycine-conjugated bile acids raises the possibility that the glycine might have been used. Comparison of the growth of skin isolates and stool isolates revealed an initial faster growth of stool isolates in anaerobic conditions but followed by a growth drop. Growth was similar in the presence and absence of BS in the media. These suggests that their metabolic routes associated with anaerobic conditions and presence of BS were already active and there was no need to reorganise their metabolism. Taurine-conjugated bile salts were not metabolised by any isolate, which is consistent with the fact that only one intestinal organism, *Bilophila wadsworthia*, is able to use taurine [355, 356]. Enzymes for biotransformations of CA into DCA and CDCA into LCA are not as wide spread in nature as enzymes for deconjugation, and only a few Clostridia are able to remove the hydroxyl from the position 7 [245, 357]. It has been reported, however, that some *Clostridium* and *Bacteroides* spp are able to use different metabolic routes that enhance bile salt degradation [354, 358, 359]. A significant rise in the concentration of DCA could be observed, and even with a high variability, it was observed too in the case of LCA. This suggests bile acid transformation of some type. Transcriptomics, proteomics and metabolomics might provide a route to identify the proteins and/or routes involved in this relationship between *S. epidermidis* and bile acids.

Other traits considered to be associated with virulence, like biofilm formation, showed similar patterns in stool and skin isolates. Small differences were observed at genomic level,

in that only two genes of known relevance, *sdrC* (whose product showed some redundancy) and *clfB*, were present in the genome of stool isolates and were absent from the skin isolates. S. *epidermidis* also showed some traits that could be interpreted as being desirable as, in addition to the production of antimicrobials active against Gram positive and negative pathogens, *S. epidermidis* F530B cells significantly reduced the adhesion of *E. coli* LMG 2092 and *S. enterica* LT2 to HT29 cells.

Overall, these results suggest the absence of major specific genomic traits among *S. epidermidis* for adaptation to the different sites within the human body. Therefore, *S. epidermidis* adaptation to human body sites might rely on gene expression, and thus, transcriptomics, proteomics and metabolomics would be the next step to understand how the metabolism reorganises to adapt to these sites, with care to avoid laboratory domestication. Moreover, it is necessary to improve the genome annotations to learn which genes and metabolic pathways are associated to the successful adaptation to each body site.

CHAPTER VII

Antimicrobial Activity of *L. amylovorus* and *L. crispatus*

7.1. Introduction

The human microbiota associated with faeces includes several species of *Lactobacillus*. *L. amylovorus* and *L. crispatus* are two of these species, both of which are members of the *L. acidophilus* group [246, 247]. Despite being typical representatives of the human GIT, they have also been linked to other body sites, like the mouth and vaginal tract [360]. Strains of both *L. amylovorus* and *L. crispatus* have been identified as bacteriocin producers in the past. Several investigations have performed on the production of bacteriocin amylovorin L471, produced by *L. amylovorus* L471 [361-365]. Lactobin A is another bacteriocin produced by *L. amylovorus* LMG P-13139 [366]. Both strains were isolated from fresh corn steep liquor, and both bacteriocins share similar amino acid residues that show homology to the *lafX* gene product, part of the lactacin F operon. In both cases, the bacteriocins were classified as class IIb [366]. On the other hand, crispacin A, a 5393 Da bacteriocin, was purified from the supernatant and cells of *L. crispatus* JCM 2009, isolated from urine [367]. A few years later, further antimicrobial activity from a bacteriocin-like compound was reported from *L. crispatus* ATCC 33820, isolated from eye [368]. More recently, a class III helveticin-M has been reported to be produced by a chicken intestine-isolate, *L. crispatus* K313 [149]. The production of this bacteriocin was carried out by heterologous expression in *E. coli* and can be considered important because class III bacteriocins/bacteriolysins are not as extensively explored as other bacteriocins. Only four class III bacteriocins from lactobacilli have been characterised to date, including enterolysin A and helveticin J. The activity of enterolysin A has been well characterised [369], but helveticin's mode of action remains unclear, although some evidence suggest that they cause impairment of cell wall and membrane in the target cells [149].

In addition to their antimicrobial activity, both *L. amylovorus* and *L. crispatus* have representatives that are considered probiotics. Assimilation and precipitation of cholesterol have been reported in both species [370-372]. Other probiotic-associated activities include production of phytase [373], alteration of body adiposity [374] and competitive exclusion of enteropathogens [375, 376]. Therefore, *L. amylovorus* and *L. crispatus* are lactobacilli species whose potential activity can be considered beneficial, not only in the human GIT, but also in animals [377] and the food industry [378].

Here, one strain of *L. amylovorus* isolated from human faeces, Lac 20, and two strains of *L. crispatus* isolated from the same source, Lac 50 and Lac 51, are studied, after showing antimicrobial activity associated with their respective supernatants and having had their genomes analysed by BAGEL (Chapter III). Different strategies to identify the antimicrobial compounds were followed, in order to isolate the putative peptide(s)/proteins responsible for antimicrobial activity. The production of SCFA by *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51 was measured after the supplementation with different carbon sources in fermentation conditions. Their adhesion and biofilm formation abilities were also studied. Given some behavioural differences that were observed between the two isolates of *L. crispatus*, some genotypic and phenotypic differences between them were studied.

7.2. Methods

7.2.1. Partial purification of bacteriocin of *L. amylovorus* Lac 20

7.2.1.1. Initial characterisation

L. amylovorus Lac 20 was grown overnight in 500 ml of culture according to previously specified conditions (Chapter II). The culture was centrifuged at 8,000 x g for 20 min and separated into pellet and supernatant fractions. The cell pellet was mixed with 50 ml 70% 2-propan-ol 0.1% TFA (IPA) and stirred for 3 h at room temperature. The sample was centrifuged at 8,000 x g for 10 min and the supernatant assayed for activity against *L. bulgaricus* LMG6502 indicator. The culture supernatant was treated further: 5 ml of sample was passed through a Millipore centrifugal filter unit containing a 10 kDa cut off membrane. Heat stability was checked by heating aliquots of sample to 55, 70, 85 °C for 20 min. 50 µl aliquots were plated on an indicator plate. The solvent stability of the antimicrobial was assessed by mixing 300 µl of supernatant with 700 µl water, 0.1% TFA, acetonitrile and acetonitrile + 0.1% TFA. The mixtures were left at room temperature for approximately 2 h before plating on the indicator plate.

7.2.1.2. Assessment of columns for peptide and protein purification

The inhibitory activity was analysed by passing the active culture supernatant through different columns. The fractions that showed activity were analysed by MALDI-TOF MS to assess potential associated masses to the inhibitory activity. 3 ml of supernatant were applied to a 3 ml, 200 mg Strata–E C18 SPE column (Phenomenex, UK) pre-equilibrated with 15% methanol and 85% water. The column was eluted with 3 ml 30% ethanol and then

3 ml 70% 2-propanol 0.1 TFA (IPA). 3 ml of supernatant was applied to a 3 ml 200 mg Strata SCX column (Phenomenex, UK) pre-equilibrated with methanol and 50 mM sodium phosphate buffer pH 7.0 following manufacturer's instructions. The column was eluted firstly with 3 ml 50 mM sodium phosphate buffer pH 7 and secondly with 3 ml 50 mM sodium phosphate buffer pH 7 containing 1M NaCl.

10 ml of culture supernatant were applied to a column containing 500 mg of Amberlite XAD beads and the column was washed with 10 ml of 50 mM sodium phosphate buffer pH 7, 30% ethanol and IPA. 20 ml of culture supernatant were applied to an Econo column containing 2 ml of SP sepharose. The sample was eluted with 10 ml of 50 mM sodium phosphate buffer pH 7, 10 ml of 50 mM sodium phosphate containing 1M NaCl pH 7, and 10 ml of 20 mM potassium phosphate 25% acetonitrile pH 2.5 containing 2M NaCl.

7.2.1.3. Cation-exchange

Cation-exchange was performed and SP sepharose was used as a first step in purification. 400 ml of sample were applied to an Econo column containing 20 ml of SP sepharose (GE Healthcare, UK). The column was eluted with 40 ml of 50 mM sodium phosphate buffer pH 7, 40 ml of 50 mM sodium phosphate buffer pH 7 containing 200 mM NaCl, 40 ml of 50 mM sodium phosphate buffer pH 7 containing 500 mM NaCl and 40 ml of 50 mM sodium phosphate buffer pH 7 containing 1000 mM NaCl. The 3 ml of the 500 and 1000 mM NaCl eluates from the SP column were re-applied on a C18 SPE column following the steps described above for this column.

7.2.2. Partial purification of bacteriocin of *L. crispatus* Lac 50

7.2.2.1. Initial characterisation

L. crispatus Lac 50 was grown overnight in 500 ml culture according to previously specified conditions (Chapter II). The culture was centrifuged at 8,000 x g for 20 min and separated into pellet and supernatant fractions. The cell pellet was mixed with 50 ml 70% 2-propanol 0.1% TFA (IPA) and stirred for 3 h at room temperature. The sample was centrifuged at 8,000 x g for 10 min and the supernatant assayed for activity against *L. bulgaricus* LMG6502 indicator plates. The solvent stability of the antimicrobial in the culture supernatant was assessed by mixing 300 µl of supernatant with 700 µl water, 0.1% TFA, acetonitrile and acetonitrile + 0.1% TFA. The mixtures were left at room temperature for approximately 2 h before plating on an indicator plate.

7.2.2.2. Reversed phase

Supernatant were applied to a 3 ml, 200 mg Strata–E C18 SPE column (Phenomenex, UK) pre-equilibrated with 15% methanol and 85% water. The column was eluted with 30% ethanol and 70% 2-propanol 0.1 TFA (IPA) later.

7.2.2.3. Cation exchange

Supernatant was applied to an Econo column containing 10 ml of SP sepharose. The column was washed with 50 mM sodium phosphate buffer, 20 mM sodium phosphate containing 500 mM NaCl pH 7 and 20 mM sodium phosphate pH 7, containing 1M NaCl.

7.2.2.4. Cation exchange with ÄKTA Fast Protein Liquid Chromatography (FPLC)

Cation exchange was performed in an ÄKTA Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, UK) using a HiPrep SP HP 16/10 column (GE Healthcare, UK) and different buffers. Start buffers were 50 mM sodium acetate, 50 mM MES and 50 mM sodium phosphate at pHs 5, 6, 6.5 and 7. Elution buffers were 50 mM sodium acetate 1 M NaCl, 50 mM MES 1 M NaCl and 50 mM sodium phosphate 1 M NaCl at pHs 5, 6, 6.5 and 7. Samples were prepared by dilution in the start buffer. Bacterial supernatant was applied to a Millipore centrifugal filter unit containing a cut off membrane of different kDa sizes and centrifuged at 4,000 x g for 10 min. A 50 µl aliquot of the retained sample was tested to check its antimicrobial activity. The rest of the sample remaining in the cut off unit was equilibrated in the start buffer by performing washes, centrifuging each time at 4,000 x g for 10 min. The liquid retained was diluted in start buffer and then filter sterilised and injected on the AKTA FPLC system. Flow rate was set up at 3 ml/min, following manufacturer's instructions. Fractions were collected for testing by well-diffusion assay.

7.2.2.5. Cation exchange with ÄKTA Pure

Cation exchange was performed in an ÄKTA Pure system (GE Healthcare, UK) using a HiPrep SP HP 16/10 column (GE Healthcare, UK) and different buffers. Start buffers were 50 mM sodium acetate and 50 mM MES at pHs 5 and 7. Elution buffers were 50 mM sodium acetate 1 M NaCl, 50 mM MES 1 M NaCl and 50 mM at pHs 5 and 7. Samples were prepared by equilibrating 50 ml of bacterial supernatant previously tested for antimicrobial activity in 2 l of start buffer. The equilibration was performed by dialysis (Snake skin 7000 MWCO, Thermo Scientific, USA) overnight at 4 ^{o}C in agitation. Flow rate was set up at 3 ml/min. Fractions were collected for testing by well-diffusion assay.

7.2.3. Protein gels

SDS gels were performed as described in section 2.3.3. Gel slices were prepared for trypsin digestion as described in section 2.3.11.

7.2.3.1. Native gels

Native gels were used to analysed supernatant samples of *L. crispatus* Lac 50 and Lac 51. NativePAGE™ Novex ®Precast Bis-Tris gels of 3-12% Bis-Tris were used (Life technologies, UK) according to manufacturer's instructions. 12.5 µl of sample were added to 2.5 µl of NativePAGE™ sample buffer (4x) and 10 µl of deionised water. The anode buffer was prepared by adding 50 ml of 20x NativePAGE™ running buffer to 950 ml of deionised water. Two cathode buffers were prepared, the dark blue by adding 50 ml of 20x NativePAGE™ running buffer and 50 ml of 20x NativePAGE™ cathode additive to 900 ml of deionised water, and the light blue by adding 50 ml of 20x NativePAGE™ running buffer and 5 ml of 20X NativePAGE™ cathode additive to 945 ml of deionised water. Samples and standard were added to the wells and gels were run for 120 min at 150 V. For staining purposes, the gels were immersed in staining solution of 0.3% Coomassie, 45% methanol, 10% acetic acid and 45% deionised water, shaking horizontally for 30 min. Then they were added to a destaining solution of 20% methanol, 10% acetic acid and 70% deionised water, shaking for 20-30 min. The destaining solution was replaced 3-5 times.

7.2.4. Genomic analyses

7.2.4.1. Sequencing and annotation

L. amylovorus Lac 20, *L. crispatus* Lac 50 and Lac 51 genomes were sequenced, assembled and annotated as explained in section 2.2.9.1. However, *L. amylovorus* Lac 20 genome was re-assembled by Dr. Lizbeth Sayavedra (Quadram Institute Bioscience), in order to find if the absence of matches in BAGEL4 were due to assembly problems. The adapter sequences were trimmed, and reads were quality filtered (Q=2) using BBduck V37.02 (Bushnell B. - sourceforge.net/projects/bbmap/). Genomes were assembled with Spades V3.11.1 using the k-mers 21,33,55,77,99, and 127 [379]. The completeness and contamination of the genomes were assessed with CheckM v1.0.9 [380]. Assembly metrics were estimated with Quast V.4.6.1. *L. amylovorus* Lac 20, *L. crispatus* Lac 50 and Lac 51 genomes were re-annotated using PATRIC V3.5.43 [381].

7.2.4.2. Genomic analyses

L. amylovorus Lac 20, *L. crispatus* Lac 50 and Lac 51 genomes were analysed as described in section 2.2.9.2. Artemis was used to study the genomic environment of specific genes.

7.2.4.3. Comparative genomics

An initial comparison between *L. crispatus* Lac 50 and Lac 51 genomes was performed by Dr. Andrea Telatin. I conducted a new comparison using Mauve [382] and regions that showed differences were studied to identify affected genes. A final comparison was conducted using Roary [383] to identify the exact number of genes that were different.

7.2.5. Analyses of probiotic traits

Some probiotic traits were measured as explained in section 2.1.6 and included autoaggregation, antioxidant activity and BSH activity. Biofilm formation abilities were measured as described in section 2.2. Organic acid quantification was performed as described in section 2.4.1.1.

7.2.6. Phenotypic comparison

BIOLOG assays were performed as described in section 2.1.9.

7.3. Results

7.3.1. Partial purification of peptides

7.3.1.1. Bacteriocin of *L. amylovorus* Lac 20

Assays with partially purified *L. amylovorus* Lac 20 extracts showed that the cell extract was inactive, while the culture supernatant had inhibitory activity against *L. bulgaricus* LMG6502 (Figure 7.1). This antimicrobial activity was stable at 55, 70 and 85°C, suggesting it is a peptide rather than a protein. The activity was retained by the 10 kDa membrane retentate and no activity was associated with the permeate. The activity was stable at low pH (0.1% TFA), indicating that reversed phase should be an appropriate technique for purification.

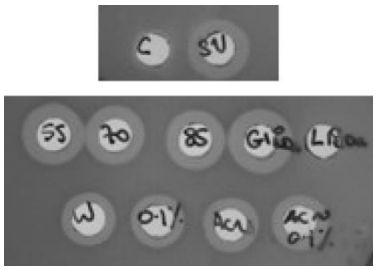


Figure 7.1. Inhibition zones of *L. amylovorus* Lac 20 extracts against indicator strain *L. bulgaricus* LMG6502 after different treatments. Cell extract (C); supernatant (SN); heat treatments at 55, 70 and 85°C (55, 70 and 85); retentate in 10 kDa cut off membrane (G10); permeate from 10 kDa cut off membrane (L10); antimicrobial stability in water (W); 0.1% TFA (0.1%); acetonitrile (ACN); acetonitrile + 0.1% TFA (ACN 0.1%).

The flow through and eluate obtained from the E C18 SPE column from both extracts showed a small zone and a very small zone was produced by the 70% 2-propanol 0.1% TFA (IPA) wash where antimicrobial activity was expected to elute (Figure 7.2). The flow through from the Strata SCX column showed a small zone of inhibition but no zone in the 1M NaCl wash where antimicrobial activity was expected to elute. These results suggested that these columns were not suited for the purification of the antimicrobial in question.

The results for the Amberlite XAD beads showed that some activity was lost in the flow through, a small inhibition zone with the sodium phosphate buffer and 30% ethanol washes and a larger inhibition zone for the IPA eluent were observed.

The results for the Econo column containing 2 ml of SP sepharose showed that a little activity was lost in the flow through but that most eluted in the NaCl containing washes (Figure 7.2) and gave the highest final activity.

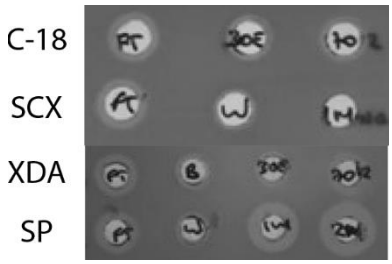


Figure 7.2. Inhibition zones of *L. amylovorus* Lac 20 extracts against indicator strain *L. bulgaricus* LMG6502 after different purification columns. C-18 row: flow through (FT); 30% ethanol (30E); IPA (70). SCX row: flow through (FT); 50 mM sodium phosphate buffer pH 7.0 (W); 1M NaCl elution (1M). XDA row: flow through (FT); 3 ml 50 mM sodium phosphate buffer pH 7.0 elution h (B); 30% ethanol (30E); IPA (70). SP row: flow through (FT); 50 mM sodium phosphate buffer pH 7.0 (W); 50 mM sodium phosphate 1M NaCl (1M); 20 mM potassium phosphate 25% acetonitrile pH 2.5 2M NaCl (2M).

The chromatograms obtained with the C18 SPE eluents did not show interesting results. However, the chromatograms obtained from the sepharose column 500 mM and 1000 mM NaCl fractions showed a 5265 Da mass that could be correlated with activity (Figure 7.3). That mass was more dominant in the 1000 mM NaCl sample but the 2000 mM NaCl sample is more active. The high salt content in the 2000 mM sample may prevent the mass from being detected, as salt content negatively impacts on MALDI TOF mass spectrometry.

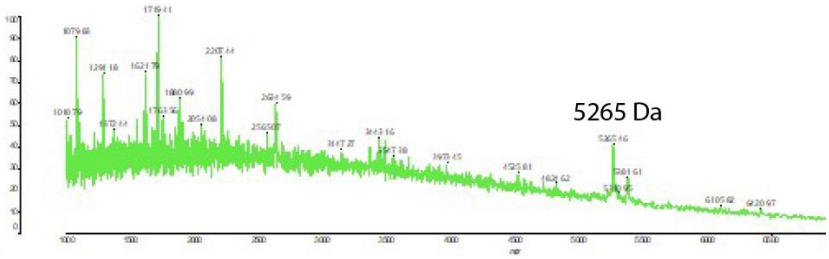
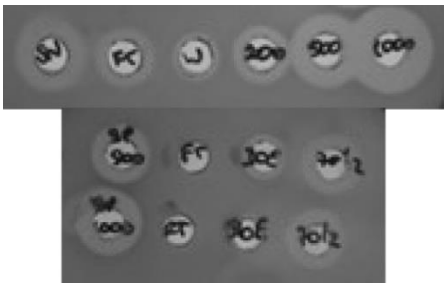


Figure 7.3. Chromatogram of fraction obtained from SP column washed with 10 ml of 50 mM sodium phosphate buffer 1M NaCl.

The cation-exchange showed that the majority of the activity eluted in the 500 and 1000 mM NaCl eluates. The chromatogram for the 500 mM NaCl sample showed the 5265 Da mass but the chromatogram for the 1000 mM sample did not (Figure 7.4).

(A)



(B)

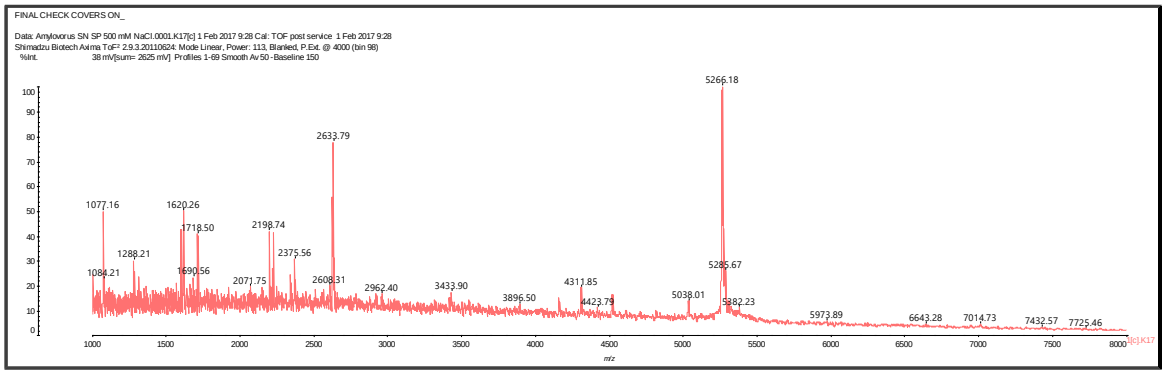


Figure 7.4. (A) Inhibition zones of *L. amylovorus* Lac 20 extracts against indicator strain *L. bulgaricus* LMG6502 after cation-exchange. Supernatant (SN); flow through (FT); 50 mM sodium phosphate buffer pH 7.0 (W); 50 mM sodium phosphate buffer pH 7.0 200mM NaCl (200); 50 mM sodium phosphate buffer pH 7.0 500mM NaCl (500); 50 mM sodium phosphate buffer pH 7.0 1000 mM NaCl (1000); Eluates from (500) re-applied to C18 SPE colum: flow through (FT); 30% ethanol (30E); IPA (70). Eluates from (1000) re-applied to C18 SPE colum: flow through (FT); 30% ethanol (30E); IPA (70). (B) Chromatogram of fraction (500) showing a mass of 5266 Da that might correspond to the potential peptide.

7.3.1.2. Protein purification from *L. crispatus* Lac 50

The supernatant and cell extract were assayed for activity on *L. bulgaricus* indicator plates and were both active, though there was little activity from the cells (Figure 7.5). The solvent stability showed that samples were stable at low pH as the 0.1% TFA sample results in the same size zone as the water sample. However, the supernatant diluted with both acetonitrile and acetonitrile 0.1% TFA lost activity, suggesting that the antimicrobial loses activity in the presence of acetonitrile. This suggests that the antimicrobial is not likely to be a peptide, but a protein.

The fractions collected from reversed phase LC and tested on the indicator plate showed that the activity bound to the C18 column, that no activity was lost with the 30% ethanol

wash and no activity eluted with IPA. As the antimicrobial is inactive when mixed with acetonitrile it is likely to also lose activity when mixed with IPA. These results show that reversed phase techniques are not suitable for purification of this antimicrobial. The fractions collected from the cation exchange column and tested in the indicator plate showed that the antimicrobial activity was retained by the column and the activity eluted in the 500 and 1000 mM NaCl samples.

Purification attempts performed by cation-exchange with ÄKTA FPLC and ÄKTA Pure were not able to separate the sample contents. Despite testing different buffers and different pH, the contents kept eluting in the unbound but with no separation. Fractions collecting the unbound molecules were tested and showed inhibitory activity by well diffusion assay against the indicator strain *L. bulgaricus* DPC5583.

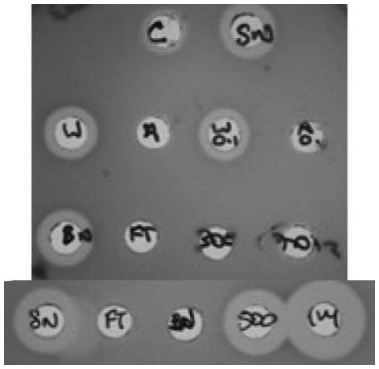


Figure 7.5. Inhibition zones of *L. crispatus* Lac 50 extracts against indicator strain *L. bulgaricus* LMG6502 after different treatments. Cells (C); supernatant (SN); antimicrobial stability in water (W); acetonitrile (A); 0.1% TFA (W 0.1); acetonitrile + 0.1% TFA (A 0.1%); C18 SPE separation: supernatant (SN); flow through (FT); 30% ethanol (30E); IPA (70). Cation exchange column: supernatant (SN); flow through (FT); 20 mM sodium phosphate buffer pH 7.0 (W); 20 mM sodium phosphate buffer pH 7.0 500 mM NaCl (500); 20 mM sodium phosphate buffer pH 7.0 1 M NaCl (1 M).

7.3.2. Protein gels

SDS gels allowed further confirmation of the molecular mass of the active antimicrobial peptides / proteins from *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51. The size corresponding to the active bands of *L. crispatus* 50 and 51, that showed inhibitory activity against *L. bulgaricus* 5583, could encode a protein, which correlates with the data obtained by the heat and protease test and the partial purification information of *L. crispatus* Lac 50

(Figure 7.6). The *L. amylovorus* supernatant showed a clear inhibition zone around 5000 Da (Figure 7.6). No inhibition zones were obtained from overlays on the native gels.

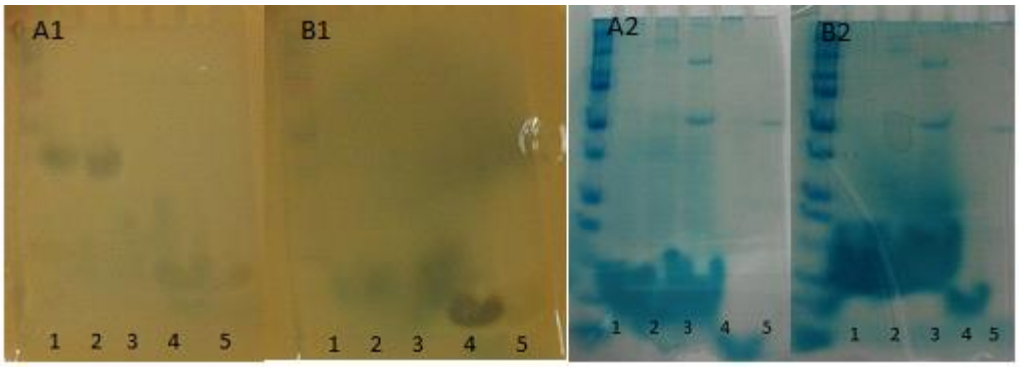


Figure 7.6. SDS gels A1 and B1 are overlaid by indicator strain *L. bulgaricus* DPC5583. A2 and B2 show the band disposition. *L. crispatus* 50 and 51 supernatants are in lanes 1 and 2. *L. amylovorus* in lane 4. Lane 3, *L. gasseri* LM19 supernatant; lane 5, contents of the 1000 mM NaCl eluate sample of *L. amylovorus* Lac 20. Standard BioRad Precision Plus protein Dual Extra Standards.

7.3.2.1. Mass analysis of proteins from gel slices

A total of 24 peptides and proteins were identified by mass spectrometry from bands excised from the region corresponding to antimicrobial activity (Appendix 4).

The peptides and proteins were shortlisted for following study, based on the presence of potential trypsin cleavage sites. Molecular mass of potential peptides derived from that cleavage were compared for match with a potential antimicrobial peptide of 5 KDa. The gene environments of the 24 matches and the potential modified peptides studied in Artemis did not show genes related to described bacteriocin clusters.

7.3.3. Study of some probiotic traits of *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51

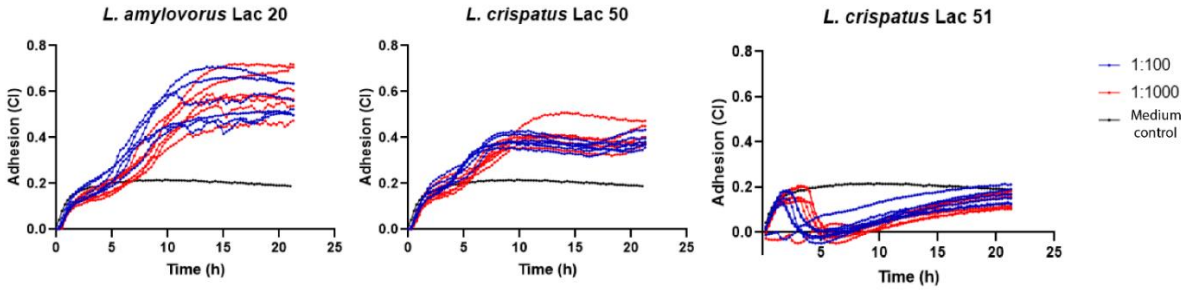
7.3.3.1. Adhesion and biofilm formation on abiotic surfaces

Adhesion to gold electrodes was measured in E-plates using RTCA using two concentrations of initial inoculum, 1:100 and 1:1000 dilutions. *L. amylovorus* Lac 20 showed the highest adhesion levels measured by cell index (CI), between 0.4-0.7 (Figure 7.7A). The levels of adhesion of *L. crispatus* Lac 50 in the E-plates were between 0.3-0.5. On the other hand, the CI of *L. crispatus* Lac 51 remained below the 0.2 level established as the medium

control, indicating that its adhesion levels at 24 h were at the same level as the medium alone (Figure 7.7A).

To test biofilm capacity, crystal violet assays were performed (Figure 7.7B) on polystyrene (PE) and glass. Due to the nature of the assay and probably the nature of the adhesion and the manipulation process, high variability was found in crystal violet on E-plate. However, when bacteria were grown on PE and glass, less variability was observed. No significant differences were observed in the crystal violet assays between the two different inoculums for the different Lactobacilli. Lac 50 generally gave the highest levels of biofilm formation.

(A)



(B)

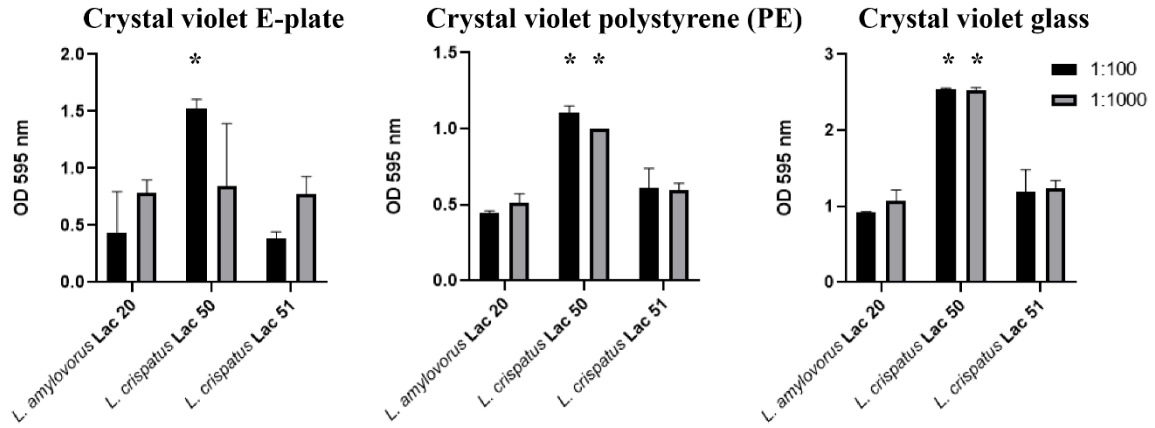


Figure 7.7. (A) Adhesion levels of *L. amylovorus* Lac 20, *L. crispatus* Lac 50 and Lac 51 measured in E-plates by RTCA. (B) Crystal violet assay to confirm biofilm formation on different abiotic surfaces (a) E-plate, (b) PE and (c) glass. Results are the mean of three samples ± SD.

7.3.3.2. Biofilm formation on a biotic surface

The HT29 cell line was used to establish the ability of the bacteria to adhere to a biotic surface. Unlike with abiotic surfaces, *L. crispatus* Lac 51 showed the highest levels of

adhesion to HT29, showing better adhesion than the positive control *L. rhamnosus* GG, while adhesion of *L. amylovorus* Lac 20 was negligible. (Figure 7.8).

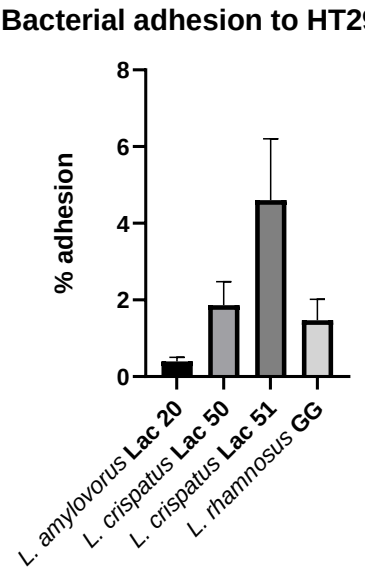


Figure 7.8. Percentage of adhesion of lactobacilli strains to HT29 cell line. Results are the mean of three samples ± SD.

7.3.3.3. Competitive exclusion of pathogens using live and dead Lactobacilli

Investigations were carried out to determine if *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and 51 could influence the adhesion levels of the enteropathogens *E. coli* LMG 2092, *S. enterica* LT2 and *C. sakazakii* LMG 5740 to HT29 cells. *L. amylovorus* Lac 20 had no significant effect on enteropathogen adhesion (Figure 7.9). However, *L crispatus* Lac 51 significantly reduced the adhesion levels of *S. enterica* LT2 and *C. sakazakii* LMG5740 to HT29 both with live and dead cells. *L. crispatus* Lac 50 showed mixed results against *S. enterica* LT2, with dead cells giving a significant reduction in adhesion but live cells having less effect, and its presence did not produce significant differences in the adherence of the other two pathogens. Adhesion of *C. sakazakii* did appear to be promoted in the presence of live *L. crispatus* Lac 50, but variability between samples meant that this difference was not significant.

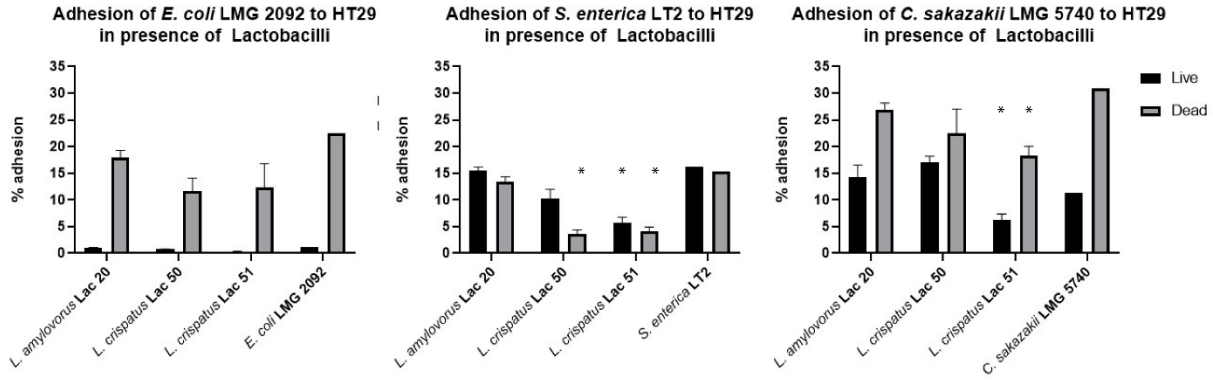
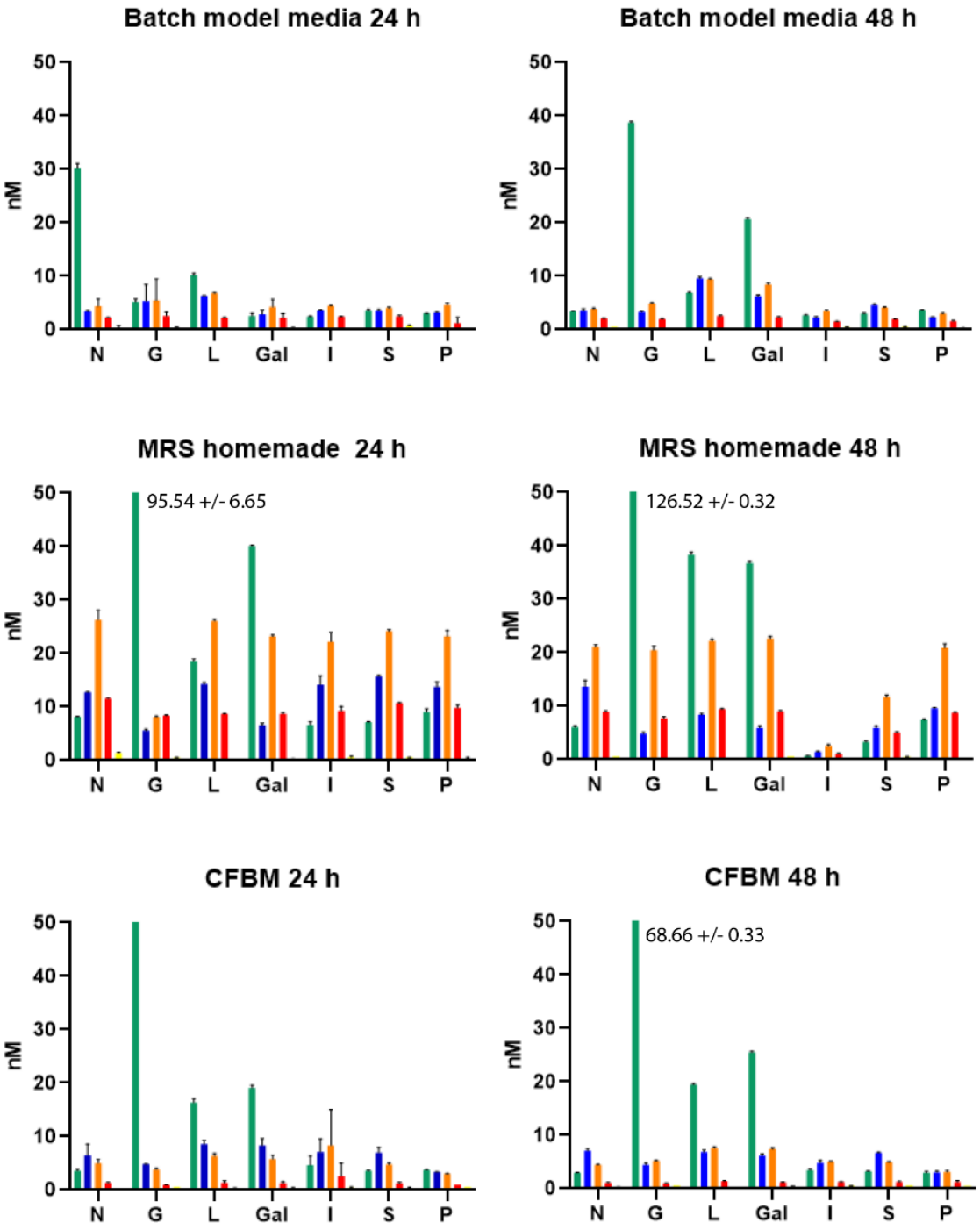


Figure 7.9. Adhesion of *E. coli* LMG 2092, *S. enterica* LT2 and *C. sakazakii* LMG 5740 to HT29 cell line alone or in the presence of live cells (black) and dead cells (grey) of *L. amylovorus* Lac 20, *L. crispatus* Lac 50 and Lac 51. Results are the mean of two measurements ± SD.

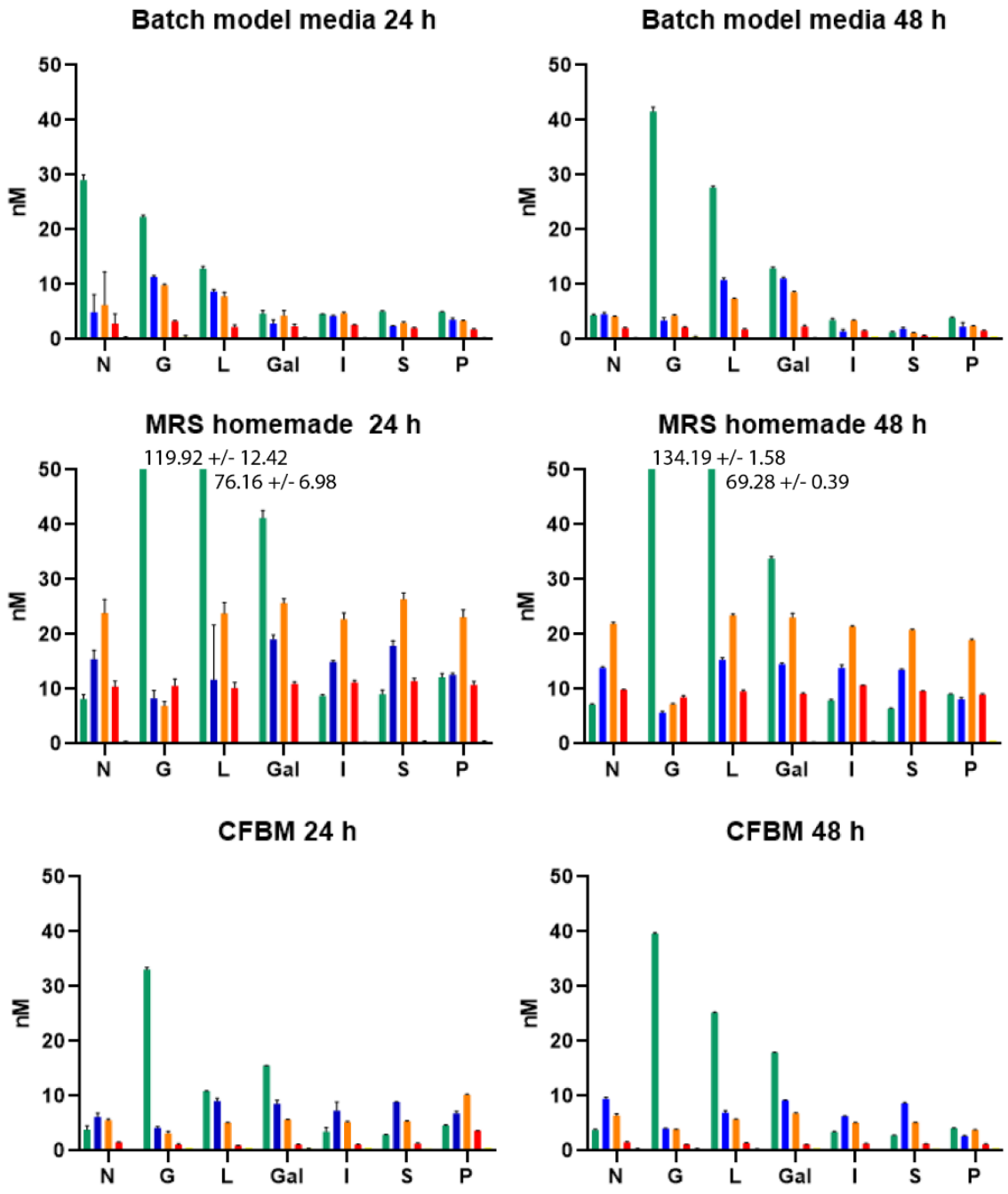
7.3.4. SCFA production

L. amylovorus Lac 20 and *L. crispatus* Lac 50 and 51 were grown in three different media (batch model media, MRS and CFBM) supplemented with different carbon sources (no supplementation, glucose, lactose, galactose, inulin, starch and pectin) and the presence of lactic, formic, acetic, propionic and butyric acids were measured at 24 and 48 h (Figure 7.10). For the three lactobacilli, the lowest levels of SCFA were found in the batch model media, while the highest levels were found in the MRS homemade media. Lactic acid was the SCFA most abundant in the three media and at the three timepoints. Overall, the media supplemented with simple sugars, more specifically glucose, lactose and galactose, showed the highest production of lactic acid. Additionally, acetic acid levels were higher, between 20 and 30 nM, in MRS. Propionic acid also showed the highest levels in MRS, with levels of 10 nM as a general rule. Butyric acid levels were very low in all combinations, between 0-2 nM.

(A)


Batch model media 24 h
Batch model media 48 h
MRS homemade 24 h
95.54 +/- 6.65
MRS homemade 48 h
126.52 +/- 0.32
CFBM 24 h
CFBM 48 h
68.66 +/- 0.33
nM
0
10
20
30
40
50
N
G
L
Gal
I
S
P

(B)


Batch model media 24 h
Batch model media 48 h
MRS homemade 24 h
119.92 +/- 12.42
76.16 +/- 6.98
MRS homemade 48 h
134.19 +/- 1.58
69.28 +/- 0.39
CFBM 24 h
CFBM 48 h
nM
0
10
20
30
40
50
N
G
L
Gal
I
S
P

(C)

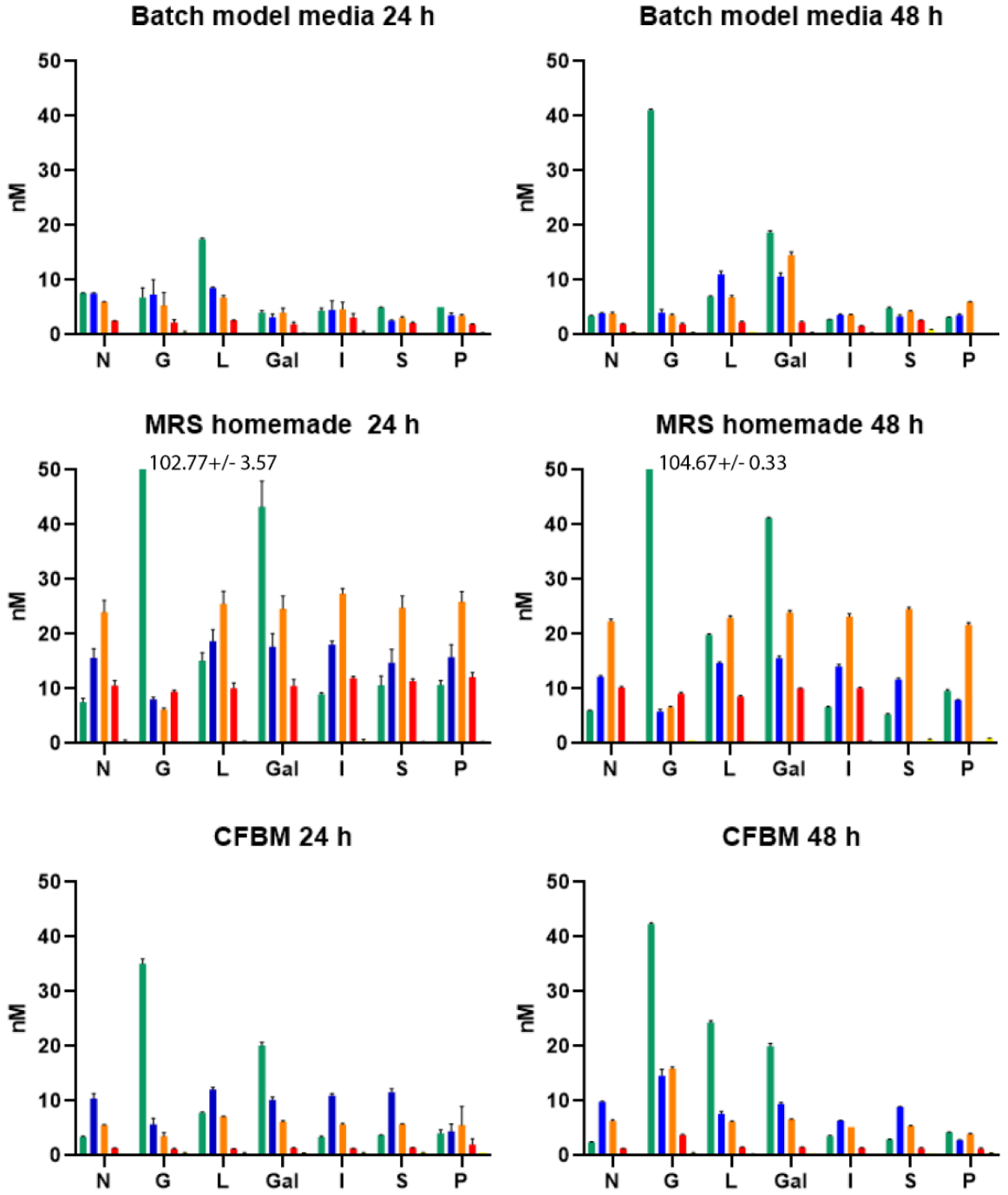


Figure 7.10. SCFA levels for fermentations with (A) *L. amylovorus* Lac 20, (B) *L. crispatus* Lac 50 and (C) *L. crispatus* Lac 51 in batch model media, MRS and CFBM supplemented with "no supplementation" (N), glucose (G), lactose (L), galactose (Gal), inulin (I), starch (S) and pectin (P) and measured at 24 and 48. Green bars represent lactic acid; blue, formic acid; orange, acetic acid; red, propionic acid; yellow, butyric acid.

7.3.5. Comparative genomics

The genomes of the two strains of *L. crispatus* (Lac 50 and Lac 51) were compared. 2187 genes were identified: 1658 genes (75.81 %) of them were classified as core genes with a probability of 99-100%; 529 genes (24.18 %) were identified as cloud genes, present in only one of the strains. Table 7.1 summarises selected genes that could explain the phenotypic differences, for example genes encoding for S-layer protein, fibronectin-binding protein and mucus-binding protein, which are commonly located on the outside of the cell, could explain the differences in behaviour and growth of these two strains.

Table 7.1. List of genes affected by differences in *L. crispatus* genomic comparison.

Gene	*L. crispatus* Lac 50	*L. crispatus* Lac 51
Cadmium resistance transporter	x	√
Endoglucanase	x	√
Fibronectin-binding protein	√	x
Glutaminase	√	x
Glycosyl transferase 2C family 2	x	√
GTP cyclohydrolase II	x	√
HAD superfamily hydrolase	√	x
Lactose permease	√	x
Lipopolysaccharide biosynthesis glycosyltransferase	x	√
Lipopolysaccharide synthesis sugar transferase	x	√
L-lactate permease	√	x
Major facilitator superfamily 2C Na-driven efflux pump	√	x
Mechanosensitive ion channel	x	√
Membrane alanine aminopeptidase	√	x
Mucus-binding protein	√	x
Nucleotidyltransferase 2FDNA polymerase for DNA repair	x	√
NUDIX family hydrolase	x	√
Permease of the major facilitator superfamily	√	x
Polysaccharide Transporter 2C PTS family	x	√
Potassium transport system protein kup	√	x
PTS system 2C cellobiose-specific IIC component	x	√

Riboflavin biosynthesis protein ribD	x	√
Riboflavin synthase2C alpha and beta subunit	√	x
Serine protease	√	x
S-layer protein	x	√
S-ribosylhomocysteine lyase	x	√
UDP-galactopyranose mutase	x	√
UDP-glucose 4-epimerase	x	√
UDP-N-acetylmuramate--L-alanine ligase	x	√

7.3.6. Antimicrobial sensitivity using BIOLOG

Based on the information obtained from the comparison and the observed behaviour of the two *L. crispatus* strains, it was hypothesised that differences on the cell surface might have an effect on other processes, such as the resistance to different antibiotics. For this reason, both *L. crispatus* Lac 50 and Lac 51 were tested for antimicrobial sensitivity using BIOLOG plates with antibiotics. Both strains grew and were inhibited by antibiotics targeting cell wall and membrane and elements inside the cell related to metabolism and cell processes (Tables 7.2 and 7.3). No patterns were observed and therefore, the results are not conclusive with respect to the influence that those differences on the cell surfaces might have on the behaviour against antibiotics. However, *L. crispatus* Lac 50 seemed to tolerate a larger number of antimicrobials, particularly those targeting the cell wall and protein synthesis and a number of toxic chemicals

Table 7.2. BIOLOG chemicals in the presence of which *L. crispatus* Lac 50 grows better than *L. crispatus* Lac 51.

Chemical	Target
Amikacin	Cell wall
Chlortetracycline	Protein synthesis 30S
Amoxicillin	Cell wall
Bleomycin	DNA topoisomerase
Colistin	Cell wall
Capreomycin	Protein synthesis
Demecloclycine	Protein synthesis 30S
Cefazolin	Cell wall
Chloramphenicol	Protein synthesis
Nalidixic acid	DNA topoisomerase
Neomycin	Protein synthesis 30S
Ceftriaxone	Cell wall
Gentamicin	Protein synthesis 30S
Cephalothin	Cell wall
Kanamycin	Protein synthesis 30S
Ofloxacin	DNA topoisomerase
Tetracycline	Protein synthesis 30S
Carbenicillin	Cell wall
Polymyxin B	Membrane
D,L-Serine hydroxamate	tRNA synthetase
5-Fluoroorotic acid	Nucleic acid analog
L-Aspartic-β-hydroxamate	tRNA synthetase
Ampicillin	DNA intercalator
Dequalinium chloride	Ion channel inhibitor, K^+
Azlocillin	Cell wall
Oxolinic acid	DNA topoisomerase
6-Mercaptopurine	Nucleic acid analog
Doxycycline	Protein synthesis 30S
Potassium chromate	Toxic anion
Cefuroxime	Cell wall
5-Fluorouracil	Nucleic acid analog
Rolitetracycline	Protein synthesis 30S
Geneticin (G418)	Protein synthesis 30S
Ruthenium red	Respiration, mitochondria
Cesium chloride	Toxic cation
Glycine	Cell wall
Thallium (I) acetate	Toxic cation
Cobalt chloride	Toxic cation
Trifluoperazine	Anti-cholinergic
Cupric chloride	Toxic cation
Moxalactam	Cell wall
Cefatoxamide	Cell wall
Phosphomycin	Cell wall
5-Chloro-7-iodo-8-hydroxy-quinoline	Chelator
Norfloxacin	DNA topoisomerase
Sulfanilamide	Folate antagonist
Trimethoprim	Folate antagonist
Dichlofluanid	(Fungicide)
Cetylpyridinium chloride	Membrane
1-Chloro-2,4-dinitrobenzene	Oxidises sulfhydryls
Diamide	Oxidises sulfhydryls
Cinoxacin	DNA topoisomerase
Streptomycin	Protein synthesis 30S
Sodium selenite	Toxic anion
Ferric chloride	Toxic cation
Glycine hydroxamate	tRNA synthetase
Chloroxylenol	(Fungicide)
Sorbic acid	Respiration, ionophore
Ketoprofen	Anti-capsule agent (biofilm)
Sodium pyrophosphate decahydrate	Chelator
Trifluorothymidine	Nucleic acid analog
Pipemidic acid	DNA topoisomerase
Azathioprine	Nucleic acid analog
Sulfisoxazole	Folate antagonist
Pentachlorophenol	Respiration, ionophore
Sodium m-arsenite	Toxic anion
Sodium bromate	Toxic anion
Lidocaine	Ion channel inhibitor, Na^+

Sodium metasilicate	Toxic anion
Sodium periodate	Toxic anion
Antimony (III) chloride	Toxic anion
Semicarbazide	Amine oxidase inhibitor
Tinidazole	Oxidising agent
Aztreonam	Cell wall
3, 5-Diamino-1,2,4-triazole (Guanazole)	Ribonucleotide reductase Inhibitor
Myricetin	DNA, RNA synthesis
5-fluoro-5'-deoxyuridine	Nucleic acid analog
2-Phenylphenol	DNA intercalator
Plumbagin	Oxidising agent
Harmane	Imidazoline binding sites
Chlorhexidine	Membrane
Umbeliferone	DNA intercalator
Cinnamic acid	Respiration, ionophore
Phenyl-methyl-sulfonyl-fluoride (PMSF)	Protease inhibitor
D,L-Thioctic acid	Oxidising agent
Lawsone	Oxidising agent
Sodium caprilate	Respiration, ionophore
Hydroxylamine	DNA damage, mutagen

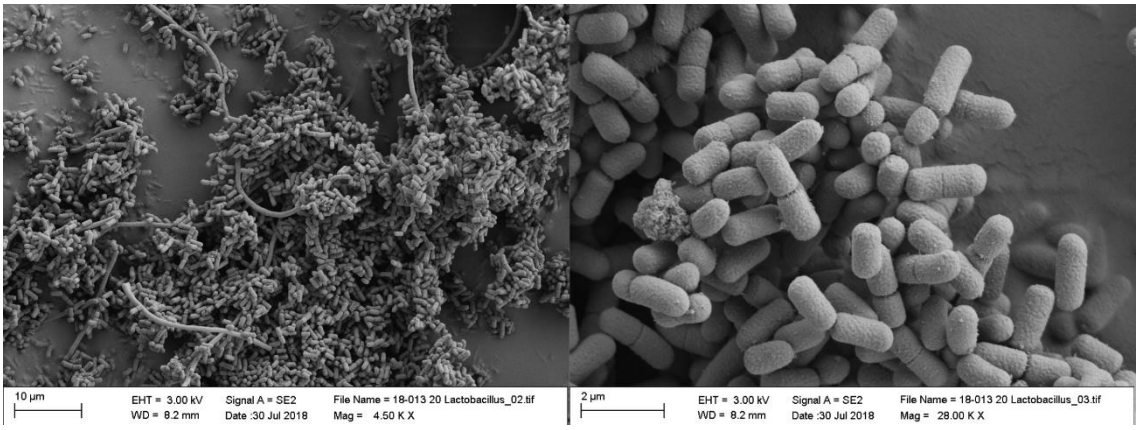
Table 7.3. BIOLOG chemicals in the presence of which *L. crispatus* Lac 51 grows better than *L. crispatus* Lac 50

Chemical	**Target**
Lincomycin	Protein synthesis 50S
Penicillin G	Cell wall
Oxacillin	Cell wall
Rifampicin	RNA polymerase
Protamine sulfate	Membrane
Thiamphenicol	Protein synthesis
Myricetin	DNA, RNA synthesis
2-Phenylphenol	DNA intercalator
Plumbagin	Oxidising agent
Josamycin	Protein synthesis 50S
Gallic acid	Respiration, ionophore
Coumarin	DNA intercalator
Iodonitro tetrazolium violet	Respiration
Phenyl-methyl-sulfonyl-fluoride (PMSF)	Protease inhibitor
Blasticidin S	Protein synthesis
Lauryl sulfobetaine	Membrane
Dihydrostreptomycin	Protein synthesis 30S
Hexammine cobalt (III) chloride	DNA synthesis
Thioglycerol	Reducing agent

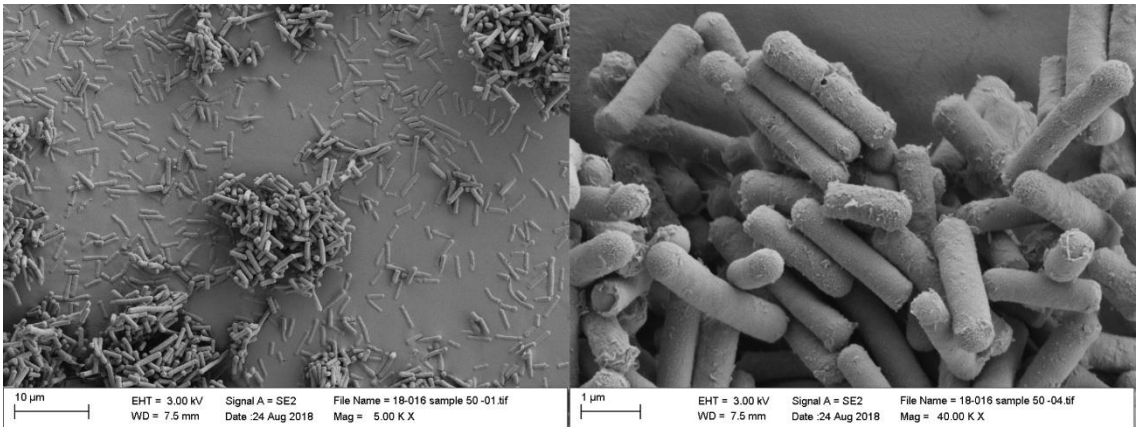
7.3.7. Microscopy

SEM was performed on cultures of *L. amylovorus* Lac 20, *L. crispatus* Lac 50 and Lac 51 (Figure 7.11). Although all three strains presented occasional long chains these were seen to be more frequent in *L. crispatus* Lac 51.

(A)



(B)



(C)

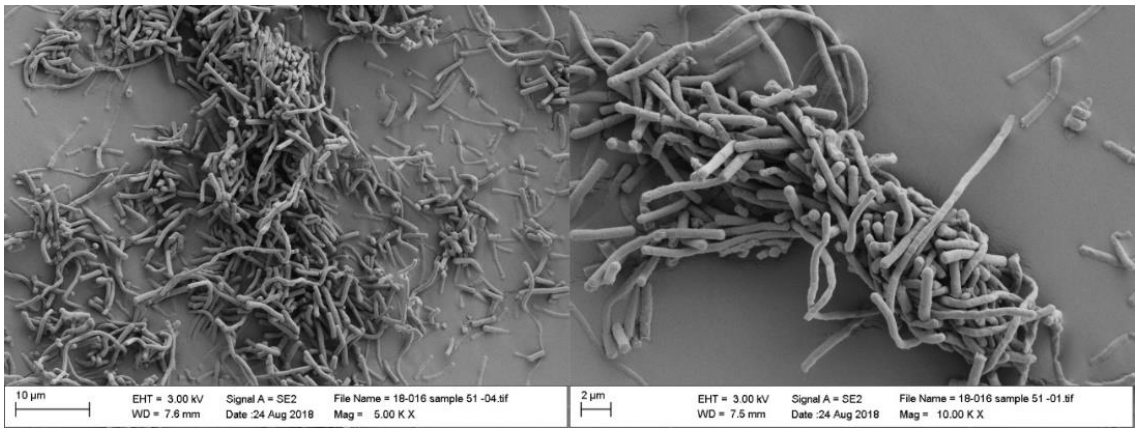


Figure 7.11. SEM images at different scales for (A) *L. amylovorus* Lac 20, (B) *L. crispatus* Lac 50 and (C) *L. crispatus* Lac 51.

7.4. Discussion

In this chapter the antimicrobial activity from cultures of *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51 was demonstrated. Different strategies were used with the aim of identifying the putative antimicrobial peptide produced by *L. amylovorus* Lac 20 and the putative antimicrobial protein produced by *L. crispatus* Lac 50, which could be the same as that produced by *L. crispatus* Lac 51. Additional characterisation of these isolates was performed to study potential probiotic traits.

The antimicrobial activity of *L. amylovorus* Lac 20 is very likely to have a peptidic origin based on gel overlay assays and size exclusion. The absence of results obtained from the BAGEL4 analysis suggests either the presence of a totally new system or the absence of key data in the databases and algorithms that could potentially identify the gene or genes related to this potential peptide. The 24 proteins and peptides, or their modifications, obtained by trypsinisation of the SDS gel bands did not match the expected 5 kDa size expected from the results of the SDS gel overlayed with the indicator strain. The manual curation of the genomic environment of these 24 proteins and peptides did not show homologies to bacteriocin operons either. The search for homologies to lactacin X, amylovorin L471 and lactobin A also did not show any matches.

It can be hypothesised, based on gel overlay assays, that the antimicrobial activity linked to *L. crispatus* Lac 50 is produced by a protein of an approximately 30 kDa. The loss of activity of the supernatant when heated and in the presence of acetonitrile also suggest a protein origin. In chapter III, section 3.3.5 summarised the five operons encoding putative antimicrobial proteins that were identified in the *L. crispatus* genomes of both Lac 50 and Lac 51 using BAGEL3. However, since the protein responsible for the observed antimicrobial activity has not been isolated yet, it has not been possible to link the activity to a structural gene and its operon. It has been reported that some bacteriocins can form aggregates of a molecular mass of around 30 kDa [384]. However, these aggregates were separated under reduced conditions by SDS gel [384]. The supernatants of *L. crispatus* Lac 50 and Lac 51 did not show these separations.

It was notable that two closely related strains of *L.* crispatus isolated from the same environment showed such differences in behaviour with regard to biofilm formation and adhesion. The differences in behaviour observed between the two strains of *L. crispatus* Lac 50 and Lac 51 could be linked to some of their genomic differences. The surface S-layer

proteins in *L. crispatus* have been connected with its ability to adhere to the human intestinal cell line HT29 [385]. A putative lactocepin S-layer and a S- layer protein precursor were found in the genome of *L. crispatus* Lac 51 but were absent in *L. crispatus* Lac 50. This might explain the higher rate of bacterial adhesion of *L. crispatus* Lac 51 to HT29 and the lower rates of adhesion of *E. coli* LMG 2092, *S. enterica* LT2 and *C. sakazakii* LMG 5740 to HT29 in the presence of *L. crispatus* Lac 51; this could be considered competitive exclusion and has been reported previously [375]. This difference in the cell surface had the potential to result in a different response to antibiotics. Despite the fact that differences were observed, since *L. crispatus* Lac 50 grew better than *L. crispatus* Lac 51 in the presence of 88 antimicrobials while *L. crispatus* Lac 51 grew better than *L. crispatus* Lac 50 in only 18, this might be due to a slower growth behaviour in Lac 51 that was observed throughout the study.

It has been observed that aggregation can be mediated by an aggregation promoting factor (APF) similar to the S-layer protein [386]. Aggregation abilities have been proven to affect the adhesion to the mucosa of *L. crispatus* and its survival in the GIT [386]. Both *L. crispatus* Lac 50 and Lac 51 exhibited similar levels of auto-aggregation *in vitro* and both have in their genome the gene encoding the APF and the putative elongation factor Tu, which has also been associated with autoaggregation and adhesion [387]. The regulation of this factor has been linked to phenotypic changes between aggregative and non-aggregative isogenic mutants of *L. crispatus* [388], so there could be other factors important to cell surface quality than just the presence or absence of a gene. More phenotypic changes involve differences in growth rates that are related to enzymes associated with carbohydrate transport and metabolism, more specifically PTS system, GTP and aminopeptidase, among others [388]. These genetic elements have been identified in regions that show differences between *L. crispatus* Lac 50 and Lac 51. This could explain the differences observed in culture phenotype between both strains, including the adhesion to biotic and abiotic surfaces, where *L. crispatus* Lac 50 is able to adhere better than *L. crispatus* Lac 51. Autoaggregation and adhesion are desirable phenotypic traits that are considered probiotic because they confer the potential to colonise the gastrointestinal tract and avoid being washed out [389].

Following results on section 3.3.6., *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51 exhibit bile salt hydrolase activity in presence of at least to one bile salt. Bile salt hydrolase

activity has been proven to interfere with bile recycling in the intestine, forcing the liver to move stored cholesterol [243]. This would help to lower cholesterol levels and it is a highly desirable characteristic that is already being exploited to design probiotic beverages [390] and has been observed in these species previously [251]. Further work would measure quantitatively the reduction rates of this BSH activity and compare it with the activity of other marketed probiotics. Antioxidant levels are a desirable trait. However, when the strains were cultured in appropriate conditions, the percentage of reduction of DPPH was very low, suggesting that further work would be needed to determine whether these strains have more antioxidant potential than that exhibited here and if it can be regulated or enhanced, as it has been observed with the SCFA, using different carbon sources. Simple sugars such as glucose, lactose and galactose, increased SCFA production, especially of lactic and acetic acids, although very little differences were observed in the production of propionic and butyric acids, which have been linked to the gut-brain axis as molecules that could be involved as regulators in a series of complex processes, like appetite and anxiety control, among others [391].

In conclusion, *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51 are isolates that show interesting capabilities in terms of antimicrobial and other probiotic characteristics and should be further studied to establish their full potential.

CHAPTER VIII

Conclusions and Future Work

The work in this PhD thesis confirms our initial hypothesis that mining bacteria from the same niche as that occupied by target pathogens will identify novel antimicrobials that are uniquely suited to act in this niche. More specifically, gut and food isolates from culture collections in QIB and Teagasc, along with new strains isolated from the same sources, showed antimicrobial activity against gut and food pathogens. Those isolates with antimicrobial activity were investigated and the ones whose activity was potentially due to bacteriocin production were followed. Bacteriocin production was confirmed, and further characterisation was performed by the identification of their biosynthetic gene clusters and purification of the associated peptides or proteins for further characterisation. These analyses involved the examination of host range, the assessment of structure and/or the gene expression response to environmental conditions. Additionally, the stability and activity of these peptides were tested in faecal fermentations in complex environments to study their effect on the native microbiota.

The initial stages of the PhD project involved the identification of bacteria that exhibited antagonistic activity against a range of pathogens that were selected based on their importance as agents of food spoilage or gut infection. 313 isolates, 17% of 2100 isolates, showed antimicrobial activity against at least one indicator strain, a percentage that was similar to those reported previously [63, 392]. The isolation of bacteria with antagonistic activity from faecal samples presented three main challenges: the bacteria were only selected because they were able to grow in the specific media, what is known as plate count anomaly. In some cases bacteria were able to exhibit antimicrobial activity, but either lost it in the later experiments or were not able to be subcultured again. The last two situations are very likely to happen due to the loss of specific factors from the faecal material that are not available when subcultured. These factors might include nutritional requirements for growth and inducers to trigger antimicrobial activity, either molecules or other bacteria. Alternative approaches developed by other groups for antimicrobial discovery include genome mining and the development of Hidden Markov Models to analyse metagenomes [393] or using heterologous gene expression to be able to express previously identified antimicrobial genes [394, 395]. The food screenings also presented difficulties related to the variety of species that were recovered. All fermented foods tested (kefir, gherkins,

yogurt and miso) were not produced by natural starter cultures, but manufactured commercially, which might decrease their bacterial diversity.

The type of bioassay was a factor that seemed to be critical for the identification of antimicrobial-producing strains. Bioassays that required contact with the indicator strain, such as the overlay and cross-streak assays, most frequently yielded antimicrobial activity, in contrast with assays using cell-free supernatant, such as the drop test, filter disc and well-diffusion assays. Only cell-free supernatant of four isolates, *L. gasseri* LM19, *L. amylovorus* Lac 20, *L. crispatus* Lac 50 and *L. crispatus* Lac 51, exhibited antimicrobial activity against at least one indicator strain. This suggests that in many instances the inter-strain contact is required to trigger antagonistic mechanisms not required in a non-competition environment. However, the lack of activity in supernatants might also be due to a low concentration of the antimicrobial [396]. Bacterial colonies are also more densely populated than liquid culture, and therefore, quorum sensing might be another mechanism underlying antimicrobial production [397]. Colony MS was performed on bacterial colonies, revealing potential peptide masses that were further studied, although this information should be considered as supportive and not as definitive with respect to antimicrobial identification.

Some of the isolates that showed interesting characteristics were the subject of genome sequencing, and the analysis of their genomes allowed us to make decisions for further work, based on the identification of putative bacteriocin gene clusters within these genomes. A total of 30 potential antimicrobials in 24 operons were predicted by BAGEL 3. However, BAGEL 3 was not able to identify bacteriocin clusters in all genomes, as was the case for *L. amylovorus* Lac 20. For other organisms, it identified operons of potential interest but which only contained some genes that represented those typically associated with bacteriocin operons. Thus, manual curation of BAGEL results is recommended to avoid false positives and, when possible, linking with experimental data. The presence of these putative bacteriocin operons and the predicted masses of their potential structural genes was correlated with the mass information obtained by colony MS, allowing a targeted selection of isolates for further study. Therefore, this combination of techniques can be used as part of a strong pipeline to identify new antimicrobials for further characterisation. *L. gasseri* LM19 was selected for deeper study following those criteria. It is a breast milk-derived bacterium whose genome encodes four bacteriocins, one of them being a novel

class IIb bacteriocin, which was named gassericin M. The work performed proved both its ability to show antagonist activity against enteropathogens and to survive in a colon environment. *L. gasseri* is a species for which several class IIb bacteriocins have been described in the past. However, many of them are similar, being variants that have received different names. A revision of the known gassericin names would be useful to improve clarity for future work. *L. gasseri* LM19 carries genes that encode bacteriocins homologous to gassericin T and gassericin S. Peptides with masses matching the expected size of the class IIb peptides encoded in the genome of *L. gasseri* LM19 were partially purified, and due to the low yield, they were also synthesised, for testing purposes. Both the fractions with the predicted masses and the synthesised peptides exhibited antimicrobial activity.

These bacteriocin genes showed a differential expression response to different carbon sources and different growth media. This line of work can be further developed to optimise the conditions for bacteriocin production. However, scale up of bacteriocin production can be a challenging process, since many elements can interfere with production, such as the presence of NaCl. Bacteriocins can also be adsorbed to the producer cells after a long fermentation process, and so specific strategies may need to be employed for different bacteriocins [364].

This work also demonstrates that *L. gasseri* LM19 is able to survive in simulated colon conditions, a characteristic that is desirable in an organism that aims to be used to control gut pathogens *in situ*. *L. gasseri* LM19 was able to express the putative bacteriocin genes in that environment and its addition showed mixed effects controlling a population of *C. perfringens* added to faecal fermentations. However, the metataxonomics profiles in the *C. perfringens* treatments where *L. gasseri* was added showed that bacterial composition changes were less acute than in the presence of *C. perfringens* alone. SCFA production measured by NMR also showed variability between the three donors, but it was increased in the *L. gasseri* LM19 treatments. The pre-established microbial communities might explain the different effects that probiotic intake has in different people [398, 399]. However, further studies would be needed in this front since, despite the suitability of colon models to assess certain characteristics like survival in colon conditions, the model does not integrate other aspects, like dietary and inflammatory factors [400].

Future work with *L. gasseri* LM19 might involve combination with prebiotics to form synbiotics. As shown here, a suitable prebiotic, like inulin or starch might increase the

production of butyrate and the gene expression of putative bacteriocins. This could increase the potential beneficial effects of *L. gasseri* LM19. Another possibility is microencapsulation, to ensure higher numbers of *L. gasseri* LM19 are able to arrive to the colon.

Lactobacilli are often found to be producers of antimicrobial activity and have been extensively studied as potential probiotics [401]. *L. amylovorus* Lac 20 and *L. crispatus* Lac 50 and Lac 51 are three isolates which were able to show antimicrobial activity and were studied further for probiotic traits. The antimicrobial activity is believed to be displayed by a peptide in the case of *L. amylovorus* Lac 20 and by a protein in the case of *L. crispatus* Lac 50 and Lac 51. However, although a number of methods were attempted for purification, further work is needed to confirm this hypothesis. The next steps could involve the use of N terminal sequencing once the peptide and proteins are separated and concentrated. With this information it would be possible to identify the structural genes in the genome. Knocking out those potential genes and correlating this with the loss of antimicrobial activity would give us confirmation that they are the correct genes. The absence of potential identities by *in silico* searches suggests that, in the case of *L. amylovorus* Lac 20, the antimicrobial molecule and machinery might be completely novel.

The phenotypic differences observed between the two *L. crispatus* strains Lac 50 and Lac 51 could also be explored further. It is likely that they are not only due to genomic differences, but also due to different responses to the environmental conditions, that could be addressed with transcriptomics and proteomics.

Nisin P, a natural nisin variant, was previously identified by genome mining in two other streptococcal species, but this work is the first report of its activity and its structure confirmation. The study of its cluster in *S. agalactiae* DPC7040 showed that the *nipPRKFEG* genes were translocated to the front of the structural gene, something that was observed previously in the nisin U operon. The study of the NisFEG proteins involved in immunity also showed that this system might have a more important role in lantibiotic immunity than previously suspected. Nisin P was purified, and its antimicrobial activity was characterised in comparison to nisin A and H. Overall, nisin P exhibited less potent antimicrobial activity than nisin A against the tested strains and in a complex environment such as a faecal fermentation. Nisin P structure was confirmed by nanoLC-MS, showing that the rings B and E were only present partially, which might explain some differences in bioactivity, based on

bioengineering studies performed in the past. Other nisin P structural features which might be related to the lower activity involve the two different amino acids in the hinge region in positions 21 and 22, or the three amino acids shorter at the C-terminal region of the molecule, when compared with nisin A. This structure seems to be specific also for the receptor, based on the higher levels of nisin P that were required to activate the nisin A promotor. Further work would include the use of nisin P and /or U as inducers with cognate receptors. The understanding of structure-activity relationships of nisin P can be applied to other lantibiotics and to the further development of more efficient antimicrobials [90].

In addition to the study of bacteriocin producers, this work provides the first genomic reference of *S. epidermidis* isolates of human stool origin. Three stool-isolated *S. epidermidis* strains were sequenced and localised in a phylogenomic tree constructed with the rest of the available *S. epidermidis* genomes from different isolation origin. Although the three genomes did not cluster in the phylogenomic tree, 44 genes were significantly associated with the stool isolates. All three stool isolates also contained a type V ACME gene cluster, linked to colonisation and evasion of the immune system, previously reported in mouth isolates. Study at the genomic level did not show major differences between stool isolates and the rest of the genome-wide *S. epidermidis* pangenome. However, phenotype comparison between stool and skin isolates showed metabolic behaviour that could indicate some level of plasticity to adapt to the different conditions within the human body. A phenotype-genotype association between the BIOLOG results on utilisation of carbon and nitrogen sources showed that some of those genes that were significantly associated with stool isolates were involved in metabolism.

Both skin and stool *S. epidermidis* isolates showed very similar growth curves when grown in the presence and absence of bile in aerobic conditions. However, stool isolates were able to grow faster in anaerobic conditions, at similar rates in the presence and absence of bile, while the growth of skin isolates was slower. In the first hours, the absence of bile was beneficial for the growth but, after that, the skin isolates grown in the presence of bile showed higher growth levels. This correlates with the depletion in the glycine-conjugated forms of the bile acids, a phenomenon that might indicate the use of these glycine molecules as nutrients. Nonetheless, no bile salt hydrolase genes were discovered through genome analysis. This indicates either the need to improve annotation of bile hydrolysing genes or that the hydrolase activity is being carried out by other enzymes or metabolic

mechanisms. The increase of secondary bile acids also suggests some kind of bile acid transformation. Further work would involve the use of transcriptomics, proteomics and metabolomics to identify the proteins and/or routes involved in this relationship between *S. epidermidis* and bile acids.

The work on *S. epidermidis* and *L. crispatus* strains shows the importance of not only thinking in terms of genomic information to understand the behaviour of human-associated bacteria, but also how their ability to adapt might rely on gene expression. Transcriptomics, proteomics and metabolomics would be the next steps to unveil and understand how *S. epidermidis* reorganises its metabolism to adapt to the environment. However, this raises the challenge of avoiding laboratory domestication. Understanding these adaptations to the different body sites might allow the discovery of new therapeutic targets and the development of effective treatments that could prevent the development of antibiotic resistance.

Overall, this work reinforces the idea that food and gut microbes are a rich source of both novel antimicrobials and new strains whose study can further our knowledge of bacterial adaptation to environment. Understanding and exploiting this adaptation would be the key for a rational design and application of antimicrobials that would help against the development of antimicrobial resistance.

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APPENDICES

Appendix 1

Table S1. Class I bacteriocins

Bacteriocin	Produced by	Isolated from	Doi / reference
Anacyclamide (AcyE)	*Anabaena* sp. SYKE763A	Lake	10.1128/AEM.01061-09
Astexin	*Asticcacaulis excentricus*	Fresh water	10.1039/c2np20085f.
Avermipeptin	*Streptomyces avermitilis* DSM 46492	Soil	10.1002/cbic.201200118 https://www.dsmz.de/catalogues/details/culture/DSM-46492.html
BLD 1648	*Bifidobacterium longum* (strain DJO10A)	Human intestine	10.1186/1471-2164-9-247
Bottromycin A2	*Streptomyces* sp	Soil	10.1093/oxfordjournals.jbchem.a12845125;251(8):2299-306.
Bottromycin D	*Streptomyces scabiei* 87.22	Soil	10.1111/j.1364-3703.2009.00561.x.
Bovicin HJ50	*Streptococcus bovis*	Raw milk	doi.org/10.1099/mic.0.26437-0
BsaA2	*Staphylococcus aureus* (strain MW2)	Septicemia	MMWR Morb Mortal Wkly Rep. 1999 20;48(32):707-10.
Butyrivibriocin	*Butyrivibrio fibrisolvens* AR10	Rumen	Appl Environ Microbiol. 1997 63(2): 394–402.
Butyrivibriocin OR79	*B. fibrisolvens* OR79A	Rumen	Appl Environ Microbiol. 1999 65(5):2128-35
Catenulipeptin	*Catenulispora acidiphila* DSM 44928	Forest soil	10.1021/cb3002446 https://www.dsmz.de/catalogues/details/culture/DSM-44928.html
Cinnamycin (Ro09-0198)	*Streptoverticillium griseoverticillatum* MAR 164CMY6, synonym of *Streptomyces cinnamoneus*	Soil	199(2):411-5. https://www.lgcstandards-atcc.org/products/all/19769.aspx?geo_country=ie#generalinformation https://www.dsmz.de/catalogues/details/culture/DSM-40114.html
Cypemycin	*Streptomyces* sp. OH-4156	Soil	10.7164/antibiotics.46.1666
Cytolysin ClyLl and ClyLs	*Enterococcus faecalis*	Gut	Cell Microbiol. 2003 5(10):661-9 10.1038/415084a
Duramycin (Leucopeptin)	*S. griseoverticillatum*, synonym of *S. cinnamoneus*	Soil	10.1021/ja01548a029 https://www.lgcstandards-atcc.org/products/all/19769.aspx?geo_country=ie#generalinformation https://www.dsmz.de/catalogues/details/culture/DSM-40114.html
Duramycin B	*Streptoverticillium* sp (strain R2075)	Soil	10.7164/antibiotics.43.1403
Duramycin C	*Streptomyces griseoluteus* R2107	Soil	10.7164/antibiotics.43.1403
Enterocin_W	*E. faecalis* NKR-4-1	Pla-ra, Thai fermented fish	10.1128/AEM.06497-11
Entianin	*Bacillus subtilis* subsp. *spizizenii*	Desert	10.1128/AEM.01962-10 https://www.dsmz.de/catalogues/details/culture/DSM-15029.html
Epidermin	*Staphylococcus epidermidis* TÜ 3298	Skin	10.1007/BF00256208 https://patents.google.com/patent/EP0431350A1/fi 10.1128/genomeA.00112-16
Epilancin 15X	*S.epidermidis* 15X154	Wound infection	10.1016/j.febslet.2005.01.083
Ericin A	*B. subtilis*	Soil	10.1111/j.1365-2958.2005.04587.x
Ericin S	*B. subtilis*	Soil	10.1111/j.1365-2958.2005.04587.x
Erythreapeptin	*Saccharopolyspora erythraea* NRRL 2338	Soil	10.1002/cbic.201200118 https://www.lgcstandards-atcc.org/products/all/11635.aspx?geo_country=ie
FrEUN1f 0188 putative linardin	*Frankia* sp. EUN1f	Root	10.1007/978-94-011-5232-7_38 10.1073/pnas.1008608107

Gallidermin	*Staphylococcus gallinarum* Tü 3928	Chicken skin	10.1023/A:1005667406165
Gardimycin (actagardine)	*Actinoplanes liguriae* ATCC 31048	Soil	10.7164/antibiotics.52.730 https://www.lgcstandards-atcc.org/products/all/31048.aspx?geo_country=ie
Geobacillin I	*Geobacillus thermodenitrificans* NG80-2	Subsurface oil reservoir	10.1073/pnas.0609650104 10.1073/pnas.1116815109
Geobacillin I like	*Geobacillus kaustophilus* HTA426	Sea mud Mariana Trench	10.1128/AEM.01669-12
Geobacillin II	*G. thermodenitrificans* NG80-2	Subsurface oil reservoir	10.1073/pnas.1116815109
Glycocin F	*Lactobacillus plantarum* KW30	Fermented corn	10.1111/j.1365-2672.1996.tb03561.x 10.1002/chem.201405692
Grisemycin	*Streptomyces griseus* NBRC 13350	Soil	10.1128/JB.00204-08 10.1128/JB.00171-11
Griseopeptin	*S. griseus* DSM 40236	Soil	10.1002/cbic.201200118 https://www.dsmz.de/catalogues/details/culture/dsm-40236.html
Haloduracin	*Bacillus halodurans* C-125	Soil	10.1073/pnas.0606088103 https://www.lgcstandards-atcc.org/products/all/BAA-125.aspx?geo_country=ie
Htur 3018 putative linardin	*Haloterrigena turkmenica* DSM 5511	Soil	https://www.dsmz.de/catalogues/details/culture/DSM-5511.html
Labyrinthopeptin A1/A3	*Actinomadura namibiensis*	Soil	10.1099/ijs.0.02286-0 10.1002/anie.200905773
Labyrinthopeptin A2	*A. namibiensis*	Soil	10.1099/ijs.0.02286-0 10.1021/bi200526q
Lacticin 3147	*Lactococcus lactis* subsp. *lactis*	Irish kefir grain	Appl Environ Microbiol. 1996 62(2):612-9.
Lacticin 481 (Lactococcin DR)	*L. lactis* subsp *lactis* CNRZ 481	Dairy isolate	https://patents.google.com/patent/CA2695487A1/no 10.1007/978-1-4615-2668-1_7
Lactocin S	*Lactobacillus sakei* L45	Dry fermented sausage	Appl Environ Microbiol. 1991 57(6):1829-34.
Lantibiotic 107891	*Microbispora* sp. 107891	Environment	https://patents.google.com/patent/CA2695487A1/no
Lariatin A	*Rhodococcus* sp. K01-B0171	Soil	10.1021/ja056780z 10.1038/ja.2007.48
Lariatin B	*Rhodococcus* sp. K01-B0171	Soil	10.1021/ja056780z 10.1038/ja.2007.48
Lichenicidin VK21	*Bacillus licheniformis* VK21	Thermal springs	10.1021/bi100871b 10.1023/A:1015463122840
Lxx19440 putative_linardin	*Leifsonia xyli* CTCB07	Plant pathogen	10.1094/MPMI.2004.17.8.827
Lxx19470 putative linardin	*L. xyli* CTCB07	Plant pathogen	10.1094/MPMI.2004.17.8.827
Macedocin	*Streptococcus macedonicus* ACA-DC 198	Greek cheese	10.1128/aem.68.12.5891-5903.2002
Macedovicin	*S. macedonicus* ACA-DC 198	Greek cheese	10.1016/j.fm.2012.09.008
McdA1	*S. macedonicus* ACA-DC 198	Greek cheese	10.1016/j.idairyj.2007.10.006
Mersacidin	*Bacillus* sp (strainHIL-Y85/54728)	Soil	10.7164/antibiotics.45.832
Michiganin A	*Clavibacter michiganensis* subsp. *michiganensis*	Tomato pathogen	10.1128/AEM.00639-06
Microbisporicin NAI-107	*Microbispora corallina*	Environment	10.1073/pnas.1008285107 10.1016/j.chembiol.2007.11.009 10.1021/bi700131x
Microcin B17 (MccB17)	*Escherichia coli* LR05	Human gut baby	10.1099/mic.0.26396-0 10.1002/prot.340010305
Microcin E492	*Klebsiella pneumoniae* RYC492	Human faeces	10.1007/BF00692715 10(1):74-85.

Microcin H47 (MccH47)	*E. coli* H47	Human faeces	10.1128/jb.172.11.6585-6588.1990
Microcin L	*E. coli* LR05	Human faeces	10.1111/j.1574-6968.2000.tb09408.x
Microcin M	*E. coli* K12 MC4100	Human faeces	10.1099/mic.0.26396-0
Microcin V (ColicinV)	*E. coli*	human faeces	10.1128/MMBR.00036-06
Microcyclamide	*Microcystis aeruginosa* NIES-298	Freshwater	10.1021/np000159p 10.1016/S0040-4039(00)76848-1
Mutacin 1140 (MutacinIII)	*Streptococcus mutans* JH1140	Spontaneous mutant of JH1000 (oral cavity)	66(6): 2743–2749 https://www.lgcstandards-atcc.org/products/all/55677.aspx?geo_country=ie
Mutacin B Ny266	*S. mutans*	Human mouth	10.1016/s0014-5793(97)00425-0 10.1139/m89-056
Mutacin II (Mutacin H-29B)	*S. mutans*	Human mouth	10.1139/m89-056 10.1186/1471-2180-6-36
Mvan 2782 putative linardin	*Mycobacterium vanbaalenii* PYR-1	Oil contaminated sediment	10.1128/JB.00215-11 10.1007/s10532-008-9189-z
NAI-802	*Actinoplanes* sp. DSM 24057	Environment	10.1038/ja.2012.92 https://patents.google.com/patent/US20140094402A1/en
Nisin A	*L. lactis*	Milk	10.1111/j.1751-7915.2010.00207.x
Nisin F	*L. lactis* F10	Freshwater catfish	10.1128/AEM.01862-07
Nisin Q	*L. lactis* 61-14	River water	10.1271/bbb.67.1616
Nisin U	*Streptococcus uberis*	Bovine mastitis	10.1128/AEM.72.2.1148-1156.2006
Nisin Z	*L. lactis* NIZO 221 86	Dairy isolate	10.1111/j.1432-1033.1991.tb16317.x
Nmul A1818 putative linardin	*Nitrosospira multiformis* ATCC 25196	Soil	10.1073/pnas.1008608107 https://www.lgcstandards-atcc.org/products/all/25196.aspx?geo_country=ie
Nosiheptide	*Streptomyces actuosus* ATCC 25421	Soil	10.1007/BF01575585 https://patents.google.com/patent/US3057779 https://www.lgcstandards-atcc.org/Products/All/25421.aspx?geo_country=ie#generalinformation
Nukacin A (NukacinISK-1)	*Staphylococcus warneri* ISK-1	Nukadoko, fermented rice bran	10.1271/bbb.64.2420
Paenibacillin	*Paenibacillus polymyxa* OSY-DF	Kimchee	10.1128/AEM.02023-06
Patellamide A	*Prochloron* sp.	Cyanobacterial symbiont	10.1073/pnas.0501424102
Pep5	*S. epidermidis*	Diagnostic material in laboratory	10.1007/BF00447005
Plantaricin W	*L. plantarum* LMG 2379	Fermented wine	10.1099/00221287-147-3-643
Plantazolicin	*Bacillus amyloliquefaciens* FZB42	Soil	10.1128/JB.00784-10
Prenylagaramide B	*Planktothrixa gardhii* NIES-596	Cyanobacteria	10.3390/md13116910
Prenylagaramide C	*P. gardhii* NIES-596	Cyanobacteria	10.3390/md13116910
Putative lantibiotic	*Streptococcus pyogenes* M1GAS	Human wound infection	10.1073/pnas.071559398
Putative lantibiotic	*S. pyogenes* SSI-1	Human septic shock	10.1101/gr.1096703
Putative lantibiotic	*S. aureus* subsp. *aureus* MSSA 476	Osteomyelitis	10.1073/pnas.0402521101
Putative lantibiotic precursor	*Bacillus cereus* ATCC 14579	Gelatine-dish exposed to the air in a cow-shed	10.1038/nature01582 10.1098/rstb.1887.0011

			https://www.lgcstandards-atcc.org/products/all/14579.aspx?geo_country=ie#generalinformation
Rp 71.955	*Streptomyces* sp AA3891	Soil	10.1007/BF00211754 https://patents.google.com/patent/EP0603030A3/en
Ruminococcin A	*Ruminococcus gnavus*	Human faeces	10.1128/aem.67.9.4111-4118.2001
Salivaricin 9	*Streptococcus salivarius* M18	Human saliva	10.1099/mic.0.044719-0
Salivaricin A	*S. salivarius* 20P3	Human saliva	Appl Environ Microbiol. 1993 59(7):2014-21
Salivaricin B	*S. salivarius* K12	Human saliva	10.1371/journal.pone.0077751 10.1128/AEM.02265-06
Salivaricin A2	*S. salivarius* K12	Human saliva	10.1128/AEM.02265-06
Salivaricin A3	*S. salivarius*	Human saliva	10.1128/AEM.72.2.1459-1466.2006
Salivaricin A4	*S. salivarius*	Human saliva	10.1128/AEM.72.2.1459-1466.2006
Salivaricin A5	*S. salivarius*	Human saliva	10.1128/AEM.72.2.1459-1466.2006
Salivaricin D	*S. salivarius* 5M6c	Human faeces	10.1128/AEM.06588-11
Salivaricin G32	*S. salivarius* G32	Human saliva	10.1155/2012/738503
Salivaricin A	*S. pyogenes* MGAS 10394	Human throat	https://www.lgcstandards-atcc.org/Products/All/BAA-946.aspx?geo_country=ie
Sap B	*Streptomyces coelicolor*	Potato	10.1016/0092-8674(91)90096-H 78:528-38
SCAB 84151 putative linardin	*S. scabiei* 87.22	Potato	10.1111/mpp.12296 http://webcat.warwick.ac.uk/record=b2340081~S15
SCAB 84201putative linardin	*S. scabiei* 87.22	Potato	http://webcat.warwick.ac.uk/record=b2340081~S15
SGR 1511 putative linardin	*S. griseus* IFO 13350	Soil	10.1128/JB.00171-11
SGR 1512 putative linardin	*S. griseus* IFO 13350	Soil	10.1128/JB.00171-11
SGR 1513 putative linardin	*S. griseus* IFO 13350	Soil	10.1128/JB.00171-11
SGR 1514 putative linardin	*S. griseus* IFO 13350	Soil	10.1128/JB.00171-11
SiamycinI (BMY-29304)	*Streptomyces* sp. AA6532	Soil	10.1007/BF00211754
SiamycinII (BMY-29303)	*Streptomyces* sp. AA3891	Soil	10.1007/BF00211754
Smb	*S. mutans* GS5	Human caries	10.1128/AAC.49.2.541-548.2005 10.1128/JB.01106-12
Sporulation-killingfactor skfA	*B. subtilis* (strain168)	Chemically induced from Marburg strain	10.1128/JB.00722-08
Staphylococcin C55 Saca A	*S. aureus* C55	Skin	J Bacteriol. 1969 97(3):985-91
Streptococcin A-FF22	*S. pyogenes*	Human throat	10.1128/aac.4.3.214
Streptococcin A-M49	*S. pyogenes*	Nephritis	J Clin Microbiol. 1976 4(3): 232–238
Sublancin 168	*B. subtilis* (strain168)	Chemically induced from Marburg strain	10.1128/JB.00722-08
Subtilin	*B. subtilis*	Soil	10.3181/00379727-61-15216
SvirD4 22614 putative linardin	*Streptomyces viridochromogenes*	Unknown (possibly soil)	10.1073/pnas.1008608107

Thermophilin 1277	*Streptococcus thermophilus* SBT1277	Raw milk	10.1111/j.1365-2672.2006.03159.x
Thiocillin	*B. cereus* (strain ATCC 14579/DSM31)	Soil	10.1073/pnas.0900008106 http://www.bacdive.dsmz.de/resultpdf.php?resultid=607
Thiocillin GE 37468	*Streptomyces* sp. Waksman and Henrici (ATCC® 55365)	Soil	10.7164/antibiotics.44.693
Thiomuracin A	*Nonomurae* sp.	Soil	10.1021/jm300783c
Thiostrepton A	*Streptomyces azureus*	Soil	10.1007/978-3-642-46403-4_18
Thuricin CD	*Bacillus thuringiensis* DPC 6431	Human faeces	10.1073/pnas.0913554107
Thuricin H (thuricin 17)	*B. thuringiensis* SF361	Honey	10.1111/j.1574-6968.2009.01749.x
Trichamide	*Trichodes miumerythraeum* ISM101	Bloom-forming marine cyanobacterium	0.1128/AEM.00380-06
Trunkamide A	*Lissoclinum sp*	Great Barrier Reef	10.1021/jo9914566
Ulithiacyclamide	*Lissoclinum patella*	Western Caroline Islands	10.1021/ja00537a053
Variacin	*Kocuria varians*	Salami	10.1046/j.1365-2672.2001.01222.x
Venezuelin	*Streptomyces venezuelae*	Soil	10.1371/journal.pbio.1000339
Thiocillin GE2270	*Planobispora rosea*	Soil	10.7164/antibiotics.44.693 10.7164/antibiotics.44.702 https://www.lgcstandards-atcc.org/Products/All/53773.aspx?geo_country=ie
Subtilosin A	*B. subtilis* (strain 168)	Chemically induced from Marburg strain	10.1128/JB.00722-08 10.1093/oxfordjournals.jbchem.a135315
Capistruin	*Burkholderia thailandensis* E264	Soil	10.1021/ja802966g https://www.lgcstandards-atcc.org/products/all/700388.aspx?geo_country=gb
Circularin A	*Clostridium beijerinckii* ATCC 25752	Soil	10.1128/aem.69.3.1589-1597.2003 https://www.dsmz.de/catalogues/details/culture/DSM-791.html
Carnocyclin A	*Carnobacterium maltaromaticum* UAL307	Fresh pork	10.1128/AEM.00817-08
Butyrivibriocin AR10	*Butyrivibriofibri solvens* AR10	Rumen	Appl Environ Microbiol. 1997 63(2): 394–402.
Enterocin AS-48	*E. faecalis* AS-48	Human faeces	10.1139/m86-141
Enterocin AS-48RJ	*E. faecalis* RJ16	Goat cheese	10.1016/j.syapm.2005.01.007
Garvicin ML	*Lactococcus garvieae* DCC43	Mallard ducks	10.1128/AEM.01173-10
Gassericin A	*Lactobacillus gasseri* LA39	Human faeces	10.1271/bbb.62.2438
Lactocyclicin Q	*Lactococcus* sp. QU12	Cheese	10.1128/AEM.02299-08
Leucocyclicin Q	*Leuconostoc mesenteroides* TK41401	Japanese pickles	10.1128/AEM.06348-11
Microcin J25(MccJ25)	*E. coli* AY25	Human faeces	10.1128/jb.174.22.7428-7435.1992
Uberolysin	*S. uberis* 42	Mastitis	10.1099/mic.0.2006/005967-0
Carnolysins A1	*Carnobacterium maltaromaticum* C2	Brazilian smoked fish	10.1016/j.ijfoodmicro.2013.12.019
Carnolysins A2	*C. maltaromaticum* C2	Brazilian smoked fish	10.1016/j.ijfoodmicro.2013.12.019
Ticin A1	*B. thuringiensis* BMB3201	Soil China	10.1128/AEM.01851-15
Ticin A2	*B. thuringiensis* BMB3201	Soil China	10.1128/AEM.01851-15
Ticin A3	*B. thuringiensis* BMB3201	Soil China	10.1128/AEM.01851-15
Formicin	*Bacillus paralicheniformis APC 1576*	Mackerel intestine	10.1099/mic.0.000340

Appendix 2

Table S2. Class II bacteriocins

Bacteriocin	Produced by	Isolated from	Doi / reference
Abp118	*L. salivarius* UCC-118	Human gut	10.1099/00221287-148-4-973
AcdB acidocin	*Lactobacillus acidophilus* M46	Unknown (reference omitted)	10.1099/13500872-141-7-1629 10.1111/j.1574-6968.1994.tb06724.x
Acidocin 8912	*L. acidophilus*	Dairy product	10.1080/00021369.1991.10870929
Acidocin J1132	*L. acidophilus* JCM1132	Human faeces	Appl Environ Microbiol. 1996 62(3):892-7
Acidocin LF221B (Gassericin K7B)	*L. gasseri* LF221B	Human faeces	10.1007/s002530051221
Acidocin A	*L. acidophilus* TK9201	Starter fermented milk	Appl Environ Microbiol. 1995 61(3): 1061–1067
AdDLP	*Anaeromyxobacter dehalogenans*	Soil	10.1371/journal.pone.0002103 10.1016/j.bbrc.2009.07.043
AFP-1	*Streptomyces tendae* Tü901	Soil	J Bacteriol. 1999 181(24): 7421–7429
Amylovorin	*Lactobacillus amylovorus* DCE 471	Corn liquor	10.1016/S0723-2020(96)80003-8
Aureocin A53	*S. aureus* A53	Pasteurised commercial milk	10.1128/aem.68.11.5274-5280.2002
Bac32	*Enterococcus faecium* VRE 200	Human faeces	10.1128/AAC.50.4.1202-1212.2006
Bac43	*E. faecium* VRE 82	Human faeces	10.1128/aem.68.11.5274-5280.2002
Bacteriocin 31	*E. faecalis* YI717	Clinical isolate	10.1128/jb.178.12.3585-3593.1996
Bacteriocin J46	*L. lactis* subsp. *cremoris* J46	Fermented milk	10.1006/anae.1996.0018
Bacteriocin like peptide associated	*S. pyogenes* M1GAS	Wound infection	10.1073/pnas.071559398
Bacteriocin like peptide associated	*S. pyogenes* SSI-1	Human septic shock	10.1101/gr.1096703
Bacteriocin LS2	*L. salivarius* BGH01	Human mouth	10.1016/j.ijantimicag.2012.04.011
Bacteriocin T8	*E. faecium* T8	Human vaginal secretions	10.1128/AEM.00436-06
Bavaricin A	*L. sakei* MI401	Sour doughs	10.1111/j.1365-2672.1993.tb02755.x
Bavaricin MN	*Lactobacillus sake* MN	Meat	62(12): 4529–4535 57(6): 1683–1688
BlpI	*Streptococcus pneumoniae* TIGR4 (previously JNR.7/87)	Human blood	10.1126/science.1061217
BlpJ	*S. pneumoniae* TIGR4 (previously JNR.7/87)	Human blood	10.1126/science.1061217
BlpM	*S. pneumoniae* TIGR4 (previously JNR.7/87)	Human blood	10.1126/science.1061217 10.1128/IAI.01775-05
BlpN	*S. pneumoniae* TIGR4 (previously JNR.7/87)	Human blood	10.1126/science.1061217 10.1128/IAI.01775-05
BlpO	*S. pneumoniae* TIGR4 (previously JNR.7/87)	Human blood	10.1126/science.1061217 10.1128/IAI.01775-05
BlpU	*S. thermophilus* 18311	Yogurt	10.1038/nbt1034
Bovicin 255 peptide	*Streptococcus* sp. LRC0255	Rumen	10.1128/AEM.67.2.569-574.2001
Brochocin C	*Brochothrix campestris* ATCC 43754	Soil	59(7): 2326–2328 https://www.lgcstandards-atcc.org/products/all/43754.aspx?geo_country=ie

BTL	*B. subtilis* B-TL2	Tobacco stems	10.1016/j.peptides.2007.11.024
Carnobacteriocin A (Piscicolin 61)	*Carnobacterium piscicola* LV61	Meat	Current Microbiology August 199429(2):63-8
Carnobacteriocin B2	*C. piscicola* LV17	Vacuum-packed meat	Appl Environ Microbiol. 1996 62(11):4095-9 Appl Environ Microbiol. 1990 56(8):2503-10
Carnobacteriocin BM1 (CarnobacteriocinB1)	*C. piscicola* LV17B	Vacuum-packed meat	10.1128/MMBR.00016-05
Carnocin CP52	*C. piscicola* CP5	Ripened cheese	Curr Microbiol. 1997 35(6):319-26
Cerein 7B	*B. cereus* Bc7	Soil	10.1111/j.1574-6968.2005.00009.x 10.1111/j.1574-6968.1999.tb08696.x
Coagulin A	*Bacillus coagulans* I4	Cattle faeces	10.1046/j.1365-2672.1998.00466.x
Colicin V	*Xylella fastidiosa* 9a5c	Infected twigs of orange tree	10.1089/omi.2005.9.43 https://www.ncbi.nlm.nih.gov/bioproject?cmd=ShowDetailView&TermToSearch=271
Curvacin A	*Lactobacillus curvatus* LTH1174	Dry sausages	10.1016/S0723-2020(11)80223-7
Curvaticin FS47	*L. curvatus* FS47	Retail meat	10.1016/0168-1605(93)90017-b Appl Environ Microbiol. 1994 60(6):2191-5
Curvaticin L442	*L. curvatus* L442	Fermented sausage	10.1007/s10482-005-9004-3
Delta-lysin I	*S. warneri* RK	Environment	10.1016/j.peptides.2008.01.017 10.1016/j.femsle.2005.03.046
Divercin RV41	*Carnobacterium divergens* V41	Construction	10.1159/000104756
Divercin V41	*C. divergens* V41	Fish viscera	10.1099/00221287-144-10-2837
Divergicin 750	*C. divergens* 750	Laboratory stock, Bundesanstalt fur Fleischforschung, Kulmbach. Germany,	10.1111/j.1574-6968.1996.tb08043.x
Divergicin A	*C. divergens* LV13	Fish	62(11):4095-9 10.1128/jb.177.11.3143-3149.1995
Divergicin M35	*C. divergens* M35	Smoked mussels	10.1016/j.ijfoodmicro.2004.04.013
Durancin TW-49M	*Enterococcus durans* QU49	Carrot	10.1111/j.1365-2672.2008.03798.x
E50-52	*E. faecium*	commercial broilers	10.1021/jf073284g
Enterocin P-like	*E. faecium* P 13	Dry fermented sausage	Appl Environ Microbiol. 1997 63(11):4321-30
EJ97enterocin	*E. faecalis* EJ97	Waste water	10.1007/s002030050678
EntA	*E. faecium* PLBC21 T136	Wood pigeons	10.1007/s00253-007-1044-3
Enterocin 1071	*E. faecalis* BFE 1071	Minipig faeces	10.1128/aem.66.4.1298-1304.2000
Enterocin A	*E. faecium* CTC492	Dry fermented sausage	Appl Environ Microbiol. 1996 62(5):1676-82
Enterocin B	*E. faecium* T136	Dry fermented sausage	10.1099/00221287-143-7-2287
Enterocin CRL35	*Enterococcus mundtii* CRL35	Cheese	10.1128/AAC.48.7.2778-2781.2004
Enterocin E-760	*Enterococcus* sp. NRRL B-30745	Chicken ceca	10.1128/AAC.01569-06
Enterocin L50	*E. faecium* L50	Dry fermented sausage	Appl Environ Microbiol. 1995 61(7): 2643–2648

Enterocin NKR-5-3A	*E. faecium* NRK-5-3	Fermented fish	10.1271/bbb.110972
Enterocin NKR-5-3D	*E. faecium* NRK-5-3	Fermented fish	10.1271/bbb.110972
Enterocin NKR-5-3Z	*E. faecium* NRK-5-3	Fermented fish	10.1271/bbb.110972
Enterocin P	*E. faecium* P13	Dry sausages	Appl Environ Microbiol. 1997 63(11):4321-30
Enterocin Q	*E. faecium* L50	Fermented sausage	10.1128/jb.182.23.6806-6814.2000
Enterocin RJ-11	*E. faecalis* RJ-11	Rice bran	10.1128/aem.69.10.5546-5553.2003
Enterocin SE-K4	*E. faecalis* K4	Grass silage	10.1128/MMBR.00016-05
Enterocin X	*E. faecium* KU-B5	Sugar apples	10.1128/AEM.02264-09
Enterocin-HF	*E. faecium* M3K31	Griffon vultures faeces	10.1021/acs.jafc.5b03882
Enterocin M	*E. faecium* AL-41	Environment	10.17221/21/2016-CJAS
EP-20	*Xenorhabdus budapestensis* NMC-10	Entomopathogenic bacteria	10.1016/j.peptides.2012.03.027
Fulvocin C	*Myxococcus fulvus* Mx f16	Soil	10.1007/bf00405407
Garvieacin Q	*L. garvieae* BCC 43578	Nham (fermented pork sausage)	10.1128/AEM.06891-11
Gassericin T	*L. gasseri* SBT 2055	Human faeces	10.1271/bbb.64.2201
GP-19	*X. budapestensis* NMC-10	Entomopathogenic bacteria	10.1016/j.peptides.2012.03.027
Halocin C8	*Halobacterium* sp AS7092	Great Chaidan Salt Lake	10.1007/s00792-003-0335-6
Hiracin JM79	*Enterococcus hirae* DCH5	Mallard duck	10.1128/AEM.02559-07 10.1016/j.ijfoodmicro.2007.04.012
Hominicin	*Staphylococcus hominins* MBBL2-9	Vagina	10.1016/j.bbrc.2010.07.024 10.1111/j.1365-2672.2009.04485.x
Enterocin 96	*E. faecalis* WHE 96	Munster cheese	10.1128/AEM.02772-08
Ipomicin	*Streptomyces ipomoeae* 91-03	Sweet potato pathogen	10.1128/AEM.01598-08
Lactacin F	*Lactobacillus johnsonii*	Human faeces	10.1007/978-1-4615-2668-1_14 60(3):1006-13
Lacticin Q	*L. lactis* QU5	Fresh corn	10.1128/AEM.02286-06
Lacticin Z	*L. lactis* Q14	Horse intestine	10.1271/bbb.70169
Lactocin 705	*Lactobacillus casei* CRL 705	Dry fermented sausage	10.1016/S0944-5013(99)80015-9
Lactococcin 972	*L. lactis* subsp. *lactis* IPLA 972	Cheese	10.1099/00221287-142-9-2393
Lactococcin A	*Clostridium perfringens* strain SM101/TypeA	Derivative of NCTC 8798 isolated from meat	J Bacteriol. 1998 180(1):136-42
Lactococcin A	*L. lactis* subsp. *cremoris* LMG 2130	Milk	173(12): 3879–3887 10.1099/ijs.0.023945-0
Lactococcin B	*L. lactis* subsp. *cremoris* 9B4	Milk	10.1099/ijs.0.023945-0 59(4):1041-8
Lactococcin G	*L. lactis* LMG2081	Pear	174(17): 5686–5692 10.1128/AEM.03988-15
Lactococcin MMFII	*L. lactis* MMFII	Dairy product	10.1111/j.1574-6968.2001.tb10924.x
Lactococcin Q	*L. lactis* QU4	Corn	10.1128/AEM.72.5.3383-3389.2006
Laterosporulin	*Brevibacillus* sp. GI-9	Soil	10.1371/journal.pone.0031498
Leucocin A (LeucocinA-UAL187)	*Leuconostoc gelidum* UAL 187	Vacuum-packaged meat.	J Bacteriol. 1991 173(23): 7491–7500
Leucocin B (LeucocinB Ta11a)	*Leuconostoc carnosum* Ta11a	Vacuum-packaged meat	10.1007/BF01570155
Leucocin_C	*L. mesenteroides* TA33a	Processed meat	10.1099/00221287-144-5-1343
Leucocin K7	*L. mesenteroides* K7	Fermented pickle	10.1007/s10529-016-2127-y

Lichenin	*B. licheniformis* 26 L-10/3RA	Buffalo rumen	10.1046/j.1365-2672.2001.01429.x
LSEI 2163	*L. casei* ATCC 334	Dairy products, emmental cheese	10.1007/s00253-012-4149-2 https://www.lgcstandards-atcc.org/products/all/334.aspx?geo_country=ie
LSEI 2386	*L. casei* ATCC 334	Dairy products, emmental cheese	10.1007/s00253-012-4149-2 https://www.lgcstandards-atcc.org/products/all/334.aspx?geo_country=ie
Mesentericin B105	*L. mesenteroides* Y105	Goat milk	10.1099/00221287-138-12-2725
Mesentericin Y105 (anti-Listeria)	*L. mesenteroides* Y105	Goat milk	10.1099/00221287-138-12-2725
Microcin C7	*E. coli* RYC25	Newborn faeces	10.1128/aac.27.5.791
Microcin I47	*E. coli* H47	Human faeces	10.1099/mic.0.26396-0
Microcin PDI	*E. coli* 25	Cattle	10.1038/srep42529
Microcin S	*E. coli* G3/10 DSM 17252	Symbioflor human	10.1155/2016/3535621 10.1371/journal.pone.0033351
MR10	*E. faecalis* MRR10-3	Uropygial gland of the hoopoe (Upupa epops).	10.1128/AEM.02940-05
Mundticin L	*E. mundtii* CUGF08	Alfalfa sprouts	10.1128/AEM.00752-09
Mundticin ATO6	*E. mundtii* ATO 6	Chicory endive	10.1016/s0005-2736(98)00086-8
Mundticin KS	*E. mundtii* NFRI 7393	Grass silage	10.1128/aem.68.8.3830-3840.2002
Mutacin IV	*S. mutans* UA140	Human caries	10.1128/AEM.67.1.15-21.2001
Penocin A	*Pediococcus pentosaceus* ATCC 25745	Plant	10.1099/mic.0.28794-0 https://www.lgcstandards-atcc.org/Products/All/25745.aspx?geo_country=ie
Piscicolin 126	*C. piscicola* JG126	Spoiled ham	Appl Environ Microbiol. 1996 62(8):2897-903
Plantaricin 1.25β	*L. plantarum* TMW 1.25	Sausage fermentation	10.1016/S0167-4781(00)00003-8
Plantaricin 423	*L. plantarum* 423	Sorghum beer	10.1046/j.1365-2672.1998.00451.x
Plantaricin A	*L. plantarum* C-11	Cucumber fermentation	10.1590/S1517-83822009000200001
Plantaricin ASM1	*L. plantarum* A-1	Mexican tortilla	10.1016/j.ijfoodmicro.2009.10.021
Plantaricin C19	*L. plantarum* C-19	Fermented cucumber	10.1016/s0168-1605(01)00482-2
Plantaricin E/F	*L. plantarum* C-11	Fermented cucumber	10.1128/aem.68.8.3830-3840.2002
Plantaricin F	*L. plantarum* BF001	Catfish fillets	10.1128/aem.68.8.3830-3840.2002
Plantaricin J/K	*L. plantarum* C-11	Fermented cucumber	10.1128/aem.68.8.3830-3840.2002
Plantaricin K	*L. plantarum* DK9	Fufu, cassava product	10.1128/aem.68.8.3830-3840.2002
Plantaricin N	*L. plantarum* C-11	Fermented cucumber	10.1128/aem.68.8.3830-3840.2002
Plantaricin NC8	*L. plantarum* NC8	Grass silage	10.1016/0963-9969(92)90121-K
Plantaricin S	*L. plantarum* LPCO10	Green olive fermentations	10.1128/aem.68.8.3830-3840.2002
Plantaricin SA6	*L. plantarum* SA6	Fermented sausages	10.1111/j.1365-2672.1995.tb03417.x
PlnK (putative)	*L. plantarum* WCFS1	Human saliva	10.1073/pnas.0337704100
Propionicin SM1	*Propionibacterium jensenii* DF1	Swiss raw milk	10.1016/S0723-2020(00)80002-8

Propionicin T1	*Propionibacterium thoenii*	Dairy product	10.1128/aem.66.10.4230-4236.2000
Propionicin F	*Propionibacterium freudenreichii* subsp. *freudenreichii* LMGT2946	Cheese starter	10.1128/AEM.70.12.7303-7310.2004
Protease-activated antimicrobial protein (PAMP)	*P. jensenii*	Dental plaque	Appl Environ Microbiol. 1992 58(1): 215–220
Putative bacteriocin	*Streptococcus gordonii* ATCC 35105 (previously classified as *Streptococcus sanguis* White in White and Niven)	Isolated from endocarditis patients	10.1128/JB.01023-07
Putative bacteriocin	*S. pyogenes* MGAS 10394	Human throat	https://www.lgcstandards-atcc.org/Products/All/BAA-946.aspx?geo_country=ie
Putative bacteriocin	*S. pyogenes* MGAS 5005	Human cerebroespinal fluid	https://www.lgcstandards-atcc.org/Products/All/BAA-947.aspx?geo_country=ie
Putative bacteriocin	*S. pyogenes* MGAS 315	Streptococcal toxic shock	https://www.lgcstandards-atcc.org/products/all/BAA-595.aspx?geo_country=ie
Putative bacteriocin	*S. mutans* UA159	Human caries	10.1073/pnas.172501299
Putative bacteriocin	*S. pyogenes* MGAS 6180	Human blood	https://www.lgcstandards-atcc.org/products/all/BAA-1064.aspx?geo_country=ie
Putative bacteriocin	*L. sakei* subsp. *sakei* 23K	French sausage	10.1038/nbt1160
Rhamnosin A	*Lactobacillus rhamnosus* strain 68	Human faeces	10.1111/j.1365-2672.2009.04539.x
Sakacin 5XSakX	*L. sakei* 5	Malted barley	10.1128/aem.69.12.7194-7203.
Sakacin A	*L. sake* Lb706	Meat product	10.1007/978-1-4615-2668-1_16
Sakacin G	*L. sake* 2512	Dry fermented sausages	10.1128/AEM.68.12.6416-6420.2002 10.1111/j.1365-2672.1994.tb01105.x
Sakacin P	*L. curvatus* strain CRL705	Fermented sausages	10.1128/JB.06416-11
Sakacin P	*L. sakei* l151	Fermented sausages	10.1007/s00253-005-0172-x
Sakacin Q	*L. curvatus* ACU-1	Dry sausages	10.1016/j.meatsci.2014.03.003
SakT	*L. sakei* 5	malted barley	10.1016/j.femsle.2004.05.011
Salivaricin P	*L. salivarius* DPC6,005	porcine intestine	10.1128/AEM.00666-06
Serracin P	*Serratia plymuthica* A30	Rotten potato tissue	10.1128/JB.01699-12
Subpeptin JM4-B	*B. subtilis* JM4	Soil	10.1007/s00284-005-0004-3
Subtilosin (SboX)	*Bacillus amyloliquefaciens*	Dairy Yogu Farm™	10.1111/j.1365-2672.2007.03626.x
Thermophilin 13	*S. thermophilus* SFi13	Nestle strain collection	10.1371/journal.pone.0059239
Thermophilin A	*S. thermophilus* ST134	In-house culture collection	10.1007/bf00172834
Thuricin S	*B. thuringiensis* subsp. *entomocidus* HD198 strain	Bacillus Genetic Stock Center collection (Columbus, Ohio).	10.1139/w06-116
Trifolitoxin	*Rhizobium leguminosarum* bv. *trifolii* Strain T24	Soil	10.1104/pp.85.2.335
Ubericin A	*S. uberis*	Bovine mastitis	10.1128/AEM.01818-07
UviB	*B. thuringiensis* serovar *israelensis* ATCC35	Sewage	10.1016/j.femsle.2005.07.008 https://www.lgcstandards-atcc.org/products/all/35646.aspx?geo_country=ie
Warnericin RC	*S. warneri* RK	Environment	10.1016/j.femsle.2005.03.046
Weisselin A	*Weissella paramesenteroides* DX	Fermented sausages	10.1016/j.enzmictec.2013.04.003
Weissellicin M	*Weissella hellenica* QU13	Barrel for pickles	10.1111/j.1365-2672.2011.05180.x

Weissellicin Y	*W. hellenica* QU13	Barrel for pickles	10.1111/j.1365-2672.2011.05180.x
Pentocin KCA1	*Lactobacillus pentosus* KCA1	Vagina	10.1371/journal.pone.0059239
BlpD / Thermophilin 9	*S. thermophilus* LMD-9	Dairy product	10.1038/nbt1034 10.1073/pnas.0607117103 10.1128/JB.00966-07 https://www.lgcstandards-atcc.org/products/all/BAA-491.aspx?geo_country=ie#generalinformation

Appendix 3

Table S3. Class III bacteriocins

Bacteriocin	Produced by	Isolated from	Doi / reference
Alveicin A	*Hafnia alvei*	Enteric bacteria from wild Australian mammals	10.1128/jb.186.6.1598-1605.2004
Alveicin B	*H. alvei*	Enteric bacteria from wild Australian mammals	10.1128/jb.186.6.1598-1605.2004
Bacteriocin	*Thermotoga maritima* MSB8	Geothermally-heated sea floors	10.1080/09593330.2010.484076
Bacteriocin 28b	*Serratia marcescens*	enterobacteria	10.1016/0923-2508(96)80293-2
Albusin B	*Ruminococcus albus* 7.	Rumen	10.1128/aem.70.5.3167-3170.2004
Carocin D	*Pectobacterium carotovorum* subsp. *carotovorum* Pcc21	Phytopathogen isolated from host plant	10.1128/AEM.03103-09 19(1):42-50
Closticin 574	*Clostridium tyrobutyricum* ADRIAT 932	NIZO food research (Ede, The Netherlands)	10.1128/aem.69.3.1589-1597.2003
Colicin	*Erwinia carotovora* subsp. *atroseptica* SCRI1043	Phytopathogen	10.1111/j.1365-2672.2009.04462.x
Colicin B	*E. coli* K12/5K	Human faeces	10.1128/MMBR.00036-06
Colicin-A	*Citrobacter freundii* CA 31	enterobacteria	10.1128/MMBR.00036-06 10.1111/j.1432-1033.1981.tb05380.x
Colicin M	*E. coli* K-12 32T 19F/T1	K-12 faecal	10.1128/MMBR.00036-06 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC429005/pdf/aac00335-0086.pdf
Halocin H4	*H. mediterranei* R4 (ATCC 33500)	Salt ponds	179(2): 548–551 https://www.lgcstandards-atcc.org/products/all/33500.aspx?geo_country=ie
Halocin S8	*Haloarchaeon* sp S8a	Great Salt Lake	10.1128/JB.182.17.4951-4958.2000
Lin M18	*Bordetella bronchiseptica* RB50	Rabbit	10.1038/ng1227
Linocin M18	*Wolinella succinogenes*	Rumen	10.1007/s10482-007-9151-9 https://www.lgcstandards-atcc.org/products/all/29543.aspx?geo_country=gb
Linocin M18	*Brevibacterium linens* M18	Cheese	10.1038/ng1227
Putative Bacteriocin family protein	*Nocardia farcinica* IFM 10152	Human bronchus	10.1073/pnas.0406410101
Putidacin L1	*Pseudomonas* sp. strain BW11M1	Rizhosphere	10.1128/jb.185.3.897-908.2003
Zoocin A	*Streptococcus zooepidemicus* strain 4881	Animal origin	10.1017/s0022172400063774

Appendix 4

Table S4. Peptides and proteins shortlisted from the trypsin digestion of the band with antimicrobial activity extracted from SDS gel from *L. amylovorus* lac 20 supernatant. GG motifs common to bacteriocin leader cleavage sites are highlighted; alternative masses are calculated with the removal of this potential leader

Peptide / protein	Annotated as	Mass (Da)	Alternative Masses (Da)
MKSKHAIYWLICLIVIVLGGWYMYGHLNRSSATSSMVSTTAKSKATAKD SSSEDSNMRTPIDWQKSSETIPYPDRSKVKDLWVKVSLKGNRTYLYDGS KIIYTMYSSGGVYQKDDKTGKMKSATPTGTFYVQAERGDSFFNQELGE GANYYVSWLNHGEYLFHSVPTKADGSYNLKEAAKLGKSTGSHGCIRLSV PDAKWMEQNLPEGTKVVIADN	Alanine carboxypeptidase	24073	21834
MIMDLQFFSHHKGGGSTANGRNSAGRRLGTKAADGSVVTAGSIIYRQ RGTHIHPGENVGRGGDDTLFAKIDGVVKFERMGRNNRKVSVYPVAE	LSU ribosomal protein L27p	9985	8298, 4647, 6334
MAFDKDAIIASLKEASISDLNDLVKAIEEEFDVSAAAPVAVAGAAGGDA AAKDSFTVELTSAGSAKVKVIKAVKDITGLGLKDAKDLVDNAPSAVKED VKEDEANDIKEKLEAAGATVTLK	LSU ribosomal Protein L7/L12	12330	4578, 7656
MALDLYKYVASIPDYPEKGIIFRDILPLMADGEAFKQATDEITAFARERN VDMVVGPEARGFIVGCPVAYELGVGFAPARKKGKLPRATVSASYQLEY GEATLQMENDSVKPGQRVLVVDDLLATGGTIGATIDMVEQMGGKVV GAAFLIELKELEGRKHLRDIDIKTLMEF	Alanine phosphoribosyl_ transferase	18784	5017, 3613
MNSTKYKRFLMVLIFMVSLWLVPQAQAAVANNAAEFTIDPVYPSEQQ ADNTGYFSLAVHPASTVPVKVKITNTNQHQAITYRLKVGNATTNPDGSI NYANFKAKKDPTAQYQLTDFIDHAKRGQKITVPANSDRTVTVNLRLPAH AFSGIVAGGVYVERLTNGANQQSGNFQTQNHFAMTLPILVTEHPHAKR IAKMQLAQVKVQKGQIAARLHNVRPVLFGKLTIHARITKAHQTKALVKK TVTNYQVAPNSAFDFVVTDPKQPLNTGHYTLTMNLQSGKRQWYFSRA FTVTASQAAPLTKRTGWLGLPLLLWLIGGGLILIILALVGVILKQRKKLSQQ	Cell surface protein precursor	37714	16737, 20880
MIQTIDLKKGMVFERGGKLLKVLQINHHKPGKGNTLMQMDIQDVRTGS IVHTTMRPSEKVEQVNVDKKNAQYLYDEGNTAVFMDMETYEQYEISDE QLTEEKKYLVENMQVQMDFVGSELVGIELPTTVVLTVEHTEPMIKGATID GGGKPATMSTGLVVNVPAFIKNGDQIVVNTMDGSYKSRA	Translation elongation factor	20629	3867
MISKPDKNKIRQRRHMRVRGKISGTAERPRLSVYRSNKNIYAQLIDDVKG VTLASASTLDSEVSGNTKTEQAAGVGALIAKRGVEKNISGVVFDRGGYLY HGRVQALAEAARENGLQF	LSU ribosomal protein L18p	12914	2406, 10411
MKLNELKPAAGSRFKRLRKGRGLSSGHGFTSGRGTKGQKAHGKTRLGFE GGQMPLYRQMPKRGFSNINRKEYAIVNLTTLNRFDDGAEVTPAVLLENG VIKNVKSGVKILGNGKLTKKLTVKANKFSASAVKAIEAAGGKTEVM	LSU ribosomal protein L15p	15486	5298
MASQSYIDPSKLDLEDQVVSINRVTKVVKGGRRLRFAALVIVGDKKGHVGF GTGKAQEVPEAIRKASAAAQKNLIEVPIVGTTIPHEVIGTYGGGKIMLKPAV EGSGVAAGGAVRNVMELAGVADVTSKRLGSNTPINVVRATFEGLKALKSA EEVSQLRGVSVDHLAE	SSU ribosomal protein S5p	17595	3263, 9840, 6034, 7061

Appendix 5

Table S5. BIOLOG chemicals that inhibit growth of *L. crispatus* Lac 50 and Lac 51

Chemical	Target
Cloxacillin	Cell wall
Erythromycin	Protein synthesis 50S ribosomal
Vancomycin	Cell wall
Novobiocin (3c)	DNA topoisomerase
Spyramycin	Protein synthesis 50S
Cetylpyridinium chloride	Membrane

Diamide (1c)	Oxidises sulfhydryls
Poly-L-lysine (2c)	Membrane
Sodium bromate (2c)	Toxic anion
Sodium periodate (2c)	Toxic anion
Harmane (2c)	Imidazoline binding sites
FCCP	Respiration, ionophore
D,L-Thioctic acid (2c)	Oxidising agent
Phenethicillin	Cell wall

Table S6. BIOLOG chemicals that have no influence on the growth of *L. crispatus* Lac 50 and Lac 51

Chemical	Target
Potassium tellurite	Toxic anion
Demecocycline	Protein synthesis 30S
Enoxacin	DNA topoisomerase
Neomycin	Protein synthesis 30S
Kanamycin	Protein synthesis 30S
Ofloxacin	DNA topoisomerase
Penimepicycline	Protein synthesis 30S
Sulfamethazine	Folate antagonist
Sulfadiazine	Folate antagonist
Sulfathiazole	Folate antagonist
Sulfamethoxazole	Folate antagonist

Nickel chloride	Toxic cation
2,2'-Dipyridyl	Chelator, lipophilic
Dequalinium chloride	Ion channel inhibitor, K^+
Geneticin (G418)	Protein synthesis 30S
Glycine	Membrane
Moxalactam	Cell wall
Sulfanilamide	Folate antagonist
5-Azacytidine	DNA methylation
Sulfisoxazole (only 51)	Folate antagonist
Semicarbazide	Amine oxidase inhibitor
Tinidazole	Oxidising agent
Coumarin (3C)	DNA intercalator

Table S.7. BIOLOG chemicals in which presence *L. crispatus* Lac 50 and Lac 51 behaved similarly

Chemical	Target
Amoxicillin	Cell wall
Cloxacillin	Cell wall
Lomefloxacin	DNA topoisomerase
Mynocycline	Protein synthesis, 30S
Nafcillin	Cell wall

Erythromycin	Protein synthesis 50S
Potassium tellurite	Toxic anion
Penimepicycline	Protein synthesis 30S
Sisomicin	Protein synthesis 30S
Vancomycin	Cell wall

Paromomycin	Protein synthesis 30S
Sulfamethazine	Folate antagonist
Sulfadiazine	Folate antagonist
Sulfathiazole	Folate antagonist
Sulfamethoxazole	Folate antagonist
Novobiocin	DNA topoisomerase
2,4-Diamino-6,7-diisopropyl-pteridine	Folate antagonist
Tobramycin	Protein synthesis 30S
Spectinomycin	Protein synthesis 30S
Spiramycin	Protein synthesis 50S
Rifampicin	RNA polymerase
Dodecyltrimethyl ammonium bromide	Membrane, detergent
Cytosine-1-beta-D-arabino-furanoside	Nucleic acid analog
Manganese chloride	Toxic cation
Trifluoperanize	Anti-cholinergic
Tylosin	Protein synthesis 50S
Cinoxacin (3c)	DNA topoisomerase
5-Azacytidine	DNA methylation
Rifamycin SV	RNA polymerase
Aluminium sulfate	Toxic cation
Chromium chloride	Toxic cation
L-Glutamic-g-hydroxamate	tRNA synthetase
Ketoprofen	Anti-capsule agent (biofilm)
Poly-L-lysine	Membrane
Sodium bromate	Toxic anion
Lidocaine	Ion channel inhibitor
Sodium metasilicate	Toxic anion
Sodium periodate	Toxic anion
Antimony (III) chloride	Toxic cation
Triclosan	Lipid synthesis, fatty acid inhibitor
Coumarin	DNA intercalator

Methyltrioctylamonium chloride	Membrane
Harmane	Imidazoline binding sites
Chlorhexidine	Membrane, electron transport
Cinnamic acid	Respiration, ionophore
Disulphiram	Nucleic acid inhibitor
FCCP	Respiration, ionophore
D,L-Thioctic acid	Oxidising agent
Phenethicillin	Cell wall
Blasticidin S	Protein synthesis
Hexammine cobalt (III) chloride	DNA synthesis