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Identification and characterisation of lactic acid bacterial secondary metabolites with functional and health benefits.

A Thesis Presented to the National University of Ireland for the degree of Doctor of Philosophy by

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Declaration

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

Signed: _____ Date: _____

Daragh Hill

Note: Recently, the lactobacilli have undergone major changes in nomenclature (Zheng et al., Int J Syst Evol Microbiol. 2020. doi: 10.1099/ijsem.0.004107). 250 former species have now been assigned to 25 new genera. Traditional taxonomic names were used when this thesis was prepared and have been retained here.

Dedication

For my Granny, Marie Hill.

Publications

Published;

Hill, D., R. P. Ross, E. Arendt and C. Stanton (2017). Chapter 4 - Microbiology of Yogurt and Bio-Yogurts Containing Probiotics and Prebiotics. Yogurt in Health and Disease Prevention. N. P. Shah, Academic Press: 69-85.

Hill, D.*, I. Sugrue*, E. Arendt, C. Hill, C. Stanton and R. P. Ross (2017). "Recent advances in microbial fermentation for dairy and health." F1000Research 6: 751.

Hill, D.*, I. Sugrue*, C. Tobin*, C. Hill, C. Stanton and R. P. Ross (2018). "The Lactobacillus casei Group: History and Health Related Applications." Frontiers in Microbiology 9: 2107.

*Contributed equally

Accepted;

Hill D, O'Connor PM, Altermann E, Day L, Hill C, Stanton C, Ross RP. Extensive bacteriocin gene shuffling in the *Streptococcus bovis/Streptococcus equinus* complex reveals gallocin D with activity against vancomycin resistant enterococci. Scientific Reports. Accepted.

Abstract

During fermentation lactic acid bacteria (LAB) produce and secrete a range of secondary metabolites. These metabolites have a range of potential applications in both medical and food sectors. They can be used to enhance a food product through the production of desirable metabolites, they can synthesise exopolysaccharides (EPS) and polyols, and LAB can impact both food and medicine through the production of potent antimicrobial compounds.

In the research described in this thesis LAB were isolated from ovine, bovine, and caprine raw milks, raw milk cheeses, honey, sheep faeces and other environmental sources to create a biobank of 799 putative LAB strains. This biobank was screened for exopolysaccharide (EPS) and polyol producing LAB for applications in dairy fermentations to produce foods with enhanced functionality and potential health benefits.

EPS production has been used to improve the functional properties of food products; a property that is increasingly important given the rising demand for clean label food ingredients. In Chapter Three the entire biobank was tested for EPS production using three phenotypic screening methods in order to identify both ropy and mucoid EPS producers. In total 466 (58%) positive strains were identified, five of which were utilised as adjunct cultures in skim milk fermentations. Following assessment of both safety and acidification characteristics, three strains were selected for inclusion as adjunct cultures in a goat milk fermentation. All three strains significantly improved the texture of the yogurt produced in comparison to the control containing only the starter culture. This work highlights the prevalence of EPS production among LAB and the advantage of using multiple screening methods for identification of EPS producing isolates.

Mannitol is a six-carbon polyol that is 50-70% as sweet as sucrose but with only half the calories. With the rising demand for low calorific dairy products there is a need for alternative sweetening methods. In Chapter Four we included a mannitol producing LAB in dairy fermentations to produce a low-calorific, sweetened

fermented product. Two hundred and sixty-one isolates of the LAB biobank were screened for their ability to produce mannitol using a high throughput HPLC-based screening method. Twenty-five strains produced mannitol, with five high mannitol producers identified that had yields of over 1.5g/L in fermentation broth. These five strains were applied in skim milk fermentations to investigate if mannitol could also be produced in milk. Small quantities of mannitol were detected in the fermented product, highlighting the potential for *in situ* production in a dairy product, albeit that production would require further optimisation for commercial application.

In Chapter Five the properties of a bacteriocin produced by *Streptococcus gallolyticus* LL009 were examined. LL009 was one of the high EPS producing strains identified in an earlier chapter. Gallocin D is a narrow spectrum two component bacteriocin with potent activity against vancomycin-resistant enterococci (VRE). It can be readily synthesized and has potential in the treatment of VRE infections. *In silico* analysis revealed that gallocin D is distinct from a previously identified *S. gallolyticus* bacteriocin, gallocin A, although the gene clusters encoding both bacteriocins share a high degree of gene synteny. The structural genes are highly variable and appear to have undergone gene shuffling with other streptococcal species.

S. gallolyticus is part of the *Streptococcus bovis/Streptococccus equinus* complex (SBSEC), which contains seven subgroups. Members of the SBSEC show high genome plasticity and in Chapter Six the bacteriocin capabilities of the complex were further examined by including all sequenced strains from each subgroup. The genomes of 105 strains were downloaded and analysed using Bagel4 to identify potential bacteriocin clusters. Forty-four strains showed at least one area of interest (AOI) with a total of 67 AOI identified, and 166 predicted structural genes. Each bacteriocin cluster was aligned using MAUVE and the 67 clusters were assigned to six subgroups, with ten remaining unassigned. The predicted structural genes within each group were compared using MUSCLE to determine whether these structural peptides are identical, variants or unrelated within and/or between species. The source of each isolate was also examined but no obvious pattern was evident. The high prevalence of structural genes among the strains led us to speculate why one

operon in a strain would harbour multiple diverse bacteriocin structural genes. Whether or not these strains are capable of producing each of these bacteriocins, there is a fitness cost associated with the presence of extra genes. If these are all transcribed at once, or production is changed depending on the niche the strain is growing in, remains unknown. In depth study on these strains involving growth in a range of conditions and mass spectrometry could help to answer some of these questions.

Chapter 1.

The *Lactobacillus casei* group: History and Health Related Applications.

Published as;

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Note: *Lactobacillus casei*, *paracasei* and *rhamnosus* have been re-named as *Lacticaseibacillus casei*, *paracasei* and *rhamnosus*. Traditional taxonomic names were used when this Chapter was published and have been retained here. (Zheng et al., Int J Syst Evol Microbiol. 2020. doi: 10.1099/ijsem.0.004107).

Abstract

The *Lactobacillus casei* group (LCG), composed of the closely related *Lactobacillus casei, Lactobacillus paracasei* and *Lactobacillus rhamnosus*, are some of the most widely researched and applied probiotic species of lactobacilli. The three species have been extensively studied, classified and reclassified due to their health promoting properties. Differentiation is often difficult by conventional phenotypic and genotypic methods and therefore new methods are being continually developed to distinguish the three closely related species. The group remain of interest as probiotics, and their use is widespread in industry. Much research has focused in recent years on their application for health promotion in treatment or prevention of a number of diseases and disorders. The LCG have the potential to be used prophylactically or therapeutically in diseases associated with a disturbance to the gut microbiota. The group have been extensively researched with regard to stress responses, which are crucial for their survival and therefore application as probiotics.

Introduction

There are more than 200 species of *Lactobacillus*, the largest and most diverse genus within the lactic acid bacteria (LAB) [1]. *Lactobacillus* spp. are part of the microbiota of humans and animals [2] where they colonize the gastrointestinal tract (GIT) and the urogenital tract [3]. They are also found in a variety of food products from fruit and vegetables [4] to a range of naturally fermented products [5, 6]. *Lactobacillus* spp. have been deployed and studied extensively as fermentation starter cultures [7] and as probiotics [8, 9]. Their long history of use in fermented products has led to their recognition as GRAS (generally recognised as safe) by the US Food and Drug Authority (FDA), and earned them a place on the QPS (qualified presumption of safety) list assembled by the European Food Safety Authority (EFSA) [10].

The *Lactobacillus casei* group (LCG), comprised mainly of the closely related *Lactobacillus casei, Lactobacillus paracasei,* and *Lactobacillus rhamnosus* species, are among some of the most studied species due to their commercial, industrial and applied health potential. Commercially, they are used to ferment dairy products, often producing foods with improved flavour and texture. They have also been found to produce many bioactive metabolites which can confer host benefits when consumed [11]. As such, many LCG strains are considered to be probiotics. One member, *L. rhamnosus* GG (LGG), is perhaps one of the most studied bacterial strains in relation to health applications [12] .

This review will discuss the complex issues relating to the taxonomy of the LCG, current research and novel applications of species and strains within the group for health promotion, and will consider stress response mechanisms crucial for probiotic survival.

A Brief Taxonomic History

The *Lactobacillus casei* group (LCG) is composed of three genotypically and phenotypically related facultatively heterofermentative species, *L. casei, L. paracasei,* and *L. rhamnosus.* The taxonomy of these species has been extensively

investigated in the past, as many strains within the group are commonly applied as starter cultures and probiotics. As species of the LCG are of significant commercial value, their relationship to one another has been the focus of much research. It is important to emphasize that distinguishing them is crucial to ensure correct evaluation of the literature and to avoid confusion between related probiotic strains in food and health-associated products. However, the taxonomic history of the LCG has been both convoluted and challenging.

Lactobacillus casei was first proposed as a novel species in 1971 [13], but this characterisation was first questioned in 1996 [14]. It was proposed that the type strain *L. casei* ssp. *casei* ATCC 393 and *L. rhamnosus* ATCC 15820 should be reclassified as the previously described *L. zeae* [15], due to high similarity to one another and separation from the rest of the LCG cluster. The name *L. paracasei* was rejected, and *L. casei* ATCC 334 was suggested as the new type strain for the species [14]. Further research continued to call into question the phylogenetic relationship between the species using more refined methods such as comparative sequence analysis of the *recA* gene [16] and 23S-5S rRNA intergenic spacer regions [17]. In 2002, it was proposed that the strain designated *L. casei* ssp. *casei* ATCC 393 should in fact be reclassified as *L. zeae* and that *L. paracasei* strains should be reclassified under *L. casei* ATCC 334 [18]. In 2008, the type strain of *L. casei* was confirmed to be the original strain ATCC 393, and the strain designated ATCC 334 was rejected as it represented a separate taxon, *L. paracasei* [19]. The revived name *L. zeae* was deemed to contravene Rules 51b (1) and (2) of the International Code of Nomenclature of Bacteria and has since been superseded by *L. casei* [19]. Current research continues to compare and reclassify known species within the group according to developed and novel methods.

Difficulties in Speciation

The guidelines for probiotics in food according to the Food and Agriculture Organization of the United Nations and the World Health Organisation (FAO/WHO) makes it mandatory that microorganisms which are used intentionally in food or animal feed must be taxonomically defined to genus and species level [20]. As species

of the LCG are present in a diverse range of habitats and are used in the production of many food products in the food industry, molecular genotyping is crucial for determining their evolution and phylogeny. The LCG contains a high number of carbohydrate utilisation gene clusters, allowing for survival and adaptation across a number of different environments [21]. Traditional methods of characterisation for most species include phenotypic analysis such as carbohydrate fermentation profiles [22], however, the gain and loss of plasmids can influence this phenotype and can make identification of strains problematic. Molecular genotyping through comparative analysis of the 16S rRNA gene is another widely used method of species identification [23]. The 16S subunit of the bacterial ribosome is commonly used for species identification and differentiation as it is both ubiquitous and contains regions which are conserved among bacteria. Hypervariable regions flanked by these conserved sequences allow for species identification based on sequence similarity, and has been used as the gold standard approach that avoids potentially confusing phenotypic identification methods [24]. However, 16S gene analysis is not always reliable for LCG species due to high sequence similarities across the three species. Denaturing gradient gel electrophoresis (DGGE), which discriminates based on DNA fragment mobility under increasingly denaturing conditions, has been applied for the identification of LCG species, but has produced confusing results when compared with restriction endonuclease analysis of total chromosomal DNA [25]. A comparison of randomly amplified polymorphic DNA (RAPD) analysis, ribotyping, and pulse field gel electrophoresis (PFGE) have all been applied for the differentiation of LCG species, but often yield contradictory results which lead to differing levels of speciation [26].

Current and Future Trends

In recent years, other methods have been employed for the classification of LCG strains, (see Table 1). A combined technique approach has been suggested to assist where conflicts occur in speciation [27]. Using species-specific PCR and high-resolution melting (HRM) analysis on 201 strains from international culture collections belonging to the *L. casei* group, it was determined that 46 strains were different from their previous classification [27].

Table 1. Recent publications differentiating species within the *L. casei* group.

Method of species differentiation/ classification	# Strains	Results	Pros & cons to method	Ref
MALDI-TOF MS analysis of protein biomarker profile with proteomic analysis using in-house database	49	100% discrimination between *L. paracasei* subspecies. Discrepancies found between probiotic product descriptions and biomarker identification.	+ Rapid, accurate, and cheap identification - Initial high cost of equipment - Requires database of known species biomarker profiles	[27]
Comparative genomics	183	Strains separated into three clades based on whole GC content, relatedness of conserved genes, and clustered based on functional genomic categories.	+ Large number of comparisons can be made with publicly available data - Potential difficulties with acquiring complete whole genomes of new strains	[28]
Multiplex PCR targeting the *mutL* gene	76	88% of strains identified, and improved characterization of 10 strains which couldn't be identified by 16S.	+ Rapid method which can be used with relative ease (PCR based assay) + Can be used as a combined approach - Can provide some ambiguous results	[29]
Species specific PCR and high-resolution melting (HRM) analysis	201	96.5% of strains identified as part of the LCG. 23.7% were in disagreement with species specific PCR.	+ Highly sensitive post- PCR technique	[30]
Phylogenetic analysis based on the *dnaJ* gene	30	*L. casei*, *L. paracasei* and *L. rhamnosus* separated, with better discrimination than the 16s rRNA gene.	+ Gene similarities of 81.7-85.5% at interspecies level allow for good separation - Requires specific kit and minisequencing technology	[31]
PCR amplification and comparison of *yycH* gene in LCG type strains	49	Strains were distinctly separated into clades according to species.	+ Average % gene identity of 78.5% among type strains allow for strong separation	[32]
Whole genome sequencing, annotation and comparative genomics	10	Differentiation of *L. casei* ATCC 393 from *L. paracasei* strains, and identification of conserved genomic islands and truncated genes in separate species.	+In depth analysis of gene clusters -Limited number of species compared	[33]
Sequence comparison of the *dnaK* gene and RFLP	46	Strains were distinctly separated into clades per species.	+ Strong separation of species + PCR based method - Poor separation of subspecies	[34]

A study was also conducted on 40 *L. casei* strains from plant material, human GITs, human blood, cheeses from different geographical regions, and unknown sources; these were characterised according to standard phenotypic methods (API

biochemical testing) and multilocus sequence typing (MLST) using six housekeeping genes, together with PFGE [28]. MLST revealed between 11 and 20 'allelic types' based on the *nrdD* and *mutL* genes, and 36 sequence types. The study determined that PFGE was an effective method for discriminating isolates, even where MLST was unsuccessful [28]. Another MLST study of 224 *L. casei* isolates from fermented foods using ten housekeeping genes found no correlation with geographic location or food source, implying a complex evolutionary history and contradicting previous studies [29]. Other single target genes used for species discrimination include housekeeping genes such as the chaperone protein gene, *dnaJ* [30], *dnaK* [31], *yycH* [32], and *mutL* [33]. Indeed, it was found that the *dnaJ* gene provided better resolution than the 16S rRNA subunit gene for comparisons within and between species, and this has been suggested as an alternative marker [30]. The gene for the DNA mismatch repair protein *mutL* has been chosen as a target for species discrimination using multiplex PCR, and was found to provide a rapid method to distinguish *L. casei, L. rhamnosus* and *L. paracasei,* though in areas where conflicting results were found, whole genome sequencing (WGS) is suggested [33].

Whole genome sequencing has advantages in terms of the amount of sequence data provided, therefore allowing numerous gene target comparisons, and *in silico* DNA-DNA hybridisation [34]. The ever-decreasing cost of WGS is pushing current research in that direction. Numerous studies have used WGS as a method for distinguishing species of the LCG, though the methods are not without difficulty. Toh *et al.* (2013) produced complete genome sequences of *L. paracasei* JCM 8130 and *L. casei* ATCC 393, along with a draft sequence of *L. paracasei* COM0101 and a re-annotated *L. rhamnosus* ATCC 53103 (otherwise known as *L. rhamnosus* GG), and compared them to other available sequences from LCG strains. Through comparative genome analysis a core of 1,682 genes was found among the LCG and extensive genome wide synteny was observed, particularly for genomic islands containing carbohydrate utilisation genes. The presence of truncated forms of the *spaCBA* pilus gene cluster previously identified in LGG was found in several *L. paracasei* strains [35]. Such genomic insights are invaluable in determining evolutionary relationships within the group and may help differentiate strains where conventional methods fail.

Comparative genomic analysis of 213 lactobacilli determined that the LCG clade harbour the most piliated species, which is unsurprising due to their well-known colonisation of the GIT [1]. Until now such pan- and core-genome approaches have largely been ignored in taxonomy, though more studies are performing comparative genomics in order to determine evolutionary relationships, which in turn provide insights into taxonomy [36]. Pan-genomic approaches are highlighting inconsistencies in current taxonomy of the group [37], and comparative genomic studies have revealed high levels of intra-species diversity within the group, even among isolates from the same niche [38] .

Isolates of *L. casei* have been found to have high levels of phenotypic and genotypic diversity, as has been demonstrated through fermentation profiling and MLST analysis [28]. Comparative genome analysis of carbohydrate utilisation clusters of *L. casei* strains found a loss of specific genes which enabled superior adaption for dairy isolates, particularly cheese strains, in the dairy niche, but decreased capacity to inhabit other niches when compared with *L. casei* 'generalists' as a result [21]. In addition to gene decay among dairy isolates, comparative genome analysis of dairy, plant, and human strains found horizontal gene transfer from other LAB has to be important in adaption of *L. casei* strains to new niches [39]. *L. paracasei* strains have been found to display similar loss of genes in dairy isolates and high diversity among strains from other sources [40]. It has been suggested that *L. rhamnosus* species have two distinct genotypes, one adapted to stable nutrient rich environments, *i.e*. milk and dairy products, and another which is more suitable to changeable environment such as the GIT [41]. A comparison of niche specificity across the LCG may be of interest in the future, in order to help unravel its taxonomic history.

Recently, novel methods have been developed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to rapidly identify and classify bacterial isolates according to their protein profiles [42]. This phenotypic proteomic approach has rapidly gained traction, and is being applied in laboratories [43], food [44], and clinical settings [45]. This method has been applied to differentiate species of the LCG after development of an in-house database of phenotypic profiles, and succeeded in identifying strains down to subspecies level

[46]. These methods are dependent on phenotypic protein profiles which may shift depending on culture conditions of the bacterium, and difficulties can occur due to initial high cost of equipment. Future approaches will likely depend on combined methods to assist in LCG species and subspecies differentiation, and without an agreed consensus on methods, the taxonomic future of LCG is likely to be as confusing as its past.

The *L casei* group and Health Related Research

The LCG contains numerous strains with proven probiotic activities [47]. Probiotics are defined as live microorganisms that, when administered in an adequate amount, confer a health benefit on the host [48]. Bacteria with associated health benefits have been researched since Elie Metchnikoff first suggested that the daily consumption of fermented milk products had a beneficial effect on human health [49]. This research has been growing steadily over the last 100 years, with great strides having been made in linking health benefits with probiotic consumption. The work on these strains has progressed beyond the use of these bacteria in fermented dairy products, or even fermented foods in general, to a wider array of technological and medicinal purposes. As the mechanisms behind their health promoting capabilities are becoming unravelled, possible applications for these strains are being developed in the food, biotechnology and medical fields [50]. This is providing evidence for future novel non-dairy probiotic foods as well as potential for microbial-based therapies for certain disorders and diseases. Some of the more promising applications for the LCG in these fields will be discussed below. The ability of a number of these strains to help to maintain a healthy microbiota may provide non-invasive adjunctive therapies for a range of disorders. They could be deployed prophylactically or therapeutically in a number of diseases linked to disturbances in the gut microbiota.

There is great potential in the fields of novel functional foods and pharmabiotics derived from the LCG. However, it should be noted that while many of these strains have been shown to have an effect *in vitro* or *in vivo* mouse models, the underlying mechanisms require further research for any future use as microbial

therapeutics.

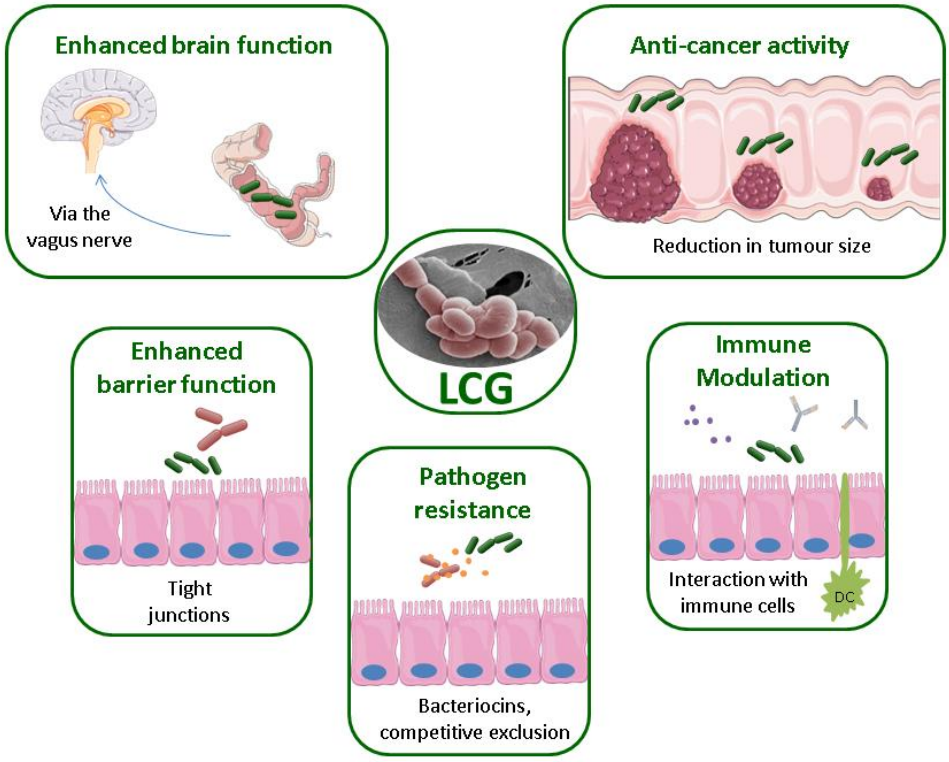

Figure 1. Methods of probiotic action by the LCG, EM of *L. paracasei* NFBC 338 [51].

Health benefits associated with the LCG have been reported for a variety of health conditions, ranging from atopic dermatitis to cancer. The mechanisms by which these bacteria directly, or indirectly, have a beneficial effect on human health are not yet fully understood and require further study. Potential mechanisms include the production of antimicrobial substances such as bacteriocins, enhancing the epithelial barrier through attachment, competition for pathogenic binding sites, or modulation of the immune system, (see Figure 1) [52]. The processing and inclusion method of these strains has also been the subject of much interest. For example, the delivery matrix has been shown to influence the effects of *L. casei* BL23 in the treatment of colitis [53]. Indeed, this strain only restored the microbiota when consumed in milk.

Allergic Disease

Rates of childhood asthma are rising which has been linked to the hygiene hypothesis [54]. Essentially, this proposes that children are exposed to fewer

microbes in infancy due to better hygiene, in addition to antibiotic exposure, birth by caesarean section and dietary changes. These factors influence development of the gut microbiota and its immune stimulation in early life. However, through modulation of the gut microbiota, probiotics have shown potential to reduce the risk of developing allergies such as atopic dermatitis. For example, the risk of developing allergy was shown to be reduced at five years of age by early life colonisation with the LCG in children of both allergic and non-allergic parents [55]. The use of probiotics such as LGG have been investigated for their potentially protective effect on children with a high risk of developing allergies such as asthma and atopic dermatitis [56]. However, this study was recently repeated and found to be ineffective for the prevention of these allergies at two years [57]. A recent meta-analysis shows that there are confounding results in this area [58]. While it is important to note this analysis included studies using multiple species and strains, it was concluded that the data overall indicates a benefit to the consumption of a probiotic, although this is dependent on many factors in addition to the specific strain used in the trials.

Brain Function

A 'psychobiotic' is a bacterium which when administered in adequate amounts can have a positive mental health benefit [59]. Members of the LCG have been involved in a number of important studies in this area. In one study focusing on the brain-gut-axis the strain *L. rhamnosus* JB1 highlighted both the ability of bacteria to positively affect brain function and also the importance of the vagus nerve in the bidirectional communication between gut microbes and the brain. Administration of JB1 directly affected the expression of GABA receptors in the brain which resulted in decreased anxiety and depressive behaviours in mice [60]. This study included healthy animals in separate laboratories with the same outcome.

In a recent study, a tablet preparation of LGG and *Biifidobacterium lactis* Bb12 as adjunctive therapy on hospitalised patients suffering from mania was effective in lowering rehospitalisation rates [61]. It is important to note this study had a number of limitations; the direct effect of the probiotic on the microbiome and inflammation of the CNS were not measured, while the medication taken by the recruits was

patient-specific. It does highlight the potential for microbial therapy in mental disorders which warrants further investment and study.

The ingestion of a combination of *L. rhamnosus* R0011 and *L. helveticus* R0052 also highlights the potential for bacteria to influence behaviour in a mouse model. The study showed that modulation of the gut microbiota can positively influence certain animal behaviours, in this case stress-induced impaired memory [62]. The normalisation of the gut microbiota through ingestion of this probiotic combination helped to prevent behaviour abnormalities caused by infection with *Citrobacter rodentium*.

Obesity

Obesity is a complex syndrome with a variety of contributing factors which influence onset. These include diet, physical activity and genetic factors, but also can be influenced by the composition of the gut microbiota. Manipulation of the microbiota through the administration of probiotics is an area that could provide interventions for the prevention and treatment of obesity. In a recent study, the probiotic *L. rhamnosus* CGMCC1.3724 was administered to obese men and women for 24 weeks. Males showed no significant difference but the female participants in the probiotic group had significant weight loss. This was found to be associated with reductions in blood leptin concentrations [63]. Obesity is also associated with changes in the gut microbiota and immune parameters. A fermented milk containing *L. casei* CRL 431 fed to mice was able to positively affect the microbiota as well as some biomarkers associated with obesity [64].

In a murine model of obesity *L. casei* Shirota was evaluated for its ability to improve weight management in comparison to Orsilat, a drug used to treat obesity [65]. The results of this study showed promise in the use of probiotic strains as an alternative method for weight management. The impact of exposure to probiotics on weight gain before birth has also been investigated. *L. rhamnosus* administered to pregnant women has been found to modify weight gain in children during the first six months of life [66].

Cancer

Cancer is a global concern with large efforts being devoted to methods to prevent and/or cure the disease. Cancer is the abnormal growth of cells beyond their usual boundaries that can invade adjoining parts of the body and/or spread to other organs [67]. Probiotics have been investigated for their potential as an adjunctive therapy and as a microbial therapy for cancer [68, 69].

The previously mentioned *L. casei* species type strain ATCC 393 was investigated in an experimental model of colon cancer using both murine and human colon carcinoma cell lines [70]. The *in vivo* model showed an approximately 80% reduction in tumour volume in mice fed live *L. casei* for 13 days. The bacteria attached to the cancer cells and reduced cancer cell viability and induced apoptotic cell death. While the mechanisms are not fully understood, this provides promising evidence for the use of *L. casei* in the treatment of cancer. *L. casei* strains have also been investigated for prophylactic treatment in late stage colorectal cancer (CRC). The main cause of death from CRC is the tumour metastasising to other organs. Cell free supernatant from *L. casei* and *L. rhamnosus* was found to lower the ability of a metastatic tumour cell line to invade *in vitro* [71].

Numerous studies have investigated strategies for how probiotic strains may be used to aid tumour reduction. *L. casei* Shirota has been studied in combination with dietary fibre for its ability to reduce the reoccurrence of tumours in colorectal cancer, with early studies showing promising results [72]. LGG has also been investigated as a synbiotic with oligofructose and *Bifidobacterium lactis* Bb12 [73]. This promising study showed that the synbiotic was capable of lowering the uncontrolled growth of intestinal cells. This is thought to be due to the presence of the synbiotic enhancing the mucosal structure, reducing exposure of epithelial cells to cytotoxic and genotoxic agents, and a decrease in colonic cell growth.

Treatment methods for cancer patients can place a large burden on the immune system and can cause side effects such as diarrhoea which, in an immunocompromised individual, can prove fatal. Probiotics have been shown to reduce the likelihood of radiation-induced diarrhoea during cancer treatment [74].

LGG has also been studied as an adjunctive therapy for cancer treatments to reduce the incidence and severity of diarrhoea [75]. It was shown that treatment supplemented with LGG capsules resulted in less grade 3/4 diarrhoea, with less abdominal discomfort reported [76].

Diarrhoea

According to the WHO diarrhoea is the second highest cause of death in children under five years old, and remains a major problem associated with antibiotics. Antibiotic-associated *Clostridium difficile* infection is the leading cause of diarrhoea in high-income countries at all ages. LCG strains have been associated with improving the symptoms and/or duration of diarrhoea in numerous studies, but specifically LGG is a promising strain in this regard. The ability of many *L. casei* strains to prevent antibiotic associated diarrhoea is associated with its ability to maintain the diversity of the gut microbiome of individuals during antibiotic treatments. This could be due to direct attachment of the strain to epithelial cells. LGG attaches to mucosal cells through the SpaCBA pilus. The SpaC adhesin is present along the entire length of the pilus in large quantities [77]. This adhesin allows both long and close adhesion of the bacterium and aids in the long-term colonisation of the strain. Vancomycin resistant enterococci, such as *Enterococcus faecium*, also bind to and colonise the gut based on a pilus with high sequence similarity to the LGG SpaCBA pilus. LGG could be used prophylactically or as direct competition in the treatment of enterococcal infections [78].

Microbial therapy through bacterial components

The future of probiotics lies not only in the administration of live bacteria but also in the possibility of utilising bacterial components to modulate the immune system. The ability of *L. casei* strains to survive passage through the digestive system and potentially colonise the gut allows some strains to have direct effects on human health. However, bacterial components are also being investigated for their health associated capabilities. In one study, a heat-killed strain of *L. casei* was administered in a mouse model of colitis. Both the heat-killed and live strains were shown to have therapeutic effects on a model of inflammatory bowel disease (IBD) [79]. The

advantage of such a scenario is promising as it eliminates the need for the strain to survive passage through the gut and could potentially provide a more consistent effect as colonisation is not required.

Exopolysaccharides (EPS) are produced by numerous bacteria, sometimes in response to stress. EPS have also been investigated for their health-promoting benefits [8, 80], such as immunoregulatory effects [81]. The LCG have long been used in fermentations whether as adjunct or starter cultures. Traditionally these would have been included for their desirable technological properties such as EPS production. Many of these technologically desirable traits have since been investigated for their probiotic potential. For example, EPS produced by *L. rhamnosus* has been shown to have immunosuppressive capabilities [82]. The probiotic strain *L. paracasei* DG has a range of health promoting properties attributed to it, including its use as an adjunctive therapy in *Helicobacter pylori* treatment and modulation of the gut microbiota. Recent suggestions that bacterial components may be the mechanism by which probiotics exert their effects sparked a study on the EPS produced by this strain. This study demonstrated that the EPS which is secreted by and covers *L. paracasei* DG may have a role in its health associated properties [83].

Interest in utilising probiotics for the treatment or prevention of disease is growing, and the studies discussed here highlight some of the potential directions for the future of health-related research in the LCG. The LCG exert many of their health benefits when ingested live and are capable of colonising the gut. To survive this transit they must have the propensity to respond to a wide array of stresses. The underlying mechanisms involved in this are becoming clearer.

The *L casei* group and stress response

As probiotics the LCG must endure many stresses during processing and remain viable on transit to their site of action, the GIT. These stresses include but are not limited to bile salts, oxidative stress, cold stress, osmotic stress, acid stress and long-term storage. Resistance to these stresses in LCG is strain dependent, with some having a high resistance to multiple stresses while others exhibit little to no resistance [84]. The LCG employ many different tactics in order to survive these

stresses, including alterations to cell membrane, metabolic pathways and upregulation of chaperone proteins [85].

Acid shock

Due to the facultative heterofermentative nature of LCG, hexose sugars are almost exclusively fermented to lactic acid. The LCG have an innate tolerance to acid stress for this reason. Probiotic bacteria must also endure an acidic environment during gastrointestinal transit. Tolerance to acid stress is particularly relevant in industry for probiotic bacteria. In acidic environments, acids can passively diffuse into cells where they can rapidly dissociate to release protons. This lowers the intracellular pH (pHi) and affects the transmembrane pH gradient and the proton motive force [86-88]. Low pHi also causes damages to proteins and DNA. The methods used to counteract acid stress in *L. casei* include the arginine deiminase pathway, F0F1 proton pump, cell membrane alterations and repair of damaged DNA and proteins. Amino acid utilization also plays a physiological role in pHi control in LAB [89].

The LCG can exploit amino acids such as arginine, histidine and aspartate during acidic conditions [90-92]. This is generally achieved by deamination of the amino acid and the expulsion of the alkaline product from the cell [86, 93]. The arginine deiminase pathway is a catabolic system found in *L. casei* that uses arginine to produce ATP, ornithine, CO_2 and NH_3 [94]. The system is comprised of three enzymes; arginine deiminase (ADI), catabolic ornithine transcarbamoylase and carbamate kinase and a singular membrane transport protein [93]. The NH_3 reacts with protons to help alkalize the extracellular environment. Acid resistant *L. casei* can overexpress proteins involved with the ADI pathway in acidic conditions [92]. The F0F1-ATPase is ubiquitous among bacteria [88]. This multicomponent enzyme is bifunctional; it can produce ATP using protons or alternately pump protons out of the cell by hydrolysing ATP [88]. In acidic conditions, acid tolerant *L. casei* show higher levels of ATPase activity than that of acid sensitive cells [92]. Bender and Marquis showed that *L. casei* had a higher ATPase activity that that of the non-acid tolerant *Actinomyces viscous* in acidic conditions [95].

Bacteria also adapt to environmental changes (stresses) by modifying their membrane composition [96]. The acid tolerant *L. casei* Zhang can increase expression of the proteins MurA and MurG, key enzymes in peptidoglycan synthesis [92]. It has been shown that bacteria alter the fatty acid composition of their cytoplasmic membrane in response to acidic conditions but different results have also been reported. Fozo et al. [97] and Wu et al. [98] reported that there is an increase in the unsaturated fat proportions while Broadbent et al. [99] have reported the opposite in that the ratio of saturated to unsaturated fatty acids increases. *L. johnsonii* has been shown to become more sensitive to acid stress when supplemented with unsaturated fatty acids [100]. These contradictory results could be due to the use of different strains or different experimental conditions. In response to DNA damage, there is an overproduction of DNA repair proteins involved in base excision repair, nucleotide excision repair, mismatch repair, and homologous recombination [92]. Proteins involved in the general stress response (DnaK, DnaJ and Hspl) and chaperone proteins (GroEL, GrpE) are also overexpressed [92, 101, 102]. These proteins are involved in the prevention of incorrect protein folding and repair of damaged proteins at the expense of ATP [103]. Many of the reactions of the cell to acid stress are energy dependent and therefore require ATP. Wu et al. [104] found that while levels of glucose-phosphotransferase system (PTS) activity dropped significantly in acid stressed cells, they were still higher than in sensitive cells. This in turn means more glucose entering glycolysis and more energy (ATP) for the cell to react to acid stress. Nezhad et al. [105] also showed that there were increased levels of proteins involved with the glycolytic pathway expressed on the cell surface of *L. casei* under acidic conditions.

Cold Shock

Post fermentation, probiotic LCG are going to encounter cold stress in the form of refrigeration. Survival of cold storage is necessary for refrigerated probiotic bacteria to reach their final destination in the human GIT. Cold is a physical stress that affects physico-chemical properties inside cells. It does this by influencing membrane fluidity, diffusion rates and interactions of macromolecules such as proteins, DNA and RNA. Cold shock proteins (CSPs) are produced by bacteria in

response to growth at temperatures below optimum conditions. CspA and CspB in *L. casei* have similar sequences to that of the C-terminal of EIIA proteins of the glucose-PTS system [106]. It has been suggested that Hpr phosphorylates these cold shock proteins to activate them in response to cold shock [107]. *L. casei* mutants unable to form Hpr ser-45 were much more sensitive to freeze/thaw when grown on glucose as compared to growth on other sugars [107]. This suggests that CSPs have a major role in metabolism at low temperatures. More cold-induced proteins (CIPs) are expressed in cold shock to maintain fluidity of the cell membrane by increasing the ratio of shorter and/or unsaturated fatty acids and to maintain DNA structure by reducing negative supercoiling.

Bile stress

Bile acids (sometimes referred to as bile salts) are secreted into the intestine of mammals to aid digestion. They also offer another hurdle for bacteria as they have antimicrobial activity. Bile is known to affect the structure of membranes, DNA, RNA and protein folding [86]. Hamon et al. [108] employed a proteomic method to evaluate bile tolerance in *L. casei* and suggested the membrane modification protein RmlC, cell wall synthesis protein NagA and NagB, molecular chaperone protein ClpP and proteins involved in central metabolism were key to bile tolerance. Bile salt hydrolase (BSH) is a protein expressed by bacteria commonly found in the intestinal tract that can deconjugate bile salts and also have the added positive effect of lowering serum cholesterol levels and reducing the risk of obesity and arthrosclerosis in the host [109]. BSH activity has been found in *L. casei* but is not always present [109-111]. There is conflicting evidence whether or not BSH increases tolerance to bile salts with many studies finding a correlation [109, 112] while many others have failed to find correlation between BSH activity and bile tolerance [113, 114].

Oxidative stress

Oxidative stress refers to the production of Reactive Oxygen Species (ROS) and accumulation in the cell. ROS include superoxide (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). ROS can lead to impairment of proteins, lipids and nucleotides, contributing to the arrest of cell growth and cell death [115]. Some LAB,

including *L. casei*, can utilize oxygen in aerobic conditions for energy production using a pathway which includes pyruvate oxidase, NADH oxidase, NADH peroxidase and acetone kinase, which results in the formation of ROS [85, 116]. Catalase is an enzyme capable of degrading H_2O_2 and therefore is a key component in oxidative stress. Indeed, the presence of oxygen has been shown to increase its activity in *L. casei* [116]. The high Mn content of *L. casei* can act as an efficient scavenger of O_2, thereby compensating for the lack of a superoxide dismutase, while there are currently no known enzymes to degrade hydroxyl radicals [88].

Osmotic Stress

High osmolality is often faced by the LCG in fermented foods. Food spoilage bacteria and pathogens are often more sensitive to high osmotic conditions compared to LAB, therefore NaCl is often added to aid indigenous or starter LAB during the fermentation process [93]. Salt stress leads to cell wall conformational changes in *L. casei*, which increases its ability to form biofilms and bind cations [117]. High salt conditions cause *L. casei* to become more susceptible to antimicrobial peptides which target cell wall synthesis [117, 118].

Storage

Before use as a probiotic, bacteria may need to be stored for long periods of time. The two main methods for preparing them for long term storage are freeze-drying and spray-drying. During freeze drying, cells are subjected to a physical stress caused by crystals forming, dehydration and osmotic stress [119]. Jofré et al.[120] found that members of the LCG survive storage better at refrigeration temperature and skimmed milk alone or supplementation with trehalose or lactose to be a suitable cryoprotectant during freeze-drying. Encapsulation of *L. casei* cells during freeze drying can also offer protection to cells during storage post freeze drying [121].

During spray drying, bacteria are exposed to many different types of stress which affect the viability of the cells during storage, including heat, dehydration, shear, osmotic and oxidative stress [119]. Microencapsulation of the cells in a

protective agent is one of the main methods of increasing viability during spray drying. *L. casei* LK-1 microencapsulated in skimmed milk shows better survivability during storage and gastric digestion than cells microencapsulated in trehalose and maltodextrin [122]. Storage temperature is also a key factor in the viability of cells following this process. An increase of storage temperature from -20 C to 4 C or from 4 C to 25 C causes a three-fold acceleration of the inactivation rate of *L. casei* LK [122]. Higher inactivation rates for *L. rhamnosus* GG were also reported at room temp following spray drying as compared to chilled temperatures [123].

Conclusion

The LCG contains the species *L. rhamnosus*, *L. casei* and *L. paracasei*, these are well-researched due to their applicability in the food, biopharmaceutical and medical industries. Despite this, the group has a long complicated taxonomic history in distinguishing and discriminating the species from each another. The taxonomic debate surrounding the LCG is likely to continue to prove interesting and challenging as sequencing and novel identification methods are developed.

Stress resistance is associated with the LCG through physical and metabolic alterations, although these resistant phenotypes are strain specific. The LCG must be able to survive a number of stress conditions if they are to be used in industry, including oxidative stress, osmotic stress, cold stress, acid stress and long-term storage. In addition to their need to tolerate these conditions in food processing, live strains must be able to survive passage through the GIT if intended for health-promoting purposes.

The health-promoting capabilities of the LCG have been documented in many studies suggesting real potential for their use in the treatment, or prevention, of a variety of diseases. Moving forward, it is essential that scientists decipher the underlying mechanisms of action involved in order to be able to apply these strains or their bacterial components as novel treatments or prophylactic interventions.

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Author Contribution

DH, IS, and CT contributed equally to the writing of the manuscript. CH, CS, and RPR contributed to manuscript revision, read, and approved the submission.

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Chapter 2.

Recent advances in microbial fermentation for dairy and health.

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Abstract

Microbial fermentation has been used historically for the preservation of foods, the health benefits of which have since come to light. Early dairy fermentations depended on the spontaneous activity of the indigenous microbiota of the milk. Modern fermentations often rely on defined starter cultures with desirable characteristics to ensure consistency and commercial viability. The selection of defined starters depends on specific phenotypes that benefit the product by guaranteeing shelf life, and ensuring safety, texture and flavour. Lactic acid bacteria can produce a number of bioactive metabolites during fermentation, such as bacteriocins, biogenic amines, exopolysaccharides and proteolytically released peptides, among others. Studies are now looking at how the fermentation of prebiotics such as fructo-oligosaccharides can help in the prevention of diseases such as osteoporosis, obesity and colorectal cancer. The potential to prevent or even treat disease through the fermentation of food is a medically and commercially attractive goal and is showing increasing promise. However, the stringent regulation of probiotics is beginning to detrimentally affect the field and limit their application.

Introduction

The fermentation of food by microbes has been employed for millennia as a process to ensure extended shelf life, and to improve the functionality texture, and flavour of food products. The first evidence of dairy fermentation exists from approximately 7,000 years ago, where early Europeans are thought to have produced cheese [1]. Methods have evolved from spontaneous fermentation by the indigenous microbial population to pre-selection of starter cultures with known attributes. Lactic acid bacteria (LAB) are the major bacteria used in food fermentations worldwide. LAB consist of a myriad of genera including but not limited to *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus* and *Enterococcus*. Though the LAB are a diverse group of bacteria many species enjoy historical "generally regarded as safe" (GRAS) and "qualified presumption of safety" (QPS) status granted by the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), respectively [2]. LAB fermentation has long been recognised to confer beneficial effects on human health through the modulation of the intestinal microbiota. These either directly or indirectly affect the host microbiota, which in turn can lead to an effect on health. The use of these bacteria in fermentations to produce functional foods has greatly increased in recent years. Consumption of fermented foods has been associated with a range of health benefits from disease prevention to enhancing the bioregulation of behavioural issues such as stress and anxiety [3-5].

While the consumption of traditional fermented foods is believed to have beneficial effects in cultures around the world, not all of these foods have been subjected to appropriate trials in which these beliefs could be credited or discredited. The benefits of fermented dairy products are being researched extensively in parts of the world, but other ethnic fermentations are also beginning to be studied in more detail [6]. These traditionally fermented foods use uncharacterised starter cultures that could possess novel properties or be useful in other fermentations, some of which will be discussed in more detail later [6-8]. The potential application of microbial fermentation is enormous both in health and in biotechnology and will be an important area of research and production in coming decades [9]. Yeasts and moulds are also prominent fermenting organisms in alcoholic and certain cheese

fermentations. The focus of this review is to look at advances in the past three years in the field of microbial fermentation with a focus on food and added health benefits of fermentation including extraneous commercial and legislative factors impacting the field (Figure 1).

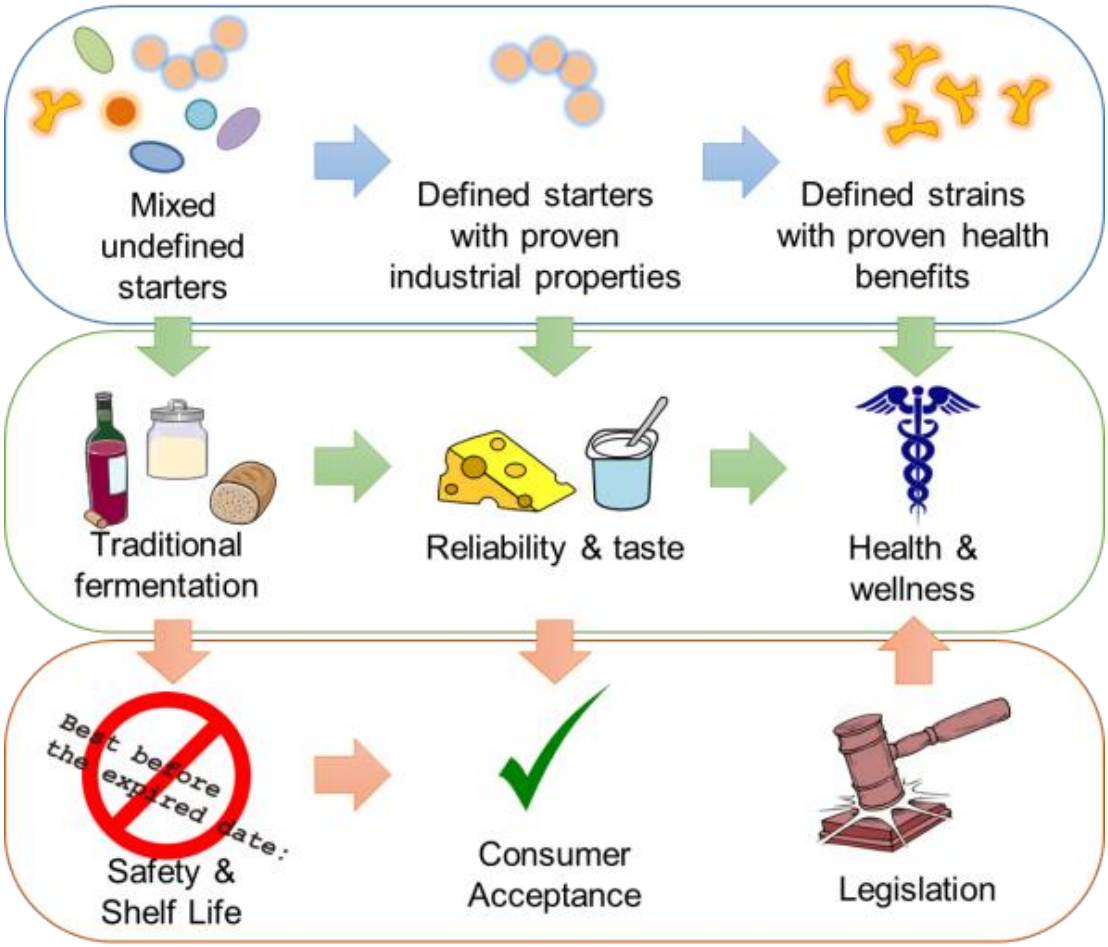

Figure 1. Schematic representing the relationships between fermenting microbes, fermented dairy products, and the consumer.

Fermentation starter cultures and by-products of fermentation

Defined starter cultures, which carry out the fermentation process, are used to ensure consistency in commercial products by using known species with desirable traits, such as a high rate of acidification through the production of lactic acid, and/or the secretion of secondary metabolites into the fermentate matrix (Figure 2). Novel starter cultures are continually in demand for the development of new commercial products along with greater characterisation of those currently in use to ensure safe

and functional products. There are many positive and negative factors that impact the selection of starter cultures in dairy fermentations such as a history of safe use; acidification rate during fermentation; exopolysaccharide production [10]; proteolytic activity, particularly during cheese production and the generation of bioactive metabolites and peptides [11, 12].

Bacteriocins

Bacteriocins are small ribosomally synthesised antimicrobial peptides against which the producer species is immune and which act against other bacteria in a bactericidal or bacteriostatic manner [13]. Great care must be taken with regards to bacteriocin production in starter cultures, as they may target other fermenting cultures or adjuncts; however, their ability to inhibit potential spoilage bacteria and pathogens can be of great use [14]**.** The identification of bacteriocinogenic strains has mostly relied on agar diffusion based assays [15]. Increasing interest in bacteriocins as alternatives to antibiotics and chemical food preservatives has led to new methods for identifying bacteriocin producers. *In silico* screening using programs such as BAGEL [16] and antiSMASH [17] enables the discovery of new bacteriocin operons where whole genome data is available. Such methods avoid any potential problems with unsusceptible indicator strains [18] and can allow for faster initial screening. Collins *et. al* [19] using an *in silico* screen of 213 lactobacilli identified 11 bacteriocins, five of which were novel [20]. Another *in silico* study which mined human gut microbiome sequence data found 74 bacteriocin gene clusters from 382 fully sequenced genomes [21]. *In silico* screens such as these rely on previously described peptides or the identification of bacteriocin accessory genes, and such it is unlikely in that initial agar diffusion-based assays will be completely replaced with *in silico* screening. However, they may represent an opportunity to search for new bacteriocins in complex microbiotas such as that of a traditional fermented product.

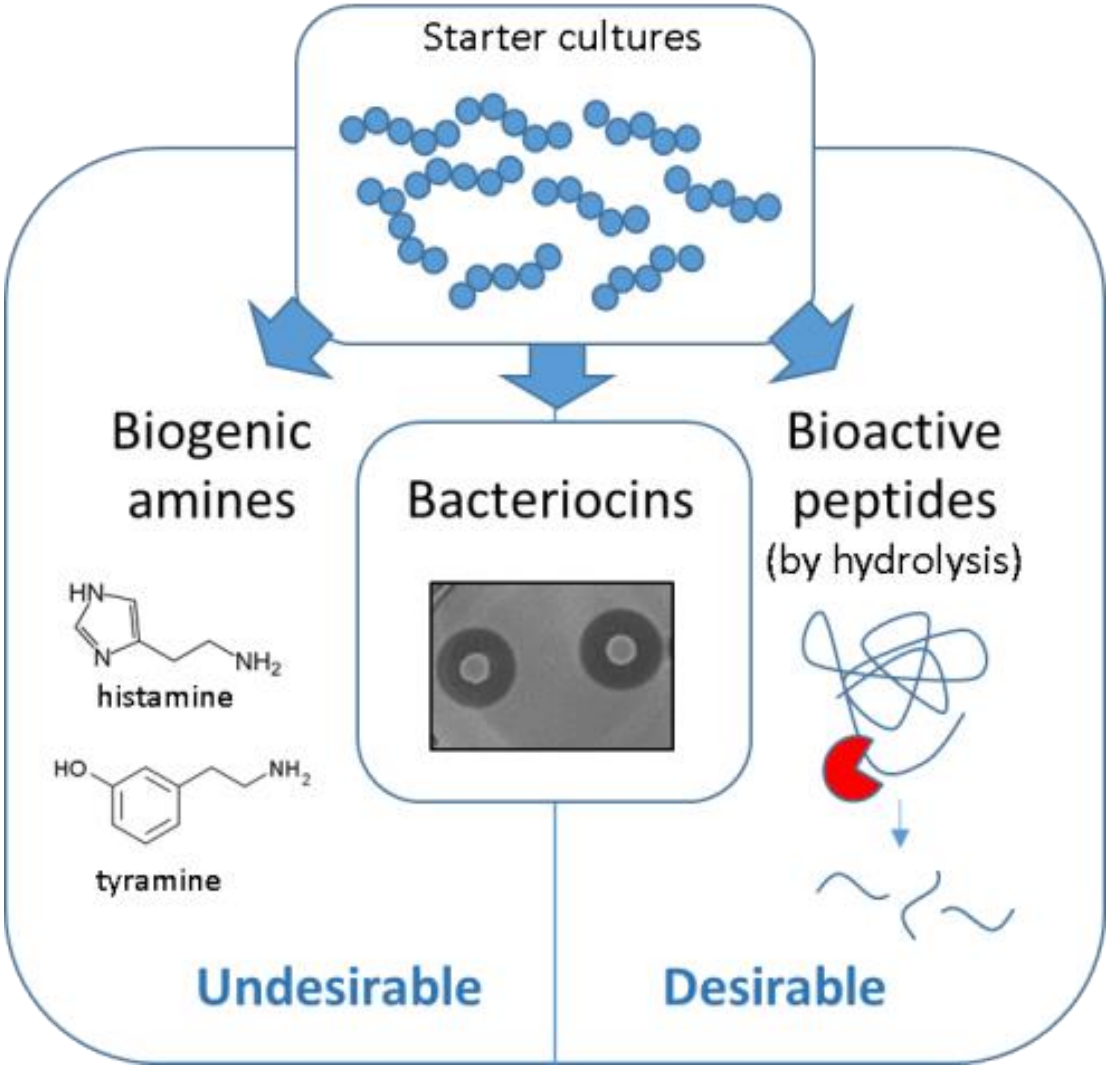

Figure 2. Desirable and undesirable bioactive metabolites produced during fermentation which can impact choice of starter cultures. Fermentation starters can produce a number of desirable and undesirable bioactive metabolites. Biogenic amines (Figure 2, left) are an undesirable product in most fermentations due to their toxicity. Bioactive peptides (Figure 2, right) produced through enzymatic release are desirable by-products due to positive biological activity. Bacteriocins (Figure 2, centre) are desirable as a known probiotic trait, but potentially undesirable in a starter culture due to possible impact on other fermenting cultures.

Biogenic amines

Biogenic amines (BAs) are biologically active, low molecular weight organic bases produced mainly through the decarboxylation of certain amino acids, which can accumulate during fermentation. Traditionally, the presence of biogenic amines

in food products is associated with undesirable microbial activity, indicating food spoilage or defective manufacture [22]. Dairy products, in particular cheese, can accumulate high levels of BAs, mainly histamine and tyramine, which are known to be toxic [23-25], but as of yet legal limits have only been set for histamine in fish products [26]. The accumulation of more than one kind of BA in products is of particular concern due to their synergistic toxicity at dietary concentrations, which has recently been demonstrated with intestinal cells *in vitro* [27]. BAs are detected in dairy products by chromatographic detection of the BA compounds, or through detection of BA-producer organisms using PCR based methods, which correlate with HPLC results [28]. In a recent study, levels of tyramine in a model cheese were reduced by 85% through the use of a bacteriophage to limit the population of BA-producing bacteria [29]. Pre-selection of starter cultures lacking BA genes in the future may be necessary to avoid unwanted build-up of BA compounds and continued avoidance of contamination, which is known to occur during post-ripening processing [30, 31].

Bioactive peptides

Bioactive peptides are encrypted in larger proteins and when released by proteolysis have been associated with health promotion through a number of mechanisms such as inhibition of angiotensin 1-converting enzyme activity, antithrombotic activity, antihypertensive activity, antioxidant activity, immunomodulation, apoptosis modulation and by opioid and anti-opioid activities [11, 32]. LAB possess a myriad of proteases and peptidases that can release encrypted peptides during milk fermentation [33] or following the ingestion of fermented products containing LAB in the intestinal lumen. In recent years, potential anticarcinogenic peptides have been found encrypted in bovine milk casein and whey proteins, including the previously known cationic lactoferricin [34]. The known cancer-preventative peptide lunasin has been found to be proteolytically released during sourdough fermentation by LAB [35]. Further research has subsequently revealed increased protease resistance during *in vitro* gastrointestinal transit in the presence of naturally occurring protease inhibitors to allow lunasin to reach the large intestine [36]. Another recent study has found that the administration of milk

fermented by a probiotic *Lactobacillus casei* strain modulated the immune response against a breast cancer tumour in a mouse model, with delayed or blocked tumour development in the fermented milk fed group as compared with unfermented milk as a control [37]. The mechanism of action has not yet been elucidated and studies of a similar nature have not yet passed the animal trial preclinical stages.

Ethnic fermented milk products

There is increasing interest in novel LAB strains isolated from ethnic fermented milk products, which would have been part of the autochthonous fermenting microbiota. Products such as matsoni, a fermented milk product of Armenian origin, and kule naoto, the traditional fermented milk product of the Maasai in Kenya, have had their previously undescribed microbiotas characterised using sequencing based analysis [38, 39]. Indeed, such products may be of great value; for example shubat, a probiotic fermented camel milk of Kazakh origin, has recently been found to demonstrate positive hypoglycaemic activity in type 2 diabetic rats [40], and the indigenous Indian fermented beverage raabadi has been investigated as a source of probiotic hypocholesterolaemic lactobacilli [41]. However, a study of mursik, a milk product from Kenya that is traditionally fermented in a gourd, has been suggested as a possible etiological factor for oesophageal cancer, because of high levels of ethanol and acetaldehyde present post fermentation [42]. This is in stark contrast to the health claims made for most commercial fermented dairy products that indicate the benefits of using safe, known starter cultures.

Prebiotics

In 2017 prebiotics were defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as *"a substrate that is selectively utilized by host microorganisms conferring a health benefit"* [43]. Prebiotics are fermented by the gastrointestinal microbiota and contribute to healthy modulation of the gut [44]. The ingestion of specific prebiotics has been shown to increase antibacterial capabilities of a probiotic strain [45]. Synbiotics are a relatively new area that involve a combination of probiotic and prebiotic in one product. The prebiotic is intended to

improve the survival/growth/performance of the probiotic, or other beneficial bacteria in the colon, which in turn has beneficial health effects on the host [46].

Currently, research is being conducted on the role of the gut microbiota in the development of cancer, with a focus on colorectal cancer [47]. While this research is in its early stage, there is evidence for the use of probiotics, prebiotics and synbiotics in the treatment or prevention of this disease. There is potential for these to act as anticarcinogens or antimutagenic agents through diet-based interventions. More detailed analysis could lead to huge strides in the prevention of cancer, but as of yet the field is open to new research [48].

Health-focused research

Research into the use of fermented foods as a potential approach to fight disease is growing, but it must be appreciated that many of these functional foods are intended to prevent disease onset, or alleviate symptoms, and not necessarily act as a curative agent [49]. This increases the burden of proof on the researcher to prove that the fermentation of the prebiotic was indeed the reason the host remained healthy. Modulation of the gut microbiota is the focus of many studies relating microbial fermentation to measurable health benefits. One emerging area of study using microbial fermentation is in osteoporosis. Osteoporosis is common in postmenopausal women and the elderly, and presents itself as weakened bones prone to breaks or fractures due to poor calcium absorption [4]. The consumption of a prebiotic fructo-oligosaccharide (FOS) has the potential to be a preventative method for osteoporosis [50]. The prebiotic is fermented in the gut, causing a drop in the pH of the lumen to such an extent that previously insoluble calcium phosphate will dissolve. This plays a beneficial role in bone mineral density [51]. The fermentation of FOS releases short-chain fatty acids and lactic acid, which cause the drop in pH. This dissolved calcium results in an increase in passive diffusion; thus it could help to treat, or even potentially prevent, the onset of osteoporosis.

Obesity is a global issue and has generated much interest in whether and how our gut bacteria could be a contributing factor in the development of this complex syndrome. On-going studies into the gut microbiota are aimed at identifying whether

a specific bacterium or bacterial group could be contributing to obesity [52]. While this is an emerging area of research, there are exciting developments on how to potentially fight this syndrome through the modulation of the gut microbiota [53]. The so called “obese microbiota profile” can be characterised as a decreased Bacteroidetes/Firmicutes ratio in individuals [52]. One study looked at the administration of prebiotics such as FOS as a potential method of reducing the likelihood of obesity by increasing the levels of “lean microbiota” through fermentation of the prebiotic in the gut. This in turn led to a decrease in the permeability of the intestine with improved tight junction integrity. While this is in early stages of research it does present a potentially new method by which obesity could be treated through microbial fermentation within the gut [54]. This area of research has great potential medically and commercially [55].

Bacteriocins, as discussed previously, are antimicrobial peptides that target and kill other bacteria, which could potentially be utilised as an anti-obesity tool. If specific strains that are contributing to obesity are identified then modulation of the gut microbiota, through introduction of specific bacteriocins or bacteriocin producers, has the potential to reduce the risk of obesity. Likewise, specific bacteriophage could be used to target such strains.

Current evidence supports the theory that microbes in the gut could be a contributing factor to mental disorders through the brain gut axis [56]. Psychobiotics are an emerging area of study on the role of the microbiota in brain health. A psychobiotic is a bacteria that, when ingested in adequate amounts, can have a positive mental health benefit [57]. The permeability of the intestinal barrier can be compromised by the westernised diet of processed foods and carbonated beverages. The bacteria present in our gut are capable of producing neurotransmitters through the metabolism of indigestible fibres, these include dopamine, noradrenalin, GABA and acetylcholine [58]. The consumption of probiotics in fermented foods could have a positive influence on maintaining the intestinal barrier and preventing chronic inflammation. Dietary interventions in adolescents with more fermented foods containing beneficial brain bacteria could help prevent the onset of depression and anxiety among other mental health issues which are becoming more prevalent.

National recommendations

Fermented foods have been consumed worldwide for thousands of years before any direct health benefits were truly understood. While the demand from consumers for functional foods is growing the national recommendations are not following suit. Now that the mechanisms by which these fermentations can beneficially affect human health are beginning to be elucidated, food guidelines around the world are slowly beginning to recommend their consumption. This inclusion is not universal, despite historical use and clinical trials proving the benefits of these fermented products in the diet. Given the strong tradition of fermented foods in Asia, it is somewhat surprising that they are not specifically included in food guidelines, albeit the Chinese Nutrition Society suggests the consumption of yogurt for those who do not tolerate lactose well.

There is a high incidence of lactose intolerance in Asian countries, and there are clinically proven studies that show the inclusion of fermented dairy foods can help to alleviate the symptoms of intolerance [59]. Japanese authorities list fermented foods in the Food of Specified Health Use (FOSHU) category, and in India the guidelines specifically encourage the consumption of fermented foods. The Indian guide highlights specifically that pregnant women should consider including more fermented foods in their diet owing to the increased bioavailability of iron that is associated with these foods.

Regulation of fermented dairy products

The regulations surrounding microbial fermentations in the food industry is beginning to have a detrimental effect on the industry as a whole. For example, there is currently no legal definition for the term “probiotics”. Unless scientific, legal, and industrial teams are all working together under one agreed definition, the term “probiotics” will begin to lose its meaning. Along with this, the general community are losing confidence in the benefits of fermented dairy products that are supplemented with probiotic/prebiotics. Since December 2012, in Europe, labelling of a probiotic was banned along with the use of health claims in any product without receiving approval from EFSA, which has yet to approve any probiotic health claim.

This is despite the numerous clinical trials proving the benefits of probiotic yogurts in health. This change has led to consumer confusion as to whether or not the claims were ever true. It is essential that labelling of fermented food products with clinically proven health benefits is permitted to allow industry to begin to profit from funding these trials, or they will begin to invest in marketing strategies rather than the much-needed research [60]. The International Dairy Federation (IDF) represents the dairy sector at relevant Codex meetings regarding the international standards for dairy products [61]. The IDF are currently involved in investigating product labelling with regard to nutritional information and health claims and how these affect the consumer's choice of different products [62]. These studies will hopefully lead to a change in labelling laws to allow for clinically proven health claims to be present on fermented dairy products.

Conclusion

Microbial fermentation holds the key to some extremely complex interactions between bacterial species and the food matrix they are fermenting. The studies highlighted in this review show the potential of utilising these microbial fermentations in a more knowledge-based fashion than that of the past. With regard to microbial fermentation in food, this represents an area with potential well beyond the extension of shelf life. The work in these areas is continuing and with the help of better regulation could lead to exciting new discoveries on managing disease symptoms through food. Though fermented products have long been associated with health promotion, the lack of regulation has been a confounding factor in consumer attitudes. Indeed, other legislation must be put in place in the near future for harmful levels of BAs in fermented dairy products, given that at present no upper limit for potentially toxic levels of histamine and tyramine are available. The search for probiotics is ongoing using both genetic and traditional screening methods [63, 64]. Probiotics have a bright future in the area of supplemented fermented foods for health promotion.

There have been numerous advances in our understanding of fermented products, the microbes which produce them, and fermentable polysaccharides in

recent years. With public opinion shifting towards healthier lifestyles and viewing chemical preservatives in a negative light, fermented products show great commercial promise. New starter cultures are being identified using more sophisticated methods to ensure their effectiveness and viability. *In silico*-based methods and research in the health-promoting activities of LAB in fermentates are on the rise, along with the characterisation of traditional products that have been associated with good health.

Author Contribution

DH and IS contributed equally to the writing of the manuscript. CH, EA, CS, and RR contributed to manuscript revision, read, and approved the submission.

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Chapter 3.

Phenotypic screening reveals a high prevalence of EPS producers in lactic acid bacteria from bovine, ovine and caprine milk.

Manuscript in preparation

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Abstract

The aim of this study was to isolate and characterise exopolysaccharide (EPS) producing lactic acid bacteria (LAB) from bovine, ovine and caprine milk sources. Seven hundred and ninety-nine isolates were recovered on LAB selective agar from a range of environmental sources, primarily raw milk from cows, sheep and goats. All isolates were screened for EPS production using three phenotypic methods, in order to identify both ropy and mucoid EPS types. In total 466 isolates (58%) were positive for EPS production by one or more of the phenotypic tests. Forty-six EPS positive strains were classified using 16S sequencing and eight strains with generally regarded as safe (GRAS) status were further selected to investigate their potential in dairy fermentations. These eight strains were evaluated for their ability to grow in milk at three temperatures (30, 37, and 42 °C). Five EPS producing strains capable of reliable growth in milk were used as adjunct cultures to CH-1, a commercial non-EPS producing starter culture, in small scale dairy fermentations. Each strain significantly improved the texture of the resulting yogurt (increasing gel strength from 1.2 to 1.5 Newtons, $p<0.0001$). Following whole genome sequencing of all eight strains and antibiotic testing, two were excluded (*S. gallolyticus* LL009 and *L. johnsonii* LL006). The remaining three strains, two *L. plantarum* strains (LL001 isolated from bovine milk and LL025 isolated from dog saliva) and *Leuconostoc mesenteroides* (LL002 isolated from bovine milk), show significant promise as adjunct strains in dairy fermentations. This work highlights the prevalence of EPS production among LAB and the advantage of using multiple screening methods for identification of EPS producing LAB.

Introduction

Exopolysaccharide (EPS) production is widespread among bacteria and has been exploited by the food industry to improve the technological and/or organoleptic quality of food products, as previously reported [1, 2]. Due to growing consumer demand for 'clean label' products, replacing additives by inclusion of EPS-producing lactic acid bacteria (LAB) to improve texture is an appealing approach. The EPS produced by LAB are diverse in their structure, size, and functionality. They can be produced inside or outside the cell and can remain attached or associated with the cell wall, i.e. capsular polysaccharide (CPS), or they can be released into the environment/supernatant [2]. EPS are divided into homopolysaccharides (HoPS), and heteropolysaccharides (HePS) depending on their composition. HoPS are constructed from one monosaccharide subunit, while HePS contain three to eight repeating units of two or more monosaccharides. The majority of EPS produced by LAB are reported to be HePS [1]. The quantity produced is dependent on both the individual strain and growth conditions. The variability in type and amount of EPS leads to different properties of the final product produced as a result of the fermentation process [3].

Different types of EPS have been used in the pharmaceutical and food industries, for example as immunomodulatory agents in the pharmaceutical industry and as thickeners for low fat dairy products [4-6]. In dairy fermentations, EPS contributes to texture, stability and sensory properties of the product [7]. Yogurts containing EPS have been shown to have a firmer body, increased creaminess and a shiny surface - properties attributed to a decrease in syneresis - and an increase in viscosity and stiffness [3, 8, 9]. This is of particular importance in goat milk yogurt which in general has weak gel strength [10]. The binding of water, and interactions with the protein network contribute to the beneficial rheological effects of EPS. Both attached and secreted EPS have been shown to contribute rheological effects on the final product, though there is large variation between studies that could be attributed to experimental methods and strain specificity [11]. The effect of attached and secreted EPS is often generalised due to limited understanding of the specific type of polysaccharide produced by the strain under investigation. A number of

characteristics influence the effect that the polysaccharide has on the final product matrix; whether it is secreted or attached (EPS/CPS), the molecular weight, the degree of branching, intrinsic viscosity, charge and amount present [3]. The inclusion of EPS-producing bacteria is not just functionally beneficial to the final product but can also result in health benefits for the consumer [12]. EPS reportedly have health-promoting properties such as immunomodulatory, cholesterol lowering, anticancer and antioxidant activity [13-15].

Common terms used to describe EPS include ropy, mucoid and slimy, although these terms are often used without a clear definition which can lead to confusion. We define ropy as a strain whose colony produces a filament when touched with an inoculation loop, and mucous type (also described in other studies as mucoid or slimy) as the formation of a shiny layer on the colonies. The terms are not mutually exclusive, bacteria can harbour multiple EPS operons that can each produce a distinct EPS type, which is not always obvious from phenotypic tests. Indeed, a single strain can produce both ropy and mucoid type EPS depending on the culturing conditions.

EPS production can be optimised by varying selected parameters such as carbon and nitrogen sources, pH control, incubation time and temperature [16]. Any number of these parameters can be investigated for their ability to increase EPS production, but cannot all be manipulated in a dairy fermentation for yogurt production. Nitrogen addition using yeast extract, and the media used for growth can lead to issues in quantification and characterisation of EPS [17, 18]. Growth at constant pH has been shown to increase EPS yield for some strains [19], but for *in situ* production in dairy fermentations, the pH will decrease over time. When changing temperature for the production of a fermented product, it is important that this does not significantly increase the fermentation time as this could have cost implications.

In this study, we isolated and screened 799 putative LAB to identify both ropy and mucoid EPS types from a range of environmental sources, primarily raw milk from cows, sheep and goats, using three phenotypic screening methods. Forty-six

EPS producing strains were identified by 16S sequencing, and eight were selected to investigate their potential in dairy fermentations. Five of these strains showed reliable growth in reconstituted skim milk; *Streptococcus gallolyticus* LL009, *Lactobacillus johnsonii* LL006, two *Lactobacillus plantarum* (LL001 and LL025) and *Leuconostoc mesenteroides* LL002. These five were chosen for whole genome sequencing and inclusion in small scale dairy fermentations for their effects on texture properties. When used as adjunct cultures in a small-scale goat milk fermentation, all five strains significantly ($p < 0.0020$) improved the gel strength of yogurt compared to the control. *L. plantarum* LL025 and *S. gallolyticus* LL009 had the largest effect, with a 25% increase in gel strength over the control yogurt. Two strains were excluded following whole genome analysis due to the presence of antibiotic resistance genes in *L. johnsonii* LL006, and genes associated with pathogenicity in *S. gallolyticus* LL009. This study thus led to the identification of two *L. plantarum* strains and one *L. mesenteroides* strain suitable for use in food production for texture improvement in dairy fermentations.

Materials and Methods

Biobank Preparation

Samples of raw bovine milk (produced in Ireland), ovine and caprine milk (produced in New Zealand), raw milk cheeses, honey, dog saliva and sheep faeces (all sourced in Ireland) were used for isolation of LAB. Each sample was streaked on LAB selective agar and incubated under the conditions outlined in Table 1. Three to five morphologically distinct colonies from each plate were re-streaked to obtain a pure culture. One colony from these plates was transferred into the appropriate broth before stocking in a 20% (v/v) glycerol solution and stored at -80°C. All isolates were routinely cultured in the relevant broth as described in Table 1.

Exopolysaccharide Screening Methods

Multiple screening methods were used to test for ropy and non-ropy type EPS [20]; ruthenium red MRS agar (RR-MRS), the loop touch test and sugar-supplemented MRS, using a representative scale (+, ++, +++) for each test. The loop

touch test was conducted on colonies formed on re-streaked agar plates. A sterile loop was touched to a single colony and slowly pulled away. A string between the loop and colony was recorded as a positive result [21]. Ruthenium red (RR) was filter sterilised and added to cooling MRS agar at 0.08%, and thoroughly mixed before pouring plates [22]. Isolates were grown from stock on the appropriate media (Table 1), and 5µL spot plated on the agar. A positive result on RR_MRS agar is a white colony, while negative colonies are stained red. Isolates that were positive for one or both of the above tests were screened using sugar-supplemented media [23]. 10% sucrose and 10% lactose solutions and MRS agar were autoclaved separately at 121°C for 15 min and mixed before plates were poured. As with the RR-MRS testing, isolates were spot plated on sugar supplemented MRS, a positive result was defined as slime forming around the spot.

Table 1. Selective agar-based screen protocol for isolation of LAB, with incubation temperatures and times

Genera	**Selective Agar**	**Re-streak Agar**	**Broth**	**Temperature**	**Incubation Time**
Streptococcus thermophilus	STA	MRS	MRS	42°C	48hrs
Lactococcus	LM-17	LM-17	LM-17	30°C	48hrs
Leuconostoc	MRS + vancomycin (30µg/ml)	MRS	MRS	37°C	48hrs
Thermophilic *Lactobacillus*	MRS pH5.4	MRS	MRS	42°C	48hrs
Mesophilic *Lactobacillus*	LBS	MRS	MRS	30°C	48hrs
Bifidobacterium	TOS-MUP	TOS	TOS/BSM	37°C	48hrs

Safety Assessment and antimicrobial activity testing

The lowest concentration completely inhibiting growth, minimum inhibitory concentration (MIC) value, of antibiotics was assessed using the VetMIC Lact-1 and Lact-2 MIC determination plates (National Veterinary Institute, Sweden). The antibiotics tested were ampicillin, penicillin, vancomycin, erythromycin, virginiamycin, tetracycline, clindamycin, chloramphenicol, kanamycin, gentamycin, streptomycin, neomycin, linezolid, rifampicin, ciprofloxacin, and trimethoprim. Briefly, colonies were suspended in maximum recovery diluent at a concentration of

~1x10^8 cfu ml^{-1} and transferred into ISO-MRS broth for a final inoculum of 5x10^5 cfu ml^{-1}. VetMIC plates were inoculated with 100µL, sealed and incubated for 24hrs. Presence of growth was determined using a backlight. For antimicrobial activity strains were grown overnight on agar plates, overlaid with MRS agar (7.5gL^{-1} agar) seeded with *L. bulgaricus* LMG6901, and incubated anaerobically overnight at 37 °C. Activity was defined by a clearing in the overlay medium.

Genome Sequencing

DNA was extracted using a Genelute bacterial genomic DNA kit (Sigma) and prepared for sequencing using a Nextera XT kit (Illumina) for library preparation. DNA was quantified using a Qubit 2.0 fluorometer. Sequencing was carried using an Illumina MiSeq platform with paired-end 2 x 300 base pair reads by the Teagasc Sequencing Centre, Teagasc Food Research Centre Moorepark. Assembly was performed *de novo* using SPADES and automatically annotating using RAST [24].

Strain Identification

Strains were identified based on 16S rRNA gene sequence analysis. Genomic DNA was extracted using a Genelute bacterial genomic DNA kit (Sigma) and amplified by PCR using the primer pair B27F/U1492R, B27F– 5' AGAGTTTGATCCTGGCTCAG 3', U1492R – 5' GGTTACCTTGTTACGACTT 3'. For 16S rRNA gene sequencing, the PD primer (5' GTATTACCGCGGCTGCTG 3') was used.

Milk fermentation

Bovine and caprine skim milk powders were reconstituted in water at 12% w/v, mixed thoroughly and left at 4°C overnight to rehydrate. Reconstituted skim milk (RSM) was heated to 85°C for 30 min and cooled to 43°C. Two g frozen pellets of commercial starter (CH-1, CHR Hansen as negative control) were added to 200 ml sterile bovine rehydrated skim milk at 43°C and stirred for 5 min to allow the solution to mix. The number of viable cells in frozen cultures is minimum 1 x 10^{10} cfu per gram. An aliquot of 1.1ml of starter culture solution was then added per 100 ml of milk.

For acidification profiling, strains were grown in MRS broth overnight and inoculated into appropriate milk at a 2% inoculum. For addition as adjuncts, cultures were grown fresh from stock overnight in skim bovine milk and added at a 2% inoculum along with the starter. Following inoculation, five minutes was allowed before distributing into containers for fermentation. The milk was distributed into 50 ml containers and fermented at 37/43°C in an incubator until a pH of 4.6 was reached and the fermentation was terminated. The yogurt samples were stored at 4°C for 21 days, n=6 per yogurt type.

Textural characteristics of the yogurt were determined on day 21 using a TA.X2 Plus Texture Analyser using a load cell of 5 kg. Back extrusion was conducted using an 35mm A/BE back extrusion cell, and the tests were performed at the following settings: test speed: 1 mm/s, post-test speed: 1 mm/s, distance: 25 mm, rate for data acquisition: 200 points per second. Gel firmness (gel strength) was defined as the maximum force during sample compression and reported in Newtons.

Statistical Analysis

Statistical analysis of texture data was completed using the GraphPad Prism 8.4.0 software. Individual paired T-tests were completed using GraphPad software for each test yogurt against the non-EPS producing control, CH-1.

Results

The overall aim of this project was to adopt a multi-screening approach to isolate EPS producing LAB from different raw milk, and environmental samples for inclusion in dairy fermentation/processing applications (Figure 1). To achieve this, 799 putative LAB were isolated and tested for EPS production (Table 2). Fifty eight percent of the isolates were positive for at least one of the EPS tests used (Figure 2). The highest positive number was found in the putative mesophilic *Lactobacillus* group.

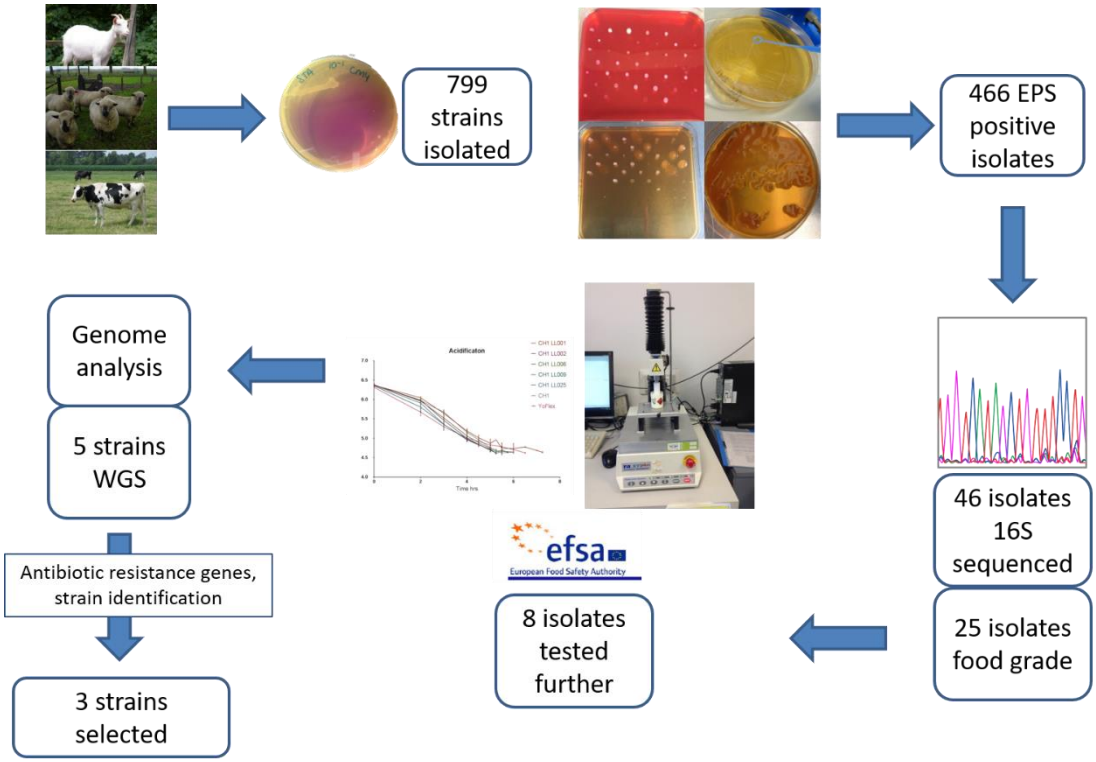


Figure 1. Summary of methods for isolation of exopolysaccharide producing lactic acid bacteria. WGS – whole genome sequencing.

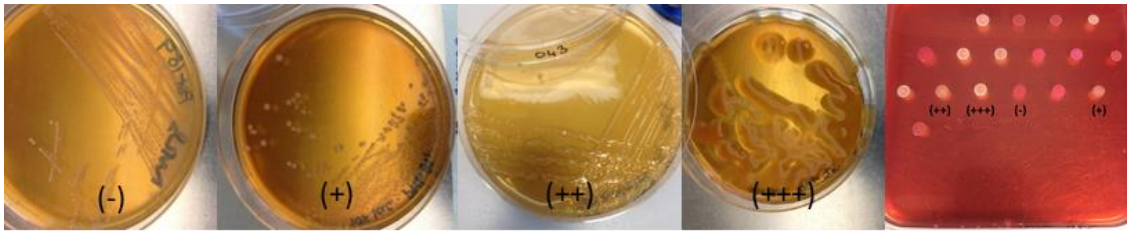
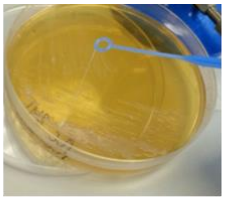
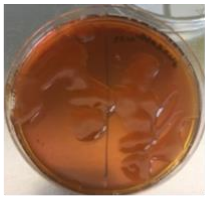


Figure 2. Example of scaling results for phenotypic agar screen. 10% sucrose supplemented media and ruthenium red agar spot testing (far right).

Of 46 isolates that were selected for species identification based on 16S rRNA gene sequencing (nine from bovine milk, 12 from caprine milk, 17 from ovine milk, and six from raw milk cheeses and two environmental), 25 were confirmed as being potentially food grade based on the closest respective BLAST hit to their 16S rRNA gene sequences [25].

Table 2. Results from screen for exopolysaccharide production. Example images are shown. Total numbers for positive result (+), negative result (-), no growth (ng), no result (nr) due to not tested, size of colony growth, or an inconclusive result.






Putative genera	Total Isolates	Loop Touch Test				Ruthenium Red Agar				10% sucrose MRS				10% Lactose MRS			
		+	-	ng	nr	+	-	ng	nr	+	-	ng	nr	+	-	ng	nr
S.thermophilus	225	56	109	8	52	66	99	48	12	15	118	32	60	3	98	61	63
Lactoccocus	251	92	91	11	57	72	109	8	62	35	142	53	21	16	159	54	22
Thermophilic *Lactobacillus*	87	33	44	1	9	63	13	3	8	16	58	4	9	6	64	5	12
Mesophilic *Lactobacillus*	68	52	7	1	8	42	25	1	0	15	52	0	1	14	53	0	1
Leuconostoc	92	56	26	1	9	52	22	2	16	6	75	2	9	8	75	2	7
Bifidobacterium	76	8	38	5	25	17	21	28	10	0	2	29	45	3	3	42	28
Overall Totals	**799**	**297**	**315**	**27**	**160**	**312**	**289**	**90**	**108**	**87**	**447**	**120**	**145**	**50**	**452**	**164**	**133**

Eight strains with positive EPS test results from a range of sources were selected for further study (Table 3). The selected strains included two from raw cow milk (*L. pentosus/plantarum* LL001, *Leuconostoc mesenteroides* LL002), two from raw sheep milk (*Lactococcus lactis* LL005, *L. johnsonii* LL006), one from raw goat milk (*Streptococcus gallolyticus/macedonicus* LL009), two from raw goat milk cheese (*L. paracasei/ casei* LL021, *L. pentosus/plantarum* LL021), and one from dog saliva (*L. pentosus/plantarum* LL025). These were all shown to be negative for biogenic amine production and non-proteolytic (data not shown). Five of the strains showed potential for antimicrobial activity when overlaid with *L. bulgaricus*. EPS production following growth in 12% skim milk was confirmed (Table 4).

Table 3. 16S rRNA identification of potential food grade strains. Results from all tests completed, positive result (+), negative result (-), not tested (nt), no growth (ng).

Isolate	16S closest relative	Source	Loop Touch Test	Ruthenium red MRS	10% lactose	10% sucrose
LL001	*Lactobacillus pentosus/plantarum*	Raw cow milk	+++	+++	-	-
LL002	*Leuconostoc mesenteroides*	Raw cow milk	+	-	-	+++
LL005	*Lactococcus lactis ssp. Lactis*	Raw sheep milk	-	-	-	+++
LL006	*Lactobacillus johnsonii*	Raw sheep milk	-	+++	+	+++
LL009	*Streptococcus gallolyticus/macedonicus*	Raw goat milk	+++	+	-	+++
LL021	*Lactobacillus paracasei/casei*	Raw goat milk cheese	+++	+++	ng	+++
LL022	*Lactobacillus plantarum*	Raw goat milk cheese	+	+++	nt	nt
LL025	*Lactobacillus pentosus/ plantarum*	Dog saliva	+++	+++	-	-
LL094	*Lactobacillus plantarum/pentosus/casei*	Raw cow milk	+	+++	nt	nt
LL115	*Lactobacillus plantarum/pentosus/casei*	Raw sheep milk	-	+	-	+++
LL299	*Streptococcus salivarius*	Raw cow milk	-	-	-	+++
LL300	*Streptococcus salivarius*	Raw cow milk	-	-	-	+++
LL317	*Lactobacillus plantarum/pentosus/casei*	Raw cow milk	+	+++	ng	ng
LL335	*Lactobacillus plantarum/pentosus/casei*	Raw cow milk	+	+++	+	+
LL478	*Lactobacillus plantarum/pentosus/casei*	Raw goat milk	+++	ng	-	-
LL537	*Lactobacillus plantarum/pentosus*	Raw goat milk cheese	+++	+++	-	-
LL572	*Streptococcus salivarius*	Raw sheep milk cheese	+	+++	-	+++
LL593	*Lactobacillus plantarum/pentosus*	Raw sheep milk	+	+++	+	+
LL594	*Lactobacillus reuteri*	Raw sheep milk	-	+++	+	+
LL602	*Lactobacillus plantarum/pentosus*	Raw goat milk	++	ng	ng	+++
LL607	*Lactobacillus plantarum/pentosus*	Raw goat milk	++	ng	ng	+++
LL625	*Lactobacillus plantarum/paraplatarum*	Raw cow milk	+	++	+	+
LL626	*Lactobacillus pentosus/plantarum*	Raw cow milk cheese	+	+++	+	+
LL664	*Leuconostoc pseudomesenteroides/mesenteroides*	Raw goat milk	++	-	-	+
LL665	*Leuconostoc pseudomesenteroides/mesenteroides*	Raw goat milk	+	-	-	-

Strains LL005 (*Lactococcus lactis*), LL021 (*Lactobacillus paracasei*/*casei*), and LL022 (*Lactobacillus paracasei*/*casei*) showed little evidence of EPS production following growth in milk and so were excluded from further study (Table 4). All strains showed varying rates of acidification during growth in 12% skim milk over 24 hr, LL009 (*Streptococcus gallolyticus*) in particular, significantly reduced the pH from pH

6.3 to pH 5, with LL021 (*Lactobacillus paracasei*/*casei*), also causing acidification to pH 5.7 (data not shown).

Table 4. Characteristics of selected eight strains. Positive result (+), negative result (-), not tested (nt).

Strain	16S closest relative	Source	Antimicrobial activity	Biogenic amine production	EPS from RSM plated on	
					SS-MRS	RR-MRS
LL001	*Lactobacillus pentosus/plantarum*	Raw cow milk	+	-	+	+
LL002	*Leuconostoc mesenteroides*	Raw cow milk	-	-	+	-
LL005	*Lactococcus lactis ssp. lactis*	Raw sheep milk	+	-	nt	nt
LL006	*Lactobacillus johnsonii*	Raw sheep milk	+	-	+	-
LL009	*Streptococcus gallolyticus/macedonicus*	Raw goat milk	+	-	+	-
LL021	*Lactobacillus paracasei/casei*	Raw goat milk cheese	+	-	nt	nt
LL022	*Lactobacillus plantarum*	Raw goat milk cheese	-	-	nt	nt
LL025	*Lactobacillus pentosus/plantarum*	Dog saliva	-	-	+	+

The five remaining strains were tested as adjunct cultures together with commercial non-EPS producing starter culture CH-1 at three fermentation temperatures (30, 37 and 42°C) to investigate the effect on fermentation time and EPS production (Figure 3) in bovine milk. Addition of the adjunct cultures at 42°C had the largest effect on the acidification rate, which was more rapid in the initial three hours, but resulted in a similar time overall for completion of the yogurt fermentation. Reducing the fermentation temperature to 37°C resulted in a similar acidification profile in the adjunct supplemented milks, with an increase in EPS production by the adjunct cultures based on the loop touch test and mucus phenotype. As expected, reduction to 30°C slowed the acidification rate while increasing overall fermentation time. Based on these data, 37°C was selected as the fermentation temperature for prototype yogurt production.

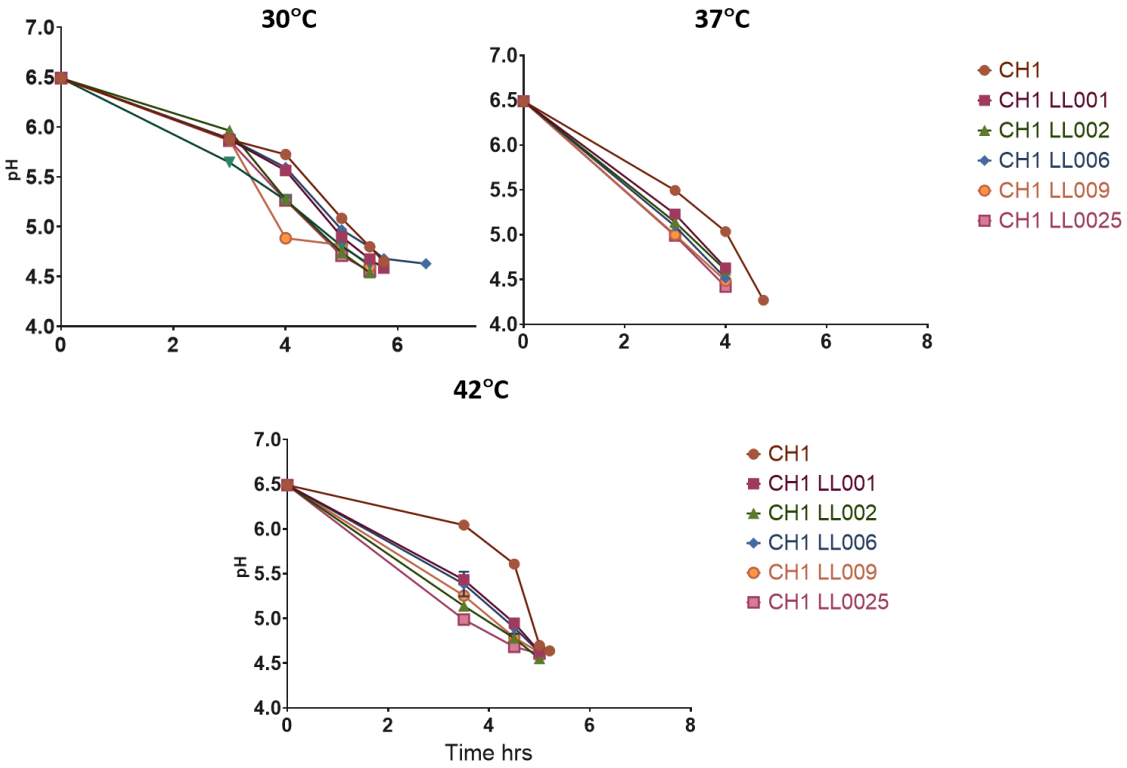


Figure 3. Temperature reduction testing. Acidification profile of EPS-producing strains as 2% adjunct in 12% (w/v) skim bovine milk at three temperatures, typical curve presented.

The five strains were included as a 2% adjunct in 12% (w/v) reconstituted skim caprine milk powder to determine the effect on yogurt texture over 21 days. The loop touch test was used throughout the fermentation as an indication of EPS production (Figure 4).

Inclusion of each of the five strains as adjunct cultures during the fermentation had positive effects on enhancing texture of the resulting yogurt (Figure 5). Gel strength is a measure of the firmness of the yogurt gel, with a higher value indicating a stronger gel. Each prototype yogurt exhibited significantly stronger gel strength ($p < 0.002$) than the control, with *L. mesenteroides* LL002, *S. gallolyticus* LL009 and *L. plantarum* LL025 having the highest gel strength (causing an increase in gel strength from 1.2 to 1.5N, $p<0.0001$), compared with the non-EPS producing negative control, CH-1 [9].

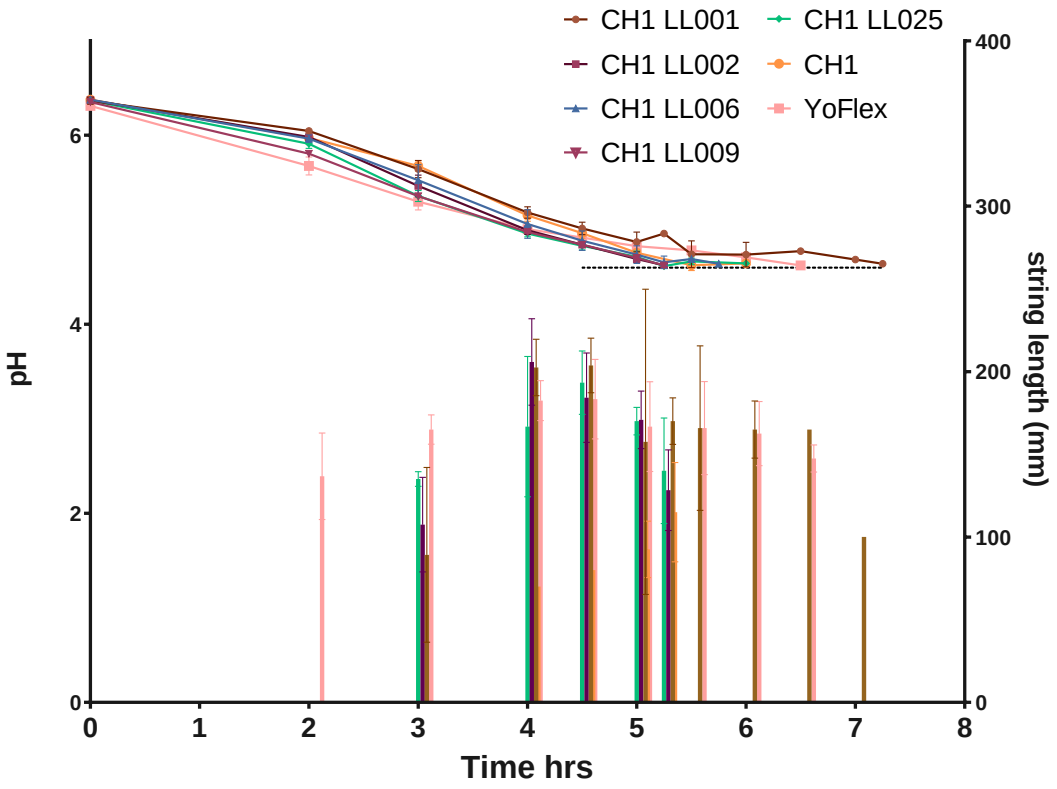


Figure 4. EPS production throughout fermentation. Acidification profile of EPS-producing strains as 2% adjunct in 12% (w/v) skim caprine milk fermented at 37°C, with string length using loop touch test during fermentation (n=6).

Whole genome sequence data allowed discrimination between the species identified in 16S rRNA gene sequencing results. From the whole genome analysis, we identified EPS encoding operons in *L. plantarum* LL001 and *L. plantarum* LL025 (Figure 6). The *eps* clusters were identified using the Artemis Comparison Tool and Mauve with *L. plantarum* WCFS1 as the reference genome [26, 27]. *L. plantarum* WCSF1 harbours four *cps* operons and these regions were investigated in detail. Both *L. plantarum* LL001 and *L. plantarum* LL025 featured the same operon structure as the reference genome. Based on previous genomic analyses of *L. plantarum* strains, this *eps* cluster was annotated as *cps4* [28].

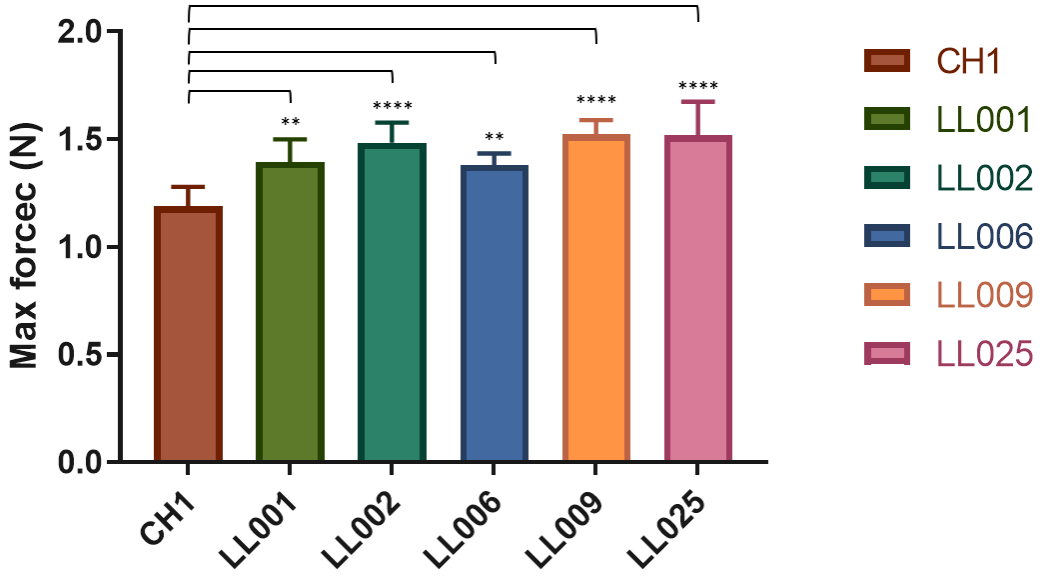


Figure 5. Texture analysis using max force. Gel strength of goat milk yogurt manufactured with EPS-producing adjunct cultures LL001, LL002, LL006, LL009 and LL025, compared with negative control CH1, **** p <0.0001, ** p < 0.0020.

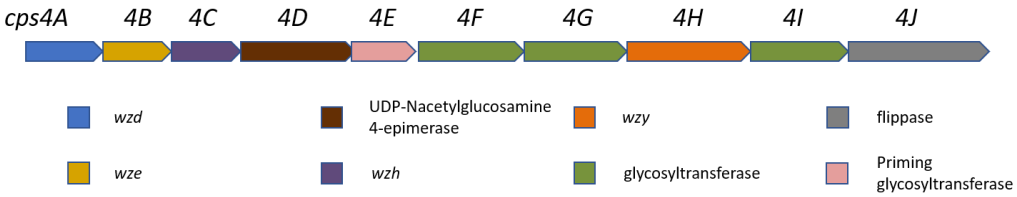


Figure 6. Representation of the exopolysaccharide operon in *L. plantarum* LL001 and LL025. Both strains are producing *cps4*, as described by Jiang *et al* [28].

LL009 was identified as *Streptococcus gallolyticus*, a potentially pathogenic species, and so is excluded from food use [29]. During the genome analysis, antibiotic resistance genes *tetW* and *ermB* were identified in *L. johnsonii* LL006. Tetracycline resistance was confirmed to be above the EFSA guideline threshold using VetMIC antibiotic resistance plates, showing resistance up to 64 mg/L whereas the cut off is 4 mg/L [30]. There were no antibiotic resistance genes detected in the other four strains (*L. plantarum* LL001, *L. mesenteroides* LL002, *S. gallolyticus* LL009, *L. plantarum* LL025) which agreed with their sensitive phenotypes.

Discussion

We evaluated the prevalence of EPS production in 799 putative LAB isolated from raw milk, raw milk products and environmental samples using three phenotypic assays. Phenotypic-based assays were chosen to facilitate simple and rapid identification when testing large biobanks. The presence of *eps* genes does not always correlate with EPS production [31] and so we did not employ sequence based methods in the initial screening phase. The high number of positive isolates identified in this study is likely due to the methods used, which aimed to detect multiple types of EPS. The high concentration of sugar included in the screen for mucous type EPS is also likely to have contributed to the high prevalence of positive EPS producing isolates recovered.

The screening methods were used in combination during the screening stage and during skim milk fermentation to monitor production. Visualisation of a slimy phenotype is frequently used in EPS screens as it identifies particularly high EPS-producing strains, the slime formed around the colonies as a result of hydrated EPS. Over 10% of isolates were positive when MRS was supplemented with sucrose, representing 18.7% of all positive isolates. The importance of carbon source is clear when comparing the number of isolates positive for EPS production in the sucrose and lactose supplemented media, at 10.9% and 6.3% respectively. Only two of the 799 isolates, LL466 and LL467 isolated from raw goat milk, were classed as high EPS producers for the lactose supplemented media, whereas thirty-three isolates were high EPS producers on sucrose containing media. This indicates a higher number of the isolates utilise sucrose as the carbon source for EPS production. The two high EPS producing isolates on lactose supplemented media were also identified as high producers using sucrose. Once the strains for further testing had been selected, both fructose and glucose were also used as carbon sources. There was no mucous EPS production seen using MRS supplemented with glucose or fructose for any of the selected isolates (data not shown).

A positive loop touch test is recorded when a string forms between the colony on a plate and a sterile loop, but scaling this phenomenon is subjective in nature and

dependent on the ropy nature of the positive control. This makes it a difficult test to standardise. The level of ropiness is a qualitative test, indicating the strain is producing EPS but does not provide information on the amounts produced. Overall, 37.1% of isolates were positive for this test which represents 63.8% of the positive isolates. During skim milk fermentation the loop touch test was also utilised, the string length was considerably longer than when using the test on colonies on a plate. This allowed for a clearer visualisation of the loop touch test result and could be compared with the non-EPS producing commercial control. There was some string formation during gelation of the control yogurt throughout fermentation, but this was increased in the adjunct yogurts (Figure 4).

The final test used for EPS production was ruthenium red-supplemented MRS. Ruthenium red stains the cell wall but cannot penetrate ropy-type EPS, resulting in white colonies, while non-EPS producing colonies stain red. There was high variability with this test, due to the degree of staining of the colonies. While some colonies were very clearly either white or red, indicating a positive or negative result a large number gave an intermediate result, leaving room for subjective interpretation. Three hundred and twenty-one isolates were positive on ruthenium-red agar, representing 67% of the total positive isolates. The results for the loop touch test and ruthenium red agar did not correlate for a large proportion of isolates, highlighting the subjective nature of both tests for detecting ropy EPS. Of the 799 isolates tested, 153 were positive for both tests, while over 300 isolates were positive for only one test. In the entire biobank, there are almost twice as many isolates positive for each individual test when compared to the number positive for both (Table 5). Including two tests for ropy EPS production reduced the number of false positives identified, and increased confidence in the outcome.

Having used multiple screening methods and scaling in each test (+, ++, +++) to select isolates, a number of EPS levels were subsequently identified by 16S rRNA sequencing. Eight strains were selected to represent a range of isolation sources and EPS positive results, taking the scaled results system into account; *L. pentosus/plantarum* LL001, *L. mesenteroides* LL002, *L. lactis* LL005, *L. johnsonii* LL006, *S. macedonicus/gallolyticus* LL009, *Lactobacillus paracasei/casei* LL021, *L.*

plantarum LL022, and *L. pentosus/plantarum* LL025. The percentage of non-generally regarded as safe (GRAS) organisms found in the study (46%) is not surprising as EPS production can also play a role in biofilm formation [32], protection against bacteriophage [33] and as a protective trait which prevents a strain being detected by the immune system in the gut [34]. Not all non-food grade strains were recognised as being pathogenic, but they did not have GRAS status. Ten of the sequenced strains isolated from raw milk samples were found to be members of the *Streptococcus bovis/Streptococcus equinus* complex (six sheep milk, three goat milk, one cow milk). These species were previously designated as *S. bovis* and are known commensals of ruminant animals [35].

Table 5. Comparing results for ropy type EPS testing. LT- loop touch test, RR – ruthenium red agar.

Putative species	**Both (LT+RR)**	**one test (LT/RR)**
S.thermophilus	36	50 (20/30)
Lactoccocus	18	128(74/54)
Thermophilic *Lactobacillus*	28	40(5/35)
Mesophilic *Lactobacillus*	34	26(18/8)
Leuconostoc	36	36(20/16)
Bifidobacteria	1	23(7/16)
Total	153	303(144/159)

The inclusion of scaling for each test was of great relevance to strain selection, as we had the objective of identifying isolates with the highest EPS production. For RR-MRS, there is no indication of EPS production levels and it was difficult to assign a phenotype to those that grew poorly and gave only a small spot on a plate. There was also a discoloration of the media to a yellow colour by a number of isolates, which made the result inconclusive. The loop touch test was easiest to visualise following the production of the yogurt prototype, although measuring the string length leaves room for error.

Each of the eight selected strains showed the ability to grow to at least some extent in bovine, and caprine skim milk, but *L. lactis* LL005, *Lactobacillus paracasei/casei* LL021, and *L. plantarum* LL022 were excluded from further testing because they showed little growth or acidification in skim milk. The EPS phenotypes

were assessed following growth in skim milk. All strains except *L. johnsonii* LL006 were positive for mucous production on sucrose-supplemented RSM, but only *L. pentosus/plantarum* LL001 and *L. pentosus/plantarum* LL025 had distinctly white colony types on RR-MRS. As mentioned above, EPS production can be influenced by media composition and pH [36]. Three strains did not show EPS production following growth in RSM but did show production from MRS: *Leuconostoc mesenteroides* LL002, *L. johnsonii* LL006, and *S. gallolyticus* LL009. For future studies we would recommend using RSM during the screening phase for EPS production.

Acidification time and acidification rate are important factors when selecting strains for the dairy industry because this determines gel formation [37]. A short fermentation time is desirable in the production of fermented dairy products such as yogurt, but sufficient time must be allowed for the gel network to form. Rapid initial acidification is desirable, with temperatures of 42°C and 37°C both resulting in promising acidification profiles and times. The lower temperature was selected for texture analysis, due to the levels of EPS production. A goat milk yogurt was chosen for testing the application of these strains as this has been shown to have weaker gel strength than cow milk yogurt, which is linked with the porous microstructure of the yogurt [10]. Prototype adjunct yogurts were prepared and tested using the loop touch test throughout the fermentation to assess potential EPS production while also measuring pH. The adjunct *L. plantarum* LL001 increased the acidification time to pH 4.6 by ~2hr and thus is unlikely to be commercially useful at this temperature. The gel strength of all prototype yogurts was significantly stronger than the negative control, indicating a positive impact of inclusion of the EPS producing adjunct strains.

Whole genome sequencing was performed on the eight selected strains, which led to the removal of *L. johnsonii* LL006 and *S. gallolyticus* LL009 as potential candidates for use in the dairy industry. All eight genomes were run through CARD, an antibiotic resistance detection tool, and antibiotic resistant genes were identified in *L. johnsonii* LL006. A resistant phenotype was confirmed using the VetMIC antibiotic resistance protocol. *S. gallolyticus* LL009 was initially selected due to the large amount of slime produced on sugar-supplemented MRS, *S. gallolyticus* is a

pathogenic species which has been previously reported to produce high amounts of capsular EPS.

Both *L. plantarum* LL001 and *L. plantarum* LL025 were identified based on their positive tests for the ropy phenotype using the loop touch test and RR-MRS. The EPS producing capability of *L. plantarum* has been characterised in detail and the responsible *cps4* locus is present in both *L. plantarum* LL001 and *L. plantarum* LL025. The *cps4* cluster contains all of the necessary genes for EPS production and is a *wzy*-dependent pathway, which is often a characteristic of ropy type EPS. Jiang (2018) described the analysis of the whole genomes of over 40 *L. plantarum* strains and designated the EPS biosynthesis as *cps* genes (capsular polysaccharide) encoding the enzymes responsible for producing surface associated polysaccharide [28]. All *L. plantarum* genomes contained *cps* gene clusters on genomic islands, regions that are prone to horizontal gene transfer. Similar clusters were found on strains from similar isolation sources. There are four types of *cps* clusters, A-D. Both *L. plantarum* strains identified in this study (LL001, LL025) lack the *cps1*, *cps2* and *cps3* clusters based on genomic analysis, characterising them as type D *cps* producing *L. plantarum* strains.

Conclusions

We isolated LAB from raw milk samples obtained from cow, goat, and sheep, raw milk cheeses, sheep faces, honey and dog saliva. Seven hundred and ninety-nine putative LAB were tested for their ability to produce EPS and 466 isolates were positive based on phenotypic testing. This highlight the prevalence of EPS production in isolates from raw milk samples. The use of multiple screening methods allowed for the isolation, and selection of isolates producing ropy and mucous type EPS. Five strains were selected and tested as adjuncts with a non-EPS producing starter culture CH-1 in prototype fermentations. Each adjunct culture significantly improved the texture of the goat milk yogurt produced. A complete *cps* operon was identified in two *L. plantarum* strains (LL001 and LL025). *L. mesenteroides* LL002, and two *L. plantarum* strains (LL001 and LL025) show potential for improving gel strength of fermented dairy products. This work highlights the benefit of using multiple phenotypic testing methods for the identification of EPS producing bacteria from

various sources that could be used in a cost-effective manner to add value in dairy fermentations.

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Author Contribution

DH performed experimental work and prepared the initial manuscript, HN, JD, EA, LD, EA, CS, RPR acquired funding and contributed to study design, manuscript revision, read, and approved the manuscript.

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Chapter 4.

Identification of mannitol producing LAB for application in fermented dairy products.

Manuscript in preparation

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Abstract

The demand for low calorie dairy products without sugar has created a need for alternative sweeteners. Mannitol is a six-carbon sugar alcohol that has 50-70% sweetness when compared to sucrose, but which contains less than half the calories (1.5 kcal/g vs 4 kcal/g, respectively). Polyol production has been investigated in the production of prototype sourdough and burger buns but to our knowledge this has not been tested in milk fermentation. We isolated lactic acid bacteria from raw milk samples and screened them for their ability to produce mannitol using a sugar supplemented medium followed by HPLC analysis. Twenty five mannitol producing strains were identified, with five strains yielding over 1.5g/L of fermentation broth defined as high producers. These strains were optimised for mannitol production in broth and tested in an experimental fermented milk product in which *Weissella paramesenteroides* LL677 emerged as the highest mannitol producing strain, generating 0.58 g/L mannitol. One whole genome sequence of *W. paramesenteroides* is available, this was downloaded and the fructose permease and mannitol dehydrogenase genes were identified. This study highlights the potential benefits of including a mannitol producing strain to reduce sugar content in dairy products.

Introduction

The global rise in obesity, type 2 diabetes and cardiovascular disease has led to increasingly health conscious consumers demanding alternative low calorie foods with reduced sugar [1]. Combined with the introduction of a sugar tax in the EU there is a need for the food industry to produce low-sugar content foods, ideally without a loss in flavour or texture of the product. Many popular zero sugar carbonated drinks use artificial sweeteners, but the fastest growing market in terms of application of these sweeteners is the dairy and frozen food sectors [1]. However, the loss in quality, texture and taste, and the high cost associated with production of sweeteners, is a limiting factor for industries trying to change their product formulations in an effort to meet the demand for products with reduced sugar content [2]. This is true for yogurt products, particularly those marketed at young children, many of which have high sugar content [3].

Sweetness is a major factor driving consumers' choice of dairy products, and so sugar is often added to reduce the acidic taste produced during fermentation which is unpalatable to many consumers. This is particularly relevant when looking at dairy products aimed at children, where many fermented products intended for school lunch boxes have high sugar contents. Non-communicable diseases are a major concern across the globe and according to WHO the most prevalent are dental diseases [4, 5]. The treatment and prevention of these diseases has improved significantly, but the consequences of dental disease can still range from serious disease to social handicap through tooth loss. Dental health-care is often expensive and so prevention is key.

While there is a significant body of evidence that yogurt consumption is beneficial to health, reformulation of a number of these products is worth further investigation [6, 7]. In an analysis of UK dairy products, it was found that only two out of 101 yogurts aimed at children were categorised as low in sugar [8]. The calorie content in many of these products could conceivably be improved through the inclusion of a mannitol producing strain.

Polyols are sugar alcohols which are naturally present in a range of fruits and vegetables and can be included in foods as low-calorific sweeteners [9]. The most commonly known polyols are xylitol, erythritol, sorbitol and mannitol. Xylitol is widely found in chewing gum and mint flavoured sweets, and mannitol is used in both the food and medical industries. This category of sweetener has a "generally regarded as safe" status and they are approved for use in foods. Polyols are typically produced by catalytic hydrogenation synthesis, enzymatic, or fermentation processes [10]. Mannitol production by microorganisms (both natural and genetically engineered) has been investigated as a means to maximise production using low cost substrates. Mannitol is a six-carbon sugar alcohol with 50-70% sweetness when compared to sucrose, but contains less than half the calories, 1.5 kcal/g vs 4 kcal/g for sucrose [2]. The sweetness relative to sucrose varies for each polyol, and so mannitol is often used as a bulking agent for texture in artificially sweetened products. The low calorific value is partly because it is incompletely absorbed in the gut, but this can lead to bloating in the colon for certain individuals such as those suffering from irritable bowel syndrome (IBS) [11]. This incomplete absorption leads to a reduction of the glycaemic response, making it a suitable sweetener for people with diabetes. Mannitol is also non-cariogenic in that it does not promote tooth decay and both of the above effects are EFSA approved health claims [12].

In bacteria, polyol production is a method for cofactor recycling and has been related to osmotic stress [13]. Studies on mannitol production by lactic acid bacteria (LAB) show that media composition and temperature are important factors in relation to yields [14]. Homofermentative LAB produce mannitol from glucose, while several heterofermentative LAB can produce mannitol directly from fructose, including members of the genera *Lactobacillus*, *Leuconostoc*, and *Oenococcus.* The highest amounts of mannitol are produced by heterofermentative species [13]. Almost all of the existing studies on the use of LAB to produce mannitol are based on producing pure mannitol for inclusion in other foods or for the pharmaceutical industry. The mannitol yield is strain-specific but *Leuconostoc* ssp. are among the most effective in their ability to convert sugars to mannitol [15]. One strain of *Leuconostoc citreum* has been reported to convert 95% of fructose to mannitol under

optimal conditions [16]. Mannitol is produced by both hetero and homofermentative LAB by separate enzymes; mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase, respectively. The current method utilised for industrial scale polyols production industrially is chemical conversion [17], to our knowledge *in situ* production in milk fermentations has not been tested [18].

In this study we used mannitol producing LAB to create a prototype product to reduce the sugar content in fermented dairy products, (Figure 1). We isolated LAB from raw milk, and environmental sources, and tested their potential for mannitol production for inclusion in a dairy fermentation. The overarching aim was to convert the added sugar in yogurt production to mannitol, to maintain a high degree of sweetness but without the same calorific and cariogenic impact.

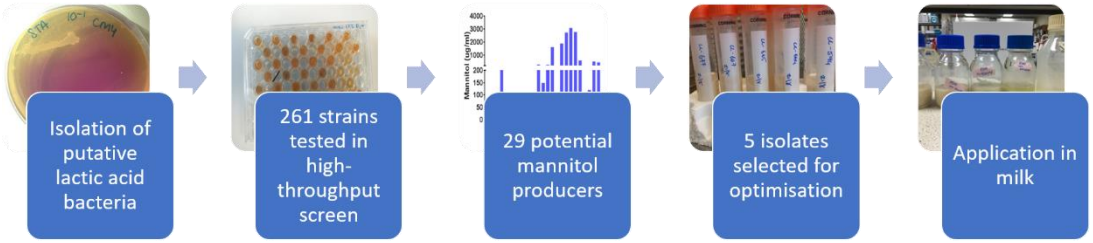


Figure 1. Workflow of methods.

Materials and Methods

Isolation of putative LAB for biobank preparation

A selective agar-based assay was used for isolation of a range of LAB (Table 1). Raw milk samples from cow, goat and sheep, honey, raw milk cheeses, and sheep faeces were streaked onto the selective agar and incubated under the appropriate conditions (Table 1). Three to five colonies from each selective medium were selected based on morphological differences and restreaked to obtain a pure culture. One colony was inoculated into the appropriate broth and incubated before stocking at -80°C in a 15% glycerol solution.

Table 1. Isolation agar and broth for LAB. STA; *S. thermophilus* selective agar, MRS; De Man, Rogosa and Sharpe, MRS_V; MRS + 30µg/ml vancomycin, LBS; *Lactobacillus* selective agar.

Isolation Agar	Broth	Putative Species	Incubation (Temp, time)
STA	B-Elliker	*S. thermophilus*	42 °C, 48h
MRS_V	MRS	*Leuconostoc*	37 °C, 48-72h
MRS pH 5.4	MRS	Thermophilic *Lactobacillus*	42 °C, 48h
LBS	MRS	Mesophilic *Lactobacillus*	30 °C, 3-5 days

High-throughput screen for mannitol production in MRS F/G

Putative *Streptoccocus thermophilus*, *Leuconostoc*, and thermophilic *Lactobacillus* were grown from frozen stocks on de Man-Rogosa-Sharpe (MRS) agar (Sigma-Aldrich, Gillingham, UK). Single colonies were inoculated in a 96-well plate with 350uL of MRS, and following overnight growth were sub-cultured at 1% into MRS F/G (Table 2) for 24 hr and tested for mannitol production.

Species identification

Isolates were identified based on 16S rRNA gene analysis. Genomic DNA was extracted using a Genelute bacterial genomic DNA kit (Sigma) and amplified by PCR using the primer pair B27F/U1492R, B27F**–** 5’ AGAGTTTGATCCTGGCTCAG 3’, U1492R – 5’ GGTTACCTTGTTACGACTT 3’. For 16s rRNA gene sequencing the PD primer (5’ GTATTACCGCGGCTGCTG 3’) was used.

Hemolysis

Tryptic Soy Agar was prepared 5% v/v sterile defibrinated horse blood was pre-warmed, added to the sterile agar and mixed well before pouring. Previously grown strains were re-streaked onto the plates and incubated for 72 hours, plates were viewed every 24 hours for lysis, defined as a clearing or discolouration of the agar.

Exopolysaccharide Screening

For exopolysaccharide (EPS) screening sugar supplemented media was utilised. 10% (w/v) sucrose and 10% (w/v) lactose supplemented MRS plates were autoclaved at 121 °C for 15 min and poured, a mucous phenotype was characterised as a positive result.

Optimising Mannitol Production in Media and Mannitol Production in Milk

High producing isolates were grown on MRS agar, at 37°C for 24 hr, and a single colony was inoculated into MRS broth overnight, after which a 1% inoculum was added to sugar supplemented MRS (Table 2) to optimise mannitol production. Three strains of mannitol producing *Lactobacillus reuteri* were included as positive controls [19]. For skim milk testing, following growth in MRS, the culture was centrifuged and washed with PBS before inoculation into reconstituted skim milk. Reconstituted skim milk was prepared at 12% and autoclaved at 115°C for 10 minutes, before cooling and adding sterilised fructose and glucose (Table 2). At regular intervals samples were taken for for serial dilution and plating for enumeration and pH analysis, 2ml was also withdrawn and treated for HPLC analysis to test for mannitol. Samples were frozen at -20°C until HPLC analysis.

Table 2. Composition of media. MRS added as per manufacturers instruction, 55g/L

	MRS	FRUCTOSE	GLUCOSE	LACTOSE	SUCROSE	RSM
MRS_5	✓	5%	5%	10%	-	-
MRS_F/G	✓	6.5%	1%	-	-	-
MRS_SUC	✓	-	-	-	10%	-
RSM	-	-	-	-	-	12%
RSM_F/G	-	6.5%	1%	-	-	12%

HPLC analysis for mannitol and sugar detection

Sample preparation and analysis of both broth and milk samples was performed according to the protocol of Sahin et al. [18]. Briefly, samples were extracted in Milli-Q water and clarified with 2.5% (w/v) carrez I and 2.5% (w/v) carrez II and filtered (0.2µm pore size). For HPLC analysis an Agilent 1260 high performance

liquid chromatography system equipped with a refractive index detector (RID) and an ultra violet-diode array detector (UV/DAD) was used. The concentration was determined over the RID (40 °C) by elution of the extract from a RezexTM RPM-Monosaccharide Pb + column (300 × 7.8 mm, Phenomenex, California, USA) at 80 °C, equipped with a guard column (Carbo-Pb, 4 × 3.0 mm, Phenomenex, California, USA), using MiliQ-water at a flow rate of 0.6 mL/min, an injection volume of 20 µL and a runtime of 45 minutes.

Results and Discussion

This study focuses on the isolation of mannitol producing bacteria and optimisation of mannitol production in broth. We identified 25 mannitol producing LAB, with five showing promise as high mannitol producers, in that they produced over 1.5g/L in broth. The aim is to investigate if *in situ* production of mannitol in a fermented milk product is possible. We identified three strains which are capable of conversion of fructose/glucose in milk to mannitol, and could represent a novel method for sugar reduction in fermented dairy products.

Screening for polyol production

The initial screen was completed using MRS-FG, MRS media supplemented with 6.5% fructose and 1% glucose. The screen was performed in 96-well plates to allow for a high throughput so that we could rapidly characterise both positive and negative strains from our LAB biobank. The biobank consists of bacteria isolated from raw milk, honey and other environmental samples. Of 261 strains tested 9.6% were positive for mannitol production (Figure 2).

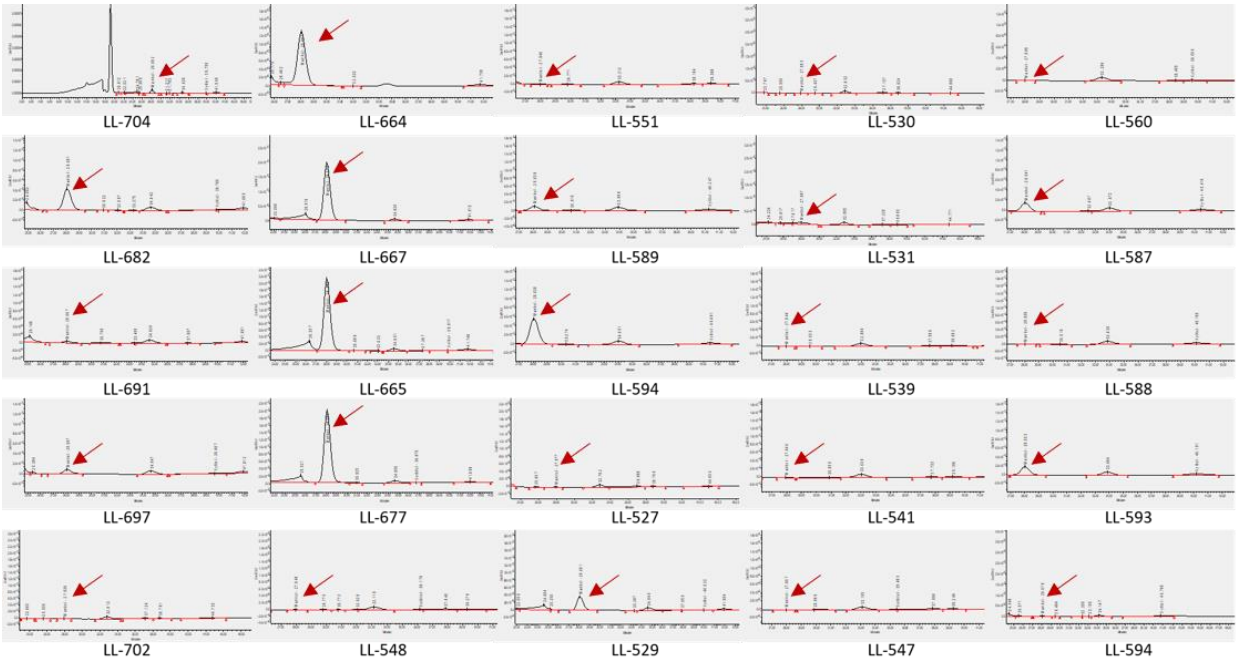
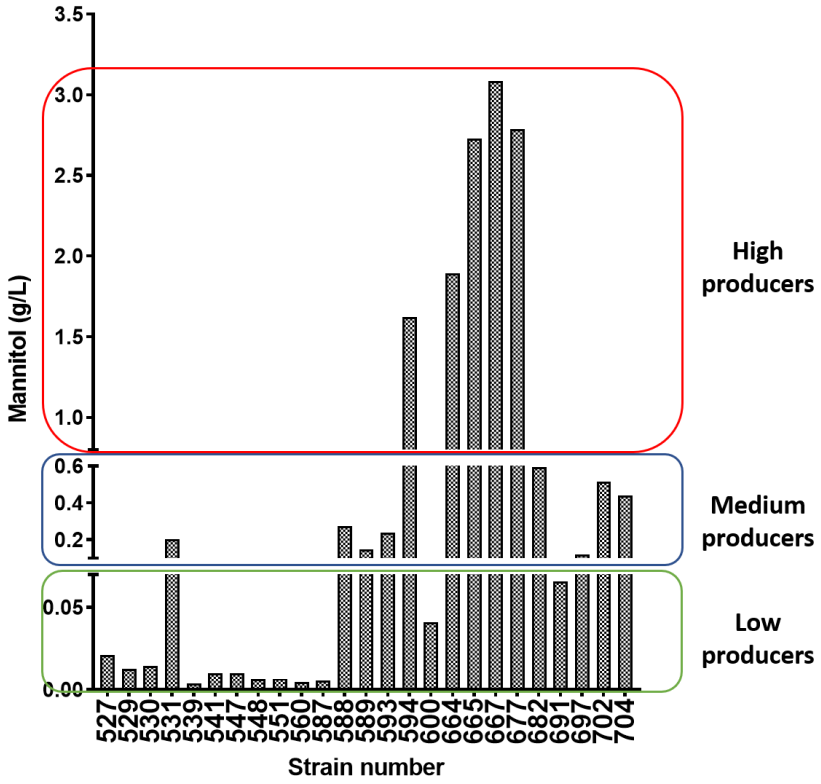


Figure 2. HPLC output for 25 mannitol producting strains. Mannitol peaks marked with arrow, strain names below.

These mannitol positive strains were grouped as low, medium and high producers (Figure 3). Twelve strains were defined as low producers yielding between 0 - 0.06 g/L, eight medium producers generated between 0.1 - 0.6 g/L, and five high producers generated more than 1.5g/L. Sequencing of the 16S rRNA gene was performed on the five high producing isolates and the species were identified as *Lc. pseudomesenteroides/mesenteroides* (n=2), *L. reuteri/fermentum*, *Weisella hellenica*, and *Weisella paramesenteroides*. *Leuconostoc* sp. have been reported to produce mannitol in multiple studies [15, 16, 20], and *Weisella* sp. were identified in a previous study that isolated polyol producing LAB from sourdough samples [21].

All high producing strains, LL-594, LL664, LL665, LL667 and LL677 were evaluated for hemolytic activity and all were non-hemolytic, although LL665 and LL667 both showed α-hemolysis. This is defined as bruising and not true lysis of the red blood cells. Exopolysaccharide (EPS) production was also evaluated using sugar supplemented media and three strains tested positive for mucous type EPS; LL594, LL665 and LL677. EPS can contribute to the rheological properties of fermented dairy products and has been associated with health effects such as cholesterol reduction [22, 23].



Strain	Source	Amount (g/L)	H/M/L
LL-527	GC	0.0212	L
LL-529	GC	0.0129	L
LL-530	GC	0.0146	L
LL-531	GC	0.2040	M
LL-539	GC	0.0038	L
LL-541	GC	0.0099	L
LL-547	GC	0.0099	L
LL-548	SC	0.0064	L
LL-551	SC	0.0067	L
LL-560	CC	0.0045	L
LL-587	SM	0.0055	L
LL-588	SM	0.2720	M
LL-589	SM	0.1486	M
LL-593	SM	0.2401	M
LL-594	SM	1.6200	H
LL-600	GM	0.0412	L
LL-664	GM	1.8949	H
LL-665	GM	2.7270	H
LL-667	GM	3.0827	H
LL-677	GM	2.7864	H
LL-682	CM	0.5936	M
LL-691	GC	0.0656	L
LL-697	CM	0.1203	M
LL-702	CC	0.5138	M
LL-704	CC	0.4399	M

Figure 3. Mannitol production by positive isolates in the initial screen in MRS_F/G. GC – raw goat milk cheese, SC – raw sheep milk cheese, CC - raw cow milk cheese, SM - raw sheep milk, GM – raw goat milk, CM – raw cow milk. H - high producer, M - medium producer, L - low producer.

Media Optimisation for Mannitol Production

The high producing strains were grown in multiple media types to optimise mannitol production. The composition of sugars in the growth medium has a significant impact on the amount of mannitol that will be produced. A combination of fructose and glucose is required for mannitol synthesis and so different combinations were tested [20]. Sucrose supplementation led to EPS production and was included to determine whether mannitol and EPS could be produced simultaneously. MRS_5 has been previously used in studies on mannitol production in sourdough and burger buns, and this media was selected to analyse the sugar consumption throughout fermentation (Table 2).

Three mannitol producing strains of *L. reuteri* were included as positive controls [19]. Very little mannitol was produced in MRS, but growth in MRS_5

resulted in similar production of 6g/L from the three positive controls and *W. paramesenteroides* 677. The positive control strains showed promising results when grown on MRS_suc but little production was detected from the newly isolated strains. Our new isolates showed highest production on MRS-FG, perhaps as expected given that MRS-FG was also used as the initial screening media and we would be biased in favour of producers which best utilise this sugar combination (Figure 4). *W. paramesenteroides* LL677 was the highest producer (23.48g/L) when supplemented with fructose and glucose.

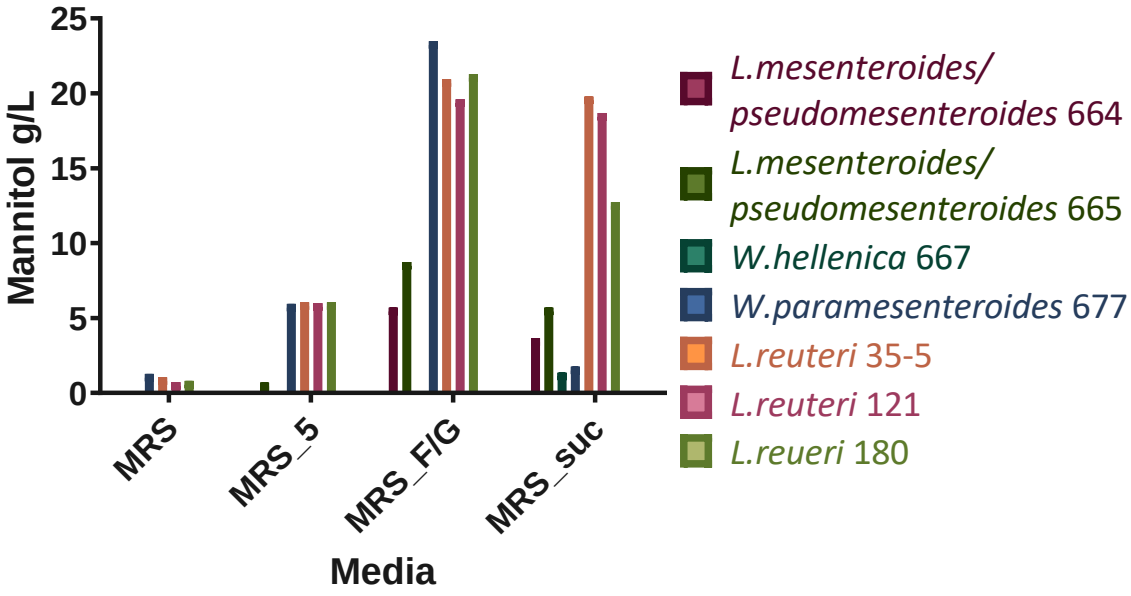


Figure 4. Results of mannitol production in multiple media types.

MRS_5 has been previously used in studies on mannitol production for sugar reduction in sourdough and burger buns [18], and so it was selected to analyse the sugar consumption throughout fermentation. All five strains resulted in a reduction in glucose and fructose after 24 hr fermentation, albeit to varying degrees (Figure 5). No fructose was detected at the end of fermentation using *W. paramesenteroides* LL677.

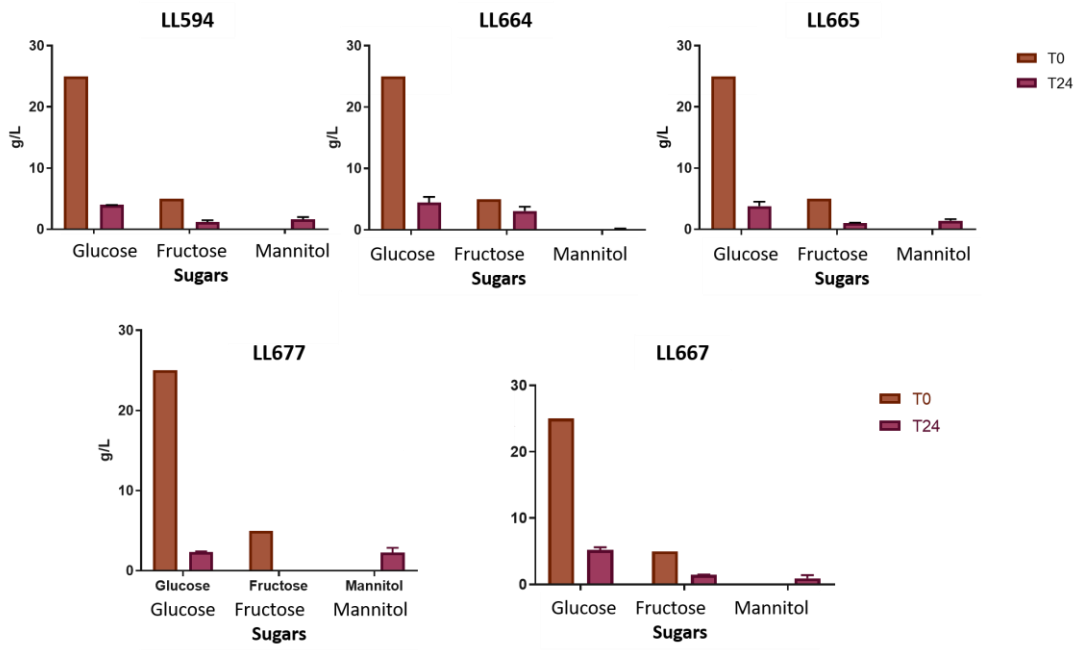


Figure 5. Sugar consumption in MRS_5 after 24hr growth

Growth characteristics in milk

We investigated mannitol production in RSM_FG by the four most promising isolates, three of which produced small amounts of mannitol. *W. paramesenteroides* LL677 once again resulted in the highest mannitol production at 0.58g/L, more than three times that of the next most promising isolate, LL664 with 0.17g/L (Figure 6). One study using *in situ* production of mannitol in a quinoa-based milk substitute, following glucose-isomerase treatment, achieved 100% conversion of fructose to mannitol, 15.6 g/L [21]. While this study is using a different strain, substrate, and conditions, it confirms that complete conversion of fructose to mannitol in an *in situ* fermentation is possible. This suggests that considerable improvement may also be achievable in our fermentation following optimisation.

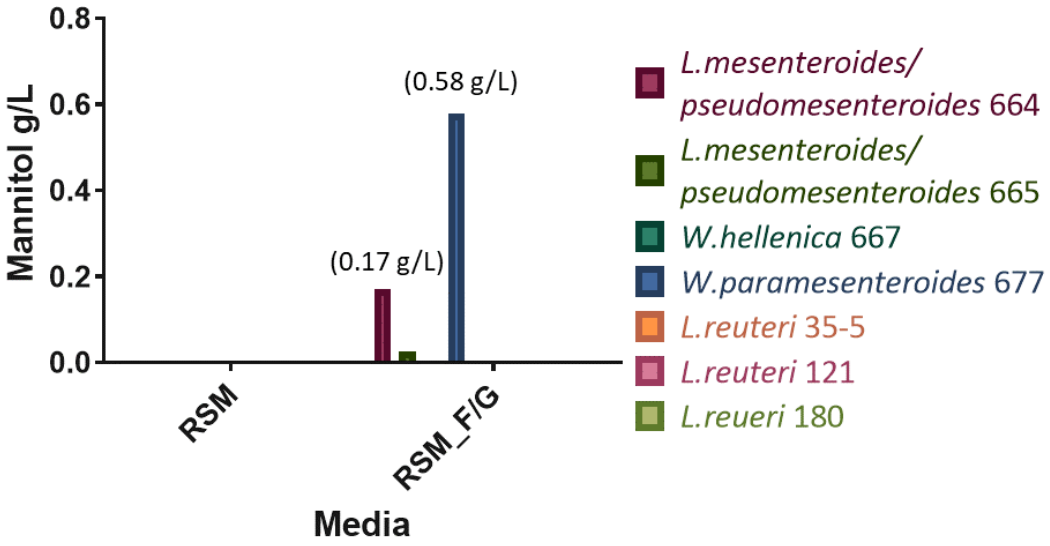
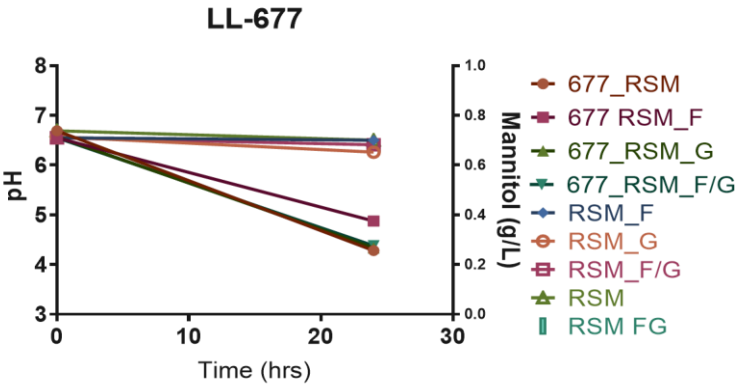


Figure 6. Mannitol production by isolates in RSM and RSM_F/G. RSM; 12% reconstituted skim milk, F/G; 6.5% fructose and 1% glucose.

We also tested the growth characteristics of *W. paramesenteroides* LL677 in supplemented RSM, where the pH was monitored over 24 hr and final cell counts were analysed at 24 hr. Sugars were included either alone or in combination to evaluate their impact on the growth characteristics in milk (12% RSM supplemented with glucose, fructose, or fructose and glucose). *W. paramesenteroides* LL677 caused the acidification of each milk type over 24 hr (Figure 7). The acidification profile was evaluated in all milk types. RSM_F had a slower initial acidification with a final pH 5, while all other RSM types had a faster acidification profile with a final pH between pH 4.1 and 4.7. Milk samples were inoculated at 9 x 10^6 cfu/ml and enumerated after 24 hr. *W. paramesenteroides* LL677 increased by two logs or more in each milk type.



Media	Cfu/ml	pH
RSM	1.65E+09	4.14
RSM_G	2.14E+09	4.43
RSM_F	4.2E+08	5.04
RSM_F/G	5.35E+08	4.73

Figure 7. Fermentation of skim milk with added sugars. With and without *W. paramesenteroides* LL677, the final pH and cfu/ml are shown in the table on the right.

Identification of the mannitol dehydrogenase gene within *W. paramesenteroides* genomes

One whole genome sequence of *W. paramesenteroides* is available online (FDAARGOS_414), this was downloaded and examined in Artemis (Accession no. GCA_002386265.1). The gene originally annotated as encoding a zinc-dependent alcohol dehydrogenase family protein, C0680_RS03205, shares high protein sequence homology with a mannitol dehydrogenase from *Leuconostoc mesenteroides*, (91% ident, 100% query cover, 96% positive). The adjacent gene, C0680_RS03210, is annotated as a hypothetical protein, but on further analysis using BLASTP the sequence was found to be almost identical to fructose permease. This is consistent with the fact that heterofermentative bacteria need intracellular fructose to produce mannitol, and so both fructose permease and mannitol dehydrogenase are required (Figure 8).

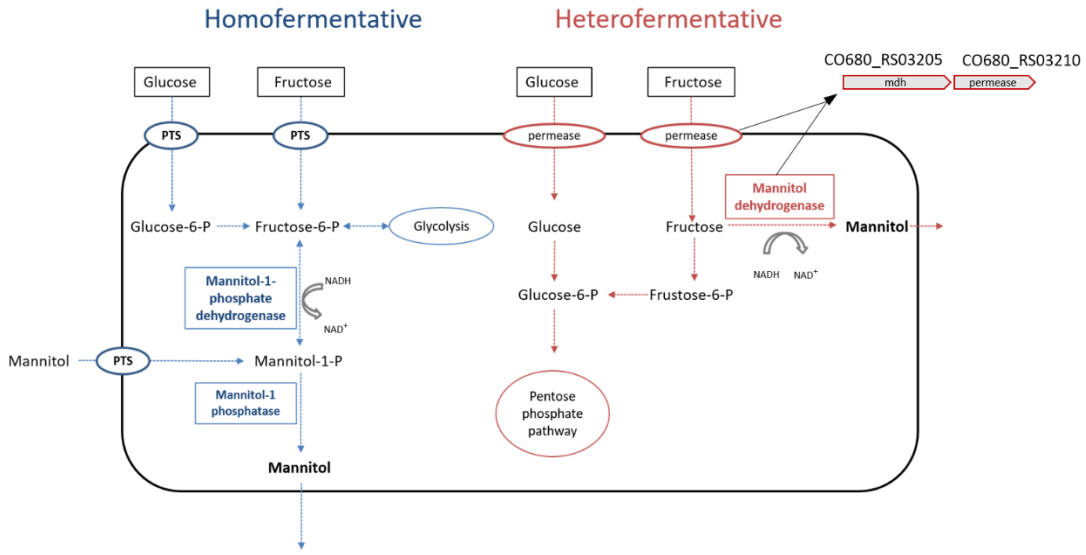


Figure 8. Mannitol production pathways. PTS- phosphotransferase system. Genes shown identified in *W. paramesenteroides* FDARRGOS_414

Conclusion

The reduction of sugar content in dairy products is problematic as added sugar contributes to the organoleptic qualities of the fermented product. Sweetness is a major driver in consumers choice of fermented dairy products, and so reformulation of these products for a healthier product is desirable. We aimed to convert added sugar into mannitol, a natural non-cariogenic low calorie sweetener

during fermentation. While this study detected small quantities of mannitol (0.58g/L) and it highlights the potential for *in situ* production of mannitol in a dairy product, further optimisation of fermentation conditions is required for application.

Acknowledgements

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Author Contribution

DH performed experimental work and prepared the initial manuscript, DH and HS completed HPLC analysis, EA, CS, RPR contributed to study design, manuscript revision, read and approved the manuscript.

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Chapter 5.

Extensive bacteriocin gene shuffling in the *Streptococcus bovis/Streptococcus equinus* complex reveals gallocin D with activity against vancomycin resistant enterococci.

Hill D, O'Connor PM, Altermann E, Day L, Hill C, Stanton C, Ross RP. Extensive bacteriocin gene shuffling in the *Streptococcus bovis/Streptococcus equinus* complex reveals gallocin D with activity against vancomycin resistant enterococci. Scientific Reports. Accepted.

Abstract

Streptococcus gallolyticus LL009 produces gallocin D, a narrow spectrum two component bacteriocin with potent activity against vancomycin-resistant enterococci. Gallocin D is distinct from gallocin A, a separate two-component bacteriocin produced by *S. gallolyticus*. Although the gene clusters encoding gallocin A and gallocin D have a high degree of gene synteny, the structural genes are highly variable and appear to have undergone gene shuffling with other streptococcal species. Gallocin D was analysed in laboratory-based experiments. The mature peptides are 3343±1 Da and 3019±1 Da, could be readily synthesized and display activity against a vancomycin-resistant *Enterococcus* strain EC300 with a MIC value of 1.56 μM. Importantly, these bacteriocins could contribute to the ability of *S. gallolyticus* to colonize the colon where they have been associated with colorectal cancer.

Introduction

With the rise of antibiotic resistant pathogens and the decreasing number of novel antibiotics, the search for alternative antimicrobials is of increasing importance [1]. Bacteriocins are potential antimicrobial candidates and consist of different classes of ribosomally-synthesized antimicrobial peptides which are either narrow or broad spectrum [2]. Narrow spectrum bacteriocins are of particular interest as targeted therapeutics since they could be expected to have minimal impact on resident microbiota [3, 4]. Bacteriocin-producing bacteria have been isolated from a range of sources including food, skin and the gastrointestinal tracts of both animals and humans [5]. Among the functions attributed to bacteriocins are competition, quorum sensing and even host signalling [6]. They are classified into multiple types; class I are lantibiotics such as nisin which are subject to post-translational modification, and class II that are unmodified or cyclic peptides. The class II bacteriocins are divided into several subgroups [7].

Class IIb are two-peptide bacteriocins where the two components are required for maximal activity. The structural genes encoding them are usually adjacently located, together with a gene encoding an immunity protein that protects the cell from being killed by its own bacteriocin [8]. Class IIb bacteriocin operons usually also contain an ABC transporter and an accessory protein. Both peptides are synthesised as pre-peptides with a leader sequence at the N terminal that is cleaved during export at a GG motif to produce the extracellular mature active peptide. This cleavage is performed by the ABC transporter or a peptidase which recognises the leader sequence and transports the peptide across the cell membrane. Two-component bacteriocins require both peptides for optimal activity and both peptides interact with one another at the same target site to form one antibacterial unit. The mode of action of these bacteriocins involves the binding of the peptides to a target in the cell membrane, leading to pore formation causing leakage and cell death.

Streptococcus gallolyticus ssp. *gallolyticus*, formerly described as *S. bovis* biotype I, is an opportunistic pathogen associated with infectious endocarditis (IE) and colorectal cancer (CRC) [9]. It is hypothesized that altered conditions in the colon

associated with cancer provide an environment in which *S. gallolyticus* is able to thrive and colonise. This ability is linked to the production of a two-component bacteriocin gallocin, for clarification referred to as gallocin A. In one study using a murine model of CRC, colonisation of tumour bearing mice by *S. gallolyticus* UCN34 was mediated by production of gallocin A encoded by two structural genes, *gallo_2020* and *gallo_2021,* hereafter designated *gllA1* and *gllA2* [10]. The bacteriocin is active against enterococci, potentially creating a niche in the colon for *S. gallolyticus* colonisation. *S. gallolyticus* UCN34, in which *gllA1* and *gllA2* were knocked out, lacked this colonisation advantage in tumour bearing mice. In this study, we describe a strain of *S. gallolyticus* which harbours distinct structural genes in place of *gllA1* and *gllA2*. These structural genes are very similar to genes encoding a bacteriocin produced by *Streptococcus infantarius* ssp *infantarius* (*Sii*) [11].

S. gallolyticus and *Streptococcus infantarius* are both members of the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) which is divided into seven subspecies; *S. gallolyticus*, *Streptococcus infantarius*, *Streptococcus gallolyticus* ssp. *macedonicus, Streptococcus gallolyticus* ssp. *pasteurianus*, *Streptococcus lutetiensis*, *Streptococcus alactolyticus* and *Streptococcus equinus* [12, 13]. SBSEC members have been isolated from human, animal and food sources, with an estimated carriage rate of 5% in healthy adults. *S. gallolyticus* has been linked with IE and CRC in humans, while *S. infantarius* is found in fermented foods, predominantly in sub-Saharan Africa [14-18]. *S. infantarius* has also been linked to CRC in an African cohort, although this did not correlate to consumption of traditional fermented food containing species of *S. infantarius*. This suggests a separation between commensal, opportunistic pathogen and food lineages [19, 20]. The SBSEC genomes have been studied for their ability to colonise in their associated niche, for example some strains such as *S. gallolyticus* retain tannin degrading abilities associated with survival in ruminant animals [18, 21, 22]. Other strains such as *S. infantarius* and *S. gallolyticus* ssp *pasteurianus* have lost this ability and have undergone a genome reduction associated with an adaption to the dairy environment [18]. This has been discussed at length for members of the SBSEC, and it has been suggested that isolation source is associated with strain lineage [23, 24].

S. gallolyticus strains have retained the largest genomes and most diverse functional capabilities and have been found in both healthy individuals and patients with underlying disease. It has been found in 74% of CRC patients and preferentially associates with tumour tissue [9, 25].

This study analysed class IIb bacteriocins in *S. gallolyticus* and *S. infantarius* and highlights the ability of these strains to adapt to their niche. Based on the discovery of bacteriocin structural genes in our new isolate *S. gallolyticus* LL009, we completed an *in silico* screen on available sequences of other SBSEC members. This analysis revealed that a similar bacteriocin operon is present in each *S. gallolyticus* strain analysed, but *S. gallolyticus* LL009 contains distinct structural and immunity genes. The bacteriocin, termed gallocin D, is a variant of a bacteriocin produced by another dairy isolate *Streptococcus infantarius* LP90 [11]. A similar bacteriocin operon is also present in members of the related *S. infantarius*. The bacteriocin produced by *S. gallolyticus* LL009, that we designate gallocin D, was synthesized and analysed for activity against a related *S. gallolyticus* strain and other clinically-relevant pathogens. It was found to have activity against clinically important pathogens, including *Streptococcus pneumoniae*, vancomycin-resistant enterococci and another *S. gallolyticus* strain.

Results

Identification of the bacteriocin produced by *Streptococcus gallolyticus* LL009

S. gallolyticus LL009 (*Sgg* LL009) was isolated from raw goat milk sourced in New Zealand; milk samples were stored at -20 °C until processing. The strain was initially isolated by streaking 10µL of milk onto *Streptococcus thermophilus* selective agar that was incubated at 42°C for 24 hours. To test for bacteriocin production, individual isolates were streaked onto BHI agar and incubated overnight at 37°C, aerobically. When overlaid with *Lb. delbrueckii* ssp *bulgaricus* LMG 6901, zones of inhibition were seen around the colonies of *Sgg* LL009 (Figure 1). No antimicrobial activity was observed when a cell-free supernatant was tested by well diffusion assay, but a zone of clearing was observed when cell-containing broth was used in this assay. Inhibitory activity was restored to the cell-free supernatant when

Tween80 was added. We designated this bacteriocin activity as gallocin D, pending further analysis.

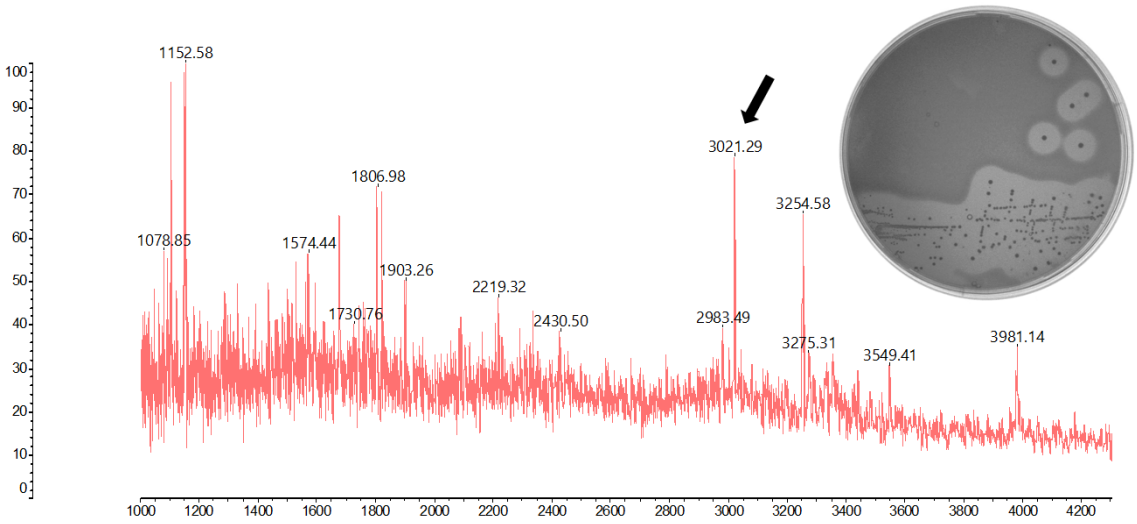


Figure 1; Detection of the 3021.29 Da gallocin D2 peptide by MALDI-TOF MS from colonies on a plate. Inset shows the antimicrobial activity of *S. gallolyticus* LL009 against *L. bulgaricus* LMG6901.

Sgg LL009 was tested for the production of capsular exopolysaccharide (EPS), a feature of some *S. gallolyticus* strains. Colonies were positive for the loop touch test and were white on ruthenium red supplemented medium, indicating a ropy type EPS [26, 27]. On sucrose supplemented medium, a large amount of EPS was produced (data not shown).

Sgg LL009 was not completely resistant to any antibiotic tested (Table 1). It is non-proteolytic in that no zones of clearing were observed surrounding colonies on 10% (w/v) reconstituted skim milk (RSM). A green colouration was observed surrounding cells on blood agar for hemolysis testing, indicative of alpha hemolysis. Alpha hemolysis is defined as bruising of the red blood cells and not true lysis.

Table 1. Antibiotic resistance. EFSA guidelines do not have specific values for *S. gallolyticus.* Values for *S. thermophilus* and the defined values for "other Gram positive" bacteria displayed.

Antibiotic	*S.gallolyticus* LL009 profile			EFSA *S.thermophilus*	EFSA other Gram +ve
Gentamicin	8	8	8	32	4
Kanamycin	128	128	128	64	16
Streptomycin	64	64	64	64	8
Neomycin	32	32	32	n/a	n/a
Tetracycline	0.5	0.5	0.5	4	2
Erythromycin	0.06	0.03	0.03	2	0.5
Clindamycin	0.12	0.06	0.06	2	0.25
Chloramphenicol	2	2	2	4	2
Ampicillin	0.25	0.25	0.25	2	1
Penicillin	0.12	0.12	0.12	n/a	n/a
Vancomycin	0.5	0.5	0.5	4	2
Quinupristin-dalfopristin	1	1	1	n/a	n/a
Linezolid	1	1	1	n/a	n/a
Trimethroprim	2	2	2	n/a	n/a
Ciprofloxacin	1	1	1	n/a	n/a
Rifampicin	0.25	0.12	0.25	n/a	n/a

In silico analysis of *Streptococcus gallolyticus* LL009

Following draft genome sequencing of the gallocin D producer *Sgg* LL009, *in silico* analysis was performed using BAGEL4 and antiSMASH to identify bacteriocin-associated genes [28, 29]. A single bacteriocin operon was identified in *Sgg* LL009 that consists almost entirely of genes also found in the gallocin A operon of strain *S. gallolyticus* DSM16831 (Figure 2A). The predicted function of each gene is shown in Table 2. However, the bacteriocin structural genes (*gllD1* and *gllD2*) and that encoding the immunity protein (*gllDI*) do not have homologues in the gallocin A producing *S. gallolyticus*. These genes are homologous to genes found in the infantaricin ABCDEFG bacteriocin cluster from *S. infantarius* LP90 (Figure 2b). The predicted structural genes in the LL009 operon are variants of infantaricin A, where *gllD1* shares 98% amino acid identity with *infA1*, while *gllD2* shares 90% amino acid identity with *infA2*. The predicted molecular weight of *gllD2*, 3019.54 Da, is consistent with the 3021.29 Da mass found by MALDI TOF mass spectrometry analysis (Figure 1). This mass could not be matched to any known bacteriocin in the bactibase database.

Table 1. *In silico* analysis of the predicted function of each gene involved in bacteriocin production

Orf	Length (aa)	Blast hit	Query cover	E-value	% Identity	Accession No.
1	75	Bacteriocin, hypothetical protein [Streptococcus gallolyticus]	100%	3.00E-45	100.00%	WP_074595821.1
2	454	two-component system, AgrA family, sensor histidine kinase AgrC [Streptococcus gallolyticus]	100%	0	99.12%	WP_074595819.1
3	255	response regulator transcription factor [Streptococcus gallolyticus]	100%	0	99.61%	WP_081341218.1
4	97	Enterocin A Immunity [Streptococcus gallolyticus]	100%	1.00E-63	100.00%	WP_074581674.1
5	75	hypothetical protein SGADD03_00501 [Streptococcus gallolyticus]	100%	1.00E-45	98.67%	WP_061458100.1
6	103	enterocin A Immunity protein [Streptococcus gallolyticus subsp. gallolyticus ATCC 43143]	100%	6.00E-68	100.00%	WP_003066564.1
7	99	DUF3884 family protein putative bacteriocin [Streptococcus gallolyticus subsp. gallolyticus ATCC 43143]	100%	2.00E-64	97.98%	WP_009854998.1
8	46	competence stimulating peptide (CSP) precursor [Streptococcus gallolyticus]	100%	1.00E-24	100.00%	AQP43123.1
9	436	histidine kinase of the competence regulon,ComD1 [Streptococcus gallolyticus subsp. gallolyticus DSM 16831]	100%	0	99.54%	WP_058621373.1
10	243	response regulator transcription factor [Streptococcus gallolyticus]	100%	3.00E-178	100.00%	WP_013643488.1
11	108	MULTISPECIES: LytTR family transcriptional regulator [Streptococcus]	100%	8.00E-72	99.07%	WP_009855002.1
12	451	Glycosyl transferase family 2 [Streptococcus gallolyticus subsp. gallolyticus ATCC BAA-2069]	95%	0	98.84%	WP_013643489.1
13	99	hypothetical protein BTR42_10805 [Streptococcus gallolyticus subsp. gallolyticus DSM 16831]	100%	1.00E-66	100.00%	AQP43128.1
14	60	hypothetical protein BTR42_10810 [Streptococcus gallolyticus subsp. gallolyticus DSM 16831]	100%	9.00E-37	100.00%	WP_013643491.1
15	69	helix-turn-helix transcriptional regulator [Streptococcus orisratti]	100%	2.00E-41	98.55%	WP_018375366.1
16	337	bacteriocin biosynthesis protein [Streptococcus gallolyticus]	100%	0	96.74%	WP_077497732.1
17	71	hypothetical protein SAMN02910295_0951 [Streptococcus gallolyticus]	100%	3.00E-40	98.59%	WP_074595803.1
18	224	InfAE-Imm [Streptococcus infantarius subsp. infantarius]	100%	2.00E-141	92.86%	WP_006531871.1
19	51	infantaricin A1 [Streptococcus infantarius subsp. infantarius]	100%	5.00E-28	98.04%	WP_006531870.1
20	74	infantaricin A2 [Streptococcus infantarius subsp. infantarius]	70%	3.00E-24	90.38%	WP_006531869.1
21	85	hypothetical protein [Streptococcus gallolyticus]	100%	3.00E-50	98.82%	WP_074628192.1
22	715	ABC-type bacteriocin transporter [Streptococcus gallolyticus]	100%	0	99.44%	WP_074595798.1
23	411	bacteriocin secretion accessory protein, partial [Streptococcus gallolyticus]	100%	0	99.27%	WP_061458107.1

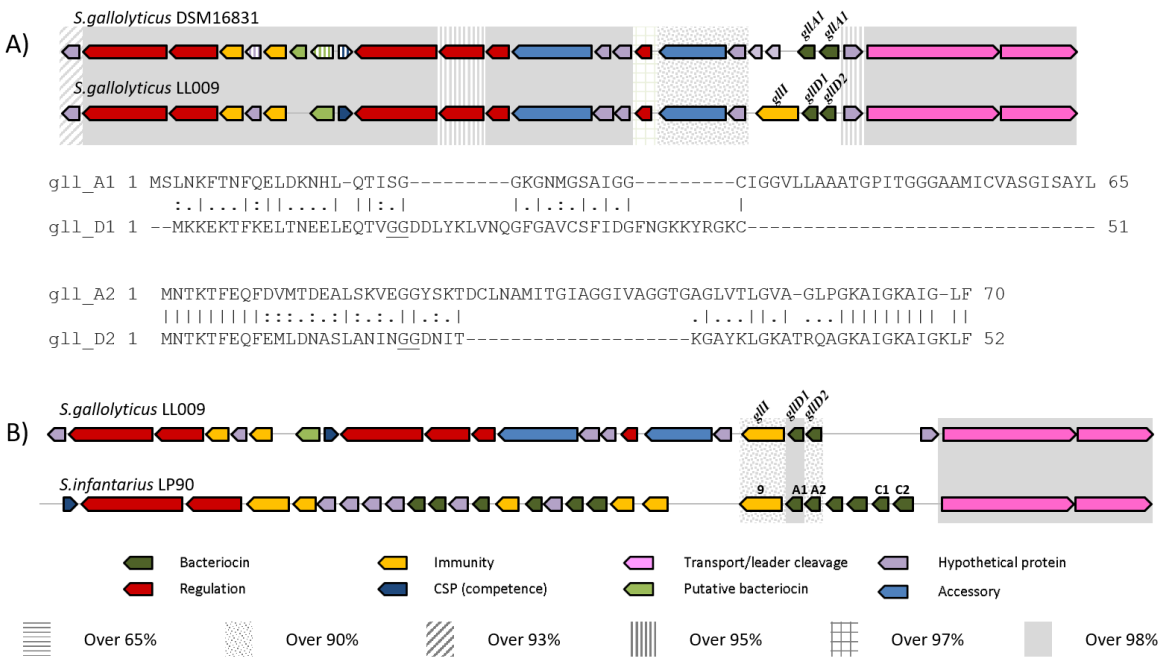


Figure 2. Organisation of the predicted operon encoding the bacteriocin. Aligned to the operon of A) type strain *S. gallolyticus* DSM 16831, with alignment of structural genes below; *gllA2* and *gllD2* show 42% identity, *gllA1* and *gllD1* share 22% identity. B) *S. infantarius* LP90 infantaricin ABCDEFG bacteriocin cluster. Genes connected by shaded boxes are homologous and the amino acid percent identity is indicated.

Nine strains of *S. gallolyticus* and *S. infantarius* with available genomic data (including *Sgg* LL009) were analysed for the presence of bacteriocin operons (Table 2). The ABC transporters are highly conserved in both species, with 97% amino acid identity (Figure 3). Infantaricin A is encoded by the infantaricin ABCDEFG bacteriocin operon in *S. infantarius* LP90. Twelve structural bacteriocin genes can be identified in the operon, ten of which make up five two-peptide bacteriocins [11]. There is significant variation in the structural gene arsenal of each strain. Each previously sequenced strain of *S. gallolyticus* contained the gallocin A structural genes, *gllA1*and *gllA2*, while all *S. infantarius* strains other than CJ18 contained a homologue of infantaricin C. The infantaricin C bacteriocin genes, *infC1* and *infC2,* in *Streptococcus infantarius* LP90 have 66% and 78% identity with *gllA1* and *gllA2*, respectively. One strain of *S. gallolyticus* ssp *macedonicus* has *gllA2* next to a gene with 60% identity to *gllA1*. *Sgg* LL009 is the only *S. gallolyticus* strain that lacks a homologue for gallocin A, and instead harbours a variant of infantaricin A, here termed gallocin D.

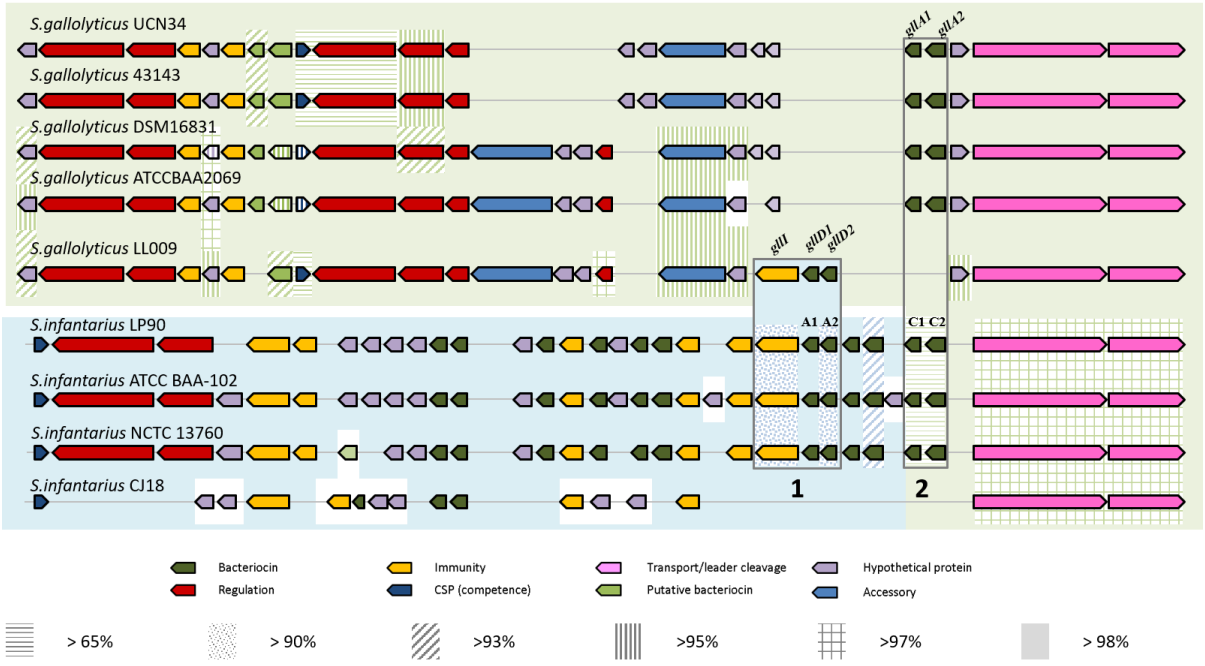
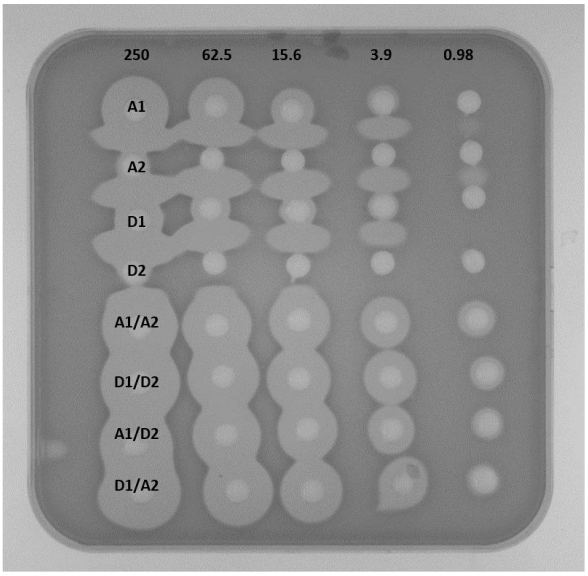
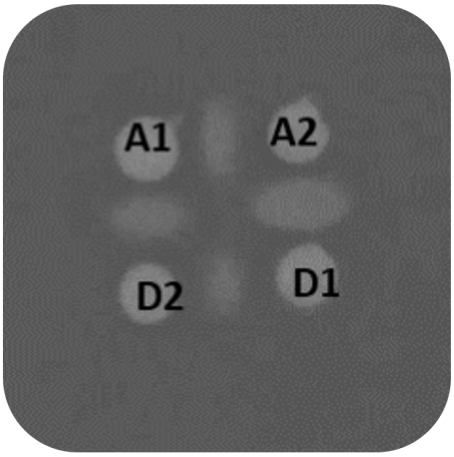


Figure 3. Comparison of the operons of all strains analysed. Genes connected by shaded boxes are homologous and the amino acid percent identity is indicated, while the white background represents genes for which no homologues have been found between strains; box 1 identifies the immunity protein and infantaricin A in *S. infantarius* strains and immunity protein and the gallocin D variant in *S. gallolyticus* LL009; box 2 identifies potential homologues for gallocin A.

Synthesis and spectrum of inhibition of Infantaricin A and gallocin D

The gallocin D1 prepeptide encoded by *gllD1* is 51 amino acids and the D2 pre-peptide encoded by *gllD2* is 52 amino acids in length, while the active peptides are predicted to be 30 and 29 amino acids, respectively. We synthesised both D1 and D2 peptides for further characterisation, the mass of the synthesized D2 peptide matched the observed mass of 3021 Da found in the colony mass sepc. The D2 peptide displays no activity in a well diffusion assay against *Lb. delbrueckii* ssp *bulgaricus,* while the D1 peptide has solo activity, which is enhanced when in a 1:1 combination with D2. When tested against pathogenic bacteria, both peptides are required for activity. The infantaricin A peptides were also synthesized and it was found that the peptides cross-complemented each other, A1/D2 or D1/A2 (Figure 4).






```
gll_D1   1 MKKEKTFKELTNEELEQTVGGDDLYKLVNQGFGAVCSFIDGFNGKKYRGKC     51
             ||||||||||||||||||||||||||||||||:||||||||||||||||
inf_A1   1 MKKEKTFKELTNEELEQTVGGDDLYKLVNQGFGAICSFIDGFNGKKYRGKC     51

gll_D1   1 MNTKTFEQFEMLDNASLANINGGDNITKGAYKLGKATRQAGKAIGKAIGKLF     52
             ||||.|||||:|||.|||||||||||||||||||||.|||||||:||||
inf_A2   1 MNTKGFEQFEILDNTSLANINGGDNITKGAYKLGKATRQAAKAIGKAVGKLF     52
```

Figure 4. Synthesised gallocin D. A) Mixing gallocin D and infantaricin A peptides indicates that any combination of the alpha and beta peptides results in activity, concentration in µM across the top. 'Rugby ball' shape seen between wells indicates where the peptides meet following diffusion into the media, showing complementary activity. B) Alignment of sequences of the prepeptides from both species, the double glycine cleavage point is underlined.

A number of indicator organisms were tested to determine the spectrum of activity, using both the overlay method on a plate and a well diffusion assay using the synthesized peptide (Table 3). The bacteriocin was active against a narrow range of indicator organisms that included the clinically important pathogens *S. pneumoniae* and vancomycin-resistant enterococci (VRE) strains, but was inactive against other unrelated pathogenic and commensal bacteria. The minimum inhibitory concentration (MIC) of the gallocin D peptide was assessed at 1.56 µM against VRE EC300.

Table 3. Indicator organisms with growth conditions. Inhibition is represented by relative activities + 0.5-2mm zone, ++ 2-4mm zone, +++ >4mm zone +, and no inhibition -.

Indicator organism	Growth Medium	Incubation Conditions	Inhibition
Actinomyces neuii LMG 19524t	BHI	37°, O_2^-	-
Actinomyces radingae LMG 15960t	BHI	37°, O_2^-	-
Enterococcus faecium EC300	BHI	37°, O_2^+	+++
Enterococcus faecium EC520	BHI	37°, O_2^+	+++
Enterococcus faecium EC533	BHI	37°, O_2^+	+
Enterococcus faecium EC571	BHI	37°, O_2^+	++
Escherichia coli DPC 6054	BHI	37°, O_2^+	-
Enterococcus faecalis E265	BHI	37°, O_2^+	+
Lactobacillus delbrueckii ssp. *bulgaricus* LMG 6901	MRS	37°, O_2^-	+++
Lactobacillus helveticus ATCC11454	MRS	37°, O_2^-	-
Listeria innocua DPC 1768	BHI	37°, O_2^+	+
Listeria monocytogenes DPC 3572	BHI	37°, O_2^+	-
Salmonella enterica ser. Typhimurium DPC 6046	BHI	37°, O_2^+	-
Staphylococcus aureus R963	BHI	37°, O_2^+	-
Streptococcus agalactiae LMG 14694	BHI	37°, O_2^+	-
Streptococcus pnuemoniae DSM 24048	BHI	37°, O_2^+	++
Streptococcus pnuemoniae DSM 14377	BHI	37°, O_2^+	+++
Streptococcus pnuemoniae DSM 20566	BHI	37°, O_2^+	+++
Streptococcus pnuemoniae DSM 25971	BHI	37°, O_2^+	++
Streptococcus gallolyticus DPC 6501	BHI	37°, O_2^+	+++

S. gallolyticus DPC6501 was isolated from a porcine jejunum in a previous study and is reported to produce a bacteriocin [30]. This strain was tested for sensitivity to *Sgg* LL009 and the synthesized gallocin D peptides. The overlay assay of *Sgg* LL009 showed zones of inhibition against *S. gallolyticus* DPC6501 and a zone of inhibition from 15µM gallocin D peptides was observed in a well diffusion assay (Figure 5).

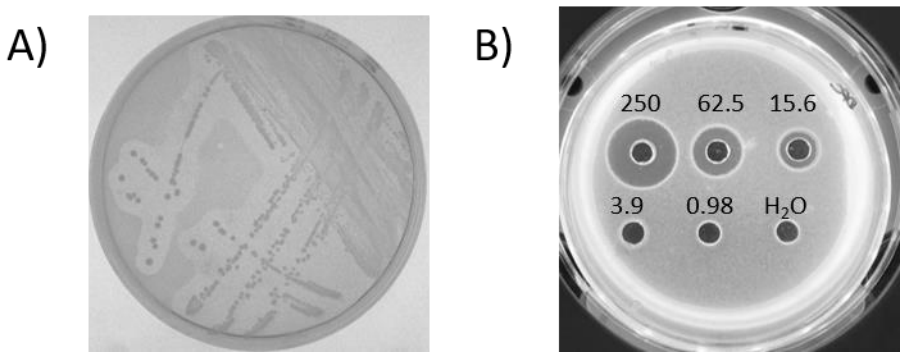


Figure 5; Testing against related *S. gallolyticus* (a) *Sgg* LL009 colonies showing inhibition of *Sgg* DPC6501 (indicator organism). (b) Well diffusion assay using synthesized gallocin D against *Sgg* DPC6501, concentration in µM indicated above each well.

Resistance Development in VRE EC300 against Gallocin D

The microtitre plates from the MIC determination experiment were plated in to check the viability of VRE EC300. Following 24 hours growth, VRE EC300 cells from the control and wells containing 100µM gallocin D, the highest concentration included, were spread plated on BHI, and then sub-cultured into fresh BHI. At 24 hours, the sub-cultured broths were serially diluted and plated. The bacteriocin treated cells showed no growth at any dilution, while the untreated cells reached 4.9 X 10^8 cfu ml $^{-1}$. At this concentration and inoculum no colonies were found following treatment with gallocin D.

For the time-kill assay, actively growing VRE EC300 at 10^8 cfu ml $^{-1}$ was treated with gallocin D at 15.6µM, 10x the MIC value. Within the first four hours, VRE EC300 was undetectable in all treated broths, while the untreated control remained at 10^8 cfu ml $^{-1}$. At 6 hours, VRE EC300 was detected in treated broths while at 24 hours the treated broths were lower than the untreated control at 10^7 cfu ml $^{-1}$ (Figure 6).

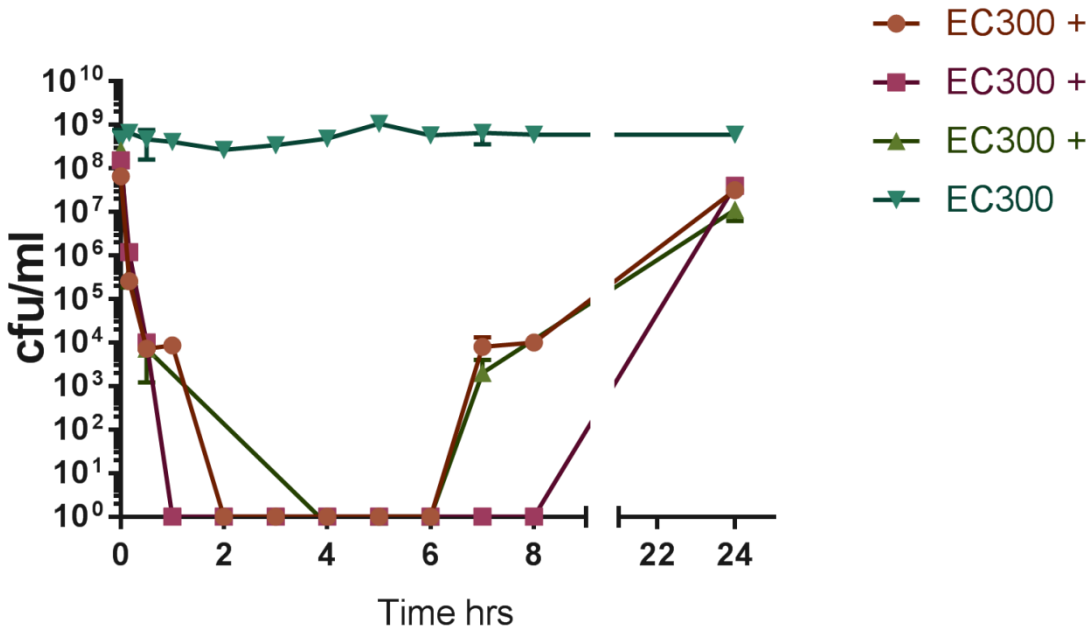


Figure 6; Kill curve of VRE EC300 using 15.6µM gallocin D. EC300 + samples contain bacteriocin (each representing a biological triplicate), EC300 has no bacteriocin (control).

Discussion

The bacteriocin operons within the *S. gallolyticus* spp. *gallolyticus* of the SBSEC were found to be generally highly conserved, with the exception of the newly isolated *Sgg* LL009 which lacks the gallocin A structural genes, and has different structural genes and a putative immunity gene at this locus. The structural genes in *Sgg* LL009 are variants of genes in an infantaricin A producing *S. infantarius* LP90 (*Sii* LP90) and its associated immunity gene. Infantaricin A is encoded by an operon with seven predicted bacteriocin structural genes, of which *infC* shows sequence homology to the gallocin A structural genes. *Sgg* LL009 is the only genome analysed found to lack genes for gallocin A/infantaricin C, and to contain a variant of previously identified infantaricin A. In a BLAST search limited to the SBSEC, no gallocin D hits were found outside of the *S. gallolyticus* or *S. infantarius* species and only one potential gallocin A homologue was identified in *S. macedonicus*.

Gallocin A is a two-peptide bacteriocin which has been reported to give *S. gallolyticus* a competitive advantage in conditions found in in the gut of patients with CRC. This bacteriocin is absent from closely related species, but we identified possible

homologues in *S. infantarius* strains, and *gllA2* was found in *S. gallolyticus* ssp *macedonicus* together with a gene with 60% identity to *gllA1*. High genome plasticity has been reported in the SBSEC and *S. gallolyticus* is reported to have retained the largest genome and highest functional capacity. Two human isolates of *S. infantarius*, NCTC 13760 and ATCC BAA-102, possess the infantaricin ABCDEFG bacteriocin operon while the dairy isolate CJ18 does not have the complete operon. *S. gallolyticus* LL009 and *S. infantarius* LP90 were isolated from goat and water buffalo raw milk samples, respectively. In previous comparative genomics studies, high numbers of IS elements were found in these species. The organisation of the operon and high sequence identity between genes suggests that transfer of bacteriocin structural genes occurred between these strains due to adaptive pressure in the microbe-rich environment of the gut [21]. *Sgg* LL009 has unique structural genes which are produced within the gallocin D operon.

The association between *S. gallolyticus* and CRC is not fully understood [31], but it is thought that the conditions in the colon when tumours are present provide a suitable niche for *S. gallolyticus* if competitors can be controlled with gallocin A. Gallocin A and gallocin D share similar characteristics and target organisms. Gallocin A production is enhanced in the presence of secondary bile acids, a known risk factor of CRC. The cell free supernatant of *S. gallolyticus* UCN34 shows no activity in a well diffusion assay in the absence of a detergent or secondary bile acids, a feature that is also observed for *Sgg* LL009 producing gallocin D [10]. *S. gallolyticus* mutants lacking *gllA1* and *gllA2* do not have the same colonisation advantage in tumour-bearing mice, leading to the conclusion that *S. gallolyticus* is not a causative factor of CRC but does promote its acceleration if pre-malignant tumours are present and the strain colonises. Both gallocin A and gallocin D target enterococci, with enhanced bacteriocin production in CRC conditions. This suggests that *Sgg* LL009 could well retain the colonisation advantage seen in *S. gallolyticus* UCN34 in CRC conditions, despite their distinct amino acid sequences.

The operons in the various *S. gallolyticus* strains were strikingly similar with the exception of the bacteriocin structural genes, suggesting that these bacteriocins would be subject to similar regulation and would be produced under similar

conditions. Whether this strain would have a protective effect in the colon is unclear. It is likely, due to their similar target organisms and production characteristics, that this strain of *S. gallolyticus* producing gallocin D would occupy the same niche as *S. gallolyticus* producing gallocin A. Further studies are required to assess if this gallocin D producing *S. gallolyticus* is able to colonise and accelerate cancer development.

This work confirms horizontal gene transfer of bacteriocin structural genes between members of the SBSEC, which have been shown to have high genome plasticity [18]. *Sgg* LL009 and *Sii* CJ18 are both dairy isolates and both show the most "unusual" operons; the structural and immunity genes of *Sgg* LL009, and *Sii* CJ18 has the lowest number of bacteriocin structural genes of all the *Sii* strains. This hints at a role for these bacteriocins, gallocin D and infantaricin A, in streptococcal strains colonising ruminant animals. Further identification of bacteriocin producers from the SBSEC could lead to further evidence of shuffling of bacteriocin structural genes.

A bacteriocin producing *S. gallolyticus* from our culture collection, *S. gallolyticus* DPC6501, is sensitive to gallocin D, which strongly indicates that the genomic annotation of the immunity protein is correct. This is the only predicted immunity protein present in *Sgg* LL009 and absent in related *S. gallolyticus* strains and also the only immunity protein shared between *Sgg* LL009 and the infantaricin A producer. *Sgg* LL009 is not inhibited by *Sgg* DPC6501; this strain has not been sequenced, but is known to be a bacteriocin producer from previous work [30]. We hypothesise that *Sgg* LL009 is not susceptible to gallocin A, but that synthesized gallocin D could provide an alternative strategy for the control of *S. gallolyticus* infections.

The application of gallocin D is not limited to its potential role in controlling the growth of other *S. gallolyticus* strains in CRC. It is a narrow spectrum bacteriocin with potent activity against VRE, opportunistic pathogens that are particularly relevant in hospital settings for patients who are immunocompromised and those under antibiotic treatment for endocarditis [32]. VRE can infect the urinary tract, surgical wounds or the bloodstream and are spread by direct contact. Many patients who develop VRE infections have underlying illnesses and due to antibiotic resistance

this infection can lead to serious problems or fatalities [33]. The kill curve of gallocin D shows it can reduce the numbers of VRE from 10^8 cfu ml $^{-1}$ to undetectable levels in two hours, though the cells regrow after 8 hours. Importantly when added to growing cells at 10^5 cfu ml $^{-1}$, which is regarded as a clinical infection, VRE EC300 did not recover and no resistant colonies were found. This suggests that the mode of action of this bacteriocin is related to cell contact; if the bacteriocin is present in the right ratio to cells the infection can be cleared. The MIC and resistance testing results for gallocin D show that it completely kills VRE EC300 when present at 10^5 cfu ml $^{-1}$, and when gallocin D is present at the MIC of 1.56µM.

Conclusions

A combination of laboratory and *in silico* analyses led to the discovery and characterisation of gallocin D, a class IIb bacteriocin with activity against VRE, *S. pneumoniae* and a related strain of *S. gallolyticus*. Gallocin D is a variant of infantaricin A produced by closely-related *S. infantarius* species. The operon is similar to those found in other *S. gallolyticus* genomes, with the exception of the structural and immunity genes. This work highlights the shuffling of bacteriocin structural genes within these closely related species. Gallocin D could be applied for treatment of VRE infections and potentially for the control of other species of *S. gallolyticus.*

Materials and Methods

Bacterial strains culture conditions

S. gallolyticus LL009 was isolated from raw goat milk produced in New Zealand on *S. thermophilus* agar at 42°C. *S. gallolyticus* LL009 was routinely cultured under aerobic conditions at 37°C, in brain heart infusion (BHI) medium (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), all other reagents were sourced from Sigma-Aldrich (Wicklow, Ireland) unless otherwise stated. Indicator strains used and their incubation conditions are listed in Table 2.

Draft Genome Sequencing

DNA was extracted using a Genelute bacterial genomic DNA kit (Sigma) and prepared for sequencing using a Nextera XT kit (Illumina) for library preparation. DNA was quantified using a Qubit 2.0 fluorometer. Sequencing was carried out using an Illumina MiSeq platform with paired-end 2 x 300 base pair reads at the Teagasc Sequencing Centre, Teagasc Food Research Centre Moorepark. Assembly was performed *de novo* using SPADES and automatically annotating using GAMOLA [34]. The whole genome sequence was run through Bagel4 and antiSMASH to search for bacteriocin genes. The genome was also compared to other available *S. gallolyticus* and *S. infantarius* genomes from NCBI listed in Table 2.

Table 2. Genomes analysed in this study.

Species	Strain	NCBI reference	Source
Streptococcus gallolyticus **ssp.** *gallolyticus*	DSM 16831	NZ_CP018822.1	koala faeces
	ATCC 43143	NC_017576.1	human blood
	ATCC BAA-2069	NC_015215.1	human blood
	UCN34	NC_013798.1	human blood
	LL009		raw goat milk
Streptococcus infantarius **ssp.** *infantarius*	CJ18	NC_016826.1	suusac fermented camel milk
	LP90	KJ475434.1	water buffalo milk
	NCTC13760	NZ_UHFP00000000.1	human faeces
	ATCC BAA-102	NZ_ABJK00000000.2	human GIT

Antimicrobial Activity assays

Sgg LL009 was grown on BHI agar and incubated overnight at 37°C. *Sgg* LL009 was assayed against various indicator organisms, listed in Table 3 along with incubation conditions**.** Plates were overlaid with MRS agar (7.5gL^{-1} agar) seeded with *L. bulgaricus* LMG6901 and incubated overnight at 37°C, anaerobically. Activity was defined by a clearing in the overlay medium.

Overnight cultures were centrifuged and filtered to obtain cell free supernatant (CFS). MRS agar was seeded with an overnight culture of *L. bulgaricus* LMG6901 and wells were made. 50µL of both broth and CFS were added to the wells. Plates were incubated overnight at 37°C. 1% Triton X-100 was added to CFS. All

assays were performed in triplicate. The well diffusion method was repeated with synthesized gallocin D, using peptides alone and in combination (1:1).

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) determinations of gallocin D (D1D2) were carried out in triplicate in microtitre plates as previously described [35]. Briefly, VRE EC300 was grown overnight in BHI broth at 37°C and subcultured at 0.5% into fresh broth. The strain was grown to OD600 of 0.5 and diluted to a final concentration of 10^5 cfu ml $^{-1}$ in a final volume of 100µL. Synthesized gallocin D peptides in a 1:1 ratio were made up to 100µM concentration and serially diluted to a concentration of 0.98µM in sterile water. The peptide solutions were added at 100µL volumes to the VRE broth. The plate was incubated at 37°C for 16 hours, the MIC was determined as the lowest concentration causing visible inhibition of growth.

Resistance Testing

In order to assess resistance development by VRE EC300, the MIC protocol was repeated. The microtitre wells containing 100µM gallocin D and control wells were plated directly and cultured in fresh BHI broth. At 24 hours the sub-cultured broths were serially diluted and plated. The whole microtitre plate was reincubated for a further 48 hours and observed for cloudiness indicative of growth.

Kill Curve

VRE EC300 was grown overnight in BHI and adjusted to a final concentration of 10^8 cfu ml $^{-1}$ in fresh BHI. Synthesized gallocin D was added at concentration of 10x MIC (15.6µM) to triplicate testing broths, and sterile water added to control VRE broths. Samples were taken at multiple time points until 8 hours and again at 24 hours, serial diluted in MRD and plated on BHI medium. Plates were incubated for 24 hours at 37°C before colonies were enumerated.

Colony Mass Spectrometry.

Colony MALDI-TOF MS (Axima TOF^2 MALDI-TOF mass spectrometer, Shimadzu Biotech) was used to determine the molecular mass of the peptides

present on the surface of colonies as follows: cells were first mixed in 70 % (v/v) 2-propanol/0.1 % TFA (IPA) and vortex mixed, the sample was separated by centrifugation at 14,000 r.p.m and the supernatant was subsequently used for analysis. A MALDI target plate was precoated with CHCA matrix solution, 0.5 µL of the supernatant from the cell extract was then placed on the target and a final layer of matrix solution was added. Positive-ion linear mode was used to identify the peptide masses on an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). The masses detected were then compared to those of known bacteriocins.

Peptide synthesis and purification

Peptides were synthesised by microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a Liberty Blue microwave peptide synthesizer (CEM Corporation. Mathews, NC, USA). Gallocin D1 was synthesized on a H-Cys(Trt)-HMPB)-ChemMatrix resin and Gallocin D2 was synthesised on H-Phe-HMPB-ChemMatrix resin (PCAS BioMAtrix Inc., Quebec, Canada). Crude peptide was purified using RP-HPLC on a Semi Preparative Jupiter Proteo C12 (10 x 250mm, 4µ, 90Å) column (Phenomenex, Cheshire, UK) running acetonitrile 0.1% TFA gradients specific to the peptide of interest. Fractions containing the desired molecular mass were identified using MALDI-TOF-mass spectrometry in positive in linear mode and were pooled and lyophilized on a Genevac HT 4X lyophiliser (Genevac Ltd., Ipswich, UK).

Growth Curve

A single colony of VRE was inoculated into BHI medium and incubated at 37°C for 16 hours, and subcultured at 1% into fresh medium. Samples were taken at 0, 2, 4, 5, 6, 7, 8 and24 hours for OD_{600} and plating. OD was read in duplicate, 100µL was serially diluted in MRD to 10^{-8} and plated on BHI agar. Plates were incubated for 24 hours and enumerated. Experiments were completed in triplicate, each with technical duplicates.

Antibiotic Resistance

The MIC value of sixteen antibiotics was assessed using the VetMIC Lact-1 and Lact-2 MIC determination plates (National Veterinary Institute, Sweden). The antibiotics tested were ampicillin, penicillin, vancomycin, erythromycin, virginiamycin, tetracycline, clindamycin, chloramphenicol, kanamycin, gentamycin, streptomycin, neomycin, linezolid, rifampicin, ciprofloxacin, and trimethoprim. Briefly, colonies were resuspended in MRD at a concentration of ~1x10^8 cfu ml^{-1} and transferred into ISO-MRS broth for a final inoculum of 5 x 10^5 cfu ml^{-1}. VetMIC plates were inoculated with 100µL, sealed and incubated for 24 hours. Evidence of growth was determined using a backlight, MIC value is the lowest concentration completely inhibiting growth. EFSA guidelines do not have specific values for *S. gallolyticus,* so values were interpreted using *S. thermophilus* values and the defined values for "other Gram positive bacteria" [36].

Exopolysaccharide Screening

Multiple screening methods were used to test for ropy and non-ropy type EPS; ruthenium red agar, the loop touch test and sucrose-supplemented MRS. Ruthenium red was filter sterilised, added to cooling MRS agar at 0.08% and mixed before pouring plates [27]. For the loop touch test, a sterile loop was touched to a single colony and slowly pulled away. A string between the loop and colony was recorded as a positive result. 10% (w/v) sucrose and 10% (w/v) lactose supplemented MRS plates were autoclaved at 121 ^oC for 15 min and poured, a mucous phenotype was characterised as a positive result.

Proteolysis

10% reconstituted skim milk (RSM) was autoclaved at 121^oC for 5 min and combined with a 3% (w/v) agar solution, autoclaved at 121^oC for 15 min, the solutions were allowed to cool to ~45°C, combined 1:1, and poured into petri dishes. Previously grown *Sgg* LL009 was re-streaked onto the RSM plates and incubated at 37°C for 48 hours, plates were viewed every 24 hours. Proteolysis was defined as clear zones surrounding colonies.

Hemolysis

Tryptic Soy Agar was prepared, 5% v/v sterile defibrinated horse blood was pre-warmed, added to the sterile agar and mixed well before pouring. Previously grown *Sgg* LL009 was re-streaked onto the plates and incubated for 72 hours, plates were viewed every 24 hours for lysis, defined as a clearing or discolouration of the agar.

Author Contributions

C.S., and R.P.R. were involved in study design, guidance with experiments, and interpretation of the results. D.H. performed the *in silico* screen, isolated the bacteriocin producer, characterized the spectrum of inhibition, performed whole-genome sequencing, and wrote the manuscript. P.M.O'C. performed MALDI-TOF MS, bacteriocin purification, and peptide synthesis. All authors took part in reviewing the manuscript and approved the final manuscript.

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Chapter 6.

In silico analysis reveals multiple diverse structural gene homologues within bacteriocin clusters in the *Streptococcus bovis/Streptococcus equinus* complex pangenome.

Abstract

With advances in high-throughput sequencing, the genomes of more and more bacterial strains are becoming accessible. Correct annotation and detailed analysis of these genomes is essential if we are to fully understand bacterial functionality. Using a combination of genome analysis and laboratory experimentation, we discovered shuffling of bacteriocin structural genes between *Streptococcus infantarius* and *Streptococcus gallolyticus* (Chapter 5). This prompted us to look further into the genomes of all sequenced strains in the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) to determine the incidence and organisation of bacteriocin genes within this taxonomic group. Bagel4 is a bioinformatic tool that was used to computationally identify putative bacteriocin operons in a total of 107 genomes. Sixty-seven areas of interest (AOIs) were identified, containing 166 predicted structural peptides, comprised of class I and class II bacteriocins. The AOIs were aligned and grouped into six types of bacteriocin clusters based on gene synteny and the putative bacteriocin structural genes within each group were analysed. Individual AOIs contained between two and fifteen structural genes in each operon. The operons containing multiple bacteriocin structural genes display remarkable diversity in their predicted mature peptide and signal sequences, however in some cases different bacteriocins shared almost identical leader sequences. It is tempting to speculate that the possession of such differing bacteriocin structures within this bacterial group offers competitive advantages to the producers in different microbial niches.

Introduction

The *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) includes seven species; *Streptococcus gallolyticus*, *Streptococcus infantarius*, *Streptococcus gallolyticus* ssp *macedonicus, Streptococcus gallolyticus* ssp *pasteurianus*, *Streptococcus lutetiensis*, *Streptococcus alactolyticus* and *Streptococcus equinus* [1, 2]. Members of the SBSEC are found in food, the environment, the gastrointestinal tract (GIT) of humans and animals and have been clinically associated with infective endocarditis [3, 4]. The genome plasticity of this complex has been studied and adaptations have been observed between animal and human isolates [5]. Bacteriocin production has been reported for a number of these species [6, 7].

Bacteriocins are ribosomally synthesized antimicrobial peptides which can act on a narrow or broad range of target organisms. The bacteriocins of lactic acid bacteria (LAB) have been studied for many years and have a long history of safe use in food. Bacteriocins can be classed into three categories; class I contain the post-translationally modified peptides (including the lantibiotics and others), class II are unmodified peptides and class III are larger enzymes that are >10kDa in size [8]. Bacteriocins are of interest due their current and potential biopreservative applications in food and as potential alternatives to antibiotics in therapeutic settings. Broad spectrum bacteriocins such as the lantibiotic nisin A can act on a wide range of target organisms, while narrow spectrum bacteriocins such as the sactibiotic thuricin CD act on only a small number of targets [9, 10]. The use of narrow spectrum bacteriocins as novel therapeutics for infectious bacteria is attractive as they would not be expected to have a significant collateral impact on commensal bacteria, unlike the widespread damage caused to the microbiota by broad spectrum antibiotics. Moreover, their gene-encoded nature means that they can be readily bioengineered and they have the potential to be used to support or complement the use of antibiotics [11-13].

Following the discovery of bacteriocin structural gene shuffling between two members of this species, *S. infantarius* and *S. gallolyticus* (Chapter Five), we analysed all bacteriocin gene clusters we could identify in sequenced members of the SBSEC.

The genomes of all 105 available strains were analysed using Bagel4 to identify potential bacteriocin operons across the SBSEC pangenome, in addition to the previously analysed *S. gallolyticus* LL009 and *S. infantarius* LP90. We found that there is the genetic capacity to encode a large number of bacteriocins in members of this complex. There were 67 areas of interest (AOI) found in 44 strains, with 117 predicted structural genes. The AOI could be aligned into six major groups based on gene synteny. The predicted peptides encoded within each group were then compared with one another. The peptides were also compared across all identified AOI. While these are all predicted bacteriocins and we do not have laboratory confirmation of production or activity, our results give an insight into the extensive carriage of bacteriocin production genes across the SBSEC pangenome.

Results and Discussion

All available NCBI genomic data for each member of the SBSEC was downloaded (as of 26/05/2020). The genomes are listed in Table 1, together with their source and genome assembly level. There were 107 genomes in total, 28 *S. gallolyticus* including strain LL009 described in Chapter Five, five *S. infantarius* including *S. infantarius* LP90 encoding the bacteriocin cluster infantaricin ABCDEFG also described in Chapter Five, 44 *S. equinus*, seven *S. macedonicus*, nine *S. lutetiensis*, 12 *S. pasteurianus*, and two *S. alatolyticus*. The FASTA sequence for each genome was downloaded and Bagel4 was used to search for bacteriocin operons. In total, 67 areas of interest (AOI) were identified in 44 of the genomes (41% of all strains analysed possessed at least one AOI), with three being the highest number of AOIs identified in any one strain (Table 1).

The species with the highest percentage of strains containing AOIs was *S. infantarius* with three of five strains (60%). *S. gallolyticus* was next highest with 15 of 28 strains (54%). AOIs were identified in five of 12 strains of *S. pasteurianus* (42%), followed by *S. equinus* with 17 of 44 strains (39%), two of seven strains of *S. macedonicus* (29%), and one of nine strains of *S. lutetiensis* (11%). There were no bacteriocin operons predicted in the two *S. alactolyticus* genomes analysed.

Table 1; Genomes analysed using Bagel4. The number of AOI identified is provided. AOI = area of interest. Hungate = The hungate 1000 rumen microbiome

Strain	Source	AOI
S. gallolyticus		
ICDDRBNRCS1	Faeces a hospitalised child	1
ATCC4314	Human blood	2
ATCCBAA2069	Human blood	3
DSM1683	Koala faeces	2
UCN34	Clinical human blood	2
LL009	Goat milk	1
FDAARGOS 666	Clinical human isolate	2
NCTC 13773	Koala faeces	2
NCTC13767	Not available	2
ICDDRB_NRC_S3	Faeces a hospitalised child	2
NTS_31106099	Human blood	0
BgPS.064.S07	Cultured Isolate from human donor	0
LMG17956	Cow gut	1
TX20005	Human biological product	3
ATCC 700065	Missing	0
LMG 15572	Goat rumen	0
VTM1R27	Hungate	0
AM24-4	Human faeces	1
VTM1R29	Hungate	0
NTS31301958	Human blood	2
VTM3R24	Hungate	0
VTM3R42	Hungate	0
NTS 31307655	Human blood - infective endocarditis	0
TM07-4AT	Human faeces	0
VTM2R47	Hungate	0
BI02	Kakapo faecal isolate	0
DD03	Propithecus verreauxi oral cavity	0
DD02	Propithecus verreauxi oral cavity	1
S.infantarius		
ATCC BAA 102	Human GIT	0
LP90	Water buffalo milk	1
CJ18	Susac fermented camel milk	1
NCTC13760	Human faeces	1
ICDDRBNRCS5	Faeces a hospitalised child	0
S. lutetiensis		
NCTC 8796	Not available	0
NCTC 13774	Not available	0
NCTC 8738	Not available	0
_033	A diarrheal child	1
NCTC8746	Not recorded	0
AM25-7	Human faeces	0
MGYG-HGUT-00088	Human gut	0
AM38-16	Human faeces	0
DD06	Lemur catta nose	0
S. pasteurianus		
ATCC 43144	Human blood	1
WUSP067	Domestic pig brain	2
NCTC 13784	Cerebrospinal fluid	1
AL101002	Duck - bacterial meningitis	1
HC-2909-2	Human blood - diseased sample	0
SPSS39-a2-mod	Cultured Isolate from human donor	0
SS_Bg39	Cultured Isolate from human donor	0
MGYG-HGUT-00246	Human gut	1
AM21-19	Human faeces	0
AF13-35	Human faeces	0
651_SPAS 52	Clinical or host-associated sample	0
GED7275A	Human vagina	0

Strain	Source	AOI
S. macedonicus		
ACA-DC 198	Greek kasseri cheese	3
679	Uncooked semi-hard cheese	1
UMBO0733	Homo sapiens	0
211 MA	“malga” (pasture) cheese	0
33MO	Curd of morlacco cheese	0
19AS	Asiago cheese	0
27MV	Monte veronese cheese	0
S. equinus		
CNUG6	Rumen fluid	2
FDAARGOS251	Human blood	2
NCTC 8140	Cow faeces	0
NCTC 11436	Human blood culture	0
NCTC 10389	Faeces	0
CNU 77 23	Rumen fluid	1
AG46	sheep rumen	1
NCTC12969	Horse faeces	0
ATCC 33317	Cow dung	0
NCTC10386	Horse faeces	0
FMD1	Forest musk deer	0
Sb17	Hungate	0
Sb10	Hungate	1
GA-1	Hungate	2
ICDDRB-NRC-S6	Stool of a healthy control child	0
AR3	Hungate	2
Sb18	Hungate	0
Sb04	Hungate	0
Ye01	Hungate	0
SN033	Hungate	1
JB1	Rumen from alfalfa fed cow	0
NBRC 12057	Bovine rumen contents	1
ATCC 700338	Human-associated habitat	2
ATCC 9812	Human-associated habitat	0
Sb05	Hungate	0
pGA-7	Hungate	0
2B	Hungate	0
ES1	Hungate	2
C277	Hungate	2
Sb13	Hungate	0
H24	Hungate	1
Sb20	Hungate	0
HC5	Cow rumen	0
MPR4	Hungate	0
Sb09	Hungate	2
pR-5	Hungate	1
SI	Hungate	2
B315	Hungate	1
JB1	Hungate	0
MPR1	Hungate	0
MPR2	Hungate	0
MGYG-HGUT-01308	Human gut	0
MGYG-HGUT-01363	Human gut	0
RUG664	Rumen uncultured genome	0
S. alactolyticus		
BL-178-WT-3A	Wild pig	0
FGM	Red junglefowl	0

All 67 AOIs were aligned using MAUVE, which identifies conserved segments between genomes by creating locally colinear blocks (LCBs) (Figure 1) [14, 15]. MAUVE identifies groups of similarly-organised genes based on homology. These LCBs could be entire bacteriocin operons, or more often represent smaller groups of genes within operons. The output of MAUVE gives LCBs the same colour regardless of their position in the different bacteriocin operons. Homologous genes can be orthologs, which occur from speciation events arising from the species last common ancestor, or xenologs which come from horizontal gene transfer events. MAUVE aligns regions of genomes that are othologous and xenologous.

AOIs were not identified in 59% of the genomes analysed. While this could be due to low sequence quality in some instances, we limited our search to AOIs identified by Bagel4. This tool does not identify all bacteriocin operons, which is evident from the inability of Bagel4 to identify an AOI in *S. infantarius* ATCC BAA 102, despite the fact that we have shown in Chapter Five that it contains a bacteriocin operon. As the study was based on the gallocin D and infantaricin A structural genes identified in Chapter Five, these operons were included together with all AOIs identified using Bagel4.

In the MAUVE alignment, all 67 AOIs were grouped based on their synteny (Figure 1). Groups were assigned where an AOI shares synteny over 50% of its length with another AOI. This resulted in six groups; Group One has eight AOIs and contains the macedocin bacteriocin cluster, Group Two has two AOIs predicted to encode sactipeptides, Group Three has six nisin U operons, Group Four has eight AOIs and is predicted to contain partial structural genes, Group Five has eight AOIs that are predicted to be Linear azol(in)e-containing peptides (LAP's), and Group Six is the largest group with 25 AOIs. There are ten AOIs which did not fit within any of the six groups. The groups are discussed in detail below.

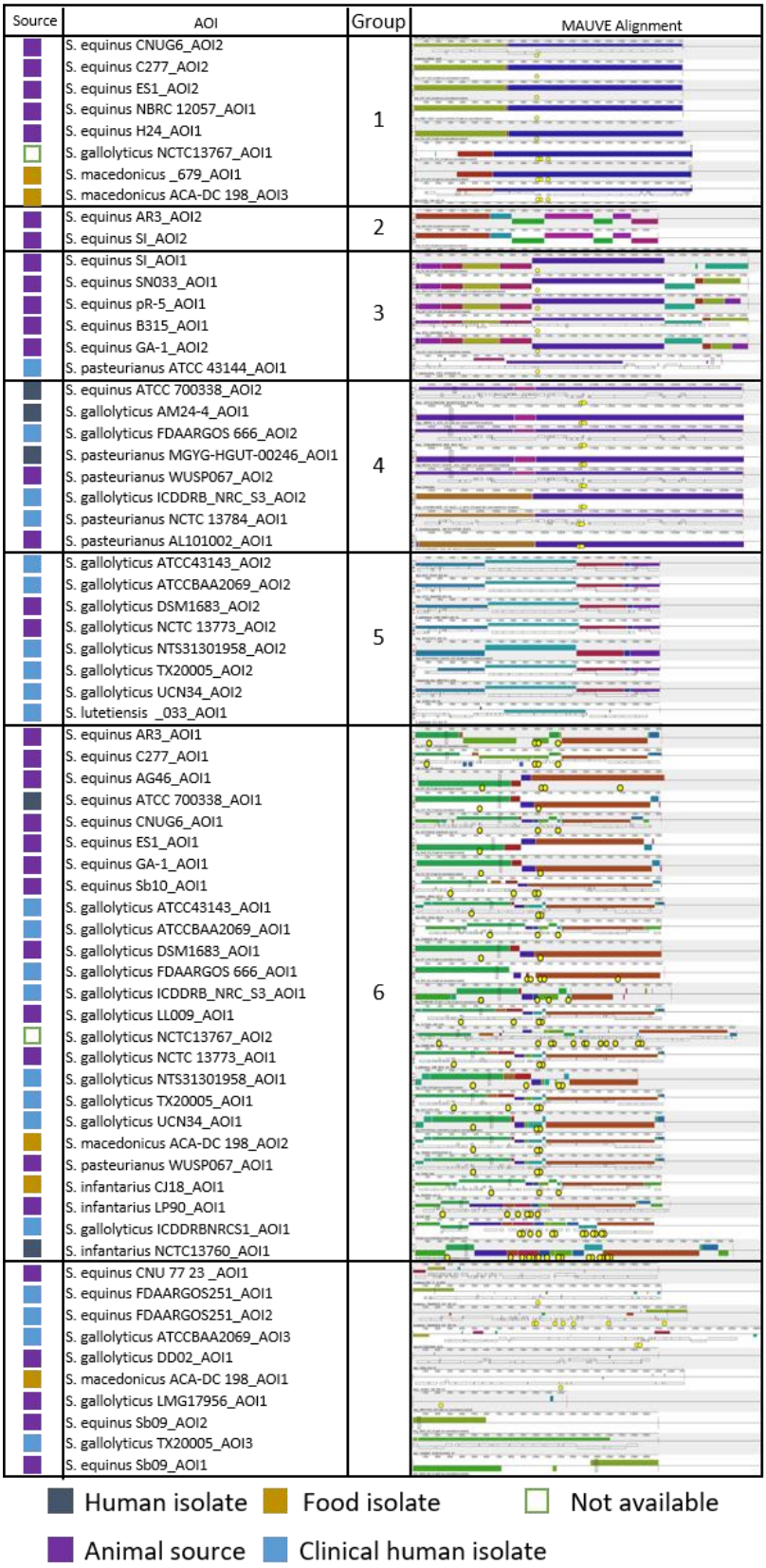

Figure 1. MAUVE alignment of all 67 AOIs grouped into similarly ordered bacteriocin operons.

Group One

Group One contains eight AOIs, five from *S. equinus* (strains C277, ES1, CNUG6, NRBC12057, H24), one from *S. gallolyticus* (NCTC13767), and two from *S. macedonicus* (679, ACADC-198). The group could be further divided into two subgroups based on their synteny. Subgroup 1a (#1-5 in Figure 2) all contain one structural gene, annotated by Bagel as J46, and subgroup 1b (#6-8 in Figure 2) contain three structural genes, two annotated as J46 and a third structural gene that encodes a bacteriocin with similarity to macedocin, McdA1 [16]. The J46 structural gene in subgroup 1a encodes a peptide that shares 66% amino acid identity with the J46 peptide in subgroup 1b. Subgroup 1b includes the macedocin gene cluster, encoding a previously identified bacteriocin described in detail by Papadelli et al [17].

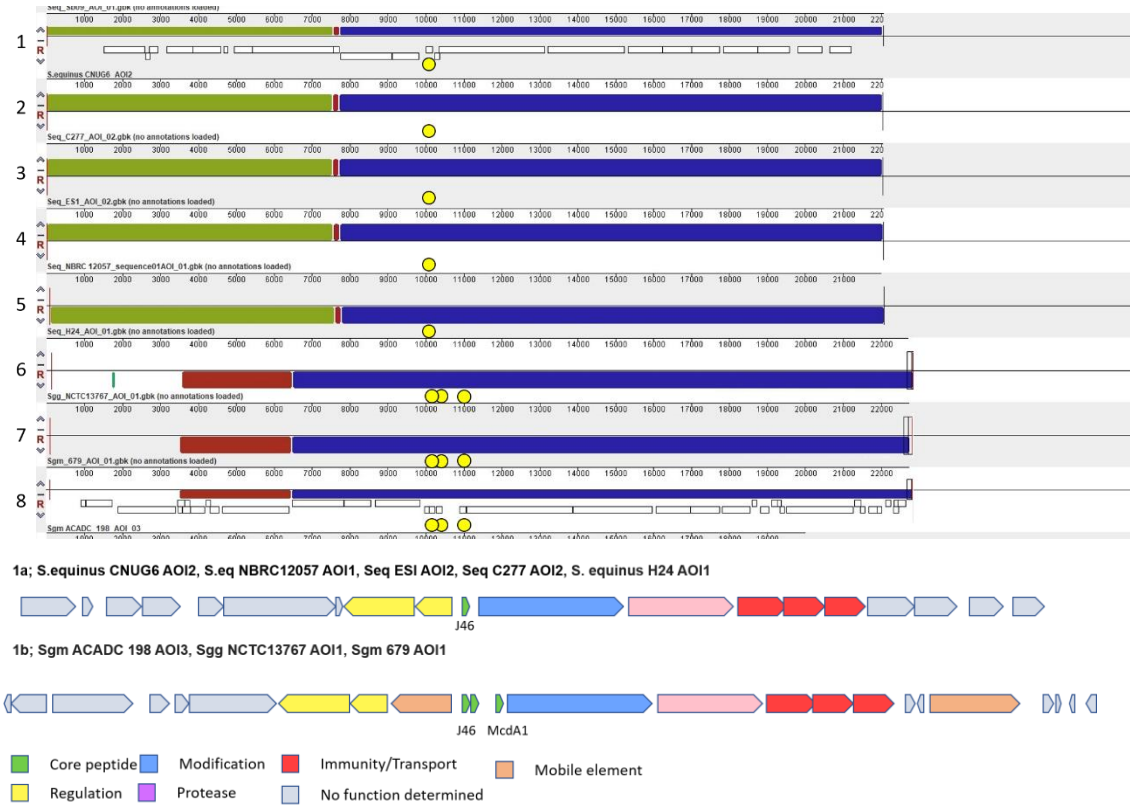

Figure 2. MAUVE analysis of Group One. a) Group One AOI MAUVE alignment, the location of putative structural peptides is marked with a yellow dot. b) Operon structure of subgroups 1a and 1b.

It is possible that subgroup 1a has lost two of the three structural genes found in subgroup 1b, or alternatively, that subgroup 1b has acquired two additional

structural genes. Both J46 genes in group 1b are 100% identical, and second J46 gene in subgroup 1b could be due to a duplication event. As described in Georgalaki *et al,* the macedocin gene cluster contains putative mobile elements which have a higher G+C content which could indicate the macedocin genes were acquired by horizontal gene transfer [17]. These mobile elements and structural genes were not identified in group 1a, which could support the theory that these additional bacteriocin structural genes in 1b were acquired by horizontal gene transfer.

Group Two

Group Two contains two bacteriocin operons predicted to encode sactipeptides as a result of the presence of a characteristic radical SAM protein [18]. Group Two only contains AOIs from two *S. equinus* strains (AR3 and SI). The synteny is perfectly conserved across the entire AOI (Figure 3). The Bagel output for these had limited annotation and despite further analysis no structural genes could be identified.

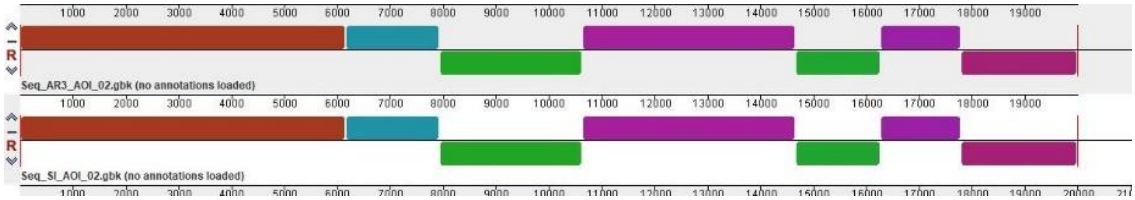

Figure 3. MAUVE analysis of Group Two. Group Two contains operons within two strains of *S. equinus* predicted to encode sactipeptides.

Group Three

Group Three contains six strains, five *S. equinus* (GA-1, SN033, pR-5, SI, B315-G597, #1-5 in Figure 4) and one *S. pasteurianus* (ATCC43144, #6 in Figure 4), and all contain a similar operon. The structural genes were aligned with Nisin U using MUSCLE and all were found to represent variants of nisin U [19]. The five *S. equinus* strains share the same structural peptide, designated as nisin UE, while *S. pasteurianus* encodes nisin UP.

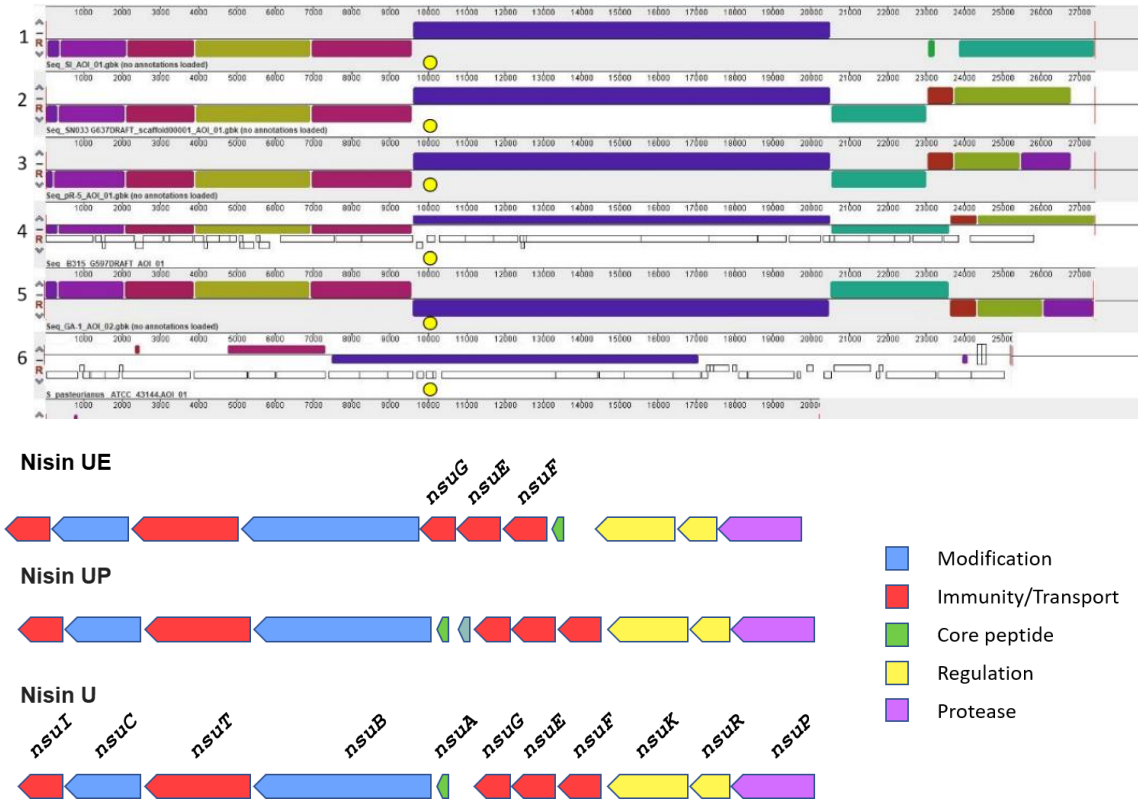

Figure 4. MAUVE analysis and operon structure of Group Three. a) Group Three AOIs, with structural genes marked with a yellow dot b) Nisin UE, nisin UP and nisin U operons compared.

The predicted nisin UE has an additional 13 amino acids at the start of the leader, and two amino acid substitutions in the mature peptide along with an additional asparagine residue at the terminal end (Figure 5). The structural gene is likely to utilise the alternative start codon GTG for valine 14 since leaders are normally conserved in length. Nisin UP in *S. pasteurianus* ATCC43144 is the same length as the original nisin U with two substitutions in the leader and three substitutions in the mature peptide. This was found to be identical to nisin P, previously isolated from *Streptococcus gallolyticus* subsp. *pasteurianus* [20]. The additional gene adjacent to *nisUP* could not be identified using BLAST, Bagel4 or Bactibase.



Figure 5. Multiple sequence alignment of nisin U, nisin P and predicted nisin U variants discovered in *S. equinus* (nisin UE) and *S. pasteurianus* (nisin UP)

Group Four

There are eight putative bacteriocin operons in this group; one from *S. equinus* ATCC 700338 #1 in Figure 6, three from *S. gallolyticus* (AM24-4 #2, FDAARGOS 666 #3, ICDDRB-NRC-S3 #6 in Figure 6), and four from *S. pasteurianus* (MGYG-HGUT-00246 #4, WUPS067 #5, NCTC 13784 #7, AL101002 #8 in Figure 6). Each operon contains two structural genes, both of which annotate as bovicin-255 (Figure 6). These genes overlap in the BAGEL output, with the larger predicted structural genes containing an internal stop codon. Both predicted structural peptides were included in a multiple sequence alignment and share significant homology with garvicin Q (Figure 7). These partial genes probably result from mutations of an intact functional garvicin Q gene and are unlikely to encode active bacteriocins.

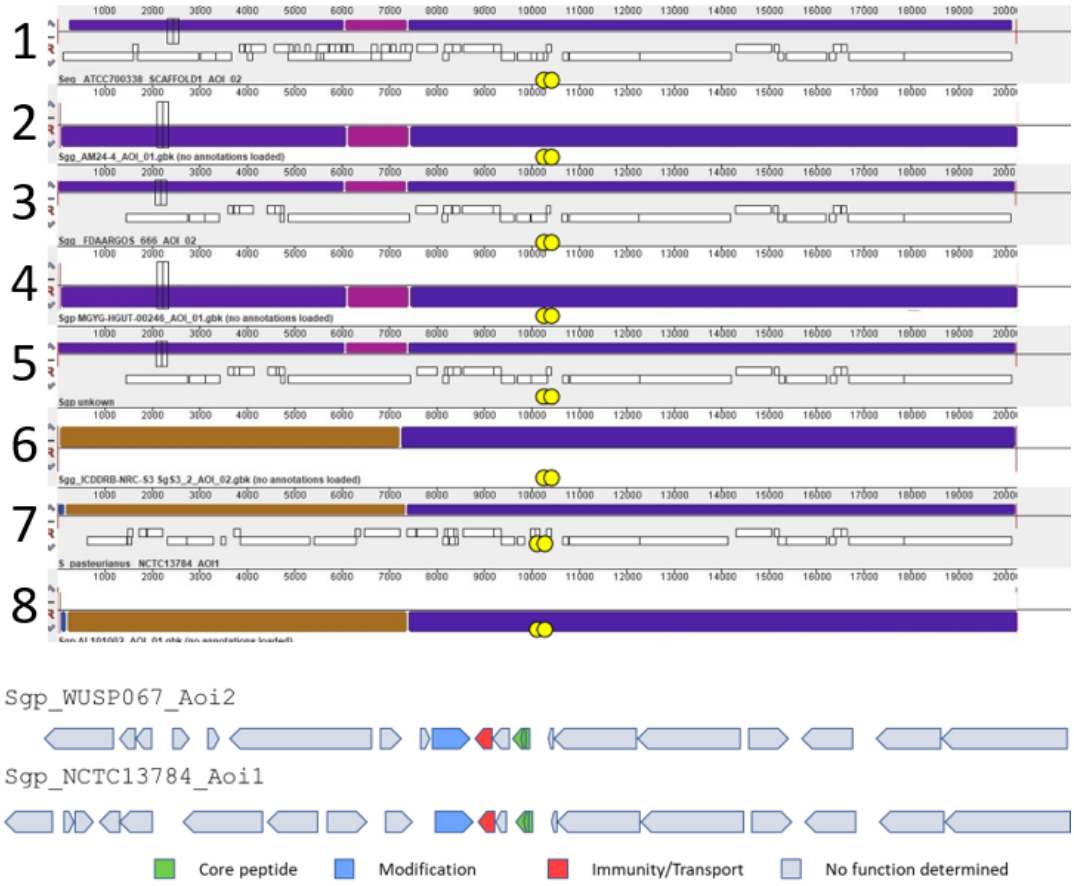

Figure 6. MAUVE analysis of Group Four. a) Group Four AOI's, with structural genes marked with yellow dots, b) representative operon structure of #1-5 operon (top), #6-8 operon (bottom).

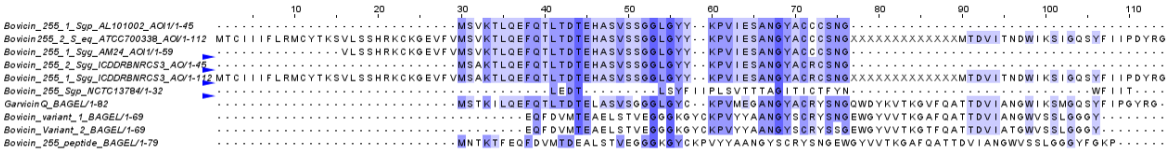

Figure 7. Multiple sequence alignment of structural genes from Group Four. (x) indicates where a stop codon interrupts the longer structural peptides.

Group Five

There are eight strains in Group Five; seven *S. gallolyticus* (#1-7 in Figure 8) and the only *S. lutetiensis* with an identified AOI (#8 in Figure 8). No structural genes were predicted by Bagel4, but non-structural bacteriocin-related genes are present, suggesting that these operons potentially encode linear azol(in)e-containing peptides (LAPs). This group of ribosomally post-translationally modified peptides are characterised by the cyclodehydration of the peptide backbone. The operons contain genes encoding proteins that are distinctive of LAPs, such as the YcaO protein which is involved in LAP biosynthesis [21]. Using Artemis, one potential structural gene could be identified in *S. gallolyticus* NCTC 13773. This gene did not show significant homology to known bacteriocin genes. Known LAP's were aligned with this gene, but these bacteriocins share little homology in general and so it is difficult to predict if this is in fact a novel LAP (Figure 9). The structural peptide identified in *S. gallolyticus* NCTC 13773 contains a high number of Cys residues, a common feature seen in LAP's.

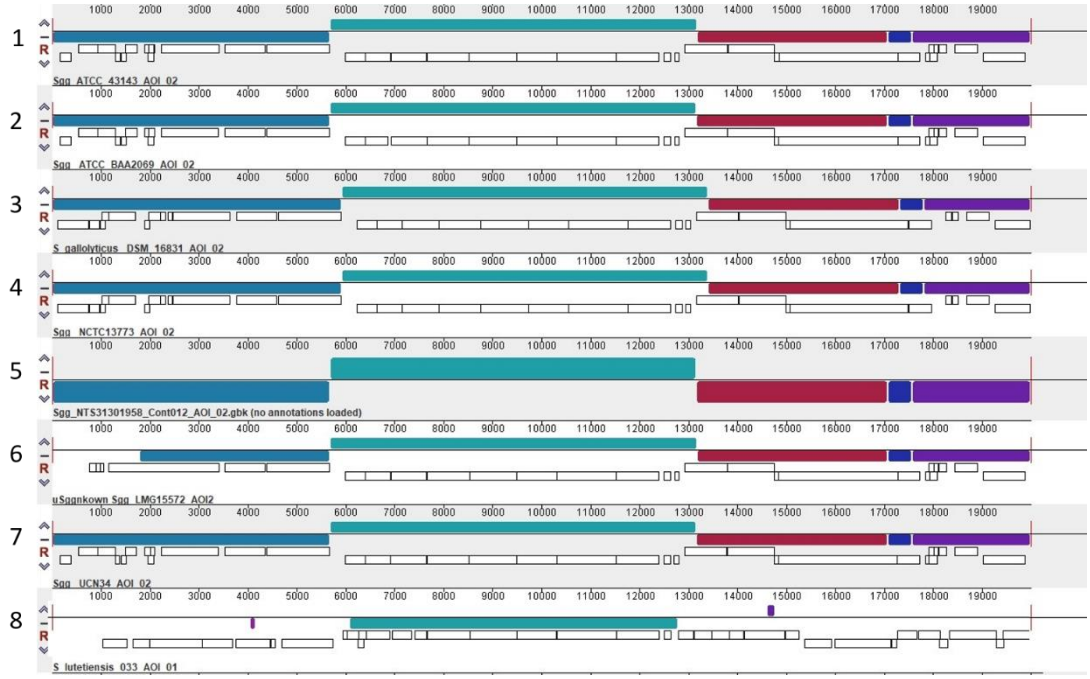

Figure 8. MAUVE analysis of Group Five. There are no structural genes identified for these in Bagel4

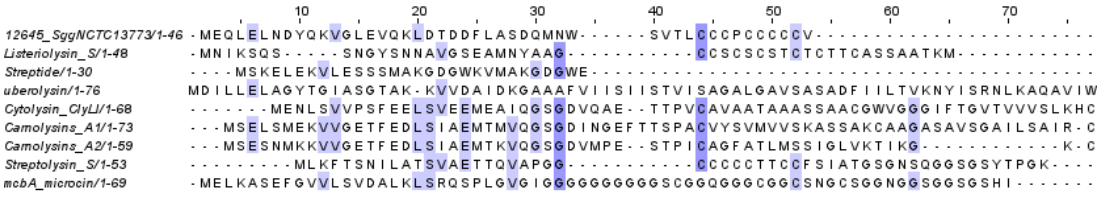

Figure 9. Multiple sequence alignment of potential LAP's. Putative novel LAP from *S. gallolyticus* NCTC 13773 (top).

Group Six

This is the largest grouping with twenty-five strains containing 117 structural genes (Figure 10). Individual AOIs contain between two and fifteen structural genes and the grouping includes both operons analysed in Chapter Four. These are similar in their gene arrangement but do not contain the same number of, or necessarily similar, structural genes. When the structural genes are aligned, it is obvious that they encode conserved leader sequences in many instances, despite the fact that the regions representing the mature peptides share little homology.

All 25 strains contain at least one peptide sharing the most common leader sequence (boxed in Figure 11). Even though the leader is conserved, there is large variation in the structural peptide which follows, which includes several known

bacteriocins such as gallocin D, gallocin A and infantaricin A (described in Chapter Five).

Since this common leader sequence is present in characterised bacteriocins that we know are produced and active, it is presumably capable of acting as a true leader. We cannot be confident that all the other predicted propeptides have functional leaders. It is intriguing to speculate as to the reason why individual strains contain so many structural genes encoding propeptides with different leaders. One possibility is that all of the structural genes, regardless of the leader region they encode, are transcribed and translated at the same time, meaning that these strains are producing multiple bacteriocins simultaneously. Alternatively, it may be that only one or a very small number of the structural genes present in a specific operon are actually expressed and exported as functional bacteriocins, and this might be dictated by the type of leader. It could also be that the additional structural genes represent a reservoir of options that could be expressed under specific conditions or in a specific niche. Could each of these bacteriocins have a different host range that is adapted to those bacteria likely to be present in a specific niche? Lastly, it could be that the alternative structural genes must be 'swapped' by recombination into a single genetic location that is expressed and as such might provide a 'bacteriocin toolbox' to the strain facilitating rapid antimicrobial evolution. As the species under investigation were isolated from different sources and we have no evidence as to which, if any, bacteriocins are produced by each strain there is no obvious answer to these questions. Some of these possibilities could potentially be resolved by conducting forensic mass spectrometry analysis under different growth conditions to determine the extent of production of each putative bacteriocin under different growth conditions.

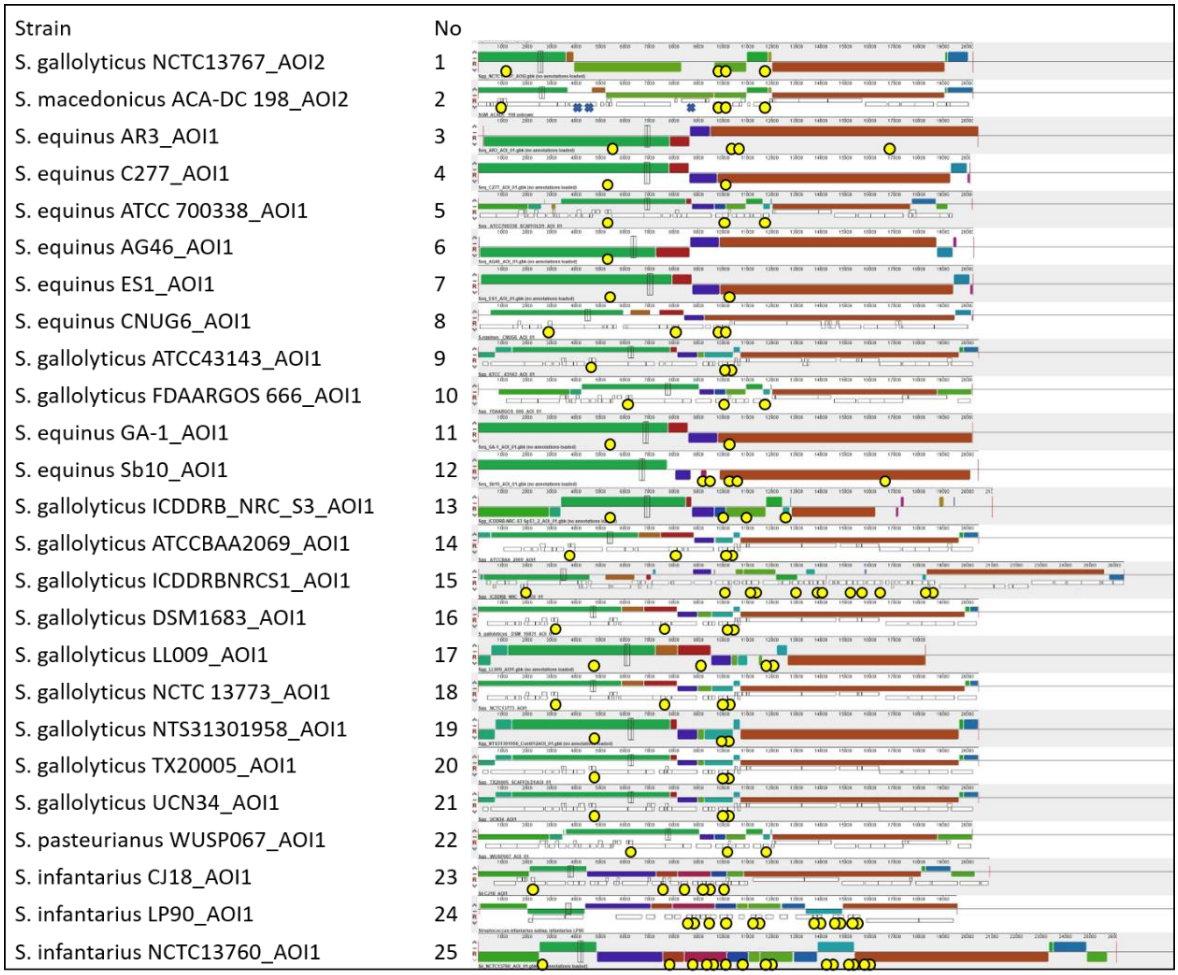

Figure 10. MAUVE analysis of Group Six. 25 AOI with 117 peptides marked with yellow dots.

The ability to produce an arsenal of bacteriocins with different target specificities could be expected to provide the strain with an advantage in different environmental niches. It would be more energetically efficient to have alternative structural genes rather than to encode entire alternative bacteriocin operons. It thus seems logical to assume that the additional structural genes must provide a competitive advantage to a strain in at least one of its 'normal' environments (other than in a test-tube). There should be a fitness cost associated with harbouring these extra genes and they could be expected to have degraded or be lost if they were not performing some useful function. Equally, if multiple genes are simply a result of random duplication events but have no function it would not be expected to find so many instances of this phenomenon in the strains we examined.

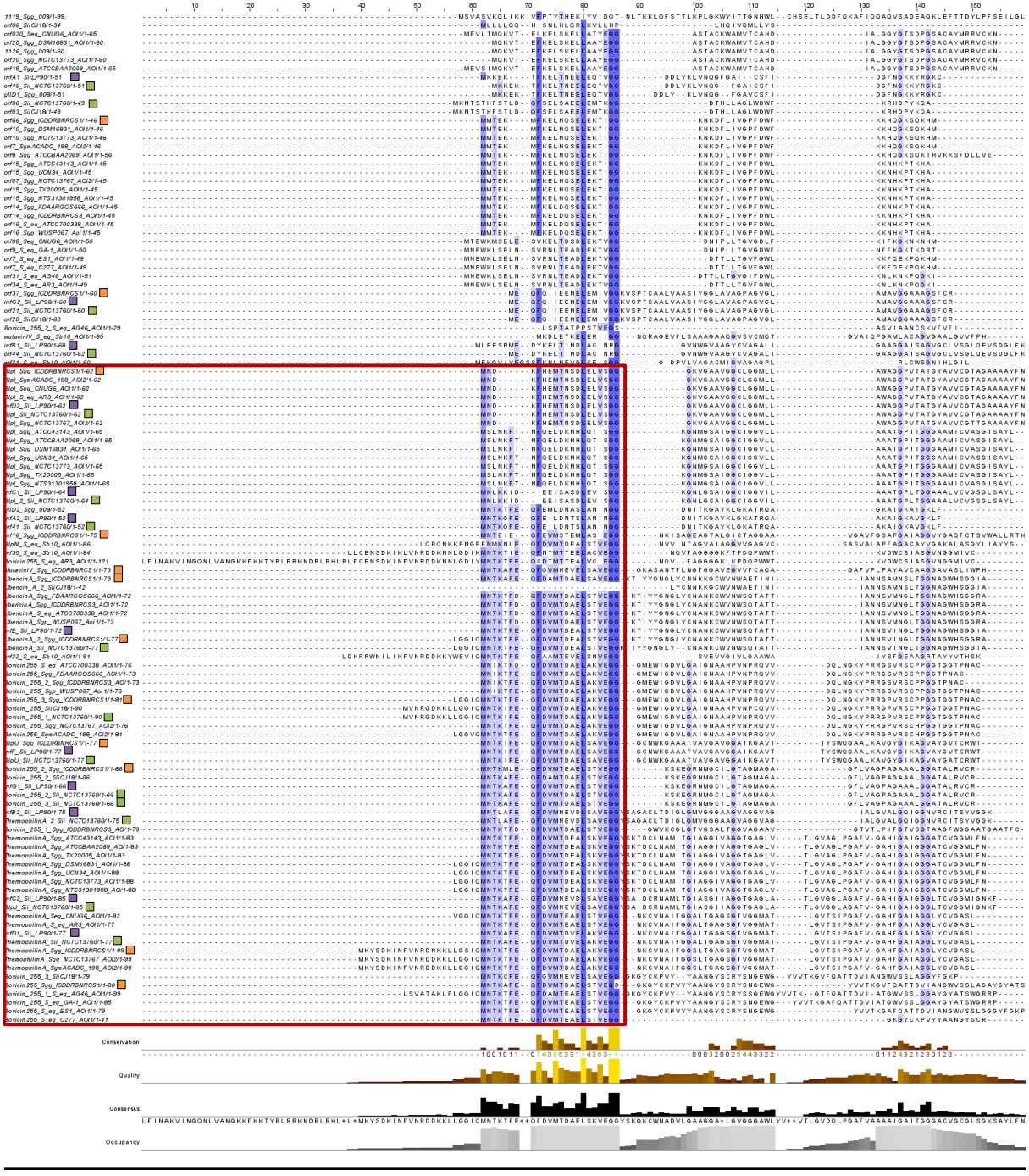

Figure 11. Multiple sequence alignment of Group Six peptides (n=117). Boxed is the most common leader sequence. Coloured squares indicate structural genes from the multi-peptide operons, 15 *S. infantarius* NCTC 13760 structural peptides in green, 12 *S. infantarius* LP90 structural peptides in purple, and 12 *S. gallolyticus* ICDDRB NRC S1 structural peptides in yellow.

AOI with no grouping

There are ten AOIs that did not fit into any of the larger groupings that are discussed here. There are some interesting features that are worth mentioning. *S. macedonicus* ACA DC 198 contains the complete operon for macedovicin [7]. *S. equinus* Sb09 also had two AOIs identified by Bagel4 that are potentially in fact one larger AOI. The beginning of AOI 9 and end of AOI 10 are the same region of the genome. Neither region contains an obvious structural gene, and none were discovered on further detailed analysis of the region. *S. equinus* FDAARGOS 251 also had two AOI identified by Bagel4 that overlap, the start of AOI 2 and end of AOI 3 (Figure 12).



Figure 12. Ten AOI which do not fit into any grouping

S. equinus CNU 77 23 has no structural gene and none was identified on further analysis of the region. The region does encode a LanKC complex, which is typically found in the class I lanthipeptides. *S. gallolyticus* DD02 also contains lanthipeptide genes encoding LanM (modification protein) and LanT (lanthionine transporter), but no obvious structural gene. *S. gallolyticus* TX20005 contains lanthipeptide genes and encodes a protein involved in cyclodehydration, but once again no structural gene could be identified.

S. gallolyticus LMG17956 encodes DysA2, a large heat-labile bacteriocin previously isolated from *S. dysgalactiae subsp. equisimilis* [22]. *S. macedonicus* ATCCBAA 2069 is predicted by Bagel4 to contain a two-component lantibiotic BHT previously identified in *S. ratti*, that is a variant of Smb isolated from *S. mutans* GS5 [23]. All three bacteriocins were aligned using MUSCLE and while *S. gallolyticus* ATCCBAA 2069 shares some similarity, it is not a close relative of these bacteriocins (Figure 13).

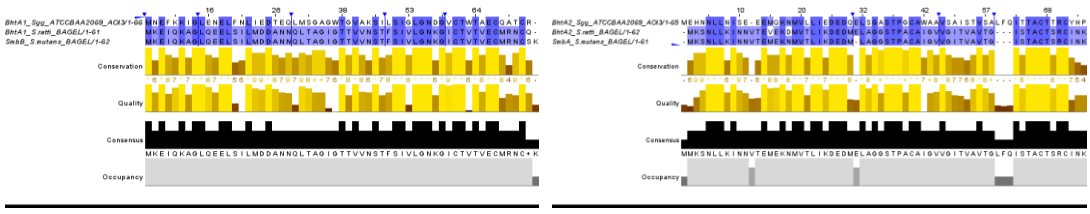

Figure 13. Multiple sequence alignment of the potential two-component lantibiotic. BhtA1 and BhtA2 from *S. gallolyticus* TX20005 and closest relative bacteriocins**.**

Peptide Comparison

All 166 structural genes were analysed, regardless of their MAUVE grouping, to determine if we could detect any association between individual structural genes and a particular niche or producing species. Only structural peptides predicted by Bagel4 were included for this part of the analysis. The leader sequence discussed in Group Six is boxed again here (Figure 14). There were 166 peptides predicted by Bagel4, but when identical peptides are made redundant, there are 82 unique peptide sequences. For clarity one name was chosen to represent each unique sequence (see Supplementary Material Table 1). The 82 peptides were aligned using the MUSCLE multiple sequence alignment tool, and a phylogram was created using Jalview. The isolation source of the strain of origin for each structural gene was also noted (Figure 15). There is no obvious pattern relating peptide sequence and isolation source. However, it must be acknowledged that the source is not always a reliable indicator of the natural niche of a strain, samples can be contaminated during collection and so it is important not to put too much weight on the recorded source. In addition, there was no available source for certain isolates.

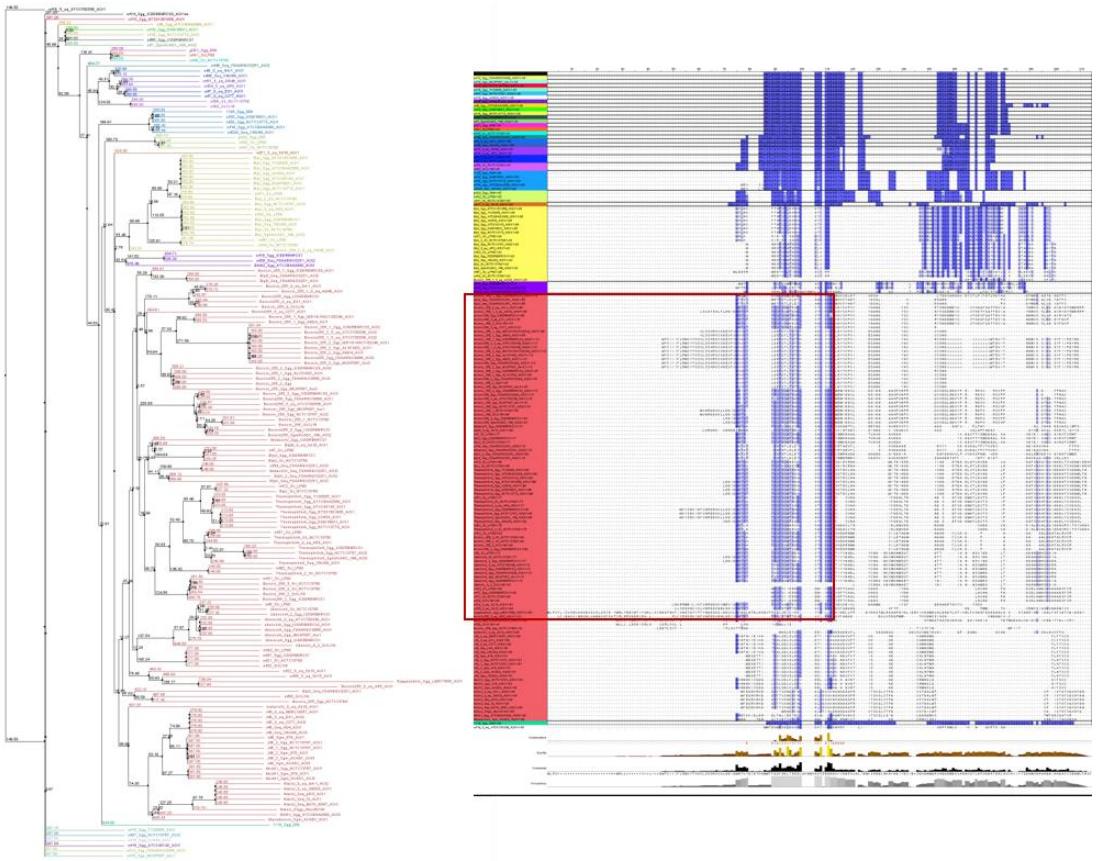

Figure 14. All 166 peptides. Phylogram of all 166 peptides and their multiple sequence alignment. Both are coloured by group.

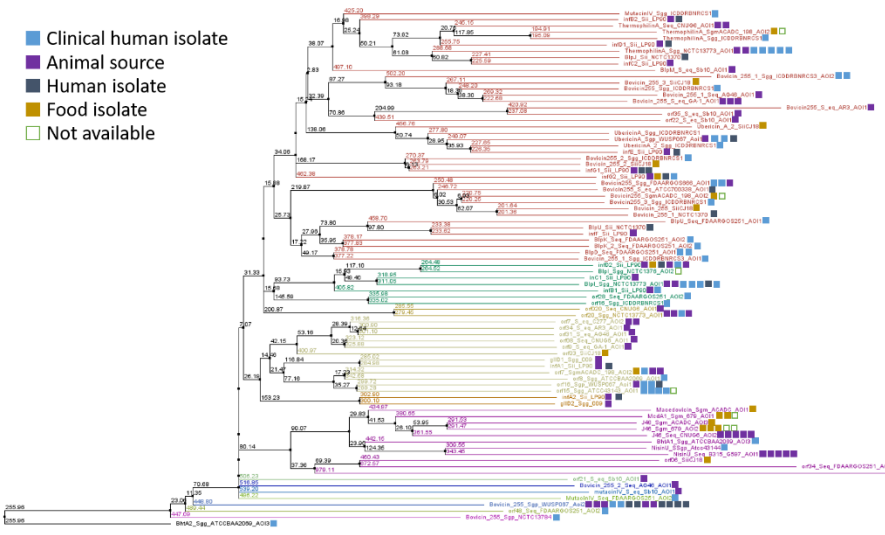

Figure 15. Phylogram of 82 peptides with isolation source indicated in the colour key, tree coloured by group using Jalview. Strains with multiple coloured boxes represent the sources of structural peptides that had been made redundant, see supplementary material.

We also mapped the parent species onto the same phylogram (Figure 16). Once again, no obvious pattern linked peptide sequences with encoding species. We next wanted to identify if the individual peptides within multi-peptide operons were present in clusters or distributed across the phylogram. The AOIs containing the highest numbers of structural peptides were marked and we found that the structural peptides are distributed across the entire phylogram with no obvious patterns.

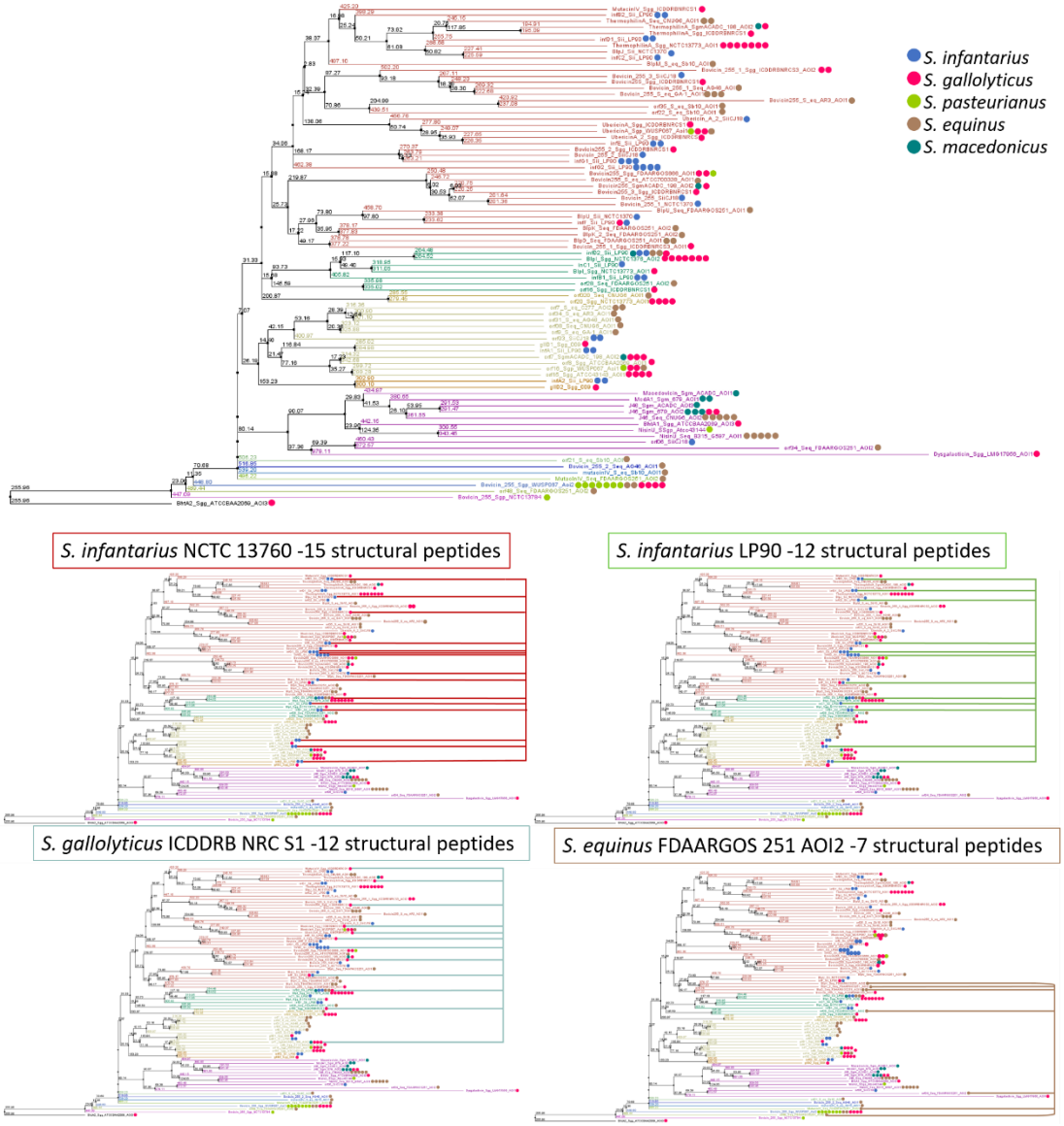

Figure 16. Phylogenetic tree of all 82 peptides predicted by Bagel4, with parent species marked. The multi-peptide operons are superimposed onto this phylogram.

S. equinus FDARRGOS 251 showed the highest degree of clustering with its seven structural peptides all mapping to the lower half of the phylogram. However, these peptides mostly share their leader regions which are common in three peptides, the fourth shows a high degree of homology and the other three peptides share little to no amino acid homology. There is little amino acid homology between the seven predicted mature peptides (Figure 17).

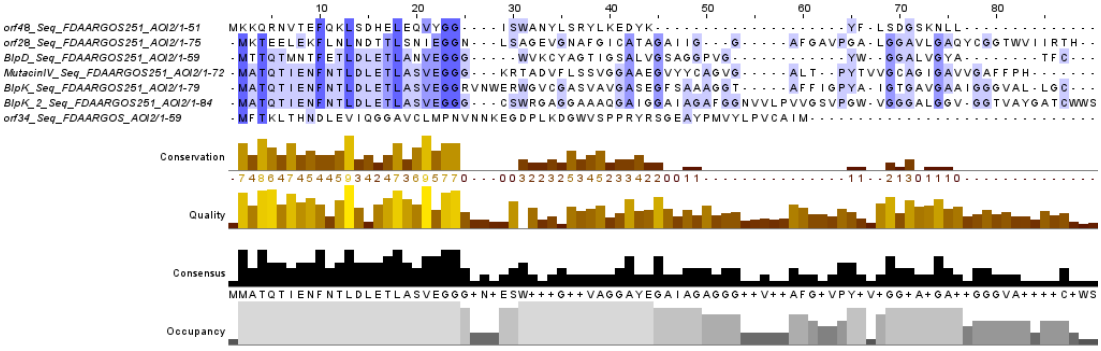

Figure 17. MSA of all seven predicted peptides from *S. equinus* FDARRGOS 251.

Conclusions

This study reveals that the genetic capacity to produce bacteriocins is widespread and diverse within the SBSEC complex. A number of strains contained multiple bacteriocin operons (AOIs identified by Bagel4) and many strains could encode multiple structural peptides. We could not find any pattern linking structural genes to either producing species or isolation source. It remains to be seen if each of these are produced, or whether they have activity against different target organisms. We have speculated as to the reasons for such a diversity and multiplicity of bacteriocin structural genes in the SBSEC pangenome. Further research is needed to investigate these hypotheses.

Materials and Methods

Bacteriocin gene cluster identification

Genome sequences to all available members of the SBSEC were run through the *in silico* bacteriocin prediction tool Bagel4. Bagel4 is a tool used to mine genomic DNA for bacteriocins, and provides a database of core peptides. Gene clusters are

identified using this core peptide database, and areas of interest (AOI) are potential bacteriocin operons within the genome. Where an AOI was detected the image and Genbank file were downloaded. These Genbank files were used as the input files for alignment using progressive MAUVE. The Artemis genome visualisation tool was used to view the bacteriocin clusters, and for further identification of putative structural genes where no bacteriocin genes were predicted in Bagel4. Artemis allows for browsing and annotation of genome sequences and provides greater detail than available in Bagel4. BlastP, Bagel4, and Bactibase were used for identification of putative bacteriocin structural genes. Bactibase is another database for bacteriocin structural peptides.

Predicted structural peptide comparison

Bacteriocin peptides were aligned using the multiple sequence alignment tool MUSCLE (Multiple Sequence Comparison by Log- Expectation) and viewed using Jalview. Phylogenetic trees were calculated by Jalview using the neighbour-joining method and coloured by group. Using the percent identity matrix, peptides showing 100% identity to another peptide were made redundant. There were 82 peptides remaining and the method was repeated.

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Supplementary Materials

Table 1. Combined peptides. Identical peptides combined following alignment of all 166 peptides using MUSCLE, Percent Identity matrix output was analysed and all 100% identical peptides combined. First name in list used for further analysis.

Peptides Combined
orf7_SgmACADC_198_AOI2/orf66_Sgg_ICDDRBNRCS1/orf10_Sgg_NCTC13773_AOI1/orf10_Sgg_DSM16831_AOI1
orf8_Sgg_ATCCBAA2069_AOI1
orf16_Sgp_WUSP067_Aoi1/orf14_Sgg_FDAARGOS666_AOI1/orf14_Sgg_ICDDRBNRCS3_AOI1/orf16_S_eq_ATCC700338_AOI1
orf15_Sgg_ATCC43143_AOI1/orf15_Sgg_UCN34_AOI1/orf15_Sgg_TX20005_AOI1/orf15_Sgg_NTS31301958_AOI1/orf07_Sgg_NCTC13767_AOI2
Macedovicin_Sgm_ACADC_AOI1
McdA1_Sgm_ACADC_AOI3/McdA1_Sgm_679_AOI1/McdA1_Sgg_NCTC1376_AOI1
J46_Sgm_ACADC_AOI3/J46_2_Sgm_ACADC_AOI3/J46_2_Sgm_679_AOI2/J46_1_Sgg_NCTC1376_AOI1/J46_2_Sgg_NCTC1376_AOI1
J46_Sgm_679_AOI2
J46_Seq_CNUG6_AOI2/J46_Seq_H24_AOI1/J46_S_eq_C277_AOI1/J46_S_eq_ES1_AOI2/J46_S_eq_NBRC12057_AOI1
BhtA1_Sgg_ATCCBAA2069_AOI3
NisinU_Sgp_Atcc43144
NisinU_Seq_B315_G597_AOI1/NisinU_Seq_SI_AOI1/NisinU_Seq_pR-5_AOI1/NisinU_S_eq_SN033_AOI1/NisinU_S_eq_GA-1_AOI2
orf48_Seq_FDAARGOS251_AOI2
Bovicin_255_Sgp_WUSP067_Aoi2/Bovicin_255_2_Sgp_WUSP067_Aoi2/Bovicin_255_2_Sgp/Bovicin255_Sgg_FDAARGOS666_AOI2/Bovicin255_2_Sgg_FDAARGOS666_AOI2/Bovicin_255_1_Sgg_AM24_AOI1/Bovicin_255_2_Sgg_AM24_AOI1/Bovicin_255_1_Sgp_AL101002_AOI1/Bovicin_255_2_Sgp_AL101002_AOI1/Bovicin_255_1_Sgp_MGYG-HGUT-00246_AOI1/Bovicin_255_2_Sgp_MGYG-HGUT-00246_AOI1/Bovicin255_1_S_eq_ATCC700338_AOI2/Bovicin255_2_S_eq_ATCC700338_AOI2
Bovicin_255_1_Sgg_ICDDRBNRCS3_AOI2/Bovicin_255_2_Sgg_ICDDRBNRCS3_AOI2
Ubericin_A_2_SiiCJ18
Bovicin255_S_eq_AR3_AOI1
orf35_S_eq_Sb10_AOI1
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BlpD_Seq_FDAARGOS251_AOI1/BlpD_Seq_FDAARGOS251_AOI2
BlpK_Seq_FDAARGOS251_AOI2
MutacinIV_Seq_FDAARGOS251_AOI2/orf34_Seq_FDAARGOS251_AOI2
BlpK_2_Seq_FDAARGOS251_AOI2
Dysgalacticin_Sgg_LMG17956_AOI1
Bovicin_255_2_S_eq_AG46_AOI1
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BlpI_Sgg_NCTC1376_AOI2
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gllD1_Sgg_009
orf03_SiiCJ18/orf06_Sii_NCTC1370
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orf34_S_eq_AR3_AOI1
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orf08_Seq_CNUG6_AOI1
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infD1_Sii_LP90/ThermophilinA_Sii_NCTC1370
ThermophilinA_Sgg_NCTC13773_AOI1/ThermophilinA_Sgg_DSM16831_AOI1/Thermoph ilinA_Sgg_ATCC43143_AOI1/ThermophilinA_Sgg_UCN34_AOI1/ThermophilinA_Sgg_ATC CBAA2069_AOI1/ThermophilinA_Sgg_TX20005_AOI1/ThermophilinA_Sgg_NTS31301958 _AOI1
BlpJ_Sii_NCTC1370
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gllD2_Sgg_009
Bovicin255_SgmACADC_198_AOI2/Bovicin_255_Sgg_NCTC1376_AOI2

Bovicin255_3_Sgg_ICDDRBNRCS1
Bovicin_255_SiiCJ18
Bovicin_255_1_NCTC1370
Bovicin255_Sgg_FDAARGOS666_AOI1/Bovicin_255_2_Sgg_ICDDRBNRCS3_AOI1/Bovicin_255_Sgp_WUSP067_Aoi1
Bovicin255_S_eq_ATCC700338_AOI1
BlpU_Sii_NCTC1370
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BlpM_S_eq_Sb10_AOI1
MutacinIV_Sgg_ICDDRBNRCS1
orf22_S_eq_Sb10_AOI1
Bovicin_255_1_Sgg_ICDDRBNRCS3_AOI1
Bovicin_255_3_SiiCJ18
Bovicin255_Sgg_ICDDRBNRCS1
Bovicin_255_1_S_eq_AG46_AOI1
Bovicin_255_S_eq_GA-1_AOI1/Bovicin255_S_eq_C277_AOI2/Bovicin255_S_eq_ES1_AOI1

Chapter 7.

General Discussion.

Lactic acid bacteria (LAB) produce a myriad of secondary metabolites which have many existing and potential applications across a range of industrial sectors, from food to pharmaceuticals to environmental [1]. The research described in this thesis was aimed at identifying important secondary metabolites with a focus on their use in functional foods; foods designed not only to fulfil nutritional needs but also to provide a health benefit to consumers. Three main secondary products were studied; exopolysaccharide (EPS), polyol and bacteriocin producing LAB were isolated and analysed for their potential use in fermented dairy products and as targeted antimicrobials. In this discussion, I will give further interpretation and significance of the results from the thesis, and present areas for potential improvement if given the opportunity to repeat, as well as potential future directions and oppurtunities for this research.

Exopolysaccharide producing bacteria

In Chapter 3, multiple agar-based testing methods were used for the isolation of EPS-producing bacteria. The inclusion of multiple screening methods was utilised to identify both ropy and mucous type EPS, as well as to potentially capture strains producing both types. Phenotypic screens such as the loop touch test are highly subjective and so a second testing method for ropy type EPS was also incorporated into the screening plan [2]. EPS production is a widespread trait found in many LAB genera and plays a vital role in such diverse areas as stress response, protection from antimicrobials and bacteriophages, and biofilm formation [3-5]. Screening for EPS production can be complicated by the fact that the production of EPS can be variable depending on the physiology of the bacterium under different conditions of growth, and the fact that multiple EPS operons can be present in a single strain that potentially can produce different types of EPS. EPS are also complex molecules that can be divided into homopolysaccharides (HoPS) and heteropolysaccharides (HePS) depending on their sugar composition, and are diverse in structure and size [6]. In milk fermentations, EPS contributes to texture, stability and sensory qualities of the final product. Yogurts containing EPS have been shown to have a firmer body, increased creaminess and a shiny surface, effects that are largely due to a decrease in syneresis and an increase in viscosity and stiffness [7]. The binding of water and

interactions with the protein network contribute to the beneficial rheological effects of EPS [8]. Capsular or non-ropy EPS can increase viscosity and reduce the effect of shearing which is desirable in a stirred yogurt product. There is a large variation in previous studies of EPS production in dairy fermentations by LAB that probably results from the use of a variety of experimental methods and strain specificity.

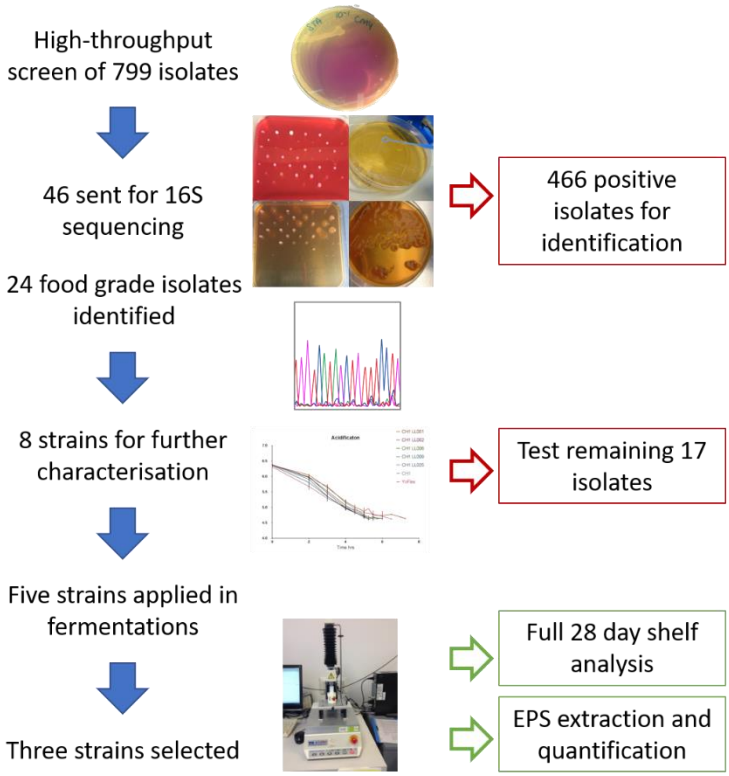

Figure 1. Chapter Three, Exopolysaccharide screen. Methods used in this thesis on the left, blue arrows. In red potential options for improvement, in green the future directions for strains identified are indicated.

Of the 799 isolates, 466 were positive in one or more of the EPS screening methods, and 46 of these EPS producers were identified by 16S rRNA sequencing. If possible, it would have been interesting to sequence the entire biobank of EPS producers. While 10% (46) of the positive isolates were identified by 16S rRNA sequencing, further sequencing of isolates would have given a better picture of the species producing EPS in our sources. Twenty-five of the identified strains represented potentially food-grade isolates and eight of these were selected for further analysis. Five strains showed reliable growth in skim milk at three temperatures (30, 37, and 42 °C) and were included as adjunct cultures together with

a commercial starter culture CH-1 in a small scale goat skim milk fermentation for texture analysis [9]. Goat milk yogurt was selected as it typically has a more porous microstructure and so has a weak gel network [10]. All five strains significantly improved the gel strength of the yogurt when compared to the control. All five were whole genome sequenced, following which two of the strains were excluded due to the presence of antibiotic resistance genes (*Lactobacillus johnsonii* LL006) or genes implicated in pathogenicity (*Streptococcus gallolyticus* LL009). Two *Lactobacillus plantarum* strains (strain LL001 isolated from bovine milk and strain LL025 isolated from dog saliva) and *Leuconostoc mesenteroides* (strain LL002 isolated from bovine milk), show significant promise as adjunct strains in dairy fermentations given their performance during fermentation and the quality of product they produced.

In conclusion, Chapter 3 confirmed that EPS production is prevalent among putative LAB from raw milk. The high number of EPS producing bacteria identified validated the decision to use multiple screening methods. Obvious next steps in this study would involve using the best three strains in a full-scale yogurt study, to ensure that the improved rheological characteristics would be maintained over a 28 day shelf-life and to measure the quantity of EPS produced [11]. It would also be interesting to characterise the specific EPS produced by each strain in terms of their structure and size, and to test for bioactivity [12, 13]. For example, characterisation of the EPS produced by these three strains would include testing to see if there are any potential health benefits. EPS has a variety of health benefits which include antioxidant and antitumor activity, cholesterol lowering effects, and immunomodulation [14-17]. Cultures for application in dairy fermentations are often freeze dried or spray dried, so evaluation of the culture following these processes would be desirable [18] this would include the survival of each strain following these processes, as well as the long term storage survival.

Mannitol producing bacteria

The objective of the research described in Chapter 4 was to identify strains capable of producing mannitol for application in fermented dairy products. We chose to use phenotypic screening methods that had proven successful in Chapter 3. The

use of PCR or gene-based screening methods could have been used since molecular methods have the advantage of being quick and relatively straight forward, but it should be acknowledged that the presence of a gene does not always guarantee that the strain will produce the associated metabolite. Primer design is also dependent on specific homology, and new pathways could not be identified, only known mechanisms can be identified using specific primers. While the presence of specific genes in a genome representing a particular biochemical pathway is a good indication that a strain can produce the end-product of that pathway, it may not be produced under the specific growth conditions found during a dairy fermentation. We chose to use a HPLC based method to identify if mannitol was produced, and quantify the amount of mannitol produced by isolates.

Mannitol production requires the input of fructose and/or glucose for production by bacteria [19]. We used MRS supplemented with fructose/glucose as our selection media, and during optimisation this was also seen to be the best sugar combination for the production of high levels of mannitol by our selected strains. This is perhaps to be expected, since these were the conditions used in the primary screening step. To improve this method, multiple sugar combinations could be included in the initial 96-well plate screen this likely would have increased the number of mannitol producing isolates identified. The overall aim of Chapter Four was to investigate if the selected strains are capable of producing mannitol in a skim milk fermentation. Sugar replacers are desirable in the industry for health-conscious consumers, and to have this sweetener produced naturally during fermentation would be an advantage over supplementation [20]. Yogurt aimed at children typically contain high amounts of sugar to appeal to children but this can contribute to problems such as dental caries and obesity, both of which are associated with increased sugar consumption [21]. While a yogurt is perceived as a healthy snack due to the umbrella effect, the sugar content is often an issue. *In situ* production of mannitol has several advantages over added sugar; it is low calorie, non-cariogenic and a natural sweetener.

Over 250 LAB were screened for mannitol production, we chose to include our putative *Leuconstoc*, *Streptococcus thermophilus*, and *Lactobacillus* for this

screen. According to the literature these are the species most likely to be capable of mannitol production [22, 23]. Ideally all 799 isolates would have been included, as we found that the selective media used was not only isolating the targeted species. This larger sample size would have almost certainly increased the number of mannitol producing bacteria. 25 strains were positive for mannitol in MRS supplemented with 6.5% fructose and 1% glucose (MRS F/G). We chose to isolate high mannitol producers in broth rather than using skim milk. While this would be a viable option we wanted to identify strains capable of producing high levels of mannitol to apply in milk fermentations. Five of these were categorised as high mannitol producers, in that they gave yields of over 1.5g/L.

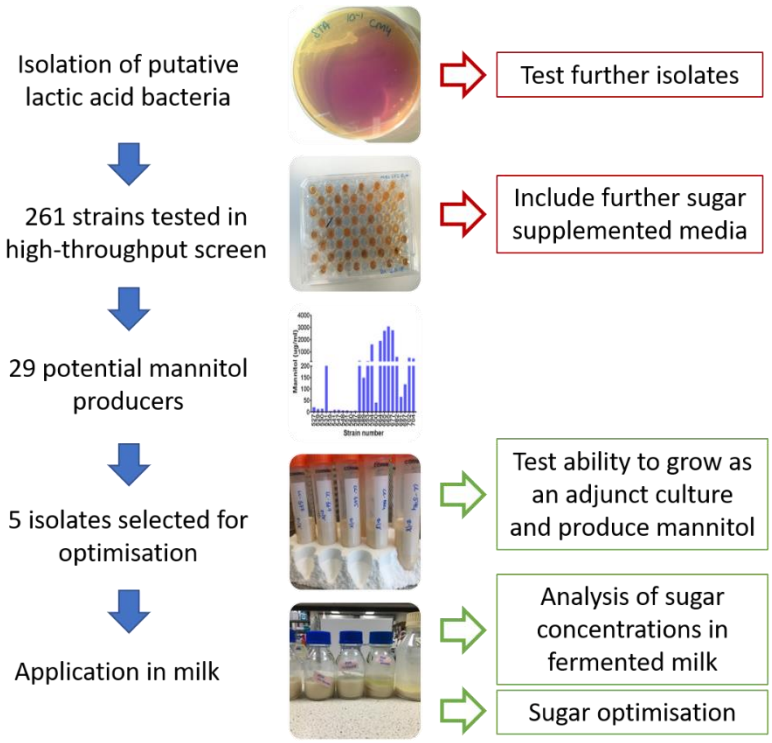

Figure 2. Chapter Four, Identification of polyol producing bacteria. Methods used on the left, in blue. In red the potential for improvement if given the opportunity to repeat. In green future directions for strains identified.

All five strains were tested in multiple sugar-supplemented media types to optimise mannitol production, *Weissella paramesenteroides* LL677 gave the highest mannitol production in MRS F/G (23.48g/L). This strain was chosen to conduct initial fermentations in skim milk supplemented with F/G, and 0.58g/L was detected following a 24 hr fermentation. This provides proof of principle that strains can

produce mannitol in a skim milk fermentation, which was the overall objective. It is obvious that significant optimisation is required before these strains could be applied in dairy fermentations. This could involve testing different concentrations and combinations of fructose and glucose, temperature optimisation and length of fermentation. Despite the low levels produced in this instance, this study shows there is potential for *in situ* production of mannitol in fermented dairy products. To continue this study, we could include further analysis of the fermented prototype for quantification of sugars at the beginning and end of the fermentation process, this would allow calculation of the conversion rate of fructose to mannitol. It would be of interest to see if mannitol is produced if the strain was added as an adjunct to a yogurt starter culture. *Lactococcus lactis* has been genetically engineered to produce mannitol and application of this in our prototype would be a potential future direction.

Phenotype based screens

The above chapters utilised phenotypic methods for the isolation of metabolite producers. The screening protocols used were successful to a degree, but were almost certainly biased by the parameters chosen when designing the screening protocol, including temperature, media and sample size. Chapter 3 illustrates that multiple methods can help to reduce bias in the screen for EPS production, but the use of multiple screening methods may not always be feasible. In Chapter 4 a polyol screening protocol was developed which allowed for high throughput growth of isolates using a 96-well plate growth method, with a single media aimed at identifying mannitol producers. The pitfalls of these methods are outlined above and potential for next steps for the strains identified. Another potential direction for these strains could be optimisation through genetic engineering or strain mutagenesis. There have been studies involving genetic engineering for mannitol production [24]. A lactate dehydrogenase inactivated *L. lactis* was engineered to co-express mannitol 1-phosphate dehydrogenase and mannitol 1-phosphatase, and achieved a 50 mol% glucose to mannitol conversion [25]. To replicate these methods for application in our dairy prototype would be an interesting approach to increase the mannitol production. Future goals for both the

EPS and polyol studies would be to conduct human studies for sensory analysis of the products.

Bacteriocin Identification

Chapter Five relied more heavily on molecular methods. This took advantage of the fact that the EPS producer *Streptococcus gallolyticus* LL009 described in Chapter 3 had been whole genome sequenced in order to identify the EPS genes. During the analysis of the genome a bacteriocin operon was identified, and this prompted a combination of *in silico* and laboratory-based experiments to characterise the bacteriocin that we subsequently named gallocin D. This a two-component bacteriocin with a narrow spectrum that includes activity against clinically-important vancomycin resistant enterococci (VRE) and *Streptococcus pneumoniae*. Gallocin D was applied to growing VRE cells at 10^5 cfu ml $^{-1}$ to represent a clinical infection, and no VRE could be recovered after 24hours, either when plated or transferred into fresh broth. When applied to growing cells at 10^8 cfu ml $^{-1}$ the VRE recovered after eight hours. Further work could include the application of this bacteriocin in urine, or synthetic urine, to test effectiveness against VRE *ex-vivo* for therapeutic application. This almost certainly could have been improved by the addition of higher levels of gallocin D. Based on the lack of gallocin D immunity genes in other available *S. gallolyticus* genomes, it is also likely that gallocin D could also be effective against pathogenic *S. gallolyticus.* It would be interesting to test gallocin D against additional VRE strains, and to determine the level, if any, at which resistance might occur. Gallocin A production by *S. gallolyticus* strains is associated with colorectal cancer and we speculate that gallocin D producing *S. gallolyticus* would occupy the same niche, however we also speculate that this gallocin D would target gallocin A-producing strains. Testing for cross immunity to gallocin A producing *S. gallolyticus* strains would allow for determining whether gallocin D could potentially have a protective effect preventing colonisation of other strains of *S. gallolyticus.*

Bacteriocin production by *S. gallolyticus* has been previously described; gallocin A is a two-peptide bacteriocin that has been reported to give *S. gallolyticus* a competitive advantage in conditions found in the gut of patients with colorectal

cancer [26]. Gallocin A shares little amino acid similarity with gallocin D. However, when the two operons were compared they were found to share high similarity apart from the structural genes and genes encoding the putative immunity proteins. On further analysis, gallocin D was found to be a variant of infantaricin A, which is part of the infantaricin ABCDEFG bacteriocin cluster produced by *S. infantarius* LP90 [27]. This operon encodes seven predicted bacteriocins, and when the operon is aligned to the gallocin D operon we found that the ABC transporters share 97% amino acid identity. We included additional strains of *S. infantarius* and *S. gallolyticus* to identify if there is further evidence of bacteriocin structural gene shuffling between species. The gallocin A operon was identified in four strains of *S. gallolyticus* with a high degree of homology, and similarly the infantaricin operon was identified in three further strains of *S. infantarius*. Gallocin D produced by *S. gallolyticus* LL009 was the only strain which appears to have acquired structural genes from another species while maintaining the operon found in *S. gallolyticus. S. gallolyticus* LL009 and *S. infantarius* LP90 were isolated from goat and water buffalo raw milk samples respectively, respectively. The high sequence identity between gallocin D and infantaricin A suggests that transfer of bacteriocin structural genes occurred between these strains or their recent ancestors, perhaps due to adaptive pressure in the microbe-rich environment of the gut.

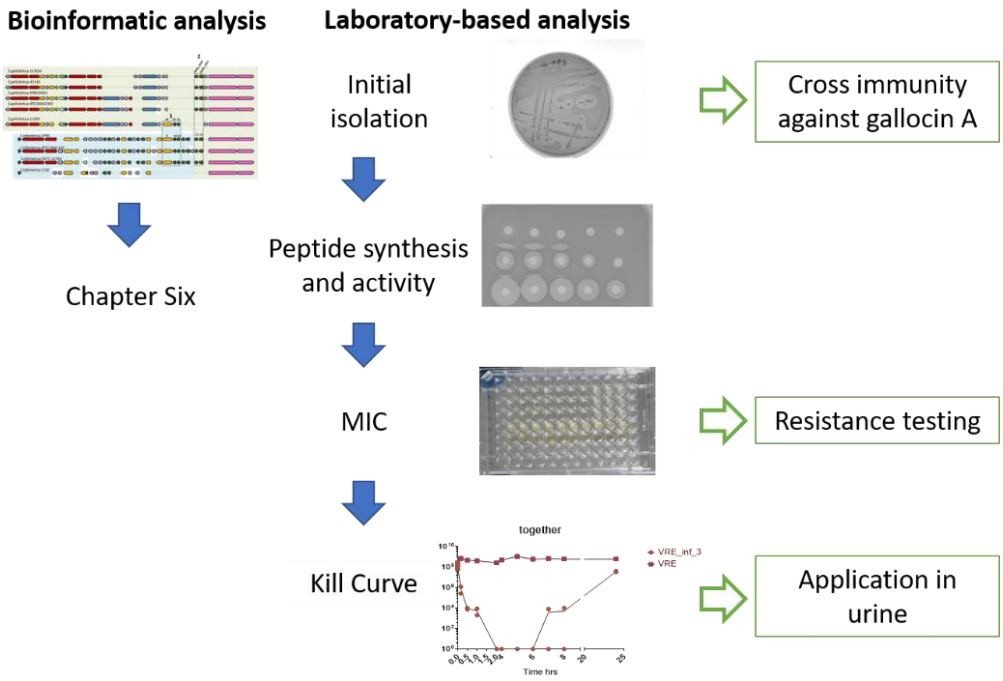

Figure 3; Identification of Gallocin D. Methods used on the left. In green future directions for application of gallocin D. Bioinformatic work was continued into Chapter Six.

In silico analysis of bacteriocin gene clusters in the SBSEC

Chapter Six extended these observations to further investigate the incidence and organisation of bacteriocin operons and structural genes within the *Streptococcus bovis/Streptococccus equinus* complex (SBSEC) [28]. All 105 sequenced genomes from the SBSEC were downloaded from NCBI and analysed using Bagel4 [29]. We included *S. gallolyticus* LL009 and *S. infantarius* LP90 bringing our total to 107 genomes; 28 *S. gallolyticus* including strain LL009, five *S. infantarius* including *S. infantarius* LP90, 44 *S. equinus*, seven *S. macedonicus*, nine *S. lutetiensis*, 12 *S. pasteurianus*, and two *S. alatolyticus*. From these we identified 67 areas of interest (AOI) containing 166 predicted structural genes. Twelve of the 67 AOI contained no predicted structural gene, and so the 166 structural genes were distributed across 55 AOI. Individual AOIs contain between two and 15 structural genes. The AOIs were aligned using MAUVE to identify similar operon structures and grouped into six operon types, with ten AOI remaining unclassified [30].

In brief, Group One are all related to the macedocin bacteriocin operon, Group Two contains sactipeptide operons, Group Three contains a nisin U operon with potential variants, Group Four is predicted to have partial structural peptides, Group Five encode Linear azol(in)e-containing peptides (LAP')s, and Group Six is the largest grouping and contains the bacteriocin operons discussed in Chapter Five. There was no obvious pattern linking AOI type and the source of each strain. However, it is important to note that source of isolates is not always a reliable indication of the natural niche of a strain.

Group Six is the largest and most diverse group consisting of 25 AOIs containing 117 structural genes. A more detailed analysis of Group Six provided further insights into the bacteriocinogenic capabilities of the SBSEC. Each of the 25 AOI contain at least one peptide with a similar leader sequence, which is present in known bacteriocins that are produced and active, confirming that this sequence can act as a functional leader. The mature peptides that follow this leader show high levels of diversity, and includes the bacteriocin identified in Chapter Five, gallocin D. We cannot be sure that the leader sequences in the other putative structural genes are functional. It is interesting to speculate why one operon would contain multiple peptides with the same leader (gallocin D leader) and some with alternative leader sequences. It may be that each of the encoded bacteriocins are transcribed, translated and exported regardless of their leader, so the strain produces multiple bacteriocins at one time. It could also be that only one or a small number of bacteriocins are produced and that this is dictated by which leader sequence is present. This would suggest the presence of a specific export machinery that only recognises the 'gallocin D' type leader. The other structural genes could represent a reservoir of bacteriocin encoding genes that are only expressed and exported when the strain is in a particular niche or under a certain set of conditions. Potentially the bacterium could 'switch' between which bacteriocins are produced depending on the circumstances of growth, potentially representing an 'antimicrobial toolbox' for the strain. This could suggest that each bacteriocin has a specific set of target organisms to allow a competitive advantage in a range of niches. Another possibility is that only

the structural gene in one genetic location is transcribed and this gene can be ‘swapped’ for another structural gene through recombination (Figure 4).

Future work would include conducting mass spectrometry on those strains that contain multiple structural genes following growth under a range of conditions to determine which of the above theories, if any, is correct. This would prove difficult if the strain produces different bacteriocins depending on the niche it is growing in, as growth in a test tube in a lab is not representative of a natural niche. The production of multiple bacteriocins in some capacity seems to be more feasible as there is a fitness cost associated with harbouring extra genes. If these genes are not transcribed, they would be expected to have undergone degradation or complete loss. If they are present due to duplication, we would have expected to see multiple copies of structural genes which share high homology, but these strains compare multiple diverse structural genes. As gallocin D was readily synthesized, these peptides could be synthesized to determine their target species. This would allow us to determine if they do in fact have different host ranges and so are likely to be utilised under different circumstances.

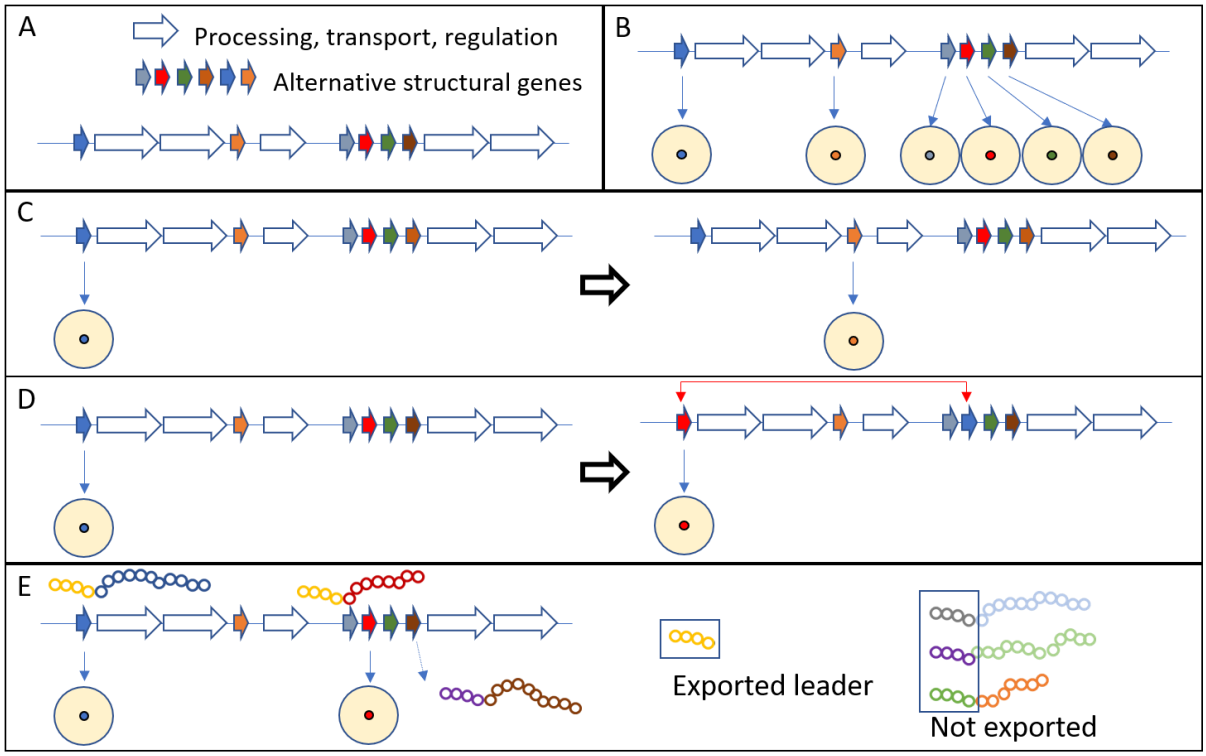

Figure 4. Possible explanation of operons with multiple structural genes. A) Hypothetical operon structure. B) All structural genes, regardless of the leader region they encode, are transcribed and translated at the same time. C) It could also be that the additional structural genes represent a reservoir of options that could be expressed under specific conditions or in a specific niche. D) Structural genes can be 'swapped' by recombination into a single genetic location that is expressed. E) One or small number of the structural genes present are actually expressed and exported as functional bacteriocins, this might be dictated by the type of leader.

Conclusion

Overall, this thesis sought to screen for bacteria capable of producing valuable secondary metabolites, using both traditional and sequence-based methods. Both approaches were successful. While much remains to be done in terms of process optimisation and strain improvement before these strains are ready for deployment in food fermentations, it has been established that strains sourced from diverse environments can lead to the identification of strains with diverse functionality and a variety of potential applications in food, functional foods and even medicine.

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