Harnessing Antimicrobial Bacteriocin Production of Probiotic Bacteria

Thesis submitted in fulfilment of the requirements of the degree of Master of Philosophy, Cardiff University.

2020

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Acknowledgements

My thanks, and acknowledgements go to:

My academic supervisor's Dr. Katja Hill and Prof. David Thomas for their excellent supervision, guidance, and encouragement throughout this research project.

My industry supervisor's Dr. Sue Plummer and Dr. Alison Jack, for their expertise, enthusiasm, support, and for offering me this great opportunity. Dr. Alison Jack has been my biggest inspiration in my scientific career as she is always so positive and passionate about research which has been a real driving force, but not only that, she has always been there come rain or shine.

The ATG members at Cardiff University for their motivation and help in our weekly meetings, especially Dr. Joana Bradunaite and Dr. Lydia Powell who tutored me on how to use the confocal microscope and carry out comstat analysis.

The technical staff at Dental School, with a special thanks to Dr. Sarah Bamford who was always around to help and chat to. As well as Dafydd Thomas, who shared some of his consumables with me and for his advice.

To the post-grads and post-docs, I met during my time at Cardiff University, who made it so enjoyable. A special thanks to the members of office 5F.06 (Elen, Katrina, Megan, Emma, Jordanna and Richard), who really supported me day-to-day with any enquiries I had and who's lunchtime chats and laughs I will never forget (especially Caterpillar Cake Club). Special shout out to Jordanna who is like a big sister to me and never fails in making me laugh until I cry.

Everyone at Cultech Ltd past and present, who I feel so lucky to work with as they are all so helpful and supportive that it feels like a work family. Especially Niall, Josh, Rhiannon, and Elizabeth.

To all my friends at home and the biomed lot for being so understanding and always being there for me and telling me ‘you can do this!’. Special thank you to my best friend of many years, Holly, she is my biggest supporter, has always been a shoulder I can lean on, and kept me sane throughout.

Lastly, to my family, for their continuous and unparalleled love and support. As well as my parents, for giving me the opportunities and experiences that made me who I am and always telling me they are proud of me no matter what.

This research was funded by a Knowledge Economy Skills Scholarships (KESS 2) grant which is a pan-Wales higher level skills initiative led by Bangor University on behalf of the HE sector in Wales. It is part funded by the Welsh Government’s European Social Fund (ESF) convergence programme for West Wales and the Valleys.

Abstract

Bacteriocins are ribosomally-synthesised antimicrobial peptides, and their production by probiotic bacteria is extremely common, particularly in *Lactobacillus* species. This can be advantageous, giving them a competitive advantage over other microorganisms. However, in the production of probiotics, they can affect recovery of all the microorganisms present, thereby, causing an underestimation of the total viable bacterial numbers in commercial products. To ensure regulatory compliance, a significant additional microbial inoculum is added to these products to compensate for poor recovery. This is a huge commercial economic burden and prompted exploration into which strains were responsible for the observed antibacterial effects. In this study, neutralised cell-free supernatants (CFSs) of lactic acid bacteria (LAB), *L. salivarius* CUL61 and *L. paracasei* CUL08 were evaluated for potential antimicrobial inhibition against themselves, other probiotic bacteria (*L. acidophilus* CUL60 and CUL21, *Bifidobacterium bifidum* CUL20 and *Bifidobacterium animalis subsp. Lactis* CUL34) and against two commercial probiotic consortia (Lab4 and Lab4b). CFSs were obtained from bacterial cultures grown in MRS broth under planktonic or biofilm growth conditions and harvested at different time-points (5, 24, 48, and 72 h) to determine the optimum growth conditions for bacteriocin production. Harvested CFSs were neutralised, filter-sterilised and concentrated (lyophilised) prior to use. Antibacterial susceptibility testing of CFSs (single-strength or 50% concentrated) was performed using growth curves, well-diffusion, microbroth dilution and biofilm formation assays. *L. salivarius* CUL61 and *L. paracasei* CUL08 showed antimicrobial activity against themselves, other probiotic bacteria, and commercial probiotic consortia. Antimicrobial activity was highest during late exponential/early stationary phase (24, 48, 72 h), and greater in liquid-medium (growth curve, microbroth dilution and biofilm formation assays) than in solid media (well-diffusion assays). Unsurprisingly, the genomes of both bacteria were found to harbour genes encoding bacteriocins. This study confirms long-observed findings during commercial production. A greater understanding of putative bacteriocin synthesised by LAB could help to improve and optimise production of mixed-population probiotics.

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List of Abbreviations

Adenosine triphosphate	ATP
Antimicrobial peptides/proteins	AMP
Antimicrobial resistance	AMR
Biofilm	B
Cell-free supernatant(s)	CFS(s)
Colony forming units	CFU
Colony forming units per millilitre	CFU ml^{-1}
Concentrated cell-free supernatant	cCFS
Confocal laser scanning microscope	CLSM
de Man-Rogosa Sharpe	MRS
Deoxyribonucleic acid	DNA
Double strength de Man-Rogosa Sharpe	dsMRS
Extracellular polymeric substance	EPS
The European Food Safety Authority	EFSA
Food and Agriculture Organisation	FAO
Gastrointestinal tract	GIT
Generally Recognized As Safe	GRAS
Hour	h
High Performance Liquid Chromatography	HPLC

International Dairy Foundation	IDF
The International Organization for Standardization	ISO
Irritable bowel syndrome	IBS
Kilo Daltons	kDa
Lactic acid bacteria	LAB
Linear azol(in)e-containing peptides	LAPs
Mannose-phosphotransferase system	Man-PTS
Minimum inhibitory concentration	MIC
Minute	min
Number of replicates	*N*
National Collection of Industrial Food and Marine Bacteria	NCIMB
Not applicable	N/A
Not determined	ND
Optical density	OD
Open reading frame(s)	ORF(s)
Overnight	O/N
Phosphate buffered saline	PBS
Planktonic	P
Polymerase chain reaction	PCR
Qualified Presumption of Safety	QPS

Quorum sensing	QS
Randomly amplified polymorphic DNA-PCR	RAPD-PCR
16S Ribosomal ribonucleic acid	16S rRNA
Ribosomally synthesized and post translationally modified peptides	RiPPs
Ribonucleic acid	RNA
Single strength cell-free supernatant	ssCFS
Single strength de Man-Rogosa Sharpe	ssMRS
Sodium Hydroxide	NaOH
Standard deviation	SD
Standard error of the mean	SEM
Scanning Electron Microscopy	SEM
Viable but non-culturable	VBNC
Whole genome sequencing	WGS
World Health Organisation	WHO
Weight per volume	w/v
Volume per volume	v/v

Chapter One

Introduction

1. Introduction

1.1 Probiotics

Probiotics are described by the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2002). Many commercially available probiotics tend to be strains of Lactobacilli and Bifidobacteria. Probiotics travel through the colon, interacting with the cells throughout the gut, as well as immune cells, nutrients, and bacterial cells to directly and indirectly, deliver benefits. The health benefits of probiotics have been demonstrated, with probiotics shown to decrease irritable bowel syndrome (IBS) symptoms, prevent high blood cholesterol levels and improve the metabolic status of type 2 diabetes (Ford et al 2014; Wang et al 2018; Raygan et al 2018). In a randomised controlled trial involving pregnant women and infants aged 0-6 months, those who were given a multi-strain probiotic were 57% less likely to develop atopic eczema and 44% less likely to develop an allergic reaction to common allergens (pollen, egg, cow's milk and house dust mite) (Allen et al 2014). It has been documented that all of the aforementioned health conditions share a similarity that has been characterised by low bacterial diversity of the gut microbiota, otherwise known as dysbiosis, when compared to the microbiota of healthy humans as reviewed by Sanders et al (2018). There is increasing evidence linking dysbiosis with human disease (Carding et al 2015). Using live biotherapeutics like probiotics, can aid by re-enriching the gut with increased numbers and species of gut bacteria. Although no standard cell count level is recognised to guarantee a positive health effect, the manipulation of microbial ecosystems using live biotherapeutics, provides a new potential preventative and treatment option for many illnesses.

1.1.1 Probiotic product specifications and their label claims

The beneficial effects of probiotics have been shown to be strain specific (Campana et al 2017). For example, *Lactobacillus reuteri* can benefit those who are lactose intolerant by aiding in lactose digestion (Ojetti et al 2010). For this reason, most commercially available probiotics are usually sold as multi-

strain products as they are found attribute synergistic effects and are more effective than single strains in competing with pathogens in the gut due to a broader spectrum of activity against a wider variety (**Table 1**) (Drago et al 1997).

Table 1. Bacterial strains found in commercially available multi-strain probiotic products

Brand name (product)	**Genus, species and strain**	**Formulation**
Bioflorin	*Enterococcus faecium* SF 68	powder, sachets
Bio K+ (25 billion)	*Lactobacillus acidophilus* CL1285 *Lactobacillus casei* Lbc80r *Lactobacillus rhamnosus* CLR2	fermented drink, capsules
Proven Probiotics (Fit for school)	*Lactobacillus acidophilus* CUL60 *Lactobacillus acidophilus* CUL21 *Bifidobacterium bifidum* CUL20 *Bifidobacterium animalis subsp. lactis* CUL 34	chewable tablet, powder sticks
OptiBac (For every day)	*Lactobacillus rhamnosus* Rosell-11 *Lactobacillus acidophilus* Rosell-52 *Lactococcus lactis* Rosell-1058 *Bifidobacterium longum* Rosell-175 *Bifidobacterium breve* Rosell-70 *Bifidobacterium bifidum* Rosell-71	capsule
Garden of Life (Once daily women's)	*Lactobacillus acidophilus snr* *Lactobacillus plantarum snr* *Lactobacillus casei snr* *Lactobacillus paracasei snr* *Lactobacillus bulgaricus snr* *Lactobacillus brevis snr* *Lactobacillus reuteri snr* *Lactobacillus salivarius snr* *Lactobacillus fermentum snr* *Lactobacillus gasseri snr* *Lactobacillus rhamnosus snr* *Bifidobacterium lactis snr* *Bifidobacterium bifidum snr* *Bifidobacterium infantis snr* *Bifidobacterium breve snr* *Bifidobacterium longum snr*	capsule

ATCC, American Type Culture Collection, snr, strain not reported

As consumers become more familiar with probiotics, there is more demand for probiotic products with numerous strains of bacteria (Stanton et al 2001). It is thought that with more variety in bacterial strains, the better the product at re-enriching the gut. Although, a meta-analysis found no significant difference between single-strain and multi-strain probiotic products, as it is not the number of strains alone that is used to prevent or treat specific diseases but the specific strains chosen (McFarland 2020).

Commonly, bacterial strains for probiotic production are identified using phenotypic tests alongside genotypic methods that incorporate the use of polymerase chain reaction (PCR), which generates large amounts of DNA amplified from a few copies. There are articles demonstrating the effective use of RAPD-PCR to recognise probiotic lactobacillus strains by using indiscriminate primers to randomly amplify small pieces of DNA using PCR. The products from the PCR are then separated by gel electrophoresis and a fingerprint is constructed (Seseña et al 2004; Schillinger et al 2003; Torriani et al 1999; Du Plessis and Dicks 1995). Schillinger et al (2003) used this method to attempt to identify *Lactobacillus* species from yoghurt by using primers for the *L. casei*, *L. acidophilus*, *L. gasseri/johnsonii* groups. They were able to assign 19 out of 20 strains unambiguously to the species *L. acidophilus*, *L. johnsonii*, *L. crispatus*, *L. casei* and *L. paracasei* (Schillinger et al 2003). However, some researchers have found that RAPD has low discriminatory powers with some ready-to-use primer kits developed for screening bacterial communities (Plengvidhya et al 2004). This indicates that this approach should be used in combination with an additional method to improve confidence in identifications such as using DNA sequencing using 16S ribosomal ribonucleic acid amplification (16S rRNA) (Chandok et al 2014). For identifying *Lactobacillus* species, this would involve sequencing a 750-base pair fragment of the 16S gene but this technique can be time-consuming (Tannock 1999).

With technological advances, a new method that has become the choice for many food commercial industries is whole genome sequencing (WGS) (Brown et al 2019). This technique can provide the complete DNA sequence of a microorganism's genome which can provide significant information of metabolic properties. A study conducted by the Steele research group and collaborators used WGS on *L. helveticus* CNRZ32 and discovered

12 genes that encode for proteolytic enzymes which play a critical role in cheese ripening (Christensen et al 1999; Broadbent and Steele 2007). In the future, WGS could become the default for identification of bacterial strains due to the fact that costs will reduce with increased demand. The information found using these methods is used to provide the consumer, and regulatory authorities, specific details on the probiotic product, including precise details on the bacterial contents such as genus, species and strain, which must all be stated on the label. This is just one of several recommended criteria to be displayed on the label of the product according to The Working Group for Probiotics and Prebiotics (Kolaček et al 2017) (**Table 2**).

Table 2. Recommended information for display on probiotic products.

Recommended Probiotic Product Label
1. Genus, species, and strain designation for each microorganism in the product
2. Minimum viable quantity of each probiotic strain at the end of the stated shelf-life*
3. The suggested dose **
4. Storage conditions
5. Company contact details for consumer information

*Described as colony forming units (CFU). **dose must provide the user with an effective quantity of probiotics that demonstrates a benefit, paired with supportive scientific evidence held by the manufacturer. Information from (FAO/WHO 2002; (IPA/CRN) 2017).

Equally important, is the minimum viable number of each probiotic strain at the end of the product shelf-life, this is also required to be stated on the label and is generally expressed as colony-forming units (CFU) per dose (**Table 2**). A study by Weese and Martin (2011), investigated the quality of 25 commercially available veterinary probiotic products. For this, the labels were inspected for the stated bacterial number and the authors determined if there were any discrepancies between this number and actual bacterial counts by

enumerating the bacterial contents of the products (Weese and Martin 2011). They found that only 8% (2/25) of the probiotic products studied had a viable bacterial count that met, or exceeded, what was stated on the label. This is not the first study to have discovered probiotic supplements having mis-leading label claims (Drago et al 2010; Morovic et al 2016; Ullah et al 2019). These findings highlight concerns for those promoting the efficacy of probiotics and raises important questions about the quality control procedures used. Bacterial count failures like these should be detected and solved by the manufacturer prior to these products going to market to uphold and maintain standards for their consumers (Hamilton-Miller and Shah 2002).

1.1.2 Quantifying probiotics

Quality control checks on probiotic supplements consist of enumerating bacterial numbers by standard plate counts, which is the gold-standard method to quantify viable bacteria in probiotic manufacturing (ISO/IDF 2010). This method is advantageous in that it is simple and easy to perform, and only viable bacterial cells will multiply into visible colonies on an agar plate. It is assumed that one colony arises from a single cell and therefore the colonies are counted and from this the CFU can be calculated (**Figure 1**). One of the main disadvantages of standard plate counts is that they are time-consuming due to the incubation period needed and this time can vary considerably depending on the bacterial strain and species being tested. Additionally, Jackson et al (2019) highlighted that the reproducibility and repeatability of plate counts can be influenced by operator-generated bias, equipment, and inter- and intra-laboratory variability. As well as this, the method used to preserve cultures such as cryopreservation or lyophilisation (a common practice in commercial probiotic production) can affect the viability of microbes in downstream processing (Bircher et al 2018). Even the initial reconstitution of lyophilised probiotic samples (reviving the bacteria), can be affected by parameters such as pH, buffering capacity, osmolarity, homogenisation intensity and formation of aggregates which can all significantly affect the resulting plate counts (Jackson et al 2019).

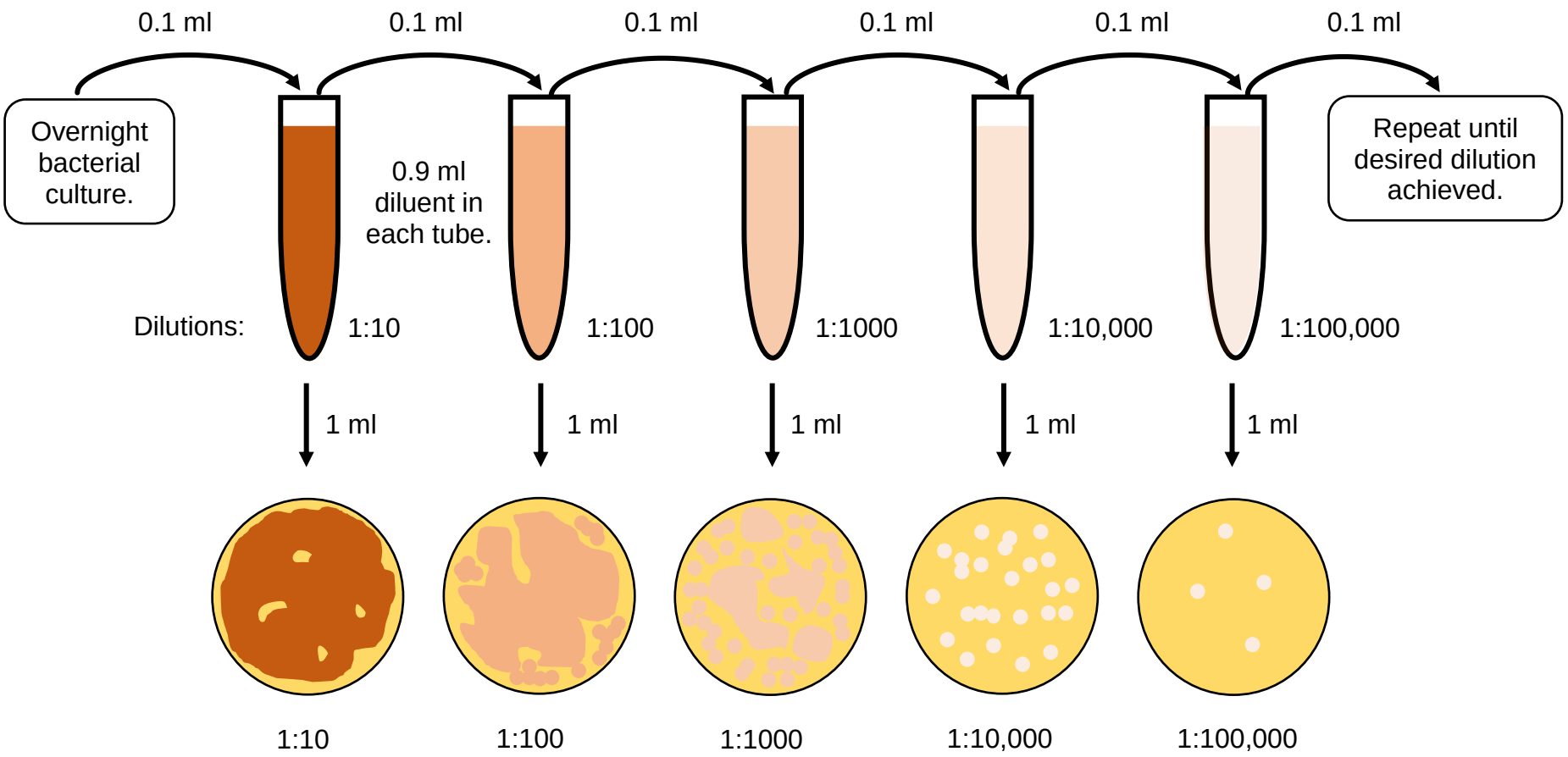


Figure 1. Standard plate count protocol for enumeration of bacteria in quality control procedures to determine total viable counts. (Figure adapted from Bauman 2009).

More recently, The International Organization for Standardization (ISO) and International Dairy Federation (IDF) have approved a new method to enumerate bacteria using flow cytometry (ISO/IDF 2015), which uses fluorescent nucleic acid stains such as the BacLight Live/Dead bacterial viability stains called SYTO 9 and propidium iodide (Gao et al 2018). Importantly, this method can differentiate between viable, non-viable and viable but non-culturable (VBNC) cells; the latter being dormant while maintaining low cellular and metabolic activity (Ramamurthy et al 2014). Detecting those VBNC cells (that would otherwise be undetected using plate count techniques), could reduce the need for manufacturers to overcompensate with overages (the process of including surplus bacterial raw material in excess of the label claim to compensate for possible losses), which comes at a considerable economic cost. Additionally, it can significantly reduce sample processing time while still maintaining high accuracy. A recent study has shown that this method was able to count approximately 0.3×10^{10} CFU/ml^{-1} more viable bacteria than plate counts (Ou et al 2017). Although this technique is gaining prominence, plate counts remain the gold-standard among customers and regulatory boards as no expensive flow cytometry equipment is required and the ease of training required.

1.1.3 Viability

A viable bacterial cell is defined as having metabolic activity, an intact cell membrane and retaining the capability for reproducing over a generally accepted time frame (Barer and Harwood 1999; Oliver 2005). Plate count techniques on an agar plate that supply the bacteria with all essential nutrients for its growth, are able to account for reproducibility (Kumar and Ghosh 2019).

It has been established that many diverse bacteria from various environments reach a VBNC state by different stressors which highlights that this state is a common mechanism adopted by bacteria to survive in these adverse conditions (Oliver 2000). Researchers have found a gene that is expressed whilst bacteria are in VBNC state called the *RpoS* gene, which regulates this state and is also responsible for their survival in stationary phase (Li et al 2014; Zhao et al 2017). Cells that are VBNC have been found to have

low metabolic activity, retain apparent cell integrity, decrease the transport of nutrients, appear dwarfed, have greater autolytic capacity than exponentially growing cells and have apparent capacity to regain *in vivo* culturability (Lahinen et al 2005; Sebastiano and Guglielmino 2012; Fakruddin et al 2013; Castellani et al 2013). Previously, bacteria in this VBNC state have resuscitated due to the presence of favourable conditions, this has been connected to foodborne outbreaks with pathogenic bacteria (Makino et al 2000; Asakura et al 2002). Resuscitating VBNC cells in probiotics to retrieve a true count of bacterial cells under culture-dependent techniques would require a number of different growth conditions, even among strains of the same species and this can be tedious and time-consuming, especially if the probiotic product contained multiple different species and strains (Pinto et al 2011). This highlights why methods such as flow cytometry are better suited for this role to achieve more accurate information on the functionality of probiotic products.

Dormancy is a state in which bacterial cells are like VBNC where they are active but nonculturable, but also they can be inactive but ultimately culturable, indicating their metabolism is slowed or completely stopped (Lahtinen et al 2005; Blinkova et al 2014). Metabolic activity has been found to be primarily respiration and fermentation (Porter et al 1995). There are two classifications of a dormant condition, endogenous or exogenous, and this is dependent on the reasons for the transition from viable to dormant (Blinkova et al 2014). Endogenous dormancy has been described as being part of the natural growth cycle and is triggered by the internal process of a bacterial cell in response to stressful habitat influences (Blinkova et al 2014). The effect of environmental elements including freezing (cryobiosis), dehydrating (anhydrobiosis), or both of these together, and the retention of osmotically active ingredients (osmobiosis) such as salts and carbohydrates like sucrose, are associated with exogenous dormancy (Blinkova et al 2012). According to Lahtinen et al (2005), this has predominantly been researched in pathogenic microorganisms but looking at their study into *Bifidobacterium* probiotic strains (*B. longum* 2C (DSM 14579), *B. longum* 46 (DSM 14583), *B. lactis* Bb012) indicated that a subpopulation of *B. longum* 2C (DSM 14579) entered a dormant or an active but non culturable stage when the LIVE/DEAD viability

count strategy used continued to vary even after two months of cells losing their ability to be cultured on agar plates (Lahtinen et al 2005).

The absence of colonies in culture-based methods using sufficient sample quantities and favourable growth conditions, is assumed to mean cell death has occurred (Keer and Birch et al 2003). There are limitations to this characterisation because as previously mentioned, bacterial cells can enter into a VBNC state or simply cannot be grown in a laboratory such as the human pathogen that causes syphilis, *Treponema pallidum* as reviewed by Radolf et al (2016). A beneficial method to determine if a cell has died, is by discovering if the membrane integrity has been lost. A way to look at the membrane integrity is using the red-fluorescent nuclear and chromosome stain, propidium iodide, which is only permeant to cells that are dead or have lost membrane integrity (Steinkamp et al 1999). As well as this, Trevors (2012) suggested additional experimentation of the gene expression immediately following cell death to determine if the cell is still alive metabolically active or not (Trevors 2012).

1.1.4 Functionality of probiotics

According to FAO/WHO, any probiotic product destined for human consumption needs to have its efficacy and effect supported by scientific evidence gathered from human trials (FAO/WHO 2002; Allen et al 2014). Alongside this, *in vitro* tests are also needed to confirm that the chosen probiotic bacteria in the product meets a range of further selection criteria (**Figure 2**). Probiotic bacteria are selected based on their ability to withstand conditions found in specific environments within the human body. For example, the low pH and high concentrations of conjugated and unconjugated bile acids found in the human digestive system can cause probiotic bacteria to become ineffective (Ruiz et al 2013). When grown with bile salts, the salts decrease the phospholipid content and the ratio of saturated to unsaturated fatty acids of the bacterial cells in comparison to the control, thereby disrupting their proton motive force and leading to cell death (Šušković et al 2000). The bile salt hydrolase gene has been found in the genomes of > 90% of *L. reuteri* and species within the *L. plantarum* and *L. delbrueckii* groups which increases

their likelihood of their survival in the GIT as reviewed by Filippis et al (2020). Consequently, it is essential that the bacteria chosen by probiotic manufacturers can resist such conditions, to ensure that their product reaches the colon in a viable state for its beneficial effects to occur (**Figure 2**).

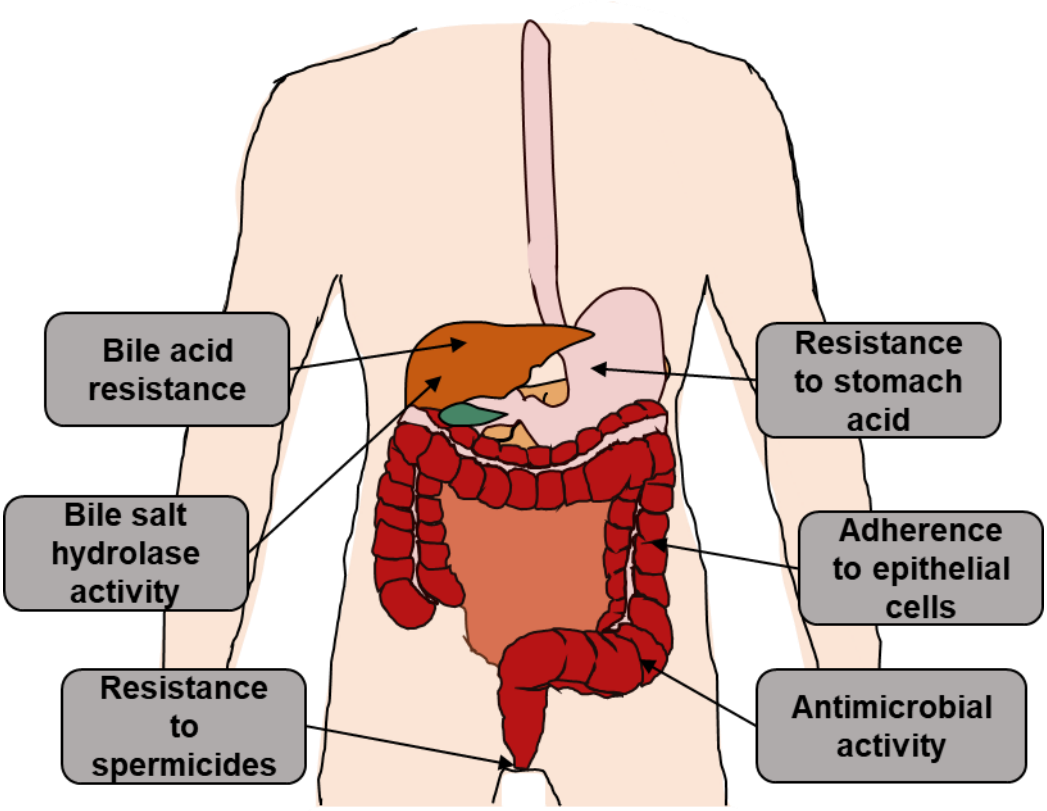


Figure 2. Functionality criteria used to select probiotic bacteria for use in probiotic products. *In vitro* tests must be carried out to prove the functionality of the probiotic species and must correlate with *in vivo* performance when consumed by humans. (Information provided by FAO/WHO 2002).

Probiotic microorganisms need to be non-pathogenic. Therefore, any virulence factors need to be carefully evaluated, so that *in vivo* transfer of antimicrobial resistance (AMR) is prevented. Some lactobacilli thrive in the gut during a pathogenic bacterial infection as several species are intrinsically resistant to vancomycin (Campedelli et al 2018). Intrinsic resistance is where bacteria are able to resist activity of a therapeutic by changing their inherent structural or functional characteristics, this type of resistance is natural.

Whereas acquired resistance is a result of a chromosomal mutation(s), or acquired by horizontal gene transfer via transformation, transduction or conjugation. In lactobacilli, the terminal D-alanine residue is replaced by D-lactate or D-serine in the muramyl pentapeptide, which prohibits vancomycin from binding and inhibiting cell wall synthesis (Delcour et al 1999). Although, there is a low-risk of transfer in intrinsic resistance or acquired resistance due to chromosomal mutation(s), information on the antibiotic resistance phenotypes of probiotic bacteria is significant, as it could be informative in healthcare settings for the treatment in rare cases of *Lactobacillus*-related bacteraemia (Gueimonde et al 2013; Salminen et al 2004). Moreover, vancomycin resistance in these species can be beneficial to the host, as some antibiotics are known to kill pathogenic bacteria as well as beneficial bacteria in the gut, which can make the gut vulnerable to opportunistic pathogens and lead to antibiotic-associated diarrhoea, commonly caused by *Clostridium difficile* (McFarland 2008; Neut et al 2017).

Adherence to human epithelial cells, as well as to mucus produced by these cells, is fundamental for bacterial colonisation of the gut and their beneficial effects to occur (Juntunen et al 2001). Probiotic products aimed at women for vaginal administration necessitate that the bacterial species be resistant to spermicides (such as *Lactobacillus rhamnosus* GR-1), to ensure that the probiotic bacteria are not killed-off before colonisation can begin (Reid and Bruce 2003).

1.2 Lactic acid bacteria (LAB)

LAB belong to the phylum *Firmicutes* and include the genera *Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weisella* (Walter 2008). LAB are a group of Gram-positive, rod- and coccus-shaped bacteria that on fermentation of carbohydrates, produce a variety of antimicrobial substances such as organic acids, hydrogen peroxide, ammonia, diacetyl (2,3-butanedione), enzymes and bacteriocins (Deegan et al 2006). LAB are facultative anaerobes, meaning that they can not only grow anaerobically, but also in the presence of oxygen, because they contain

enzymes such as superoxide dismutase and peroxidase which are capable of detoxifying peroxide radicals to hydrogen peroxide (Tomusiak-Plebanek et al 2018). Generally, LAB can be found naturally in dairy products such as milk and yoghurt, as well as being present in humans, specifically in the mouth, gastrointestinal tract (GIT; ileum and colon) and vagina (Ravel et al 2011; Shimada et al 2015; Berstad et al 2016).

1.2.2 Function of LAB in the gastrointestinal tract (GIT)

1.2.2.1 Inhibiting pathogens

LAB are able to inhibit pathogens that are found in the GIT. They do this by both secreting inhibitory substances and out-competing with them for available resources in the gut of the host (**Table 3**).

Table 3. Inhibitory compounds produced by LAB that can kill pathogenic bacteria found in the gastrointestinal tract (GIT).

Product	Example of LAB species	How it is synthesised	Mode of action	Reference
Lactic acid	*Lactobacillus plantarum*	End-product of homolactic fermentation	Targets cell wall, cytoplasmic membrane, replication, and protein synthesis	(Russo et al 2017)
Diacetyl	*Lactococcus lactis ssp. lactis*	By-product from citrate uptake and metabolism	Interferes with arginine-binding protein in Gram-negative bacteria	(Jay et al 2005; García-Quintáns et al 2008)
Hydrogen peroxide	*Lactobacillus crispatus*	Produced in the presence of oxygen	Use of superoxide anion chain reaction enhancing toxic oxidation damage	(Vallor et al 2001; Mitchell et al 2015)
Carbon dioxide	*Lactobacillus brevis*	By-product of heterolactic fermentation	Creates an anaerobic environment that is unfavourable to aerobic bacteria, preventing growth	(Singh 2018)
Reuterin	*Lactobacillus reuteri*	Product of glycerol metabolism by *L. reuteri*	Inhibits DNA replication	(Helal et al 2016)

A previous study found that *Lactobacillus acidophilus* and *Lactobacillus fermentum* were able to inhibit the growth of several pathogens including *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Bacillus cereus* (Tsai et al 2005).

Lactic acid is the primary by-product by homofermentative LAB, lowers the pH of the surrounding microenvironment, generating adverse conditions for many pathogenic bacteria to survive as reviewed by Pessione (2021). This is advantageous for humans because, while the lactic acid inhibits invading harmful bacteria, it does not affect the epithelial cells found in the digestive tract (Allen and Flemström 2005). Organic acids like lactic acid, kill pathogenic bacteria by targeting the cell wall and membrane, as well as their metabolic functions (Surendran et al 2017; Zhitnitsky et al 2018) (**Table 3**). These characteristics make LAB attractive for their role in probiotic products.

1.3 Bacteriocins produced by LAB

Bacteriocin production by probiotic bacteria is very common, particularly in strains of *Lactobacillus* species. They are a diverse group of low molecular weight, ribosomally synthesised antimicrobial peptides (AMPs) produced by a diverse number of bacteria. Bacteriocins produced by a bacterium that inhibit bacteria of the same species are said to have a narrow spectrum of activity. These types of AMPs are advantageous to the bacterium producing them, as they give them a competitive edge in their respective ecological niches. An example is pyocin S5, which was described to inhibit several *P. aeruginosa* strains but was unable to exhibit antibacterial activity against *E. coli* and *S. aureus* (Ling et al 2010). In contrast, when bacteriocins are capable of inhibiting bacteria of a different genera, they show broad spectrum activity. *Lactococcus garvieae* produces a bacteriocin called garvicin KS that could inhibit 19 species of Gram-positive bacteria (Chi and Holo 2018) Broad-spectrum bacteriocins are advantageous in the field of food preservation and food spoilage to inhibit prevalent pathogenic species found in these niches (Kaboré et al 2013). Bacteria able to produce bacteriocins are protected by immunity peptides located within the same operon as the bacteriocin synthesis genes (Oppegård et al 2007; Todorov 2009).

1.3.2 Classification of bacteriocins of Gram-positive bacteria

There has been much discussion in the literature with regards to what criteria should be used to classify the bacteriocins of Gram-positive bacteria. Over the years, this has led to inconsistencies and uncertainty in their classification. Bacteriocins have been classified based on their method of excretion, activity, mode of action or lantibiotic amino acid structure. An undefined classification system has led to several bacteriocins confusingly being classified into multiple classes as reviewed by Ramu et al (2015). However, the use of several different classification schemes is impractical and the need for a universally adopted classification scheme of bacteriocins produced by LAB is therefore vital. Previously, bacteriocins were classified into 12 different classes based on their structural similarity, phylogenetic relationships, and consensus protein motif sequences (Zouhir et al 2010). More recently (Alvarez-Sieiro et al 2016), classified bacteriocins into 3 classes based on the biochemical and genetic characteristics of their affiliates (**Figure 3**).

1.3.2.1 Class I bacteriocins

This class of bacteriocins are post-translationally modified peptides which are small (<10kDa) and heat-stable.

Class Ia bacteriocins undergo extensive modification by enzymes during biosynthesis, yielding mature peptides with unique structures and uncommon amino acids (Alvarez-Sieiro et al 2016). For example, a group of class I bacteriocins known as lanthipeptides are produced after modification with thioether amino acids known as lanthionine and/or β-methyllanthionine, as well as dehydroalanine, dehydrobutyrine, and/or D-alanine (Alkhalili and Canbäck 2018). These bacteriocins can also be referred to as lantibiotics. The most well-known lantibiotic (discovered in 1928) is nisin, which is produced by the bacterium *Lactococcus lactis* and is used against food-borne pathogens (Shin et al 2016).

Class Ib bacteriocins have a structure whereby the N- and C-terminal residues are covalently linked by a peptide bond depicting them as circular. Hence, they have become known as head-to-tail cyclised or circular

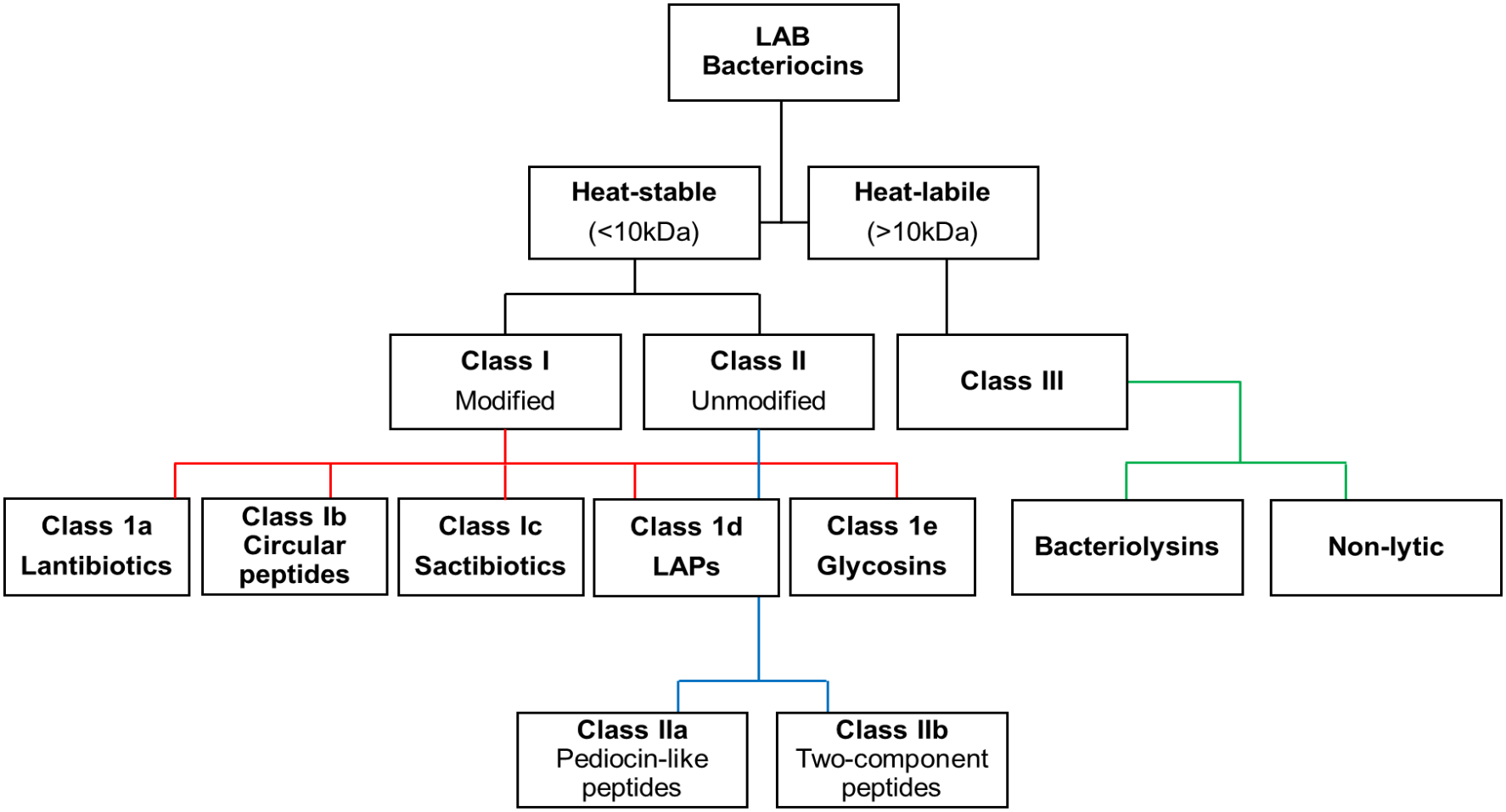


Figure 3. Classification scheme proposed for bacteriocins synthesised by lactic acid bacteria (LAB). (Figure adapted from Alvarez-Sieiro 2016).

bacteriocins (Perez et al 2018). Recently, a circular bacteriocin was purified from *L. plantarum* N1326 (found in olives) known as plantaricyclin A (Borrero et al 2017).

Class Ic bacteriocins can be characterised by their intramolecular thioether bond between the sulphur-to- α-carbon linkages of a Cys residue (Arnison et al 2013). It is a relatively new group of bacteriocins and are known as sactipeptides (sactibiotics when they have antimicrobial activity). The first sactipeptide discovered was subtilosin A, in *Bacillus subtilis* 168 in 1985 (Babasaki et al 1985), however it's complete structure was not resolved until years later (Kawulka et al 2003). This type of bacteriocin has yet to be identified in LAB, although, efforts to identify these sactipeptide bacteriocins in LAB have been described by Rea et al (2010). This involved inspecting the DNA of LAB for homologue genes of the two-component sactibiotic 'thuricin CD'. Thuricin CD was discovered in *Bacillus thuringiensis* (Rea et al 2010).

Class Id are bacteriocins that are linear azol(in)e-containing peptides (LAPs). They undergo extensive post-translational modification which converts cysteine, serine, and threonine structures into various combinations of heterocyclic rings of thiazole, xazole, and methyloxazole, respectively (Collins et al 2017). Streptolysin S is an example of a class Id bacteriocin that undergoes modification by the enzyme complex SagBCD (Lee et al 2017). It has been possible to find homologs of this complex in *L. crispatus* DSM 20584 and *L. intestinalis* DSM 6629 (Collins et al 2017).

Glycosins are **class Ie bacteriocins** and their name is derived from the glycosylated residue(s) on the cysteine residues contained in the structure (Arnison et al 2013). This type of bacteriocin has been isolated from *L. plantarum* KW30 (Stepper et al 2011).

1.3.2.2 Class II bacteriocins

This class encompasses peptides that are also small and heat-stable like class I bacteriocins, but they are unmodified. This class is divided into two subclasses class IIa and class IIb.

Class IIa bacteriocins, are pediocin-like peptides, and are named after the first of their kind found in 1987, pediocin PA-1 (Gonzalez and Kunka 1987).

Pediocin-like class IIa bacteriocins are also referred to as listeria-active peptides due to their role in inhibiting *Listeria* species. The structure of these peptides normally consists of two distinct domains connected by a flexible hinge. The first domain is the hydrophilic N-terminal domain, which contains a disulphide bridge and the conserved amino acid sequence of YGNGV/L (V or L can change). The second amphiphilic C-terminal domain is less conserved but has a role in target cell specificity (Uteng et al 2003; Kjos et al 2011). In the C-terminal domain most class IIa bacteriocins contain a single disulphide bond between cysteine residues, but in some cases, a few can present with an extra disulphide bond which not only stabilises the 3D structure of this domain, but also gives the species an increased competitive edge due to higher potency at higher temperatures and a wider antimicrobial spectrum (Richard et al 2006).

Class IIb bacteriocins are like class IIa bacteriocins in that they are cationic and contain amphiphilic and/or hydrophilic regions. However, they contain two-components that although separate, usually must work synergistically and in equal amounts to have an optimal antimicrobial effect (Zacharof and Lovitt 2012). For instance, lactococcin G was the first two-peptide bacteriocin to be isolated (Nissen-Meyer et al 1992). It was found that individually the two peptides, termed alpha, and beta, had no antimicrobial activity, but when they were combined in a 1:1 ratio, a potent antimicrobial effect was observed (Moll et al 1996). The genes of the two peptides are found next to each other on the same operon, alongside a gene encoding for a single immunity peptide, highlighting the fact that they act as one entity (Franz et al 2002; Oppegård et al 2007).

1.3.2.3 Class III bacteriocins

Class III bacteriocins are large (>30 kDa) molecular weight proteins which can be destroyed or deactivated by heat. Helvetican J is a well-known class III bacteriolysin produced by the strain *Lactobacillus helveticus* 481 (Joerger and Klaenhammer 1990). Like enterolysin A, it possesses a structure that links a threonine-proline-rich region to the N-terminal and to a putative C-terminal

recognition domain (Nilsen et al 2003). These bacteriocins work by degrading the peptidoglycan layer of the cell wall.

Lastly, there are also non-lytic bacteriocins, which unlike bacteriolysins, exhibit their effect without causing concomitant cell lysis. Instead they inhibit DNA and protein biosynthesis, as is the case of caseicin harvested from *L. casei* (Müller and Radler 1993).

1.3.3 LAB bacteriocins and their mechanism of action

The structure of the cell wall in Gram-positive bacteria allows AMPs to be secreted directly into the external environment, whereas those secreted by Gram-negative bacteria such as *E. coli*, cannot be extracellularly secreted and instead remain in the periplasm (Schneewind and Missiakas 2012). Once secreted, the effects of bacteriocins can be either bactericidal (causing cell death) or bacteriostatic (inhibiting cell growth).

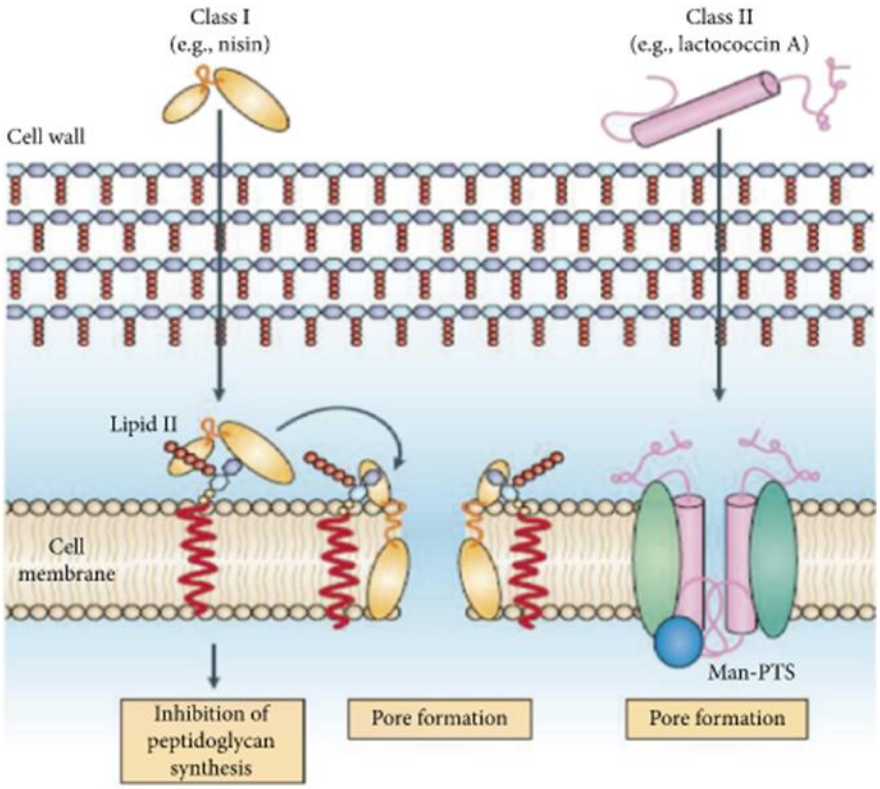


Figure 4. Mechanism of action of class I and class II bacteriocins against Gram-positive bacteria (Cotter 2012).

The mode of action of LAB bacteriocins has been investigated and it has been concluded that most involve cell-envelope-associated mechanisms. Some bacteriocins interact with negatively-charged phospholipids in the cytoplasmic membrane via electrostatic interactions. This causes damage to the membrane and subsequent pore formation in the membrane occurs. Consequently, charged elements and small molecules (potassium and phosphate ions, ATP, and amino acids) leak out, causing a decline in the proton motive force, ultimately leading to cell death (Parada et al 2007). Other bacteriocins, such as those in class I and II, inhibit cell-wall synthesis by binding to Lipid II (Linnett and Strominger 1973), while pore-forming bacteriocins that cause cell death are those that target the mannose phosphotransferase system (Man-PTS) (Diep et al 2007).

1.3.4 Applications in food preservation

It is the AMPs secreted by LAB, that are attractive for exploitation as bio-preservatives in foods such as vegetables, dairy products, and meats (Singh 2018). A bio-preservative is the use of a microorganism and/or its natural products to prevent spoilage and prolong shelf-life, to maintain food safety and quality (Yost 2014). Bacteriocins produced by LAB are used to inhibit the presence and prevent the proliferation of foodborne pathogenic bacteria. In addition, these metabolites have the added benefit of being 'Generally Recognised as Safe' (GRAS) by the FDA for human consumption and strains such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and certain strains of *Streptococcus* have been approved as 'Qualified Presumption of Safety' (QPS) by the European Food Safety Authority (EFSA) (EFSA 2007). As bacteriocins are found naturally and can target strain-specific species such as those that cause food spoilage (e.g. *Bacillus cereus* and *Clostridium perfringens*) it is likely that they are preferred by consumers over chemical preservatives, particularly due to the association of such chemicals with behavioural problems (Buka et al 2011). As well as the fact that bacteriocins from LAB are GRAS, they are also effective at low concentration and importantly, they do not change the nutritional or flavour properties of the food

they are used to treat and they are thought to be easily degraded by proteases in the GIT (Sarika et al 2019).

The application of nisin as a bio-preservative involves inhibiting the growth of *Clostridium botulinum* spores and toxin formation in pasteurised cheese (FDA 1988). Similarly, pediocins produced by the LAB species *Pediococcus*, have also proven to be useful as bio-preservatives (Cintas et al 2002). Indeed, pediocins have shown to be more successful than nisin against the food-borne pathogens *S. aureus* and *L. monocytogenes* and have also been found to enhance the shelf-life of raw buffalo milk (Verma et al 2017). Furthermore, lacticins, which are only produced by specific strains of *L. lactis,* have also been shown to inhibit food spoilage bacteria (Martínez-Cuesta et al 2010). So far, only two such bacteriocins have been isolated; lacticin 3147 (class IIb) and lacticin 481 (class I; (Piard et al 1992; McAuliffe et al 1998)). Lacticin 3147 was found to prevent late blowing in cheese by *Clostridia*, while lacticin 481 could kill *L. fermentum* within 240 minutes (min) (Martínez-Cuesta et al 2010).

1.4 Harnessing and detecting bacteriocins produced by LAB

1.4.2 Optimisation and storage

Bacteriocin production is highly dependent on the growth medium, culture conditions (pH, temperature, incubation, atmosphere, and time) and the type of microbial strain used (**Table 4**). In a study by Turgis et al (2016), they looked at the influence of temperature (25, 30, 37 and 45°C), pH (from 4-11), carbon sources (glucose, lactose, galactose, sucrose, fructose and maltose) and various nitrogen sources (casein peptone E1, kosher casein peptone, tryptone plus, tryptone N1, casein-meat peptone E2, meat peptone N2, gelatine peptone N2, gelatine peptone N3, malt extract R2, yeast extract, malt extract R3, pea peptone, wheat peptone E430, soy peptone AM41, wheat peptone E1, vegetable peptone, vegetable peptone ET1, soy peptone A3SC and casein peptone plus) on the growth of *L. lactis* MM19 for the optimal production of nisin. It was found that nisin production could be increased by 6.7 times if *L. lactis* (MM19) was grown in MRS broth which contains glucose as a carbon source, an initial pH of 9 for the broth, temperature of 30°C and the addition of

wheat peptone E430 (Turgis et al 2016) (**Table 4**). In this way, carrying out preliminary experiments like this where the growth conditions are varied for bacteriocin-producing strains can be very useful to have optimal production of bacteriocins for down-stream production in food and clinical industries. Bacteriocins such as pediocins, possess many advantageous properties, one being that they can retain their activity, even after several months of being refrigerated, frozen or lyophilised (Bari et al 2005).

Table 4. Growth conditions used for optimal bacteriocin expression in LAB

Bacteriocin	Producer organism	Growth conditions	Reference
Nisin	*Lactobacillus lactis* MM19	24 h, MRS broth (pH 9), wheat peptone E430, 30°C,	Turgis et al (2016)
LiN333	*Lactobacillus casei*	48 h, Tomato base broth (TBB), 37°C	Ullah et al (2017)
HW01	*Pediococcus acidilactici* HW01	20 h, MRS broth, 30°C	Ahn et al (2017)
Crispacin A	*Lactobacillus crispatus* JCM 2009	16 h, MRS broth, 37°C	Tahara and Kanatani (1997)
Enterocin NKR-5-3 A, B, C and D.	*Enterococcus faecium* NKR-5-3	22 h, M17 broth, 30°C	Ishbashi et al (2012)

1.4.3 Bacterial biofilms

Bacteria can form multi-cellular and complex communities on moist or wet surfaces which are enclosed in a matrix of extracellular polymeric substances (EPS) called a biofilm (Bisht and Wakeman 2019). A common example of a biofilm is dental plaque. In this community, whether it be mono- or multi-species microorganisms, bacteria are involved in complex social interactions that can influence gene regulation using a quorum sensing (QS) signalling system (Nadell et al 2009). QS is strictly controlled by cell density and occurs based on the ability of a signal molecule to diffuse and attach to cognate receptors on the membranes of neighbouring bacterial cells (Li and Tian

2012). Due to this, QS activation is strongly associated with bacterial growth type (**Fig. 5A**).

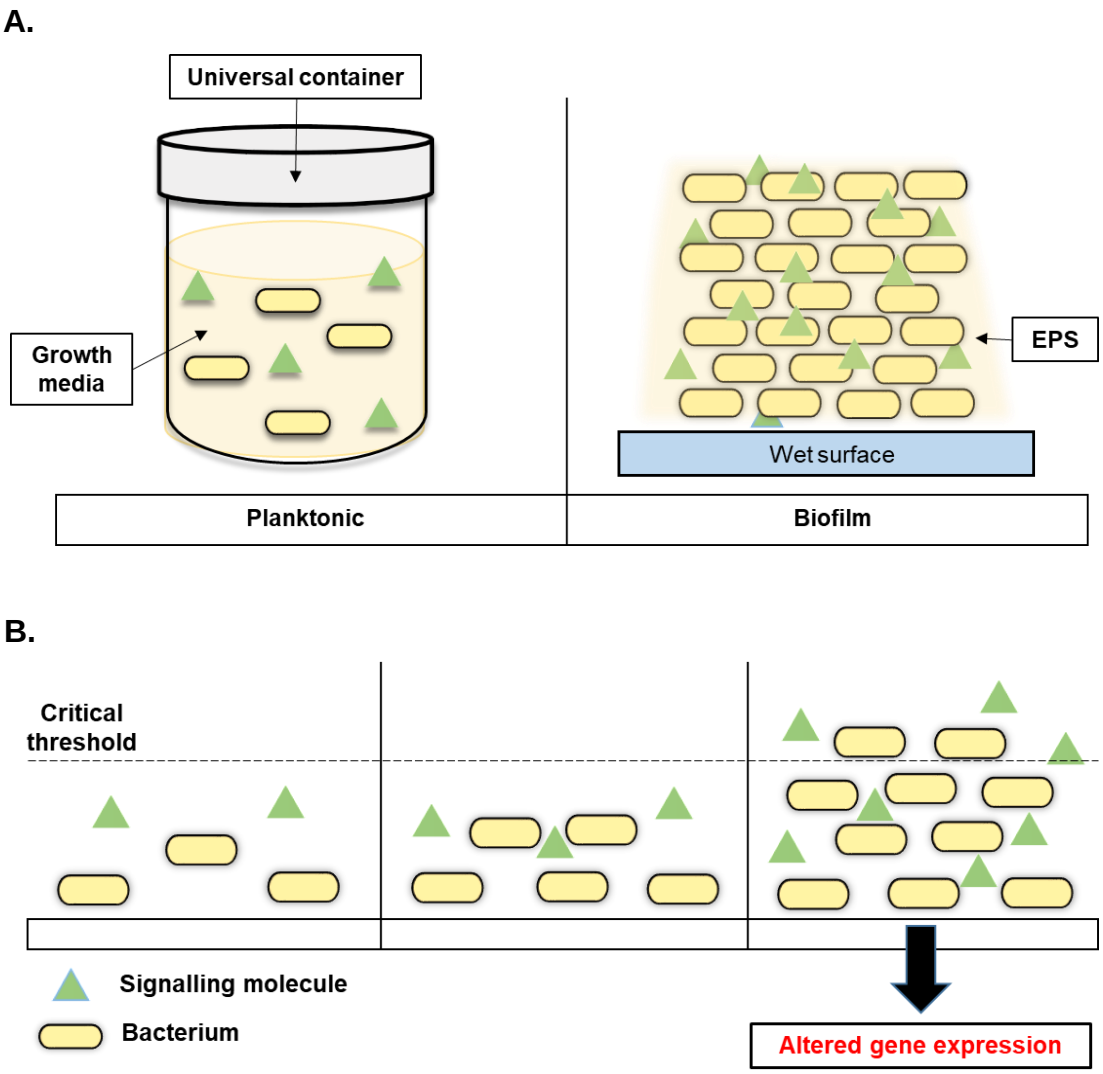


Figure 5. (**A**) Planktonic vs. biofilm gene expression (**B**) Altered gene expression in biofilms regulated by quorum sensing is activated when a critical threshold is reached.

Looking at planktonic growth in a liquid culture, signal molecules face difficulties in attachment, because they are not concentrated enough and have diffusion limitations due to spatial distributions (Ikuma et al 2013). However, in biofilms there are environmental advantages, like the presence of EPS, which allow signal molecules to diffuse to receptors more easily. Specific quorum sensing genes allow the bacteria to sense the concentration of the chemical

signals to monitor the cell-population density and diversity. Once these chemical signals reach a critical threshold, they can affect the behaviour of the biofilm population by triggering changes in gene expression (**Fig 5B**). In Gram-negative bacteria such as *P. aeruginosa,* the signal molecules are commonly homoserine lactones, whilst in Gram-positive bacteria, they are commonly small secreted peptides (Monnet and Gardan 2015).

1.4.4 Detecting bacteriocins in LAB

1.4.4.1 Culture-based assays

To find and identify novel bacteriocins in LAB that have target specificity for industrial applications, it is important to have suitable and reliable methods of detection. When LAB are grown in broth growth medium, they secrete any products into the environment during propagation; the combination of these products and the spent media is called a supernatant (Koohestani et al 2018). The supernatant can be filter-sterilised to remove bacterial cells and the now cell-free supernatant (CFS) can be used in testing to detect for bacteriocins. CFS can be purified using methods such as ammonium sulphate precipitation followed by High-Performance Liquid Chromatography (HPLC) (Goh and Phillip 2015). Various treatments of the CFS or purified bacteriocin to be analysed can undergo various treatments with specific enzymes (e.g. proteinase K, lysozyme, lipase, catalase, lyticase, trypsin and peptidase) or sodium hydroxide and heat to rule out inhibition from the other products secreted by LAB and confirm that it is highly likely the observed antagonistic effect originates from a potential bacteriocin. Bacteriocins can diffuse into semi-solid or solid culture media (Ramu et al 2015). Therefore, detection of bacteriocins from LAB has traditionally involved culture-dependent agar plate assays against common foodborne pathogens such as *E. coli* and *L. monocytogenes* (Gao et al 2016; Pasteris et al 2014) such as the agar-spot method and the well-diffusion assay (Hernández et al 2005; Tagg and McGiven 1971).

1.4.4.2 Molecular screening methods – Genome mining

Molecular screening methods that involve genome mining can be used to directly identify bacteriocins in LAB strains. WGS of a bacterial strain can give access to the genome whereby bacteriocin genes can be mined. Genome mining of LAB strains for bacteriocins can be carried out using a web-based bacteriocin genome-mining tool known as BAGEL4 (http://bagel4.molgenrug.nl/). This site is a sequence database repository for bacterial strains, containing whole genome sequences uploaded to the developed webserver. The site enables users to inspect prokaryotic DNA for antimicrobial peptide motifs involved in the biosynthesis of ribosomally synthesised and post-translationally modified peptides (RiPPs) as well as bacteriocins (van Heel et al 2018). BAGEL4 is freeware, with an easy-to-use web interface. From there, bacteriocin gene clusters can be further characterised using a second database known as BACTIBASE, with a range of tools for bacteriocin characterisation. This database can give more information on the protein properties of specific bacteriocins including mass, net charge, isoelectric point etc. (Hammami et al 2010). However, a disadvantage to using these databases is they can only detect bacteriocins that have already been characterised previously.

1.5 Background to the project

This project was sponsored by the health supplements company Cultech Ltd (Port Talbot, UK) who manufacture probiotic and nutritional products. As the popularity in probiotics increases, so do customer demands. Combinations of microorganisms are frequently requested, and the generation of multi-strain probiotic supplements is required. These multi-strain products are generated from commercially produced pure strain inocula. However, when combined, they can demonstrate a significant reduction in bacterial numbers when compared against the predicted bacterial input of the original raw material. There can be as much as a 65% reduction of the total microbial original biomass. A disadvantage of multi-strain probiotics may be reduced efficacy due to antagonistic intra- and inter- species inhibition. This inhibition has been shown to be due to antimicrobial peptides called bacteriocins, that are known to be produced by *Lactobacillus* strains. Bacteriocins can be beneficial to the bacteria, in that they provide them with a competitive advantage over other microorganisms in their ecological niches. However, in mixed populations such as in the production of probiotics, they have been suggested to contribute to the reduced recovery of all the organisms present, thereby potentially causing the underestimation of the total bacterial numbers in products. This became an issue for Cultech, as part of their production quality control processes whereby the CFU from the viable counts, was not matching up to the label claim of products and so an additional microbial input is required in order to meet the label claim, which come at an economic burden. This prompted this study into the inter-strain and inter-species interactions of these probiotic consortia. To comply with the appropriate regulatory authorities who are demanding accurate quantification of probiotic products. A greater understanding of these inhibitory effects is needed, to not only maximise production efficacy, but to also support the commercial position of the probiotic product in the marketplace.

1.6 Aims

The aims of this study were to investigate potential antibacterial activity in the form of bacteriocins in a range of LAB strains used in the preparation of probiotic supplements. To identify, not only which strains could be producing putative antimicrobials, but also which probiotic strains they had antimicrobial activity against.

The specific aims and objectives of the project were:

- To develop screening techniques to identify and quantify antimicrobial-producing strains.

- To use planktonic and biofilm growth conditions, as well as different incubation time-points, to assess both biomass production and expression/production of antimicrobial substances in probiotic strains.

- To isolate putative antimicrobials and determine their antimicrobial range and efficacy by screening them against a range of probiotic bacteria.

- To use genome mining tools like BAGEL4 to identify putative bacteriocin genes within the bacterial genome of probiotic strains to evaluate potential bacteriocin-associated genes that could be the cause of any observed antimicrobial effects.

Chapter Two

Materials and Methods

2 Methods and Materials

2.1 Bacterial strains and culture conditions

All bacterial strains used within this study were supplies and lyophilised by Cultech Ltd (Port Talbot, UK) and were maintained at -20°C (**Table 5**).

Table 5. Probiotic bacterial strains used in this study.

Bacterial strain
Lactobacillus paracasei CUL08 (NCIMB 30154)
Lactobacillus salivarius CUL61 (NCIMB 30211)
Premix consortia
Acidophilus Premix comprised of: *L. acidophilus* CUL21 (NCIMB 30156), *L. acidophilus* CUL60 (NCIMB 30157),
Bifid Premix comprised of: *Bifidobacterium bifidum* CUL20 (NCIMB 30153), *Bifidobacterium animalis subsp. lactis* CUL34 (NCIMB 30172).
Lab4 comprised of: *L. acidophilus* CUL21 (NCIMB 30156), *L. acidophilus* CUL60 (NCIMB 30157), *B. bifidum* CUL20 (NCIMB 30153), *B. animalis subsp. lactis* CUL34 (NCIMB 30172).
Lab4b comprised of: *L. salivarius* CUL61 (NCIMB 30211), *L. paracasei* CUL08 (NCIMB 30154), *B. bifidum* CUL20 (NCIMB 30153), *B. animalis subsp. lactis* CUL34 (NCIMB 30172).

NCIMB, National Collection of Industrial, Food and Marine Bacteria.

To revitalise the microorganisms, overnight (O/N) cultures were made with approximately 1 mg of lyophilised bacterial strain which was grown in 10 ml of MRS broth (Oxoid, Basingstoke, UK) for 20 h at 37°C, under anaerobic conditions (10% carbon dioxide, 10% hydrogen, 80% nitrogen) (De Man et al 1960).

2.2 Screening of probiotic species for antimicrobial activity

The antimicrobial activities of the probiotic species were initially determined using the perpendicular streak plate method on MRS agar (Cappuccino and Sherman 2004). The optical density at 600 nm (OD_{600}) of a 20 h bacterial culture in MRS broth was diluted with MRS broth to 0.1. A single-line streak inoculation of the bacterial suspension was made in the centre of the agar plate and allowed to dry under a Class 1 laminar flow hood, before incubating the plates anaerobically at 37°C for 24 h (10 µl loop; red lines; **Figure 6**).

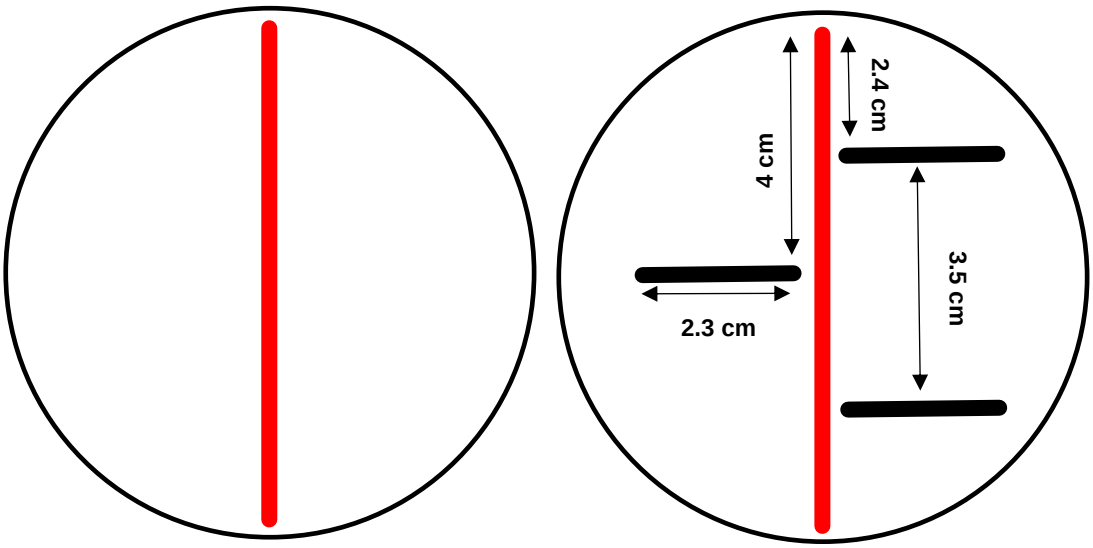


Figure 6. Streaking plan for screening probiotic species for their antimicrobial activity, using the perpendicular streak plate technique. (**A**) Placement of antimicrobial producing probiotic strain (red line); (**B**) Placement of the susceptibility test organisms (black lines) following 24 h incubation of the vertical streak.

After incubation, these steps were repeated with all the probiotic species to test the survival of the probiotic microorganisms against each other and themselves. However, in this case, a perpendicular streak (90° to the original streak) was made (black lines; **Figure 6**). The plates were then re-incubated for a further 24 h, under the same conditions. Any zones of inhibition (from the perpendicular streak to the original vertical streak) were then measured for each probiotic species. Mean zones of inhibition (mm) were calculated from *n* = 3 repeats. *Bifid* Premix was used as a negative control.

2.3 Detecting lactic acid production in probiotic species

Overnight cultures of the probiotic bacteria and consortia (**Table 5**) were made as previously described (**Section 2.1**). Aliquots of the O/N cultures were diluted to an OD_{600} of 0.1, and using a sterile loop (10 µl), a single-line streak inoculation of the diluted bacterial culture was made in the centre of an MRS agar plate supplemented with bromocresol purple indicator (0.12 g/L) (Sobrun et al 2012). The streak was then dried under a Class 1 laminar flow for 5 min and the plates incubated anaerobically for 24 h at 37ºC. The *Bifid* Premix was used as a control. Streaks that displayed a yellow zone (emanating) from the bacterial colonies grown were measured. After *n* = 4 repeats, the mean and standard error of the mean (SEM) of the yellow zones were calculated. These results were then compared to the inhibition seen from the perpendicular streak plate method to determine if they correlated.

2.4 Growth curve assays to determine growth phases of *L. salivarius* CUL61 and *L. paracasei* CUL08 for bacteriocin harvesting

Cultures of *L. salivarius* CUL61 and *L. paracasei* CUL08 were grown for 20 h in MRS broth anaerobically at 37°C to reach 1 $x10^9$ and 3 $x10^8$ CFU ml^{-1} for *L. salivarius* CUL61 and *L. paracasei* CUL08, respectively. Bacterial cells were adjusted to 1 x 10^6 CFU ml^{-1} in MRS broth (using *n* = 3 independent cultures) in a microtiter plate and then incubated anaerobically at 37°C, with optical density readings (A_{600}) taken every hour for 24 h. Each growth curve was repeated in triplicate. The growth characteristics (growth rate, doubling time and maximum carrying capacity were analysed using the Growthcurver

package in R Studio (Comprehensive R Archive Network, [CRAN]) as previously described; (Sprouffske 2016).

2.5 Generation of cell-free supernatants (CFS)

2.5.1 Generation of planktonic CFS

L. salivarius CUL61 and *L. paracasei* CUL08 O/N cultures were subcultured (150 µl) in MRS broth (150 ml) and incubated anaerobically for 5, 24, 48 or 72 h at 37°C, (*n* = 3). An uninoculated MRS broth-only control was incubated alongside the cultures. Supernatants were harvested by centrifugation at 2504 x *g* for 30 min at 4°C. To eliminate any antibacterial effects of organic acids, the supernatants were pH neutralised by adjustment to pH 7.0 with 5 M NaOH solution using a HI-2211 Bench Top pH & mV Meter (Hanna instruments, Bedfordshire, UK). After supernatants were neutralised to pH 7, they were filter-sterilised (0.22 µm, Sigma-Aldrich, Dorset, UK) to remove all bacterial cells and produce cell-free supernatants (CFS). A loopful of both 5 and 24 h CFS was inoculated onto an MRS agar plate and incubated anaerobically for 48 h to confirm sterility. The CFS (10 ml) was then aseptically aliquoted into falcon tubes and frozen (-80°C) until used. Total viable counts were carried out on O/N cultures (10^{-6} and 10^{-7}), the inoculated media before incubation (10^{-3} and 10^{-4}) and the bacterial cultures after incubation for both 5 h (10^{-3} and 10^{-4}) and 24 h (10^{-6} and 10^{-7}), to ascertain the approximate number of bacteria that cause antibacterial effects (**Appendix 1**).

2.5.2 Generation of biofilm CFS

Overnight cultures of *L. salivarius* CUL61 and *L. paracasei* CUL08 were subcultured (40 µl) in MRS broth (40 ml) in $T75^2$ tissue culture flasks (Greiner, Gloucestershire, UK), and incubated anaerobically at 37°C for 1 h, including an MRS broth-only control, (*n* = 3). After 1 h, the culture supernatant was removed from the $T75^2$ flasks and aseptically replaced with 40 ml fresh MRS broth to ensure that only attached, biofilm growing cells remained. The biofilms were then incubated for a further 4 or 23 h (to give 5 or 24 h biofilm cultures respectively). Biofilms and supernatants were then harvested by scraping the flask floor with a cell scraper (10 mm, Starlab, Milton Keynes, UK) and

centrifuged at 2504 x *g* for 30 min at 4°C. The supernatant obtained from the biofilm cultures was then pH-adjusted to pH 7.0, filter-sterilised, aliquoted, and frozen as previously described for the planktonic cultures (**Section 2.5.1**). Purity plates were again performed for both 5 and 24 h CFS and total viable counts determined as previously described above (**Section 2.5.1**).

2.6 Lyophilisation and concentration of CFS

Single strength CFS (ssCFS) were concentrated by lyophilisation as described by (Bermudez-Brito et al 2013). For this, ssCFS were aliquoted (20 ml) into petri dishes and frozen for a minimum of 4 h at -80°C. Primary drying was conducted at -20°C for 24 h and then at -4°C for 24 h. Then, secondary drying was completed at 10°C for 4 h.

Table 6. Concentrations of cCFSs and their respective value in percentage (%)

cCFS Lyophilised Concentration	New cCFS Values for Lyophilised Concentration (%)
X 20	100
X 10	50
X 5	25
X 2.5	12.5
X 1.25	6.25
X 0.63	3.13
X 0.31	1.56
X 0.16	0.78
X 0.08	0.39
X 0.04	0.2
X 0.02	0.1
X 0.01	0.05
0	0

Samples were then concentrated (X 20) by re-suspending each dried pellet into 1 ml of sterile distilled water (dH_2O) and swirling. This concentrated ssCFS was denoted X 20 cCFS (and will also be referred to as the 100% concentration: **Table 6**).

2.7 Growth inhibition of probiotic species using growth curves

Growth curves were performed to determine the antimicrobial effects of probiotic CFSs on planktonic bacterial growth. For this, *L. salivarius* CUL61 and *L. paracasei* CUL08 ssCFS (5 and 24 h, planktonic and biofilm) and cCFS (24 h planktonic) were used as follows. For experimental consistency, O/N cultures of each bacterial strain and premix consortia (**Table 5**) were grown for 20 h in double strength MRS broth (dsMRS) anaerobically at 37°C. The cultures were then adjusted to an OD_{600} of 0.1 with dsMRS. Next, ssCFS or cCFS (100%) was added to flat-bottom 96-well microtiter plates (100 µl per well), alongside dsMRS (80 µl per well), which was used to compensate for nutrients within the spent medium. The adjusted bacterial cultures were then inoculated into the wells (20 µl per well) giving a final concentration of 50% for both ssCFS and cCFS. Growth controls containing dsMRS (80 µl per well), diluted bacterial culture (20 µl per well) and ssMRS (single strength MRS; 100 µl per well) were also performed. Plates were incubated at 37°C, anaerobically, with low shaking (5 Hz, amplitude 15 mm) and optical density readings were taken every hour (OD_{600}, Thermo Scientific Multiskan GO) for 24 h (*Acidophilus* Premix, *L. salivarius* CUL61, Lab4 and Lab4b) or 72 h (*Bifid* Premix). Replicate *L. paracasei* CUL08 plates were incubated aerobically at 37°C for 72 h without shaking. All tests were repeated in triplicate.

Following incubation, the growth characteristics were analysed using the Growthcurver package in R Studio (Comprehensive R Archive Network, [CRAN]) as previously described (**Section 2.4**).

2.8 Determination of minimum inhibitory concentrations (MICs) using microbroth dilution assay

MICs were determined by broth microdilution assays adapted from (Jorgensen and Turnidge 2015). First, dilutions of the cCFS in the range 50% - 0.05%

were prepared by the addition of cCFS (105 µl) and double strength Iso-sensitest broth (Oxoid, Basingstoke, UK): MRS broth (Iso: MRS, 9:1 ratio) (95 µl) to column 1 of flat-bottomed 96-well microtiter plates. Columns 2-12 were loaded with double strength Iso: MRS broth (100 µl) and two-fold serial dilutions of the cCFS samples were carried out down the plate from column 1 to 11. O/N cultures of probiotic species were diluted in double strength Iso: MRS broth to an OD_{600} of 0.1. The diluted bacterial cultures were added to each well of the microtiter plates containing the cCFS serial dilutions (10 µl). Each plate included a positive growth control (10 µl bacterial inoculum added to 100 µl of two-fold Iso: MRS broth) as well as a broth sterility control (110 µl). The final volume of each well was 110 µl. Plates were sealed with parafilm and incubated anaerobically at 37°C for 24 h and MICs were determined as the lowest concentration (%) at which there was no visible growth relative to the control by eye. Experiments were repeated in triplicate.

2.9 Antimicrobial testing using a well diffusion assay

Overnight cultures of probiotic species (**Table 5**) were adjusted to an OD_{600} = 0.1 and then the bacterial suspensions (as test indicator microorganisms) were pipetted aseptically (1 ml per 20 ml) into sterile molten (50°C) MRS agar (0.7% w/v). After gentle mixing, the inoculated medium, was poured into petri dishes, allowed to set. Using a sterilised 7 mm cork-borer, four wells (equi-distant apart) were cut into the agar plate before adding 35 µl of either ssCFS or cCFS to the wells. The well-diffusion plates were then sealed with parafilm and incubated anaerobically for 24 h at 37°C. The disinfectant Virkon (1%; Fisher Scientific, Leicestershire, UK) was used as a positive control. After incubation, bacteriocin activity was determined by wells that displayed a ‘no bacterial growth’ halo around them, (signifying a zone of inhibition) which were measured. Mean zones of inhibition (mm) were calculated from *n* = 3 repeats by measuring the diameter of the well (size of the well was included in the measurements).

2.10 Effect of 24 h planktonic *Lactobacillus* cCFS on biofilm formation

L. salivarius CUL61, *L. paracasei* CUL08, *Bifid* Premix and Lab4b consortium biofilms treated with cCFS were analysed using CLSM and LIVE/DEAD *Bac*Light staining (bacterial viability kit; Invitrogen, Paisley, UK) as previously described (Khan et al 2012). The LIVE/DEAD *Bac*Light stain contains SYTO 9 dye (stains LIVE cells, green) and propidium iodide (staining DEAD cells, red).

Overnight cultures of probiotic species were diluted in ssMRS to an OD_{600} of 0.1. Then, 10 µl of the diluted cultures was inoculated into a glass-bottomed 96-well plate that contained ssMRS broth (78.75 µl) with 100% cCFS (11.25 µl), to give a final total volume per well of 100 µl. The plates were sealed with parafilm and incubated for 24 h at 37°C anaerobically. After incubation, the supernatant was carefully removed from the wells and 2 µl of each stain (red-fluorescent propidium iodide and green fluorescent SYT09) was added to 1 ml PBS (v/v) and mixed. The stain was added to each test sample (4 µl), incubated in the dark (10 min), before imaging using CLSM with a Leica TSC SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany) using a X 63 oil immersion lens. Five z-stack images (with a step size of 0.79 µm) were taken per well. Following this, the biofilm images were quantified using COMSTAT image analysis software (Heydorn 2000) to determine mean biofilm biomass. Other conditions such as biofilm thickness and roughness was also quantified, and these results can be found in Appendix 2.

2.11 *In silico* genome analysis of *Lactobacillus* strains to identify putative bacteriocin genes

Using the web-based tool BAGEL4, FASTA files for *L. salivarius* CUL61 and *L. paracasei* CUL08 were uploaded onto the database and analysed to identify potential bacteriocin clusters (van Heel et al 2018). Following this step, any genes found to encode a bacteriocin were explored using BACTIBASE for further characterisation (Hammami et al 2010).

2.12 Statistical analysis

Excel (Microsoft Corp., USA) or GraphPad Prism 8.3.1 (GraphPad Software Inc., La Jolla, USA) were used to perform statistical analysis. All experiments were carried out three times (unless otherwise stated), and results were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). The following tests were used including the Shapiro-Wilk test to check for normality of data and the nonparametric Kruskal-Wallis test performed at the 95% confidence interval and Dunn's multiple comparisons method (well-diffusion assays). $P < 0.05$ was deemed significant.

Chapter Three

Results

3 Results

3.1 Perpendicular streak plate assay

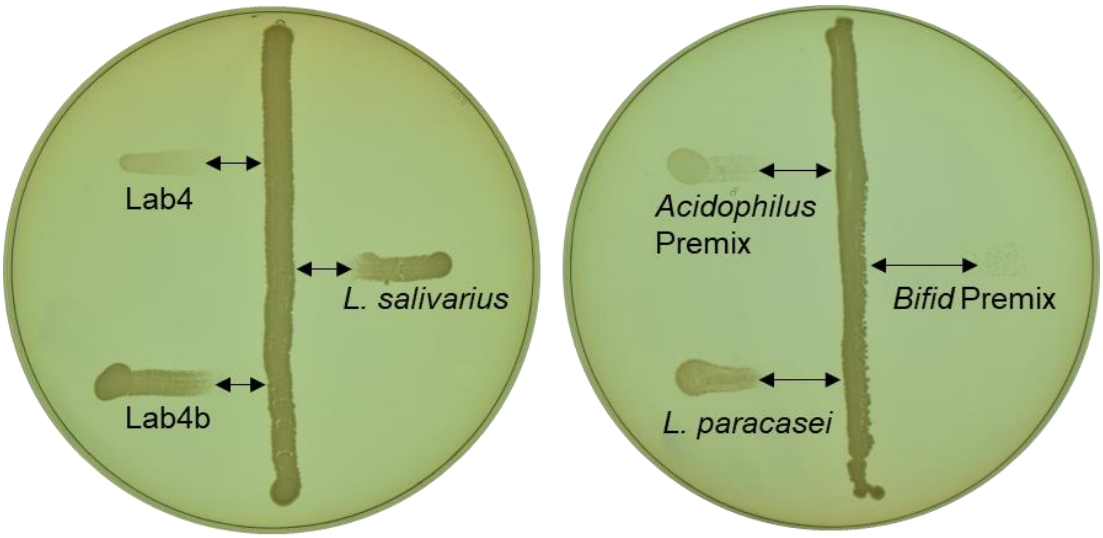


Figure 7. Perpendicular streak plate of *L. salivarius* CUL61 (vertical streak) displaying antibacterial activity against (**A**) Lab4, Lab4b and *L. salivarius* CUL61 (itself); (**B**) *Acidophilus* Premix, *L. paracasei* CUL08 and *Bifid* Premix (horizontal streaks). Antibacterial activity was determined by a zone of inhibition from the vertical streak to the perpendicular streak (indicated by the arrows shown) and measured in mm (n = 3).

An example of the streak plate assay is shown in **Fig 7,** with mean measurements (n = 3) for all organisms tested given in **Table 7**. It was apparent that *L. salivarius* CUL61 inhibited all the probiotic strains tested, including itself. In addition, *L. salivarius* CUL61 not only inhibited the individual microorganisms found in Lab4b, but the Lab4b consortia as well. *L. paracasei* CUL08 also inhibited all strains tested including itself, but at lower levels compared to *L. salivarius* CUL61. These effects also appeared to be greater against individual organisms (*Acidophilus* Premix and itself) and less so against the mixed consortia tested. The *Bifid* Premix was unable to inhibit any of the probiotic microorganisms or itself but was inhibited by all other strains and the two consortia, which is consistent with the predictions of no bacteriocins.

Table 7. Perpendicular streak plate assay showing mean inhibition zones (mm) measured from the vertical streak to the test streak (*n* = 3) for all the LAB strains tested.

Microorganism	Mean Inhibition (mm)					
	Acidophilus **Premix**	***L. paracasei*** **CUL08**	***L. salivarius*** **CUL61**	***Bifid*** **Premix**	**Lab4**	**Lab4b**
**L. paracasei* CUL08	**5.8 ± 0.44**	**5.5 ± 0.29**	2.7 ± 0.33	**10.3 ± 0.33**	4.3 ± 0.33	2.0 ± 0.58
**L. salivarius* CUL61	**9.7 ± 0.33**	**9.8 ± 0.17**	**6.2 ± 0.17**	**12.7 ± 0.33**	**6.5 ± 0.50**	**5.2 ± 0.17**
**Acidophilus* Premix	2.0 ± 0.577	3.2 ± 0.44	ND	**7.2 ± 1.17**	ND	ND
**Bifid* Premix	ND	ND	ND	ND	ND	ND
*Lab4	3.8 ± 0.17	2.5 ± 0.29	ND	**6.7 ± 0.88**	ND	ND
*Lab4b	**8.5 ± 0.76**	**9.7 ± 0.17**	**6.2 ± 0.17**	**13.2 ± 0.93**	**7.0 ± 1.53**	**5.7 ± 0.67**

*Organisms used for the vertical streak. ND, not detected. Mean ± SEM. Numbers in bold indicate considerable inhibitory zones (antimicrobial effects).

The Lab4b consortia showed similar inhibition to *L. salivarius* CUL61, indicating that even in the consortia, *L. salivarius* CUL61 and *L. paracasei* CUL08 were able to cause inhibition.

3.2 Acid diffusion of LAB using MRS agar supplemented with bromocresol purple indicator

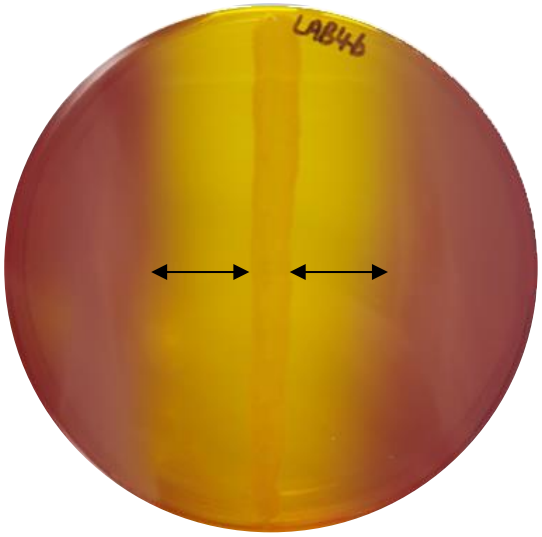


Figure 8. Diffusion of acid from commercial consortia (Lab4b) into MRS agar supplemented with bromocresol purple indicator. The yellow colour change in the agar indicates where the pH of the agar has been lowered due to acid secretion. This area was measured from the middle of the vertical bacterial streak (to left and right as indicated by the arrows shown, mm) and the mean was calculated from *n* = 4

Acid secreted by probiotic bacteria (from the vertical bacterial streak) lowers the pH of the agar causing a colour change from purple (pH 6.8) to yellow (pH 5.2 and below). This yellow colour change of the agar was measured (on either side of the streak from the same point on each plate; **Fig 8**). The mean values from *n* = 4 repeats are shown in (**Table 8**). From these results it could be determined that *L. salivarius* CUL61 secreted the largest amount of acid (7.1 mm), > Lab4b > *L. paracasei* CUL08 > *Acidophilus* Premix > with acid production being undetected in the negative control *Bifid* Premix.

Table 8. Mean acid diffusion of probiotic LAB and consortia.

Bacteria/Consortia	Mean Lactic Acid Diffusion (mm)
L. paracasei CUL08	5.4 ± 0.13
L. salivarius CUL61	7.1 ± 0.06
Acidophilus Premix	4.5 ± 0.23
Bifid Premix	ND
Lab4	2.8 ± 0.18
Lab4b	5.6 ± 0.21

ND, not detected. Values displayed as mean of *n* = 4 repeats ± SEM.

To determine what effects were not derived from simple acid production, the mean results from the acid diffusion assay (**Table 8**) were subtracted from those of the perpendicular streak plates results (**Table 7**). The final values calculated removed inhibition that could be caused by acid secreted from the LAB, suggesting any remaining inhibition (mm) was the due to other extracellular products, such as bacteriocins (**Table 9**).

Even with the effect of acid removed, Lab4b was still seen to inhibit all the probiotic bacteria and consortia tested. Lab4 showed inhibition against the *Acidophilus* Premix and *Bifid* Premix (1.1 and 4.0 mm, respectively). Additionally, the *Acidophilus* Premix inhibited *Bifid* Premix (2.6 mm). Both *L. paracasei* CUL08 and *L. salivarius* CUL61 showed inhibition against the same probiotic species (*Acidophilus* Premix, *L. paracasei* CUL08 and the *Bifid* Premix), although, *L. salivarius* CUL61 demonstrated a greater antibacterial effect.

Table 9. Mean inhibition of products secreted from probiotic bacteria and consortia with acid-effect removed.

Microorganism	Mean Perpendicular Streak Plate Inhibition – Mean Lactic Acid Diffusion = Potential Putative Bacteriocin Activity (mm)					
	***Acidophilus* Premix**	***L. paracasei* CUL08**	***L. salivarius* CUL61**	***Bifid* Premix**	**Lab4**	**Lab4b**
****L. paracasei* CUL08**	**0.4**	**0.1**	ND	**4.9**	ND	ND
****L. salivarius* CUL61**	**2.6**	**2.7**	ND	**5.6**	ND	ND
****Acidophilus* Premix**	ND	ND	ND	**2.7**	ND	ND
****Bifid* Premix**	ND	ND	ND	ND	ND	ND
***Lab4**	**1.1**	ND	ND	**4.0**	ND	ND
***Lab4b**	**2.9**	**4.1**	**0.6**	**7.6**	**1.4**	**0.1**

*Organisms used for the vertical streak. ND, not detected.

3.3 Growth curves of *L. salivarius* CUL61 and *L. paracasei* CUL08 to discover growth phases

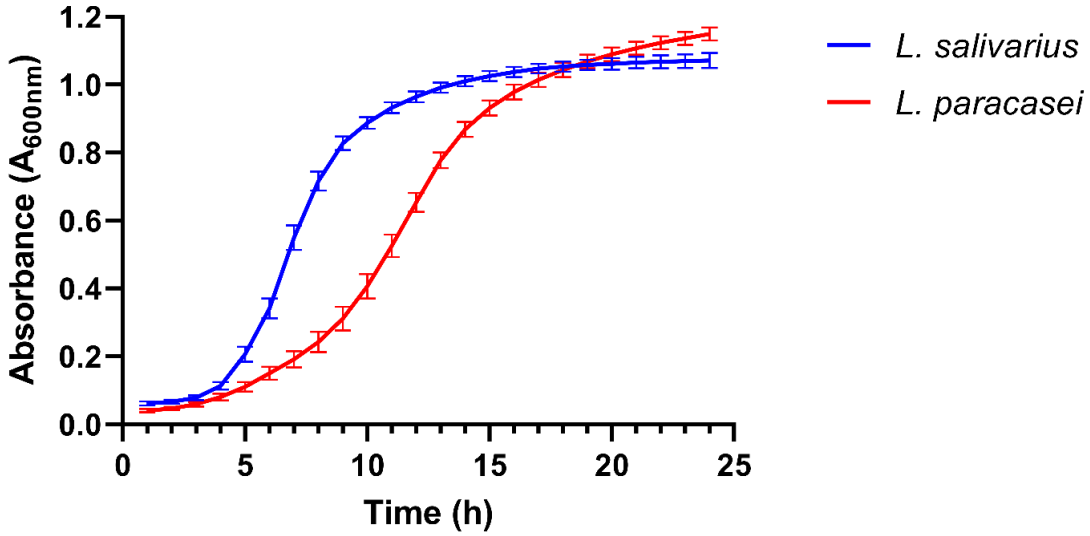


Figure 9. Growth curves of *L. salivarius* and *L. paracasei* grown anaerobically in MRS broth for 24 h at 37°C. (Data kindly provided by Dr A. Jack, Cultech Ltd).

Expression of antimicrobial peptides and bacteriocins are regulated by quorum sensing pathways and as such, these peptides are commonly produced at stationary phase. Therefore, the growth characteristics of both *L. salivarius* CUL61 and *L. paracasei* CUL08 were investigated to ensure that the CFS was collected within the correct growth phase to harness potential bacteriocins. *L. salivarius* CUL61 and *L. paracasei* CUL08 showed a lag time of 3 and 5 h, reaching stationary phase after 10 and 16 h, respectively (**Fig 9**). Growth rates were 0.755 h^{-1} and 0.451 h^{-1} and doubling times were 1.001 h^{-1} and 1.169 h^{-1} for *L. salivarius* CUL61 and *L. paracasei* CUL08, respectively. The maximum possible population size (K) for *L. salivarius* CUL61 was determined to be 0.92 and for *L. paracasei* CUL08 1.54. Hence, in the beginning, *L. salivarius* CUL61 grew more quickly than *L. paracasei* CUL08, with both strains reaching parity at 18 h, and *L. paracasei* CUL08 then superseding *L. salivarius* CUL61 in terms of population size by 24 h.

3.4 Generation of bacterial CFS

Initial CFSs were obtained from both planktonically and biofilm grown *L. salivarius* CUL61 and *L. paracasei* CUL08 at only two time-points (5 and 24 h). These single-strength and concentrated CFS samples were tested for their antimicrobial activity using antimicrobial susceptibility methods (microbroth dilution, well-diffusion, and growth curve assays). Subsequently, ssCFS samples of *L. salivarius* CUL61 were then produced over a time course for bacteriocin production and harvested at 48 and 72 h. These samples were then tested for antimicrobial activity in growth curve assays to determine whether bacteriocin production continued after 24 h.

3.5 Characterisation of the antimicrobial activities of *Lactobacillus* ssCFS and cCFS

3.5.1 Analysis of antagonistic CFS effects using growth curves

3.5.1.1 *Acidophilus* Premix growth curves

The growth curves show that untreated *Acidophilus* Premix had a growth lag time of approximately 3 h, and that at 10 h, bacterial growth began to slow and enter stationary phase (**Figs. 10A and B**).

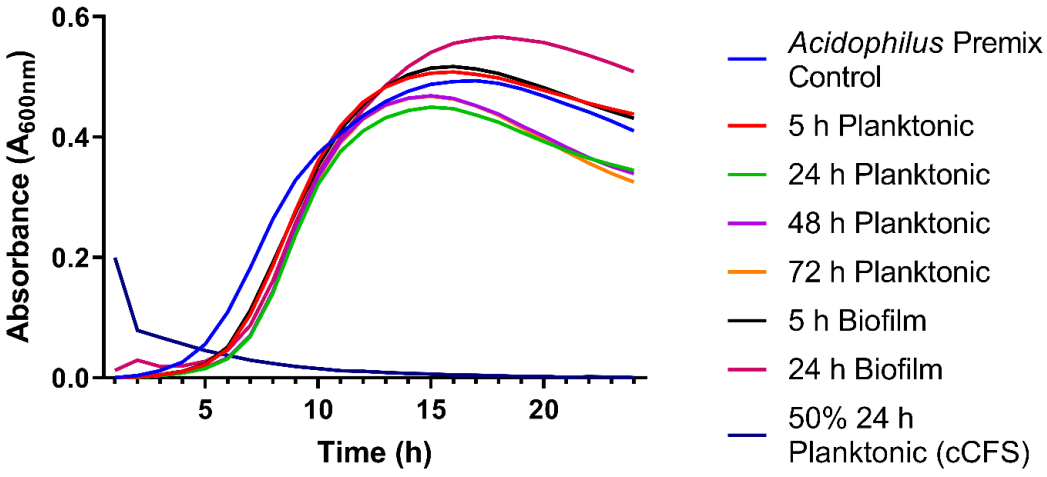


Figure 10A. Effect of *L. salivarius* CUL61 (5, 24, 48 and 72 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *Acidophilus* Premix (24 h) (*n* = 3).

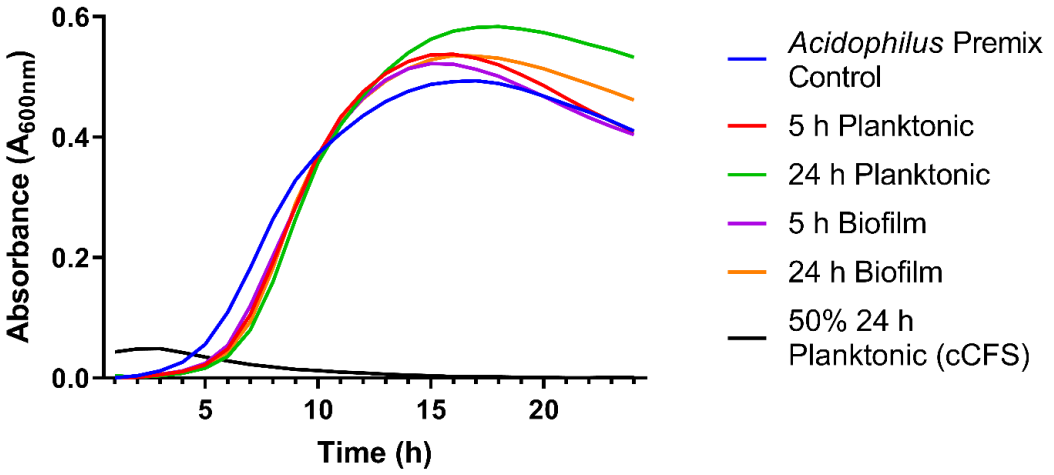


Figure 10B. Effect of *L. paracasei* CUL08 (5 and 24 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *Acidophilus* Premix (24 h) (*n* = 3).

Following treatment with ssCFS, a slight increase in the length of the lag phase (to ca. 5 h and 4 h for *L. salivarius* CUL61 and *L. paracasei* CUL08 CFS respectively) was noted (**Figs. 10A and B**). Despite this longer lag phase, all the growth curves for the ssCFS-treated *Acidophilus* Premix were very similar, reaching comparable growth rates and maximum population sizes to those of the control (**Table 10**). Perhaps noteworthy is the fact that for the for *L. salivarius* CUL61 biofilm CFSs, the maximum population sizes were slightly higher than those of the control (≥0.465 K), whilst those of the planktonic ssCFS were almost all slightly lower, indicating slight overall inhibition of *Acidophilus* Premix. This effect was not seen with the *L. paracasei* CUL08 CFSs, where instead, CFS treated suspensions appeared to have similar or slightly higher maximum population sizes to that of the control (≥0.465 K). In contrast to the ssCFS, the 24 h planktonic cCFS dramatically affected the growth of *Acidophilus* Premix demonstrating 100% cell death at ≥17 h.

Table 10. Effect of ssCFS and 50% cCFS on growth characteristics of *Acidophilus* Premix (derived from the Growthcurver package in R).

CFS Type				Growth Characteristics		
Bacteria	**Conc.**	**Growth Type**	**Time-point (h)**	**Doubling Time (h^{-1})**	**Growth Rate (h^{-1})**	**MCC (K)**
L. sal	ss	B	5	0.88	0.787	0.486
			24	1.03	0.671	0.534
	ss	P	5	0.83	0.831	0.482
			24	0.67	1.028	0.408
			48	0.65	1.065	0.419
			72	0.64	1.079	0.414
	c		24	**0**	**0.014**	**0.01**
L. par	ss	B	5	0.83	0.834	0.478
			24	0.86	0.808	0.51
	ss	P	5	0.8	0.869	0.492
			24	0.98	0.705	0.563
	c		24	**0**	**0.012**	**0.007**
Growth control	N/A	N/A	N/A	1.01	0.689	0.465

ss, single strength, c, 50% concentrated, B, biofilm, P, planktonic, N/A, Not applicable, MCC, Maximum carrying capacity. Numbers in bold indicate substantial inhibitory zones (antimicrobial effects).

3.5.1.2 *L. paracasei* CUL08 growth curves

The growth curves show that untreated *L. paracasei* CUL08 began exponential phase at ca. 2 h, and that this stage lasted for 15-16 h before entering stationary phase (**Fig 11**) showing that it had a much slower growth rate than *Acidophilus* Premix (**Fig 10**). In contrast to *Acidophilus* Premix and

the growth control, the *L. salivarius* CUL61 and *L. paracasei* CUL08 ssCFSs appeared to have a more positive effect on the growth of *L. paracasei* CUL08, demonstrating very short lag phases, with growth shown to almost immediately enter into exponential phase at 1 h (**Figs. 11A and B**), although both *L. salivarius* CUL61 and *L. paracasei* CUL08 ssCFSs extended the length of the doubling time compared to the control (all ≥4.11 h^{-1}; **Table 11**).

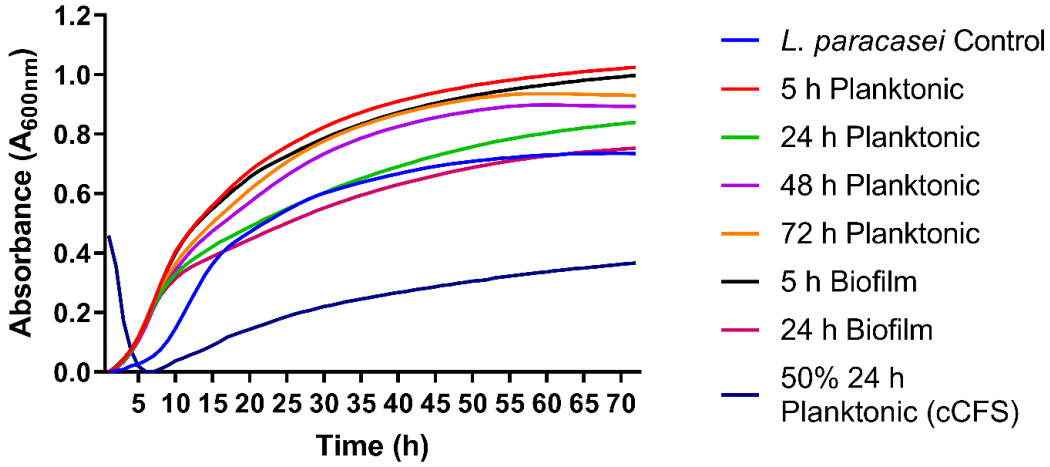


Figure 11A. Effect of *L. salivarius* CUL61 (5, 24, 48 and 72 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *L. paracasei* CUL08 (72 h) (*n* = 3).

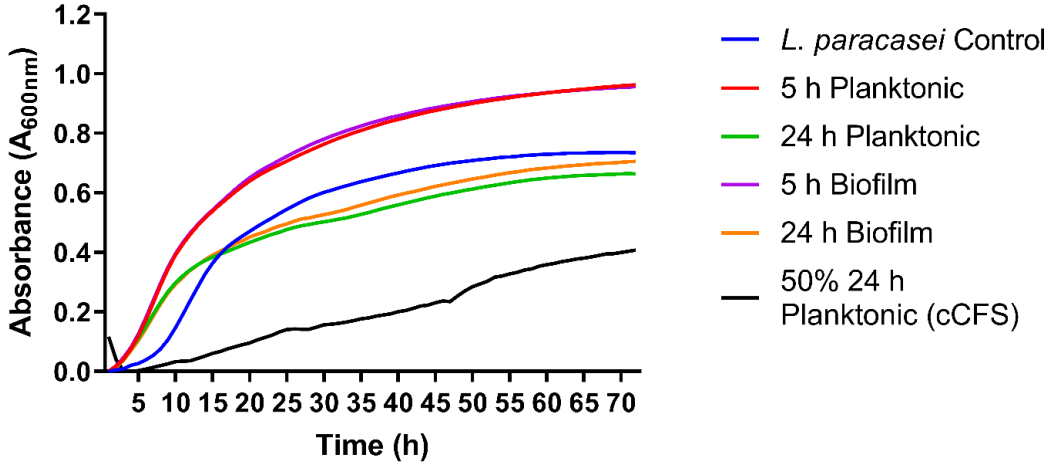


Figure 11B. Effect of *L. paracasei* CUL08 (5 and 24 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *L. paracasei* CUL08 (72h) (*n* = 3).

Interestingly, both types of 5 h ssCFS appeared to slightly stimulate the growth of *L. paracasei* CUL08, as seen by the increase in maximum population from 0.707 K (control) to 0.913 K and 0.912 K (5 h biofilm and planktonic respectively) whilst both types of 24 h ssCFS slightly inhibited growth in comparison to the growth control (**Table 11**).

Table 11. Effect of ssCFS and 50% cCFS on growth characteristics of *L. paracasei* CUL08 (derived from the Growthcurver package in R).

CFS Type				Growth Characteristics		
Bacteria	**Conc.**	**Growth Type**	**Time-point (h)**	**Doubling Time (h^{-1})**	**Growth Rate (h^{-1})**	**MCC (K)**
L. sal	ss	B	5	5.32	0.130	0.943
			24	7.25	0.096	0.725
	ss	P	5	5.08	0.136	0.974
			24	7.27	0.095	0.804
			48	5.36	0.129	0.882
			72	5.17	0.134	0.919
	c		24	**13.21**	**0.052**	**0.41**
L. par	ss	B	5	4.97	0.140	0.913
			24	6.44	0.108	0.669
	ss	P	5	5.24	0.132	0.912
			24	6.51	0.106	0.633
	c		24	**10.99**	**0.063**	**0.481**
Growth control	N/A	N/A	N/A	4.11	0.169	0.707

ss, single strength, c, 50% concentrated, B, biofilm, P, planktonic, N/A, Not applicable, MCC, Maximum carrying capacity. Numbers in bold indicate substantial inhibitory zones (antimicrobial effects).

Again, (as with *Acidophilus* Premix), in contrast to the ssCFS, the 24 h *L. salivarius* CUL61 planktonic cCFS had the biggest effect on the growth of *L. paracasei* CUL08. However, this reduced bacterial growth rate (<0.169 h^{-1} for the control) was only apparent for the first 7 and 3 h (for *L. salivarius* CUL61 and *L. paracasei* CUL08 respectively). After these time-points, *L. paracasei* CUL08 resumed exponential growth, albeit at a much-reduced growth rate, producing a marked increase in doubling time from 4.11 h^{-1} (control) to 13.21 and 10.99 h^{-1} (*L. salivarius* CUL61 and *L. paracasei* CUL08 respectively) but never reaching the same population size (0.41 and 0.481 K respectively) as the growth control (0.707 K).

3.5.1.3 *L. salivarius* CUL61 growth curves

The growth curves show that untreated *L. salivarius* CUL61 had a lag phase of ca. 3 h, beginning stationary phase at 10 h (**Fig 12**). Both *L. salivarius* CUL61 and *L. paracasei* CUL08 ssCFSs appeared to have little effect on bacterial growth compared to the control (**Figs. 12A and B**).

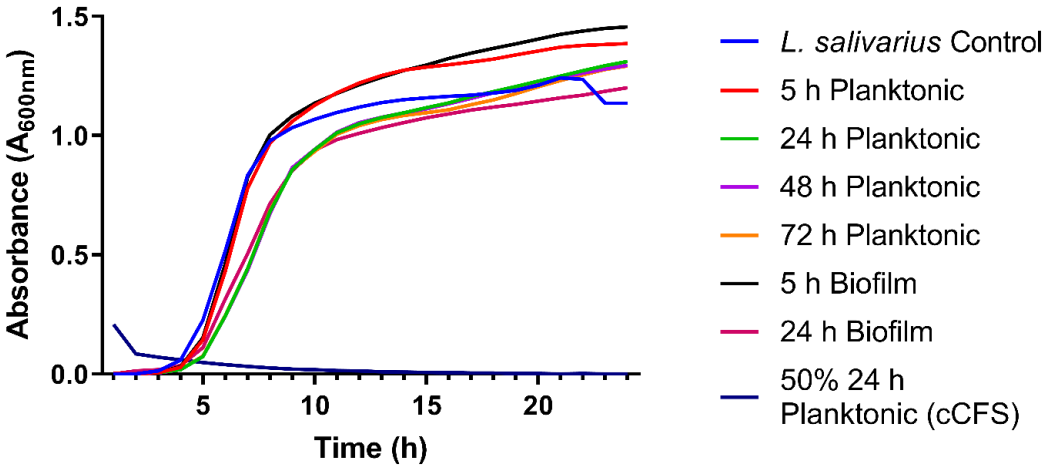


Figure 12A. Effect of *L. salivarius* CUL61 (5, 24, 48 and 72 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *L. salivarius* CUL61 (24 h). (*n* = 3)

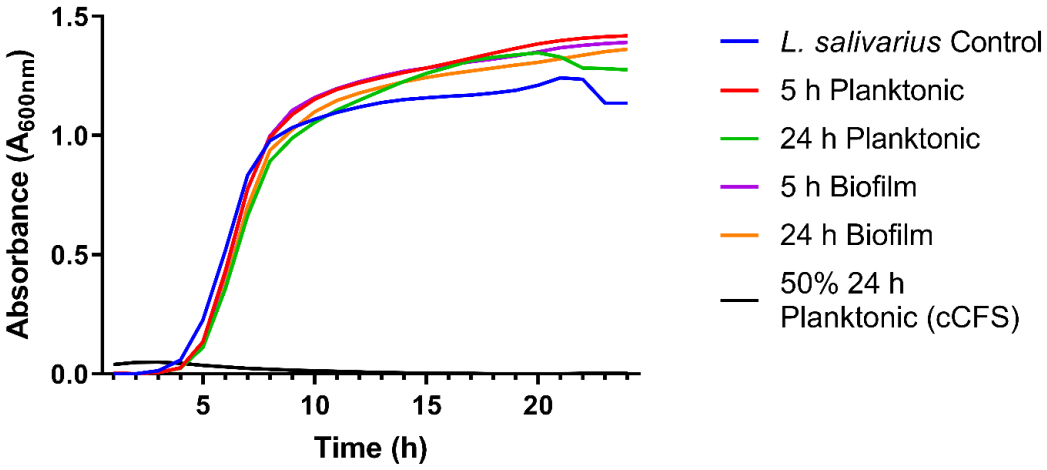


Figure 12B. Effect of *L. paracasei* CUL08 (5 and 24 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *L. salivarius* CUL61 (24 h) (*n* = 3).

In fact, very similar growth curves were produced for all the agents tested, although both reduced growth rates and longer doubling times were obtained for all the test agents compared to the controls using the Growthcurver software (**Table 12**). For *L. salivarius* CUL61 CFSs, the only exception to this was during exponential growth, where only the 5 h biofilm and planktonic showed equivalent growth to the control; the other curves all showing slightly reduced exponential growth (**Fig 12A**). Similarly, to *Acidophilus* Premix (**Fig. 10A and B**), both the *L. salivarius* CUL61 and *L. paracasei* CUL08 24 h planktonic cCFSs dramatically reduced the growth of *L. salivarius* CUL61, resulting in 100% cell death at ≥ 17 h.

Table 12. Effect of ssCFS and 50% cCFS on growth characteristics of *L. salivarius* CUL61 (derived from the Growthcurver package in R).

CFS Type				Growth Characteristics		
Bacteria	**Conc.**	**Growth Type**	**Time-point (h)**	**Doubling Time (h^{-1})**	**Growth Rate (h^{-1})**	**MCC (K)**
L. sal	ss	B	5	0.87	0.797	1.344
			24	0.980	0.705	1.118
	ss	P	5	0.830	0.833	1.311
			24	1.040	0.665	1.196
			48	1.010	0.687	1.189
			72	1.000	0.694	1.172
	c		24	**0**	**0.029**	**0.011**
L. par	ss	B	5	0.81	0.861	1.327
			24	0.92	0.754	1.274
	ss	P	5	0.74	0.936	1.309
			24	0.81	0.857	1.272
	c		24	**0**	**0.033**	**0.007**
Growth control	N/A	N/A	N/A	0.63	1.108	1.262

ss, single strength, c, 50% concentrated, B, biofilm, P, planktonic, N/A, Not applicable, MCC, Maximum carrying capacity. Numbers in bold indicate substantial inhibitory effects (antimicrobial effects).

3.5.1.4 *Bifid* Premix growth curves

The growth curves showed that the untreated *Bifid* Premix had a lag phase of ca. 6 h before entering exponential growth at 7 h (**Figs. 13A and B**). Again, very similar effects were seen following treatment with either *L. salivarius* CUL61 or *L. paracasei* CUL08 ssCFS. All these test agents produced curves

with much reduced exponential growth rates, extending them from ca. 18 h to 25 h. This is reflected in the R analysis showing the growth rate of the control as 1.41 h-1 compared to those of the ssCFSs which were significantly reduced to between ~0.4-0.55 h^{-1}, with the largest effect coming from 5 h biofilm samples (**Table 13**). The doubling time was also considerably increased.

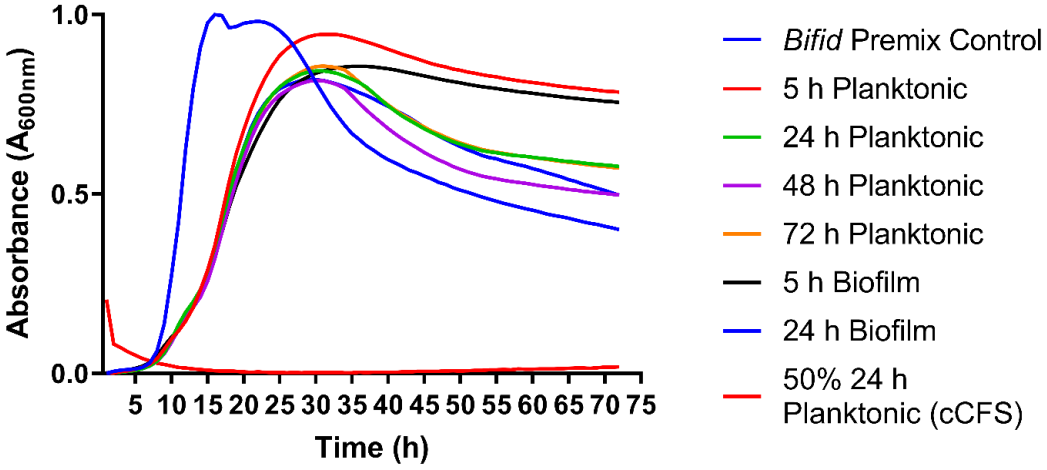


Figure 13A. Effect of *L. salivarius* CUL61 (5, 24, 48 and 72 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *Bifid* Premix (72 h) (*n* = 3).

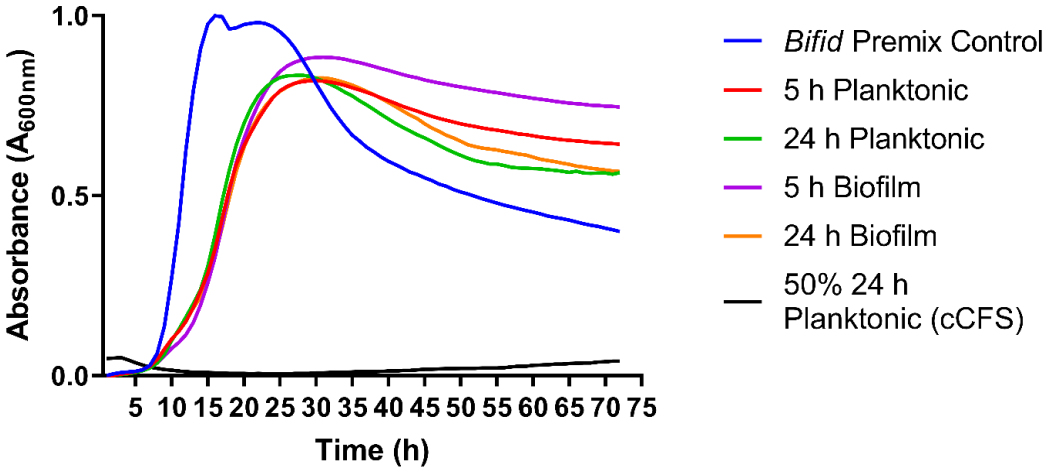


Figure 13B. Effect of *L. paracasei* CUL08 (5 and 24 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of *Bifid* Premix (72 h) (*n* = 3).

Table 13. Effect of ssCFS and 50% cCFS on growth characteristics for *Bifid* Premix (derived from the Growthcurver package in R).

CFS Type				Growth Characteristics		
Bacteria	**Conc.**	**Growth Type**	**Time-point (h)**	**Doubling Time (h^{-1})**	**Growth Rate (h^{-1})**	**MCC (K)**
L. sal	ss	B	5	2.17	0.32	0.81
			24	1.41	0.439	0.669
	ss	P	5	1.72	0.404	0.861
			24	1.52	0.455	0.692
			48	1.42	0.487	0.633
			72	1.54	0.449	0.694
	c		24	**0**	**0.022**	**0.006**
L. par	ss	B	5	1.59	0.444	0.814
			24	1.26	0.548	0.674
	ss	P	5	1.59	0.436	0.725
			24	1.26	0.548	0.674
	c		24	**24.2**	**0.029**	**0.075**
Growth control	N/A	N/A	N/A	0.49	1.41	0.648

ss, single strength, c, 50% concentrated, B, biofilm, P, planktonic, N/A, Not applicable, MCC, Maximum carrying capacity. Numbers in bold indicate substantial inhibitory zones (antimicrobial effects).

Despite ssCFS affecting the growth of the bacteria, the maximum population size was not significantly affected, and in comparison to the control, the final population size was slightly higher when treated with ssCFS. *L. salivarius* CUL61 and *L. paracasei* CUL08 cCFSs were able to inhibit the *Bifid*

Premix completely. It is unclear whether the slight increase in OD observed in **Fig 13B** at 30 h was due to regrowth of *Bifid* Premix or not.

3.5.1.5 Lab4 consortium growth curves

Like *L. paracasei* CUL08 in **Figs. 11A and 11B,** the growth curves for the untreated Lab4 consortium showed a very short lag phase (1 h) before entering exponential phase and with stationary phase beginning at ca. 11 h (**Figs. 14A and B**).

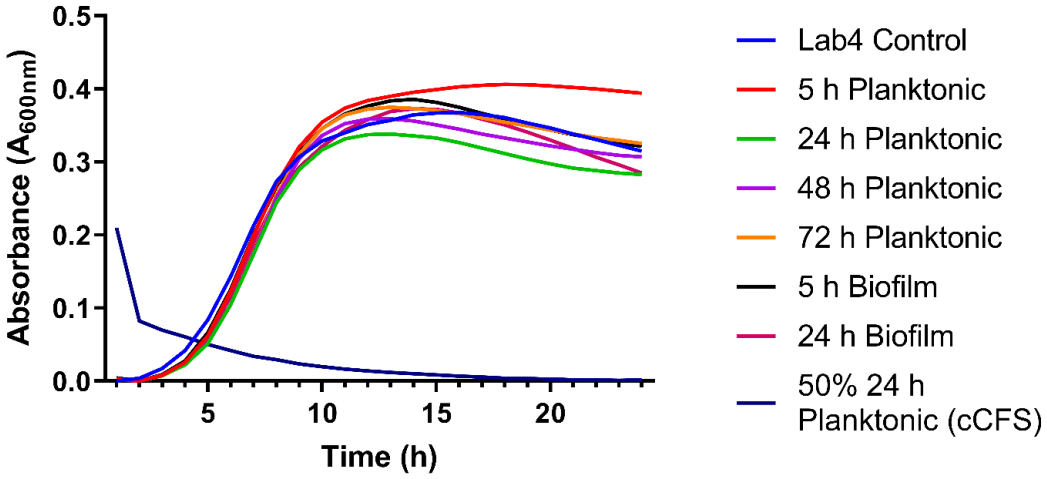


Figure 14A. Effect of *L. salivarius* CUL61 (5, 24, 48 and 72 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of Lab4 (24 h) (n = 3).

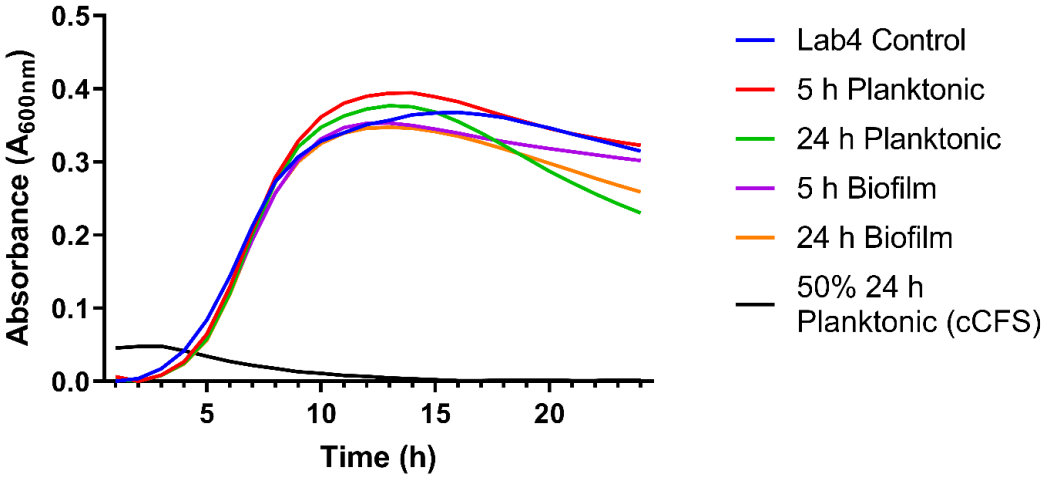


Figure 14B. Effect of *L. paracasei* CUL08 (5 and 24 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of Lab4 (24 h) (n = 3).

Table 14. Effect of ssCFS and 50% cCFS on growth characteristics of the Lab4 consortium (derived from the Growthcurver package in R).

CFS Type				Growth Characteristics		
Bacteria	**Conc.**	**Growth Type**	**Time-point (h)**	**Doubling Time (h^{-1})**	**Growth Rate (h^{-1})**	**MCC (K)**
L. sal	ss	B	5	0.76	0.912	0.358
			24	0.77	0.9	0.34
	ss	P	5	0.9	0.77	0.399
			24	0.67	1.036	0.313
			48	0.68	1.015	0.336
			72	0.71	0.97	0.355
	c		24	**0**	**0**	**0**
L. par	ss	B	5	0.71	0.974	0.331
			24	0.64	1.083	0.315
	ss	P	5	0.71	0.977	0.364
			24	0.59	1.173	0.322
	c		24	**0**	**0.028**	**0.005**
Growth control	N/A	N/A	N/A	0.85	0.82	0.349

ss, single strength, c, 50% concentrated, B, biofilm, P, planktonic, N/A, Not applicable. Numbers in bold indicate substantial inhibitory effect (antimicrobial effects).

Neither the *L. salivarius* CUL61 nor the *L. paracasei* CUL08 ssCFSs had much of an effect on doubling time or growth rate in comparison to the control (**Table 14**). Although the lag phase and exponential phase of the ssCFS gave very similar growth curves to the control, it was only once the

bacteria reached stationary phase that some distinct differences (to the final population sizes) were observed. The 5 h planktonic *L. salivarius* CUL61 ssCFSs showed the greatest maximum population sizes and faster growth rates (0.399 K and 0.977 respectively) for Lab4 in comparison to the control (0.349 K and 0.82 h-1 respectively). The 24 h planktonic ssCFSs showed the most inhibition of Lab4, as seen by the reduced maximum population size from 0.349 K (control) to 0.313 and 0.322 K (for *L. salivarius* CUL61 and *L. paracasei* CUL08 respectively). Again, 100% cell death was achieved by the 24 h planktonic cCFSs.

3.5.1.6 Lab4b consortium growth curves

The growth curves showed that the untreated Lab4b consortium had a 2 h lag phase before entering a relatively short exponential phase of ca. 4 h, followed by a lengthy stationary phase (17 h). Following treatment with *L. salivarius* CUL61 and *L. paracasei* CUL08 ssCFSs, the growth curves produced were very similar to those of *L. salivarius* CUL61 (seen in **Figs. 12A and B**), with little effect seen on bacterial growth compared to the control (**Figs. 15A and B**).

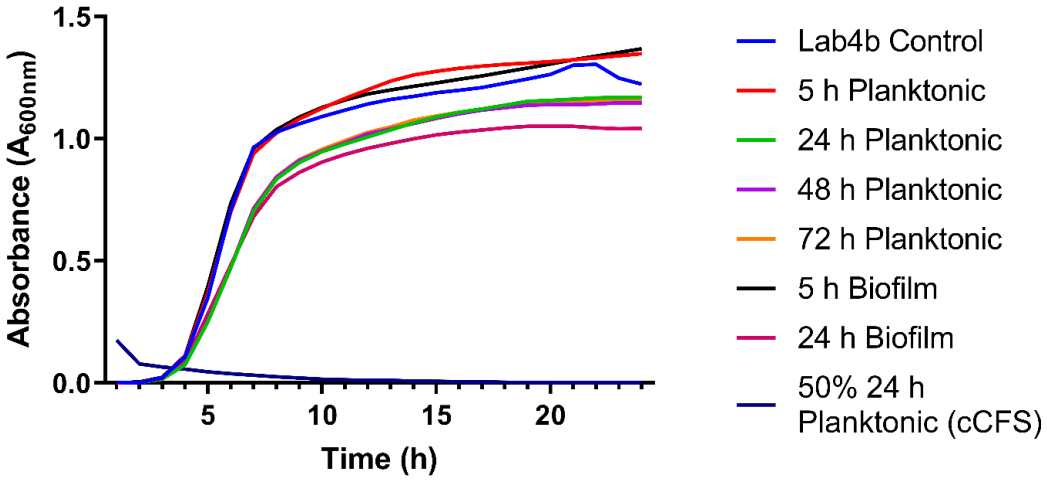


Figure 15A. Effect of *L. salivarius* CUL61 (5, 24, 48 and 72 h) ssCFS (planktonic and biofilm and cCFS (50% 24 h planktonic) on growth of Lab4b (24 h) (n = 3).

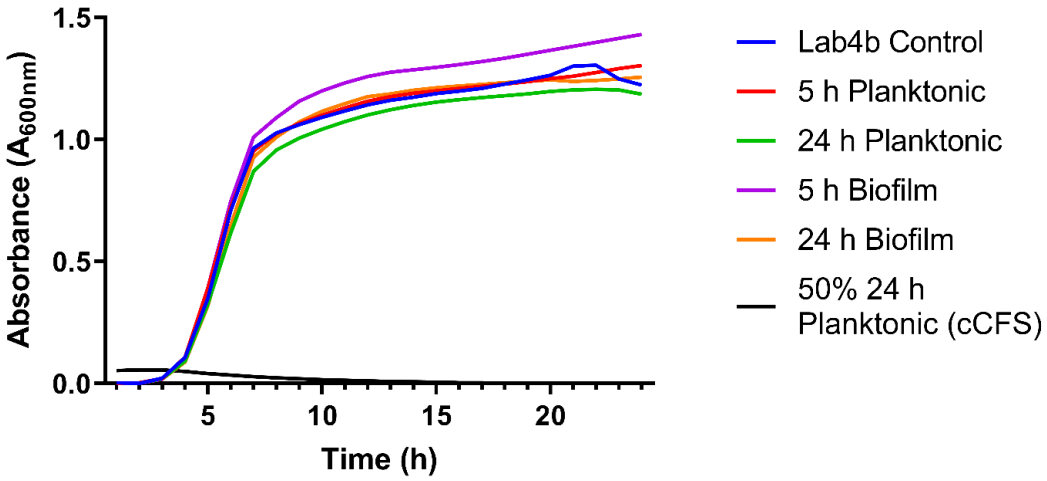


Figure 15B. Effect of *L. paracasei* CUL08 (5 and 24 h) ssCFS (planktonic and biofilm) and cCFS (50% 24 h planktonic) on growth of Lab4b (24 h) (*n* = 3).

This was particularly apparent for the *L. paracasei* CUL08 ssCFSs. In addition, the *L. salivarius* CUL61, 5 h planktonic and biofilm ssCFSs produced virtually the same growth as the control. In comparison**, Figs. 15A and B** and the Growthcurver output (**Table 15**), both showed that the 24, 48 and 72 h planktonic ssCFSs all had a slight inhibitory effect on Lab4b. Again, the 24 h planktonic cCFSs inhibited all Lab4b growth after 24 h.

Table 15. Effect of ssCFS and 50% cCFS on growth characteristics for the Lab4b consortium (derived from the Growthcurver package in R).

CFS Type				Growth Characteristics		
Bacteria	**Conc.**	**Growth Type**	**Time-point (h)**	**Doubling Time (h^{-1})**	**Growth Rate (h^{-1})**	**MCC (K)**
L. sal	ss	B	5	0.77	0.9	1.256
			24	0.89	0.775	1.019
	ss	P	5	0.84	0.827	1.278
			24	0.99	0.703	1.114
			48	0.93	0.749	1.101
			72	0.94	0.739	1.111
	c		24	**0**	**0**	**0.011**
L. par	ss	B	5	0.74	0.932	1.321
			24	0.7	0.992	1.21
	ss	P	5	0.71	0.978	1.21
			24	0.76	0.918	1.155
	c		24	**0**	**0.032**	**0.009**
Growth control	N/A	N/A	N/A	0.67	1.035	1.201

ss, single strength, c, 50% concentrated, B, biofilm, P, planktonic, N/A, Not applicable, MCC, Maximum carrying capacity. Numbers in bold indicate substantial inhibitory effect (antimicrobial effects).

A summary table of the main findings from the growth curve assays is given in **Table 16** showing the antagonistic effects of 24 h *L. salivarius* CUL61 and *L. paracasei* CUL08 planktonic cCFS against the individual probiotic bacteria, themselves and the two consortia. Overall, *L. salivarius* CUL61 24 h

planktonic cCFS caused 100% cell death against itself, *Acidophilus* Premix, *Bifid* Premix, Lab4 and Lab4b. However, against *L. paracasei* CUL08, there was re-growth after 3-5 h. In the case of *L. paracasei* CUL08 24 h planktonic cCFS, this treatment also caused 100% cell death against *L. salivarius* CUL61, *Acidophilus* Premix, Lab4 and Lab4b. Against itself, the effect was similar to that of 24 h *L. salivarius* CUL61 planktonic cCFS, and despite an immediate rapid decline in growth, there was good regrowth within 3-5 h. Similarly, the *Bifid* Premix also showed a slight regrowth but only after 30 h.

Table 16. Summary table showing the overall effects of 24 h *L. salivarius* CUL61 and *L. paracasei* CUL08 planktonic cCFS on planktonic bacterial growth in the growth curves.

cCFS Bacteria	Test strain(s)	Overall Effect
	L. paracasei CUL08	Time-limited cell death, followed by good regrowth after 3-5 h
L. salivarius CUL61	*L. salivarius* CUL61	100% cell death
	Acidophilus Premix	100% cell death
L. paracasei CUL08	*Bifid* Premix	100% cell death, (followed by slight regrowth after 30 h)*
	Lab4	100% cell death
	Lab4b	100% cell death

**L. paracasei* cCFS only

3.5.2 Minimum inhibitory concentrations (MIC) of *L. salivarius* CUL61 and *L. paracasei* CUL08

Table 17. MIC of *L. salivarius* CUL61 and *L. paracasei* CUL08 cCFS needed to inhibit themselves, probiotic bacteria and consortia (expressed as % cCFS)

Microorganism	24 h cCFS Tested (%)			
	L. salivarius		***L. paracasei* CUL08**	
	P	**B**	**P**	**B**
L. paracasei CUL08	**12.5**	**12.5**	50	**12.5**
L. salivarius CUL61	50	50	50	50
Acidophilus Premix	**12.5**	**12.5**	50	25
Bifid Premix	**12.5**	**6.25**	**12.5**	**12.5**
Lab4	25	25	25	25
Lab4b	50	50	50	50

50% indicates addition of 100% cCFS to two-fold Iso-sensitest: MRS broth (9:1). (1:1, v/v). P, planktonic, B, biofilm. MIC values for cCFS with the most effective antimicrobial activity (≤12.5%) are in bold.

The MIC results indicated that neither *L. salivarius* CUL61 nor *L. paracasei* CUL08 (planktonic or biofilm) ssCFS inhibited any of the strains tested including themselves (data not shown). However, after concentrating the CFS (100%) by lyophilisation, the cCFS of *L. salivarius* CUL61 and *L. paracasei* CUL08 were both able to inhibit all the strains tested including themselves (**Table 17**), although to varying degrees. When *Acidophilus* Premix was treated with cCFS, *L. salivarius* CUL61 cCFS (planktonic and biofilm) proved to be more effective at inhibiting bacterial growth than *L. paracasei* CUL08 cCFS (planktonic and biofilm), as the MICs were half that of *L. paracasei* CUL08 (50% and 25%, respectively) compared to 12.5% for both for *L. salivarius* CUL61 (**Table 17**). These results were very similar to those of *L. paracasei* CUL08 when also treated with cCFS, except against this strain, the

L. paracasei CUL08 cCFS (biofilm) inhibited *L. paracasei* CUL08 at a slightly lower concentration (12.5%). For *L. salivarius* CUL61, Lab4, and Lab4b, all *Lactobacillus* cCFS's had the same MIC values, indicating that for these bacterial strains and consortia, all cCFS carried the same inhibitory strength. All the cCFSs also inhibited the *Bifid* Premix, but *L. salivarius* CUL61 cCFS (biofilm) inhibited this strain at the lowest recorded concentration (6.25%). Interestingly, although the growth curves showed little difference between the biofilm and planktonic cCFS samples, the MIC revealed that, when there was a difference, although it was small, (one or two dilutions at most) the biofilm sample was always the more potent. i.e. *L. salivarius* CUL61 (biofilm) against the *Bifid* Premix; *L. paracasei* CUL08 (biofilm) against *Acidophilus* Premix and *L paracasei.*

3.5.3 Detection of inhibition of CFS by agar well-diffusion assay

Initial experiments to determine antimicrobial activity using the ssCFS in well diffusion assays proved unsuccessful. This was the case, regardless of any alterations to the protocol made, which included: use of different growth media such as MRS agar, Brucella Agar with 5% horse blood (CLSI, US standard) and Wilkins-Chalgren Agar with 5% horse blood (BSAC, UK standard); increasing the size of the well in the agar plates from 6 mm to 12 mm to increase the amount of ssCFS used in the assay (50 to 100 µl respectively); and whether the test bacteria were inoculated onto the agar surface followed by addition of the ssCFS samples to the wells or vice versa with pre-incubation of the ssCFS in the well for 1 h before inoculation of the test organism. Only after concentration of the ssCFSs and addition of the indicator bacteria to soft agar to make pour plates, could some antimicrobial activity be detected.

Using this adapted well-diffusion assay, it was observed that both *L. salivarius* CUL61 and *L. paracasei* CUL08 5 h and 24 h ssCFS and 5 h cCFSs still did not show any inhibition against the probiotic bacteria or either consortia (data not shown). However, this was not the case for the 24 h cCFSs. Here, both planktonic and biofilm *L. salivarius* CUL61 24 h cCFSs were found to inhibit both *Acidophilus* Premix and *L. paracasei* CUL08 (producing zones of inhibition ≥ 9.3 and ≤ 11.3 mm) when compared to the control (**Figs. 16A and**

B). *L. paracasei* CUL08 planktonic and biofilm 24 h cCFSs also inhibited *Acidophilus* Premix. However, in the case of *L. paracasei* CUL08 24 h planktonic cCFS versus *L. paracasei* CUL08 itself there was no inhibition seen (**Fig. 16**). Although, the *L. paracasei* CUL08 cCFSs demonstrated a slightly lower antimicrobial effect to the *L. salivarius* CUL61 cCFSs (producing zones of inhibition ≥ 9 and ≤ 9.9 mm).

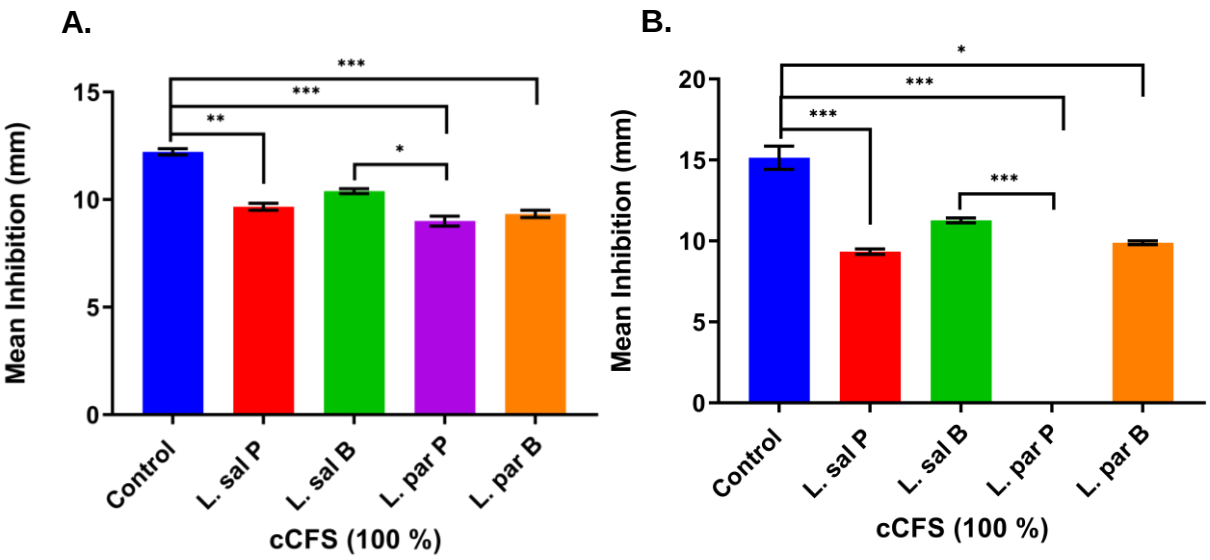


Figure 16. Mean inhibition of 24 h *L. salivarius* CUL61 planktonic (*L. sal* P) and biofilm (*L. sal* B) cCFS (100%) and *L. paracasei* CUL08 24 h planktonic (*L. par* P) and biofilm (*L. par* B) against (**A**) *Acidophilus* Premix and (**B**) *L. paracasei* CUL08 after 24 h. (*significantly different; *n* = 3; **P* < 0.05; ***P* < 0.001, ****P* < 0.0001).

Again, (as seen with the MICs) the biofilm cCFSs had a slightly stronger antagonistic effect in comparison to planktonic samples which were significant (**Figs. 16A and B**: *L. salivarius* CUL61 biofilm vs. *L. paracasei* planktonic *P* = 0.0124 and *P* = <0.0001, respectively). Furthermore, none of the 24 h cCFSs were able to inhibit *L. salivarius* CUL61, *Bifid* Premix, Lab4 or Lab4b in this assay, despite inhibition being seen in other experiments.

3.6 Anti-biofilm effects of cCFS using biofilm formation assays and Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) images of single (*L. paracasei* CUL08*, L. salivarius* CUL61) and mixed-species (*Bifid* Premix, Lab4b) biofilms

grown in glass-bottomed 96-well plates and then stained with LIVE/DEAD cell viability dyes highlighted that the control (untreated) wells produced a high bacterial biomass (**Figs 17** and **18**). A small number of dead (red) cells were evident in all the biofilms, although these were greatest in the case of the *Bifid* Premix and Lab4b biofilms (**Figs 17C; 18D**).

The effect of 12.5% 24 h planktonic *Lactobacillus* cCFS was tested in biofilm formation assays against single species of LAB (*L. salivarius* CUL61 and *L. paracasei* CUL08), dual-species (*Bifid* Premix) and mixed-species (Lab4 and Lab4b consortia) biofilms. The CLSM images following treatment with 12.5% *L. salivarius* CUL61 and *L. paracasei* CUL08 24 h planktonic cCFS (quantified using COMSTAT image analysis software) showed a distinct reduction in live bacterial biofilm biomass in both the *Bifid* Premix (**Figs. 17A** and **C**) and *L. salivarius* CUL61 (**Figs. 18A** and **C**) biofilms, with the greatest antimicrobial effect on the *Bifid* Premix biofilms (<0.24 $\mu m^3/\mu m^2$ and <0.20 $\mu m^3/\mu m^2$ respectively).

However, these same effects for either 12.5% *L. salivarius* CUL61 or *L. paracasei* CUL08 24 h planktonic cCFSs, were not seen in the *L. paracasei* CUL08 or Lab4b biofilms (**Figs 17D** and **18D**), where the bacterial biomass was much higher in comparison, indicating that the antimicrobial treatment was far less effective. Unfortunately, the *L. paracasei* CUL08 control in **Fig 17B and D** showed an unusually low bacterial biomass. After repeating the experimental conditions and staining the biofilm with crystal violet, it became apparent that the biofilm had been sloughed from the surface of the control, and that the reduced biomass seen in the control was likely to be an artefact caused by sample preparation for the CLSM imaging, possibly due to the washing steps. These crystal violet staining results (**Fig 19**) demonstrated that the biomass of the control was larger as expected, and that the *L. paracasei* CUL08 biofilms were unaffected by treatment with 12.5% *Lactobacillus* 24 h planktonic cCFS (n = 3). Both treatments had similar effects on the bacterial biomass of the multi-species Lab4b biofilms (**Figs 17B** and **D**).

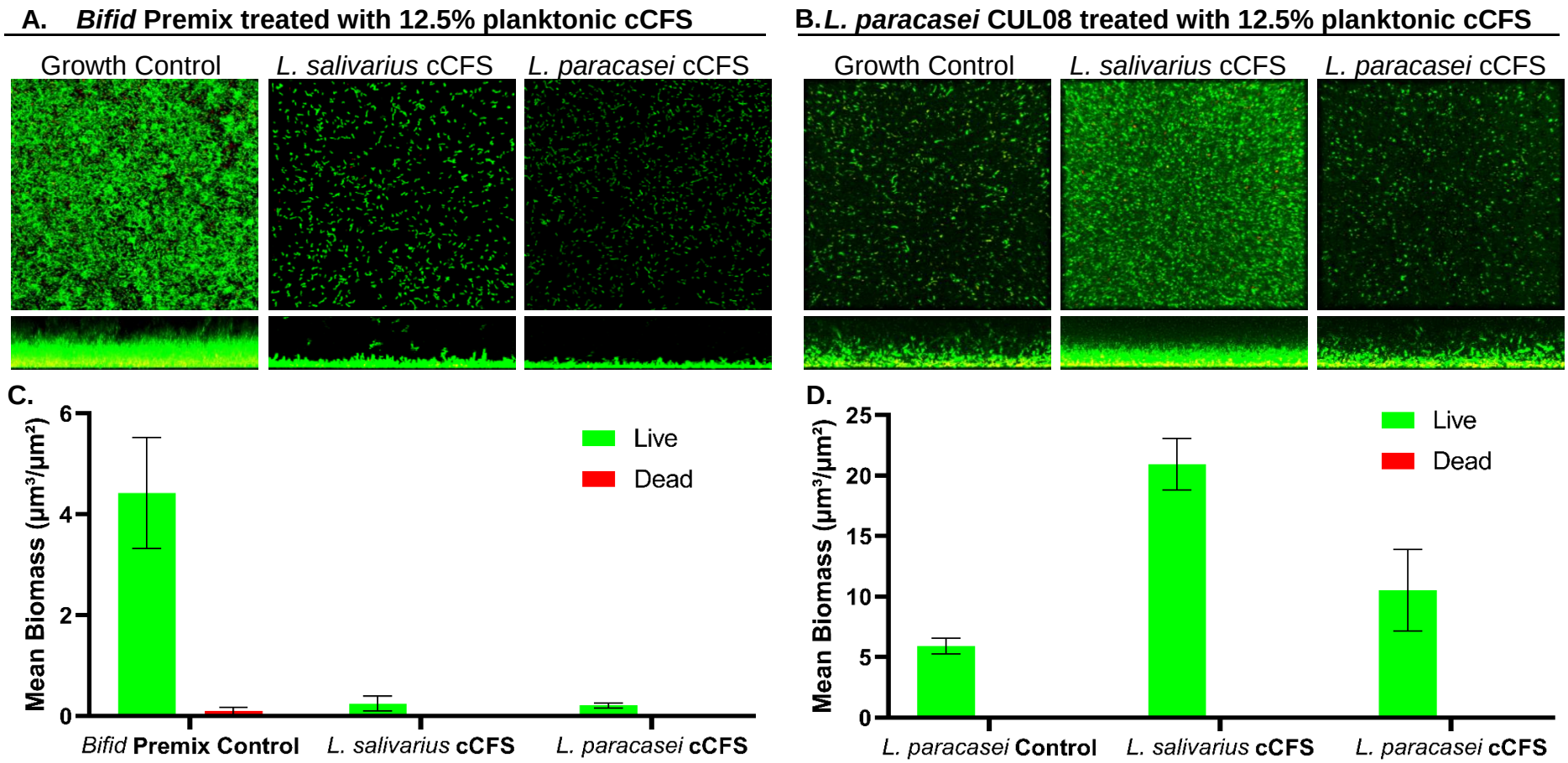


Figure 17. CLSM images of LIVE/DEAD staining of 24 h single and dual-species biofilms grown with and without treatment of 12.5% 24 h planktonic *Lactobacillus* cCFS (*L. salivarius* CUL61 cCFS and *L. paracasei* CUL08 cCFS): (**A**) *Bifid* Premix biofilms. (**B**) *L. paracasei* CUL08 biofilms. Comstat analysis showing mean biofilm biomass of LIVE (green) and DEAD (red) cells (*n* = 5 images each from *n* = 1 replicate): (**C**) *Bifid* Premix biofilms. (**D**) *L. paracasei* CUL08 biofilms. (Error bars depict SD).

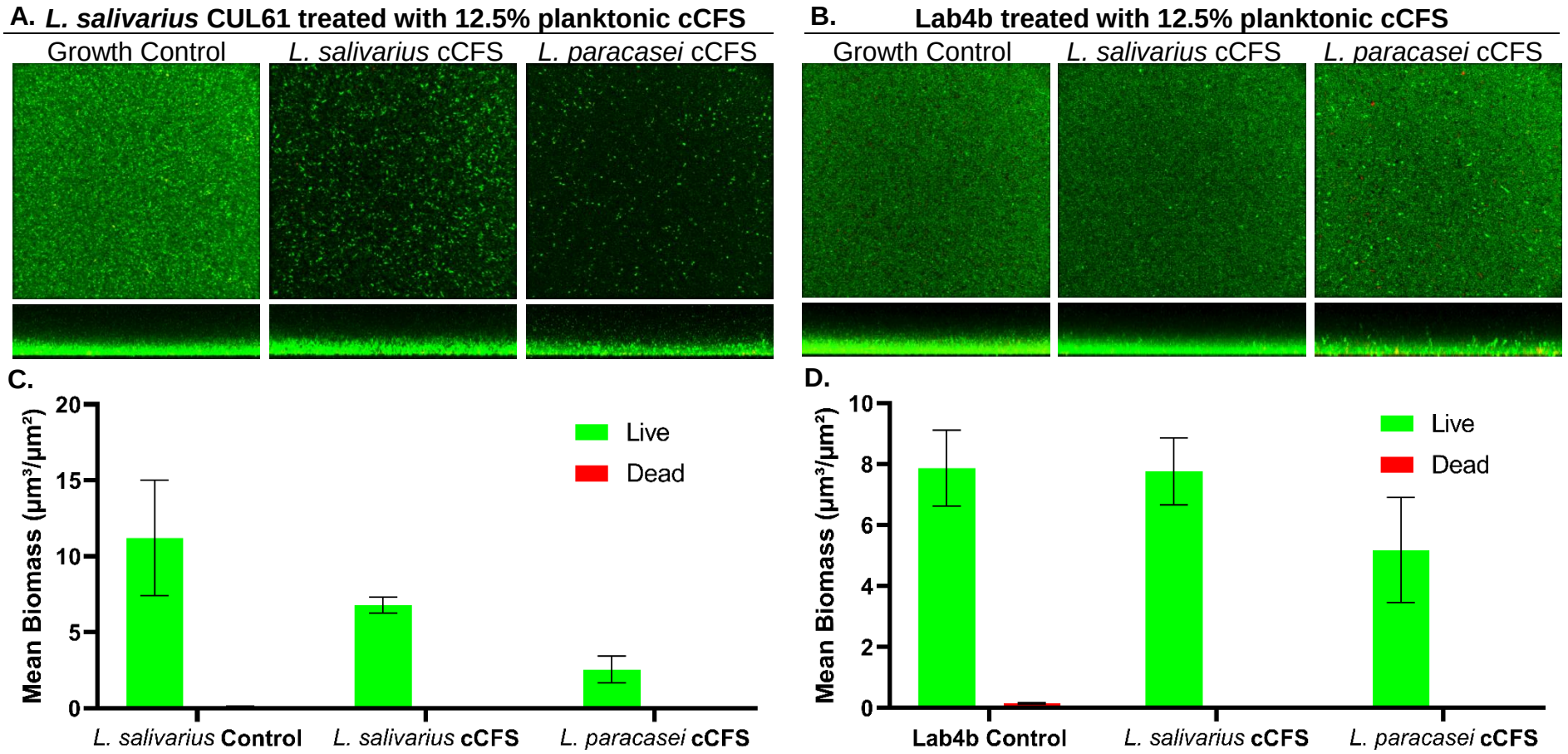


Figure 18. CLSM images of LIVE/DEAD staining of 24 h single and mixed-species biofilms grown with and without treatment of 12.5% 24 h planktonic *Lactobacillus* cCFS (*L. salivarius* CUL61 cCFS and *L. paracasei* CUL08 cCFS): (**A**) *L. salivarius* CUL61 biofilms. (**B**) Lab4b biofilms. Comstat analysis showing mean biofilm biomass of LIVE (green) and DEAD (red) cells (n = 5 images each from n = 1 replicate): (**C**) *L. salivarius* CUL61 biofilms. (**D**) Lab4b biofilms. (Error bars depict SD).

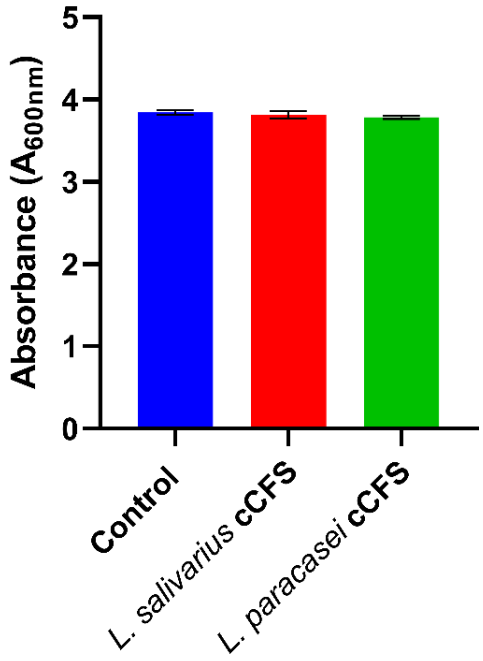


Figure 19. Confirmatory test of the biofilm biomass of *L. paracasei* CUL08 (Blue; *L. paracasei* CUL08 growth control, Red; *L. paracasei* CUL08 treated with 12.5% *L. salivarius* CUL61 24 h planktonic cCFS, Green; *L. paracasei* CUL08 treated with 12.5% *L. paracasei* CUL08 24 h planktonic cCFS) determined using crystal violet staining (*n* = 3).

Overall, in the biofilm formation assay, 12.5% *L. paracasei* CUL08 24 h planktonic cCFS demonstrated the biggest antagonistic effect on biofilm biomass of *Bifid* Premix, *L. salivarius* CUL61 and Lab4b, but against itself there was no effect. Also, 12.5% *L. salivarius* CUL61 24 h planktonic cCFS inhibited *Bifid* Premix and itself at this concentration although perhaps using a bigger concentration would have had a bigger anti-biofilm effect against *L. paracasei* CUL08 and Lab4b.

3.7 Genome mining of CFS bacterial strains for putative bacteriocin genes

Bacteriocin producers can be positively identified through *in silico* investigations using database tools like BAGEL4 and BACTIBASE. BAGEL4 mines bacterial genomic DNA for putative bacteriocin genes, RiPPs and even immunity genes and transporters. FASTA files of *L. salivarius* CUL61 and *L.*

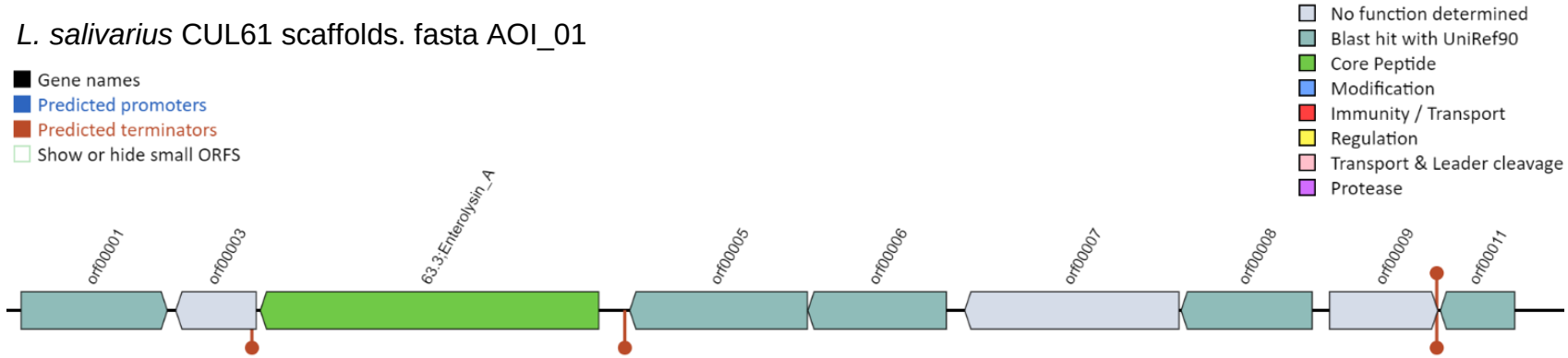
paracasei CUL08 were uploaded to the online database where they were cross-referenced against known bacteriocin genetic clusters. A single area of interest (AOI) was identified in the genome of *L. salivarius* CUL61 (**Fig 20 A**), while three AOI's were found in *L. paracasei* CUL08 (**Figs. 21A, B** and **C**). The AOI for *L. salivarius* CUL 61 identified a gene cluster for a single bacteriocin, (enterolysin A) on contig 10 of the genome sequence.

The first AOI (AOI 1) for *L. paracasei* CUL08 (**Fig 21A**) identified five genes encoding for bacteriocins on contig 9 of its genome sequence. These included, carnocin CP52 and enterocin X (beta chain) which appears twice in two different positions of the genome (**Figs 21A)**. As well as this, two genes that code for bacteriocins that are both named bacteriocin IIc (orf00023 and orf00049; **Fig 21A** and **D**) indicating that these genes code for a class IIc bacteriocin but have yet to be investigated further and named.

In the second AOI (AOI 2) for *L. paracasei* CUL08, a gene cluster for a bacteriocin called LSEI 2163 was found in the genome sequence (**Figs. 21B** and **E**). Additionally, two genes (entA-Immun) were identified that encode for immunity proteins and a third gene (*HlyD*) was found that is involved in protein transportation (**Figs. 21B** and **E**).

The third AOI (AOI 3) given from the BAGEL4 analysis output, identified no gene clusters encoding for putative bacteriocins (green) or immunity peptides/transport (red) on contig 23 of the genome sequence, although BAGEL4 did indicate an unknown gene (orf00029) representing an uncharacterised protein that could encode for a class of bacteriocins known as the sactipeptides (**Figs. 21C** and **F**).

A.

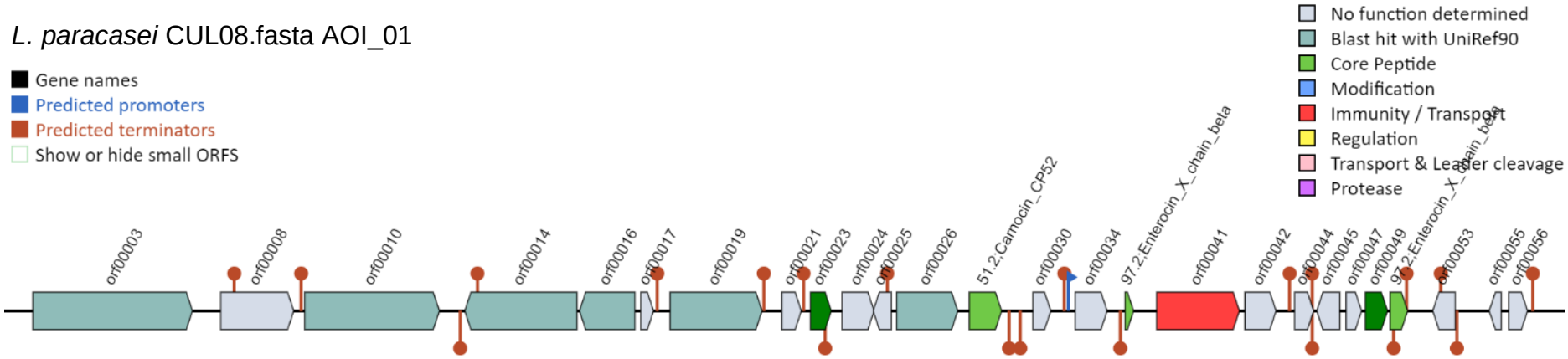


B.

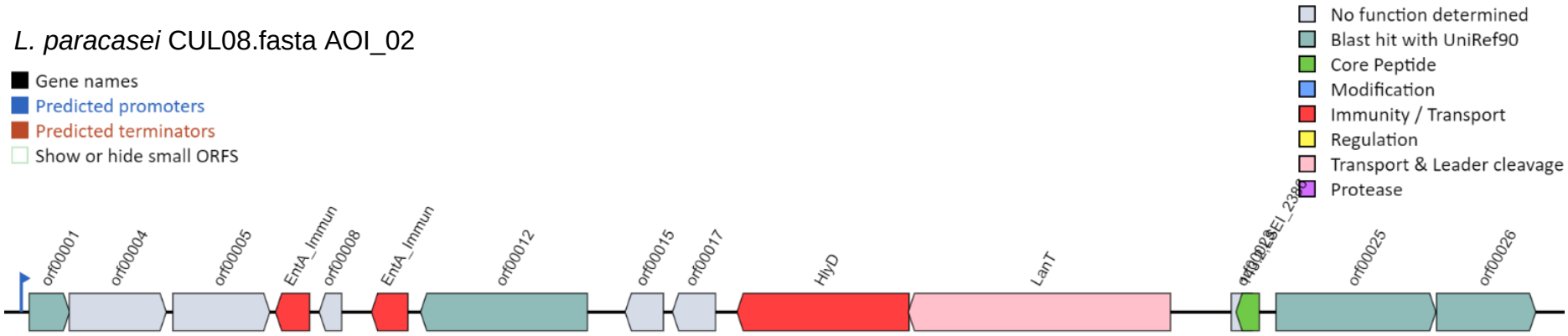
Name	Function	Motifs
orf00001	Quinolone resistance protein NorA	
orf00003		
63.3;Enterolysin_A	63.3;Enterolysin_A	
orf00005	Putative O-antigen transporter	
orf00006	UDP-galactopyranose mutase	
orf00007		
orf00008	Lipopolysaccharide 1,6-galactosyltransferase	
orf00009		
orf00011	Beta-1,6-galactofuranosyltransferase WbbI	

Figure 20. Output from BAGEL4 depicting a putative bacteriocin gene on the genome of *L. salivarius* CUL61 (contig 9) (**A**) AOI 1 (**B**). Open reading frame (orf) function. Genes encoding core peptides (bacteriocins) indicated (green; **A** and **B**).

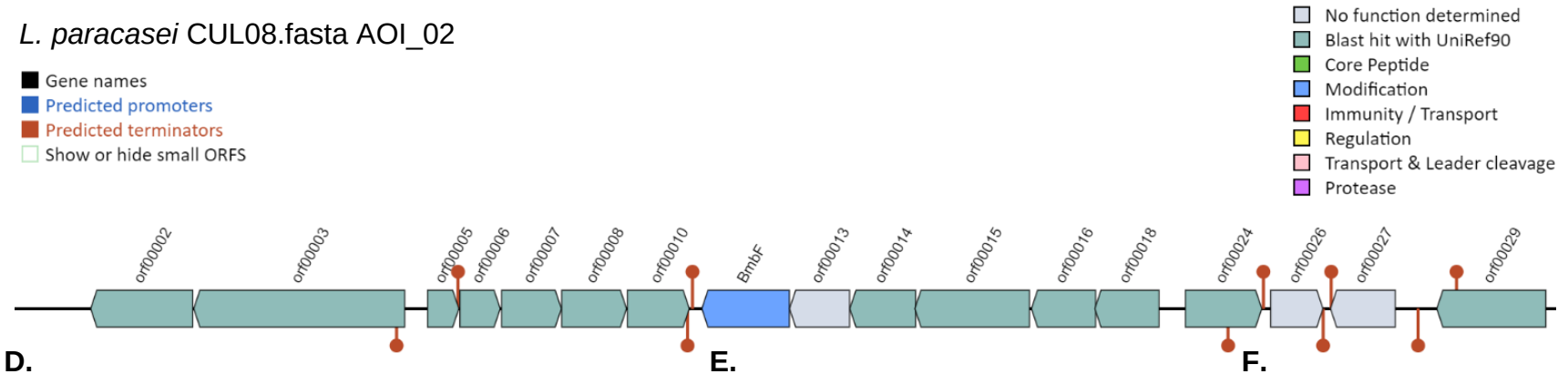
A.


L. paracasei CUL08.fasta AOI_01
Gene names
Predicted promoters
Predicted terminators
Show or hide small ORFS
No function determined
Blast hit with UniRef90
Core Peptide
Modification
Immunity / Transport
Regulation
Transport & Leader cleavage
Protease
orf00003
orf00008
orf00010
orf00014
orf00016
orf00017
orf00019
orf00021
orf00023
orf00024
orf00025
orf00026
51.2:Carnocin_CP52
orf00030
orf00034
97.2:Enterocin_X_chain_beta
orf00041
orf00042
orf00044
orf00045
orf00047
orf00049
97.2:Enterocin_X_chain_beta
orf00053
orf00055
orf00056

B.


L. paracasei CUL08.fasta AOI_02
Gene names
Predicted promoters
Predicted terminators
Show or hide small ORFS
No function determined
Blast hit with UniRef90
Core Peptide
Modification
Immunity / Transport
Regulation
Transport & Leader cleavage
Protease
orf00001
orf00004
orf00005
EntA_Immun
orf00008
EntA_Immun
orf00012
orf00015
orf00017
HlyD
LanT
orf00025
orf00026

C.



D.

Name	Function
orf00003	Divalent metal cation transporter MntH
orf00008	
orf00010	Fumarate hydratase class II
orf00014	Probable NADH-dependent flavin oxidoreductase YqiG
orf00016	Uncharacterized oxidoreductase SSP1627
orf00017	
orf00019	Oxidoreductase YdhF n=471 RepID=YDHF_ECOLI
orf00021	
orf00023	Bacteriocin_IIc;
orf00024	
orf00025	
orf00026	Probable maltose O-acetyltransferase
51.2;Carnocin_CP52	51.2;Carnocin_CP52
orf00030	
orf00034	
97.2;Enterocin_X_chain_beta	97.2;Enterocin_X_chain_beta
orf00041	P71468_LACPL PlnI (Immunity protein PlnI, membrane-bound protease CAAX family)
orf00042	
orf00044	
orf00045	
orf00047	
orf00049	ComC; Bacteriocin_IIc;
97.2;Enterocin_X_chain_beta	leaderLanM; ComC; Lactococcin; L_biotic_typeA; Bacteriocin_IIc; 97.2;Enterocin_X_chain_beta

E.

Name	Function
orf00001	hypothetical protein pMRC01_047
orf00004	
orf00005	
EntA_Immun	Putative bacteriocin immunity protein -
orf00008	
EntA_Immun	SpiA (Putative sakacin P immunity protein) (OrfY protein)
orf00012	Uncharacterized protein HI_0108
orf00015	
orf00017	
HlyD	Accessory factor for ABC-transporter PlnH
LanT	Transport/processing ATP-binding protein ComA
orf00023	
143.2;LSEI_2386	143.2;LSEI_2386
orf00025	Bacteriocin production related histidine kinase
orf00026	response regulator PlnD

F.

Name	Function
orf00002	RNA polymerase sigma-54 factor
orf00003	Transcriptional regulatory protein LevR
orf00005	PTS system mannose-specific EIIAB component
orf00006	PTS system mannose-specific EIIAB component
orf00007	PTS system sorbose-specific EIIC component
orf00008	PTS system mannose-specific EIID component
orf00010	Acetyl esterase
BmbF	Anaerobic sulfatase-maturating enzyme
orf00013	
orf00014	Serine/threonine-protein kinase pkn1
orf00015	Arylsulfatase
orf00016	PTS system mannose-specific EIID component
orf00018	PTS system sorbose-specific EIIC component
orf00024	HTH-type transcriptional regulator GalR
orf00026	
orf00027	
orf00029	Uncharacterized protein ORF8 in nfe locus (Fragment)

Figure 21. Output from BAGEL4 depicting putative bacteriocin genes on the genome of *L. paracasei* CUL08 (**A**) AOI 1 (contig 9) (**B**) AOI 2 (contig 22) (**C**) AOI 3 (contig 23). Open reading frame (orf) function (**D**) AOI 1, (**E**) AOI 2 and (**F**) AOI 3. Genes encoding core peptides (bacteriocins) indicated (green; **D** and **E**), immunity peptides and transport (red; **E**).

After discovering that *L. salivarius* CUL61 and *L. paracasei* CUL08 contained bacteriocin gene clusters and immunity peptides (*L. paracasei* CUL08 only), it was possible to then characterise these bacteriocins further by using the web-based database BACTIBASE by searching for the gene name that was found from the BAGEL4 output, on the database. A summary of these findings are shown in **Table 18** and additional supplementary material can be found in Appendix 3.

Table 18. BACTIBASE output of bacteriocins identified from BAGEL4 analysis.

Bacterial stain	**Bacteriocin**	**Bacteriocin classification**	**Originating producer bacterial species**
L. salivarius CUL61	Enterolysin A	class III	*Enterococcus faecalis*
L. paracasei CUL08	Carnocin CP52	class IIa	*Carnobacterium piscicola*
L. paracasei CUL08	Enterocin X (beta)	class IIb	*Enterococcus faecium*

Chapter Four

Discussion

4 Discussion

Cultech manufactures probiotic supplements and as the popularity in probiotics increases, so do customer demands. Combinations of microorganisms are frequently requested, and the generation of multi-strain probiotic supplements is required. These multi-strain products are generated from commercially produced pure strain inocula. However, when combined, they can demonstrate a significant reduction in bacterial numbers when compared against the predicted bacterial input of the original raw material. However, this is not the first case of multi-strain probiotic products that produce viable bacterial counts lower than expected using agar-based plate count techniques. Kumar and Ghosh (2019) highlighted that this effect can be linked to competition for nutrients among the bacterial strains present which inhibits growth and it could be the presence of a bacteriocin from bacteria in the products which causes inhibition of growth (Kumar and Ghosh 2019).

To ensure regulatory compliance, a significant additional microbial input is added to these products, to compensate for the poor recovery and ensure that the label claim is still met at the end of the shelf-life. This is a huge economic burden to the company. It was hypothesised that the lower bacterial counts observed might be due to the presence of antimicrobial peptides such as bacteriocins produced by LAB themselves. The Lab4b consortium is comprised of four bacterial strains and the present study aimed to determine if two of the strains, *L. paracasei* CUL08 and *L. salivarius* CUL61, found in Lab4b, were the cause of this underestimation of bacteria numbers. It has previously been reported in the literature that both these LAB strains produced bacteriocins (Pangsomboon et al 2006; Barrett et al 2007; Vera et al 2009; Ge et al 2016).

To determine the antagonistic activities of the probiotic bacteria at Cultech Ltd, the bacteria were initially screened for their antibacterial activity using the perpendicular streak plate assay. This assay highlighted that the *Bifid* Premix was the most sensitive indicator. Also, that the mixed consortia, Lab4b, was the most active against the other probiotic bacteria and itself, with *L. salivarius* CUL61 having similar results and *L. paracasei* CUL08 closely behind. Chapman et al (2012), used a similar cross-streaking assay and found

that probiotic strains were able to inhibit each other, and specifically, that lactobacilli showed the greatest inhibition of the probiotic species tested (Chapman et al 2012). Other researchers had previously found that *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 were able to inhibit both Gram-positive and Gram-negative bacteria using this method (Coman et al 2014). The streak plate method proved to be more sensitive than the ssCFS samples used in the agar-well diffusion assay. However, the antibacterial activity observed on the perpendicular streak plates was produced from live cells, so this will have comprised of many inhibitory compounds such as lactic acid, acetic acid, diacetyl etc. Therefore, the observed antibacterial activity was enhanced by these other compounds and cannot have arisen solely because of putative bacteriocins alone (Lertcanawanichakil and Sawangnop 2008). Nevertheless, it gave a good initial screening into which bacteria could be responsible for the plate count inhibition.

To try and distinguish any effects arising from putative bacteriocins alone, MRS agar was supplemented with bromocresol purple indicator, and these plates were used to determine how much of the inhibition was due to the acid-effect. Acid diffusion (i.e. produced by the LAB) could be successfully visualised and quantified from the single streak inoculation by the colour change of the agar from purple to yellow. Subsequently subtracting these values from those of the perpendicular streak plate, this experiment highlighted that *L. paracasei* CUL08, *L. salivarius* CUL61, *Acidophilus* Premix, Lab4 and Lab4b consortia, secreted other antibacterial products that were not due to acid-effects alone. It was clear at this point that *L. salivarius* CUL61 and *L. paracasei* CUL08 were involved in the poor recovery of bacteria in plate counts of the Lab4b consortium as they as they had the largest antibacterial effect against the other probiotic bacteria, especially the *Bifid* Premix. In addition to this, this assay emphasised the need to neutralise the pH of CFSs to ensure that any antibacterial effects seen in future assays, were not from acid origin.

When harvesting CFSs from bacterial cultures for putative bacteriocin extraction, it has previously been noted that optimal production is significantly influenced by the culture conditions used (temperature, pH, incubating time, medium). Bacteriocin production is regulated by QS and this typically occurs

in stationary phase of bacterial growth, as reported by other researchers (Guerra and Pastrana 2003; Todorov and Dicks 2004). After analysing the growth curves of *L. salivarius* CUL61 and *L. paracasei* CUL08, it was identified that late exponential, early stationary phase was achieved at the 24 h time point. In contrast, other studies have found that bacteriocin expression would not occur in earlier time points (Barman et al 2018). As a result, initial CFSs were extracted at 5 h (to act as a no bacteriocin control) and 24 h from both *L. salivarius* CUL61 and *L. paracasei* CUL08. Additional time-points for *L. salivarius* CUL61 were harvested at 48 and 72 h to determine if antibacterial effects continued after stationary phase. Similar time course studies have also been used by other researchers with some success (Martinez et al 2013; Lü et al 2014; Georgieva et al 2015; An et al 2017).

Malheiros et al (2015) found that the optimum temperature and pH for bacteriocin production from *L. sakei* (2a) was between 25°C and 30°C, but only when the pH of the MRS broth was between pH 5.0 and 5.5. In addition, at temperatures above 37°C, no bacteriocin was detected (Malheiros et al 2015). Furthermore, several studies have reported that MRS broth was the most optimal medium for bacteriocin production by LAB due to the high glucose content (Todorov and Dicks 2004; De Kwaadsteniet et al 2005). Yang et al (2018) disclosed that the highest bacteriocin production occurred when LAB (*L. curvatus* [Arla-10], *E. faecium* [JFR-1], *L. paracasei* subsp. paracasei [JFR-5] and *S. thermophilus* [TSB-8]) were grown in MRS broth, at pH 6.2, and 37°C (Yang et al 2018). This led to the use of MRS broth at a pH of 6.2 and an incubation temperature of 37°C in this study. Additionally, both planktonic and biofilm modes of growth were chosen as factors to explore in this study to determine if the antibacterial effects, differed markedly in either.

Initially extracted 24 h ssCFS showed some antibacterial effects in growth curves assays by extending the lag phase of bacterial growth and reducing the bacterial population slightly. Similar inhibitory effects have previously been noted by others, for example, *L. kefiranofaciens* DH101 was found to prolong the lag phase of *C. sakazakii* ATCC 29544 by 3 h (Kim et al 2018). The effect of 5 h samples was opposite to that of the 24 h samples, and in contrast, promoted growth of the indicator bacteria. One reason for this could be that 5 h ssCFS would contain a higher nutrient content, as ≥ 24 h

CFSs had longer incubation times, propagating a larger number of bacterial cells, resulting in a higher volume of spent medium and potentially, a higher concentration of bacteriocins. In agreement with the latter, other researchers have found that a co-culture of inducer bacteria such as *L. crispatus* NRRL B-30884 (LWP 252) and *L. acidophilus* NRRL B-30510 (LWP 320) with signal peptides and low concentrations of growth media can greatly enhance bacteriocin production (Svetoch et al 2010). This observation suggests that when bacteria are subjected to stress, such as competing for resources and starvation conditions, bacteria will develop strategies to adapt and survive. This is likely to have been the case in the 24, 48 and 72 h CFS samples, as the cultures would be in stationary phase, therefore competition between the bacteria would be increased. Remarkably, bacteria use bacteriocins to compete with not only unrelated or closely related bacteria, but also to attack genetically identical cells (sibling cells) within the same colony. This is termed 'fratricide' and could explain why *L. salivarius* CUL61 and *L. paracasei* CUL08 were also able to inhibit themselves (Be'er et al 2009).

No inhibition was found using ssCFS in the well-diffusion assays, despite efforts to change/optimise the protocol, and the evidence from the growth curves assays showing that antimicrobial activity was definitively present. This prompted the change in protocol to concentrate the ssCFSs by lyophilisation. It was only then that antibacterial effects were confirmed, with complete inhibition observed against *L. salivarius* CUL61, *Acidophilus* Premix (*L. acidophilus* CUL60 & 21), Lab4 and Lab4b in the growth curves assays by planktonic and biofilm *L. salivarius* CUL61 and *L. paracasei* CUL08 cCFSs. Similar findings were reported by Arena et al (2016), who found that CFS harvested from *L. plantarum* exhibited no inhibition against the pathogenic bacteria tested until it was concentrated 10-fold (Arena et al 2016).

After analysing the *L. salivarius* CUL61 ssCFS and cCFS growth curve results, the most dramatic differences were seen between 5 h and 24 h CFSs. In comparison, the 24, 48 and 72 h time points proved to be very similar in their antimicrobial activity. So, due to time constraints of the project, the 48 and 72 h planktonic CFSs were not investigated further in this project. Instead, looking at 5 h and 24 h CFS samples from *L. salivarius* CUL61 and *L.*

paracasei CUL08 it was evident (at least at these growth conditions) that the antimicrobial substance production was optimal at stationary phase (≥ 24 h).

The results from the well diffusion, growth curve and microbroth dilution assays suggested that *L. salivarius* CUL61 and *L. paracasei* CUL08 could be harbouring putative bacteriocins with broad-spectrum activity. This coincides with another study that used neutralised CFS from *L. plantarum* which was shown to be able to inhibit a wide range of *Lactobacillus* species including *L. acidophilus, L. rhamnosus, L. leishmanii, L. plantarum* G1 and *L. casei* G3 (Seatovic et al 2011).

Comparing all results from the susceptibility testing highlighted the fact that antagonistic effects of the CFS were not always consistent, even in different assays. In the assays using liquid-medium, the indicator bacteria and consortia tested were sensitive to ssCFS (growth curve assays) and cCFS treatment (growth curves and microbroth dilution assays). On the other hand, in solid-medium (agar), these antibacterial effects were only seen against *L. paracasei* CUL08 and *Acidophilus* Premix, despite the use of 100% cCFS in this assay, equivalent to double the concentration of cCFS used in the microbroth dilution assays, i.e. 50%. Other investigators have had similar findings (Saadatzadeh et al 2013). The reason for this could be due to simple diffusion effects, where if the antimicrobial substance was unable to completely diffuse through the agar, which might be a particular problem for larger (>10 KDa) class III bacteriocins, one of which was identified in the genome of *L. salivarius* CUL61 (enterolysin A). Additionally, the interaction of the bacteriocins with specific components of the media can reduce its antimicrobial activity (Arena et al 2016; Azevedo et al 2018). Comparing the results from growth curve, well diffusion and microbroth dilution assays highlights the importance of using more than one bioassay to analyse the potential antimicrobial activity of putative bacteriocins in LAB strains to achieve valid and reliable results.

Interestingly, in the planktonic assays (growth curves and microbroth dilution assays), *L. salivarius* CUL61 cCFSs showed a greater antagonistic effect compared to *L. paracasei* CUL08 cCFSs. However, in the biofilm formation assay, this was not the case. Instead, *L. paracasei* CUL08 planktonic 24 h cCFS reduced the biomass of the biofilms considerably more.

This was the case even though the concentration used (12.5%) was lower than that of the MIC (50%); essentially the concentration required to inhibit planktonically grown *L. salivarius* CUL61, *L. paracasei* CUL08 and Lab4b. The antibiofilm action of lactobacilli is due to their capability to compete and prevent the adherence of pathogenic microorganisms to biological surfaces such as in the gut (Monteagudo-Mera et al (2019). It has been discovered that they do this by secreting metabolites which contain biosurfactants that decrease the hydrophobicity of the surface substratum, which in turn prevent advancements in adhesion and desorption (Rodrigues et al 2006). Sambanthamoorthy et al (2014) reported for the first time that biosurfactants harvested from *L. jensenii* and *L. rhamnosus* were able to significantly reduce the development of *A. baumannii*, *E. coli* and *S. aureus* biofilms (Sambanthamoorthy 2014). According to Mathur et al (2018), lower concentrations of an antimicrobial are required to inhibit biofilm formation compared to disruption of a fully established biofilm, so it would also be interesting to see whether these cCFSs are effective against established biofilms (Mathur et al 2018).

Importantly, the results of the BAGEL4 analysis directly supported the hypothesis that the inhibitory effects seen in agar plate counts could be due to bacteriocins secreted by these bacteria. *L. paracasei* CUL08 was found to carry several class II bacteriocins and *L. salivarius* CUL61*, a* class III bacteriocin. It was not yet possible to determine if any of the identified bacteriocin genes were expressed under the CFS culture conditions chosen in this study, given the time constraints of the project. However, if time allowed, it would be worth following-up with molecular methods to investigate gene expression of enterolysin A in *L. salivarius* CUL61 and carnocin CP5 and enterocin X (beta) in *L. paracasei* CUL08. Also, as different genes are up-regulated (via QS pathways) in biofilm versus planktonic culture and with this study already showing that the biofilm CFSs had slightly higher antibacterial effects, investigating gene expression under these different growth conditions would also be useful.

Other future investigations of this project could involve looking into the growth curves for the *Bifid* Premix treated with *L. paracasei* CUL08 cCFS. *Bifid* Premix showed a slight upturn in growth at 30 h after virtually complete inhibition. It was unclear whether this was actual regrowth of *Bifid* Premix or

not. Given more time, it would have been good to investigate this further and extend the growing time to see whether the *Bifid* Premix could recover and might be explored as part of future studies. Additionally, this could highlight whether the antibacterial effect seen was bacteriostatic or bactericidal, which could help to determine the actual class of bacteriocin involved. Another technique that could be useful in determining the mode of action of the antimicrobial substance is Scanning Electron Microscopy (SEM), which might be able to observe the direct effect on cell morphology.

As well as this, due to time and resource constraints (insufficient cCFS remained to do repeats) the biofilm formation assay was only completed once (n = 1), so to draw clearer conclusions, further repeats are required.

Future studies could perform larger-scale isolation or purification of the growth by-products and characterisation of their specific properties. Given that *L. salivarius* CUL61 was found to only encode a single bacteriocin, it is likely that its purification and characterisation would be the simplest, and this could begin with ammonium sulphate precipitation of the CFS, followed by separation of the purified samples via HPLC (Lü et al 2014). The compounds could then be structurally characterised using SDS PAGE and Coomassie blue staining to separate the individual proteins by size (kDa) (An et al 2017). Having large samples of the antimicrobial substance would enable further characterisation e.g. sensitivity to proteolytic enzymes, heat, and pH, which should be sufficient to determine if the substance is of proteinaceous form and then potentially which class of bacteriocin is expressed by *L. salivarius* CUL61 (Lü et al 2014; Barman et al 2018). Large scale purification could also highlight novel bacteriocins that may not have been detected using genome mining techniques (i.e. unassigned putative ORFs), as databases such as BAGEL4 only detect bacteriocin genes that have been previously characterised.

Substantial proof and successful identification of potential bacteriocins secreted by *L. salivarius* CUL61 and *L. paracasei* CUL08 could have applications in food preservation as bacteriocins such as nisin are already used to inhibit food borne pathogens such as *L. monocytogenes*, *S. typhimurium* or *E. coli* (Moshtaghi et al 2018). As CFSs carry no risk to human health, they can readily be used in food manufacturing processes as food preservatives. Interestingly, a property of the class IIa carnocin bacteriocins is

that they are *Listeria* active i.e. they possess antimicrobial efficacy against *Listeria* spp., (Mathieu et al 1994), therefore *L. paracasei* CUL08 could be an excellent candidate for this. Subsequently, it would be interesting to discover the extent of the antimicrobial spectrum by testing the cCFSs against other Gram-positive bacteria and Gram-negative bacteria such as wound bacteria (*S. aureus* and *P. aeruginosa*) and *Fusobacterium nucleatum* which has been shown to be associated with the development of colorectal cancer (Wu et al 2019)

There are clinical benefits that can be derived from the CFSs of *Lactobacillus* species. For example, CFSs from some LAB display antimicrobial activity against *C. difficile,* suggesting an alternative therapy for the treatment of gut disorders caused by this species (Lee et al 2013). They have also been shown to have positive immunomodulatory activities and have even been suggested as an adjunctive treatment for cancer (Marco et al 2018; Wan et al 2014). Antioxidant and cholesterol-reducing activities have also been described (Xing et al 2015; Kim et al 2008).

5 Conclusions

LAB are known to secrete bacteriocins into their environment to give them a competitive advantage over other species in terms of space and nutrients. This study demonstrated that an antimicrobial substance, possibly a bacteriocin, was also the case with the probiotic LAB used commercially by the probiotics company Cultech Ltd, and that the probiotic bacteria themselves, were possibly responsible for the unexpected low recovery of bacteria from their multi-strain probiotic products. It was concluded that both *L. salivarius* CUL61 and *L. paracasei* CUL08 caused an antibacterial effect against the probiotic bacteria and consortia tested, as well as showing inhibition against themselves. This antibacterial effect was augmented when combined in the multi-strain product, Lab4b, where they are competing for resources. Furthermore, this antibacterial activity was proven to not just be down to acid production alone as the CFS was pH neutralised and inhibition was still found.

Antimicrobial activity was optimal in CFS samples collected in late exponential/early stationary phase. It was clear from this study that

concentration of the ssCFS samples was essential to ensure that their antimicrobial activity could be detected. Both concentrations (ssCFS and cCFS) of planktonic and biofilm CFSs isolated from *L. paracasei* CUL08 and *L*. *salivarius* CUL61, showed antimicrobial activity in the susceptibility assays, and in a few cases, biofilm CFSs of both the *Lactobacillus* strains, showed a slightly greater effect than the planktonic CFSs samples, indicating that growing the strains as biofilms could increase the expression of the antimicrobial substance. This evidence, alongside the identification of specific genes for putative bacteriocins within the genomes of both LAB strains, gives indication that the antibacterial activity could be due to bacteriocins. In order to establish what exactly is contributing to the poor recovery of the expected total microbial input in multi-strain probiotic preparations, more investigation is required. This could include large scale isolation of proteinaceous substances in the ssCFS using ammonium sulphate precipitation and HLPC, and exploration into the expression of the specific bacteriocin genes identified in *L. salivarius* CUL61 and *L. paracasei* CUL08 through molecular techniques. Only in this way can Cultech hope to find a useable solution to this expensive, ongoing commercial problem.

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7 Appendix

7.1 Appendix 1 - Plate drop counts to determine total viable counts of cell-free supernatant (CFS) cultures

To deduce the number of viable bacterial cells in the cultures for CFS harvesting, serial dilutions were carried out up to a dilution of 10^{-7} before being plated onto the surface of MRS agar plates (Oxoid, Basingstoke, UK) (5 x 10 µl droplets for the 10^{-6} and 10^{-7} dilutions) and allowed to dry. The plates were incubated anaerobically for 48 h at 37°C. To determine CFU, the individual colonies grown in drops were counted and added together. The CFU ml^{-1} were calculated as follows:

CFU ml^{-1} = Number of colonies for a dilution x 20 x dilution factor.

Then, mean values and standard error of the mean from *n* = 3 repeats were then determined (**Table 19**).

Table 19. Mean total viable counts of *L. salivarius* CUL61 and *L. paracasei* CUL08 from ssCFS growth cultures.

Bacterial Strain	Growth Conditions	Growth Culture Time (h)					
		5			24		
		O/N	*Pre-incubation	After Incubation	O/N	*Pre-incubation	After Incubation
L. salivarius CUL61:	P	$1.36 \times 10^9 \pm 1.10 \times 10^8$	$1.63 \times 10^6 \pm 8.13 \times 10^4$	$3.91 \times 10^6 \pm 2.12 \times 10^5$	$1.29 \times 10^9 \pm 9.02 \times 10^7$	$1.33 \times 10^6 \pm 1.68 \times 10^5$	$1.39 \times 10^9 \pm 1.07 \times 10^8$
	B	$1.46 \times 10^9 \pm 5.33 \times 10^7$	$1.68 \times 10^6 \pm 8.08 \times 10^4$	$2.60 \times 10^7 \pm 3.76 \times 10^6$	$1.39 \times 10^9 \pm 2.03 \times 10^8$	$1.59 \times 10^6 \pm 8.23 \times 10^4$	$1.55 \times 10^9 \pm 1.04 \times 10^8$
L. paracasei CUL08:	P	$3.02 \times 10^8 \pm 8.78 \times 10^7$	$4.07 \times 10^5 \pm 1.26 \times 10^5$	$1.09 \times 10^6 \pm 2.26 \times 10^5$	$1.51 \times 10^8 \pm 9.69 \times 10^6$	$2.11 \times 10^5 \pm 1.60 \times 10^4$	$3.84 \times 10^8 \pm 2.89 \times 10^7$
	B	$4.73 \times 10^8 \pm 7.13 \times 10^7$	$4.62 \times 10^5 \pm 5.58 \times 10^4$	$1.36 \times 10^6 \pm 2.26 \times 10^5$	$3.04 \times 10^8 \pm 2.35 \times 10^7$	$4.24 \times 10^5 \pm 1.90 \times 10^4$	$4.60 \times 10^8 \pm 5.59 \times 10^7$

P, planktonic, B, biofilm, O/N, Overnight culture. Values displayed as mean of *n* = 3 repeats ± SEM. * Pre-incubation indicates 1000-fold dilution of O/N culture in MRS broth.

7.2 **Appendix 2 -** Anti-biofilm effects of cCFS using biofilm formation assays and Confocal laser scanning microscopy (CLSM)

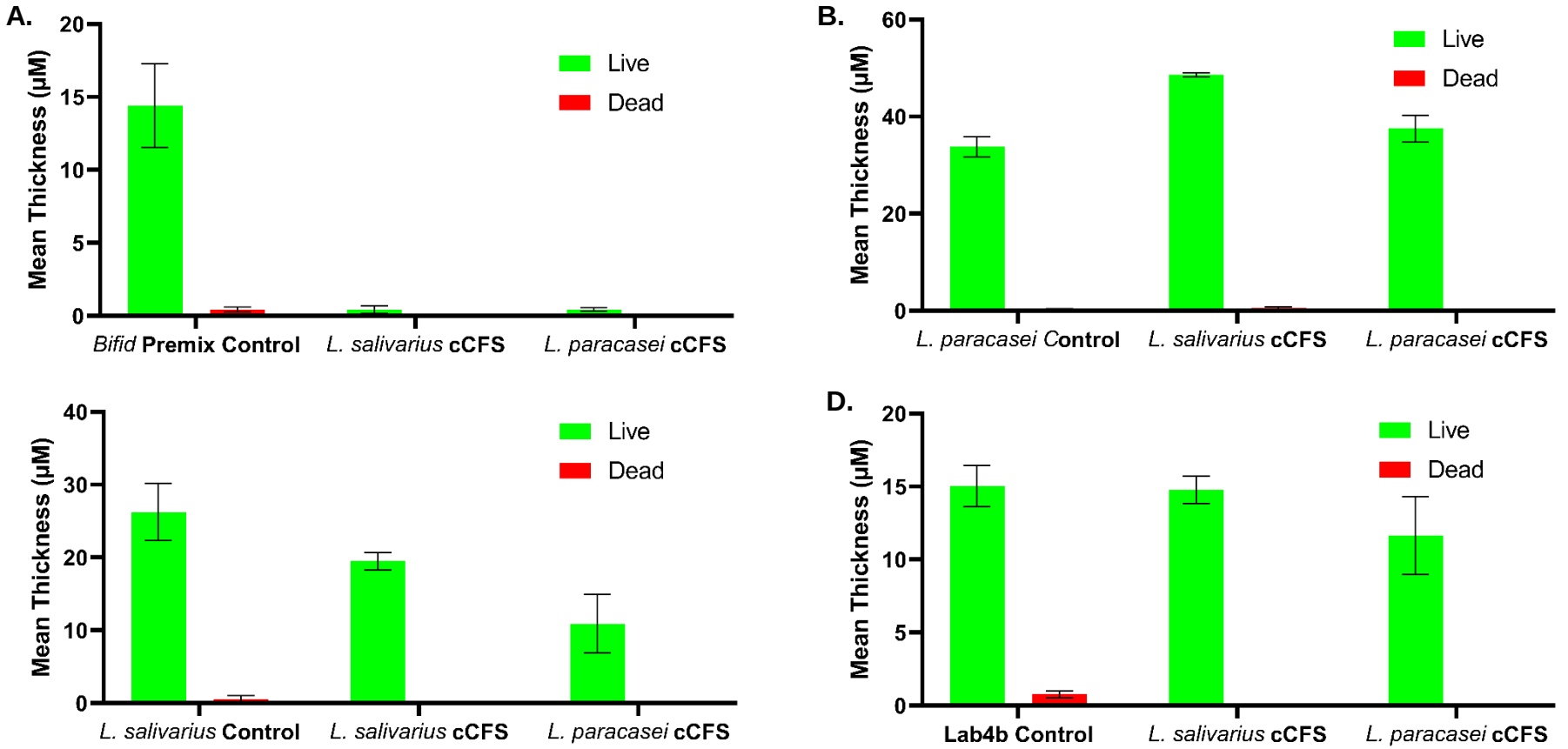

Figure 22. Comstat analysis showing mean thickness of LIVE (green) and DEAD (red) cells (*n* = 5 images each from *n* = 1 replicate): (**A**) *Bifid* Premix (**B**) *L. paracasei* CUL08 (**C**) *L. salivarius* CUL61 (**D**) Lab4b biofilms grown for 24 h with and without treatment of 12.5% 24 h planktonic *L. salivarius* CUL61 and *L. paracasei* CUL08 cCFS. (Error bars depict SD).

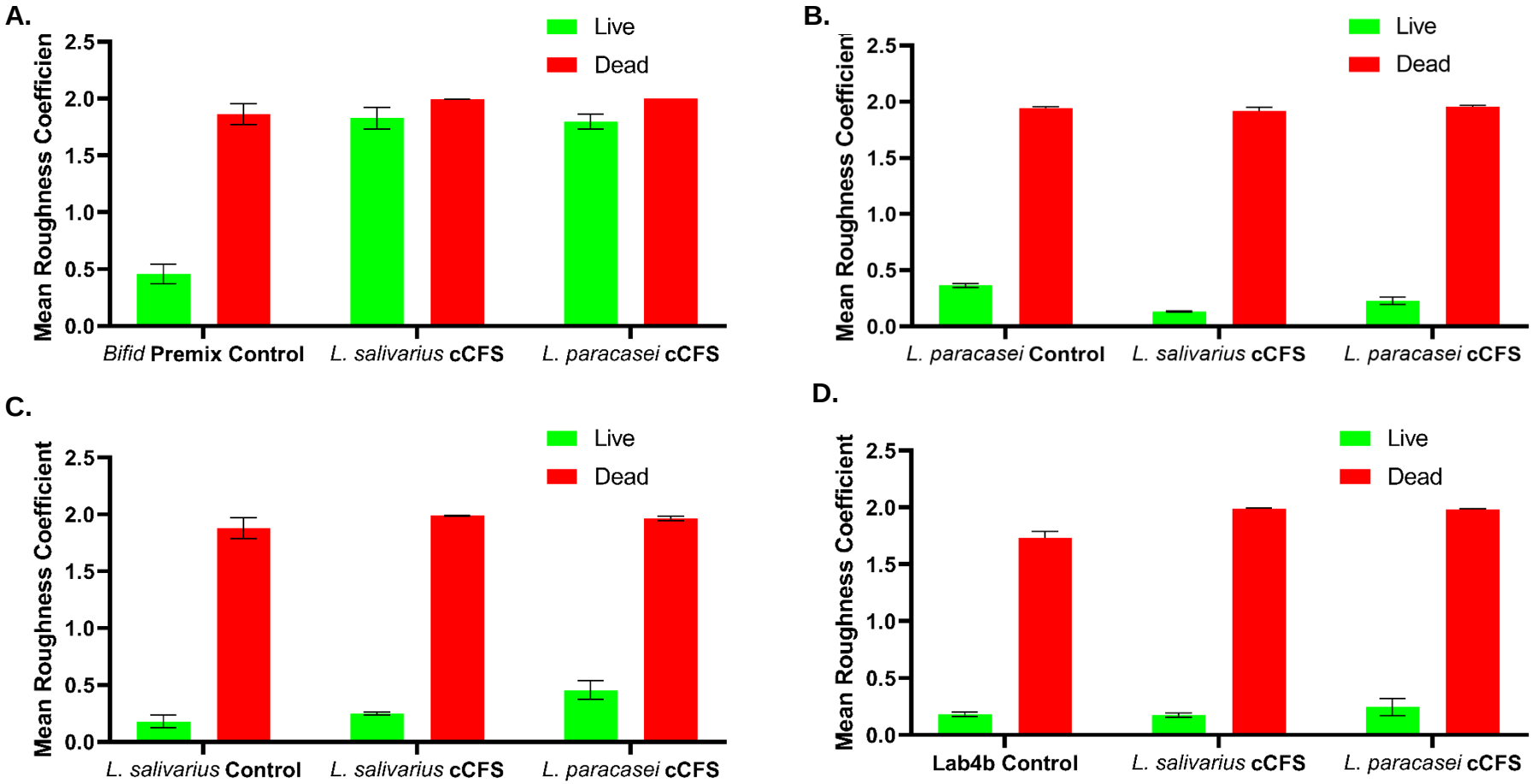

Figure 23. Comstat analysis showing mean roughness coefficient of LIVE (green) and DEAD (red) cells (*n* = 5 images each from *n* = 1 replicate): (**A**) *Bifid* Premix (**B**) *L. paracasei* CUL08 (**C**) *L. salivarius* CUL61 (**D**) Lab4b biofilms grown for 24 h with and without treatment of 12.5% 24 h planktonic *L. salivarius* CUL61 and *L. paracasei* CUL08 cCFS. (Error bars depict SD).

7.3 Appendix 3 - Characteristics of the putative bacteriocin genes found in the genome of *L. salivarius* CUL61 and *L. paracasei* CUL08

All figures and data obtained were found using the database BACTIBASE (Hammami et al 2010).

7.3.1 *L. salivarius* CUL61: enterolysin A

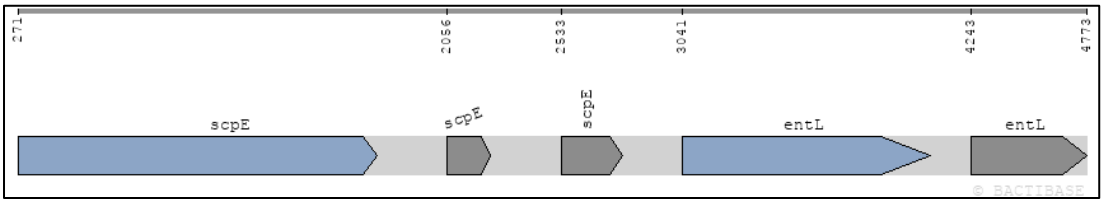

Figure 24. Gene structure of enterolysin A.

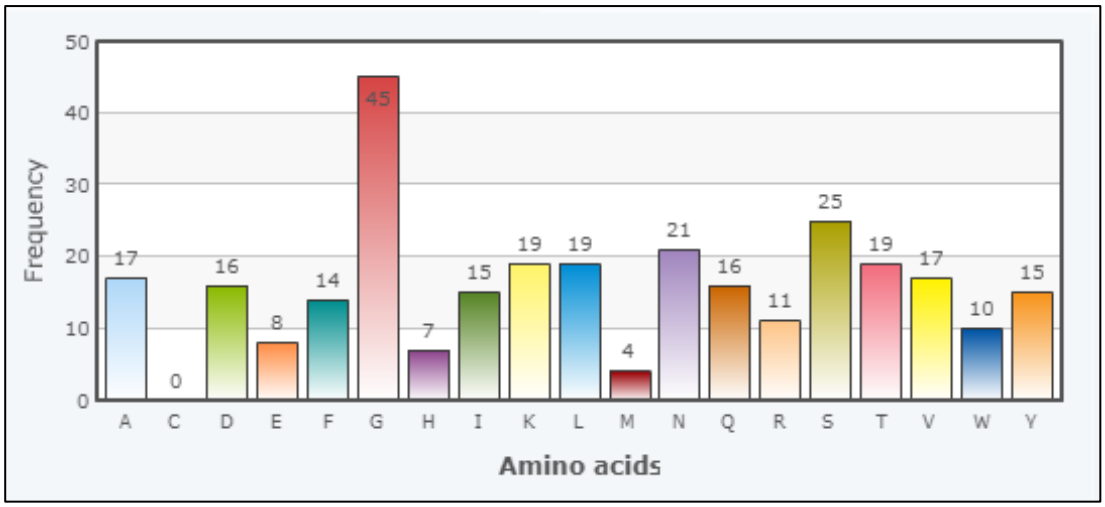

Figure 25. Amino acids composition of enterolysin A.

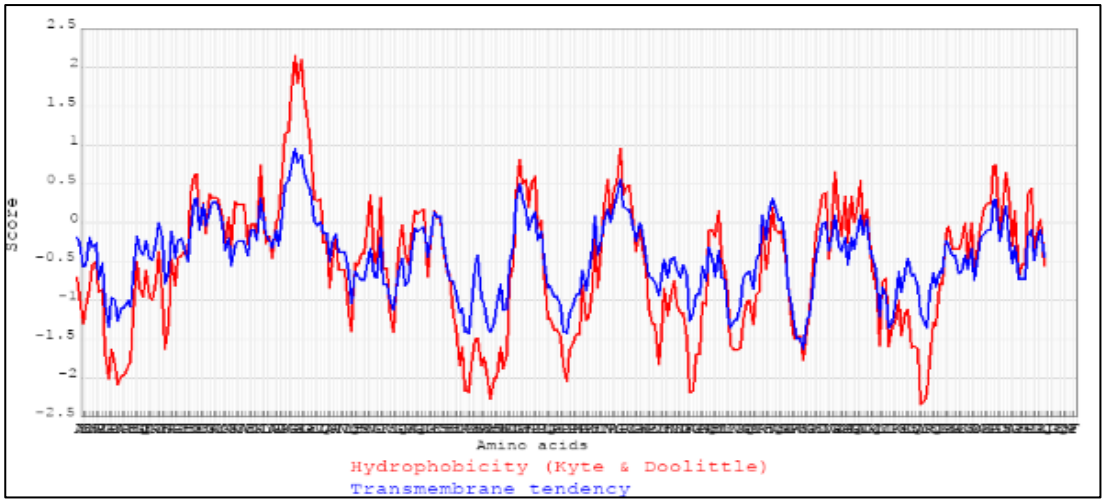

Figure 26. Hydrophobicity of enterolysin A.

Table 20. Physicochemical data on enterolysin A

Composition of enterolysin A	
Formula	C_{1552} H_{2329} N_{429} O_{461} S_4
Absent amino acids	C
Common amino acids	G
Mass (Da)	34524.69
Net charge	+13
Isoelectric point	9.64
Basic residues	37
Acidic residues	24
Hydrophobic residues	92
Polar residues	125
Aliphatic residues	51
Tiny residues	87
Boman Index	-476.75
Hydropathy Index	-0.57
Aliphatic Index	62.94
Instability Index	23.5 (stable)
Half Life	Mammalian: 4.4 h Yeast: >20 h *E. coli*: >10 h
Extinction Coefficient	77350 M^{-1} cm^{-1}
Absorbance 280nm	245.56

7.3.2 *L. paracasei* CUL08: carnocin CP52

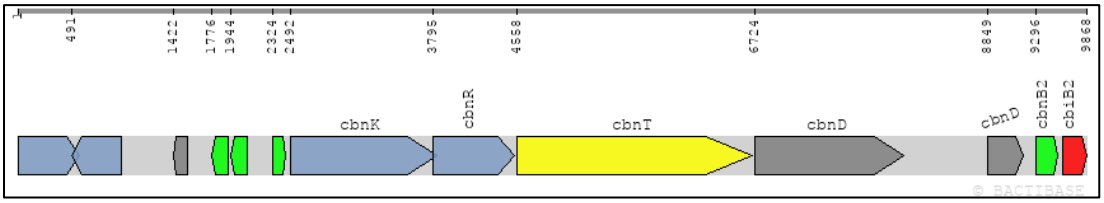

Figure 27. Gene structure of carnocin CP52.

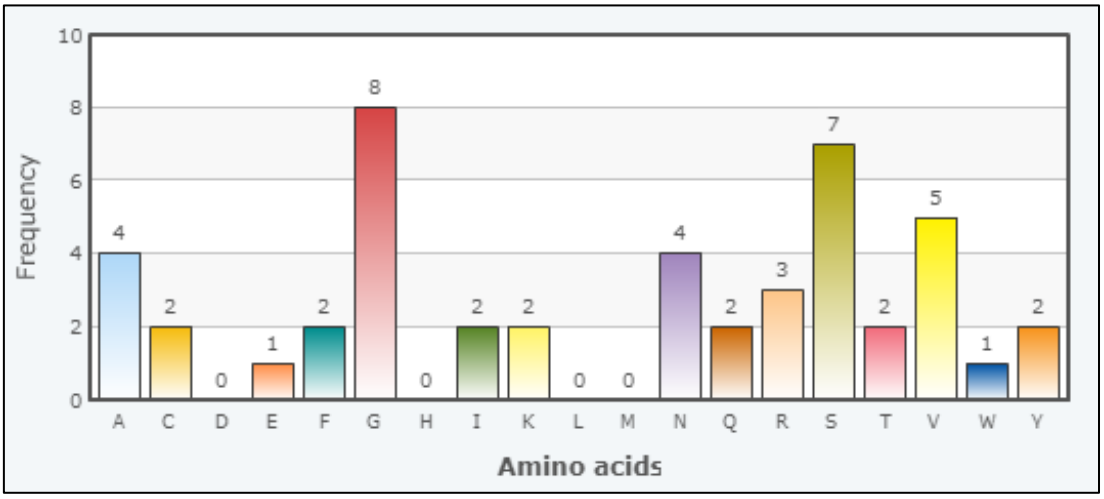

Figure 28. Amino acid composition of carnocin CP52.

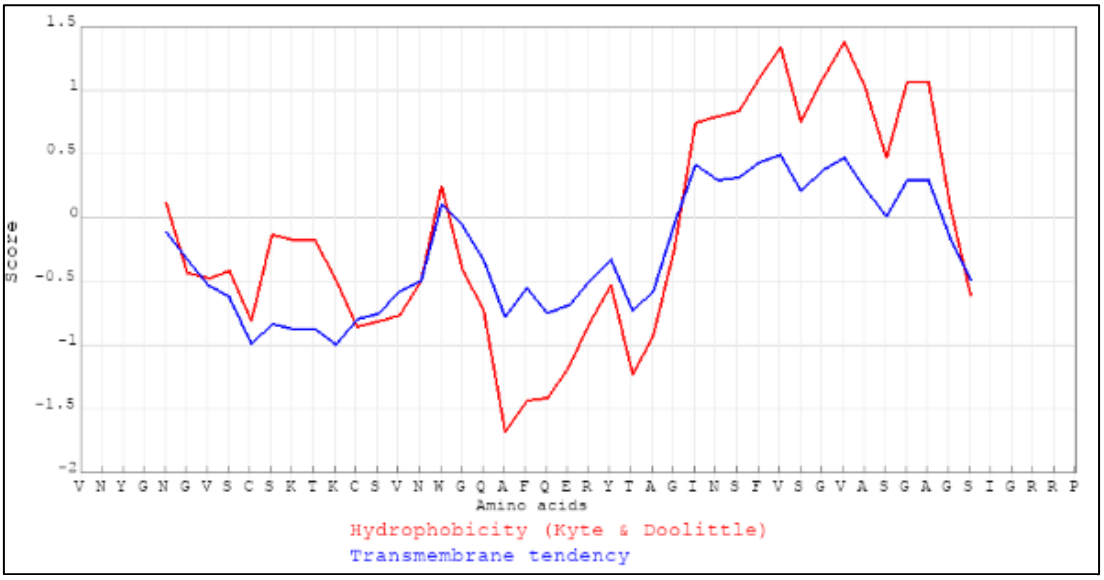

Figure 29. Hydrophobicity of carnocin CP52.

Table 21. Physicochemical composition of carnocin CP52

Composition of carnocin CP52	
Formula	C_{213} H_{332} N_{66} O_{68} S_2
Absent amino acids	D, H, L, M
Common amino acids	G
Mass (Da)	4988.27
Net charge	+4
Isoelectric point	9.96
Basic residues	5
Acidic residues	1
Hydrophobic residues	14
Polar residues	25
Aliphatic residues	7
Tiny residues	19
Boman Index	-73.88
Hydropathy Index	-0.28
Aliphatic Index	54.79
Instability Index	18.67 (stable)
Half Life	Mammalian:100 h Yeast: >20 h *E. coli*: >10 h
Extinction Coefficient	8605 M^{-1} cm^{-1}
Absorbance 280nm	183.09

7.3.3 *L. paracasei* CUL08: enterocin X (beta)

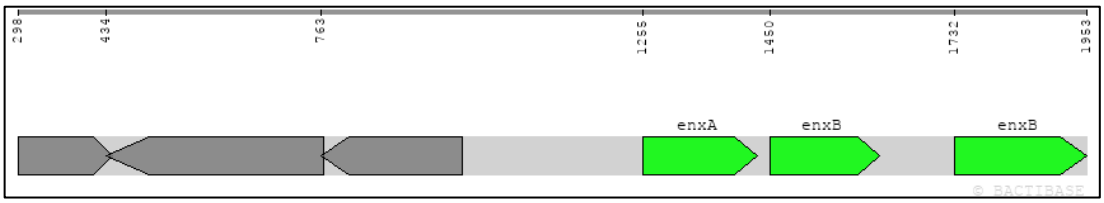

Figure 30. Gene structure of enterocin X (beta).

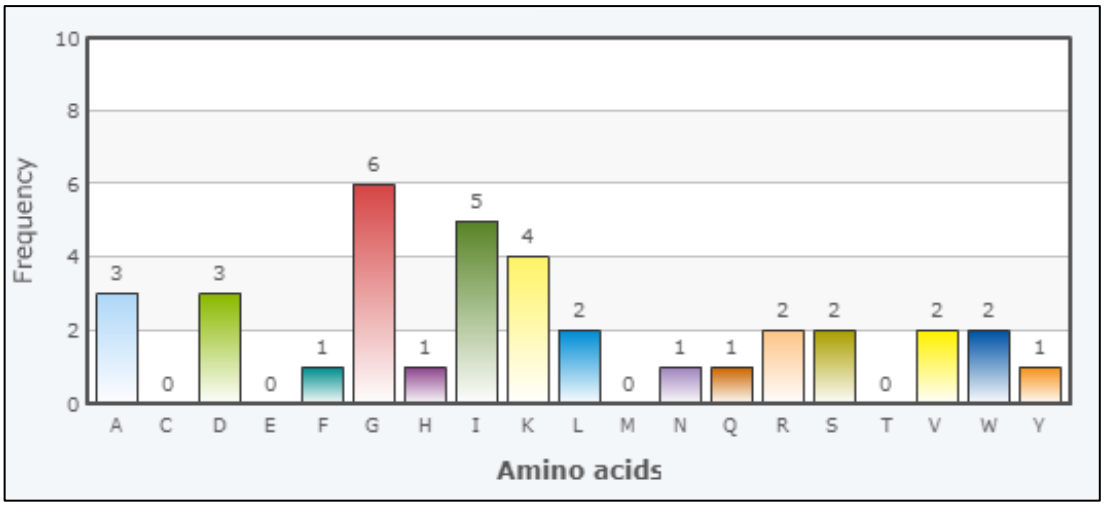

Figure 31. Amino acid composition of enterocin X (beta).

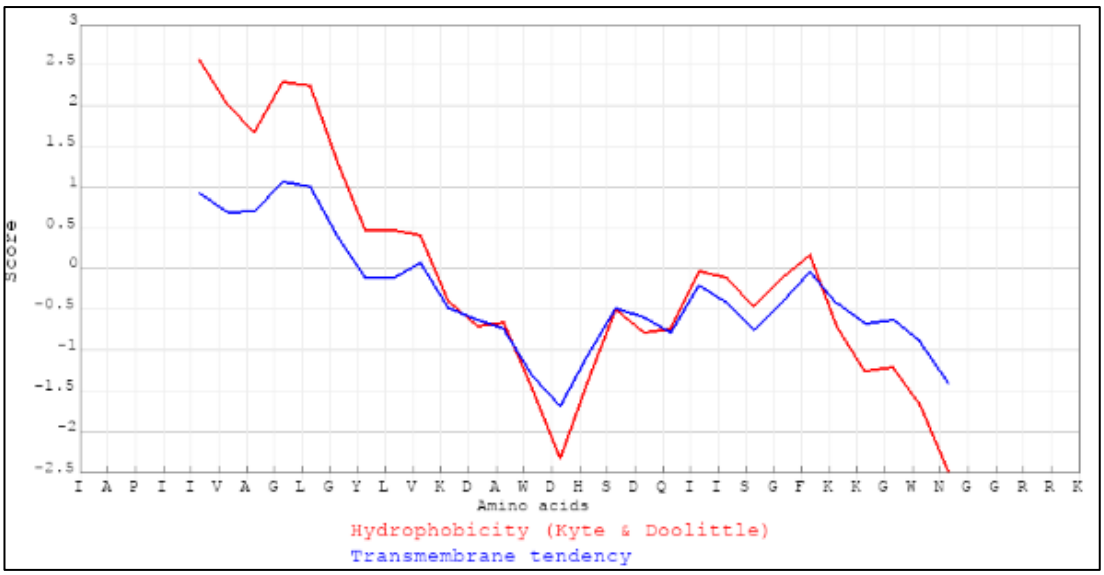

Figure 32. Hydrophobicity of enterocin X (beta).

Table 22. Physiochemical composition of enterocin X (beta).

Composition of enterocin X (beta)	
Formula	C_0 H_0 N_0 O_0 S_0
Absent amino acids	C, E, M, T
Common amino acids	G
Mass (Da)	4
Net charge	+4
Isoelectric point	10.48
Basic residues	7
Acidic residues	3
Hydrophobic residues	15
Polar residues	10
Aliphatic residues	9
Tiny residues	11
Boman Index	-40.75
Hydropathy Index	-0.197
Aliphatic Index	97.57
Instability Index	26.64 (stable)
Half Life	Mammalian: 20 h Yeast: 30 min *E. coli*: >10 h
Extinction Coefficient	12490 M^{-1} cm^{-1}
Absorbance 280nm	346.94