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Inhibition of Listeria Monocytogenes by Human Gut Bacteria

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Inhibition of *Listeria monocytogenes* by human gut bacteria

A thesis presented to Cork Institute of Technology for the Degree of Masters of Science by

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Declaration

I hereby declare that the research and the findings presented in this thesis is entirely my own work. The work presented has not been submitted for any other degree, either at Cork Institute of Technology or elsewhere. Every effort was made to clearly indicate the contributions of others through citations referring to literature in the text as well as acknowledgement of collaborative research in relevant sections.

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Thesis abstract

Listeria monocytogenes is a foodborne pathogen, which continues to be problematic to the food industry due to its ability survive within food products and persist within food processing environments. The growing trend towards natural food preservatives and antimicrobial agents increases the need for the development of novel, natural anti-*Listeria* agents. Evidence presented in the literature suggests that the human gut microbiota is a reservoir of novel antimicrobial agents. It was therefore hypothesized that novel anti-*Listeria* agents are produced by human gut-derived bacteria. The objective of this study was to isolate and characterize gut bacteria with anti-*Listeria monocytogenes* activity.

A total of 23 fecal banks of human origin were screened for antagonistic activity against *L. monocytogenes* 10403S. The results of the initial screening have shown that 1,569 gut-derived isolates have demonstrated various levels of anti-*Listeria* activity. Following an extensive screen 59 gut-derived isolates were initially shortlisted and were identified mostly as *Enterococcus* spp. By a combination of MALDI-TOF MS analysis and 16S rRNA gene sequencing. The data collected allowed a further shortlisting of strains from 59 to 16 based on differences observed in the colony morphology, the size and appearance of the zones of inhibition observed in the deferred antagonism assays and well diffusion assays as well as spectrum of activity of each isolate against a number of indicator strains. The antimicrobials produced by 11 out of 16 shortlisted gut derived isolates were identified as peptides using a proteinase K assay. The anti-*Listeria* activity of the shortlisted isolates was examined in a model broth co-culture experiment. Based on the results obtained the antimicrobials produced by two *Enterococcus faecium* isolates were selected for further investigation. The activity of the antimicrobials was investigated in dairy food homogenate models and stainless steel biofilm assays. The findings of the food trials have shown a reduction of *Listeria* numbers by ~0.6 Log CFU/mL in natural yogurt treated with cell free supernatant (CFS) and a reduction of ~1.5 log CFU/mL observed in cheddar cheese treated with whole cell extract (WCE) following a 3 hr incubation. The anti-biofilm assays demonstrated a reduction in biofilm formation of stainless steel by 70% when treated with CFS and 92.42% when treated with WCE.

The experiments presented in this thesis confirm that bacteria from the human gut produce anti-*Listeria* compounds which can control or reduce the numbers of *Listeria* in model co-cultures and in food homogenates. The antimicrobials isolated in this study have also shown the ability to reduce *Listeria* biofilm formation. Future efforts should focus on identifying the antimicrobials produced by all of the short-listed strains (through a combination of genome sequencing and peptide purifications). It is hoped that any novel antimicrobial that is discovered have an application in the food industry to reduce the significant threat posed by *L. monocytogenes*. These antimicrobials could be used as a natural preservative in food products or packaging or incorporated into a spray to control biofilm in the food processing environment.

Literature review

Inhibition of *Listeria monocytogenes* by human gut bacteria

Contents

1. Introduction

L. monocytogenes is a causative agent of a severe foodborne disease; listeriosis (Mead *et al.* 1999; Scallan *et al*. 2011). The disease is particularly dangerous for immunocompromised individuals, the elderly and pregnant women. Despite a relatively low number of outbreaks the mortality rate is high reaching 30% and is accountable for 16-28% of foodborne disease related deaths in the US annually (Mead *et al*. 1999; Scallan *et al*. 2011). The pathogen is very problematic especially in the food industry where it can survive various processing hurdles such as extreme temperature, pH, or osmotic pressure (Hill *et al*. 2002). Additionally the bacterium is capable of biofilm formation (section 2.4), which makes its eradication even more difficult and allows for its persistence in the food processing environment and contamination of food products (Bagge-Ravn *et al*. 2003; Gunduz & Tuncel 2006; Sharma & Anand 2002). Novel approaches / methods to inhibit and control *L. monocytogenes* are urgently required.

The human gut microbiome is home to approximately 10^{14} bacteria (Gill *et al.* 2006), which provide protection to the host by a mechanism known as colonization resistance. Direct competition for nutrients, production of short chain fatty acids or bacteriocins as well as modulation of the host immune system can all inhibit gut colonization by enteric pathogens (Kamada *et al* 2013). This review provides an overview of some of the antimicrobials produced by gut bacteria and it summarizes the evidence that shows that gut bacteria can inhibit *L. monocytogenes*.

2. *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, non-spore forming facultative anaerobe (Jamali *et al.* 2013; Zhang *et al*. 2007). Murray *et al*. (1926) were first to describe *L. monocytogenes* when the pathogen was isolated from infected rabbits. For years *L. monocytogenes* was regarded as an animal pathogen as human infections with the bacterium were sporadic. Schlech *et al*. (1983) linked the transmission of the pathogen to food. Since its discovery *Listeria* has been extensively researched which makes it one of the best-characterized pathogens (Cossart 2007; Lebreton *et al*. 2016).

2.1 Pathogenicity of *L. monocytogenes*

L. monocytogenes is highly infectious when ingested with an infectious dose of 100-1,000 cells (Almeida *et al*. 2013; Drevets and Bronze, 2008; Zhang *et al*. 2007). Outbreaks are mostly associated with transmission via food (Carpentier & Cerf 2011). Pregnant women, infants, elderly and immunocompromised people belong to the most susceptible group and are under increased risk of developing listeriosis upon consumption of contaminated food (Jamali *et al.* 2013). Despite the fact that the foodborne disease listeriosis caused by *L. monocytogenes* is not as frequent as those associated with *Salmonella* or *Campylobacter* its high mortality makes it a pathogen of high concern to the food industry (Donovan 2015; Mead *et al.* 1999) *L. monocytogenes* can have a long incubation period especially in pregnant women (Goulet *et al*. 2013), which makes it problematic not only to identify the pathogen, but also to trace the source of infection. Septicaemia, meningitis and various infections of the central nervous system are often associated with listeriosis. The disease is particularly severe in pregnant women where it may cause complications such as spontaneous abortion, foetal death or stillbirth (Rocourt & Cossart 1997).

Infection with *L. monocytogenes* can cause serious consequences to the host, depending on their susceptibility. Severity can vary from infections of the central nervous system and /or blood stream, gastroenteritis, abortion, brain infections or materno-foetal infections (Carpentier & Cerf 2011; Toledo-Arana *et al.* 2009). The disease can also take the form of severe listeriosis (Almeida *et al.* 2013; Carpentier & Cerf 2011; Gilbreth *et al.* 2005). *Listeria'*s ability to cause disease relies on a number of factors. Those include its capability to attach and invade the gastrointestinal epithelium as well as the ability to withstand the immune response of the host (Corr *et al*. 2007). Although the topic is extensively researched, the behavior of the pathogen once it invades the gastrointestinal tract is not fully known (Corr *et al.* 2007).

L. monocytogenes has 13 known serotypes, but it has been estimated that >50% causes of listeriosis are caused by strains with serotype 4b and that the majority of strains isolated from food are strains of serotype 1/2a (Gilbreth *et al*. 2005). One factor which allows *L. monocytogenes* to adapt to various hurdles inside the human body, is its ability to withstand a wide range of pH changes from pH 4.6 to pH 9.5

which enables it to survive during gastric passage (Carpentier & Cerf 2011; Almeida *et al.* 2013; Gandhi & Chikindas 2007). *L. monocytogenes* is also known for being able to survive the environmental conditions found in the human immune system (Hill *et al.* 2002; Mead *et al*. 1999) and is highly osmotolerant as it can endure NaCl concentrations of 10-20% (w/v) and has the ability to withstand water activity of 0.92 (Carpentier & Cerf 2011). It is believed that *L. monocytogenes* ability to adapt to changing environmental factors aided by assimilation of the information about the external environment to which the bacterium is exposed (Hill *et al.* 2002; Mead *et al.* 1999).

It is estimated that ingestion of less than 1,000 cells of *L. monocytogenes* can cause infection in people belonging to the susceptible group (Drevets and Bronze, 2008). When ingested the bacterial cells infect the gastro-intestinal epithelium and becomes blood borne, the bacterium can then infect the spleen, liver and the lymphatic system (Drevets and Bronze, 2008). It can then invade the nervous system; in the case of pregnant women it can cross over the placental barrier and infect the foetus. Listeriosis has a high fatality and hospitality rates however it can be treated with antibiotics if detected early (Williams and Nadel, 2001). Typically listeriosis is treated with a combination of antibiotics including penicillin and ampicillin combined with gentamicin (Hof *et al.* 1997; Marco *et al.* 2000; Safdar and Amstrong 2003).

As described by Vázquez-Boland *et al.* (2001) the clinical consequence of infection with *L. monocytogenes* is highly dependent on three main variable conditions. Those include; the ingested dose of the pathogen, the pathogenicity of the ingested strain of *Listeria* and the immunological health of the infected individual. It is believed that ingestion of a low dose of *L. monocytogenes* by an individual who is immunocompetent and does not fall into the susceptibility group should have no adverse effects on their health other than increasing anti-*Listeria* immunity. However ingestion of a high dose of the bacterium can be harmful and may result in fever, gastroenteritis and potentially an invasive infection. In contrast immunocompromised and fatigued individuals cannot display an immune response powerful enough to limit the propagation of the pathogen in the liver, which is the main organ associated with *L. monocytogenes* infection. As a consequence those individuals are susceptible to development of an invasive *Listeri*a infection even at a lower dose. Additionally an unsuccessful reduction of *L. monocytogenes* growth in the liver cells in those

individuals is likely to result in an increased critical mass of the bacterium present in the hepatocytes and subsequent release of bacterial cells into the blood stream. Furthermore this can result in local infections of the main secondary organs associated with *Listeria* infections such as the placenta and the brain, in critically immunocompromised individuals the infection can become septicemic (Vázquez-Boland *et al.* 2001). The pathophysiology of *L. monocytogenes* infection is shown in **Fig.1.**

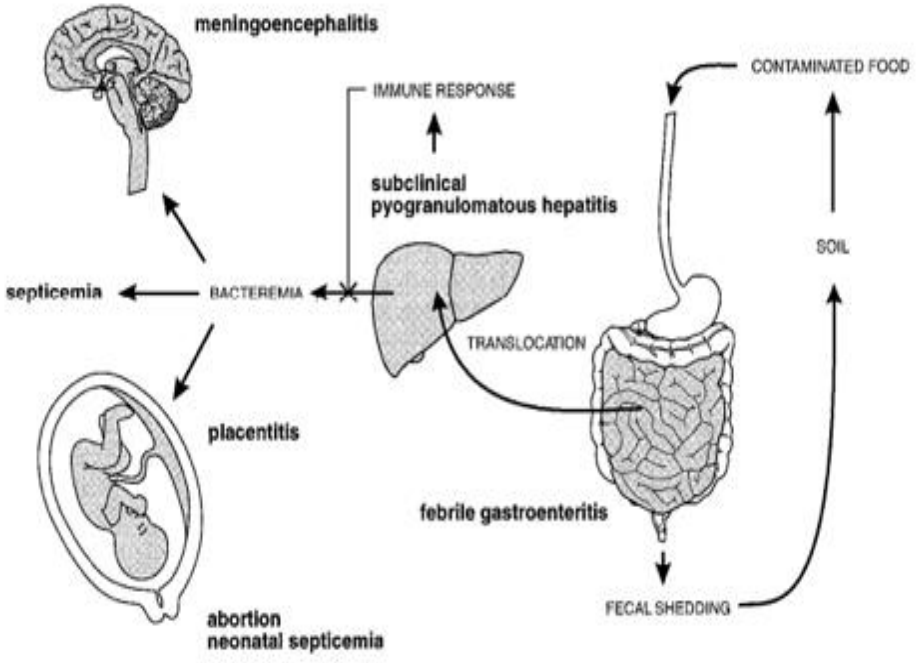


Fig.1 A schematic representation of the pathophysiology of *Listeria* infection. (Figure taken from Vázquez-Boland *et al*. 2001).

2.2. Outbreaks of listeriosis

There are a number of factors which can impact the prevalence of *L. monocytogenes* and listeriosis outbreaks; those include the globalization of the food industry and growing need for imported ethnic foods. The evolution of eating habits leaning towards consumption of minimally processed food and an increased demand

for refrigerated and ready to eat convenience foods all affect the occurrence of listeriosis outbreaks in recent years (Rocourt and Bille, 1997). The foods commonly associated with *Listeria* outbreaks are soft cheese and other dairy based foods such as Latin style fresh cheese or Mexican style cheese, smoked fish, pates, hot-dogs, fresh vegetable and ready to eat foods (de Castro *et al*. 2012; FDA 2017; Gillesberg Lassen *et al*. 2016; Jackson *et al*. 2018; Morris & Ribeiro 1991; Pinto *et al*. 2010; Ricci *et al.* 2018; Schwartz *et al.* 1988; Zhu *et al.* 2017). The common feature of these food products, which makes them favourable for the pathogen growth are the intrinsic properties of those products. Those include moderate water activity and reasonably low microflora which can survive in the storage conditions of such food products and can compete with *Listeria* (Swaminathan and Gerner-Smidt, 2007). Some of the more recent listeriosis outbreaks include four state outbreak in the US linked to raw milk cheese, which caused 8 hospitalizations and 2 deaths and resulted in recall of the product (CDC 2017). Another smaller outbreak was caused by raw organic milk contaminated with *L. monocytogenes* where 2 people from 2 different states were hospitalized and one mortality was recorded, there was no recalls of the contaminated product associated with the outbreak as the causative agent of the foodborne disease was not recognized until 2 years later (CDC 2016). Makino *et al.* (2005) reported an outbreak of *L. monocytogenes* associated with consumption of cheese. In this case 86 people were infected with the pathogen and 38 developed clinical symptoms associated with listeriosis, it was the first reported case of listeriosis outbreak in Japan. Koch *et al.* (2010) described an outbreak reported in Germany where 189 individuals were infected with *L. monocytogenes* due to consumption of contaminated cheese made with pasteurized milk. One of the largest *Listeria* outbreaks ever recorded took place in South Africa between the January of 2017 and March of 2018. There has been 978 confirmed cases of listeriosis and 183 reported deaths. The outbreak has been linked to a ready to eat sausage product. *Listeria monocytogenes* Sequence Type 6 (ST6) has been determined as the main contaminant found in the food processing plants, however 9% of listeriosis reported have been associated with a different strain of *Listeria* suggesting multiple outbreaks. Further investigations are ongoing ("WHO Listeriosis – South Africa," 2018).

Despite continuous advances in food technology the changing food trends are shifting towards less processed ready to eat products. Additionally changes in the life spans of the older, more susceptible population group mean that *L. monocytogenes* amongst other foodborne pathogens will remain an issue to the food industry (Begley and Hill 2015; Newell 2010).

2.3 Stress tolerance and persistence in the food industry

L. monocytogenes is capable of survival and growth over a wide range of environmental conditions with a broad scope of temperatures ranging from 1-45 °C. Additionally it has the ability to survive and grow at refrigeration temperatures (1-4°C) which make it very problematic to control its growth in food products, bearing in mind that refrigeration is one of the most common methods used for shelf life extension for many food products (Almeida *et al.* 2013; Carpentier & Cerf 2011).

L. monocytogenes is often found in the environment with important sources of the bacterial contamination including water and soil. The survival of the pathogen in soil can be affected by a number of factors including presence of competing microbiota and the composition of the soil. Additionally its persistence in the environment can be affected by weather (NicAogáin & O'Byrne 2016; Swaminathan & Gerner-Smidt 2007). Exposure to contaminated feed can then infect the animals, which shed the bacterium in their faeces (Swaminathan and Gerner-Smidt, 2007). The bacterium can then further spread to the environment and contaminate crops when animal manure containing *Listeria* is spread onto fields as a fertilizer (Schlech *et al.* 1983; Szymczak *et al.* 2014). Resulting contaminated animal or plant material can introduce the bacterium into food processing plants where undetected it can grow to high numbers and can potentially contaminate food product even post processing (Guerini *et al.* 2007; Leong *et al.* 2014). The bacterium can then enter the food chain and infect consumers (NicAogáin & O'Byrne 2016). A schematic of transition of *L. monocytogenes* from the agricultural environment to the food chain is depicted in **Fig.2** below.

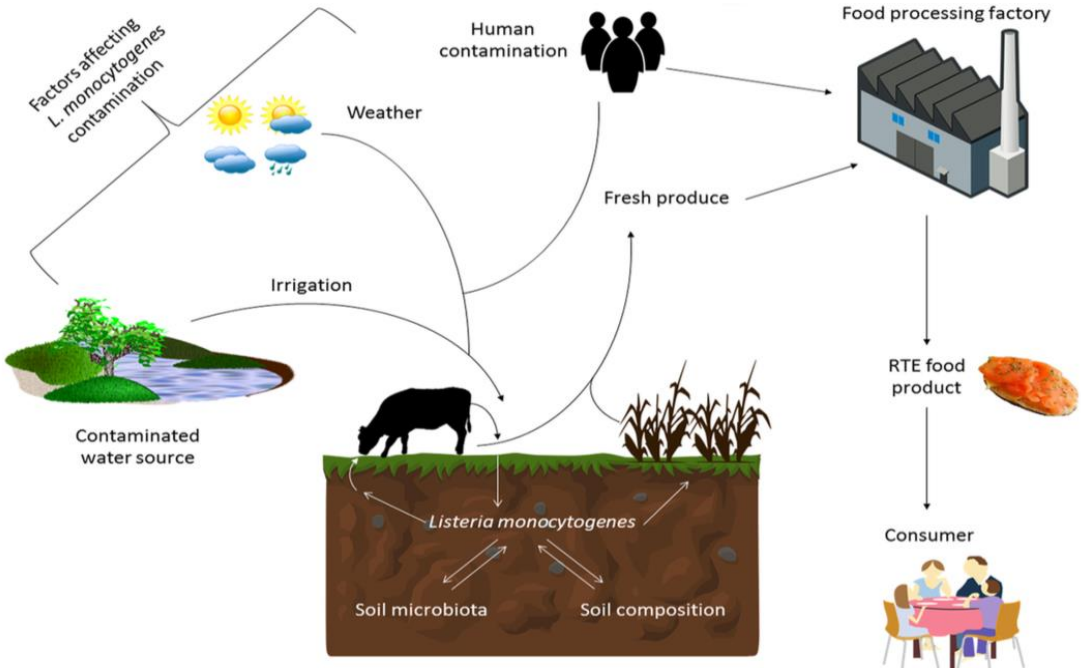


Fig. 2 The survival and transmission of *Listeria monocytogenes* in the agricultural environment, the food industry and its subsequent entry of the food chain (This figure was taken from NicAogáin & O'Byrne 2016).

A number of studies have been performed within food processing environments, the data collected in those studies shows that the occurrence of *L. monocytogenes* within those environments can be either endemic or transient (Carpentier & Cerf 2011; Martinez *et al*. 2003; Norton *et al.* 2001; Rørvik *et al.* 2003). The transient or passing populations of *Listeria* are often introduced to the food processing environments on contaminated raw materials such as raw meat and are often depleted once the source of contamination is gone. The endemic, more persistent *Listeria* populations can develop on surfaces and may come in contact with the food product, examples of such contact surfaces include conveyer belts or slicers (Klausner & Donnelly 1991; Scollon *et al.* 2016). Scollon *et a*l. (2016) reported cross-contamination of raw onions with *L. monocytogenes* from slicers used for product preparation. The contaminated product was linked to a number of recalls of products containing contaminated onion. *Listeria* was also reported to attach to surfaces with poor hygiene such as drainage areas, storage areas and walls as reported by Gunduz & Tuncel (2006). Once established in a food processing environment *Listeria* populations can form biofilms and persist for prolonged periods of time as reported by Unnerstad *et al*. (1996) who recovered the same clone of *L. monocytogenes* from Scandinavian dairy and cheese manufacturing plant over a period of 7 years.

Hilliard *et al*. (2018) compared a selection of 1,300 Irish *L. monocytogenes* isolates from food processing environments and food samples and used pulse-field gel electrophoresis and whole genome sequencing methods in order to compare the genomes of those isolates to 25 clinical isolates associated with outbreaks of listeriosis in Ireland during time period for 2013 to 2015. The study has found that 64% of the clinical pulsotypes of isolates were previously identified in food or in the food processing environments. Additionally the genomes of five matching isolates were sequenced and revealed correlations between the genotypes and the pulsotypes of the isolates. The findings of the study show that there are close similarities in the *L. monocytogenes* strains currently responsible for infections with listeriosis and the isolates found in the food environment suggesting that contamination of food products associated with the disease is potentially caused by endemic populations of *L. monocytogenes* found in the food industry.

2.4 *Listeria* biofilms

Some bacteria are able to form biofilm (i.e. embed themselves in a self-produced polymer matrix) that allows adherence to biotic or abiotic surfaces. Biofilms can arise in nearly any environment where the viable microorganism capable of biofilm formation is present (Kumar and Anand, 1998). Biofilms are of concern to the food industry as they frequently contain pathogenic bacteria, which increases the risk of post-processing contamination of the product and subsequent product spoilage while posing a serious threat to the public health (Shi & Zhu 2009; Singh *et al*. 2017). A number of studies have shown that that formation of biofilm in food industry settings is one of the key factors contributing to post processing product contamination (Bagge-Ravn *et al.* 2003; Gunduz & Tuncel 2006; Sharma & Anand 2002).

Biofilms can be found on a broad range of surfaces including water system piping, equipment, stainless steel surfaces, conveyer belts and storage areas and storage tanks, floor drains and hand trucks (Di Bonaventura *et al.* 2008; Donlan 2002; Kumar & Anand 1998; Mafu *et al.* 1990; Wong 1998).

Listeria biofilms often display an increased resistance to disinfectants, sanitizers and other antimicrobial agents, which makes their elimination a challenging task for the food manufacturers (Mah & O'Toole 2001; Lundén *et al.* 2003; Romanova *et al.* 2002). A study by Cabeça *et al.* (2012) demonstrated that *Listeria* biofilm is resistant to cleaning with chemical agents such as iodine disinfectant**,** biguanide disinfectant**,** quaternary ammonium compounds disinfectant, peracetic acid, sodium hypochlorite disinfectant and can persist on stainless steel. The results of the study are presented in **Fig.3**.

Listeria has the ability to form monoculture biofilms as well as mixed culture biofilm (Bremer *et al.* 2001). A study carried out by Bremer *et al*. (2001) investigated *Listeria*`s ability to form biofilms in a monoculture and as part of a mixed culture biofilm with *Flavobacterium*. The findings of the study showed that when grown in a mixed culture the number of *Listeria* a cells attached to stainless steel has significantly increased when compared to monoculture biofilm. Additionally it was noted that that the survival period of *Listeria* cells in the mixed culture biofilm was significantly longer than that of monoculture biofilm (Bremer *et al.* 2001). However some microorganisms found in the food processing environment can have the opposite effect. A study carried out by Carpentier & Chassaing (2004) focused on isolation of microorganisms found in 7 various food processing plants following cleaning and disinfection procedures. A total of 29 bacterial strains were isolated and the isolates were allowed to form a mixed culture biofilm with *Listeria* on stainless steel coupons and the influence of those isolates on the biofilm formation was measured. The data showed that 16 of the bacterial isolates have in fact reduced the number of CFU of *Listeria* found in the biofilm while 4 had a positive impact on biofilm formation resulting in increased number CFU of *Listeria* attached to the surface of the stainless steel coupons (Carpentier and Chassaing, 2004). Findings of such studies are of high importance to the food industry as many microorganisms and pathogenic bacteria such as *Listeria* are often found in various parts of food processing plants where there is an increased likelihood of formation of mixed culture biofilm.

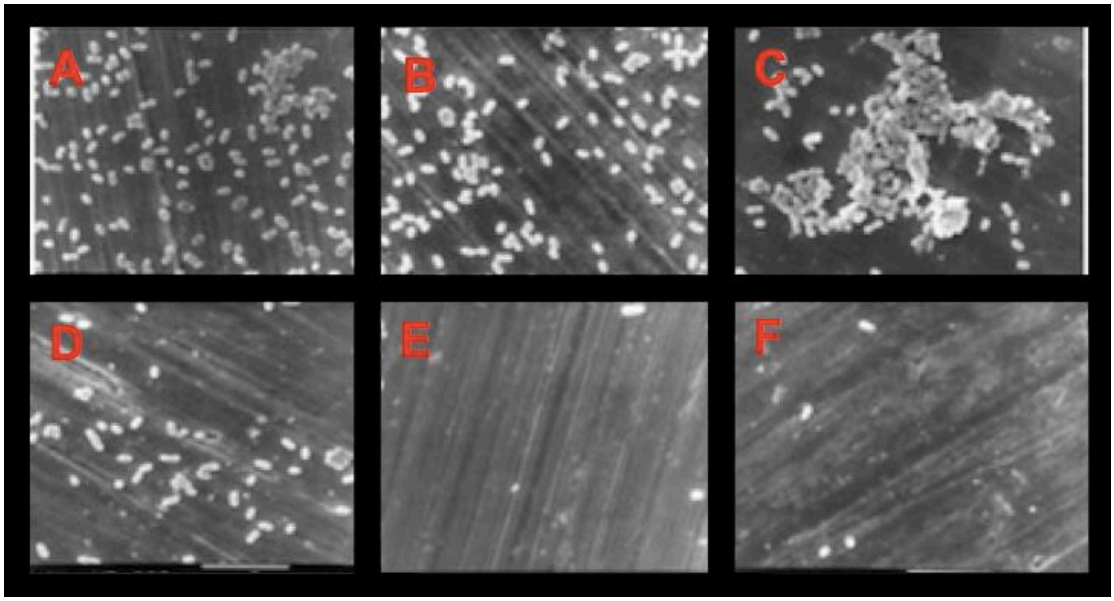


Fig. 3 Scanning electron micrograph of *L. monocytogenes* biofilm on stainless steel surface (x5,000) **(A)** biofilm before treatment with disinfectants **(B)** after treatment with iodine disinfectant **(C)** after treatment with biguanide disinfectant **(D)** after treatment with quaternary ammonium compounds disinfectant **(E)** after treatment with peracetic acid **(F)** after treatment with sodium hypochlorite disinfectant. (This figure was adapted from Cabeça *et al*. 2012).

3.0 Human gut microbiota

The human gut microbiome comprises between 10^{13} and 10^{14} microorganisms (Gill *et al*. 2006). The gut microbiota is mainly composed of members of two phyla, namely Firmicutes and Bacteroidetes (Arumugam *et al.* 2011). In recent years new advancement in research of culturomics and metagenomics of the human gut allowed for greater research of the gut microbiome and its functions. Due to its beneficial functions it has been referred to as an "essential" (Eckburg *et al.* 2005) or the "forgotten" organ (O'Hara & Shanahan 2006). A number of studies support the theory that the gut microbiome has a massive impact on numerous aspect of human health in areas such as metabolism, immune function, physiology and nutrition (Cénit *et al.* 2014; Eckburg *et al.* 2005; Guinane & Cotter 2013).

3.1 Cultivation of the gut microbiota

Investigation of the composition and functions of the gut microbiota is a challenging task. Traditional methods of cultivation of gut microbiota are practically completely culture dependent. This has provided huge benefits when cultivating so called "easily grown microbial species" which can grow under lab conditions, however cultivation of anaerobic species is still very limited. Traditional methods allow for culturing only 10-50% of the bacteria found in the human gut**,** which are considered to be "easy to culture" (Eckburg *et al.* 2005). Overall cultivation of the majority of the gut bacteria from what is considered as a "normal" gut, is very ineffective especially when considering the fact that the understanding of carbon sources necessary for microbial growth within the gut is very limited and therefore difficult to reproduce within a lab set up (Cénit *et al*. 2014; Lagier *et al*. 2012).

The majority of studies examining the microbial populations of the gastrointestinal microflora focus predominantly on the analysis of faecal samples as those are easy to obtain (Atya *et al.* 2015; Awaisheh *et al*. 2013; Birri *et al.* 2010; Birri *et al.* 2013). However the structural and functional differences between the mucosal and faecal micro flora are not fully known (Eckburg *et al.* 2005). There are a number of the restrictions associated with the culture based approach, which can to an extent be overcome due to the recent developments such as of cultivation methods of microorganisms with more fastidious needs, such developments include microbial culturomics of the gastrointestinal microflora (Lagier *et al.* 2016).

Research in the field of cultivation of gut bacteria has led to the development of new culturing methods combined with the use of matrix–assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF) and 16S rRNA gene sequencing for identification and amplification of the growing bacterial colonies with the aim of identifying previously unidentified organisms. Lagier *et al*. (2012) carried out a study on two African stools, where 212 various culture conditions were investigated including different physicochemical conditions such as pre-incubation in sterile stool extract, pre-incubation in rumen fluid or pre-incubation in blood, all with the aim to copy the natural environments for the gut bacteria. This methodology allowed for recovery of 32,500 colonies which have been identified as 340 bacterial

species, 174 of which were previously not described in the human gut. The methodology used by this approach focused on omission of the microorganisms, which can be cultured using the traditional culture based approach and are predominant in the gut. The abundant population of easy to culture organisms were eliminated with the use of phage cocktails, antibiotics or filtration methods. Elimination of those organisms allows for supplementary growth of the microbes of interest and allows for their identification. In more detail antibiotics were added to the culture media in order to eliminate the sensitive microorganisms present in the samples and allow for identification of the resistant ones. New approaches were developed in order to advance the use of "classic selective media". Alternative strategies were developed in order to identify the proteobacteria present in the samples, one of the major obstacles encountered was the fact that under the aerobic condition the population of *E. coli* was immensely dominant. In order to overcome that problem and allow for identification of less abundant microorganisms a cocktail of lytic bacteriophages targeting *E. coli* was added to the culture. This allowed for identification of *Enterobacter massiliensis,* a previously unknown member of enterobacterial species, which could not be detected with the use of traditional axenic culture approaches. Other effective strategies which allowed for the removal of major, dominant bacterial populations present in the samples was the use of active filtration with successive membranes with pore sizes ranging from 5 to 0.2 lm. This approach identified 8 new bacterial species. The final approach used focused on exploiting the physical characteristics of some bacteria, passive filtration was applied and allowed the identification of 3 motile bacteria previously not associated with the human gut. The study used MALDI -TOF MS for rapid identification of colonies, colonies not identified with that approach were identified with 16S rRNA sequencing (Lagier *et al.* 2012).

3.2 Metagenomics and 16S rRNA sequencing to identify microbes in the gut-microbiota.

Metagenomics is defined as a genetic analysis of an entire microbial population from an environmental sample (Handelsman, 2004). With the aid of bioinformatics and genomic sequence based studies, metagenomic studies look at all the genes present in a given environment and can detect novel genes, novel proteins from already known

protein families and novel small molecules with antimicrobial activities (Chen & Pachter 2005; Handelsman 2004; Petrosino *et al.* 2009). Mammals including humans are considered metagenomic due to the fact that their composition extends past their own genetic material and comprises of the genetic material of the microbes associated with them. This is especially evident when talking about the mammalian gastrointestinal tract (Ley *et al.* 2008). It is anticipated that the human gastrointestinal tract comprises over 2,000 different microbial species and accumulates over 10^{14} microbial cells the collective microbial genome is estimated to be over 9 million and its metabolic activity is equivalent to an organ (Bocci 1992; Hugon *et al.* 2015; Thursby & Juge 2017; Yang *et al.* 2009) .

The 16S rRNA gene is widely distributed amongst all prokaryotic organisms and for that reason it is regularly used for identification of microorganisms at taxonomic level. The data collected from 16S rRNA analysis supplies information regarding the microbial composition found in a given ecosystem. However it does not provide data in relation to the viability of the microorganisms present or their potential functions (Eckburg *et al.* 2005; Hooper *et al.* 2012; Kurokawa *et al.* 2007; Qin *et al*. 2010). It should also be noted that the 16S based approaches are limited to bacterial identification as viruses and parasites do not possess the 16S rRNA gene (Cénit e*t al.* 2014). Similarly 18S rRNA is the eukaryotic nuclear homologue of the 16S rRNA gene found in prokaryotic organism and can be used for identification of eukaryotes (Field *et al.*1988).

In order to comprehend the gut environment, the gut biome and its complexity extensive studies such as The European Metagenomics of the human intestinal tract (MetaHIT) and the US Human Microbiome Project (HMP) were undertaken. The objectives of the (MetaHIT) project are to establish the associations between bacterial genes present in the human gut and human health and disease. The study focused mostly on obesity and inflammatory bowel disease (IBD), both of which are of increasing importance not only in Europe but globally. As part of the project a reference catalog containing bacterial genomes recovered from the human intestine was created. Over all 3.3 million non-redundant genes of microbial origin were identified form 124 Europeans (Qin *et al.* 2010). Additionally bioinformatics tools such as SmashCommunity were developed to act as stand-alone metagenomic annotation and

analysis pipelines, by provision of tools to approximate the quantitative functional and phylogenetic composition of the metagenomes from the gut and to provide visual representation of the analyses (Arumugam *et al.* 2010). Bioinformatics tools for organization, storage and interpretation of the gathered information were also developed. The frequencies of bacterial genes present in both healthy and sick individuals were determined in order to gain insight into any possible correlations of the bacterial genome carried by an individual to health and disease. Methods were developed to study the functions of the bacterial genes, which are associated with disease and the causal host-microbiome interactions. The findings of the project highlighted the differences in the distribution of anti-inflammatory bacterial species in the guts of healthy individuals versus the guts of sick individuals associated with IBD (Dusko Ehrlich and MetaHIT consortium, 2010). The findings of the project should allow for development of prognostic and diagnostic tools for gut microbiota modulation for health optimization. More information about the project can be found at http://www.metahit.eu.

The HMP project has created genomic sequence databases of over 2,200 strains isolated from the human body. The samples have been obtained from a test group of 300 healthy adults aged 18 to 40. The samples were collected from five main body locations including the gastro intestinal tract, urogenital tract, nasal cavity, skin and the oral cavity. Over all 11,000 samples were collected. The findings of the project were published in two main publications (Consortium, 2013) and (Human Microbiome Project Consortium *et al.* 2012) general information about the HMP is available at http://hmpdacc.org.

3.3 Functions of the gut microbiota

The gut microbiota confers gut colonization resistance to its host. The principle of colonization resistance is that the indigenous gut microbiota makes it difficult for foreign often invasive and pathogenic, bacteria to invade the gut, as it would have to displace the already well-established commensal bacteria. Additionally the gut microbiota protects the host from overgrowth of pathobionts, which can occur as a

result of disturbance of the healthy gut microbiome (Buffie & Pamer 2013; Hooper *et al.* 2012; Kamada Nobuhiko *et al*. 2013).

In an extensive review on the role of gut microbiota and chronic gastrointestinal disease, Guinane & Cotter (2013) highlight the importance of what is considered to be a "healthy gut microbiota" in the context of health and intestinal disease. The review emphasizes the fact that while a healthy gut microbiota confers benefits to the host its disruption may be a detrimental factor conferring development of a number of intestinal disorders such as a range of bowel disorders, obesity, diabetes and cancer **(Fig 4).** It is also proposed that manipulation of the human gut microbiome by controversial methods such as faecal transplants can have a therapeutic effect in treatment of aforementioned gastrointestinal disorders (Guinane & Cotter 2013).

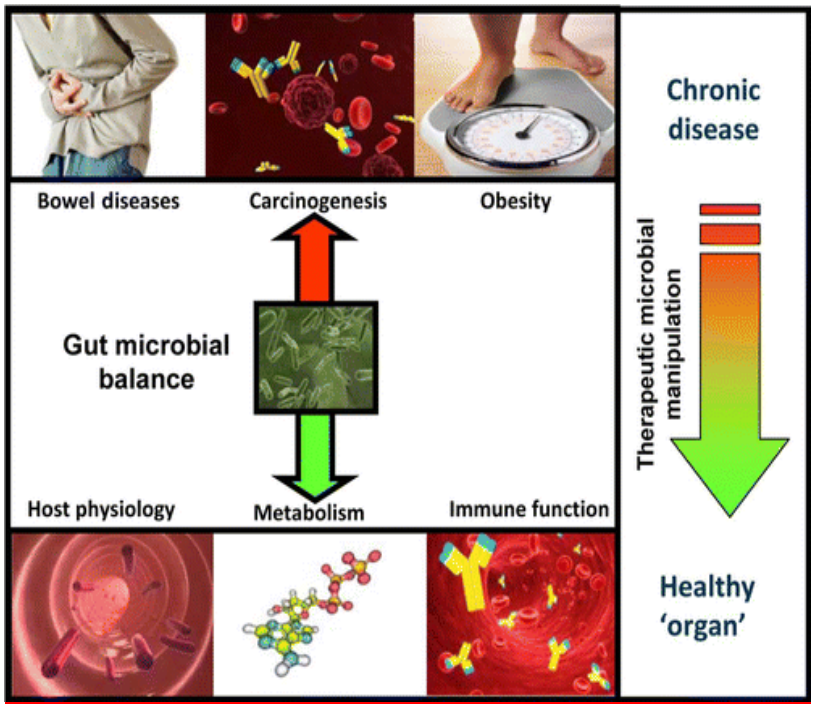
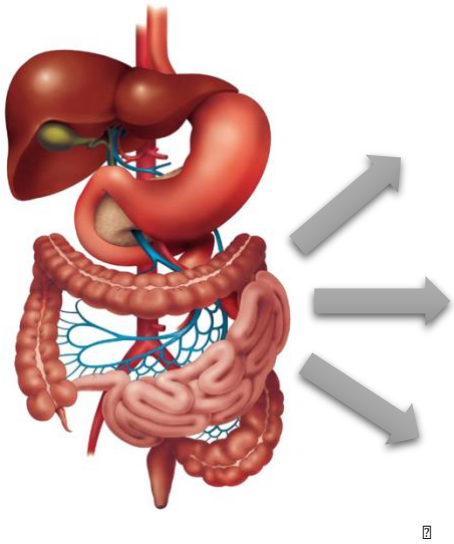


Fig.4 The role of a healthy gut microbiota in health and intestinal disease. (This figure was taken from Guinane & Cotter 2013).

The diversity of highly specific metabolites produced by the human gut microbiota and the complexity of their effects on the host have been discussed by Mousa *et al*. (2017), who has reviewed two decades of research in the area of the human gut microbiota, its effect on health and disease of the host as well as the taxonomic

diversity of the gut. The importance of the metabolic compounds such as amino acids, lipids and glycolipids, post-transitionally modified peptides as well as non-ribosomal peptides, oligosaccharides, polyketides and terpenoids and their functions in various aspects of human health such as cytotoxicity, immunomodulation, antimicrobial protection via production of antimicrobial peptides and production of antioxidants have been highlighted (Mousa *et al*. 2017).

It is also understood that the complex often symbiotic relationships between the human gut and the gut microbiome as well as organism present in different parts of our bodies have shaped our evolution, immunity to disease and behaviors for thousands of years suggesting that humans are super-organisms formed by those complex interactions as discussed by Kramer & Bressan (2015).



Protective functions	Structural functions	Metabolic functions
Pathogen displacement	Barrier function	Control intestinal epithelial cell differentiation and proliferation
Nutrient competition	Induction of IgA	Metabolize dietary carcinogens
Receptor competition	Apical tightening of tight junctions	Synthetize vitamins e.g. Biotin, folate
Production of antimicrobial factors e.g. bacteriocins, lactic acid	Immune system development	Ferment non-digestible dietary residues and endogenous epithelial-derived mucus

Fig. 5 Functions of the human gut microbiota (Figure adapted from O'Hara & Shanahan 2006).

4. Antimicrobials produced by gut bacteria

The production of antimicrobial agents is usually considered as a manifestation of competition in bacteria found in the same or similar environmental

niches (Cray *et al.* 2013; Mitri & Richard Foster 2013). Kommineni *et al.* (2015) has investigated the role of conjugative plasmid pPD1 responsible for bacteriocin expression in niche dominance by *Enterococcus* spp. in a mouse model colonized with *E. faecalis*. The experiment revealed that the *E. faecalis* harboring the pPD1 plasmid has replaced the native Enterococci present in the mouse gut, which did not have the pPD1 plasmid. It was also reported that while in the intestine the pPD1 plasmid was transferred to other *E. faecalis* strains. Overall the results have shown that the murine gut was colonized with *E. faecalis* harboring the pPD1 plasmid while the other strains of Enterococci including vancomycin-resistant Enterococci were eliminated, effectively showing that bacteriocin expression can significantly influence the niche competition within the gastrointestinal tract.

Antimicrobials are also often considered as signals used for communication with other microorganisms (Abrudan *et al.* 2015). A study demonstrating that microorganisms can use antibiotics as signals, which can be used to harmonize social interaction with bacteria inhabiting the same niche was carried out by Abrudan *et al.* (2015). Such behavior has been observed in antibiotic producing *Streptomyces*. The study has shown that the production of antimicrobial agents has noticeably increased due to competitive interactions between strains. Additionally it has been observed that bacteria can have a number of responses to signals sent by competing strains such as increase in the rate of production of antimicrobial agents, or suppression of antimicrobial production by the competing strains, which effectively decreases the direct threat posed by the competing strain (Abrudan *et al.* 2015)

It is also suggested that antimicrobial activity within dense microbial populations can act as an effective approach in maintaining biodiversity of a niche. A theoretical model developed by Czaran *et al.* (2002) looked at antibiotic production and how it shapes the structure and diversity of microbial communities within a spatially limited multispecies model. The model specified up to 14 different antimicrobials, it also specified that each bacterial clone can have three types; Killer type - it can excrete the antimicrobial while being resistant to it, resistant type – a bacterium which cannot produce the antimicrobial but is resistant to it and a sensitive type - a cell with no resistance or ability to produce the antimicrobial. The model takes into account the metabolic cost of antimicrobial production/ resistance with the metabolic cost being the

lowest in the sensitive strains and the highest in the killer strains. The model also shows that a niche dominance hierarchy; killer cells are dominant over the resistant cells, the resistant cells are dominant over sensitive cells and sensitive cells can be dominant over the killer cells due to the fact that their metabolic cost is the lowest. The model explains that shifts in the dominance can be made through acquisition of novel toxin production genes as well as the corresponding resistance genes by horizontal gen. At one extreme it can be assumed that every strain in the model represents a different species in a microbial community. This interpretation can be considered if novel antimicrobial systems are present at low levels and the horizontal gene transfer is relatively rare in that case species diversity is promoted. On the other side of the spectrum the model can also be applied to an environment representative of different variants of the same bacterial species. This scenario applies fast evolution of toxicity systems between strains due to more likely horizontal gene transfers and gene recombination processes resulting in polymorphic species. The intermediate and the most natural interpretation of the model can be applied to a bacterial community made of different species, where some may share common antimicrobial systems with low gene recombination rates again promoting species diversity within a niche (Czaran *et al.* 2002).

Despite the fact that the human gut microbiota and its interactions with the host are not fully understood and explored yet it is considered as a rich source of novel metabolites and antimicrobials. In a review on the molecules produced by the human gut microbiota, Donia and Fischbach (2015) discussed the fact that the spectrum of microbial metabolites found in the human gut is as broad as that of any ecosystem, however it was emphasized that a lot of those metabolites are produced specifically to accommodate interactions with the host such as production of novel antimicrobials conferring protection for the host (Donia and Fischbach, 2015). Bacteria found in the human gut utilize various mechanisms, which allow them to outcompete and antagonize other bacteria found in their ecological niche. There are a number of antimicrobials and metabolites, which can be produced by the gut bacteria in order to directly inhibit or limit growth of their competition. The activity of such antimicrobial agents can be very specific and target one organism in particular or non-specific and target a broader range of microorganism (Donia and Fischbach, 2015).

4.1. Non-peptide antimicrobials produced by gut bacteria.

Gut bacteria have been shown to produce non-peptide antimicrobials such as organic acids, hydrogen peroxide (H_2O_2), ammonia, diacetyl, phenolic compounds and carbon dioxide (CO_2) (Imlay 2008; Jay, 1982; Donia and Fischbach 2015).

Lactic acid and other organic acids such as propionic and acetic acids produced by the lactic acid bacteria (LAB) found in the human gut lack specificity in their spectrum of activity. Their mode of action is based on intrusion into the cytoplasm membrane and subsequent suppression of the active transport system within the targeted cell (Pessione, 2012). Another example of a non-specific, non-peptide antimicrobial metabolite produced by the gut bacteria is H_2O_2. Imlay (2008) discusses the toxicity of superoxide and H_2O_2 to bacterial cells. The antimicrobial effect of H_2O_2 is attributed to its ability to oxidize the cell as well as its molecular components. A study by Pridmore *et al*. (2008) has shown that H_2O_2 producing *L. johnsonii* NCC 533 (La1) isolated from human intestine kills *Salmonella enterica* serovar *Typhimurium* SL1344 in an *in vitro* model. Similar observations were reported by Hertzberger *et al*. (2014). It should also be noted that H_2O_2 can be used by bacteria in combination with lactic acid as previously reported for intestinal strains *L. johnsonii* NCC 533 and *L. johnsonii* NCC 933 and vaginal isolate *L. gasseri* KS120 (Atassi & Servin 2010 Pridmore *et al*. 2008). The gut microbiota can also produce other antimicrobial compounds such as diacetyl (Jay, 1982) ammonia, phenolic compounds and CO_2 which are also often associated with the antagonistic activity of the gut microbiome. However due to the fact that their activity is not specific their applications in the food industry as well as therapeutic uses are limited.

4.1.1 Short chain fatty acids (SCFA)

The human gut microbiota can confer pathogen resistance by producing short chain fatty acids (SCFA). Production of SCFA involves the break down and oxidation of non-digestible carbohydrates by the gut microbiota present in the anaerobic lumen effectively releasing short chain fatty acids as byproducts of fermentation. There are a number of biological pathways, which can result in formation

of SCFA. Commonly the complex carbohydrates are broken down into simple sugars, which are then fermented primarily by Bacteroidetes to form organic acids such as SCFA and hydrogen. *Clostridium* spp. and other bacteria capable of butyrate production are the second line of fermenters in the gut (Hooper *et al*. 2002; Sun and O'Riordan, 2013). The three main SCFA produced in the intestine are acetate propionate and butyrate, all of which carry out different functions in various organs. Acetate is mostly used in the peripheral tissues of the human body such as cardiac muscle and the skeletal tissues (Cummings and Macfarlane, 1997). Butyrate is predominantly metabolized by the intestinal epithelium. It is then transformed to ketone bodies and oxidized to carbon dioxide (Pennington 1952). It is estimated that butyrate provides 60-70% of the energy needs to the colonic epithelium (Roediger, 1980). It has also been shown that the metabolism of butyrate can prevent the oxidation of glucose (Fleming *et al.* 1991). Propionate on the other hand is carried to the liver through the portal vein. Studies on ruminants have shown that it plays a significant role as a precursor of gluconeogenesis in the glucose uptake in ruminants (Bergman *et al*. 1966), although its function in the human metabolism is not fully understood (Hooper et al., 2002). While SCFA's play very important role for the hosts energy homeostasis and immune functions they also act as one of the main carbon sources for endogenous gut microbiota (Fischbach and Sonnenburg, 2011) and when present at elevated concentrations can become toxic to bacteria (Sun and O'Riordan, 2013).

A number of studies have shown that toxic effects of SCFAs were assigned to non-ionized forms of the fatty acids in question, which is more likely to occur at low pH such as that in the gastric tract. For example a study carried out by Baskett & Hentges (1973) has shown inhibition of *Shigella flexneri* by acetic acid caused by interference with the bacterium's ability of intracellular accumulation of glutamic acid and glucose, resulting in inhibition of glucose metabolism and subsequent inhibition of the bacterium. The main process, which is understood to take place for SCFA dependent toxicity relies on the entry of the fatty acids into the bacterial cytoplasm. The non-ionized fatty acids are uncharged and small in size, thus are believed to be able to easily diffuse across the bacterial membranes (Ricke, 2003). Once the fatty acids are present inside the bacterial cytoplasm which normally has a pH of 6.5-7.5 the non-ionized acids diffuse causing a buildup of SCFA anions and protons (Lambert and Stratford, 1999). Subsequently the introduction of protons into the cytoplasm acidifies

the intracellular compartments of the cell and degenerates the proton motive force as described by (Axe and Bailey, 1995) for *E. coli* and its ability to transport acetate and lactate across its cytoplasmic membrane. This in turn can have a detrimental effect on the metabolic reactions and energy conservation of the cell which has been demonstrated by (O'Byrne *et al*. 2002) where the growth *E. coli* K12 has been inhibited by the reduction of its intracellular pH resulting from treatment with acetic acid. The buildup of SCFA anions inside the cytoplasm can also cause serious implication to the physiology of the cells including changes in the osmotic balance of the cell. SCFA mediated damage to *E. coli* MG1655 cell membranes has been shown by (Royce *et al.* 2013). The toxicity induced by SCFA frequently results in inhibition of growth due to pleiotropic defects taking place in the cellular processes. These defects are likely to differ depending on the organism, metabolic pathways and environmental conditions (Sun and O'Riordan, 2013). The effectiveness of SCFA growth suppression has been demonstrated *in vitro* where the growth of *E. coli* strain O157:H7 was significantly reduced (Shin *et al.* 2002). A study by Sun *et al.* (2012) has shown that elevated levels of butyrate have the capability to impede virulence factor production in *L. monocytogenes* thus implying that SCFA have a protective impact against infection with *L. monocytogenes.* Additionally SCFA especially those present in the gut in high quantities such as propionate and butyrate are responsible for down regulation of (SP1) pathogenicity genes of *S. typhimurium*, which are necessary for successful invasion of the human gut (Gantois *et al*. 2006; Lawhon *et al.* 2002). The toxic effects of SCFA build up have been used by the food industry in order to control microbial populations and improve food safety (Carpenter & Broadbent 2009; Ricke 2003). The addition of SCFA to animal feed has been practiced with the aim to prevent or reduce the colonization of vector animals such as chickens, which often shed pathogenic organisms such as *Salmonella* subsequently contaminating the food product (Van Immerseel *et al.* 2005).

4.2. Antimicrobial peptides produced by gut bacteria

Bacteria have the ability to produce antimicrobial peptides, those peptides can be specific in their antimicrobial activity, meaning that that they can target specific microorganism, which are often found in the same environmental niche as the peptide

producing bacteria. Antimicrobial peptides can be categorized based on the location in the bacterial cell where they were synthetized; i.e. they can be ribosomal or non-ribosomal.

Non-ribosomal peptides are secondary metabolites produced by synthesis of multi-functional peptides with enzymes. Examples of well-known non-ribosomal antimicrobial peptides include polymyxin, penicillins and vancomycin, which are currently used as antibiotics (Hancock and Chapple, 1999). Despite the fact that non-ribosomal peptides belong to a quite large group of bacterial metabolites across a number of various environments there is a relatively low number of non-ribosomal peptides identified and characterized from the human gut (Donia and Fischbach, 2015).

Ribosomal antimicrobial peptides are typically made up of 10 to 50 amino acids. Their antagonistic activity and specificity against bacteria is based on the interactions between the peptide, the cell membranes and cell walls of bacterial cells being attacked (Bahar & Ren 2013; Zhang & Gallo 2016). The levels of antimicrobial peptide production in the gut as well as their bioavailability are dependent on the producing strain as well as the environmental conditions. Identification and detection of such compounds can be especially problematic when talking about the gut as the physical environment and the limited bioavailability of such peptides can act as obstacles. Modern developments in the field of bioinformatics can be used to recognize and categorize genetic clusters found in samples from the human gut. Metagenomic studies of the human gut microbiota such as those carried out by Cimermancic *et al*. (2014), Donia *et al*. (2014), Drissi *et al*. (2015) and Walsh *et al*. (2015) all suggest that the human gut is source of novel antimicrobial peptides.

4.2.1 Bacteriocins

Bacteriocins are an example of ribosomally synthesized antimicrobial peptides. The first known bacteriocin "colicin" was discovered in 1925 as reported by Cascales *et al.* (2007). Since the first discovery bacteriocins have been isolated and characterized from a number of various environments including that of the human gut (Czaran *et al.* 2002). Bacteriocin production is regarded as one of the key factors

contributing to obtaining bacterial dominance at a niche level, which is often seen in dense microbial populations such as that of the human intestine (Cotter *et al.* 2005; Dobson *et al*. 2011; Guinane & Cotter 2013; O'Shea *et al*. 2009; Czaran *et al.* 2002; Kommineni *et al*. 2015). It is anticipated that production of at least one bacteriocin is a common trait in most bacteria (Dobson et al., 2011; Klaenhammer, 1988). While a high number of bacteriocins has been identified from food sources a considerable amount of bacteriocins have also been isolated from human gut microbes as can be seen in **table 1**. Additionally Drissi *et al*. (2015) suggests that there is a great number of potential antimicrobial producers in the human intestine. A database of 317 bacterial genomes holding 1,359 bacteriocin sequences was generated in the study, out of which 1050 were identified as bacteriocins from Gram-positive bacteria. Overall 962 of the uncovered bacteriocins were synthesized by Firmicutes, one of the most common phyla in the human gut (Arumugam *et al.* 2011).

Table 1. Examples of bacteriocins isolated from human gut bacteria and their spectrums of activity.

Group	**Bacteriocin**	**Producer (gut strain)**	**Antimicrobial activity**	**Reference**
Class IId	Acidophilucin A	*L. acidophilus* LAPT1060	*Lactobacillus* spp.	Toba *et al*. 1991
Class IIa	Avcin A	*E. avium* 208/XA83	*Listeria, Enterococcus, Lactobacillus, Leuconostoc, Pediococcus, Carnobacterium*	Birri *et al*. 2010
Class IIb	Bacteriocin B3A-B3B	*Enterococcus faecalis* B3A-B3B	*L. monocytogenes, S. aureus,* methicillin-resistant S. *aureus* (MRSA) *C.perfringens*	Al-Seraih *et al.* 2017
Class IIb	Abp118	*L. salivarius* UCC 118	*L. innocua, B. coagulans L. monocytogenes EGDe L.monocytogenes LO28*	Flynn *et al*. 2002 Corr *et al.* 2007
Class IId	ESL5	*E. faecalis* SL-5	*P. acnes*	Kang *et al*. 2009
Class IIa	Bifidocin B	*B.bifidum* NCFB1454	*L. plantrum, L. monocytogenes*	Yildirim *et al*. 1999
Class IIa	Enterocin A	*E. faecium* DPC6482	*L. monocytogenes*	O Shea *et al.* 2009
Class IIa	*Gassericin*	*L. gasseri LA39*	*L. acidophilus* JCM 5342,	Toba *et al.* 1991

			L. plantarurn JCM 1149, *L. gasseri* LA 32, LA 33, LA 38 and LA 39	
Class IId	Gassericin T	*L. gasseri* SBT2055	Not stated	Kawai *et al.* 2000
Class IId	Gassericin KT7	*L. gasseri* KT7	*B. cereus, C. piscicola, C. botulinum C. perfringens, E. faecalis, L. acidophilus L.delbrueckii, L. helveticus, L. plantarum, L. sake, L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. mesenteroides L. ivanovii, L. monocytogenes, M. avus, P.pentosaceus, S. aureus*	Zhu *et al*. 2000
Class IIb	Microcin M	*E. coli* Nissle 1917	*E.coli*	Patzer *et al.* 2003
Class Ia	Nisin O	*B. obeum* A2-162	*C. perfingenes*	Hatziioanou *et al*. 2017
Class Ia	Nisin Z	*P. acidilactici* MM33	Vancomycin-resistant *Enterococci* (VRE)	Millette *et al*. 2008
Class II	Pediocin PA-1	*P. acidilactici* UVA1	*L. monocytogenes*	Mathys *et al*. 2007
Class II	Pediocin PA-1AcH	*L. lactis* MM19	Vancomycin-resistant *Enterococci* (VRE)	Millette *et al*. 2008
Class Ia	Rumionococcin A	*C. nexile, R. gnavus* E1	*C. perfringens C. difficile*	Dabard *et al*. 2001
Class I	Thuricin CD	*B. thuringiensis* DPC6431	*C. difficile*	Rea *et al*. 2010

Bacteriocins can be sub-grouped based on their spectrum of inhibitory activity. A narrow spectrum of activity is generally used a measure of targeting a specific bacterium, often pathogenic, without affecting the natural bacterial population of the niche. Bacteriocins can tackle some of the issues associated with conventional antibiotics such as antibiotic resistance and are more often looked at as therapeutics for applications in the veterinary and human industry (Chikindas *et al*. 2018; Dobson *et al*. 2012; Montalban-Lopez *et al*. 2011). A number of studies have shown antimicrobial

properties of bacteriocins as an effective measure in controlling the populations of gastrointestinal pathogens *in vivo* (Corr *et al*. 2007), such as *Salmonella* spp. (Casey *et al*. 2004) and *C. jejuni* (Stern *et al*. 2006). A study by Corr *et al*. (2007) has shown the protective effect of a bacteriocin delivered from a probiotic bacteria *L. salivarius* UCC118 against infection with *Listeria* in mice. A study by Rea *et al*. (2011) has shown that the sactibiotic group of bacteriocins such as thuricin CD can inhibit bacteria such as *C. difficile* in a model of the distal colon while causing no adverse effects on the desired members of the gut microbiota. On the other hand a broad-spectrum bacteriocin can have a therapeutic potential targeting a number of microorganisms (Langdon *et al*. 2016).

Additionally bacteriocins have a great potential to be used in the food industry as preservatives due to their low levels of toxicity. Presently there are two bacteriocins, which are commercially used in food preservation, those are pediocin PA1 (MicrogardTM, ALTA 2431, Quest) and nisin (Nisaplin, Danisco) (Simha *et al.* 2012).

Pucci *et al*. (1988) have demonstrated that pediocin PA-1 can inhibit the growth of *L. monocytogenes* in half and half cream (commercially available pasteurized), cottage cheese and in cheese sauce. 10^2-10^4 CFU/mL of *L. monocytogenes* and 100AU/mL of pediocin PA-1were added to cream and the cheese sauce. 300g cottage cheese portions were supplemented with 10AU/g and 50AU/g of pediocin PA-1. All samples were kept at 4°C. *Listeria* counts for half and half cream and cheese sauce were taken immediately after experimental set up after 1 day, 7 days and 14 days. *Listeria* counts for cottage cheese were taken after 24 hr and 7 days. Rapid growth of *L. monocytogenes* was observed in the negative controls (i.e. food product without pediocin PA-1) of half and half cream and in cheese sauce while *Listeria* numbers decreased in the cottage cheese. Supplementation of the food systems with pediocin PA-1 has resulted in a rapid decrease in *Listeria* numbers in all tested foods.

Benech *et al.* (2002) has demonstrated an anti-*Listeria* effect of addition of capsulated nisin Z in to cheddar cheese as well as its' *in situ* production by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis UL719 used as the starter culture. The cheeses were produced on a pilot scale with 10^5 – 10^6 CFU/mL of *L. innocua* added to the milk used for cheese making, capsulated nisin Z at 300 IU/g was added to one cheese while

nsin Z producing strain was added to the second cheese. *Listeria* number were reduced by 3 log CFU/g in the cheddar with capsulated nisin Z and 1.5 log CFU/g in cheese made with nisin Z producing starting culture immediately after cheese production was complete. Following a 6 month ripening period the numbers of *L. innocua* in the cheese made with encapsulated nisin Z were reduced to <10 CFU/g additionally nisin Z maintained 90% of its initial activity. The cheese made with a nisin Z producing starter culture had 10^4 CFU/g of *Listeria* and maintained 12 % of its initial activity.

Evidence also highlights the numerous novel applications of bacteriocins in the food industry. It has been suggested that bacteriocins or bacteriocin producers can be potentially integrated into food packaging, providing protection from spoilage agents (Castellano & Vignolo 2006; Parada *et al*. 2007). This can be applied to already existing food packaging solutions such as modified atmosphere packaging MAP and can further extend their effectiveness in food protection (Liserre *et al* 2002). Liserre *et al* (2002) has demonstrated the effectiveness of application of a bacteriocin producing *Lactobacillus sake* to MAP. Bioactive food packaging infused with nisin has shown to reduce the numbers of LAB present in sliced cheese and ham. Additionally the packaging has shown to be effective in reduction of foodborne pathogens such as *L. inocua* by ~2 logs in both cheese and ham and *S. aureus* by ~1.5 log in cheese and ~ 2.8 logs in ham, similar results were obtained when vacuum packing the products. As described by Scannell *et al*. (2000) lacticin 3147 was also immobilized and applied to cellulose based food packaging and demonstrated activity for duration of 3 months (Scannell *et al*. 2000). Furthermore *L. curvatus* CRL705 isolated from dry cured sausage, producing bacteriocins lactocin 705 and lactocin AL705 has been reported to reduce growth of *B. thermosphacta* and *L. innocua* in vacuum packed meat discs as described by Castellano & Vignolo (2006). A study carried out by Chopra *et al.* (2015) has demonstrated that sonorensin, a bacteriocin isolated from *B. sonorensis* isolate of marine soil origin can effectively control the growth of *L. monocytogenes* and *S. aureus* in food. Its application was demonstrated in a low density polyethylene (LDPE) film, which was used as a coating on raw chicken meat spiked with *L. monocytogenes* or *S. aureus* the samples were stored at 4°C, a controls of meat samples set up with untreated films were also set up. All samples were observed for presence of unpleasant smell and any visible signs of bacterial growth at set time intervals. The applications of the sonorensin coated (LDPE) films were also investigated for applications in spoilage

prevention during storage of fresh vegetables. An experiment was set up where fresh tomatoes were packed in the active (LDPE) films and stored at 4°C, again controls were set up with untreated (LDPE) films. The tomatoes were observed for visible signs of spoilage and decay. The results of both experiments are presented in **Fig.6**.

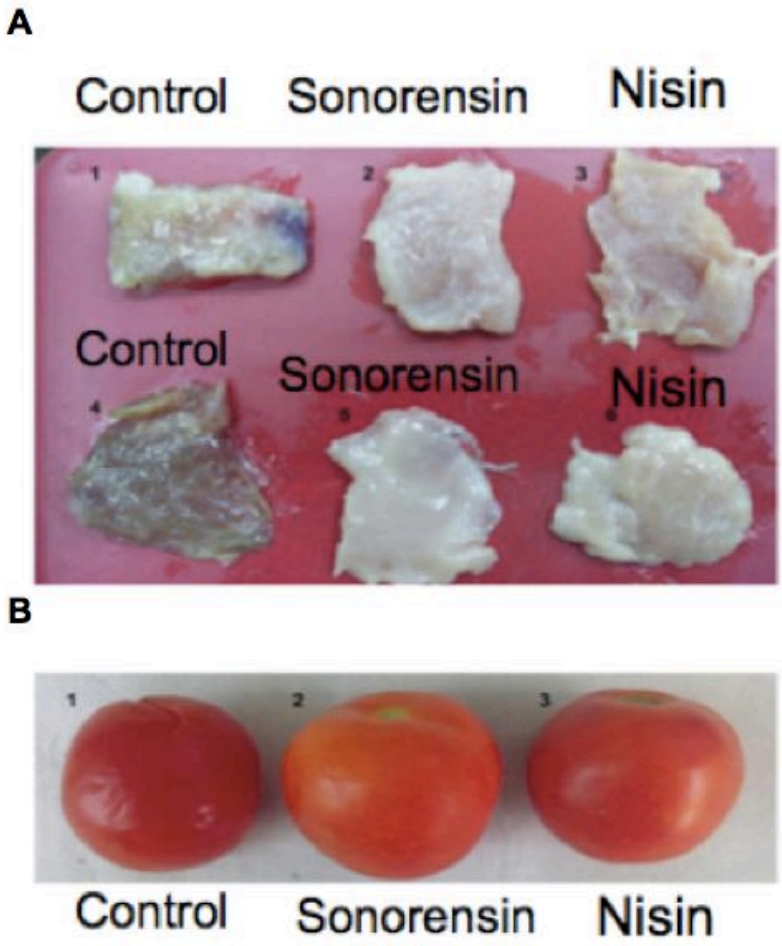


Fig.6 Results of the study carried out by Chopra *et al*. (2015). Preservative effect of coated low density polyethylene (LDPE) film during the storage of **(A)** meat **(B)** tomatoes. **(A)** Meat samples were spiked with *L. monocytogenes* (1–3) and *S. aureus* (4–6). Spoilage of meat is visible in meat samples packaged in control LDPE films (1 & 4) whereas no spoilage was observed in samples packaged with sonorensin (2 & 5) and nisin (3 & 6) coated LDPE films**. (B)** Tomato samples (1) packaged in untreated LDPE films showed signs of spoilage in contrast to no spoilage in case of tomatoes packaged in sonorensin (2) and nisin (3) coated LDPE films. (This figure was taken from Chopra et al. (2015).

Incorporation of bacteriocin producing bacteria into food products has also been investigated by McAuliffe *et al.* (1999). Here a lacticin 3147 producing *L. lactis* DPC4286 was used as a starter culture in the making process of cottage cheese. The

aim of the study was to investigate the anti-*Listeria* properties of the bacteriocin when produced *in situ*. The cheese was inoculated with 10^4 CFU/g of *L. monocytogenes* Scott A and stored at 4°C, the results obtained in the assay demonstrated a 99% reduction in the numbers of *Listeria* cells following a 5 day storage period. A control of non-lacticin 3147 cottage cheese was also inoculates with *L. monocytogenes* Scott A, however the results showed no reduction in the number of *Listeria* cells.

Bacteriocins can also be potentially applied as anti-biofilm agents. A study carried out Al-Seraih *et al.* (2017) has shown anti-biofilm properties of bacteriocins. Here bacteriocin B3A-B3B produced by human gut isolate *E. faecalis* B3A-B3B has demonstrated a significant reduction of *L. monocytogenes* biofilm formation on stainless steel.

4.3 Identification of antimicrobials from the gut.

Identification of novel antimicrobials such as bacteriocins from the human gut is often carried out with methods, which rely on detection of antagonistic activity of the producing strain against the indicator strain. There are a number of variations which can be applied to the assays while the principle remains the same (Hoover & Harlander 1993). In deferred antagonism assays the selected potential antimicrobial producing bacteria are grown on agar and subsequently overlaid with a layer of semi-soft or sloppy agar seeded with an indicator strain. Here a positive result is indicated with a zone of inhibition. A well diffusion assay implements the use of cell free supernatant (CFS) of the producing strain and adding it to wells cut out in agar seeded with an indicator strain. The CFS diffuses out into the agar surrounding the wells and if antimicrobial peptide is present a visible zone of inhibition is produced (Hoover & Harlander 1993). A study by O'Shea *et al.* (2009) is an example of a small-scale study using culture-based approaches in order to isolate novel antimicrobials from the gut. In total 278 gastrointestinal isolates were screened for activity agains *L. innocua* DPC3572, *L. bulgaricus* , *L.innocua* DPC3572 and *L. bulgaricus* LMG 6901 in a well diffusion assay. Subsequent characterization of the isolates revealed 23 individual strains producing bacteriocin like antimicrobials and three class II bacteriocins identified as enterocin A, salivaricin A and salivaricin P.

Recent developments in molecular tools allow for identification of clusters of putative bacteriocins in genomes from environmental samples. Public software and databases which are most commonly used for research such as BAGEL and BACTIBASE provide large numbers of genetic sequences (Hammami *et al.* 2010; van Heel *et al.* 2013). Walsh *et al.* (2015) used an i*n silico* approaches in their screening methodologies in order to mine for potential bacteriocin cluster in bacterial genomic sequences. BAGEL3 (i.e. a bacteriocin-mining tool) was used to identify possible bacteriocins. Consequently the resultant genome clusters were manually annotated and an evaluation of potential bacteriocin genes was carried out. As a result 74 bacteriocin clusters were identified form 130 putative producers.

While metagenomic *in silico* methods allow for identification of antimicrobials produced by previously uncultured residents of the gut microbiota, their subsequent characterization and evaluation of possible application may not always be possible (Hiergeist *et al.* 2015).

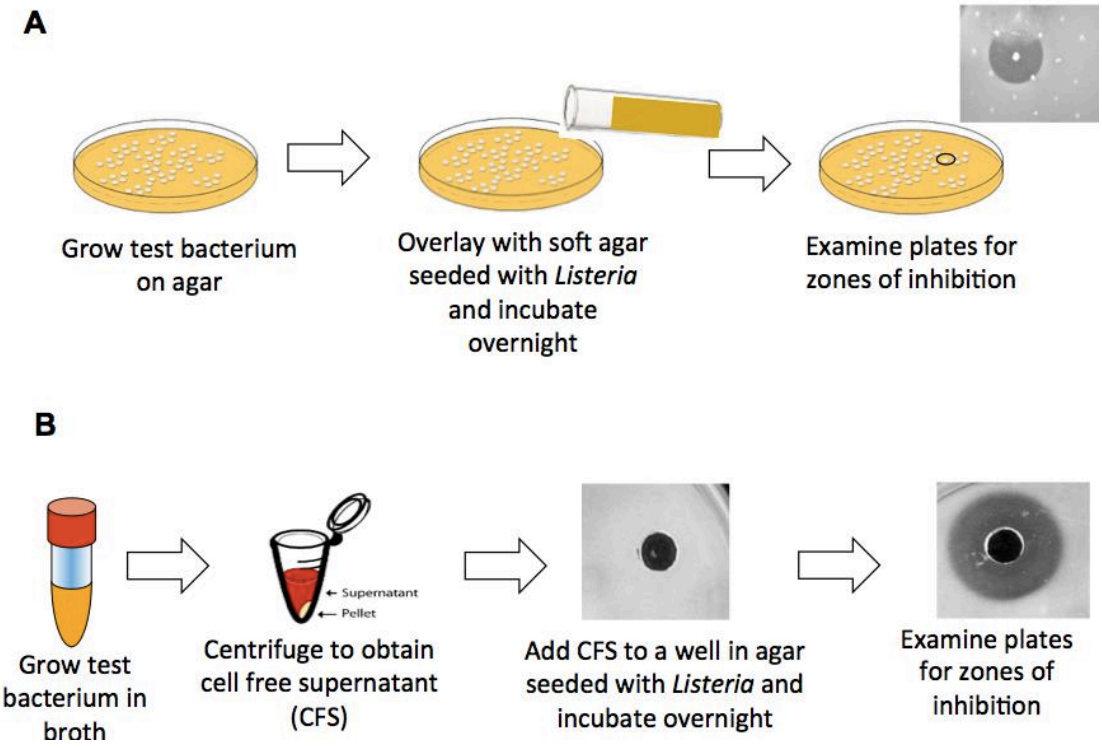


Fig.6 Bioassays used for identification of antimicrobials produced by the gut isolates **(A)** a deferred antagonism assay **(B)** well diffusion assay (This figure was obtained from Dr. Máire Begley).

5. Evidence that gut microbiota inhibits *Listeria.*

Studies dating back to the 1970s suggest that the gut microbiome has the ability to protect the host from becoming infected with *L. monocytogenes*. Zachar & Savage (1979) carried out experiments with germ free mice (GF) and specific pathogen free (SPF) mice. Both groups were inoculated with *L. monocytogenes*. Following a 24 hr incubation period the bacterium colonized colon and the cecum tissue of the GF mice. In contrast the SPF remained healthy, suggesting that the presence of the gut microbiota in the SPF mice had a protective effect and successfully inhibited *L. monocytogenes* colonization.

Corr *et al.* (2007) has demonstrated that a human isolate *L. salivarius* UCC118 has the ability to produce bacteriocin Abp118 which can sucessfully inhibit infection with *L. monocytogenes* EGDe and *L. monocytogenes* LO28 in a mouse model by 99%. The *in vivo* trials were carried out as follows; mice were were fed with the probiotic strain *L. salivarius* UCC118 at a concentration of 1 x10^9 CFU per mice for a duration of 3 or 6 days prior to administration of 2 x 10^9 CFU of *L.monocytogenes* EGDe per mouse. A placebo test group was fed with phosphate buffered saline (PBS) for 3 days prior to infection with the pathogen. In order to demonstrate that *Listeria* was in fact inhibited by bacteriocin Abp118 two controls were set up in the experiment. In one mice model was infected with a mutant stain of *L. salivarius* UCC118 unable to produce the bacteriocin, a second control was set up with *L. monocytogenes* engineered to express immunity protein AbpIM associated with imunity to to Abp118. The results have demonstrated that the mice treated with a mutant strain of *L. salivarius* UCC118 prior to infection with *L. monocytogenes* became infected with the pathogen. Furthermore no effect of antimicrobial treatment was observed mice infected with *L. monocytogenes* expressing immunity to the antimicrobial suggesting that the anti-*Listeria* activity was in fact mediated by bacteriocin Abp 118. Additionally typical infection associated with *L. monocytogenes* was observed in the placebo group fed with PBS.

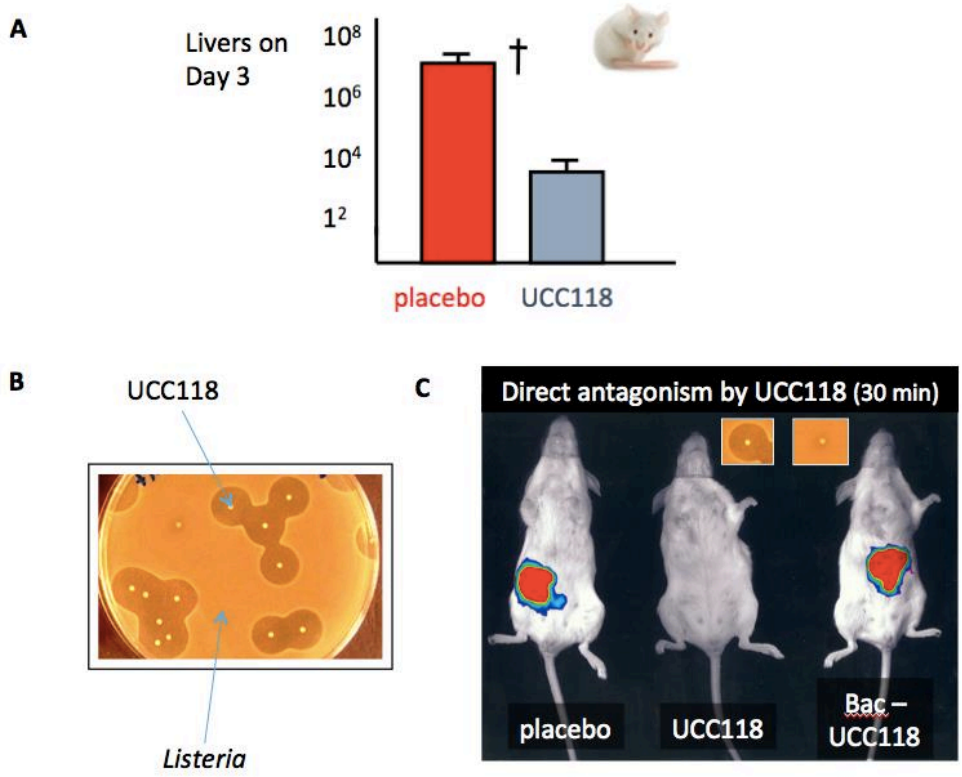


Fig. 7 Results of the study carried out by Corr *et al*. (2007) **(A)** Feeding mice with *L. salivarius* UCC118 can reduce the infectivity of *L.monocytogenes* by 99% in an animal model. **(B,C)** the antinfective activity was linked to bacteriocin produced by *L. salivarius* UCC118. (This figure was obtained from Prof. Colin Hill and was originlly adapted form Corr *et al.* 2007).

A study performed by Becattini *et al.* (2017) has demonstrated that the gut microbiota of mice can noticeably reduce the colonization of the gut lumen with pathogenic *L. monocytogenes* 10403S. The study has demonstrated that antibiotic treatment prior to infection with the intestinal pathogen effectively increases the growth of the pathogenic bacterium in the intestine and the susceptibility to disease. In the experiment mice treated with streptomycin (STREPTO) 24 hr prior to being infected with 10^2 CFU of *L. monocytogenes*. High levels of *Listeria* colonization were observed in the intestines of those animals, additionally the test animals displayed other symptoms of including faecal shedding of the pathogen exceeding a 10 day period, weight loss, diarrhea and general symptoms of distress. It was observed that the pathogen had penetrated the intestinal epithelium and spread into the mesenteric lymph nodes and subsequently to the spleens and livers of the antibiotic treated animals, this however was not observed in a control group of animals fed with PBS suggesting that a healthy gut microbiota i.e. not treated with antibiotics can provide colonization

resistance against *L. monocytogenes.* Similar results were observed in mice who were given a single dose of clindamycin (MNVC) and were infected with a sub-lethal dose of *L. monocytogenes* 24 hr following the antibiotic treatment. It was observed that clindamycin treatment resulted in an increased numbers of *Listeria* cells in the intestinal lumen and tissue.

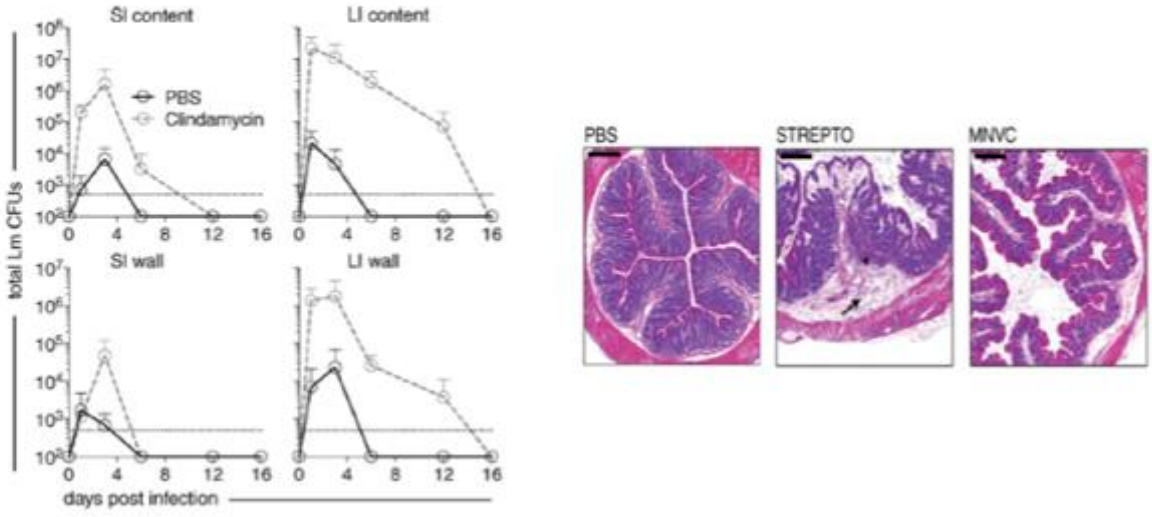


Fig. 8 Antibiotic treatment predisposes to severe *L. monocytogenes* infection. Mice were treated with a single i.p. injection of clindamycin or PBS and then orally infected with *L. monocytogenes*. The bar charts show the number of *L. monocytogenes* (Lm) in the small intestine (SI) and large intestine (LI) walls and contents after 24hrs. The pictures show stains of colonic tissue 3 days after infection. In this experiment mice were treated with streptomycin (STREPTO) or clindamycin (MNVC). (This figure was taken from Becattini *et al.* 2017).

Similar findings were observed by Czuprynski & Balish (1981) who also demonstrated that the gut microbiota of healthy rats had a protective effect against *L. monocytogenes* as the bacterium did not colonize the guts of rats with indigenous gut microbiota. The study has also revealed that antibiotic treatment increases the susceptibility to *L. monocytogenes* infection and that gut microbiota delivers defense against *L. monocytogenes* infections in immune-compromised hosts.

6. Conclusions and future directions

L. monocytogenes is a foodborne pathogen, which is commonly found in the environment. It has an outstanding ability to persist in various environments and withstand exposure to harsh conditions of food processing and other environmental stresses such as extreme temperatures, pH and water activity. Furthermore *Listeria* has the ability to persist in the food processing environments due to it's ability to form biofilms in so-called harbourage sites which are difficult to clean. When growing in a biofilm form *Listeria* shows increased levels of resistance to antimicrobial agents. Such persistence in the food industry can potentially lead to post processing contamination of food product, which is particularly dangerous especially in ready to eat foods as *Listeria* causes a rare but highly infective foodborne disease listeriosis.

The fact that *Listeria* persists in the environment makes its elimination from the food chain very difficult. Efforts are therefore focusing on controlling the pathogen in the food processing environment and in food products. Methods of control under investigation include different sanitizers, photodynamic inactivation, bacteriophage and bacteriocins (NicAogáin & O'Byrne 2016).

Novel antimicrobial compounds produced by the members of the human microbiome could be purified and potentially applied as a measure for decreasing and controlling *Listeria* populations in the food industry. Potential applications of such antimicrobials include reducing biofilm formation on stainless steel and other surfaces. Additionally gut derived antimicrobials can be potentially used as a natural preservatives or food ingredients and can be incorporated into food packaging with the aim of extending the shelf life and safety of the products associated with outbreaks of *Listeria.*

Chapter 1

Isolation and identification of human gut-derived bacteria with anti – *Listeria monocytogenes activity*

INTRODUCTION

In recent years the human microbiota has been researched in great detail. Evidence found in numerous studies shows that the human gut microbiota has an impact on many aspects of human health including immune function, nutrition, physiology and metabolism (Cénit *et al*. 2014; Eckburg *et al*. 2005; Guinane & Cotter 2013). A growing number of studies support the theory that the gut microbiota plays an important role in the predisposition and susceptibility to diseases by carrying out protective functions (Cénit *et al*. 2014). It has been proposed that antimicrobials produced by commensal human bacteria play a fundamental role in establishing a long term symbiotic relationship with the host (Zheng *et al*. 2015).

Experiments with animal models have provided evidence to suggest that intestinal microbes provide defense against *L. monocytogenes* infection. Zachar & Savage (1979) performed experiments with two groups of mice; one gnotobiotic and one specific pathogen free (SPF) where the indigenous microbiota was present. Both groups were orally inoculated with ~ 100 CFU/mL of *L. monocytogenes*. After 24hr *L. monocytogenes* colonized the gnotobiotic mice and reached 10^5 to 10^7 bacterial cells per gram of stomach tissue and 10^8 to 10^9 bacterial cells per gram of cecum and colon tissue. On the other hand, the SPF mice stayed healthy after oral inoculation with up to 5 x 10^7 bacterial cells with *L. monocytogenes* isolated from the gastrointestinal tract of only one of a total of 27 SPF mice. The study showed that establishment of *L. monocytogenes* was prevented by the presence of the indigenous gut microbiota in the SPF mice. Similar findings were observed by Czuprynski & Balish (1981) who demonstrated that *L. monocytogenes* colonization of germ-free rats reached 10^{10} to 10^{11} bacterial cells per gram of dry weight within 24 hr following oral inoculation. Here the indigenous microbial flora present in healthy rats also demonstrated a protective effect against *L. monocytogenes* as the bacterium did not colonize the guts of rats with indigenous gut microbiota. It has also been shown that treatment with antibiotics increases the predisposition to becoming infected with *L. monocytogenes* and that the gut microbiota delivers defense against *L. monocytogenes* infections in immune-compromised hosts (Becattini *et al*. 2017). As already mentioned in the literature review, it is suggested that the gut microbiome is a source of large numbers of bacteriocin-producing bacteria (Drissi *et al.* 2015).

The antimicrobial properties of bacteriocins have shown to be an effective measure in controlling the populations of GI pathogens *in vivo* (Corr *et al*. 2007; Guinane & Cotter 2013). Studies have shown that *L. monocytogenes* (Corr *et al*. 2007), *Clostridium difficile* (Rea *et al.* 2011), *Salmonella* spp. (Casey *et al*. 2004) and *Campylobacter jejuni* (Stern *et al*. 2006) can all be controlled with the use of bacteriocins. Antimicrobial production has been demonstrated by bacterial species isolated from the human gut (Al-Seraih *et al*. 2017; Birri *et al*. 2010; Birri *et al*. 2013; Booth *et al*. 1977; İspirli *et* al. 2015; O'Shea *et al*. 2009) and many more. This provides a strong basis on which a hypothesis can be made that screening of the gut microbiota has the potential for successful isolation and identification of novel antimicrobials such as bacteriocins (O'Shea e*t al*. 2009). The aim of the current study is to isolate anti-*Listeria* bacteria of human-gut origin, identify them and characterize the antimicrobials produced by the selected gut-derived isolates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial strains used in this study and the growth conditions used for their cultivation are listed in **Table1.**

Table 1. Growth conditions used for cultivation of bacterial strains used in the study. Bacterial cultures were grown in brain heart infusion (BHI) broth/ agar (Oxoid), or M17 agar/broth (Lab M) supplemented with 1% (w/v) glucose (Oxoid) i.e. GM17.

Strain	Growth media	Incubation temperature	Source	Comment/ Reference
Listeria monocytogenes 10403S	BHI	37°C	UCC culture collection	Serotype 1/2a, isolated from human skin lesion
Listeria monocytogenes EGDe	BHI	37°C	UCC culture collection	Serotype 1/2a, isolated from rabbit tissue
Listeria monocytogenes F2365	BHI	37°C	UCC culture collection	Serotype 4b, a cheese isolate from 1985 Californian listeriosis outbreak
Listeria monocytogenes LO28	BHI	37°C	UCC culture collection	Serotype 1/2 c, isolate from faeces of a healthy pregnant woman
Lactococcus lactis NZ9700	GM17	30°C	CIT culture collection	Nisin producer
Lactococcus lactis HP	GM17	30°C	CIT culture collection	Indicator strain sensitive to nisin
Listeria innocua 13568t	BHI	37°C	Teagasc Moorepark culture collection	
Enterococcus faecium DPC1146	BHI	37°C	Teagasc Moorepark culture collection	Enterocin A producer (O'Keeffe et al., 1999)
Enterococcus faecium DPC6482	BHI	37°C	Teagasc Moorepark culture collection	Enterocin A producer (O'Shea et al., 2009)
Enterococcus faecium EM342-BC-1	BHI	37°C	Teagasc Moorepark culture collection	Enterocin B producer
Micrococcus luteus	BHI	37°C	UCC culture collection	
Staphylococcus aureus ATTC6538	BHI	37°C	CIT culture collection	

Salmonella enteritidis	BHI	37°C	CIT culture collection	
Pseudomonas aeruginosa DSM1128	BHI	37°C	CIT culture collection	
Bacillus subtilis	BHI	37°C	CIT culture collection	

CIT- Cork Institute of Technology, **UCC**- University College Cork

Assembly of human faecal bacterial banks. The faecal samples were acquired from a previous study in our lab and were originally obtained from 23 lean male donors with a body mass index of <25 (Clarke et al., 2014). The samples were stored at -80°C until ready to use. The faecal samples were thawed on ice and 1 g portions of each were aseptically transferred into sterile plastic bags and sealed. All subsequent manipulations were performed in an anaerobic chamber (Don Whitley). 9 mL of Maximum Recovery Diluent (MRD) (Merck) was added to each 1 g sample of faeces and samples were manually agitated until all soluble constituents were dissolved. The resulting faecal water was serially diluted in MRD and 100µL of each dilution was spread plated in triplicate onto Wilkins-Chalgren Anaerobe (WCA) agar (Oxoid). Plates were incubated anaerobically at 37°C for 48 hr. All colonies obtained for an individual faecal sample were then pooled and transferred to a sterile stock bottle (i.e. 1.5 mL WCA broth was pipetted onto the surface of a plate, colonies were scraped into the broth using a sterile plastic spreader. The resultant broth containing bacteria was removed by pipetting and transferred onto the surface of another plate of colonies from the same faecal sample. This was continued until all colonies for that faecal sample were recovered). 500µL of sterile 80% (v/v) glycerol (final concemtration 40%) was added to the stock tubes and these faecal bacterial banks were stored at -80°C.

Selection of a *Listeria monocytogenes* indicator strain. A deferred antagonism assay was carried out in order to determine the sensitivity of the indicator strains *L. monocytogenes* EGDe*, L. monocytogenes* F2365*, L. monocytogenes* L028 and *L. monocytogenes* 10403S to nisin. The nisin producing strain *Lactococcus lactis* NZ 9700 was grown overnight in GM17 broth at 30°C for 18 hr. After the incubation time was competed 20µL of the overnight was spotted onto the centre of a GM17 plate in triplicate, the plates were then left to dry in a biosafety cabinet and incubated overnight

at 30°C. Simultaneously, individual colonies of the indicator strains were inoculated into 10 mL of fresh BHI broth and incubated at 37°C for 18 hr. After the incubation time was completed the spotted GM17 plates were examined for contamination and exposed to a 30 min UV light treatment in a CL-1000 Ultraviolet Crosslinker (UPV) in order to kill off the bacteriocin producing cells. The plates were then overlaid with 10 mL of BHI sloppy 0.7% (w/v) agar seeded with 100μL of indicator overnight cultures and incubated at 37°C for 18 hr. After the incubation was competed the zones of inhibition were measured (including the bacterial spot).

Identification of gut-derived bacteria that directly antagonize *L. monocytogenes.* When required, faecal bacterial banks were removed from the -80°C freezers and thawed on ice. 100μL of the bank was serially diluted in MRD and aliquots were plated onto WCA agar and incubated anaerobically at 37°C for 48 hr. Individual colonies were manually transferred into WCA broth in 96 well plates i.e. each colony was picked with a sterile pipette tip and transferred to an individual well of a 96 well plate containing WCA broth. The 96 well plates were incubated at 37°C for 24 hr after which wells were checked for turbidity and 2μL aliquots were transferred onto WCA agar plates using a multichannel pipette. The plates were incubated anaerobically at 37°C for 24 hr. All dilutions, plating, incubations etc. were performed in an anaerobic chamber. Plates were checked for growth, removed from the anaerobic chamber and exposed to UV light in a CL-1000 Ultraviolet Crosslinker for 45 min in order to kill all viable cells. The plates were subsequently overlaid with sloppy BHI agar 0.7% (w/v) seeded with 1% *L. monocytogenes* 10403S overnight culture. The plates were incubated aerobically at 37°C for 24 hr after which they were examined for zones of inhibition.

Selection of gut-derived strains for further investigation and stocking. 59 gut-strains, which inhibited *Listeria* i.e. produced zones of inhibition in the screening assay, were selected for further investigation. These strains were chosen on the basis of their colony morphologies and zone sizes i.e. it was hoped that by selecting strains with different appearance and zone sizes that different type of bacteria would be identified. The 59 strains were obtained from the 96 well master stock plates. The plates were removed from the -80° C freezer and left to thaw out in a biosafety cabinet for 15 min. The plates were then transferred into an anaerobic hood and once fully thawed out the contents of each well of interest were mixed with a sterile pipette tip. 20μL aliquots

of each were transferred into 10 mL WCA broth. Inoculated broth was then mixed with a vortex and incubated anaerobically at 37° C for 24 hr. Overnight cultures were vortexed and streaked onto WCA agar plates. Plates were incubated anaerobically at 37°C. Cell morphology was recorded after 24 hr and after 72 hr incubation. Any sample containing more than one colony type was re-streaked on fresh WCA agar. Stocks were prepared by adding 500µL aliquots of fresh overnight cultures to 500µL of 80% (v/v) glycerol in sterile stock tubes. These stocks are stored at -80° C for long-term storage.

Confirmation of the anti-*Listeria* activity of the gut-derived strains. Shortlisted gut- derived isolates were grown overnight at 37°C in 10 mL of WCA broth. 5µL aliquots of overnight cultures were then spotted onto the center of fresh WCA agar plates and incubated anaerobically at 37°C for 24 hr. Following the incubation the WCA agar plates were checked for growth and were exposed to UV light in a CL-1000 Ultraviolet Crosslinker for 45 min in order to kill any viable cells. The plates were then overlaid with 5 mL BHI sloppy 0.7% (w/v) agar that was seeded with 1% *L. monocytogenes* 10403S overnight culture. The plates were incubated at 37°C for 24 hr after which they were checked for the presence of zones of inhibition. Zones were measured with a Vernier calipers.

Gram staining. All gut – derived isolates were streaked onto fresh WCA agar plates and incubated anaerobically at 37°C for 24 hr. Individual colonies were then picked and a standard Gram stain procedure was carried using a Staining Kit (Sigma Aldrich) in accordance to the manufacturers' instructions.

MALDI-TOF MS. All gut-derived isolates were anaerobically inoculated from WCA broth onto fresh WCA agar and incubated anaerobically at 37°C for 24 hr. Individual colonies of each isolate were deposited onto the surface of a MALDI target plate in duplicate (one thick and one thin coat of sample for increased chance of identification). Each well of the target plate was coated with 1µL of matrix solution and left to dry at room temperature for 5 min. The target plate was then inserted into the Burker Daltonik MALDI Biotyper located at Cork University Hospital (CUH). The data collected was classified in accordance to Burker Taxonomy database of CUH. The database allowed for identification of microorganisms by ranking the mass of cell

proteins into peaks, then the peaks were ranked based on the score of the peak against the database. A score of 2.3-3.0 indicated a highly probable species identification, 2.99-2.00 indicated a secure identification and a probable species identification, 1.99-1.70 indicated a probable genus identification and a score lower than 1.69 indicated no reliable identification.

16S rRNA partial gene sequencing. Individual colonies from WCA agar plates were grown anaerobically in 10 mL WCA broth 37°C for 24 hr. Genomic DNA was extracted from 1.5 mL of overnight culture using the BlueElute Bacterial GenomicKit (Sigma Aldrich) in accordance with the instructions given by the manufacturer. Extracted genomic DNA was quantified using a NanoDrop (Thermo Scientific). Genomic DNA was used as a template in PCRs with universal 16S rRNA primers (CO1 5' AGTTTGATCCTGGCTCAG3' and CO2 5'TACCTTGTTACGACTT3'). The Platinum PCR super mix (Invitrogen) was used according to manufacturers' instructions. PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and subsequently quantified with the Qubit dsDNA HS Assay kit (Molecular Probes Life Technologies) again in accordance to instructions provided by the manufacturer. Purified PCR products were sequenced by GATC Biotech using the 16S primers CO1 and CO2. Homology searches were performed using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Investigation of the anti-*Listeria* activity of cell free supernatants and whole cell extracts. Individual colonies of gut-derived isolates from taken from WCA agar were inoculated into fresh WCA broth and incubated anaerobically for 24 hr at 37°C. 2 mL aliquots of each overnight culture were centrifuged at 12,000 rpm for 6 min. 1 mL aliquots of the cell free supernatant (CFS) were removed from the cell pellet and transferred into a fresh sterile eppendorf tubes and were re- centrifuged at 12,000 rpm for 6 min. 500µL aliquots of the CFS were then transferred into fresh sterile Eppendorf tubes. The approximate pH of CFS was measured with pH strips (Macherery – Nagel). Cell pellets were used to obtain whole cell extracts (WCE) as described by Field et al. (2012). For the WCE preparation the cell pellets were re-suspended in 2.5 mL of 70% IPA 0.1% TFA (isopropanol + trifluoroacetic acid) and placed on a shaking incubator (9,000 rpm) for 3 hr at room temperature. Tubes were then centrifuged (6,500

rpm x 10 min) and 1 mL was removed and transferred to fresh sterile Eppendorf tubes. BHI agar was seeded with *L. monocytogenes* 10403S i.e. aliquots of a *L. monocytogenes* 10403S overnight culture (0.5% inoculum) were added to freshly autoclaved BHI agar that was cooled to ~50°C and poured into sterile Petri dishes. The plates were allowed to set for 20 min inside a biosafety cabinet after which wells were bored in the agar with a sterile P200 pipette tip. 45µL of the CFS and WCE extracts were added to individual wells. The CFS and WCE from the nisin producer *L. lactis* NZ9700 were added to the wells as positive controls. The plates were incubated for 24 hr at 37°C after which they were examined for zones of inhibition.

Deferred antagonism assays to examine the spectrum of activity of gut-derived strains. The ability of the 59 gut-derived strains to inhibit a selection of indicator strains, namely *L. monocytogenes* 10403S, *L. monocytogenes* F2365, *L. monocytogenes* LO28, *L. monocytogenes* EGDe, *L. innocua, E. faecium, E. hirae, S. aureus, S. enteritidis, B. subtilis, E. coli*, *M. luteus, L. lactis* HP and *P. aeruginosa*, were examined by performing agar-based deferred antagonism assays. *L. lactis* HP was streaked onto GM17 agar and incubated at 30°C for 18 hr. All other strains (the 59 gut-derived isolates and the indicator stains) were taken out of -80°C stock onto fresh BHI agar and incubated at 37°C for 18 hr. The 59 gut-derived strains were then grown in BHI broth and incubated at 37°C for 18 hr. 50µL aliquots were spotted onto the center of fresh BHI agar plates and plates were incubated 37°C for 18 hr. The spot plates were UV treated for 30 min in a CL-1000 Ultraviolet Crosslinker, the plates were then overlaid with 5 mL of sloppy 0.7% (w/v) BHI/ GM17 agar seeded with 1% inoculum of an overnight of the relevant indicator strain (grown in BHI/GM17 at 37°C/ 30°C as appropriate). The overlaid plates were incubated at 37°C for 18 hr and examined for zones of inhibition.

Cross immunity deferred antagonism assays. Selected gut-derived isolates and an enterocin A producer *E. faecium* DPC 6482 and an enterocin B producer *E. faecium* EM342-BC-1 were taken out of stock onto fresh BHI agar and incubated at 37°C for 18 hr. Fresh colonies were transferred into 10 mL of fresh BHI broth and were incubated at 37°C overnight. 10µL aliquots of the bacterial cultures were then spotted onto BHI agar and incubated at 37°C for 18 hr. The plates were subsequently UV

treated for 30 min in a CL-1000 Ultraviolet Crosslinker and overlaid with 5 mL of sloppy BHI 0.7% (w/v) agar seeded with 1% inoculum of other gut-derived strains originating from the same faecal bank / or the enterocin A/ B producing strains. The plates were then incubated at 37°C for 18 hr after which they were examined for zones of inhibition.

Deferred antagonism assays in the presence of proteinase K. Deferred antagonism assays were carried out using the previously described method except with the addition of proteinase K (Sigma- Aldrich). All short-listed gut-derived strains were spotted onto two separate plates, grown overnight and subsequently UV treated for 30 min in a CL-1000 Ultraviolet Crosslinker. One plate was then left untreated as a control. On the other plate 2µL of a proteinase K stock (50 mg/mL) was spotted next to the bacterial spot. All plates were incubated at 37°C for 1hr. Plates were then overlaid with 0.7% (w/v) sloppy BHI agar seeded with 1% *L. monocytogenes* 10403S or *E. faecium* DPC 6482 overnight cultures and were incubated at 37°C for 18 hr after which they were examined for zones of inhibition.

PCRs with primers for enterocin genes. Selected gut-derived strains and enterocin A and enterocin B producers were grown overnight in BHI broth at 37°C for 18 hr. DNA was extracted from 1.5 mL of overnight cultures with the BlueElute Bacterial Genomic Kit (Sigma Aldrich) in accordance with the instructions given by the manufacturer. The sequences of the genes responsible for the production of enterocin A (primers TH 10 and TH11) were obtained from Aymerich et al., (1996) and O'Shea et al., (2009) and were synthesized by Eurofins Genomics. The gene sequences responsible for production of enterocin B, enterocin L50A, enterocin L50B, enterocin P, duracin A, duracin B, duracin Q and hiracin JM79 were obtained from the NCBI nucleotide search engine and GenBank (https://www.ncbi.nlm.nih.gov/nuccore/). Oligonucleotide primers were designed for these genes with Snap Gene, (sequences are provided in **Table 2**) and were synthesized by Eurofins Genomics. The PCR conditions used for detection of the enterocin A gene were as follows: initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C x 1 min, annealing at 47°C x 1 min and extension at 72°C x 1 min, followed by a final extension step at 72°Cx 1 min. The PCR products were run on a 2.5% agarose gel containing SYBR Safe (Invitrogen) at 100V for 1hr and visualized under UV light

miniBIS pro (DNR Bio-Imagining Systems). The approximate size of products was determined by comparison to the molecular weight marker HyperLadder IV (Bioline). The same conditions were used for detection of enterocin B, enterocin P duracin A, duracin B, duracin Q and hiracin JM79 except the annealing temperature was set to 50°C, similarly a change to the annealing temperature was made in attempts to amplify enterocin L50A and enterocin L50B genes (annealing temperature set to 49°C).

Table 2. Sequence of primers used to detect known bacteriocin genes

Primer name	Primer sequence	Bacteriocin encoded by the structural gene targeted	Sequence accession number	Reference
TH10	5'GATTATGAAACATTTAAAAATTTTGTC 3'	Enterocin A	-	(O' Shea et al., 2009) (Aymerich et al., 1996)
TH11	5' CAA GAA TAT CAG AAT ATT TAG G3'	Enterocin A	-	(O' Shea et al., 2009) (Aymerich et al., 1996)
EntcinB_Fr.	5' GTTATTCCTCATTCAGAGTTCC 3'	Enterocin B	U87997.1	This study
EntcinB_ Rv.	5'GAAGAGAAAAAACTCGTAATGAG3'	Enterocin B	U87997.1	This study
EntcinL50A_Fr.	5' GCTTCTTTTGGACTCATAACC 3'	EnterocinL50A	AJ223633.1	This study
EntcinL50A_Rv.	5' GCGTTAAGCCGAATGTTTAC 3'	EnterocinL50A	AJ223633.1	This study
EntcinL50B_Fr.	5' GGCTTGATATAGTTGCATTTCATC 3'	EnterocinL50B	AJ223633.1	This study
EntcinL50B_Rv.	5' CTATCATTAACTAAATTTTGGGGTGG 3'	EnterocinL50B	AJ223633.1	This study
EntcinP_ Fr.	5' GACACACGATTTTCTAGGGAATG 3'	Enterocin P	AF005726.1	This study
EntcinP _Rv.	5' AGTTCCCATACCTCGCAAAC 3'	Enterocin P	AF005726.1	This study
DurA_Fr	5'CACGGGTATAGCAAGCTC3'	Duracin A	HQ696461.1	This study
DurA_Rv	5' CATCCCCTATATTTTTGGCTCT 3'	Duracin A	HQ696461.1	This study
DurB_Fr.	5' GTTGGGTAGATTGGAATAAAGCTTC3'	Duracin B	HQ696461.1	This study
DurB_Rv.	5' GCTGGGCTATAAAACACCATTG 3'	Duracin B	HQ696461.1	This study
DurQ_ Fr.	5' CCATGATACAGTCATGTTGTAATAC3'	Duracin Q	AB298307.1	This study
DurQ_Rv.	5' CTTCCAGATACTCTTAGATGATAAGC3'	Duracin Q	AB298307.1	This study
HirJM79_Fr.	5' GGCGTTACTTGGTAGTATCG3'	Hiracin JM79	DQ664500.1	This study
HirJM79_Rv.	5' CACCTTCGGTGAAATTGTTG3'	Hiracin JM79	DQ664500.1	This study

RESULTS

Assembly and preparation of human bacterial banks. A total of 23 bacterial banks from 22 human faecal samples have been created and stored at -80°C (two separate samples of faeces number 26 were collected). The number and morphology / appearance of the colonies were noted. It was recorded that on average 10^7 CFU/ mL of cells were recovered from each faecal bank (data not shown). A variety of colony morphologies were also observed; small white pinpoint colonies, beige irregular colonies, large irregular semi-transparent colonies etc.

Selection of a *L. monocytogenes* indicator strain. *L. monocytogenes* 10403S demonstrated the highest sensitivity to nisin, with an average zone of inhibition of 23 mm, when compared to the average zones of inhibition produced by *L. monocytogenes* EGDe of 19mm (**Fig.1**), *L. monocytogenes* F2365 has a zone of inhibition of 16 mm and *L. monocytogenes* L028 has an average zone of inhibition of 17.3 mm. It was therefore decided to use *L. monocytogenes* 10403S as the indicator for the screening assays with the bacterial faecal banks.

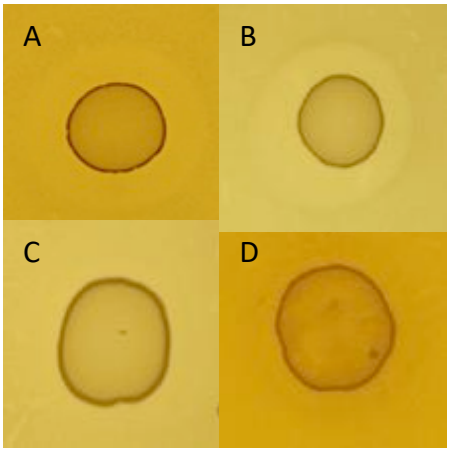


Fig.1 *Lactococcus lactis* NZ9700 (a nisin producer) was grown overnight in GM17 broth and incubated at 30° C. Following the incubation 10μL aliquots of the overnight culture were spotted in the center of fresh GM17 agar plates and incubated overnight at 30° C. The plates were then UV treated for 30 minutes in order to kill any viable cells. The UV treated plates were subsequently overlaid with fresh BHI sloppy agar seeded with 1% inoculum of **(A)** *L. monocytogenes* 10403S **(B)** *L. monocytogenes* EGDe **(C)** *L. monocytogenes* L028 **(D)** *L. monocytogenes* F2356. The plates were subsequently incubated at 37° C overnight and examined for zones of inhibition.

Identification of gut-derived bacteria strains that directly antagonize *L. monocytogenes.* The ability of gut-derived bacteria to inhibit *L. monocytogenes* 10403S was investigated using agar-based deferred antagonism assays as described in Materials and Methods. Differences were observed between the different faecal banks. For example, for 3 faecal banks only one distinct zone of inhibition was observed for one of the samples whereas 127 colonies from another faecal sample produced distinct zones of inhibition **(Fig.2)**. It was noted that several of the producing colonies looked similar so it is likely that particular gut strains are represented multiple times on the plates. A total of 4,065 colonies from 23 faecal banks were screened for anti- *Listeria* activity **(Table 3)**. Direct antagonism of *L. monocytogenes* 10403S with distinct clear zones of inhibition was seen in 443 colonies from 11 of the screened faecal bacterial banks. Hazy zones of inhibition were observed in 1,126 colonies. The remaining 2,496 colonies did not demonstrate anti- *Listeria* activity under the conditions tested. Overall 1,569 colonies demonstrated activity against *Listeria* (clear distinctive zones or hazy zones).

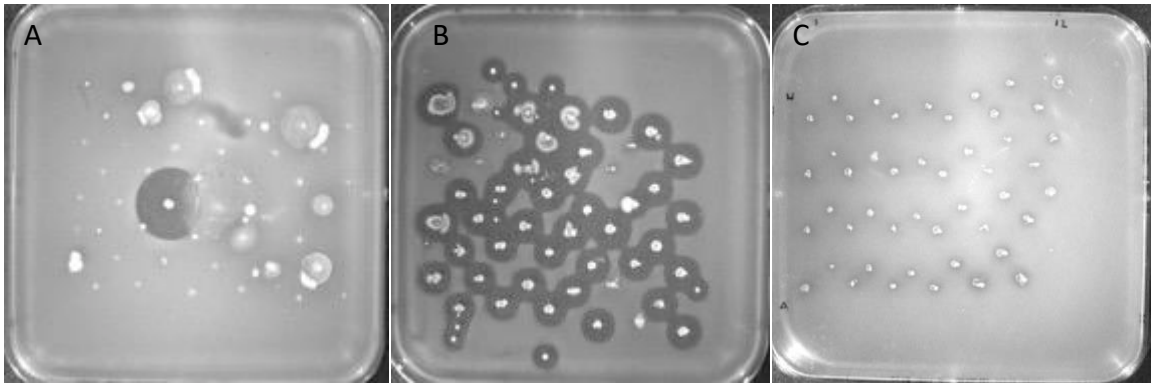


Fig.2 Representative pictures of plates showing results obtained for deferred antagonism assays with gut-derived bacterial strains from 3 different faecal samples using *L. monocytogenes* 10403S as the indicator strain. The gut derived- bacteria were grown on agar and subsequently UV treated and overlaid with sloppy agar seeded with *L. monocytogenes*. Plates were incubated overnight and examined for zones of inhibition. **(A)** Only one colony in this faecal bank produced a clear, distinct zone of inhibition. 17 colonies produced hazy zones of inhibition (not shown in picture) **(B)** 127 colonies from this faecal bank produced clear, distinct zones of inhibition. 9 colonies produced hazy zones (not shown in picture) **(C)** 168 colonies from this faecal bank produced hazy zones of inhibition. 12 colonies produced clear, distinct zones of inhibition (not shown in picture).

Table 3. Number of colonies and different types of zones of inhibition observed in the initial screening of the bacterial faecal banks.

Bank no.	No. of colonies examined	No. of distinct zones	No. of hazy zones
102	135	53	67
104	180	13	38
105	141	0	45
107	192	0	0
110	168	0	0
111	142	121	0
113	179	127	9
117	236	0	0
119	227	1	17
121	249	0	0
122	157	0	70
123	302	0	156
126 stock 1	266	0	266
126 stock 2	190	0	139
128	122	0	11
129	142	0	49
130	144	1	7
131	191	2	84
132	94	0	0
133	142	2	0
134	132	7	0
136	190	12	168
175	144	104	0
TOTAL	4,065	443 (11%)	1,126 (28%)

Selection of strains for further investigation. In total, of the 1569 colonies that have shown anti- *Listeria* activity, 59 were chosen for further analyses. For each faecal bank all active colonies were compared to each other so that the maximum number of colonies showing different cell morphology and variety in the zone of inhibition size and appearance were selected in order to maximize the number of potentially different bacterial isolates and antimicrobials. The 59 strains were grown from the 96 well stock plates in broth and streaked onto agar to ensure purity. Colonies from pure cultures were regrown and were re-stocked. The anti-*Listeria* activity of strains was re-investigated using deferred antagonism assays. Different types of zones of inhibition were observed **(Fig.3)**. These varied from clear distinctive zones of inhibition, hazy zones of inhibition to a slight clearing in the agar just where the bacterial spot had grown.

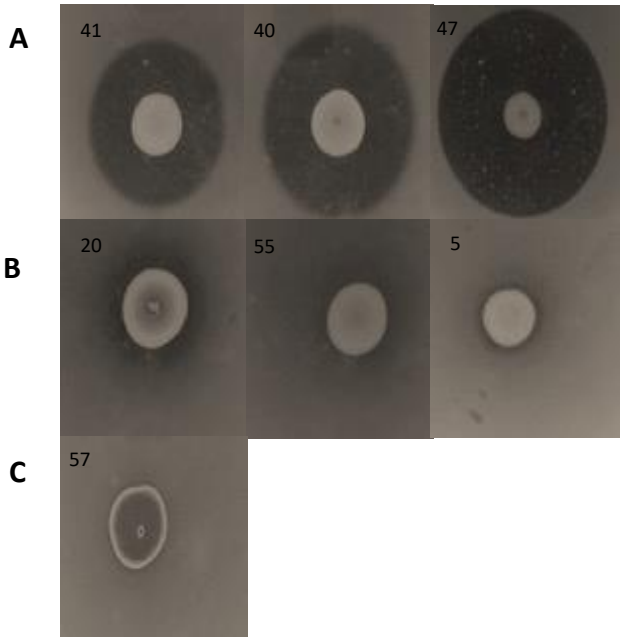


Fig. 3 Representative pictures of the deferred antagonism assays that were carried out to confirm the anti-*Listeria* activity of gut-derived bacterial strains**.** The gut derived-bacteria were grown on agar and subsequently UV treated and overlaid with sloppy agar seeded with *L. monocytogenes* 10403S. Plates were incubated overnight and examined for zones of inhibition. (**A)** – Clear, defined zones of inhibition, **(B)** - Hazy zones of inhibition, (**C)** - Inhibition only within the area of the spot. The number in the top left corner indicates the gut-derived isolate shown in the panel.

Identification of gut-derived strains. Gram stains revealed that all of the strains under investigation were Gram-positive and cocci in shape. A combination of two approaches was used to identify the gut-derived strains. At first the isolates were identified with MALDI–TOF MS, which was carried out on fresh colonies and results suggest that the majority of strains were *Enterococcus* (**Table 4**). This approach identified 39 of the shortlisted isolates while the remaining 20 isolates were not identified. The remaining isolates were putatively identified by sequencing part of the 16S rRNA gene. Overall 33 of the isolates were sequenced in order to confirm the reliability of identification obtained by MALDI TOF MS (**Table 4).** 56 of the isolates were identified as *Enterococcus* spp. while the remaining 3 isolates were identified as S*treptococcus* spp.

Table 4. Identification of the gut- derived isolates based on the results obtained from combination of MALDI-TOF MS readings as well as the results obtained from the partial 16S genome sequencing.

Strain ID	Screening ID	Isolate ID obtained from MALDI –TOF MS	MALDI – TOF MS score	Isolate ID obtained from partial 16S gene sequencing	Accession number of closest BLAST homology
32	102.1.E3	*Enterococcus faecium*	2.217	*Enterococcus faecium*	MH127510.1
53	102.1.F6	*Enterococcus faecium*	1.878	*Enterococcus faecium*	MH127529.1
12	102.2.D2	*Enterococcus faecium*	2.296	*	N/A
24	102.2.D6	*Enterococcus faecium*	2.056	*	N/A
64	102.3.C5	*Enterococcus faecium*	2.367	*	N/A
21	102.3.C9	*Enterococcus faecium*	2.245	*	N/A
20	104.5.A1	-	-	*Enterococcus hirae*	KY950612.1
18	104.5 G7	-	-	*Enterococcus hirae*	MF975716.1
5	104.5.A9	*Enterococcus hirae*	1.946	*Enterococcus hirae*	MF108171.1
51	104.5.H10	-	-	*Enterococcus hirae*	KY950612.1
48	111.1.A1	-	-	*Enterococcus durans*	MF357679.1
44	111.1.C1	-	-	*Enterococcus faecium*	KT598442.1
4	111.1.G1	-	-	*Enterococcus faecium*	KY129997.1
6	111.1.G11	-	-	*Enterococcus faecium*	MH127529.1
8	111.1.H10 A	-	-	*Enterococcus faecium*	MF424775.1
45	111.2.A11	*Enterococcus faecium*	2.041	*	N/A
42	111.2.A3	*Enterococcus faecium*	2.136	*	N/A
33	111.2.H6	-	-	*Enterococcus faecium*	KY129997.1
37	111.3.A11 A	-	-	*Enterococcus faecium*	LC193724.1
49	111.3.B2	-	-	*Enterococcus faecium*	KY129997.1
1	111.3.G7	*Enterococcus faecium*	2.181	*	N/A
71	111.3.H10	*Enterococcus faecium*	2.172	*	N/A
9	111.3.H2	*Enterococcus faecium*	1.991	*	N/A
40	113.1.A1	*Enterococcus faecium*	2.227	*Enterococcus faecium*	MF354616.1
72	113.1.D2	-	-	*Enterococcus faecium*	KY129997.1
46	113.1.F12	*Enterococcus faecium*	2.056	*	N/A
7	113.2.A1	*Enterococcus faecium*	2.369	*	N/A
3	113.2.A7	*Enterococcus faecium*	1.831	*	N/A
28	113.2.H8	*Enterococcus faecium*	1.996	*Enterococcus faecium*	CP019770.1
27	113.3.B8	*Enterococcus faecium*	1.952	*	N/A
10	113.4.A3	*Enterococcus faecium*	2.195	*	N/A
47	113.4.D8	*Enterococcus faecium*	2.049	*	N/A
17	113.4.H12	*Enterococcus faecium*	2.049	*	N/A
15	119.1.E5	*Enterococcus faecium*	2.422	*Enterococcus faecium*	MH127530.1
66	130.2.C1	*Streptomyces phaeochromogenes*	1.357	*Streptococcus mitis*	LT707616.1
73	133.1.C7	-	-	*Streptococcus mutans*	LC193724.1

70	133.2.A1 (A)	*Streptococcus mutans*	1.914	*Streptococcus mutans*	CP013237.1
54	134.1.B6	*Enterococcus faecium*	2.318	*Enterococcus faecium*	KF25454.1
43	134.2.A11	*Enterococcus faecium*	2.001	*Enterococcus faecium*	KY962871.1
2	134.2.A5 (A)	*Enterococcus faecium*	2.38	*	
62	134.2.A7 (A)	-	-	*Enterococcus faecium*	KY962897.1
60	134.2.E3	-	-	*Enterococcus faecium*	KT598442.1
58	134.2.E.9	*Enterococcus faecium*	2.049	*	N/A
39	134.3.A9	*Enterococcus faecium*	2.281	*	N/A
57	136.4.A9	-	-	*Enterococcus avium*	KP645382.1
29	136.4.B2A	-	-	*Enterococcus faecium*	MF357615.1
52	136.4.A11	-	-	*Enterococcus faecium*	LC193724.1
63	136.4.A1	-	-	*Enterococcus faecium*	KP645382.1
38	175.1.A5	*Enterococcus faecium*	1.595	*Enterococcus faecium*	MF357685.1
13	175.1.C9 (A)	*Enterococcus faecium*	2.269	*	N/A
11	175.1.G7 (A)	*Enterococcus faecium*	1.651	*Enterococcus faecium*	MH111696.1
41	175.1.H12 (A)	*Enterococcus faecium*	1.982	*	N/A
61	175.1.H2	*Enterococcus faecium*	1.631	*Enterococcus faecium*	MH111695.1
55	175.2.A1	*Enterococcus faecium*	1.862	*	N/A
31	175.2.C3 (A)	*Enterococcus faecium*	2.11	*	N/A
36	175.2.G9	*Enterococcus faecium*	2.364	*	N/A
16	175.3.B10	*Enterococcus faecium*	2.306	*	N/A
14	175.3.H10	*Enterococcus faecium*	2.084	*	N/A
34	175.3.A1 (A)	-	-	*Enterococcus faecium*	KY129997.1

(-) No match in the database, * 16S sequencing was not carried out for that strain, **N/A** not applicable. Note: screening IDs were assigned based on the 96 well plate number and well position that the strain was obtained from e.g. 102.1.E3 is a strain from faecal bank 102, 96 well plate number 1, well E3.
The (A) in Screening IDs refers to stock A of that strain when more than one stock was made.

Investigation of the nature of the antimicrobial compounds. Agar well diffusion assays were carried out to investigate whether the antimicrobial activity is primarily associated with the cells or if the antimicrobial is secreted into the growth medium. The CFS and WCE from overnight cultures were prepared and examined for their anti-*Listeria* activity in agar well diffusion assays. Differences were observed in the zone sizes for CFS and WCE when different strains were compared **(Fig.4)**. For example, for samples 1 and 2 zones of inhibition were observed for both CFS and WCE, but for sample 1 the zone for the CFS was bigger than the zone for the WCE, while the opposite was the case for sample 2. The findings of the experiments presented in **Table**

5 have shown that the distinct zones of inhibition were observed for CFS from 45 of 59 isolates while zones were not observed for the other 14. For the 14 for which zones were not observed for CFS, it is possible that the antimicrobial compound may not be secreted into the medium at all, or is secreted but the concentration is too low to be detected using the agar diffusion assay. It was noted that the WCE of 4 of the strains (isolates 20, 18, 5, 73) demonstrated anti-*Listeria* activity whereas their corresponding CFS did not demonstrate activity suggesting that in these cases the antimicrobial compounds may be primarily cell-associated.

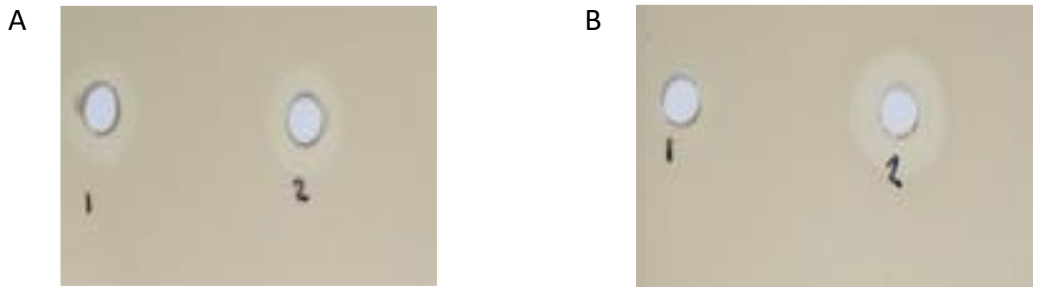


Fig.4 Representative pictures of agar well diffusion assays with **(A)** CFS and **(B)** WCE, prepared from two gut-derived strains. Agar was seeded with 1% *L monocytogenes* 10403S, wells were bored in the agar to which either CFS or WCE from gut-derived strains was added. Plates were incubated overnight at 37°C, after which they were examined for zones of inhibition. Wells labelled 1= gut derived isolate 4, wells labelled 2 = gut derived isolate 15.

Table 5. The pH of the cell free supernatants (CFS) extracted from the isolates and results of a well diffusion assay carried out with CFS and whole cell extract (WCE) from gut derived isolates, the zones were measured in (mm) including the size of the well (5 mm).

Strain ID	Screening ID	Isolate ID	CFS pH	Zone size obtained with CFS (mm)	Zone size obtained with WCE (mm)
32	102.1.E3	*E. faecium*	7	0	0
53	102.1.F6	*E. faecium*	7	0	0
12	102.2.D2	*E. faecium*	7	0	0
24	102.2.D6	*E. faecium*	7	0	0
64	102.3.C5	*E. faecium*	7	0	0
21	102.3.C9	*E. faecium*	7	0	0
20	104.5.A1	*E. hirae*	7	0	6
18	104.5 G7	*E. hirae*	7	0	6
5	104.5.A9	*E. hirae*	7	0	6
51	104.5.H10	*E. hirae*	7	hh	6
48	111.1.A1	*E. durans*	7	11	7
44	111.1.C1	*E. faecium*	7	9	9
4	111.1.G1	*E. faecium*	7	11	6
6	111.1.G11	*E. faecium*	7	11	7
8	111.1.H10 A	*E. faecium*	7	10	7
45	111.2.A11	*E. faecium*	7	11	7
42	111.2.A3	*E. faecium*	7	10	0
33	111.2.H6	*E. faecium*	7	9	7
37	111.3.A11 A	*E. faecium*	7	9	9
49	111.3.B2	*E. faecium*	7	9	6
1	111.3.G7	*E. faecium*	7	12	7
71	111.3.H10	*E. faecium*	7	11	7
9	111.3.H2	*E. faecium*	7	10	7
40	113.1.A1	*E. faecium*	7	7	9
72	113.1.D2	*E. faecium*	7	7	7
46	113.1.F12	*E. faecium*	7	8	7
7	113.2.A1	*E. faecium*	7	9	7
3	113.2.A7	*E. faecium*	7	8	8
28	113.2.H8	*E. faecium*	7	7	8
27	113.3.B8	*E. faecium*	7	8	8
10	113.4.A3	*E. faecium*	7	8	10
47	113.4.D8	*E. faecium*	7	11	0
17	113.4.H12	*E. faecium*	7	6	7
15	119.1.E5	*E. faecium*	7	11	15
66	130.2.C1	*S. mitis*	7	0	0
73	133.1.C7	*S. mutans*	7	0	7
70	133.2.A1 (A)	*S. mutans*	7	0	0
54	134.1.B6	*E. faecium*	7	13	14

43	134.2.A11	*E. faecium*	7	12	11
2	134.2.A5 (A)	*E. faecium*	7	13	13
62	134.2.A7 (A)	*E. faecium*	7	13	14
60	134.2.E3	*E. faecium*	7	13	14
58	134.2.E.9	*E. faecium*	7	11	15
39	134.3.A9	*E. faecium*	7	15	15
57	136.4.A9	*E. avium*	7	0	0
29	136.4.B2A	*E. faecium*	7	15	15
52	136.4.A11	*E. faecium*	7	12	6
63	136.4.A1	*E. faecium*	7	0	0
38	175.1.A5	*E. faecium*	7	7	8
13	175.1.C9 (A)	*E. faecium*	7	7	7
11	175.1.G7 (A)	*E. faecium*	7	7	7
41	175.1.H12 (A)	*E. faecium*	7	7	9
61	175.1.H2	*E. faecium*	7	7	7
55	175.2.A1	*E. faecium*	7	7	8
31	175.2.C3 (A)	*E. faecium*	7	7	7
36	175.2.G9	*E. faecium*	7	7	8
16	175.3.B10	*E. faecium*	7	7	7
14	175.3.H10	*E. faecium*	7	7	7
34	175.3.A1 (A)	*E. faecium*	7	7	8

hh- hazy halo, no clear define zone of inhibition.

Deferred antagonism assays to examine the spectrum of activity of gut-derived strains. In order to investigate the inhibition spectrum of the 59 shortlisted gut-derived isolates, deferred antagonism assays were carried out with 14 different indicator strains. **Fig. 5** illustrates representative images showing various images with types of zone sizes observed in the test. **Table 6** displays detailed results of the assay with Gram-positive and Gram- negative indicator strains. The results showed that 57 out of 59 of the shortlisted gut-derived isolates inhibited *L. monocytogenes* 10403S again confirming the anti-*Listeria* activity that was observed in the initial screen. The remaining 2 isolates (isolates 66 and 73) did not produce zones. It should be noted that those two isolates have previously confirmed anti-*Listeria* activity, but produced very small hazy zones/halos of inhibition and have shown varied growth rates under the experimental conditions used.

The other *Listeria* strains that were tested were also inhibited by the 57 gut isolates – it was noted that the zones of inhibition produced against *L. monocytogenes* EGDe were closest in size to those produced against *L. monocytogenes* 10403S, smaller zones were observed against *L. monocytogenes* F2365 and the least activity was observed against *L. monocytogenes* LO28. It can be seen from the figure that differences the antimicrobial activity varied greatly between various indicator strains. The results obtained for *L. monocytogenes* F2365 showed that 46 of the isolates have shown activity while 13 have not shown activity against this strain of *Listeria.* The results acquired when using *L. monocytogenes* L028 have shown that 15 showed activity while the remaining 44 have shown no activity. The results obtained when using *L. monocytogenes* EGDe as an indicator strain have showed that 42 of the isolates have shown activity against this indicator strain while the remaining 17 have shown no activity. The results obtained when using *L. innocua* as an indicator strain established that 50 of the isolates have demonstrated activity under the experimental conditions. 58 out of 59 strains also inhibited some of the other Gram-positive bacteria tested (*Enterococci*, *M. luteus*, *L. lactis*) while no activity was observed for isolate 66. The results obtained when using *E. hirae* as an indicator strain have showed that 38 have shown activity against this indicator. 58 of the isolates have demonstrated activity when using *E. faecium* as an indicator. The results obtained when using *L. lactis* HP as an indicator revealed that 57 of the isolates produced zones of inhibition, with the majority being small and hazy zones. 54 of the isolates have produced zones of inhibition when using *M. luteus* as an indicator strain, again the majority of observed zones were small and hazy. No activity was observed against *B. subtilis* and the Gram-negative bacteria tested (*S. enteritidis*, *E. coli*, and *P. aeruginosa*). **Fig. 5** shows a representative set of results obtained for gut-derived strain number 29.

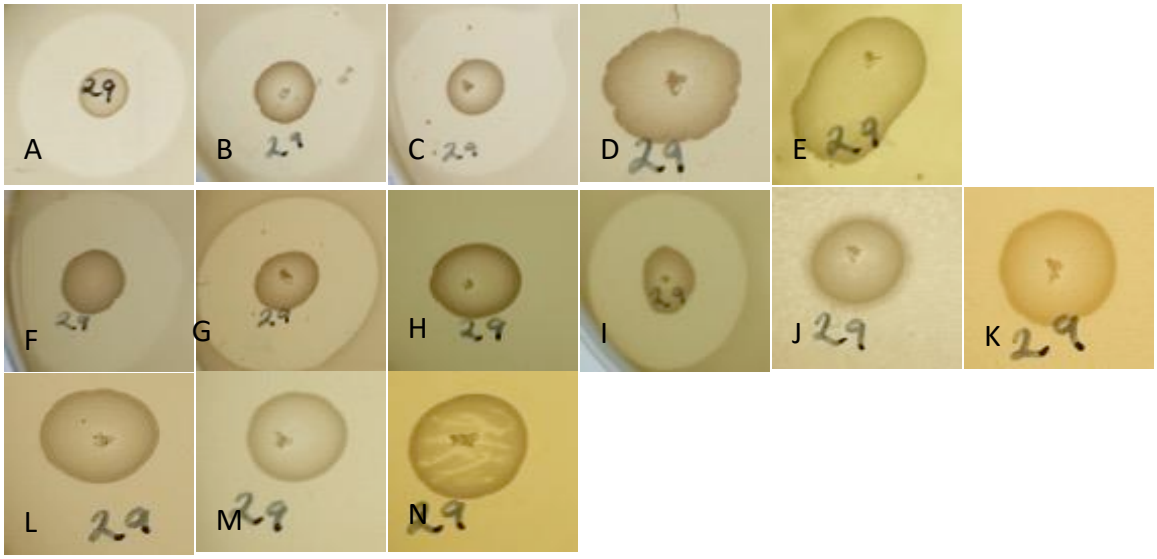


Fig.5 Deferred antagonism assays showing the spectrum of activity of gut-derived isolate number 29 against various indicator strains. Isolate 29 was grown overnight in BHI broth, spotted onto BHI agar and incubated at 37°C overnight. Spot plates were then UV treated and overlaid with 5 ml of sloppy agar seeded with 1% inoculum of the following indicator strains: **(A)** *L. monocytogenes* 10403S, **(B)** *L. monocytogenes* F2356, **(C)** *L. monocytogenes* EGDe **(D)** *L. monocytogenes* L028, **(E)** *L. innocua*, **(F)** *E. faecium* **(G)** *E. hirae* **(H)** *L. lactis* HP **(I)** *M. luteus*, **(J)** *Bacillus subtilis*, **(K)** *S. aureus,* **(L)** *Salmonella enteritidis*, **(M)** *E. coli* and **(N)** *P. aeruginosa*. The plates were then incubated at 37°C for 18 hours and checked for zones of inhibition.

Table 6. Results of deferred antagonism assays that investigate the spectrum of activity of the 59 shortlisted isolates against Gram-positive indicator strains; *L. monocytogenes* 10403S, *L. monocytogenes* F2365, *L. monocytogenes* L028, *L. monocytogenes* EGDe, *L. innocua*, *E. faecium, E. hirae, B. subtilis, L. lactis* and *M. luteus* and Gram-negative strains *S. aureus, S. enteritidis, E. coli* and *P. aeruginosa.*

Strain ID	Sample number	Isolate ID	10403S	F2365	L028	EGDe	*L. innocua*	*E. faecium*	*E. hirae*	*B. subtilis*	*L. lactis* HP	*M. luteus*	*S.aureus*	*S. enteritidis*	*E. coli*	*P. aeruginosa*
32	102.1.E3	*E. faecium*	hh	-	-	-	hh	-	+	-	hh	+	-	-	-	-
53	102.1.F6	*E. faecium*	hh	-	-	-	hh	-	+	-	hh	+	-	-	-	-
12	102.2.D2	*E. faecium*	hh	-	-	-	hh	-	+	-	hh	+	-	-	-	-
24	102.2.D6	*E. faecium*	hh	-	-	-	hh	-	+	-	hh	+	-	-	-	-
64	102.3.C5	*E. faecium*	hh	-	-	-	hh	-	+	-	hh	+	-	-	-	-
21	102.3.C9	*E. faecium*	hh	-	-	-	hh	-	+	-	hh	+	-	-	-	-
20	104.5.A1	*E .hirae*	hh	+h	-	+	-	+	+h	-	hh	+	-	-	-	-
18	104.5 G7	*E. hirae*	hh	hh	-	-	-	+h	-	-	hh	-	-	-	-	-
5	104.5.A9	*E. hirae*	hh	-	-	-	-	+	+h	-	hh	-	-	-	-	-
51	104.5.H10	*E. hirae*	+	-	-	-	-	+	+h	-	hh	-	-	-	-	-
48	111.1.A1	*E. durans*	+	hh	-	+	+	+	-	-	+	hh	-	-	-	-
44	111.1.C1	*E. faecium*	+	+	-	-	+	+	-	-	+	hh	-	-	-	-
4	111.1.G1	*E. faecium*	+	+	+h	+	+	+	+h	-	hh	+	-	-	-	-
6	111.1.G11	*E. faecium*	+	+h	-	+h	+	+	+h	-	hh	hh	-	-	-	-
8	111.1.H10A	*E. faecium*	+	+h	-	+h	+	+	+h	-	hh	hh	-	-	-	-
45	111.2.A11	*E. faecium*	+	+	+h	+	+	+	+h	-	hh	+	-	-	-	-
42	111.2.A3	*E. faecium*	+	+h	-	+h	+	+	+h	-	hh	+h	-	-	-	-
33	111.2.H6	*E. faecium*	+	+h	-	+h	+	+	+h	-	hh	hh	-	-	-	-

37	111.3.A11A	*E. faecium*	+	+	-	+	+	+	-	-	+	hh	-	-	-	-
49	111.3.B2	*E. faecium*	+	+h	-	+	+	+	-	-	+	hh	-	-	-	-
1	111.3.G7	*E. faecium*	+	+	+h	+	+	+	+h	-	hh	+	-	-	-	-
71	111.3.H10	*E. faecium*	+	+h	-	hh	+	+	+h	-	hh	hh	-	-	-	-
9	111.3.H2	*E. faecium*	+	+h	-	+h	+	+	+h	-	hh	hh	-	-	-	-
40	113.1.A1	*E. faecium*	+	+	-	+	+	+h	+h	-	hh	hh	-	-	-	-
72	113.1.D2	*E. faecium*	+	+	-	+	+	+	-	-	+	+	-	-	-	-
46	113.1.F12	*E. faecium*	+	hh	-	hh	+	+h	-	-	hh	hh	-	-	-	-
7	113.2.A1	*E. faecium*	+	+	+h	-	+	+	+h	-	hh	hh	-	-	-	-
3	113.2.A7	*E. faecium*	+	hh	-	+	+	+h	-	-	hh	hh	-	-	-	-
28	113.2.H8	*E. faecium*	+	+	-	+	+	+h	+h	-	hh	hh	-	-	-	-
27	113.3.B8	*E. faecium*	+	+	-	+	+	+h	+h	-	hh	hh	-	-	-	-
10	113.4.A3	*E. faecium*	+	hh	-	+	+	+h	-	-	hh	hh	-	-	-	-
47	113.4.D8	*E. faecium*	+	+	hh	+	+	+	-	-	+	+	-	-	-	-
17	113.4.H12	*E. faecium*	+	hh	-	+	+	+h	-	-	hh	hh	-	-	-	-
15	119.1.E5	*E. faecium*	+	+	+h	+	+	+	+	-	+	+	-	-	-	-
66	130.2.C1	*S. mitis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
73	133.1.C7	*S. mutans*	-	-	-	-	-	-	-	-	hh	hh	-	-	-	-
70	133.2.A1 (A)	*S. mutans*	hh	-	-	-	-	-	-	-	hh	hh	-	-	-	-
54	134.1.B6	*E. faecium*	+	+	+h	+	+	+	+	-	hh	+	-	-	-	-
43	134.2.A11	*E. faecium*	+	+	+	-	+	+	+	-	hh	hh	-	-	-	-
2	134.2.A5 (A)	*E. faecium*	+	+	+h	+	+	+	+	-	hh	+	-	-	-	-
62	134.2.A7 (A)	*E. faecium*	+	+	+h	+	+	+	+	-	hh	+	-	-	-	-
60	134.2.E3	*E. faecium*	+	+	+h	+	+	+	+	-	hh	+	-	-	-	-
58	134.2.E.9	*E. faecium*	+	+	+h	+	+	+	+	-	hh	+	-	-	-	-

39	134.3.A9	*E. faecium*	+	+	+h	+	+	+	+	-	hh	+	-	-	-	-
57	136.4.A9	*E. avium*	hh	-	-	+	-	-	-	-	-	-	-	-	-	-
29	136.4.B2A	*E. faecium*	+	+	+	+	+	+	+	-	hh	+	-	-	-	-
52	136.4.A11	*E. faecium*	+	+	+	+	+	+	+h	-	+	+	-	-	-	-
63	136.4.A1	*E. faecium*	hh	-	-	-	-	-	-	-	hh	hh	-	-	-	-
38	175.1.A5	*E. faecium*	+	+h	-	+	+	+h	hh	-	hh	hh	-	-	-	-
13	175.1.C9 (A)	*E. faecium*	+	+h	-	+	+h	+h	hh	-	hh	hh	-	-	-	-
11	175.1.G7 (A)	*E. faecium*	+	+h	-	-	+	+h	hh	-	hh	hh	-	-	-	-
41	175.1.H12 (A)	*E. faecium*	+	+h	-	+	+	+h	hh	-	hh	hh	-	-	-	-
61	175.1.H2	*E. faecium*	+h	+h	-	+	+	+h	hh	-	hh	hh	-	-	-	-
55	175.2.A1	*E. faecium*	+	+	-	+	+	+h	-	-	+	hh	-	-	-	-
31	175.2.C3 (A)	*E. faecium*	+	+	-	+	+	+h	-	-	+	hh	-	-	-	-
36	175.2.G9	*E. faecium*	+	+h	-	+	+	+h	hh	-	hh	hh	-	-	-	-
16	175.3.B10	*E. faecium*	+	+h	-	+	+	+h	-	-	+	hh	-	-	-	-
14	175.3.H10	*E. faecium*	+	+	-	+	+	+h	-	-	+	hh	-	-	-	-
34	175.3.A1 (A)	*E. faecium*	+	+	-	+	+	+h	-	-	+	hh	-	-	-	-

(+) Indicates a clear distinctive zone of inhibition, **(+h)** indicates a zone of inhibition with a hazy outline, **(hh)** indicates a hazy halo around the bacterial growth without a clear distinctive zone of inhibition **(-)** indicates no zone of inhibition.

The colony morphology, spectrum of activity and the sizes of the zones of inhibition produced against specific indicator strains, the results obtained in MALDI-TOF analysis and partial 16S rRNA sequencing as well as the results of well diffusion assay with CFS and WCE of the shortlisted gut-derived isolates originating from the same faecal banks were compared in detail in order to narrow down the number of isolates for further investigation. Isolates displaying almost identical results were assumed to be the same and only one of the set of isolates displaying a given set of characteristics was further shortlisted; e.g. isolates 40, 46, 73, 28, 27, 10 and 17 from faecal bank 113 all displayed almost identical results when overlaid with the indicator strains, so only one of those isolates (40) was shortlisted. Isolates 72 and 47 also from faecal bank 113 produced much bigger clearer zones of inhibition than the remaining isolates from that faecal bank (data not shown), but their zones of inhibition were almost identical to each other so only one of those two isolates (72) was shortlisted. Based on the data collected it was established that there are potentially two different types of bacteria in faecal bank 113, isolates 40 and 72 were selected for further testing. **Table 7** displays the list of 17 gut-derived isolates selected for further investigation, 15 of which were identified as *Enterococci* spp. and the remaining two were identified as *Streptococcus* spp.

Table 7. List of 17 shortlisted isolates selected for further testing based on the results obtained by comparison of cell morphology, spectrum of activity, bacterial identity and the results of well diffusion assays with CFS and WCE.

Strain ID	Screening ID	Identity
32	102.1.E3	*E. faecium*
12	102.1.D2	*E. faecium*
20	104.5.A1	*E. hirae*
18	104.5.G7	*E. hirae*
48	111.1.A1	*E. durans*
4	111.1.G1	*E. faecium*
40	113.1.A1	*E. faecium*
72	113.1 D2	*E. faecium*
15	119.1. E5	*E. faecium*
66	130.2.C1	*S. mitis*
73	133.2.A1A	*S. mutans*
54	134.1.B6	*E. faecium*
43	134.2.A11	*E. faecium*
57	136.4.A9	*E. avium*
52	136.4.A11	*E. faecium*
29	136.4.B2	*E. faecium*
38	175.1.A5	*E. faecium*

Cross immunity deferred antagonism assays. Cross immunity deferred antagonism assays with a known enterocin A producer, *E. faecium* DPC 6482, and a known enterocin B producer, *E. faecium* EM3-42-BC-1, were carried out in order to investigate if any of the 15 short-listed gut-derived isolates identified as *Enterococcus spp*. produced either of these bacteriocins. The main principle of this experiment is the fact that any bacterium producing a given bacteriocin is immune to it. Therefore, if cross immunity between shortlisted gut isolates and known enterocin producers were observed it could be deduced that the gut strain is likely to produce that particular bacteriocin.

Fig. 6 Representation of the results obtained from this experiment. The complete set of data collected from this experimental approach can be seen in **Table 8.**

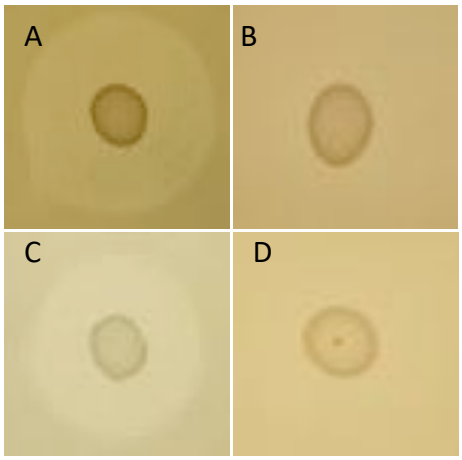


Fig.6 A representative figure of the cross-immunity deferred antagonism assays. **(A)** Enterocin A producing strain overlaid with *L. monocytogenes* 10403S showing a distinct zone of inhibition. **(B)** Enterocin A producer overlaid with itself, showing no zone of inhibition and demonstrating immunity to enterocin A. **(C)** Enterocin A producer overlaid with a gut derived isolate, a zone of inhibition indicates lack of immunity and suggests that the peptide produced by the indicator gut strain is not enterocin A. **(D)** Enterocin A producer overlaid with a gut-derived isolate, a lack of zone of inhibition suggests that the gut strain produces enterocin A.

The data collected shows that the cross-immunity experiments suggest that 4 of the 15 short-listed *Enterococcus* (gut-derived strains 15, 54, 43 and 29) are likely to produce enterocin A and B, i.e., no zones of inhibition were produced when the gut strains were overlaid with the enterocin A and B producers (results in **Table 9**), additionally no zones were produced when the enterocin A and B producers were overlaid with the gut-derived isolates (results in **Table 8**) suggesting immunity to the produced antimicrobials. A total of 11 isolates did not

produce results indicative of immunity; i.e., produced zones of inhibition when overlaid with enterocin A and B producers. It was noted that gut-derived isolates 18 and 20 are sensitive to enterocin A and B (**Table 8**) but the enterocin A and B producers are immune to the antimicrobial(s) produced by isolates 18 and 20 (**Table 9**).

Table 8. Cross immunity deferred antagonism assay carried out with an enterocin A producing *E. faecium* DPC 6482 and enterocin B producing *E. faecium* EM342-BC-1 spotted onto BHI agar overlaid with gut- derived strains, zone sizes measured in mm.

	Gut-derived isolate number														
	32	**12**	**20**	**18**	**48**	**4**	**40**	**72**	**15**	**54**	**43**	**57**	**52**	**29**	**38**
DPC 6482	27	26	25	26	25	26	27	23	0	0	0	18	24	0	23
EM342-BC-1	30	30	29	28	27	30	30	28	0	0	0	20	26	0	28

Table 9. Cross immunity deferred antagonism assay carried out with gut-derived isolates spotted onto BHI agar and overlaid with enterocin A producing *E. faecium* DPC 6482 and enterocin B producing *E. faecium* EM342-BC-1 zones of inhibition measured in mm.

Strain ID	**Screening ID**	**Isolate ID**	**Indicator *strain.* *E. faecium* DPC 6482**	**Indicator strain *E. faecium* EM342-BC-1**
32	102.1.E3	*E. faecium*	13	13
12	102.2D2	*E. faecium*	13	12
20	104.5.A1	*E. hirae*	0	0
18	104.5.G7	*E. hirae*	0	0
48	111.1.A1	*E. duranus*	18	18
4	111.1.G1	*E. faecium*	16	18
40	113.1A1	*E. faecium*	11	11
72	113.1.D2	*E. faecium*	13	21
15	119.1.E5	*E. faecium*	0	0
54	134.1.B6	*E. faecium*	0	0
43	134.2.A11	*E. faecium*	0	0
57	136.4.A9	*E. avium*	0	0
52	136.4.A11	*E. faecium*	19	26
29	136.4.B2	*E. faecium*	0	0
38	175.1.A5	*E. faecium*	0	12

Since a number of the gut-derived isolates selected for further experimentation were obtained from the same faecal banks and their identities were confirmed to be the same by either 16S rRNA sequencing or MALDI-TOF sequencing, an additional test was carried out in order to reduce the possibility of shortlisting two identical isolates. A deferred antagonism assay was carried out with the gut- derived isolates from the same faecal sample where each strain was spotted onto agar and overlaid with the other strain(s). Again the main principle behind this experiment was the fact that if two isolates were identical they should be immune to each other's antimicrobial agents (Mélançon and Grenier, 2003). As can be seen in **Table**

10 the results suggest that strains 32 and 12 isolated from the same faecal bank number 102 are likely to be identical. The results are visualized in **Fig.7** and show that zones of inhibition were not observed when the isolates were overlaid with each other, while antagonistic activity was observed when the isolates were overlaid with a sensitive indicator strain of *E. faecium*. Strains 48 and 4 have also demonstrated cross-immunity, however, those gut-derived isolates were identified as being different (*E. durans* and *E. faecium*) so both were retained for further investigation. Similarly, isolates 43 and 54 showed cross-immunity. However, at the time the assay was carried out experiments to establish the identity of isolate 54 were still underway (described in Chapter 1) so both isolates were retained for further investigation. At this stage the total number of the shortlisted isolates was reduced from 17 to 16. Strain 32 was eliminated from further investigation.

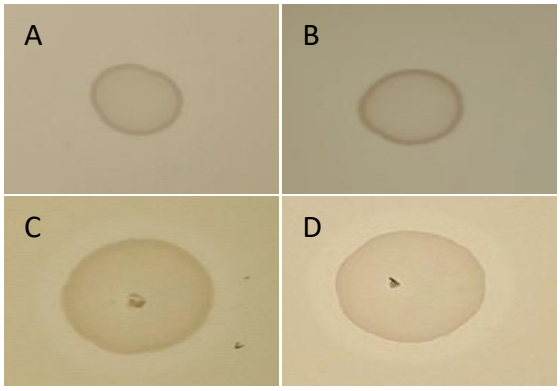


Fig.7 A representative figure showing the results of deferred antagonism cross immunity assays. **(A)** Gut-derived isolate 12 overlaid with gut-derived isolate 32. A zone of inhibition was not observed. **(B)** Gut-derived isolate 32 overlaid with gut-derived isolate 12 also from faecal bank 102. A zone of inhibition was not observed.
(C) A positive control showing that isolate 12 produces a zone of inhibition when overlaid with *Enterococcus faecium*. **(D)** A positive control showing that isolate 32 produces a zone of inhibition when overlaid with *Enterococcus faecium*.

Table 10. Results of a cross immunity assay of faecal isolates derived from the same faecal banks. Faecal isolates were grow in 10ml of fresh BHI broth overnight at 37°C. 10 µL aliquots of each of the cultures were then spotted onto fresh BHI agar and were incubated overnight at 37°C. The plates were then UV treated for 30 minutes in order kill any viable cells. UV treated plates were overlaid with 5ml of fresh 0.7% (w/v) agar sloppy BHI agar seeded with 1% inoculum of a faecal isolate originating from the same faecal bank. The plates were incubated overnight at 37°C and zones of inhibition were recorded. Zone sizes in (mm) include the size of the well (5mm)

Spotted strain			**Strain in the overlay**			
Screening ID	**Strain ID**	**Sample ID**	**Indicator Screening ID**	**Indicator strain ID**	**Indicator ID**	**Zone size**
102.1.E3	32	*E. faecium*	102.1.D2	12	*E. faecium*	0
102.2.D2	12	*E. faecium*	102.1.E3	32	*E. faecium*	0
104.5A1	20	*E. hirae*	104.5.G7	18	*E. hirae*	13
104.5.G7	18	*E. hirae*	104.5.A1	20	*E. hirae*	hh
111.1.A1	48	*E. durans*	111.1G1	4	*E. faecium*	0
111.1.G1	4	*E. faecium*	111.1.A1	48	*E. durans*	0
113.1.A1	40	*E. faecium*	113.1.D2	72	*E. faecium*	hh
113.1.D2	72	*E. faecium*	113.1.A1	40	*E. faecium*	17
134.1.B6	54	*E. faecium*	134.2.A11	43	*E. faecium*	0
134.2.A11	43	*E. faecium*	134.1.B6	54	*E. faecium*	0
136.4.A9	57	*E. avium*	136.4.A11	52	*E. faecium,*	0
136.4. A11	52	*E. faecium*	136.4.A9	57	*E. avium*	28
136.4A9	57	*E. avium*	136.4.B2	29	*E. faecium*	0
136. 4. B2	29	*E. faecium*	136.4.A9	57	*E. avium*	21*
136.4 A11	52	*E. faecium*	136.4.B2	29	*E. faecium*	23
136.4.B2	29	*E. faecium*	136.4.A11	52	*E. faecium*	29

(hh) Represents a hazy halo around the colony where no clear definite zone of inhibition is present, **(*)** represents a zone of inhibition with no definite outline.

Deferred antagonism assays with the final 16 short-listed strains in the presence of proteinase K. In order to investigate if the compounds responsible for antimicrobial activity were protein in nature, deferred antagonism assays were carried out in the presence of proteinase K. **Fig.8** shows the results obtained for one gut-derived isolate (strain no. 48).

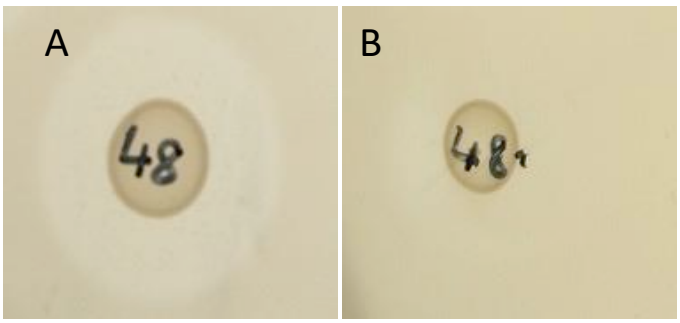


Fig. 8 A representative image of the results obtained for the protease assay with gut-derived strain no. 48. The strain was spotted onto BHI agar in duplicate, grown overnight and UV treated. 2µL of proteinase K was spotted adjacent to the bacterial spot on one plate, the plate was incubated at 37°C for 1 hr and then overlaid with sloppy (0.7%) BHI agar seeded with 1% L. *monocytogenes* 10403S incubated at 37°C for 18 hours. **(A)** Control showing the anti-*Listeria* activity of gut strain no.43 (i.e. when no enzyme is present). **(B)** Proteinase K was spotted adjacent to the bacterial spot (indicated by the black spot).

For 10 of the 16 shortlisted strains the zones of inhibition were measurably reduced by proteinase K when overlaid with *L. monocytogenes* 10403S. The effect of proteinase K on the remaining 6 strains could not be conclusively determined by the assay, as the zones of inhibition obtained were small and/or hazy when using *L. monocytogenes* 10403S as an indicator strain. As the cross-immunity deferred antagonism assays showed that large zones of inhibition were observed when gut-derived isolates were tested against the enterocin A producers *E. faecium* DPC 1146 / *E. faecium* DPC 6482 (data not shown), the proteinase K assays were also carried out using those strains as indicators. These assays revealed that the zone of inhibition for one additional strain (strain 12) was visibly reduced by the presence of proteinase K. Overall a total of 11 out of 16 gut-derived isolates were confirmed to produce antimicrobials that are peptide in nature. The zones of inhibition produced by the remaining 5 isolates were too small to allow the observation of reduction of zone sizes in the experimental conditions used as can be seen in **Table 11.**

Table 11. Results obtained from protease assay with 16 shortlisted gut-derived isolates. The strains were spotted onto BHI agar in duplicate, grown overnight and UV treated. 2μL of proteinase K was spotted adjacent to the bacterial spot on one plate, the plate was incubated at 37°C for 1 hr and then overlaid with sloppy (0.7%) BHI agar seeded with 1% L. *monocytogenes* 10403S or *E. faecium* DPC 6482/ 1146 and incubated at 37°C for 18 hour

(+) Strain ID	Screening ID	Isolate ID	Reduction of antimicrobial activity by proteinase K
12	102.1.D2	*E. faecium*	+
20	104.5.A1	*E. hirae*	ND
18	104.5.G7	*E. hirae*	ND
48	111.1.A1	*E. durans*	+
4	111.1.G1	*E. faecium*	+
40	113.1.A1	*E. faecium*	+
72	113.1 D2	*E. faecium*	+
15	119.1. E5	*E. faecium*	+
66	130.2.C1	*S. mitis*	?
73	133.2.A1	*S. mutans*	?
54	134.1.B6	*E. faecium*	+
43	134.2.A11	*E. faecium*	+
57	136.4.A9	*E. avium*	?
52	136.4.A11	*E. faecium*	+
29	136.4.B2	*E. faecium*	+
38	175.1.A5	*E. faecium*	+

Indicates a positive results i.e. a visible reduction of the size of the zone of inhibition by proteinase K, **ND-** the effect of proteinase K on the zone size could not be determined.

PCRs with primers for enterocin, duracin and hiracin genes. PCRs with a number of enterocin, duracin and hiracin specific primers was carried out. The results obtained for strains 15, 43, and 29 are in agreement with the results obtained in the cross immunity assay with enterocin A and B producers **(Table 8 and 9)** indicating that these 3 isolates have the genes needed for synthesis of enterocin A and enterocin B. Overall, isolates 15, 29, 40 and 43 were positive for enterocin A genes, while testing of isolates 12, 15, 29, 43, 52 and 54 confirmed the presence of enterocin B genes (data not shown). One of the main limitations of the PCRs was the lack of positive controls; the enterocin A and enterocin B PCRs were the only two PCRs which had positive controls. Due to the lack of controls, the remaining PCRs could not be optimized despite careful primer design as well as continuous efforts to optimize the protocols, i.e., running the PCRs with a variety of annealing temperatures. Taking into account that a number of PCRs including enterocin P, enterocin L50A, enterocin L50B, duracin A, duracin B, duracin Q as well as hiracin JM79 were not optimized and despite obtaining faint bands, the presence of genes of interest was not confirmed.

DISCUSSION

A total of 23 bacterial banks were created from 23 human faecal samples obtained from 22 donors. A number of various colony types was observed while preparing the faecal banks, those varied from small white pin point colonies, single white and beige colonies, "fuzzy" type of colonies to irregularly wrinkled white colonies, colonies varied in size and shape from circular to irregular, differences in color of the colonies were also observed varying from semi- transparent, white, beige, yellow to red (data not shown). While some of the faecal banks showed a variety of colony morphologies, others contained colonies that were mostly similar in appearance. As reported by several studies in the literature (Harmsen *et* al. 2002; Sender *et al*. 2005; Thiel & Blaut 2005) , the number of bacterial cells per gram of faeces varies between 10^{10} to 10^{11} CFU/g of dry/wet faeces. The number of bacterial cells recovered from the faecal samples in the current study was lower at around 10^7 CFU/g of faeces. This may be attributed to the fact that the faecal banks were not fresh and have been previously thawed out and frozen a number of times. Literature shows that significant statistical differences should not be observed when comparing the microbiota recovered from fresh faecal samples vs. samples frozen for long term storage (Fouhy *et al.* 2015; Kruse *et al*. 2015). However, it has also been reported that the time between fecal sample collection and its subsequent freezing for long term storage during which the samples are stored at refrigerator temperature can be critical to the microbial composition prior its freezing (Choo *et al.* 2015). Resuscitation of cells post freezing can also be lower when comparing to fresh samples especially with respect to less abundant organisms sensitive to freezing such as *Bifidobacterium* (Fouhy *et al.* 2015). All of the above can contribute to the fact that the major bacterial species recovered in the initial screening were identified as Enterococci as those are considered to be easy to culture in laboratory conditions. It should also be considered that the experimental conditions used for screening is not optimal for all of the bacteria present in the faecal banks. The incubation conditions and media used in the study could potentially be the limiting factor for growth of some of the faecal isolates, especially in light of the fact that it is estimated that only 10-20 % of the gut microbiota are cultivable in laboratory conditions (Eckburg *et al*. 2005). Additionally the experimental design used in the initial screening was limited to the number of colonies which could be picked off the plate. This could have potentially lead to missing/not picking up colonies showing activity against *L. monocytogenes.* Use of a robotic colony picker would allow for greater precision and could end up with picking up a higher number of isolates showing antagonistic activity. However, due to the nature of the

samples the initial screening was carried inside an anaerobic hood which did not allow for the use of a robot.

The bacterial banks were screened for antagonistic activity against *L. monocytogenes* 10403S. Major differences were observed in the activity of the faecal isolates from different faecal banks (**Table 3**). The fact that the majority (61%) of the gut derived isolates did not show any activity in the experimental conditions used for screening does not necessarily mean that those isolates were not producing any antimicrobials. Literature frequently shows that the production of antimicrobials such as bacteriocins is often inducible in specific environmental conditions such as competition with other microorganisms present in the specific environmental niche; i.e., co-culture with particular microorganisms, or the presence of certain autoinducer peptides can induce bacteriocin production (Maldonado-Barragán *et al.* 2013). Lactic acid bacteria (LAB) often have an optimum temperature range at which the bacteriocin production may be induced (Cintas *et al*. 1995; Mortvedt-Abildgaa *et al*. 1995; Nilsen *et al*. 1998). This principle also applies to pH, as an optimal pH is regularly required in order for a bacteriocin to be produced (Biswas *et al.* 1991; Mortvedt-Abildgaa *et al*. 1995). It should be noted that only one indicator strain was used in the current study meaning that only the bacteria producing anti- *Listeria* compounds were picked out. It is possible that the gut isolates could produce antimicrobials active against other enteric pathogens but were not the focus of this study. The current study screened 4,065 colonies, of which only 443 screened colonies produced clear distinctive zones of inhibition against the indicator strain. An additional 1,126 colonies have produced hazy zones of inhibition with a total of 1569 (39%) of isolates displaying some level of antagonistic activity against the indicator strain. Whilst this appears as a relatively small proportion of the isolates, it is actually much higher than the relative (%) of active isolates found in similar studies. In contrast, a study carried out by O'Shea *et al*. (2009), which included over 40,000 colonies of mammalian intestinal origin, only 278 demonstrated antagonistic activity against the indicator strains *L. innocua* DPC3572 and *L. bulgaricus* LGM 6001; i.e. 0.695% of the screened colonies. Similarly, in a study carried out by Lakshminarayanan *et al*. (2012), where 70,000 colonies from 266 samples of faeces of elderly subjects were screened, activity was recorded for 273 screened isolates, representing 0.4% of all screened isolates. For this study, *L. innocua* DPC3572 was one of several indicators used and was employed as an indicator in the screening of 123 samples. Another similar study by Al-Seraih *et al*. (2017) performed at a smaller scale, screened 500 colonies isolated from

infant faeces and found 70 isolates active against *L. innocua* ATTC51742, which represent 14 % of all the colonies screened.

Anti-*Listeria* bacteriocins have previously been reported to be isolated from bacterial strains of human gastrointestinal origin; i.e. *Lactobacillus salivarius* UCC118 producing bacteriocin Abp118 active against *L. monocytogenes* (Corr *et al.* 2007) or *Bifidobacterium* BIR-0304, BIR- 0307 and BIR-0349 isolated from human faeces, with a broad spectrum of activity isolated by Collado *et al.* (2005). Anti-*Listeria* activity of bacteriocins produced by members of the *Enterococcus* spp. isolated from humans has also been reported (Al Atya *et al.* 2015; Birri *et al*. 2010; Birri *et al*. 2013; İspirli *et al*. 2015). Additionally metagenomic analysis of the human gut microbiota showed that *Enterococcus* have between one and 12 bacteriocin related genes in their genomes (Drissi *et al.* 2015). It is possible that the reason that the majority of the isolates with anti-*Listeria* activity were *Enterococcus* may be due to the fact that they are easy to culture and/or they produce antimicrobials such as class IIa bacteriocins with very potent anti-*Listeria* activity. Bacteria that produce antimicrobials with low activity may not have been detected by our screen. Enterococci have also been shown to produce more than one bacteriocin simultaneously. Vandera *et al*. (2018) examined 11 isolates of *E. faecium* four of those isolates were shown to produce enterocin A, two were shown to produce two enterocins namely enterocin A and enterocin B, while the remaining five isolates were shown to produce 3 bacteriocins enterocin A, enterocin B and enterocin P. Similar findings were observed by Aspri *et al*. (2017) who has examined 3 *E. faecium* isolates all of which have shown production of two bacteriocins enterocin A and enterocin B. Additionally Du *et al*. (2017) has shown that *E. durans* 152 can produce two bacteriocins duracin 152A (a derivative of enterocin L50A) and enterocin L50B.

The main objective when performing the well diffusion assay with both the CFS and the WCE was to identify the nature of the antimicrobial compounds synthetized by the gut-derived isolates. The main principle behind the experimental set up was based on the idea that if the antimicrobial compound is cell associated it remains bound to the cell wall of the producing cell, however if the nature of the antimicrobial is extracellular it is secreted into the surrounding growth medium (Barbour and Philip, 2014). Thus if the antimicrobial was excreted to the surrounding growth medium, higher levels of antimicrobial activity should be observed in the CFS of the gut isolate. On the other hand, if the antimicrobial compound is cell bound it is not readily extracted into the surrounding medium, thus the zone of inhibition produced by

the CFS would not be as great. In such cases the antimicrobial is "stripped" off the cell by shaking the cells for 3 hr in 70%IPA0.1% TFA and the resulting zone of inhibition produced by the WCE would be greater in size. The results of the current study have shown that the CFS of 43 of the gut-derived isolates have shown antagonistic activity against *Listeria* suggesting that the antimicrobial produced by those isolates was excreted into the surrounding media. The WCE of 5 of the isolates has demonstrated anti-*Listeria* activity while the CFS of the corresponding isolates has shown no activity suggesting that the antimicrobial produced by those strains is primarily cell bound.

As reported in the literature, the bacteriocins and bacteriocin like antimicrobials produced by Gram-positive bacteria often have a narrow spectrum of activity normally with activity against other Gram-positive bacteria (Jack *et al.* 1995). A few reported bacteriocins are capable of inhibiting Gram-negative bacteria. Examples of such bacteriocins include; plantaricin 35d synthetized by *Lactobacillus plantarum* showing antagonistic activity against *Aeromonas hydrophila* (Messi *et al*. 2001; Parada *et al*. 2007), the bacteriocin thermophylin, synthetized by *Streptococcus thermophilus* showing antagonistic activity against *Yersinia enterocolitica*, *E. coli*, *Yersinia pseudotuberculosus, S. typhimurium* as well as Gram-positives such as *L. monocytogenes* and a number of *Bacillus* spp. (Ivanova *et al*. 1998; Parada *et al*. 2007) and bacteriocin ST151BR synthetized by *L. pentosus* ST151BR active against *Pseudomonas aeruginosa, E. coli* and Gram- positive *L. sakei* and *L. casei* (Parada *et al*. 2007; Todorov & Dicks 2004). The results obtained in this study suggest that the isolates have a narrow spectrum of activity against Gram–positive bacteria, which is a common trait of class II bacteriocins, including the many enterocins produced by *Enterococcus* spp. (Jack *et al.* 1995). A number of enterocins have been reported to show a narrow spectrum of activity. Enterocin produced by *E. faecium* FAIR-E198 has shown antimicrobial activity against *L. monocytogenes*, while activity was not reported against *B. cereus* or *S. aureus* (Nascimento *et al.* 2010). Similar findings are documented by Ennahar & Deschamps (2000) where enterocin A produced by *E. faecium* EFM01 has shown activity against 13 *L. monocytogenes*, with significant inhibition of *L. sake* and enterococcal strains and a relatively low activity against *Pediococcus* strains with no activity against *S. aureus* or *Leuconostoc* strains. Al-Seraih *et al*. (2017) has shown that the enterocins isolated from infant faeces had a narrow spectrum of activity. Antagonistic activity was recorded against Gram-positive bacteria including *Listeria, Clostridium, Bacillus* while antagonistic activity was not observed against *S. newport, S.*

heidelberg, *E. coli* and *P. aeruginosa.* Nes *et al*. (2014) reviews enterococcal bacteriocins, their activity and contribution to niche control.

Lactic acid bacteria are known for production of bacteriocins as well as specific immunity proteins, which deliver self-protection from the toxic effects of the bacteriocins they produce. The production of those proteins can also deliver cross-immunity against associated bacteriocins (Mélançon and Grenier, 2003). This trait was observed in a number of gut delivered strains under investigation, conferring cross immunity not only to enterocin A and enterocin B producers in 4 gut derived isolates but also to each other in some cases, allowing for shortlisting of the isolates.

A deferred antagonism assay was performed with addition of proteinase K in order to investigate if any of the antimicrobials produced by the gut-derived isolates were peptide/ protein in nature. Sensitivity to proteolytic enzymes indicates that the antimicrobials are protein in nature. The bacteriocins synthetized by LAB are often completely deactivated by proteases (Kumar *et al*. 2010). The current study shows that the antimicrobials produced by 11 out of 16 gut-derived isolates were inactivated by proteinase K suggesting that those were peptide in nature. Inactivation of enterocins has been previously shown in a study carried out by Kumar *et al.* (2010) where enterocin LR/6 isolated from *E. faecium* LR/6 isolated from rhizosphere was found to show sensitivity to proteolytic enzymes such as proteinase K. Similar findings were shown in a study carried out by Liu *et al.* (2008) were *Enterococcus* spp. producing enterocin E- 760 isolated from caecum of boiler chickens showed sensitivity to proteinase K. Similarities were also observed by O'Shea *et al*. (2009) where *E. faecium* DPC6482 isolated from neonate faeces demonstrated sensitivity to proteinase K.

CONCLUSIONS

A total of 4,065 colonies from 23 faecal bacterial banks were screened for anti-*Listeria* activity. Overall 1,569 (39%) colonies have demonstrated various levels of antagonistic activity against *L. monocytogenes*, whereas 2,496 (61%) colonies did not show any activity under the experimental conditions used. The isolates showing activity were then shortlisted to 59 based on the size and appearance of the zone of inhibition and its size, colony morphology as well as the faecal bank it ordinated from. The identity of the 59 gut-derived strains with anti-*Listeria* activity has been established. The results of the 16S rRNA gene sequencing, MALDI-TOF, deferred antagonism assays (against a variety of indicator strains), well diffusion assays with CFS and WCE suggested that there were 17 different strains amongst the 59 gut-derived isolates. A cross immunity deferred antagonism assays of faecal isolates originating from the same faecal bank allowed for further shortlisting of the gut-derived isolates from 17 to 16 based on the basis that two isolates were cross-immune. Deferred antagonism assays with proteinase K suggested that the anti-*Listeria* activity in 11 of the 16 strains was likely to be due to an antimicrobial peptide production. The results of the cross immunity assays and PCRs have shown that 4 of the 16 strains seem to have the genes responsible for production of enterocin A while 6 tested positive for presence of genes responsible for production of enterocin B**.** The data collected to date suggests 10 of the 16 short listed gut -derived isolates may potentially produce novel bacteriocins or perhaps variants of already known enterocins.

Chapter 2

Further investigation of the anti-*Listeria monocytogenes* activity of selected human gut derived bacteria

INTRODUCTION

Ensuring a safe food supply is a constant challenge for the food industry. The use of antimicrobial peptides such as bacteriocins produced by LAB, which have the ability to target common foodborne pathogens without causing any adverse effects and ease of their digestion have received attention (Cleveland *et al*. 2001; Mills *et al.* 2011). Bacteriocins have a wide range of applications in the food industry, they can be used as bio-preservatives in foods either by themselves or can be combined with other preservation methods; i.e., hurdle technology (De Vuyst and Leroy, 2007; Perez *et al*. 2014). Currently there are only two bacteriocins, which are available for commercial use in food preservation, those are nisin (Nisaplin, Danisco) and pediocin PA1 (MicrogardTM, ALTA 2431, Quest) (Simha *et al*. 2012), meaning that there is a need for the discovery of new highly active and relatively cheap bacteriocins, which can be used as food preservatives.

Enterococcus spp. are known for their production of enterocins, which include a varied group of bacteriocins in relation to both their spectrum of activity and their classification (Egan *et al*. 2016). The majority of LAB are considered as generally regarded as safe (GRAS) and can be incorporated into food, however, in the case of bacteriocinogenic enterococci, there are safety concerns (Barlow *et al.* 2007). Bearing those concerns in mind, the use of purified enterocins for food applications is considered as a safer option than using the producing strains (Silva *et al.* 2018). There are a number of novel food technology developments which can employ the use of enterocins in food preservation. Bioactive food packaging has a potential application where bacteriocins, or bacteriocin producing strains, can potentially be integrated into the packaging itself, which acts as an anti-spoilage measure and can significantly extend the shelf life of the product (Castellano & Vignolo 2006; Parada *et al*. 2007). This can be applied to already established packaging materials. The effectiveness of such packaging has been demonstrated by Liserre *et al*. (2002), where a bacteriocin-producing *Lactobacillus sake* has been applied to modified atmosphere packaging (MAP). Similarly, bioactive packaging with nisin has shown a reduction of LAB in sliced ham and cheese in MAP packaging. It has also been observed that the packaging reduced the numbers of *L. innocua* by ~2 logs in both cheese and ham and *S. aureus* by ~1.5 log in cheese and ~ 2.8 logs in ham, with comparable results obtained when vacuum packing the products (Scannell *et al.* 2000), thus suggesting great potential for commercialization of bioactive packaging. Comparably, *Lactobacillus curvatus* CRL705 isolated from dry cured sausage, producing lactocin 705 and lactocin AL705,

have decreased the growth of *Bacillus thermosphacta* and *L. innocua* in vacuum packed meat discs (Castellano and Vignolo, 2006).

Biofilm formation is an on-going issue in the food-processing environment and is of concern to the food industry as it can often cause contamination of the food being processed on a production line. Biofilms are often formed in areas such as stainless steel processing equipment, conveyer belts and storage area surfaces. *Listeria* biofilms are difficult to remove and, if undetected, allow the bacterium to contaminate manufactured food product (Gandhi & Chikindas 2007) and potential spread of a foodborne disease (Wong, 1998). Bacteria growing in a biofilm often display higher levels of resistance to unfavorable environments such as heat or sanitizers (Gandhi & Chikindas 2007; Pan *et al*. 2006; Van der Veen & Abee 2011). Naturally occurring biofilms like those found in the food industry are often composed of mixed bacterial species. Those have been shown to have a greater resistance to cleaning agents and sanitizers (Bremer *et al.* 2001; Carpentier & Chassaing 2004). A significant reduction of *Listeria* biofilm on stainless steel by enterocin B3A-B3B produced by a gut-derived *Enterococcus faecalis* B3A-B3B has been shown by Al-Seraih *et al*. (2017), suggesting that there is a potential for the development of novel anti-biofilm cleaning sprays with a view to application in the food industry and prevention of product contamination.

The aim of this study was to further examine the inhibitory activities of gut-derived isolates against *L. monocytogenes* 10403S. They were examined in broth co-culture experiments, in food models and their ability to inhibit biofilm formation on stainless steel was also investigated.

MATERIALS AND METHODS

Broth based co-culture experiment. All shortlisted gut-derived isolates identified as *Enterococcus spp*. and *L. monocytogenes* 10403S were grown overnight in BHI broth at 37°C. 1 mL of each overnight culture was centrifuged and washed in sterile ¼ strength Ringers' solution. Co-culture experiments and controls were set up in 10 mL volumes of BHI broth with 100µL of washed bacterial cells of *L. monocytogenes* 10403S and selected gut-derived isolate combined in each tube. Growth controls were set up for every experiment i.e. one tube containing *L. monocytogenes* 10403S alone and tubes containing the individual gut-derived isolates only. A positive control was set up with a known enterocin A producer *Enterococcus faecium* DPC 6482. All cultures were incubated at 37°C and aliquots removed, serially diluted and plated on both BHI and Listeria Selective Agar Oxford Formulation (Oxoid) (LSA) at T=0 hr, T=8 hr and T=24 hr. Plates were incubated at 37°C for 48 hr after which they were counted. Plates were re-incubated and counted again after 72 hr in case there was any further growth on them. Plate counts were performed on washed cells to determine the initial inocula.

Food trials. Dairy products; (Tesco Everyday Value French brie, Tesco soft cheese, Charleville Freshly Crafted Select Red Cheddar, Avonmore cottage cheese, Greek Cheese Tesco Feta and Yoplait natural yoghurt) were purchased from a local supermarket in Cork city and were analyzed as follows: a 50 g aliquot of brie cheese was aseptically weighed out and transferred into a sterile stomacher bag (Seward) inside a laminar flow hood. 100 mL of sterile PBS, i.e., a 1:2 dilution of cheese was added, the bag was sealed and the contents were homogenized in a Tekmar Stomacher Lab Blender STO 80 for 5 min at the maximum speed. Subsequently the cheese homogenate was transferred into sterile 50 mL tubes and washed *L. monocytogenes* 10403S cells (1% inoculum, i.e. initial inoculum of ~ 10^7 CFU/mL) was added and vortexed for 1 min. (The *L. monocytogenes* 10403S cells were washed in the manner described previously. Briefly 1 mL of *L. monocytogenes* 10403S overnight culture was centrifuged at 13,000 rpm for 5 min, the supernatant was then removed and the pellet was re-suspended in 1mL of sterile ¼ strength Ringers solution). 900µL samples were taken from the inoculated cheese homogenates and serially diluted in 900µL sterile ¼ strength Ringers solution). Then 10 µL aliquots of each dilution 10^{-1} to 10^{-9} were spotted onto LSA agar cheese homogenate samples were then immediately placed in a 37°C incubator and were incubated

for 3 hr in total, with 100 µL samples removed, serially diluted and 10µL aliquots of diluted sample spot plated onto LSA. Counts performed at T=1 hr and T= 3 hr. The LSA plates were incubated at 37°C for 48 hr. plate counts were then obtained and CFU/ mL were calculated. The same protocol was used for number dairy products. The dilution factor used for various cheeses was dependent on the texture and matrix of each cheese. Natural yogurt and soft cheese were diluted 1:1 with sterile PBS solution; cheddar cheese, cottage cheese and feta cheese were diluted 1:3 with sterile PBS solution. The experimental set up is displayed in **(Table 1)** The CFS and WCE were prepared as previously outlined **(Chapter 1)** and their anti-*Listeria* activity was confirmed by carrying out a well diffusion assays.

Table 1. The experimental set-up of the model food trials.

Tube contents	**Comment**
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes*.	Negative control to examine the growth of *L. monocytogenes* in the food.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL sterile BHI broth.	Negative control for test samples with added CFS.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL 70%IPA0.1%TFA.	Negative control for test samples with added WCE.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL of CFS from a known Enterocin A producer.	Comparison sample.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL of WCE from a known Enterocin A producer.	Comparison sample.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL of CFS from isolate 15.	Test sample.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL of WCE from isolate 15.	Test sample.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL of CFS from isolate 43.	Test Sample.
3 mL of cheese homogenate inoculated with 1% *L. monocytogenes* + 100 µL of WCE from isolate 43	Test Sample.
3 mL of uninoculated homogenized cheese sample.	Negative control – to confirm that *Listeria* cells were not present in the food matrix prior to inoculation.

Microtiter plate biofilm assays. Biofilm assays were carried out as described by (Begley et al., 2009). *L. monocytogenes* 10403S was grown overnight in 10 mL of BHI broth at 37°C. Overnight culture were centrifuged at 8,000g x 6 min, the supernatant was discarded and the cell pellet was re-suspended in ¼ strength Ringers' solution and samples were re-

centrifuged. The supernatant was removed and pellets were re-suspended in 10 mL fresh BHI broth. 100µl of the resulting washed cells was added to 10 mL fresh BHI broth and cells were mixed by vortexing. 180µL was subsequently transferred into individual wells of a sterile 96 well flat bottom plate (Sarstedt cat no. 82.1581.001). 2µL of CFS from gut-derived strains prepared as previously described in chapter 1 was added to specific wells. Plates were sealed with parafilm in order to prevent evaporation and incubated statically at 37°C for 18 hr. All experiments were performed in triplicate using three biological repeats. Sterile broth alone was used as a negative control. *Listeria* alone was used as a positive control. After the incubation step, the contents of wells were removed and each well was washed three times with 200µL of sterile water to remove unattached or loosely bound cells. The plate was allowed to dry adjacent to a Bunsen burner for 30 min after which attached cells were stained by the addition of 200µL of a 1% aqueous crystal violet solution (Sigma Aldrich) for 45 min. The stain was removed and wells were washed three times with sterile water. Bound stain was re-solubilized in 200µL 96% ethanol and OD595 nm was determined.

Examination of biofilm formation by *L. monocytogenes* on stainless steel. An assay for examining the formation of *Listeria* biofilm on stainless steel was devised. Stainless steel coupons (2 cm x 2 cm) were obtained from Dr. Hugh O` Donnell from the Department of Biomedical Engineering in CIT. The coupons were autoclaved in freshly prepared phosphate buffer pH4, they were then scraped with sterile cotton swabs and 100% ethanol in order to remove bacterial from the surface of the coupons, they were then were left to dry by the Bunsen flame. The coupons were subsequently aseptically placed into individual wells of a 6 well microliter plate (Starstedt cat no. 83.1839) and 50 mL Falcon tubes. 3mL of fresh BHI broth inoculated (1%) with washed *Listeria* cells was added to each well of the 6 well plates and 15 mL of fresh BHI broth that was inoculated (1%) with washed *Listeria* cells was added to each Falcon tubes. Each experiment was set up in triplicate and incubated at 37°C (as described below) for 16 hr and biofilm were examined. One plate and one set of tubes were incubated statically and one plate and one tube were incubated in a shaking incubator at 100 rpm. Biofilms were measured in all plates and tubes by staining with crystal violet and de-colorization with 96% ethanol and measuring absorbance at 595 nm. Finally one plate and one tube were incubated statically. One plate and one tube were incubated in a shaking incubator at 100 rpm. Biofilm were measured in the last set of samples by sonication of the coupons and performing plate counts. The sonication method described by Bjerkan et al. (2009) was followed. In

summary, after the washing step the coupons were submerged in 5ml of sterile ¼ strength Ringers solution and were sonicated in a Soniprep 150 ultrasonic disintegrator (MSE, UK Ltd) for 1 minute on each side (in order to remove the biofilm formed by *Listeria* cells bound to the surface of the coupons) 100µL of the resulting cell suspension was then serially diluted in 900µL of sterile ¼ strength Ringers solution. 10µL spots of each dilution and the neat undiluted samples were spotted onto fresh LSA agar and were incubated at 37°C for 48hr.

Examination of the effects of gut-derived antimicrobials on *Listeria*'s biofilm formation on stainless steel coupons. Sterile stainless steel coupons were aseptically placed into individual wells of a 6 well microliter plate. The coupons were then covered with 3 mL of fresh BHI broth that had been seeded with 1% inoculum of washed *L. monocytogenes* 10403S cells. Various aliquots (10µL, 30µL and 100µL) of CFS or WCE from isolate 15, isolate 43 or enterocin A producing *E. faecium* DPC6482 were added to separate wells. The contents of each well was mixed by pipetting the well contents up and down a few times as well as swirling of the plate manually. The plates were then incubated at 37°C for 16 hr. Following the incubation, the stainless steel coupons were removed from the wells and were washed three times in 5 mL phosphate buffer saline (PBS). The coupons were then left to dry for 30 min. The coupons were subsequently stained with 1% crystal violet solution for 45 min and were then washed three times with sterile PBS and dried at room temperature for 30 min. The stain bound to the biofilm was then decolorized by application of 5 mL 96% ethanol and the biofilm was quantified by measuring its absorbance at 595 nm.

Antimicrobial peptide purification. Bacteriocin purification was performed in accordance to the method described by Aspri et al. (2017) with slight modifications. Pure cultures of gut-derived isolates 15 and 43 were grown on BHI agar and then aerobically grown overnight in 5 mL of BHI broth at 37°C, the culture was then transferred to 30 mL of BHI broth and incubated for 8 hr at 37°C, following the incubation the culture was transferred to 2 L of fresh BHI broth and was incubated overnight at 37°C. The resulting cultures were then centrifuged at 8,280 g for 20 min. The cell pellets retained and re-suspended in 250 mL of 70% isopropanol 0.1% TFA. The cell suspensions were then stirred at room temperature for 3-4 hr and were centrifuged as described above; the supernatants were retained for further analysis. The IPA was eliminated from the supernatant using rotary evaporation (Buchi, R-300, Flawil, Switzerland). The samples were then passed through a 12 mL, 2 g Strata–E C18 SPE column

(Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 12 mL 30% ethanol and then 20 mL 70% isopropanol 0.1 TFA (IPA). Well diffusion assays were carried out with *L. innocua* DPC 3572 as the indicator strain to show the activity of the sample at different stages of purification. Mass spectrophotometer analysis of each sample was then carried out with Axima TOF^2 (Shimadzu Biotech, Manchester, UK) mass spectrophotometer. Each sample was then concentrated and passed through an analytical Proteo Jupiter (4.6 x 250 mm, 90Å, 4µ) RP-HPLC column (Phenomenex, Cheshire, UK) with a 25-55% acetonitrile 0.1% TFA and 90% acetonitrile 0.1% TFA (buffer B). The fractions of the eluent were collected at 1-min intervals and were monitored at 214 nm. The anti-*Listeria* activity of the fractions was assayed by carrying out a well diffusion assay on *L. innocua* DPC 3572 indicator plates. The molecular masses of the active fractions were analyzed on a mass spectophotometer and were then lyophilized with a Genevac HT4X lylophiliser (Genevac Ltd, Ipswich, England).

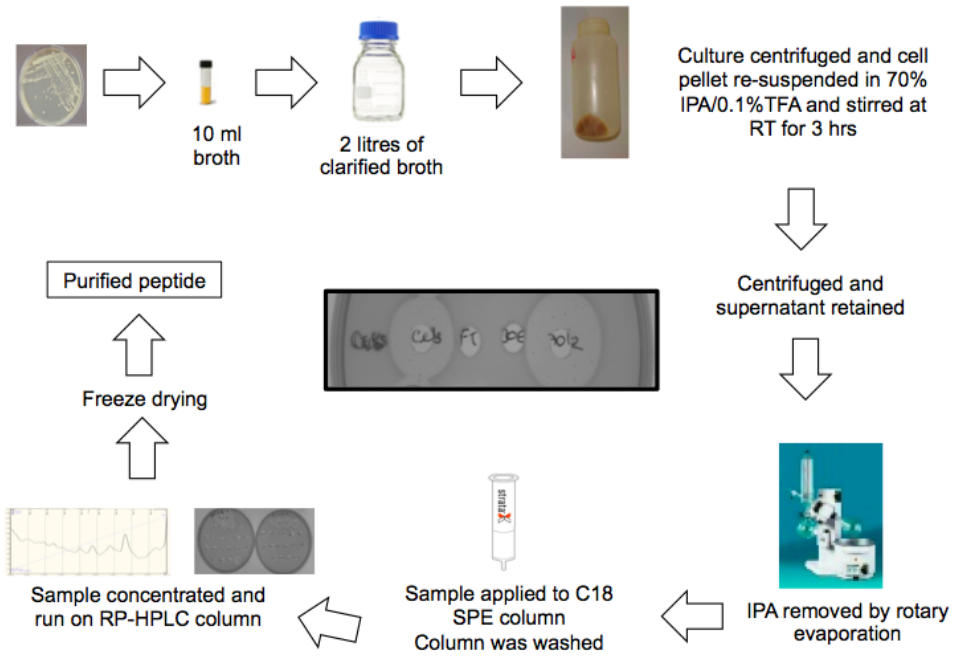


Fig.1 A schematic representation of peptide purification methods. (This figure was obtained from Dr. Máire Begley).

Assessment of the anti-*Listeria* activity of partially purified peptides. Lyophilized preparations of anti-*Listeria* peptides were weighed and re-suspended in 1 mL sterile water (the final concentration was 18.1 mg/mL for gut- derived isolate 15 and 7.91mg/mL for gut derived isolate 43). Well diffusion assays were carried out with various

Listeria strains. Briefly fresh BHI agar was made, once the temperature of the molten agar was ~ 50°C aliquots of agar were seeded with 1% inoculums of overnight cultures of *L. monocytogenes* 10403S, *L. monocytogenes* L028, *L. monocytogenes* EGDe, *L. monocytogenes* F2356 and *L. innocua* 13568t grown in BHI broth .The agar was then left to dry and 5 mm wells were cut in each plate. 10µL aliquots of antimicrobial preparations were then added to wells and plates were incubated at 37°C for 16 hr following the incubation plates were examined for zones of inhibition.

Arbitrary Units (AU)/mL of activity against *L. monocytogenes* 10403S. Lyophilized preps were re-suspended in sterile water and dilutions were made using sterile water (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048). 10µL aliquots of each dilution were then added to individual wells of a BHI agar plate that was seeded with 1% *L. monocytogenes* 10403S. Plates were incubated at 37°C for 16 hr after which they were examined for zones of inhibition and the AU/ mL were calculated by multiplying the dilution factor of the last clear zone of inhibition by reciprocal of 20.

RESULTS

Investigation of the ability of gut-derived isolates to inhibit *Listeria* in a co-culture model broth system. The ability of 14 of the short-listed gut derived- isolates identified as *Enterococcus spp*. to inhibit *Listeria* when co-cultured in broth was examined as outlined in the Materials and Methods section. A positive control, i.e., a known enterocin A producer, *E. faecium* DPC6482, from our culture collection was included for comparison purposes. A co-culture assay was carried out in a model broth system. A variety of results were obtained in the assay (**Fig.2)**. It should be noted that the initial *L. monocytogenes* inoculum at T=0 was established as Log 7 CFU/mL. Following 8 hr incubation, the number of *Listeria* cells in the negative control showed a ~2 log increase in the number of cells. The positive control brought about a decrease in the number of *Listeria* cells by ~ 3 log following 8 hr incubation. A number of isolates showed a reduction in the number of *Listeria* cells; co-culture with isolates 54, 43 and 15 all demonstrated a reduction by ~3 log. Isolates 29, 52, 72 and 38 showed a ~2.5 log, ~ 2 log, ~ 1 log and ~0.5 log decrease in the number of *Listeria* cells, respectively. Isolates 4, 12, 18,20, 40 and 57 all showed a slight increase in the numbers of *Listeria,* but the counts were lower than the –ve control. Based on these results and the findings presented in Chapter 1, two isolates (15 and 43) were selected for further investigation.

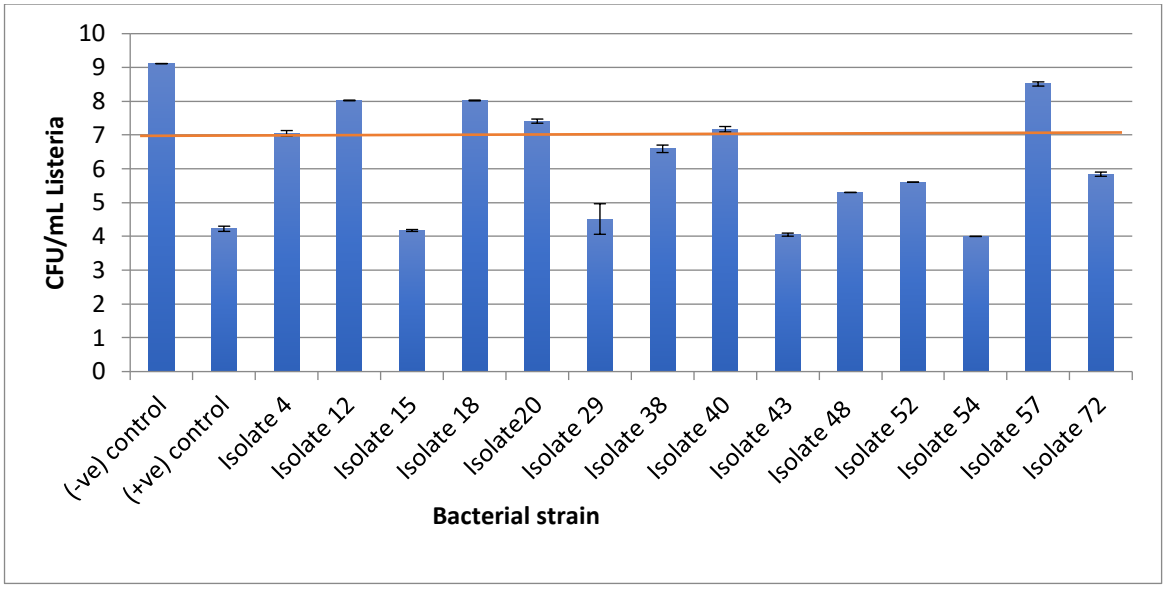


Fig.2 Inhibition of *L. monocytogenes* 10403S by a known enterocin A producer *E. faecium* DPC 6482 and 14 gut-derived isolates in a broth-based co-culture experiments. The bar chart shows *L. monocytogenes* 10403S counts in each experiential set up at T8 hr. (-ve) control = *L. monocytogenes* 10403S alone. (+ve) control = *L. monocytogenes* 10403S + enterocin A producing *E. faecium* DPC 6482. The red line indicates the initial number of *L. monocytogenes* 10403S (i.e. counts at T0 =Log 7 CFU/mL).

Investigation of antimicrobial activity of CFS and WCE from gut isolates 15 and 43 in model food trials.

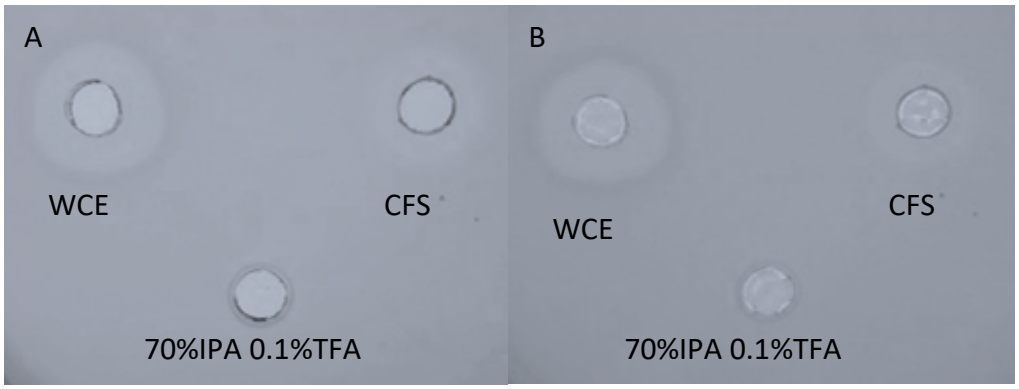


Fig.3 A representative figure showing the zones of inhibition obtained in a well diffusion assay carried out in BHI agar seeded with 1% *L. monocytogenes* 10403S showing the difference in activity displayed by whole cell extract (WCE) vs. cell free supernatant (CFS) of gut derived isolates (A) 15 and (B) 43. 70% IPA 0.1% TFA was added to the wells as a negative control.

Fig. 3 visualizes the difference in the anti- *Listeria* activity observed in the CFS vs. WCE from the selected gut- derived isolates. A noticeable difference can be seen in the size and the appearance of the zones when comparing WCE vs. CFS. The results obtained for both gut- derived isolate 15 and 43 were the same; the zone of inhibition obtained for WCE was recorded as 15mm while the one obtained for CFS was 11mm. The negative control shows that IPA used in extraction of the WCE does not contribute to the antagonistic activity of the extract.

The CFS and WCE from isolates 15 and 43 as well as a known enterocin A producer *E. faecuim* DPC 6482 were added to various dairy foods otlined in the materials and methods section. *Listeria* counts were determined at various time points with a view of examination of the anti- *Listeria* acitivty of the isolated enterococcal antimicrobials in food matrices. A food trial performed in a model cheddar homogenate (**Fig.4 –A)** shows that treatment with 100 μL of CFS from gut derived isolates 15 and 43 as well as an enterocin A producing strain did not inhibit the growth of *Listeria* in the cheddar model. However, the results do show that *Listeria* growth was hindered in comparison to the negative control with no antimicrobial agent present. The trial carried out in a model soft cheese homogenate (**Fig.3-B)** shows a reduction of growth by ~0.3 log in samples treated with isolate 15 and the enterocin A producer, while *Listeria* present in the sample did not grow following the treatment with CFS from isolate 43. The trial carried out in a model natural yogurt homogenate (**Fig.4-C)** shows that treatment CFS from gut derived isolates 15 and 43 reduced the number of *Listeri*a cells in the sample by ~0.5 log and ~0.6 log, respectively, while the reduction observed by the enterocin A producer was ~0.4 log. For the model feta and brie (**Fig.4-D, Fig.4-E)**, the effect of the treatment with CFS from gut derived isolates as well as the enterocin A producing strain could not be determined as the initial number of *Listeria* added to the samples was reduced in all samples, including the negative controls, suggesting that *Listeria* was inhibited by the constituents of the food products. **Fig.4-F** shows the results obtained in a cottage cheese homogenate. Here the treatment with CFS from gut-derived isolate 43 and the enterocin A-producing strain inhibited the growth of *Listeria* keeping the cell numbers the same as the initial inoculum following a 3 hr incubation from the initial inoculum levels while CFS from gut-derived isolate15 showed to have no inhibitory effect.

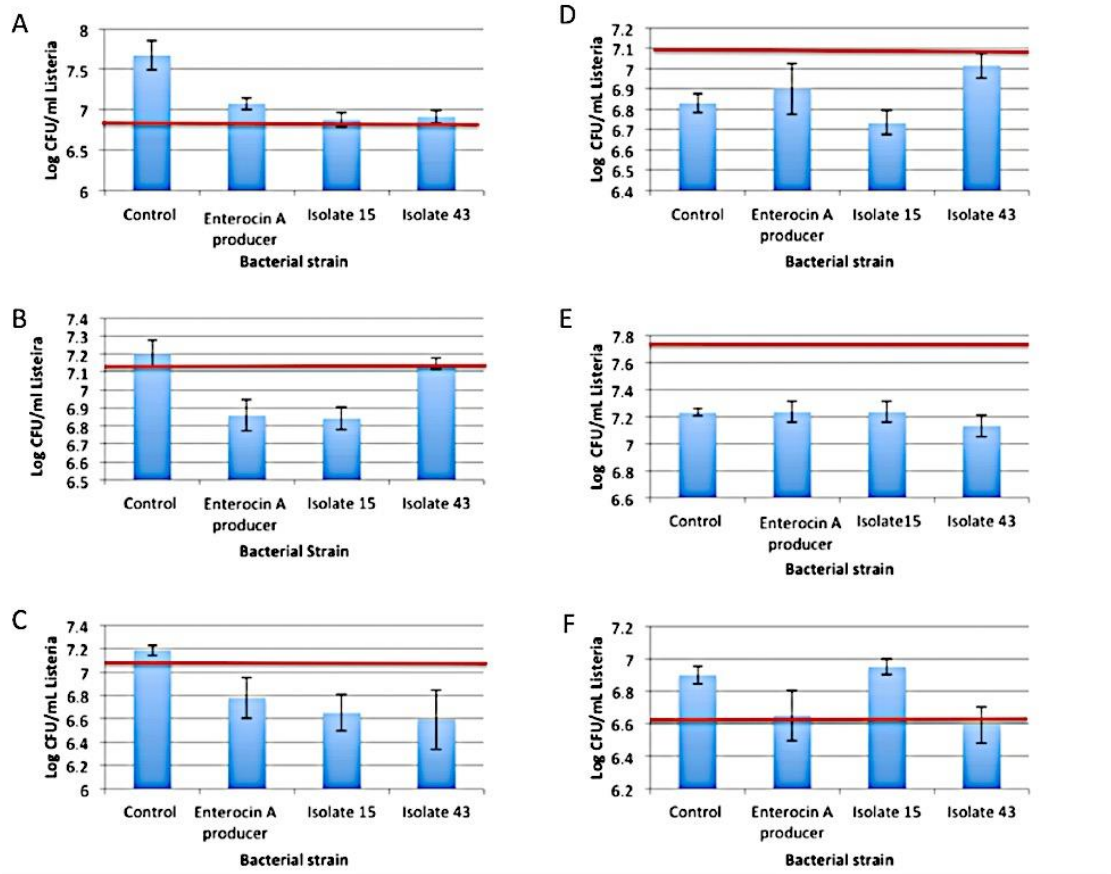


Fig.4 Inhibition of *L. monocytogenes* 10403S by the CFS from a known enterocin A producer *E. faecium* DPC6482 and gut derived isolates 15 and 43.The bar charts show *L. monocytogenes* 10403s counts at T3hr. The red lines indicate the initial *Listeria* counts at T0hr. Control - dairy product homogenate inoculated with *L. monocytogenes* 10403S + 100 µL of sterile BHI broth, which acted as the negative control. Enterocin A producer– dairy product homogenate inoculated with *L. monocytogenes* 10403S + 100 µL of CFS from *E. faecium* DPC 6482 which acted as a positive control. Test samples- dairy product homogenate inoculated with *L. monocytogenes* 10403S + 100 µL of CFS from gut-derived isolates 15 and 43; **(A)** cheddar, **(B)** soft cheese, **(C)** natural yogurt, **(D)** feta, **(E)** brie, **(F)** cottage cheese.

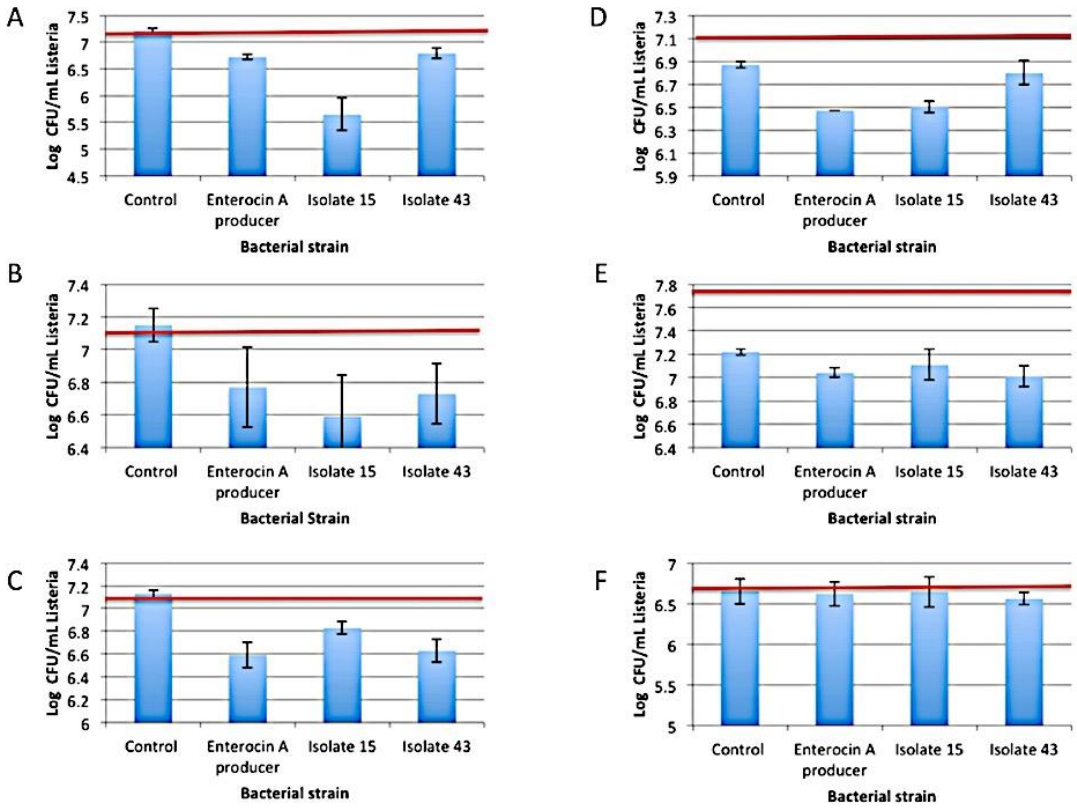


Fig. 5 Inhibition of *L. monocytogenes* 10403S by the WCE from a known enterocin A producer *E. faecium* DPC6482 and gut derived isolates 15 and 43.The bar charts show *L. monocytogenes* 10403s counts at T3hr. The red line indicates the initial *Listeria* counts at T0hr. Control - dairy product homogenate inoculated with *L. monocytogenes* 10403S + 100 µL 70%IPA0.1%TFA, which acted as a negative control. Enterocin A producer – dairy product homogenate inoculated with *L. monocytogenes* 10403S + 100 µL of WCE from *E. faecium* DPC 6482, which acted as a positive control. Test samples- dairy product homogenate inoculated with *L. monocytogenes* 10403S + 100µL of WCE from gut-derived isolates 15 and 43; **(A)** cheddar, **(B)** soft cheese, **(C)** natural yogurt, **(D)** feta, **(E)** brie, **(F)** cottage cheese.

Fig. 5 shows the results obtained when samples were treated with WCE from gut-derived isolates. A model cheddar homogenate treated with WCE from gut-derived isolates 15 and 43, as well as the enterocin A producer, can be seen in **Fig.5-A.** Treatment with WCE from isolate 15 resulted in a reduction by ~1.5 log, WCE from isolate 43 reduced the number of *Listeria* by 0.4 log, and the enterocin A producer brought about a reduction of ~0.5 Log. **Fig.5-B** shows the results obtained in a model soft cheese homogenate. The numbers of *Listeria* were decreased by treatment with all test samples; with a reduction by Log ~0.5 log by WCE from

isolate 15 and a reduction of ~0.4 log by isolate 43. The results acquired from trial in a model natural yogurt homogenate are presented in **Fig.5-C.** The numbers of *Listeria* were decreased by treatment with WCE from all test samples by ~0.5 log. **Fig.5-D** shows the results obtained in the feta homogenate model. The reduction in the number of *Listeria* observed in the sample treated with WCE isolate 15 and the positive control was ~0.6 log and ~0.3 log by isolate 43. The results obtained from the trial carried out in a model brie homogenate seen in **Fig.5-E** suggest that the treatment with WCE had a minimal to no effect on *Listeria* numbers. It can also be concluded that treatment of the cottage cheese seen in **Fig.5-F** had no impact on the numbers of *Listeria* present in the sample.

Microtiter biofilm assay. A biofilm assay was carried out in order to investigate whether the antimicrobials showing antagonistic activity against *Listeria* isolated in this study have anti- biofilm properties. The results obtained in the microtiter biofilm assay carried out with CFS from 14 shortlisted gut-derived isolates identified as *Enterococcus* are summarized in **Table 2** and show that 3 (21%) of the tested isolates contributed to biofilm reduction greater than 75% when comparing it directly to the negative control. Additional 5 (36%) of the tested isolates have showed biofilm reduction between 50-75% in comparison the negative control, 3 (21%) of the tested isolates have shown biofilm reduction between 20-50%, while 1 (7%) of the tested isolates showed a biofilm reduction < 10%. Additionally 2 of the tested isolates (14%) contributed to an increase in biofilm formation. Overall the assay has demonstrated the ability of the CFS extract from selected gut derived isolates to prevent biofilm formation on plastic surfaces.

Table 2. Summarised results obtained in the microtiter biofilm assays where 180µL of BHI broth was inoculated with washed *L. monocytogenes* 10403S cells and 20µL of CFS from selected gut-derived isolates, incubated overnight at 37° C for 18 hr, washed, stained with 1% aqueous crystal violet and quantified at 595nm. The % values in the table show the % increase (+) or decrease (-) in OD595nm compared to the OD595nm obtained for the control (i.e. *L. monocytogenes* 10403S in broth alone). Biofilm assays were performed in triplicate with three biological repeats and repeated twice (i.e. a total of 9 biological repeats. The results represented in the Table are averages of one set of biological repeats but are representative of all 3 experiments).

Isolate ID	Effect on biofilm formation
4	- 94.69%
12	- 53.84%
15	- 69.13%
18	- 5.53%
20	- 27.47%
29	- 81.94%
38	+ 41.57%
40	- 68.45%
43	- 20.59%
48	- 70.97%
52	- 53.17%
54	- 90%
57	- 37.50%
72	+ 7.98%

Assessment of biofilm formation by *Listeria* on stainless steel coupons and examination of anti-biofilm properties of CFS and WCE from gut-derived isolates 15 and 43. Various methods were assessed for examining biofilm formation by *Listeria* on stainless steel coupons. The most reproducible results were obtained when experiments were performed under static incubation conditions after which biofilm were stained with crystal violet and the biofilm quantified by decolorizing with ethanol and OD measured. Representative images of biofilm experiments and corresponding quantification of results can be seen in **Fig.6.** It was observed that *Listeria* could form biofilm on the coupons and biofilm formation was prevented when increasing amounts of WCE from selected gut-derived isolates.

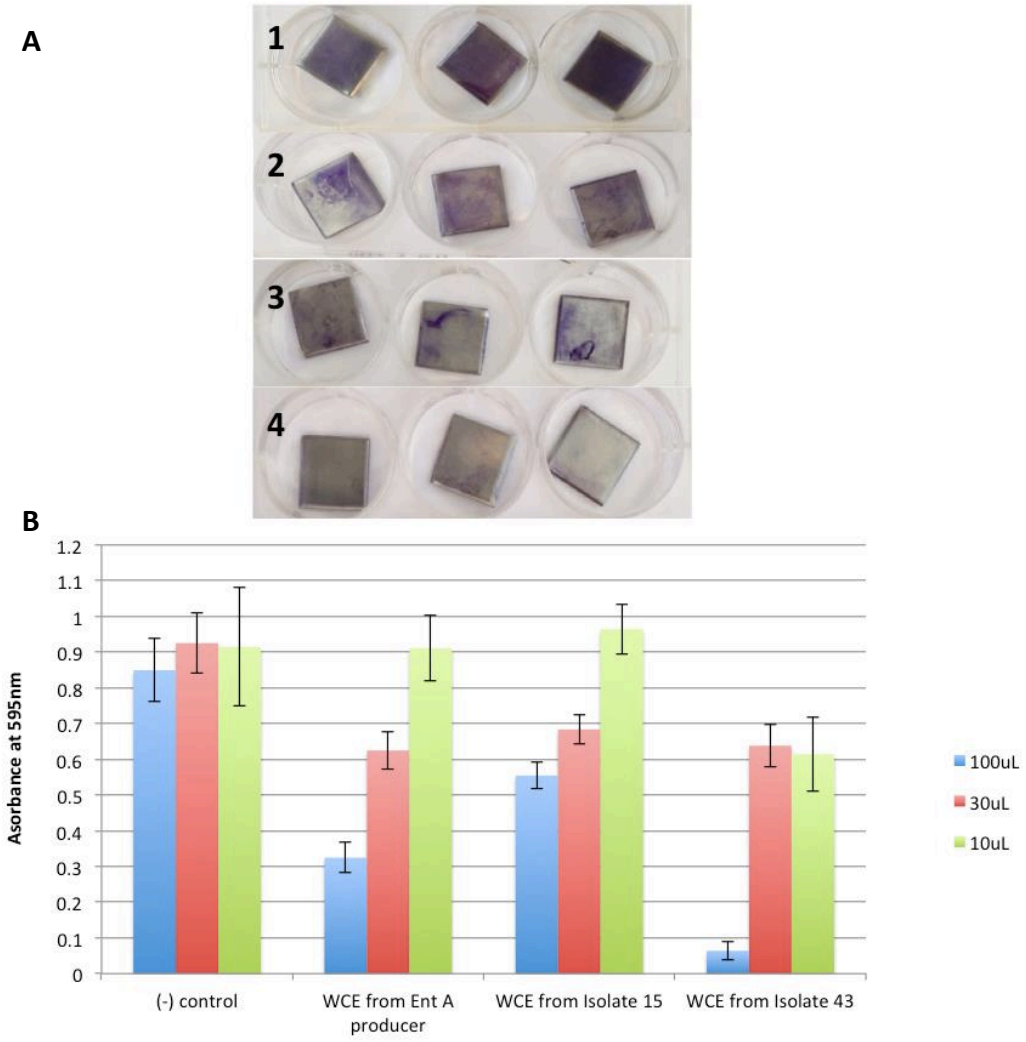


Fig6. (A) Biofilm formation by *Listeria* on stainless steel coupons. Stainless steel coupons were covered in fresh BHI broth seeded with 1% of washed *L. monocytogenes* 10403S cells +/- WCE extract from gut derived isolate 43. **(1)** Control: *Listeria* + 100µL 70% IPA0.1%TFA, **(2)** *Listeria* + 10 µL WCE from gut-derived isolate 43, **(3)** *Listeria* + 30µL WCE from gut-derived isolate 43 **(4)** *Listeria* + 100µL WCE from gut-derived isolate 43. **(B)** A representative chart displaying a reduction in biofilm formed by *Listeria* on stainless steel (2cm x 2cm) coupons. Stainless steel coupons were covered in fresh BHI broth seeded with 1% of washed *L. monocytogenes* 10403S cells. The resulting cultures underwent treatment with WCE from gut-derived isolates 15 and 43. **(-)** Control =70% IPA0.1%TFA added to the culture. Test samples = WCE from gut-derived isolates 15, 43 or enterocin A producing strain were added to the culture in a 100 µL, 30 µL or 10 µL volume.

Fig.6-A shows biofilm formation visualized with 1% crystal violet stain. As seen in the image a strong biofilm was produced by the negative control containing untreated *Listeria*

culture visible in row (1). Row (2) shows biofilm formed following treatment with 10 µL of the WCE from isolate 43, here a small difference can be seen. Row (3) represents biofilm formation following a treatment with 30 µL of WCE from isolate 43 a notable difference can be seen here. Row (4) shows biofilm treated with 100 µL of WCE of isolate 43, here barely any biofilm can be seen. The results of the stainless steel biofilm assay are represented in **Fig. 6-B** as can be seen the results correspond to the visual representation of the data in **Fig.5-A**. Again showing that the reduction of biofilm formed on stainless steel increases with the volume of added WCE. Based on the results of this assay it can be concluded that the WCA of gut - derived isolate 43 causes most biofilm reduction.

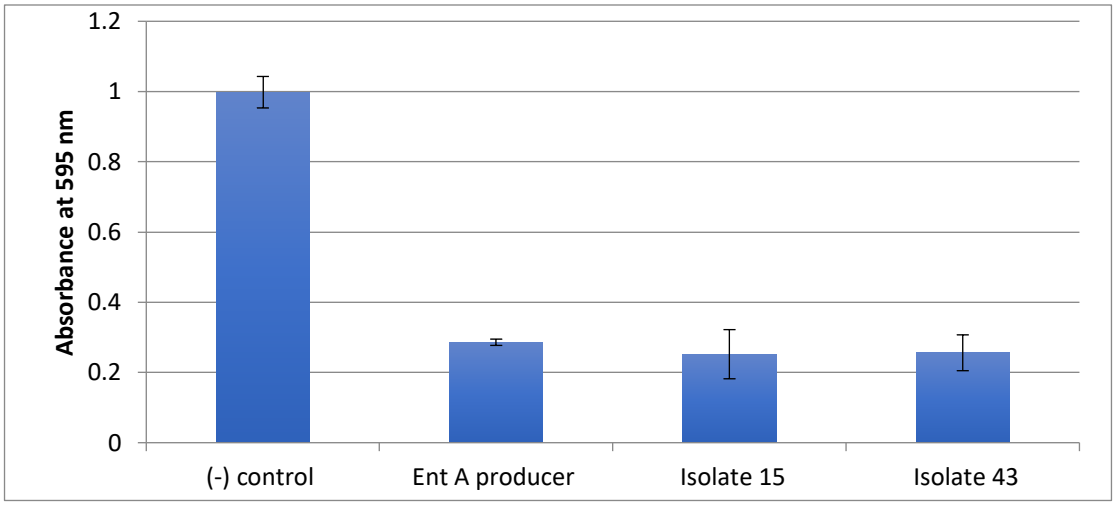


Fig.7 Biofilm assays using stainless steel (2cm x 2cm) coupons. Stainless steel coupons were covered in fresh BHI broth seeded with 1% of washed *L. monocytogenes* 10403S cells and 100µL CFS from gut-derived isolates 15 and 43, (-) Control =100µL of sterile BHI broth added to the culture, no CFS from gut- derived strain added

As seen in **Fig.**7the assay was also carried out with addition of 100 µL of CFS from isolates 15, 43 a and an enterocin A producing strain. The results obtained from this experiment showed that all three test samples have the ability to reduce biofilm by ~70%.

Partial purification of the antimicrobial peptides produced by gut-derived isolates 15 and 43. Efforts focused on purifying the antimicrobial peptide(s) produced by isolates 15 and 43 from whole cell extracts, as the agar well diffusion assay shown in **Fig.3** suggested that the antimicrobial produced by the strains may be primarily cell associated. A combination of column chromatograph and RP-HPLC was used. The anti-*Listeria* activity at

each stage of the purification process was assessed by agar well diffusion assays. Mass spectrophotometry was performed at various stages in order to determine the masses of any peptides present. Previous experiments outlined in chapter 1 suggested that isolates 15 and 43 potentially produce enterocin A. This was confirmed by peptide purification. Data collected during various steps of the purification process can is represented in **Fig.8** and **Fig 9.** One of the first steps of the purification was the separation of the CFS from the cells this was carried out in accordance to the materials and methods section, following the initial centrifugation step the cells were re-suspended in 70% isopropanol 0.1% TFA the cells were then shaken for 3-4 hours and centrifuged again, the resulting supernatant referred to as WCE was retained and isopropanol was removed from it using a rotary evaporator, the concentrate was then passed through Starta – C18 column, the column was then washed with 30% Ethanol and the bound peptide was then eluted with 70% isopropanol. During each step fractions were collected in order to show that no activity was lost at any of the stages**. Fig.8-A** and **Fig. 9-A** show results of a well diffusion assay carried out in a *L. innocua* DPC3572 indicator plate showing that no activity was lost during the extraction of the antimicrobial peptides from gut-derived isolates 15 and 43, respectively. The first collected fraction (1) represented the WCE extracted from the cells as can be seen in both figure the fraction has showed activity. This was an expected result as at this stage the active antimicrobial peptide was stripped off the cell surface and was suspended in the resulting WCEs. The WCE was then passed through the column. At this stage the active peptide was expected to bind to the column. As seen in (2) no activity was detected in the WCE flow through, meaning that all active peptide was indeed bound to the column at that stage. The next stage involved a washing step with 30% ethanol in order to remove any unbound peptides and other contaminants, a fraction of the flow through has been collected and is activity was checked in the well diffusion assay (3) once again no activity was observed, which means that all the active peptide remained bound to the column at this stage and no activity was lost. The final stage of the column chromatography was elution with 70% isopropanol, at this stage the peptide bound to the column should be eluted; therefore activity should be seen in the fraction collected at this step, which can be seen in well labelled (4). The eluted peptide was then analyzed using a mass spectrophotometer in order to determine if masses of any known bacteriocins could be identified. The mass chromatograms showing results obtained from the analysis of the 70% isopropanol eluent obtained following column chromatography the results from isolates 15 and 43 are displayed in **Fig.8-B** and **Fig.9-B**.

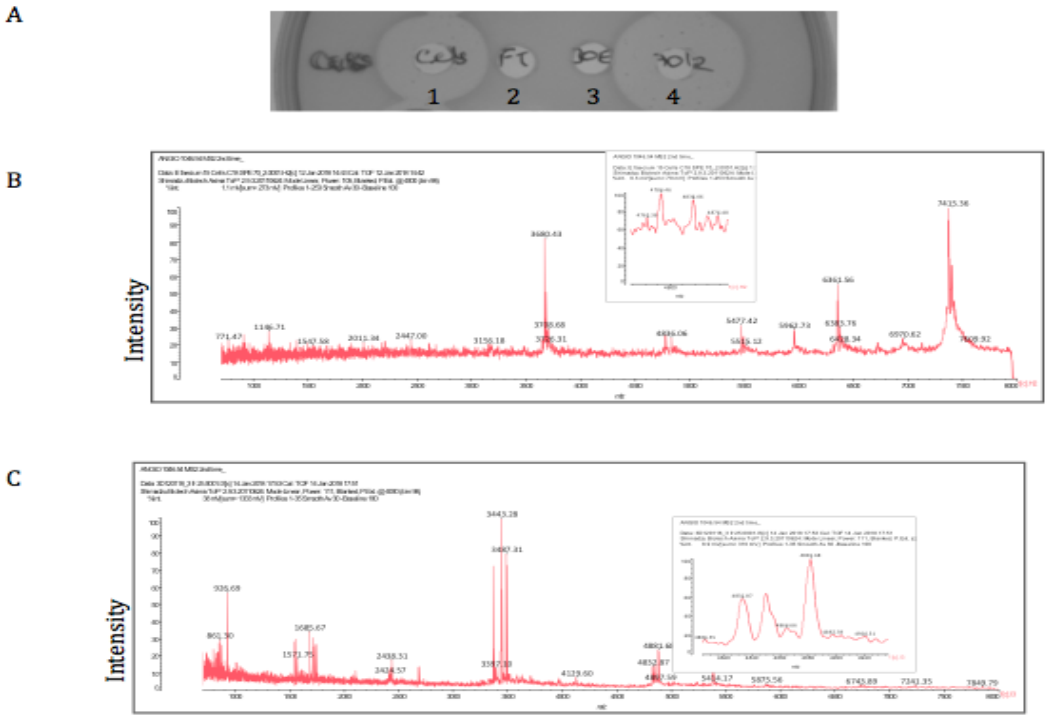


Fig.8 A figure showing various steps of purification of the antimicrobial peptide from gut derived isolate 15**. (A)** a well diffusion assay carried out with BHI agar inoculated with 1% *L. innocua* DPC3572. 50µL aliquots from various fractions from column chromatography were added to the wells. **(1**) Whole cell extract (WCE) from 2 L of overnight gut derived isolate 15 grown in BHI broth. **(2)** WCE eluted through Starta-E C18 column **(3**) 30% ethanol wash **(4)** 70%IPA elution. **(B)** A mass chromatogram showing the active IPA eluent of an antimicrobial peptide produced by the gut-derived isolate 15 showing a peak at 4836. 06Da. **(C)** A mass chromatogram showing the active fraction from gut-derived isolate 15 collected during RP-HPLC purification showing a peak at 4832 Da.

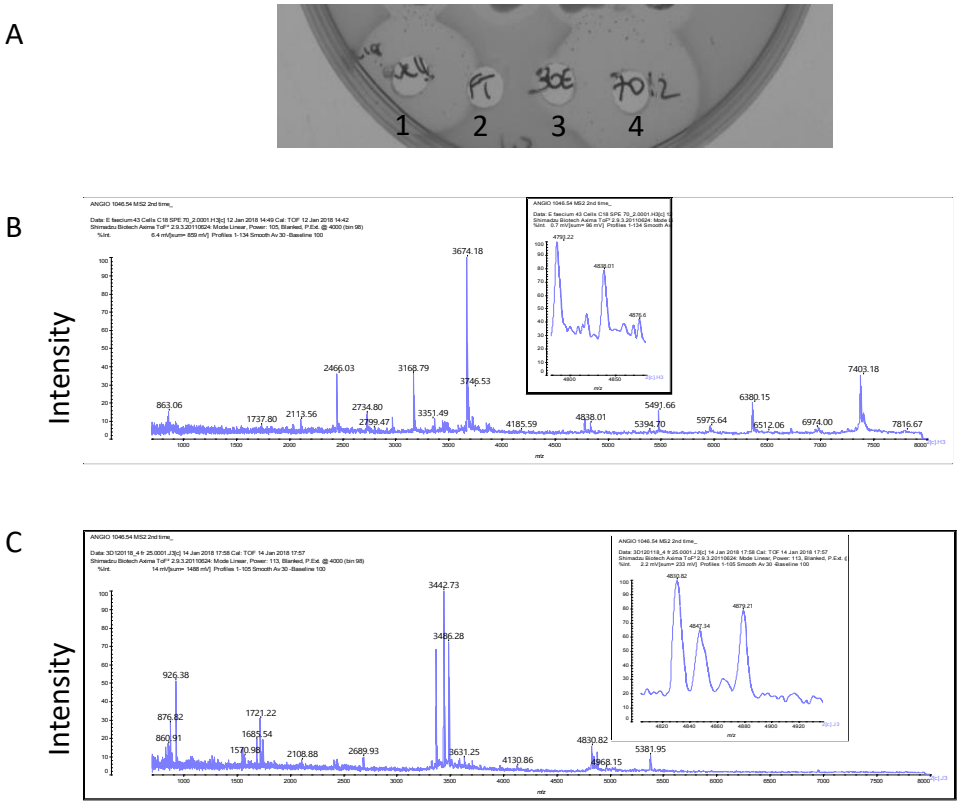


Fig.9 A figure showing various steps of purification of the antimicrobial peptide from gut derived isolate 43. **(A)** as well diffusion assay carried out with BHI agar inoculated with 1% *L. innocua.* 50µL aliquots from various fractions of column chromatography peptide purification were added to the wells. **(1)** Whole cell extract (WCE) from 2 L of overnight gut derived isolate 43 grown in BHI broth. **(2)** WCE eluted through Starta-E C18 column **(3)** 30% ethanol wash **(4)** 70%IPA elution. (B) A mass chromatogram showing the active IPA eluent of an antimicrobial peptide produced by the gut derived isolate 43 showing a peak at 4838.01 Da. **(C)** analysis showing the active fraction from gut-derived isolate 43 collected during RP-HPLC purification showing a peak at 4838.82 Da.

The mass chromatogram presented in **Fig. 8-B** displays a peak at 4836.06 Da, which is a mass relatively similar to that of enterocin A (4832.61 Da). Similar findings were obtained following mass spectra analysis of the active eluent from isolate 43 **Fig. 9-B** here a peak at 4838.01 Da was observed, again showing a mass very close to that expected of enterocin A. Due to the fact that the mass spectra readings have indicated peaks showing masses close to that of enterocin A the active 70% isopropanol eluents from isolates 15 and 43 were then passed through a RP-HPLC column with fractions collected at 1 min intervals. Each resulting fraction was when examined for anti-*Listeria* activity in a well diffusion assay with an *L. innocua* DPC3572 indicator plate (data not shown) in both samples activity was observed in fractions 24-26, with highest activity observed in fraction 25 meaning that the active peptide came off the column in those fractions. Based on the results obtained in the well diffusion assay

fraction 25 was chosen from both isolates and were analyzed on the mass spectra seen in **Fig8-C** and **Fig.9-C**. The mass chromatogram shows a peak at 4832.87 Da for the antimicrobial isolated from gut derived isolate 15 and 4838.82 Da for the antimicrobial isolated from gut derived isolated 43. The masses of the isolated antimicrobials suggest that their identities are enterocin A with a mass of 4832.61 Da.

Determination of activity of the partially purified antimicrobial from shortlisted gut isolates against various *Listeria* indicator strains and AU/ mL arbitrary units of activity. The anti–*Listeria* activity of the antimicrobial preparation was confirmed against a number of *Listeria* strains including; *L. monocytogenes* EGDe, *L. monocytogenes* L028, *L. monocytogenes* 2365, *L. monocytogenes* 10403 and *L. innocua* in a well diffusion assay. A two-fold dilution-well diffusion/ critical dilution well diffusion assay was carried out in order to investigate the Arbitrary Units (AU) of the purified bacteriocins. The AU/mL are standard units used to measure the activity of antimicrobial agents such as bacteriocins. As can be seen in **Fig. 10-A** and **Fig.10-B** both preps demonstrated activity against all tested strains of *Listeria.* The results obtained in are in agreement with the previous findings (chapter 1). The results show that the partially purified peptide from isolate 15 shows antagonistic activity against *L. monocytogenes* 10403S with a visible zone of inhibition when diluted a by a dilution factor of 1:256 the results obtained can be seen in **Fig. 10-C** The AU/mL were calculated to be 5120 AU/mL. Similar results were observed when measuring the AU/mL units of the purified peptide from isolate 43, here once again last clear zone of inhibition was noted in sample diluted by a factor of 1: 256, again resulting with the AU/mL units to be calculated as 5120 AU/mL as seen in **Fig.10-D**

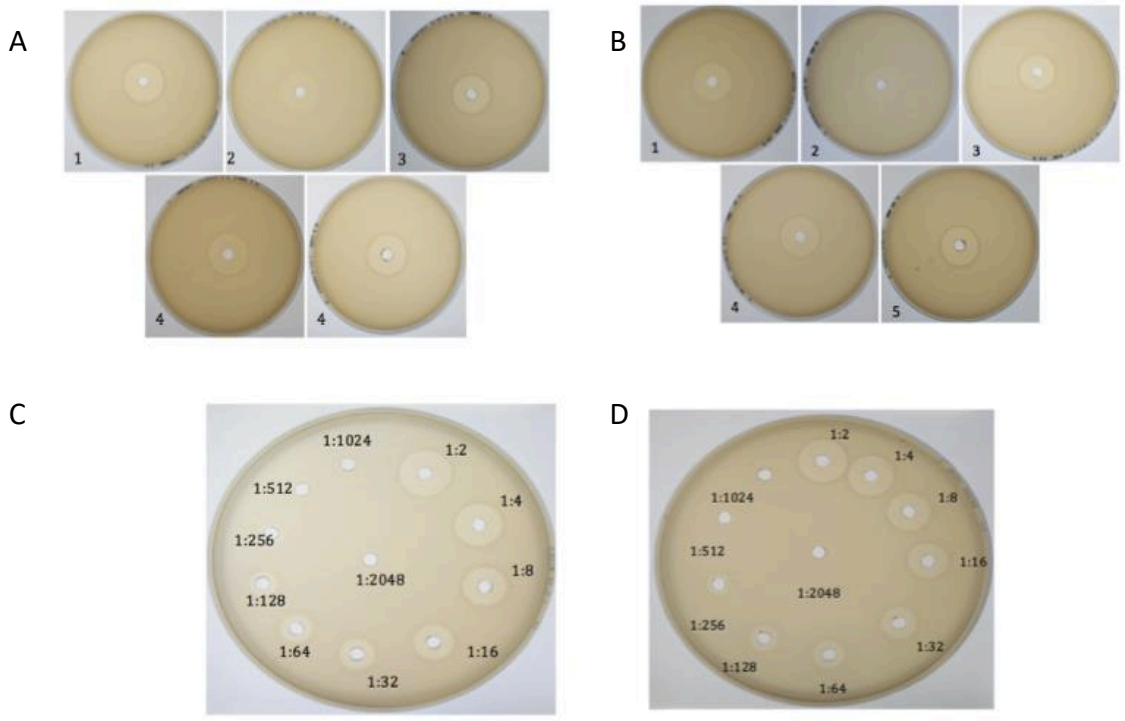


Fig. 10 Results of a well diffusion assay carried out with crude antimicrobial preparations from **(A)** gut-derived isolate 15 and **(B)** isolate 43. The lyophilised peptide was re-suspended in 1 mL of sterile water resulting in a final concentration of 18.1 mg/mL for isolate 15 and 7.91mg/mL for isolate 43. 10µL was added to the wells and plates were incubated overnight at 37° C. The indicators used were **(1)** *L. monocytogenes* EGDe **(2)** *L. monocytogenes* L028 **(3)** *L. monocytogenes* F2365 **(4)** *L. monocytogenes* 10403S and **(5)** *L. innocua*. Panels **(C)** and **(D)** show a well diffusion showing the anti-*Listeria* effect of the crude antimicrobial prep from gut-derived isolate 15 seen in panel **(C)** and gut- derived isolate 43 seen in panel **(D)**. 5 mm wells cut in BHI agar seeded with 1% *L. monocytogenes* 10403S inoculum were filled with 10 µL aliquots of the diluted antimicrobial preparations and incubated at 37° C overnight.

DISCUSSION

Investigation of the ability of gut-derived isolates to inhibit *Listeria* in a co-culture model broth system. Due to the fact that the inhibitory spectrum of bacteriocins frequently includes common foodborne pathogenic bacteria, there is growing interest in their application as natural food additives (Vuyst, 2014). The potential of the application of enterocins as food additives or their in situ production during food fermentation have been investigated by (Leroy *et al.* 2003). The results obtained in the current study have demonstrated that the antimicrobial agents produced by *Enterococcus* isolated from the human gut have the ability to reduce the growth of *Listeria* in a co-culture assay by up to 3 log CFU/mL following an 8 hr incubation in BHI broth, thus suggesting their potential application in the food industry. It should be noted that gut-derived isolates 15, 43 and 54 showing the highest levels of antagonistic activity have tested positive for cross immunity with enterocin A and enterocin B (chapter 1), suggesting that the antimicrobials produced by those gut-derived strains may be enterocin A and B. Fimland *et al*. (2002) has proposed that cross immunity between class IIa bacteriocin producers such as *E. faecium* can occur in situations where the bacteriocins or the immunity proteins fit to subgroups with related sequences. Suggesting that the antimicrobials produced by those gut-derived isolates may also be variants or enterocin A and B or may be closely related to those bacteriocins. The results obtained in the co-culture assay show antagonistic activity similar to that displayed by the enterocin A producing strain *E. faecium* DPC 6482. It should be taken into account that the initial *Listeria* inoculum was at 10^7 CFU/ mL, which is much higher than the usual numbers of *Listeria* normally isolated from food products, e.g., Moosavy et al. (2014) has reported to isolate on average 40 CFU/mL of *L. monocytogenes* in raw milk used for production of Lighvan cheese. The findings of this study are comparable to findings observed in studies carried out with LAB found in the literature (Pleasants *et al.* 2001), where a model co-culture broth system of *L. monocytogenes* and a bacteriocin producing *L. sakei* 706 showed that co-culture with a bacteriocin producing strain has detrimental effects on the numbers of *Listeria* present in the sample. A study carried out by (Khay *et al*. 2013) examined a co- culture of *L. monocytogenes* CECT 4032 and *E. durans* E204 in skimmed milk, the results show that *Listeria* population was completely eliminated following a 16 hr incubation with the antimicrobial producing strain of *E. durans.* A co-culture in BHI broth treated with CFS from *E. durans* 4032 showed a rapid a rapid decrease in the number of *Listeria* cells observed in the first 4 hr after incubation, similar to that obtained in the current study. The findings of the current study, as well as evidence found in the literature suggest that the antimicrobials produced by LAB, namely

Enterococcus spp. have a protective potential, which may be utilized in foods susceptible to contamination with *Listeria*.

Investigation of antimicrobial activity of CFS and WCE from gut isolates 15 and 43 examined in model food trials. Dairy based foods especially semi-hard and soft cheeses are believed to be some of the most significant at-risk products associated with outbreaks of foodborne listeriosis, and are often recognised as the main channel for human infection with *L.monocytogenes* (Hunt *et al*. 2012; Lahou and Uyttendaele, 2017). Bacteriocins isolated from LAB often display antagonistic activity against pathogenic bacteria in food (Lewus *et al.* 1991). In the current study the effect of CFS and WCE of two gut-derived isolates was investigated in fermented dairy products**.** The aim of this experiment was to gain insight if the antimicrobials produced by the gut-derived isolates have a potential as food preservatives i.e. being used as potential food additives. It is important to realize that even though an antimicrobial shows activity in a broth based system that does not mean that one can assume activity in a food system. Food constituents such as proteins or lipids or the intrinsic properties of the food matrix such as the a_w or the pH of the food material can alter antimicrobial and its effectiveness in the food product (Davidson *et al.* 2013).

While variation of results and different rates of *Listeria* growth have been observed across different dairy media used, this is not surprising. Relative variation in growth of *Listeria* in various cheese media has previously been noted by Lahou & Uyttendaele (2017). The key findings of the current study show that, when treated with CFS from the gut-derived isolates, the numbers of *Listeria* were unaffected, or affected to a minimal degree when growing in cheddar, feta, brie and cottage cheese. The greatest reduction in the numbers of *Listeria* was observed in the natural yogurt homogenate with a 0.6 log CFU/mL reduction following a 3 hr incubation period. In contrast treatment with WCE from the gut-derived isolates was more effective, with a reduction of ~1.5 log CFU/mL in the cheddar homogenate. The resutls obtained in the current study showed that the anti-*Listeria* activity of the WCE was higher than that of CFS in most food media. This however was not the case in all food types as the opposite was observed in the natural yogurt model. A similar experimental approach was undertaken by Huang *et al*. (2013), who investigated the use partially purified enterocin RM6 in a cottage cheese homogenate inoculated with 10^5 CFU/mL *Listeria*. This resulted in a reduction in *Listeria* numbers by 4 logs in 30 min post treatment and below detectable levels after 26 hr incubation period. While slight differences in the experimental set-up exist, i.e., the current

study used a higher initial *L. monocytogenes* inoculum and the antimicrobials used to treat the cheese homogenates were at a lower purity level, the results obtained by Huang *et al*. (2013) suggest that similar level of antagonistic activity against *L. monocytogenes* can be expected if the assay was to be repeated with a semi purified antimicrobial peptide from gut-derived isolates.

The literature shows a number of approaches in which the anti-*Liseria* activity of bacteriocins can be assessed in a dairy foods. Those include; spraying of the bacteriocin producing strain onto the surface of the cheese as described by Izquierdo *et al*. (2009) where bacteriocin producing *E. faecium* WHE 81 isolated from cheese was sprayed onto the surface of a Munster cheese at the start of the ripening process. The experiment has shown that while in some Munster soft cheese samples a number of *Listeria* cells did survive on the surface of the cheese it could not grow in the presence of the bacteriocin. The ability of using bacteriocin producers as starter cultures has also been investigated in great detail Maisnier-Patin *et al*. (1992) investigated making of camembert cheese with a nisin producing *L. lactis* subsp. *lactis* starter culture. Liu *et al*. (2008) has demonstrated the anti- *Listeria* potential of heterogous LAB starter culture producing enterocin in cottage cheese manufacture. Cárdenas *et al*. (2016) investigated fermented cheese models with a starter culture of bacteriocin producing *E. faecium* CECT 8849 isolated from human milk. A study carried out by Leroy *et al*. (2003) has investigated the applicability of *E. faecium* RZS C5 isolated from cheese to be used as a starter culture in chedar cheese manafacture. Vandera *et al*. (2017) showed that a cheese isolate *E. faecium* KE82 entirely inhibited *L. monocytogenes* in a raw milk sample following incubation at 37°C for 6 hr, while the further incubation at 18°C for 66 hr has significantly inactivated the pathogen. However, there is still a lot of controversy when considering using gut-derived enterococcal isolates for such applications, thus the activity of the antimicrobials produced by those isolates can sometimes be harnessed by instead adding a (semi-) purified bacteriocin as a food ingredient during product manufacture. A recent study Ribeiro *et al.* (2017) has reported the effectiveness of a semi-purified enterocin produced by *E. faecalis* strain isolated from cheese in reduction of *L. monocytogenes* in freshly made cheese. The observed reduction was dose dependent, with the highest dose reducing the numbers of *Listeria* below levels of detection. Similarly, Davies *et al.* (1997) showed that the introduction of nisin to ricotta type cheese during its production can inhibit the growth of *L. monocytogenes* for 8 to 10 weeks, while cheese made without addition of nisin accumulated unsafe levels of *Listeria* 1-2 weeks post production. The findings of the current study as well as the evidence found in the literature

show that there is a great potential for enterococcal bacteriocins to be used in the food industry as food preservatives or can be incorporated into the food products as starter cultures.

Examination of the effects of gut-derived antimicrobials on *Listeria* biofilm formation on stainless steel coupons *L. monocytogenes* imposes a serious threat to the food industry due to its ability to adapt to extreme environmental changes and therefore being able to withstand numerous hurdles to which it may be exposed during food processing treatment (Hill *et al.* 2002). It has been established that despite harsh processing conditions *Listeria* can be found in various parts of the dairy processing plant (Waak *et al.* 2002). Therefore biofilm formation is of concern to the food industry as it can often cause contamination of the food being processed on a production line. Biofilms are often formed in areas such as stainless steel equipment and utensils, processing equipment, conveyer belts as well as storage area surfaces (Gandhi & Chikindas 2007; de Oliveira *et al*. 2010). Bacteria growing in a biofilm from often display higher levels of resistance to unfavorable environments such as heat, sanitizers or disinfecting agents (Gandhi & Chikindas 2007; Wong 1998). It has been documented that *L. monocytogenes* is capable of sustaining growth in a monoculture biofilm as well as being a part of a mixed culture biofilm. The study by Bremer *et al*. (2001) has determined a significant increase in the number of *L. monocytogenes* cells attached to stainless steel surfaces when forming a mixed culture biofilm with *Flavobacterium* when compared to the number of cells attached to the stainless steel surface when in a monoculture biofilm, it has also been determined that the survival time of *L. monocytogenes* has increased significantly in a mixed culture. This is of concern to the food industry as the possibility of a mixed biofilm formation are quite high, especially in industrial environments (Bremer *et al*. 2001; Gandhi & Chikindas 2007). It is also argued that the persistence of *L. monocytogenes* in food industry premises and equipment is not a result of strain specific biofilm formation, but a consequence of poor hygiene in harborage sites (Carpentier & Cerf 2011). Insignificant sanitation and cleaning procedures especially of food based product contact surfaces, processing environments and the equipment used has been indicated as one of the major causes contributing to outbreaks of foodborne diseases especially those associated with *L. monocytogenes* or *Salmonella* (Chmielewski and Frank, 2003). Generally speaking, upon the completion of biofilm formation the bacterial cells become irreversibly adhered to the surface and often require challenging chemical or mechanical treatments, which are not always feasible in the food industry setting (Al-Seraih *et al*. 2017). A novel natural, sustainable and safe group of anti- biofilm agents can potentially be established with a view of food safety. Bearing in mind recent research in the

field of bacteriocins which are promising candidates for such application (Al-Seraih *et al*. 2017). The biofilm assays carried out in this study show a significant biofilm reduction with up to 92.42% reduction of biofilm on stainless steel surface when treated with WCE and up to 70% biofilm reduction when threated with CFS from the gut derived isolates. The findings of the current study are comparable to those described by (Al-Seraih *et al*. 2017) who examined the anti-biofilm activity of B3A-B3B enterocin produced by *E. faecalis* B3A-B3B isolated from infant faeces.

Partial purification of the antimicrobial peptides produced by gut-derived isolates 15 and 43. Bacteriocin producing LAB often have complex nutritional needs which need to be meet in order to confer bacterial growth and bacteriocin production, this causes difficulties in purification (Guyonnet *et al*. 2000; Li *et al*. 2002). The difficulties are mostly associated with the fact that bacteriocins are often secreted into the growth medium. In the current study, the LAB isolates were grown in BHI broth, which is high in peptides of animal origin. Since the antimicrobial peptides produced by LAB are often produced in low quantities the peptides of animal origin present in the medium can sometimes be a preventive factor in the identification of the antimicrobials with mass spectrophotometry methods. This has been previously reported by Carroilssen-Mackay *et al*. (1997) and was also the case in the current study. Because the identification of the antimicrobial peptides has been problematic when the gut derived- isolates were grown in BHI broth, the activity of the antimicrobials was also examined when gut-derived isolates were grown in different media (tryptic soy broth (TSB), tryptone yeast extract (TY) broth and De Man, Rosa and Sharpe (MRS) broth), however antimicrobial activity was not as high as that observed with BHI broth (data not shown). Mass spectrophotometric identification of the active peptides present in the CFS was also attempted when the gut-isolates were grown in clarified BHI broth (data not shown). However, this was unsuccessful due to a high number of background masses corresponding to the peptides of animal origin present in medium used in the purifications medium itself.

Due to the fact that bacteriocins belong to a particularly diverse family of peptides, specific purification protocols must be often designed for each of them (Balciunas *et al.* 2013). The current study has encountered a number of problems while trying to purify the antimicrobials produced by gut-derived isolates. A method previously described by (O'Shea *et al*. 2009) has been attempted a number of times (data not shown) and has been unsuccessful.

Due to the fact that bacteriocins are secreted into the growth medium, the majority of bacteriocin purification methodologies begin with a concentration step, where the bacteriocins are concentrated in the cell free supernatant. The main objective of such procedures is to decrease the working volume of the antimicrobial being purified rather than to deliver a highly pure peptide (Guyonnet *et al*. 2000). A number of protocols have been devised for purification purposes that involve ammonium sulfate precipitation (Dezwaan *et al*. 2007; Dündar *et al*. 2015), absorption-desorption (Yang *et al.* 1992), and/or organic solvent extraction (Burianek and Yousef, 2000).

In the current study, the bacteriocins have been extracted with the organic solvent extraction method and were separated by adsorption onto a C18 column and subsequent RP-HPLC as described by (Aspri *et al.* 2017). The purifications have allowed for detection and partial purification of enterocin A. However, both gut-derived isolates have tested positive for both enterocin A and enterocin B in a cross immunity assay carried out with known bacteriocin producers (see chapter 1). The presence of enterocin A and enterocin B was also detected for those isolates in a PCR (data not shown).

CONCLUSIONS

A model broth co-culture experiment was set up with 14 shortlisted gut-derived isolates identified as *Enterococcus*. The results obtained in the co-culture experiment have showed that the numbers of *L. monocytogenes* were reduced by up to 3 log CFU/mL following an 8 hour incubation. Based on the results obtained in the co-culture assay two isolates (15 and 43) were chosen for further experimentation. Food trials with CFS and WCE from the selected isolates were carried out in cheddar cheese, soft cheese, natural yogurt, feta, brie and cottage cheese. The results of those trials showed a ~ 0.6 log CFU/mL of reduction in the number of *Listeria* cells in natural yogurt when treated with CFS. Treatment with WCE has shown to be more effective than treatment with CFS for most dairy products (with the exception of natural yogurt) with the highest reduction of ~1.5 log CFU/mL observed in cheddar cheese following a 3 hr incubation. The anti-biofilm activity of the antimicrobials produced by the gut- derived isolates was also examined showing a biofilm reduction of 92.42% when treated with WCE and 70% when treated with CFS. The antimicrobials produced by isolates 15 and 43 have been partially purified and provisionally identified as enterocin A with masses of 4832 Da and 4838.82 Da respectively. Their activity against *L. monocytogenes* EGDe, *L. monocytogenes* L028, *L. monocytogenes* F2365, *L. monocytogenes* 10403S and *L. innocua* has been confirmed in a well diffusion assay. The arbitrary units of activity of the partially purified enterocin A has been calculated to be 5120 AU /mL. The data collected suggest that the antimicrobials isolated in the current study have a potential for applications as food preservatives or anti-biofilm agents. However, further experimentation is necessary.

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Thesis Summary

L. monocytogenes continues to be a major problem to the food industry. Its versatility in terms of environmental conditions it can survive and its ability to form biofilms allows it to persist in food and in the food-processing environments. Novel methods are needed to control *L. monocytogenes*, which could be used as food preservatives or antimicrobials in industry settings such as washes or sprays or anti-biofilm agents. Gut bacteria have been shown to produce antimicrobials and based on the evidence presented in sections 4 and 5 of the literature review we postulated that gut microbiota may produce novel anti-*Listeria* compounds. The aim of this thesis study was to isolate strains with anti- *Listeria* activity and characterize them.

To our knowledge this is the first large scale screen for anti-*Listeria* compounds from the human gut. The work presented in chapter one focused on isolation and characterization of gut bacteria with anti-*Listeria* activity from 23 human faecal banks. A total of 4,065 colonies were screened in deferred antagonism assays and anti- *Listeria* activity was observed for 1,569 colonies. Isolates displaying various levels of anti-*Listeria* activity were compared to each other and shortlisted to a total of 59 gut- derived isolates for further examination on the basis of colony morphology, faecal bank of origin and the level of anti-*Listeria* activity. The shortlisted isolates were identified with a combination of MALDI-TOF analysis and partial 16S rRNA gene sequencing and the vast majority were identified as *Enterococci* spp. A total of three isolates were identified as *Streptococcus* spp. Various assays were carried out in order characterize the antimicrobials produced by the gut-derived isolates. Deferred antagonism assays with a variety of indicator strains was carried out to establish the inhibitory spectrum of activity of the isolates and showed that the antimicrobials produced by the gut- derived isolates have a narrow spectrum of activity, showing antagonism mostly to other closely related Gram-positive bacteria such while activity against Gram-negative organisms was not observed. The nature of the antimicrobials (i.e. cell bound vs. extracellular) was investigated in well diffusion assays with cell free supernatants (CFS) and whole cell extracts (WCE) showing that the antimicrobials produced by majority of the gut-derived isolates secreted their antimicrobials into the growth medium. Based on the data collected it was determined that there were possibly 17 different strains present amongst the 59 shortlisted isolates, and those 17 isolates were selected for further investigation. Cross immunity deferred antagonism assays with gut-derived isolates originating from the same faecal banks showed a cross immunity in two isolates and allowed for further shortlisting of the gut-derived isolates to a total of 16. The proteinaceous nature of the antimicrobials was investigated by proteinase

K deferred antagonism assays and showed that the anti-*Listeria* activity of 11 out of the 16 shortlisted isolates was likely due to production of an antimicrobial peptide. Further cross immunity assays with known bacteriocin producers revealed that 4 of the gut-derived isolates were cross-immune to enterocin A and enterocin B. PCRs confirmed the presence of enterocin A and B structural genes in the strains.

The key focus of the work presented in chapter two was the further investigation of the anti-*Listeria* activity of selected human gut isolates. The anti-*Listeria* activity of the isolates was investigated in a model broth co-culture assay and results showed a reduction in the numbers of *Listeria* by up to 3 log CFU/ mL after an 8 hr incubation period. Based on previous studies in our lab we knew that the purification of antimicrobial peptides from enterococci was challenging, and that the existing purification methods routinely used in our lab for the purification of other antimicrobial peptides (i.e. the bacteriocins nisin and lacticin 3147) would require optimization. For this reason and given the time restrictions of the current Masters research project it was decided to focus on two strains in order to optimize purification methods. Isolates 15 and 43 were chosen due to the fact that biggest zones of inhibition were produced by those isolates and while the purifications were underway other experiments (food trials and biofilm assays) were carried out with CFS and WCE in order to optimize those experiments and obtain preliminary results with crude antimicrobial extracts.

The ability of CFS and WCE prepared from the selected isolates were investigated for their ability to inhibit *L. monocytogenes* in cheddar cheese, soft cheese, natural yogurt, feta, brie and cottage cheese. The results obtained in these food trials have shown a ~0.6 log CFU/mL reduction in the numbers of *Listeria* in a natural yogurt homogenate treated with CFS and ~1.5 log CFU/mL reduction in cheddar cheese homogenate treated with WCE. Additionally anti-biofilm activity of the extracts was investigated on stainless steel and results showed a biofilm reduction of 92.42 % following a WCE treatment and a 70% reduction following treatment with CFS. The antimicrobials of the two selected isolates were then partially purified and provisionally identified as enterocin A.

The data collected to date shows that the antimicrobials isolated in the current study have potential applications for novel applications in the food industry such as food preservatives or as anti-biofilm agents. Future work should focus on sequencing the genomes of all of the shortlisted gut-derived isolates, the genomes should then be analysed with

bacteriocin gene cluster mining tools such as BAGEL3 in order to find out what antimicrobial genes are present and if any of them may encode any novel antimicrobials (as determined by homology searches and literature searches). If novel antimicrobials were identified their purification and optimization of purification methods should be prioritized. The antimicrobials should be then assessed for toxicity and checked for their safety in food applications, if safe potential food applications as food preservatives, active packaging or cleaning agents should be investigated as the applications of enterocins of human gut origin into the food industry is a relatively unexplored area of research.

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