An Investigation into Antimicrobial Production in the *Lactobacillus* Genus and the Fish Microbiome

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

by

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Declaration

I hereby certify that this material, which I submit for assessment on the programme of study leading to the award of Ph.D. is my own work along with the below contributions and has not been submitted for another degree, either at University College Cork or elsewhere.

Contributions:

Chapter 2: Ms. Paula O'Connor carried out mass spectrometry and peptide purification, Dr. Orla O'Sullivan carried out genome assembly.

Chapter 3: Ms. Paula O'Connor carried out mass spectrometry and peptide purification, Dr. Orla O'Sullivan carried bioinformatics analysis

Chapter 4: Ms. Paula O'Connor carried out mass spectrometry and peptide purification, Dr. Beatriz Mesa Pereira carried out vector design and heterologous expression in the *Lactobacillus* host.

Chapter 5: Dr. Calum Walsh carried out bioinformatics analysis

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List of Publications

Book Chapters

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Research Papers

Collins, F.W., O'Connor, P.M., O'Sullivan, O., Rea, M.C., Hill, C. and Ross, R.P., 2016. Formicin–a novel broad-spectrum two-component lantibiotic produced by *Bacillus paralicheniformis* APC 1576. *Microbiology*, *162*(9), pp.1662-1671.

Collins, F.W., O'Connor, P.M., O'Sullivan, O., Gómez-Sala, B., Rea, M.C., Hill, C. and Ross, R.P., 2017. Bacteriocin Gene-Trait matching across the complete *Lactobacillus* Pan-genome. *Scientific Reports*, *7*(1), p.3481.

Collins, F.W., Mesa-Pereira, B., O'Connor, P.M., Rea, M.C., Hill, C. and Ross, R.P., 2018. Reincarnation of Bacteriocins From the Lactobacillus Pangenomic Graveyard. *Frontiers in microbiology*, *9*.

Abbreviations

3-HPA	-	3-hydroxypropionaldehyde
CPM	-	Copies per Million
Dha	-	2,3-didehydroalanine
Dhb	-	2,3-didehydrobutyrine
DOM	-	Dissolved Organic Matter
EF-Tu	-	Elongation Factor Tu
GDHt	-	Adenosylcobalamin-dependent Glycerol Dehydratase
GRAS	-	Generally Regarded as Safe
HMM	-	Hidden Markov Model
IPTG	-	Isopropyl ß-D-1-thiogalactopyranoside
LAB	-	Lactic Acid Bacteria
LAP	-	Linear azol(in)e-containing peptide
MAG	-	Metagnomic Assembled Genome
MALDI TOF	-	Matrix Assisted Laser Desorption/Ionization
MS	-	Mass Spectrometry
NRPS	-	Non-ribosomal Peptide Synthetase
PAGE	-	Polyacrylamide Gel Electrophoresis

PCoA	-	Principle Coordinate Analysis
PKS	-	Polyketide Synthase
POM	-	Particulate Organic Matter
RiPPs	-	Ribosomally Synthesised Post-translationally modified Peptides
SAM	-	S-adenosylmethionine
SDS	-	Sodium Dodecyl Sulfate
TMA	-	Trimethylamine
TOMM	-	Thiazole/oxazole-modified Microcin
WDA	-	Well Diffusion Assay

Abstract

This thesis outlines a study of the identification and characterisation of antimicrobials from two primary sources; the *Lactobacillus* genus and the microbiome of fish. Through the incorporation of a wide variety of techniques, this study successfully demonstrates how a variety of methods can be used for the identification and production of novel antimicrobials.

Chapter 1 gives an overview of the variety of different antimicrobials which can be produced by the lactic acid bacteria (LAB) which can play an important role in a number of processes, such as the preservation of fermented foods. These antimicrobials can vary from organic acids, such as lactic and acetic acid, to antimicrobial peptides known as 'bacteriocins'.

Chapter 2 outlines the identification of a novel bacteriocin known as 'formicin' through traditional colony isolation and screening methods. Formicin was identified from *Bacillus paralicheniformis* APC1576, an antimicrobial producing strain isolated from the intestine of a fish. Using a combination of mass spectrometry and genomic screening, formicin was found to be a two-peptide lantibiotic, displaying antimicrobial activity against a broad range of Gram positive microbes.

In Chapter 3, through a combination of *in silico* and lab-based screening of the *Lactobacillus* pangenome, it was possible to determine the extent and diversity of bacteriocins encoded and produced by the genus. This study shows that bacteriocin production may not be as prevalent as previously believed, however of the bacteriocins which were identified from within the genomes, many were found to be novel. By screening the strains identified as harbouring bacteriocin-related genes, five novel active bacteriocins were identified.

Many strains of lactobacilli were found to encode bacteriocins, however upon analysis, these failed to display *in vitro* antimicrobial activity. Often the regulation of bacteriocin operons, or the loss of key bacteriocin-associated genes, results in the failure of these strains to produce bacteriocins when tested *in vitro*. Chapter 4 outlines a method for the heterologous expression of a

particular class of bacteriocins, the Class IIa ‘pediocin-like’ bacteriocins. Here, using an expression designed for Class IIa bacteriocins, it was possible to produce eight novel bacteriocins identified from genomic data.

In Chapter 5 shotgun metagenomic sequencing is used to characterise the compositional and functional properties of the intestinal microbiome of deep sea fish. Here it can be seen how bacteria have adapted to live in this environment by encoding systems to relieve the stress associated with life at higher pressures. The study also outlines the diversity of the potential antimicrobials which may be produced within the microbiome of such fish whilst also highlighting an apparent lack of genes associated with known mechanisms of antibiotic resistance.

Overall, the results of this work demonstrate the effectiveness of a variety of methods for identifying novel antimicrobials from a range of bacterial sources.

Chapter 1: Antimicrobials from Lactic Acid Bacteria and their Potential Applications

Book Chapter: Lactic Acid Bacteria: Microbiological and Functional Aspects, Fifth Edition 2019 (Accepted)

Abstract

The antimicrobial activity of lactic acid bacteria (LAB) has been utilised throughout recent human history. The fermentation of foods is an ancient method of preservation and often relies on the production of antimicrobials from bacteria such as LAB to inhibit the growth of spoilage microbes and pathogenic strains. Such antimicrobials include bacteriocins, organic acids, hydrogen peroxide and ethanol; these can be particularly useful for industrial use due to their potent activity and low toxicity. As consumers move away from the use of synthetic preservatives and food treatments, bacteriocins and organic acids represent obvious natural alternatives for food preservation. Similarly in the medical field where antibiotic resistant superbugs are becoming an increasing concern, antimicrobials from LAB represent potentially novel treatments for infections where classical antibiotics may no longer be effective in the future. This chapter will outline the different antimicrobials produced by LAB and discuss their potential applications in the food processing and medical industries.

Introduction

Lactic acid bacteria (LAB) are a non-sporulating group of Gram-positive bacteria found in environments ranging from food to the GI tract. LAB have been utilised for millennia due to their ability to preserve food and are key to the production of many fermented foods such as yoghurt, cheese, sourdough bread, fermented vegetables such as sauerkraut and fermented meats. The metabolites produced by the LAB from the breakdown of the original substrate (i.e. milk, meat or vegetable carbohydrates and proteins) both may alter the properties of the product whilst potentially inhibiting the growth of competing spoilage microbes (Ross et al., 2002). A range of antimicrobial metabolites can be produced by LAB (Figure 1), primarily in the form of organic acids such as lactic acid, which acidifies the product thus inhibiting the growth of competing microbes (Peréz-Dıaz et al., 2013). Many LAB have also been shown to produce antimicrobial peptides known as bacteriocins which target and kill sensitive competing microbes .

The World Health Organization (WHO) estimated that 600 million food-borne illnesses occurred in 2010, leading to an estimated 420,000 deaths (World Health Organization (2015)). The implementation of systems to improve food safety is of high importance. Antimicrobials produced by LAB have been shown to inhibit a wide range of food spoilage bacteria and the addition of bacteriocins such as nisin to food can effectively reduce the levels of pathogens in a variety of products (O'Sullivan et al., 2002).

Another major issue is the escalating crisis of antimicrobial resistant pathogens, already a cause of an estimated 700,000 deaths per year, a figure predicted to rise to 10 million deaths per year by 2050 (O'Neill, 2014). Coupled with this is a paucity of new antibiotics to target such pathogens (World Health Organization, 2017). The antimicrobials produced by

LAB show potential for applications in the medical sector, with many shown to effectively inhibit multi-drug resistant strains (Okuda et al., 2013). Whilst much research has focused on the use of these antimicrobials in food, further work needs to be done to realise their potential in medicine (Cotter et al., 2013). This chapter reviews the range of antimicrobials produced by LAB, outlining their potential applications in industrial and medical settings.

Bacteriocins

Bacteriocins are heat stable antimicrobial peptides produced by bacterial cells and, unlike traditional antibiotics, are gene-encoded and ribosomally synthesised. The antimicrobial spectrum of these bacteriocins can vary, ranging from broad spectrum targeting a wide range of bacterial species to narrow spectrum inhibiting only closely related bacterial specie. LAB are recognised as prominent bacteriocin producers (Zacharof and Lovitt, 2012). Whilst a wide variety of bacteriocins exist, a common genetic architecture is found in many of the associated gene clusters. The structural gene in bacteriocin operons encodes the active peptide, this usually contains an N-terminal leader sequence with important functions during bacteriocin production. When translated within the cell the leader sequence reduces or abolishes the antimicrobial activity of the bacteriocin, preventing the cell from being killed by its own antimicrobial. In certain bacteriocins the leader can also be recognized by modification enzymes which can then direct the post translational modification of the active bacteriocin peptide. The leader is also crucial for bacteriocin secretion whereby dedicated ATP-binding cassette (ABC) bacteriocin transporters recognize the leader sequence and cleave it at a specific motif (i.e Gly-Gly) as it is being exported from the cell, thus releasing an active bacteriocin (Oman and van der Donk, 2010). Bacteriocins generally have their own secretion system encoded within the

operon, which can consist of single or multiple ABC transporters. These transporters also often include protease domains for cleavage of the bacteriocin signal sequence (Havarstein et al., 1995). Some bacteriocin have been shown to be secreted via the Sec translocase system (Herranz and Driessen, 2005). Bacteriocin operons also encode genes encoding immunity proteins which prevent the strains being killed by their own bacteriocins (Draper et al., 2012). Bacteriocin production can also be under tight regulation in producer cells due to the presence of a two component regulatory system within the operon which has an important role in quorum sensing (Rohde and Quadri, 2006, van der Ploeg, 2005). Certain bacteriocins also have key genes encoding modification and accessory proteins; however the presence of these depends on the class of bacteriocin.

LAB produce a wide variety of different bacteriocins which can be grouped into classes based on their structure, genetics and mode of action (Table 1). Many classification systems for bacteriocins have been proposed, but we will follow the system outlined by Cotter *et al.* as this has probably been the most used classification system in recent years (Cotter et al., 2013).

Class I

Class I bacteriocins undergo enzymatic post translational modification. Such modifications have important structural and functional roles for these bacteriocins. Class I bacteriocins can be further broken down into subclasses based on how the peptides are modified. For this chapter we will only discuss the classes which contain LAB derived bacteriocins.

Lantibiotics

The lantibiotics are the largest group of modified bacteriocins, and are produced by a range of LAB. These bacteriocins are termed lantibiotics due to the presence of lanthionine and methyllanthionine bridges within the peptides. Serine and threonine residues can be dehydrated to form 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb). Dha and Dhb can then react with the thiol group on cysteine resides within the peptide, forming internal thiother crosslinks known as lanthionine and methyllanthionine bridges, respectively (Willey and van der Donk, 2007). These dehydration and cyclisation reactions are catalysed by modification enzymes encoded within the bacteriocin operon. These modifications can be carried out by two separate enzymes (LanB and LanC) in type 1 lantibiotics, or a single enzyme (LanM) in type 2 lantibiotics (Marsh et al., 2010). Type 3 and 4 lantibiotics also exist but these are much less common.

Nisin is a type I lantibiotic produced by strains of *Lactococcus lactis* and represents one of the more well-known and commercialised bacteriocins. Nisin is a 34 amino acid lantibiotic, containing 5 (methyl)lanthionine ring structures along with three dehydrated residues (two Dha and one Dhb) (Hooven et al., 1996). Nisin interacts with lipid II in sensitive cells where the globular N-terminus binds to a pyrophosphate moiety in lipid II. Once bound

the linear C-terminal tail of nisin inserts into the bacterial membrane which results in the formation of a pore complex. This pore causes leakage of cytoplasmic material from the bacterial cell which leads to cell death (‘t Hart et al., 2015).

TOMMs

The thiazole/oxazole-modified microcins (TOMMs) are another member of the class I post-translationally modified bacteriocins. TOMMs all contain heterocycles derived from cysteine, serine, and threonine residues (Cox et al., 2015). Linear azol(in)e-containing peptides (LAPs) are a group of TOMMs produced by LAB. Streptolysin S from *Streptococcus pyogenes* is one such LAP however, unlike typical bacteriocins, it is a potent cytolytic toxin and virulence factor. Streptolysin modification is carried out by a complex of three key enzymes encoded within the operon. SagC is a cyclodehydratase which then removes hydrogens from these modified amino acids resulting in the formation of thiazoline, oxazoline and methyloxazoline rings. SagB is a dehydrogenase which coverts the thiazoline and (methyl)-oxazoline residues into thiazole, oxazole and methyloxazole heterocycles, respectively. SagC is a cyclodehydratase which then removes hydrogens from these modified amino acids resulting in the formation of thiazoline, oxazoline and methyloxazoline rings. A third protein, SagD, aids in the formation of the SagBCD complex (Lee et al., 2008). Despite a lack of characterised LAPs from other LAB, operons have been found in some *Lactobacillus*, *Lactococcus* and *Oenococcus* strains (Collins et al., 2017, Alvarez-Sieiro et al., 2016).

Thiopeptides are another family of TOMM bacteriocins, in addition to azoline and azole residues these can also contain Dha and Dhb residues due to the dehydration of Ser and Thr residues by LanB-like enzymes encoded within the thiopeptide operon (Zhang and Liu,

2013). In a recent *in silico* screen of the human microbiome metagenomic data a novel thiopeptide called 'lactocillin' was identified and characterised from a *Lb*. *gasseri* strain. This is active against a range of pathogens and represents a potential novel antimicrobial compound for the treatment of vaginal infections (Donia et al., 2014).

Glycocins

The glycocins are a family of post-translationally modified bacteriocins characterised by the glycosolation of amino acid residues on the peptide. The glycocins are a relatively small family of bacteriocins; however several have been isolated from LAB. Glycocin F is a 43 amino acid bacteriocin produced by *Lb. plantarum* KW30 (Norris and Patchett, 2016) and is one of the more studied of these bacteriocins. It is composed of two α-helices held together by two disulphide bonds followed by a C-terminal tail. The peptide contains two β-linked N-acetylglucosamine moieties attached to the oxygen and sulphur residues on Ser18 and Cys43 respectively, this S-glycosolation on the Cys residue is very rare (Venugopal et al., 2011, Brimble et al., 2015).

Class II

Class II bacteriocins differ from class I in the fact they do not undergo extensive post-translational modification. These bacteriocins are further classified based on their composition and structure.

Class IIa

The class IIa or 'pediocin-like' bacteriocins are single unmodified peptides with characteristic anti-listerial activity. These bacteriocins all contain a highly conserved N-terminal YGNGV motif often followed by a Cys residue involved in the formation of a

disulphide bridge, the peptides contain a less conserved hydrophobic C-terminus. An accessory protein encoded within the bacteriocin operon ensures correct disulphide bond formation and peptide folding (Oppegard et al., 2015). These peptides work by initially binding to the cell using the extracellular loop of the mannose phosphotransferase sugar uptake system as a receptor (Kjos et al., 2010). Once bound to the cell, the more hydrophobic C-terminus penetrates the cell membrane where it then forms a pore complex, thus causing leakage from the cell and eventual cell death (Papagianni and Anastasiadou, 2009).

Class IIb

The class IIb bacteriocins are two-peptide unmodified bacteriocins where both peptides are generally required in equal amounts for optimum antimicrobial activity. These bacteriocins are typically encoded by adjacent genes in the bacteriocin operon.(Nissen-Meyer et al., 2011). Despite being composed of two quite different peptides, these bacteriocins interact to act as a single antimicrobial entity. Most of these two-peptide bacteriocins contain GxxxG motifs which are involved in helix-helix interactions; this may allow the two peptides to interact and form a single functional unit (Fimland et al., 2008, Nissen-Meyer et al., 2010). These permeabilize the membrane of sensitive target cells to small molecules which results in cell death (Nissen-Meyer et al., 2010). Many of the class IIb bacteriocins which have been identified are produced by LAB, and appear to be the most prevalent type of bacteriocins encoded by lactobacilli (Collins et al., 2017)

Class IIc

Class IIc bacteriocins are circular peptides, linked by N-terminal to C-terminal covalent bonds (Cotter et al., 2005). The circularised structure aids in the pH and thermal stability of

these peptides and helps confer resistance to proteolytic enzymes. These bacteriocins act by inducing ion permeation in the membrane of sensitive cells resulting in a loss in membrane potential and cell death (Hemu et al., 2016, Gálvez et al., 1991). Many of the class IIc bacteriocins which have been discovered are produced by LAB. The mechanism of peptide circularisation is still unclear and the enzymes involved have yet to be identified.

Class IId

The class IId bacteriocins are a group of single peptide, non-pediocin like bacteriocins. They bear no homology to pediocin-like bacteriocins and are not modified, acting as a heterogeneous group of peptides which don't fit in other classes. Many diverse bacteriocins from LAB are found within this class. Bactofencin is one such bacteriocin, it is a small unmodified cationic bacteriocin produced by *Lb. salivarius* DPC6502 (O'Shea et al., 2013). One interesting subgroup of these bacteriocins is a group displaying homology to the bacteriocin lactococcin 972. Lactococcin 972 is bacteriocin produced by *Lc. lactis* subsp. *lactis* IPLA 972 and acts as a homodimer. This is a growing group of bacteriocins, several of which are encoded by LAB, with a unique mode of action and may warrant separate classification in the future (Collins et al., 2017, Letzel et al., 2014).

Bacteriolysins (Formerly Class III Bacteriocins)

Bacteriolysins are large heat sensitive antimicrobial proteins and due to their size and structure these proteins they are no longer considered bacteriocins. Several such peptides have been shown to be produced by LAB, such as enterolysin A (Nilsen et al., 2003), zoocin A (Heath et al., 2004), millericin B (Beukes et al., 2000) and helveticin J (Joerger and Klaenhammer, 1990). Bacteriolysins work by degrading the cell wall of sensitive cells. The N-terminus of these proteins contains a catalytic domain homologous to those of cell wall

degrading enzymes, the C-terminus then contains a potential cell wall binding domain (Nilsen et al., 2003, Heath et al., 2004).

Bacteriocin Applications

The antimicrobial activity of bacteriocins coupled with their low toxicity makes them attractive compounds for industry and the fact that they are naturally produced by GRAS organisms may also render them more acceptable to health conscious consumers. A limited number of bacteriocins are already in use in the food industry such as nisin (Younes et al., 2017) and carnocyclin A which is marketed as Micocin® (Liu et al., 2014). However, the potential exists to widen the scope of applications, particularly as antimicrobials in the medical field (Cotter et al., 2013). Indeed, research into bacteriocin applications is increasing: the granting of bacteriocin-related patents increased by 66% between 2010-2015 compared to the previous five years, covering a range of applications from biomedical research to nanotechnology (López-Cuellar et al., 2016).

Food Preservation and Safety

The use of bacteriocins in the food industry has primarily focused on food biopreservation and safety. Bacteriocins offer a natural alternative to the addition of chemical preservatives to food and have been shown to improve the flavour of certain fermented foods (Younes et al., 2017, O'Sullivan et al., 2002). Due to the peptide nature of bacteriocins they are generally digested in the gut upon ingestion, thus eliminating potential downstream effects on the microbiota as compared to traditional antibiotics (Umu et al., 2017).

Bacteriocins themselves are used in food manufacture in at least three ways (Figure 2), the first of which is the addition of partially purified bacteriocins to the food product (Chikindas et al., 2018). Nisaplin® (Danisco), a dried powder containing 1.82% nisin is one such product (Gough et al., 2017). Nisin was awarded generally regarded as safe (GRAS) status by the FDA in 1988 and has been approved by the World Health Organization as a food additive and was also assigned the E number E234 (Younes et al., 2017). Nisin is one of the most commonly used bacteriocin food preservatives, its broad spectrum of activity, heat stability and history of effectiveness making it an attractive option for the food industry. The addition of Nisaplin® to cottage cheese was shown to effectively control levels of *Listeria monocytogenes* (Ferreira and Lund, 1996). Nisaplin® was also shown to immediately reduce *L. monocytogenes* levels by 3 log CFU/g in queso fresco, a non-fermented cheese (Lourenço et al., 2017).

The second method by which bacteriocins may be added to food is as crude fermentates containing the active bacteriocins. One such example is ALTA® 2351 (Kerry Bioscience) a pediocin containing fermentate which has been shown to reduce *L. monocytogenes* numbers in raw sausage batter over 60 days (Knipe and Rust, 2009). Another popular example is microGARD™ (Danisco), a product of the fermentation of skimmed milk by LAB. This is extensively used in industry and has been shown to inhibit the spoilage of dairy products such as cottage cheese and yoghurt (Makhal et al., 2015, Salih et al., 1990).

The third approach is to use bacteriocin-producing strains as starter or protective cultures in fermented foods. One advantage of this is that *in situ* production of bacteriocins by starter cultures reduces the need for the addition of external preservatives. The use of bacteriocin producing cultures has been shown to be effective in the biopreservation of a

variety of foods such as fish (Gómez-Sala et al., 2016), meat (Diaz-Ruiz et al., 2012), vegetables (Settanni and Corsetti, 2008) and dairy products (Mills et al., 2017). One such example is the use of the nisin producer *Lc. lactis* subsp. *lactis* IFO12007 in the production of miso, a fermented soybean paste. The strain was used as a starter culture and was shown to inhibit the growth of *Bacillus subtilis*, even when this spoilage bacteria is inoculated at a concentration of 10^6 cells/g (Kato et al., 1999). The combination of a nisin and lacticin 3147 producing *Lc. lactis* strain with a plantaricin producing *Lb. plantarum* strain served as a protective culture in cheese production causing a reduction of *Listeria* numbers to 0.3 log CFU/g compared to 2.9 log CFU/g in the non-bacteriocin producing control (Mills et al., 2017). This study highlighted how bacteriocin-producing cultures can be used to stack bacteriocins in a food system thus creating multiple antimicrobial hurdles.

Bacteriocins are useful as part of a hurdle technology in combination with other methods for food preservation (Hsiao et al., 2016). The use of other compounds and treatments together with the addition of bacteriocins may also lead to synergistic antimicrobial activity. Organic acids for example can help increase the net charge of bacteriocins at low pH, thus aiding in bacteriocin translocation through the cell wall. This potential interaction is particularly useful in LAB which often produce these acids and bacteriocins concurrently (Mills et al., 2011). The role of organic acids as antimicrobials will be discussed later in this chapter. The use of outer membrane permeabilizing agents such as EDTA can also extend the range of activity of these bacteriocins to include Gram-negative bacteria such as *E. coli* O157:H7 in food (Ananou et al., 2005). The combination of lacticin 3147 and the lactoperoxidase system in powdered infant formula, for example, was shown

to inhibit the growth of pathogenic *Cronobacter* species up to 12 hours after rehydration of the milk formula (Oshima et al., 2012).

Packaging and materials

Another potential use for bacteriocins is their incorporation into packaging and other materials such as nanofibers. In cases where food comes into contact with the antimicrobial packaging, bacteriocins diffuse from the packaging thus inhibiting the growth of surface microorganisms. The antimicrobial packaging thus serves as an extra hurdle in food processing to improve safety, prevent food spoilage and extend product shelf life. The incorporation of bacteriocins into packaging can have advantages over direct bacteriocin addition to food through the reduction of non-specific binding of the bacteriocins to food components rather than the targeted bacterial strains, and by reducing the risk of bacteriocin degradation and inactivation in the food matrix (Laridi et al., 2003).

There are several ways in which bacteriocins can be incorporated into such materials. Bacteriocins can be simply coated or absorbed onto a polymer or incorporated directly into the polymer matrix for packaging (Deshmukh and Thorat, 2013). Numerous studies on a range of foods have shown the effectiveness of nisin incorporated into food packaging (Irkin and Esmer, 2015). For example, the use of packaging containing immobilised nisin was shown to reduce *Listeria innocua* and *Staphylococcus aureus* levels as well as those of other microbes in sliced cheese and ham in modified atmosphere packaging, thus improving shelf life (Scannell et al., 2000).

Bacteriocin producing strains as probiotics

Probiotics are defined as live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host (Pineiro and Stanton, 2007).Bacteriocin production can be considered an important probiotic trait due to the potential of such strains to inhibit pathogenic bacteria in the GI tract (Dobson et al., 2012). An example of this was demonstrated by Corr *et al.* who showed that the bacteriocin-producing *Lactobacillus salivarius* UCC118 strain displayed the ability to protect mice against infection with *L. monocytogenes* whilst a non-bacteriocin-producing mutant failed to show the same effect (Corr et al., 2007). Millette *et al.* also demonstrated that the nisin Z producing *Lc. lactis* MM19 and pediocin PA-1 producing *P. acidilactici* MM33 reduced vancomycin-resistant enterococci (VRE) in infected mice (Millette et al., 2008). Bacteriocins may also help strains establish themselves in a complex environment by helping them out-compete the resident microbiota in a particular niche (Dobson et al., 2012). The production of the bacteriocins blpMN by *Str. pneumoniae* was shown to aid the establishment of the strain in the mouse nasopharynx (Dawid et al., 2007). The administration of an *E. faecalis* strain harbouring the bacteriocin 21 encoding plasmid pPD1 was able to colonize and outcompete VRE lacking pPD-1in infected mice, thus indicating the role bacteriocin production may play in the establishment producing strains in the host (Kommineni et al., 2015). Bacteriocins may also act as useful signalling peptides between the bacteria themselves and also with the host. The production of plantaricins by *Lb. plantarum* WCFS1 for example was shown to be linked to a change in the levels of the cytokines interleukin 10 and 12 from peripheral blood mononuclear cells which may offer protection against colitis (van Hemert et al., 2010, Foligne et al., 2007). The benefits of bacteriocin production in probiotic LAB however is influenced by the producers ability to actively establish itself and produce bacteriocins in the host, a trait which is not always guaranteed. The pediocin PA-1 producing strain *P.*

acidilactici UL5 for example failed to establish itself in the mouse intestinal microbiota as the strain was not detectable in faecal samples two days after administration. This *Pediococcus* strain was originally isolated from fermented sausage and therefore may simply not have been well adapted to survive and establish in the GI tract of the host (Dabour et al., 2009).

Medical applications of bacteriocins

With the increasing prevalence of antibiotic resistant pathogens, bacteriocins represent a potential novel treatment for the control of these pathogens due to their potency and low toxicity. Much of the work which has been done in this field has involved the use of animal models, thus more clinical human research must be completed to determine the actual efficacy of bacteriocins in clinical applications. Nonetheless, the results of trials using model systems are promising. Intravenous injections of nisin, for example, was shown to be more effective than vancomycin treatment in *Str. pneumoniae* infected mouse models (Goldstein et al., 1998). Due to its inhibitory activity against *Helicobacter pylori* nisin can be used in the treatment of peptic ulcers and has been commercialized for treatments of gastric *Helicobacter* infections (Dicks et al., 2011). The stability and activity of nisin at low pH makes this a potentially useful antimicrobial for the treatment of this gut pathogen. The narrow spectrum of inhibition of pediocin-like bacteriocins could also be useful in the treatment of listeriosis which is extremely dangerous to pregnant women and immunocompromised individuals. Intra-gastric administration of pediocin PA-1 in mice was shown to reduce *Listeria* levels and slow pathogen translocation to other organs whilst having no effect on the intestinal microbiota (Dabour et al., 2009).

Bacteriocins can also be used in oral and respiratory health, for example a nisin-containing mouthwash was found to reduce gingivitis in beagle dogs (Howell et al., 1993). Nisin F was also shown to be effective against *S. aureus* infections in the respiratory tracts of immunosuppressed rats, a remedy which could be beneficial in the treatment of respiratory diseases in immunocompromised and cystic fibrosis patients (De Kwaadsteniet et al., 2009). Bacteriocins also have potentially useful roles in skin care by modulating the skin microbiome and inhibiting pathogens. The bacteriocins ESL5 produced by *E. faecalsi* SL-5 displayed antimicrobial activity against *Propionibacterium acnes*, a key factor in the pathogenesis of acne vulgaris. The incorporation of the concentrated bacteriocin into a topical lotion was shown to significantly reduce inflammatory lesions in treated patients in comparison to a placebo (Kang et al., 2009).

One of the issues associated with the use of bacteriocins in medical applications is that they can be degraded proteolytically in the body, thus reducing their effectiveness (Rink et al., 2010). Encapsulation of the bacteriocins and the use of drug delivery systems may circumvent this, allowing bacteriocins to be delivered directly to the area of interest in the host (Leserman et al., 1980). Encapsulation of bacteriocins may allow for their slow and extended release resulting in prolonged antimicrobial activity whilst also protecting the peptide from degradation in the body (Langer and Folkman, 1976).

A similar concept can be used in a clinical setting and the incorporation of bacteriocins into nanofibers and materials has numerous potential uses. Electrospinning is one such method whereby bacteriocins such as nisin can be incorporated into the core of nanofibres. The use of such nisin-eluting nanofibres in wound dressings has been shown to significantly reduce the bacterial load of *S. aureus*-induced skin infections of a wound in a murine model.

This represents a potentially useful medical barrier against the acquisition of skin infections (Heunis et al., 2013). The incorporation of these antimicrobials into dressings and fibres extends the potential use of bacteriocins in the medical field.

Veterinary applications of bacteriocins

Bacteriocins also display potentially useful benefits for animal care. One such application is the use of bacteriocins to treat mastitis in lactating animals. Mastitis is the inflammation of the mammary gland as a result of the infection with pathogenic microbes. A study by Cao *et al.* used an intramammary infusion of nisin to treat mastitis in dairy cows; the results for the nisin treatment had a clinical cure rate similar to that of gentamycin treatment. Nisin, however, is a food grade product with very few associated issues if it enters the food chain, as opposed to antibiotics such as gentamycin (Cao et al., 2007). A nisin based teat sealer was shown to reduce *S. aureus* and *E. coli* levels by 3.9 log and 4.22 log respectively after a one-minute exposure to the formula, a result comparable to conventional chemical treatments. The nisin formula however displayed a lower potential for skin irritation in comparison to the chemical treatments (Sears et al., 1992). A lacticin 3147 containing fermentate was used as a teat dip for mastitis prevention in dairy cows. Here teats were first coated with a pathogen before being treated with the teat dip for ten minutes, the lacticin 3147 containing fermentate was shown to reduce *Staphylococcus*, *Str. dysgalactiae* and *Str. uberis* levels by 80%, 97% and 90% respectively (Klostermann et al., 2010).

The antimicrobial activity of bacteriocins may also be useful for the treatment of GI tract infections in a range of animals. A bacteriocin OR-7 from *Lb. salivarius* was shown to reduce the colonization of chickens by *Campylobacter jejuni*. The purified bacteriocin was encapsulated and incorporated into chicken feed, chicks were then challenged with *Campylobacter jejuni* strains. Bacteriocin treatment was shown to greatly reduce pathogen colonization (Stern et al., 2006). An *in vitro* model of swine intestinal fermentations displayed the potential of pediocin A to inhibit the growth of pathogenic clostridia in the intestine, again representing an alternative to the use of traditional antibiotics in animal husbandry (Casadei et al., 2009).

Bacteriocins also represent an alternative to the addition of subclinical levels of antibiotics to animal feed as growth promoters. Dietary nisin was shown to increase feed conversion and body weight gain in broiler chickens. While the exact mechanism of action is unclear, nisin was shown to modulate the gut microbial ecology, thus the authors postulated that it may be a result of improved nutrient absorption and utilization or due to an improved immune response to pathogenic *Eimeria* parasites (Józefiak et al., 2013). Similarly the addition of pediocin A to the feed of chickens challenged with *Clostridium perfringens* improved their growth and feed conversion rates (Grilli et al., 2009).

Reutericyclin

Reutericyclin is an antimicrobial *N*-acylated tetrameric acid produced by a number of *Lb. reuteri* strains (Gänzle, 2004). Reutericyclin is produced by the combined activity of a non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) encoded within the *Lb. reuteri* genome. It is unique in the fact that is one of the very few functional NRPS/PKS systems described in LAB (Lin et al., 2015). Reutericyclin acts as a proton-ionophore and

dissipates the transmembrane ΔpH of sensitive cells by translocating protons across the cytoplasmic membrane (Gänzle, 2004). It is active against a range of Gram-positive bacteria including pathogens such as *Clostridium difficile* and MRSA (Cherian et al., 2014).

Reutericyclin producing *Lb. reuteri* strains have a number of potential uses in both food production and as potential probiotics due to the antimicrobial activity of the compound. Reutericyclin production in sourdough has been shown to help *Lb. reuteri* persist in the environment and remains active against sensitive strains in the dough (Gänzle and Vogel, 2003). This suggests that such producing strains may have potential as starter cultures for food preservation (Gänzle and Vogel, 2003). As a potential probiotic trait, a reutericyclin producing strain was found to subtly alter the fecal microbiota of weanling pigs, however no effect on clostridial toxins in host faeces was observed after treatment (Yang et al., 2015).

Antimicrobial metabolites

In certain cases, metabolic waste products and intermediates produced by LAB during fermentation can themselves display antimicrobial activity. These antimicrobial metabolites can play an important role in food preservation by limiting the growth of spoilage and pathogenic microbes (Ross et al., 2002). The composition and characteristics of these antimicrobials will be further discussed below.

Organic Acids

Organic acids are a by-product of LAB metabolism, however in several cases these have been shown to possess antimicrobial activity. The primary acids produced by LAB are lactic acid and acetic acid, however others such as formic acid can also be produced (Lindgren and Dobrogosz, 1990). Their antimicrobial activity may be primarily due to the lowering of the

internal pH of sensitive cells. In the uncharged form these acids are lipid permeable and thus can freely diffuse into the cell's cytoplasm (Hirshfield et al., 2003). Once in the cytoplasm they can dissociate causing an accumulation of anions, lowering the cells internal pH (pHi) (Salmond et al., 1984). This can affect numerous processes in the cells and can lead to internal enzyme denaturation. The increased anion concentration within the cell can also lead to an increase in the transportation of potassium ions into the cell (Roe et al., 2002). This influx of ions increases the turgor pressure within the cell, and in order to balance this, glutamate is then transported out of the cells which results in the disruption of the cells' osmolarity and thus inhibits cell growth (Warnecke and Gill, 2005). A drop in pH can also induce changes in the fatty acid composition of the cells membrane (Cotter and Hill, 2003). The inhibition of cells by weak acids is not however solely due to lowering the cells pHi, as different acids can have specific effects on cells; for instance the treatment of *E. coli* cells with formate or acetate leads to distinctive transcriptional responses between the two acids. Acetate was shown to induce the production of proteins found in the RpoS regulon which is an important controller of the bacterial stress response, while formate caused reduced steady state expression of these genes (Kirkpatrick et al., 2001). Acetic, lactic and citric acids were also shown to display different levels of antimicrobial activity against *L. monocytogenes* even when cells had identical pHi values (Young and Foegeding, 1993). Such differences may be explained by the distinctive anion pools within the cells after treatments with each acid, the particular lipophilicity of each acid may also affect cells differently as was shown in yeast cells (Hirshfield et al., 2003, Capozzi et al., 2009).

Lactic acid is the primary organic acid produced by LAB and, as with other organic acids, its activity is not solely due to the lowering of the cells pHi (Mols and Abee, 2011). Upon

exposure to lactic acid, *Bacillus cereus* displayed an altered expression of 196 genes which are not associated with the general response to acidic shock, again indicating a more specific mode of action for this molecule. Rather, it was shown to alter the metabolism of these cells by controlling the expression of genes involved in amino acid metabolism (Mols et al., 2010). In addition lactic acid may also induce oxidative stress within cells (Mols et al., 2010, Mols and Abee, 2011, Abbott et al., 2009).

Acetic acid was also found to have a large impact on gene expression in *Bacillus cereus*, altering the regulation of 1430 genes, affecting a variety of pathways involved in oligopeptide and amino acid transport and metabolism which is similar to the response seen for lactic acid. Acetic acid also altered carbohydrate transport and metabolism in cells, with genes involved in glucose, fructose, lichenan and trehalose transport and metabolism being down-regulated (Mols et al., 2010). As with lactic acid, acetic acid may also inhibit cells by causing oxidative stress (Mols et al., 2010, Mols and Abee, 2011)

Applications

Organic acids can be used in a variety of applications, and due to their broad spectrum of activity and their 'generally regarded as safe' (GRAS) status they are particularly suited to the food industry (Chai et al., 2016). Solutions containing organic acids are used in the meat processing industry in the US and Canada for carcass decontamination (Loretz et al., 2011). They may also be added to juices and beverages where they can serve as biopreservatives and as acidity regulators (Quitmann et al., 2013). The neutralization of these organic acids can produce salts which are useful due to their wide spectrum of activity against a range of pathogens and food spoilage microbes such as *E. coli* O157, MRSA and *Pseudomonas aeruginosa* (Lee et al., 2002, McWilliam Leitch and Stewart, 2002). The sodium salts of these

organic acids in particular can be used to improve the shelf life of products such as meat, poultry and fish and to control the growth of pathogens (Sallam, 2007, Maca et al., 1997). Levels up to 4.8% by weight of formulation of sodium and potassium lactate are permitted in food to inhibit microbes (Juneja and Thippareddi, 2004). These may also be useful if incorporated into food packaging whereby a controlled release may prolong a product's shelf life by inhibiting spoilage microorganisms (Wang et al., 2015). Often these salts may have benefits beyond their antimicrobial activity, sodium lactate for example can also act as a humectant and emulsifier (Brewer et al., 1991). Organic acids may also be highly useful in animal feed systems as reducing the microbial load of feedstuffs there is an increase in nutrients available to the host. The production of these acids may also reduce the production of ammonia by spoilage microbes and also reduce the pH of the digesta (Dibner and Buttin, 2002).

The combination of treatment with organic acids and other compounds can greatly enhance the antimicrobial activity of both and can lead to potential novel treatments for food preservation. The combination of lactic acid with the phenolic compound carvacrol has been shown to have synergistic antimicrobial activity against *Shigella sonnei* in infected lettuce leaves (Chai et al., 2016). The combination of organic acids and transition metals was also found to be highly synergistic with an up to a 1000 fold increase in antimicrobial activity whilst greatly also improving the effective range of activity against many pathogenic bacteria. Here the organic acids form complexes with these transition metals, increasing the permeability of the metals which leads to an increase in their intracellular concentration. The addition of organic acids to copper sprays currently used in plant and crop treatment could thus greatly increase their antimicrobial potency (Zhitnitsky et al., 2017). Synergistic

antimicrobial activity between different organic acids and UV-A radiation has also been reported even when used at sub-lethal levels (de Oliveira et al., 2017).

The production of these acids by LAB is in itself an important commercial trait for food fermentations. It is the production of lactic acid and other organic acids which helps in the preservation of fermented foods (Leroy and De Vuyst, 2004). While acids have important functional roles in the fermentation of many products (i.e. separation of curds and whey in cheese manufacture), these acids also reduce the growth of spoilage microbes by lowering the pH of the food to a prohibitive level. LAB are involved in the fermentation of a wide range of food, such as fermented meats (salami), fermented vegetables (kimchii, sauerkraut) and fermented dairy products (kefir, yoghurt). Thus, fermentation by LAB provides a cheap and cost effective method for food preservation whilst also often enhancing flavour and nutritional qualities (Ezeji and Ojimelukwe, 1993).

Reuterin

Reuterin is an antimicrobial compound which is an intermediate in the metabolism of glycerol in certain species. The name is derived from its most notable producer, *Lb. reuteri*, however several other *Lactobacillus* species have also been shown to produce this compound, as well as cells from other genera such as certain strains of *Klebsiella* (Martin et al., 2005, Sauvageot et al., 2000, Schütz and Radler, 1984, Slininger et al., 1983). Reuterin is composed of a mixture of 3-hydroxypropionaldehyde (3-HPA), its dimer and its hydrated form (Vollenweider and Lacroix, 2004). 3-HPA is an intermediate in the breakdown of glycerol to 1, 3-propanediol. Glycerol is first converted to 3-HPA by the adenosylcobalamin-dependent glycerol dehydratase (GDHt), 3-HPA can then be further broken down into 1, 3-propanediol by an NAD^+-dependant oxidoreductase (Liu and Yu, 2015). *Lb. reuteri* is a

particularly useful producer of reuterin due to its ability to tolerate larger concentrations of the compound compared to other producing species (Vollenweider and Lacroix, 2004). Reuterin inhibits sensitive cells by inducing oxidative stress as the reactive aldehyde in reuterin reacts with thiol groups of small molecules and proteins which can lead to their inactivation (Schaefer et al., 2010). Reuterin has a broad spectrum of activity, inhibiting a wide range of both Gram-positive and Gram-negative bacteria along with yeasts, moulds and protozoa (Cleusix et al., 2007).

Applications

Due to its broad spectrum of activity, reuterin may potentially be a useful antimicrobial. Reuterin treated mice infected with *Trypanosoma brucei brucei* displayed a 61% reduction in parasitemia levels and had an increased survival rate after a 7 day treatment (Yunmbam and Roberts, 1993). Due to its low potential toxicity in the body along with its inhibitory spectrum against food borne pathogens and spoilage bacteria, reuterin could also be used in combination with other treatments as a potential food preservative (Fernández-Cruz et al., 2016). When added to a Spanish curdled milk product reuterin alone displayed little antimicrobial activity against *L. monocytogenes* or *S. aureus*, however when used together with nisin and the lactoperoxidase system there was synergistic inhibition of these pathogens (Arqués et al., 2008). Reuterin-producing *Lb. reuteri* strains may also serve as potential probiotics. *Lb. reuteri* itself has been shown to survive gastric transit and has the ability to colonise the intestine (Vollenweider and Lacroix, 2004). Models of the colonic epithelium have shown that reuterin production improves the protection offered by *Lb. reuteri* against the adherence, invasion and intracellular survival of *Salmonella enterica* serovar Typhimurium in a model system (De Weirdt et al., 2012). The availability of

glycerol in the human intestine remains unclear, however, a *Lb. reuteri* strain was shown to produce reuterin in the gut of gnotobiotic mice following a cecal glycerol injection (Morita et al., 2008).

Hydrogen Peroxide

Several LAB have also been found to produce hydrogen peroxide in the presence of oxygen (Hertzberger et al., 2014, Schellenberg et al., 2012, Hütt et al., 2016). The exact mode of action for the antimicrobial activity of H_2O_2 is not completely understood; however it is most likely a combination of DNA damage, protein oxidation and membrane disruption of the target cell (Tamarit et al., 1998, Imlay et al., 1988). This can be due to the production of reactive hydroxyl radicals formed by Fenton's reaction (Linley et al., 2012). These hydroxyl radicals cause breaks in DNA due to their reaction with the methyl groups of thymine (Engevik and Versalovic, 2017, Di Mascio et al., 1989). The small molecular size of the molecule allows it to easily enter the cells where it can react with internal proteins and DNA, the activity of H_2O_2 can also be affected by whether the compound is in liquid or gaseous form (Finnegan et al., 2010). H_2O_2 is thought to be more effective against Gram-positive than Gram-negative bacteria and anaerobic strains are thought to be more sensitive to the compound as they lack the peroxidases and catalases encoded by aerobic bacteria which allows them to break down H_2O_2 (McDonnell and Russell, 1999). The activity of H_2O_2 can also be enhanced synergistically by acting together with the lactic acid produced by these bacteria (Atassi and Servin, 2010). Here, the membrane damage induced by lactic acid may make cells more susceptible and sensitive to the activity of H_2O_2 (Engevik and Versalovic, 2017).

Applications

The antimicrobial activity associated with H_2O_2 production makes it a potentially useful probiotic trait and its importance can be especially seen in the vaginal microbiota. H_2O_2 producing lactobacilli have been associated with protection against the acquisition of bacterial vaginosis and their absence is associated with a greater risk of acquiring HIV-1 infection (Hawes et al., 1996, Martin Jr et al., 1999). H_2O_2 production may also support colonization of the vagina by producing strains (Vallor et al., 2001). Whilst H_2O_2 production has been associated with colonisation, it has been shown that the levels produced by such strains may not be sufficient to inhibit the growth of vaginal pathogens and that lactic acid may be playing a greater antimicrobial role (O'Hanlon et al., 2010, O'Hanlon et al., 2011, Gong et al., 2014). Rather than acting as an antimicrobial H_2O_2 may have a more important immunomodulatory role in the vagina, lowering the levels of pro-inflammatory cytokines. This may explain the positive correlation between H_2O_2 producing lactobacilli and reduced bacterial vaginosis levels (Mitchell et al., 2015).

Ethanol

Ethanol is another antimicrobial product resulting from the metabolic reactions of certain LAB. Alcohols such as ethanol are commonly used as disinfectants due to their broad spectrum of activity, inhibiting bacterial cells, fungi and viruses. Ethanol is thought to damage cell membranes and denature proteins which disrupts crucial cell processes (McDonnell and Russell, 1999). Whilst ethanol at high concentrations displays potent antimicrobial activity, the levels produced by cells *in vivo* are unlikely to reach high enough concentrations to act as an effective antimicrobial (Sissons et al., 1996, Elshaghabee et al., 2016). Despite this, ethanol produced by LAB can display additive or synergistic effects when

combined with other antimicrobial such as lactic acid which is also produced by these strains (Oh and Marshall, 1993).

Diacetyl

Diacetyl is a metabolic product from LAB which has also been shown to display antimicrobial activity. Diacetyl is an alternative minor product of the metabolism of some of these strains and can be formed by the spontaneous oxidation of acetolactate, an intermediate in the conversion of pyruvate to acetoin (Gänzle, 2015, Cocaign-Bousquet et al., 1996). The formation of diacetyl by LAB can be beneficial for fermented foods and other products due to its butter-like aroma, diacetyl has also been found to display antimicrobial activity against a range of bacteria. Whilst its activity is not as strong as other antimicrobials (Olasupo et al., 2003), it can inhibit the growth of Gram-negative bacteria which are typically unaffected by bacteriocins from LAB such as pediocin PA-1 or nisin (Kang and Fung, 1999, Gao et al., 1999). Gram positive strains tend to be less sensitive to diacetyl activity (Lanciotti et al., 2003). The concentration of diacetyl which is normally found in fermented foods is much lower than that required for antimicrobial activity and is unlikely to play a large role in bacterial inhibition in the environment (Clark and Winter, 2015, Helander et al., 1997). Continued exposure to diacetyl in an industrial setting has also been associated with lung disease which makes the molecule less attractive as an additive (Clark and Winter, 2015, Harber et al., 2006)

Conclusion

Antimicrobials produced by LAB could potentially represent an untapped resource in regards to food processing and medical applications. With increasing concerns over food safety and the increase of multidrug resistant superbugs there is an urgent need for novel treatments. In this respect, bacteriocins, in particular, offer much potential. Many studies have focused on nisin, outlining its efficacy against important pathogens; it is also approved as a food additive and is extensively used in certain food processing industries. As we have seen, LAB produce a plethora of bacteriocins with potential to follow the same route as nisin for food safety and medicinal applications.

Another advantage of bacteriocins, over other antimicrobials, is that they can be easily altered through genetic manipulation owing to their gene-encoded nature (Field et al., 2015). Modification of the nisin peptide for example has already led to the identification of mutants with enhanced antimicrobial activity (Healy et al., 2013). This may allow scientists to overcome some of the deficiencies associated with bacteriocins such as their degradation and stability in food matrices and the body. The narrow spectrum of activity of certain bacteriocins also allows pathogens to be targeted more directly with less collateral damage to the rest of the microbiota (Rea et al., 2010).

Whilst production of purified antimicrobial compounds from LAB could prove to be economically unfeasible, LAB themselves serve as antimicrobial micro-factories, producing a range of antimicrobial compounds *in situ*. This feature of LAB is particularly suited to their role as starter cultures in food fermentations and as probiotics in the realms of host health.

To date, much of the research into bacteriocins and other antimicrobials from LAB has focused on the food industry but the antibiotic resistance crisis has placed an urgent need on the development of novel antimicrobials. Furthermore, our knowledge of the

importance of our microbiota clearly indicates that such therapies must impart minimal damage to the host's microbiota. In this regard, LAB antimicrobials offer a potential option in the development of narrow-spectrum antimicrobial compounds which impart their pathogen-inhibiting effects with minimal consequences for the mammalian host.

Future research should now focus on expanding the LAB antimicrobial repository beyond the handful which have made it into the commercial and medical realms with an emphasis on progressing these molecules towards intelligently-designed studies which provide evidence of their safety and efficacy in practical situations and towards clinical and field trials for their debut into the domain of evidence-based medicine.

Figures and Tables

Figure 1. LAB produce a variety of antimicrobials which can inhibit and kill sensitive microbes. Compounds such as lactic acid and diacetyl are metabolic waste products which can also act as antimicrobials. Bacteriocins are antimicrobial peptides which may be produced by LAB to target and inhibit competing bacteria.

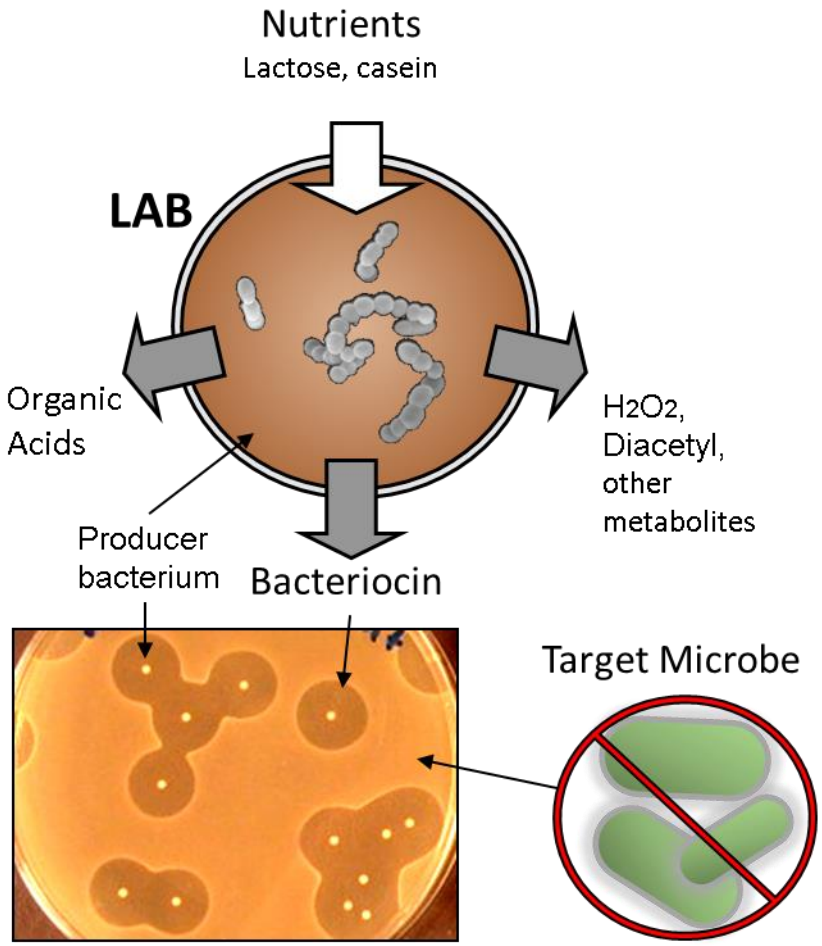

Figure 2. LAB can be utilised in food biopreservatoin in several ways. (a) LAB can be used as starter cultures in the production of fermented foods, whereby the *in situ* production of bacteriocins and other antimicrobials inhibits the growth of spoilage microbes. (b) Bacteriocins from LAB cultures can also be concentrated into purified and semi-purified food additives for use as preservatives in the food processing industry.

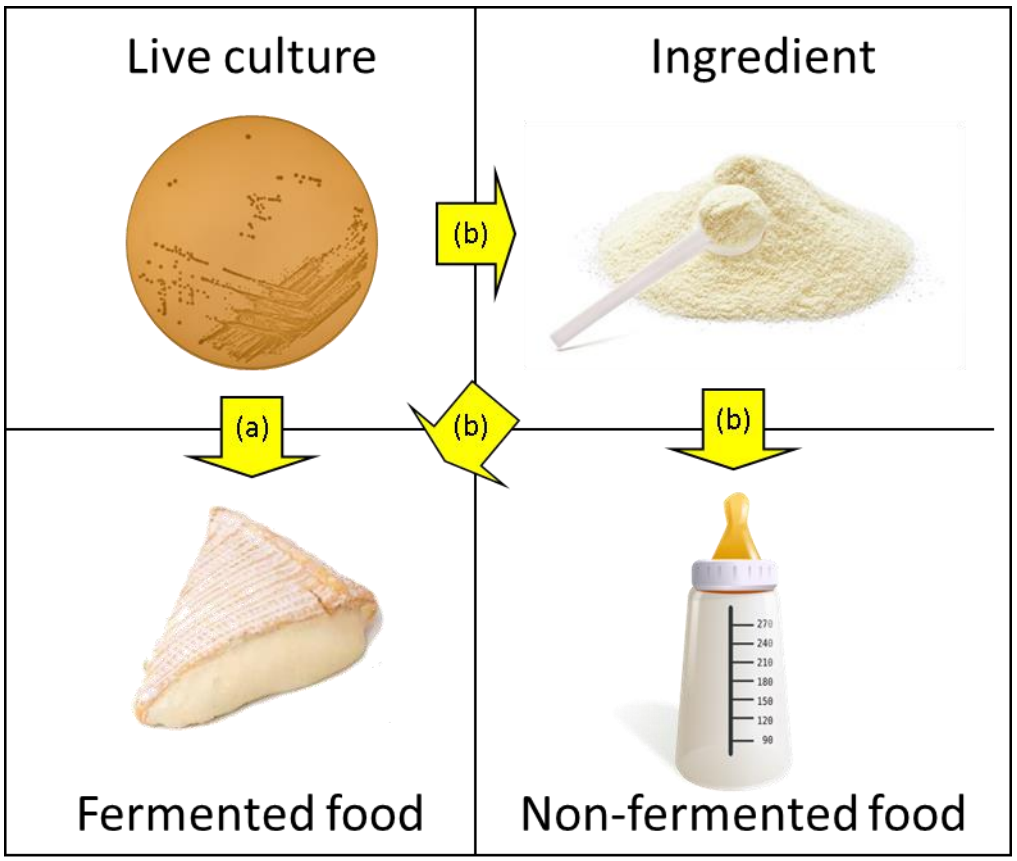

Table 1. Notable bacteriocins produced by LAB

Class I	Producer	Notable Targets	Reference
Lantibiotics			
Nisin	*Lc. lactis* subsp. *lactis*	*L. monocytogenes*, *S. aureus, Str. pyogenes*	(Rogers and Whittier, 1928)
Mutacin B-Ny266	*Str. mutans* Ny266	*Enterococcus* sp., *Staphylococcus* sp., *Propioniibacterium acnes*	(Mota-Meira et al., 1997)
Lacticin 3147	*Lactococcus lactis* DPC3147	*L. monocytogenes*, *E. faecalis, S. aureus*	(Ryan et al., 1996)
TOMM			
Streptolysin	*Str. pyogenes*	Erythrocytes, leukocytes, platelets	(Todd, 1938)
Lactocillin	*Lb. gasseri* JV-V03	*Gardnerella vaginalis, Corynebacterium aurimucosum, E. faecalis*	(Donia et al., 2014)
Glycocins			
Glycocin F	*Lb. plantarum* KW30	*Enterococcus* sp., *Streptococcus* sp., *Bacillus* sp.	(Kelly et al., 1996)
Class II			
Class IIa			
Pediocin PA-1	*P. acidilactici* PAC-1.0	*L. monocytogenes, Lactobacillus* sp., *Clostridium tyrobutyricum*	(Henderson et al., 1992)
Enterocin A	*E. faecium* CTC492	*L. monocytogenes, E. faecalis, Cl.tyrobutyricum*	(Aymerich et al., 1996)
Class IIb			
ABP-118	*Lb. salivarius* subsp. *salivarius* UCC118	*Bacillus coagulans, L. monocytogenes, L. innocua*	(Flynn et al., 2002)
Plantaricin S	*Lb. plantarum* LPCO10	*Propionibacterium* sp., *Cl. tyrobutyricum, E. faecalis*	(Jimenez-Diaz et al., 1993)
Class IIc			
Carnocyclin A	*Carnobacterium maltaromaticum* UAL307	*S. aureus, L. monocytogenes, E. faecalis*	(Martin-Visscher et al., 2008)
Acidocin B	*Lb. acidophilus* M46	*L. monocytogenes, Cl. sporogenes, Brochothrix thermosphacta*	(Leer et al., 1995)
Class IId			
Bactofencin A	*Lb. salivarius* DPC6502	*S. aureus, L. monocytogenes*	(O'Shea et al., 2013)
Lactococcin 972	*Lactococcus lactis* subsp. *lactis* IPLA 972	*Lactococcus* sp., *Lb sake*	(Martinez et al., 1996)

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Chapter 2: Formicin – A Novel Broad Spectrum Two-Component Lantibiotic Produced by *Bacillus paralicheniformis* APC 1576

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Abstract

Bacteriocins represent a rather underutilised class of antimicrobials, despite often displaying activity against many drug resistant pathogens. Lantibiotics are a post-translationally modified class of bacteriocins, characterised by the presence of lanthionine and methyllanthionine bridges. In this study, a novel two-component lantibiotic was isolated and characterised. Formicin was isolated from *Bacillus paralicheniformis* APC 1576, an antimicrobial producing strain originally isolated from the intestine of a mackerel. Genome sequencing allowed for the detection of the formicin operon, and from this the formicin structural genes were identified, along with those involved in lantibiotic modification, transport and immunity. The identified bacteriocin was subsequently purified from the bacterial supernatant. Despite the degree of conservation seen amongst the entire class of two-component lantibiotics, the formicin peptides are unique in many respects. The formicin α peptide is far less hydrophobic than any of the equivalent lantibiotics, and with a charge of plus two it is one of the most positively charged α peptides. The β peptide is unique in that it is the only such peptide with a negative charge due to the presence of an aspartic acid residue in the C-terminus, possibly indicating a slight variation to the mode of action of the bacteriocin. Formicin also displays a broad spectrum of inhibition against Gram positive strains, inhibiting many clinically relevant pathogens such as *Staphylococcus aureus, Clostridium difficile* and *Listeria monocytogenes*. The range of inhibition displayed against many important pathogens indicates a potential therapeutic use against such strains where antibiotic resistance is such a growing concern.

Introduction

With the increased prevalence of many drug resistant bacterial strains, the development of new antimicrobials is becoming a growing necessity. One such class of antimicrobials which appear to be underrepresented in clinical applications are bacteriocins (Cotter et al., 2013). Unlike traditional antibiotics, bacteriocins are gene encoded and ribosomally-synthesised peptides. This makes them suitable for genetic manipulation, with the potential for novel and specialised drug design (Gillor et al., 2005). The spectrum of inhibition of bacteriocins can range from broad to narrow spectrum, the latter of which may allow for highly targeted antibacterial therapies which may reduce the collateral damage associated with the use of broad spectrum antibiotics (Rea et al., 2011).

The lantibiotics (*lan*thionine containing *antibiotics*) comprise a well-studied class of bacteriocins, the most notable of which is nisin (Rogers, 1928) which is commonly used as a food preservative. Lantibiotics are classified based on the presence of lanthionine or methyllanthionine bridges. In these peptides, serine and threonine residues are post-translationally modified and dehydrated to form 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) residues. The thiol group of a cysteine residue subsequently reacts with the Dha or Dhb residues resulting in the formation of lanthionine or methyllanthionine thioether cross-links (Xie and van der Donk, 2004).

The lantibiotic gene cluster encodes an array of genes required for the modification, regulation and transport of the bacteriocin. Lantibiotics are divided into classes depending on the mechanism by which they are synthesised. Class I lantibiotics encode the enzymes LanB and LanC within the bacteriocin operon, where LanB catalyses the dehydration of the serine and threonine residues, whilst LanC catalyses the cyclization of the lanthionine rings. In Class II lantibiotics, LanM alone catalyses both dehydration and cyclization of the lantibiotics (Willey and van der Donk, 2007). LanR and LanK play key roles in the regulation of lantibiotic production (Lee et al., 2011). Once the mature lantibiotic is produced, its cleavage and transport is carried out by LanP and LanT

respectively (Escano et al., 2015). In some cases, LanT can carry out both leader sequence cleavage and peptide secretion functions (Furgerson Ihnken et al., 2008). Immunity to lantibiotics can be afforded by immunity proteins such as the lipoprotein LanI which likely binds the secreted lantibiotic, and the ABC transporter LanFEG which transports bacteriocin peptides from the membrane to the extracellular medium. Here LanF binds and hydrolyses ATP which provides the energy required for the transport of the bacteriocin through the LanEG membrane complex (Stein et al., 2005, Takala et al., 2004, Alkhatib et al., 2012). For a review on this class of bacteriocins see (Willey and van der Donk, 2007).

Within the lantibiotic class of bacteriocins exists a small subgroup of two-component lantibiotics. Such bacteriocins are produced by an array of genera, including *Staphylococcus* and *Lactobacillus* (Navaratna et al., 1998, Holo et al., 2001). Interestingly, of the few two-component lantibiotics which have been described, two of these bacteriocins identified prior to this study are produced by *Bacillus* species. Bacilli species are known to produce a vast range of antimicrobials, whether antibiotics (e.g. gramicidin, bacitracin) or bacteriocins (e.g. thuricin CD, mersacidin) (Katz and Demain, 1977, Rea et al., 2010, Chatterjee et al., 1992). The currently identified two-component lantibiotics include lacticin 3147 (*Lactococcus lactis*) (Ryan et al., 1996), lichenicidin (*Bacillus licheniformis*) (Begley et al., 2009, Dischinger et al., 2009), haloduracin (*Bacillus halodurans*) (McClerren et al., 2006), enterocin W (*Enterococcus faecalis*) (Sawa et al., 2012), plantaricin W (*Lactobacillus plantarum*) (Holo et al., 2001), BHT (*Streptococcus rattus*) (Hyink et al., 2005), Smb (*Streptococcus mutans*) (Yonezawa and Kuramitsu, 2005) and staphylococcin C55 (*Staphylococcus aureus*) (Navaratna et al., 1998). In this subclass of bacteriocins, the two peptides produced tend to act synergistically and usually display negligible antimicrobial activity on their own.

The mode of action of lacticin 3147 identifies a likely model for the mode of action of similarly structured lantibiotics. The α peptide of lacticin 3147 (Ltnα) resembles the globular lantibiotic mersacidin, mirroring its activity by binding to lipid II which acts as an important docking molecule. Binding to lipid II results in a conformational change of Ltnα, which presents a site to

which the β peptide (Ltnβ) can then bind. Ltnβ resembles an elongated lantibiotic, which, once recruited by Ltnα, inserts itself into the target membrane, inducing pore formation, which results in cell death. Here the cooperative activity of both peptides is necessary for optimal antimicrobial activity as the stability of the total bacteriocin-lipid II complex is important for both pore formation and the inhibition of cell wall biosynthesis (Martin et al., 2004, Wiedemann et al., 2006).

In this study we extend the class of two-component lantibiotics by identifying a novel bacteriocin known as formicin which is produced by a marine isolate, *Bacillus paralicheniformis* APC 1576. Whilst this lantibiotic resembles the previously described two-component lantibiotics, it contains a number of features which differentiate it from the rest of the class.

Results

Isolation of *B. paralicheniformis* APC 1576

B. paralicheniformis APC 1576 was isolated from the intestinal microbiota of a freshly caught mackerel. In an initial screen for bacteriocin production the strain was found to inhibit *L. delbrueckii* subsp. *bulgaricus* LMG 6901 in an overlay assay (Fig. 1(a)). In addition, cell free supernatants (CFS) also inhibited *L. delbrueckii* subsp. *bulgaricus* LMG 6901 in a well diffusion assay indicating that the antimicrobial substance was secreted by the cells into the media (Fig. 1(b)). Colony MS was used to determine the molecular masses of the peptides produced by the cell, however, the detected peptide masses (Fig. 1(c)) failed to match any previously characterised bacteriocin, including lichenicidin, a bacteriocin produced by *B. licheniformis* (Begley et al., 2009). Moreover, more than one source of antimicrobial activity was found following purification of the antimicrobial peptides. MALDI-TOF MS identified a molecule with a mass of 1422.54 Da, which displayed activity against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 once purified; this mass correlates closely with that of bacitracin which is encoded on the genome. The production of more than one antimicrobial from *Bacillus* species is not unexpected. Therefore, in order to identify all potential antimicrobials with activity against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 the genome of *B. paralicheniformis* APC 1576 was sequenced.

Identification of a Novel Two-Component Lantibiotic Operon

Once the draft genome was obtained, the sequence was analysed with BAGEL3 and antiSMASH to identify the antimicrobials encoded. Gene clusters encoding the antibiotics bacitracin, surfactin and fengycin were found within the genome. The strain likely produces at least one of these antimicrobials, as antifungal activity was also observed against *Aspergillus niger* in overlay assays (data not shown).

A novel lantibiotic operon was also identified within the genome of the strain (Fig. 2). This operon spans approximately 17 kb and was located on a single contig of the draft bacterial genome.

Two putative lantibiotic-encoding structural genes were identified on this operon. ORF1 (*frcA1*) encodes a 66 amino acid peptide and ORF3 (*frcA2*) encodes a 71 amino acid peptide. Analysis of the prepropeptides (including the bacteriocin leader sequence) of these lantibiotics shows the formicin A1 prepropeptide displayed 47.8% amino acid identity to the unmodified haloduracin A1 equivalent and 35.9% identity to the lantibiotic mersacidin. As the putative bacteriocin appears to be a two-component bacteriocin, two lantibiotic modification enzymes should be present. The order of the genes in the operon would suggest that ORF2 (*frcM1*) is the modification enzyme associated with *frcA1*. Upon analysis, this ORF displayed 38.7% identity to the haloduracin HalM1 modification enzyme. The second lantibiotic gene, ORF3 (*frcA2*), appears to resemble the elongated β peptides of the other two-component lantibiotics which are involved in membrane insertion (Wiedemann et al., 2006). Upon analysis, formicin A2 revealed 42.4% identity with the unmodified lichenicidin LchA2 prepropeptide. ORF4 (*frcM2*) encodes the modification enzyme which follows this structural peptide, and displayed 33.6% identity to the lichenicidin LchM2 modification enzyme.

ORF5 located downstream of LchM2 is predicted to encode a lantibiotic transporter, displaying 52.5% identity to the haloduracin transporter, HalT. In addition to its function in bacteriocin transport, a sequence encoding a C39 peptidase domain (cd02425) can also be found within the gene, this is likely involved in the cleavage of the leader sequence from the prebacteriocin. BLAST analysis of ORF6 identified the gene as encoding a hypothetical protein; the sequence, however, did show 28.4% identity to LanY encoded within the lichenicidin operon (Begley et al., 2009). ORFs 7, 8 and 9 all encode ABC transporter related peptides, as do ORFs 11, 12 and 13. These are likely to be involved in bacteriocin immunity. ORF7 and ORF11 both encode domains resembling that of the ABC-binding cassette domain of the bacitracin-resistance transporter (cd03268) and displayed 44.5% identity to each other. Instead of the common Q-loop motif found in the nucleotide binding domains of such transporters, both these proteins instead encode an E-loop motif which is indicative of lantibiotic immunity proteins (Okuda et al., 2010, Alkhatib et al., 2012). Each of the other components encode ABC-2 type transporter domains (cl21474). The presence of

these gene clusters may suggest a dual mechanism of bacteriocin immunity. Immunity to the lichenicidin bacteriocin is thought to follow a similar mechanism, with two transporters being encoded, with one showing homology to the bacitracin transporter (Dischinger et al., 2009). Such mechanisms, however, do not confer a general immunity against all two-component lantibiotics, as both the producers of lichenicidin (*B.* licheniformis ATCC 14580) and lacticin 3147 (*Lactococcus lactis* subsp. *lactis* DPC 3147) displayed sensitivity to formicin (Table 1).

ORF10 (*frcR*), which splits the transporter clusters, encodes a LanR equivalent transcriptional regulator. This gene encodes helix-turn-helix XRE-family domains, crucial for binding DNA and regulating gene expression. This LanR type protein displayed 49.4% and 60.3% identity to the regulators found within the lichenicidin and haloduracin operons, respectively. ORF14 (*frcP*) encodes a lanthionine specific protease displaying 29.8% identity to LicP found in the lichenicidin operon. As in lichenicidin, the LanT-like ORF (*frcT*) likely cleaves the N-terminal glycine leader sequence from both propeptides upon transport, whilst the LanP-like protease (*frcP*) possibly cleaves the six newly exposed N-terminal amino acids from the β peptide to generate the mature bacteriocin (Tang et al., 2015). The final ORF found in the gene cluster encodes a DNA damage inducible protein.

Bacteriocin Structure Prediction and Analysis

The spectrum of activity and characteristics of the bacteriocin could not be determined from the crude bacteriocin supernatant alone due to the interference from the other antimicrobials produced by the strain. Thus, to determine the activity of formicin, it was necessary purify the bacteriocin from the cell free supernatant. Using the predicted masses of the lantibiotic structural peptides identified from genomic data it was possible to determine if the formicin peptides were present in active HPLC-derived fractions using MALDI-TOF MS.

From the purified peptides, masses of 3254.34 Da and 2472.06 Da were detected for the α and β peptides, respectively. The predicted mass of the Frcα peptide based on the amino acid sequence from the genome is 3310.80 Da; the difference between the predicted and observed

masses correlates with the loss of three water residues which is most likely associated with the formation of lanthionine and methyllanthionine bridges, and also the possible formation of one disulphide bond, resulting in a predicted mass of 3254.80 Da. Due to the similarities between the two, the structure of Halα was used as a basis for the prediction of the structure of Frcα. Based on the Halα template the formation of a lanthionine bridge may occur between Ser-7 and Cys-17, while methyllanthionine bridges could form between Thr-18 and Cys-23, and Thr-20 and Cys-27, whilst Ser-26 remains unaltered. In addition, a disulphide bridge is also likely to form between Cys-1 and Cys-8 (Fig. 3).

The second mass determined by MALDI-TOF MS relates to the β peptide of the bacteriocin. Due to the presence of the extra LanP serine protease encoded in the bacteriocin operon and the similarity formicin displays to haloduracin and lichenicidin it is likely that the first six amino acids following the lantibiotic leader sequence are also cleaved from the formicin peptide. Once these amino acids are discounted the predicted mass of the peptide is 2614.95 Da, a difference of 142.89 Da from the mass detected by MALDI-TOF MS. This mass difference corresponds closely with the loss of 144 Da which would be associated with 8 dehydration reactions. Using the β peptides of lichenicidin and lacticin 3147 as templates, we predicted that the peptide is most likely to form bridges between Thr-1 and Cys-8, Thr-13 and Cys-17, Ser-19 and Cys-22, and Thr-23 and Cys-26. This would result in Thr-2, Ser-4, Ser-5 and Thr-10 being dehydrated to their respective Dha and Dhb residues, whilst Ser-24 remains unaltered (Fig. 3).

The purified peptides were screened against a range of indicator organisms to determine the spectrum of inhibition (Table 1). Purified formicin inhibited 29 of the 35 indicator strains screened, exhibiting a broad spectrum of activity against a range of bacterial genera including lactobacilli and enterococci, and notable pathogens such as *Staphylococcus aureus, Listeria monocytogenes, Clostridium difficile* and *Bacillus subtilis*. The Frcα peptide alone at a concentration of 50 μM also displayed antimicrobial activity against a number of indicators, whilst Frcβ alone displayed no detectable antimicrobial activity (Fig. 1d) .

In terms of thermostability, the bacteriocin retained a high degree of activity after treatment at 100˚C for thirty minutes, displaying a reduction in the size of the zone of inhibition of approximately 28%. Activity was, however, lost after treatment for fifteen minutes at 121˚C. The bacteriocin was also found to be susceptible to digestion by α-chymotrypsin and proteinase K, indicating its proteinaceous nature.

Homology between Bacteriocins

The previously described two-component lantibiotics all display a degree of homology with certain conserved residues found throughout. As a result, sequence comparisons of these structural peptides were carried out with formicin to determine if this conservation extended to the new bacteriocin (Fig. 4). The results indicate that formicin complies with the conservation that is seen amongst the other bacteriocins. The mersacidin like α peptides display the greatest levels of conservation, this reflects the shared mode of action in specifically binding to lipid II. This homology, especially in the lanthionine and methyllanthionine bridge forming regions, confers a structural similarity in each of the peptides. The broader role of the β peptides in membrane insertion is reflected in a greater degree of divergence in the composition of these peptides. The regions of conservation which are seen amongst the β peptides extend to Frcβ also, with the C-terminus of the peptides showing a relatively conserved pattern of lanthionine and methyllanthionine bridge formation. The N-terminus of the β peptides display a much lower degree of conservation, despite this, these N-terminus regions are rich in hydrophobic amino acids which likely play an important role in membrane insertion and pore formation.

Discussion

Formicin represents a novel member of the class of two-component lantibiotics. This class of bacteriocins are themselves unusual given the lipid II binding and pore forming activities of the bacteriocin are performed by two separate peptides, whilst certain lantibiotics such as nisin and subtilin have the ability to carry out both functions on a single peptide. It is unclear as to whether these two-component lantibiotics have evolved due to a divergence of a nisin like lantibiotic into two separate genes after a duplication event or whether they have come about due to the convergence of a mersacidin like lipid II binding lantibiotic and a pore forming lantibiotic. If the latter is the case, it is interesting as to how such different peptides would have evolved to depend on each other for antibacterial activity, and in some cases lose the activity each would have shown on its own.

Sequencing of *B. paralicheniformis* APC 1576, allowed for the elucidation of the formicin bacteriocin operon (Fig. 2). Analysis of the bacteriocin operon identified two lantibiotic structural genes (*frc*A1 and *frc*A2) and two modification enzymes (*frc*M1 and *frc*M2) which convert the formicin structural peptides to the mature lantibiotics. Transport and leader cleavage is likely to be carried out by *frc*T, whilst *frc*P may act as a further protease, cleaving 6 N-terminal amino acids from Frcβ. ORFs 7, 8, 9 and 11, 12, 13 all predict to encode ABC transporters which are likely to comprise the strain's immunity mechanism, protecting itself from attack by its own bacteriocin.

Comparative analysis of the bacteriocin structural genes allow for the homology between bacteriocins to be determined (Fig. 4). In the case of both Frcα and Frcβ, the closest homologs are the haloduracin α and β mature peptides, displaying 71% and 39% identity respectively. Such homology reflects the close relationship of the two producers as both belong to the Bacillus genus. The differences between the formicin and lichenicidin peptides are surprisingly large, given that both are produced from related species, with the α peptides displaying 46% identity and the β peptides 36%. This would suggest that both strains may have acquired these operons independently. The

layout of the formicin operon itself differs from that of the previously characterised two-component lantibiotics, transcription of the formicin operon would appear to be unidirectional whereby the genes for the structural peptides are separated by those encoding the LanM modification enzymes, an arrangement which seems to be unique to formicin. Both the haloduracin and lichenicidin structural genes (Figure 2) would likely be transcribed in opposite directions, possibly indicating that gene inversion may have taken place. Such differences again display the evolutionary divergence seen between this class of bacteriocins.

Analysis of the primary structure of these peptides indicates that some key differences exist between the formicin peptides and other members of the class, despite such strong regions of homology found throughout. The α peptide of formicin, for example, contains only five hydrophobic amino acids, whilst others in the class contain an average of nine. Whilst hydrophobic residues are crucial for membrane activity in certain bacteriocins, it has been suggested that it is the charged residues of these lantibiotics which control binding to lipid II as opposed to hydrophobic interactions. This indicates that binding of formicin to lipid II is not compromised despite its lower hydrophobicity, a fact which is supported by the activity of the α peptide independent of the β peptide (Hsu et al., 2003, Fimland et al., 2006). As with the α peptides from enterocin W and plantaricin W, the α peptide of formicin contains six charged amino acids, with an overall positive charge of plus two, rendering them amongst the most highly charged in the class. These charged residues not only affect the structure of the peptide but the higher positive charge may lead to an increased affinity for the anionic bacterial membrane. The formicin β peptide differs most when compared to other lantibiotic β peptides with regards to charge. As is common in this class, the N-terminal tails of the β peptides are composed largely of hydrophobic residues, crucial for membrane insertion and pore formation. Whilst the previously described β peptides all contain a positively charged C-terminus, containing Lys and Arg residues, formicin is unique in that it encodes a negatively charged β peptide. The lone charged residue found in the peptide is the penultimate C-terminal Asp residue. This portion of the peptide is believed to be involved in the interaction

between the α and β peptides (Wiedemann et al., 2006), thus this negative residue may suggest an increased affinity for the positively charged α peptide, possibly representing a stronger complex compared to previously described pairs.

The tertiary structure of these peptides has an important functional role in the antimicrobial activity of these lantibiotics. Analysis of the N-terminus of Frcα suggests the formation of a disulphide bridge between Cys-1 and Cys-8. Whilst this has been shown to be inessential for antimicrobial activity, it may reduce the degradation of the peptide once secreted (Cooper et al., 2008). Of the lantibiotic rings believed to be formed in Frcα, only the C ring is thought to be essential, with alterations abolishing all activity completely in both haloduracin and lacticin 3147 (Cooper et al., 2008, Cotter et al., 2006). The B ring found in these α peptides, has been shown to be unnecessary, which is unusual given the high degree of conservation amongst such bacteriocins, including mersacidin. Disruption of the A ring in haloduracin has been shown to reduce, but not eliminate activity, thus showing this region is important but not essential for the antibacterial activity of the bacteriocin (Cooper et al., 2008). As per analysis of the haloduracin β peptide, the A ring of the peptide has been found to be dispensable, whilst loss of the C and D rings led to a reduction in activity but not total elimination. Disruption of the B ring could not be achieved without disruption of the other ring structures (Cooper et al., 2008).

Conclusion

In this study, formicin, a novel member of the class of two-component lantibiotics has been identified. Key regions of homology, primarily those involved in lanthionine and methyllanthionine bridge formation, seen throughout this class have been shown to be extended to formicin. Such homology is expected to confer a similar mode of action to all lantibiotics in this class, with the α peptide of the bacteriocin binding to lipid II and subsequently recruiting the β peptide for membrane insertion and pore formation. Whilst formicin likely conforms to such mechanisms, there are certain key variations which differentiate it from the rest of the class. The reduction of hydrophobicity of Frcα, and the unusual negative charge of Frcβ make formicin a unique member of the two-component lantibiotics. Further studies are required to determine the effects of such changes on the activity of the bacteriocins, as it is recognised that charge and hydrophobicity play a central role in the activity of these lantibiotics, and in bacteriocins in general. Formicin itself displays a broad range of inhibition, inhibiting several clinically relevant Gram-positive pathogens, such as *C. difficile*, *S. aureus* and *L. monocytogenes*. With the continued progression of antibiotic resistance in pathogenic bacteria, the discovery of novel therapies against such agents is a priority and since the bacteriocin is produced by a species long associated with biotechnology applications, a straightforward route towards large scale processing of the readily purified peptides is anticipated. Thus formicin represents a potential novel antimicrobial therapy against a range of pathogenic bacteria.

Material and Methods

Isolation of Bacteria from Fish Samples

Marine fish were caught off the coast of Ireland and stored on ice prior to analysis. The intestinal contents of the fish and a sample of the skin and gills were aseptically removed. Samples were suspended in maximum recovery diluent (MRD) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), serial dilutions were then plated on brain heart infusion (BHI) agar (Merck, Darmstadt, Germany), and marine media 2216 (Difco Laboratories, Detroit, MI) and incubated aerobically at 30˚C for three days. Colonies were isolated from these plates and analysed for antimicrobial activity using deferred antagonism assays, whereby spots of the bacterial cultures were overlaid with 10 ml de Man, Rogosa and Sharpe (MRS) agar (Difco Laboratories, Detroit, MI) seeded with 25 µl of a *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 overnight culture. Colonies which displayed significant zones of inhibition were further characterised.

In this study, the strain of interest, *B. paralicheniformis* APC 1576, was isolated from the intestinal tract of a mackerel (*Scomber scombrus*) and grown on BHI aerobically at 37˚C. The strain was identified by 16S rRNA sequencing using the UniF (5’ -AGAGTTTGATCCTGGCTCAGG- 3’) and UniR (5’ -ACGGCAACCTTGTTACGAGT- 3’) primers to amplify the sequence. PCR products were cleaned using an illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK) and subsequent sequencing was completed by Cogenics (Essex, UK).

Colony Mass Spectrometry

Colony matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Axima TOF^2 MALDI-TOF mass spectrometer, Shimadzu Biotech, Manchester, UK) was used to determine the molecular mass of the peptides produced as follows: cells were first mixed in a 70% 2-propanol 0.1% TFA (IPA) and vortexed, the sample was separated by centrifugation and the supernatant was subsequently used for analysis. A MALDI target plate was precoated with CHCA

matrix solution, 0.5 µl of the supernatant from the cell extract was then placed on the target and a final layer of matrix solution was added. Positive-ion reflectron mode was used to identify the peptide masses. The masses detected were then compared to those of known bacteriocins.

Draft Genome Sequencing

Genomic DNA was extracted using the GenElute bacterial genomic kit (Sigma-Aldrich, Wicklow, Ireland) and the Nextera XT DNA kit (Illumina, San Diego, CA, USA) was used for library preparation. The DNA was quantified using a Qubit® 2.0 fluorometer. Sequencing was performed using Illumina's MiSeq platform using paired-end 2 x 300 base pair reads in the Teagasc Sequencing Centre, Teagasc Food Research Centre Moorepark. Reads were assembled *de novo*, using SPADES (version 3.1.1), resulting in 70 contigs. Open reading frames (ORFs) were identified and annotated using Prokka (version 1.1). Further manual annotation was implemented with ARTEMIS and Artemis Comparison Tool (ACT). Genomic data is available from GenBank/EMBL under accession no. LXPD00000000

Bacteriocin Identification

The bacteriocin mining tool BAGEL3 was used to identify the bacteriocin operons encoded in the genome (van Heel et al., 2013). BAGEL3 scans small ORFs to identify potential bacteriocin encoding genes. The surrounding genes are then analysed for other bacteriocin related components such as transporters and immunity proteins, thus allowing the entire bacteriocin operon to be identified (de Jong et al., 2006). The program antiSMASH was also used to identify antibiotic and secondary metabolite encoding genes within the genome, as these compounds are often associated with the *Bacillus* genus (Medema et al., 2011). Sequence alignments of the bacteriocin were performed using the Clustal Omega software.

Bacteriocin Purification

Cultures of *B. paralicheniformis* APC 1576 were grown statically overnight in 400 ml volumes of BHI broth aerobically at 37˚C. The cell-free supernatant was passed through a column containing 30g

of Amberlite XAD-16 beads (Sigma-Aldrich, Wicklow, Ireland). The column was washed with 250 ml of 35% ethanol and antimicrobial activity eluted with 250ml of IPA. The IPA was removed via rotary evaporation and the sample was then applied to a 10g, 60ml Strata C_{18}-E solid-phase extraction (SPE) column (Phenomenex, Cheshire, UK). The SPE column was washed with 90ml of 35% ethanol and 90ml of IPA. The IPA was once again removed via rotary evaporation from the eluent and the sample applied to a semi prep Proteo Jupiter HPLC column (10 x 250 mm, 90Å, 4µm) running a 27.5-65% acetonitrile 0.1% TFA gradient where buffer A was 0.1% TFA and buffer B was 90% acetonitrile 0.1% TFA. Fractions were collected at 1 minute intervals and were subsequently analysed with MALDI-TOF MS and agar well diffusion assays as described below using *L. delbrueckii* subsp. *bulgaricus* LMG 6901 as the target organism to identify active fractions containing peptides of interest.

Antimicrobial Assays

The antimicrobial activity of the isolated peptides was analysed using well diffusion assays against a range of indicator organisms (Table 1). Briefly, this involved seeding 20 mls of the appropriate agar with 50µl of an overnight indicator culture, the agar was allowed to cool and 7 mm wide wells were then bored in the agar. The purified bacteriocin peptides were dried to a powder and diluted separately in potassium phosphate buffer pH 6.8 to a concentration of 50µM. The combination of these peptides in a 1:1 ratio thus gave a total bacteriocin concentration of 25µM for each peptide. Fifty microliters of this solution was then placed in wells in the indicator plate, these were subsequently incubated overnight under the appropriate growth conditions as outlined in Table 1.

Peptide Stability

The stability of the bacteriocin was determined using purified peptides. To determine the active temperature range of the lantibiotic, 25µM aliquots of the bacteriocin were treated at 60˚C, 70˚C, 80˚C, 90˚C and 100˚C for 30 minutes, a sample was also treated at 121˚C for 15 minutes. These samples were then tested for inhibitory activity against *L. delbrueckii* subsp. *bulgarcus* LMG 6901 in

well diffusion assays as previously described. To determine the susceptibility of the bacteriocin to proteases, 5 µM aliquots of the α and β peptides were treated separately with proteinase K and α-chymotrypsin each at a concentration of 10 mg/ml (Sigma-Aldrich, Wicklow, Ireland). Samples were incubated at 37˚C for three hours followed by treatment at 100˚C for ten minutes to inactivate these proteases. Both bacteriocin peptides were then combined post treatment to give a final total concentration of 2.5µM, these were then screened against *L. delbrueckii* subsp. *bulgarcus* LMG 6901 in well diffusion assays to determine the antimicrobial activity.

Figures and Tables

Figure 1. Formicin identification and activity. (a) Deferred antagonism assay against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 identified *B. licheniformis* APC 1576 as an antimicrobial producer. (b) Antibacterial activity of the *B. licheniformis* APC 1576 cell free supernatant against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 in a well diffusion assay. (c) Colony MALDI-TOF MS displaying the masses of the peptides produced by *B. licheniformis* APC 1576, allowing identification of the antimicrobials produced (3255.92 Da = Frc α (formicin); Frcβ is not seen using colony MALDI-TOF MS; 1423.94 Da = Bacitracin). (d) Combined and individual activity of purified formicin peptides.

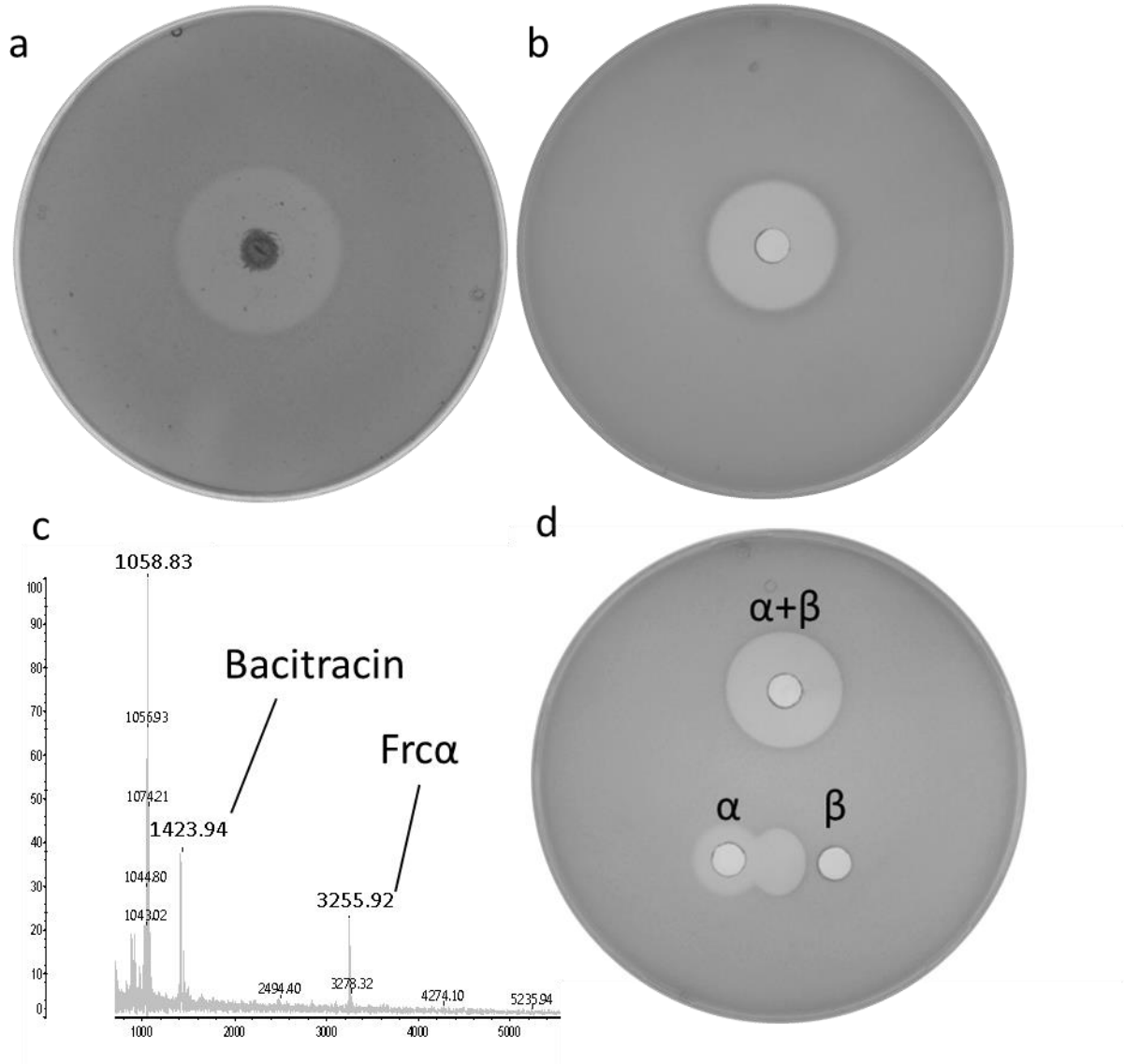


Figure 2. Formicin Operon. Visualisation of the formicin, lichenicidin and haloduracin bacteriocin gene clusters. Clear bacteriocin homologs are identified using the accepted nomenclature for describing lantibiotics. For formicin, *frcA1* and *frcA2* encode the putative bacteriocins, *frcM1* and *frcM2* encode the accompanying modification enzymes and *frcT* and *frcP* are involved in bacteriocin transport and leader cleavage. Similar nomenclature is used for lichenicidin (*lic*) and haloduracin (*hal*) genes. Genes are colour-coded as per BAGEL3, indicating the putative role of each protein.

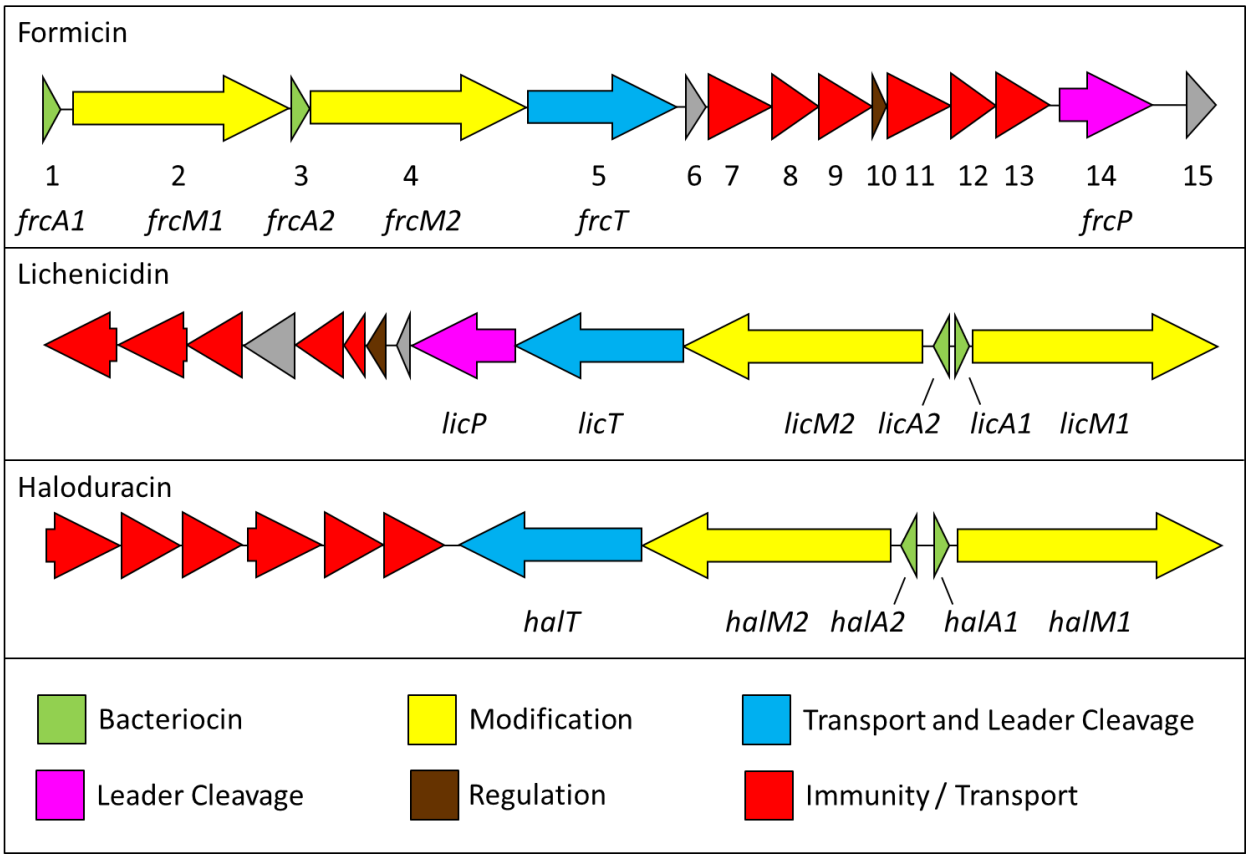
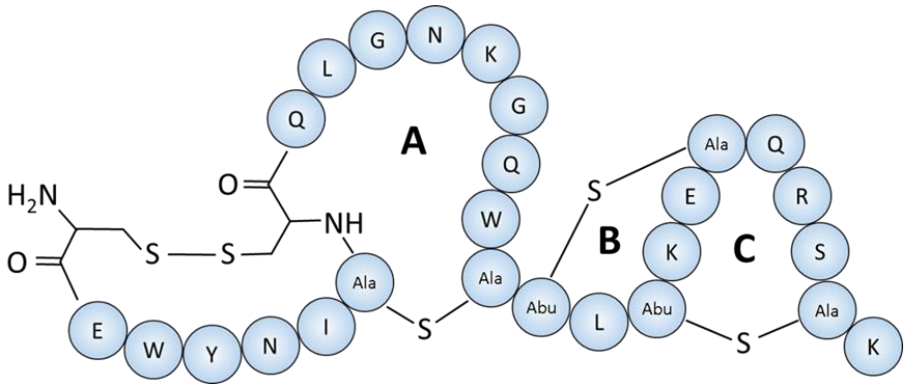


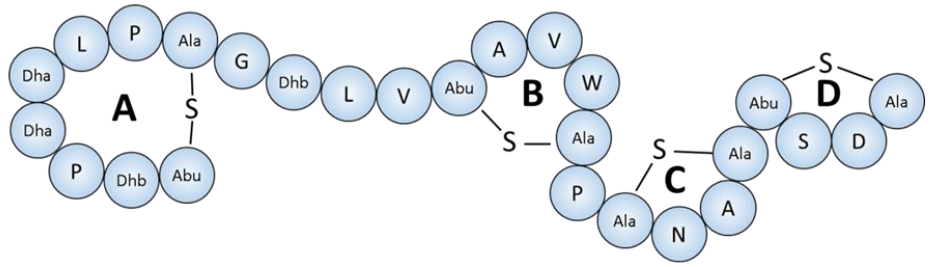
Figure 3. Lantibiotic Structure Prediction. The structures of the formicin α and β peptides were predicted using the Halα and Licβ peptides, respectively, as templates. The conservation of key amino acids suggests a structural homology between the peptides. The rings formed from lanthionine and methyllanthionine bridges are labelled alphabetically, with the N-terminal ring of Frcα excluded as it is predicted to be formed via a disulphide bond. The bacteriocin prepropeptides are shown below each structure, with likely dehydrated serine and threonine residues indicated in red.



MSKIEAWKNPVARMNSQIVSPAGDLMDELSDSEMEMLAGGCEWYNISCQLGNKGQWCTLTKECQRSCK

Leader Cleavage

Frcα

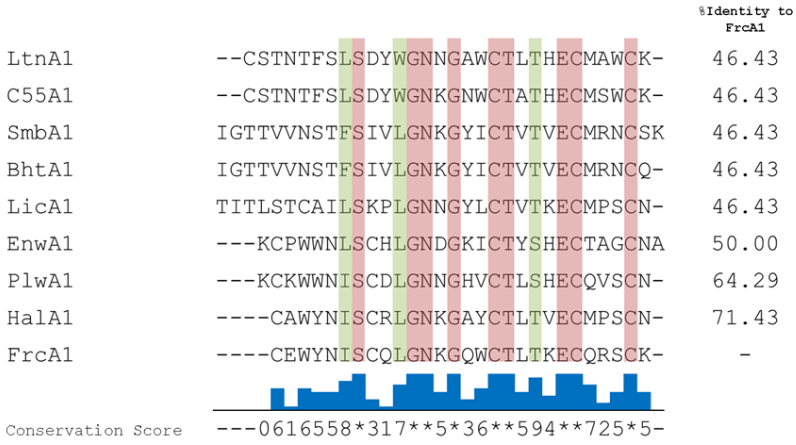


MSHREMAAIYRDANKRANLEFSNPVGEVNEEELKNLAGAADVTPHTTPSSLPCGTLVTAVWCPSNACTSDC

Leader Cleavage Leader Cleavage

Frcβ

Figure 4. Sequence Alignment of Formicin Structural Peptides. Using Clustal Omega, the formicin peptides FrcA1 (a) and FrcA2 (b) were aligned against the previously described two-component bacteriocins. The percentage amino acid identities of each peptide with the formicin peptides are shown. The conservation score between the peptides were calculated with Clustal Omega for the alignments containing less than 25% gaps, * represent a score of 10. The sequences in bold represent the six amino acids cleaved from the N-terminus of these peptides by LanP proteases.



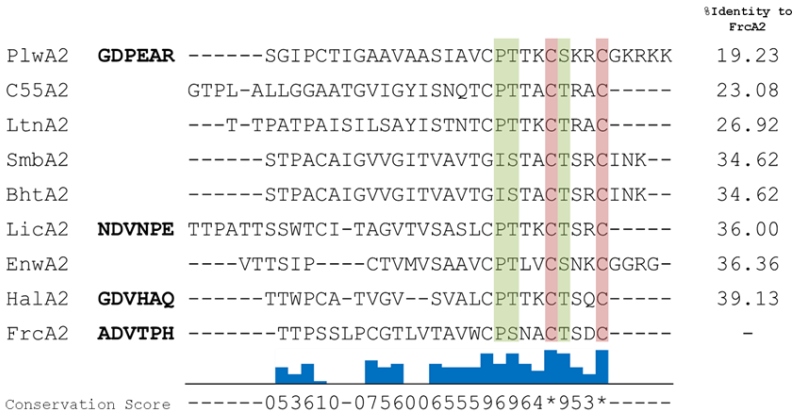


Table 1. Growth conditions of indicator strains and inhibition spectrum of formicin pure peptides following well diffusion assays (- = No activity, + = 0.5-1.5mm inhibition zone, ++ = 2-3.5mm inhibition zone, +++ = ≥ 4mm inhibition zone).

		Growth Conditions			
Species	Strain	Temp (˚C)	Atmosphere	Growth Media	Inhibition
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901	37	Anaerobic	MRS	+++
L. delbrueckii subsp. *lactis*	LMG 7942	37	Anaerobic	MRS	+++
L. amylovorus	LMG 9496	37	Anaerobic	MRS	+
L. fermentum	LMG 6902	37	Anaerobic	MRS	++
L. agilis	LMG 9186	37	Anaerobic	MRS	++
L. casei	LMG 6904	37	Anaerobic	MRS	++
L. amylophilus	DSM 20533	37	Anaerobic	MRS	+++
L. acidophilus	LMG 9433	37	Anaerobic	MRS	-
L. buchneri	DSM 20057	37	Anaerobic	MRS	-
Enterococcus faecium	DPC 4898	37	Anaerobic	MRS	++
E. faecalis	LMG7397	37	Anaerobic	MRS	++
E. saccharolyticus	DPC 4902	37	Anaerobic	MRS	++
E. mundtii	LMG 10748	37	Anaerobic	MRS	+
Lactococcus lactis	HP	30	Aerobic	LM17	+++
L. lactis subsp. *lactis*	DPC 3147	30	Aerobic	LM17	+++
Micrococcus luteus	DPC 6275	30	Aerobic	BHI	+++
Listeria innocua	DPC 3572	37	Aerobic	BHI	++
L. monocytogenes	DPC 5788	37	Aerobic	BHI	+
L. monocytogenes	DPC 6893	37	Aerobic	BHI	+
L. monocytogenes	DPC 6894	37	Aerobic	BHI	+
Bacillus cereus	DPC 6087	37	Aerobic	BHI	+
B. subtilis	DPC 6551	37	Aerobic	BHI	++
B. subtilis	LMG 8198	37	Aerobic	BHI	+
B. licheniformis	DSM 13	37	Aerobic	BHI	++
Pseudomonas aeruginosa	APC 2064	37	Aerobic	BHI	-
Staphylococcus chromogenes	APC 82	37	Aerobic	BHI	-
S. aureus	C55	37	Aerobic	BHI	-
S. aureus	R963	37	Aerobic	BHI	+
Streptococcus mutans	APC 1076	37	Aerobic	BHI	+++
Clostridium indolis	DPC 6345	37	Anaerobic	RCM	++
C. histolyticum	DPC 6344	37	Anaerobic	RCM	++
C. sporogenes	DPC 6341	37	Anaerobic	RCM	++
C. difficile	ATCC 1382	37	Anaerobic	RCM	+
C. perfringens	LMG 11264	37	Anaerobic	RCM	++
Salmonella enterica	APC 174	37	Aerobic	BHI	-

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Chapter 3: Bacteriocin Gene-Trait matching across the complete *Lactobacillus* Pan-genome.

Mass spectrometry and peptide purification was carried out by Ms. Paula O'Connor, initial BAGEL screen was carried out by Dr. Orla O'Sullivan

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Abstract

Lactobacilli constitute a large genus of Gram-positive lactic acid bacteria which have widespread roles, ranging from gut commensals to starters in fermented foods. A combination of *in silico* and laboratory-based screening allowed us to determine the overall bacteriocin producing potential of representative strains of each species of the genus. The genomes of 175 lactobacilli and 38 associated species were screened for the presence of antimicrobial producing genes and combined with screening for antimicrobial activity against a range of indicators. There also appears to be a link between the strains environment and bacteriocin production, with those from the animal and human microbiota encoding over twice as many bacteriocins as those from other sources. Five novel bacteriocins were identified belonging to differing bacteriocin classes, including two-peptide bacteriocins (muricidin and acidocin X) and circular bacteriocins (paracyclicin). In addition, there was a clear clustering of helveticin type bacteriolysins in the *Lactobacillus acidophilus* group of species. This combined *in silico* and *in vitro* approach to screening has demonstrated the true diversity and complexity of bacteriocins across the genus. It also highlights their biological importance in terms of communication and competition between closely related strains in diverse complex microbial environments.

Introduction

Bacteriocins are ribosomally-synthesised antimicrobial peptides which generally act by inducing pore formation or inhibiting cell wall synthesis in target cells (Cotter et al., 2005). Some bacteriocins, such as nisin, have found widespread applicability as bio preservatives in food systems where they have been used for decades. Moreover, bacteriocin production can also be a key probiotic trait (Dobson et al., 2012, Walsh et al., 2008), and bacteriocins have been suggested as potential alternatives to antibiotics in the future (Cotter et al., 2013). The *Lactobacillus* genus has a long association with bacteriocin production, with numerous bacteriocins isolated from such species (O'Shea et al., 2013, Holo et al., 2001). Originally bacteriocin producers were isolated from functional screens against selected target strains, but many studies now rely on prior *in silico* screening, using tools such as BAGEL (Begley et al., 2009, McClerren et al., 2006). BAGEL scans the bacterial genome for putative bacteriocin open reading frames (ORFs) and also analyses surrounding ORFs to search for possible biosynthetic genes, immunity genes and transporters (de Jong et al., 2010). Whilst the areas of interest identified by BAGEL represent potential bacteriocin operons, this does not always translate into functional bacteriocin production for many reasons including problems with mutation, regulation or target specificity.

There are varying accounts on the extent of bacteriocin production in the environment. While numerous accounts assume ubiquity in production (Gillor et al., 2008, Inglis et al., 2013), a definitive analysis has yet to focus on clarifying the actual extent of bacteriocin production. In this study, we elucidate the bacteriocinogenic potential of representative species of the *Lactobacillus* genus and some related genera; i.e. the *Lactobacillus* Genus Complex. Previously Sun *et al*. (Sun et al., 2015) analysed the genomes of 175 *Lactobacillus* species and 38 closely related species, carrying out a screen for putative bacteriocin operons using the BAGEL bacteriocin mining tool. Despite no longer formally being considered as bacteriocins, large (>30kDa) helveticin-like antimicrobial proteins were also included in the study. Based on those results, we analysed strains which were identified as

encoding putative bacteriocin operons for *in vitro* production using well diffusion assays (WDAs) and MALDI TOF MS. Well diffusion assays were used to detect antimicrobial production whilst MALDI TOF MS and SDS PAGE were used to identify the masses of the bacteriocins. Peptide masses identified by MS were correlated with the theoretical masses of bacteriocins identified by BAGEL to confirm the identity of the anti-microbial. We reinforced the BAGEL results with BLAST searches for key lantibiotic and sactibiotic enzymes using specific sequences employed in previous studies against this new dataset of *Lactobacillus* genomes (Begley et al., 2009, Marsh et al., 2010, Goto et al., 2010). This redundancy allows for a more comprehensive analysis of bacteriocin gene clusters in the sequenced strains.

Results

Distribution of Bacteriocin Operons

Several studies have completed bacteriocin screens on diverse and unrelated species of bacteria (Begley et al., 2009, Walsh et al., 2015, Murphy et al., 2011). The aim of this study was to focus primarily on the lactobacilli and investigate the distribution of bacteriocin genes across this single large important genus. From the information identified by BAGEL, we used a phylogenetic tree to visualise the distribution of bacteriocin operons within the genus (Figure 1). Historically the *Lactobacillus* genus has a long association with bacteriocin production. While this study focuses on the type strain of each *Lactobacillus* species, Table 1 identifies those bacteriocins which have been previously identified and characterised from all strains in the *Lactobacillus* Genus Complex. In all, 66 bacteriocins have been characterised from lactobacilli previously, which would suggest a high degree of production within the genus. It is notable that the production of these unique bacteriocins is, in fact, restricted to 16 different species.

Visualisation of the distribution of bacteriocins throughout the *Lactobacillus* Genus Complex shows that there is a clear clustering of helveticin-like operons amongst the *L. acidophilus* branch of species, indicating that such genes have been retained from a common ancestor (Figure 1). Despite being previously classified as class III bacteriocins, these proteins are now termed bacteriolysins and are considered a distinct group of antimicrobials. Whilst these proteins are ribosomally synthesised, they are much larger than classical Gram positive bacteriocins (approx.30 kDa) and are heat labile. Helveticin J is the only previously characterised bacteriolysin from lactobacilli (Joerger and Klaenhammer, 1986), but here we show that these genes are actually highly prevalent across the genus, with 43 potential homologs identified from 23 strains (for alignment results see Supplementary Figure 1). Of the 18 strains in the *L. acidophilus* group, 36 helveticin homologs were distributed amongst 16 of these strains. While certain strains can encode up to four helveticin homologs, there is insufficient homology between those to suggest recent gene duplications. The

high degree of homology (in some cases greater than 99%) between some structural genes encoded by different strains does indicate that horizontal gene transfer of helveticin homologs has occurred; such a mechanism may also explain the presence of these genes in the six strains outside of the *L. acidophilus* group (Figure 1).

The environment from which these strains have been isolated also seems to correlate with their bacteriocinogenic potential (Supplementary Table 1). For example, of the strains isolated from an animal or human origin 37.5% were identified as encoding a complete bacteriocin or helveticin like operon in BAGEL or BLAST screens (21 of 56 strains). This figure for strains isolated from non-animal source (food, plants, environmental and alcohol/wine products) displays an over two-fold reduction at 16.67% (25 of 150 strains). This result suggests that the bacteriocin production may prove to be a competitive advantage for strains from complex environments such as the microbiota of humans and animals.

Diversity of Bacteriocins Identified

Bacteriocins are a diverse and varied group of antimicrobials, which use different systems for bacteriocin modification, transport and immunity. *In silico* analysis allows us to determine which types of bacteriocins the lactobacilli can synthesise. To analyse the diversity of the bacteriocins encoded by lactobacilli, an *in silico* screen was first carried out on the genome of each strain followed by *in vitro* screening of each bacteriocin encoding strain to identify antimicrobial activity against a range of indicators (Table 2). MALDI TOF MS and SDS PAGE allowed us to determine the mass and subsequently the identity of the bacteriocins produced by the strains (Supplementary Figure 2). The bacteriocin classification scheme devised by Cotter *et al.* (Cotter et al., 2005, Cotter et al., 2013) was used to distinguish between the different classes of bacteriocins.

Class I

Class I bacteriocins are comprised of ribosomally synthesised, post-translationally modified bacteriocins (RiPPs)(Cotter et al., 2013). Originally restricted to lantibiotics, this class has now been extended to include other post-translationally modified bacteriocins such as sactibiotics.

Lantibiotics

Lantibiotics are a group of bacteriocins characterised by the presence of lanthionine and methyllanthionine bridges. Here, serine and threonine residues are converted to 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), respectively, which then react with the thiol group found in cysteine residues, forming lanthionine or methyllanthionine thioether cross-links (McAuliffe et al., 2001). Currently three lantibiotics have been attributed to the *Lactobacillus* genus; lactocin S (Mortvedt et al., 1991), plantaricin C (Gonzalez et al., 1994) and the two peptide lantibiotic plantaricin W (Holo et al., 2001).

The BAGEL screen of the *Lactobacillus* dataset identified three further lactobacilli encoding lantibiotic structural peptides (Table 3). Of these, potential production was only identified in *L. taiwanensis* DSM 21401 which encodes a type I lantipeptide (a lantibiotic which doesn't display antimicrobial activity), characterised by the presence of LanB and LanC modification enzymes. What is unusual about this peptide is the fact the structural gene is small compared to other lantipeptides, with the mature peptide predicted to contain only 14 amino acids. Despite a lack of demonstrated antibacterial activity against the range of indicators tested, MALDI TOF MS did identify a mass which correlates with the predicted mass of the mature lantipeptide. The lack of antimicrobial activity may simply imply that the indicator organisms tested were not sensitive, or that the putative lantipeptide has a signalling rather than a bacteriocidal role.

A further type I lantibiotic operon was identified by BAGEL in the strain *L. amylovorus* DSM 20531. This strain appears to encode a complete lantibiotic operon which contains the required modification enzymes and an ABC transporter. *L. gastricus* DSM 16045 was found to encode a Lan C

homolog but a LanB homolog was absent from the operon which is necessary for initial dehydration of serine and threonine residues. The production of either of these bacteriocins was not detected *in vitro.*

Lantibiotic operons were also identified in some of the other genera studied. *Pediococcus damnosus* DSM 20331 was found to encode and produce a class II lantibiotic. This strain has previously been found to produce the partially characterised lantibiotic pediocin PD-1 (Bauer et al., 2005). From genomic data used in this study, the sequence of the pediocin PD-1 gene has now been elucidated, showing a high similarity to the lantibiotic plantaricin C (PlnC) (Turner et al., 1999). Due to the similarity between the two bacteriocins, pediocin PD-1 likely shares a common mode of action with PlnC whose activity has been shown to be as a result of the combination of pore formation and inhibition of lipid II synthesis (Wiedemann et al., 2006). *P. claussenii* DSM 14800 was also shown to encode pediocin PD-1, however, this strain failed to display bacteriocin production. The *Carnobacterium maltaromaticum* strains DSM 20722 and DSM 20730 were also both found to encode the two-component lantibiotic carnolysin, however the *in vitro* production of this bacteriocin was not seen in either strain (Tulini et al., 2014).

To supplement the results of BAGEL searches, previous *in silico* lantibiotic screens were repeated on the new *Lactobacillus* dataset. We used the modification enzymes NisC, LtnM1 and VenL as drivers in the BLAST search for novel lantibiotics (Marsh et al., 2010, Begley et al., 2009, Goto et al., 2010). *L. gallinarum* DSM 10532, *L. crispatus* DSM 20584 and *P. cellicola* DSM 17757 were all found to harbour a NisC homolog, despite not being identified by BAGEL. However, upon examination of the surrounding genes, no potential structural genes were identified. Strains identified in BLAST searches as encoding LanM homologs had also been identified by BAGEL. No homolog of the novel lanthionine synthase VenL was identified in the BLAST screen.

Sactibiotics

The sactibiotics are a growing class of bacteriocins characterised by the presence of unusual sulphur to α-carbon linkages. These modifications are carried out by radical S-adenosylmethionine (SAM) proteins which catalyse the formation of these thioether bonds (Fluhe et al., 2012, Mathur et al., 2015). To analyse the prevalence of potential sactibiotic operons within the lactobacilli, the sequences for the radical SAMs associated with a two-component sactibiotic thuricin CD (TrnC and TrnD) were used as drivers in a BLAST analysis of the genomes available(Rea et al., 2010, Murphy et al., 2011). Only two radical SAMs were found resembling those associated with thuricin CD. *L. mali* DSM 20444 was found to encode one such SAM, however, analysis of the operon failed to identify a potential structural gene. *Kandleria vitulina* DSM 20405 appears to encode a complete sactibiotic operon, encompassing a structural gene, transporter and associated radical SAM, however, no biological activity could be attributed to this strain with the panel of indicators tested. BAGEL further identified two potential sactibiotic related radical SAM proteins in *C. maltaromaticum* DSM 20342 and DSM 20722 but no potential structural genes for these enzymes were apparent.

TOMMs

Thiazole/oxazole modified microcins (TOMMs) are a class of RiPPs which are now included with the class I bacteriocins. These peptides undergo extensive post-translational modification, where cysteine, serine and threonine residues are converted into the corresponding heterocycles; thiazole, oxazole and methyloxazole, respectively (Molloy et al., 2011). TOMMs exist in gene clusters, with components encoding several factors involved in transport, modification and immunity. Using streptolysin as an example, the modification of the structural peptide is the result of the activity of the SagBCD enzyme complex, encompassing a cyclodehydratase (SagC), a dehydrogenase (SagB) and a docking protein (SagD) (Lee et al., 2008). Whilst SagBCD clusters are described as being relatively widespread amongst prokaryotes, no TOMM has yet been identified from a *Lactobacillus* species (Lee et al., 2008). In our study *L. crispatus* DSM 20584, *L. intestinalis* DSM 6629 and *Oenococcus kitaharae* DSM 17330 were identified by BAGEL as encoding homologs of the SagBCD gene cluster.

Whilst the operons in *O. kitaharae* DSM 17330 and *L. intestinalis* DSM 6629 appear to be complete, the *L. crispatus* DSM 20584 TOMM operon appears to lack a structural gene, however, the structural gene for similar operons has been found to be some distance from the SagBCD homologs previously (Haft, 2009). Of these three strains, *L. crispatus* DSM 20584 was the only one found to display antimicrobial activity; the source of this activity, however, remains unclear.

Class II

Class II bacteriocins are small heat stable peptides which are not subject to extensive post translational modification, most of which act to permeabilize the membrane of target cells (Cotter et al., 2005). This class of bacteriocins is further subdivided based on the structure and activity of the peptides.

Class IIa

Class IIa or 'pediocin-like' bacteriocins display a narrow range of antimicrobial activity, particularly displaying strong anti-listerial activity. Such bacteriocins encompass a highly conserved YGNGV/L N-terminal motif followed by cysteine residues which can form a disulphide bridge. Unlike the N-terminus, the C-terminus is less conserved and is likely involved in membrane insertion and pore formation (Fimland et al., 2005). These bacteriocins likely act by using the mannose-phosphotransferase system on sensitive cells as a receptor (Ramnath et al., 2000).

Despite having a long association with this class of bacteriocins, surprisingly only 3 *Lactobacillus* strains were found to encode what appear to be complete class IIa bacteriocin operons, containing structural, immunity and transport genes (Table 4a). Of these, *L. hordei* DSM 19519 displayed bacteriocin production against six of the indicators tested. From MALDI TOF MS and BAGEL results, the production of coagulin was confirmed. This 44 amino acid bacteriocin was originally isolated from *Bacillus coagulans* and closely resembles the bacteriocin pediocin PA-1, differing by a single amino acid due to a N41T substitution (Hyronimus et al., 1998, Le Marrec et al., 2000). The presence of a further pediocin-like operon was noted within the *L. hordei* genome,

encoding a structural peptide displaying 74% amino acid identity to plantaricin 423. Production of this bacteriocin however was not seen.

Numerous lactobacilli identified in this study were found to carry partial pediocin-like operons, often containing the bacteriocin structural gene and associated immunity protein but lacking the appropriate transporters (Table 4b). One potential explanation is that when a strain acquired the gene for pediocin resistance that the neighbouring small bacteriocin structural gene was also transferred, whilst the larger transporters were not.

Although not included in the *Lactobacillaceae* family, several *Carnobacterium* strains were included in the preceding genomic study (Sun et al., 2015). Numerous bacteriocins have been attributed to this genus previously (Quadri et al., 1994, Tulini et al., 2014). While the source of antimicrobial activity from *C. maltaromaticum* DSM20342 is unclear, C*. maltaromaticum* DSM 20722 was found to produce the class IIa bacteriocin cbnB2 and cbnBM1, the class IId bacteriocin cbnX was also produced by the strain (Tulini et al., 2014). CbnB2 contains an N2Y mutation which was also previously seen by Tulini *et al.* (Tulini et al., 2014).

Class IIb

The class IIb bacteriocins are comprised of unmodified two peptide bacteriocins, whose activity is dependent on the synergistic activity of both peptides which interact to form a single antimicrobial unit (Nissen-Meyer et al., 2010). These bacteriocins are likely to act by forming membrane spanning pores which result in the leakage of small molecules from the cell. Such bacteriocins tend to contain conserved GxxxG or AxxxA motifs which are responsible for close helix interactions between each bacteriocin peptide (Nissen-Meyer et al., 2010). A wide range of class IIb bacteriocins were identified by BAGEL in this study (Table 5).

L. murinus DSM 20452 was one of the strains which demonstrated bacteriocin production. MALDI TOF MS identified masses which correlate with a two-peptide bacteriocin identified by BAGEL

(muricidin). Both peptides of muricidin display homology to the class IIa bacteriocin plantaricin S, with the α peptide displaying 41% amino acid identity to pln Sα and the β peptide 48% to pln Sβ. The β peptide found here however lacks the AxxxA motif found in pln Sβ, a sequence which has been shown to be important for helix-helix interactions in pln S (Soliman et al., 2011).

Another two-peptide bacteriocin (acidocin X) was also identified from *L. acidophilus* DSM 20079. Correlation between the bacteriocins identified by BAGEL and MALDI TOF MS results led to the identification of two, bacteriocin like, peptides. The first of these was a 35 amino acid peptide displaying 53% identity with the enterocin X β peptide. The second peptide was not identified in BAGEL and was found by manual analysis of the bacteriocin operon, this displays 25% identity to the enterocin X α peptide.

Class IIc

Class IIc bacteriocins are also known as circular bacteriocins due to the covalent linkage of the N- and C-termini. The compact circular structure of these bacteriocins can contribute to their temperature and pH stability (Gabrielsen et al., 2014). These circular bacteriocins permeabilize the target cell membrane, resulting in a loss of membrane potential which leads to cell death (Van Belkum et al., 2011). Despite having similar modes of action, this class of bacteriocins are further broken down into two subgroups, based on the isoelectric point of the peptides and the conservation seen amongst the groups (Acedo et al., 2015). Currently, there are two examples of class IIc bacteriocins produced from lactobacilli, both of which belong to subgroup II. Originally identified as two separate class IIc bacteriocins, Gassericin A (*L. gasseri* LA39) and reutericin 6 (*L. reuteri* LA6) have now been shown to be identical (Kawai et al., 2001, Arakawa et al., 2010). Acidocin B (*L. acidophilus* M46), originally thought to be linear, has also been recently reclassified as a circular bacteriocin. *Leuconostoc mesenteroides* TK41401 has also been shown to produce leucocyclicin Q, a subgroup I circular bacteriocin.

From the analysis carried out in this study, *L. paracasei* subsp. *paracasei* DSM 5622 was found to produce a potential class IIc bacteriocin (paracyclicin), with a structural gene displaying 64% amino acid identity to butyrivibriocin AR10 (Kalmokoff et al., 2003). The operon contains a putative ABC permease, ATPase and a protein belonging to the DUF 95 protein family, all of which have been associated with the gene clusters of circular bacteriocins (Gabrielsen et al., 2014). Upon purification of the bacterial supernatant, a mass of 5905.75 Da was identified as the causative agent of antimicrobial activity. This mass correlates closely with the predicted mass of the mature bacteriocin structural peptide which is calculated as 5906.87 Da. It is clear that paracyclicin belongs to the subgroup II circular bacteriocins, due to a high level of conservation found within the group (Table 6). Despite this conservation, this novel bacteriocin does display variation in certain conserved regions which is not seen in the rest of the class. *L. nodensis* DSM 19682 was also found to encode one such potential bacteriocin, however, no antimicrobial activity was observed with this strain.

Class IId

Class IId bacteriocins are single peptide, linear bacteriocins which do not display homology to the pediocin like bacteriocins (Cotter et al., 2013). This class of bacteriocins displays a high degree of diversity and numerous class IId bacteriocins have been characterised from lactobacilli previously (Table 1). *In silico* analysis of the *Lactobacillus* dataset identified numerous novel structural genes (Table 7) with several shown to be produced.

L. paralimentarius DSM 13961 was one such strain displaying the production of a class IId bacteriocin (paralimenterocin). The paralimenterocin structural gene identified encodes a 44 amino acid single peptide bacteriocin whose closest homolog appears to be the relatively uncharacterised bacteriocin BacSJ2-8 to which it has 77% identity (Kojic et al., 2010). The mode of action of both of these bacteriocins remains unclear.

L. equicursoris DSM 19284 is also highly likely to produce a novel class IId bacteriocin (equicursorin). The strain displayed antimicrobial production upon analysis, but MALDI TOF MS did

not identify an associated mass. *In silico* BAGEL analysis identified three putative bacteriocin operons, two of which encoded larger bacteriolysins of approximately 30kDa, the remaining operon encodes a homolog of lactococcin 972. SDS PAGE analysis of the concentrated culture supernatant identified a mass between the 5kDa and 10kDa markers which displayed antimicrobial activity once overlaid with *L. delbrueckii* subsp. *bulgaricus* LMG 6901 (Supplementary Figure 2). This mass correlates well with the predicted mass (approximately 7kDa) of the lactococcin 972 homolog 'equicursorin'. Lactococcin 972 is unique with respect to its activity in comparison to other class II bacteriocins. These bacteriocins do not induce pore formation in the cells but instead act by binding to lipid II and inhibiting septum formation. Lactococcin 972 is also unusual in that it's biologically active form is as a homodimer (Martinez et al., 2000, Martinez et al., 2008). Given that only two such bacteriocins have been identified, it was surprising that four further lactococcin 972-like operons were identified in genomic dataset screened in this study (Table 7 (b)). An *in silico* screen carried out by Letzel *et al.* (Letzel et al., 2014) identified 9 further Lactococcin 972 operons in anaerobic bacteria, thus due to the expansion of this group, these bacteriocins may warrant a separate classification, given their unique mode of action when compared to other class II bacteriocins.

Bacteriolysins (Formerly Class III Bacteriocins)

In the *Lactobacillus* dataset, a number of homologs of the bacteriolysin helveticin (Joerger and Klaenhammer, 1986) were found to be encoded, with several displaying *in vitro* antimicrobial activity. The approximate size of these proteins was determined using SDS PAGE overlay assays, as MALDI TOF MS wasn't used to determine the size of these larger proteins. Several strains encoded numerous helveticin homologs, however, SDS PAGE overlays were not able to identify which of these homologs was actually produced as all had masses of approximately 37 kDa (Supplementary Figure 3).

L. intestinalis DSM 6629 was shown to produce one of these helveticin homologs, with four potential structural genes found within the genome ranging from 38% to 67% amino acid identity to

helveticin J. *L. kitasatonis* DSM 16761 also produced a helveticin like peptide, the strain encodes two such proteins displaying 35% and 41% identity to helveticin J. Two *L. amylovorus* strains (DSM 16698 and DSM 20531) were shown to produce a helveticin homolog. *L amylovovrus* DSM 16698 encodes four of such proteins, whilst *L. amylovorus* DSM 20531 encodes three. Both share a single identical helveticin homolog but it is unclear whether this is the protein produced by both strains. *L. kalixensis* DSM 16043 also produces a helveticin-like protein, with 3 homologs encoded within the genome displaying, 34%, 49% and 50% amino acid identity to helveticin J.

BAGEL also identified a helveticin homolog (77% identity to helveticin J) from *L. crispatus* DSM20584. Interestingly, analysis of the results of a previous exoproteomic study identified the secretion of this protein (Johnson et al., 2016). The antimicrobial activity of the strain in this study was determined to be due to a small peptide by an SDS PAGE overlay assay, this is most likely a lactacin F homolog (Fremaux et al., 1993) or else a novel TOMM like peptide.

Discussion

This study gives the first complete assessment of bacteriocin production across the *Lactobacillus* Genus Complex, combining both *in silico* and laboratory based screening methods. This combination of approaches allows for a more representative estimation of bacteriocin production to be calculated. Well-diffusion assays and MALDI TOF MS allows for the confirmation of *in vitro* bacteriocin production by cells. Bacteriocin production however can be a highly regulated process, with strains requiring specific conditions and environments to induce production of these antimicrobials (Diep et al., 2000, Maldonado-Barragan et al., 2013). Such regulations would make it extremely difficult to identify the bacteriocins found here using *in silico* screens if we were to rely on *in vitro* screening methods alone. Thus, the use of BAGEL and BLAST bacteriocin screens allows us to identify these bacteriocin operons from the *Lactobacillus* Genus Complex without the shortcomings and restrictions of laboratory based screens.

In silico analysis has allowed us to determine the overall bacteriocinogenic potential of the *Lactobacillus* genus. Of the 213 strains analysed, 51 were identified by BAGEL or in BLAST screens as harbouring what appears to be a complete bacteriocin or helveticin like operon, a prevalence of 23.94%. If we focus on the lactobacilli, of the 175 strains analysed only 25 were found to encode bacteriocin operons (14%). If helveticin operons and those of previously characterised bacteriocins are included, of the *Lactobacillus* species analysed 30% were found to encode at least one antimicrobial. This figure of 30% is surprisingly high given that lactobacilli are not associated with the production of more traditional antibiotics formed by non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). Given the extent of bacteriocin production within the genus, the production of antimicrobials by these means may be unnecessary, especially given the size of such NRPS and PKS operons and the subsequent energy it would take for their production. Thus, bacteriocin production may supplant the need for NRPS and PKS enzyme complexes in certain genera.

There was a high degree of novelty within the bacteriocins identified by BAGEL in this study. Of all the structural genes identified here, 73% had not previously been characterised. Screening of these strains identified five novel functional bacteriocins (muricidin, acidocin X, paracyclicin, paralimenterocin and equicursorin) from a range of bacteriocin classes. In addition, five novel producers of helveticin-like peptides were also identified. The abundance of homologs of helveticin-like bacteriolysins encoded by lactobacilli is surprising given how little these proteins have been characterised to date. The observation that most strains in the *L. acidophilus* group encode helveticin homologs with significant homology suggests that this trait was derived from a common ancestor and then disseminated by horizontal transfer. Apart from narrow spectrum antimicrobial activity, no other function has been ascribed to these proteins. The role these proteins play in the life cycle of this narrow branch of strains warrants further study.

The variety and distribution of bacteriocins throughout the genus is interesting when compared to the results of other *in silico* screens which were carried out. Letzel *et al*. (Letzel et al., 2014) used BAGEL and other tools to screen the genomes of 211 anaerobes for bacteriocin encoding genes (no lactobacilli were included in the screen, and helveticin like proteins were excluded). Of these 211 strains, just over 25% were found to encode a bacteriocin like peptide. Thus despite the differences in the make-up of the datasets, there is a similar level of bacteriocin encoding genes found in both groups. While the overall levels may be similar, the diversity of the bacteriocins encoded differs greatly. Of the bacteriocins encoded in the anaerobic dataset, 78% were found to be class I modified bacteriocins, while in the *Lactobacillus* Genus Complex this value is only 17%. One similarity between these sets of results, however, is the presence of lactococcin 972 like bacteriocins. 9 novel homologs were identified in the anaerobic bacteria, this result taken with the number of novel homologs identified from the lactobacilli suggests that this group of unique bacteriocins merit their own class of bacteriocins in the future given their unique mode of action and increasing prevalence.

In a bioinformatics screen of *Bacillus* species for bacteriocin operons (Zhao and Kuipers, 2016), the overall level of bacteriocins encoded by such strains was much higher, with 583 putative bacteriocin operons encoded in the genomes of 328 strains. 89% of these strains, covering 50 different species encode a bacteriocin, a much higher level than seen in the anaerobic bacteria and the lactobacilli. The diversity of encoded bacteriocins again differs to that of the lactobacilli with 66% of operons identified here encoding class I bacteriocins. This difference suggests that there is not an even distribution in the types of bacteriocins across genera, with the lactobacilli in particular relying on the production of class II bacteriocins in comparison to other groups. A similar high prevalence of bacteriocin operons can be found in the cyanobacteria, with 145 putative bacteriocin gene clusters being identified in 43 of the 58 complete and partial genomes screened (Wang et al., 2011). It must be remembered, however, that in both studies these operons were not manually analysed so, in reality, overall levels may be lower.

The inter-species diversity of bacteriocin production can be seen in a screen carried out, whereby the genomes of 169 *Streptococcus mutans* strains were screened by BAGEL for bacteriocin operons (Liu et al., 2016). 211 bacteriocin operons were found distributed amongst 157 strains, of which 32 were lantibiotic operons. These results show that despite carrying out a comprehensive analysis of bacteriocin production in lactobacilli, a high level of diversity within each species can still result in novel bacteriocins being identified.

The environment from which strains are isolated may also influence their bacteriocinogenic potential. 37.5% of strains isolated from human and animal microbiomes encoded bacteriocins or bacteriolysins, this is over twice the value for strains isolated from food, wine and beer, plants and the environment at 16.67%. The microbiota of animals is a complex environment with microbes under constant competition for nutrients and resources (Kostic et al., 2013). Bacteriocin production can provide a competitive advantage for strains, allowing them inhibit sensitive strains thus reducing competition and allowing them to establish themselves in a complex community (Dobson et al.,

2012, Walsh et al., 2008, Gillor et al., 2009). This may suggest why a greater proportion of lactobacilli from these environments encode bacteriocins. Environments such as fermented foods would provide a much narrower niche for the growth of microbes. Less competition here may negate the need for these bacteria to expend energy on bacteriocin production.

Given the association of lactobacilli with probiotics and food production, the knowledge of their potential to produce antimicrobials is of great value (Sanders and Klaenhammer, 2001). Bacteriocin production may increase their ability to establish themselves in a community such as the gut, or provide a natural mechanism to inhibit the growth of food spoilage microorganisms (Walsh et al., 2008, Yang et al., 2014). Thus bacteriocin production can prove a useful trait for an industrially important group of bacteria. Previously, the isolation of bacteriocins from lactobacilli relied on intensive laboratory screens of individual cultures. The use of tools such a BAGEL and BLAST however now allow for the rapid identification of bacteriocin operons within strains, and with the increasing availability of genomic data, these tools are becoming more relevant.

Materials and Methods

Bacteriocin Identification

The bacteriocin mining tool BAGEL2 was used to identify putative bacteriocin operons(de Jong et al., 2010) and the genome visualisation tool ARTEMIS was subsequently used for manual analysis of the bacterial genomes(Rutherford et al., 2000). To determine the degree of novelty in the bacteriocins identified by BAGEL2, BLASTP searches were done for each putative bacteriocin peptide against those identified in the BAGEL screen. The levels of identity described in this study are derived from Clustal Omega. For bacteriocin analysis using specific "driver" sequences, the BLASTP program was used using default parameters. The driver sequences used were NisC (GenBank Accession no. CAA79470.1), LtnM1 (GenBank Accession no. NP_047321.1), VenL (GenBank Accession no. AEA03262.1), TrnC and TrnD from *Bacillus thuringiensis* DPC 6431.

Bacterial Strains

The bacterial strains screened for bacteriocin production and the conditions for growth are listed in Supplementary Table 2. Anaerocult A gas packs (Merck, Darmstadt, Germany) were used to generate anaerobic conditions.

Bacteriocin Assays

Bacteriocin activity was analysed via well diffusion assays against the indicator organisms listed in Supplementary Table 3. Briefly, each strain screened was grown in broth under the appropriate conditions. The cell free supernatant of each culture was prepared by centrifuging the fully grown culture at 4000 RCF for 20 minutes, the pH was adjusted to pH7 using sodium hydroxide to negate any antimicrobial activity which may be caused by the acidity of the cell free supernatants. Fifty microlitres of an overnight culture of each indicator was then added to 20 ml of the appropriate media containing 1.5% agar. Plates were allowed cool and the 7mm wide wells were bored into the

agar. Fifty microliters of the cell free supernatant of the strains being tested was then placed in a well. These indicator plates were refrigerated for two hours prior to incubation.

Mass Spectrometry (MS)

MALDI TOF colony mass spectroscopy was carried out on each of the strains as previously described to identify masses of putative bacteriocins (Field et al., 2010). Here colonies were first mixed with a 70% propan-2-ol 0.1% TFA solution to elute bacteriocin from the cell. Following centrifugation, the subsequent supernatant was spotted on the target pre-coated with CHCA matrix solution. A further layer of matrix solution was then added on top of this supernatant. An Axima TOF^2plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) was used to identify the peptide masses using positive-ion reflectron mode.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS PAGE was used for the identification of higher molecular weight antimicrobial proteins (bacteriolysins). Cultures were grown overnight in broth and the cell free supernatants were prepared as described above. The proteins from the bacterial supernatant were precipitated by addition of ammonium sulphate salts up to a concentration of 50%. The precipitate was collected by centrifugation and resuspended in water. Supernatants were then incubated with TruPAGE™ LDS sample buffer (Sigma-Aldrich, Wicklow, Ireland) for 10 minutes at 70˚C. Samples were run on 12% acrylamide gels at 30 mA, together with Precision Plus Protein™ Dual Xtra prestained protein standards (Bio-Rad, Hertfordshire, UK) which were used to estimate molecular mass with a range of 2-250kDa. The completed gels were divided in two, one half was stained using the EZBlue™ staining reagent (Sigma-Aldrich). The other half was washed with 1% tween-80 (Sigma-Aldrich) for 45 minutes, followed by three 5 minute washes in distilled water. This gel was overlaid with soft MRS agar (0.8% agar), seeded with 0.25% of an overnight culture of *L. delbrueckii* subsp. *bulgaricus* LMG

6901. The plate was incubated overnight to determine the mass of any antimicrobial proteins produced.

Bacteriocin Purification

Carnobacteriocins CbnB2, CbnBM1 and CbnX

Carnobacterium maltaromaticum DSM 20722 was grown overnight in TSA broth, 100 ml of the supernatant was passed through a 5g, 20ml Strata C_{18}-E solid-phase extraction (SPE) column (Phenomenex, Cheshire, UK). The column was washed with 20ml of 30% ethanol and 20ml of 70% 2-propanol (IPA) 0.1% TFA. The 70% IPA eluent was concentrated and applied to a Semi Prep Proteo Jupiter RP-HPLC column (10 x 250mm, 90Å, 4µm) (Phenomenex, Cheshire, UK) running a 20-55% gradient whereby buffer B was 90% acetonitrile. MALDI TOF MS was carried out on fractions to identify the presence of the peptides of interest.

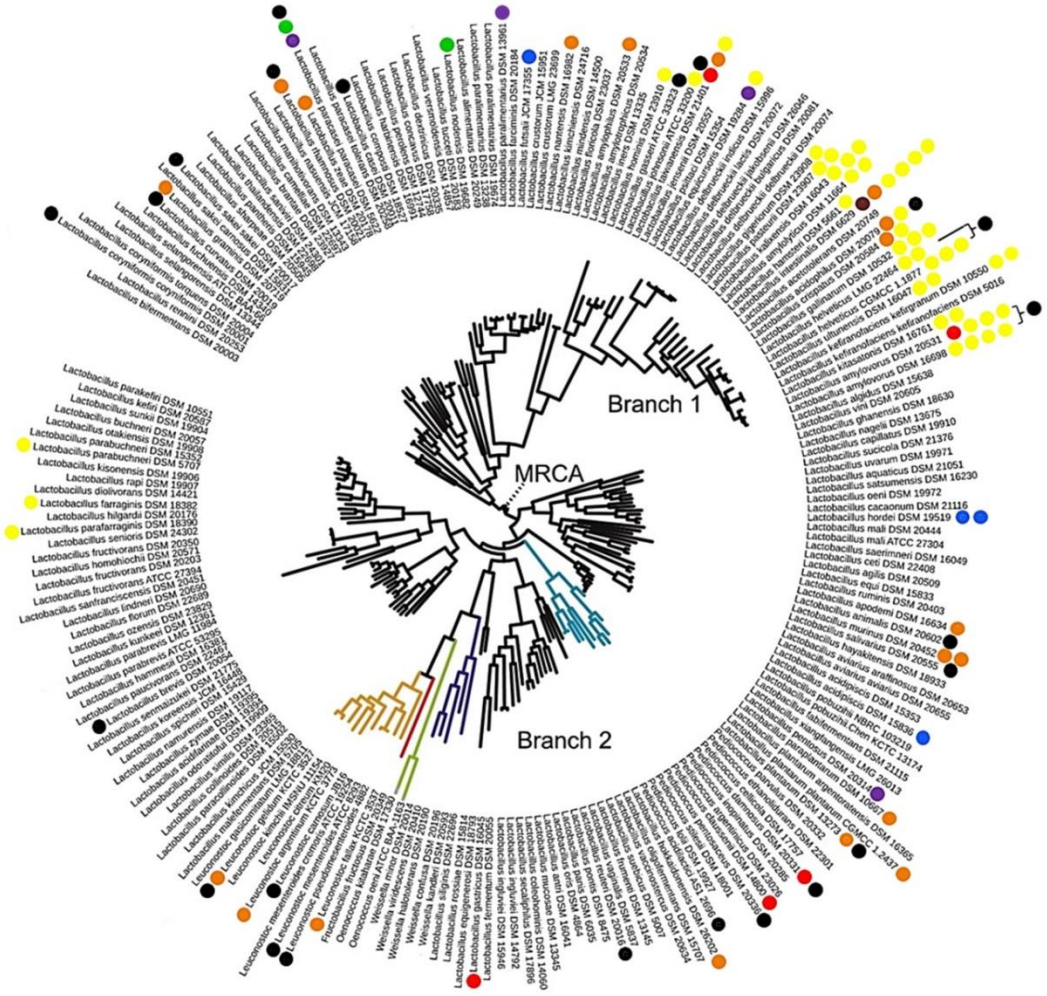
Paracyclicin

L. paracasei subsp. *paracasei* DSM 5622 was grown overnight in MRS broth. Culture supernatant was passed through a column containing 60g Amberlite XAD beads and washed with 400ml of 50% ethanol and the antimicrobial peptide eluted with 400ml of 70% IPA 0.1% TFA. The IPA was removed and the eluent passed through a 5g, 20ml C18 SPE column pre-equilibrated with methanol and water. The column was washed with 30ml of 50% ethanol and activity eluted with 30 ml of IPA. The IPA was removed from the C18 SPE IPA eluent and the sample applied to a semi preparative Vydac C4 Mass Spec (10 x 250 mm, 300Å, 5µ) RP-HPLC column (Grace, Columbia, USA) running an acetonitrile and propan-2-ol gradient described as follows: 5-55% buffer B and 0-5% buffer C over 25 minutes followed by and 55-19% buffer B and 5-81% buffer C over 60 minutes, 19-5% buffer B and 81-95% buffer C over 5 minutes where buffer A is Milli Q water containing 0.1% TFA, buffer B is 90% acetonitrile 0.1% TFA and buffer C is 90% propan-2-ol 0.1% TFA. Eluent was monitored at 214nm and fractions were collected at 1 minute intervals. Fractions were assayed

using well diffusion assays against *L. delbrueckii* subsp. *bulgaricus* LMG 6901. MALDI TOF MS was used to determine the mass of the antimicrobial peptide.

Figures and Tables

Figure 1. Distribution of complete bacteriocin operons amongst the Lactobacillus Genus Complex (Adapted from Sun *et al.*)



Legend:	
Species for which a bacteriocin has been previously characterised	
Class I Bacteriocin	
Class IIa Bacteriocin	
Class IIb Bacteriocin	
Class IIc Bacteriocin	
Class IId Bacteriocin	
TOMM	
Helveticin	

Table 1. Bacteriocins characterised from species within the *Lactobacillus* Genus Complex

Bacteriocin	Subclass	Producing strain	Origin	Reference
Class I				
Plantaricin W (α and β)	II	*Lactobacillus plantarum* LMG 2379	Wine	(Holo et al., 2001)
Plantaricin C	II	*L. plantarum* LL441	Cabrales cheese	(Gonzalez et al., 1994)
Lactocin S[a]	II	*L. sakei* L45	Sausages	(Mortvedt et al., 1991)
Pediocin PD-1	II	*Pediococcus damnosus* NCFB1832	Lager Beer	(Green et al., 1997)
Glycocin F	Glycocin	*L. plantarum* KW30	Fermented corn	(Venugopal et al., 2011)
Class II				
Acidocin A	IIa	*L. acidophilus* TK9201	Fermented milk (starter)	(Kanatani et al., 1995)
Curvaticin L442	IIa	*L. curvatus* L442	Greek fermented sausage	(Xiraphi et al., 2006)
Curvaticin 13	IIa	*L. curvatus* SB13	Sausages	(Sudirman et al., 1993)
Sakacin P (variant)[b]	IIa	*L. curvatus* LTH1174	Fermented meat	(Cocolin and Rantsiou, 2007)
Plantaricin BM-1	IIa	*L. plantarum* BM-1	Fermented meat	(Zhang et al., 2013)
Plantaricin C19	IIa	*L. plantarum* C19	Fermented cucumber	(Atrih et al., 2001)
Plantaricin 423	IIa	*L. plantarum* 423	Sorghum (beer)	(Van Reenen et al., 2003)
Sakacin P[c]	IIa	*L. sakei* LTH673	Cured meat	(Tichaczek et al., 1994)
Sakacin A[d]	IIa	*L. sakei* Lb706	Meat	(Holck et al., 1992)
Sakacin G[e]	IIa	*L. sakei* 2512	Food origin	(Simon et al., 2002)
Sakacin X[f]	IIa	*L. sakei* 5	Malt	(Vaughan et al., 2003)
Bavaricin A	IIa	*L. sakei* MI1401	Sourdough	(Larsen and Norrung, 1993)
Bavaricin MN	IIa	*L. sakei* MN	Meat (bovine)	(Kaiser and Montville, 1996)
Bacteriocin L-1077	IIa	*L. salivarius* L-1077	Intestine (broilers)	(Svetoch et al., 2011)
Leucocin A[gh]	IIa	*Leuconostoc geldium* UAL 187	Vacuum-packed meat	(Hastings et al., 1991)
Leucocin C	IIa	*Leuc. mesenteroides* TA33a	Spoiled vacuum-packed meat	(Papathanasopoulos et al., 1997)
Leucocin 10C[h]	IIa	*Leuc. mesenteroides* 10	Malted barley	(Vaughan et al., 2001)
Leucocin 683Y	IIa	*Leuc. mesenteroides* 683	Malted barley	(Vaughan et al., 2001)
Mesentericin Y105	IIa	*Leuc. mesenteroides* subsp. *mesenteroides* Y105	Goats milk	(Hechard et al., 1992)
Pediocin PA-1 (ACH)[i]	IIa	*P. acidilactici* PAC1.0	Meat	(Gonzalez and Kunka, 1987)
Pediocin SA-1	IIa	*P. acidilactici* NRRL B5627	Meat	(Anastasiadou et al., 2008b)
Penocin A	IIa	*P. pentosaceus* ATCC 25745	Plants	(Diep et al., 2006)
Pediocin SM-1	IIa	*P. pentosaceus* Mees 1934	Meat	(Anastasiadou et al., 2008a)
Weissellin A	IIa	*Weissella paramesenteroides* DX	Sausage	(Papagianni and Papamichael, 2011)
Lactobin A[j]	IIb	*L. amylovorus* LMG P-13139	Corn liquor	(Contreras et al., 1997)
Brevicin 174 (*breB* and *breC*)	IIb	*L. brevis* 174A	Iyokan (fruit)	(Noda et al., 2015)
Lactocin 705 (Lac705α and Lac705β)	IIb	*L. casei* CRL 705	Meat	(Vignolo et al., 1996)
Acidocin LF221 (LF221A and LF221B)[k]	IIb	*L. gasseri* LF221	Faeces (child)	(Majhenič et al., 2004)
Gasericin T (GatA and GatX)	IIb	*L. gasseri* SBT2055	Faeces (human)	(Kawai et al., 2000)
Lactacin F (LafA and LafX)[l]	IIb	*L. johnsonii* VPI11088	Intestine (human)	(Fremaux et al., 1993)
Sakacin T (SakTα and SakTβ)[m]	IIb	*L. sakei* CTC372	Sausages	(Aymerich et al., 2000)
Plantaricin E/F (PlnE and PlnF)	IIb	*L. plantarum* C11	Fermented cucumber	(Diep et al., 1996)
Plantaricin J/K (PlnJ and PlnK)	IIb	*L. plantarum* C11	Fermented cucumber	(Diep et al., 1996)
Plantaricin S (Plsα and Plsβ)[n]	IIb	*L. plantarum* LPCO10	Green olives	(Stephens et al., 1998)

Table 1. Continued

Bacteriocin	Subclass	Producing strain	Origin	Reference
Plantaricin NC8 (PLNC8α and PLNC8β)	IIb	*L. plantarum* NC8	Ensilage	(Maldonado et al., 2003)
Salivaricin ABP-118 (Abp118α and Abp118β)	IIb	*L. salivarius* UCC118	Intestine (human probiotic)	(Flynn et al., 2002)
Salivaricin CLR 1328 (Salα and Salβ)	IIb	*L. salivarius* CLR1328	Vagina (human)	(Vera Pingitore et al., 2009)
Salivaricin P (Sln1 and Sln2)	IIb	*L. salivarius* DPC6005	Intestine (pig)	(Barrett et al., 2007)
Salivaricin T (SalTα and SalTβ)	IIb	*L. salivarius* DPC6488	Intestine (neonate)	(O'Shea et al., 2011)
Acidocin B	IIc	*L. acidophilus* M46	Food origin	(Leer et al., 1995)
Gassericin A[o]	IIc	*L. gasseri* LA39	Faeces (child)	(Kawai et al., 1998)
Leucocyclicin Q	IIc	*Leuc. mesenteroides* TK41401	Japanese pickles	(Masuda et al., 2011)
Acidocin 8912	IId	*L. acidophilus* TK8912	Dairy origin	(Tahara et al., 1992)
Brevicin 27	IId	*L. brevis* SB27	Sausages	(Benoit et al., 1997)
Lactocin MXJ 32A	IId	*L. coryniformis* MXJ 32	Fermented vegetables	(Lu et al., 2014)
Curvalicin BAP2	IId	*L. curvatus* CWBI-B28	Meat	(Ghalfi et al., 2010)
Curvaticin FS47	IId	*L. curvatus* FS47	Meat	(Garver and Muriana, 1994)
Sakacin Q (variant)[p]	IId	*L. curvatus* LTH1174	Fermented meat	(Cocolin and Rantsiou, 2007)
Bacteriocin SJ2-8	IId	*L. paracasei* BGSJ2-8	Home-made cheese	(Lozo et al., 2007)
Paracin C	IId	*L. paracasei* CICC 20241	Probiotic	(Pei et al., 2013)
Plantaricin 1.25 α	IId	*L. plantarum* TMW1.25	Fermented sausages	(Remiger et al., 1999)
Plantaricin 1.25 β	IId	*L. plantarum* TMW1.25	Fermented sausages	(Remiger et al., 1999)
Plantaricin 149	IId	*L. plantarum* NRIC 149	Pineapple	(Kato et al., 1994)
Plantaricin 163	IId	*L. plantarum* 163	Fermented vegetables	(Hu et al., 2013)
Plantaricin A	IId	*L. plantarum* C11	Fermented cucumber	(Nissen-Meyer et al., 1993)
Plantaricin ASM1	IId	*L. plantarum* A-1	Corn bread	(Hata et al., 2010)
Plantaricin JLA-9	IId	*L. plantarum* JLA-9	Suan-Tsai (Chinese fermented cabbage)	(Zhao et al., 2016)
Plantaricin ST31	IId	*L. plantarum* ST31	Sourdough	(Todorov et al., 1999)
Sakacin Q[q]	IId	*L. sakei* LTH673	Fermented dry sausage	(Mathiesen et al., 2005)
Salivaricin L	IId	*L. salivarius* DPC6488	Intestine (neonate)	(O'Shea et al., 2011)
Plantaricin Y	IId	*L. plantarum* 510	Koshu vineyard	(Chen et al., 2014)
Rhamnosin A	IId	*L. rhamnosus* 68	Intestinal microbiota (human)	(Dimitrijevic et al., 2009)
Bactofencin A	IId	*L. salivarius* DPC6502	Intestine (porcine)	(O'Shea et al., 2013)
Bacteriocin LS2	IId	*L. salivarius* BGHO1	Oral (human)	(Busarcevic and Dalgalarrondo, 2012)
Leucocin B	IId	*Leuc. mesenteroides* TA33a	Spoiled vacuum-packed meat	(Papathanasopoulos et al., 1997)
Mesentericin 52B[r]	IId	*Leuc. mesenteroides* FR52	Raw Milk	(Revol-Junelles et al., 1996)
Leucocin N	IId	*Leuc. pseudomesenteroides* QU 15	Nukadoko	(Sawa et al., 2010)
Leucocin Q	IId	*Leuc. pseudomesenteroides* QU 15	Nukadoko	(Sawa et al., 2010)
Weissellicin 110	IId	*Weissella cibaria* 110	Plaa-Som	(Srionnual et al., 2007)
Weissellicin L	IId	*W. hellenica* 4-7	Sian-sianzih	(Leong et al., 2013)
Weissellicin M	IId	*W. hellenica* QU 13	Pickel barrel	(Masuda et al., 2011)
Weissellicin Y	IId	*W. hellenica* QU 13	Pickel barrel	(Masuda et al., 2011)
Lactacin B[s]	-	*L. acidophilus* N2	Food origin	(Barefoot and Klaenhammer, 1983)
Bacteriocin TSU4	-	*L. animalis* TSU4	Intestine (fish)	(Sahoo et al., 2015)

Table 1. Continued

Bacteriocin	Subclass	Producing strain	Origin	Reference
Curvalicin BAP3	-	*L. curvatus* CWBI-B28	Meat	(Ghalfi et al., 2010)
Gassericin E	-	*L. gasseri* EV1461	Healthy vagina (human)	(Maldonado-Barragan et al., 2016)
Plantacin B	-	*L. plantarum* NCDO1193	Dairy origin	(West and Warner, 1988)
Plantaricin F	-	*L. plantarum* BF001	Spoiled cat fish filets	(Fricourt et al., 1994)
Plantaricn T	-	*L. plantarum* LPCO10	Green olives	(Jimenez-Diaz et al., 1993)
Bacteriocin SMXD51	-	*L. salivarius* SMXD51	Faeces (chicken)	(Messaoudi et al., 2012)
Salivaricin B	-	*L. salivarius* M7	Food origin	(Ten Brink et al., 1994)
Bacteriolysin				
Helveticin J		*L. helveticus* NCDO481	Dairy origin	(Joerger and Klaenhammer, 1986)

Characterised bacteriocins with identicle amino acid sequences: [a]Sakacina M/lactocin S from *L. sakei* 148 (Sobrino et al., 1992, Skaugen et al., 1997). [b]Varient of sakacin P from *L. curvatus* L442 (Cocolin and Rantsiou, 2007). [c]Sakacin 674 from *L. sakei* 674 (Holck et al., 1994). [d]Curvacin A from *L. curvatu*s LTH1174 (Tichaczek et al., 1993) and sakacin K from *L. sakei* CTC 494 (Hugas et al., 1995). [e]Bacteriocin R1333 from *Lb. sakei* R1333 (Todorov et al., 2011). [f]Sakacin X from *L. curvatus* 2711 (Hequet et al., 2007) and *L. curvatus* CRL705 (Hebert et al., 2012). [g]Leucocin A-TA33a from *Leuonostoc mesenteroides* TA33a (Papathanasopoulos et al., 1997) and Leucocin B-Ta11a from *Leuc. carnosum* Ta11a (Felix et al., 1994). [h]Leucocin A-4010 and Lecucocin B-4010 from *Leuc. carnosum* 4010 (Budde et al., 2003). [i]Also produced by *L. plantarum* WHE92 (Ennahar et al., 1996). [j]Amilovorin L471 from *L. amylovorus* DCE471 (De Vuyst et al., 2004). [k]Gassericin K7 (K7A y K7B) from *L. gasseri* K7 (Peternel et al., 2010). [l]Lactacin F from *L. acidophilus* 30SC (Oh et al., 2011).[m]Sakacin T (SakTα and SakTβ) from *L. sakei* 5 (Vaughan et al., 2003), *L. curvatus* 2711 (Hequet et al., 2007) and *L. curvatus* CRL705 (Hebert et al., 2012). [n]Also produced by *L. pentosus* B96 (Hurtado et al., 2011). [p]Reutericin 6 from *L. reuteri* LA6 (Kawai et al., 2001).[p]Varient of sakacin Q from *L. curvatu*s L442 (Cocolin and Rantsiou, 2007) and *L. curvatus* CRL705 (Hebert et al., 2012). [q]Sakacin Q from *L. sakei* Lb674 (Mathiesen et al., 2005) and sakacin Q from *L. curvatus* CRL705 (Hebert et al., 2012). [r]Mesentericin B105 from *Leuc. mesenteroides* subsp. *mesenteroides* Y105 (Héchard et al., 1999). [s]Acidocin J1132 from *L. acidophilus* JCM1132 (Tahara et al., 1996). The - symbol represents bacteriocins which were difficult to accurately classify based on information retrieved.

Table 2. Spectrum of inhibition of bacteriocin producing strains against a range of indicator strains

Bacteriocin Producers	Strain (DSM)	Activity of Bacteriocin Producers vs. Indicator Organisms*								
		L. delbrueckii subsp. *bulgaricus*	*L. delbrueckii* subsp. *lactis*	*L. amylovorus*	*L. casei*	*L. plantarum*	*L. rhamnosus*	*Listeria innocua*	*Enterococcus saccharolyticus*	*E. mundtii*
L. paralimentarius	13961				++	+++	++		+	+++
L. murinus	20452	+								
L. hordei	19519	++	++			++		+++	+++	+++
L. intestinalis	6629	+++		+						
L. paracasei subsp. *paracasei*	5622	+	+							
L. acidophilus	20079	++	++	+					+	
L. agilis	20509							+		
L. crispatus	20584	++	+	+						
L. equicursoris	19284	++								
L. pentosus	20314	+								
L. kalixensis	16043	+								
L. amylovorus	20531	+								
L. kitasatonis	16761	++								
P. damnosus	20331	+++								
C. maltaromaticum	20342	+	+							
C. maltaromaticum	20722	++	+					+	++	

Activity of pH neutralised cell free supernatants from bacteriocin producers in agar well diffusion assay. Inhibition of indicators is described in radius (mm) of the zone of inhibition in WDA, scores are as follows: + = 0.5 – 2mm, ++ = 2.5 – 5mm, +++ = >5mm

Table 3. Potential Lantibiotic/Lantipeptide Structural Peptides

Species	Strain	Potential Unmodified Lantibiotic/Lantipeptide Sequence
L. taiwanensis	DSM 21401	TSTGCCNGPSKLQG
L. amylovorus	DSM 20531	AKSYSAYSSCSCVNPPCPIATMD
L. gastricus	DSM 16045	GTETAQSTPAISRVTLSIARKSSAKCISWISFSAGGLNSYKSKC
P. damnosus (Pediocin PD-1)	DSM 20331	KKIKKSSSGDICTLTSECDHLATWVCC

Table 4 (a). Structural Genes for Complete Class IIa Operons

Species	Strain	Structural Peptide	Homolog (%Amino Acid Identity)
L. hordei	DSM 19519	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC	Coagulin (100%)
		KYYGNGVSCTKKHGCKVNWGQAFTCSVNRFANFGHGNC	Plantaricin 423 (74%)
L. acidipiscis	DSM 15836	KYYGNGLHIPKHGKPYINWGQAIQSIGKISYHGWVNGITSGAAGVGRH	Hiracin JM79 (44%)
L. futsaii	JCM 17355	KYYGNGVSCGKHTCKVNWGQAWNESVNRWGNSWVNGLTGLRQH	Plantaricin 423 (57%)
C. maltaromaticum	DSM 20722	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	Carnobacteriocin cbn BM1 (100%)
		VYYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	Carnobacteriocin cbn B2 (98%)

(b) Structural Genes for Incomplete Class IIa Operons

Species	Strain	Structural Peptide	Homolog (%Amino Acid Identity)
L. agilis	DSM 20509	SRYYGNGITCGKHKCTVNWGQAWTCGVNRLANFGHGNC	Plantaricin 423 (73%)
L. aquaticus	DSM 21051	KNYGNGVYCTKKHGYKVDWGQAWSIIGNNSAANSTTRGAAGWKSK	Avicin A (74%)
L. rennini	DSM 20253	KYYGNGVSCSKHSCSVDWGKALTCTINNGAMAWTTGGHQGNHKC	Pediocin Ach/PA-1 (89%)
L. ruminis	DSM 20403	KYYGNGVYCGKHKCRVDWGQAWGCSVNRWGAAVGTGGKATIGHC	Pediocin Ach/PA-1 (55%)
P. pentosaceus	DSM 20336	KYYGNGLYCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC	Pediocin Ach/PA-1 (93%)
C. maltaromaticum	DSM 20342	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	Carnobacteriocin cbn BM1 (100%)

Table 5. Potential Class IIb Structural Genes

Species	Strain	Structural Peptide		
L. murinus	DSM 20452	YNRLAGQIGHYTGKAVIVGATVLGIASLF KRGLGYHIVDAVVSFGKGFLDAF	Produced *in vitro*	(Muricidin)
		YDIEKALWGGYGYQLGWRNKWNLSHRYFKI GVPGWYYGMLWKIGVSGYKHRKDIMNGFDRGFNNYPK		
L. acidophilus	DSM 20079	SNNIFWTRVGVGWAAEARCMIKPSLGNWTTKAVSCGAKGLYAAVRG VAPIVYPIAGYVMKQMFEHSDQIIKGFKRGWKKYK	Produced *in vitro*	(Acidocin X)
L. taiwanensis	DSM 21401	NRWGDTVLSAASGAGTGIKACKSFGPWGMAICGSNRRLFWLYS RNNWQTNVGGAVGSAMIGATVGGTICGPACAVAGAHYLPILWTGVTAATGGFGKIRK		
L. crispatus	DSM 20584	NRWTNAYSAALGCAVPGVKYGKKLGGVWGAVIGGVGGAAVCGLAGYVRKG SKGKGRNNWAGNTIGIVSSAATGAALGSAICGPGCGFVGAHWGAVGWTAVASFSGAFGKIRK		
L. nantensis	DSM 16982	SFKGFVQGFINGLTGKKH KGPWNYKTGYNLGKWISKRF		
L. apodemi	DSM 16634	YDIEKALWKGYGYQLGWRSKWNLSHRYFKI GVPGWYYSMLWKIGVSGYKHRKDIMSGFDKGFNNYPK		
L. plantarum	DSM 13273	RRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK GAWKNFWSSLRKGFYDGEAGRAIRR		
		FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG		
L. plantarum subsp. *plantarum*	CGMCC 1.2437	RRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK GAWKNFWSSLRKGFYDGEAGRAIRR		
		FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG		
L. paraplantarum	DSM 10667	FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG		
L. intestinalis	DSM 6629	RHSVPYSYGYQSGRGFKGAAAAYNIIKTVASFFE KRKKHHPWYWSIQEFGRGFLAGLASKYNL		
L. rhamnosus	DSM 20021	IGPLAIPVAAILGFLATDAWSHADELVAGVKQGWERS DNGNLWTFIGKAIGSTARSWAEGAMFAPAIGPAKEIVDKLNGN		
L. zeae	DSM 20178	NAWGNAVNGALNGAATGARFGKNLGPWGMIGGMALGAGIGGYFGYNG RNTWQQNVSGVAGAAAGGAALGAVVGGPAGAFLGAHYGPILWTAVTGFTGGF		
Leuc. fallax	KCTC 3537	CPLLPIVVTVAASGAHFVAKDGWNHLDQIRSGWRKSGNSKW STDGSWEDFGAGLHKTVNTVIYAGTTVARAHTRSHQRCFTGNKW		

Table 6. Alignment of Class IIc Subgroup II Bacteriocins

Bacteriocin	Structural Peptide
Gassericin A	IYWIADQFGIHLATGTARKLLDAMASGASLGTAFAAILGVTLPAWALAAAGALGATAA
Acidocin B	IYWIADQFGIHLATGTARKLLDAVASGASLGTAFAAILGVTLPAWALAAAGALGATAA
Butyrivibriocin AR10	IYFIADKMGIQLAPAWYQDIVNWVSAGGTLTTGFAIIVGVTVPAWIAEAAAAFGIASA
L. paracesei subsp. *paracasei* DSM 5622 (Paracyclicin)	IYFIANKLGIHLAPGWYQDMVNYVSAGGSLAGAFSVVAGVTLPAWIVPIATAFGAVSA
L. nodensis DSM 19682	-IWIAGLFGIHLDNSLESKLVSGILNGGSAAGVFAAMLGITLPAWAAAAATAMGATAA
	:** :**:* . .::. : *.: *: : *:*:*** * *:* .:*

* = Positions with a single conserved residue

: = Conservation between groups with strongly similar properties, scoring > 0.5 in the Gonnet PAM 250 matrix

. = Conservation between groups with weakly similar properties, scoring ≤ 0.5 in the Gonnet PAM 250 matrix

Table 7. Potential Class IId Structural proteins

Species	Strain	Structural Protein
L. paralimentarius	DSM 13961	NFFGGSNGYSWRDKKGHWHYTVTSGVSSTVAQIIGNGWGSAGAPGVGQR
L. pentosus	DSM 20314	KSNTYSLQMGSVVRTATKIFKKMEW
L. hokkaidonensis	DSM 26202	VTLSVATHSKNGLKKFFKWVRKL
L. xiangfangensis	LMG 26013	KLVKLYTAEPYTFYRDTRTKKIVMRQTTGYSAHLQHVIADGWVRSAHL
L. paracasei	DSM 5622	DSIRDVSPTFNKIRRWFDGLFK
L. murinus	DSM 20452	YDIEKALWGGYGYQLGWRNKWNLSHRYFKI
Leuc. kimchii	IMSNU 11154	KSFWSWASDASSWLSGPQQPNSPLLKKKR
Leuc. geldium	KCTC 3527	KRVYIPNGNGAWLDSNTGKGGVDWNVAVPALGSIMVNGWAQNGPLAHLHP

(b) Potential Class IId Lactococcin 972 Homologs

Species	Strain	Structural Protein
L. equicursoris (equicursorin)	DSM 19284	GGTWNYGVGSKYVWSYYSHNSKTHKASVEGKYYVTSGWIKEKTQARASAAKAAAGNQSYYDVK
L. amylophilus	DSM 20533	GGTWNYGVGLTGTFGYSDYLHNSKTHSASVGRTKSDCNKVTKTKGVWAQSKYTKIPPTGLNYWWSVS
L. graminis	DSM 20719	GGTWYSGFSGTKVYSQYYHGSKKHSATAKNGWGAGVRNTQKAGIWAYSSVNSTLTGNKTYWAVY
L. hamsteri	DSM 5661	GGVWNYGVGKKYVWSYYSHHRLTHKSSVEGKYYSSSGWVSPGTEARASAEKAQHGNKSYFDVE
Leuc. argentinum	KCTC 3773	GGDWRHGVGSYYVWSYYFHNYRNHSSSVSGQYFASSGRTSPGYDAQASAPKSLFGNKAYYDFW

Supplementary Table 1. Source of Strains

Species Name	StrainID	Source	Antimicrobial Encoded
Lactobacillus agilis	DSM-20509	Environment	
Lactobacillus aquaticus	DSM-21051	Environment	
Lactobacillus concavus	DSM-17758	Environment	
Lactobacillus coryniformis torquens	DSM-20004	Environment	
Lactobacillus paracollinoides	DSM-15502	Environment	
Lactobacillus sharpeae	DSM-20505	Environment	
Weissella kandleri	DSM-20593	Environment	
Carnobacterium divergens	DSM-20623	Food	
Carnobacterium maltaromaticum	DSM-20342	Food	
Carnobacterium maltaromaticum	DSM-20722	Food	Yes
Lactobacillus acetotolerans	DSM-20749	Food	
Lactobacillus acidifarinae	DSM-19394	Food	
Lactobacillus acidipiscis	DSM-15836	Food	Yes
Lactobacillus acidipiscis	DSM-15353	Food	
Lactobacillus algidus	DSM-15638	Food	
Lactobacillus alimentarius	DSM-20249	Food	
Lactobacillus bifermentans	DSM-20003	Food	
Lactobacillus capillatus	DSM-19910	Food	
Lactobacillus casei	DSM-20011	Food	
Lactobacillus collinoides	DSM-20515	Food	
Lactobacillus crustorum	LMG-23699	Food	
Lactobacillus crustorum	JCM-15951	Food	
Lactobacillus curvatus	DSM-20019	Food	
Lactobacillus delbrueckii bulgaricus	DSM-20081	Food	
Lactobacillus delbrueckii delbrueckii	DSM-20074	Food	
Lactobacillus delbrueckii indicus	DSM-15996	Food	
Lactobacillus delbrueckii lactis	DSM-20072	Food	
Lactobacillus farciminis	DSM-20184	Food	
Lactobacillus frumenti	DSM-13145	Food	
Lactobacillus fuchuensis	DSM-14340	Food	
Lactobacillus futsaii	JCM-17355	Food	Yes
Lactobacillus hammesii	DSM-16381	Food	
Lactobacillus harbinensis	DSM-16991	Food	
Lactobacillus helveticus	CGMCC-1.1877	Food	Yes
Lactobacillus kefiranofaciens kefiranofaciens	DSM-5016	Food	
Lactobacillus kefiranofaciens kefirgranum	DSM-10550	Food	Yes
Lactobacillus kefiri	DSM-20587	Food	
Lactobacillus kimchicus	JCM-15530	Food	
Lactobacillus kimchiensis	DSM-24716	Food	
Lactobacillus kisonensis	DSM-19906	Food	
Lactobacillus koreensis	JCM-16448	Food	
Lactobacillus mindensis	DSM-14500	Food	

Supplementary Table 1. Continued

Lactobacillus namurensis	DSM-19117	Food	
Lactobacillus nantensis	DSM-16982	Food	Yes
Lactobacillus nodensis	DSM-19682	Food	Yes
Lactobacillus odoratitofui	DSM-19909	Food	
Lactobacillus otakiensis	DSM-19908	Food	
Lactobacillus panis	DSM-6035	Food	
Lactobacillus parabrevis	LMG-11984	Food	
Lactobacillus parabrevis	ATCC-53295	Food	
Lactobacillus paracasei tolerans	DSM-20258	Food	
Lactobacillus parakefiri	DSM-10551	Food	
Lactobacillus paralimentarius	DSM-13238	Food	
Lactobacillus paralimentarius	DSM-13961	Food	Yes
Lactobacillus perolens	DSM-12744	Food	
Lactobacillus plantarum plantarum	CGMCC-1.2437	Food	Yes
Lactobacillus pontis	DSM-8475	Food	
Lactobacillus rapi	DSM-19907	Food	
Lactobacillus rossiae	DSM-15814	Food	
Lactobacillus sakei carnosus	DSM-15831	Food	
Lactobacillus sanfranciscensis	DSM-20451	Food	
Lactobacillus secaliphilus	DSM-17896	Food	
Lactobacillus selangorensis	DSM-13344	Food	
Lactobacillus selangorensis	ATCC-BAA-66	Food	
Lactobacillus senmaizukei	DSM-21775	Food	
Lactobacillus siliginis	DSM-22696	Food	
Lactobacillus spicheri	DSM-15429	Food	
Lactobacillus suebicus	DSM-5007	Food	
Lactobacillus sunkii	DSM-19904	Food	
Lactobacillus tucceti	DSM-20183	Food	
Lactobacillus versmoldensis	DSM-14857	Food	
Lactobacillus xiangfangensis	LMG-26013	Food	
Lactobacillus zymae	DSM-19395	Food	
Lactococcus lactis	LMG-7760	Food	
Leuconostoc argentinum	KCTC-3773	Food	Yes
Leuconostoc carnosum	JB16	Food	
Leuconostoc citreum	KM20	Food	
Leuconostoc fallax	KCTC-3537	Food	Yes
Leuconostoc gasicomitatum	LMG-18811	Food	
Leuconostoc gelidum	KCTC-3527	Food	Yes
Leuconostoc kimchii	IMSNU-11154	Food	
Leuconostoc mesenteroides	ATCC-8293	Food	
Leuconostoc pseudomesenteroides	4882	Food	
Pediococcus argentinicus	DSM-23026	Food	
Weissella halotolerans	DSM-20190	Food	
Weissella minor	DSM-20014	Food	
Weissella viridescens	DSM-20410	Food	

Supplementary Table 1. Continued

Atopobium minutum	DSM-20586	Human/Animal	
Atopobium rimae	DSM-7090	Human/Animal	
Carnobacterium maltaromaticum	DSM-20730	Human/Animal	Yes
Kandleria vitulina	DSM-20405	Human/Animal	Yes
Lactobacillus acidophilus	DSM-20079	Human/Animal	Yes
Lactobacillus amylophilus	DSM-20533	Human/Animal	Yes
Lactobacillus amylotrophicus	DSM-20534	Human/Animal	
Lactobacillus amylovorus	DSM-20531	Human/Animal	Yes
Lactobacillus amylovorus	DSM-16698	Human/Animal	Yes
Lactobacillus animalis	DSM-20602	Human/Animal	
Lactobacillus antri	DSM-16041	Human/Animal	
Lactobacillus apodemi	DSM-16634	Human/Animal	Yes
Lactobacillus aviarius araffinosus	DSM-20653	Human/Animal	
Lactobacillus aviarius aviarius	DSM-20655	Human/Animal	
Lactobacillus brantae	DSM-23927	Human/Animal	
Lactobacillus brevis	DSM-20054	Human/Animal	
Lactobacillus ceti	DSM-22408	Human/Animal	
Lactobacillus coleohominis	DSM-14060	Human/Animal	
Lactobacillus crispatus	DSM-20584	Human/Animal	Yes
Lactobacillus equi	DSM-15833	Human/Animal	
Lactobacillus equicursoris	DSM-19284	Human/Animal	Yes
Lactobacillus equigenerosi	DSM-18793	Human/Animal	
Lactobacillus fermentum	DSM-20055	Human/Animal	
Lactobacillus gallinarum	DSM-10532	Human/Animal	Yes
Lactobacillus gasseri	ATCC-33323	Human/Animal	
Lactobacillus gastricus	DSM-16045	Human/Animal	Yes
Lactobacillus gigeriorum	DSM-23908	Human/Animal	Yes
Lactobacillus hamsteri	DSM-5661	Human/Animal	Yes
Lactobacillus hayakitensis	DSM-18933	Human/Animal	
Lactobacillus hominis	DSM-23910	Human/Animal	Yes
Lactobacillus iners	DSM-13335	Human/Animal	
Lactobacillus ingluviei	DSM-15946	Human/Animal	
Lactobacillus ingluviei	DSM-14792	Human/Animal	
Lactobacillus intestinalis	DSM-6629	Human/Animal	Yes
Lactobacillus jensenii	DSM-20557	Human/Animal	
Lactobacillus johnsonii	ATCC-33200	Human/Animal	Yes
Lactobacillus kalixensis	DSM-16043	Human/Animal	Yes
Lactobacillus kitasatonis	DSM-16761	Human/Animal	Yes
Lactobacillus mucosae	DSM-13345	Human/Animal	
Lactobacillus murinus	DSM-20452	Human/Animal	Yes
Lactobacillus oligofermentans	DSM-15707	Human/Animal	
Lactobacillus oris	DSM-4864	Human/Animal	
Lactobacillus pantheris	DSM-15945	Human/Animal	
Lactobacillus parabuchneri	DSM-5707	Human/Animal	Yes
Lactobacillus psittaci	DSM-15354	Human/Animal	
Lactobacillus rennini	DSM-20253	Human/Animal	

Supplementary Table 1. Continued

Lactobacillus reuteri	DSM-20016	Human/Animal	
Lactobacillus ruminis	DSM-20403	Human/Animal	
Lactobacillus saerimneri	DSM-16049	Human/Animal	
Lactobacillus salivarius	DSM-20555	Human/Animal	
Lactobacillus saniviri	DSM-24301	Human/Animal	
Lactobacillus senioris	DSM-24302	Human/Animal	
Lactobacillus ultunensis	DSM-16047	Human/Animal	Yes
Lactobacillus vaccinostercus	DSM-20634	Human/Animal	
Lactobacillus vaginalis	DSM-5837	Human/Animal	
Olsenella uli	DSM-7084	Human/Animal	
Fructobacillus fructosus	DSM-20349	Plant	
Lactobacillus buchneri	DSM-20057	Plant	
Lactobacillus cacaonum	DSM-21116	Plant	
Lactobacillus camelliae	DSM-22697	Plant	
Lactobacillus coryniformis coryniformis	DSM-20001	Plant	
Lactobacillus dextrinicus	DSM-20335	Plant	
Lactobacillus diolivorans	DSM-14421	Plant	
Lactobacillus fabifermentans	DSM-21115	Plant	
Lactobacillus floricola	DSM-23037	Plant	
Lactobacillus florum	DSM-22689	Plant	
Lactobacillus ghanensis	DSM-18630	Plant	
Lactobacillus graminis	DSM-20719	Plant	Yes
Lactobacillus hokkaidonensis	DSM-26202	Plant	Yes
Lactobacillus hordei	DSM-19519	Plant	Yes
Lactobacillus manihotivorans	DSM-13343	Plant	
Lactobacillus nasuensis	JCM-17158	Plant	
Lactobacillus ozensis	DSM-23829	Plant	
Lactobacillus plantarum	DSM-13273	Plant	Yes
Lactobacillus plantarum argentoratensis	DSM-16365	Plant	
Lactobacillus pobuzihii	NBRC-103219	Plant	
Lactobacillus pobuzihii.Chen	KCTC-13174	Plant	
Lactobacillus sucicola	DSM-21376	Plant	
Lactobacillus taiwanensis	DSM-21401	Plant	Yes
Lactobacillus thailandensis	DSM-22698	Plant	
Lactobacillus uvarum	DSM-19971	Plant	
Lactobacillus vini	DSM-20605	Plant	
Pediococcus lolii	DSM-19927	Plant	
Pediococcus parvulus	DSM-20332	Plant	
Pediococcus stilesii	DSM-18001	Plant	
Weissella confusa	DSM-20196	Plant	
Lactobacillus fructivorans	DSM-20203	Unknown	
Lactobacillus paracasei paracasei	DSM-5622	Unknown	Yes
Lactobacillus pasteurii	DSM-23907	Unknown	Yes
Lactobacillus pentosus	DSM-20314	Unknown	Yes
Lactobacillus rhamnosus	DSM-20021	Unknown	Yes
Leuconostoc mesenteroides cremoris	ATCC-19254	Unknown	

Supplementary Table 1. Continued

Pediococcus acidilactici	AS1-2696	Unknown	
Lactobacillus amylolyticus	DSM-11664	Wine/Alcohol Products	Yes
Lactobacillus composti	DSM-18527	Wine/Alcohol Products	
Lactobacillus delbrueckii jakobsenii	DSM-26046	Wine/Alcohol Products	
Lactobacillus farraginis	DSM-18382	Wine/Alcohol Products	Yes
Lactobacillus fructivorans	ATCC-27394	Wine/Alcohol Products	
Lactobacillus fructivorans	DSM-20350	Wine/Alcohol Products	
Lactobacillus helveticus	LMG-22464	Wine/Alcohol Products	Yes
Lactobacillus hilgardii	DSM-20176	Wine/Alcohol Products	
Lactobacillus homohiochii	DSM-20571	Wine/Alcohol Products	
Lactobacillus kunkeei	DSM-12361	Wine/Alcohol Products	
Lactobacillus lindneri	DSM-20690	Wine/Alcohol Products	
Lactobacillus malefermentans	DSM-5705	Wine/Alcohol Products	
Lactobacillus mali	DSM-20444	Wine/Alcohol Products	
Lactobacillus mali	ATCC-27304	Wine/Alcohol Products	
Lactobacillus nagelii	DSM-13675	Wine/Alcohol Products	
Lactobacillus oeni	DSM-19972	Wine/Alcohol Products	
Lactobacillus parabuchneri	DSM-15352	Wine/Alcohol Products	
Lactobacillus parafarraginis	DSM-18390	Wine/Alcohol Products	Yes
Lactobacillus paralimentarius	DSM-19674	Wine/Alcohol Products	
Lactobacillus paraplantarum	DSM-10667	Wine/Alcohol Products	Yes
Lactobacillus paucivorans	DSM-22467	Wine/Alcohol Products	
Lactobacillus sakei sakei	DSM-20017	Wine/Alcohol Products	
Lactobacillus satsumensis	DSM-16230	Wine/Alcohol Products	
Lactobacillus similis	DSM-23365	Wine/Alcohol Products	
Lactobacillus zeae	DSM-20178	Wine/Alcohol Products	Yes
Oenococcus kitaharae	DSM-17330	Wine/Alcohol Products	
Oenococcus oeni	ATCC-BAA-1163	Wine/Alcohol Products	Yes
Pediococcus cellicola	DSM-17757	Wine/Alcohol Products	
Pediococcus claussenii	DSM-14800	Wine/Alcohol Products	Yes
Pediococcus damnosus	DSM-20331	Wine/Alcohol Products	Yes
Pediococcus ethanolidurans	DSM-22301	Wine/Alcohol Products	
Pediococcus inopinatus	DSM-20285	Wine/Alcohol Products	
Pediococcus pentosaceus	DSM-20336	Wine/Alcohol Products	

Supplementary Table 2. Bacterial strains screened for bacteriocin production

Species	Strain	Growth Medium	Condition	Temp (˚C)
Carnobacterium maltaromaticum	DSM 20342	TSA	Aerobic	30
Carnobacterium maltaromaticum	DSM 20722	TSA	Aerobic	30
Carnobacterium maltaromaticum	DSM 20730	TSA	Aerobic	30
Lactobacillus acidipiscis	DSM 15836	MRS + Vitamin soln.	Anaerobic	30
Lactobacillus acidophilus	DSM 20079	mMRS	Anaerobic	37
Lactobacillus agilis	DSM 20509	mMRS	Anaerobic	38
Lactobacillus amylophilus	DSM 20533	MRS	Aerobic	30
Lactobacillus amylovorus	DSM 16698	MRS	Anaerobic	37
Lactobacillus amylovorus	DSM 20531	MRS	Anaerobic	37
Lactobacillus apodemi	DSM 16634	MRS	Anaerobic	37
Lactobacillus aquaticus	DSM 21051	MRS	Anaerobic	37
Lactobacillus buchneri	DSM 20057	MRS	Aerobic	37
Lactobacillus casei	DSM 20011	MRS	Aerobic	30
Lactobacillus composti	DSM 18527	MRS	Anaerobic	30
Lactobacillus coryniformis subsp. *coryniformis*	DSM 20001	MRS	Aerobic	30
Lactobacillus coryniformis subsp. *torquens*	DSM 20004	MRS	Aerobic	30
Lactobacillus crispatus	DSM 20584	mMRS	Anaerobic	37
Lactobacillus equicursoris	DSM 19284	MRS	Anaerobic	37
Lactobacillus fabifermentans	DSM 21115	MRS	Anaerobic	30
Lactobacillus fuchuensis	DSM 14340	MRS	Aerobic	20
Lactobacillus futsaii	JCM 17355	MRS	Aerobic	30
Lactobacillus gastricus	DSM 16045	MRS	Anaerobic	37
Lactobacillus graminis	DSM 16045	MRS	Aerobic	30
Lactobacillus hamsteri	DSM 5661	MRS	Anaerobic	37
Lactobacillus harbinensis	DSM 16991	MRS	Anaerobic	37
Lactobacillus helveticus	CGMCC 1.1877	MRS	Anaerobic	37
Lactobacillus helveticus	LMG 22464	MRS	Anaerobic	37
Lactobacillus hokkaidonensis	DSM 26202	MRS	Anaerobic	25
Lactobacillus hordei	DSM 19519	MRS	Anaerobic	30
Lactobacillus intestinalis	DSM 6629	MRS	Aerobic	37
Lactobacillus johnsoni	DSM 10533	MRS	Anaerobic	37
Lactobacillus kalixensis	DSM 16043	MRS	Aerobic	37
Lactobacillus kimchicus	JCM 15530	MRS	Aerobic	37

Supplementary Table 2. Continued

Species	Strain	Growth Medium	Condition	Temp (˚C)
Lactobacillus kimchiensis	DSM 24716	MRS	Anaerobic	25
Lactobacillus kitasatonis	DSM 16761	MRS	Anaerobic	37
Lactobacillus mali	DSM 20444	MRS	Aerobic	30
Lactobacillus mindensis	DSM 14500	MRS	Anaerobic	30
Lactobacillus murinus	DSM 20452	MRS	Aerobic	37
Lactobacillus nantensis	DSM 16982	mMRS + 1% maltose + 0.5% YE	Aerobic	30
Lactobacillus nodensis	DSM 19682	MRS	Anaerobic	30
Lactobacillus otakiensis	DSM 19908	MRS	Anaerobic	30
Lactobacillus parabuchneri	DSM 5707	MRS	Aerobic	30
Lactobacillus paracasei subsp. *paracasei*	DSM 5622	MRS	Aerobic	30
Lactobacillus paracasei subsp. *tolerans*	DSM 20258	MRS	Aerobic	30
Lactobacillus parafarraginis	DSM 18390	MRS	Anaerobic	30
Lactobacillus paralimentarius	DSM 13961	MRS	Aerobic	30
Lactobacillus paralimentarius	DSM 13238	MRS	Aerobic	30
Lactobacillus paraplantarum	DSM 10667	MRS	Aerobic	30
Lactobacillus pasteurii	DSM 23907	MRS	Anaerobic	37
Lactobacillus pentosus	DSM 20314	MRS	Aerobic	30
Lactobacillus plantarum	DSM 13273	MRS	Aerobic	37
Lactobacillus plantarum subsp. *argentoratensis*	DSM 16365	MRS	Anaerobic	30
Lactobacillus plantarum subsp. *plantarum*	DSM 20174	MRS	Aerobic	30
Lactobacillus rennini	DSM 20253	MRS + CAA	Aerobic	30
Lactobacillus reuteri	DSM 20016	MRS	Aerobic	37
Lactobacillus rhamnosus	DSM 20021	MRS	Aerobic	37
Lactobacillus rossiae	DSM 15814	MRS + 1% maltose + 1% YE	Anaerobic	30
Lactobacillus ruminis	DSM 20403	MRS	Anaerobic	37
Lactobacillus similis	DSM 23365	MRS	Anaerobic	37
Lactobacillus taiwanensis	DSM 21401	MRS	Anaerobic	37
Lactobacillus xiangfangensis	LMG 26013	MRS	Aerobic	30
Lactobacillus zeae	DSM 20178	MRS	Aerobic	37
Leuconostoc fallax	DSM 20189	MRS	Aerobic	30
Leuconostoc gasicomitatum	LMG 18811	MRS	Anaerobic	22
Leuconostoc gelidum subsp. *gelidum*	DSM 5578	MRS	Anaerobic	25
Leuconostoc lactis	DSM 8581	MRS	Aerobic	30

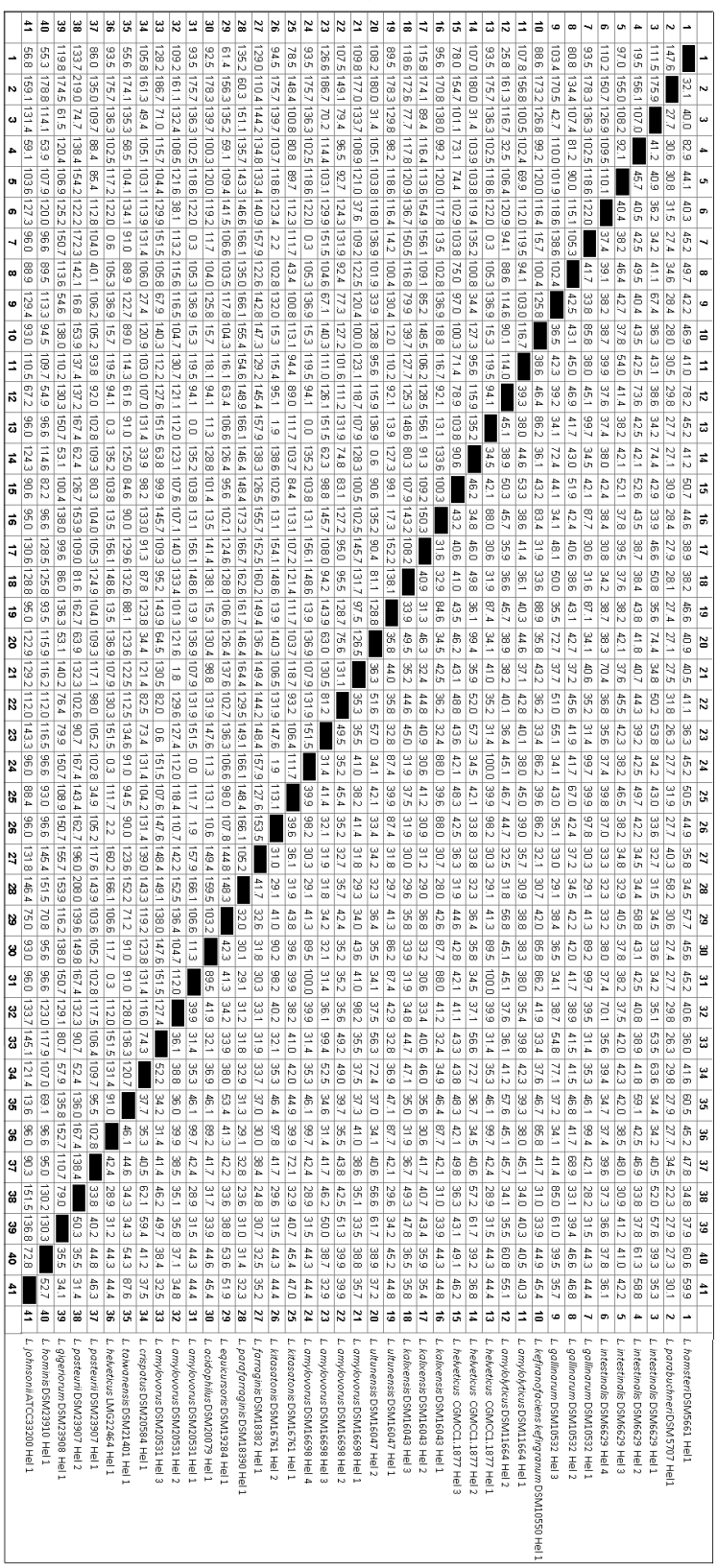
Supplementary Table 2. Continued

Species	Strain	Growth Medium	Condition	Temp (˚C)
Oenococcus kitaharae	DSM 17330	mMRS + 10% tomato juice	Anaerobic	30
Oenococcus oeni	ATCC-BAA 1163	MRS	Anaerobic	37
Pediococcus cellicola	DSM 17757	MRS	Anaerobic	30
Pediococcus claussenii	DSM 14800	MRS pH 5.7	Aerobic	30
Pediococcus damnosus	DSM 20331	mMRS pH 5.7	Anaerobic	26
Pediococcus ethanolidurans	DSM 22301	MRS	Anaerobic	37
Pediococcus pentosaceus	DSM 20336	MRS	Aerobic	30
Pediococcus stilesii	DSM 18001	MRS	Anaerobic	30

Supplementary Table 3. Indicator Strains

Species	Strain	Growth Medium	Conditions	Temp (˚C)
Enterococcus faecium	LMG 11423	MRS	Anaerobic	37
E. mundtii	LMG 10748	MRS	Anaerobic	37
E. saccharolyticus	LMG 11427	MRS	Anaerobic	37
Lactobacillus acidophilus	LMG 9433	MRS	Anaerobic	37
L. agilis	LMG 9186	MRS	Anaerobic	37
L. amylovorus	LMG 9496	MRS	Anaerobic	37
L. casei	LMG 6904	mMRS	Anaerobic	37
L. crispatus	LMG 9479	MRS	Anaerobic	37
L. delbrueckii subsp. *bulgaricus*	LMG 6901	MRS	Anaerobic	37
L. delbrueckii subsp. *lactis*	LMG 7942	MRS	Anaerobic	37
L. fermentum	LMG 6902	MRS	Anaerobic	37
L. johnsonii	DSM 10533	MRS	Anaerobic	37
L. plantarum	LMG 6907	MRS	Anaerobic	37
L. rhamnosus	GG	MRS	Anaerobic	37
Listeria innocua	DPC 3572	BHI	Aerobic	37
Micrococcus luteus	DPC 6275	BHI	Aerobic	30
Staphylococcus aureus	DPC 5246	BHI	Aerobic	37
Salmonlella typhimurium	LT2	BHI	Aerobic	37

Supplementary Figure 1. Divergence and percentage identity of helveticin homologs encoded by *Lactobacillus* species (Clustal ω)



Supplementary Figure 2. Colony MALDI TOF MS and SDS PAGE profiles of the peptides produced by the cells indicate the mass and identity of each bacteriocin produced

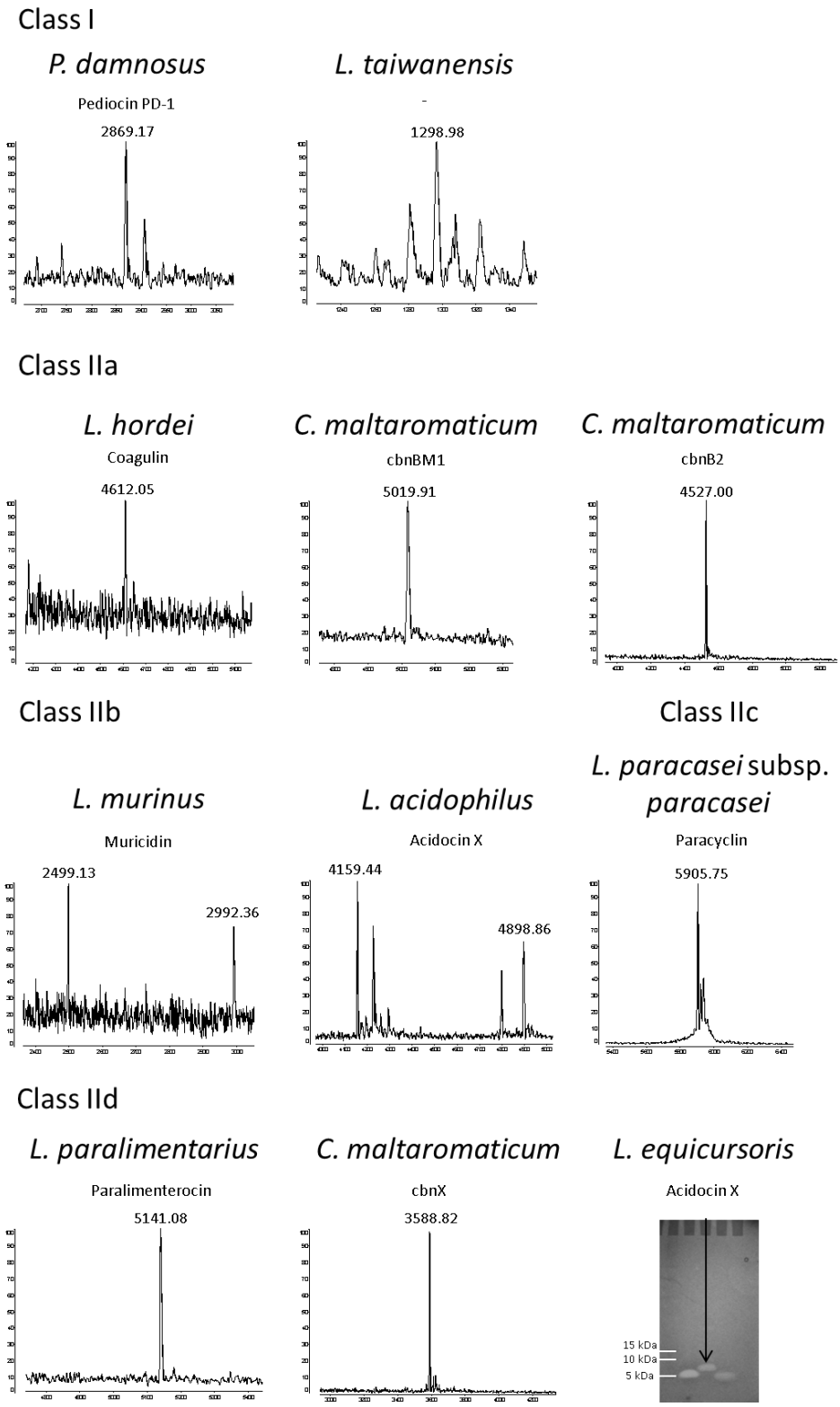
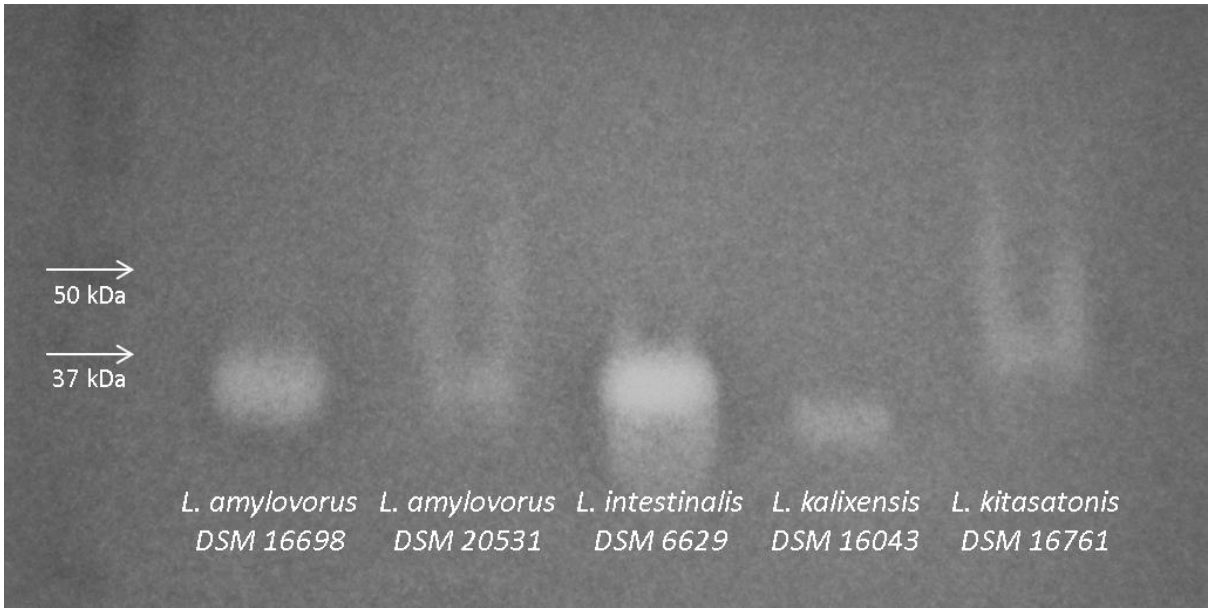


Figure 3. An SDS PAGE gel overlayed with the indicator *L. delbrueckii* subsp. *bulgaricus* shows the mass of the antimicrobial helveticin-like peptides produced by these lactobacilli.



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Chapter 4: Reincarnation of Bacteriocins from the *Lactobacillus* Pangenomic Graveyard

Mass spectrometry and peptide purification was carried out by Ms. Paula O'Connor, vector design and heterologous expression in *Lactobacillus* host was carried out by Dr. Beatriz Mesa Pereira

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Abstract

Bacteria commonly produce narrow spectrum bacteriocins as a means of inhibiting closely related species competing for similar resources in an environment. The increasing availability of genomic data means that it is becoming easier to identify bacteriocins encoded within genomes. Often, however, the presence of bacteriocin genes in a strain does not always translate into biological antimicrobial activity. For example, when analysing the *Lactobacillus* pangenome we identified strains encoding ten pediocin-like bacteriocin structural genes which failed to display inhibitory activity. Nine of these bacteriocins were novel whilst one was identified as the previously characterised bacteriocin 'penocin A'. The composition of these bacteriocin operons varied between strains, often with key components missing which are required for bacteriocin production, such as dedicated bacteriocin transporters and accessory proteins. In an effort to functionally express these bacteriocins, the structural genes for the ten pediocin homologs were cloned alongside the dedicated pediocin PA-1 transporter in both *Escherichia coli* and *Lactobacillus paracasei* heterologous hosts. Each bacteriocin was cloned with its native leader sequence and as a fusion protein with the pediocin PA-1 leader sequence. Several of these bacteriocins displayed a broader spectrum of inhibition than the original pediocin PA-1. We show how potentially valuable bacteriocins can easily be 'reincarnated' from *in silico* data and produced *in vitro* despite often lacking the necessary accompanying machinery. Moreover, the study demonstrates how genomic datasets such as the *Lactobacillus* pangenome harbour a potential "arsenal" of antimicrobial activity with the possibility of being activated when expressed in more genetically amenable hosts.

Introduction

Bacteria exist in complex communities, under constant competition from other strains and species for nutrients and space. The production of antimicrobial peptides known as bacteriocins has been shown to be one means by which such strains can gain a competitive advantage (Kommineni et al., 2015). Bacteriocins can be broad spectrum, inhibiting a variety of bacteria, or narrow spectrum where they inhibit primarily closely related species. As closely related species are more likely to occupy the same environmental niche as the producer, these bacteriocin genes could offer a potentially useful armoury of antimicrobials in helping the producer establish itself in such a niche.

The increasing availability of genomic data has changed the way we identify and study bacteriocins in communities. Bioinformatic screening tools such as BAGEL (de Jong et al., 2010) and antiSMASH (Weber et al., 2015) can now process vast amounts of genomic data to search for antimicrobial operons and genes (Walsh et al., 2015, Letzel et al., 2014). This allows us to identify previously uncharacterised bacteriocins and antibiotics, and to understand the extent of which strains encode these natural weapons for targeting competitors. In many of these cases however, the bacteriocin genes appear to be inactive antimicrobial relics which are unlikely to play an active role given the degradation of the surrounding accessory genes. Mutations within genes, loss of key genes within operons and tight transcriptional regulation can all prevent cells from producing these antimicrobials. This means that many such strains harbour potentially useful bacteriocins that are destined to remain uncharacterised due to a lack of *in vitro* production. One method to overcome this issue is to clone the antimicrobial operon into a host where expression can be controlled. This allows for the natural bacteriocin regulation to be circumvented and/or gene loss to be overcome, thus ensuring production of otherwise unavailable antimicrobials for further characterisation and potential exploitation.

In this study we focused on the expression of potential class IIa bacteriocins from members of the *Lactobacillus* Genus Complex. Bacteriocins are divided into different classes based on their

modifications, structure and mode of action. Class II bacteriocins are not subject to extensive post-translational modification and the class IIa bacteriocins are single peptides with a highly conserved 'YGNGV' N-terminal sequence. These bacteriocins (also known as 'pediocin-like' bacteriocins) are relatively narrow spectrum and display high levels of activity against *Listeria* species (Cotter et al., 2005). They bind to the mannose phosphotransferase transport system in sensitive strains and subsequently induce pore formation which leads to cell death (Zhou et al., 2016). The effective production of these bacteriocins depends on the production of several other associated proteins (Fimland et al., 2005). For example, an associated ABC transporter must be produced by cells in order to transport the bacteriocin outside the cell, and an immunity protein is also required to protect the producing strain from being killed by its own bacteriocin (Drider et al., 2006). Certain class IIa bacteriocins also require an accessory protein for correct disulphide bond formation (Oppegard et al., 2015). These operons may also have a three-component regulatory system which regulates expression of the bacteriocin. Here, when the concentration of an inducer peptide encoded within the operon reaches a certain level it signals a transmembrane histidine kinase which in turn activates a response regulator which initiates transcription of the bacteriocin operon (Ennahar et al., 2000).

The *Lactobacillus* Genus Complex analysed in this study encompasses the *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Fructobacillus* and *Oenococcus* genera and numerous pediocin-like bacteriocins have been associated with this grouping (Collins et al., 2017). Previously Sun *et al*. (Sun et al., 2015) sequenced the genomes of 213 strains belonging to this complex, these were then subject to both *in silico* and *in vitro* bacteriocin screening (Collins et al., 2017). Numerous strains from that study were found to encode bacteriocin operons but did not show *in vitro* bacteriocin production. Several of these strains encoded complete operons for pediocin-like bacteriocins, whilst more had incomplete operons harbouring just structural and immunity genes. Whereas regulatory issues may prevent expression of bacteriocins in strains harbouring complete operons, those without the necessary transporter systems seem unlikely to be produced. It is unclear as to why such

strains encode intact bacteriocin and immunity genes whilst lacking the machinery needed to actually produce and secrete these antimicrobials. One potential explanation for this is that these strains may have maintained the bacteriocin immunity gene under selective pressure from these antimicrobials. The neighbouring bacteriocin encoding gene is small and may have simply been maintained due its proximity to the immunity gene, the much larger transport machinery may then have been lost.

In this study we use an expression systems derived by Mesa Pereira *et al*. (Mesa-Pereira et al., 2017) to reincarnate these bacteriocins, allowing for *in vitro* production. It was found that for production of pediocin PA-1, only the structural gene and the transporter were necessary for bacteriocin production and secretion. As some of our putative novel bacteriocins lacked an associated transporter, the pediocin PA-1 transporter was used in each case. Bacteriocin peptides were detected by mass spectrometry and activity was identified by screening against a range of indicator organisms. The systems used have allowed us to isolate seven novel bacteriocins and represents a unique and rapid way of producing bacteriocins. This method allows us to reincarnate otherwise ineffectual antimicrobial relics identified solely by *in silico* methods.

Results

Identification of 'Silent' Pediocin Homologs

Previous work performed by our group analysed bacteriocin production in 213 *Lactobacillus* and related species (Collins et al., 2017). *In silico* screening identified the presence of bacteriocin operons (or remnants of operons) encoded within the genome of a large number of these strains. Strains encoding bacteriocins were then tested for *in vitro* bacteriocin production but several strains failed to display antimicrobial activity (Collins et al., 2017, Sun et al., 2015). This study focused on the class IIa bacteriocins; eight strains were found to encode ten of these pediocin-like bacteriocins but failed to display any antimicrobial production. Of these ten potential bacteriocins identified, nine were novel and one was identified as penocin A, a bacteriocin previously characterised from *P. pentosaceus* ATCC 25745 (Diep et al., 2006) (Table 1) .

Typically at least four genes are commonly required for the production of a class II bacteriocin; a structural gene, a transporter, an immunity gene and a gene encoding an accessory protein (Drider et al., 2006). The operons for the ten "silent" class IIa bacteriocins differed in their composition, with several lacking the genes for some of the necessary bacteriocin associated components (Figure 1). Each of these nine novel bacteriocins were named based on the species which produced them; e.g. ruminicin produced by *L. ruminus*. Of the operons identified, only those encoding hordeiocin and ruminicin were found to encode all four key components for bacteriocin production. The acidicin operon also appeared to be complete as the structural peptide doesn't contain any cysteine residues for disulphide bond formation, thus the accessory protein is likely unnecessary. The futcin operon was the only other such operon which encoded an ABC transporter for bacteriocin transport and leader cleavage. The remaining operons were composed of two to three genes encoding just the structural gene and immunity protein, with the agilicin operon also encoding an accessory protein.

Heterologous Cloning

As there was no identifiable bacteriocin production from these eight strains, it was decided to express these novel bacteriocins first in an *E. coli* host. A modified pETcoco^{TM}-2 vector, pMPB1, generated by our group was used for bacteriocin expression (Mesa-Pereira et al., 2017). It was previously found that only the structural gene and the ABC transporter are required to express pediocin PA-1 using this system. As over half of the bacteriocins identified here lack an associated ABC transporter, the pediocin PA-1 transporter PedD was used for the expression of each of these bacteriocins. PedD contains an intrinsic domain for cleavage of the bacteriocin leader sequence, and as these transporters rely on this leader sequence for peptide processing each of these bacteriocins were cloned separately using both their native leader sequence and as a fusion protein containing the pediocin PA-1 leader. Recursive PCR was used to generate the genes for each of these fusion proteins; genes for the natural bacteriocins and the PedD transporter were amplified by PCR (Prodromou and Pearl, 1992). An In-Fusion reaction was then used to assemble and join these PCR products to the enzyme-digested pMPB1 vector. For production in *L. paracasei* NFBC 338, a pNZ44-derived vector was used; in this case the bacteriocin was cloned as a fusion with the pediocin leader along with the PedD transporter. PCR products for these reactions were obtained using positive pMPB1 plasmids as a template. In-Fusion reaction was then used to assemble and join these PCR products to the enzyme-digested pNZ44 vector. The vectors used are shown in Supplementary Table 1.

Production

E.coli Tuner^{TM} (DE3) cells transformed with the newly constructed plasmids were grown in LB supplemented with glucose to maintain a low-copy number and bacteriocin expression was induced by co-incubation of the clones with 50µM IPTG for three hours. Wells diffusion assays (WDAs) were used to identify bacteriocin activity from the bacterial supernatants and cell lysates. Bacteriocins were exclusively found in the bacterial supernatant. Antimicrobial activity was lower in cases where

the native leader of the bacteriocin sequence was used; with only four of the nine strains displaying zones of inhibition against a sensitive indicator (pediocin 20336a has an identical leader to pediocin PA-1). For expression in *L. paracasei* NFBC 338, cells were transformed by electroporation with the bacteriocin-containing pNZ44 vectors. Positive transformants were grown overnight and the neutralised cell free supernatant was used to identify bacteriocin production in WDAs.

Of the bacteriocins expressed with the pediocin PA-1 leader sequence in *E. coli*, eight of the ten displayed anti-microbial activity from the cellular supernatant against at least one indicator organism (Table 2). Purification of the bacteriocin peptides from the *E. coli* bacterial supernatant and MALDI-TOF mass spectrometry were used to determine the mass of the active peptides. These masses correlated closely with the predicted mass of the bacteriocin structural peptide, allowing us to identify the bacteriocin produced. This approach confirmed the production of nine of the ten bacteriocins, aquaticin was not identified using these methods, potentially due to low levels of production by the cells (Supplementary Figure 1). The percentage amino acid identity of these bacteriocins in comparison to pediocin ranges from 29.5% to 93.2%. Production levels of bacteriocins were shown to vary between the *E. coli* and *L. paracasei* expression systems. *E. coli* transformants actually displayed greater or equal bacteriocin activity compared to those produced by *L. paracasei* (Table 3). The only exceptions were the original pediocin PA-1 which was used as a control and rennicin B which was produced in very low quantities by the *E. coli* producer where production was only noted after purification of the bacteriocin from the cellular supernatant. Interestingly, *L. paracasei* NFBC 338 failed to produce futcin which was produced by *E. coli*.

Spectrum of Activity

The spectrum of activity was determined using WDAs against 32 indicator strains for bacteriocins produced by the *E. coli* transformants encoding the pediocin leader (Table 2). Despite being of the same class of bacteriocins, these novel antimicrobials display a varying spectrum of activity. Interestingly, pediocin 20336a and hordeiocin inhibited the growth of a larger number of

the indicators tested than pediocin PA-1. Aquaticin and rennicin B failed to display antimicrobial activity in the crude supernatant from the *E. coli* heterologous host, whilst acidicin displayed activity exclusively against *L. mali* DSM 20444 in this assay. Many of these bacteriocins, however, can inhibit a wide range of Gram-positive bacteria including potential pathogens such as *Enterococcus faecalis*. As with most class IIa bacteriocins, these also display potent anti-listerial activity. Interestingly, *L. mali* DSM 20444 appears to also be an extremely sensitive indicator for testing the activity of class IIa bacteriocins here and may prove a safer alternative over the use of *Listeria* strains to test the activity of these bacteriocins.

Bacteriocin Structures

Class IIa bacteriocins tend to have a relatively conserved structure. However, some of these novel bacteriocins display key differences in the typical conserved regions associated with previously characterised class IIa bacteriocins. A three-stranded β-sheet structure can be found at the N-terminus, this is often stabilised by a conserved disulphide bridge formed between two cysteine residues at the N-terminus. Because aquaticin has only a single cysteine residue and acidicin completely lacks cysteine residues, these bacteriocins would be unable to produce the conserved disulphide bond. The C-terminus is less conserved and can be composed of one or two α-helices and an elongated C-terminal tail which can fold back on the α-helix, forming a hairpin-like structure. A C-terminal disulphide bond can stabilise this hairpin structure in certain bacteriocins and pediocin 20336a, rennicin A, rennicin B, hordeiocin, agilicin and ruminicin all have the ability to form this disulphide bond (Fimland et al., 2005).

The N-terminal 'YGNGV/L' region is highly conserved in these peptides, interestingly agilicin is the only such bacteriocin where the valine or leucine residue in this sequence has been replaced by an isoleucine. These peptides also contain a conserved hinge region (VD/NWGXA) which separates the N-terminal β sheet configuration from the C-terminal α helix (Uteng et al., 2003). Acidicin

contains a valine to isoleucine substitution in this motif, a modification only previously seen in lactococcin MMFII (Ferchichi et al., 2001).

Discussion

Due to the growing availability of genomic data and the improvement of software a growing number of novel bacteriocins are being identified (Letzel et al., 2014, Zhao and Kuipers, 2016). There continues however, to be a disconnect between the identification of these genes and the actual production of these bacteriocins *in vitro*. Whilst metagenomic data provides the ability to detect these genes, this is often not correlated to the isolation and characterisation of the producing strain. Thus, whilst bacteriocin genes are being discovered at a much greater rate from metagenomic data, the isolation of these antimicrobials themselves has proved more difficult.

One method to bridge the gap between the discovery of bacteriocin genes and their *in vitro* production is to heterologously express these genes in a new host. This could prove particularly valuable for strains that are non-culturable or that are extremely difficult to grow in the laboratory. Developing and optimising cloning techniques for individual bacteriocins requires time, which can make it a laborious task when working with a large number of potential bacteriocin genes identified in a genomic screen. The methods used by Mesa-Pereira *et al.* (Mesa-Pereira et al., 2017) and in this study provide a rapid mechanism to express non-lantibiotic bacteriocins such as class IIa and class IId bacteriocins. It has been determined that the presence of the bacteriocin structural gene and the bacteriocin transporter is sufficient to express these bacteriocins using this system (Mesa-Pereira et al., 2017). This provides a quick and easy method to produce such bacteriocins, even if the original operons identified are incomplete. Using this method, it was possible to express novel bacteriocins identified from *in silico* screening of the *Lactobacillus* Genus Complex, despite many of these lacking the obligatory genes required for bacteriocin production by the parent strains. It is unclear as to why such strains encode intact bacteriocin and immunity genes whilst lacking the machinery needed to actually produce and secrete these antimicrobials. One potential explanation for this is that these strains may have maintained the bacteriocin immunity gene under selective pressures and that the

neighbouring small bacteriocin encoding gene may also have been maintained whilst the larger transport machinery was lost.

The bacteriocin expression system used is based on the pediocin PA-1 operon, using the associated transporter PedD to transport the expressed bacteriocin from the cell. Each of the ten bacteriocins described here was cloned in an *E. coli* heterologous host alongside this transporter using both its native leader sequence and as a fusion containing the pediocin PA-1 leader as opposed to its own. Of the ten bacteriocins studied here, nine were novel. The bacteriocins varied from 93.2% to 29.5% amino acid identity to pediocin PA-1. Nine of the ten bacteriocins displayed antimicrobial activity (aquaticin activity was not seen and the peptide was not identified after purification and MALDI-TOF mass spectrometry); the production of these bacteriocins shows the flexibility in the PedD transporter and its ability to secrete several bacteriocins. Production levels of these bacteriocins in an *E. coli* host was greater when they were expressed with the pediocin PA-1 leader sequence rather than their own, this is not surprising given that PedD has evolved to cleave the pediocin PA-1 leader. Four bacteriocins were produced and secreted using their native leader which reflects a degree of redundancy in the specificity of the cleavage domain in the PedD transporter.

The bacteriocins produced displayed a varying spectrum of activity despite all belonging to the class IIa bacteriocins. Pediocin PA-1 is an important commercial additive used in food production in the form of powdered fermentates such as ALTA® 2351 (Kerry Bioscience) for the inhibition of *Listeria* species as well as other food spoilage and pathogenic bacteria. The discovery of novel bacteriocins here with a greater inhibitory range indicates that alternative bacteriocins may prove to be more effective additives in food; it also opens up the possibility for extending the use of these bacteriocins for alternative applications such as potential therapeutic uses. Further studies into the effect the structure of these bacteriocins can have on bacteriocin activity may also allow for targeted peptide engineering to improve activity and extend their range of inhibition in the future.

The structure of these bacteriocins can be affected by differences in sequences of these peptides. The N-terminal β-sheet structure of these bacteriocins can be stabilised by the presence of a disulphide bridge, aquaticin and acidicin, however, lack the ability to form such a bond. Sit *et al.* previously found that this disulphide bridge can be removed from class IIa bacteriocins; this reduces but does not eliminate the peptides' inhibitory activity (Sit et al., 2012). This may explain the lower levels of activity seen for aquaticin and acidicin. The hydrophobic/amphiphilic C-terminus of these peptides is less conserved than the N-terminus. It is involved in membrane insertion, resulting in pore formation, and also determines the spectrum of activity of the bacteriocins (Johnsen et al., 2005). The C-terminus is composed of an α-helix followed by a C-terminal tail which forms a hairpin and folds back upon the α-helix. This motif can be stabilised by the presence of a C-terminal disulphide bridge which makes the structure less flexible. Class IIa bacteriocins lacking this second disulphide bridge tend to be more heat sensitive and can undergo unfolding , making them less active at 37˚C (Kaur et al., 2004). Acidicin, futcin, aquaticin and penocin A all lack the ability to form this disulphide bridge, which may explain the lower levels of activity seen for these bacteriocins, as the majority of the indicator organisms used here are grown at 37˚C. In certain bacteriocins, which lack the ability to form this disulphide bridge, the interaction between tryptophan residues found just after the hinge region and at the C-terminus can stabilise the hairpin fold (Fimland et al., 2002). Penocin A and aquaticin both have terminal tryptophan residues which would compensate for an absent disulphide bond. Futcin has a tryptophan residue at position 33 in the mature peptide; however this is not predicted to be involved in the stabilisation of the hairpin fold; acidicin lacks a stabilising terminal tryptophan residue altogether. This may suggest that such an extended hairpin structure does not form in these bacteriocins. Structural differences between these bacteriocins may not only affect their inhibitory activity but also may affect the ability of the pediocin transporter to secrete these bacteriocins. The bacteriocins which display the greatest divergence form pediocin PA-1 were, however, shown to be secreted here indicating a level of redundancy in the transporter which may extend to the production of other unmodified bacteriocins. This potential extension of

the classes of bacteriocins secreted by this system is supported by the activity of the transporter EnkT from *E. faecium* NKR-5-3 which is involved in the transport of a class IIa bacteriocin, two peptides of a class IIb bacteriocin and also an inducer peptide (Sushida et al., 2018). This emphasises the potential flexibility of these bacteriocin ABC transporters.

The novel bacteriocins described here cluster into different groups upon alignment. Pediocin, pediocin 20336a, rennicin A and rennicin B display between 84-93% homology to each other. Despite being 90.9% identical to pediocin PA-1, rennicin B did not display activity in the crude supernatant from the *E. coli* heterologous host; it did however display equal levels of activity compared to rennicin A when expressed in *L. paracasei* NFBC 338. Two amino acid substitutions in rennicin B may explain this as they occur in important structural regions for the bacteriocin. The Gly29-Ser29 substitution is found in the C-terminal α-helix of the peptide which is involved in membrane insertion. The substitution of a non-polar amino acid for a larger polar one here may affect the formation of the helix and membrane insertion. A Gly36-Ser36 substitution occurs in a double glycine motif which follows the α-helix. This motif may provide the flexibility for the C-terminal tail to fold back upon the helix (Fimland et al., 2002), this flexibility may be lost due to the substitution with a larger serine residue.

Hordeiocin, agilicin, futcin and ruminicin to a lesser extent, also cluster together, displaying between 60-76% amino acid identity. Hordeiocin, agilicin and ruminicin all display a relatively broad spectrum of activity (inhibiting between 18-14 strains), whilst futcin is more narrow spectrum (inhibiting nine strains) which again may be due to a lack of a C-terminal stabilising disulphide bridge. Penocin A, acidicin and aquaticin lack a high degree of similarity to each other and the other bacteriocins produced.

Through analysis of the data from previous *in silico* bacteriocin screens there is the potential to use these cloning systems to a far greater extent to increase the current repertoire of unmodified class II bacteriocins. Whilst this study focuses on the *Lactobacillus* Genus Complex, numerous other

studies have analysed large amounts of genomic data from other sources to identify novel bacteriocins. Zheng *et al*. (Zheng et al., 2015) screened 700 shotgun metagenomic datasets from the Human Microbiome Project for the presence of bacteriocin operons. Of the 4875 putative bacteriocin genes found here, there were 3048 potential class II bacteriocins including 50 class IIa pediocin homologs, all of which represent potential candidates for use in this expression system. Similarly Alvarez-Sieiro *et al*. screened 238 genomes of lactic acid bacteria using BAGEL3, from this they identified 785 putative bacteriocin genes of which 514 encoded potential unmodified bacteriocin genes containing 31 class IIa homologs (Alvarez-Sieiro et al., 2016). Other such *in silico* screening studies have identified 209 further unmodified bacteriocin genes (Walsh et al., 2015, Liu et al., 2016, Azevedo et al., 2015, Kjos et al., 2011). While duplications and false positives are likely to occur in these datasets, even if a small proportion of these genes can be analysed using this expression system it represents a significant extension of the class II bacteriocins.

Thus, whilst *in silico* genomic studies can lead to the identification of bacteriocins, often this research is not carried forward for the characterisation of these antimicrobials. The simple system used here outlines how bacteriocin genes identified through *in silico* screening of the *Lactobacillus* Genus Complex could easily be heterologously expressed. Bacteriocins which otherwise would not have been produced by the original strain due to tight regulation of the operon or loss of necessary genes were able to be produced and studied. Ten class IIa bacteriocins were studied here, nine of which were novel. Nine of these bacteriocins were produced and secreted by the PedD transporter, despite showing less than 30% identity to pediocin PA-1, which reflects the permissiveness of the transporter in secreting these peptides. These novel bacteriocins notably extend the group of class IIa bacteriocins, however these would likely not have been produced and analysed if not for the expression systems used here. This has allowed us to reincarnate these bacteriocin relics, and provides the capacity to identify and produce a vast range of novel bacteriocins identified by other *in silico* screens which may otherwise be destined to remain uncharacterised in their genomic graveyard.

Materials and Methods

Strains and Culture Conditions

Bacterial strains and growth conditions used are displayed in Supplementary Table 2. *E. coli* HST08 Stellar™ cells (Takara BIO USA, Inc., Mountain View, CA) were used for normal cloning methods, *E. coli* BL21 Tuner™ (DE3) cells (Novagen, EMD Millipore, Billerica, MA) were used for expression of the transformed genes. *E. coli* strains were grown in Luria-Bertani (LB) media containing 50µg/ml of ampicillin for plasmid selection. IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Fisher Scientific, Dublin, Ireland) was added to the growth media to induce gene expression. L-(+)-arabinose and D -(+)-glucose (Sigma Aldrich, Arklow, Ireland) were also added to the media to control plasmid levels in recombinant cells. *L. paracasei* NFBC 338 cells were grown in modified MRS media containing 0.05% cysteine and 10µg/ml chloramphenicol (Sigma Aldrich, Arklow, Ireland).

Molecular Cloning and Gene Expression

Total genomic DNA was extracted from the bacteriocin encoding strains using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, Arklow, Ireland). Primers were designed for amplification of the bacteriocins and transporter genes as outlined in the In-Fusion HD cloning protocol (Takara BIO USA, Inc., Mountain View, CA). For amplification of genes containing the bacteriocin along with the native leader sequence, the genes were amplified by PCR from the original genomic DNA of the host strain using CloneAmp Hifi PCR premix (Takara BIO USA, Inc., Mountain View, CA). Recursive PCR was used for the synthesis of fusion genes containing the pediocin PA-1 leader sequence joined to the bacteriocin structural gene (Prodromou and Pearl, 1992). In both cases, the fragment containing the transporter encoded gene (*pedD)* was amplified using pMPB1 pedD Fw1 and pMPB1 pedD Rv1 using *P.acidilactici* LMG 2351 as a DNA template. The oligonucleotides used in this study are listed in Supplementary Table 3. PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purificatoin kit (GE Healthcare, UK), these were then inserted into the linearized *Sph*I-*Avr*II

pMPB1 vector using In-Fusion HD cloning Plus. The pMPB1 vector is based on the commercial plasmid pETcoco™-2 which allows a dual control of expression; at transcriptional level by IPTG induction and for amplification at the DNA replication level by L-arabinose (Sektas and Szybalski, 2002).

The constructions were transformed into Stellar™ competent cells and colonies were selected on LB agar plates containing 50µg/ml of ampicillin and 0.2% D-glucose. The transformants were confirmed by colony PCR reactions. L-arabinose was used to increase the plasmid copy number in cells and plasmid DNA was isolated using the NucleoSpin plasmid kit (Macherey-Nagel, Duren, Germany) to be analysed subsequently by double digestion and sequencing. For bacteriocin expression, Tuner™ (DE3) *E. coli* cells were transformed with the bacteriocin encoding vectors of interest. The transformants were then grown in LB containing 50µg/ml of ampicillin and 0.2% glucose which maintains the plasmid in a single copy state. Once cells had grown to an OD_{600} of 0.5-0.7, IPTG was added at a concentration of 50 µM to induce expression of the bacteriocin genes.

For expression in *L. paracasei* NFBC 338 the pNZ44 plasmid was used which contains the p44 constitutive promoter. Each bacteriocin was cloned containing the pediocin promoter and the pedD transporter, PCR fragments were amplified from positive Stellar™ transformants using the pNZ44 pedA FW and pNZ44 pedD RV oligonucleotides. These were inserted into the linearized *Nco*I-*Hind*III pNZ44 vector using In-Fusion HD cloning Plus. Constructions were cloned into Stellar™ competent cells and confirmed as before. For expression, *L. paracasei* NFBC 338 cells were transformed with the bacteriocin-containing plasmids by electroporation. Transformants were grown overnight in MRS containing 0.05% cysteine and 10µg/ml of chloramphenicol.

Bacteriocin Assays

Bacteriocin activity was determined using WDAs. For Tuner™ (DE3) *E. coli* transformants both the cell supernatant and the cell lysates were analysed for antimicrobial activity, whilst only the

supernatants were analysed for *L. paracasei* NFBC 338 cells. Cell supernatants were isolated by centrifugation of the liquid cultures at 4000 RCF for 15 minutes, these supernatants were then filtered using 0.20 µm membrane filters. For cell lysates, cell pellets were resuspended in 5 ml of phosphate-buffered saline (PBS), cells were lysed by sonication using a MSE Soniprep 150 (MSE, London, UK). Lysates were subsequently centrifuged at 4000 RCF for 15 minutes and the resulting supernatants were filtered using 0.20µm membrane filters. For the indicator plates, 50µl of an overnight culture of the indicator strain was added to the appropriate media containing 1% agar. Plates were cooled and 7mm wells were bored in the agar. 50µl of the cell supernatants/lysates being tested were added to each well and plates were refrigerated for 2 hours prior to incubation under the appropriate conditions. WDAs were carried out in triplicate for each bacteriocin.

Bacteriocin Purification and Mass Spectrometry

Purification and analysis was carried out for bacteriocins encoding the pediocin PA-1 leader sequence from Tuner^{TM} (DE3) *E. coli* transformants. 85ml of each culture supernatant was applied to 2ml SP sepharose columns (GE Healthcare, UK) pre-equilibrated with 25ml 20mM potassium phosphate buffer 25% acetonitrile, pH 2.5. Columns were washed with 20ml 20mM potassium phosphate buffer 25% acetonitrile, pH 2.5, the bacteriocins analysed eluted from columns with 25ml 20mM potassium phosphate buffer 25% acetonitrile containing 2 M KCl, pH 2.5. Eluents were passed through a 6ml, 500mg Strata–E C18 SPE column pre-equilibrated (Phenomenex, Cheshire, UK) with methanol and water. The column was washed with 6ml 30% ethanol and then 6ml 70% 2-propanol 0.1 TFA (IPA). MALDI TOF colony mass spectroscopy was carried out on the eluents using an An Axima TOF^{2}plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) in positive-ion reflectron mode.

Figures and Tables

Figure 1. Class IIa Bacteriocin Operons

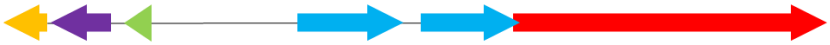
Bacteriocin	Operon	Size (kb)
Hordeiocin		6.5
Ruminicin		5.5
Acidicin		5.2
Futcin		4.7
Agilicin		1.2
Aquaticin		1.7
Rennicin A		0.6
Rennicin B		0.2
Pediocin 20336a		0.6
Penocin A		0.5

Table 1. Bacteriocins analysed and percentage identity to pediocin PA-1

Encoding Strain	Bacteriocin	Leader	Structural Gene	Structural Gene % Amino Acid Identify to Pediocin PA-1
P. acidilactici LMG 2351	Pediocin PA-1 (PedA)	-----MKKIEKLTEKEMANIIGG	-KYYGNGVTCG-KHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC---	-
P. pentosaceus DSM 20336	Pediocin 20336a (Ped20336)	-----MKKIEKLTEKEMANIIGG	-KYYGNGLYCG-KHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC---	93.18
L. rennini DSM 20253	Rennicin B (RenB)	----------MLSKEELTQVNGG	-KYYGNGVSCG-KHSCSVDWGKATTCTINNSAMAWATSGHQGNHKC---	90.91
L. rennini DSM 20253	Rennicin A (RenA)	----------MLSKEELTQVNGG	-KYYGNGVSCS-KHSCSVDWGKALTCTINNGAMAWTTGGHQGNHKC---	88.64
L. hordei DSM 19519	Hordeiocin (Hrd)	-----MKKEIELSEKELVRIIGG	-KYYGNGVSCTKKHGCKVNWGQAFTCSVNRFANFGH-G------NC---	56.76
L. agilis DSM 20509	Agilicin (Agl)	MSDK-MENKKKLTTADLAKVTGG	SRYYGNGITCG-KHKCTVNWGQAWTCGVNRLANFGH-G------NC---	56.76
L. ruminis DSM 20403	Ruminicin (Rum)	--------MRQLSEKELKKIMGG	-KYYGNGVYCG-KHKCRVDWGQAWGCSVNRWGAAVGTGGKATIGHC---	54.55
L. aquaticus DSM 21051	Aquaticin (Aqu)	-------------------MNGG	-KNYGNGVYCTKKHGYKVDWGQAWSIIGNNSAANSTTRGAAGWKSK---	47.73
P. pentosaceus DSM 20336	Penocin A (PenA)	-----MTEIKVLNDKELKNVVGG	-KYYGNGVHCG-KKTCYVDWGQATASIGKIIVNGWTQHGPWAHR-----	47.62
L. futsaii JCM 17355	Futcin (Fut)	MKGRYVNMKKVIDENSLSLISGG	-KYYGNGVSCG-KHTCKVNWGQAWNESVNRWGNSWVNGLTGLRQH----	44.19
L. acidipiscis DSM 15836	Acidicin (Acd)	-----------LSLEESSSVIGG	-KYYGNGLHIPKHGKPYINWGQAIQSIGKISYHGWVNGITSGAAGVGRH	29.55
			: ** : ****: : ::**:* :	

Table 2. Spectrum of Bacteriocin Activity

Species	Strain	PedA	Acd	RenB	Hrd	Ped 20336	Aqu	RenA	PenA	Fut	Rum	Agl
Bacillus cereus	DPC 6087											
Enterococcus faecalis	LMG 7397	4.33±0.47			2.67±0.47	4±0		3±0	0.67±0.47	1.67±0.47	1.17±0.24	2.17±0.24
E. faecium	DPC 4898											
E. saccharolyticus	DPC 4902	6±0			3.67±0.47	5.67±0.47		2.33±0.47	1.33±0.94	2±0	2±0	3.33±0.47
E.mundtii	LMG 10748	5±0			2.67±0.47	4.67±0.47		3.33±0.47	1.33±0.94	1.83±0.24	2±0	3±0
Lactobacillus ingluvei	DSM 15946											
L. amylovorus	DSM 20531										0.67	
L. apodemi	DSM 16654	3.33±0.47			2.67±0.47	3.67±0.47		0.67±0.47		0.33±0.47	1.67±0.47	2.67±0.47
L. bulgaricus	LMG 6901											
L. crustorum	JCM 15951	0.33±0.47			1±0	1±0					0.33±0.47	0.33±0.47
L. delbrueckii indicus	DSM 15996											
L. delbrueckii lactis	LMG 7942											
L. farraginis	DSM 18382											
L. amylophilus	DSM 20509				2.50±0.41	0.33±0.47			0.67±0.47		1.33±0.47	
L. intestinalis	DSM 6629											
L. kimchicus	JCM 15530				3±0	0.33±0.47						
L. mali	DSM 20444	7.67±0.47	1.50±0.41		4.33±0.47	6.33±0.47		1±0	1.50±1.08	2.17±0.85	2±0	4.50±0.41
L. nodensis	DSM 16982				3.67±0.47	1.67±0.47						3.50±0.71
L. paralimentarius	DSM 13961	0.67±0.47			1±0.47	1±0.47						
L. plantarum	DSM 13273	0.33±0.47			1.33±0.47	1.33±0.47						0.33±0.47
L. salivarius	DPC 6502											
Leuconostoc fallax	DSM 20189											
Listeria innocua	DPC 3572	8±0			4.67±0.47	7.33±0.47		6.33±0.94	2.50±0.41	2.00±0	3.33±0.47	5±0
Li. monocytogenes	DPC 6893	3±0			2±0	2.67±0.47		2±0	0.67±0.47		1±0	2±0
Li. monocytogenes	DPC 6894	5±0			4±0	4.33±0.47		2±1.41	0.67±0.47	0.83±0.24	2±0	4±0
Li. monocytogenes	WSL 1416	3.33±0.94			2±0.82	3.33±0.94		2.67±0.47	0.67±0.47	1±0	1±0	2±0.82
Pseudomonas aeruginosa	APC 2064											
Pediococcus stilesii	DSM 18001	1±0			1±0	1±0			0.67±0.47			
P. ethanolidurans	DSM 22301	5.67±0.47			3.67±0.47	5.67±0.47		1.50±0.41	2±1.41	3±0	2.17±0.24	3.33±0.47
P. clausenii	DSM 14800				2±0	0.67±0.47			1±0.82		1±0	0.67±0.47
Staphylococcus aureus	C55											
Salmonella typhimurium	LT2											
Total No. of Strains Inhibited		14	1	0	18	18	0	10	12	9	14	14

Antimicrobial activity is calculated as the radius of the zone of inhibition from a well diffusion assay measured in millimetres. PedA, pediocin PA-1. Acd, acidicin. RenB, rennicin B. Hrd, hordeiocin. Ped 20336, pediocin 20336a. Aqu, aquaticin. RenA, rennicin A. PenA, penocin A. Fut, futcin. Rum, ruminicin. Agl, agilicin.

Table 3. Bacteriocin Activity (BU/ml) vs *L. innocua* DPC3572

	E. coli Tuner (DE3)	*L. paracasei* NFBC338
PedA	+	++
RenA	+	+
Rum	+++	+++
Agl	++	++
Aqu	-	-
RenB	-	+
Acd	-	-
Ped 20336	+	+
Fut	+	-
Hrd	+++	++
PenA	++	+

Bacteriocin units per ml (BU/ml) were calculated as the inverse of the highest dilution of bacterial supernatant showing activity against *L. innocua* DPC3572 in a well diffusion assay. + = 0 – 160 BU/ml, ++ = 320 – 2560 BU/ml, +++ = 5120 – 10240 BU/ml

Supplementary Table 1. Vectors Used

Plasmid	Characteristics
pETcoco™	Plasmid with T7*lac* promoter for gene expression and araC-P_{BAD} promoter for amplification. Ap^r.
APC2313	pMPB1, pETcoco™-2 with modified MCS. Ap^r.
APC2666	pMPB1, *pedApedD*. Ap^r.
APC3139	pMPB1, *ped20336pedD*. Ap^r.
APC3140	pMPB1, *renApedD*. Ap^r.
APC3141	pMPB1, *PLrenApedD*. Ap^r.
APC3142	pMPB1, *renBpedD*. Ap^r.
APC3143	pMPB1, *PLrenBpedD*. Ap^r.
APC3144	pMPB1, *hrdApedD*. Ap^r.
APC3145	pMPB1, *PLhrdApedD*. Ap^r.
APC3146	pMPB1, *aglApedD*. Ap^r.
APC3147	pMPB1, *PLaglApedD*. Ap^r.
APC3148	pMPB1, *rumApedD*. Ap^r.
APC3149	pMPB1, *PLrumApedD*. Ap^r.
APC3150	pMPB1, *aquApedD*. Ap^r.
APC3151	pMPB1, *PLaquApedD*. Ap^r.
APC3152	pMPB1, *penApedD*. Ap^r.
APC3153	pMPB1, *PLpenApedD*. Ap^r.
APC3154	pMPB1, *futApedD*. Ap^r.
APC3155	pMPB1, *PLfutApedD*. Ap^r.
APC3156	pMPB1, acd*ApedD*. Ap^r.
APC3157	pMPB1, *PLacdApedD*. Ap^r.
APC3489	pNZ44, MCS, Cl^r
APC3490	pNZ44, *pedApedD*. Cl^r.
APC2328	pNZ44, *ped20336pedD*. Cl^r.
APC2622	pNZ44, *PLrenApedD*. Cl^r.
APC2623	pNZ44, *PLrenBpedD*. Cl^r.
APC2763	pNZ44, *PLhrdApedD*. Cl^r.
APC2764	pNZ44, *PLaglApedD*. Cl^r.
APC3349	pNZ44, *PLrumApedD*. Cl^r.
APC3639	pNZ44, *PLaquApedD*. Cl^r.
APC3488	pNZ44, *PLpenApedD*. Cl^r.
APC3640	pNZ44, *PLfutApedD*. Cl^r.
APC3641	pNZ44, *PLacdApedD*. Cl^r.

*MCS, multiple cloning site. Ap^r, ampicillin resistant. Cl^r, chloramphenicol resistance. *PL*, pediocin PA-1 leader sequence fusion gene.

Supplementary Table 2. Bacterial Strains and Growth Conditions

Species	Strain	Growth Media	Temp. (˚C)	Conditions
Bacteriocin Encoding Strains				
Lactobacillus acidipiscis	DSM 15836	MRS	30	Anaerobic
L. agilis	DSM 20509	MRS	37	Anaerobic
L. aquaticus	DSM 21051	MRS	37	Anaerobic
L. futsaii	JCM 17355	MRS	30	Aerobic
L. hordei	DSM 19519	MRS	30	Anaerobic
L. rennini	DSM 20253	AAM	30	Aerobic
L. ruminis	DSM 20403	MRS	37	Anaerobic
Pediococcus acidilactici	LMG 2351	MRS	30	Aerobic
P. pentosaceus	DSM 20336	MRS	30	Aerobic
Host Strains				
Escherichia coli	HST08 Stellar™	LB	37	Aerobic
E. coli	BL21 Turner™ (DE3)	LB	37	Aerobic
L. paracasei	NCC 338	mMRS	37	Anaerobic
Indicator Strains				
Bacillus cereus	DPC 6087	BHI	37	Aerobic
Enterococcus faecalis	LMG 7397	MRS	37	Anaerobic
E. faecium	DPC 4898	MRS	37	Anaerobic
E. saccharolyticus	DPC 4902	MRS	37	Anaerobic
E.mundtii	LMG 10748	MRS	37	Anaerobic
L. amylophilus	DSM 20509	MRS	30	Aerobic
L. amylovorus	DSM 20531	MRS	37	Anaerobic
L. apodemi	DSM 16654	MRS	37	Anaerobic
L. bulgaricus	LMG 6901	MRS	37	Anaerobic
L. crustorum	JCM 15951	MRS	30	Aerobic
L. delbrueckii indicus	DSM 15996	MRS	37	Anaerobic
L. delbrueckii lactis	LMG 7942	MRS	37	Anaerobic
L. farraginis	DSM 18382	MRS	30	Anaerobic
L. ingluvei	DSM 15946	MRS	37	Anaerobic
L. intestinalis	DSM 6629	MRS	37	Aerobic
L. kimchicus	JCM 15530	MRS	37	Aerobic
L. mali	DSM 20444	MRS	30	Aerobic
L. nodensis	DSM 16982	MRS	30	Anaerobic
L. paralimentarius	DSM 13961	MRS	30	Anaerobic
L. plantarum	DSM 13273	MRS	37	Aerobic
L. salivarius	DPC 6502	MRS	37	Anaerobic
Leuconostoc fallax	DSM 20189	MRS	30	Aerobic
Listeria innocua	DPC 3572	BHI	37	Aerobic
Li. monocytogenes	DPC 6893	BHI	37	Aerobic
Li. monocytogenes	DPC 6894	BHI	37	Aerobic
Li. monocytogenes	WSL 1416	BHI	37	Aerobic
Pseudomonas aeruginosa	APC 2064	BHI	37	Aerobic
Pediococcus ethanolidurans	DSM 22301	MRS	37	Anaerobic
P. stilesii	DSM 18001	MRS	30	Anaerobic
P. clausenii	DSM 14800	MRS	30	Aerobic
Staphylococcus aureus	C55	BHI	37	Aerobic
Salmonella typhimurium	LT2	BHI	37	Aerobic

Supplementary Table 3. Primers Used

Primer Name	Sequence
pMPB1 pedA Fw1	AGAAGGAGATATAAGCATGAAAAAAATTGAAAAATTAACTG
pedApedD Rv1	CAAAACCATATTAACCAGGTGATTATTGATGCCAGCTCAG
pMPB1 renA Fw1	AGAAGGAGATATAAGCATGTTAAGTAAAGAAGAGCTAAC
renApedD Rv1	CAAAACCATATTAACCAGGTGATTAGCATTTATGATTTCC
pMPB1 penA Fw1	AGAAGGAGATATAAGCATGACTGAAATTAAAGTACTAAACG
penApedD Rv1	CAAAACCATATTAACCAGGTGATTATCTATGTGCCCAAGGCCCG
pMPB1 hrdAFw1	AGAAGGAGATATAAGCATGAAGAAAGAAATAGAATTGTCAG
hrdApedD Rv1	CAAAACCATATTAACCAGGTGATTAACAATTACCATGGCCAAAATTTG
pMPB1 acdA Fw1	AGAAGGAGATATAAGCCTATCTTTAGAAGAGTCTAGTAG
acdApedD Rv1	CAAAACCATATTAACCAGGTGATTAATGTCGGCCTACTCCAGCAG
pMPB1 futA Fw1	AGAAGGAGATATAAGCATGAAAGGGAGATATGTCAATATG
futApedD Rv1	CAAAACCATATTAACCAGGTGATTAATGTTGGCGTAATCCTGTTAATC
pMPB1 aglA Fw1	AGAAGGAGATATAAGCATGAGTGATAAAATGGAAAACAAG
aglApedD Rv1	CAAAACCATATTAACCAGGTGATTAGCAGTTACCATGACCAAAGTTG
pMPB1 aquA Fw1	AGAAGGAGATATAAGcATGAATGGAGGAAAAAATTATGG
aquApedD Rv1	CAAAACCATATTAACCAGGTGATTACTTGCTTTTCCATCCTGCAG
pMPB1 rumA Fw1	AGAAGGAGATATAAGCATGAGACAACTTTCCGAAAAAG
rumApedD Rv1	CAAAACCATATTAACCAGGTGATTAGCAATGGCCGATTGTAGCC
pMPB1 pedD Fw1	TCACCTGGTTAATATGGTTTTGTAACCAATGTAAAAGG
pMPB1 pedD Rv1	GCTCGAGTGCGGCCTAGGCTATTCTTGATTATGAATTAACC
pNZ44 pedA Fw1	GGAGGCGCTTCCATGAAAAAAATTGAAAAATTAACTG
pNZ44 pedD Rv1	GGTTCAAAGAAAGCTCTATTCTTGATTATGAATTAACC
Recursive Primers	
pMPB1 PedL Fw1	AGAAGGAGATATAAGCATGAAAAAAATTGAAAAATTAACTGAAAAAGAAATGGCCAATATCATTGGTGG
PLrenA Rv2	CAACCGAGCAAGAATGCTTACTACACGAAACACCATTGCCATAATATTTACCACCAATGATATTGGCC
PLrenA Fw3	GCATTCTTGCTCGGTTGACTGGGGTAAAGCTTTGACTTGTACCATTAATAATGGTGCAATGGCTTGG
PLrenApedD Rv4	CCATATTAACCAGGTGATTAGCATTTATGATTTCCTTGGTGACCACCTGTGGTCCAAGCCATTGCACCAT
PLpenA Rv2	CCACATAGCAAGTCTTTTTACCACAATGCACTCCGTTACCGTAATACTTACCACCAATGATATTGGCC
PLpenA Fw3	AAAAGACTTGCTATGTGGACTGGGGACAAGCTACAGCTAGCATTGGAAAAATTATAGTGAACGGATGG
PLpenApedD Rv4	CCATATTAACCAGGTGATTATCTATGTGCCCAAGGCCCGTGTTGTGTCCATCCGTTCACTATAAT
PLhrdA Rv2	CCATGTTTCTTTGTACAGCTAACTCCATTTCCATAGTATTTCCCACCAATGATATTGGCC
PLhrdA Fw3	CTGTACAAAGAAACATGGTTGCAAAGTAAATTGGGGACAAGCTTTCACTTGCAGCGTTAATCGT
PLhrdApedD Rv4	CCATATTAACCAGGTGATTAACAATTACCATGGCCAAAATTTGCAAAACGATTAACGCTGCAAGT
PLacdA Rv2	CCCCAATTAATATATGGTTTTCCATGTTTAGGAATATGAAGACCATTACCATAGTATTTGCCACCAATGATATTGGCC
PLacdA Fw3	ACCATATATTAATTGGGGACAAGCTATACAATCAATAGGCAAAATTTCATACCATGGTTGGGTTAATGGT
PLacdApedD Rv4	CCATATTAACCAGGTGATTAATGTCGGCCTACTCCAGCAGCGCCACTAGTTATACCATTAACCCAACCATG
PLfutA Rv2	CCAGTTTACTTTACATGTATGTTTTCCACAAGAAACTCCGTTACCGTAGTACTTACCACCAATGATATTGGCC
PLfutA Fw3	ACATGTAAAGTAAACTGGGGACAAGCCTGGAACGAAAGTGTTAATCGTTGGGGTAATTCATGGGT
PLfutApedD Rv4	CCATATTAACCAGGTGATTAATGTTGGCGTAATCCTGTTAATCCATTTACCCATGAATTACCCCAA
PLaglA Rv2	CTTATGTTTGCCACAAGTAATACCGTTACCATAATATCTACTTCCACCAATGATATTGGCC
PLaglA Fw3	ACTTGTGGCAAACATAAGTGCACAGTTAACTGGGGGCAAGCTTGGACTTGCGGAGTTAAC
PLaglApedD Rv4	CCATATTAACCAGGTGATTAGCAGTTACCATGACCAAAGTTGGCAAGGCGGTTAACTCCGCAAGTCCA
PLaquA Rv2	CTACTTTATAACCATGCTTTTTTGTACAGTAGACTCCATTTCCATAATTTTTTCCACCAATGATATTGGCC
PLaquA Fw3	GCATGGTTATAAAGTAGACTGGGGACAGGCTTGGTCAATTATTGGGAACAATTCGGCAGCGAATTCG
PLaquApedD Rv4	CCATATTAACCAGGTGATTACTTGCTTTTCCATCCTGCAGCTCCACGAGTTGTCGAATTCGCTGCCGAATT
PLrumA Rv2	CACGCGGCACTTGTGCTTGCCGCAGTAAACGCCGTTTCCATAATACTTGCCACCAATGATATTGGCC
PLrumA Fw3	AAGCACAAGTGCCGCGTGGACTGGGGACAGGCATGGGGATGCAGTGTCAACAGATGGGGCGCCGCAGTA
PLrumApedD Rv4	CCATATTAACCAGGTGATTAGCAATGGCCGATTGTAGCCTTGCCGCCGGTTCCTACTGCGGCGCCCCATCT

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Chapter 5: Life in Depth: the Resilient and Adaptive Nature of the Microbiome of Deep-Sea Fish.

Bioinformatic analysis was carried out by Dr. Calum Walsh

Abstract

The deep sea is a vastly underexplored environment, with research relating to deep sea fauna being rare relative to investigations of other species living in more accessible habitats. This difference in understanding is even more profound at the microbiological scale. Here shotgun metagenomic sequencing was employed to characterise the compositional and functional diversity of the intestinal microbiome of deep-sea fish and to assess the capacity of the microbiome of deep-sea fish to produce a diverse range of natural products. It was established that the microbiomes of these fish assemble into three clear clusters that, when analysed functionally, suggest an important link between host diet and microbiome composition. From the metagenomic data it was also possible to construct 46 high-quality genomes from individual microbial species found. These genomes highlight the adaptions of microbes to life at these depths, displaying a greater capacity for protein and DNA synthesis, processes which can be inhibited by high pressure. We found that antibiotic resistance appears to be rare within these microbiomes, with most of these devoid of detectable antibiotic resistance gene clusters. The ability of these microbes to bioluminesce also appears to be lower than expected, despite predictions that it has an important role in the life cycle of microbes at these depths. Ultimately, metagenomic analysis has begun to shed light on the life and complexity of these microbial communities which otherwise would have remained in the dark.

Introduction

The deep sea is an extreme environment characterised by high pressures, low temperatures, low light and low nutrient levels. Nevertheless, it is estimated the deep sea harbours one of the largest pools of microbes in aquatic systems, accounting for almost 75% of the oceanic prokaryotic biomass (Aristegui et al., 2009). This relative abundance of microbes is primarily due to the sheer size of the habitat. The primary source of carbon and energy for these microbes is likely to be descending particulate organic matter (POM) from the ocean surface (Herndl and Reinthaler, 2013). Microbes have evolved to take advantage of this POM by encoding a greater number of genes involved in surface associated processes (DeLong et al., 2006). As POM descends through the water column it is degraded and metabolised by microbes through the activity of extracellular enzymes. The digestion of this POM releases dissolved organic matter (DOM) into the surrounding water which then may be utilised by free-living bacteria (Cho and Azam, 1988). Chemoautotrophy represents another method by which deep-sea microbes can obtain their energy in the absence of light (Pachiadaki et al., 2017, Cavanaugh, 1983).

Despite representing the largest ecosystem on the planet, the microbial composition of the deep sea remains vastly underexplored compared to surface water and terrestrial environments (Jebbar, 2015). Using metagenomic sequencing approaches, it is possible to characterise the entire microbial population within a sample. This is particularly suitable for the deep sea due to the difficulties in culturing microbes from such an environment. Metagenomic studies completed to date have focused on the characterisation of the microbial communities at different depths in the water column, both as particle-attached and free-living communities (DeLong et al., 2006, Salazar et al., 2016, Terahara et al., 2016). Deep-sea prokaryotic bacterial populations tend to be dominated by members of the Gammaproteobacteria, Alphaproteobacteria, Deltaproeobacteria, Actinobacteria and Thaumarchaeota, estimated to account for approximately 40% of the microbes identified. The remaining species constitute the rare biosphere that may vary between sampling sites and encodes

greater diversity with a high degree of novelty (Salazar et al., 2016). These relatively unexplored microbial populations offer a new source of novel bacterial compounds and processes.

To date, sequencing strategies to study deep-sea microbes have focused on free-living microbes in the water column. However, the intestines of fish represent one of the densest nutrient sources in the deep, therefore the bacteria that compose the microbiome of these fish represent one of the most concentrated bacterial communities in this vast environment. Several studies have investigated the composition of the intestinal microbiota in marine fish, but these have primarily focused on fish of commercial interest (Star et al., 2013, Dehler et al., 2017). The composition of the microbiome of deep-sea fish is likely to be unique given the environmental challenges faced by both host and microbes. The microbiota of fish can have an important role in their health and development, with a symbiotic relationship often forming between the two with the microbiome contributing, for example, through the breakdown of complex food sources, releasing metabolites that can be utilised by the host (Egerton et al., 2018).

Deep-sea microbes display adaptions to overcome the effects of high pressure and low nutrient availability (Vezzi et al., 2005). These microbes have also developed traits that may confer competitive advantages when vying for nutrients. Indeed, marine bacteria are a well-known source of naturally produced antibiotics, allowing these strains to inhibit potential competitors (Borchert et al., 2016). Marine bacteria may also benefit from bioluminescence through an increased likelihood of being ingested by a deep-sea fish or invertebrate, thus gaining access to the nutrient-rich intestine of the host (Zarubin et al., 2012). Certain deep-sea fish can also form a symbiotic relationship with bioluminescent microbes, harbouring them within light organs that they can use to attract prey or to evade predators (Widder, 2010).

Here shotgun metagenomic sequencing was used to characterise the microbiome of deep-sea fish. There was a focus on fish caught at a depth of approximately 1000m, albeit that 8 of the 47 samples analysed were captured at a depth of 850m. Samples were taken from the offshore waters

of Ireland, Scotland and Iceland and in international waters of the Grand Banks of Newfoundland, with a water sample also taken off the Irish coast. Metagenomic sequencing allowed for the identification of the microbial composition of the microbiome of these deep-sea fish, as well as carry out functional potential profiling of these microbiomes. From this data, it was possible to construct 46 high-quality microbial genomes. A comparison between these deep-sea-derived bacteria and closely-related reference genomes allowed for the identification of potential adaptions required for life at these depths. From the metagenomic data the potential of the microbiome to produce a wide diversity of antimicrobials, as well as bioluminescence-associated genes, was recognised. The potential frequency of antibiotic resistance in these microbiomes was also determined, providing an interesting opportunity to assess the distribution of resistance genes in an environment that is likely not exposed to commercial antibiotics.

Results and Discussion

Compositional Analysis

Despite the relative lack of research carried out on the microbiome of deep-sea fish, using the taxonomic classification tool Kaiju it was possible to determine the microbial composition of the intestinal microbiome from the shotgun metagenomic data (Menzel et al., 2016). Proteobacteria are highly prevalent, followed to a lesser extent by Firmicutes, Actinobacteria and Bacteroidetes, results which mirror the microbiomes of other marine fish (Figure 1) (Givens et al., 2015, Roeselers et al., 2011, Sullam et al., 2012). Host diet has been shown to have an important influence on the composition of the microbiome. A lack of plant life at this depth due to the absence of sunlight means that all fish sampled were carnivorous, feeding on a variety of fish, crustaceans and other invertebrates. This carnivorous lifestyle has been associated with higher levels of Proteobacteria and lower levels of diversity compared to omnivorous and herbivorous fish (Sullam et al., 2012, Givens et al., 2015, Ingerslev et al., 2014).

Analysis of the distribution of phyla across the microbiome of a variety of deep-sea fish (Supplementary Table 1) revealed a significant clustering of the samples into three primary clusters; cluster A, cluster B and cluster C. This is clearly seen in a principle coordinates analysis (PCoA) plot of the phylogenetic similarity between samples (Figure 2a). Figure 2b displays the proportional phylogenetic composition of the microbiome for each cluster. Cluster A shows a relatively similar abundance of three main bacterial phyla, the Proteobacteria, Firmicutes and Bacteroidetes. In cluster B, Bacteroidetes are almost completely replaced by Actinobacteria and the Ascomycota and Basidiomycota fungal phyla, along with the Euryarchaeota to a lesser extent. Here, there appears to be an almost mutually exclusive relationship between the Bacteroidetes phylum and the combination of the Actinobacteria/Ascomycota/Basidiomycota phyla, as in the majority of the samples the abundance of one appears to be associated with the absence of the other. Similarly the abundance of Actinobacteria, Ascomycota and Basidiomycota appear to be related, as higher

proportions of Actinobacteria mostly correlate with increased levels of these fungal phyla. In cluster C, the Bacteroidetes phylum again appears to be mostly replaced by Proteobacteria.

The eukaryotic composition of the marine microbiome has remained largely unexplored, as the majority of previous sequencing studies have relied on 16S ribosomal RNA amplicon sequencing (Marden et al., 2017). In this dataset, the Basidiomycota and Ascomycota comprise 2.92% and 4.85% of the combined microbiome, respectively. This is much greater than the fungal levels in the metagenomic sequences from the human microbiome, which are estimated to be as low as 0.01% of the reads identified (Nash et al., 2017). Previous studies analysing the fungal populations in the deep sea have found fungi to be present at a relatively low abundance and to be primarily composed of a low diversity of yeast genera belonging to the Basidiomycota and Ascomycota (Peay et al., 2016). The relatively high abundance of fungal sequences identified here suggests that the intestine of these fish can act as a reservoir for marine yeasts in the deep.

Focusing on the archaeal populations in these fish, the Euryarchaeota comprise 4.14% of the reads identified across the microbiomes. This is not surprising as this phylum has been shown to dominate amongst archaea in certain deep-sea environments (Zhang et al., 2016a, Flores et al., 2012). In particular, the *Methanosarcina* genus of the Euryarchaeota appears to be widespread throughout the microbiomes of the fish. These archaea can metabolise compounds such as hydrogen, carbon dioxide and trimethylamine (TMA) produced in the GI tract of the host resulting in methane formation (Nkamga et al., 2017).

The SUPER-FOCUS program was used to determine the potential functional properties of each sample. SUPER-FOCUS uses a three-level classification system, where Level 1 uses a broad-level form of classification such as ‘carbohydrate metabolism’, whilst Levels 2 and 3 focus in on more specific functions and pathways, with Level 3 being the most in-depth (Silva et al., 2015). By mapping the potential functional similarities between samples, we can see that different phylogenetic clusters also group together (Figure 3a). There is an almost exclusive separation between the bacterially

dominated clusters A and C and cluster B, which contains greater levels of the Ascomycota and Basidiomycota. This separation between clusters is also clearly seen when the functional diversity between samples is plotted on a PCoA plot. There is a clear significant difference between the clusters dominated by bacteria (clusters A and C) which map closely to each other compared to that of cluster B (Figure 3b). In fact, the more in-depth functional predictions (Supplementary Figure 1) make clear that the distinction between functions is largely due to the greater abundance of eukaryotic processes encoded within the microbiomes of cluster B. More specifically, there is a greater abundance of processes related to the eukaryotic RNA polymerase III and eukaryotic respiration systems in samples from cluster B, while samples from clusters A and C show a greater capacity for microbial-associated processes such as cell wall and peptidoglycan biosynthesis. Clusters A and C also show possible adaptions of the bacteria within the microbiome to the deep-sea environment, such as a high capacity for flagellum production, which can be required in the deep for effectively finding nutrient sources in the open ocean (Myka et al., 2017). The significantly greater capacity for the production of Ton and Tol transport systems in clusters A and C reflects potential bacterial adaptions for the utilisation of dissolved organic matter and inorganic nutrients in the deep, as these transporters play an important role in nutrient acquisition in marine bacteria (Figure 3c)(Tang et al., 2012).

There also appears to be metabolic differences between cluster B and clusters A and C. For example, cluster B shows a much greater capacity for carbohydrate and protein metabolism, whilst clusters A and C show a significantly greater capacity for chitin degradation **(Figure 3c)**. Chitin is thought to be an extremely important food and nitrogen source in nutrient-poor environments such as the deep, and its degradation tends to be bacterially driven, which associates with the greater abundance of functions associated with chitin and N-actetylglucosamine utilization in the bacteria dominated microbiomes of clusters A and C (Keyhani and Roseman, 1999, Beier and Bertilsson, 2013). Chitin is a polymer of β1,4-linked N-acetylglucosamine and plays an important structural role in marine invertebrates (Aunkham et al., 2018). Along with other fish, invertebrates form an

important part of the food chain in the deep-sea environment. Due to the role diet plays in the development of the fish microbiome (Piazzon et al., 2017), it is tempting to suggest that fish which feed more on crustaceans and other invertebrates will have microbiomes similar to that of clusters A and C whose constituents can utilise the available chitin and its components in the intestines. Chitin derived metabolites released by chitin-degrading microbes can be potentially used by the host and by other bacteria in the environment, thus potentially forming an important symbiotic relationship between microbiome and host (Beier and Bertilsson, 2013, Egerton et al., 2018). The significantly greater proteolytic capacity of cluster B suggests a diet composed more of fish rather than these invertebrates. As all fish sampled here are carnivorous, typically consuming any available prey, it is difficult to definitively suggest a relationship between the functional capacity of the microbiome and the diet of the host.

From these results, it appears that the microbiome of deep-sea fish can fall into three primary phylogenetic clusters. Often environmental differences between the habitats of animals plays an important role in microbiome development, but the deep sea is a more stable environment compared to land and surface waters and therefore is less likely to play such a selective role in the development of such defined clusters. This is highlighted by the fact that these clusters were identified from fish sampled from different locations at different time points. A species of fish can also harbour each of these microbiome clusters, as was the case for *Alepocephalus bairdii*, so it is unclear if host genetics plays a role in microbiome selection. Therefore, given the potential link between clusters and their metabolic potential, it seems most likely that host diet plays a major role in the development of the microbiome.

A core microbiome has been suggested for certain fish species (Sullam et al., 2012). To test if this hypothesis extends to unrelated fish in a deep-sea habitat, the microbial species which were present in over half of all samples with a relative abundance >1% were identified. There are three species which fulfil these criteria; *Ralstonia solanacearum, Peptoclostridium difficile* and *Methanosarcina*

mazei (Supplementary Figure 2). Whilst *Ralstonia solanacearum* is a known plant pathogen, members of the genus have previously been associated with the fish microbiome (Carda-Diéguez et al., 2014). *R. solanacearum* is a member of the Proteobacteria and its abundance here contributes towards the overall dominance of this phylum in the fish. The species also has been shown to survive prolonged starvation in water, a trait which may help such bacteria survive in the deep (Álvarez et al., 2008). *Peptoclostridium difficile* (*Clostridium difficile*), a well-known human pathogen, has also been shown to be prevalent across the microbiomes of these fish. The *Clostridium* genus has been shown to dominate the microbiome of certain fish and can have an important symbiotic role in the gut of the host, especially in herbivorous fish (Clements et al., 2007, Egerton et al., 2018). *Clostridium* species have even been incorporated into commercial probiotics for aquaculture, aiding in improved stress tolerance and immune function (Taoka et al., 2006). Whilst certain *Clostridium* species are well known in fish, *Peptoclostridium difficile* has not been previously associated with the marine fish microbiome (Egerton et al., 2018). The production of spores by clostridial species also allows these bacteria to survive deep-sea conditions and may allow cells to germinate once they reach the intestine of the host. Whilst the shotgun metagenomic strategy used here allows us to identify bacteria at a strain level, the 16S amplicon sequencing strategies employed previously when studying these environments does not reach such a resolution, thus potentially explaining the non-detection of *P. difficile*. The abundance of the *Methanosarcina* genus in the microbiomes of these fish can be partly attributed to the prevalence of *M. mazei* in the majority of samples. The metabolism of *M. mazei* may explain its prevalence as methanogenic species can metabolise a variety of substances produced in the GI tract of the host, such as carbon dioxide, hydrogen and also TMA (Youngblut et al., 2015).

Reconstruction of Metagenome-Assembled Genomes

The assembly of near-complete individual bacterial genomes from metagenomic data allows us to gain insights into the adaptions of these microbes to life in the deep-sea. These metagenomic-assembled genomes (MAGs) are created by assembling metagenomic data and grouping similar contigs likely to be from the same strain of microbe into "bins" that can then be used to assemble a putative genome (Bowers et al., 2017, Tully et al., 2018). Whilst MAGs lack the purity and completion of individually sequenced bacterial genomes they do provide information about strains which previously may not have been possible to isolate or sequence (Parks et al., 2017).

Initial assembly and binning resulted in the formation of 234 MAGs from the metagenomic data. To ensure a high quality in the draft genomes created, we removed MAGs which had an estimated completeness of less than 80% and also an estimated contamination greater than 5%. This left 46 high-quality MAGs distributed across nine phyla; the Proteobacteria, Fusobacteria, Euryarchaeota, Bacteroidetes, Fibrobacteres, Spirochaetes, Tenericutes, Deferribacteres and Firmicutes (Supplementary Figure 3, Supplementary Table 2). Given that only one of these could be classified to species level, they represent a source of genomic information for potentially numerous new taxa of microbes.

In order to identify possible adaptions of these deep-sea microbes, the potential functional properties of some of these MAGs were compared to those of their most closely-related reference genome in the NCBI database (Supplementary Table 3). The SUPER-FOCUS program was used to perform a functional analysis on these genomes, with numerous significant differences being identified between these deep-sea microbes and their surface-derived relatives (Supplementary Table 4) (Silva et al., 2015).

One of the clearest adaptions seen between these MAGs compared to related reference genomes is the greater frequency of genes involved in protein synthesis and degradation

(Supplementary Table 4). High hydrostatic pressure is known to have an effect on protein synthesis, leading to the dissociation of ribosomal subunits and the denaturation of the ribosomes (Groß and Jaenicke, 1990, Niven et al., 1999, Lauro and Bartlett, 2008). High pressure has been shown to induce the upregulation of protein synthesis machinery as a method of counteracting the reduced translational capacity of the cells under such conditions (Drews et al., 2002, Pavlovic et al., 2005). Similarly, the significantly increased capacity of the deep-sea strains for protein synthesis may reflect adaptions enabling them to negate the detrimental effects of high pressure on protein synthesis. An increase in the capacity for protein degradation of these MAGs compared to reference genomes may provide a means to reuse proteins denatured by the effects of high pressure. Lower available carbohydrate levels found at these depths may also lead to a greater reliance on protein metabolism as an energy source.

The significantly increased levels of heat-shock proteins encoded by these MAGs (Supplementary Table 4) reflect similar adaptions seen in piezophilic bacteria where these heat-shock proteins can aid in protein folding and stabilisation (Sato et al., 2015). Similar to protein synthesis, DNA synthesis is also a highly pressure-sensitive process, where the initiation of DNA synthesis can be inhibited by these conditions (Abe et al., 1999, El-Hajj et al., 2009). The significantly increased capacity of the MAGs assembled here for DNA replication again represents another possible adaption for these bacteria to overcome the effects of their high-pressure environment on DNA synthesis.

Membrane damage represents one of the primary mechanisms by which high hydrostatic pressure can cause the death of cells (Pagán and Mackey, 2000). High pressures can reduce membrane fluidity and permeability due to the tighter packing of phospholipid acyl chains found within the membrane (Macdonald, 1984, Ritz et al., 2000). Higher levels of unsaturated fatty acids in the membrane of these bacteria can increase membrane fluidity due to their expanded conformation and reduced melting temperature, this can help negate the effect of high hydrostatic

pressure on bacterial membranes (Allen et al., 1999). The MAGs assembled here display a significantly increased capacity for the metabolism of unsaturated fatty acids, thus reflecting their ability to potentially increase membrane fluidity in comparison to the related reference genomes. The effects of high pressure on bacterial membranes has also been shown to influence the composition of membrane transporters (Vezzi et al., 2005). Here we see a significantly decreased level of membrane transporters in the MAGs compared to reference genomes. This may reflect the reduced nutrient availability in the deep. The transport of large molecules across membranes has been shown to be inhibited by high pressures, thus these pressure adapted microbes may have adapted by reducing their reliance on such transporters (Abe and Horikoshi, 2000).

High pressure can also induce oxidative stress in bacterial cells by inducing a metabolic imbalance which causes the formation of reactive oxygen species (ROS) (Aertsen et al., 2005, Guyet et al., 2018). Microbes with a reduced capacity to negate the effects of ROS display an increased sensitivity to high hydrostatic pressure. These MAGs encode systems offering greater protection from ROS, thus outlining another potential adaption of these deep-sea microbes to pressure-induced stress (Supplementary Table 4) (Aertsen et al., 2005).

Limited nutrients in the deep may also affect the functional capacities of these microbes. In certain deep-sea bacteria carbohydrate metabolism appears to be overrepresented within the genome. This gives these microbes the flexibility to adapt to a variety of carbon sources which they might encounter (Vezzi et al., 2005). This adaption is reflected here also, whereby the MAGs have a greater capacity for carbohydrate metabolism compared to the reference genomes (Level 2). Similarly, these MAGs were found to have a significantly greater capacity for siderophore synthesis, most likely in response to the low concentration of dissolved iron found in the deep. Increased siderophore synthesis allows these bacteria to utilize the majority of the iron available in the environment, which is required for growth (Smedile et al., 2013).

Antibiotic Resistance

The microbiome of animals can be an important source of antibiotic resistance genes which could potentially be transferred to, or found in, potential pathogens (Argudín et al., 2017). Whilst antibiotic resistance is a major issue in medicine which has been exacerbated by the human use of antibiotics, it is in fact an ancient phenomenon (D'Costa et al., 2011). The natural production of antimicrobials by certain species has always been followed by competing strains developing resistance (Barlow and Hall, 2002). The microbiome of deep-sea fish is an interesting sample set for studying the distribution of antibiotic resistance. Due to the relative isolation of these fish from human activity, the identification of any resistance genes here may give an insight into the development of such resistance mechanisms in an environment where exogenous antibiotics may be very rare.

The metagenomic data from the intestinal microbiome of each fish was analysed for the presence of antibiotic resistance genes using the Resistome Analyzer software. The relative abundance of resistance genes varied between samples, with 29 of the 48 metagenomic samples containing no detectable genes for antibiotic resistance (Figure 4). The resistance profile of these genes is dominated by resistance to a class of antibiotics known as elfamycins, which target elongation factor TU (EF-Tu) in bacterial cells (Prezioso et al., 2017). EF-Tu is critical for bacterial translation as it delivers the correct amino acyl-tRNA (aa-tRNA) to the ribosome. Once the EF-Tu-aa-tRNA complex binds to the ribosome, EF-Tu hydrolyzes a GTP molecule thus allowing it dissociate from the aa-tRNA/ribosome complex so translation can continue (Yikilmaz et al., 2014). Elfamycins can inhibit this process by primarily two mechanisms, either by preventing EF-Tu from dissociating from the ribosome once the aa-tRNA has been delivered, or by inhibition of complex formation between EF-Tu with the aa-tRNA (Parmeggiani et al., 2006b, Parmeggiani et al., 2006a).

Potential resistance to elfamycins was identified in 18 of the metagenomic samples analysed, in each case due to mutations in the EF-Tu encoding gene *tufA.* As most bacteria encode two virtually

identical copies of the EF-Tu genes, levels of resistance due to differences in these genes may be relatively overrepresented (Zuurmond et al., 1999). Elfamycin resistance, however, is naturally found in the environment occurring across a broad spectrum of prokaryotes (Cammarano et al., 1982, Wörner et al., 1983). The mutations identified here by this programme as a result may simply be a result of natural variation in the *tufA* genes rather than resistance due to the exposure of these strains to these antibiotics. EF-Tu has been identified as possibly having an important role in maintaining protein synthesis response to high-pressure treatment of bacteria, thus potential adaptions of these genes to this environment may confer upon them a resistance to these antibiotics (Drews et al., 2002). Elfamycins themselves are not used therapeutically so it is extremely unlikely that these fish and their associated microbiomes would have encountered these antibiotics in their environments as a result of human activity (Prezioso et al., 2017).

There is a much lower prevalence of other potential antibiotic resistance genes identified in this metagenomic data. Potential multi-drug resistant genes were identified at low levels in the metagenomic sequences from 3 fish. These were all due to a potential mutation in the CRP regulator which regulates the transcription of a large number of genes in the cell. Mutations to CRP may result in lower levels of regulation leading to an increased expression of the MdtEF multidrug efflux pump, thus increasing the capacity of strains to export antibiotics from the cell (Nishino et al., 2008). Potential rifampicin resistance was detected in two samples due to differences in the *rpoB* gene encoding for the RNA polymerase β subunit. Rifampicin binds to RNA polymerase blocking elongation of the RNA chain, although mutations to the *rpoB* gene can alter the structure of the protein and reduce binding of rifampicin to the complex (Campbell et al., 2001). Potential flouroquinolone resistance was identified in two samples. One of these displays an alteration in the *gyrA* gene encoding a subunit of the bacterial DNA gyrase complex, which is involved in negative supercoiling of DNA through cleavage and ligation of DNA strands. Quinolones act by binding to the DNA gyrase and blocking ligation of the DNA, thus resulting in the cleavage of bacterial DNA and inhibition of DNA replication, eventually leading to cell death. Mutations to the DNA gyrase genes

reduce the affinity of these proteins towards fluoroquinolones (Hawkey, 2003). One of these samples, however, was found to encode the quinolone resistance protein, which binds to DNA gyrase and inhibits binding of a quinolone antibiotic thus offering resistance (Tran et al., 2005). Low levels of a potential tetracycline resistance encoding gene were identified in one sample whilst low levels of potential resistance to aminoglycosides were found in another.

The majority of the resistance genes identified here may be due to natural variations in the sequences of key genes and rather than acquired forms of antibiotic resistance. A previous functional metagenomic study identified the ocean as being a vast reservoir of antibiotic resistance; however, only 28% of the genes providing resistance to antibiotics in that study were previously identified as antibiotic resistance inducing genes. Many of the genes which did confer resistance when heterologously expressed had roles similar to characterised resistance genes, although their primary function was not associated with resistance. It may have simply been increased expression of such genes (i.e. transporters, oxidoreductases and hydrolases) in the new host which resulted in an acquired resistance to antibiotics (Hatosy and Martiny, 2015). With this in mind, our study relied on a database of previously identified genes; therefore it is possible that there is an underrepresentation of the levels of resistance found in the microbiota of these fish if they possess novel mechanisms which are hitherto unknown.

Natural Products

The production of antimicrobials such as antibiotics and bacteriocins by bacteria in the microbiome provide a mechanism by which these strains can establish themselves and compete in the host. Through the suppression of competitors, these antimicrobials give the producer access to a greater pool of nutrients within the GI tract (McNally and Brown, 2015).

Marine microbes have shown to be an excellent source of novel antibiotics and natural products, in particular secondary metabolites such as polyketide synthases (PKSs) and nonribosomal peptide

synthetases (NRPSs) (Udwary et al., 2007, Letzel et al., 2017). This is reflected in the greater capacity of the MAGs identified here for secondary metabolism compared to reference strains (Supplementary Table 4). PKSs and NRPSs are modular enzymes involved in the synthesis of a range of secondary metabolites, many of which display antimicrobial activity. The domains in these enzymes are involved in the selection, modification and combination of the subunits required for the production of polyketides and nonribosomal peptides (Wang et al., 2014). These enzyme complexes can produce a vast array of different molecules, and the diversity of which is determined by these key domains in the enzyme complexes that define which subunits are incorporated into the growing molecule. In NRPSs each adenylation (A) domain binds and activates a specific amino acid which is to be incorporated into the peptide chain. In PKSs, the ketosynthase (KS) domain carries out a condensation reaction involving a malonyl donor unit (Lee et al., 2015, Cane and Walsh, 1999). Whilst such domain-encoding enzymes can be involved in other cellular processes, the diversity of the A and KS domains found in the metagenomic data is a reflection of the diversity of the natural products which can be produced by microbes (Borchert et al., 2016, Fang et al., 2010).

While much of the work on marine bacterial natural products has focused on invertebrate hosts, the intestinal microbiome of fish and marine mammals has been shown to be a potential reservoir for the production of novel antimicrobials and other secondary metabolites. In that respect, the intestinal microbiome of these deep-sea fish may represent a previously neglected source of such compounds (Ochoa et al., 2017, Sanchez et al., 2012). A hidden Markov model (HMM) was used to identify A and KS domains found in the metagenomic data from the intestinal microbiota of deep-sea fish. Figure 5 shows the phylogenetic assignment of these A and KS domains identified by the HMM at a phylum level. Both the KS and A domains display a similar distribution across phyla being dominated by Proteobacteria and, to a lesser extent, Bacteroidetes. Whilst much work has been carried out on the identification and characterisation of natural products from marine microbes, most research has focused on the Actinobacteria, which themselves are responsible for most of the antibiotics used clinically today (Murphy et al., 2012, ul Hassan and Shaikh, 2017). In this study

however, the Actinobacteria only comprise a small proportion of the strains encoding A and KS domains despite their relatively high prevalence in the microbiome of these fish.

A growing number of natural products are now being identified from Proteobacteria, representing potential novel antibiotics and medicines in the treatments of diseases such as cancer and Alzheimer's disease (Zhang et al., 2016b, Offret et al., 2016). Interestingly, marine Proteobacteria have been shown to produce certain natural products whose structure and synthesis differs from those encoded by Actinobacteria (Timmermans et al., 2017). 34% and 40.8% of the unique A and KS domains, respectively, identified by the HMM in this study were identified as proteobacterial sequences. When assessing the abundance of these genes in the microbiome (by determining the number of reads from these domains per million total reads in the metagenomic sample), it is apparent that proteobacterial genes are even more abundant (62.3% of the A domain and 70.5% of the KS domain-encoding reads identified). These proteobacterial A and KS domains are distributed across 304 different genera, highlighting the diversity in the potential natural products which may be produced throughout the phylum.

17.5% of the identified A and 20.1% of the KS domains were encoded by Bacteroidetes-asscoiated strains. There has been relatively little research of the production of natural products from this phylum, however certain species have been shown to encode and produce such secondary metabolites (Oku et al., 2008, Murphy et al., 2012). Firmicutes account for 9.8% and 11% of the A and KS domains encoded here respectively. This is not unusual as Firmicutes have a long association with the production of natural products, with 31% of strains within the phylum estimated to encode an NRPS or PKS gene cluster (Wang et al., 2014). The *Bacillus* genus, in particular, has shown to be a rich source of non-ribosomal peptides, lipopeptides and polyketides (Aleti et al., 2015, Felnagle et al., 2008). It is interesting that the Spirochaetes have a relatively high distribution and abundance of A and KS domains, given that this phylum has not been previously shown to encode NRPS or PKS

operons. Much of the work on this phylum, however, has focused on pathogenic strains (Letzel et al., 2013).

A significant number of the A and KS domains identified could not be assigned to a phylum, however when we look at the abundance at which these genes were found in the host they only accounted for 3.2% and 3.9% of the reads associated with the A and KS domains. This is also seen for reads attributed to the less prevalent phyla which are grouped together as 'other'. This high level of diversity, coupled with the low abundance of these genes, shows that these less common microbes, often known as the 'rare biosphere', can be an important source of potentially novel natural products and further work should be carried out on the isolation and cultivation of such strains.

This *in silico* identification of potential natural product genes can be important as it enables researchers to discover potential novel drug candidates from strains which in the past may have been difficult to grow or may not have produced the compound when tested. The novel genes identified here may also be used to mine for related sequences and compounds in future screening work, this, combined with heterologous expression methods could potentially allow researchers to produce these novel secondary metabolites for further study and characterisation (Timmermans et al., 2017, Yamanaka et al., 2014)

Bioluminescence

Bioluminescence can be an important strategy for deep-sea bacteria to establish themselves in the host microbiome, often forming symbiotic relationships where they are maintained in certain light organs of the host. The host then uses the light produced by these bacteria to attract prey and avoid predators (Widder, 2010). Despite this, many deep-sea bacteria are bioluminescent and yet do not form a symbiotic relationship with the light organs of a host. These bacteria are often associated with POM, and their bioluminescence indicates the presence of food to zooplankton and other fish. Once ingested, these bacteria can replicate in the more nutritious environment of the host's gut. The

host also provides a means for dissemination of the bacteria over a large area through their faeces (Zarubin et al., 2012). These bacteria use quorum sensing to restrict bioluminescence until the cells have reached a high enough concentration, they will then continue to glow in the presence of oxygen (Haddock et al., 2010). Bioluminescence is catalyzed by a heterodimeric luciferase enzyme composed of α and β subunits. This luciferase enzyme uses molecular oxygen to oxidize a reduced flavin mononucleotide and a long-chain aliphatic aldehyde resulting in the release of photons of light (Dunlap, 2014). The genes required for luciferase activity are encoded within the *lux* operon. The *luxA* and *luxB* genes encode for both subunits of the luciferase enzyme, whilst the remaining genes of the operon encode the fatty acid reductase complex involved in aldehyde production, the flavin reductase enzyme, and also regulatory genes involved in quorum sensing (Lin et al., 1998, Stevens and Greenberg, 1997).

Bioluminescent bacteria have frequently been found in the GI tracts of marine fish, where they have shown the ability to survive gastric transit and proliferate extensively in the nutrient rich intestinal contents of the host (Ruby and Morin, 1979). Our results give an indication of the diversity and abundance of these luciferase genes in the intestinal microbiome of these fish and their environment. Using a Hidden Markov model, sequences related to the *luxA* luciferase genes were identified in the metagenomic data (Supplementary Table 5). The distribution of these genes is much lower than anticipated, with only 11 of the fish samples and the water sample containing homologs to the *luxA* gene. 27 *luxA*-like genes were distributed amongst these 12 samples and of these, 24 could be traced back to the genus of the encoding bacterial strain. Many of these bacteria belong to the *Phosphobacterium*, *Shewenella* and *Aliivibrio* genera, all of which are known marine bacteria previously associated with luciferase activity. Interestingly, *luxA* homologs were also identified in members of the *Methanolinea*, *Phaeobacter* and *Agrobacterium* genera, none of which have been previously found to produce bioluminescence.

The relatively low level of luciferase-like genes in these samples is quite surprising. If the theory that bioluminescence is used by these bacteria as an aid to become established in the host microbiota of deep-sea fish, then it would be expected that such luciferase-like clusters would be much more prevalent in the samples analysed in this study. Luminescence genes were still identified at a relatively high abundance in the deep water sample analysed, which suggests that these bacteria were outcompeted by non-luminous strains or have not colonized the intestine of the host once ingested.

Conclusion

The life of deep-sea marine microbes is shaped by low nutrient availability, high pressure, darkness and low temperatures, which create a harsh environment for free-living cells. The intestines of deep-sea fish provide one of the few nutrient dense environments in such habitats. Here, we identified the microbes which can take advantage of such environments, establishing themselves within the intestinal microbiome of the host. The identification and functional analysis of three primary clusters of the fish microbiome provide a potential link between host diet and microbiome development, a relationship made more likely given that diet is one of the more conspicuous aspects of the lifestyle of deep-sea fish which is prone to fluctuation. The adaptations of these deep-sea microbes are clear when a comparison is made between the genomes of microbes derived from the deep-sea microbiome and their most closely related sequenced strains. The greater ability to deal with pressure related stress is seen in their significantly increased capacity for protein and DNA synthesis as well as their significantly increased capacity to negate the effects of oxidative stress, all of which are related to pressure resistance. The capacity for these microbes to produce potentially novel secondary metabolites was also identified. Here we identified domains related to natural product synthesis distributed throughout the microbiomes, even in phyla previously not associated with the production of secondary metabolites, again highlighting the deep as a reservoir for novel compounds with potential uses in industry and medicine. Whilst the production of secondary metabolites appeared to be widespread, the levels of antibiotic resistance identified here were surprisingly low. Antibiotic resistance is thought to be ubiquitous, however resistance genes were not identified in the majority of the microbiomes (Pärnänen et al., 2016). Given the uniqueness of the dataset however, it is possible that novel resistance genes may simply have been missed in the homology-based search employed. The levels of bioluminescent genes (*luxA*) also were quite low, given their potential importance in the life cycle of many marine microbes (Zarubin et al., 2012). This may suggest that the production of bioluminescence may suit a more transient lifestyle of

certain microbes, rather than one which relies on a more long-term establishment in the host microbiome.

This study gives a detailed insight into the compositional and functional diversity of microbes harboured in the intestine of deep-sea fish. We have identified a previously untapped source of novel compounds and processes which warrants much further research. Thus, whilst the appearance of the deep-sea fish themselves often provoke much interest and enthusiasm, the not-so-visible bacteria they harbour within have become equally as well adapted to life in the deep.

Material and Methods

Sample Collection and DNA Isolation

Fish were caught from the offshore waters of Ireland, Scotland and Iceland and in international waters off the Grand Banks of Newfoundland at a depth of approximately 1000m, eight samples were caught at a depth of 850m. Once caught, the intestinal contents from these fish were isolated and stored for analysis using the OMNIgene GUT kit (DNA genotek, Ontario Canada). Total DNA was extracted from the stored samples using the PowerFecal DNA Isolation kit as per the manufacturer's protocol (Qiagen).

Whole-metagenome Shotgun Sequencing

The Nextera XT DNA Library Preparation kit (Illumina) was used for the preparation of whole-metagenome shotgun libraries. NextSeq libraries were sequenced on the Illumina NextSeq 500, with a NextSeq 500/550 High Output Reagent Kit v2 (300 cycles), in accordance with the standard Illumina sequencing protocols resulting in over 600 million read pairs.

Panphlan (v. 1.2.2.2) was used to build a pangenome from all freely-available fish genome sequences (Supplementary Table 6). Host contamination was removed from the raw whole-metagenome sequencing reads by Bowtie2 (v. 2.2.9) alignment against this pangenome (Scholz et al., 2016, Langmead and Salzberg, 2012). The remaining reads were converted from fastq to BAM format using SAMtools (v. 1.5) and duplicate reads were removed using Picard Tools (v. 2.7.1) (https://github.com/broadinstitute/picard) (Li et al., 2009). Low quality reads were removed using the trimBWAstyle.usingBam.pl script from the Bioinformatics Core at UC Davis Genome Center (https://github.com/genome/genome/blob/master/lib/perl/Genome/Site/TGI/Hmp/HmpSraProcess/trimBWAstyle.usingBam.pl). Specifically, bases with a quality score less than Q30 were trimmed and resulting reads shorter than 105bp were discarded. The resulting fastq files were converted to fasta using the fq2fa script packaged with IDBA-UD (v. 1.1.1)(Peng et al., 2012).

Statistical Analysis

Statistical analysis was performed in R (v. 3.4.4) (https://www.R-project.org/) and significance was accepted as $p < 0.05$ (FDR-adjusted). Figures were generated using a combination of Hclust2 (https://bitbucket.org/nsegata/hclust2), GraPhlAn (https://bitbucket.org/nsegata/graphlan/wiki/Home), and the pheatmap (v. 1.0.10) (https://CRAN.R-project.org/package=pheatmap), ggplot2 (v. 2.2.1) (Wickham, 2016) and cowplot (v. 0.9.2) (https://CRAN.R-project.org/package=cowplot) packages for R.

Composition

Taxonomic classification of paired-end reads was performed by comparison against the NCBI's nr database using Kaiju (v. 1.5.0) and functional analysis was performed using SUPER-FOCUS (v. 0.27) with DIAMOND alignment against the default DB_98 database. The vegan package (v. 2.5-1) (https://CRAN.R-project.org/package=vegan) was used to calculate alpha diversity, dissimilarity matrices and perform PERMAOVA (Menzel et al., 2016, Silva et al., 2015). Differences in alpha diversity and relative abundances of microbial taxa between groups were detected using the Kruskal-Wallis test implementation in the compareGroups package (v. 3.3.4) (10.18637/jss.v057.i12).

Reconstruction of Metagenome-assembled Bins

Assembly of metagenomes was performed using the 'meta-sensitive' preset option of megahit (v. 1.1.2)(Li et al., 2015). Binning of metagenome-assembled genomes was performed using the 'supersensitive' preset option of MetaBat2 (v. 2.12.1) (Kang et al., 2015). The completeness and contamination of these bins were assessed using the 'lineage_wf' workflow of CheckM (v. 1.0.7)(Parks et al., 2015). MAGs with completeness ≥ 80% and contamination ≤ 5% were deemed 'high-quality', inserted into the tree of microbial life using PhyloPhlAn and their taxonomy was predicted using the -t flag (Segata et al., 2013).

To identify possible adaptations for survival in the gut of deep-sea fish, MAGs that could be taxonomically classified at genus or species-level were compared to their closest identifiable relative

in the NCBI database (Supplementary Table 3). For MAGs identified a genus level, PhyloPhlAn was used to identify the reference genome to which it was most closely related. This was deemed to be the most closely-related species in the database so all available genomes of that species were included in the subsequent analysis. MAGs and reference genomes were annotated using Prokka (v. 1.12) (Seemann, 2014). The .faa files generated were used as the input for PhyloPhlAn, while the .ffn files were used as the input for functional analysis of ORFs using SUPER-FOCUS.

The Mann–Whitney *U* test implemented in the compareGroups package for R was used to detect statistically significant differences between the functional potential of these MAGs and their related reference genomes. This was performed on SEED levels 1, 2, and 3 of the SUPER-FOCUS output.

Antimicrobial Resistance

Antimicrobial resistome analysis was performed using Resistome Analyser pipeline (https://github.com/cdeanj/resistomeanalyzer). Reads were taxonomically classified using Kaiju search against the nr database and normalised for sequencing depth across samples as copies per million reads (CPM).

Natural Products and Bioluminescence

Assembled metagenomes were translated in all six reading frames using the EMBOSS (v. 6.6.0) transeq script with option -frame 6 and were searched using Hidden Markov Models obtained from the pfam database (Punta et al., 2011). PF00296 was used to search for luciferase activity, PF00501 was used to search for NRPS-associated adenylation domains, and PF00109 was used to search for PKS-associated ketosynthase domains. The Hmmer (v. 3.1) (http://hmmer.janelia.org/) hmmsearch script was used to identify potential matches using an e-value cutoff of 0.1. The abundance of each contig containing a significant hit was calculated by aligning all reads against the assembled metagenomes using Bowtie2 and calculating coverage using the samtools idxstats script. These contigs were taxonomically classified using Kaiju as previously described.

Figures and Tables

Figure 1. Complete phylum-level phylogenetic composition of the combined microbiomes of the deep-sea samples.

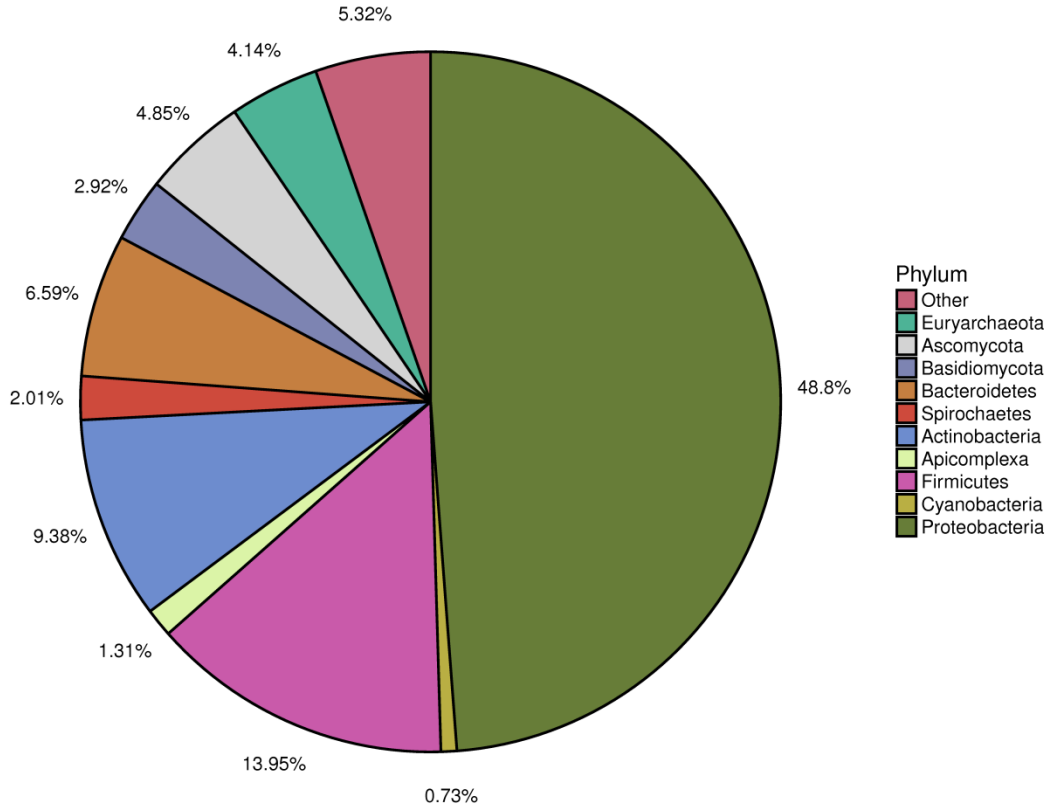
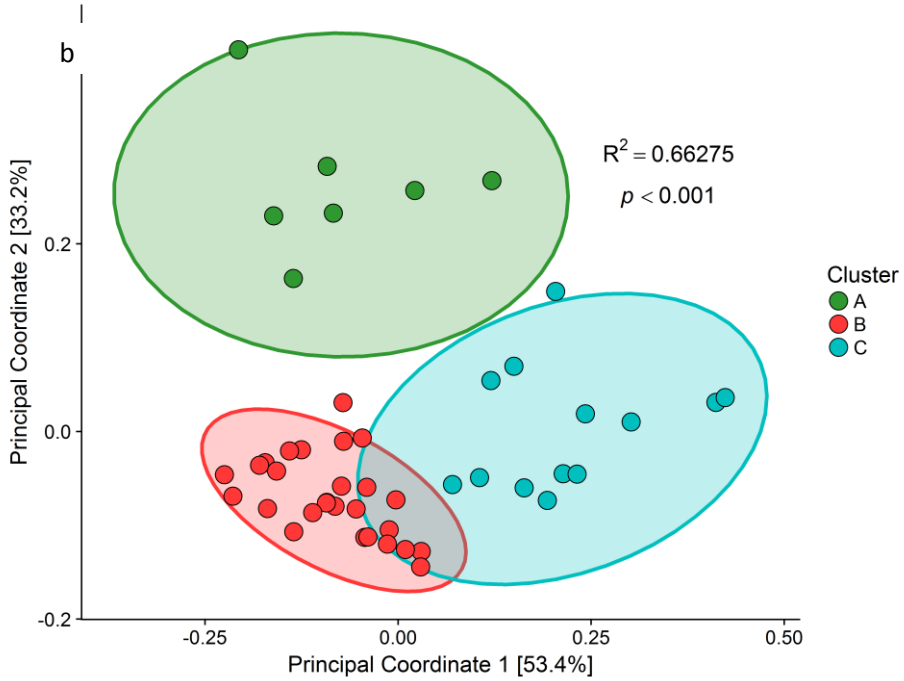
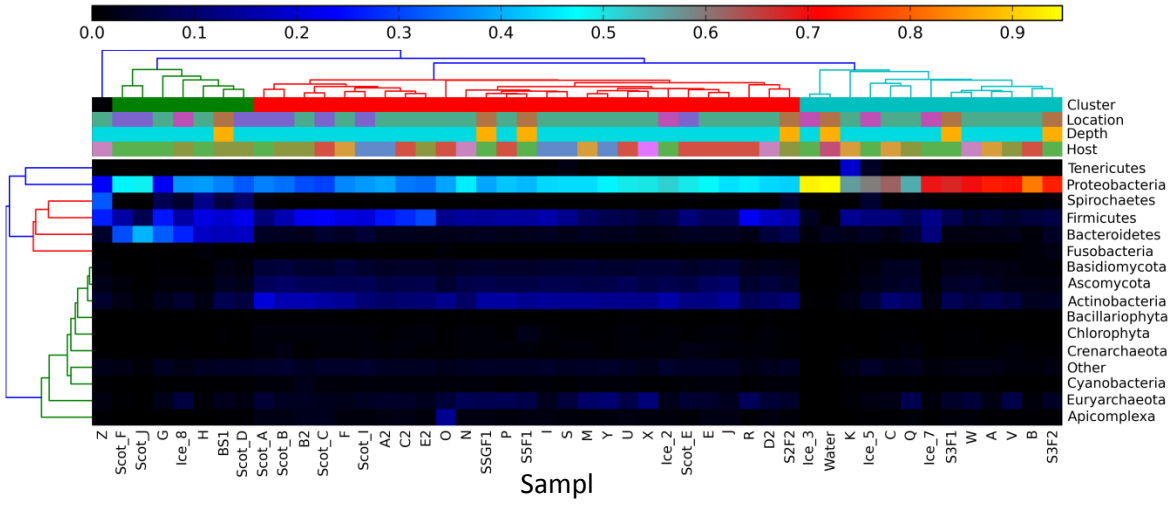


Figure 2. Phylum-level compositional analysis of microbiome samples. a) PCoA plot displaying the similarities between the phylogenetic composition of the microbiomes of deep-sea fish. b) Heat-map outlining the microbial composition of each metagenomic sample.





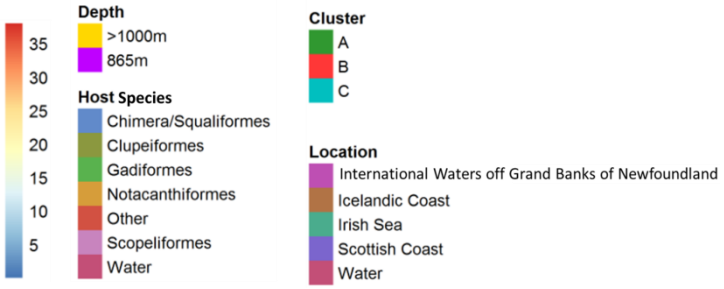
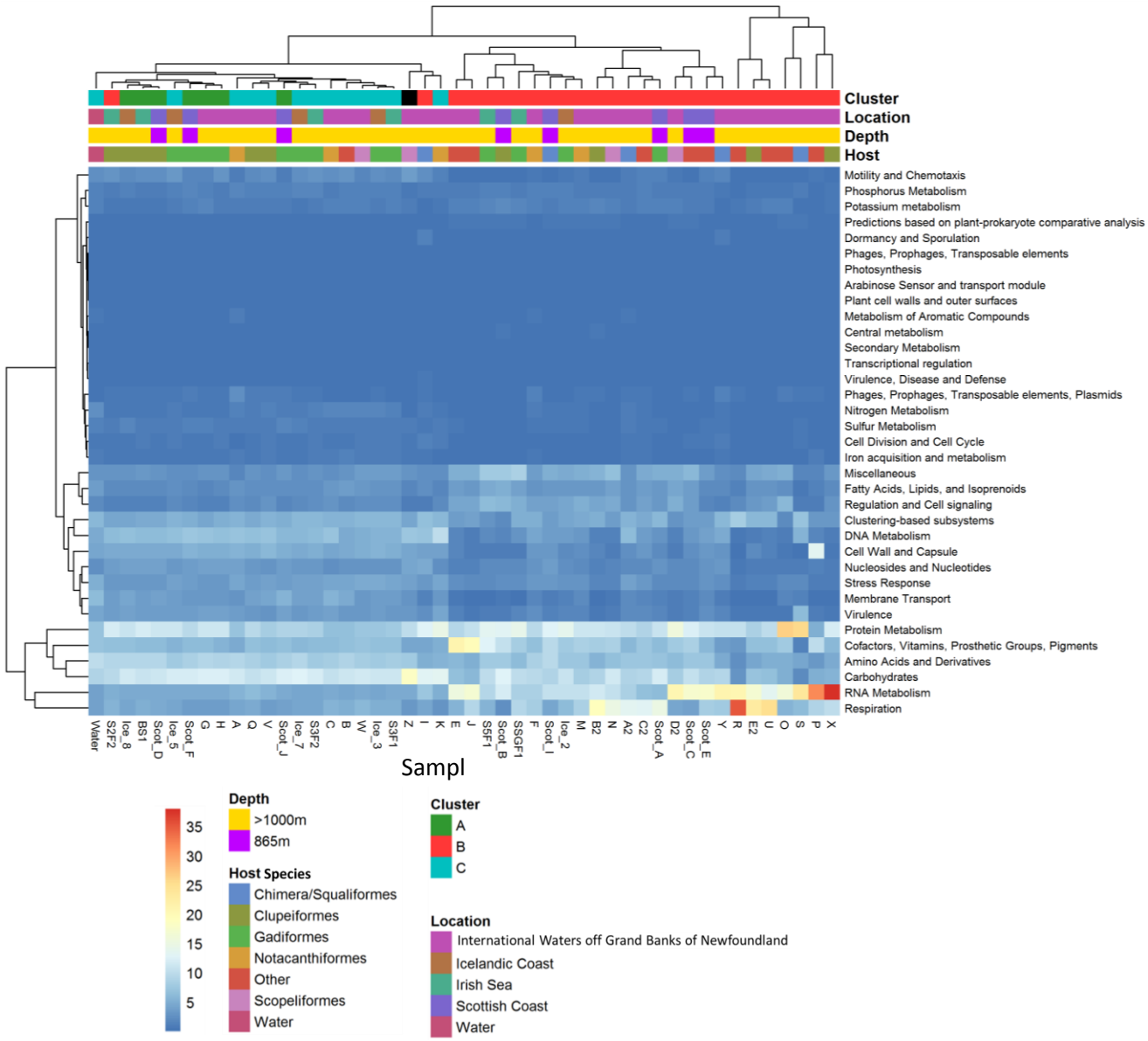
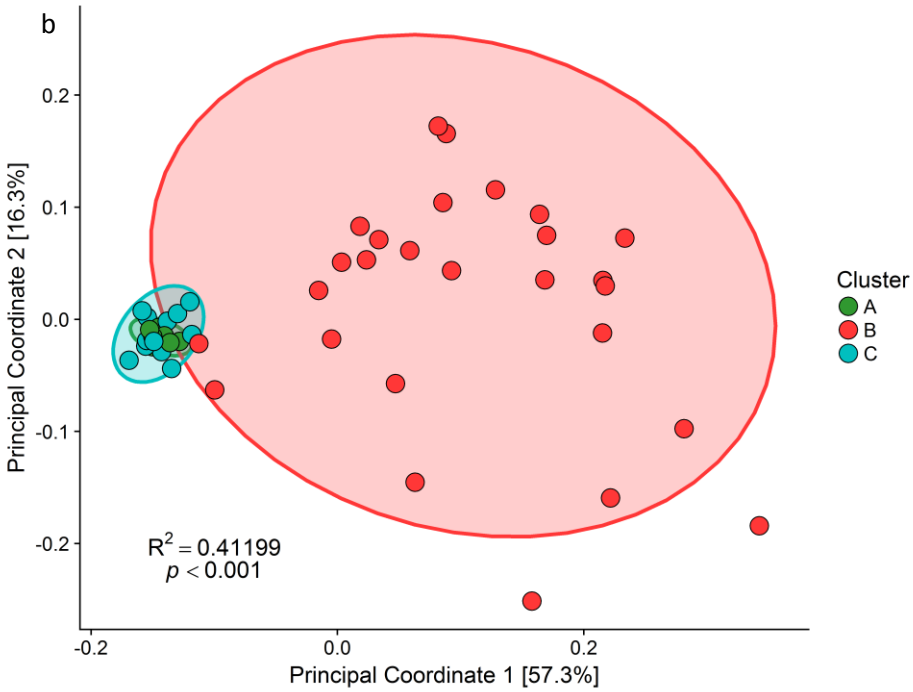


Figure 3. SUPER-FOCUS functional classification of the metagenomic data for the microbiomes of deep-sea fish. a) Heatmap outlining the functional capacity of each of the metagenomic samples. b) PCoA plot of the functional diversity of each sample derived from SUPER-FOCUS Level 1 results, grouped by compositional cluster. c) Selected differentially abundant functions between clusters potentially associated to host diet, significant differences were found between all pairwise alignments, p-values can be found in **Supplementary Table 7**.

a




b
Principal Coordinate 2 [16.3%]
0.2
0.1
0.0
-0.1
-0.2
$R^2 = 0.41199$
$p < 0.001$
-0.2
0.0
0.2
Principal Coordinate 1 [57.3%]
Cluster
A
B
C

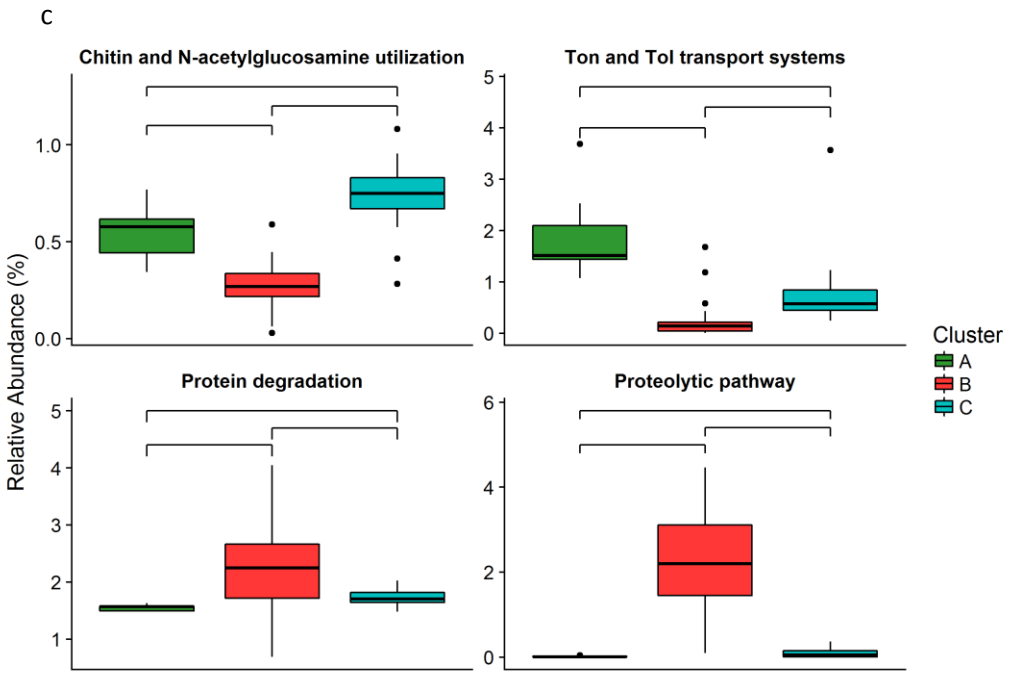

c
Chitin and N-acetylglucosamine utilization
Ton and Tol transport systems
Protein degradation
Proteolytic pathway
Relative Abundance (%)
Cluster
A
B
C

Figure 4. Abundance and distribution of antibiotic resistance genes in the microbiome of deep-sea fish

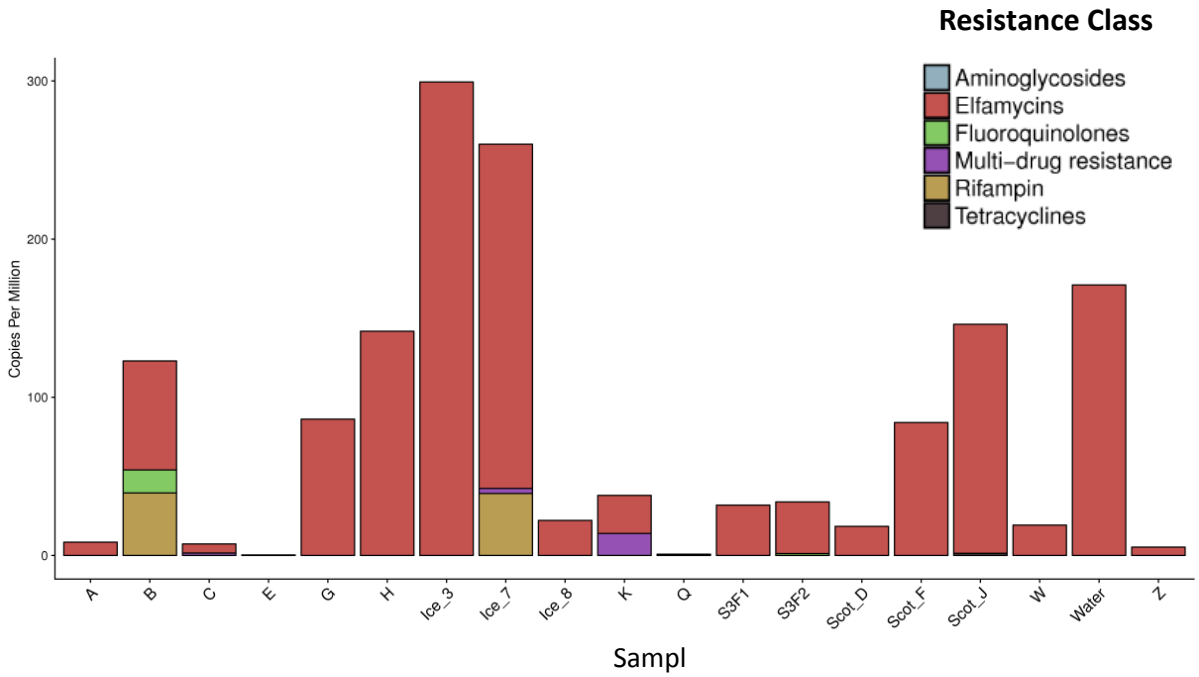
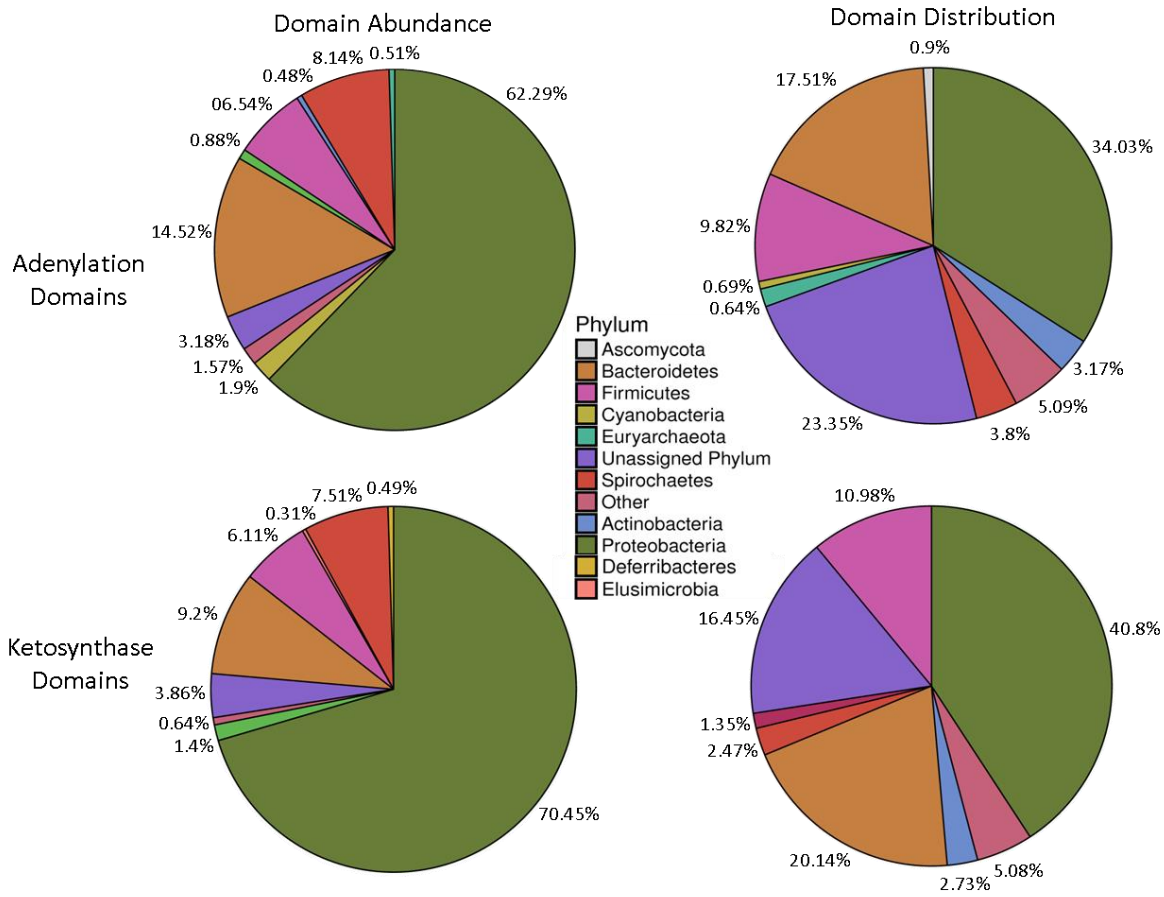
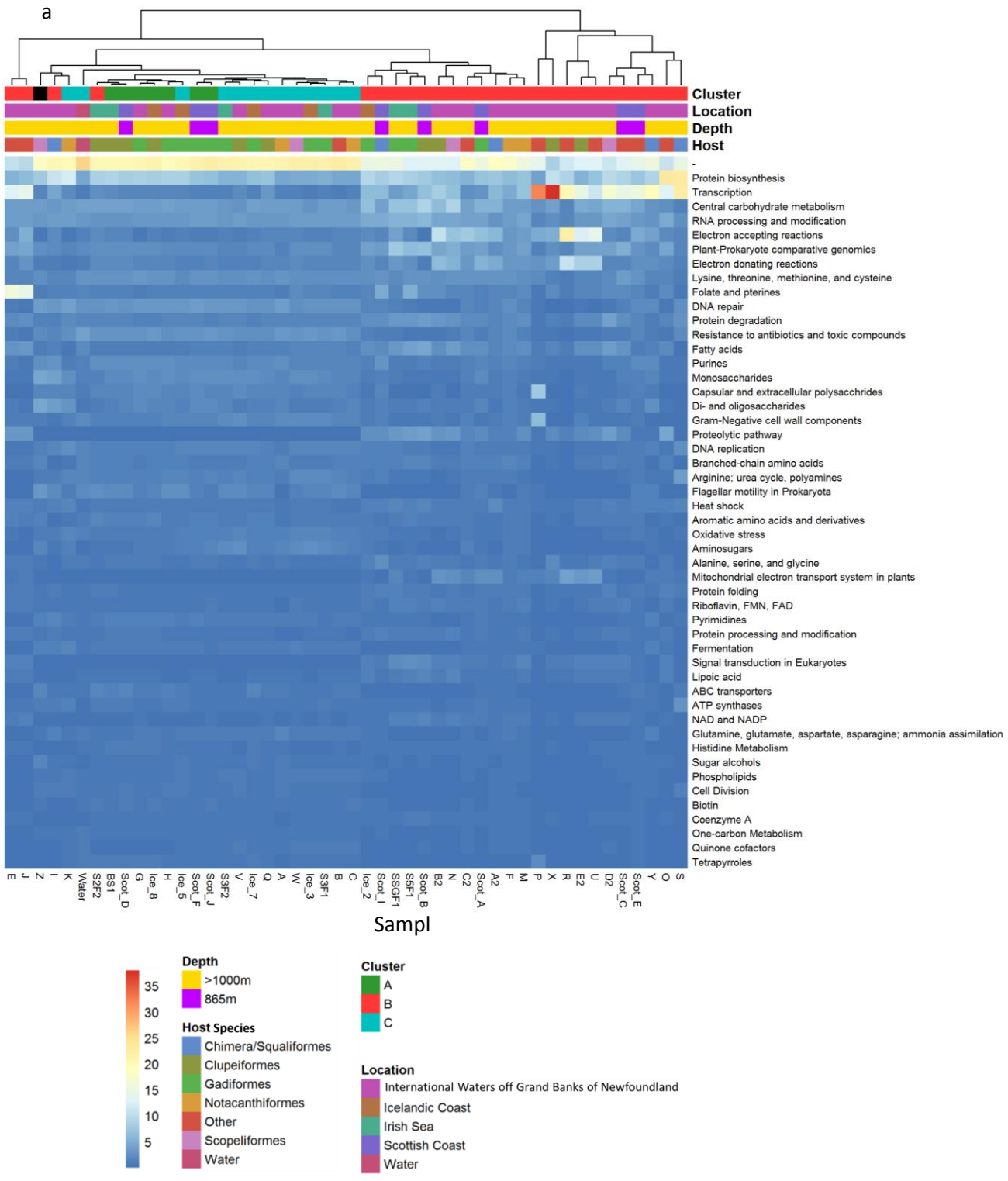
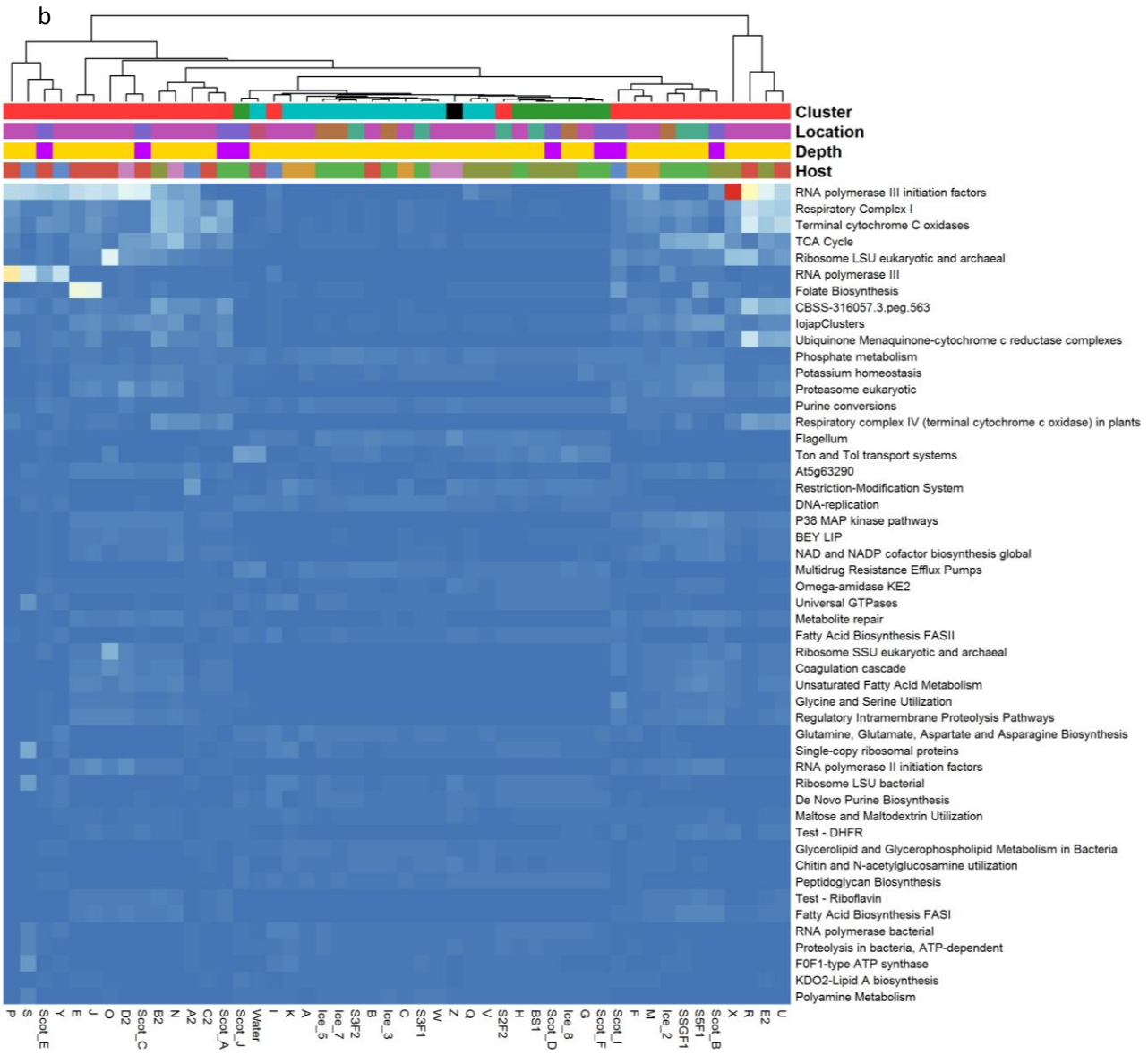


Figure 5. Phylogenetic assignment of adenylation (top) and ketosynthase domains (bottom) identified in the metagenomic data. Domain abundance refers to the proportional number of copies of each domain in the metagenome. Domain distribution refers to the number of unique domains identified in the metagenome.



Supplementary Figure 1. Level 2 (a) and Level 3 (b) SUPER-FOCUS functional analysis results for the microbiomes of the deep-sea samples. For the ease of visualisation, only the 50 most abundant functions at each level are included.




b
Cluster
Location
Depth
Host
RNA polymerase III initiation factors
Respiratory Complex I
Terminal cytochrome C oxidases
TCA Cycle
Ribosome LSU eukaryotic and archaeal
RNA polymerase III
Folate Biosynthesis
CBSS-316057.3.peg.563
IojapClusters
Ubiquinone Menaquinone-cytochrome c reductase complexes
Phosphate metabolism
Potassium homeostasis
Proteasome eukaryotic
Purine conversions
Respiratory complex IV (terminal cytochrome c oxidase) in plants
Flagellum
Ton and Tol transport systems
At5g63290
Restriction-Modification System
DNA-replication
P38 MAP kinase pathways
BEY LIP
NAD and NADP cofactor biosynthesis global
Multidrug Resistance Efflux Pumps
Omega-amidase KE2
Universal GTPases
Metabolite repair
Fatty Acid Biosynthesis FASII
Ribosome SSU eukaryotic and archaeal
Coagulation cascade
Unsaturated Fatty Acid Metabolism
Glycine and Serine Utilization
Regulatory Intramembrane Proteolysis Pathways
Glutamine, Glutamate, Aspartate and Asparagine Biosynthesis
Single-copy ribosomal proteins
RNA polymerase II initiation factors
Ribosome LSU bacterial
De Novo Purine Biosynthesis
Maltose and Maltodextrin Utilization
Test - DHFR
Glycerolipid and Glycerophospholipid Metabolism in Bacteria
Chitin and N-acetylglucosamine utilization
Peptidoglycan Biosynthesis
Test - Riboflavin
Fatty Acid Biosynthesis FASI
RNA polymerase bacterial
Proteolysis in bacteria, ATP-dependent
F0F1-type ATP synthase
KDO2-Lipid A biosynthesis
Polyamine Metabolism
P
S
Scot_E
Y
E
J
O
D2
Scot_C
B2
N
A2
C2
Scot_A
Scot_J
Water
I
K
A
Ice_5
Ice_7
S3F2
B
Ice_3
C
S3F1
W
Z
Q
V
S2F2
H
BS1
Scot_D
Ice_8
G
Scot_F
Scot_I
F
M
Ice_2
SSGF1
S5F1
Scot_B
X
R
E2
U

Sampl

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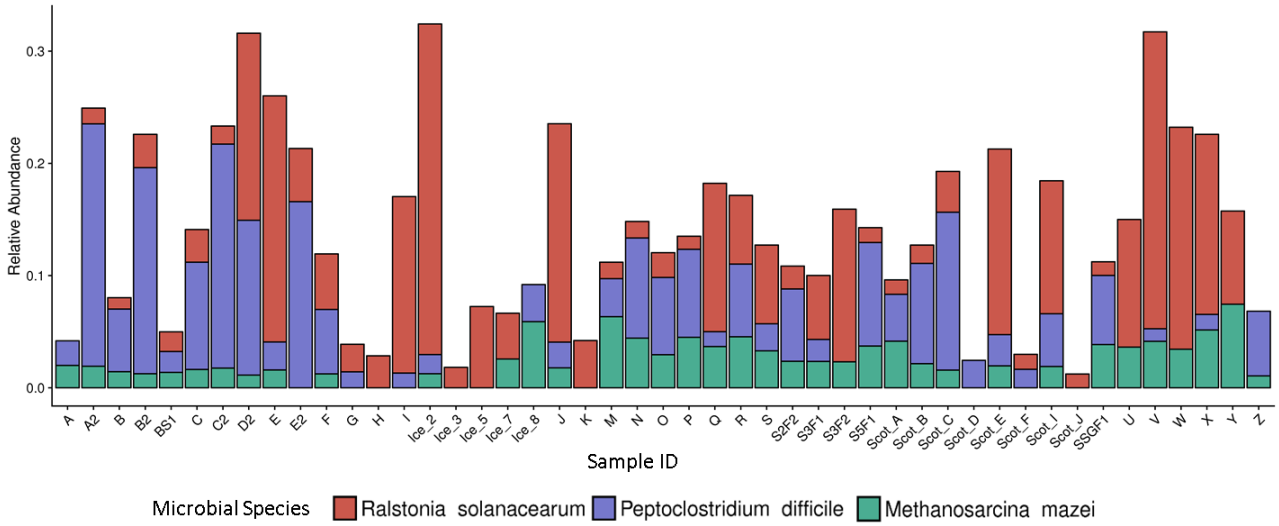
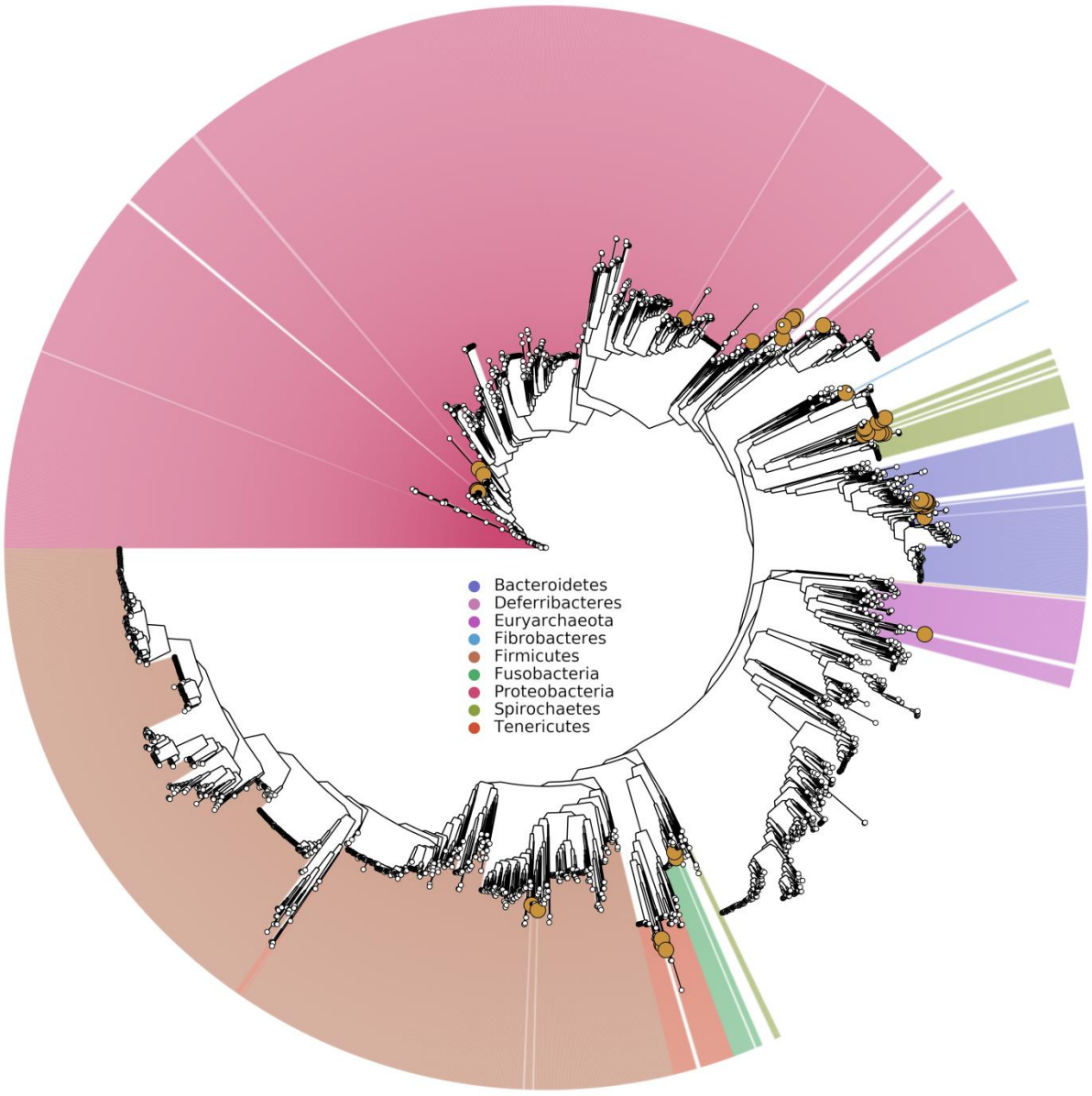
Supplementary Figure 2. Core microbiome of deep see fish, encompassing microbial species present at an abundance of >1% in over half of all samples.



Supplementary Figure 3. All 46 high-quality MAGs placed into the tree of microbial life using PhyloPhlAn. MAGs are marked in gold and phyla which are predicted to contain at least one MAG are denoted by colour.



Supplementary Table 1. Deep-sea fish species sampled

Source	Code	Fish Species	Depth
International Waters off the Grand Banks of Newfoundland	A	*Polycanthonotus rissoanus*	>1000m
	B	*Bathypterois dubius*	
	C	*Notacanthus chemnitzii*	
	E	*Anoplogaster cornuta*	
	F	*Polycanthonotus rissoanus*	
	G	*Coryphaenoides rupestris*	
	H	*Gaidropsaurus ensis*	
	I	*Centroscymnus coelolepis*	
	J	*Anoplogaster cornuta*	
	K	*Notacanthus chemntzii*	
	M	*Polycanthonotus rissoanus*	
	N	*Bathysaurus ferox*	
	O	*Simenchelys parasitica*	
	P	*Centroscyllium fabricii*	
	Q	*Alepocephalus agassizii*	
	R	*Eurypharynx pelecanoides*	
	S	*Apristurus sp.*	
	U	*Stomias boa boa*	
	V	*Alepocephalus bairdii*	
	W	*Bathysaurus ferox*	
	X	*Bathylagus euryops*	
	Y	*Centroscymnus coelolepis*	
	Z	*Scopelosaurus lepidus*	
	A2	*Hydrolagus affinis*	
	B2	*Pachystomias microdon*	
	C2	*Cottunculus thomsonii*	
	D2	*Lampadena speculigera*	
	E2	*Pachystomias microdon*	
Scottish Waters	Scot A	*Mora moro*	865m
	Scot B	*Argentina silus*	
	Scot C	*Aphanopus carbo*	
	Scot D	*Alepocephalus bairdii*	
	Scot E	*Galeus murinus*	
	Scot F	*Coryphaenoides rupestris*	
	Scot I	*Chimaera monstrosa*	
	Scot J	*Halargyreus johnsonii*	
Icelandic Waters	Ice #2	*Macrourus berglax*	>1000m
	Ice #3	*Lepidion eques*	
	Ice #5	*Trachyrincus murrayi*	
	Ice #7	*Trachyrincus murrayi*	
	Ice #8	*Alepocephalus bairdii*	
Irish Waters	S5F1	*Molva dypterygia*	>1000m
	S2F2	*Alepocephalus bairdii*	
	SSGF1	*Coelorinchus occa*	
	S3F2	*Lepidion eques*	
	BS1	*Alepocephalus bairdii*	
	S3F1	*Lepidion eques*	
	Water	Irish Deepsea water	

Supplementary Table 2. Metagenomic assembled genomes constructed from metagenomic data

Bin	Level of Classification	Classification	Completeness (%)	Contamination (%)
B_bin_3	Genus	*Psychromonas*	92.61	2.16
B_bin_7	Class	Fusobacteria	86.64	4.49
C_bin_2	Family	Vibrionaceae	94.29	3.29
G_bin_1	Order	Thermoplasmatales	83.87	1.61
G_bin_11	Genus	*Treponema*	92.76	4.02
G_bin_17	Genus	*Alistipes*	96.94	0
G_bin_20	Genus	*Treponema*	94.25	0.57
G_bin_28	Species	*Fibrobacter succinogenes*	85.16	1.1
G_bin_34	Family	Vibrionaceae	97.03	0.14
G_bin_38	Genus	*Alistipes*	95.53	0
G_bin_4	Genus	*Alistipes*	87.91	2.17
G_bin_40	Genus	*Alistipes*	98.4	3.82
G_bin_46	Genus	*Alistipes*	84.82	1.12
G_bin_5	Genus	*Alistipes*	86.94	2.3
G_bin_54	Class	Deltaproteobacteria	88.49	0.65
G_bin_55	Genus	*Alistipes*	96.39	0.96
G_bin_6	Genus	*Treponema*	96.8	4
H_bin_10	Genus	*Brachyspira*	85.39	0
H_bin_11	Genus	*Mycoplasma*	96.15	0.64
H_bin_14	Genus	*Treponema*	93.47	1.57
H_bin_2	Genus	*Sulfurimonas*	87.14	1.56
H_bin_25	Genus	*Treponema*	98.4	3.2
H_bin_26	Genus	*Desulfovibrio*	94.56	0.59
H_bin_28	Genus	*Brachyspira*	86.57	3.73
H_bin_31	Order	Alteromonadales	87.57	1.35
H_bin_4	Class	Fusobacteria	92.13	1.12
H_bin_5	Genus	*Desulfovibrio*	95.86	0
H_bin_8	Family	Deferribacteraceae	88.64	2.59
I_bin_2	Genus	*Epulopiscium*	92.17	0.67
Ice_5_bin_4	Genus	*Mycoplasma*	97.18	1.68
Ice_7_bin_3	Family	Vibrionaceae	93.33	1.62
Ice_7_bin_5	Order	Clostridiales	93.51	1.51
Ice_7_bin_7	Class	Alphaproteobacteria	99.32	0.2
K_bin_1	Genus	*Mycoplasma*	97.31	2.42
Q_bin_3	Family	Vibrionaceae	90.5	3.69
Scot_D_bin_10	Genus	*Treponema*	80.09	4.43
Scot_D_bin_13	Genus	*Mycoplasma*	82.53	0.77
Scot_D_bin_14	Genus	*Desulfovibrio*	98.82	2.96
Scot_D_bin_15	Genus	*Brachyspira*	86.25	1.27
Scot_D_bin_16	Genus	*Brachyspira*	97.6	4.27
Scot_D_bin_5	Genus	*Alistipes*	95.35	0.42
Scot_J_bin_10	Genus	*Desulfovibrio*	84.94	2.27
Scot_J_bin_2	Genus	*Alistipes*	89.85	3.21
Scot_J_bin_3	Phylum	Bacteroidetes	87.14	2.38
Z_bin_2	Genus	*Brachyspira*	87.35	1.12
Z_bin_4	Genus	*Treponema*	94.99	0.74

Supplementary Figure 3. Reference sequences for MAG comparison

RefSeq Accession Number	Species
GCA_002007365.1	*Epulopiscium* sp.
GCA_001995005.1	*Epulopiscium* sp.
GCA_002007375.1	*Epulopiscium* sp.
GCA_001994995.1	*Epulopiscium* sp.
GCF_000786575.1	*Alistipes inops*
GCF_000733685.1	*Mycoplasma iowae*
GCF_000227355.1	*Mycoplasma iowae*
GCA_001983545.1	*Epulopiscium* sp.
GCF_000413055.1	*Treponema maltophilum*
GCF_000219725.1	*Treponema caldarium*
GCA_001983535.1	*Epulopiscium* sp.
GCA_001983455.1	*Epulopiscium* sp.
GCA_001983515.1	*Epulopiscium* sp.
GCA_002007285.1	*Epulopiscium* sp.
GCA_001983475.1	*Epulopiscium* sp.
GCA_001983465.1	*Epulopiscium* sp.
GCA_002007295.1	*Epulopiscium* sp.
GCA_001983555.1	*Epulopiscium* sp.
GCF_000171335.1	*Epulopiscium* sp.
GCA_002007305.1	*Epulopiscium* sp.
GCA_002007315.1	*Epulopiscium* sp.
GCF_000482725.1	*Psychromonas arctica*
GCF_000319185.1	*Brachyspira pilosicoli*
GCF_000296575.1	*Brachyspira pilosicoli*
GCF_900445975.1	*Brachyspira pilosicoli*
GCF_900445955.1	*Brachyspira pilosicoli*
GCF_002865865.1	*Brachyspira pilosicoli*
GCF_000325665.1	*Brachyspira pilosicoli*
GCF_000143725.1	*Brachyspira pilosicoli*
GCF_000265365.1	*Alistipes finegoldii*
GCF_000321205.1	*Alistipes ihumii*
GCF_003149465.1	*Chitinophaga* sp.
GCF_000156375.1	*Desulfovibrio piger*
GCA_001917195.1	*Desulfovibrio piger*
GCF_900116045.1	*Desulfovibrio piger*
GCA_003538575.1	*Desulfovibrio piger*
GCF_000195755.1	*Desulfovibrio vulgaris* str. Hildenborough
GCF_000166115.1	*Desulfovibrio vulgaris*
GCF_000015485.1	*Desulfovibrio vulgaris*
GCF_000021385.1	*Desulfovibrio vulgaris*

Supplementary Table 4. Significant functional differences identified by SUPER-FOCUS between reference genomes and deep-sea

Functional Assignment	Enrinched in	p-value
Level 1		
DNA Metabolism	MAG	0.01768
Phages, Prophages, Transposable elements, Plasmids	MAG	0.00579
Protein Metabolism	MAG	0.00086
Secondary Metabolism	MAG	0.0228
Clustering-based subsystems	Reference	0.00333
Cofactors, Vitamins, Prosthetic Groups, Pigments	Reference	0.04457
Dormancy and Sporulation	Reference	0.02779
Membrane Transport	Reference	3.00E-05
Level 2		
Biologically active compounds in metazoan cell defence and differentiation	MAG	0.02879
CO2 fixation	MAG	0.01752
Central carbohydrate metabolism	MAG	0.04017
DNA replication	MAG	0.03811
Fatty acids	MAG	0.0226
Gram-Negative cell wall components	MAG	0.01594
Hypothetical in Lysine biosynthetic cluster	MAG	0.01865
Oxidative stress	MAG	0.00037
Pathogenicity islands	MAG	0.0014
Proline and 4-hydroxyproline	MAG	0.0458
Protein and nucleoprotein secretion system, Type IV	MAG	0.00805
Protein biosynthesis	MAG	0.00128
Protein degradation	MAG	0.01013
Pyridoxine	MAG	0.03288
Quorum sensing and biofilm formation	MAG	0.00195
Regulation of virulence	MAG	0.03278
Ribosome-related cluster	MAG	0.04005
Siderophores	MAG	0.02157
Translation	MAG	0.01289
Uni- Sym- and Antiporters	MAG	0.00332
Clustering of 2 heat shock proteins, phosphoenolpyruvate carboxykinase and a putative hydrolase	MAG	0.04005
ABC transporters	Reference	<0.00001
Coenzyme A	Reference	0.00167
Di- and oligosaccharides	Reference	0.00116
Hypothetical protein possible functionally linked with Alanyl-tRNA synthetase	Reference	0.00532
Isoprenoid/cell wall biosynthesis: predicted undecaprenyl phosphate	Reference	1.00E-05
Isoprenoids	Reference	0.00088
Lipoic acid	Reference	0.04864
NAD and NADP	Reference	0.01163
Nucleotidyl-phosphate metabolic cluster	Reference	0.00074
Periplasmic Stress	Reference	0.01521
Phospholipids	Reference	0.00016
Polysaccharides	Reference	2.00E-05
Probably GTP or GMP signaling related	Reference	0.02641
Protein processing and modification	Reference	0.00052
RNA processing and modification	Reference	0.04189
Riboflavin, FMN, FAD	Reference	0.02493
Ribosomal Protein L28P relates to a set of uncharacterized proteins	Reference	0.0033
Signal transduction in Eukaryotes	Reference	0.02409
Spore DNA protection	Reference	0.00632
TRAP transporters	Reference	0.01304
Tetrapyrroles	Reference	0.02493
Toxins and superantigens	Reference	0.00329
Two related proteases	Reference	0.00512

Supplementary Table 4. Continued

Functional Assignment	Enrinched in	p-value
Level 3		
(GlcNAc)2 Catabolic Operon	MAG	0.02068
A Gammaproteobacteria Cluster Relating to Translation	MAG	0.04005
Arginine Deiminase Pathway	MAG	0.01275
At5g37530 (CsdL protein family)	MAG	0.0184
Autoinducer 2 (AI-2) transport and processing (lsrACDBFGE operon)	MAG	0.00662
Biogenesis of cbb3-type cytochrome c oxidases	MAG	0.04532
Biphenyl Degradation	MAG	0.0143
CBSS-203122.12.peg.188	MAG	0.00598
CBSS-211586.1.peg.2357	MAG	0.04532
CBSS-243265.1.peg.198	MAG	0.00283
CBSS-318161.14.peg.2599	MAG	0.04532
CBSS-320372.3.peg.6046	MAG	0.01017
CBSS-323850.3.peg.3269	MAG	0.01865
Catechol branch of beta-ketoadipate pathway	MAG	0.01201
Cluster Ytf and putative sugar transporter	MAG	0.0302
DNA topoisomerases, Type II, ATP-dependent	MAG	0.00961
De Novo Purine Biosynthesis	MAG	0.00961
Dimethylarginine metabolism	MAG	0.04175
Glutamate dehydrogenases	MAG	0.00265
Glutathione-dependent pathway of formaldehyde detoxification	MAG	0.04005
Histidine Degradation	MAG	0.01177
Hydrogenases	MAG	0.03261
KDO2-Lipid A biosynthesis	MAG	0.03665
Lipopolysaccharide assembly	MAG	0.00269
Lipopolysaccharide assembly cluster	MAG	0.03278
Lipopolysaccharide-related cluster in Alphaproteobacteria	MAG	0.01201
Lysine degradation	MAG	0.00103
Mannose-sensitive hemagglutinin type 4 pilus	MAG	0.01865
Mycobacterium virulence operon involved in DNA transcription	MAG	0.0048
Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	MAG	0.00193
Oxidative stress	MAG	0.00066
Oxygen stress response / Human gut microbiome	MAG	0.00277
Phosphonate metabolism	MAG	0.00903
Plasmid replication	MAG	0.00117
Polyadenylation bacterial	MAG	0.01627
Proline, 4-hydroxyproline uptake and utilization	MAG	0.00247
Propionyl-CoA to Succinyl-CoA Module	MAG	0.0069
Proteasome bacterial	MAG	0.00171
Protection from Reactive Oxygen Species	MAG	0.00094
Proteolysis in bacteria, ATP-dependent	MAG	0.01013
Proton-dependent Peptide Transporters	MAG	0.0069
Putative sugar ABC transporter (ytf cluster)	MAG	0.0104
Pyridoxin (Vitamin B6) Biosynthesis	MAG	0.02822
Rcs negative regulator IgaA	MAG	0.04005
RecA and RecX	MAG	0.01865
Resistance to fluoroquinolones	MAG	0.01425
Restriction-Modification System	MAG	0.03613
Ribosome LSU bacterial	MAG	0.01328
Ribosome SSU bacterial	MAG	2.00E-04
Rubrerythrin	MAG	0.01201
Serine-glyoxylate cycle	MAG	0.01959
Staphylococcal pathogenicity islands SaPI	MAG	0.00197
Steroid sulfates	MAG	0.02879
Succinate dehydrogenase	MAG	0.00452
TCA Cycle	MAG	9.00E-05
TenI-like tautomerase	MAG	0.00375
Test Pyridoxin B6	MAG	0.04153

Supplementary Table 4. Continued

Functional Assignment	Enrinched in	p-value
Level 3		
Threonine anaerobic catabolism gene cluster	MAG	0.03097
Translation elongation factor G family	MAG	0.00226
Translation initiation factors bacterial	MAG	0.00306
Type IV pilus	MAG	0.04915
Ubiquinone Menaquinone-cytochrome c reductase complexes	MAG	0.04005
Unsaturated Fatty Acid Metabolism	MAG	0.03278
YebC	MAG	0.01225
acinetobacter tca	MAG	0.00769
tRNA aminoacylation, Gly	MAG	0.02229
tRNA aminoacylation, Ile	MAG	0.04783
tRNA aminoacylation, Leu	MAG	0.00447
tRNA aminoacylation, Met	MAG	0.01822
tRNA-methylthiotransferase containing cluster	MAG	0.03842
2-phosphoglycolate salvage	Reference	0.01711
5-FCL-like protein	Reference	0.04693
A Gram-positive cluster relating ribosomal protein L28P to a set of uncharacterized proteins	Reference	0.0033
A TRAP transporter and a hypothetical	Reference	0.01304
ABC transporter alkylphosphonate (TC 3.A.1.9.1)	Reference	<0.00001
ABC transporter branched-chain amino acid (TC 3.A.1.4.1)	Reference	0.00988
ABC transporter oligopeptide (TC 3.A.1.5.1)	Reference	1.00E-05
ATP-dependent efflux pump transporter Ybh	Reference	0.00157
Adenosyl nucleosidases	Reference	0.01888
Alanine biosynthesis	Reference	0.00035
Alkanesulfonates Utilization	Reference	0.0331
Alpha-Amylase locus in Streptocococcus	Reference	0.00943
Ammonia assimilation	Reference	0.00018
Arginine and Ornithine Degradation	Reference	0.01657
Arsenic resistance	Reference	0.02603
At1g69340 At2g40600	Reference	0.01202
At3g50560	Reference	0.02905
At4g10620 At3g57180 At3g47450	Reference	0.00255
Bacterial Cytoskeleton	Reference	0.0101
CBSS-176299.4.peg.1292	Reference	0.02641
CBSS-222523.1.peg.1311	Reference	0.00074
CBSS-257314.1.peg.488	Reference	0.00532
CBSS-257314.1.peg.676	Reference	0.00512
CBSS-262719.3.peg.410	Reference	1.00E-05
CBSS-292415.3.peg.2341	Reference	0.01841
CBSS-56780.10.peg.1536	Reference	0.00233
CBSS-83331.1.peg.3039	Reference	1.00E-05
CBSS-84588.1.peg.1247	Reference	5.00E-05
CMP-N-acetylneuraminate Biosynthesis	Reference	0.00355
Carbon Starvation	Reference	0.02602
Cell Division Cluster	Reference	0.00716
Cell division-ribosomal stress proteins cluster	Reference	0.00011
Cobalamin	Reference	4.00E-05
Coenzyme A Biosynthesis	Reference	0.02438
Competence or DNA damage-inducible protein CinA and related protein families	Reference	0.00319
Cyanobacterial Circadian Clock	Reference	0.01112
Cysteine Biosynthesis	Reference	0.045
D-allose utilization	Reference	0.02905
D-gluconate and ketogluconates metabolism	Reference	0.04805
D-ribose utilization	Reference	0.0155
DNA repair, bacterial MutL-MutS system	Reference	4.00E-05
Dipicolinate Synthesis	Reference	0.00157
EC 3.4.11.- Aminopeptidases	Reference	0.00126
ECF class transporters	Reference	<0.00001
Energy-conserving hydrogenase (ferredoxin)	Reference	0.01409

Supplementary Table 4. Continued

Functional Assignment	Enrinched in	p-value
Level 3		
FeFe hydrogenase maturation	Reference	0.00238
Flagellar motility	Reference	0.00895
Flavodoxin	Reference	0.0015
Formaldehyde assimilation: Ribulose monophosphate pathway	Reference	0.04255
Fructooligosaccharides(FOS) and Raffinose Utilization	Reference	1.00E-04
Glutamine, Glutamate, Aspartate and Asparagine Biosynthesis	Reference	0.04936
Glutathione-regulated potassium-efflux system and associated functions	Reference	0.00303
Glutathione: Non-redox reactions	Reference	<0.00001
Glycerate metabolism	Reference	0.00063
Glycerolipid and Glycerophospholipid Metabolism in Bacteria	Reference	0.00016
Glycogen metabolism	Reference	1.00E-05
Glycolate, glyoxylate interconversions	Reference	0.00951
Group II intron-associated genes	Reference	0.00682
HPr catabolite repression system	Reference	0.00011
Heme, hemin uptake and utilization systems in GramPositives	Reference	0.00549
High affinity phosphate transporter and control of PHO regulon	Reference	0.00034
Hydantoin metabolism	Reference	0.00208
IojapClusters	Reference	0.00097
Isoprenoid Biosynthesis	Reference	6.00E-04
Isoprenoid Biosynthesis: Interconversions	Reference	0.01009
Isoprenoinds for Quinones	Reference	0.02655
L-Arabinose utilization	Reference	0.0091
L-ascorbate utilization (and related gene clusters)	Reference	0.03055
L-rhamnose utilization	Reference	0.01157
Lipoprotein Biosynthesis	Reference	0.00511
Mannitol Utilization	Reference	0.0166
Menaquinone biosynthesis from chorismate via 1,4-dihydroxy-6-naphthoate	Reference	0.01537
Methicillin resistance in Staphylococci	Reference	0.03978
Methylglyoxal Metabolism	Reference	0.00157
Methylthiotransferases	Reference	0.0052
Molybdenum cofactor biosynthesis	Reference	0.01271
Molybdopterin cytosine dinucleotide	Reference	0.00672
Multidrug Resistance Efflux Pumps	Reference	0.00193
Murein Hydrolases	Reference	<0.00001
Mycobacterium virulence operon involved in unknown function with a Jag Protein, YidC and YidD	Reference	0.00555
Mycobacterium virulence operon involved in fatty acids biosynthesis	Reference	0.00157
NAD and NADP cofactor biosynthesis global	Reference	0.0188
Nitrate and nitrite ammonification	Reference	0.0303
Nitrogen fixation	Reference	0.04327
Nonmevalonate Branch of Isoprenoid Biosynthesis	Reference	0.01068
NusA-TFII Cluster	Reference	0.00057
One-carbon metabolism by tetrahydropterines	Reference	0.00822
Osmoregulation	Reference	0.00418
Peptidoglycan biosynthesis--gjo	Reference	<0.00001
Periplasmic Stress Response	Reference	0.02092
Phage tail fiber proteins	Reference	0.00453
Phd-Doc, YdcE-YdcD toxin-antitoxin (programmed cell death) systems	Reference	0.00193
Phenylalanine and Tyrosine Branches from Chorismate	Reference	0.00292
Plastidial (p)ppGpp-mediated response in plants	Reference	0.02409
Poly-gamma-glutamate biosynthesis	Reference	0.02623
Polyprenyl Diphosphate Biosynthesis	Reference	0.00025
Polysaccharide deacetylases	Reference	1.00E-05
Protein chaperones	Reference	0.03687
Protocatechuate branch of beta-ketoadipate pathway	Reference	<0.00001
Pseudouridine Metabolism	Reference	0.04199
Putative hemin transporter	Reference	0.00933
Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate	Reference	0.01056

Supplementary Table 4. Continued

Functional Assignment	Enrinched in	p-value
Level 3		
Queuosine exploration RZ	Reference	0.0238
Queuosine-Archaeosine Biosynthesis	Reference	0.02801
Quinate degradation	Reference	0.00039
Quinone oxidoreductase family	Reference	0.02946
RNA 3'-terminal phosphate cyclase	Reference	0.00074
RNA processing and degradation, bacterial	Reference	0.00228
RP Bacterial Cell Division	Reference	0.03246
Raj MurE	Reference	<0.00001
Recycling of Peptidoglycan Amino Acids	Reference	0.00053
Respiratory dehydrogenases 1	Reference	<0.00001
Riboflavin, FMN and FAD metabolism Extended	Reference	0.00439
Ribosomal protein S12p Asp methylthiotransferase	Reference	0.00177
Ribosome biogenesis bacterial	Reference	5.00E-04
Rrf2 family transcriptional regulators	Reference	0.01606
SCIFF peptide maturase system	Reference	0.00148
Selenocysteine metabolism	Reference	0.02257
Serine Biosynthesis	Reference	0.03019
SigmaB stress responce regulation	Reference	<0.00001
Signal peptidase	Reference	0.01969
Small multidrug resistance strays	Reference	0.00329
Spore Core Dehydration	Reference	0.00045
Sporulation Cluster	Reference	0.00051
Sporulation draft	Reference	0.00074
Sporulation gene orphans	Reference	0.0088
Sporulation-related Hypotheticals	Reference	0.00108
Streptococcal Hyaluronic Acid Capsule	Reference	0.01712
Streptolysin S Biosynthesis and Transport	Reference	0.00329
Sucrose utilization	Reference	2.00E-05
Terminal cytochrome d ubiquinol oxidases	Reference	0.00826
Terminal cytochrome oxidases	Reference	0.01905
Test Q	Reference	0.01849
Thiamin biosynthesis	Reference	0.00018
Thiamin biosynthesis LDP	Reference	0.00215
Tn552	Reference	0.00682
Transcription initiation, bacterial sigma factors	Reference	0.03244
Transport of Nickel and Cobalt	Reference	0.024
Tricarboxylate transport system	Reference	0.00391
Urea decomposition	Reference	0.00305
Urease subunits	Reference	0.02716
Xylose utilization	Reference	0.00131
YgjD and YeaZ	Reference	0.01656
YjeE	Reference	0.00178
YrdC-YciO-Sua5 and associated protein families	Reference	1.00E-05
dcernst CoA Salvage	Reference	0.00636
t(6)A synthesis in bacteria	Reference	0.00325
tRNA aminoacylation, Thr	Reference	0.04417
tRNA mods Archaea	Reference	0.04643
tRNA processing	Reference	0.00374
tRNA-dependent amino acid transfers	Reference	0.01409

Supplementary Table 5. *LuxA* homologs identified

Sample	Reads	CPM	Level of Classification	Classification
B	25	0.555619094	Species	*Shewanella hanedai*
B	11	0.244472401	Species	*Photobacterium phosphoreum*
B	28	0.622293385	Genus	*Aliivibrio*
BS1	34	3.110886639	Species	*Methanolinea tarda*
Ice_3	740	156.0052536	Species	*Photobacterium phosphoreum*
Ice_7	12	0.666977182	Species	*Shewanella hanedai*
Ice_7	14	0.778140045	Species	*Shewanella hanedai*
S3F1	177	24.35908093	Species	*Photobacterium phosphoreum*
S3F1	53	7.293962086	Species	*Photobacterium phosphoreum*
S3F2	10	1.317664024	Species	*Aliivibrio logei*
S3F2	8	1.054131219	Genus	*Aliivibrio*
Scot_D	798	27.43080596	Order	Bacteroidales
Scot_F	43	2.204323345	Species	*Agrobacterium* sp. 13-626
Scot_J	12	1.269158744	Genus	*Aliivibrio*
Scot_J	30	3.172896861	Genus	*Photobacterium*
Scot_J	19	2.009501345	Species	*Photobacterium phosphoreum*
Scot_J	90	9.518690584	Species	*Photobacterium phosphoreum*
Scot_J	760	80.38005382	Genus	*Photobacterium*
Scot_J	72	7.614952467	Species	*Photobacterium phosphoreum*
W	17	0.694240766	Species	*Photobacterium phosphoreum*
W	570	23.27748452	Genus	*Photobacterium*
Water	528	102.6523581	Species	*Shewanella piezotolerans*
Water	6	1.166504069	Genus	*Epsilonproteobacteria bacterium* LFT 1.7
Water	13	2.527425483	Genus	*Phaeobacter*
Water	3	0.583252034	Species	*Mycobacterium* sp.
Water	4	0.777669379	Domain	Bacteria
Z	10	0.249029768	Species	*Photobacterium phosphoreum*

Supplementary Table 6. Composition of fish pangenome

Species	RefSeq Accession
Anguilla anguilla	GCA_000695075.1
Anguilla japonica	GCA_000470695.1
Astyanax mexicanus	GCA_000372685.2
Branchiostoma floridae	GCF_000003815.1
Callorhinchus milii	GCF_000165045.1
Ctenopharyngodon idellus	PRJEB5920 (BioProject)
Cynoglossus semilaevis	GCF_000523025.1
Dicentrarchus labrax	GCA_000689215.1
Electrophorus electricus	GCA_003665695.2
Gadus morhua	GCA_000231765.1
Gasterosteus aculeatus	GCA_000180675.1
Larimichthys crocea	GCF_000972845.1
Lates calcarifer	GCA_001010145.1
Latimeria chalumnae	GCA_000325985.2
Lepisosteus oculatus	GCF_000242695.1
Neolamprologus brichardi	GCF_000239395.1
Nothobranchius furzeri	GCF_001465895.1
Notothenia coriiceps	GCF_000735185.1
Oreochromis niloticus	GCF_001858045.1
Oryzias latipes	GCA_002234675.1
Periophthalmus magnuspinnatus	GCA_000787105.1
Petromyzon marinus	GCA_002833325.1
Poecilia formosa	GCF_000485575.1
Scleropages formosus	GCF_001624265.1
Sinocyclocheilus graham	GCF_001515645.1
Takifugu flavidus	GCA_000400755.1
Takifugu rubripes	GCF_000180615.1
Tetraodon nigroviridis	GCA_000180735.1
Thunnus orientalis	GCA_000418415.1
Xiphophorus maculates	GCF_002775205.1

Supplementary Table 7. p-values for statistical comparisons of SUPER-FOCUS functional outputs between clusters

Function	Cluster Comparison		
	A vs. B	A vs. C	B vs. C
Chitin and N-acetylglucosamine utilization	7.77E-04	4.33E-02	1.07E-05
Ton and Tol transport systems	2.45E-04	2.28E-03	1.49E-04
Protein degradation	1.59E-02	1.47E-02	1.59E-02
Proteolytic pathway	8.55E-05	4.33E-02	8.10E-06

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Chapter 6: General Discussion

As the spread of antibiotic resistance continues, the risks associated with microbial infections are becoming of much greater concern. The need for new therapies for the prevention and treatment of such infections is clear, but the development of novel antimicrobials and new pipelines has dwindled (Burki, 2017). Bacteriocins are one class of antimicrobials which have been generally overlooked when it comes to tackling the issue of antibiotic resistance. These antimicrobial peptides can exhibit potent inhibitory activity, often against clinically important pathogens even at nanomolar concentrations (Cotter et al., 2013). A narrow spectrum of antimicrobial activity for some bacteriocins allows them to target specific pathogens with minimal collateral damage to the host microbiome (Rea et al., 2011). The *in situ* production of bacteriocins by probiotic bacteria or members of the host microbiome also forms the basis for treatment and prevention of intestinal infections (Corr et al., 2007).

Whilst the use of alternative antimicrobials such as bacteriocins and organic acids remains negligible in medicine, the use of cultures which produce these antimicrobials for the safe preservation of foods has been established for millennia (Ross et al., 2002). In particular, the lactic acid bacteria (LAB) have a long association with food preservation, having important functional and protective roles in the production of fermented foods such as yoghurt, cheese, sauerkraut and kimchi. These LAB have been shown to produce a range of metabolites which can lead to the inhibition of potential spoilage microbes. Organic acids are a by-product of LAB metabolism which lowers the pH of the food source, thus inhibiting the growth of spoilage microbes while also modulating the flavour of the food (Rhee et al., 2011). Bacteriocins offer a much more targeted approach for enhancing food safety. Bacteriocins can be introduced into fermented foods either by *in situ* production by starter cultures, or else by the exogenous addition of crude fermentates or purified bacteriocins (Cotter et al., 2005). Nisin, for example, was awarded generally regarded as safe (GRAS) status by the FDA in 1988, and the safe commercial use for decades of this lantibiotic as a food preservative demonstrates the potential of such antimicrobials for wide-scale use in industry (Younes et al., 2017).

In this study we utilised a variety of mechanisms for the identification and characterisation of novel bacteriocins and antimicrobials. Traditionally large microbial screening studies were performed against a range of target organisms to identify *in vitro* antimicrobial activity. As genomic sequencing has become more affordable and prevalent, current antimicrobial screens can take an *in silico*-based approach using genomic searches. These genomic screening methods allow for a much more targeted approach for the identification of new antimicrobials. It is much less labour intensive compared to traditional colony screening, with a much higher success rate. The issue with genomic screening, however, is that these homology-based searches depend on the sequences of previously characterised antimicrobials, meaning that novel classes of antimicrobials may be overlooked due to their lack of similarity to previously characterised genes. Also, antimicrobials identified in large *in silico* screens are often not produced when tested *in vitro*, which can result a failure to characterise these potentially useful molecules. Here, we utilised both *in silico* and *in vitro* approaches for the identification and characterisation of bacteriocins and discuss their benefits and drawbacks.

In Chapter 2, traditional colony-based screening was used to isolate an antimicrobial-producing strain, *Bacillus paralicheniformis* APC1576. A wide number of colonies were initially isolated from the intestine of marine fish, which were then screened against a *Lactobacillus bulgaricus* strain to identify antimicrobial production. The fact that only one strain out of the thousands screened was identified as producing a novel bacteriocin demonstrates the low success rate of colony-based screening. Once this strain was identified as producing a novel antimicrobial, genomic sequencing of the strain was still required to identify the bacteriocin produced, formicin. Due to the high level conservation seen amongst the class of two-peptide lantibiotics, the bacteriocin genomic screening tool BAGEL easily identified this novel bacteriocin (de Jong et al., 2010). This study shows how traditional screening methods still have a place for the identification of novel antimicrobials, however, the combination of these methods with *in silico* screening provides a more elegant solution.

In Chapter 3 we adopted this targeted approach, using the genomic data of strains composing the *Lactobacillus* Genus Complex to screen for new bacteriocins and bacteriolysins. The BAGEL screening tool was used for the identification of bacteriocins encoded within the genomes of these strains (de Jong et al., 2010). Many of these strains have been long investigated for bacteriocin production, often due to their association with food preservation. Through *in silico* screening, this study provided an excellent overview of the genetic capacity of these strains to produce bacteriocins. We combined this with functional screening for antimicrobial production against a range of indicator organisms, this resulted in the identification of five novel bacteriocins. While these bacteriocins require further study and characterisation, some do offer potential industrial uses. Paralimenterocin, for example, can inhibit the growth of certain enterococci, and as the encoding strain was initially isolated from kimchii, it may have a protective role as a starter culture in the fermentation of vegetables. The combination of the initial genomic screen followed by the functional screen greatly increased the success rate in the identification of these novel bacteriocins. This approach is more efficient method for identifying new antimicrobials, cutting down the number of cultures to be screened through initial genomic homology-based searches.

In Chapter 4, we attempted overcame one of the main hurdles associated with genomic-based screening, which is the failure of many strains to produce the bacteriocins encoded within their genomes. Bacteriocin synthesis can be an energetically costly task for many bacteria, thus their production is often under tight regulation by the encoding strain (Uzelac et al., 2015). In the case of several of the bacteriocins identified here however, many key genes required for the synthesis and transport of the peptides were missing from the bacteriocin operon. To overcome the inability of such strains to produce a bacteriocin we used a heterologous expression system in which the pediocin PA-1 ABC transporter was cloned alongside a range of novel pediocin-like bacteriocins. We were able take advantage of the high degree of homology within this pediocin-like bacteriocin subclass. Of the novel class IIa bacteriocins identified from the *Lactobacillus* Genus Complex, their relatively high degree of homology to the bacteriocin pediocin PA-1 means that the pediocin-

associated transporter has the ability to secrete these new bacteriocins from the cells. Whilst this system works well for the class IIa bacteriocins, further work is required to determine the effectiveness of such an approach using different classes of bacteriocins. The study does show, however, how the *in silico* identification of novel antimicrobials can be translated into biological production using the correct expression systems. The benefits of this approach are shown in the fact that some of these class IIa bacteriocins expressed heterologously display a greater range of antimicrobial activity compared to pediocin PA-1, a commercially used bacteriocin.

A microbiome-based approach was used in Chapter 5 for the characterisation of microbial communities found in the intestinal tracts of deep-sea fish, as well as identifying their potential ability for the production of secondary metabolites. Whilst the production of antimicrobials is one mechanism by which microbes can influence the microbiome, often it is larger scale environmental changes which control which species occupy any given niche. This is seen with the potential identification of a link between the diet of the host and the metabolic capabilities of the microbes they harbour within their gut. The screen for genes related to the production of secondary metabolites shows the potential diversity of natural products which may be synthesised in the microbiome of these fish. Given how these bacteria display such adaptions to their environment, their apparent lack of resistance towards common antibiotics suggests that any natural products encoded within these communities may be highly novel. Studies such as this, however, need to be followed up with the heterologous expression of the identified operons, or the isolation of microbes from the source which can then be screened for *in vitro* antimicrobial production.

Taken together, this thesis outlines several approaches which can be used for the identification of novel antimicrobials. Reliance solely on either traditional or *in silico* screening methods limits the possibility of a successful outcome. Traditional wet lab screening is made much more efficient when combined with initial genomic screens, the only downside of which is the potential oversight of completely novel compounds due to the reliance on homology-based searches. *In silico* screening, if

not followed up by the expression and characterisation of the antimicrobial produced, offers little benefit when trying to develop new therapies targeting antibiotic resistant microbes. We show that when combined correctly, these approaches can lead to the identification of a range of new antimicrobials so that the full antimicrobial capability of the strains can be fully harnessed.

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