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Harnessing the bacteriocin-producing capacity of the gut with a view to controlling microorganisms that have been associated with obesity and related metabolic disorders

A thesis presented to the National University of Ireland for the degree of Doctor of Philosophy

By

James William Hegarty BSc

Teagasc Food Research Centre, Moorepark, Fermoy Co. Cork, Ireland

School of Microbiology, University College Cork, Cork, Ireland

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Research supervisors:

Dr. Paul D. Cotter, Dr. Caitriona M. Guinane, Prof. Colin Hill, Prof. R. Paul Ross



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Declaration

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

Signed:_____

James Hegarty

Date: _____

Abstract

Obesity is a complex syndrome associated with a number of serious implications for human health including cardiovascular disease, type-2 diabetes and musculoskeletal disorders. Although diet, lifestyle factors and host genetics are certainly key factors, the gut microbiota in obesity and related metabolic conditions has received considerable attention. The "Obesibiotics" project was developed following a proof of concept study which demonstrated that antimicrobials, and indeed bacteriocins, could be used to alter the gut microbiota with respect to obesity. The aim of this project within the scope of "Obesibiotics" was to (1) harness the bacteriocin-producing capacity of the gut and (2) develop a bacteriocin-producing probiotic that can contribute to the prevention/treatment of obesity and related metabolic disorders.

Firstly, bacteriocin production among a selection of commercial probiotic products was examined. This study resulted in the identification of 8 distinct bacteriocin-producing isolates from 8 distinct products. All were identified as *Lactobacillus acidophilus*, with antimicrobial activity attributed to the narrow spectrum, class II bacteriocin, lactacin B. This investigation suggests that the commercial bacteriocin-producing probiotics are not very heterogeneous and that bacteriocin production is not being optimally harnessed as a probiotic trait.

To further expand the variety of bacteriocin-producing probiotics, and in particular, in relation to identifying strains with activity against obesity-associated targets, a culture-based screen was undertaken using faecal samples from lean donors. Screening was performed using both aerobic and anaerobic conditions with a selection of indicators. Four lead bacteriocin-producing isolates were identified, *Streptococcus salivarius* DPC6988, *Streptococcus mutans* DPC7039, *Enterococcus faecalis* DPC7040, and *Streptococcus agalactiae* DPC7041, which successfully

inhibited species enriched in type 2 diabetic patients. As a consequence of the nature of the producing strain, *S. salivarius* DPC6988 was selected for further investigation.

We next investigated the impact of this bacteriocin-producing *S. salivarius* DPC6988, and a non-producing control, *S. salivarius* HSISS4 on gut microbial populations, to which *Clostridium ramosum* DSM1402 was added, using an *ex vivo* model of the distal colon. Although both strains altered microbial populations over the 24 hr fermentation period, a number of beneficial changes were attributed to DPC6988 only.

Finally, the ability of DPC6988 to alter the gut microbiota and mitigate the metabolic abnormalities with respect to obesity in a DIO mouse model was examined. C57BL/6 mice were fed a HFD or LFD for a period of 12 weeks followed by an 8 week intervention period. Despite alterations to the gut microbiota, treatment with *S. salivarius* did not result in improvements to weight gain or metabolic health, suggesting a higher dosage or longer intervention maybe needed. Future studies with this strain may also employ obesity-promoting species to fully investigate the potential of DPC6988 to control weight gain.

Overall this thesis resulted in the identification of a number of baceriocin-producing gut microbes. Further work with *S. salivarius* DPC6988 will be necessary to understand the extent to which this strain can contribute to the prevention/treatment of obesity and related disorders and, more generally, to optimally harness the ability of bacteriocin-producing strains to beneficially change the composition of the gut microbiota.

Publications

James W. Hegarty, Caitriona M. Guinane, R. Paul Ross, Colin Hill, Paul D. Cotter. 2016. Bacteriocin production: a relatively unharnessed probiotic trait? F1000 Research 2016, 5(F1000 Faculty Rev):2587

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Glossary of Terms

μg/mL	Microgram per millilitre
μL	Microliter
μM	Micromolar
BAGEL	Bacteriocin genome mining tool
BHI	Brain heart infusion broth
BLAST	Basic local alignment search tool
BMI	Body mass index
Bp	Base pair
BSA	Bovine serum albumin
CDAD	*Clostridum difficile*-associated disease
CFS	Cell free supernatant
CFU	Colony forming unit
CFU/g	Colony forming unit per gram
CFU/mL	Colony forming unit per millilitre
CMS	Colony mass spectrometry
CRC	Colorectal cancer
Da	Daltons
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority

ELISA	Enzyme-linked immunosorbent assay
FAA	Fastidious anaerobe agar
FAO	Food and Agriculture Organisation
FFA	Free fatty acids
FLASH	Fast length adjustment of short reads to improve genomic assemblies
g	Grams
GBS	Group B streptococci
gDNA	Genomic DNA
GI	Gastrointestinal
GIT	Gastrointestinal tract
gM17	Glucose M17 broth
H_2	Hydrogen gas
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HFD	High-fat diet
HMP	Human Microbiome Project
HNSCC	Head and neck squamous cell carcinoma
HPLC	High performance liquid chromatography
hrs	Hours
IL-10	Interleukin-10
IL-2	Interleukin-2
ISO	International Organization for Standardization

IST	Iso-sensitest
LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
LFD	Low-fat diet
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
man-PTS	Mannose phosphotransferase
mg/mL	Milligrams per millilitre
MIC	Minimum inhibitory concentration
mins	Minutes
mL	Millilitre
mM	Millimolar
mMRS	Modified de Man-Rogosa-Sharpe broth
MRD	Maximum recovery diluent
MRI	Magnetic resonance imaging
MRS	de Man-Rogosa-Sharpe broth
N_2	Nitrogen gas
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
nm	Nanometre
O_2	Oxygen gas
OD	Optical density
OTU	Operational taxonomic unit

PBGC	Putative bacteriocin gene cluster
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
pH	Power of hydrogen
PSI-BLAST	Position-specific iterated basic local alignment search tool
PyNAST	Python nearest alignment space termination
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative PCR
QPS	Qualified presumption of safety
RCM	Reinforced clostridial medium
ROP	Retro-orbital puncture
RP	Reverse phase
rpm	Revolutions per minute
rRNA	ribosomal ribonucleic acid
SAM	S-adenosylmethionine
sec	Seconds
SEM	Mean plus standard error
SIHUMI	Simplified human intestinal microbiota
SPE	Solid phase extraction
T	Time

T2D	Type 2 diabetes/diabetic
TC	Total cholesterol
TFA	Trifluoroacetic acid
TG	Triglycerides
v/v	Volume per volume
w/v	Weight per volume
WCAA	Wilkins-Chalgren anaerobe agar
WHO	World Health Organisation

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Chapter 1

Bacteriocin production: a relatively unharnessed probiotic trait?

A version of this literature review is published in F1000 Research 2016, 5(F1000 Faculty Rev):2587

1.1 Abstract

Probiotics are “live microorganisms which, when consumed in adequate amounts, confer a health benefit to the host”. A number of attributes are highly sought after among these microorganisms, including immunomodulation, epithelial barrier maintenance, competitive exclusion, production of short-chain fatty acids and bile salt metabolism. Bacteriocin production is also generally regarded as a probiotic trait, but it can be argued that, in contrast to other traits, it is often considered a feature that is desirable, rather than a key, probiotic trait. As such, the true potential of these antimicrobials has yet to be realised.

1.2 Introduction

1.2.1 What are probiotics?

Probiotics are "live microorganisms which, when consumed in adequate amounts confer a health benefit to the host" (Hill, Guarner et al. 2014). The majority of probiotic species in commercial use today are members of the genera *Lactobacillus* or *Bifidobacterium*, but can also include *Escherichia coli* strain Nissle 1917, some strains of *Enterococcus* and *Streptococcus*, and yeasts such as *Saccharomyces boulardii* (Soccol, Vandenberghe et al. 2010, Guarner, Khan et al. 2012). These can be used individually or combined to form multi-species/strain mixtures (Chapman, Gibson et al. 2011, Chapman, Gibson et al. 2012).

It is of importance that a number of criteria are met when selecting strains for probiotic applications. Viability of the strain is crucial, and in the majority of cases, the strain must survive processing, storage, and ultimately, passage through the gastrointestinal tract (GIT), while retaining the original health promoting effects. In particular, GIT survival depends on the ability to withstand the low pH of the stomach and tolerate the presence of bile acids. A daily oral administration of $1x10^9$ colony forming units has frequently been regarded as the optimal probiotic dosage based on the recovery of probiotic organisms in faeces, although in some cases higher doses may be needed (Tannock 2003). Ideally, potential probiotics should be of human origin and must also be safe for human consumption i.e. be non-pathogenic and non-toxigenic. A qualified presumption of safety (QPS) approach was introduced as a pre-market safety assessment for selected groups of microorganisms (Barlow, Chesson et al. 2007). This concept provided a universal assessment system for use within the jurisdiction of the European Food Safety

Authority (EFSA) as a way of assessing microorganisms purposely introduced into the food chain. The principal is that if a taxonomic group does not present safety concerns it can be granted QPS status, thereby reducing the number of obstacles to its commercial development. Key factors include identity, the associated body of knowledge, an absence of virulence factors, and the establishment of antibiotic resistance breakpoints. Aside from these practical considerations, strain selection is an important factor when studying the effects of probiotics as many of the health benefits are strain dependant. The ability of different probiotic strains to impart different health benefits has been reviewed extensively (Ouwehand, Salminen et al. 2002, Özdemir 2010, Wolvers, Antoine et al. 2010, Delzenne, Neyrinck et al. 2011, Reid, Younes et al. 2011, Petschow, Doré et al. 2013, Sanders, Guarner et al. 2013) and, so, are summarised only briefly below.

The majority of these health benefits that have been attributed to probiotics include (i) immunomodulation of host functions, including the innate and acquired immune system, (ii) maintaining the intestinal barrier, and (iii) having a direct effect on other microorganisms such as commensals and/or pathogens, or products thereof (Oelschlaeger 2010, Nagpal, Kumar et al. 2012). Many probiotics can produce antimicrobial substances directly themselves, in addition to stimulating host cells to produce antimicrobials. These antimicrobials consist of organic acids, including lactic acid and acetic acid which function by reducing the pH of the lumen, bacteriocins, nitric oxide and hydrogen peroxide, and contribute to the ability of the probiotic to become established in its niche, control pathogenic microbes and change microbiota composition (O'Shea, Cotter et al. 2012, Tejero-Sariñena, Barlow et al. 2012). Bacteriocin production as a probiotic trait is the central focus of this review.

1.2.2 What are bacteriocins?

Bacteriocins are small, heat-stable, ribosomally synthesised antimicrobial peptides, produced by bacteria that are active against other bacteria and to which the producer has a specific immunity mechanism (Cotter, Hill et al. 2005). These peptides exhibit considerable diversity with respect to their size, structure, mechanism of action, inhibitory spectrum, immunity mechanisms and target cell receptors (Gillor, Etzion et al. 2008). Indeed, for example, many bacteriocins have a narrow spectrum of activity, displaying antimicrobial activity against strains that are closely related to the producer, while others display antimicrobial activity across a broad variety of different genera (Cotter, Hill et al. 2005). The regulation of bacteriocin production can be complex, in some instances being influenced by environmental conditions, such as pH, temperature and growth medium, (Fernandez, Le Lay et al. 2013, Guinane, Piper et al. 2015, Turgis, Vu et al. 2016). Autoinduction (Kuipers, Beerthuyzen et al. 1995, Kleerebezem, Bongers et al. 2004) and induction in the presence of target microbes (Tabasco, Garcia-Cayuela et al. 2009) can also occur.

1.3 Bacteriocin classification

Despite the diversity among bacteriocins, they can generally be classified into one of two groups on the basis of whether they undergo post-translational modifications (Cotter, Hill et al. 2005). This approach was further updated by Cotter *et al*. (Cotter, Ross et al. 2013) and divides class I (modified) bacteriocins into the following subgroups: lantibiotics, linaridins, linear azol(in)e-containing peptides, cyanobactins, thiopeptides, lasso peptides, sactibiotics, glycocins and modified microcins. Class II (unmodified) bacteriocins consist of five subgroups: four correspond to the unmodified lactic acid bacteria (LAB) bacteriocins and one corresponds to the

unmodified microcins and includes class IIa (pediocin-like), IIb (two-peptide bacteriocins), IIc (circular bacteriocins), IId (linear non-pediocin-like) and IIe (microcin E492-like bacteriocins). Although not discussed below but worth mentioning are the bacteriolysins; these are large, heat labile, antimicrobial proteins, and were previously regarded as class III bacteriocins (Rea, Ross et al. 2011). A selection of bacteriocins are described below, based on either the association of the bacteriocin with members of lactic acid bacteria, or the probiotic potential of the bacteriocin-producing strain.

Class I bacteriocins

1.3.1 The lantibiotics/lanthipeptides

The lantibiotics, a name derived from the term lanthionine-containing antibiotics, are small peptides that undergo distinctive post-translational modifications involving the initial dehydration of serine and threonine residues in the precursor peptide to give dehydroalanine and dehydrobutyrine (Arnison, Bibb et al. 2013). The subsequent interaction of these dehydro-residues with an intrapeptide cysteine results in the formation of the eponymous amino acids lanthionine or β-methyl lanthionine, giving lantibiotics their characteristic structural features (Cotter, Hill et al. 2005, Marsh, Hill et al. 2012). The term lanthipeptides is used to capture all peptides that contain these structures, with lantibiotics, i.e., those with antimicrobial activity, being a sub-group therein. Currently the lanthipeptides are divided into four subclasses/types based on the nature of the modification enzymes that carry out these distinctive reactions (Marsh, Hill et al. 2012, Zhang, Yu et al. 2012). Subclass (type) 1 and 2 are the focus here as subclass 3 and 4 generally do not display antimicrobial activity (Arnison, Bibb et al. 2013). For subclass 1 lantibiotics, modifications are carried out

by two enzymes; LanB, a lanthionine dehyratase which performs the dehydration reaction and LanC, a lanthionine synthase that is responsible for cyclization. For subclass 2 peptides, both of these reactions are carried out by a single, LanM, enzyme. Examples of gut-associated subclass 1 (LanBC) lantibiotics include nisin H (O'Connor, O'Shea et al. 2015), nisin F (De Kwaadsteniet, Ten Doeschate et al. 2008) and salivaricin D (Birri, Brede et al. 2012) while ruminococcin A is a subclass 2 (LanM) lantibiotic (Dabard, Bridonneau et al. 2001). Other lantibiotics of human origin are saliviaricin A2 and salivaricin B, isolated from the oral cavity and produced by the oral probiotic *Streptococcus salivarius* K12 (Burton, Chilcott et al. 2006). The isolation of salivaricin A2 from the gut has also been reported (O'Shea, Gardiner et al. 2009).

The antimicrobial activity of many lantibiotics is due to one, or both, of two mechanisms of action, i.e. (i) inhibition of cell wall biosynthesis by targeting lipid II, the membrane bound cell wall precursor and/or (ii) pore formation disrupting the integrity of the cell membrane (Chatterjee, Paul et al. 2005, Bierbaum and Sahl 2009). Nisin, one of the most well-known and best studied lantibiotics, functions using both mechanisms of action. However, activity is generally limited against Gram-negative bacteria due to the protection conferred by the outer membrane (Stevens, Sheldon et al. 1991, Helander and Mattila-Sandholm 2000).

1.3.2 The sactibiotics

The sactibiotics are another group of post-translationally modified bacteriocins but differ with respect to the modifications that they undergo, being altered to contain sulphur to α-carbon linkages (Mathur, Rea et al. 2015). This reaction is catalysed by S-adenoslymethionine (SAM). These radical SAM proteins carry out the cleavage of

adenosylmethionine, a reaction that results in combining SAM with an unusual iron sulphur cluster (Fontecave, Atta et al. 2004). Bacteriocins within the sactibiotic class that have been identified to date exhibit a rather narrow spectrum of inhibition. Subtilosin A produced by *Bacillus subtilis* 168 and thuricin CD produced by *Bacillus thuringiensis* DPC6431 are just two examples of bacteriocins from this class (Babasaki, Takao et al. 1985, Rea, Sit et al. 2010). The activity of subtilosin A has been examined against *Gardnerella vaginalis*, and the formation of transient pores by subtilosin A was revealed as the mechanism of action which lead to cell death of *G. vaginalis* (Noll, Sinko et al. 2011).

Class II bacteriocins

1.3.3 Class IIa

Class II bacteriocins are also small (<10kDa) and can be heat stable, but unlike class I bacteriocins are not subject to post-translational modifications (Cotter, Hill et al. 2005). As mentioned previously class II bacteriocins consist of five subgroups, the first of these are the class IIa, pediocin-like peptides. This subgroup is among the most widely studied of the class II bacteriocins, many of which are produced by lactic acid bacteria (LAB) (Nissen-Meyer, Rogne et al. 2009). A key feature among bacteriocins belonging to this class is their particularly potent ability to inhibit *Listeria monocytogenes* (Katla, Naterstad et al. 2003). They range in size from 37-55 amino acid residues and contain the conserved "pediocin box" Y-G-N-G-V/L within the N-terminal region as well as two cysteine residues joined by a disulphide bridge. Furthermore, some bacteriocins within this class contain a second disulphide bridge (Nissen-Meyer, Rogne et al. 2009, Rea, Ross et al. 2011). Class IIa bacteriocins include leucocin A (Hastings, Sailer et al. 1991), pediocin PA-1 (from which this

subgroup of bacteriocins derived its name) (Henderson, Chopko et al. 1992) and sakacin P (Tichaczek, Nissen-Meyer et al. 1992). Examples of gut associated class IIa bacteriocins include coagulin, produced by *Bacillus coagulans* I_4 and avicin A produced by two strains of *Enterococcus avium* (Birri, Brede et al. 2010). Other studies have detected class IIa bacteriocins, which were initially identified in non-gut environments, in gut samples, such as enterocin A produced by *Enterococcus faecium* DPC6482 (O'Shea, Gardiner et al. 2009). Class IIa bacteriocins function by permeabilising the cell membrane leading to a disruption in the proton motive force and, ultimately, cell death (Chikindas, García-Garcerá et al. 1993, Drider, Fimland et al. 2006). This involves the use of the mannose phosphotransferase (man-PTS) system as a receptor. More specifically, the peptides require a short region containing an extracellular loop in the N-terminal region of the IIC protein, a structural component of the man-PTS complex, to target sensitive cells (Kjos, Salehian et al. 2010).

1.3.4 Class IIb

Class IIb bacteriocins consist of two peptides, both of which are required for optimal antimicrobial activity. Lactococcin G was among the first bacteriocin of this class to be identified (Nissen-Meyer, Holo et al. 1992), with a number of others identified since. The two peptides of class IIb bacteriocins are synthesised to contain a 15-30 residue N-terminal leader that is cleaved at the C-terminal side by an ABC transporter which also moves it across the membrane (Oppegård, Rogne et al. 2007, Nissen-Meyer, Oppegård et al. 2010). These bacteriocins function to permeabilise the cell membrane upon contact, leading to the efflux of small molecules and death of the cell (Nissen-Meyer, Rogne et al. 2009). Indeed, *Lactobacillus salivarius* UCC118 (NCIMB 40829 LSUCC118), a strain isolated from the ileal-caeca region

of the human gastrointestinal (GI) tract, produces the two-component bacteriocin Abp118 which is capable of inhibiting a number of foodborne and medically significant pathogens (Dunne, Murphy et al. 1999, Flynn, van Sinderen et al. 2002). Another example is the closely related salivaricin P bacteriocin produced by the porcine gut isolate *L. salivarius* DPC6005 (Barrett, Hayes et al. 2007).

1.3.5 Class IIc

Class IIc bacteriocins, also known as the circular bacteriocins, are characterised by an amide bond between the N- and C-termini. Due to the presence of this circular formation, these bacteriocins display resistance to many proteases and are temperature stable (Maqueda, Sánchez-Hidalgo et al. 2008, Van Belkum, Martin-Visscher et al. 2011). Many of the bacteriocins from this class range in size from 58-78 amino acids and include examples such as gassericin T, isolated from human faeces (Kawai, Saitoh et al. 2000) and garvicin ML isolated from mallard ducks (Borrero, Brede et al. 2011). Circular bacteriocins have a broad spectrum of inhibition, with activity generally directed against Gram-positive bacteria belonging to the phylum Firmicutes. Along with other class II bacteriocins, they are believed to function by disrupting the integrity of the cell membrane (Van Belkum, Martin-Visscher et al. 2011). As discussed in a review by Gabrielsen *et al*. (2014), it is unclear if circular bacteriocins require a receptor molecule for target recognition. However, it was suggested that concentration may affect the mode of action of circular bacteriocins, where activity at a high concentration is non-specific, but may be specific when concentrations of the bacteriocin are low (Gabrielsen, Brede et al. 2014). A specific receptor, such as the maltose ABC transporter, is responsible for sensitivity to the class IIc bacteriocin, garvicin ML (Gabrielsen, Brede et al. 2012).

1.3.6 Class IId

Class IId bacteriocins show no significant similarity to other class II bacteriocins and are known as the unmodified, linear, non-pediocin-like bacteriocins. Bacteriocins are placed into this class simply because they do not fit the criteria to be part of subgroups IIa, b or c as set by the various classification schemes (Iwatani, Zendo et al. 2011, Cotter, Ross et al. 2013). This subgroup therefore contains a heterogenous group of bacteriocins produced by a wide variety of strains from various ecological niches (Rea, Ross et al. 2011). As mentioned by Iwantai *et al*. (2011) this class may be further subdivided into three groups as follows, (1) sec-dependant bacteriocins, whereby a signal peptide is secreted by the general secretory pathway, (2) leaderless bacteriocins and (3) non sub-grouped bacteriocins (Iwatani, Zendo et al. 2011). One of the best characterised class IId bacteriocins is lactococcin A, produced by some strains of *Lactococcus lactis*. This bacteriocin displays a narrow spectrum of inhibition, acting exclusively against other lactococci by increasing the permeability of the cell membrane in a proton motive force-dependant manner, resulting in solutes leaking across the membrane leading to cell death (Diep, Skaugen et al. 2007). Other class IId bacteriocins include salivaricin L (O'Shea, O'Connor et al. 2011). Salivaricin L is a one-component class IId bacteriocin with anti-listerial activity, produced by *L. salivarius* DPC6488 and was isolated from infant faeces. The novel bactofencin A produced by *L. salivarius* DPC6502, isolated from the porcine gut, does not share similarities with any known bacteriocin and displays a narrow spectrum of inhibition with activity against some medically significant pathogens (O'Shea, O'Connor et al. 2013).

1.4 Strategies to identify new bacteriocins

1.4.1 Traditional methods

A number of approaches can be taken to identify novel bacteriocin-producing probiotics (Marsh, Hill et al. 2012). Traditional culture-based methods, such as the deferred antagonism assay, are frequently employed. For this assay, samples to be screened are plated on a suitable growth medium and grown under appropriate conditions before overlaying colonies with an indicator organism. Bacteriocin production is observed as a zone of growth inhibition surrounding the producing isolate. However, traditional approaches have their disadvantages, with one major limitation arising from the difficulty or inability to culture some microorganisms, resulting in potentially novel bacteriocin-producing strains being overlooked. Bacteriocin production is also tightly regulated and the associated gene cluster may be switched off under the culture-based conditions used in the screen (Kleerebezem 2004). Furthermore, the narrow spectrum of inhibition often associated with bacteriocins may result in production not being detected if just a limited number of indicator organisms are used during screening. As a consequence, a common feature among culture-based screens is the low frequency with which bacteriocin producers are isolated. Although culture-based strategies suffer from these limitations, they continue to be used to identify novel bacteriocins, and many studies have employed this approach in the search for bacteriocins from different GI sources (O'Shea, Gardiner et al. 2009, Birri, Brede et al. 2012, Lakshminarayanan, Guinane et al. 2013). Some investigations have relied on the use of a more targeted approach aimed at isolating a bacteriocin producer with antimicrobial activity against a particular pathogen (Rea, Sit et al. 2010). Using this traditional approach, the screening of a

faecal sample from a healthy infant led to the identification, purification and characterisation of salivaricin D, produced by *Streptococcus salivarius* 5M6c, a lantibiotic which shares significant homology with the nisins (Birri, Brede et al. 2012). A screen of mammalian intestinal samples by O'Shea *et al*. led to the eventual isolation and characterisation of the class IId bacteriocin, bactofencin A, by *L. salivarius* DPC6502 (O'Shea, Gardiner et al. 2009, O'Shea, O'Connor et al. 2013) and the more recently described lantibiotic, nisin H, produced by the gut-derived *Streptococcus hyointestinalis* DPC6484 (O'Connor, O'Shea et al. 2015).

1.4.2 Bioinformatic approaches

An alternative strategy in the search for novel bacteriocins employs the use of *in silico* screens. This approach makes use of data generated from whole genome and metagenome sequencing projects, and screens for highly conserved regions in bacteriocin gene clusters such as genes encoding bacteriocin modification enzymes. This approach bypasses many of the limitations associated with traditional culture-based methods in the search for novel bacteriocin-producing microbes, as the initial need to grow the isolate and detect production is removed. This approach has been used in the past to identify many novel lantibiotics (Begley, Cotter et al. 2009) and to study the diversity of type 1 lantibiotic gene clusters from among genome sequenced bacteria (Marsh, O'Sullivan et al. 2010). In one example, Murphy *et al*. undertook an *in silico* screen for novel sactibiotic/thuricin CD-like gene clusters using TrnC and TrnD radical SAM proteins as driver sequences to identify homologous gene clusters (Murphy, O'Sullivan et al. 2011). Mining of genomes was performed using the BlastP webserver and resulted in the identification of 99 TrnC and 53 TrnD homologs, with further analysis leading to the identification of 15 novel clusters not

previously determined to be potential bacteriocin-like clusters. While many investigations have used a BLAST based approach in the search for novel clusters, the web-based bacteriocin genome mining tool BAGEL has also been a very valuable resource (van Heel, de Jong et al. 2013). BAGEL searches both directly for bacteriocin structural genes and indirectly for bacteriocin associated genes. An *in silico* investigation by Walsh *et al*. used this genome-mining tool to screen the GI tract subset of the Human Microbiome Project (HMP) reference genome database (Walsh, Guinane et al. 2015). From 382 reference genomes, just 59 encoding putative bacteriocin gene clusters (PBGC's) were identified, and within these strains, 74 gene clusters were described. From the PBGC's identified, the most common were the bacteriolysins, which are lytic proteins (Rea, Ross et al. 2011), followed by the lantibiotics and sactipeptides. This study also identified PBGC's in species not previously associated with bacteriocin production, including *Bacteroides uniformis* and *Roseburia intestinalis*. Zheng *et al*. also identified putative bacteriocins from HMP metagenomic data sets (Zheng, Gänzle et al. 2015). The aim of this study was to examine the diversity and distribution of bacteriocins from different body sites and reveal the relationship between bacteriocin clusters and the taxonomic structure of bacterial communities. Using protein sequences from the BAGEL3 database as driver sequences, a PSI-BLAST approach was used to identify gene clusters. PSI-BLAST searches a database for proteins with distant similarity to a query sequence (Schäffer, Aravind et al. 2001). This method identified 4875 putative bacteriocins from the human microbiome including 802 class I bacteriocins, 3048 class II bacteriocins and 1025 bacteriolysins. Interestingly, Zheng *et al*. reported that the gut contained the lowest density of putative bacteriocin genes when compared to other sites including the vagina, the airway and the oral cavity, with the highest density

arising from the oral cavity. This low density may have been due to differences in the ecology of different body sites, and it was proposed that bacteriocins play important roles in enabling bacteria to occupy several body sites, and develop a commensal relationship with the human host (Zheng, Gänzle et al. 2015). With the increasing amount of sequence data that is being generated, these investigations highlight the value of *in silico* based approaches in the search for novel bacteriocins. However, it is worth noting that the correlation between the *in silico* identification of gene clusters and production of the bacteriocin remains putative until inhibitory action is demonstrated, either due to production by the natural host or by heterologous expression of the genes.

1.5 Bacteriocin-producing probiotics

Antimicrobial/bacteriocin production may contribute to probiotic functionality through three different mechanisms (Dobson, Cotter et al. 2012). Firstly, as colonizing peptides, bacteriocins aid the survival of the producing strain in the gut environment (Walsh, Gardiner et al. 2008). Secondly, bacteriocins function through direct inhibition of the growth of pathogens (Corr, Li et al. 2007), and, finally, bacteriocins may serve as signalling peptides/quorum sensing molecules in the intestinal environment (van Hemert, Meijerink et al. 2010). However, although bacteriocin production is generally regarded as a probiotic trait, it can be argued that, in contrast to other traits, it is often considered a feature that is desirable, rather than a key probiotic trait. As such, the true potential of these peptides for gut health, and indeed other applications (Drider, Bendali et al. 2016), has yet to be realised.

As previously discussed, the majority of probiotic species in commercial use today are members of the genera *Lactobacillus* or *Bifidobacterium*. However, despite the

health-promoting attributes associated with *Bifidobacterium* spp. and their potential ability to produce these antimicrobials, there is limited information available regarding functional bacteriocin production by bifidobacteria (Martinez, Balciunas et al. 2013). This raises the question: is bacteriocin production a rare trait among bifidobacteria or are bacteriocin-producing bifodobacteria being overlooked or not being effectively harnessed? Interestingly, while examining the diversity and distribution of bacteriocins from different body sites, Zheng *et al*. reported the absence of bacteriocins produced by *Bifidobacterium* spp. in the gut, despite bifidobacteria accounting for up to 10% of the microbiome (Zheng, Gänzle et al. 2015). Walsh *et al*. identified just two novel putative bacteriocin gene clusters, belonging to the lantibiotic class, from two *Bifidobacterium* spp. during a screen of the GI tract subset of the Human Microbiome Project reference genome database (Walsh, Guinane et al. 2015), again emphasizing the rarity of production among this genus. Other probiotics include specific strains of *Streptococcus* spp., *Lactococcus* spp., *Enterococcus* spp. as well as the *Escherichia coli* strain Nissle 1917 and yeasts such as *Saccharomyces boulardii* (Guarner, Khan et al. 2012, Sanders, Guarner et al. 2013). As lactococci are not typically regarded as gut-associated microorganisms and the use of enterococci as probiotics is controversial, for the purposes of this review we have focused on reviewing what is known about bacteriocin production from among probiotic lactobacilli and streptococci of human origin and discussing the extent to which this trait is valued when commercialising associated strains (Figure 1). Although not typically regarded as a probiotic species, *Bacillus thuringiensis* DPC6431 has also been examined for its probiotic potential and will be reviewed below.

1.5.1 Probiotic lactobacilli

The mechanism by which bacteriocin production contributes to probiotic functionality among species of *Lactobacillus* has been the focus of a number of studies. Plantaricin EF is a bacteriocin produced by strains of *Lactobacillus plantarum* and is composed of two secreted peptides (plnE and plnF). van Hemert *et al*. reported that genes required for plantaricin production and transport contributed to the immunomodulatory effects of *L. plantarum* WCFS1 on peripheral blood mononuclear cells (PBMCs) (van Hemert, Meijerink et al. 2010). Here it was suggested that antimicrobial peptides of bacterial origin may play a similar role to that of antimicrobial peptides of human origin, such as defensins, secreted in the gut which are known to modulate immune responses. Using the same strain of *L. plantarum*, Meijerink *et al*. established that six of the eight genes that modulate the dendritic cell cytokine response were involved in bacteriocin production or secretion (Meijerink, Van Hemert et al. 2010). The beneficial impact of using *Lactobacillus johnsonii* La1 to control *Helicobacter pylori* colonization was also previously examined (Gotteland, Andrews et al. 2008).

A number of strains of *L. salivarius* which possess probiotic traits have been identified, and the genus is also associated with the production of a number of class II (a, b and d) bacteriocins (Stern, Svetoch et al. 2006, O'Shea, Gardiner et al. 2009, O'Shea, O'Connor et al. 2011, Svetoch, Eruslanov et al. 2011, O'Shea, O'Connor et al. 2013). *L. salivarius* UCC118 (NCIMB 40829 LSUCC118) is a very well characterised strain that has been studied with a view to potential probiotic applications and that notably produces the class II, two-peptide bacteriocin Abp118 (Flynn, van Sinderen et al. 2002). Abp118 displays a relatively broad spectrum of antimicrobial activity against a number of food-borne and medically significant

pathogens (Dunne, Murphy et al. 1999). This probiotic strain was the focus of particular attention when it was employed in an important 'proof-of-concept' study which proved that bacteriocin production is indeed a probiotic trait, by virtue of its ability to protect mice against *L. monocytogenes* infection (Corr, Li et al. 2007). Notably, protection was not provided by a non-bacteriocin-producing derivative of UCC118. Furthermore, a strain of *L. monocytogenes*, in which the Abp118 immunity gene was expressed, was not inhibited by the bacteriocin and caused infection in mice that had received a dose of the probiotic (Corr, Li et al. 2007). The impact of this bacteriocin-producing probiotic, and the non-producing mutant thereof, on the gut microbiota of pigs and Balb/c mice has also been examined. In this study, an effect was observed on members of the phylum Firmicutes which was believed to be associated with bacteriocin production (Riboulet-Bisson, Sturme et al. 2012). Also of relevance to this section, but discussed further below, was an investigation by Murphy *et al*. which examined the impact of two antimicrobials on the metabolic abnormalities associated with obesity in a DIO mouse model. Abp118, in the form of the bacteriocin-producing probiotic *L. salivarius* UCC118, was chosen as one of the antimicrobials and its effect on the gut microbiota was also examined (Murphy, Cotter et al. 2012).

As the ability to adhere to intestinal epithelium can play a role in probiotic functionality, this strain has also been examined to assess the influence of adhesion to intestinal epithelial cells on gene expression. Notably, bacteriocin gene expression was induced upon adhesion to epithelial cells, possibly through a mechanism whereby the presence of an induction peptide at a high enough local concentration triggers bacteriocin production. The phenomenon was observed for the UCC118 wild-type strain but not an *srtA* mutant, as disruption of the sortase gene *srtA* results

in significantly lower levels of adhesion (van Pijkeren, Canchaya et al. 2006), following exposure to Caco-2 cells (O'Callaghan, Buttó et al. 2012).

Despite the fact that bacteriocins produced by potential probiotic strains have significant promise as alternative treatments to target clinically relevant pathogens, the degree to which they are expressed under the harsh conditions within the GI tract has not been studied in great detail. For the same reason, strategies have not been developed to ensure that bacteriocin production is triggered within this environment. It has only been established that certain bacteriocins produced by *L. salivarius* strains can indeed be produced within many of the stressful conditions encountered in the gut (Guinane, Piper et al. 2015, Guinane, Lawton et al. 2016). In a recent investigation by Guinane *et al*., the impact of environmental factors on the bacteriocin promoter in gut derived *L. salivarius*, including the strain UCC118, was assessed (Guinane, Piper et al. 2015). More specifically the putative bacteriocin promoter regions of three class IIb bacteriocins, Abp118, salivaricin P and T were fused to a reporter gene and promoter activity was examined under various environmental conditions. In each case promoter activity was weak during growth analysis in MRS broth, which may have been due to insufficient levels of inducing peptide production. The response of these promoter regions to environmental stresses, including low pH, salt and bile, was also examined. However no significant increase in promoter activity was observed. The presence of an induction peptide was the only factor that induced production of the class IIb bacteriocin. It is notable that, despite UCC118 being perhaps the probiotic strain in which the benefits of bacteriocin production are clearest, the strain has yet to be brought to market.

Another *L. salivarius*-produced bacteriocin that has been the focus of investigation is bactofencin A, a class IId bacteriocin. This bacteriocin is unusual in that it does not

share significant homology with previously characterised bacteriocins but instead is more similar to a group of eukaryotic antimicrobial peptides (O'Shea, O'Connor et al. 2013). Bactofencin A has a relatively broad spectrum of activity, inhibiting two clinically significant pathogens: *Staphylococcus aureus* and *L. monocytogenes* and, due to the unmodified nature of this bacteriocin and the associated ability to generate large volumes in a synthetic form through peptide synthesis, has the potential to serve as a viable alternative to antibiotics in the maintenance of animal husbandry (O'Shea, O'Connor et al. 2013). Additionally, in the aforementioned investigation of bacteriocin promoter activity, it was observed that the bactofencin A promoter exhibited considerable activity regardless of the presence or absence of the associated antimicrobial peptide, while exposure to conditions that mimic the GI tract, such as the presence of gastric fluid and low levels of salt, further induced expression (Guinane, Piper et al. 2015). The impact of the bactofencin A-producing strain on intestinal populations and microbial diversity in a simulated model of the distal colon has been examined. The conditions in the distal and proximal colon can be mimicked using *ex vivo* fermentation systems and have previously been used to study the effects of antimicrobials on microbial composition (Dobson, Crispie et al. 2011, Rea, Dobson et al. 2011, Guinane, Lawton et al. 2016). The presence of the bactofencin A-producing strain altered the proportions of a number of important gut genera including *Fusobacterium*, *Bacteroides* and *Bifidobacterium*, resulting in a positive, albeit subtle, effect on gut populations (Guinane, Lawton et al. 2016).

Despite the research described above, bacteriocin production among commercial probiotic lactobacilli has, in general, not been studied in great detail and the information available regarding which commercial probiotics produce bacteriocins, and which bacteriocins are produced most frequently, is limited. *Lactobacillus*

acidophilus probiotics, several of which are employed for use in commercial products (Shah 2007, Bull, Plummer et al. 2013), are somewhat exceptional in this regard in that two such strains, NCFM and LA-5, are known to produce the bacteriocin lactacin B (Sanders and Klaenhammer 2001, Tabasco, Garcia-Cayuela et al. 2009). This bacteriocin has a narrow spectrum of activity, capable of inhibiting other lactobacilli and *Enterococcus faecalis* (Barefoot and Klaenhammer 1984). Notably, with respect to this commentary, the contribution of lactacin B, if any, to probiotic functionality has not been determined.

1.5.2 *Streptococcus salivarius*

S. salivarius is a well-characterised human commensal of the oral cavity (Aas, Paster et al. 2005) and has been found to colonise within just a few hours of birth (Pearce, Bowden et al. 1995). It is also a common inhabitant of the gut, particularly the stomach and jejunum. Some strains of *S. salivarius* have gained attention because of their role as safe and effective probiotics, and have been employed to promote a healthy oral microbiota (Burton, Chilcott et al. 2005, Burton, Wescombe et al. 2006). As reviewed by Wescombe *et al*., strain K12 is the model *S. salivarius* probiotic and is available in commercial preparations (BLIS K12; BLIS Technologies, Otago, New Zealand). K12 was initially selected because of its ability to inhibit the pathogen *Streptococcus pyogenes*, but now several other the health-promoting effects have been noted (Wescombe, Hale et al. 2012). Unsurprisingly, the criteria frequently employed to assess the safety and health-promoting properties of probiotics have been examined in this strain (Burton, Wescombe et al. 2006, Burton, Chilcott et al. 2010). *S. salivarius* K12 was found to produce two bacteriocins belonging to the lantibiotic subgroup of class I bacteriocins, i.e. salivaricin A2 and salivaricin B. In a study which examined the effects of this strain on oral malodour parameters,

administration of this bacteriocin-producing probiotic, following an antimicrobial mouthwash, reduced the oral volatile sulphur compound levels produced by bacteria implicated in halitosis. *In vitro* testing also demonstrated that *S. salivarius* K12 inhibited various strains associated with this condition (Burton, Chilcott et al. 2006). Due to the ability of this strain to supress the growth of *S. pyogenes*, a common cause of pharyngitis and tonsillitis, and its ability to inhibit the growth of potential pathogens of the ear and oral cavity which cause infections such as acute otitis media, the capacity of strain K12 to reduce the incidence of these infection-causing microorganisms *in vivo* was examined in a study by Di Piero *et al*. (Di Pierro, Donato et al. 2012). *S. salivarius* K12 was delivered orally *via* a slow release tablet containing $5X10^9$ colony forming units and, while there were limitations specified in this study, including not being randomized or blinded and the absence of a placebo, administration of this oral probiotic appeared to reduce the incidence of recurrent cases of bacterial throat and ear infections in children. A follow up study which again aimed to study the effects of this strain observed similar results, with a reduced incidence of infection (Di Pierro, Colombo et al. 2014). Moving away from the oral cavity, Patras *et al*. recently investigated the ability of *S. salivarius* K12 to inhibit group B streptococci (GBS) (Patras, Wescombe et al. 2015), including isolates suspected of causing disease in newborns and colonising isolates from the vaginal tract of pregnant women. Some of these activities were dependant, or partially dependent, on the presence of a megaplasmid that encodes the salivaricin A2 and salivaricin B bacteriocins (Patras, Wescombe et al. 2015).

Other strains of *S. salivarius* examined for their probiotic application include M18, which contains a megaplasmid encoding a number of bacteriocins (Heng, Haji-Ishak et al. 2011). To evaluate its probiotic potential, the ability of this strain to prevent or

reduce the risk of dental caries and influence dental health was examined in a randomized double-blind, placebo controlled trial (Burton, Drummond et al. 2013). The persistence of this strain in saliva was also investigated and was revealed to be dose dependant (Burton, Wescombe et al. 2013). This study demonstrated *in vitro* transfer of the bacteriocin-encoding megaplasmids between two strains of *S. salivarius*. This may allow the enhancement of probiotic strains by transferring the megaplasmid from those that persist poorly but demonstrate strong bacteriocin production to indigenous *S. salivarius* that persist strongly but demonstrate poor bacteriocin production (Burton, Wescombe et al. 2013). Additionally, the identification of novel bacteriocins, including salivaricin 9 (Wescombe, Upton et al. 2011) and the recently identified salivaricin E (Walker, Heng et al. 2016), from this species continues to enhance the probiotic potential of *S. salivarius*.

1.5.3 *Bacillus thuringiensis* DPC6431

Some gut isolates produce bacteriocins with promising applications but, for one reason or another may not meet the requirements to be regarded as appropriate for probiotic use. For it to be considered a probiotic trait, bacteriocin production must occur *in situ* by the producing strain. Additionally, if it has potential implications for human health or fails to meet QPS status, selection for probiotic use becomes more challenging. The thuricin CD-producing *B. thuringiensis* DPC6431 is an example of a promising bacteriocin-producing strain, but the apparent absence of *in situ* bacteriocin production limits its probiotic potential.

This strain was identified following a search for alternative strategies to combat the gut pathogen *Clostridium difficile*. *C. difficile* is a causative agent of nosocomial infection and cases of *C. difficile*-associated-disease (CDAD) (Owens, Donskey et

al. 2008). The primary cause of CDAD is antibiotic treatment which eradicates the beneficial microbiota, allowing the opportunistic pathogen to flourish. Screening of over 30,000 colonies resulted in the detection of a single colony that prevented the growth of *C. difficile* in an overlay. The isolated colony was identified as *Bacillus thuringiensis* DPC6431 which produced the narrow spectrum thuricin CD (Rea, Sit et al. 2010). The impact of thuricin CD was further investigated using a distal colon model to assess the effects of broad and narrow spectrum antimicrobials on *C. difficile* and gut populations. More specifically, the activity of purified thuricin CD (90μM) but not the producing strain was compared against the antibiotics metronidazole and vancomycin and the purified bacteriocin lacticin 3147 in a faecal fermentation over a 24 hr period. Thuricin CD was as efficient as the broad spectrum antimicrobials at killing *C. difficile* but did not significantly impact on the other components of the gut microbiota (Rea, Dobson et al. 2011). Ultimately, for it to be active *in vivo*, thuricin CD would need to be present in the colon in a bioavailable form. Rea *et al*. investigated the bioavailability of thuricin CD in the GIT through oral administration in pigs and mice as well as rectal administration in mice (Rea, Alemayehu et al. 2014). Trn-β, one of the peptides of the two component thuricin CD, was found to be degraded by digestive enzymes following oral administration of thuricn CD to pigs. Furthermore, investigation to determine if spores of *B. thuringiensis* DPC6431 could germinate and produce thuricin CD following administration in mice revealed no evidence of spore germination as almost all spores were shed. Survival and efficacy was also examined using rectal administration which proved to be a promising mode of delivery (Rea, Alemayehu et al. 2014). Although evidence of *in situ* production by this strain is lacking, and therefore its capacity to be used as a bacteriocin-producing probiotic is currently

unlikely, this bacteriocin is noteworthy as it represents a number of the traits of an ideal gut bacteriocin, that is, can be used to target gut pathogens, has a narrow spectrum of inhibition and does not impact the beneficial populations.

1.6 Novel Targets

The ability of bacteriocins to modulate the gut microbiota by targeting undesirable components without having a negative impact on the beneficial populations is an attractive trait. The role by which a bacteriocin could regulate niche competition among enterococci or between enterococci and the intestinal microbiota was examined by Kommineni *et al* (Kommineni, Bretl et al. 2015). Here it was demonstrated that *E. faecalis* containing the conjugative pPD1 plasmid, which expresses bacteriocin 21, both replaced indigenous enterococci and outcompeted *E. faecalis* which lacked the plasmid, while the transfer of this plasmid to other *E. faecalis* strains enhanced their survival in the intestine. Finally, vancomycin-resistant enterococci were cleared following subsequent colonisation with *E. faecalis* harbouring a conjugation-defective pPD1 mutant (Kommineni, Bretl et al. 2015). These results do indeed demonstrate that bacteriocin production by commensal bacteria contributes to niche competition and an alternative therapeutic approach to eliminating intestinal colonisation by multidrug-resistant bacteria may be provided by bacteriocins delivered by commensals (Kommineni, Bretl et al. 2015).

Janek *et al*. observed a high frequency of bacteriocin production among nasal *Staphylococcus* strains with highly variable antimicrobial activity against other nasal members, suggesting a need to inhibit different competitors (Janek, Zipperer et al. 2016). The diverse activity spectra of bacteriocins within the nose may facilitate the ability of a bacterial species to dominate the resident populations, suggesting the

development of probiotics that could promote a desirable microbiota composition and eliminate pathogens such as *S. aureus* (Janek, Zipperer et al. 2016).

The majority of studies to date, focus on bacteriocin-producing probiotics that can inhibit well established gut pathogens. Next-generation sequencing technologies continue to provide a more thorough understanding of the role of the gut microbiota in GI health and, as a result, new targets are emerging. The use of a targeted approach can help to provide further insights into such studies by establishing whether increases in specific taxa are the cause, or a consequence, of such diseases. More specifically, in instances where the link between the putative pathogen and disease is not clear, the targeted removal of the microbe by bacteriocin-based approaches can establish aetiology. Even more significantly, if the target microbe is established to be a pathogen, the bacteriocin can also be employed to prevent/treat disease. However, as with many other aspects, the harnessing of bacteriocin-producing strains to this end has remained a focus of academic research only, here we provide some examples of ways in which these bacteria could be applied.

1.6.1 Metabolic Health

Obesity is a complex syndrome and has a number of serious implications for human health including cardiovascular disease, type 2 diabetes (T2D) and musculoskeletal disorders. Obesity develops from a prolonged imbalance of energy intake and expenditure and, while lifestyle choices, diet and host genetics are key factors, the role of the gut microbiota in obesity and overall metabolic health has received considerable attention in recent years. Although the specific populations are under investigation, initially it was noted that the gut microbiota of genetically obese mice have been associated with an increase in the phylum Firmicutes and a decrease in the

phylum Bacteroidetes (Ley, Bäckhed et al. 2005, Turnbaugh, Ley et al. 2006). However, there is conflicting evidence in human studies with regard to what the key populations involved are (Schwiertz, Taras et al. 2010).

More recent research has specifically highlighted populations that may play a role in obesity or in T2D (Turnbaugh, Bäckhed et al. 2008, Larsen, Vogensen et al. 2010, Qin, Li et al. 2012, Everard, Belzer et al. 2013, Karlsson, Tremaroli et al. 2013, Le Chatelier, Nielsen et al. 2013). In a metagenome-wide association study of 345 Chinese individuals with T2D and non-diabetic controls, specific populations were enriched in the T2D group and precise genetic and functional components of the gut metagenome associated with T2D were highlighted. This study revealed that T2D patients had a moderate degree of microbial dysbiosis, accompanied by a decline in butyrate-producing bacteria and an increase in several opportunistic pathogens. Among the species that were enriched in T2D patients were several members of the *Clostridum* genus including *Clostridium ramosum*, *symbiosum* and *hathewayi* (Qin, Li et al. 2012). The gut metagenome of European women with normal, impaired and diabetic glucose control has also been examined (Karlsson, Tremaroli et al. 2013). Here, similar findings were observed with a decrease in butyrate-producing *Roseburia* species and an increase in *Clostridium clostridioforme* in T2D metagenomes. However, differences were observed between the two studies, Qin *et al*. observed an enrichment of Proteobacteria in T2D patients, whereas an increase in *Lactobacillus gasseri* and *Streptococcus mutans* was identified in the T2D cohort by Karlsson *et al*. These investigations can serve as an initial starting point when selecting targets that may play a role in T2D and obesity.

There have been other studies that have more specifically established the role of a particular species or strain in obesity and T2D. Fei and Zhao (2013) demonstrated

the role of the endotoxin-producing *Enterobacter cloacae* B29 in inducing obesity and insulin resistance in germfree mice (Fei and Zhao 2013). In this study mono-association of germfree C57BL/6J mice with this strain, previously isolated from the gut of a morbidly obese individual, induced obesity and insulin resistance in mice fed a high-fat diet. The same effect was not observed in control mice on a high-fat diet. It was also shown by Woting *et al*. (Woting, Pfeiffer et al. 2014) that *Clostridium ramosum*, a species previously shown to be enriched in T2D patients (Qin, Li et al. 2012), promoted obesity in a gnotobiotic mouse model fed a high-fat diet. More specifically, gnotobiotic mice received a simplified human intestinal microbiota (SIHUMI) of eight bacterial species including *C. ramosum*, a SIHUMI without *C. ramosum*, or received *C. ramosum* alone and were fed either a high-fat or low-fat diet. Mice associated with *C. ramosum* either alone or in the presence of the SIHUMI gained more body weight and fat storage than mice without *C. ramosum*, and was believed to promote obesity through enhanced intestinal glucose and lipid absorption (Woting, Pfeiffer et al. 2014). Bacteriocins produced within the gut with specific activity against some these microorganisms may be effective in beneficially balancing metabolic health.

Murphy *et al*. examined the strategy of using two antimicrobial-based approaches, vancomycin and the bacteriocin-producing probiotic *L. salivarius* UCC118, to target the metabolic abnormalities associated with obesity in a diet-induced obesity (DIO) mouse model (C57BL/J6 mice) and their impact on the gut microbiota (Murphy, Cotter et al. 2012). The ability of the previously mentioned *L. salivarius* UCC118 strain to inhibit a number of Firmicutes was part of the logic behind investigating its ability to control weight gain in DIO mice. Both antimicrobial strategies altered the gut microbiota, but in distinct ways, and only treatment with vancomycin resulted in

an improvement in the metabolic abnormalities associated with obesity. It was notable, however, that the bacteriocin-producing probiotic more successfully controlled weight gain in mice fed a high-fat diet when compared to the non-producing mutant used. However, this effect was short lived and eventually this difference decreased to below significant levels. Nevertheless, a key point that emerged from this study was the ability to target the gut microbiota using antimicrobials such as bacteriocins as a method for treating certain disease states, though the choice and specificity of action of the bacteriocin is critical.

It would seem that bacteriocins have the potential to serve as a method for treating obesity and related disorders or, at the very least, discriminate between cause and effect. Taking the study by Murphy *et al*. (Murphy, Cotter et al. 2012) as proof-of-concept and, as the role of specific microbes in obesity continues to emerge, such as the aforementioned, equivalent, but more targeted studies can be carried out.

1.6.2 Cancer

There have been some suggestions that bacteriocins can be employed as anticancer agents, either through their impact on cancerous cells or through the inhibition of bacteria associated with the initiation of disease (Kaur and Kaur 2015). One such study focused on the impact of nisin on head and neck squamous cell carcinoma (HNSCC) cell apoptosis and cell proliferation *in vitro* and *in vivo* in murine oral cancer (Joo, Ritchie et al. 2012). It revealed that treatment with increasing concentrations of nisin induced increasing DNA fragmentation and apoptosis on three different cancer cell lines. In the oral cancer mouse model, groups receiving nisin showed reduced tumour volumes through activation of CHAC1 expression when compared with controls, while pre-treating with nisin prior to and three weeks after tumour cell inoculation led to the same effect (Joo, Ritchie et al. 2012). It was

suggested that in this study the selective action of nisin arose from structural differences in the composition of the plasma membranes between HNSCC cells and primary keratinocytes. Although it was the nisin peptide rather than the bacteriocin-producing strain that was used, it would be interesting if strains capable of producing nisin or its variants could be used in a similar manner.

In the context of inhibiting potentially cancer-causing microbes, we refer to the example of *Fusobacterium nucleatum* (Allen-Vercoe, Strauss et al. 2011). Though initially regarded as a component of the oral cavity, *F. nucleatum* is also present in the gut and has been linked to playing a part in different GI disorders such as colorectal cancer (CRC), inflammatory bowel disease and appendicitis (Swidsinski, Dörffel et al. 2009, Strauss, Kaplan et al. 2011, Castellarin, Warren et al. 2012, Kostic, Chun et al. 2013). The mechanism by which *F. nucleatum* is thought to promote CRC has been investigated (Rubinstein, Wang et al. 2013, Gur, Ibrahim et al. 2015). As members of the genus *Fusobacterium*, and in particular *F. nucleatum*, play a role in numerous disease states as mentioned above, they represent an ideal target for bacteriocin-producing probiotics, but yet again, this potential has yet to be harnessed.

1.7 Future Perspectives

This review highlights the potential for bacteriocins and bacteriocin-producing probiotics as novel therapeutic treatments in many disease states, including the targeting of newly emerging pathobionts involved in a variety gut disorders. While there is an abundance of knowledge on the application of bacteriocin-producing strains with probiotic potential in an *in vitro* setting, less is known of their impact in an *in vivo* environment and even less again with regard to their relevance to human health. This is undoubtedly the primary hurdle that needs to be overcome in order for

the potential of the multitude of bacteriocin-producing strains that continue to be identified using traditional methods (O'Shea, Gardiner et al. 2009, Rea, Sit et al. 2010, Birri, Brede et al. 2012) or bioinformatic approaches (Walsh, Guinane et al. 2015, Zheng, Gänzle et al. 2015) to be realised.

In addition to identifying new targets, recent studies have identified *Akkermansia muciniphila* (Dao, Everard et al. 2016) and *Faecalibacterium prausnitzii* (Miquel, Leclerc et al. 2015) that correlate positively with gut health, as well as a decline in butyrate-producing *Roseburia* species in certain disease states (Qin, Li et al. 2012, Karlsson, Tremaroli et al. 2013), which may play a role in future probiotic applications alongside the more traditional strains currently employed. The capacity to produce a bacteriocin by such microbes was demonstrated by Hatziioanou *et al*., who highlighted the first example of a bacteriocin-like substance produced by *Roseburia faecis* M72/1 (Hatziioanou, Mayer et al. 2013). Additionally, *in silico* screens may prove useful in identifying putative bacteriocin gene clusters from these genera/species, such as the sactipeptide-like cluster from *Roseburia intestinalis* L1-82 (Walsh, Guinane et al. 2015). It will be necessary to determine if these potential probiotics of the future have the ability to produce bacteriocins that can contribute to human health and if this potential can be more effectively harnessed than has been the case to date. Until such time that as this occurs, bacteriocin production will continue to be regarded as a probiotic trait in theory rather than in commercial reality.

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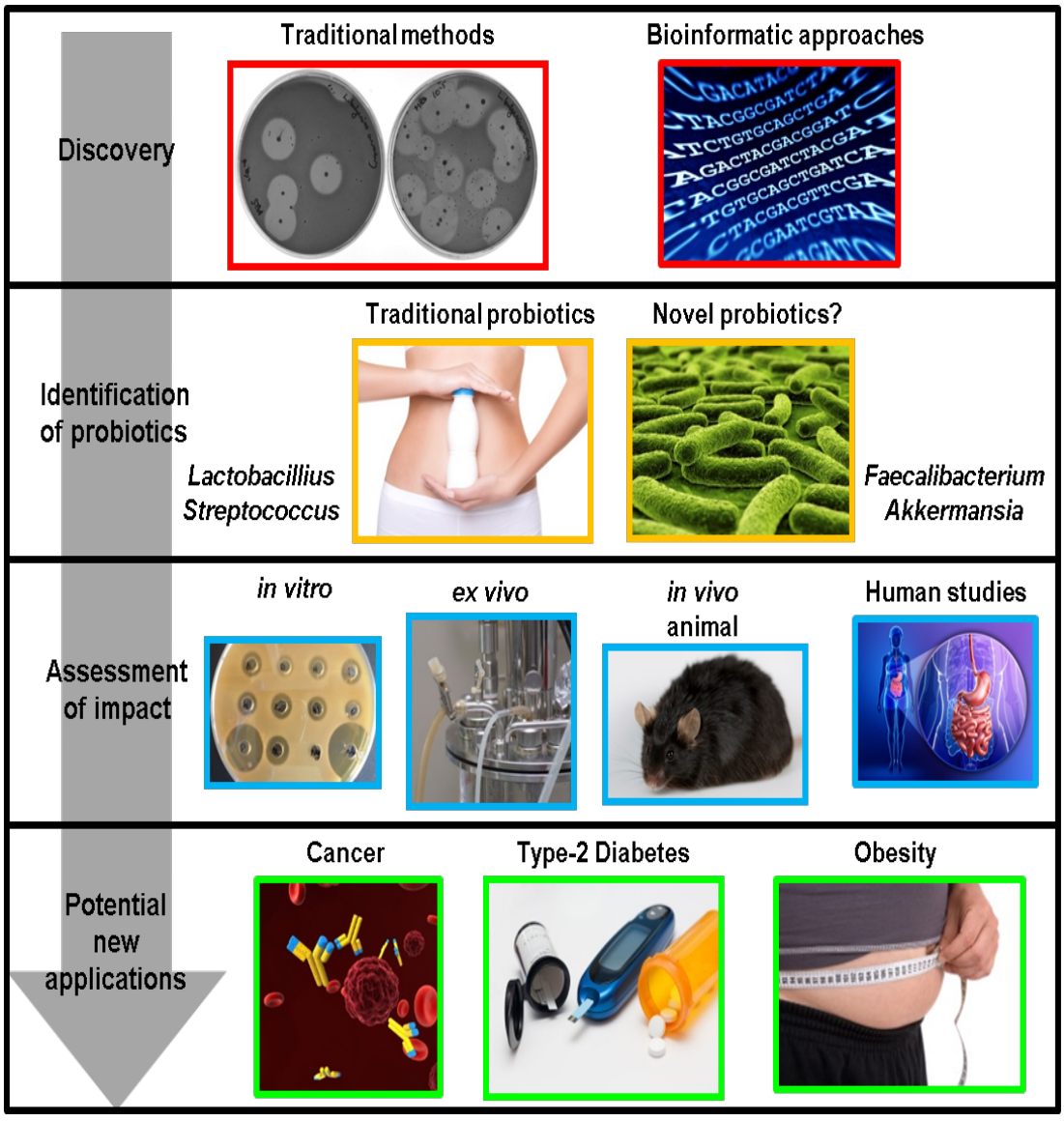
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Figure 1 Bacteriocins, from discovery to potential probiotic application. Strategies to identify new bacteriocins include culture-based methods and newer bioinformatics-based approaches. These can lead to the identification of bacteriocin-producing strains from traditionally utilised, or novel, probiotic species. The impact of a bacteriocin-producing strain on health can be assessed using *in vitro*, *ex vivo* and *in vivo* methods and, depending on the outcome, has the potential to be applied to prevent or treat various disease states



Chapter 2

Lack of heterogeneity in bacteriocin production across a selection of commercial probiotic products

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2.1 Abstract

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host. Bacteriocin production has often been mooted as a desirable probiotic trait, and in specific cases, has been shown to promote probiotic survival within the GI tract, contribute to the control of pathogens and even influence host gene expression in the gut. However, it is not clear what proportion of probiotic strains routinely found in commercial products produce bacteriocins, and additionally it is not known which bacteriocins are produced most frequently. To address this, we conducted a culture-based assessment of the bacteriocinogenic ability of bacterial strains found in a variety of commercially available probiotic products. We detected 8 bacteriocin-producing isolates from 16 tested products. Interestingly, in all cases the isolates were *Lactobacillus acidophilus* and the bacteriocin produced was identified as the narrow spectrum class II bacteriocin, lactacin B. The apparent absence of other bacteriocin-producing strains from across these products suggests a lack of heterogeneity in bacteriocin production within probiotic products and suggests that bacteriocin production is not being optimally harnessed as a probiotic trait.

2.2 Introduction

Probiotics have been defined by the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) as live microorganisms that, when consumed in adequate amounts, provide a health benefit for the host (Hill, Guarner et al. 2014). These health benefits have been attributed to the ability of individual strains to, for example, maintain intestinal barrier function, perform immunomodulation and/or direct inhibition of pathogens through the production of antimicrobial substances (Mennigen and Bruewer 2009, Oelschlaeger 2010). In the latter case, the antimicrobial substances can include organic acids such as lactic and acetic acid, hydrogen peroxide and bacteriocins.

To date, the majority of probiotic strains belong to the *Lactobacillus* and *Bifidobacterium* genera, but others such as *Escherichia coli* Nissle 1917, some strains of *Enterococcus* and yeasts such as *Saccharomyces boulardii* are also employed as probiotics (Guarner, Khan et al. 2012). Furthermore, many probiotic products can contain a mixture of more than one species. These multi-strain products may, when compared to a single strain product, have the advantage of achieving a wider range of health benefits (Chapman, Gibson et al. 2011). The viability of probiotic species is an important factor in the selection of strains for use, as strains must survive in the food product or capsule and during passage through the gastrointestinal tract, while retaining the original health promoting effects (Tuomola, Crittenden et al. 2001, Selle and Klaenhammer 2013).

Bacteriocins are bacterially produced, ribosomally-synthesized, small, heat stable antimicrobial peptides that are active against other bacteria and to which the producing organism is immune (Cotter, Hill et al. 2005). They can have a broad or

narrow spectrum of inhibition (Nes, Yoon et al. 2007) and are divided into two classes (Class I and Class II) based on whether or not they undergo post-translational modifications (for review see Cotter *et al*. 2013 (Cotter, Ross et al. 2013)) and have potential applications in both food and clinical settings (Hassan, Kjos et al. 2012, Perez, Zendo et al. 2014). Bacteriocin production can contribute to probiotic functionality though three distinct mechanisms (Dobson, Cotter et al. 2012). Bacteriocins can facilitate the introduction and survival of the producer within the complex environment of the gut (Walsh, Gardiner et al. 2008), the production of these antimicrobials may also inhibit the growth of pathogens (Corr, Li et al. 2007) and finally, bacteriocins may function as signalling peptides/quorum sensing molecules in the gut environment (van Hemert, Meijerink et al. 2010).

Bacteriocin production has often been regarded as an important attribute in the selection of probiotic strains, however, it has only been over the last decade that a number of studies have definitively demonstrated the ability of a bacteriocin-producing strain to impact on gut microbial communities and/or influence the health of the host in a positive way (Millette, Cornut et al. 2008, Murphy, Cotter et al. 2012, Guinane, Lawton et al. 2016). Among the strains used commercially, *Lactobacillus acidophilus* NCFM (Sanders and Klaenhammer 2001), *L. acidophilus* LA-5 (Tabasco, Garcia-Cayuela et al. 2009) and *Streptococcus salivarius* K12 (Burton, Wescombe et al. 2006) are examples of well-characterised probiotic strains known to produce bacteriocins. Both *L. acidophilus* NCFM and LA-5 produce the class II bacteriocin, lactacin B (Sanders and Klaenhammer 2001, Tabasco, Garcia-Cayuela et al. 2009), and the oral cavity probiotic *S. salivarius* K12 produces two bacteriocins, salivaricin A2 and salivaricin B, both belonging to the lantibiotic class (class I) of bacteriocins (Hyink, Wescombe et al. 2007).

Although there have been studies comparing species identification to label accuracy among commercial probiotic products (Weese and Martin 2011, Lewis, Shani et al. 2015) or evaluating inter-species inhibition between probiotic strains (Chapman, Gibson et al. 2012), there appears to be limited data relating to the incidence of bacteriocin production among commercial probiotic products. Thus, the aim of this study was to assess the frequency of bacteriocin production, and the identity of the associated bacteriocins, from among a selection of these products.

2.3 Materials and Methods

2.3.1 Sample Collection

Sixteen commercial products were selected based on being labelled as probiotic products (13 products) or containing potentially probiotic species (3 products). Each product was assigned a letter of identification and information regarding the identity of the species purported to be present according to the product labels is highlighted in Table 1.

2.3.2 Strains, media and growth conditions

Strains from each probiotic product were cultured anaerobically at 37°C for 48 hrs on deMan, Rogosa, Sharpe (MRS; Difco Laboratories, Detroit, MI, USA) agar supplemented with 0.05% L-cysteine (w/v) (Sigma, St. Louis, MO, USA) (modified MRS; mMRS). Anaerobic conditions were maintained in anaerobic jars using Anaerocult A gas packs (Merck, Darmstedt, Germany). Indicator strains used and their respective growth conditions are listed in Table 2. Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) was added when required at 1.5% (w/v; solid media) or 0.75% (w/v; sloppy media).

2.3.3 Isolation of bacteriocin-producing strains

Serial 10-fold dilutions using maximum recovery diluent (MRD, Oxoid) were prepared for each product and 100 µL of the selected dilutions were spread plated on mMRS agar in triplicate. Plates were incubated anaerobically at 37°C for 48 hrs after which time colony forming units (CFU) were recorded. Bacteriocin production was assessed using the deferred antagonism assay method (Lewus, Kaiser et al. 1991). For this purpose, plates were overlaid with the indicator strain seeded in agar (0.75%

w/v) and incubated anaerobically or aerobically for a further 24 hrs. Indicator strains used for overlaying included *Lactobacillus delbrueckii* subsp. *bulgaricus* DPC5383 (MRS agar), *Listeria innocua* DPC3572 (BHI agar) and *Bifidobacterium longum* DPC8809 (mMRS agar) (Table 2). Bacterial colonies that produced zones of inhibition were picked and re-streaked on fresh mMRS agar before culturing in mMRS broth for 24 hrs at 37°C. The isolates were then stocked in a final concentration of 40% glycerol (w/v) (Sigma) and stored at -80°C until further characterisation.

2.3.4 Antimicrobial activity assay

Antimicrobial activity was further assessed with the cell free supernatant (CFS) from the potential bacteriocin-producing isolates using the agar well diffusion method (Ryan et. al 1996). To address the possibility that inhibition resulted from the production of acid, the CFS was adjusted to a neutral pH (pH 6.8-7.0). The inhibitory spectrum was examined targeting *L. bulgaricus* DPC5383, *L. innocua* DPC3572, *Lactobacillus fermentum* DPC6193, *Lactococcus lactis* HP, *Lactococcus lactis* DPC1363, *Streptococcus agalactiae* LMG14694, *Enterococcus faecalis* EF1 and *Salmonella enterica* serovar typhimurium LT2 DPC6048 (Table 2). Plates were examined to identify zones of inhibition following overnight incubation. Cross immunity assays were performed between test isolates, and using another lactacin B producer, *L. acidophilus* EM066-BC-T3-3 (Lakshminarayanan, Guinane et al. 2013), using the agar well diffusion method.

2.3.5 Evaluation of heat and enzyme sensitivity

Neutralised CFS was utilised to assess the heat and enzyme sensitivity of the bacteriocin produced. The heat stability was assessed by heating the neutralised CFS

to 100°C for a period of 30 mins. The untreated CFS was used as a control. To determine the protease sensitivity, the CFS was mixed with an equal volume of proteinase K (50 mg/ml^{-1}; Sigma) and incubated at 37°C for 2 hrs. CFS mixed with an equal volume of sterile water was used as a control. Antimicrobial activity was then assessed by the agar well diffusion method using *L. bulgaricus* DPC5383 as the sensitive indicator.

2.3.6 Molecular characterisation of isolates

Genomic DNA was extracted from bacteriocin-producing isolates using the GenElute Bacterial Genomic DNA Kit (Sigma) as per the manufacturer's instructions. The 16S rRNA gene was amplified using the universal primers CO1 and CO2 (Simpson, Stanton et al. 2003), while Sanger sequencing was used to determine the sequence of the complete 16S rRNA gene (Beckman Coulter Genomics, Essex, UK). Identification at species level was determined by nucleotide alignments (>98% nucleotide identity) with deposited sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Screening for the presence of the lactacin B structural gene was achieved using previously designed *lacB* forward and reverse primers (Tabasco, Garcia-Cayuela et al. 2009). Pulse Field Gel Electrophoresis (PFGE) of isolates was carried out as previously described (Simpson, Stanton et al. 2002) using *Apa*1 as the restriction endonuclease (New England Biolabs, Ipswich, MA, USA). A low range molecular weight DNA marker (9.42-194.0 Kb, New England Biolabs) was used to determine band sizes. An isolate similarity dendogram was generated using Bionumerics version 7.5 (Applied Maths, Belgium) by the unweighted pairgroup method with arithmetic mean (UPGMA) with tolerance and optimization settings of 1%.

2.4 Results

2.4.1 Screening of probiotic products for bacteriocin production

Sixteen commercial products were sourced to assess the frequency of bacteriocin production from among the strains present therein. The range of products included 8 yoghurts and 8 supplements (Table 1). Product labels indicated they included species of either: *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and/or *Lactococcus*. In addition, 2 products, designated as C and K, did not specify the species. In some instances specific strain names were provided including *L. acidophilus* LA-5, a known producer of the bacteriocin lactacin B (Tabasco, Garcia-Cayuela et al. 2009), and *Bifidobacterium lactis* BB-12.

Bacteriocin production by colonies screened from the 16 products was tested using the deferred antagonism assay. Once duplicates were removed, well diffusion assays with neutralised CFS were performed, ultimately resulting in the identification of 8 putative bacteriocin-producing isolates, sourced from 8 distinct commercial products (Table 1). In each case the putative bacteriocin-producing strain was selected based on activity against the indicator *L. bulgaricus* DPC5383. No isolates displaying bacteriocin activity were detected from the colonies screened against *L. innocua* DPC3572 or *B. longum* DPC8809 by the methods employed in this study.

2.4.2 Molecular characterisation of bacterial isolates

16S rDNA sequence analysis identified all 8 of the bacteriocin-producing isolates as *L. acidophilus*. Of these, all isolates presented a similar PFGE pattern (Figure 1). Furthermore all isolates identified as *L. acidophilus* were recovered from

commercial products that did indeed claim to contain *L. acidophilus* as a species within the product (Table 1).

2.4.3 Further analysis of bacteriocin-producing isolates

Additional assays with the bacteriocin-producing strains established that the antimicrobial activity of the 8 *L. acidophilus* isolates against *L. bulgaricus* was eliminated following treatment with proteinase K, suggesting a bacteriocin was responsible for the observed inhibition. Testing of the CFS from 8 of the *L. acidophilus* isolates established that the antimicrobial was heat stable at 100°C. The spectrum of inhibition was assessed with a collection of 8 indicators, but all isolates tested only displayed activity against *L. bulgaricus* (Table 1). Test isolates did not display activity against a lactacin B producer, *L. acidophilus* EM066-BC-T3-3, and were immune to the antimicrobial activity of the lactacin B producer.

2.4.4 Identification of bacteriocins

Primers specific to the structural gene of lactacin B were used for PCR amplification. As strain *L. acidophilus* LA-5 was listed within one product, a known producer of lactacin B (Tabasco, Garcia-Cayuela et al. 2009), primers specific to this structural gene were employed for detection of the lactacin B structural gene within the strains isolated in this study. PCR amplification with the lactacin B primers confirmed the presence of the structural gene by producing a product of 181bp (data not shown). All *L. acidophilus* strains isolated in this study were confirmed to contain the *lacB* structural gene by PCR.

2.5 Discussion

Despite bacteriocin production being regarded as a probiotic trait, there is limited information available regarding the frequency of production from among strains contained in commercially produced probiotic products. To address this, the aim of this study was to assess the frequency of bacteriocin production from a selection of commercial products and to determine which bacteriocins are produced most frequently. Although not exhaustive, this study does provide an interesting insight into the heterogeneity, or lack thereof, of bacteriocins produced by a range of commercial probiotics.

Screening yielded 8 bacteriocin-producing isolates from 16 products tested in this study. The lack of heterogeneity among those isolates was notable, with 8 displaying a similar PFGE profile and all of these possessing the lactacin B structural peptide determinant, *lacB*. It could be argued that screening for bacteriocins was restricted by the limitations associated with the choice of target organisms, but *L. bulgaricus* DPC5383 is regarded as a particularly bacteriocin-sensitive strain (Casey, Casey et al. 2004, O'Shea, Gardiner et al. 2009) and would be expected to be an appropriate indicator to screen for a broad variety of Gram-positive bacteriocins.

In the present study, the narrow spectrum of activity of the associated bacteriocin and PCR results are consistent with the production of lactacin B, a class II (i.e. unmodified) bacteriocin produced by some strains of *L. acidophilus* (Klaenhammer 1993). Indeed, several strains of *L. acidophilus* have been employed for use in commercial probiotics including LA-5, NCFM, DDS-1 and SBT-2026 (Shah 2007, Bull, Plummer et al. 2013), of which NCFM and LA-5 are known to produce lactacin B. Furthermore, the isolates tested from this study were found to be cross-

immune to the antimicrobial activity of another lactacin B producer, *L. acidophilus* EM066-BC-T3-3 (Lakshminarayanan, Guinane et al. 2013). This is consistent with the presence of a lactacin B operon, including the associated immunity gene. This class II bacteriocin was previously found to have a narrow spectrum of inhibition, hindering the growth of other *Lactobacillus* strains and *Enterococcus faecalis* only (Barefoot and Klaenhammer 1983). The isolates tested in this study failed to inhibit *E. faecalis* EF1 in a well diffusion assay but this may be as a consequence of choice of target strain. Taking into account the strain information from product D and the fact the many of these strains share an almost identical pulsotype following PFGE, it is likely that these strains are at least very similar to LA-5, a known producer of lactacin B (Tabasco, Garcia-Cayuela et al. 2009).

It was noted that products A, B, G, J, M also claimed to contain *L. acidophilus* but no bacteriocin production was observed from these products, suggesting that different strains of *L. acidophilus* that do not produce a bacteriocin may have been used. Two probiotic supplements also contained *L. bulgaricus*. This is interesting from the point of view of inter-species inhibition between strains in the products, assuming the strain used in both cases was indeed a probiotic. The *L. bulgaricus* indicator used in this screen was inhibited by the bacteriocin produced by *L. acidophilus* strains, which in turn, may also inhibit the *L. bulgaricus* present in the product. It is interesting to note the absence of bacteriocin production amongst the strains present in the multi-species supplements. There is some evidence of putative bacteriocin production by *B. lactis* BB-12 (Saleh, Kholif et al. 2004), though no activity was demonstrated here.

Despite the number of bacteriocin-producing probiotics for which the beneficial effects have been established, many are still not commercially available. Ultimately,

this study established that bacteriocin production is a feature among products containing *L. acidophilus* and highlighted a lack of diversity in bacteriocin production among the strains tested. This raises the question as to whether bacteriocin production is being optimally harnessed within the probiotic industry.

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Table 1 Commercial products screened

Product	Product type	Species labeled as per packaging information	Bacteriocin producing isolate	Identified bacteriocin	Indicator inhibited
A	Supplement	*Lactobacillus acidophilus* *Lactobacillus rhamnosus* *Bifidobacterium longum* *Sacchharomyces boulardii*	ND	ND	
B	Supplement	*Bifidobacterium breve* *Bifidobacterium infantis* *Bifidobacterium longum* *Lactobacillus acidophilus* *Lactobacillus bulgaricus* *Lactobacillus casei* *Lactobacillus rhamnosus* *Streptococcus thermophilus*	ND	ND	
C	Yoghurt	Live probiotic yoghurt cultures	ND	ND	
D	Yoghurt	*Bifidobacterium* BB12 *L. acidophilus* LA5	*L. acidophilus*	Lactacin B	*L. bulgaricus*
E	Yoghurt	*L. casei*	ND	ND	
F	Yoghurt	*L. casei* *Bifidus*	ND	ND	
G	Supplement	*Lactobacillus acidophilus* *B. bifidum* *L. rhamnosus* *L. plantarum* *B. breve* *B. longum* *L. casei* *L. lactis* *L. bulgaricus* *L. salivarius*	ND	ND	
H	Yoghurt	*Lactobacillus acidophilus* *Bifidobacterium longum* *Streptococcus thermophilus*	*L. acidophilus*	Lactacin B	*L. bulgaricus*
I	Yoghurt	*Lactobacillus bulgaricus* *Streptococcus thermophilus* *Bifidobacterium longum* *Lactobacillus acidophilus*	*L. acidophilus*	Lactacin B	*L. bulgaricus*
J	Supplement	*Lactobacillus rhamnosus* Rosell-11 *Lactobacillus acidophilus* Rosell-52 *Bifidobacterium longum* Rosell-175 *Lactococcus lactis* Rosell-1058 *Bifidobacterium breve* Rosell-70 *Bifidobacterium bifidum* Rosell-71	ND	ND	
K	Yoghurt	Live cultures	*L.acidophilus*	Lactacin B	*L. bulgaricus*
L	Yoghurt	*L. acidophilus* *Bifidobacterium*	*L. acidophilus*	Lactacin B	*L. bulgaricus*
M	Supplement	*L. casei* *L. rhamnosus* *L. acidophilus* *Streptococcus. thermophilus* *L. plantarum* *B. breve* *B. longum* *B. bifidum*	ND	ND	
N	Supplement	*Lactobacillus acidophilus* *Bifidobacterium lactis*	*L.acidophilus*	Lactacin B	*L. bulgaricus*

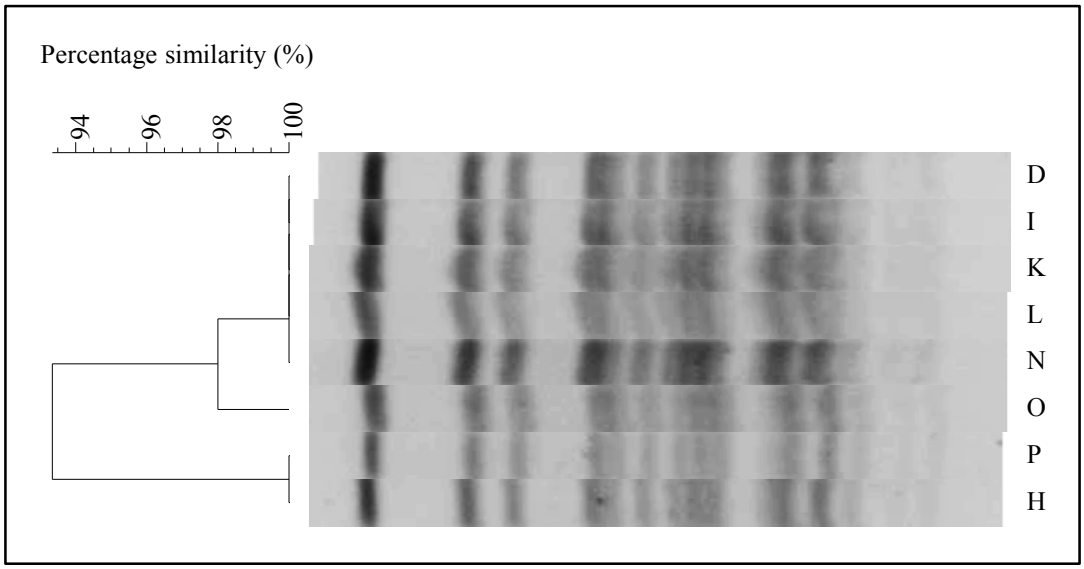
		Lactobacillus casei *Lactobacillus bulgaricus* *Streptococcus thermophilus* *Bifidobacterium longum*			
O	Supplement	*Lactobacillus acidophilus* *Lactobacillus casei* *Lactobacillus bulgaricus* *Bifidobacterium lactis* *Bifidobacterium longum* *Streptococcus thermophilus*	*L.acidophilus*	Lactacin B	*L. bulgaricus*
P	Supplement	*Lactobacillus acidophilus* *Bifidobacterium* BB-12 *Lactobacillus bulgaricus* *Lactobacillus salivarius*	*L. acidophilus*	Lactacin B	*L. bulgaricus*

(ND – Not detected)

Table 2 Indicator strains used and their growth conditions

Indicator strain	Culture Medium	Temperature	Conditions
Bifidobacterium longum DPC8809	mMRS	37°C	Anaerobic
Enterococcus faecalis EF1	BHI	37°C	Aerobic
Lactobacillus bulgaricus DPC5383	MRS	37°C	Anaerobic
Lactobacillus fermentum DPC6193	MRS	37°C	Anaerobic
Lactobacillus acidophilus EM066-BC-T3-3	MRS	37°C	Anaerobic
Lactococcus lactis HP	GM17	30°C	Aerobic
Lactococcus lactis DPC1363	M17	30°C	Aerobic
Listeria innocua DPC3572	BHI	37°C	Aerobic
Salmonella enterica serovar typhimurium LT2 DPC6048	BHI	37°C	Aerobic
Streptococcus agalactiae LMG14694	BHI	37°C	Aerobic

Figure 1 Dendogram of PFGE patterns using the restriction enzyme *Apa*1 for each lactacin B-producing isolate. The % similarity between each pulsotype is highlighted.



Chapter 3

Identification of antimicrobial producers from the gut with activity against type 2 diabetes and/or obesity-associated microbial populations

3.1 Abstract

Obesity is a complex syndrome, associated with a number of serious implications for human health including type 2 diabetes and cardiovascular disease. Obesity develops from a prolonged imbalance of energy intake and energy expenditure and while lifestyle factors, diet and exercise are key factors in obesity, the role of the microbes of the gut in obesity and associated metabolic complications has also been the focus of increased attention in recent years. Although the specific populations involved are still the subject of debate, some specific obesity-associated microbial targets such as *Clostridium ramosum,* have also emerged. The intestinal microbiota is also considered a rich source of potentially health-promoting, antimicrobial producers, many with the ability to modulate specific components of the intestinal communities. Indeed, previous research from our group suggests that antimicrobials can be used to manipulate the gut microbiota with a view to treating metabolic disorders. This current study aimed to identify antimicrobial producers, in particular, novel gut-associated bacteriocins, which can target documented obesity-associated microbes. The methodology involved a culture-based screen for bacteriocin producers from within faecal samples of lean donors. Screening of over 43,000 colonies resulted in the detection of 14 isolates, from which 4, *Streptococcus salivarius* DPC6988, *Streptococcus mutans* DPC7039, *Streptococcus agalactiae* DPC7040, and *Enterococcus faecalis* DPC7041, were the focus of a more in-depth analysis. Whole genome sequencing of these isolates and subsequent *in silico* screening of draft genomes using BAGEL3 revealed a number of putative bacteriocin gene clusters of interest.

3.2 Introduction

Bacteriocins are ribosomally synthesised, small, heat-stable antimicrobial peptides produced by bacteria and to which the producer has a specific immunity mechanism (Cotter, Hill et al. 2005). They can have a broad or narow spectrum of inhibtion and can be divded into two classes, class I (modified) and class II (unmodified) (Cotter, Ross et al. 2013). Bacteriocin production is often regarded as a probiotic trait, potentially contributing to functionality through a variety of mechanisms (Dobson, Cotter et al. 2012, Hegarty, Guinane et al. 2016), including aiding colonisation (Walsh, Gardiner et al. 2008), directly inhibiting the growth of pathogens/pathobionts (Corr, Li et al. 2007, Millette, Cornut et al. 2008) or functioning as signalling peptides (van Hemert, Meijerink et al. 2010). Current strategies for the identificion of bacterions include traditional culture-based methods and newer *in silico* based approaches (Walsh, Guinane et al. 2015, Zheng, Gänzle et al. 2015). Traditionally, culture-based methods such as the deferred antagonism assay have been used successfully for screens of intestinal samples (O'Shea, Gardiner et al. 2009, Birri, Brede et al. 2012, Lakshminarayanan, Guinane et al. 2013) and food sources (Masuda, Zendo et al. 2012, Ishibashi, Seto et al. 2015) to yield novel baceriocins (O'Shea, O'Connor et al. 2013, Collins, O'Connor et al. 2016).

Obesity is associated with a number of serious implications for human health including cardiovascular disease, type 2 diabetes and various musculoskeletal disorders. It is a complex syndrome, influenced by factors such as host susceptibility, lifestyle, diet and environmental conditions, but can be generally viewed as an imbalance in energy intake and expenditure. The role of the gut microbiota in obesity

and related metabolic disorders has received considerable attention in recent years (Ley, Bäckhed et al. 2005, Turnbaugh, Ley et al. 2006, Turnbaugh, Bäckhed et al. 2008, Larsen, Vogensen et al. 2010, Qin, Li et al. 2012, Everard, Belzer et al. 2013, Karlsson, Tremaroli et al. 2013, Le Chatelier, Nielsen et al. 2013). The specific populations that contribute to obesity are the subject of debate and conflicting evidence exsists (Schwiertz, Taras et al. 2010). The gut microbiota of genetically obese mice was initially associated with an increased abundance of the phylum Firmicutes and a decrease in the phylum Bacteroidetes (Ley, Bäckhed et al. 2005, Turnbaugh, Ley et al. 2006). However, potential roles for particular species, such as *C. ramosum* (Woting, Pfeiffer et al. 2014) and *Enterobacter cloacae* B29 (Fei and Zhao 2013) have more recently been proposed. Furthermore, ongoing advances with respect to next-generation sequencing technologies, have the potential to reveal the identity of other taxa associated with obesity. Indeed, a metagenome wide association study by Qin *et al*. highlighted specific populations that were enriched in type 2 diabetic (T2D) patients and non-diabetic controls (Qin, Li et al. 2012), while Karlsson *et al*. undertook a similar investigation (Karlsson, Tremaroli et al. 2013). *C. ramousm*, *Clostridium bolteae*, *Clostridium symbiosum* (Qin, Li et al. 2012), *Lactobacillus gasseri* and *Clostridium clostridioforme* (Karlsson, Tremaroli et al. 2013) were just some of the populations enriched in the T2D cohorts.

The use of antimicrobials with a view to impacting on obesity through modulation of the gut microbiota has been investigated previously. Murphy *et al*. examined the impact of two antimicrobials, vancomycin and the bacteriocin-producing *Lactobacillus salivarius* UCC118, on the metabolic abnormailites associated with diet-induced obesity and on the gut microbial composition (Murphy, Cotter et al. 2012). Both antimicrobials altered the gut microbiota, but in distinct ways, and only

vancomycin resulted in an improvement in the metabolic abnormalites associated with obesity. A key point that emerged from this study was the ability of antimicrobials such as bacteriocins to modulate the gut microbiota, though the choice and target specificity are likely to be crucial (Murphy, Cotter et al. 2012, Murphy, Clarke et al. 2013).

To this end, here we describe a culture-based screen to identify bacteriocin-producing gut microbes from lean donors (BMI<25) with probiotic potential that can be used to target obesity-linked species.

3.3 Materials and Methods

3.3.1 Sample collection, bacterial strains and culture conditions

Faecal samples were acquired from a previous study of 23 lean male donors with a body mass index (BMI) of less than 25 (Clarke, Murphy et al. 2014). Culturing/screening was performed both aerobically at 37°C for 24 hrs using BHI agar (Merck, Darmstedt, Germany) and anaerobically at 37°C for 48 hrs using Wilkins-Chalgren anaerobe agar (WCAA) (Oxoid Ltd, Basingstoke, Hampshire, UK). In the latter case, anaerobic conditions were maintained through the use of an anaerobic chamber (10% CO_2, 10% H_2, 80 % N_2). Target strains used for screening and antimicrobial characterisation, and their respective growth conditions, are listed in Table 1. *Clostridium symbiosum* DSM934 and *Bacteroides intestinalis* DSM17393, selected for testing anaerobic isolates, were cultured using RCM (Merck). Indicators chosen for screening of aerobic isolates were *Lactobacillus bulgaricus* DPC5383, cultured using MRS (Difco Laboratories, Detroit, MI, USA), *Listeria innocua* DPC3572 and *Salmonella enterica* serovar *typhimurium* LT2 DPC6048, grown using BHI (Merck).

3.3.2 Screening for bacteriocin-producing isolates

Faecal samples were homogenized with maximum recovery diluent (MRD) (Oxoid) under anaerobic conditions, serial 10-fold dilutions were prepared and 100 µL of the appropriate dilutions were plated in duplicate on WCAA and incubated anaerobically at 37°C for 48 hrs. Plates were examined for colony forming units after 48 hrs before overlaying with indicator strains using the deferred antagonism method (Harris, Daeschel et al. 1989, Lewus, Kaiser et al. 1991) using 0.75% agar (w/v) and

incubating for a further 24 hrs anaerobically. A high-throughput method was chosen for use in the aerobic screen which employed the QPix2 colony picking robot (Genetix, Hampshire, UK) and plating on Q-trays (Molecular Devices, Sunnyvale, CA, USA). Each Q-tray was filled with 250 mL BHI agar (1.5% w/v) and allowed to set prior to plating. Serial 10-fold dilutions were prepared for each faecal sample and 1 mL was plated onto BHI agar set in each Q-tray (Molecular Devices). Following aerobic incubation at 37°C for 24 hrs, approximately 2000 colonies were picked from each sample using the QPix2 colony picking robot (Genetix), inoculated into 384 well microtiter plates containing BHI freezing medium and incubated aerobically at 37°C for 24 hrs. After incubation, colonies from 384 well plates were re-stamped in quadruplicate on BHI agar set in Q-trays (Molecular Devices) and incubated aerobically at 37°C for 24 hrs (Collins, Joyce et al. 2010). Plates were overlaid with the respective indicators seeded in 200 mL agar (0.75% w/v) and incubated for a further 24hrs (Table 1). Bacterial colonies displaying zones of inhibition in a deferred antagonism assay were picked and re-streaked on agar plates before culturing overnight in broth. Isolates were then stocked in a final concentration 40% (w/v) glycerol (Sigma, St. Louis, MO, USA) for further characterisation.

3.3.3 Antimicrobial activity assay

The antimicrobial activity of the potential bacteriocin-producing isolates was assessed against a number of indicator strains including *C. symbiosium* DSM934, *Clostridium bolteae* DSM15670, *Clostridium ramosum* DSM1402, *Clostrdium difficile* DPC6217, *L. innocua* DPC3572, *B. intestinalis* DSM17393, *L. bulgaricus* DPC5383, *Streptococcus agalacitae* LMG14694, and *Enterococcus faecalis* EF1

(Table 1) using the deferred antagonism assay (Harris, Daeschel et al. 1989, Lewus, Kaiser et al. 1991). Plates were examined for zones of inhibition following overnight incubation. The activity of known bacteriocin producers, i.e., *Lactococcus lactis* DPC3147, *L. lactis* NZ9700, *Lactobacillus salivarius* UCC118, *L. salivarius* DPC6005, *L. salivarius* DPC6488, *L. salivarius* DPC6502, *Bacillus thuringiensis* DPC6431 (Table 1) against T2D-enriched species was also assessed using the well diffusion method (Ryan, Rea et al. 1996). Plates were examined for zones of inhibition following overnight incubation.

3.3.4 Genetic characterisation of isolates

Genomic DNA (gDNA) was extracted from putative bacteriocin-producing isolates using the GenElute Bacterial Genomic DNA Kit as per the protocol (Sigma). Amplification of the 16S rRNA gene using the universal primers CO1 and CO2 (Simpson, Stanton et al. 2003) followed by Sanger sequencing (Beckman Coulter Genomics, Essex, UK) to determine the complete sequence of the 16S rRNA gene was used to identify bacterial isolates. Analysis of sequence data was performed using Lasergene v8 software. Nucleotide alignments (>98% nucleotide identity) with species deposited in the NCBI database were used to confirm species identity. *Enterococcus* isolates were further typed using species-specific primers targeting the *sodA* gene (Jackson, Fedorka-Cray et al. 2004).

3.3.5 Colony Mass Spectrometry

Colony mass spectrometry (CMS) was used to determine the molecular mass of the peptides. Colonies were mixed with 50 µL 70% propan-2-ol 0.1% TFA, vortexed and centrifuged at 14,000 rpm for 30 seconds. Matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) was performed on the cell

supernatant using an Axima TOF^2 MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 µL aliquot of matrix solution was used to pre-coat the target. The solution was allowed to dry and 0.5 µL of sample solution was deposited onto the pre-coated spot. A 0.5 µL aliquot of matrix solution was added to the deposited sample and allowed to dry. The sample was subsequently analysed in positive-ion linear mode. The theoretical mass was then compared to the mass of known bacteriocins.

To determine the mass of the bacteriocin produced by *S. salivarius* DPC6988 a modified version of the method previously described by Hyink *et al*. was used (Hyink, Wescombe et al. 2007). An overnight culture of *S. salivarius* DPC6988 was swabbed across BHI agar plates and incubated aerobically at 37°C. Plates were overlaid with MRS agar (0.75% w/v) seeded with *L. bulgaricus* DPC5383 and subsequently incubated overnight before checking for zones of inhibition. A small section of agar was removed from each plate to allow for collection of exudate which was generated by repeated freezing of agar plates at -20°C and thawing at room temperature. Exudate was examined for antimicrobial activity in a well diffusion assay, and, if active applied to a 6 mL, 500 mg, Strata-E C18 SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The columns were washed with 6 mL 30% ethanol and then 6 mL 70% propan-2-ol 0.1% TFA. Eluents from C18 SPE columns were assayed for antimicrobial activity using *L. bulgaricus* as an indicator. Mass spectrometry was performed on the active fractions as described above.

3.3.6 Whole genome sequencing of lead bacteriocin-producing isolates

Genomic libraries were prepared on gDNA using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). The purity and concentration of the genomic DNA was established using the NanoDrop (ThermoFisher Scientific, Dublin, Ireland) and Qubit® 2.0 Fluorometer (ThermoFisher Scientific, Dublin, Ireland) as per the protocol. Whole genome sequencing of lead isolates was performed using Illumina's MiSeq platform with the MiSeq V3 600 cycles Paired Ends kit at Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland using paired-end 2x300bp reads. FastQC (Andrews 2010) was used to examine the resulting reads and trimming of low quality bases and Illumina adaptors was performed using Trim-Galore (Krueger 2015). Assembly of contigs from paired-end reads was achieved using SPAdes Genome Assembler (Bankevich, Nurk et al. 2012). Prodigal (Hyatt, Chen et al. 2010) was used to predict open reading frames of the draft genome, and the RAST annotation server (Aziz, Bartels et al. 2008) performed complementary gene calling and automated annotation. BAGEL3 software was used to screen draft genomes for putative bacteriocin operons (van Heel, de Jong et al. 2013). The predicted bacteriocin gene clusters were then further analysed using the Artemis genome browser (Carver, Berriman et al. 2008) and the BlastP web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4 Results

3.4.1 Antimicrobial activity of known bacteriocin producers

The activity of a number of well characterised bacteriocins, lacticin 3147 (*L. lactis* DPC3147), nisin (*L. lactis* NZ9700), Abp118 (*L. salivarius* UCC118), salivaricin P (*L. salivarius* DPC6005), salivaricin T/L (*L. salivarius* DPC6488), bactofencin A (*L. salivarius* DPC6502) and thuricin CD (*B. thuringiensis* DPC6431), was assessed against species enriched in patients with T2D and/or obese individuals, i.e., *C. ramosum*, *C. symbiosum*, *C. bolteae*, *C. hathewayi* and *B. intestinalis*, in a well diffusion assay (Table 2). No inhibition was detected against the Gram-negative *B. intestinalis* and, overall, nisin was found to be most active demonstrating the largest zones of inhibition against 3 of the 4 *Clostridium* species tested (Table 2). Thuricin CD demonstrated activity against all *Clostridium* strains with the exception of *C. ramosum*. The bacteriocins produced by different strains of *L. salivarius* did not exhibit activity against of the T2D/obesity-associated targets.

3.4.2 Screening and isolation of antimicrobial-producing isolates

An anaerobic screen of over 15,000 isolates from the faecal samples of 23 lean donors for activity against *C. symbiosum* DSM934 or *B. intestinalis* DSM17393 resulted in the detection of 1 distinct antimicrobial-producing isolate demonstrating inhibition against *C. symbiosum*. A second isolate was found to exhibit antimicrobial activity prior to overlaying with the indicators, preventing the growth of surrounding colonies, and was included in subsequent analyses. No activity was detected against *B. intestinalis* during screening. An aerobic screen of over 28,000 colonies from the same donors for activity against *L. bulgaricus*, *L. innocua* and *S. typhimurium*, as an

initial screen for bacteriocin production, resulted in the identification of 12 distinct isolates. None of these showed activity against *S. typhimurium*.

3.4.3 Genetic characterisation of bacterial isolates

Identification of the 14 antimicrobial-producing isolates was carried out by 16S rDNA sequence analysis. Lactic acid bacteria (LAB), particularly species of *Enterococcus* and *Streptococcus,* were most commonly identified. *Streptococcus mutans*, *Streptococcus agalactiae* and *Streptococcus salivarius* were among the streptococci isolated. *Enterococcus* isolates that could not be unambiguously assigned through 16S analysis were further differentiated using species-specific primers and found to be *Enterococcus faecalis* and *Enterococcus faecium*. *Pediococcus acidilactici*, which is also a LAB, and *Staphylococcus epidermidis* were also identified.

3.4.4 Further investigation of antimicrobial spectrum

The inhibitory spectrum of the 14 antimicrobial-producing isolates was further assessed using the deferred antagonism assay against a range of indicators, including species enriched in T2D/obesity. *S. mutans* DPC7039, *S. agalactiae* DPC7040 and *E. faecalis* DPC7041 demonstrated the widest spectrum of inhibition, and were regarded as lead isolates due to inhibition of *Clostridium* species associated with T2D/obesity (Table 3). *S. salivarius* DPC6988, which demonstrated activity against *C. ramosum*, *C. symbiosum*, *L. bulgaricus* and *S. agalactiae*, was deemed to be of greatest interest due to the non-pathogenic nature of the producer (Table 3). Among the other producers, it was also notable that *E. faecalis* DPC7041 displayed haemolytic activity when streaked across (7% v/v) blood agar plates (data not shown).

3.4.5 Genomic analysis of lead antimicrobial producers

The genome sequence of the 4 lead isolates, *S. salivarius* DPC6988, *S. mutans* DPC7039, *S. agalactiae* DPC7040, and *E. faecalis* DPC7041, was determined. Following contig assembly, the draft genomes were screened using the software BAGEL3 to reveal a number of putative bacteriocin gene clusters (PBGCs) (Figure 1). These were distributed as follows: *E. faecalis* DPC7041; 2 PBGCs, *S. salivarius* DPC6988; 3 PBGCs, *S. agalactiae* DPC7041; 1 PBGC, and *S. mutans* DPC7039; 1 PBGC.

E. faecalis DPC7041

BAGEL3 identified two PBGCs in the *E. faecalis* DPC7041 draft genome (Figure 1). The first (PBGC 1), a 6 gene subclass II lantibiotic-like cluster, contained a *lanM* and *lanT*. Two putative structural genes were also identified, one of which had a double glycine leader, and BlastP analysis revealed two putative conserved domains (both TIGR03893), resembling, but distinct from the family that includes the lantibiotics mersacidin and lichenicidin. The second (PBGC 2), revealed a 6 gene cluster resembling a cytolysin-like operon. Alignment of the two modified peptides identified using BAGEL3 with the structural subunits of cytolysin, CylLl and CylLs (Van Tyne, Martin et al. 2013), resulted in 98.53% and 98.39% amino acid identity respectively.

S. salivarius DPC6988

Three PBGCs were identified in *S. salivarius* DPC6988 (Figure 1). The first (PBGC 1), revealed a 7 gene cluster which resembled that encoding salivaricin A, a subclass II lantibiotic. BlastP analysis and alignment of the putative structural peptide with

the salivaricin A2 structural peptide (Wescombe, Upton et al. 2006) revealed 100% amino acid identity. The second PBGC (PBGC 2) contained a 9 gene streptin-like cluster, a subclass I lantibiotic. BlastP analysis revealed 100% identity to the streptin structural peptide (Wescombe and Tagg 2003). A 6 gene cluster (PBGC 3), resembling a subclass IV lanthipeptide with a putative *lanL* modification gene, was also identified (Figure 1).

S. agalactiae DPC7040

The genome of *S. agalactiae* DPC7040 was determined to have just one PBGC (Figure 1). This 12 gene cluster resembled that of a nisin-like operon. BlastP analysis of the putative structural gene revealed 93% amino acid identity with that of nisin U2 (Wirawan, Klesse et al. 2006). Subsequent alignment of the putative structural peptide from *S. agalactiae* DPC7040 with that of nisin P, identified in *S. pasteurianus* (Zhang, Yu et al. 2012), revealed 100% amino acid identity.

S. mutans DPC7039

The draft genome of *S. mutans* DPC7039 revealed one PBGC containing 8 genes which included one putative regulation gene and two putative immunity/transport genes, and was associated with a number of other undefined genes. Manual annotation and BlastP analysis did not reveal a putative structural gene and no hits to any known bacteriocins were identified (Figure 1).

3.4.6 Identification and purification of bacteriocins

The mass of the peptides produced by *S. salivarius* DPC6988 and *S. agalactiae* DPC7040 were determined by colony mass spectrometry. This revealed that *S. agalactiae* DPC7040 produced a compound with a molecular mass of 2989 Da,

which corresponds to the mass of nisin P (Figure 2a). *S. salivarius* DPC6988 produced a compound with a molecular mass of 2366 Da, which corresponds to the mass of salivaricin A2 (Figure 2b).

3.5 Discussion

The ability of two antimicrobials, vancomycin and the bacteriocin-producing probiotic *L. salivarius* UCC118, to modulate the microbiota in a DIO mouse model was demonstrated by Murphy *et al*. (Murphy, Cotter et al. 2012). Building on this previous investigation, the aim of this screen was to isolate bacteriocin-producing gut microbes that can be used to target T2D or obesity-associated populations, and initial targets selected included, *C. ramosum*, *C. bolteae*, *C. symbiosum*, *C. hatheywai* and *B. intestinalis*. While it is not yet clear if these microorganisms contribute to T2D or obesity-associated phenotypes, the role of *C. ramosum* in promoting obesity in a gnotobiotic mouse model has been demonstrated (Woting, Pfeiffer et al. 2014). In instances where a role in T2D or obesity has not been established, the potential exists to use antimicrobials which target these microorganisms to elucidate their role, if any.

In this study, the initial screening of over 43,000 colonies under both aerobic and anaerobic conditions resulted in the detection of just 14 distinct isolates displaying antimicrobial activity against the target strains selected. This low isolation frequency is consistent with that observed across other screening studies (Rea, Sit et al. 2010, Lakshminarayanan, Guinane et al. 2013) and may be attributed to the limitations of culture-based screens as previously highlighted (O'Shea, Gardiner et al. 2009). No bacteriocin activity was detected during screening against the Gram-negative indicators used, however, this was not surprising as the Gram-negative outer membrane provides protection against many bacteriocins (Gao, van Belkum et al. 1999).

Under the conditions used in this screen, all except one of the isolates identified was a LAB, with *Streptococcus* and *Enterococcus* species being most common. The four lead isolates were selected on the basis of the breadth or specificity of antimicrobial activity against T2D enriched species and included *S. salivarius* DPC6988, *S. mutans* DPC7039, *S. agalactiae* DPC7040 and *E. faecalis* DPC7041.

S. salivarius is a well-established human commensal, typically associated with the oral cavity (Aas, Paster et al. 2005) but also inhabits the gut (Pearce, Bowden et al. 1995). They are known to produce a number of bacteriocins (Nes, Diep et al. 2007, Wescombe, Heng et al. 2009) and the isolation of these bacteriocins from the intestine has previously been documented (O'Shea, Gardiner et al. 2009, Birri, Brede et al. 2012). We identified 3 PBGCs within the genome of DPC6988 including a salivaricin A2-encoding cluster (Wescombe, Upton et al. 2006), a streptin-like cluster (Wescombe and Tagg 2003), and a third, LanL-like cluster. The streptin operon has previously been reported to contain an immunity gene located at the beginning of the cluster (Wescombe and Tagg 2003), however, no immunity gene was detected from the streptin-like cluster identified in DPC6988. Strains of *S. salivarius* are capable of encoding multiple bacteriocin operons (Wescombe, Burton et al. 2006), but, to our knowledge, this particular combination of bacteriocin gene clusters has not been identified in other *S. salivarius* strains. Despite the clusters identified in *S. salivarius* DPC6988, salivaricin A2 was confirmed as the bacteriocin that was actively produced suggesting it was also responsible for the inhibitory activity.

S. agalactiae, a member of the group B streptococci, is a commensal bacterium that colonises the gastrointestinal and genitourinary tracts. However, strains of *S. agalactiae* also cause severe infections in both adults and neonates (Glaser, Rusniok

et al. 2002). Information on bacteriocin production by this species is limited. Although there are some strains of bovine *S. agalactiae* that have been shown to harbour a nisin U operon (Richards, Lang et al. 2011), these were unable to produce nisin U possibly due to truncation of the *nsaB* gene by an internal insertion sequence (Richards, Lang et al. 2011). It is also notable that the production of nisin variants by other *Streptococcus* species has previously been reported. These include nisin U by *Streptococcus uberis* (Wirawan, Klesse et al. 2006) and nisin H by *Streptococcus hyointesinalis* (O'Connor, O'Shea et al. 2015). BAGEL3 predicted one PBGC in the draft genome of *S. agalactiae* DPC7040, which consisted of a 12 gene nisin-like cluster. The mass of this peptide, determined by colony mass spec, was 2989 Da which correlates with the mass of nisin P (Zhang, Yu et al. 2012). This bacteriocin was first identified in *Streptococcus pasteurianus* by Zhang *et al*., where it was suggested that the strain may be able to produce a nisin analog similar to nisin U, and was designated nisin P (Zhang, Yu et al. 2012). Although not referred to as nisin P, this operon was also detected in *Streptococcus suis*. The structural gene identified was identical to that of nisin P, however, the mass of the peptide produced was 2780.249 Da (Wu, Wang et al. 2014). To our knowledge, the production of nisin P by *S. agalactiae* DPC7040 represents the first example of bacteriocin production by this species.

Commensal enterococci are among the dominant members of LAB in mammals and other animals (Nes, Diep et al. 2007). With the exception of cytolysin, a two-peptide lantibiotic produced by certain strains of *E. faecalis*, many of the bacteriocins produced by enterococci belong to the class II bacteriocin group, and the majority of these are referred to as enterocins (Nes, Diep et al. 2007). *E. faecalis* DPC7041 contained two PBGCs, a cytolysin-like cluster and a second putative lantibiotic

cluster. Though this second putative cluster did not display similarity to any known bacteriocins, putative conserved domains were detected within the two structural peptides resembling, but distinct from the family that includes the bacteriocins mersacidin and lichenicidin. The mass of the structural peptide produced by this strain could not be determined by colony mass spec. However, the fact that this strain demonstrates haemolytic activity when cultured on blood plates suggests the production of cytolysin, a known haemolytic bacteriocin produced by *E. faecalis* (Booth, Bogie et al. 1996).

S. mutans, a species linked with dental caries (Selwitz, Ismail et al. 2007), is typically associated with the oral cavity, and has also been associated with bacteriocin production. Baceriocins produced by *S. mutans* are generally referred to as mutacins (Nes, Diep et al. 2007). The isolation of a mutacin II-producing *S. mutans* strain from the gut of elderly subjects has been reported previously (Lakshminarayanan, Guinane et al. 2013). BAGEL3 and manual annotation of *S. mutans* DPC7039 revealed one PBGC. However, this bears no resemblance to any known bacteriocin gene clusters. Furthermore, as with *E. faecalis* DPC7041, the mass of the active peptide of *S. mutans* could not be determined and so the identity of the antimicrobial produced is, as yet, unclear.

This study again highlights the limitations associated with the use of traditional agar-based methods when searching for bacteriocin-producing microbes. Such approaches, may therefore only allow for the detection of a fraction of potential bacteriocin-producing gut microbes within a complex environment such as the gastrointestinal (GI) tract. These challenges may be overcome through the use of bioinformatic approaches that bypass requirements such as the need for growth. However, even bacterioicin gene clusters identified using these methods remain

putative until such as time as activity can be confirmed. Further work targeting the growth of difficult-to-culture microbes (Browne, Forster et al. 2016) may also enhance the isolation of bacteriocin-producing gut microbes using traditional approaches. Although approximately 43,000 colonies were screened against 5 indicators, only 4 lead isolates were taken forward, and, despite the inhibition of several species enriched in T2D and their status as "lead isolates" in this study, the association of *S. agalactiae* DPC7040, *S. mutans* DPC7039 and *E. faecalis* DPC7041 with pathogenicity would hinder their application as probiotics. However, the non-pathogenic nature of *S. salivarius*, and the ability of DPC6988 to inhibit *C. ramosum*, means that it merits further attention.

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Table 1 Indicator strains/bacteriocin producers used and their respective growth conditions

Strain	Culture Medium	Temp. (°C)	Conditions	Notes
Clostridium symbiosum DSM934	FAA (7% blood)/RCM	37	Anaerobic	T2D-enriched
Clostridium ramosum DSM1402	FAA (7% blood)/RCM	37	Anaerobic	T2D-enriched
Clostridium bolteae DSM15670	FAA (7% blood)/RCM	37	Anaerobic	T2D-enriched
Clostridium hathewayii DSM13479	FAA (7% blood)/RCM	37	Anaerobic	T2D-enriched
Bacteroides intestinalis DSM17393	FAA (7% blood)/RCM	37	Anaerobic	T2D-enriched
Clostridium difficile DPC6217	FAA (7% blood)/RCM	37	Anaerobic	Indicator
Lactobacillus bulgaricus DPC5383	MRS	37	Anaerobic	Indicator
Listeria innocua DPC3572	BHI	37	Aerobic	Indicator
Enterococcus faecalis EF1	BHI	37	Aerobic	Indicator
Streptococcus agalactiae LMG14694	BHI	37	Aerobic	Indicator
Salmonella typhimurium LT2 DPC6048	BHI	37	Aerobic	Indicator
Lactococcus lactis NZ9700	gM17	30	Aerobic	Bacteriocin producer
Lactococcus lactis DPC3147	gM17	30	Aerobic	Bacteriocin producer
Lactobacillus salivarius UCC118	MRS	37	Anaerobic	Bacteriocin producer
Lactobacillus salivarius DPC6005	MRS	37	Anaerobic	Bacteriocin producer
Lactobacillus salivarius DPC6488	MRS	37	Anaerobic	Bacteriocin producer
Lactobacillus salivarius DPC6502	MRS	37	Anaerobic	Bacteriocin producer
Bacillus thuringiensis DPC6431	BHI	37	Aerobic	Bacteriocin producer

Table 2 Inhibitory spectrum of known bacteriocins against T2D enriched indicators using the well diffusion assay

	Indicator				
Bacteriocin	*C. ramosum*	*C. hathewayii*	*C. bolteae*	*C. symbiosum*	*B. intestinalis*
Lacticin 3147	9mm	10mm	7mm	9mm	NZ
Nisin	10mm	14mm	13mm	15.5mm	NZ
Thuricin CD	NZ	15mm	10mm	9.5mm	NZ
Abp118	NZ	NZ	NZ	NZ	NZ
Salivaricin P	NZ	NZ	NZ	NZ	NZ
Salivaricin T/L	NZ	NZ	NZ	NZ	NZ
Bactofencin A	NZ	NZ	NZ	NZ	NZ

NZ; no zone

Table 3 Inhibitory spectrum of lead antimicrobial-producing isolates using the deferred antagonism assay

	Indicator									
Bacteriocin-producing isolate	*C. ramosum*	*C. bolteae*	*C. symbiosum*	*C. hathewayi*	*B. intestinalis*	*C. difficile*	*L. bulgaricus*	*L. innocua*	*S. agalactiae*	*E. faecalis*
S. mutans	+	+	+	+	-	+	+	-/hazy	+	+
E. faecalis	+	+	+	+	+	+	+	+	+	+
S. salivarius	+	-	+*	-	-	+*	+	-	+	-
S. agalactiae	+	+	+	+*	-	+	+	-	+	hazy

+; positive for inhibition, -; negative for inhibition, *; denotes faint inhibition, hazy inhibition

Figure 1 Diagrammatic representation of the putative bacteriocin gene clusters for each lead isolate identified in BAGEL3

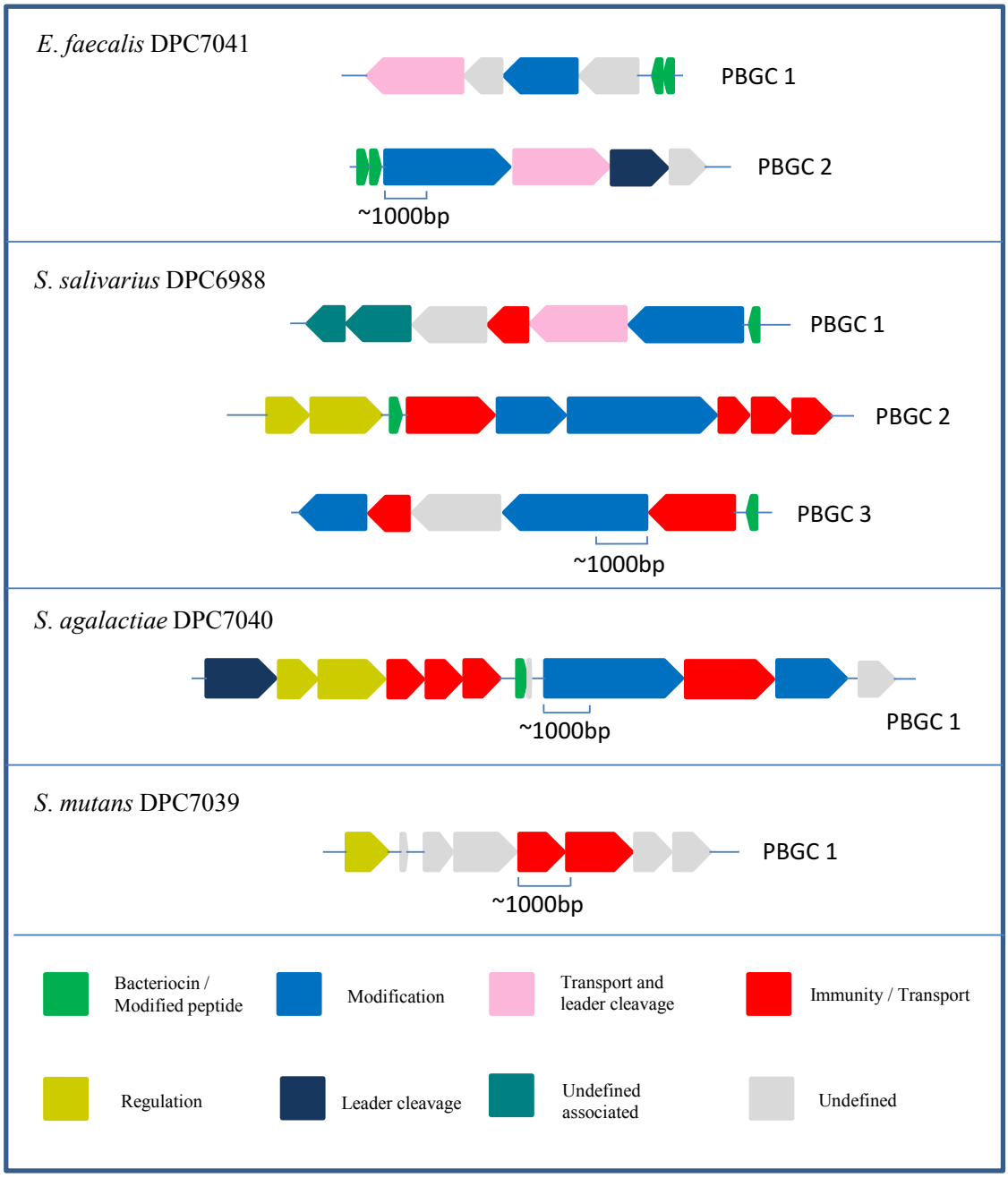
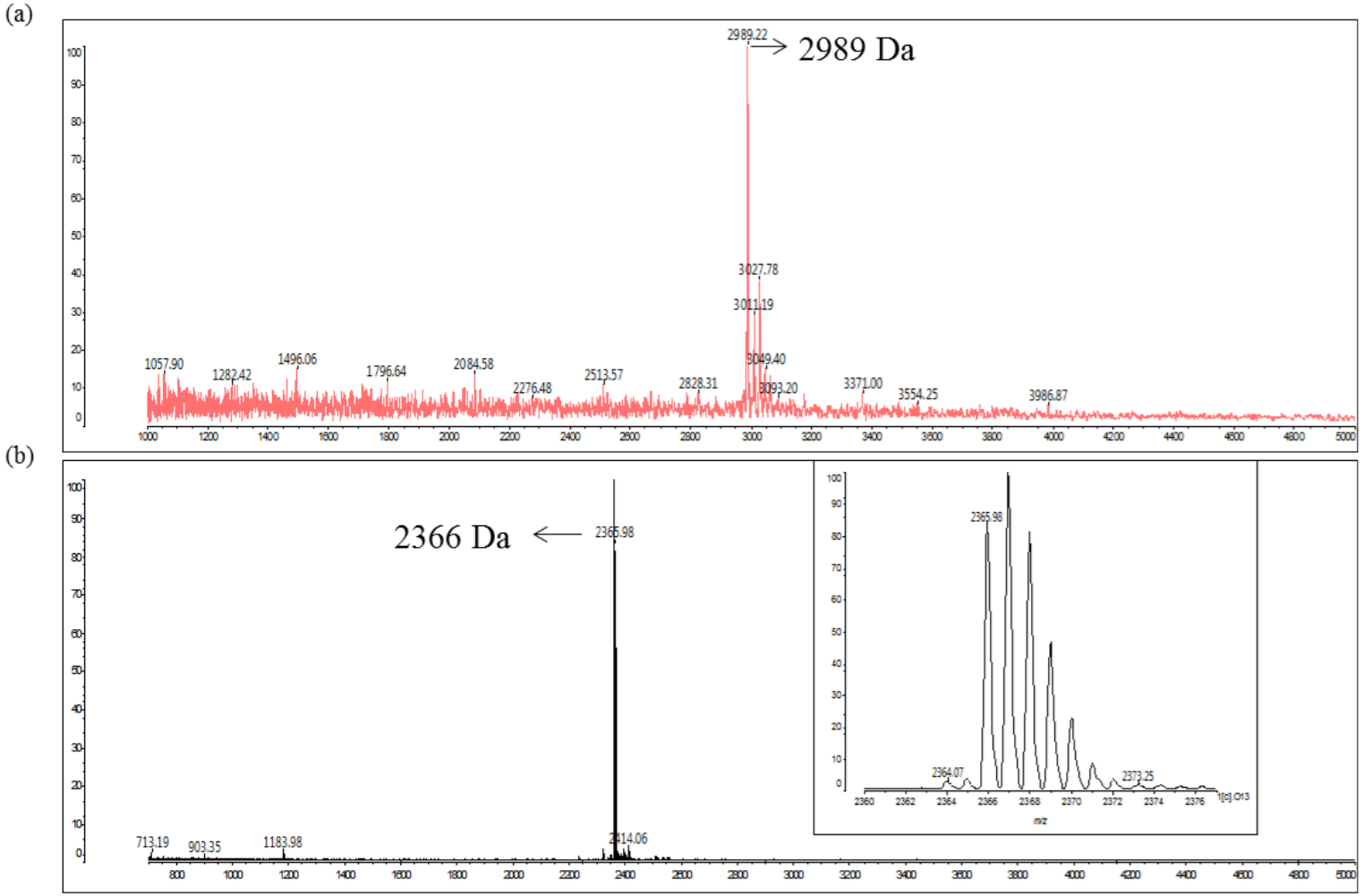


Figure 2 CMS data of (a) nisin P produced by *S. agalactiae* DPC and (b) salivaricin A2 produced by *S. salivarius* DPC6988



Chapter 4

Investigation of the impact of the bacteriocin-producing *Streptococcus salivarius* DPC6988 on gut microbial populations

4.1 Abstract

Bacteriocin-producing probiotics merit investigation as alternative treatments for obesity and related metabolic disorders through manipulation of the gut microbiota. *Ex vivo* fermentation systems can provide valuable initial insights into the impact of antimicrobials and antimicrobial-producing probiotics on gut populations. Despite the benefits of this approach, there remain only a few such studies. Here, an *ex vivo* system was used to investigate the impact of *Streptococcus salivarius* DPC6988, a salivaricin A2-producing strain of gut origin, and *S. salivarius* HSISS4, a non-bacteriocin-producing control strain, on intestinal populations and overall microbial diversity. It was revealed that, regardless of bacteriocin production the introduction of either strain had an impact on gut populations. However, treatment with DPC6988 resulted in overall decreased abundances of *Clostridum* spp. and an uncultured genus of the *Erysipelotrichaceae* family, when compared to either the control vessel or that containing HSISS4. These findings are intriguing as both taxa have previously been associated with an obese phenotype and suggest that further investigations are merited.

4.2 Introduction

The intestinal microbiota is considered a rich source of bacteriocin-producing isolates, which in turn have the potential to be employed as probiotics to shape gut microbial composition to, for example, eliminate pathogens or influence metabolic health. However, despite this potential, there is surprisingly limited information available regarding their impact on gut microbial composition.

Streptococcus salivarius is a well-established human commensal of the oral cavity (Aas, Paster et al. 2005) and a typical inhabitant of the gut (Pearce, Bowden et al. 1995). Some strains of *S. salivarius* have been studied for their probiotic potential, and bacteriocin production has been regarded as a key trait with respect to related applications. Strain K12, regarded as the model *S. salivarius* probiotic, produces two bacteriocins and is available in commercial preparations. Initially selected for its role to counteract *Streptococcus pyogenes*, several other health promoting effects have been since noted (Wescombe, Hale et al. 2012). Although bacteriocin-producing *S. salivarius* have been isolated from the GI tract (O'Shea, Gardiner et al. 2009, Birri, Brede et al. 2012), their impact on the microbial composition of the gut has not been reported.

We previously documented the isolation of *S. salivarius* DPC6988, which contains a number of putative bacteriocin gene clusters, from the gut (Thesis Chapter 3). This strain is capable of producing the bacteriocin salivaricin A2 and demonstrates inhibitory activity against obesity-linked species including *Clostridium ramosum* DSM1402 and, to a lesser extent, *Clostridium symbiosum* DSM934 (Thesis Chapter 3). Salivaricin A2 is a lantibiotic and is also produced by *S. salivarius* K12 (Wescombe, Upton et al. 2006, Hyink, Wescombe et al. 2007) and other strains.

Indeed, the isolation of a salivaricin A2-producing *S. salivarius* from the gut has also been reported (O'Shea, Gardiner et al. 2009), and suggests that bacteriocin production confers a selective advantage within the intestinal environment. The inhibitory spectrum of this bacteriocin has been examined (Hyink, Wescombe et al. 2007, O'Shea, Gardiner et al. 2009) and was found to include several pathogenic oral *Streptococcus* species but activity against gut associated indicators was limited (Hyink, Wescombe et al. 2007, O'Shea, Gardiner et al. 2009). However, it is important to note that production on laboratory media may not reflect activity within the complex anaerobic environment of the gut and, additionally, effects of a bacteriocin on overall microbial composition, including taxa that are not easy to culture, is not clear (Guinane, Lawton et al. 2016).

Obesity is a complex syndrome associated with a number of serious implications for human health including cardiovascular disease and type 2 diabetes. The role of the gut microbiota in obesity and related metabolic disorders has received considerable attention (Turnbaugh, Bäckhed et al. 2008, Qin, Li et al. 2012, Everard, Belzer et al. 2013, Karlsson, Tremaroli et al. 2013, Le Chatelier, Nielsen et al. 2013). One such study, by Woting *et al*. (Woting, Pfeiffer et al. 2014), demonstrated the ability of *C. ramosum* to promote obesity in a gnotobiotic mouse model. Our previous observations that *S. salivarius* DPC6988 displays antimicrobial activity against *C. ramosum* raises the possibility of the use of DPC6988 as part of a broader approach to control obesity. Such an application would rely on the ability of DPC6988 to be active in a gut environment and, thus, here we perform *ex vivo* investigations to this end. *Ex vivo* systems can mimic the distal or proximal colon and have been used previously to investigate the effects of bacteriocins on microbial composition (Dobson, Crispie et al. 2011, Rea, Dobson et al. 2011, Guinane, Lawton et al. 2016).

They are particularly valuable in these circumstances as *in vivo* experiments to determine the impact of a bacteriocin on the gut microbiota can be expensive and have associated ethical concerns (Williams, Walton et al. 2015). Ultimately, the aim of this study was to assess the impact of DPC6988 on microbial populations, which included an introduced *C. ramosum* DSM1402 strain, using a model of the distal colon.

4.3 Material and Methods

4.3.1 Bacterial strains and culture conditions

S. salivarius strains used in this study, DPC6988 (Thesis Chapter 3) and HSISS4 (Van den Bogert, Boekhorst et al. 2013), were grown under aerobic conditions at 37°C in BHI (Merck, Darmstedt, Germany) media. *C. ramosum* DSM1402 and *C. symbiosum* DSM934 were grown anaerobically at 37°C on Fastidious anaerobe agar (FAA) supplemented with defibrinated horse blood (7% v/v) (Cruinn) or in reinforced clostridial medium (RCM) (Merck). *Lactobacillus bulgaricus* DPC5383 was grown under anaerobic conditions at 37°C in MRS (Difco Laboratories, Detroit, MI, USA). Anaerobic conditions were maintained through the use of an anaerobic chamber (Don Whitley, West Yorkshire, UK) or an anaerobic jar using Anerocult A gas packs (Merck). Where solid media was required, 1.5% agar (w/v) (Oxoid Ltd., Basingstoke, Hampshire, UK) was added. Rifampicin (Sigma-Aldrich, St. Louis, MO, USA) was used in BHI selective medium where required at a concentration of 100 μg/mL for rifampicin resistant strains.

4.3.2 Purification of salivaricin A2

Salivaricin A2 was purified from exudate collected from *S. salivairius* DPC6988 containing active fractions of the peptide, as described in Thesis Chapter 3. Briefly, to generate purified peptide for use in minimum inhibitory concentration assays, collection of the exudate was scaled up by plating on BHI agar set in Q-trays (Genetix, Hampshire, UK) and assayed for antimicrobial activity. HPLC analysis of the exudate was achieved by applying aliquots of the sample to a semi preparative Proteo Jupiter (10 x 250nm, 90Å, 4 μ) RP-HPLC column (Phenomenex, Chesire,

UK) running a 25-50% acetonitrile 0.1% TFA over 30 minutes where buffer A is water containing 0.1% TFA and buffer B is 90% acetonitrile 0.1% TFA. Eluent was monitored at 214nm and fractions were collected at 1 minute intervals before assaying for activity. MALDI-TOF MS was performed as previously described (Thesis Chapter 3) to determine if active fractions contained the correct mass before pooling and repeating HPLC, using a longer and shallower gradient (30-46%), to purify the peptide further. Pure fraction were pooled, lyophilised and stored at -20°C.

4.3.3 Minimum inhibitory concentration assays

Replicate minimum inhibitory concentration (MIC) assays using purified salivaricin A2 were performed in 96-well microtitre plates as previously described by Field *et al*. (Field, Quigley et al. 2010). Briefly, wells were pre-treated with bovine serum albumin (BSA) prior to addition of growth medium. To achieve this, 200 µL of phosphate buffered saline (PBS) containing 1% (w/v) BSA was added to each well and plates were incubated at 37°C for 45 minutes. Wells were subsequently washed twice with PBS and allowed to dry before the addition of 100 µL of the appropriate growth medium. A 4X working stock of purified salivaricin A2 was prepared and 100 µL added to the first well of the plate resulting in starting concentrations of 20 µM and 40 µM (*C. ramosum* DSM1402), 20 µM and 5 µM (*C. symbiosum* DSM934), and 1 µM and 0.8 µM (*L. bulgaricus* DPC5383) and two-fold serial dilutions of the peptide were made. Target strains were grown overnight under appropriate conditions, subcultured into fresh broth and grown to an OD 600 nm of ~0.5, before diluting to achieve a final concentration of 10^5 CFU/mL in 200 µL in each well. Plates were incubated overnight under appropriate conditions. The MIC was determined as the lowest peptide concentration that prevented visible growth.

4.3.4 Antibiotic susceptibility of *S. salivarius* DPC6988

The antibiotic resistance of *S. salivarius* DPC6988 was evaluated using the VetMIC system as per the protocol stated in ISO 10932 (Determination of the minimum inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria) and as previously described (Huys, D'Haene et al. 2010) using Lact-1 and Lact-2 plates. Each microtitre plate contains a series of 2-fold dilutions of each antibiotic over 10 wells. Briefly, colonies of *S. salivarius* were picked and re-suspended in 2 mL of sterile saline (0.85% NaCl w/v) until an OD at 625 nm of 0.16 – 0.2 was reached. The resulting bacterial suspension was diluted 1000 times in IST-M17 medium (90% IST medium, Oxoid, 10% M17 medium, Difco) to achieve the desired cell density of approximately $3x10^5$ CFU/mL. 100µl of the suspension was added to each well of the microtitre plate in duplicate and plates were incubated at 37°C for 48 hrs. The MIC was defined as the lowest antibiotic concentration which prevented visible growth.

4.3.5 Creation of rifampicin resistant *S. salivarius*

S. salivarius strains were cultured in BHI broth aerobically at 37°C overnight, and 100 µL of culture was plated on BHI agar plates containing varying concentrations of rifampicin (8-100 µg/mL). Resistant isolates were picked and subsequently passaged to raise resistance to a concentration of 100 µg/mL. Cultures were streaked on BHI agar plates containing 100 µg/mL rifampicin. Single isolates were picked from the plate and grown again overnight in BHI broth containing rifampicin at a concentration of 100 µg/mL. Glycerol stocks (40% v/v final concentration) were made for both *S. salivarius* strains and stored at -80°C. The bacteriocin-producing ability of the resistant *S. salivarius* DPC6988 was subsequently checked to confirm

that the creation of the resistant strain did not affect activity using the deferred antagonism assay (Harris, Daeschel et al. 1989, Lewus, Kaiser et al. 1991).

4.3.6 Preparation of a faecal standard

Preparation of the faecal standard inoculum for the fermentation vessels was performed as previously described (O'Donnell, Rea et al. 2016). Briefly, donors adhered to strict criteria; all volunteers were healthy and had not received antibiotic treatment in the six months before donation. Fresh samples were stored for 1-2 hours at 4°C before subsequent processing in an anaerobic chamber under anoxic conditions (10%H_2, 0% O_2, 0% N_2). A total of 200 g of faeces from the donations provided was placed in a large stomacher bag in an anaerobic chamber. An equal volume of reduced 50 mM phosphate buffer with 0.05% (w/v) cysteine hydrochloride (Sigma Aldrich, Ireland) was added and the resulting faecal slurry homogenised and sieved. The filtered slurry was then centrifuged at 4000 rpm for 25 min in a Sorvall 3000 centrifuge and re-suspended in 50 mM phosphate buffer under anaerobic conditions. The resulting suspension was adjusted with sterile glycerol to a final concentration of 25% and stored at -80°C. Prior to use the samples were defrosted at 37°C before inoculation into the fermentation vessels.

4.3.7 Distal colon model

Faecal medium was prepared as previously described (Fooks and Gibson 2003). The medium (185 mL) was autoclaved and added to each of the required Multifors fermentation vessels (Infors, UK). The pH of the medium was adjusted to 6.8 and sparged with oxygen-free N_2 for at least 2 hrs to ensure anaerobic conditions were established. Fermentations were performed over a 24 hr period at 37°C. Conditions within each vessel were maintained at pH 6.8 by the automatic addition of 2.5 M

NaOH or 1 M HCL, sparged with oxygen-free N_2 and stirred continuously at 100 rpm. At the beginning of each fermentation, individual vessels (triplicate runs) were inoculated with either the rifampicin resistant bacteriocin-producing *S. salivarius* DPC6988 or non-producing *S. salivarius* HSISS4. Additionally, the bacteriocin-sensitive indicator *C. ramosum* DSM1402 was added to all vessels. Control vessels contained the indicator organism in the absence of either *S. salivarius* strains. Overnight cultures of rifampicin resistant *S. salivarius* strains were washed twice in maximum recovery diluent (MRD, Oxoid) and adjusted to the correct concentration prior to inoculation of the fermentation vessels. Samples were withdrawn from each fermentation vessel at time points 0, 6, 24 hrs (T0, T6, T24 respectively). Viable counts of *S. salivarius* strains from one run were enumerated on BHI agar plates containing 100 μg/mL rifampicin.

4.3.8 Culture-based analysis

One mL of sample from each fermentation vessel at the time points T0, T6 and T24 was serially diluted in MRD (Oxoid) and appropriate dilutions were subsequently plated. Enumeration of rifampicin resistant *S. salivarius* strains, representing one run, was performed by plating on BHI agar containing 100 μg/mL rifampicin. Following overnight incubation at 37°C, colonies were enumerated and the log cfu/ml calculated for both strains at each time point.

4.3.9 Preparation of DNA for high-throughput sequencing

One mL of sample from each time point was taken for DNA extraction. Samples were withdrawn after the addition of *C. ramosum* and *S. salivarius* to vessels and added to a cryovial (Starstedt, Wexford, Ireland) containing a zirconia/silica bead mix (Stratech Scientific, UK) and transferred to -80°C until required. Total

metagenomic DNA was extracted using a modified protocol which combined a bead beating step with the QIAmp Fast DNA Stool Mini Kit (Qiagen, Crawley, UK) as described previously (Fouhy, Deane et al. 2015). Quantification of DNA was performed using the Quibit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Using the 16S metagenomic sequencing library protocol, 16S rRNA amplification and MiSeq sequencing of V3-V4 variable region of the 16S rRNA gene was amplified from 27 faecal extracts (Illumina, San Diego, CA, USA) as described previously (Fouhy, Deane et al. 2015). Samples were sequenced on the MiSeq sequencing platform, using the 2 x 250 cycle V2 kit, in the Teagasc sequencing facility following standard Illumina sequencing protocols.

4.3.10 Bioinformatic analysis

FLASH (fast length adjustment of short reads to improve genomic assemblies) was used to merge paired-end reads. Quality filtering (quality score > 19) followed by the removal of mismatched barcodes and sequences below length threshold of joined reads was achieved using QIIME (version 1.8) (Caporaso, Kuczynski et al. 2010). USEARCH x7-64bit was used to perform de-noising and chimera detection as well as clustering into operational taxonomic units (OTU's) (Edgar 2010). OTU's were aligned using PyNAST (python nearest alignment space termination) (Caporaso, Kuczynski et al. 2010) and assignment of taxonomy was performed using BLAST against the SILVA SSURef database release 111. As a result of using a faecal standard, taxonomic profiles are represented as the mean of triplicate vessels (with the exception of HSISS4 T6, which is the average of two runs). Alpha and beta diversities, calculated based on weighted and unweighted UniFrac distances matrices, were generated using QIIME. Visualisation of principal coordinate analysis

(PCoA) plots was achieved using EMPeror v0.9.3-dev. As the sample number was too low, statistical analysis could not be performed.

4.3.11 Quantitative PCR (qPCR)

Total bacterial numbers were determined using absolute quantification by qPCR using the Roche LightCycler 480 II platform. To quantify total 16S bacterial counts, a standard curve was created using 10^{10} to 10^{3} copies of 16S rRNA/µL. Amplification of samples was performed using the forward primer F1 (5'ACTCCTACGGGAGGCAGCAG) and the reverse primer R1 (5'ATTACCGCGGCTGCTG) and KAPA Lightcycler 480 mix (KAPA Biosystems Ltd., Bedford Row, London, UK) according to manufacturer instructions. All samples, negative controls (where template DNA was replaced with PCR-grade water) and standards were run in triplicate. Colony forming units (CFU) were calculated from the copy number results from each qPCR run using the previously described formula (Quigley, McCarthy et al. 2013). Statistical analysis was performed using PASW Statistics 18 on triplicate runs using the Independent-Samples T-test. Significance was established as $p<0.05$. Absolute quantification of *C. ramosum* numbers was attempted using previously published primers for the *C. ramosum* subgroup (sg-Cram171-F; GACACTGCATGGTGACC and sg-Cram626-R; GGTTTCTATGGCTTACTG) (Matsuki 2007). gDNA from *C. ramosum* was used to create a standard curve. qPCR conditions were as follows; pre-incubation, 95°C for 3 mins, amplification consisting of 45 cycles at 95°C for 10 sec, 58°C for 20 sec, 72°C for 15 sec, melting curve at 95°C for 5 sec and 63°C for 1 min, 97°C continuously and a final cooling at 40°C for 10 sec. All samples, negative controls

(using gDNA from *Clostridium bolteae* DSM15670, or where template DNA was replaced with water) and standards were run in triplicate.

4.4 Results

4.4.1 Antibiotic resistance in *S. salivarius* DPC6988 is below cut-off values

As a result of its probiotic potential, and subsequent examination into its impact on microbial populations, the qualified presumption of safety (QPS) status of this strain was investigated by assessing the antibiotic susceptibility of *S. salivarius* DPC6988 using VetMIC Lact-1 and Lact-2 plates for 16 antibiotics. Microbiological cut-off values, set out by the European Food Safety Authority (EFSA), were used to determine resistance. As cut-off values have not been determined by EFSA for *S. salivarius*, for the purposes of comparison cut-off values for *S. thermophilus* were used. MIC's for each antibiotic is presented in Table 1. These results establish that *S. salivarius* DPC6988 is below the microbiological cut-off values for *S. thermophilus* for all of the antibiotics tested, i.e., ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol.

4.4.2 Minimum inhibitory concentration determination reveals specific activity of salivaricin A2 against *C. ramosum* and *C. symbiosum*

MIC assays were performed to investigate the concentration at which the purified salivaricin A2 peptide prevents the growth of a number of target strains. Indicators were selected based on previous inhibition in a deferred antagonism assay. The minimum concentration of salivaricin A2 required to inhibit the growth of *C. ramosum* was 20-40 μM. The concentration required to prevent the growth of *C. symbiosum* was 2.5-5 μM, while *L. bulgaricus* was 0.05 μM.

4.4.3 Survival of DPC6988 in a distal colon model

A distal colon model was employed to assess the impact of DPC6988 or a non-bacteriocin-producing control, *S. salivarius* HSISS4, relative to a non *S. salivarius*-containing control, on a representative gut microbial population, including *C. ramosum* which was spiked into the gut sample. Rifampicin resistant derivatives of the *S. salivarius* strains were generated to facilitate their culture based detection and, following their inoculation, samples were taken at T0, T6 and T24 and plated. Following the addition of DPC6988 at 7.8 log CFU/mL at T0, cells grew to 8.9 log CFU/mL by T6. Similarly, HSISS4 was added at 7.8 log CFU/mL at T0 and cells grew to 8.5 log CFU/mL by T6. Both DPC6988 and HSISS4 cell numbers fell to below detectable levels at T24.

4.4.4 Impact of *S. salivarius* on gut microbial populations

To determine if the introduction of *S. salivarius* DPC6988 and *S. salivarius* HSISS4 to the model colon environment had an impact on total bacterial numbers, total 16S rRNA levels at T0, T6 and T24 hrs were determined by qPCR (Figure 1). It was established that the addition of the *S. salivarius* strains did not significantly impact on total bacterial numbers at T0 and T24 relative to the control, with the exception of T6 within the DPC6988 and HSISS4-containing vessels which had significantly lower total bacterial numbers compared to the control. The average counts across all vessels were 8.08 log - 8.31 log CFU/mL for T0, 8.37 log - 8.87 log CFU/mL for T6 and 7.75 log - 8.36 log CFU/mL for T24. Attempts to quantify *C. ramosum* levels, through use of the sg-Cram171-F; GACACTGCATGGTGACC/sg-Cram626-R; GGTTTCTATGGCTTACTG primer pair (Matsuki 2007), were unsuccessful as it was found that the primers lacked sufficient specificity as evidenced by the

generation of amplicons when a *C. bolteae* negative control was employed (data not shown). *C. bolteae* was not previously used as a control when detecting *C. ramosum* numbers (Tana, Umesaki et al. 2010).

16S rRNA amplicon sequencing was performed to determine the composition of the bacterial communities at T0, T6 and T24. Samples were withdrawn at T0 after the addition of *C. ramosum* and *S. salivarius* to vessels. At T0 and T6 the composition of all vessels at phylum level was relatively similar (Supplementary figure 1). By T24 both test vessels had a lower abundance of Actinobacteria (DPC6988; 4.1%, HSISS4; 15.2%) and a higher abundance of Firmicutes (DPC6988; 88.9%, HSISS4; 74.9%) relative to the control (Actinobacteria; 30.4%, Firmicutes; 64.3%) (Supplementary Figure 1). Within this data it is also notable that at T24 the DPC6988 treated samples had lower proportions of Actinobacteria and a higher abundance of Firmicutes than those containing HSISS4 (Supplementary Figure 1).

At family level, at T0, the composition of each vessel was similar (Figure 2). However, it was apparent that the abundance of *Streptococcaceae* was greater in the DPC6988 and HSISS4 vessels than the control. Additionally, at T6 and T24 both test vessels had a greater proportion of *Streptococcaceae* when compared to the control vessel, and, at each time-point the abundance of this family was almost identical (DPC6988; T0=10.3%, T6=45.9%, T24=16.1%, HSISS4; T0=11.4%, T6=46.6%, T24=16.1%) (Figure 2). At T6 both test vessels had a lower abundance of *Clostridiaceae* 1, *Lachnospiraceae* and *Erysipelotrichaceae* and increased proportions of *Nocardiaceae* when compared to the control vessel, while at T24 both the DPC6988 and HSISS4 treated communities had reduced abundances of *Bifiobacteriaceae*, *Coriobacteriaceae*, *Clostridiaceae* 1, *Lachnospiraceae* and *Erysipelotrichaceae*, and a greater abundance of *Veillonellaceae* relative to the

control. Despite a relatively similar bacterial composition in the DPC6988 and HS1SS4 treated vessels at each time-point, DPC6988 treated vessels had a lower abundance of *Bifidobacteriaceae* (T6, T24), *Erysiplelotrichaceae* (T6, T24), *Clostridiaceae* 1 (T24) and a higher abundance of *Bacteroidaceae* (T6) when compared to HSISS4. Both vessels were dominated by *Veillonellaceae* at T24, however the proportion of reads assigned to this family was greater in DPC6988 (DPC6988; 55.7%, HSISS4; 28.1%) (Figure 2).

The compositional changes at genus level were reflective of those at family level. At all time-points a higher abundance of *Streptococcus* was observed in the test vessels when compared to the control vessel (Figure 3). This is particularly evident at T6 where this genus dominated (DPC6988 45.9% and HSISS4 46.6%). Both DPC6988 and HSISS4 vessels had lower proportions of *Bifidobacterium*, *Clostridium sensu stricto* 1, *Faecalibacterium*, an uncultured genus of the *Erysipelotrichaceae* family and a greater abundance of *Rhodococcus* at T6 when compared to the control vessel, with minor differences among other genera represented. At T24, the proportions *Bifidobacterium*, *Clostridium sensu stricto* 1 and an uncultured genus of the *Erysipelotrichaceae* family were lower in the DPC6988 and HSISS4 vessels, while both test vessels had a higher abundance of an uncultured genus of the *Veillonellaceae* family relative to the control (Figure 3). The overall composition between the two test vessels was relatively similar at T0 and T6. However, by T24, the changes to the composition were more noticeable. DPC6988 had a lower abundance of *Bifidobacterium* (T6, T24), an uncultured genus of the *Erysipelotrichaceae* family (T6, T24) and *Clostridium sensu stricto* 1 (T24) relative to the HSISS4 treated vessel. Although an uncultured genus of the *Veillonellaceae* family dominated both test vessels at T24, the abundance of this genus was higher in

the DPC6988 vessel (55.1%) when compared to the non-bacteriocin-producing strain used (27.6%) (Figure 3).

4.4.5 Impact of *S. salivarius* on intestinal microbial diversity

The impact of the introduction of *S. salivarius* on alpha and beta diversity in gut populations was also examined. Alpha diversity was determined for all samples using the Chao1 richness estimate, the Simpson's diversity index, the Shannon index and by estimating the number of observed species (Table 2). At T0, all values were relatively similar between vessels across all diversity measures with some minor differences observed (Table 2). At T6, the values for the Simpson's diversity index and Shannon index were lower for both treatment vessels when compared to the control vessel. However, the DPC6988 test vessel had a higher Chao1 value and number of observed species when compared to both the HSISS4 and control vessels (Table 2). By T24, all diversity values were lower in the DPC6988 and HSISS4 vessels when compared to the control. Additionally, it is evident that the DPC6988 vessel had lower Simpson and Shannon values when compared to the HSISS4 vessel (Table 2).

To visualise the impact on beta diversity, PCoA plots were created based on weighted UniFrac distance matrices (Figure 4). It is evident at T0 that data points representing each sample cluster together. All data points from each vessel also cluster together at the T6 time point. However by T24, the DPC6988 and HSISS4 test vessels cluster away from one another and from the control group, data points for which are co-located with the corresponding T6 samples.

4.5 Discussion

The ability of some bacteriocins to modulate the gut microbiota by specifically targeting undesirable bacterial components is an attractive trait. Additionally, it has been suggested that such bacteriocins could be employed to promote improved metabolic health and may serve as an alternative therapeutic treatment for obesity and other syndromes and diseases (Murphy, Cotter et al. 2012). Indeed, it was previously demonstrated by Rea *et al*. that the bacteriocin thuricin CD was effective at inhibiting *Clostridium difficile* in a model of the distal colon without impacting the resident populations (Rea, Dobson et al. 2011). Guinane *et al*. also recently established that bactofencin A had a positive, albeit subtle, effect on gut populations (Guinane, Lawton et al. 2016).

The aim of this study was to investigate the impact of the bacteriocin-producing *S. salivarius* DPC6988 on gut microbial populations by use of a simulated distal colon model. Such models can be used to assess if the producing strain survives and is functional within the complex colon environment. The impact of a number of bacteriocin-producing strains of *S. salivarius* has been studied within the oral cavity (Wescombe, Hale et al. 2012). However, it is not clear how such strains contribute to functionality within the GI tract or how they influence gut microbial composition. To our knowledge, this is the first report investigating the impact of a *S. salivarius* strain on gut populations.

Due to the probiotic potential of *S. salivarius* DPC6988, the resistance of this strain to a number of clinical antibiotics was first assessed using the VetMIC system. A qualified presumption of safety (QPS) approach was introduced by the European Food Safety Authority (EFSA) as a way of assessing microorganisms deliberately

introduced into the food chain. This approach was developed to ensure that only strains that lacked transferrable resistance determinants would be employed (Panel 2012). As no microbiological cut-off values for the antibiotics tested are currently in place for *S. salivarius*, cut-off values for the phylogenetically similar *S. thermophilus* (Kawamura, Hou et al. 1995) were used. In all cases *S. salivarius* DPC6988 was below these cut-off values.

In an attempt to determine if DPC6988 actively produces salivaricin A2 in a model of the distal colon, the bacteriocin sensitive indicator *C. ramosum* DSM1402 was added to each vessel. Despite efforts to determine the numbers of DSM1402, lack of primer specificity prevented accurate determination of *C. ramosum* numbers specifically. However, 16S rRNA-based analysis was employed to assess the impact on the gut microbiota in general. It is apparent from the taxonomic analysis that, regardless of whether a bacteriocin was produced or not, the introduction of *S. salivarius* strains (DPC6988 and HSISS4) had an impact on the *ex vivo* gut microbial composition. Although both treated vessels were similar with regard to a number of important populations that were altered such as *Bifidobacterium*, *Faecalibacterium*, *Clostridium sensu stricto* 1 and uncultured genera of the *Erysipelotrichaceae* and *Veillonellaceae* families when compared to the control, the extent to which these populations were altered differed between the DPC6988 and HSISS4 vessels. Both treated vessels differed considerably with respect to the control vessel, not least because of the dominance of *Streptococcus* populations, which is particularly evident at T6 and can be attributed to the introduction of *S. salivarius*. An increase in *Lactobacillus* populations was also observed by Guinane *et al*. following inoculation with *Lactobacillus* strains in a model of the distal colon (Guinane, Lawton et al. 2016). Despite the decrease in *Bifidobacterium* populations, which have been well

documented for their role as beneficial microbes (Picard, Fioramonti et al. 2005), the alterations of populations such as *Clostridium* spp. and uncultured members of *Erysipelotrichaceae* suggest a positive overall impact on the gut microbiota with respect to obesity and metabolic health. An increase in several *Clostridium* spp. has been observed in patients with type 2 diabetes (Qin, Li et al. 2012, Karlsson, Tremaroli et al. 2013), while an increase in Erysipelotrichi, a class within the Firmicutes, was observed in mice fed a Western diet (Turnbaugh, Bäckhed et al. 2008, Turnbaugh, Ridaura et al. 2009) suggesting a role for these bacteria in obesity development. Indeed *C. ramosum*, which was shown to promote obesity in a gnotobiotic mouse model (Woting, Pfeiffer et al. 2014), is a member of the Erysipelotrichi. Both test vessels, particularly the DPC6988-treated vessel, contained high proportions of uncultured members of *Veillonellaceae* relative to the control at T24. The increase in these populations is not surprising as it has been established that *Veillonella* are capable of utilizing substrates such as lactate produced by Gram-positive facultative anaerobes such as streptococci as an energy source (Mikx and Van der Hoeven 1975, Zoetendal, Raes et al. 2012).

DPC6988 was previously demonstrated to inhibit *C. ramosum* DSM1402 (*Erysipelotrichaceae* – Cluster XVIII (Rajilić-Stojanović and de Vos 2014)) and to a lesser extent *C. symbiosum* DSM934 (a member of *Clostridium* cluster XIVa (Van den Abbeele, Belzer et al. 2013)) in a deferred antagonism assay (Thesis Chapter 3). As bacteriocin production can be affected by choice of growth medium and various environmental conditions (Guinane, Piper et al. 2015, Turgis, Vu et al. 2016), as well as altered rates of diffusion through the overlaid agar, MIC assays were performed using purified salivaricin A2 to determine the minimum concentration at which visible growth is prevented. This inhibition of *Clostridium* spp. by means of a

bacteriocin, as demonstrated using various assays, may account for the greater decrease in abundance of *Clostridum sensu stricto* 1 (Cluster 1 (Gupta and Gao 2009)) and an uncultured *Erysipelotrichaceae* genus when compared to the HSISS4 vessel.

Microbial diversity was also examined to determine the impact of the bacteriocin-producing *S. salivarius* DPC6988 on species richness and diversity in the model colon. It is evident that a decrease in diversity was observed over time following treatment with DPC6988 and HSISS4 when compared to the control. This is most likely due to the relative dominance of *Streptococcus* and uncultured *Veillonellaceae*. Despite an initial decrease in diversity in the control vessel, most likely due to the relative increase in *Clostridium sensu stricto* 1, diversity remained relatively stable by T24 with some minor fluctuations across the diversity measures. The weighted UniFrac distance matrix, as demonstrated using a PCoA plot, was used to investigate beta diversity. It is apparent that at T0 all samples cluster together. This establishes that the use of a faecal standard was effective at providing a baseline microbiota to enable comparisons across vessels. Despite the dominance of *Streptococcus* at T6, all vessels clustered together at this time point. However, by T24 it is evident that samples begin to cluster separately, with the T24 control data points clustering by all of the T6 time points. Again, this implies that populations within the control vessel remained relatively stable over time. Both test vessels also begin to cluster separately by T24. Given the similarities between the *S. salivarius* strains, it is assumed that these differences are at least partially due to the key difference between the strains, i.e., bacteriocin production.

In conclusion, this study represents, to our knowledge, the first example investigating the impact of a bacteriocin-producing *S. salivarius* on gut microbial

populations. Regardless of whether a bacteriocin was produced or not, the introduction of *S. salivarius* strains had an effect on populations. However, a number of desirable changes were attributed to DPC6988 only, suggesting that bacteriocin production by the strain can lead to specific modulation of the gut microbiota and further investigations to determine if the strain can bring about similar changes *in vivo*, and potentially impact on obesity and metabolic health, are merited.

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Table 1 Antibiotic resistance of *S. salivarius* DPC6988

Antibiotic	**MIC (μg/mL)**
Gentamicin	4
Kanamycin	32
Streptomycin	16
Neomycin	16
Tetracycline	1
Erythromycin	2
Clindamycin	0.06
Chloramphenicol	2
Ampicillin	0.5
Penicillin	1
Vancomycin	1
Quinupristin-dalfopristin	1
Linezolid	1
Trimethoprim	8
Ciprofloxacin	2
Rifampicin	0.25

Table 2 Estimates of alpha diversity for fermentation vessels at each time point

Sample	Chao1 richness estimate	Simpson's diversity	Shannon index	Number of observed species
Control T0	492.71 ± 44.46	0.97 ± 0.01	6.42 ± 0.27	460 ± 64
Control T6	406.68 ± 16.32	0.91 ± 0.03	4.82 ± 0.30	370 ± 7
Control T24	387.48 ± 27.79	0.93 ± 0.03	4.89 ± 0.35	330 ± 42
DPC6988 T0	512.08 ± 14.25	0.97 ± 0.00	6.27 ± 0.04	487 ± 10
DPC6988 T6	437.02 ± 30.07	0.77 ± 0.02	3.88 ± 0.14	390 ± 25
DPC6988 T24	331.55 ± 73.39	0.65 ± 0.14	2.99 ± 0.69	286 ± 54
HSISS4 T0	494.32 ± 19.85	0.96 ± 0.01	6.18 ± 0.24	458 ± 30
HSISS4 T6	383.92 ± 10.41	0.74 ± 0.13	3.54 ± 0.68	339 ± 0
HSISS4 T24	339.31 ± 66.01	0.82 ± 0.17	4.15 ± 1.42	303 ± 62

Figure 1 Total bacterial numbers (log CFU/mL) as determined by 16S rRNA qPCR analysis for each fermentation vessel at T0, T6 and T24 hrs

; Control, ; DPC6988, ; HSISS4.

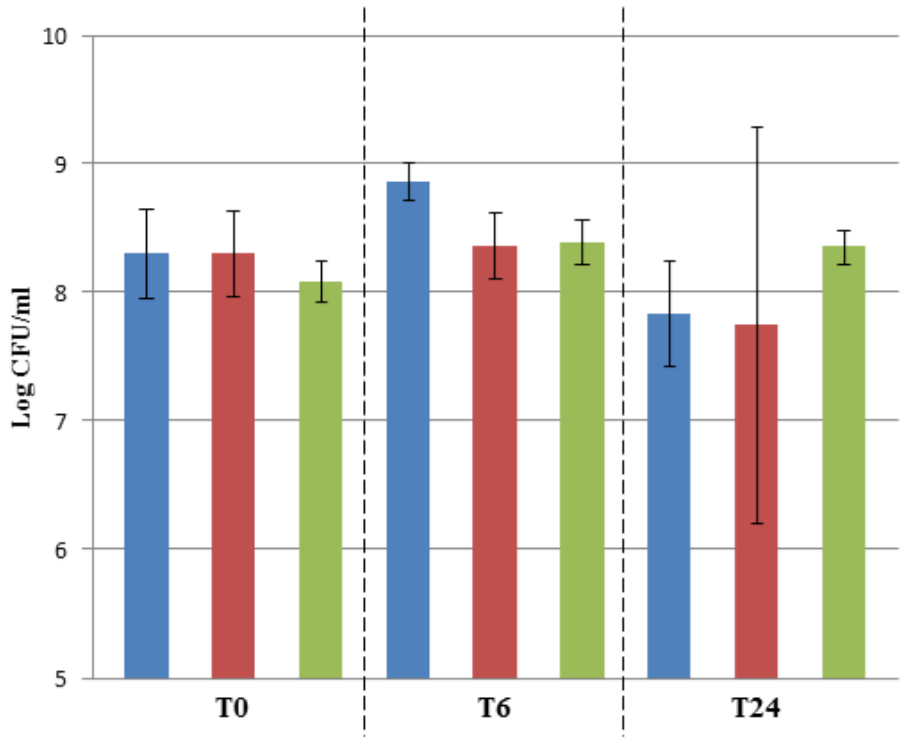


Figure 2 Relative abundances at family level for the control, DPC6988 and HSISS4 treated vessels at T0, T6 and T24 (represented as the mean of triplicate runs, HSISS4 T6 is the average of two runs)

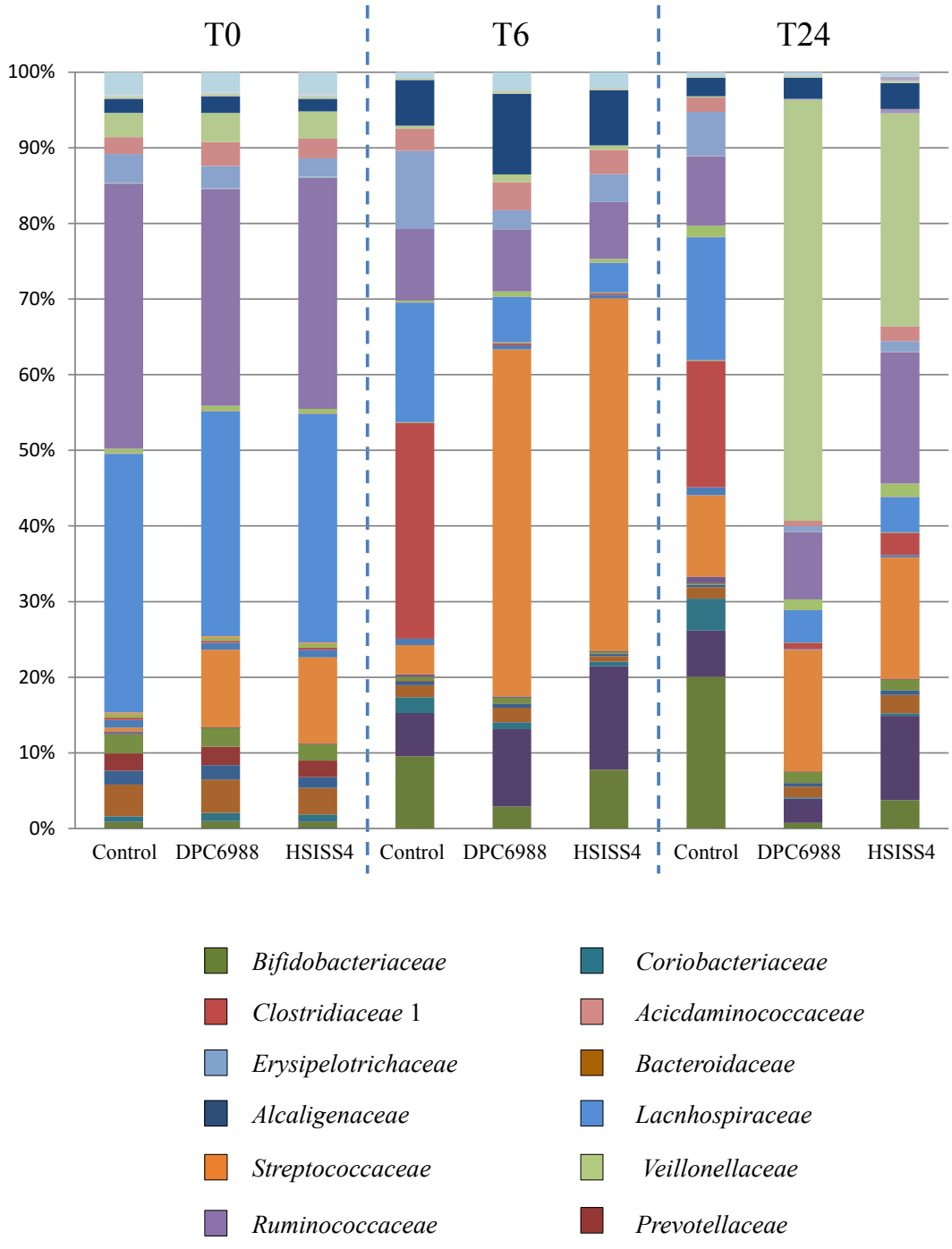


Figure 3 Relative abundances at genus level for the control, DPC6988 and HSISS4 treated vessels at T0, T6 and T24 (represented as the mean of triplicate runs, HSISS4 T6 is the average of two runs)

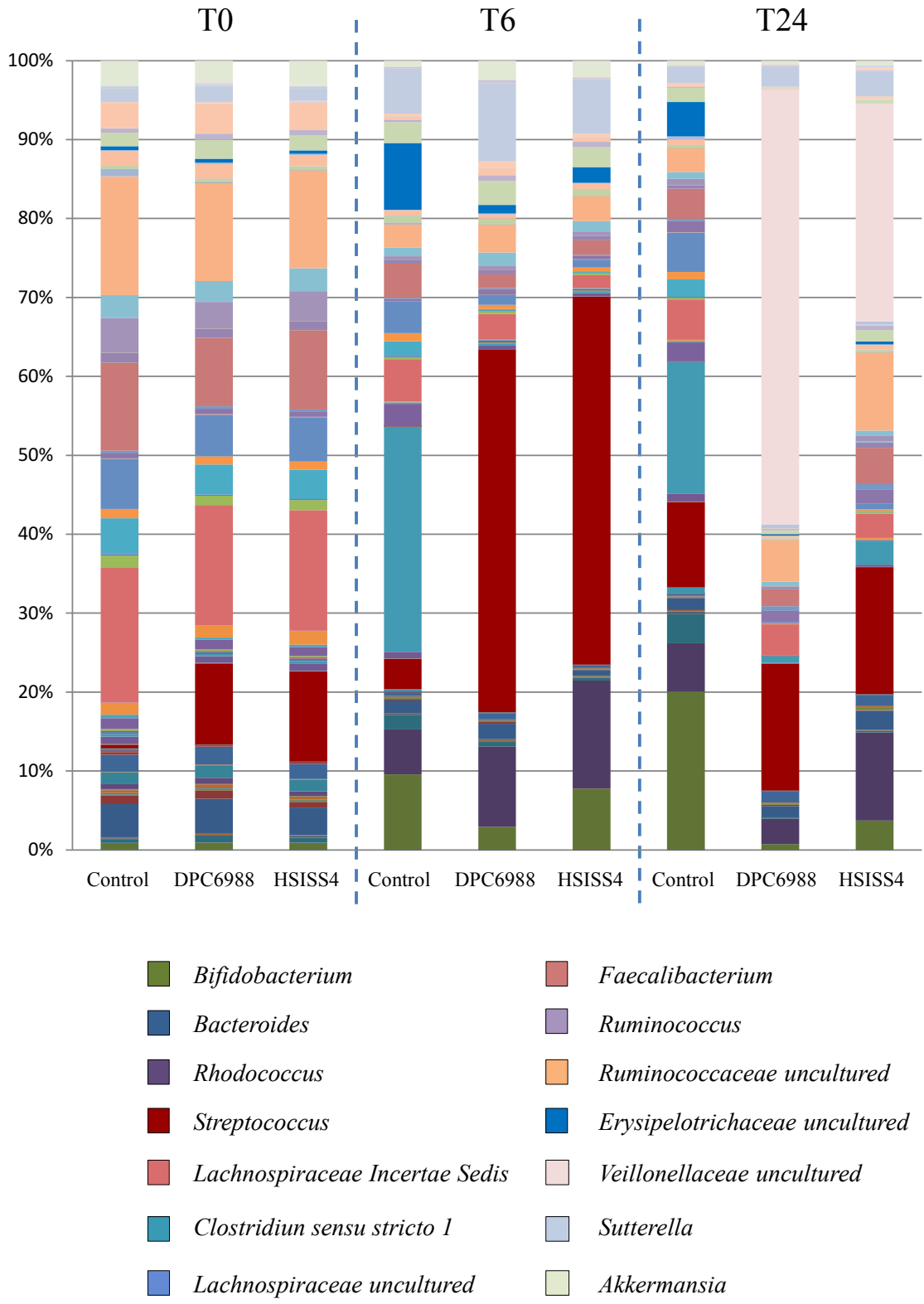
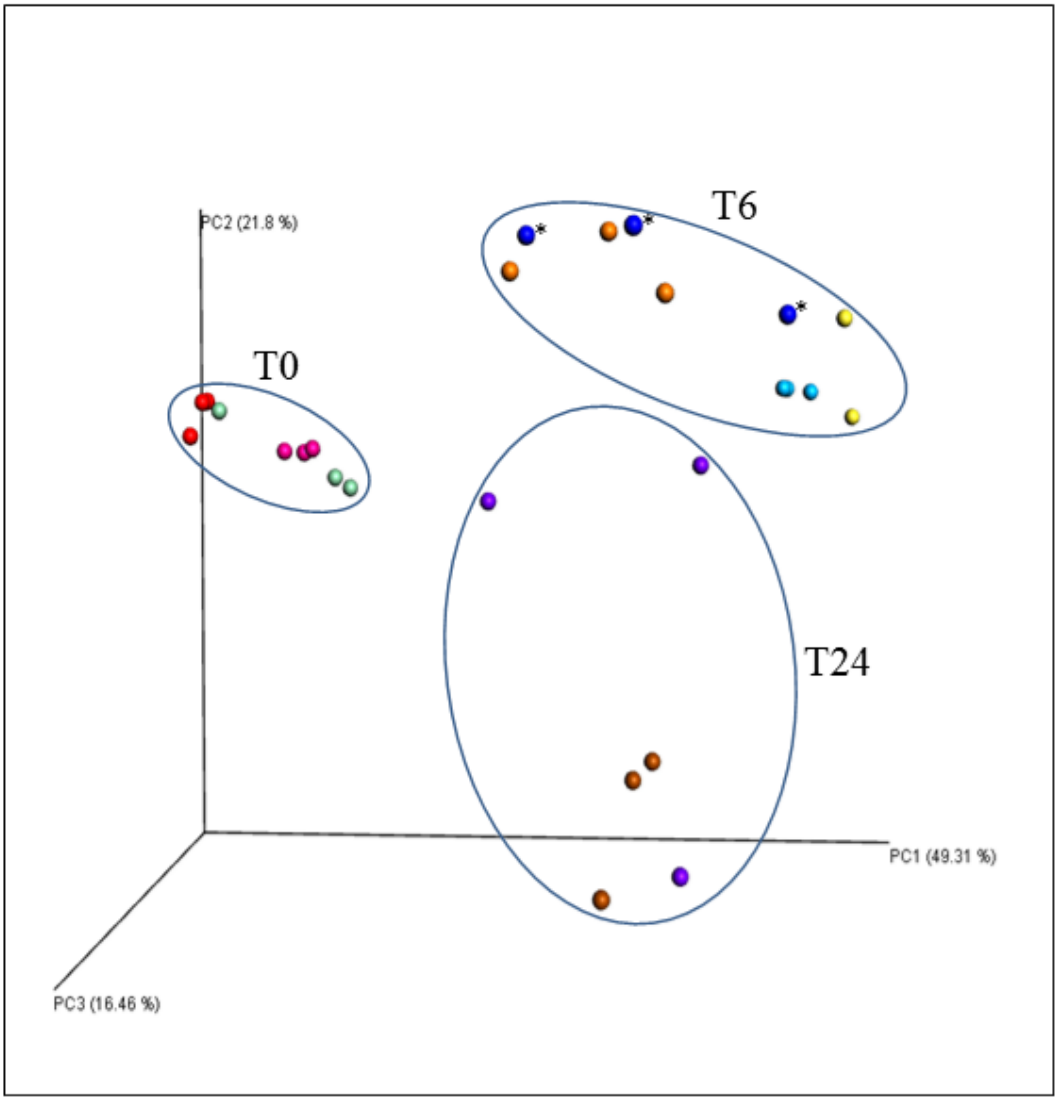
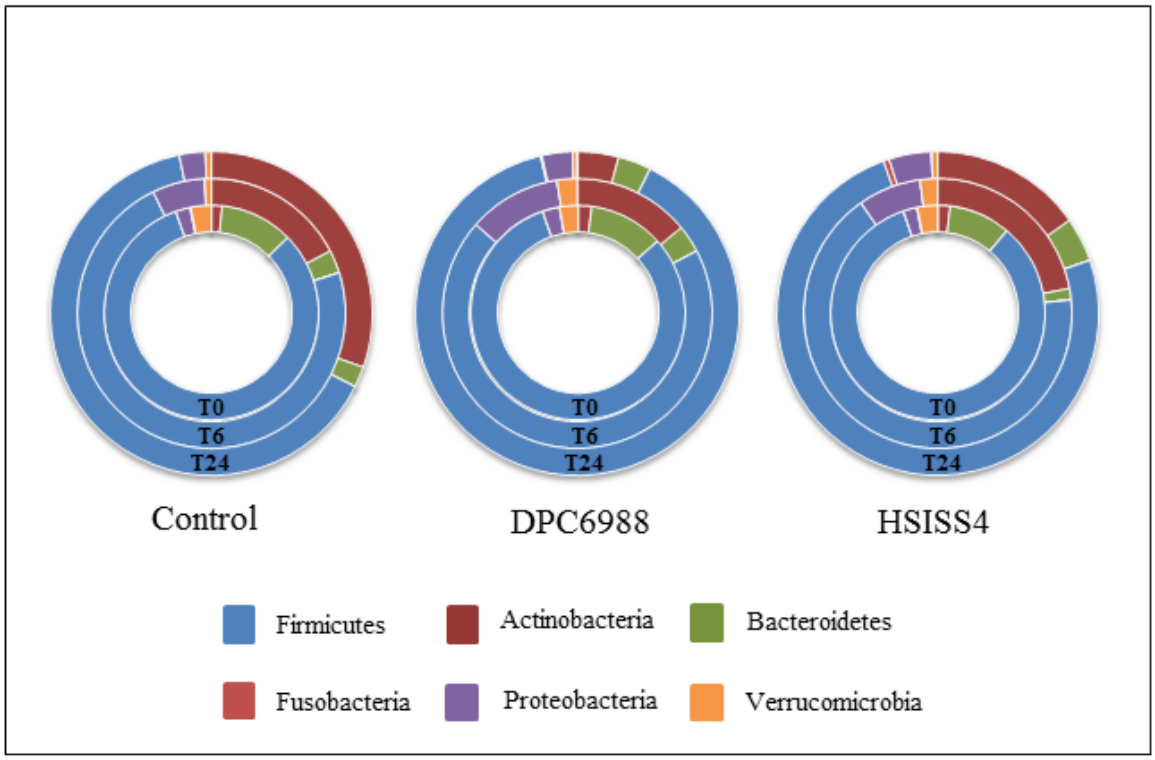


Figure 4 PCoA analysis based on weighted unifrac distances matrices at T0, T6 and T24 for each fermentation vessel. ●; Control T0, ●; DPC6988 T0, ●; HSISS4 T0, ●; Control T6, ●; DPC6988 T6, ●; HSISS4 T6, ●; Control T24, ●; DPC6988, ●; HSISS4 T24. *; denotes control T24 samples which cluster within the T6 ellipse.



Supplementary figure 1 Relative abundances at phylum level for the control, DPC6988 and HSISS4 treated vessels at T0, T6 and T24 (represented as the mean of triplicate runs, HSISS4 T6 is the average of two runs)



Chapter 5

To investigate the effects of the bacteriocin-producing *S. salivarius* DPC6988 on the metabolic abnormalities associated with obesity in DIO mouse model and its effect on the composition of the gut microbiota as a potential driver of these abnormalities

5.1 Abstract

Bacteriocin-producing probiotics may prove effective as alternative treatments for obesity and related metabolic disorders through manipulation of the gut microbiota. Targeting obesity-linked species may lead to an improved intestinal balance and in turn gastrointestinal health. As such, the aim of this study was to examine the effect of *Streptococcus salivarius* DPC6988, a salivaricin A2-producing strain of gut origin, on the metabolic abnormalities in a DIO mouse model, and its impact on the gut microbiota as a potential driver of these abnormalities. Such models are, to an extent, reflective of the changes observed in obese patients, and can be used to demonstrate if a probiotic could be used to mitigate the symptoms of metabolic syndrome. C57BL/6 mice were fed either a LFD or HFD for 12 weeks followed by an 8 week intervention period, consisting of either the bacteriocin-producing *S. salivarius* DPC6988 or non-producing equivalent *S. salivarius* HSISS4. Despite alterations to the gut microbiota, *S. salivarius* DPC6988 administration did not confer any improvements to weight gain or overall metabolic health, with the exception of lower triglyceride levels when compared to HFD controls. Although *S. salivarius* DPC6988 previously demonstrated inhibitory activity against the obesity-promoting *C. ramosum* DSM1402, it is not known if *C. ramosum* is naturally present in the gut microbiota of DIO mice, or if perhaps targeting a single obesity-promoting species alone is not sufficient to result in an improvement. Further studies with murine models of *C. ramosum* induced obesity or human studies with individuals with high levels of *C. ramosum* will be required to more accurately determine the merits of using DPC6988 as a strain as part of anti-obesity interventions.

5.2 Introduction

Obesity is a complex syndrome, with a number of serious implications for human health such as cardiovascular disease and type 2 diabetes, and is associated with an overall imbalance of energy intake and expenditure. In recent years the role of the gut microbiota in obesity and related metabolic disorders has received considerable attention (Turnbaugh, Bäckhed et al. 2008, Qin, Li et al. 2012, Karlsson, Tremaroli et al. 2013, Le Chatelier, Nielsen et al. 2013) and, initially, the gut microbiota of genetically obese mice was associated with an increase in the phylum Firmicutes and a decrease in the phylum Bacteroidetes (Ley, Bäckhed et al. 2005, Turnbaugh, Ley et al. 2006). However, conflicting evidence exists in human studies with regard to the key populations involved (Schwiertz, Taras et al. 2010). Nonetheless, more recent research has specifically highlighted key populations that may play a part in obesity and related metabolic disorders. Indeed, other studies have more specifically established the role of a particular microbe in promoting obesity. Fei and Zhao demonstrated that *Enterobacter clocae* B29, an endotoxin producer, induced obesity and insulin resistance in germfree mice (Fei and Zhao 2013). Furthermore, *Clostridium ramosum*, a species previously shown to be enriched in patients with type 2 diabetes, promoted obesity in a gnotobiotic mouse model, possibly through a mechanism where the upregulation of small intestinal glucose and fat transporters contributed to increased body fat deposition (Woting, Pfeiffer et al. 2014). As such, the gut microbiota represents a realistic target for the treatment of obesity and related metabolic disorders, through the removal of obesity-promoting microbes.

A number of alternative therapeutic treatments to current strategies to treat obesity such as probiotics, prebiotics and antibiotics have the potential to positively

influence the gut microbiota and in turn host metabolic health. Murphy *et al*. examined the strategy of using antimicrobials, the bacteriocin-producing probiotic *Lactobacillus salivarius* UCC118 and the antibiotic vancomycin, to impact on the metabolic abnormalities associated with obesity in a diet-induced-obesity (DIO) mouse model *via* modulation of the microbiota as a potential driver of these abnormalities (Murphy, Cotter et al. 2012). Both antimicrobials altered the gut microbiota but in distinct ways, and, although reductions in weight gain were evident among mice that received the probiotic, these effects were short-lived and only treatment with vancomycin resulted in an improvement in the metabolic abnormalities associated with obesity (Murphy, Cotter et al. 2012). Despite this, this important proof-of-concept study does indeed demonstrate that antimicrobials can be used to alter the microbiota with respect to obesity, although the choice and specificity of action is critical.

We previously reported the isolation of the bacteriocin-producing *Streptococcus salivarius* DPC6988 (Thesis Chapter 3). This potential probiotic produces the bacteriocin salivaricin A2, which demonstrates inhibitory activity against *Clostridium* spp. enriched in T2D patients, one of which includes the aforementioned *C. ramosum* DSM1402, an obesity-promoting species. The impact of DPC6988 on gut microbial populations in a simulated model of the distal colon was also examined, and, relative to the control and non-bacteriocin-producing strain used, displayed decreased proportions of *Clostridium* spp. and uncultured *Erysipelotrichaceae*, i.e. taxa that have been associated with obesity (Thesis Chapter 4).

The aim of this study was to evaluate the effects of *S. salivarius* DPC6988 on body weight and metabolic health in a DIO mouse model when compared to a non-

producing control strain. Such models can be used to establish if a probiotic could be used to alleviate the symptoms of metabolic syndrome, as the changes observed in DIO mice are to some degree reflective of the changes observed in obese patients (Vickers, Jackson et al. 2011). Animals used in DIO models display increased body weight over time characterised primarily by an observed increase in fat mass. While they do not typically develop diabetes, they do present symptoms including insulin resistance, glucose intolerance and elevated plasma leptin when compared with controls on a standard diet. The impact of *S. salivarius* DPC6988 on obesity associated metabolic abnormalities and gut microbial composition is examined here. Using this approach it was established that *S. salivarius* administration did not result in improvements to metabolic health despite alterations to the gut microbial composition, and further highlights the need to more specifically target obesity-promoting components.

5.3 Material and Methods

5.3.1 Animals

Male C57BL/6 mice, 3-4 weeks old, were acquired from Harlan Laboratories (Netherlands) and quarantined for one week followed by acclimatisation for a further week. Animals were housed under a controlled environment with a temperature of 23±2°C, humidity 50±20%, 15-20 fresh air changes per hour and a light/dark cycle of 12 hrs at Syngene International Ltd. Experimental protocols were conducted in compliance with the Institutional Animal Ethics Committee (IAEC) Protocol No: Syngene/IAEC/625/06-2015.

5.3.2 Experimental design

To evaluate the impact of the bacteriocin-producing *S. salivarius* DPC6988, and non-producing control strain, *S. salivarius* HSISS4, on metabolic abnormalities and gut microbial composition in a DIO mouse model, six week old male C57BL/J6 mice (10 per group) were fed either a low-fat diet (LFD; control; 10% calories from fat; RD12450B), a high-fat diet (HFD; 45% calories from fat; RD12451) for a period of 20 weeks or a high-fat diet for 20 weeks supplemented with an 8-week intervention period of *S. salivarius* administration, from week 12 (T0) to week 20 (T8) weeks. *S. salivarius* administration was achieved through resuspension of the freeze-dried powder once daily in the animal's drinking water. Water consumption was recorded each day.

5.3.3 *S. salivarius* production

Preparation of *S. salivarius* strains for freeze-drying was performed as previously described (Murphy, Cotter et al. 2012). Briefly, *S. salivarius* cultures were grown

overnight at 37°C in BHI broth (Merck, Darmstedt, Germany). Cells were harvested by centrifugation and washed twice with PBS (Sigma-Aldrich, St. Louis, MO, USA) before being resuspended in 10% w/v trehalose (Sigma) and freeze-dried. Vials containing the freeze-dried *S. salivarius* strains were stored at -20°C until required and powders were resuspended in water for administration to the mice. To determine the approximate numbers of *S. salivarius* present in each vial, the log cfu/ml was calculated for each strain after one week storage at -20°C. Counts for *S. salivarius* DPC6988 ranged from 9.8-10 log CFU/mL, while *S. salivarius* HSISS4 ranged from 9.6-10 log CFU/mL.

5.3.4 Parameters assessed

Body weight and food consumption were assessed once every three days following the initiation of *S. salivarius* administration. To determine body fat and lean mass composition mice were subjected to Echo MRI (Model no.700) at T0, 4 and 8 of the intervention. At the end of the study, mice were euthanized and internal organs (liver, spleen, duodenum, jejunum, ileum and caecum) and fat pads (epididymal, renal, mesenteric, subcutaneous and brown) were collected, weighed and stored at -80°C.

5.3.5 Preparation of DNA for high throughput sequencing

Faecal pellets were collected from each mouse for DNA extraction at T0, T4, and T8 of the intervention and stored at -80°C. Total metagenomic DNA was extracted from faecal pellets and caecal contents using the QIAmp Fast DNA Stool Mini Kit as per the protocol (Qiagen, Crawley, UK). Quantification of DNA was performed using the Quibit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Using the 16S metagenomic sequencing library protocol, 16S rRNA amplification and MiSeq

sequencing of V3-V4 variable region of the 16S rRNA gene was amplified from 120 faecal extracts and 40 caeca (Illumina, San Diego, CA, USA) as described previously (Doyle, Gleeson et al. 2016). Samples were sequenced on the MiSeq sequencing platform, using a 2 x 250 cycle V2 kit, in the Teagasc sequencing facility following standard Illumina sequencing protocols.

5.3.6 Bioinformatic analysis

FLASH (fast length adjustment of short reads to improve genomic assemblies) was used to merger paired-end reads. Quality filtering (quality score > 19) followed by the removal of mismatched barcodes and sequences below length threshold of joined reads was achieved using QIIME (version 1.9.1) (Caporaso, Kuczynski et al. 2010). USEARCH v7 (64bit) was used to perform de-noising and chimera detection as well as clustering into operational taxonomic units (OTU's) (Edgar 2010). OTU's were aligned using PyNAST (python nearest alignment space termination) (Caporaso, Kuczynski et al. 2010) and assignment of taxonomy was performed using BLAST against the SILVA SSURef database release 123. Alpha and beta diversities, calculated based on weighted and unweighted UniFrac distances matrices, were generated using QIIME. Visualisation of principal coordinate analysis (PCoA) plots was achieved using EMPeror v0.9.3-dev.

5.3.7 Metabolic markers

To determine random blood glucose and plasma insulin measurements, blood samples were collected from each group using the retro-orbital puncture (ROP) method once every 2 weeks during *S. salivarius* administration. Blood glucose was determined using a glucometer (One touch Ultra 2; Johnson & Johnson) and plasma insulin measured by an ELISA kit (Merck Millipore). To enable plasma analysis,

animals were fasted for approximately 6 hours and blood was collected using the ROP method. The plasma sample from each mouse was analysed for total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), free fatty acids (FFA) and high density lipoprotein (HDL).

5.3.8 Statistical analysis

To determine if statistically significant differences occurred, non-parametric analysis was performed using the Mann Whitney U and Kruskal-Wallis tests using the R statistical software package (Version 1.0.44). Data are represented as mean values with their SEM unless otherwise stated. Statistical significance was accepted as $p<0.05$.

5.3.9 Quantitative PCR (qPCR)

Total bacterial numbers (16S rRNA copies per gram of wet stool) were determined at T0 and T8 using absolute quantification by qPCR using the Roche LightCycler 480 II platform. Samples from three mice were used as an initial check to determine bacterial numbers. To quantify total 16S bacterial numbers, a standard curve was created using 10^{10} to 10^{3} (faecal) or 10^{9} to 10^{4} (caecal) copies of 16S rRNA/µl. Amplification of samples was performed using the forward primer F1 (5'ACTCCTACGGGAGGCAGCAG) and the reverse primer R1 (5'ATTACCGCGGCTGCTG) and KAPA LightCycler 480 mix (KAPA Biosystems Ltd., Bedford Row, London, UK) according to manufacturer instructions. All samples, negative controls (where template DNA was replaced with PCR-grade water) and standards were run in triplicate. Copies of 16S rRNA/g of wet stool were calculated using a previously described method (Zhang, DiBaise et al. 2009).

Absolute quantification of *S. salivarius* numbers was performed using *S. salivarius* specific primers, S. sal GtfP-F; CTGCATCACGTTCCAAGATATC (this study) and S. sal GtfP-R; GCGATGAGCCAAGCTGAAG (Srinivasan, Gertz Jr et al. 2012). gDNA from *S. salivarius* was used to create a standard curve. qPCR conditions were as follows; pre-incubation at 95°C for 3 mins, amplification consisting of 40 cycles at 95°C for 10 sec, 58°C for 20 sec, 72°C for 15 sec, melting curve at 95°C for 5 sec, 63°C for 1 min, 97°C continuously and a final cooling at 40°C for 10 sec. All samples, negative controls (using gDNA from *S. agalactiae* DPC7040 and *S. mutans* DPC7039, or where template DNA was replaced with water) and standards were run in triplicate.

5.4 Results

5.4.1 The effect of *S. salivarius* administration on weight gain

It is evident that mice fed a high-fat diet for a period of 20 weeks gained significantly more body weight when compared to controls on a LFD. The increase in body weight of DIO mice compared to lean controls (Day 139 HFD; 47.3±0.9g vs LFD; 34±0.8g; $p<0.001$) (Fig 1) is mostly attributed to an increase in fat mass by week 20 (18.1±0.6 vs 8.0±0.4g; $p<0.001$) (Fig 2). At all time-points investigated neither *S. salivarius* treatment had a significant effect on bodyweight when compared to controls on a HFD alone (Fig 1). Additionally, echo MRI results at T0, T4 and T8 of the intervention revealed that neither treatment had a significant effect on fat mass when compared to DIO mice (Fig 2). At all time-points investigated no significant decrease in body weight or fat mass was observed in mice receiving the bacteriocin-producing DPC6988 when compared to the non-producing strain HSISS4 (Fig 1 and 2).

5.4.2 Impact of *S. salivarius* treatment on markers of metabolic health

The impact of *S. salivarius* DPC6988 and *S. salivarius* HSISS4 on the metabolic abnormalities associated with a DIO model were examined at different times during the intervention period. At all time-points investigated, neither *S. salivarius* treatment resulted in an improvement in blood glucose levels or plasma insulin levels when compared to DIO mice on a HFD (Supplementary Fig 1). However, it was noted that triglyceride (TG) levels in DPC6988 treated mice, but not HSISS4 treated mice, were significantly lower ($p<0.05$) when compared to HFD controls. Neither *S. salivarius* treatment resulted in improvements in the levels of TC, HDL,

LDL and FFA when compared to controls, (Supplementary Fig 2 and 3). For all parameters tested no significant differences were observed in mice receiving the bacteriocin-producing strain DPC6988 when compared to the non-producing strain HSISS4 (Supplementary Fig 1, 2 and 3).

5.4.3 Microbial diversity

To investigate the impact of HFD feeding and *S. salivarius* treatment on overall microbial diversity in the DIO mouse model, alpha and beta diversities were investigated. Alpha diversity was determined for all groups using the Chao1 richness estimate, the Simpson's diversity index, the Shannon index and by estimating the number of observed species at T4 and T8 of the intervention (Table 1). At both time-points investigated, HFD feeding had no significant effect on the faecal or caecal microbiota for each of the alpha diversity measures when compared to LFD controls. To investigate the impact of the bacteriocin-producing strain on microbial diversity, DPC6988 treated mice were compared with DIO mice receiving HSISS4. At T4, the Shannon index ($p<0.05$) was significantly lower in the DPC6988 treated group, while the number of observed species ($p<0.05$) was significantly higher at T8 when compared to mice receiving HSISS4. Microbial diversity was also examined between these two groups for the caecal microbiota. Chao1 values ($p<0.01$) and the number of observed species ($p<0.05$) were lower in DIO mice receiving the bacteriocin-producing strain relative to mice receiving HSISS4. Microbial diversity between the HFD DIO control group and mice receiving DPC6988 was also investigated. At T4 and T8 of the intervention no significant differences were observed between alpha diversity measures. Within the caecum, Chao1 values ($p<0.001$) and the number of observed species ($p<0.001$) were significantly higher in mice treated with the bacteriocin-producing strain (Table 1) relative to HFD controls.

To investigate the impact on beta diversity, PCoA plots were created based on weighted UniFrac distance matrices (Fig 3). This determines if treatment groups and time-points cluster together. At the beginning of the intervention all groups cluster together regardless of diet (Figure 3a). At T4, all data points, irrespective of diet or *S. salivarius* intervention, appear to shift slightly in the same direction (Fig 3b). However by T8, with the exception of some outliers, all data points tend to be more similar to that of T0 and a further shift in diversity was not evident (Fig 3c). It is also apparent that, at all time-points investigated, the faecal and caecal microbiota cluster separately (Fig 3d).

5.4.4 The effect of *S. salivarius* administration on gut composition

DIO mice received *S. salivarius* treatment for a period of 8 weeks consisting of either the bacteriocin-producing *S. salivarius* DPC6988 or non-bacteriocin-producing equivalent *S. salivarius* HSISS4. To determine the impact of the bacteriocin-producing strain relative to the non-producing control on the faecal microbiota composition, both treatment groups were compared at T4 and T8 weeks. T0 was included to establish a baseline but results were not compared. At phylum level DPC6988 treated mice had a lower proportion of Tenericutes (T4 $p<0.01$, T8 $p<0.05$) and Deferribacteres (T4 $p<0.05$) when compared to mice receiving HSISS4. No significant differences in the abundances of the dominant phyla were observed between the two treatment groups at each time-point (Supplementary Fig 4). The number of significant families at each time-point was examined (Supplementary Fig 5 and 9). Populations previously demonstrated to be altered in obesity or type 2 diabetes, or, which are susceptible to the action of salivaricin A2, are highlighted below. DIO mice receiving DPC6988 were associated with a lower proportion of *Bacteroidaceae* (T4 $p<0.001$, T8 $p<0.01$), uncultured *Bacteroidales* S24-7 (T4

p<0.05), *Ruminococcaceae* (T4 p<0.05) and *Enterococcaceae* (T4 p<0.01, T8 p<0.05) relative to mice receiving HSISS4. *Lactobacilliaceae* (T4 p<0.05), *Clostridiaceae* 1 (T4 p<0.01) and *Rikenellaceae* (T8 p<0.01) were significantly greater in DPC6988 treated mice (Supplementary Fig 5 and 9). The numbers of significant genera altered were investigated (Fig 4 and 5). DPC6988 treated mice were associated with lower *Bacteroides* (T4 p<0.001, T8 p<0.01), an uncultured bacterium of *Bacteroidales* S24-7 (T4 p<0.05), *Parabacteroides* (T4 p<0.01, T8 p<0.01), *Ruminococcaecae Incertae Sedis* (T4 p<0.05) and an uncultured genus of the *Erysipleotrichaceae* family (T4 p<0.01, T8 p<0.01), and greater abundances of *Alistipes* (T8 p<0.05), *Lactobacillus* (T4 p<0.05), *Ruminoccocus* (T8 p<0.01), *Rikenellaceae* RC9 (T8 p<0.01) and *Subdoligranulum* (T8 p<0.01) relative to HSISS4 treated mice. *Streptococcus* was significantly lower in the DPC6988 treated group at T8 when compared to mice receiving HSISS4 (p<0.05) (Fig 4 and 5).

The composition of the caecal microbiota of *S. salivarius* treated mice was examined to investigate the impact of the bacteriocin-producting strain on microbial composition. At phylum level DPC6988 treated mice were associated with a lower abundance of Tenericutes (p<0.05) and Cyanobacteria (p<0.05) (Supplementary Fig 6). Again, populations previously shown to be altered in an obese or type 2 diabetic environment, or, which are susceptible to salivaricin A2 are highlighted below. *Bacteroidaceae* were lower (p<0.01) in mice receiving the bacteriocin-producing strain (Supplementary Fig 7 and 10). At genus level, an uncultured genus of the *Erysipelotrichaceae* family (p<0.05), *Bacteroides* (p<0.01), *Parabacteroides* (p<0.01) and *Ruminococcaceae Incertae Sedis* (p<0.05) were lower in DPC6988 treated mice, while *Clostridium sensu stricto* 1 (p<0.05), *Odoribacter* (p<0.01) and

Subdoligranulum ($p<0.05$) were greater when compared to mice receiving HSISS4 (Supplementary Fig 8 and 11).

5.4.5 The effect of the bacteriocin-producing *S. salivarius* DPC6988 relative to HFD DIO controls

To examine the impact of the bacteriocin-producing strain on the composition of the faecal microbiota with respect to DIO mice receiving a HFD alone, both groups were compared at T4 and T8 of the intervention period. No significant differences were detected between phyla at T4 of the intervention. DPC6988 treated mice had a greater abundance of Candidate division TM7 at T8 ($p<0.001$) and no significant change was detected in the abundance of Firmicutes or Bacteroidetes at each time-point (Supplementary Fig 4). The number of significant families altered at each time-point was examined (Supplementary Fig 5 and 12). DIO mice receiving *S. salivarius* treatment were associated with a greater abundance of *Bacteroidaceae* (T8 $p<0.05$), *Bacteroidales* S24-7 (T8 $p<0.01$), *Enterobacteriaceae* (T8 $p<0.01$), *Prevotellaceae* (T8 $p<0.05$) and lower proportions of *Clostridiaceae* 1 (T4 $p<0.001$) relative to HFD controls (Supplementary Fig 5 and 12). The numbers of significant genera altered between the two groups were examined (Fig 4 and 6). At genus level, DIO mice receiving *S. salivarius* administration were associated with a greater abundance of *Streptococcus* (T4 $p<0.01$, T8 $p<0.01$), *Enterobacter* (T8 $p<0.01$), *Ruminococcus* (T8 $p<0.05$), *Bacteroides* (T8 $p<0.05$) and an uncultured bacterium of *Bacteroidales* S24-7 (T8 $p<0.01$) and lower abundances of *Turicibacter* (T4 $p<0.05$, T8 $p<0.05$), *Clostridium sensu stricto* 1 (T4 $p<0.001$), *Prevotella* (T4 $p<0.05$), *Ruminococcaecae Incertae Sedis* (T4 $p<0.05$), and *Lactococcus* (T8 $p<0.05$) (Fig 4 and 6) when compared to HFD controls.

Within the caecum, Actinobacteria ($p<0.05$) was significantly lower in mice receiving *S. salivarius* administration (Supplementary Fig 6). *Bifidobacteriaceae* was lower in mice receiving DPC6988 administration ($p<0.05$) (Supplementary Fig 7 and 13). At genus level, *Bifidobacterium* ($p<0.05$), an uncultured genus of the *Lachnospiraceae* family ($p<0.05$) and *Turicibacter* ($p<0.01$) were significantly lower in mice receiving *S. salivarius* treatment, while *Lactococus* ($p<0.05$) and *Subdoligranulum* ($p<0.05$) was significantly greater when compared to HFD controls (Supplementary Fig 8 and 14).

5.4.6 *S. salivarius* administration does not impact total bacterial numbers

Absolute quantification was performed to determine if treatment with *S. salivarius* DPC6988 or *S. salivarius* HSISS4 had an impact on total bacterial numbers within the faecal environment at T0 and T8, or caecum during the intervention. It was established that treatment with either *S. salivarius* strain did not significantly impact total numbers at T0 or T8 within the faecal environment or caecum relative to HFD controls (Supplementary table 2). Additionally no significant differences were observed between mice receiving DPC6988 when compared to mice treated with HSISS4 (Table 2).

Attempts to accurately quantify *S. salivarius* numbers using the previously described primer pair were unsuccessful due background amplification and a lack of primer specificity as evidenced by the generation of amplicons when a *S. mutans* negative control was employed (Supplementary Fig 15). However an increase in absolute quantification concentration at T4 and T8 is apparent in mice receiving *S. salivarius* treatment when compared to HFD controls (Supplementary table 2)

5.5 Discussion

This study builds upon an investigation by Murphy *et al*. (Murphy, Cotter et al. 2012) to examine the effects of the potential probiotic *S. salivarius* DPC6988, a salivaricin A2 producer, on the metabolic abnormalities associated with obesity in a DIO mouse model, and its effect on the composition of the gut microbiota as a potential driver of these abnormalities. We previously reported the ability of this strain to inhibit the obesity-promoting *C. ramosum* DSM1402 (Thesis chapter 2) and observed decreased abundances of an uncultured genus of the *Ersyipelotrichaceae* family and *Clostridium sensu stricto* 1 in a model of the distal colon using a faecal fermentation system when compared to controls (Thesis chapter 3). This strain therefore represented a potential alternative therapeutic treatment to current strategies to treat obesity including weight-loss surgery that could be used to target obesity-promoting microbes in a DIO mouse model.

Bacteriocins may play a significant role in determining the composition of the microbiota and, in turn, health. In this study, alterations to the gut microbial composition of DIO mice due to administration of a bacteriocin-producing strain did not lead to any improvements in weight gain or overall metabolic health in general, but intriguingly, reduced levels of triglycerides relative to DIO controls were observed. As salivaricin A2 has previously been shown to demonstrate inhibitory activity against obesity-promoting species such as *C. ramosum*, it is possible that such benefits may only be apparent in instances where levels of *C. ramosum* are high.

Although HFD feeding has previously been shown to reduce microbial diversity (Turnbaugh, Bäckhed et al. 2008, Murphy, Cotter et al. 2012), it was evident from

the alpha diversity measures that the HFD had no significant impact on diversity, possibly due to only relatively minor alterations in the composition (not shown). DPC6988 treatment was characterised by a relatively minor impact on diversity within the faecal microbiota, and therefore, a longer treatment period may be needed to observe a more noticeable effect. A similar result was observed in a study by Park *et al*., which examined the effect of the putative probiotic *Lactobacillus plantarum* HAC01 with respect to fat mass, immunometabolic biomarkers and dysbiosis in a DIO mouse model (Park, Ji et al. 2016). This investigation used microbial ribosomal RNA rather than gDNA to analyse the gut microbiota to investigate active populations, and, despite receiving HAC01 orally for 8 weeks, a clear distinction between treatment groups was not observed for the alpha diversity measures examined (Park, Ji et al. 2016). In the present study however, the effect on diversity was more noticeable within the caecum, with a decrease relative to HSISS4 and increase when compared to DIO controls, and may be due to differences between sites.

It is also apparent from the beta diversity that, regardless of diet or treatment, all groups tended to cluster together at all time-points, and implies that HFD feeding or *S. salivarius* administration did not dramatically affect the microbiota. Although it was evident from the compositional analysis that differences were observed in DPC6988 treated mice when compared to HFD DIO controls or HSISS4 treated mice, beta diversity results may indicate that diet was a factor in driving changes and a higher dosage of the bacteriocin-producing strain may be needed to observe a more noticeable effect. It is also worth noting that samples from all groups separated by site i.e. the faecal and caecal microbiota clustered separately. This is not surprising as the caecal microbiota has previously been shown to differ from the faecal

microbiota with respect to abundance of populations present (Marteau, Pochart et al. 2001) and highlights the importance of examining different sites during intervention studies.

To determine if the bacteriocin-producing *S. salivarius* DPC6988 had an effect on the gut microbiota, the composition of mice receiving the bacteriocin-producing strain was compared to mice receiving a non-producing equivalent. It is evident that, regardless of bacteriocin production, the introduction of *S. salivarius* strains had an effect on microbial composition, and, at both time-points examined, it is also apparent that a number of significant differences exist between DPC6988 and HSISS4 at a taxonomic level. During the intervention, populations previously shown to be altered in an obese or type 2 diabetic environment were examined to determine if any alterations to these taxa by *S. salivarius* DPC6988 resulted in an improved metabolic state. However, as previously mentioned any alterations to such populations did not result in improvements to metabolic health. Despite obesity being associated with lower levels of *Bacteroidetes* (Turnbaugh, Ley et al. 2006), no apparent differences in this phylum were evident in the treated groups, however, during the *S. salivarius* intervention DPC6988 treated mice had lower proportions of *Bacteroides* and *Bacteroidales* S24-7. Certain members of the *Bacteroides* genus have previously been demonstrated as being enriched in obesity or type 2 diabetes (Qin, Li et al. 2012) and may represent a possible target in the treatment of obesity. A greater abundance of other obesity/type 2 diabetes enriched populations, including *Subdoligranulum*, *Ruminococcus* and *Rikenellaceae* (Kim, Gu et al. 2012, Qin, Li et al. 2012), in mice receiving DPC6988 administration was also observed. However, it should be noted that, although enriched, it is unknown if these populations contribute to obesity or flourish in an obese environment.

Despite the inhibition of certain species of *Lactobacillus* by salivaricin A2 (O'Shea, Gardiner et al. 2009), DPC6988 treated mice were associated with a greater abundance of *Lactobacillus* when compared to HSISS4 treated mice. Possible alterations promoting competition within the microbiota may have contributed to this greater abundance, though strain specific effects should also be taken into account. DIO mice were associated with high levels of *Lactobacillus* (LFD vs HFD not shown), and while studies have demonstrated an elevation of *Lactobacillus* populations in obese mice (Murphy, Cotter et al. 2012), obese patients (Million, Maraninchi et al. 2012), or type 2 diabetic patients (Karlsson, Tremaroli et al. 2013), Murphy *et al*. suggested that lactobacilli do not relate to the risk of obesity and that strain-specific effects should be taken into account. However, Karlsson *et al*. observed enriched levels of *Lactobacillus gasseri* in type 2 diabetic patients that correlated positively with clinical biomarkers for type 2 diabetes suggesting a possible role for certain strains of *Lactobacillus* in obesity. Nonetheless, this bloom in *Lactobacillus* was detected in the faecal microbiota only, and may be reflective of the effects of diet and treatment on gut microbiota composition at different sites, or differences in microbial composition between the two sites.

The impact of the bacteriocin-producing strain relative to DIO controls on the composition of the gut microbiota was examined. DPC6988 treated mice had increased abundances of *Bacteroides* and an uncultured bacterium of *Bacteroidales* S24-7 relative to DIO controls. However, mice receiving DPC6988 had lower proportions of these populations when compared to the non-producing equivalent, HSISS4, indicating it may have been the introduction of *S. salivarius* that had a greater effect on microbial composition and accounted for the higher levels rather than bacteriocin production. Salivaricin A2 also has a relatively narrow spectrum of

inhibition and would not be expected to have a considerable effect on a number of populations. Mice receiving the bacteriocin-producing strain also had a higher abundance of *Streptococcus* at T4 and T8 when compared to HFD control mice. This is unsurprising as a greater abundance of this genus would be expected in mice receiving treatment with of *S. salivarius*. A similar effect was observed using a model of the distal colon in which faecal fermentation vessels inoculated with *Lactobacillus salivarius* DPC6502 were marked by an increase in *Lactobacillus* (Guinane, Lawton et al. 2016), and additionally, Kwok *et al*. demonstrated a significant increase in *Lactobacillus* in humans following treatment with *L. plantarum* P-8 (Kwok, Guo et al. 2015). Absolute quantification of *S. salivarius* numbers was performed to determine if this bacterium colonised the gut of DIO mice receiving treatment during the intervention. Despite issues relating to primer specificity, a greater absolute quantification concentration was evident in mice which received *S. salivarius* treatment at T4 and T8 when compared to HFD controls, suggesting colonisation following consumption of *S. salivarius*. Further optimisation will be necessary to more accurately determine *S. salivarius* numbers.

In conclusion, this investigation builds upon previous work using *L. salivarius* UCC118 to treat obesity, in which Murphy *et al*. highlighted the importance of the target and specificity of action of the bacteriocin. Although this study began to address these important points through the use of a bacteriocin-producing strain with activity against obesity-promoting species, no improvement in body-weight or overall metabolic health was observed, with the exception of lower triglyceride levels in DPC6988 treated mice. Despite *S. salivarius* DPC6988 demonstrating inhibitory activity against *C. ramosum*, it was not known if *C. ramosum* was present naturally in the gut microbiota of DIO mice, and previous attempts to determine

numbers using a qPCR based approach were unsuccessful (Thesis Chapter 4). Therefore, either administering *C. ramosum* to DIO mice, or, the use of a model such as that used by Woting *et al.* (Woting, Pfeiffer et al. 2014), may be important steps to determine if *S. salivarius* DPC6988 would be effective in treating obesity. Additionally, it should be noted that, perhaps targeting *C. ramosum* alone may not be sufficient to significantly improve the metabolic abnormalities associated with obesity as other microbes may be involved, or a longer intervention may be needed. It is promising, however, that treatment with the potential probiotic strain DPC6988 resulted in no adverse health effects and was capable of modulating the gut microbiota. Nonetheless, more work will be necessary to determine the role of specific microbes in promoting obesity, which will further enable the selection of specific bacteriocin-producing probiotics, either as single strains or a multi-strain cocktail, which can be used to treat such disorders.

5.6 References

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Table 1 Alpha diversity measures for each group during the intervention. Data represented as the mean ± standard deviation

Time/Group	Chao1	Simpson	Shannon	Observed species
T0 LFD	665.77±77.82	0.96±0.01	5.85±0.22	614±76
T0 HFD	598.35±129.16	0.96±0.03	5.68±0.69	540±117
T0 DPC6988	769.76±51.01	0.96±0.01	5.90±0.27	723±56
T0 HSISS4	680.72±52.06	0.95±0.01	5.82±0.27	611±58
T4 LFD	647.96±154.63	0.95±0.02	5.70±0.47	593±142
T4 HFD	607.59±231.94	0.93±0.07	5.51±1.17	548±223
T4 DPC6988	657.59±163.65	0.94±0.04	5.51±0.96	591±148
T4 HSISS4	691.73±80.49	0.96±0.02	6.12±0.40	615±73
T8 LFD	710.21±77.18	0.97±0.00	6.28±0.14	610±66
T8 HFD	653.96±89.37	0.95±0.04	5.87±0.72	579±82
T8 DPC6988	713.11±144.54	0.95±0.05	5.93±0.92	626±132
T8 HSISS4	648.05±135.66	0.96±0.05	6.08±0.95	562±111
Caecum LFD	624.77±133.74	0.91±0.04	5.17±0.58	564±121
Caecum HFD	555.51±52.88	0.88±0.08	4.99±0.95	497±47
Caecum DPC6988	821.22±130.76	0.88±0.08	5.05±0.80	755±136
Caecum HSISS4	1010.35±91.71	0.90±0.05	5.24±0.55	901±76

Figure 1 Weight gain over the 8 week intervention period in LFD; —, HFD; — fed mice and DPC6988; —, HSISS4; —, treated mice. Data represented as the mean ± SEM, n = 10.

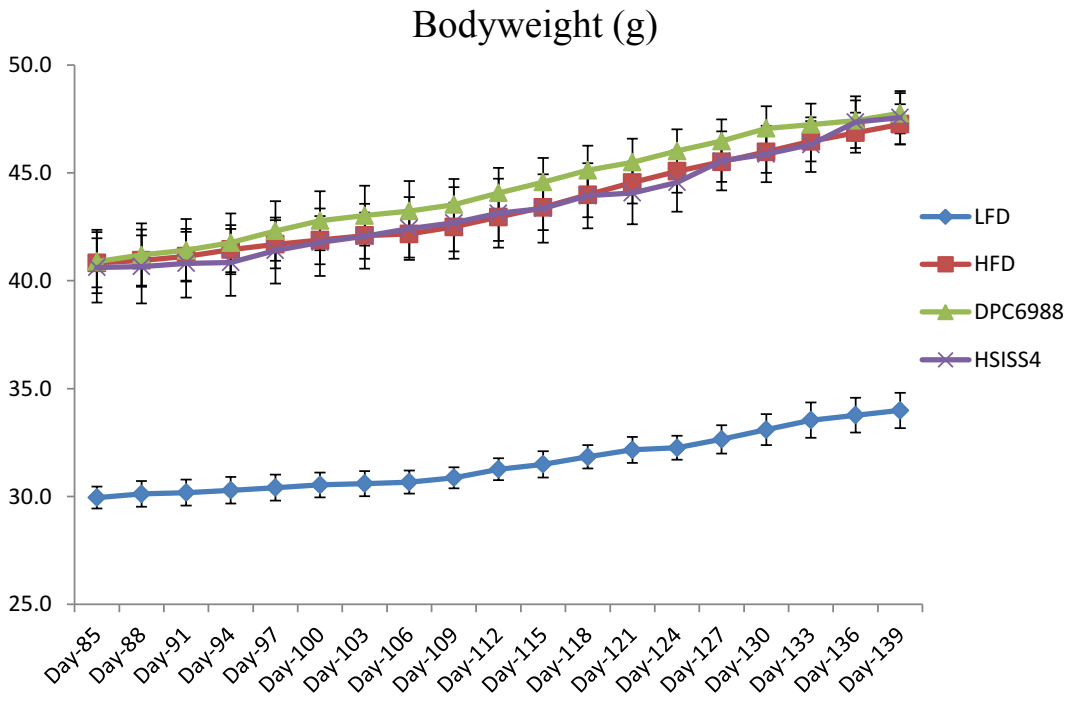


Figure 2 Assessment of fat mass composition at T0, T4 and T8 of the intervention period. LFD; , HFD; , DPC6988; , HSISS4; . Data represented as the mean ± SEM, n = 10.

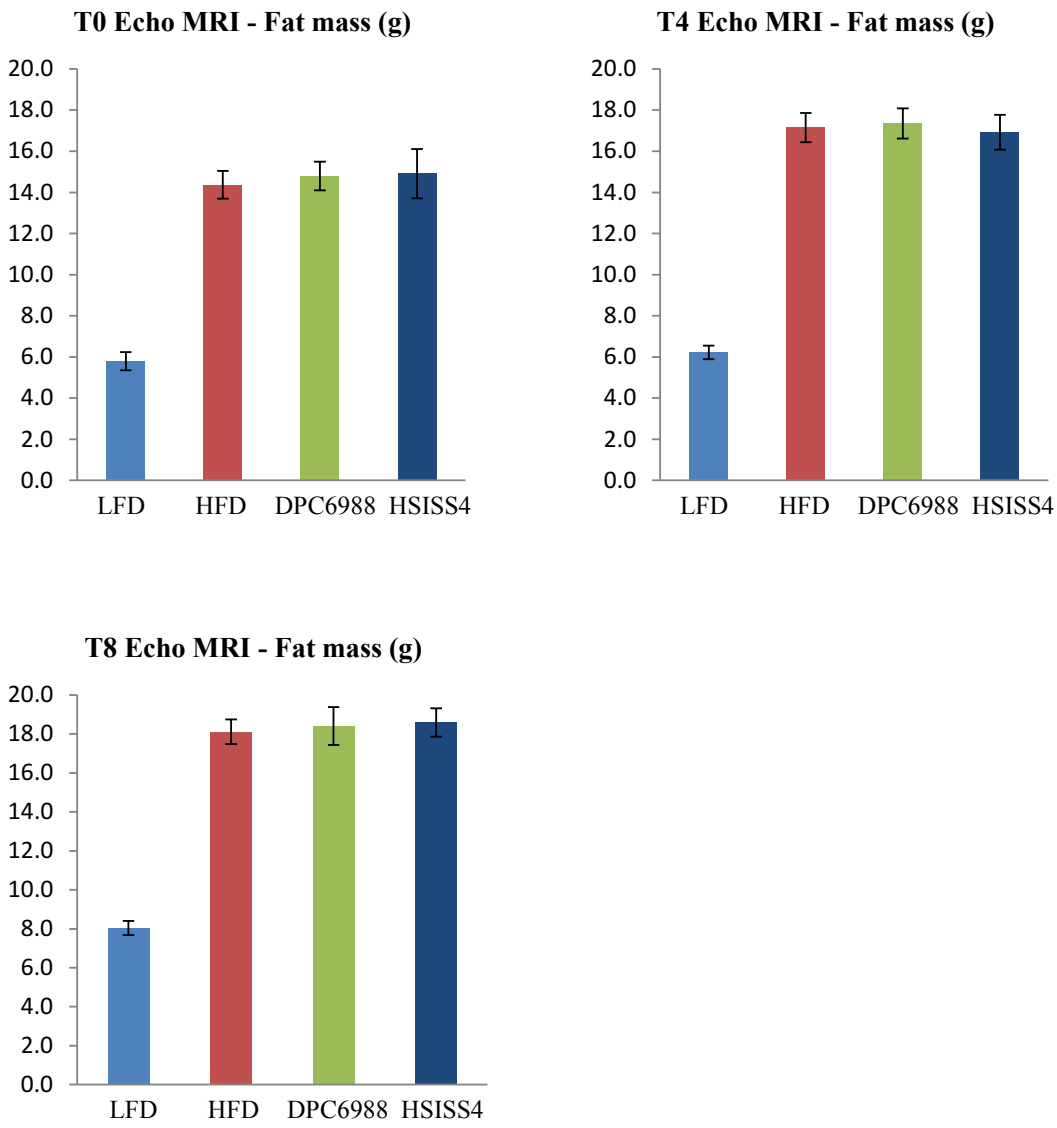


Figure 3 PCoA analysis based on weighted UniFrac distance matrices at T0; (a), T4; (b) and T8; (c) of the intervention for each group. Figure 3(d) compares the faecal (T0, T4 and T8) and caecal microbiota. Faecal microbiota LFD; ●, HFD; ●, DPC6988; ●, HSISS4; ●. Caecal microbiota LFD; ●, HFD; ●, DPC6988; ●, HSISS4; ●.

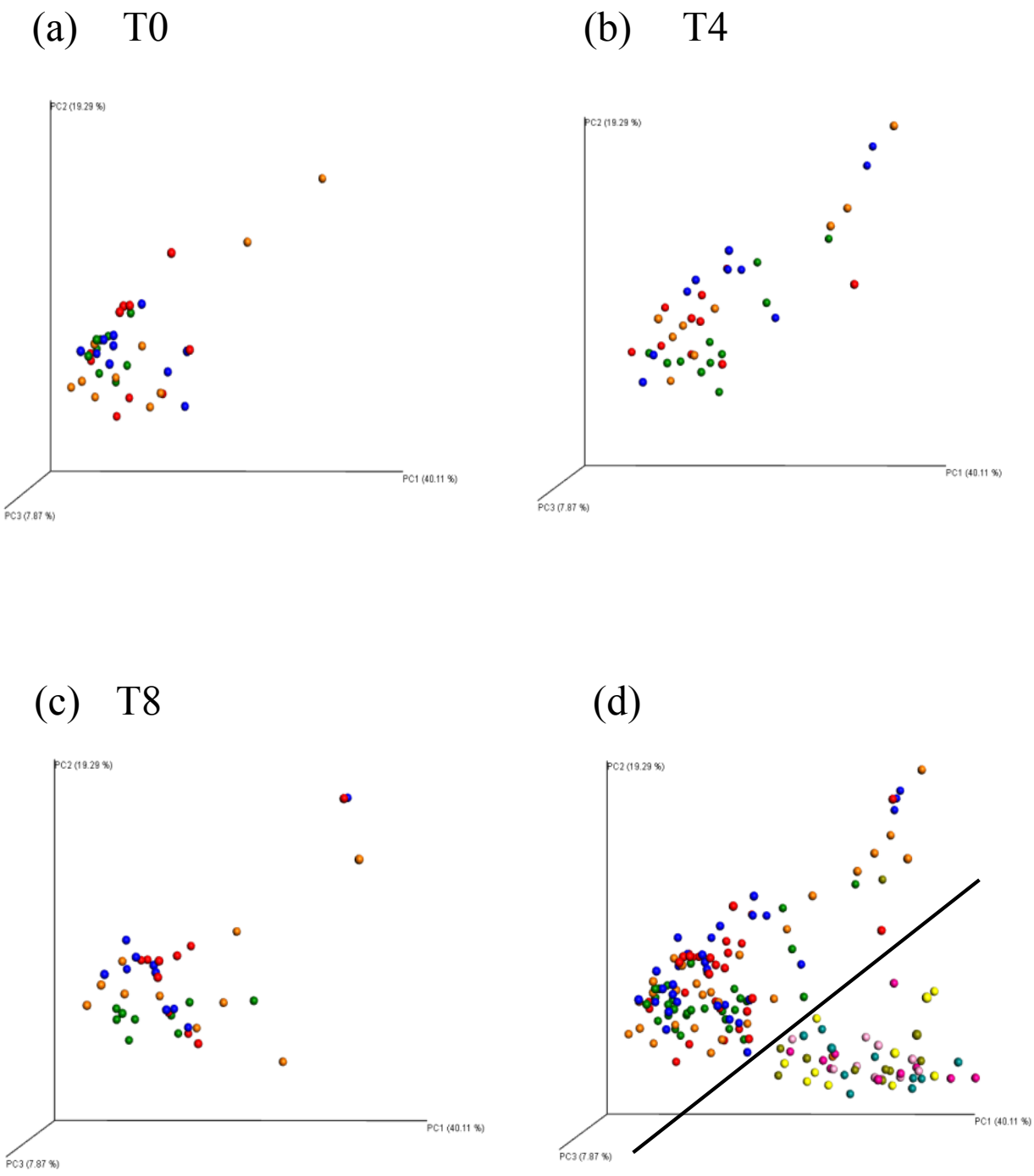


Figure 4 Relative abundances at genus level for the LFD, HFD, DPC6988 and HSISS4 groups at T0, T4 and T8 of the intervention within the faecal microbiota. Data represented as the mean number of mice for each group (n=10).

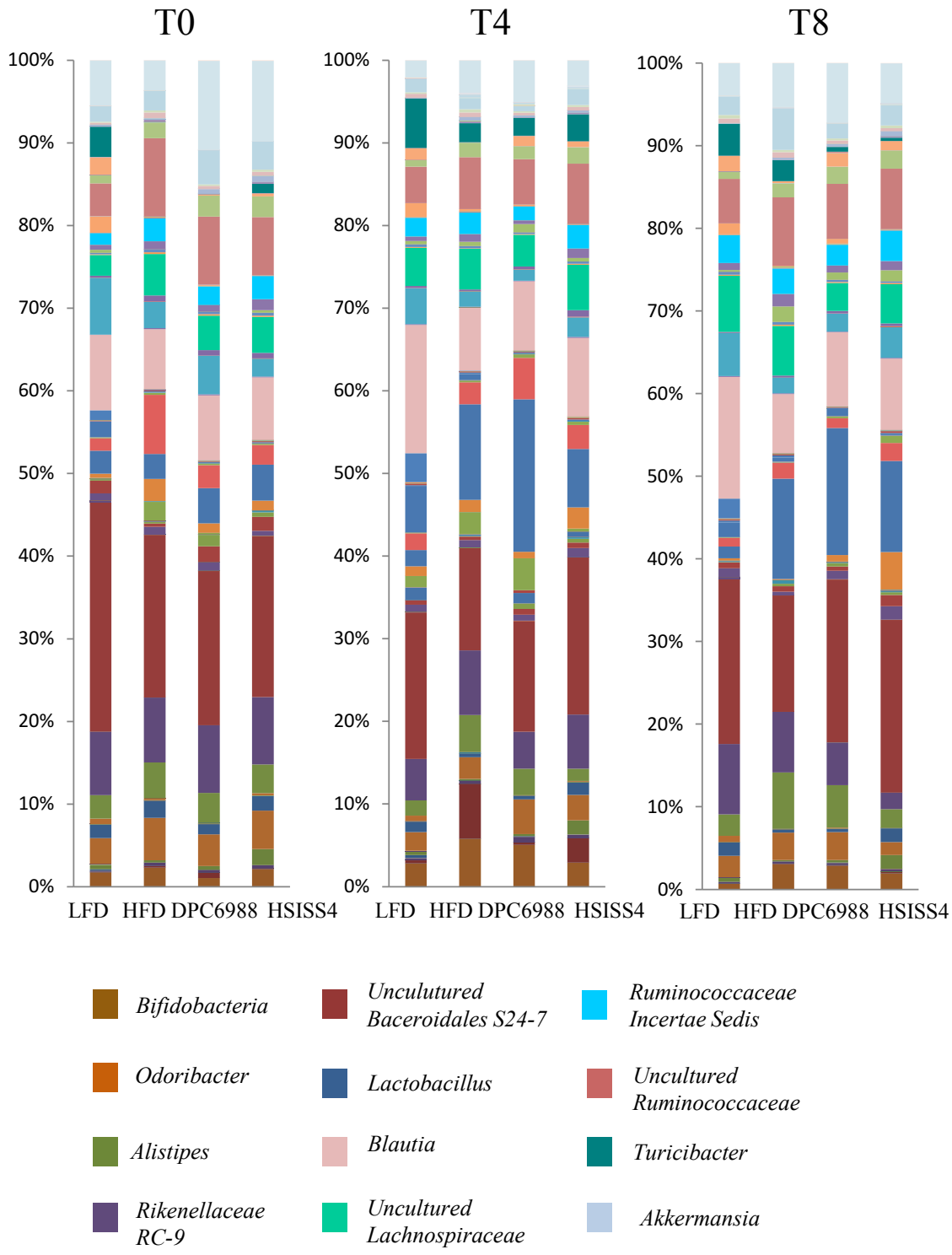


Figure 5 Significant changes in the faecal microbiota of DPC6988 treated mice relative to non-producing controls, HSISS4. Significantly lower genera, p<0.05; , p<0.01; , p<0.001; . Significantly higher genera, p<0.05; , p<0.01; , p<0.001; , in DPC6988 treated mice.

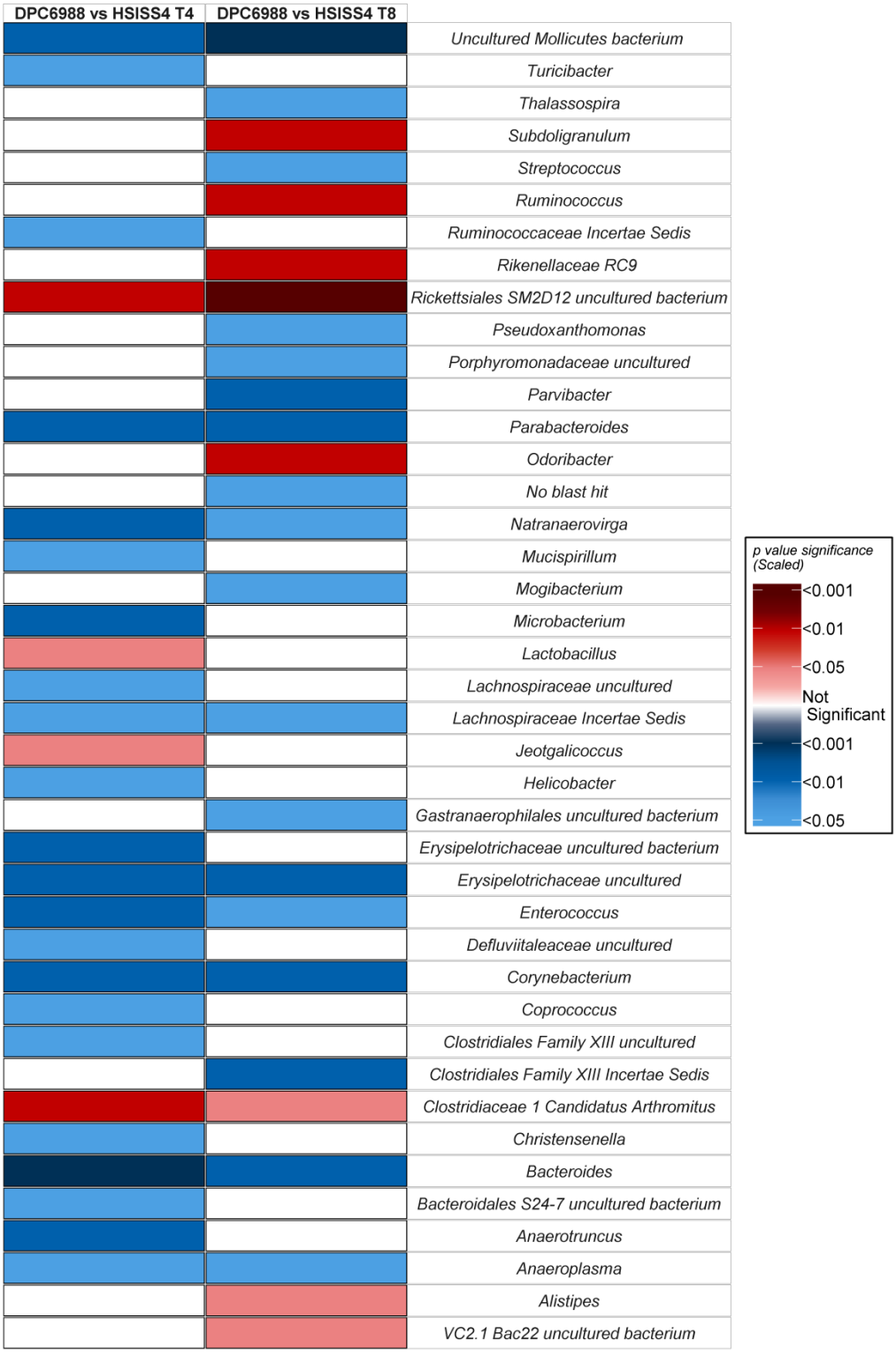
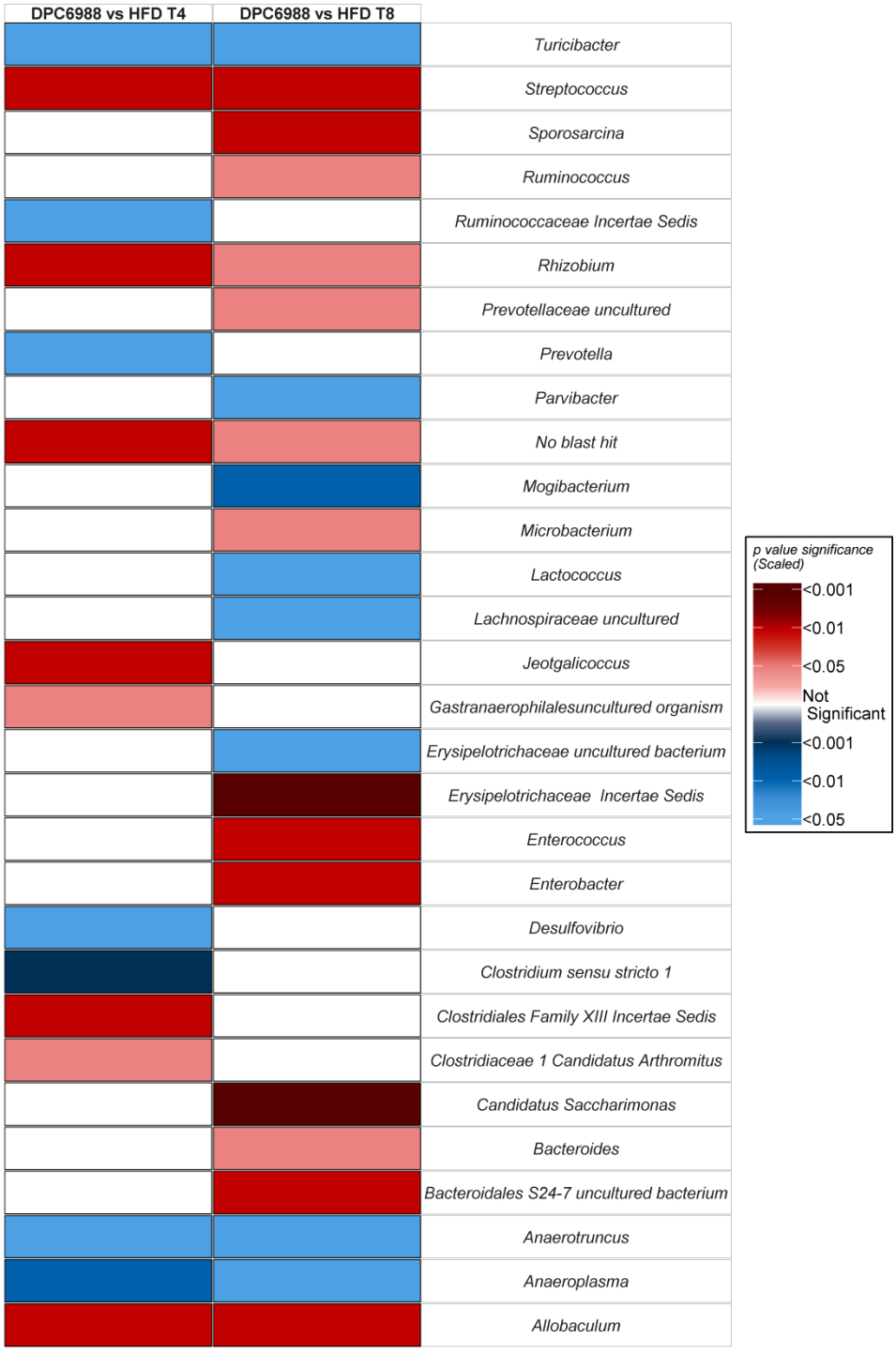


Figure 6 Significant changes in the faecal microbiota DPC6988 treated mice relative to HFD controls. Significantly lower genera, $p<0.05$; , $p<0.01$; , $p<0.001$; , significantly higher genera, $p<0.05$; , $p<0.01$; , $p<0.001$; , in DPC6988 treated mice.



Supplementary table 1 Total bacterial numbers within faecal (T0 and T8) and caecal environment

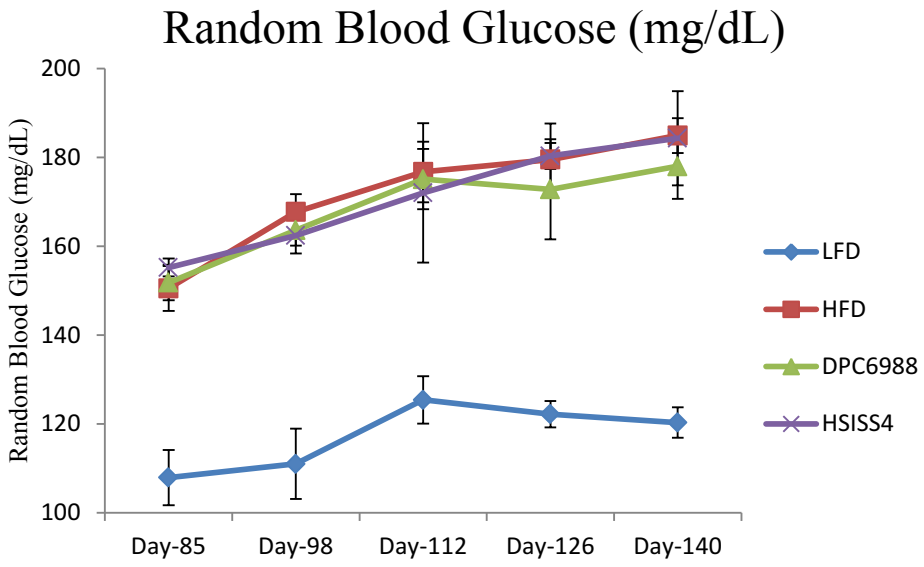
Sample	16S rRNA copies/g of wet stool	Standard deviation
Faecal – HFD T0	$6.9x10^9$	$9.07x10^9$
Faecal – DPC6988 T0	$2.9x10^{10}$	$3.4x10^{10}$
Faecal – HSISS4 T0	$6.18x10^9$	$5.94x10^{10}$
Faecal – HFD T8	$7.43x10^9$	$1.91x10^9$
Faecal – DPC6988 T8	$4.73x10^{10}$	$5.67x10^{10}$
Faecal – HSISS4 T8	$3.13x10^{10}$	$1.1x10^{10}$
Caecal – HFD	$1.5x10^9$	$1.07x10^9$
Caecal – DPC6988	$1.37x10^9$	$1.61x10^9$
Caecal – HSISS4	$5.59x10^9$	$6.83x10^9$

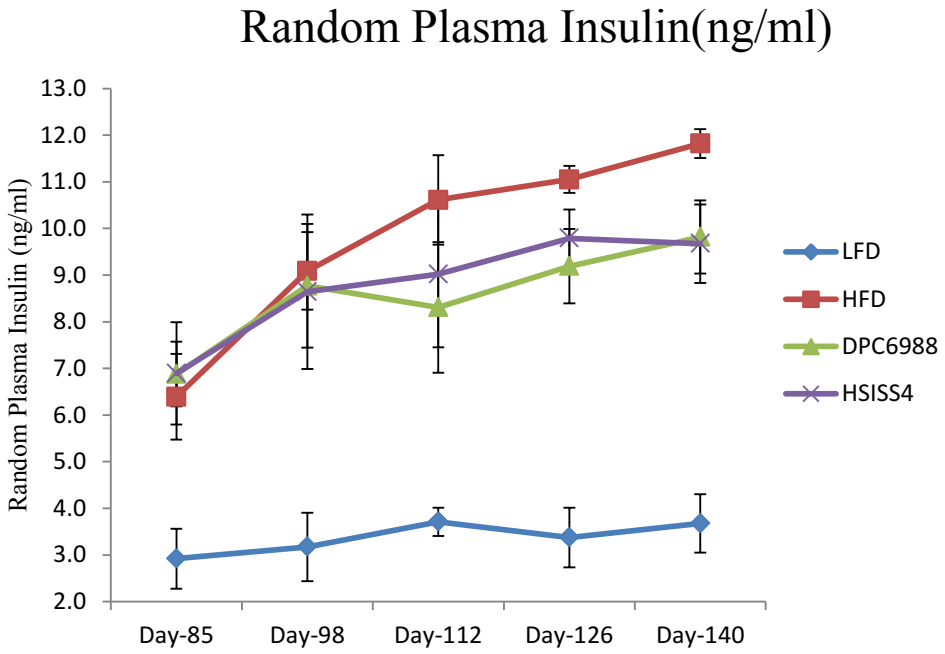
Supplementary table 2 Absolute quantification of *S. salivarius* at T0, T4 and T8 of the intervention

Sample	Concentration	Standard deviation
T0 – HFD	$9.71x10^3$	8283.7
T0 – DPC6988	$1.12x10^4$	4021.77
T0 – HSISS4	$1.09x10^4$	14295.42
T4 – HFD	$3.65x10^4$	17254.85
T4 – DPC6988	$1.38x10^6$	813417.48
T4 – HSISS4	$1.32x10^6$	241936.63
T8 – HFD	$3.58x10^4$	39810.11
T8 – DPC6988	$1.06x10^6$	397935.92
T8 – HSISS4	$1.33x10^6$	374477.41
S. mutans	563	335.4
S. agalactiae	4580	101.49

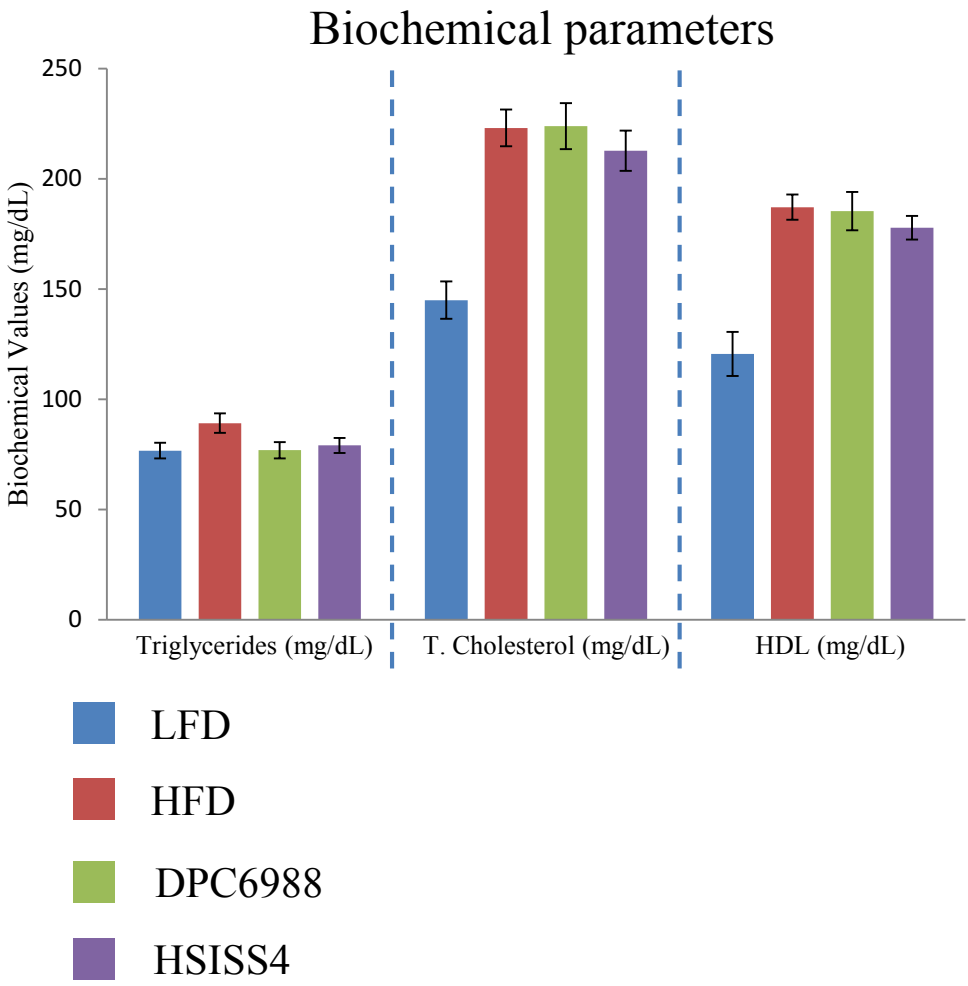
Absolute quantification concentration values represent the average of triplicate mice. T8 HFD is the average of two mice.

Supplementary figure 1 Random blood glucose and random plasma insulin measurements during the 8 week intervention period in LFD;—, HFD;— fed mice and DPC6988;—, HSISS4; —, treated mice. Data represented as the mean ± SEM, n = 10. Day 85 (T0) represents the start of the intervention.

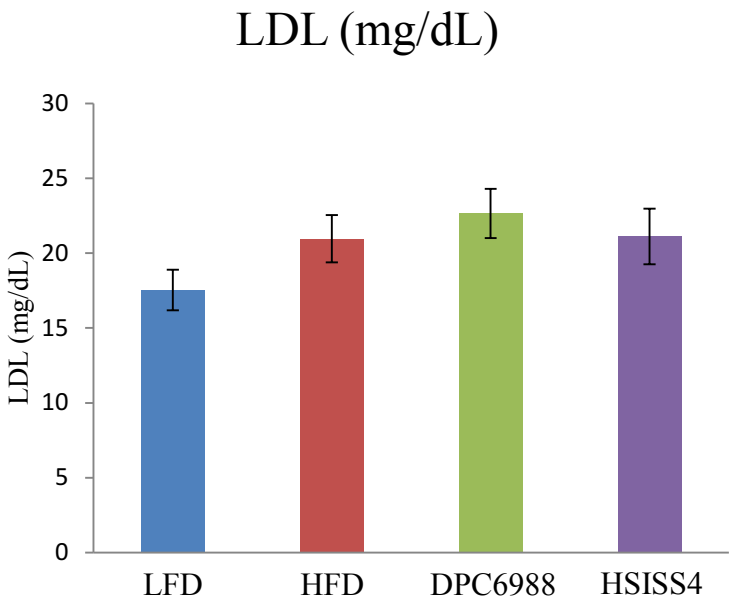


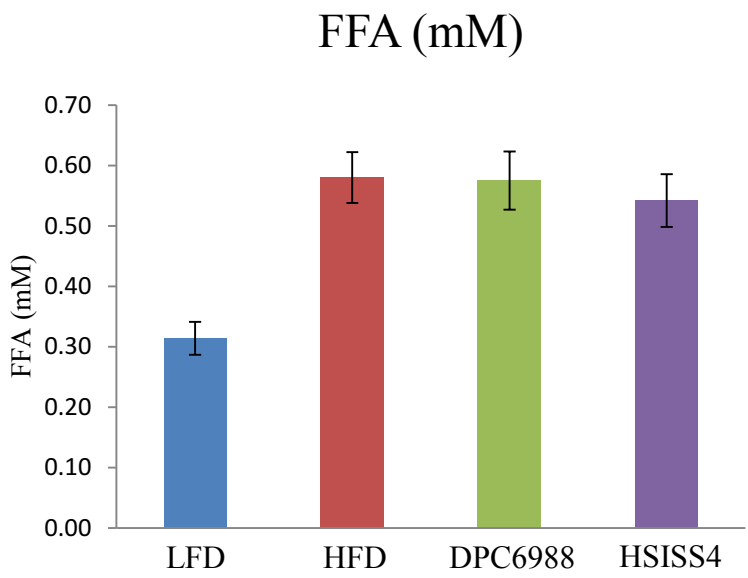


Supplementary figure 2 Biochemical parameters assessed at the end of the intervention of period. LFD; , HFD; , DPC6988; , HSISS4; . Data represented as the mean ± SEM, n = 10.

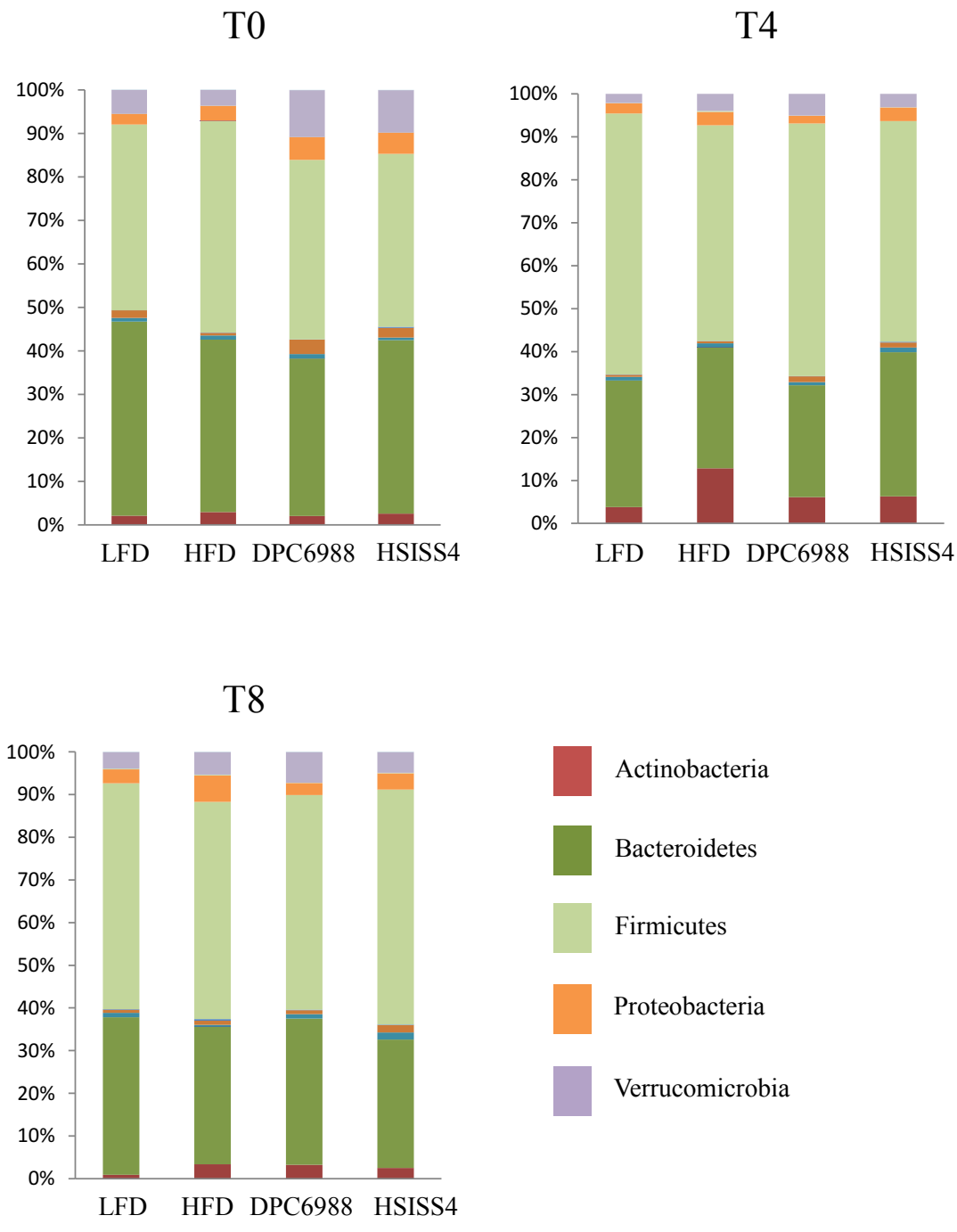


Supplementary figure 3 Biochemical parameters assessed at the end of the intervention of period. LFD; , HFD; , DPC6988; , HSISS4; . Data represented as the mean ± SEM, n = 10.

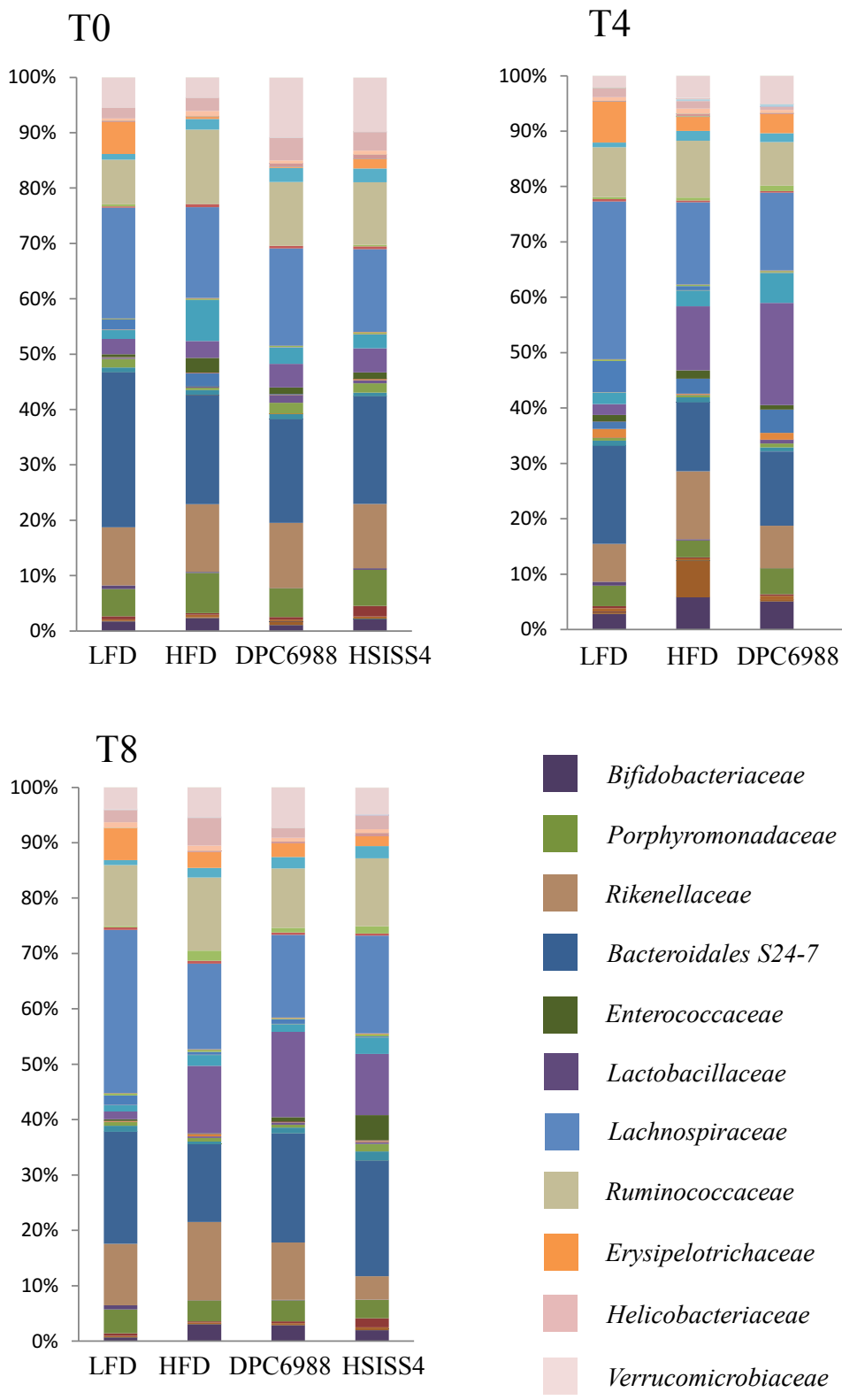




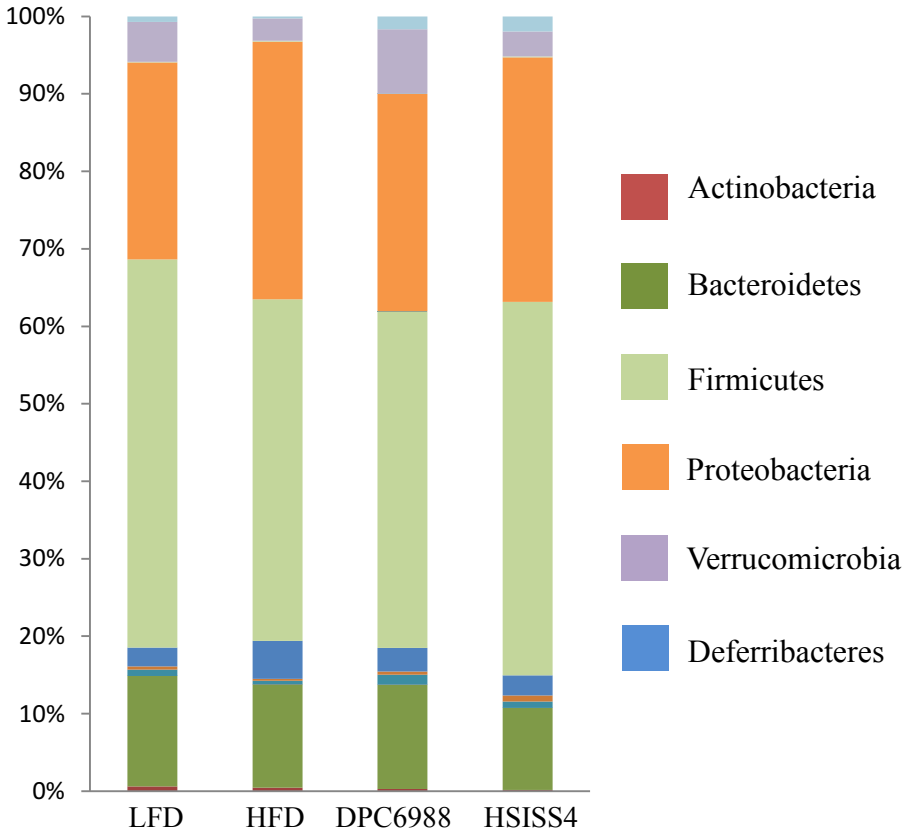
Supplementary figure 4 Relative abundances at phylum level for the LFD, HFD, DPC6988 and HSISS4 groups at T0, T4 and T8 of the intervention within the faecal microbiota. Data represented as the mean number of mice for each group (n=10).



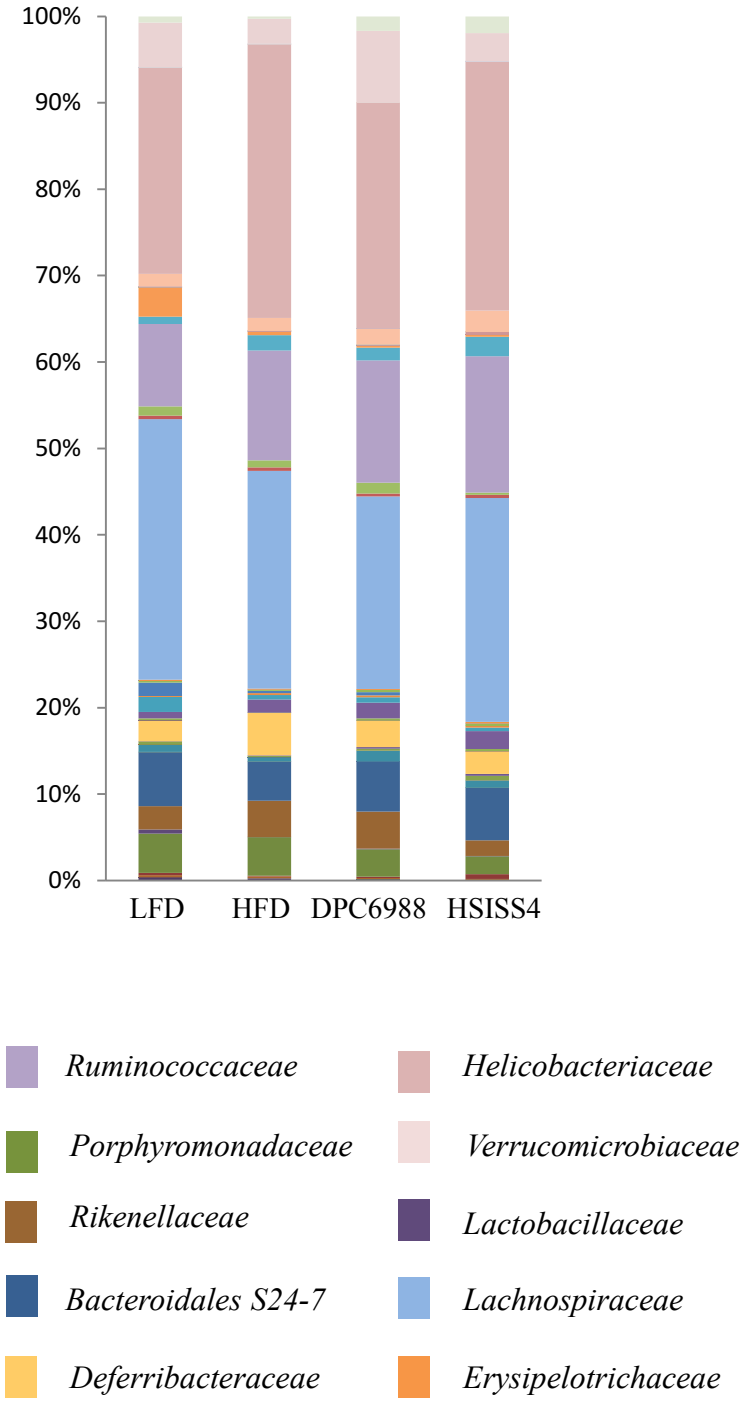
Supplementary figure 5 Relative abundances at family level for the LFD, HFD, DPC6988 and HSISS4 groups at T0, T4 and T8 of the intervention within the faecal microbiota. Data represented as the mean number of mice for each group (n=10).



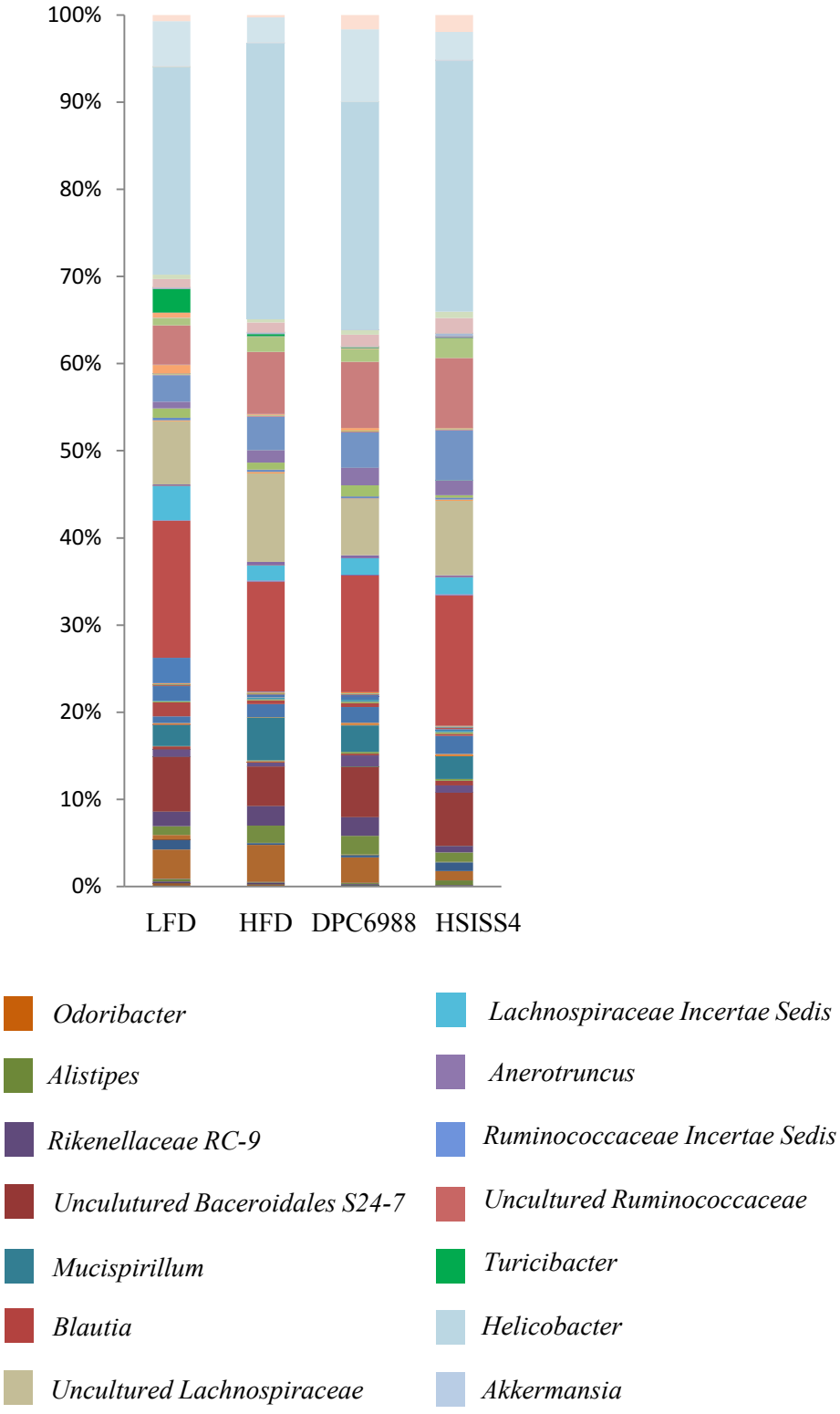
Supplementary figure 6 Relative abundances at phylum level for the LFD, HFD, DPC6988 and HSISS4 groups within the caecum. Data represented as the mean number of mice for each group (n=10).



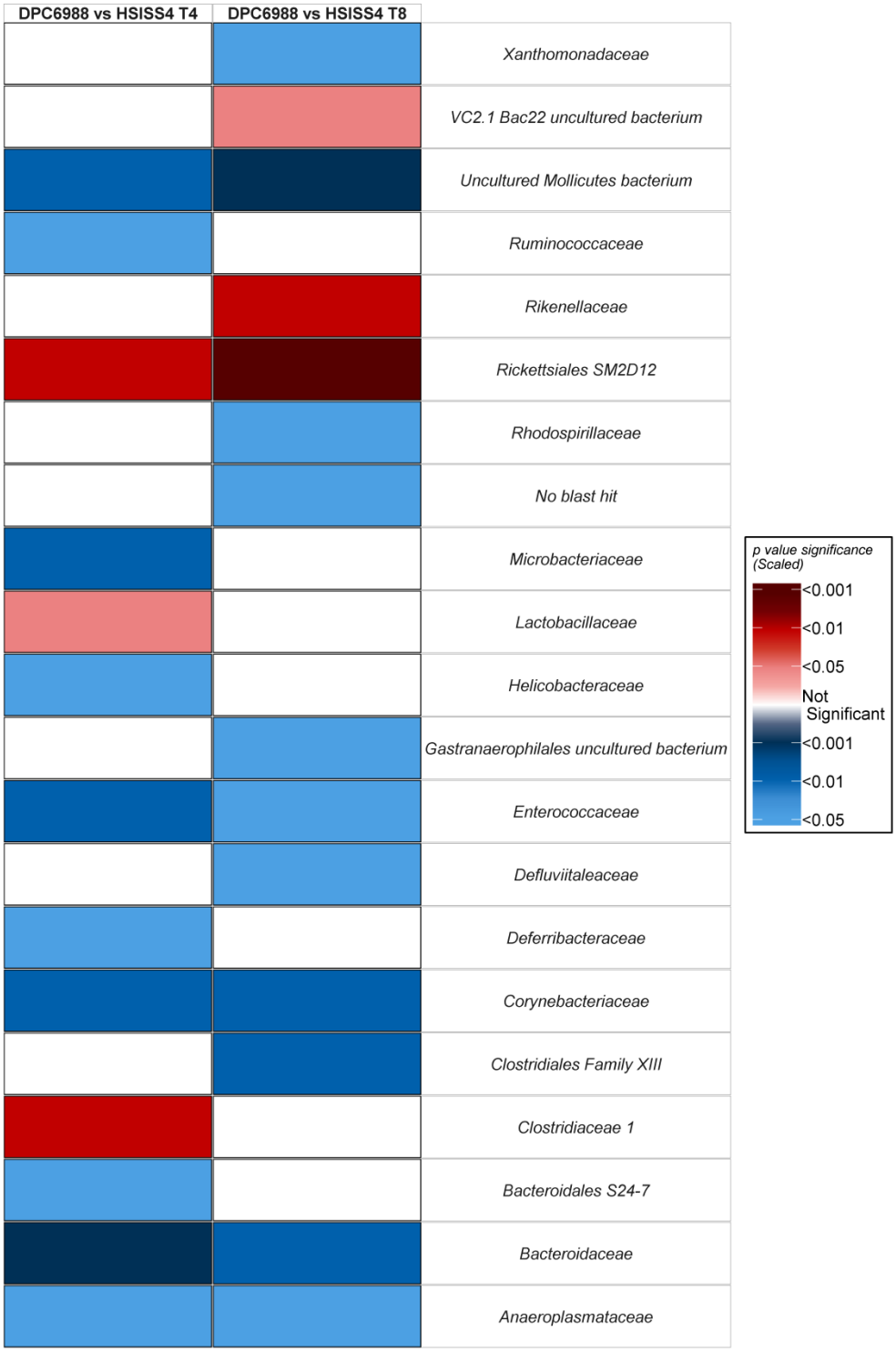
Supplementary figure 7 Relative abundances at family level for the LFD, HFD, DPC6988 and HSISS4 groups within the caecum. Data represented as the mean number of mice for each group (n=10).



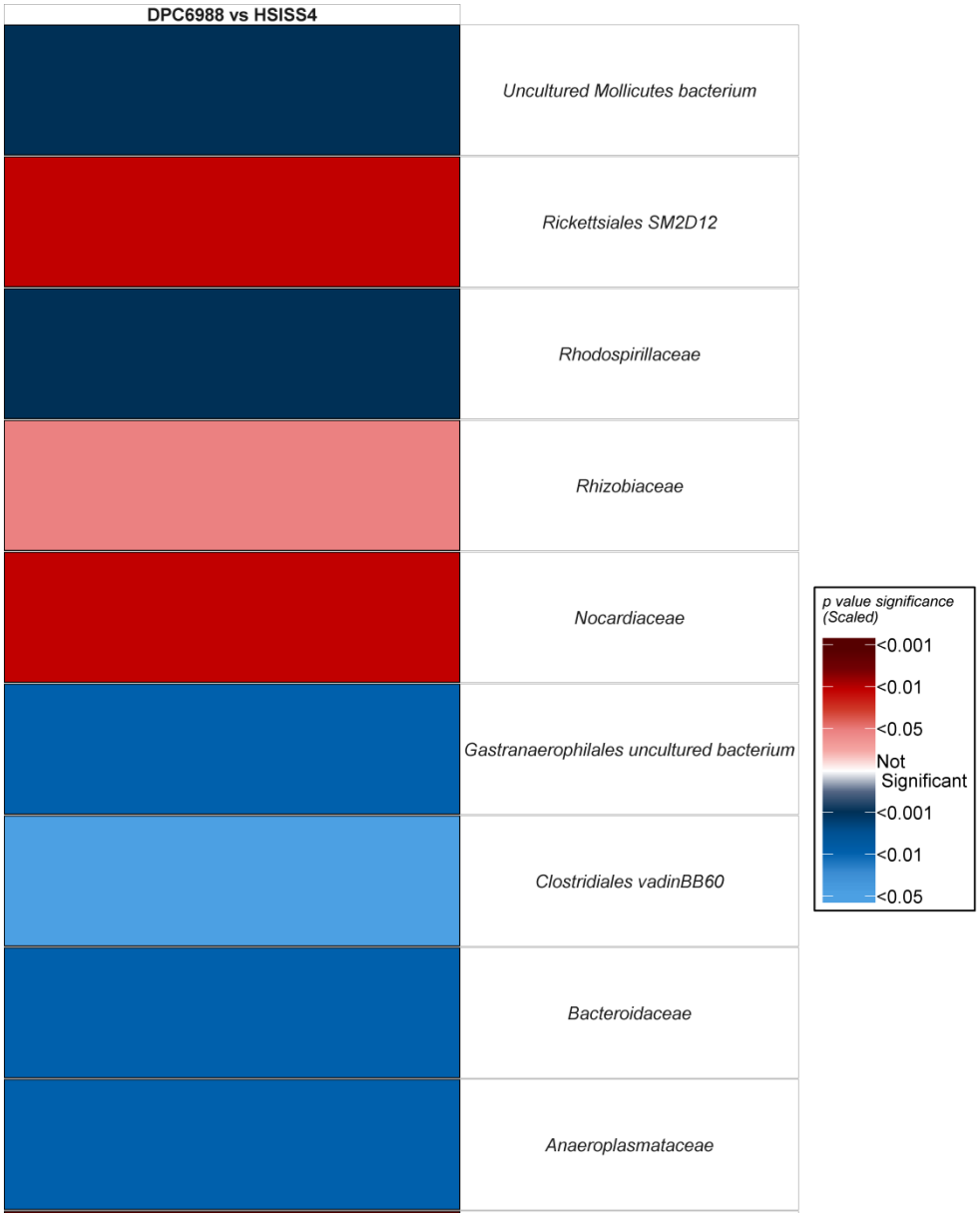
Supplementary figure 8 Relative abundances at genus level for the LFD, HFD, DPC6988 and HSISS4 groups within the caecum. Data represented as the mean number of mice for each group (n=10).



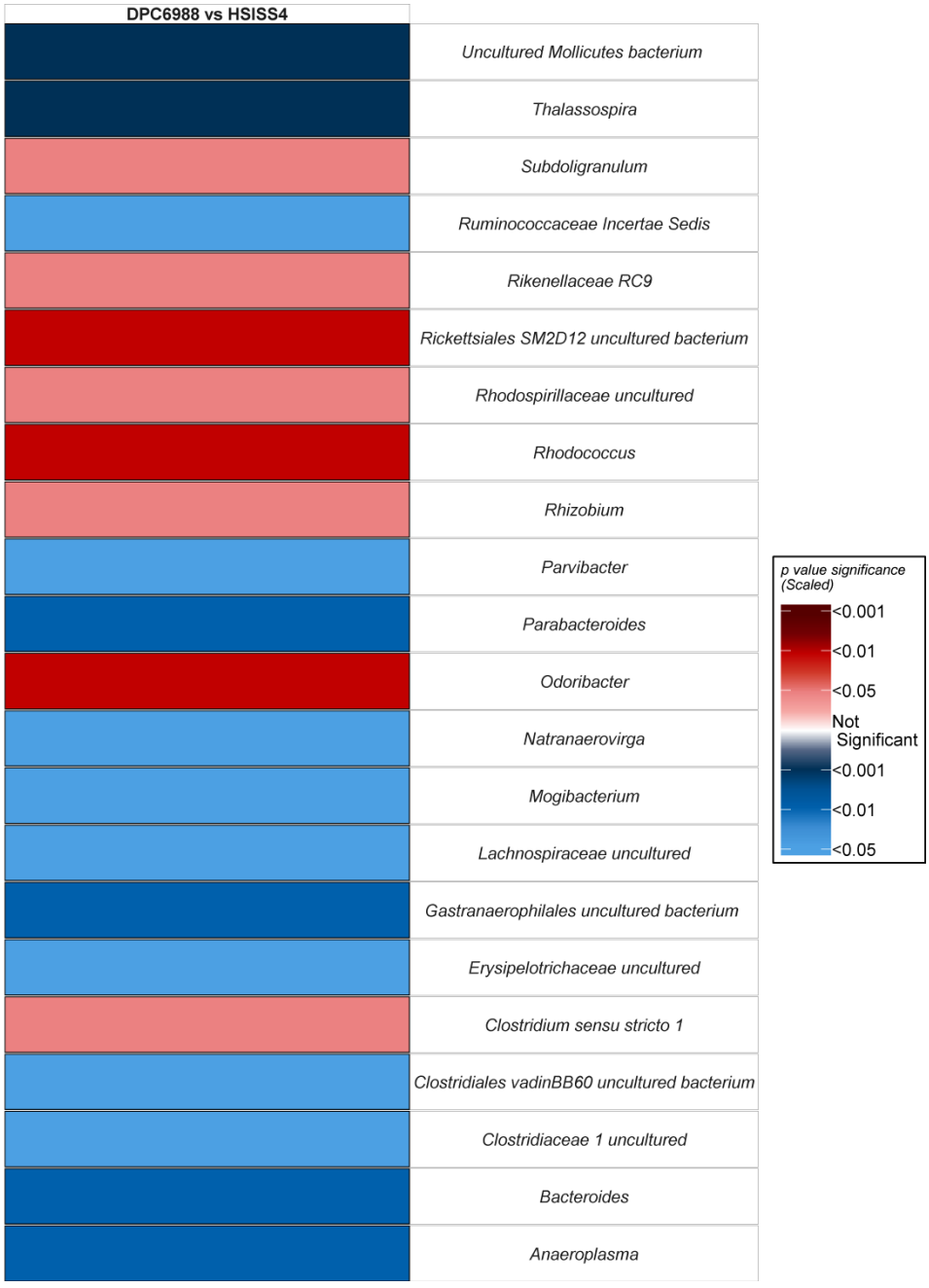
Supplementary figure 9 Significant changes in the faecal microbiota of DPC6988 treated mice relative to non-producing controls, HSISS4. Significantly lower families p<0.05;, p<0.01;, p<0.001;, significantly higher families p<0.05; , p<0.01; , p<0.001; , in DPC6988 treated mice.



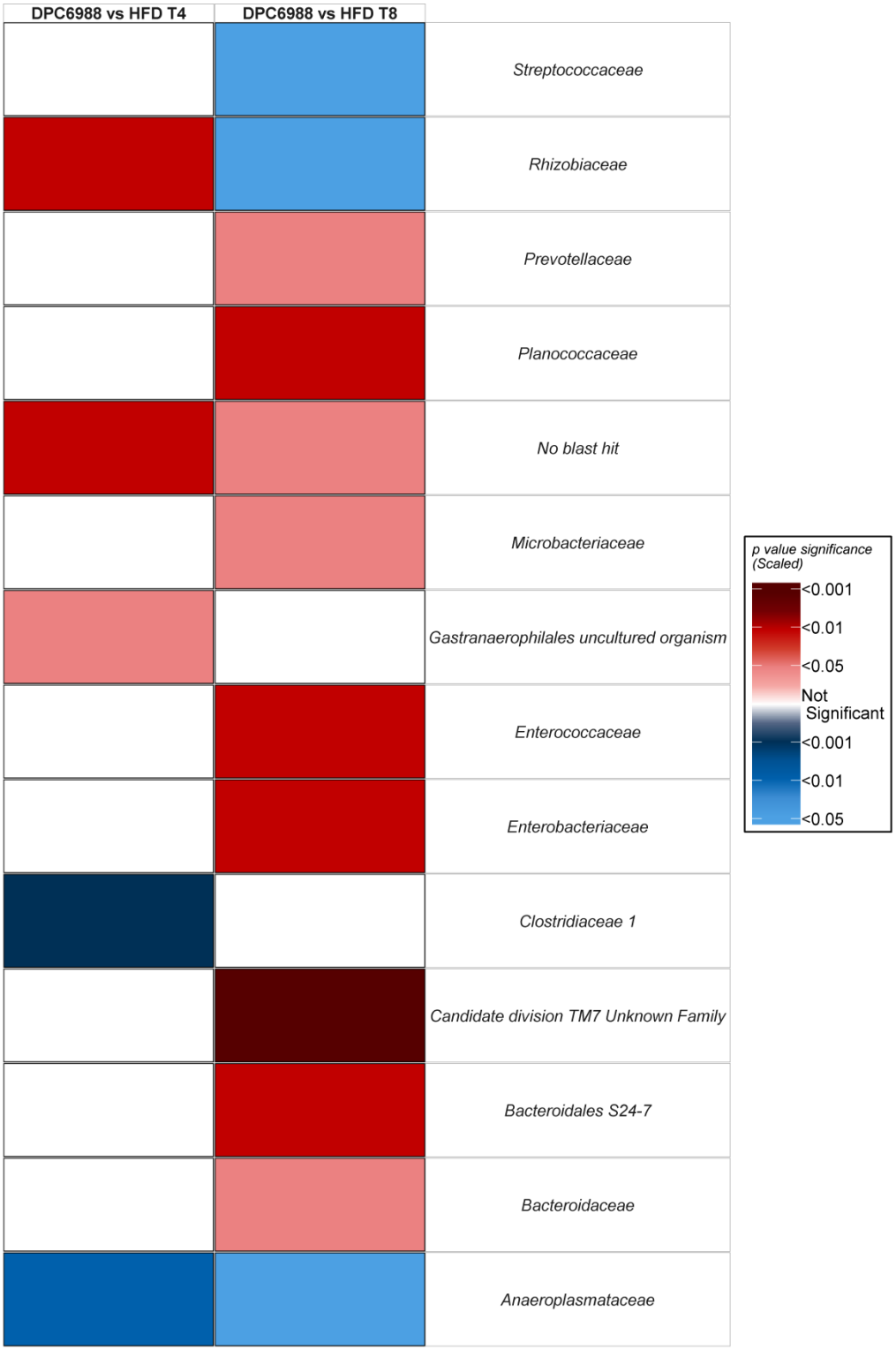
Supplementary figure 10 Significant changes in the caecum of DPC6988 treated mice relative to non-producing controls, HSISS4. Significantly lower families p<0.05; , p<0.01; , p<0.001; , significantly higher families p<0.05; , p<0.01; , p<0.001; , in DPC6988 treated mice.



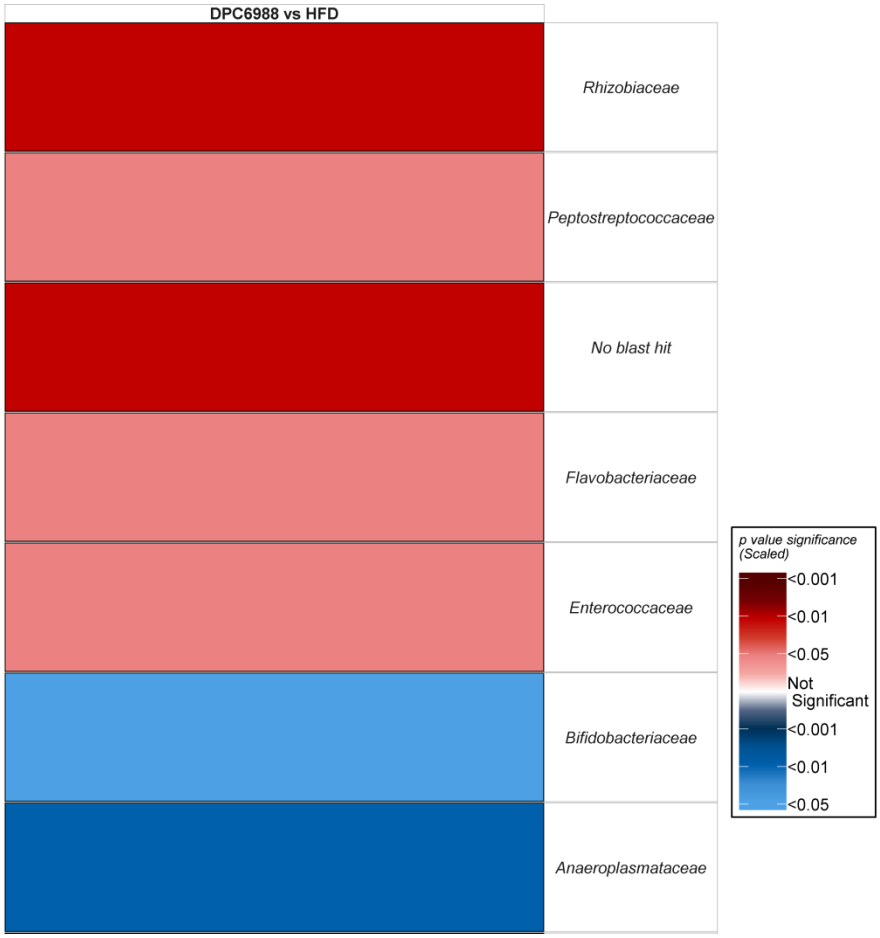
Supplementary figure 11 Significant changes in the caecum of DPC6988 treated mice relative to non-producing controls, HSISS4. Significantly lower genera p<0.05; , p<0.01; , p<0.001; , significantly higher genera p<0.05; , p<0.01; , p<0.001; , in DPC6988 treated mice.



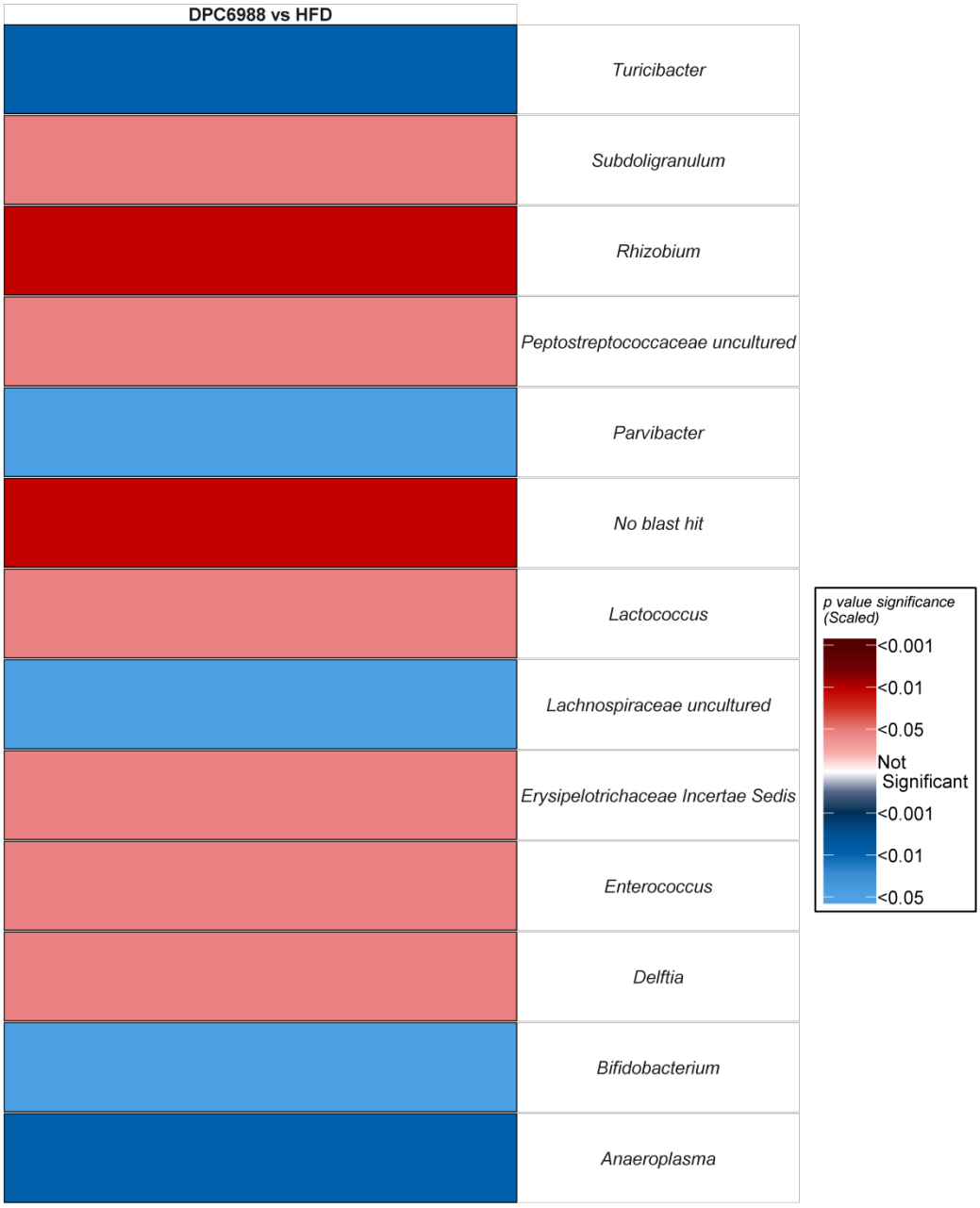
Supplementary figure 12 Significant changes in the faecal microbiota of DPC6988 treated mice relative to HFD controls. Significantly lower families, $p<0.05$; , $p<0.01$; , $p<0.001$; , significantly higher families, $p<0.05$; , $p<0.01$; , $p<0.001$; , in DPC6988 treated mice.



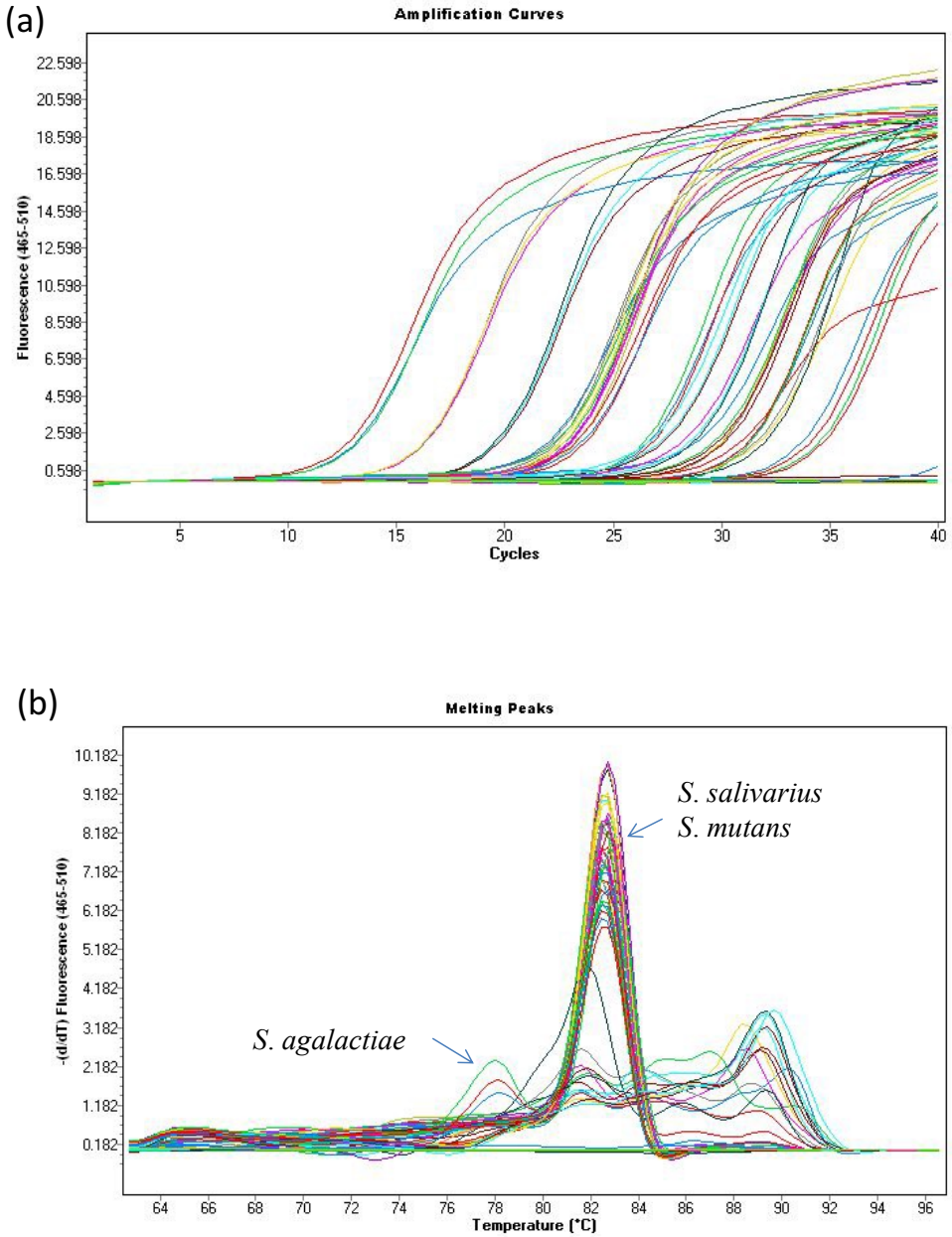
Supplementary figure 13 Significant changes in the caecum of DPC6988 treated mice relative to HFD controls. Significantly lower families, p<0.05;, p<0.01;, p<0.001; , significantly higher families p<0.05; , p<0.01; , p<0.001; , in DPC6988 treated mice.



Supplementary figure 14 Significant changes in the caecum of DPC6988 treated mice relative to HFD controls. Significantly lower genera, p<0.05; , p<0.01; , p<0.001; , significantly higher genera, p<0.05; , p<0.01; , p<0.001; , in DPC6988 treated mice.



Supplementary figure 15 Absolute quantification of *S. salivarius* numbers. (a) Amplification curves of standards, samples from T0, T4 and T8 of the intervention and negative controls. (b) Melting curves produced using the S. sal primer pair. Non-specific amplification is evidenced by the melting peak generated using the negative control DNA from *S. agalactiae*, generating a melting peak at 78°C, and *S. mutans*, generating a peak at ~82°C, at which the melting peak for *S. salivarius* was also observed.



General Discussion

Obesity, a global epidemic, is a complex syndrome associated with a number of serious consequences for human health including, type 2 diabetes, cardiovascular disease and musculoskeletal disorders. In 2014, over 1.9 billion adults aged 18 and over were overweight, with more than 600 million of these being obese. Furthermore, Ireland is on course to be the most obese country in Europe by 2030. Ultimately, the threat posed by this obesity epidemic and associated implications cannot be overstated. The "Obesibiotics" project was developed following a proof-of-concept study which examined the strategy of using vancomycin and the bacteriocin-producing probiotc *Lactobacillus salivarius* UCC118 to treat obesity (Murphy, Cotter et al. 2012). The aims of this project, within the scope of "Obesibiotics", were to (1), harness the bacteriocin-producing capacity of the gut, and (2), target and assess the individual components of the gut microbiota with respect to obesity and related metabolic diseases, and develop bacteriocin-producing probiotics that can contribute prevention/treatment of such disorders. Current strategies to treat obesity include weight loss medications, or other more severe treatments such as gastric banding or gastric bypass. Thus, the idea of taking a bacteriocin-producing probiotic that could be used in combination with improvements to lifestyle and diet is therefore attractive.

In the literature review for this thesis, the contribution of bacteriocin production to probiotic functionality, recent developments relating to bacteriocin-producing probiotics and potential novel areas in which bacteriocin-producing probiotics could be employed are all explored.

Despite being regarded as a probiotic trait, bacteriocin-production among commercial probiotic species has, in general, not been studied in great detail. Using a culture-based approach, the aim of Chapter 2 was to screen for bacteriocin-producing

isolates among a selection of commercially available probiotic products. Although bacteriocin-production was evident, the lack of heterogeneity among those that were identified was noteworthy. Screening resulted in the detection of 9 bacteriocin-producing isolates from 9 distinct products. All were speciated as *Lactobacillus acidophilus*, and all isolates produced lactacin B, with bacteriocin production appearing only to be a feature among certain strains of *L. acidophilus* and in products in which this strain is present. This study suggests that bacteriocin production as a probiotic trait is underutilised among commercial strains, and implies the harnessing of bacteriocin-producing strains to this end remains primarily the focus of academic research.

The primary aim of this PhD project was to harness to the bacteriocin-producing capacity of the gut. To achieve this, a culture-based screen was undertaken with a view to identifying bacteriocin-producing isolates that could be used to target obesity/T2D enriched populations (Chapter 3). This screen was successful as a number of isolates were identified and characterised, which were capable of inhibiting species enriched in T2D patients. This study also provides further evidence that the gut microbiota is indeed a valuable source of bacteriocin-producing isolates, which, in the case of the "Obesibiotics" project, could function as a potential probiotic treatment for obesity and related metabolic conditions. Perhaps the most noticeable drawback associated with this screen was the identification of isolates, which due to their potential for associated pathogenicity, could not be taken forward with a view to applied research. It is also anticipated that further advances designed to grow previously 'unculturable' microbes will lead to the identification of novel bacteriocin-producing gut microbes prompting the use of "next-generation probiotics". Indeed, as new targets continue to emerge with regards to obesity and

related metabolic disorders, existing and newly developed strategies to harness bacteriocin producing strains have the potential to yield significant rewards.

We next investigated the influence of *S. salivarius* DPC6988, a lead bacteriocin-producing strain from the screen of gut isolates, and non-producing control, *S. salivarius* HSISS4, in a model of the distal colon. To our knowledge, this study represents the first example investigating the impact of a *S. salivarius* strain on gut populations, and provides insight into how the strain survives and functions within this complex environment. Although both strains were capable of altering microbial composition in a model of the distal colon, a number of desirable changes were attributed to DPC6988 only. Given the similarities between the strains used, it was assumed that any differences in microbial composition were in part due to the key difference between strains, i.e. bacteriocin production. Possibly, the biggest limitation associated with this study was an inability to determine numbers of the bacteriocin sensitive indicator, *C. ramosum*, and further efforts are necessary to address this issue. The application of higher-throughput bioreactor models can increase the ease with which future studies are performed, and allow for larger studies with a greater sample number.

The final chapter of this thesis explored the approach of using *S. salivarius* DPC6988 in a DIO mouse model. Despite alterations to the gut microbiota, no improvements in weight gain, or the metabolic markers examined were observed. While this strain does indeed alter the gut microbiota, though not to the same extent as what was observed in Chapter 4 using a distal colon model, a higher dosage of *S. salivarius* DPC6988 or perhaps a longer intervention maybe needed. Furthermore, *C. ramosum* was not administered to these mice and, thus, it is anticipated that future

studies will involve the use of this obesity-associated species in a manner similar to that described previously (Woting, Pfeiffer et al. 2014).

To conclude, this project resulted in the identification of a number of bacteriocin-producing gut microbes. Further work with one such lead isolate, *S. salivarius* DPC6988, is required to determine its true potential as a strain that can contribute to controlling weight gain. We expect that, as the gut microbiota in obesity continues to receive attention, new obesity-promoting targets will continue to emerge. This will allow for the development of strategies to target potential bacteriocin-producing probiotics, or use existing producing strains, either alone or in the form a multi-strain mixture, and establish a selection of tailored probiotics that can represent another component in a multi-pronged approach to control weight gain and of obesity.

References

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