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Encapsulation technologies for delivery of bioactive compounds to the Gastrointestinal Tract

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

Doctor of Philosophy

by

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Declaration

I declare that this thesis is my own, original work and has not been submitted in whole or in part for any other *degree.* All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism. This work was carried out under the supervision of Prof. R. Paul Ross, Dr. Mary Rea and Prof. Colin Hill in Food Biosciences, Teagasc Food Research Centre, Moorepark, Co. Cork, Ireland, APC Microbiome Institute, University College Cork, Ireland, and the School of Microbiology, University College Cork, Ireland.

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Catherine O'Reilly

Author Contributions

All of the work herein was performed by the author with the following exceptions:

Chapter 2.

Dr. Órla O'Sullivan performed the bioinformatic analysis.

Paula M O'Connor performed the HPLC analysis.

Chapter 3.

Dr. Órla O'Sullivan performed part of the bioinformatic analysis. Paula M O'Connor performed the mass spectrometry analysis.

Chapter 4.

Dr.Órla O'Sullivan performed part of the bioinformatic analysis. Paula M O'Connor performed the mass spectrometry analysis.

Chapter 5.

Mairéad Coakley was involved in the planning, organisation and implementation of the animal trial. Harsh Mathur, Cecilia Soria and Matthew Aijuka assisted with sample collection. Paula M. O'Connor performed the mass spectrometry analysis. Veronica Peterson and Ciara O'Donovan carried out the bioinformatic and statistical analysis of the sequencing data. Animal husbandry was carried out with assistance from the Pig Development Department, Teagasc, Moorepark.

Abbreviations

CyA	Cyclosporine A
GI	Gastrointestinal
GIT	Gastrointestinal tract
TNBS	246-trinitrobenzenesulfonic acid
DAMPA	4-amino-4-deoxy-N-methylpteroic acid
5-ASA	5-aminosalicylic acid
6-MP	6-mercaptopurine
AMPs	Antimicrobial peptides
AHR	Aryl Hydrocarbon Receptor
BSH	Bile salt hydrolase
$CPDG_2$	Carboxypeptidase-G2
CDI	Clostridioides difficile Infection
CRC	Colorectal cancer
CAR	Constitutive androgen receptor
CD	Crohn's disease
DEX	Dexamethasone
DHFR	Dihydrofolate reductase
EMA	European Medicines Agency
FMT	Faecal Microbiota Transplants
FXR	Farnesoid X receptor
FMT	Feacal Microbial Transplantation
FDA	Food and Drug Administration
GI	Gastrointestinal
GF	Germ-free
GC	Glucocorticoids
HPLC	High Performance Liquid Chromatography
HDMTX	High-dose Methotrexate
HPA	Hypothalamic-Pituitary-adrenal
IBD	Inflammatory Bowel Disease
KO	Knock Out
MTX	Methotrexate

NTC	No treatment control
OTUs	Operational Taxonomic Units
PPAR	Peroxisome proliferator-activated receptors
PWSD	Poorly water soluble drugs
PXR	Pregnane X receptor
RA	Rheumatoid Arthritis
SCFA	Short Chain Fatty Acid
SpA	Spondyloarthritis
SASP	Sulfasalazine
TMP	Trimethoprim
TNF	Tumour necrosis factor
UC	Ulcerative Colitis
UST	Ustekinumab
XPGs	Xenobiotic-processing genes

Thesis Abstract

Abstract

Ulcerative Colitis and other inflammatory bowel diseases (IBD) are increasingly linked to changes in the gut microbiome. In addition, gastrointestinal illnesses caused by infections by gut pathogens such as *Clostridioides difficile* (*C. difficile*) have been associated with antibiotic use. Indeed, Over-use of antibiotics is increasingly adding to the build-up of antibiotic resistance. This is leading to increased ineffectiveness of currently used antibiotics and consequently there is an urgent need to develop alternative treatments to traditionally administered antibiotics. The aim of this thesis was to investigate the effect of colon targeted bioactives (pharmabiotics) on the composition and function of the gut microbiota.

Chapter 1 of the thesis is a literature review focused on the interaction of orally administered drugs for the treatment of IBD with the gut microbiota and with the host. The review highlights how understanding and manipulating these interactions may enhance treatment outcomes in the future and bring about new opportunities for precision medicine where microbiome/drug-host interactions are taken into account.

Cyclosporine, a calcineurin inhibitor, has a protective role in the treatment of Ulcerative Colitis by down-regulating pro-inflammatory cytokine production. It is currently used for the treatment of refractory ulcerative colitis. Cyclosporine has traditionally been administered intravenously, however; prolonged systemic exposure can cause toxicity. Chapter 2 details a study in which we assessed the effect of a newly developed cyclosporine containing formulation on the composition and functionality of the gut microbiota. This novel delivery system was developed by Sigmoid Pharma (now Sublimity Therapeutics Ltd) to

encapsulate cyclosporine for targeted delivery to the colon. In this way the concentration of cyclosporine delivered could be reduced. The results demonstrate that while the composition of the microbiota remains unaffected, the functionality of the microbiota may be changed given that levels of short chain fatty acids were affected significantly.

Nisin is a bacteriocin with a broad spectrum of activity against Gram-positive bacteria including the gut pathogen *C. difficile*. In Chapter 3 we assessed the activity of nisin at a range of concentrations from 0-500 μM against *C. difficile* in an *ex vivo* model colon fermentor and determined the minimum inhibitory concentration at which *C. difficile* growth is suppressed (50μM) while having minimal impact on the faecal microbiota. This study opens up the potential of nisin as a therapeutic for clostridial gut infections.

Chapter 4 outlines the impact of fidaxomicin, nisin, thuricin CD (at equimolar concentrations) and a no treatment control on the gut microbiota in the model colon system, Fidaxomicin is used primarily in patients not responding to the traditional antibiotics routinely used to treat *Clostridioides difficile* Infection (CDI). Nisin and thuricin CD are bacteriocins with broad or narrow spectrum of activity, respectively. Fidaxomicin and nisin were found to have a broader spectrum of activity that was not selective just for *C. difficile* and related species, while thuricin CD exhibited a largely selective spectrum of activity limited primarily to *C. difficile.*

Nisin is the only commercially available highly purified bacteriocin which is approved safe for use in food and for human consumption. Because of its peptide nature it is sensitive to proteolytic digestion. In Chapter 5 the ability of nisin in both an encapsulated and unencapsulated form to reach the lower porcine GIT,

was investigated. This study disproved the dogma that nisin is broken down in the mammalian gut by demonstrating that intact nisin survives intestinal transit where it can modify the gut microbiota. In particular, Gram-positive bacteria of the Firmicutes Phylum were proportionally reduced which also was associated with reductions in some short chain fatty acid – particularly butyrate. Importantly, these changes to the gut microbiota were reversed quickly following cessation of feeding the bacteriocin.

Overall, this work evaluates the usefulness of encapsulating bioactives for targeted release to treat GIT illnesses. These studies also demonstrate the potential of bacteriocins as an alternative to antibiotics in the treatment of GI pathogens such as *C. difficile* and as antimicrobial tools to modify the composition of the microbiota *in vivo* despite their proteinaceous nature.

Chapter 1

The interaction of the human microbiota with orally administered drugs for Inflammatory Bowel Disease and new opportunities for precision medicine

Abstract

Inflammatory Bowel Disease (IBD) describes chronic, inflammatory disorders of the gastrointestinal tract (GIT) that includes Crohn's disease (CD) and Ulcerative Colitis (UC). The cause of IBD is unknown in many cases but can be attributed to a combination of factors including genetic susceptibility, altered immune response, a shift in microbial composition/microbial insults and environmental influences. The main classes of drugs used to treat IBD include 5-aminosalicylic acid (5-ASA), sulfasalazine (SASP), corticosteroids, immunomodulatory drugs, anti-tumour necrosis factor-alpha (TNF-α) inhibitors, anti-integrins, anti-IL12/23, anti-IL23 and JAK inhibitors. Treatments that manipulate the gut microbial composition such as antibiotics, prebiotics, probiotics and other dietary interventions are also used. Faecal Microbial Transplantation (FMT) is another experimental treatment which aims to restore bacterial balance. Research into the role of the gut microbiota in IBD dates back over 20 years and has intensified in the last ten years. Studies investigating drug-gut microbiota interactions have found that certain drugs require the gut microbiota to convert a prodrug into its active form or they can alter gut microbiota composition and function. Other studies have elucidated that the baseline gut microbiota composition can affect the efficacy of drug treatment and thus could potentially be used as a biomarker for treatment success. The aim of this review is to discuss the interaction of orally administered drugs for the treatment of IBD with the gut microbiota and with the host. The effect of these drugs on gut pathogens thought to participate in IBD is also discussed. Finally, we highlight how understanding and manipulating these

interactions may enhance treatment outcomes in the future and bring about new opportunities for precision medicine.

Introduction

Gut commensal bacteria provide numerous benefits to the host such as maintenance of gut barrier integrity, education of the host immune response, metabolism of indigestible food components and production of nutrients including vitamins and short chain fatty acids (SCFA) (Round and Mazmanian 2009). In certain circumstances, some gut bacteria can also impact negatively on host health (often termed pathobionts) (Jochum and Stecher 2020), and perturbations in the balance of the gut microbiota can trigger pro- and anti-inflammatory responses in the host (Cerf-Bensussan and Gaboriau-Routhiau 2010; Heimesaat, et al. 2007; Packey and Sartor 2009). The human microbiota consists of 12 different phyla, the majority of which (93.5%) belong to the phyla Firmicutes, Bacteroides, Proteobacteria, and Actinobacteria. In healthy subjects, the dominant phyla are the Firmicutes and Bacteroidetes (Lozupone, et al. 2012). The composition of the gut microbiota is influenced by age, diet, antibiotics and orally administered medication consumed for a variety of medical conditions such as Inflammatory Bowel Disease (IBD). IBDs are chronic, inflammatory disorders of the gastrointestinal tract and include Crohn's disease (CD) and ulcerative colitis (UC). Drugs used to treat IBD include sulfasalazine (SASP), 5-aminosalicylic acid (5-ASA), corticosteroids, immunomodulatory drugs such as methotrexate (MTX), azathioprine (AZA), 6-mercaptopurine (6-MP), immunosuppressants such as calcineurin inhibitors (cyclosporine (CyA) and tacrolimus) and anti-tumour necrosis factor-alpha (TNF-α) inhibitors (infliximab, adalimumab, certolizumab) (Ardizzone, et al. 2010). Some of the above drugs, such as cyclosporine, for example, are poorly soluble. Furthermore, there is an increasing demand to develop novel methods to withstand drug release in the

upper gastrointestinal tract for release in the large or small intestine. This is particularly relevant for IBD-targeting drugs where the intestine is the target site.

While the exact etiology of IBD is unknown, a combination of genetic susceptibility, environmental factors, an altered immune response (which could be triggered by re-exposure to a pathogen), shifts in microbial composition or loss of tolerance to resident gut microbes are thought to be involved. Whether the microbial composition of a person affected by IBD is the cause or the result of the disease itself and how the treatments help to ameliorate shifts in microbial composition are yet to be fully understood. Additionally, studies have shown that the gut microbiota can alter the metabolism of certain orally administered drugs and in turn affect their efficacy and toxicity. Indeed, there is also increasing interest in designing drugs that specifically target the microbiome to help prevent such diseases. In this way, the microbiome represents a 'drugable target' – a malleable trait.

The aim of this review is to address human gut microbiota interactions with orally administered drugs for the treatment of IBD and the consequences of these interactions for patient outcome with a focus on gut microbiota composition, essentially Pharmacomicrobiomics. Following a summary of xenobiotic metabolism in the human body, we present six commonly used treatments for IBD. These drugs can alter gut microbiota composition and functionality or may depend on the gut microbiota for efficacy such as conversion of the prodrug into its active form. For certain treatments, gut microbiota composition prior to treatment can also be a predictor of patient responsiveness, enabling a delineation of patients into responders and non-responders, opening the way for precision medicine through modulation of a patient's baseline microbiota. This could be

achieved through live biotherapeutics, antimicrobials, nutrients or combinations of the same.

Oral bioavailability and xenobiotic metabolism

Oral ingestion is the most frequently used route for drug administration due to convenience and simplicity, which in turn increases patient compliance. It is also the most complex route for administration because these drugs tend to be lipophilic and poorly water soluble and have to survive passage through the GIT to reach their target site. The result of these hurdles is low and variable bioavailability. Oral bioavailability of a compound is extremely important when developing orally administered drugs. The absolute oral bioavailability (*F*) of a particular compound is defined as the fraction of that compound reaching the bloodstream after oral administration (Hinderliter and Saghir 2014). Approximately 40% of the oral drugs which are marketed as immediate release (IR) are classed as practically insoluble (<100g/ml) (Lipinski, et al. 2001). In contrast, a drug that is administered intravenously for example is 100% bioavailable (Tardif and Brodeur 2005). The general routes of drug administration are depicted in Figure 1. Rectal administration is an alternative route of drug administration which can virtually eliminate exposure to gastrointestinal enzymes. Rectal administration of 5-aminosalicylic acid (5-ASA) suppositories and enemas target different regions of the distal colon and are effective for treatment of proctitis and distal colitis (Lawrance 2011).

Bioavailability of an orally administered drug is affected by many factors. The efficacy of an orally administered drug is dependent on its ability to dissolve in the gastrointestinal fluid, remain intact in the lumen, pass through the epithelial membrane and complete first pass metabolism (Jamei 2009). Physiological variables include the rate of gastric emptying, gastric mobility, blood flow and transit time in the intestine; composition and pH of gastrointestinal fluid, secretion, reabsorption, enzymes (such as peptidases) and bile secretion. The type of food and fluids ingested, disease, age, gender and race also cause variations in drug bioavailability (Jamei, Turner and Yang 2009). The complex factors affecting oral drug metabolism have been studied extensively. Stability of the drug as it transits the digestive tract is vital for efficacy and bioavailability. The development of coating formulations to aide delivery of active pharmaceuticals to the colon is an active area of research in the pharmaceutical industry. Polysaccaharides such pectin, cyclodextrin, dextrans, inulin, and amylose have been studied as potential colon-specific drug carrier systems (Sinha and Kumria 2001). Coating levels and drug solubilities also influence drug release. Additionally, faecal bacteria and bacterial enzymes can affect drug release. For example, higher levels of bacterial α-amylase can influence drug release with drugs coated with amylase. Fermentation of non-starch polysaccharides by the colonic bacteria is exploited in certain formulations that use non-starch polysaccharide coatings. Formulations such as CyCol® (cyclosporine), COLAL-PRED® (steroid) and Mesasal® (Mesalazine) to treat IBD have been developed in an attempt to deliver an active ingredient to the target area. These formulations may also increase bioavailability while decreasing systemic exposure to the drug.

The metabolism of xenobiotics (biotransformation) occurs mainly in the liver after their absorption from the gastrointestinal tract (Gu and Manautou 2012). The four types of biotransformation reactions are Phase I - hydrolysis, reduction or oxidation and Phase II - conjugation. Different types of xenobiotic-metabolizing enzymes are involved in these reactions depending on the chemical composition of the xenobiotics (Parkinson and Ogilvie 2008). Phase II biotransformation is catalyzed often by transferase enzymes that perform conjugating reactions. Included in the phase II reaction schemes are glucuronidation, sulfation, methylation, acetylation, glutathione conjugation, and amino acid conjugation. The endogenous molecule or moiety is usually hydrophilic (highly polar). These conjugation reactions result in increased xenobiotic hydrophilicity (having an affinity for water). The result of a combination of Phase I and II reactions enables xenobiotics to be excreted into urine or bile (Parkinson and Ogilvie 2008).

While the liver is the primary site of xenobiotic metabolism and biotransformation, metabolism starts at the intestinal level and this has a significant impact on drug efficacy and safety. Drugs that are weakly permeable and have poor solubility have most contact with the host microflora in the large intestine as this is where the gut microbiota density is highest. There is an estimated 10^3 bacteria per gram (10^3/g) in the duodenum10^4/g in the jejunum, 10^7/g in the ileum and 10^{12}/gram in the colon (Sekirov I. 2010). Hence, the colon is the site of the GI tract where the interplay with orally ingested drugs, prodrugs and their conjugated metabolites largely occurs.

This interaction between the gut microbiota and xenobiotics can change the way drugs are metabolised by the host (Maurice, Haiser and Turnbaugh 2013). There are numerous ways that the gut microbiota interacts with orally ingested drugs

and the gut microbial composition can potentially be altered significantly by xenobiotic exposure (Wilson and Nicholson 2017). In addition, gastrointestinal infections and antibiotic use can cause pathological changes in gut microbial composition and function (Linskens, et al. 2001; Sartor 2010; Hua, Schneider and Keely 2015). Such alterations can affect microbial enzymatic secretion important in triggering drug release for microbial-dependent drug delivery systems. In the case of diarrhoea caused by gastrointestinal infections, a considerable loss of biodegradable dosage forms may occur, due to insufficient time for drug activation or release (Sartor 2010; Albenberg and Wu 2014; Hua 2020).

The gut microbiota may produce enzymes such as amylases, pectinases and β-d-glucosidases for example that can react (interact) with drugs and degrade them directly (Figure 2). They can produce enzyme-inducing metabolites such as p-cresol, for example, that change the host-drug metabolic pathways and the activity of the drug. Microbial *p*-Cresol reduces the host's ability to metabolize acetaminophen by titrating away hepatic sulfotransferase activity (Clayton, et al. 2009). Microbes may compete with ingested drugs for host metabolizing enzymes and alter the activity of the host’s drug-metabolizing enzymes such as cytochrome P450s (CYP450s), conjugators and drug transporters (Haiser and Turnbaugh 2013) and the nuclear receptors that control their expression, for example, pregnane X receptor (PXR), constitutive androgen receptor (CAR), aryl Hydrocarbon Receptor (AHR) and farnesoid X receptor (FXR) (Collins and Patterson 2020). A study by Fu et al., (2017) studied genes encoding xenobiotic-metabolizing enzymes from several regions of the intestine (duodenum, jejunum, and ileum) and the liver in both conventional and germ-free (GF) mice. They found that there was differential expression across all categories of genes

encoding xenobiotic-metabolizing enzymes between the two groups. Additionally, many cytochrome P450 (CYPs) levels were decreased in the intestinal tissue and surprisingly also in the liver in the germ-free mice when compared to the conventional mice. They concluded that the absence of intestinal bacteria in the germ-free mice altered the expression of a large number of xenobiotic-processing genes (XPGs) in the host intestine, some of which were specific to certain areas of the intestine. Cyp3a was downregulated in both the liver and intestine of GF mice, which probably contributes to altered xenobiotic metabolism in these animals (Fu, et al. 2017).

Most of the reactions with which the gut microbiota are associated are reduction and hydrolytic reactions. These generate non-polar low molecular weight by-products. The other known reactions are dehydroxylation, demethylation, decarboxylation, acetylation, deconjugation, deamination and dealkylation (Haiser and Turnbaugh 2013).

The gut microbiota also interacts with host derived bile acids and can affect the release of poorly water soluble drugs and influence their effectiveness. Cholic acid (CA) and chenoxycholic acid (CDCA) are made from cholesterol by hepatocytes in the liver (Begley, Gahan and Hill 2005). These primary bile acids are conjugated to glycine or taurine prior to secretion from the liver to the intestine upon where they come into contact with the microbial enzymes bile salt hydrolase (BSH) and 7-α-dehydroxylase (Enright, et al. 2017). Since bile acid metabolism is mediated *in vivo* by the microbial enzymes mentioned above, Enright et al., (2017) theorized that pertubations in the gut microbiota could cause alterations in the pharmakokinetic profiles of certain poorly water soluble drugs. To investigate the effect of microbial bile acid metabolism on the solubility of

orally-injested drugs they assessed the solubility capacity of conjugated versus deconjugated and primary versus secondary bile salts using nine poorly water soluble drugs (PWSD) as model solutes. The results from the study found that bile salt hydrolase activity had a relatively minor impact on micellar solubilisation capacity of bile salt micelles for some PWSDs through deconjugation of the steroidal core. The activity of microbial 7-α-dehydroxylase significantly affected the solubilisation capacity of bile salt micelles for all nine PWSDs in the study ($p<0.05$). This study shows that bile acid modifications by host and microbe lead to altered capacity of intestinal bile salt micelles. The clinical relevance of this is that treatment with antibiotics or disruption of the gut microbiota can change the composition of the bile acid pool and thereby alter drug absorption and bioavailability (Enright, et al. 2017).

Members of the gut microbiota are mostly anaerobic thus they do not use oxygen as a terminal electron acceptor for respiration (Arkhipova and Akimenko 2005). As reduction and hydrolytic reactions by bacteria are the most common reactions involved in xenobiotic metabolism, reduction reactions may facilitate anaerobic respiration by increasing the range of alternative electron acceptors available to the microbes for respiration. Alternatively, hydrolytic xenobiotic metabolism can directly provide substrates for microbial growth (Spanogiannopoulos, et al. 2016).

The Main Classes of IBD Drugs and their interaction with the Microbiota

Sulfasalazine and bacterial azoreduction

Sulfasalazine (SASP) is a prodrug made of sulfapyridine linked to 5-aminosalicylic acid (5-ASA) through an azo bond. Although very rarely used in clinical practice currently, it has historical significance and has been used as a treatment for IBD since it's development in 1940. It is an example of a prodrug that is reduced by the gut microbiota. Sulfasalazine is split in the colon by bacterial azoreductases to release 5-ASA and sulfapyridine (Cooke 1969) (Figure 3). The mechanism of action of the drug is not fully understood but is thought to be due to an antibacterial effect of sulfapyridine, an antioxidant effect of 5-ASA or an effect of 5-ASA on prostaglandin metabolism (Azad Khan, Piris and Truelove 1977; Hazenbe, et al. 1982; Zheng, et al. 2017). After oral ingestion, sulfasalazine is partially absorbed in the jejunum, however, the remaining drug passes to the colon where it is bacterially reduced. Azoreductases are a group of enzymes present in mammals, prokaryotes and eukaryotes and the enzymatic reaction results in the cleavage of an azo bond. Electron donation by NADH or NAD(P)H is necessary for reduction by azoreductases (Wang, et al. 2010). Azoreductases are predominantly found in the cytosol but have been shown to be secreted by bacteria when they are exposed to azo dyes (Morrison and John 2015).

Bacterial azoreductases are known to activate two classes of drugs. These are the azo drugs that are used to treat IBD such as sulfasalazine and also nitrofuran antibiotics (Ryan 2017). Enzymes with azoreductase activity have been identified and characterized from a wide variety of bacteria and characterisation of

azoreductases encoded by anaerobic gut bacteria is important in the development of new azo prodrugs targeting intestinal diseases (Ryan 2017). Many gut bacteria are found to have azoreductase and nitroreductase activity - mainly from the *Clostridium* genus. These include *Clostridium perfringens*, *C. leptum*, *C. paraputrificum, Eubacterium* sp and *C. clostridiiforme* (Rafii, Franklin and Cerniglia 1990; Morrison, Wright and John 2012; John and Morrison 2015). Interestingly, some probiotic bacteria have been shown to enhance the intestinal metabolism of sulfasalazine when co-administered with the drug. An *in vitro* study compared the activity of sulfasalazine alone and sulfasalazine in the presence of probiotic cultures of *Lactobacillus acidophilus, Bifidobacterium lactis* and *Lactobacillus rhamnosus* (in freeze-dried form) which were incubated anaerobically in rat faecal contents. The researchers reported higher recovery of sulfapyridine in the probiotic-containing group suggesting that these species are capable of azoreductase activity (Lee, et al. 2012).

Examples of other related prodrugs which are activated by azoreduction are Olsalazine and Balsalazide. An oxygen-insensitive azoreductase from *Pseudomonas aeruginosa* (paAzoR1) has been described that activates the azo prodrug balsalazide (an aminosalicylate) with relatively high specificity compared with common reference azo dyes (Wang, et al. 2007).

A study by Zheng et al (2017) investigated the relationship between SASP, IBD, and the gut microbiome. They used 2,4,6-trinitrobenzenesulfonic acid (TNBS) to induce experimental colitis in rats and analyzed the microbiota in the faecal samples. The three groups in the experiment included a control group (treated with ethanol + saline), a model group (treated with TNBS-ethanol + saline) and a SASP group (treated with TNBS-ethanol + SASP). Then 16S gene sequencing

and shotgun sequencing were performed to assess the effects of these treatments on gut microbiota composition and function. The study found that SASP treatment restored the TNBS-induced gut dysbiosis as shown by an increased amount of SCFA-producing bacteria and lactic acid-producing bacteria in addition to a decreased abundance of Proteobacteria in the SASP-treated group. SASP also modulated the dysregulated function of the TNBS-induced colitis to resemble that of the control group as shown by increased capacity for basic metabolism (carbohydrate metabolism, citrate cycle) and a decrease in the oxidative stress (riboflavin, sulfur, cysteine) as well as bacterial pathogenesis (cell motility and secretion, bacterial motility proteins, flagellar assembly). Additionally, a higher proportion of *Mycoplasma* was observed in the SASP group. Mycoplasma has been associated with destructive effects on fertility in humans (Taffet and Das 1983; Jamalizadeh Bahaabadi, et al. 2014) which is reversible upon withdrawal of SASP or the use of an alternative 5-ASA compound without the Sulfapyridine component (such as Mesalamine) (Chatzinoff, et al. 1988). This study provides insight into the specific microbial clades and pathways linked with SASP metabolism and its mechanism for treatment of IBD.

Influence of aminosalicylates on gut bacteria

Aminosalicylates have been used to treat IBD for over 60 years. Mesalazine (also known as Mesalamine, 5-aminosalicylic acid or 5-ASA) is a conventional treatment for IBD. 5-ASA as mentioned above is used for inducing and maintaining remission in mild to moderate forms of UC and acts at the

inflammatory and epithelial level in the colonic mucosa (Nielsen and Munck 2007; C. Andrews, et al. 2011). This form of the drug is not associated with Sulphapyridine (as is the case with SASP). The mechanism of action of Mesalazine for the treatment of IBD is still not completely understood, however, it is thought to downregulate inflammation and reduce inflammatory cytokine release by activating nuclear receptors (i.e., the gamma form of peroxisome proliferator-activated receptors (PPARS) (Rousseaux, et al. 2005). It affects a range of signalling pathways and mediators which are involved in leucocyte chemotaxis and function (eicosanoids, IL-8, NF-kB and PPAR-y) and epithelial defence (scavenging free radicals, fortifying epithelial resistance to injury, promoting cellular proliferation and inhibition of apoptosis) (Nielsen and Munck 2007).

According to the Biopharmaceutics Classification System, Mesalazine is categorized as a BCS class IV drug (low solubility, low permeability). It is slightly soluble in water and following its oral administration is poorly absorbed (approximately 25%) (Kumar, et al. 2018). Most of the oral mesalazine products on the market have a pH dependent coat which enables it to withstand the release of 5-ASA in the stomach (which has a low pH) and be released in the intestine with a higher pH (Bayan and Bayan 2020). Mesalazine comes in a variety of different forms and preparations, the most common currently used ones are listed in Table 1.

Some studies have also shown that 5-ASA (Mesalazine) alters the gut microbial composition. *Ex vivo* studies have found that it can inhibit the growth of gut *Bacteroides* and *Clostridium* species grown in pure culture (Gertzen, et al. 1985). Andrews et al., (2011) measured the effect of Mesalazine treatment on faecal

microbiota composition, mucosal proteolytic activity and clinical efficacy in 14 patients with irritable bowel syndrome-diarrhea (IBS-D). For this purpose, proteolytic activity in recto-sigmoid mucosal biopsies along with IBS symptoms' score and absence of overt gastrointestinal mucosal inflammation or ulceration before and after Mesalazine treatment were measured. The results of the study showed that Mesalazine treatment caused the responding patients to have a more similar faecal microbiota to each other than non-responders. Prior to treatment, 95% of the microbiota in the patients' stool samples were made up of the Firmicutes and Bacteroidetes. Following Mesalazine treatment there was an increase in the abundance of Firmicutes from 62+/- 8% (mean +/- S.E.M) at baseline to 71% +/- 9% with treatment. The Bacteroidetes microbiota decreased from 33 +/- 7% at baseline to 23 +/- 9% with treatment. These changes were not statistically significant, however (possibly due to the small number of patients in the study). The findings from this study suggest that Mesalazine treatment has an antibacterial effect. Following the termination of treatment the microbial community levels returned to levels observed prior to treatment. The effects observed occurred at 5-ASA concentrations that are similar to those achieved in the distal intestinal tract in patients with IBD (Andrews, et al. 2011). It is worth noting that the clinical benefits observed were not sustained after the four week treatment period and the changes in gut microbiota composition returned to levels comparable to pre- treatment levels.

Kaufman et al., (2009) studied the effect of 5-ASA using Mesalazine on bacterial gene expression by assessing its effects on a random promoter library representing the genome of *Salmonella enterica* serovar *Typhimurium*, a model enteric bacterium. In total, 45 constructs, which were representative of 38 unique

promoters, were responsive to 5-ASA. These genes were involved in bacterial invasion, cellular metabolism, and stress resistance. Some of the identified genes have homologs among commensal Gram-negative enteric bacteria. Interestingly, 5-ASA downregulated invasiveness genes in *Salmonella*. This correlated with the ability of 5-ASA to attenuate the invasiveness of *S.enterica* serovar *Typhimurium* exposed to cultured epithelial monolayers.

Mesalazine may have the same effects on other members of the intestinal flora. The effect of 5-ASA in downregulating invasiveness genes could result in a decrease in bacteria that adhere to and invade colonic mucosa which modulates the interactions between commensal gut bacteria and the host. Indeed, as already mentioned, the drug has been shown to inhibit the growth of gut-derived *Bacteroides* and *Clostridium* species in pure culture (Gertzen, et al. 1985). It also inhibits the growth of gut pathogens associated with IBD such as *Campylobacter concisus* which has been implicated as an initiator of the disease in some IBD patients (Zhang, et al. 2014).

Another way that the gut microbiota and Mesalazine are linked is through PPARs. PPARs are ligand-activated transcription factors which regulate various metabolic processes such as fatty acid metabolism (Oh, Visvalingam and Wahli 2019) and they play an important role in regulating inflammation, fibrosis and immunity (Clark 2002). PPARγ is highly expressed in the colon predominantly in the epithelial surface layer and is lowly expressed in patients with UC (Lefebvre, et al. 1999). Studies have demonstrated that the anti-inflammatory effects of 5-aminosalicylic acid (5-ASA) in patients with ulcerative colitis were mediated by PPARγ activation and in this way Mesalamine acts as a PPARγ agonist (Bertin, et al. 2013). In addition, the SCFAs Butyrate and propionate produced by gut

bacteria are strong stimulators of phosphorylation-dependent PPAR-γ transcriptional activity and hence are also considered as PPARγ agonists (Korecka, et al. 2013), (Nepelska, et al. 2017). Other studies have reported the benefits of attenuating inflammation in colitis through the supplementation of probiotics and dietary interventions with the use of prebiotics, resulting in a significant increase in PPAR-γ expression in the colon (Viladomiu, et al. 2013).

A study by Peyri-Biroulet et al; (2010) showed that the colonic mucosa of PPAR γ mutant animals was defective in killing several major components of the intestinal microbiota, including *Bacteroides fragilis*, *Enterococcus faecalis*, *Candida albicans* and *Escherichia coli*. In this study they showed that PPAR-γ-signaling may also contribute to shaping the intestinal environment by activating the expression of beta-defensins (Antimicrobial peptides AMPs). (Peyrin-Biroulet, Beisner, et al. 2010). Indeed, the transmission of *Enterococcus faecalis* from mother to child can be activated and regulated by PPARγ in primary murine colonic epithelial cells (Are, et al. 2008). The findings from these studies further support the concept that the effects of Mesalazine on the gut microbiota composition can be caused directly by interactions with the drug itself or is secondary to the known effects of Mesalazine on mucosal immune function.

Methotrexate (MTX)

Methotrexate (MTX) is an immunosuppressive drug that has been used effectively for over 20 years in patients with CD. It is used increasingly in combination with anti-TNF agents to prevent immunogenicity. MTX is also used as a treatment in UC; however, conflicting results mean that more data is

necessary to definitively assess its clinical efficacy (Herfarth, et al. 2016). MTX is a folate antagonist with anti-proliferative and anti-inflammatory activity. MTX is metabolised to polyglutamated forms intracellularly by the liver, erythrocytes and various tissues. These polyglutamates act as inhibitors of dihydrofolate reductase (DHFR) and thymidylate synthetase. Dihydrofolate reductase reduces dihydrofolate (FH2) to tetrahydrofolate (FH4). Tetrahydrofolate is the active precursor of the active folate cofactors necessary for the synthesis of pyrimidines and purines which are essential components of DNA (Chabner, et al. 1985). Similar to other immunosuppressants MTX may cause unwanted side effects such as nausea, stomatitis, liver toxicity (Feagan and Alfadhli 2004) and in rare cases high dose MTX can cause kidney injury (Bleyer 1977). Folic acid supplementation is recommended with MTX treatment which may ameliorate some unwanted side effects associated with treatment (Siegal and Sands 2005).

Orally administered Methotrexate is metabolized by intestinal bacteria to the inactive metabolite 4-amino-4-deoxy-N-methylpteroic acid (DAMPA) which accounts for less than 5% loss of the oral dose. It is thought that DAMPA is formed from MTX that is excreted via bile into the intestinal tract where it is hydrolyzed by bacterial carboxypeptidases and subsequently reabsorbed. DAMPA is believed to be an inactive metabolite of MTX as it is not an effective inhibitor of the MTX target enzyme dihydrofolate reductase (Donehower, et al. 1979; Widemann, Sung, et al. 2000). Shin et al., (1974) showed that *Lactobacillus casei* was also able to metabolize Methotrexate mainly to 4-amino-4-deoxy-10-methylpteroic acid (DAMPA) (Shin, Buehring and Stokstad 1974). Studies in mice have also shown that MTX administered parenterally interacts with the intestinal microbiota due to biliary secretion.

In the 1970's the enzyme carboxypeptidase-G1 ($CPDG_1$) was isolated from *Pseudomonas stutzeri* (McCullough, Chabner and Bertino 1974), (Chabner, Johns and Bertino 1972) and cloned into *E.coli* and the recombinant form Carboxypeptidase-G2 ($CPDG_2$) was developed. It was found to lower plasma MTX concentrations in a small number of patients with brain tumours who had been treated with a high dose of MTX (Abelson, Ensminger and Kufe 1979; Abelson, Kufe and Skarin 1981). In 21 patients with MTX-induced renal dysfunction, $CPDG_2$ lowered plasma MTX concentrations within 15 minutes of administration by >98% (Green 2012). When administered to patients with High-dose Methotrexate (HDMTX)-induced renal dysfunction, $CPDG_2$ metabolized circulating MTX to the inactive metabolite DAMPA thus providing an alternate route of elimination to renal excretion. Glucarpidase is an FDA approved recombinant form of $CPDG_1$ that hydrolizes the amide linkage on circulating Methotrexate to form glutamate and DAMPA (Widemann, Balis, et al. 1997). It is approved for use in both adult and pediatric patients with renal dysfunction who have levels of plasma MTX above 1µM/L (Rattu, et al. 2013).

A study by Nayak et al., (2019) used a combination of *in vitro* culturing, humanized mouse models and longitudinal analysis to assess the effect of MTX on the human gut microbiota. The results of the studies found that MTX treatment caused a significant, dose-dependent shift in the human gut microbial community structure and richness after a single day with doses comparable those for the treatment of cancer and arthritis in gnotobiotic mice. rRNA gene sequencing revealed a decrease in Bacteroidetes and Verrucomicrobia and an increase in Firmicutes and Proteobacteria. Longitudinal analyses of the gut microbiotas of Rheumatoid Arthritis (RA) patients revealed MTX-induced alterations in the

relative abundance of resident bacteria which were associated with patients which had an positive response to treatment. Because the shift was only observed in drug responders, Nayak et al theorized that MTX results in a decrease in Bacteroidetes which may permit other bacterial phyla to expand in relative abundance, and that these shifts could alter the inflammatory potential of the microbiome. Furthermore, experiments in gnotobiotic mice showed that these shifts lead to reduced inflammation. 38 of the 45 (84%) tested isolates exhibited either growth inhibition or alterations in growth curve parameters upon exposure to MTX at concentrations that are predicted to be found in the human gut. Following from these results they investigated whether MTX acts through the canonical mechanism-of-action established in eukaryotic cells or if it had an indirect effect on bacterial physiology. By using RNA-seq transcriptomics they found that MTX induced a profound shift in gene expression in the isolate that was most sensitive to MTX, *B. thetaiotaomicron*, affecting 83% of genes in the transcriptome. They also observed notable changes at the transcriptional level of *C. asparagiforme* which was resistant to the growth inhibitory effects of MTX (Nayak, Alexander and Stapleton-Grey 2019).

Studies have shown that MTX can bind DHFR homologues in *Escherichia coli* (*E. coli*) and *Lactobacillus casei* (Bolin, et al. 1982). Interestingly, *E. coli* were found to be resistant to any growth inhibitory effects of MTX due to the expression of a multi-drug efflux transporter TolC19. Overexpression of DHFR in a strain of *E coli* that lacks TolC rescues it from the effects of MTX, suggesting that MTX interferes with DHFR in the cellular context (Kopytek, et al. 2000).

MTX is structurally similar to the antibiotic trimethoprim (TMP). Both drugs work by inhibiting dihydrofolate reductase in prokaryotic and eukaryotic cells. Guðmundsdóttir et al; (2020) measured exponential growth rates for two pairs of clinical isogenic TMP resistant (TMP^R) and TMP susceptible *E. coli* across a range of MTX concentrations. They examined whether exposure to MTX could lead to de novo TMP^R evolution. To do this they selected spontaneous mutants from overnight cultures with and without exposure to MTX, plated on selective agar at high MTX concentrations and checked for TMP cross-resistance.

The results of their study found that MTX affects selection and evolution of TMP^R determinants at clinically relevant concentrations. MTX can mediate selection of any antibiotic resistance determinant when TMP^R is co-localized on a mobile genetic element (often the case in clinical *E. coli* isolates) (Guðmundsdóttir, et al. 2020).

In summary, the gut microbiota directly interact with MTX to convert it into a different form. Additionally, MTX can have direct effect on the gut microbiota by altering the composition but also has an impact at the cellular level of certain bacterial species.

Glucocorticoids and Steroids

Glucocorticoids (GC) are a class of corticosteroid hormone. They are synthesized in the human body in the adrenal cortex and secreted into the blood. The naturally occurring glucocorticoid in humans is hydrocortisone (cortisol); it is synthesized from its precursor cortisone. Glucocorticoid drugs inhibit inflammatory processes and are routinely used for the treatment of IBD (Van der Velden 1998).

Glucocorticoids are the therapy of choice for the treatment of bouts of IBD (Ford, et al. 2011). GCs are not useful to prolong remission however and are not recommended for long term use (O'Donnell and O'Morain 2010). They are effective in relieving symptoms but do not generally induce mucosal healing in Crohn's disease (Gorard, et al. 1993).

Dexamethasone (DEX) is a potent synthetic glucocorticoid. It is used routinely as an immunosuppressive and anti-inflammatory agent (Faubion, et al. 2001). DEX induces anti-inflammatory effects through reducing NF-κB activity (Scheinman, et al. 1995).

Glucocorticoids (GC) can promote the synthesis of mucins (Das, et al. 2013). Mucins are large glycoproteins expressed on the luminal epithelial surface and protect the intestinal mucosa from microbial damage, enzymes and chemical substances (Bevins and Salzman 2011). Certain studies have recently highlighted the link between some aspects of the GC signalling pathways and their effects on the gut microbiota (Buren, et al. 2008; Lutgendorff, Akkermans and Söderholm 2008; O'Mahony, et al. 2009). For this reason, Huang et al explored the relationship between GCs, the intestinal microbiota and colonic physiology in mice. Mice were treated with DEX by injection once a day for 10 days (acute treatment) or once every 3 days over 4 weeks (chronic treatment). DEX treatment caused a substantial shift in the gut microbiota composition which was evident in both the acute and chronic treatment groups. Assessment of beta diversity revealed microbial-based clustering in DEX-treated animals but not in saline-treated controls. Following DEX treatment, the relative abundance of *Bifidobacterium* from the Actinobacteria phylum was significantly increased and the relative abundance of *Lactobacillus* was also increased when compared to

controls. On the other hand, a specific genus of gut bacteria known as *Mucispirillum* which is a colonic mucin degrader was noticeably absent after treatment with DEX. Furthermore, expression of *muc2*, which encodes the predominant mucin gene expressed in colonic goblet cells (Tytgat, et al. 1995) (Johansson, et al. 2008), in the proximal colon was significantly lower in DEX-treated mice. This suggests that there is a relationship between GC, the altered gut microbiota, and mucin regulation in the intestine (Huang, et al. 2015). The human mucin (Muc) family consists of several secreted (e.g. Muc1) or transmembrane (e.g. Muc2) Proteins. Muc2 is a leading component in the maintenance of colonic health (Van der Sluis, et al. 2006). Expression of *muc1* and *muc3* were also significantly lower after DEX treatment in mice (Huang, et al. 2015). Furthermore, DEX had no impact on mucin gene expression in Germ-Free (GF) mice, however, conventionalizing GF mice with microbiota from a DEX-treated donor led to a significant increase in both Muc1 and Muc2 expression in the proximal large intestine. This suggests that the microbiota have a key role in the regulation of colonic mucin synthesis (Huang, et al. 2015). To test the effect of the glucocorticoid-altered gut bacteria on colonic inflammation, cecal contents from DEX- or saline-treated Muc2-Knock Out (KO) mice were conventionalized into GF IL 10-KO mice. Of note, in the absence of Muc2, the gut microbiota was shown to confer a pro-inflammatory, colitogenic phenotype despite DEX treatment and GF IL 10-KO mice are a model of genetic susceptibility to colitis (Huang, et al. 2015). The DEX-mediated shifts in composition resulted in a microbiota that conferred protective effects in the IL 10 KO mice. Interestingly, the saline-treated microbiota led to a pronounced reduction in *muc2* gene expression relative to the DEX-treated microbiota. Taken together they

hypothesized that glucocorticoids may balance Muc2 expression as part of its anti-inflammatory mechanisms, decreasing its expression under healthy conditions in germ-free mice while under diseased conditions preventing further reduction to critically damaging levels thus preventing the exacerbation of colitogenic symptoms in IL10-KO mice. This study highlights the potential for using microbiota-targeted therapeutics for the treatment of bowel diseases.

Noguera *et al* (2018) studied the effect of glucocorticoids on the gastrointestinal microbiome of wild birds. In free-living vertebrates, the Hypothalamic-Pituitary-adrenal (HPA) axis is a neuroendocrine pathway that induces the release of stress hormones i.e., glucocorticoids in response to exposure to stress. Activation of the HPA axis experimentally demonstrated here by elevating corticosterone levels in gull chicks resulted in a generalized reduction of some components of the gastrointestinal microbiome, including some Operational Taxonomic Units (OTUs) that contain potential pathogenic species of bacteria in birds notably *Mycoplasma* and *Microvirga* (Noguera, et al. 2018). Additionally, chicks treated with corticosterone were more similar to each other in their microbial community composition than non-treated chicks. They found that elevated corticosterone levels in wild gull chicks reduced the abundance of some members of the bacterial gastrointestinal microbiome, notably, there was a reduction in the Firmicutes phylum which is the most abundant bacterial phyla in gull chicks. In addition, they found that corticosterone-implanted chicks grew less than control chicks. Firmicutes have been associated with the breakdown of complex biomolecules in vertebrates creating an additional energy source to the host (Flint, Bayer, et al. 2008). They hypothesized that this reduction in the abundance of

such beneficial commensal bacteria in corticosterone-implanted chicks may be attributed to the growth reduction in these birds.

Wu et al (2018) investigated the effects of glucocorticoid treatment on lipid metabolism, the gut microbiota and the involvement of the circadian clock in rats. Ninety male Wistar rats were divided into three experimental groups: a control group that received sterile water daily; a DEX 0.01mg/kg body weight per day treated group and a DEX 0.05mg/kg body weight per day treated group. The changes in biological rhythm, glycolipid metabolism, and gut microbiota were evaluated. Using 16S rRNA sequencing they found that there was a decrease in diversity of the rat cecal contents in the DEX-treated groups most notably they observed an increase in the relative abundance of Proteobacteria. Analysis of the faecal microbiota via 16S rRNA gene sequencing showed that there was a decrease in the relative abundance of the Firmicutes, Bacteroidetes, and Actinobacteria phyla in the DEX-treated groups compared to the control. In addition, the relative abundance of *Clostridiales* and *Lactobacillus* were decreased in the DEX-treated group. The relative abundance of β-Proteobacteria and Verrucomicrobia were unchanged with DEX treatment. They also observed that long term DEX exposure, especially at the higher concentrations, caused a reduction in colonic mucus secretion and down-regulation of Muc 2 and Muc 3 expression (which are related to mucin secretion). The rats in the DEX treated group showed slow body growth and a reduction in food intake. Increased abundance of Proteobacteria has been associated with a pro-inflammatory state in IBD (Mukhopadhya, et al. 2012).

Other locally acting steroids such as budesonide have potent and highly effective anti-inflammatory effects in the treatment of IBD. As mentioned earlier GCs have

limitations and adverse effects associated with long-term use. Additionally, GCs have been shown to have harmful effects in experimental colitis. With this in mind, Ocón et al., (2016) investigated the mechanism and possible implications of this phenomenon. To test this, they treated regular, and microbiota depleted (germ free) DSS colitis mice with Budesonide. They found that there was increased bacterial translocation to extraintestinal tissues in the DSS colitis mice (with a microbiota) with Budesonide treatment. They found that Budesonide weakened mucosal barrier function by interfering with epithelial dynamics and dampened the immune response in the context of significant mucosal injury, causing sepsis. This was in contrast to the microbiota depleted mice in which the DSS inflammatory response was greatly diminished. These findings suggest that Budesonide and possibly other GCs exerts a dual role in the inflamed intestine. This may be a contributing factor in limiting clinical usefulness of corticoids in inflammatory bowel disease and they suggest that this may play a role clinically by limiting their efficacy and usefulness in IBD patients (Ocón, et al. 2016).

Janus kinase (JAK) inhibitors (Tofacitinib)

Janus kinase (JAK) inhibitors are small molecules which act intracellularly and can modulate the response of a variety of proinflammatory cytokines implicated in UC pathogenesis. This makes them a completely novel type of therapy for the treatment of immune-mediated IBDs (including UC) which is in contrast to other currently available biologic agents (Ferrante and Sabino 2020)**.** Inhibitors of JAK-STAT signaling have emerged as a new therapeutic focus in IBD.

Tofacitinib is a first in class JAK 1/3 inhibitor and has been approved for use by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Hablot et al; (2020) recently presented their preclinical data showing that tofacitinib affects both the gut microbiota and mucosal immunity. In this study they tested the effect of tofacitinub on collagen induced arthritic (CIA) mice on the gut microbiota and the intestinal mucosa. 16S rRNA gene sequencing revealed alterations in beta-diversity (but not alpha diversity) in the periods of greatest disease activity. They also noted a decrease in potentially pathogenic members of the phyla Proteobacteria and Chlamydiae in tofacitinib-treated mice. They also observed an increase in some beneficial members of the Firmicutes and Actinobacteria phyla (Hablot, et al. 2020). The results from this study posit some interesting questions on whether the anti-arthritic effects of tofacitinib ILC3s are driven by the microbiota. Additionally, does disruption of mucosal immunity and hence disruption of the gut microbiota cause rare infections upon JAK inhibitor administration? The preliminary data from this study raises interesting questions. Follow on studies of the microbiota in tofacitinib-treated patients are required with the aim at identifying theragnostic faecal biomarkers (Hablot, et al. 2020).

Another drug which acts as a JAK/STAT inhibitor is TAK-242. TAK-242 is a small molecule suppressor of pathogen-induced release of inflammatory cytokines and acts by inhibiting TLR-4-mediated signalling. It also shows inhibitory effects on the production of nitric oxide (NO) or tumour necrosis factor (TNF)-alpha induced by the TLR4-specific ligand LPS (Kawamoto T 2008). TAK-242 markedly alleviated colonic lesions and DSS-induced colitis symptoms by inhibiting IL-17 release, promoting IL-10 release, downregulating TLR4 and JAK2/STAT3 mRNA and protein expression and increasing JAK2/STAT3

phosphorylation. Additionally, TAK-242 significantly increased the relative abundance of *Akkermansia*. Wang et al hypothesized that *Akkermansia* could ameliorate DSS-induced UC by regulating certain critical inflammatory responses, including pro- and anti-inflammatory cytokines. They further speculated that *Akkermansia* or other bacteria could induce protective effects of TAK-242 against colitis. Further studies need to be performed to confirm whether the effects of TAK-242 on colitis are due to the regulation of *Proteobacteria* and/or *Akkermansia.* (Wang J 2020)

In summary, the efficacy of JAK kinase inhibitors may be due in part in their ability to restore normal mucosal barrier function, permeability to macromolecules. It may also be attributed to regulation of the host immune response to and interactions with gut bacteria.

Non-orally Administered drugs and interactions with the microbiota

Other drugs which are non-orally administered for the treatment of IBD interact with and influence the gut microbiota. Monoclonal antibody therapy is the cornerstone inflammatory diseases including inflammatory bowel diseases (Baumgart and Sandborn 2012; Feuerstein and Cheifetz 2014).

Ustekinumab (UST) is a monoclonal antibody that is active against the shared p40 subunit of IL12 and IL23. It has been approved for the treatment of Crohn's disease (CD) and ulcerative colitis (UC) (Riol-Blanco, et al. 2010). Doherty et al (2018) investigated whether the microbiome could be used as a biomarker for response to anti-IL-23 therapy in patients with Crohn's disease. To do this they used 16S rRNA gene sequencing of faecal microbiota in subjects who were

recruited as part of a Phase II clinical trial. The purpose of the trial was to demonstrate the efficacy and safety of UST for the treatment of CD which was refractory to anti-TNF drugs (Sandborn, et al. 2008). They quantified disease severity with faecal microbiota composition and compared patients who responded to treatment to patients who did not respond to therapy. Response was defined by the Crohn's Disease Activity Index (CDAI) which is the standard method used to evaluate clinical symptoms and disease severity in CD. Remission of CD corresponds to a CDAI score below 150 points, (150-220 = mild to moderately active; 221-450 = moderate to severely active; 451-1100 = severely active to fulminant disease). In this study, response to UST treatment was accepted as a decrease in the CDAI score by 100 points or remission. Stool samples were collected before commencing treatment and during treatment at 4, 6 and 22 weeks and microbial compositions were investigated. Researchers found that the relative abundance of *Escherichia/ Shigella* spp was lower in subjects with remission after 6 weeks compared with subjects with active CD. In addition, *Faecalibacterium* and *Bacteroides* were significantly more abundant in the baseline (before treatment) stool samples from the subjects who were in remission six weeks after treatment than patients with active CD (p-values = 0.003 and 0.022, respectively). The Beta-Diversity of the microbiotas were significantly different between responders and non-responders (p=0.017) before treatment. Additionally, the median baseline microbial diversity in subjects with remission after 6 weeks was 1.7 times higher than in treated subjects with active CD (P=0.020) (Doherty, et al. 2018). *Faecalibacterium* frequently occurred in their models and was associated with health, comprising up to 5% of the relative abundance in healthy individuals, and was generally rare in CD patients. Each

subject in remission 6 weeks after UST therapy had measurable *Faecalibacterium* present in their baseline samples. This supports the hypothesis that *Faecalibacterium* impacts CD pathogenesis. It may even be beneficial to administer *Faecalibacterium* as a probiotic during therapy. *Escherichia/Shigella* also occurred frequently in their models and is known to be associated with inflammation and has been shown to be associated with CD. The results support the theory that the microbial composition before treatment together with changes due to therapeutic response has the potential to act as a biomarker for response or non-response to UST treatment.

Vedolizumab is a monoclonal antibody that targets the $\alpha_4\beta_7$ integrin and selectively prevents the infiltration of leucocytes into the gastrointestinal submucosa (Soler, et al. 2009). Ananthakrishnan et al., (2017) demonstrated the ability to predict response to anti-integrin treatment using the gut microbiome. Their study highlighted the role of both microbial taxonomy and functional pathways that may be relevant to treatment (Ananthakrishnan, et al. 2017).

Anti-Tumour Necrosis Factor (TNF) therapies have transformed IBD treatment. They have been shown to induce mucosal healing, minimize the use of steroids, reduce hopitalizations and surgeries and improve overall quality of life in IBD patients (Peyrin-Biroulet and Lémann 2011). The effectiveness of these therapies is limited, however. Up to 30% of IBD patients are non-responsive to treatment (show no clinical benefit after induction therapy). A further 30-40% of patients may lose responsiveness within one year requiring them to switch to another biologic or require dose-escalation (Papamichael, et al. 2015).

TNF is a cell signalling protein cytokine involved in inflammation whose primary role is regulation of immune cells. TNF signalling drives the expression of acute-phase proteins and influences cell migration, proliferation and cell death. TNF-alpha can have beneficial as well as harmful effects in the GIT. TNF-alpha has been associated with inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), leading to the development of anti-TNF therapies for IBD patients (Behm and Bickston 2008). TNF-alpha inhibitors are used for the treatment of a number of other inflammatory diseases including rheumatoid arthritis (RA) (Olsen and Stein 2004), spondyloarthritis (SpA) (Menegatti, et al. 2020) and psoriasis (Yost and Gudjonsson 2009). Commonly used TNF-α inhibitors include infliximab, adalimumab, golimumab and certolizumab. A study using TRUC (T-bet-/- RAG2-/- ulcerative colitis) mice investigated the effect of TNF- α inhibitors on gut microbial composition (Rooks, et al. 2014). These mice develop colitis which is partly caused by dysregulated colonic TNF-α production and can be ameliorated with TNF-α neutralizing antibodies or infusion of immunosuppressive Regulatory T cells (Tregs) (Garrett, et al. 2007). In their study, (Rooks, et al. 2014) reported an increase in the abundance of Firmicutes and a decrease in proportions of Bacteroidetes in TRUC mice (mice that develop a UC-type disease) when treated with anti-TNF antibodies post colitis compared to untreated and antibiotic-treated mice. Another study investigated the effect of TNF-α inhibitors on the faecal microbiota composition of spondyloarthritis (SpA) patients. There are overlapping similarities between SpA and IBD in terms of gut inflammation in some patients. In this study, (Bazin, et al. 2018) used 38 stool samples from 19 (SpA) patients before treatment and three months after treatment with TNF-α inhibitors. Patients

who didn't respond to treatment had drastic differences in microbiota composition at order level, before and after treatment. In contrast, patients who responded to treatment displayed only few mild differences before and after treatment. This suggests that patients who responded to treatment had more stable microbial compositions than non-responders. Upon comparing Alpha-Diversity as assessed by Shannon and Simpson indices the researchers observed a difference between the microbiota of responders (R) and non-responders (NR). Alpha-diversity of NR was lower before treatment compared to those who responded to treatment. A high proportion of *Burkholderiales* before treatment was associated with a good clinical response to treatment and a high proportion of *Dialister* sp. was associated with a good clinical response after three months. There were a number of taxa that differed in proportion at the two time-points (prior to treatment and three months post treatment), with four decreasing and 15 increasing at three months. These differences were not significant after multiple-test correction (possibly due to the small cohort size). Patients that responded well to treatment had few taxa changes after treatment, and changes were moderate, with most of the taxa (6 out of 8) being reduced. In contrast, for each of the non-responding patients, many bacterial orders exhibited drastic changes with the corresponding taxa. These results suggest that patients who did not respond to anti-TNF-α treatment had high disease activity and unstable microbiota composition. This suggests that patients with reduced initial microbiota diversity are more likely to fail to respond to the anti-TNF-α treatment. All these results suggest possible biomarkers for anti-TNF-α efficacy in SpA patients (Bazin, et al. 2018). In summary, the modifications observed in microbiota composition as a result of TNF-α inhibitors could be caused by

indirect or direct effects. These treatments down-regulate inflammation, heal the digestive mucosa restoring normal structure of digestive epithelium and restore tolerance functions toward mucosal microbiota, all of which could either indirectly or directly change microbiota composition. More *In vitro* studies are needed to test the direct effects of TNF-α inhibitors on the gut microbiota.

Summary

To summarize, there are various ways the microbiota can interact with orally-ingested drugs for the treatment of IBD. Pharmamicrobiomics and its relevance to and role in precision medicine and targeted therapy will lead the way in this ever expanding field. Some drugs alter the microbial composition either directly or indirectly. Table 2 gives an overview of the drugs highlighted in this review and possible opportunities for more personalized approach to treatment.

The gut microbiota can modulate prodrugs such as sulphazalzine and convert them into their active form. In the case of DEX this modulation appears significant for efficacy. DEX can also reduce virulence of microbiota constituents by affecting gene expression. MTX was developed to target human dihydrofolate reductase (DHFR), it does not specifically to target the human enzyme however. *In vivo* MTX may not only affect community composition but it can also affects the human gut microbiota at a transcriptional and metabolic level. Further studies are needed to investigate these transcriptional and metabolic changes further.

The microbiota can also be used as a biomarker of efficacy as with TNF inhibitors and Ustekinumab. In such cases, it could be feasible to induce alterations to the microbiota, essentially converting non-responders to responders

to ensure an effective response. Performing Faecal Microbiota Transplants (FMT) in these patients with a ‘responder faecal microbiota’ may induce remission of the disease following drug treatment. Further research into faecal microbiota as a source of biomarkers for predicting therapeutic response could eventually allow for the screening of patients using stool samples at diagnosis to better inform treatment decisions for a wide range of diseases. Prior to the initiation of therapy, patients could have their faecal microbiome analysed. The microbial community data could then be used to direct the modification of a patient’s microbiota during treatment with the goal of improving treatment outcomes.

Understanding the factors underpinning such pharmacokinetic variability is of value in an industry and healthcare system progressively adopting a personalized medicine approach. There are various ways to manipulate the structure of these xenobiotics to make them more soluble and more bioavailable. Drugs can be administered as phosphate or sulfate ester prodrugs such as Sulfasalazine, for example, which was mentioned earlier.

The accelerating advent of modern pharmaceuticals characterized by poor solubility provides increasing need to consider the intraluminal factors affecting the bioavailability of biopharmaceuticals when developing new drugs. Monitoring the solubilization capacity and production of bile salts by both the host and the gut microbiota should be considered to predict with more accuracy the intestinal bioavailability and efficacy of drugs.

Follow-on longitudinal analysis in patients given a variety of treatment regimens would help to further clarify on the gut microbiome common clinical confounders.

This review highlights the importance of taking a broader look at toxicology of non-antibiotic drugs for our associated microbial communities.

In conclusion, these studies discussed in this review demonstrate the importance and value of continued research and use of integrated studies to begin to elucidate the causality and mechanism for these complex drug-microbiome-host interactions.

The observed drug-induced changes in microbial community structure associated with patient response are providing a crucial tool to elucidate the initiation of therapy and a first step towards determining which bacterial taxa can prove beneficial to successful treatment. Increasing evidence shows that the microbiota can be used to predict response to for IBD treatment specifically and can result in more personalized treatment and faster achievement of remission and increasing patients' quality of life.

Recently developed mechanisms such as metabolomics for identifying the xenobiotic metabolizing capabilities of gut microbes are still in their infancy but will be invaluable in the future of understanding the role the microbiota play in the metabolism of orally injested drugs for the treatment of IBD.

Chapter 1. Figures

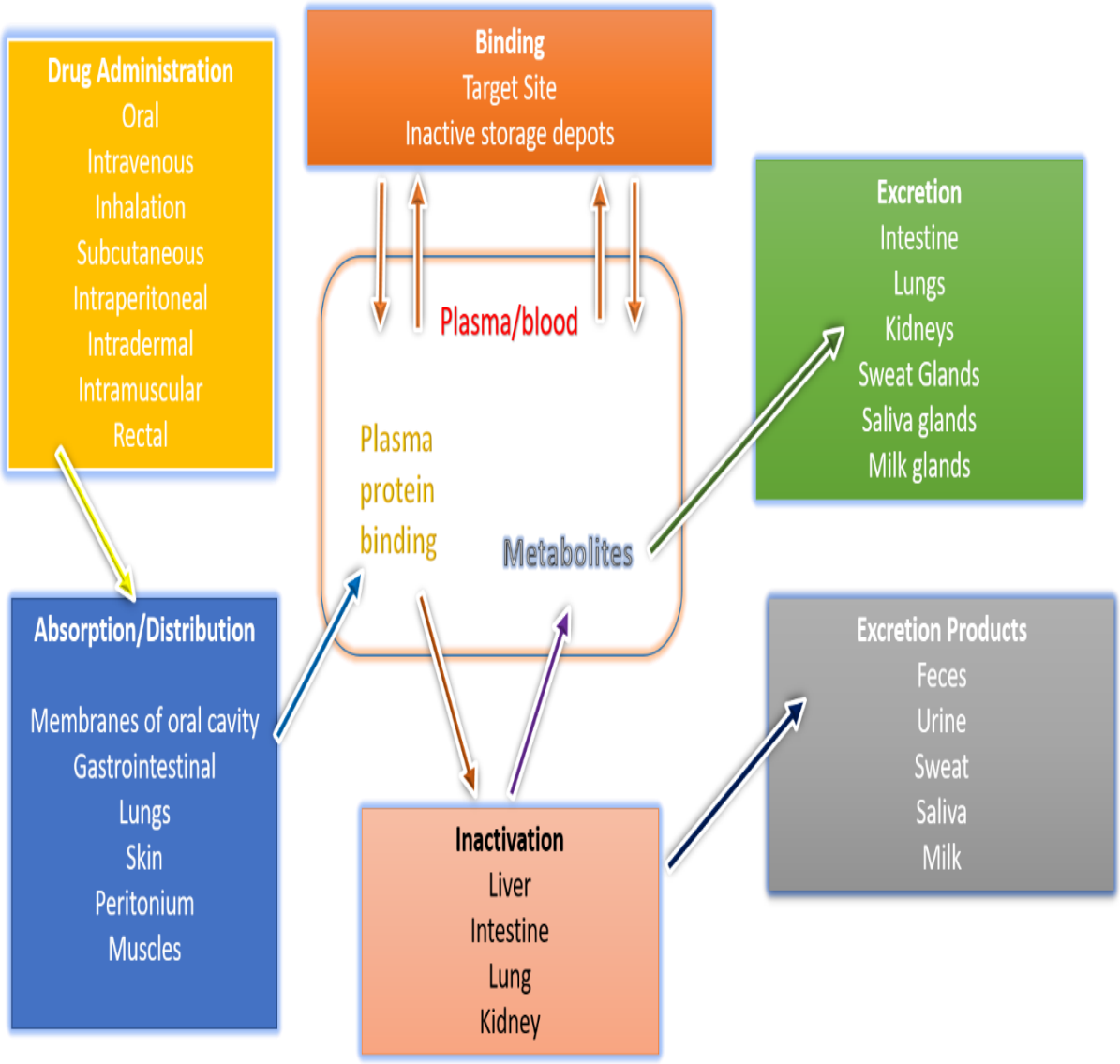


Figure 1. Drug Metabolism in the Body.

The different routes that drugs can be administered include oral, intravenous, subcutaneous, intraperitoneal, intradermal, intramuscular and rectal. After administration of the drug it is absorbed into the circulation, then distributed to various tissues throughout the body; broken down or metabolized and finally, eliminated or excreted.

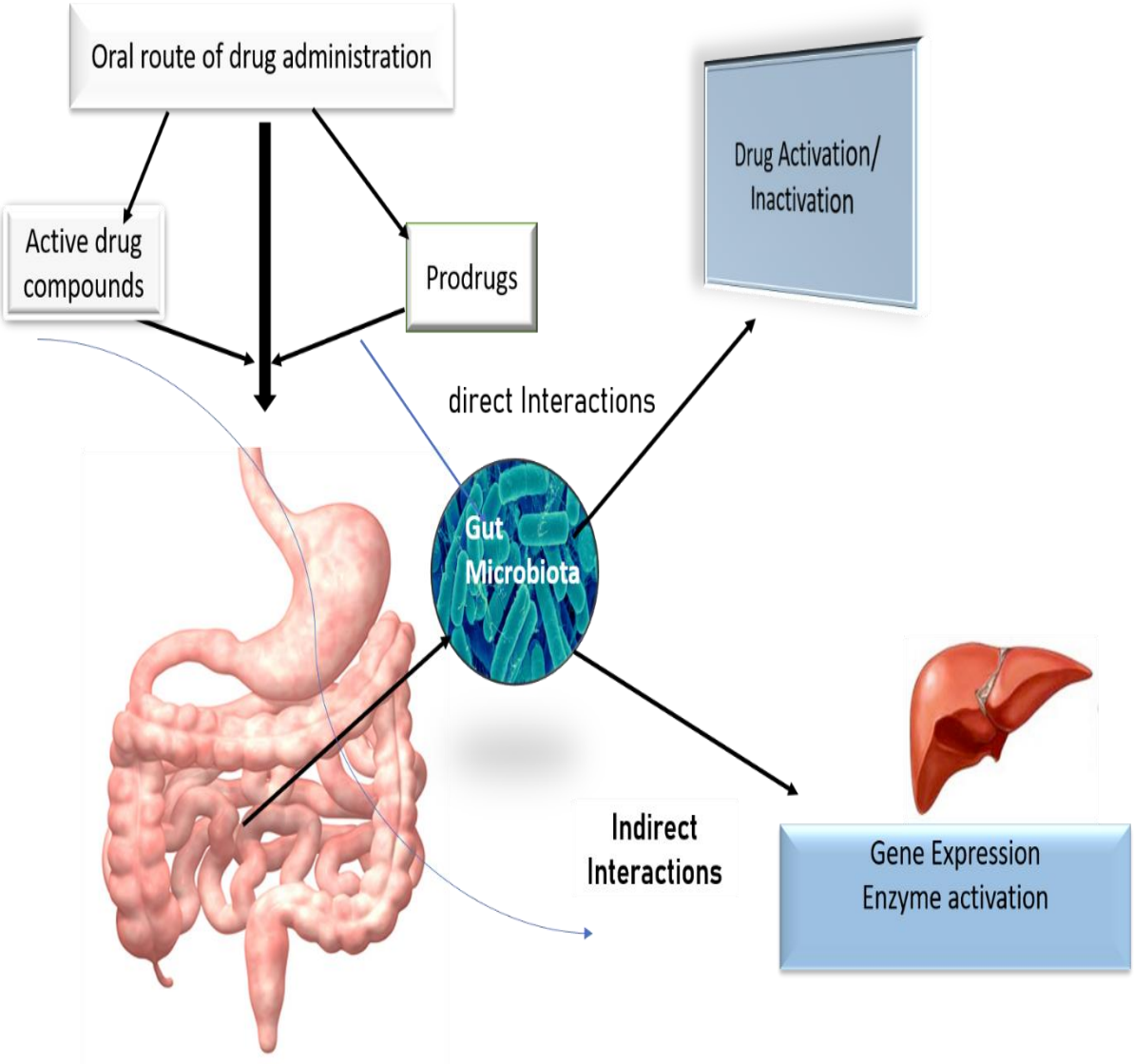


Figure 2. Oral drug administration and possible interactions with gut microbiota

The gut microbiome can influence xenobiotic metabolism by both direct and indirect mechanisms which modulates efficacy, absorption, and bioavailability of the compounds. Direct mechanisms include activation (for example conversion of prodrugs into active drugs) and deactivation. Xenobiotics may also bind directly to bacterial cells. Indirect mechanisms include enterohepatic cycling, as microbial biotransformation of compounds conjugated in the liver releases the conjugated group, reactivating the compound. Additionally, host hepatic CYP genes expression (involved in xenobiotic metabolism) is also influenced by the gut microbiome.

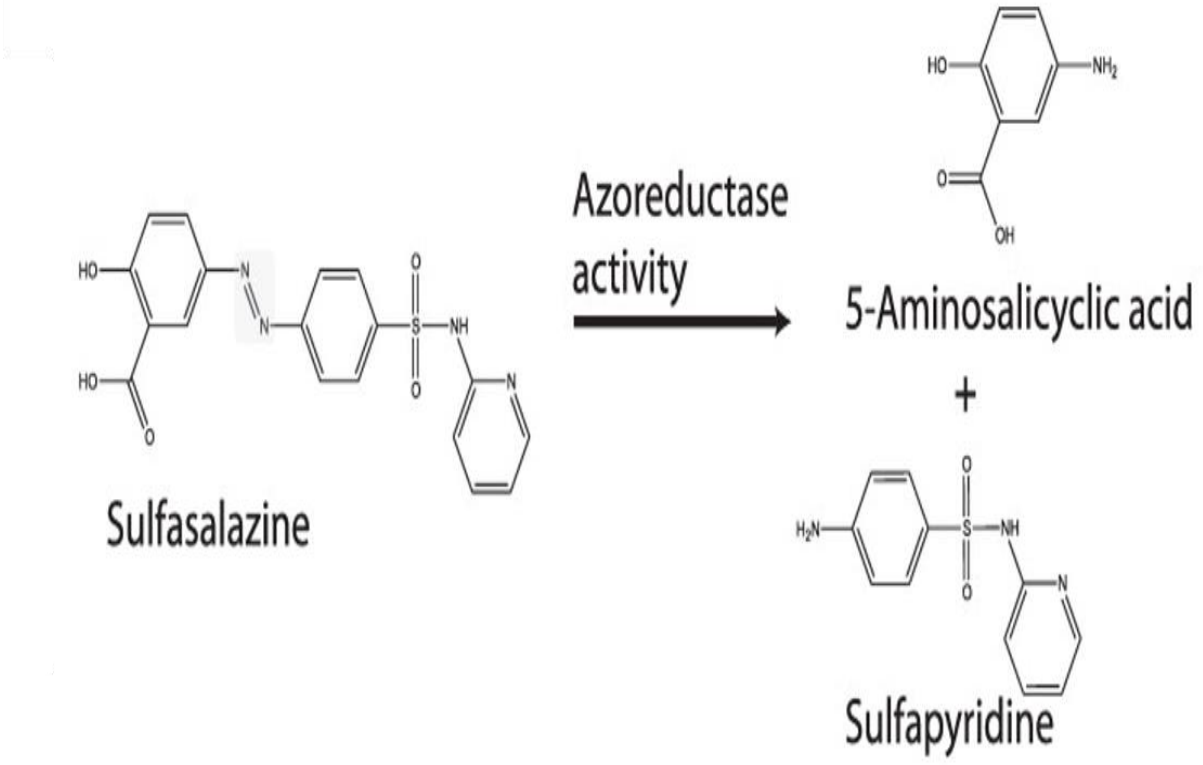


Figure 3. Azoreduction of Sulfasalazine

Sulfasalazine is metabolized by the azoreductase activity of bacteria in the gut resulting in the release of sulfapyridine and 5-aminosalicylate.

Table 1. Commonly used medicinal forms of Mesalazine (British National Formulary (BNF) updated March 2021)

Drug Name	Medicinal Form
Mesavant XL	Modified release tablet
Pentasa	Modified release tablet
Salofalk	Foam
Asacol MR	Gastroresistant tablet
Octasa MR	Gastroresistant tablet
Salofalk	Gastroresistant tablet
Pentasa	Suppository
Salofalk	Suppository
Pentasa	Modified release granules
Salofalk	Modified release granules
Pentasa	Enema
Salofalk	Enema

Drug	Principle modes of action	Direct or indirect effects on gut microbiota	Known Biotransformation of compoundby gut microbiota	Gut Microbiota Interactions	Opportunities for precision medicine
Sulfasalazine (SASP)	Antibacterial effect (sulfapyridine component) Downregulation of inflammation (5-ASA component)	Both	Azoreduction	Split by bacterial azoreductases to release 5-ASA and sulfapyridine	Assessment of baseline microbiota composition prior to and also during treatment to enhance metabolism of drug and also to assess presence of Mycoplasma for example to avoid negative side effects associated with fertility Probiotic use during treatment to enhance metabolism of the drug
5-aminosalicylic acid (5-ASA) (Mesalamine)	Downregulation of inflammation by reduction of inflammatory cytokine release promotion of cellular proliferation and inhibition of apoptosis Possible antibacterial effect	Both	Azoreduction	Production of N-acetyl-5-aminosalicylic acid	Assessment of baseline microbiota composition prior to and also during treatment to enhance metabolism of drug Probiotic use during treatment to enhance metabolism of the drug Assessment of effect on bacterial gene expression e.g invasiveness genes during treatment
Corticosteroids (Dexamethasone)	Inhibition of inflammatory processes Induction of mucosal healing	Indirect			Monitoring of colonic mucin synthesis (gene expression) and cytokine level regulation and Assessment of shifts in gut bacteria composition and gene expression associated with inflammatory response or mucosal healing
Immunomodulatory drug (Ustekinumab)	Monoclonal antibody active against IL12 and IL23	Both			Possible biomarker for drug efficacy and patient response. increased *Faecalibacterium* and *Bacteroides* in patients could enhance efficacy of Ustekinumab
Anti-tumour necrosis factor-alpha (TNF-α) inhibitors	Reduction in inflammation reducing TNF signalling which drives the expression of acute-phase proteins and influences cell migration, proliferation and cell death.	Both			Assessment of baseline microbiota composition prior to and also during treatment to enhance treatment efficacy and use as biomarkers to predict anti-TNF-α efficacy e.g high proportion of *Burkholderiales* before treatment was associated with a good clinical response to treatment and a high proportion of *Dialister* sp. was associated with a good clinical response after three months.
Immunosuppressant Methotrexate	folate antagonist anti-proliferative and anti-inflammatory activity	Both	Hydrolysis	Production of 4-amino-4-deoxy-N10-methylpteroic acid	Assessment of baseline microbiota composition prior to and also during treatment to enhance possible microbiota biomarkers for MTX efficacy carboxypeptidase-G1 ($CPDG_1$) treatment to reduce renal injury for use in both adult and pediatric patients with renal dysfunction who have levels of plasma MTX

Table 2. Drugs used for the treatment of Inflammatory Bowel Disease, interactions with the gut microbiota and possible opportunities for precision medicine

References

Abelson, H., Ensminger, W., & Kufe, D. (1979). High-dose methotrexate-carboxypeptidase G1 - a selective approach to the therapy of central nervous system tumors. In B. G. Kisliuk RL, *Chemistry and Biology of Pteridines* (pp. 629–633). New York: Elsevier North Holland.

Abelson, Kufe, & Skarin. (1981). Treatment of central nervous system tumors with methotrexate. *Cancer Treat Rep, 65*(suppl 1), 137–140.

Albenberg, L., & Wu, G. (2014). Diet and the intestinal microbiome: associations, functions, and implications for health and disease. *Gastroenterology, 146*(6), 1564-1572.

Ananthakrishnan, A., Luo, C., Yajnik, V., Khalili, H., Garber, J., Stevens, B., Xavier, R. (2017). Gut Microbiome Function Predicts Response to Anti-integrin Biologic Therapy in Inflammatory Bowel Diseases. *Cell Host Microbe, 21*(5), 603-610.e3. doi:10.1016/j.chom.2017.04.010

Andrews, C., Griffiths, T., Kaufman, J., Vergnolle, N., Surette, M., & Rioux, K. (2011). Mesalazine (5-aminosalicylic acid) alters faecal bacterial profiles, but not mucosal proteolytic activity in diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther, 34*(3), 374-83. doi:10.1111/j.1365-2036.2011.04732.x

Andrews, Griffiths, T., Kaufman, J., Vergnolle, N., & Surette, M. a. (2011). Mesalazine (5-aminosalicylic acid) alters faecal bacterial profiles, but not mucosal proteolytic activity in diarrhoea-predominant irritable bowel syndrome. *Alimentary Pharmacology & Therapeutics, 34*, 374-383. doi:10.1111/j.1365-2036.2011.04732.x

Ardizzone, S., Cassinotti, A., Manes, G., & Bianchi Porro, G. (2010). Review: Immunomodulators for all patients with inflammatory bowel disease? *Therapeutic Advances in Gastroenterology, 3*(1), 31-42. doi:10.1177/1756283X09354136

Are, A., Aronsson, L., Wang, S., Greicius, G., Lee, Y., Gustafsson, J., Arulampalam, V. (2008). Enterococcus faecalis from newborn babies regulate endogenous PPARγ activity and IL-10 levels in colonic epithelial cells. *Proceedings of the National Academy of Sciences, 105*(6), 1943-1948. doi:10.1073/pnas.0711734105

Arkhipova, O., & Akimenko, V. (2005). Unsaturated organic acids as terminal electron acceptors for reductase chains of anaerobic bacteria. *Microbiology, 76*, 725–737.

Azad Khan, A., Piris, J., & Truelove, S. (1977). An experiment to determine the active therapeutic moiety of sulphasalazine. *Lancet, 2*, 892–895. doi:10.1016/S0140-6736(77)90831-5

Baumgart, D., & Sandborn, W. (2012). Crohn's disease. *The Lancet, 380*(9853), 1590-605. doi:10.1016/S0140-6736(12)60026-9

Bayan, M., & Bayan, R. (2020). Recent advances in mesalamine colonic delivery systems. *Futur J Pharm Sci, 6*(43). Retrieved from https://doi.org/10.1186/s43094-020-00057-7

Bazin, T., Hooks, K., Barnetche, T., Truchetet, M., Enaud, R., Richez, C., . . . Schaeverbeke, T. (2018). Microbiota Composition May Predict Anti-Tnf Alpha Response in Spondyloarthritis Patients: an Exploratory Study. *Scientific Rep, 8*, 5446. doi:10.1038/s41598-018-23571-4

Begley, M., Gahan, C., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiol Rev, 29*(4), 625-51.

Behm, B., & Bickston, S. (2008). Tumor necrosis factor-alpha antibody for maintenance of remission in Crohn's disease. *Cochrane Database of Systematic Reviews*(1). doi:10.1002/14651858.CD006893

Bertin, B., Dubuquoy, L., Colombel, J., & Desreumaux, P. (2013). PPAR-gamma in ulcerative colitis: a novel target for intervention. *Curr Drug Targets, 14*(12), 1501-7. doi:10.2174/13894501113149990162

Bevins, C., & Salzman, N. (2011). Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nature Reviews Microbiology, 9*(5), 356-68.

Bleyer, W. (1977). Methotrexate: clinical pharmacology, current status and therapeutic guidelines. *Cancer Treatment Reviews, 4*(2), 87-101.

Bolin, J., Filman, D., Matthews, D., Hamlin, R., & Kraut, J. (1982). Crystal structures of Escherichia coli and Lactobacillus casei dihydrofolate reductase refined at 1.7 A resolution. I. General features and binding of methotrexate. *Journal of Biological Chemistry, 257*(22), 13650-13662.

British National Formulary (BNF). (updated March 2021). London: BMJ Group. Retrieved from https://bnf.nice.org.uk

Buren, J., Lai, Y., Lundgren, M., Eriksson, J., & Jensen, J. (2008). Insulin action and signalling in fat and muscle from dexamethasone-treated rats. *Arch Biochem Biophys, 474*, 91-101. doi:10.1016/j.abb.2008.02.034

Cerf-Bensussan, N., & Gaboriau-Routhiau, V. (2010). The immune system and the gut microbiota: friends or foes? *Nat Rev Immunol*, 735-44. doi:10.1038/nri2850

Chabner, B., Allegra, C., Curt, G., Clendeninn, N., Baram, J., Koizumi, S., .Jolivet, J. (1985). Polyglutamation of Methotrexate. *The Journal of Clinical Investigation, 76*, 907-912.

Chabner, Johns, & Bertino. (1972). Enzymatic cleavage of methotrexate provides a method for prevention of drug toxicity. *Nature, 239*, 395–397.

Chatzinoff, M., Guarino, J., Corson, S., Batzer, F., & Friedman, L. (1988). Sulfasalazine-induced abnormal sperm penetration assay reversed on changing to 5-aminosalicylic acid enemas. *Digestive diseases and sciences, 33*(1), 108-110.

Clark, R. (2002). The role of PPARs in inflammation and immunity. *Journal of leukocyte biology, 71*(3), 388-400. doi:10.1189/jlb.71.3.388

Clayton, T., Baker, D., Lindon, J., Everett, J., & Nicholson, J. (2009). Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proceedings of the National Academy of Sciences, 106*(34), 14728-14733.

Collins, S., & Patterson, A. (2020). The gut microbiome: an orchestrator of xenobiotic metabolism. *Acta Pharm Sin B, 10*(1), 19-32. doi:10.1016/j.apsb.2019.12.001

Cooke, E. (1969). Faecal flora of patients with ulcerative colitis during treatment with salicylazosulphapyridine. *Gut, 10*, 565–568. doi:10.1136/gut.10.7.565

Das, I., Png, C., Oancea, I., Hasnain, S., Lourie, R., Proctor, M., . . . McGuckin, M. (2013). Glucocorticoids alleviate intestinal ER stress by enhancing protein folding and degradation of misfolded proteins. *J Exp Med, 210*(12), 12.

Doherty, M., Ding, T., Koumpouras, C., Telesco, S., Monast, C., Das, A., . . . Schloss, P. (2018). Fecal microbiota signatures are associated with response to ustekinumab therapy among Crohn's disease patients. *mBio, 9*, e02120-17. doi:10.1128/mBio.02120-17

Donehower, R., Hande, K., Drake, J., & Chabner, B. (1979). Presence of 2, 4-diamino-N10-methylpteroic acid after high-dose methotrexate. *Clinical Pharmacology & Therapeutics*. *26*(1), 63-72.

Enright, E. F. (2017). Impact of Gut Microbiota-Mediated Bile Acid Metabolism on the Solubilization Capacity of Bile Salt Micelles and Drug Solubility. *Molecular pharmaceutics, 14*(4), 1251–1263. Retrieved from https://doi.org/10.1021/acs.molpharmaceut.6b01155

Enright, E., Joyce, S., Gahan, C., & Griffin, B. (2017). Impact of gut microbiota-mediated bile acid metabolism on the solubilization capacity of bile salt micelles and drug solubility. *Molecular pharmaceutics, 14*(4), 1251-1263.

Faubion, W. J., Loftus, E. J., Harmsen, W., Zinsmeister, A., & Sandborn, W. (2001). The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology, 121*, 255–260. doi:10.1053/gast.2001.26279

Feagan, B., & Alfadhli, A. (2004). Methotrexate in inflammatory bowel disease. *Gastroenterol Clin North Am, 33*(2). doi:10.1016/j.gtc.2004.03.001

Ferrante, M., & Sabino, J. (2020). Efficacy of JAK inhibitors in Ulcerative Colitis. *J Crohns Colitis, 14*((Supplement 2)), S737-S745. doi:10.1093/ecco-jcc/jjz202

Feuerstein, J., & Cheifetz, A. (2014). Ulcerative colitis: epidemiology, diagnosis, and management. *InMayo Clinic Proceedings, 89*(11), 1553-1563.

Flint, H., Bayer, E., Rincon, M., Lamed, R., & White, B. (2008). Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat. Rev. Microbiol, 6*, 121-131. doi:10.1038/nfmicro1817

Ford, A., Bernstein, C., Khan, K., Abreu, M., Marshall, J., Talley, N., & Moayyedi, P. (2011). Glucocorticosteroid therapy in inflammatory bowel disease: systematic review and meta-analysis. *American Journal of Gastroenterology, 106*(4), 590-9. doi:10.1038/ajg.2011.70

Fu, Z., Selwyn, F., Cui, J., & Klaassen, C. (2017). Intestinal Expression of XPGs in Germ-Free Mice. *Drug Metabolism and Disposition, 45*(12), 1225-1238. Retrieved from https://doi.org/10.1124/dmd.117.077313

Gallant C, K. P. (1986). Oral glucocorticoids and their complications. A review. *J Am AcadDermatol, 14*, 161–177. doi:10.1016/S0190-9622(86)70018-2

Garrett, W., Lord, G., Punit, S., Lugo-Villarino, G., Mazmanian, S., Ito, S., . . . Glimcher, L. (2007). Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell, 131*(1), 33-45. doi:10.1016/j.cell.2007.08.017

Gertzen, H., Kjellander, J., Sundberg-Gillar, B., & Jarrnerot, G. (1985). *In vitro* effects of sulphasalazine, azodisal sodium, and their metabolites on Clostridium difficile and some other faecal bacteria. *Scandinavian journal of gastroenterology, 20*(5), 607–612. doi:10.3109/00365528509089704

Gorard, D., Hunt, J., Payne-James, J., Palmer, K., Rees, R., Clark, M., Silk, D. (1993). Initial response and subsequent course of Chrohn's disease treated with elemental diet or prednisolone. *Gut, 34*(9), 1198-202. doi:10.1136/gut.34.

Grant. I R. (2005). Zoonotic potential of Mycobacterium avium ssp. paratuberculosis:the current position. *J. Appl. Microbiol, 98*, 1282–1293.

Green, J. M. (2012). Glucarpidase to combat toxic levels of methotrexate in patients. *Therapeutics and Clinical Risk Management, 8*, 403–13. doi:10.2147/TCRM.S30135

Greenstein RJ, S. L. (2007). On the action of 5-amino-salicylic acid and sulfapyridine on M. avium including subspecies paratuberculosis. *PLoS One, 2*(6), e516. doi:10.1371/journal.pone.0000516

Gu, X., & Manautou, J. (2012). Molecular mechanisms underlying chemical liver injury. *Expert Rev Mol Med, 3*(14), e4. doi:10.1017/S1462399411002110

Guðmundsdóttir, J., Fredheim, E., Koumans, C., Hegstad, J., Tang, P., Andersson, D., Johnsen, P. (2020). The chemotherapeutic drug methotrexate selects for antibiotic resistance. *bioRxiv*.

Hablot, J., Ferhat, M., Lavelle, A., Salem, F., Taieb, M., Medvedovic, J., Moulin, D. (2020). Tofacitinib treatment alters mucosal immunity and gut microbiota during experimental arthritis. *Clin Transl Med, 10*(5), e163. doi:10.1002/ctm2.163

Haiser, H., & Turnbaugh, P. (2013). Developing a metagenomic view of xenobiotic metabolism. *Pharmacol Res, 69*(1), 21-31. doi:10.1016/j.phrs.2012.07.009

Hazenbe, M., Bakker, M., Both-Patoir, H., Ruseler-van Embden, J., & Schröder, A. (1982). Effect of sulphasalazineon the human intestinal flora. *J. Appl. Bacteriol, 52*, 103–107. doi:10.1111/j.1365-2672.1982.tb04379.x

Heimdal K, H. H. (1992). High incidence of serious side effects of high-dose dexamethasone treatment in patients with epidural spinal cord compression. *J Neurooncol, 12*(2), 141-4. doi:10.1007/BF00172664

Heimesaat, M., Fischer, A., Siegmund, B., Kupz, A., Niebergall, J., Fuchs, D., . . . Bereswill, S. (2007). Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via Toll-like receptors 2 and 4. *PloS one, 2*(7), e662. doi:10.1371/journal.pone.0000662

Herfarth, H., Kappelman, M., Long, M., & Isaacs, K. (2016). Use of methotrexate in the treatment of inflammatory bowel diseases (IBD). *Inflammatory bowel diseases, 22*(1), 224-233. doi:10.1097/MIB.0000000000000589.

Hinderliter, P., & Saghir, S. (2014). *Pharmacokinetics.* (P. Wexler, Ed.) Academic Press. Retrieved from https://doi.org/10.1016/B978-0-12-386454-3.00419-X.

Honap, S. J.-H.-T. (2020). Anti-Mycobacterium paratuberculosis (MAP) therapy for Crohn's disease: an overview and update. *Frontline Gastroenterology*.

Hua, S. (2020). Advances in Oral Drug Delivery for Regional Targeting in the Gastrointestinal Tract-Influence of Physiological, Pathophysiological and Pharmaceutical Factors. *Frontiers in pharmacology, 11*, 524.

Hua, S. M., Schneider, J., & Keely, S. (2015). Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue. *Nanomedicine: nanotechnology, biology and medicine, 11*(5), 1117-1132.

Huang, E., Inoue, T., Leone, V., Dalal, S., Touw, K., Wang, Y., Chang, E. (2015). Using corticosteroids to reshape the gut microbiome: implications for inflammatory bowel diseases. *Inflamm Bowel Dis, 21*(5), 963–972. doi:10.1097/MIB.0000000000000332.

Jamalizadeh Bahaabadi, S., Mohseni Moghadam, N., Kheirkhah, B., Farsinejad, A., & Habibzadeh, V. (2014). Isolation and molecular identification of Mycoplasma hominis in infertile female and male reproductive system. *Nephro-urology monthly, 6*(6), e22390. doi:10.5812/numonthly.22390

Jamei, M., Turner, D., & Yang, J. (2009). Population-Based Mechanistic Prediction of Oral Drug Absorption. *AAPS J, 11*(2), 225-237. doi:10.1208/s12248-009-9099-y

Jochum, L., & Stecher, B. (2020). Label or Concept – What Is a Pathobiont? *Trends in Microbiology, 28*(10), 789-792. Retrieved from https://doi.org/10.1016/j.tim.2020.04.011

Johansson, M., Phillipson, M., Petersson, J., Velcich, A., Holm, L., & Hansson, G. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A, 105*(39), 15064-15069. doi:10.1073/pnas.0803124105

John, & Morrison. (2015). Non-classical azoreductase secretion in Clostridium perfringens in response to sulfonated azo dye exposure. *Anaerobe, 34*, 34-43. doi:10.1016/j.anaerobe.2015.04.007

Karl JP, H. A.-F. (2013). Effects of Psychological, Environmental and Physical Stressors on the Gut Microbiota. *Front Microbiol, 9*. doi:10.3389/fmicb.2018.02013

Kaufman J, G. T. (2009). Effects of Mesalamine (5-5-Aminosalicylic Acid) on Bacterial Gene Expression. *Inflammatory Bowel Diseases, 15*(7), 985–996. doi:10.1002/ibd.20876

Kawamoto T, I. M. (2008). TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *European journal of pharmacology, 584*(1), 40-8. doi:10.1186/s13075-020-2097-2

Kopytek, S., Dyer, J., Knapp, G., & Hu, J. (2000). Resistance to methotrexate due to AcrAB-dependent export from Escherichia coli. *Antimicrobial agents and chemotherapy, 44*(11), 3210-3212.

Korecka, A., de Wouters, T., Cultrone, A., Lapaque, N., Pettersson, S., Doré, J., Arulampalam, V. (2013). ANGPTL4 expression induced by butyrate and rosiglitazone in human intestinal epithelial cells utilizes independent pathways. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 304*(11), G1025-37. doi:10.1152/ajpgi.00293.2012

Kumar, M., Kaushik, S., Saini, V., Kumar, C., Bhatt, S., Malik, A., & Pandurangan, A. (2018). Formulation development and evaluation of colon targeted beads of mesalamine. *J Drug Des Res, 5*(2), 1067.

Lawrance, I. (2011). Topical agents for idiopathic distal colitis and proctitis. *J Gastroenterol Hepatol, 26*(1), 36-43. doi:10.1111/j.1440-1746.2010.06497.x.

Lee, H., Zhang, H., Orlovich, D., & Fawcett, J. (2012). The influence of probiotic treatment on sulfasalazine metabolism in rat. *Xenobiotica, 42*(8), 791-7.

Lefebvre, A., Paulweber, B., Fajas, L., Woods, J., McCrary, C., Colombel, J., . . . Desreumaux, P. (1999). Peroxisome proliferator-activated receptor gamma is induced during differentiation of colon epithelium cells. *Journal of Endocrinology, 162*(3), 331-40. doi:10.1677/joe.0.1620331

Linskens, R., Huijsdens, X., Savelkoul, P., Vandenbroucke-Grauls, C., & Meuwissen, S. (2001). The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. *Scandinavian Journal of Gastroenterology, 36*(234), 29-40.

Lipinski, C., Lombardo, F., Dominy, B., & Feeney, P. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev, 46*(1-3), 3-26. doi:10.1016/s0169-409x(00)00129-0

Lozupone, C., Stombaugh, J., Gordon, J., & al, e. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, 220–230. doi:10.1038/nature11550

Lutgendorff, F., Akkermans, L., & Söderholm, J. (2008). The role of microbiota and probiotics in stress-induced gastro-intestinal damage. *Curr Mol Med, 8*(4), 282-98. doi:10.2174/156652408784533779

Maurice, C., Haiser, H., & Turnbaugh, P. (2013). Xenobiotics shape the physiology and gene expression of the active human gut microbiome. *Cell, 152*, 39-50.

McCullough, J., Chabner, B., & Bertino, J. (1974). Purification and properties of carboxypeptidase G 1. *J Biol Chem, 246*, 7207–7213.

Menegatti, S., Guillemot, V., Latis, E., Yahia-Cherbal, H., Mittermüller, D., Rouilly, V., Leloup, C. (2020). Immune response profiling of patients with spondyloarthritis reveals signalling networks mediating TNF-blocker function in vivo. *Annals of the Rheumatic Diseases*. doi:10.1136/annrheumdis-2020-218304

Morrison, J., & John, G. (2015). Non-classical azoreductase secretion in Clostridium perfringens in response to sulfonated azo dye exposure. *Anaerobe, 34*, 34-43. doi:10.1016/j.anaerobe.2015.04.007

Morrison, Wright, & John. (2012). Identification, Isolation and characterization of a novel azoreductase from Clostridium perfringens. *Anaerobe, 18*(2), 229-34. doi:10.1016/j.anaerobe.2011.12.006

Mukhopadhya, I., Hansen, R., El-Omar, E., & Hold, G. (2012). IBD-what role do Proteobacteria play? *Nat Rev Gastroenterol Hepatol, 9*(4), 219-30. doi:10.1038/nrgastro.2012.14

Nayak, R., Alexander, M., & Stapleton-Grey, K. (2019). Perturbation of the human gut microbiome by a non-antibiotic drug contributes to the resolution of autoimmune disease. *bioRxiv*, 600155.

Nepelska, M., De Wouters, T., Jacouton, E., Béguet-Crespel, F., Lapaque, N., Doré, J., Blottière, H. (2017). Commensal gut bacteria modulate phosphorylation-dependent PPARγ transcriptional activity in human intestinal epithelial cells. *Scientific reports, 7*(1), 1-3.

Nielsen, O., & Munck, L. (2007). Drug Insight: aminosalicylates for the treatment of IBD. *Nat Rev Gastroenterol Hepatol, 4*, 160–170. doi:10.1038/ncpgasthep0696

Noguera, J., Aira, M., Pérez-Losada, M., Domínguez, J., & Velando, A. (2018). Glucocorticoids modulate gastrointestinal microbiome in a wild bird. *R Soc Open Sci, 5*(4), 171743. doi:10.1098/rsos.171743

Ocón, B., Aranda, C., Gámez-Belmonte, R., Suárez, M., Zarzuelo, A., Martínez-Augustin, O., & Sánchez de Medina, F. (2016). The glucocorticoid budesonide has protective and deleterious effects in experimental colitis in mice. *Biochem Pharmacol, 116*, 73-88. doi:10.1016/j.bcp.2016.07.010

O'Donnell, S., & O'Morain, C. (2010). Therapeutic benefits of budesonide in gastroenterology . *Therapeutic advances in chronic disease, 1*(4), 177-86. doi:10.1177/2040622310379293

Oh, H., Visvalingam, V., & Wahli, W. (2019). The PPAR–microbiota–metabolic organ trilogy to fine-tune physiology. *The FASEB Journal, 33*(9), 9706-30. doi:10.1096/fj.201802681RR

Olsen, N., & Stein, C. (2004). New drugs for rheumatoid arthritis. *New England Journal of Medicine, 350*(21), 2167-79. doi:10.1056/NEJMra032906

O'Mahony, S., Marchesi, J., Scully, P., Codling, C., Ceolho, A., Quigley, E., . . . Dinan, T. (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for Irritable Bowel Syndrome and psychiatric illnesses. *Biological Psychiatry, 65*, 263–267. doi:10.1016/j.biopsych.2008.06.026

Packey, C., & Sartor, R. (2009). Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. *Curr Opin Infect Dis, 22*(3), 292-301. doi:10.1097/QCO.0b013e32832a8a5d.

Papamichael, K., Gils, A., Rutgeerts, P., Levesque, B., Vermeire, S., Sandborn, W., & Vande Casteele, N. (2015). Role for therapeutic drug monitoring during induction therapy with TNF antagonists in IBD: evolution in the definition and management of primary nonresponse. *Inflammatory bowel diseases, 21*(1), 182-97. doi:10.1097/MIB.0000000000000202

Parkinson, A., & Ogilvie, B. (2008). Biotransformation of xenobiotics. In L. Casarett, & J. e. Doull, *Casarett and Doull's Toxicology* (Vol. 7, pp. 161-304). New York: McGraw-Hill.

Peppercorn M A, G. P. (1972). The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. *J PharmacolExpTher, 181*, 555.

Peppercorn, M. A. (1984). Sulfasalazine: pharmacology, clinical use, toxicity, and related new drug development. *Annals of Internal Medicine, 101*(3), 377-386.

Peyrin-Biroulet, L., & Lémann, M. (2011). Review article: remission rates achievable by current therapies for inflammatory bowel disease. *Aliment Pharmacol Ther, 33*(8), 870-9. doi:10.1111/j.1365-2036.2011.04599.x.

Peyrin-Biroulet, L., Beisner, J., Wang, G., Nuding, S., Oommen, S., Kelly, D., Grandjean, T. (2010). Peroxisome proliferator-activated receptor gamma activation is required for maintenance of innate antimicrobial immunitity in the colon. *Proceedings of the National Academy of Sciences, 107*(19), 8772-7. doi:10.1073/pnas.0905745107

Qasem, A. E. (2020). Anti-MAP Triple Therapy Supports Immunomodulatory Therapeutic Response in Crohn's Disease through Downregulation of NF-κB Activation in the Absence of MAP Detection. *Biomedicines, 8*(11), 513.

Rafii, F., Franklin, W., & Cerniglia, C. (1990). Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. *Applied and environmental microbiology, 56*(7), 2146-51.

Rattu, M., Shah, N., Lee, J., Pham, A., & Marzella, N. (2013). Glucarpidase (Voraxaze), a Carboxypeptidase Enzyme for Methotrexate Toxicity. *Pharmacy and Therapeutics, 38*(12), 732-744.

Riol-Blanco, L., Lazarevic, V., Awasthi, A., Mitsdoerffer, M., Wilson, B., Croxford, A.,. Oukka, M. (2010). IL-23 receptor regulates unconventional IL-17 producing T cells that control bacterial infections. *J Immunol, 184*, 1710-1720. doi:10.4049/jimmunol.0902796

Rioux KP Bibiloni R, T. G. (2005). Aminosalicyclates alter the dominant phylogenetic profile of fecal bacteria in mice. *Gastroenterology , 128,* A663.

Rooks, M., Veiga, P., Wardwell-Scott, L., Tickle, T., Segata, N., Michaud, M., . . . Morgan, X. (2014). Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. *ISME J, 8*, 1403-17. Retrieved from http://dx.doi.org/10.1038/ismej.2014.3

Rosner., J. (1985). Nonheritable resistance to chloramphenicol and other antibiotics induced by salicylates and other chemotactic repellents in Escherichia coli K-12. *Proceedings of the National Academy of Sciences of the United States of America, 82*(24), 8771-8774. doi:10.1073/pnas.82.24.8771

Round, J., & Mazmanian, S. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature reviews Immunology, 9*(5), 313–323. doi:10.1038/nri2515

Rousseaux, C., Lefebvre, B., Dubuquoy, L., Lefebvre, P., Romano, O., Auwerx, J., & ... & Desreumaux, P. (2005). Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator–activated receptor-γ. *The Journal of experimental medicine, 201*(8), 1205-1215.

Ryan, A. (2017). Azoreductases in drug metabolism. *Br J Pharmacol, 174*(14), 2161-2173. doi:10.1111/bph.13571

Sandborn, W., Feagan, B., Fedorak, R., Scherl, E., Fleisher, M., Katz, S., . . . Rutgeerts, P. (2008). Ustekinumab Chrohn's Disease Study Group. A randomised trial of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Chrohn's disease. *Gastroenterology, 135*, 1130-1141.

Sartor, R. (2010). Genetics and environmental interactions shape the intestinal microbiome to promote inflammatory bowel disease versus mucosal homeostasis. *Gastroenterology, 139*(6), 1816-1819.

Scanu A M, B. T.-T. (2005). Detection and Isolation of Mycobacterium avium Subspecies paratuberculosis from Intestinal Mucosal Biopsies of Patients with and without Crohn's Disease in Sardinia. *American Journal of Gastroenterology, 100*(7), 1529-1536.

Scheinman, R., Gualberto, A., Jewell, C., Cidlowski, J., & Baldwin, A. J. (1995). Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Mol Cell Biol, 15*(2), 943-953. doi:10.1128/mcb.15.2.943

Schwab, M., Reynders, V., Loitsch, S., Shastri, Y., Steinhilber, D., Schröder, O., & Stein, J. (2008). PPARγ is involved in mesalazine-mediated induction of apoptosis and inhibition of cell growth in colon cancer cells. *Carcinogenesis, 29*(7), 1407-14. doi:10.1093/carcin/bgn118

Sekirov I., R. S. (2010). Gut microbiota in health and disease. *Physiol. Rev, 90*, 859–904. doi:10.1152/physrev.00045.2009

Shin, Y., Buehring, K., & Stokstad, E. (1974). The Metabolism of Methotrexate in Lactobacillus casei and Rat Liver and the Influence of Methotrexate on Metabolism of Folic Acid. *The Journal of Biological Chemistry, 249*, 5772-5777.

Siegal, C., & Sands, B. (2005). Review article: practical management of inflammatory bowel disease patients taking immunomodulators. *Alimentary Pharmacology & Therapeutics, 22*, 1-16. Retrieved from https://doi.org/10.1111/j.1365-2036.2005.02520.x

Sinha, V., & Kumria, R. (2001). Polysaccharides in colon-specific drug delivery. *International Journal of Pharmaceutics*, *224*(1-2), 19-38. Retrieved from https://doi.org/10.1016/S0378-5173(01)00720-7.

Soler, D., Chapman, T., Yang, L., Wyant, T., Egan, R., & Fedyk, E. (2009). The binding specificity and selective antagonism of vedolizumab, an anti-alpha4beta7 integrin therapeutic antibody in development for inflammatory bowel diseases. *J Pharmacol Exp Ther, 330*(3), 864-75. doi:10.1124/jpet.109.153973

Spanogiannopoulos, P., Bess, E., Carmody, R., & Turnbaugh, P. (2016). The microbial pharmacists within us: a metagenomic view of xenobiotic metabolism. *Microbiology, 14*(5), 273-287. doi:10.1038/nrmicro.2016.17

Swidsinski A, L.-B. V. (2007). Azathioprine and mesalazine-induced effects on the mucosal flora in patients with IBD colitiS. *Inflammatory bowel diseases, 13*(1), 51–56. doi:10.1002/ibd.20003

Taffet, S., & Das, K. (1983). Sulfasalazine. *Digestive diseases and sciences*, *28*(9), 833-842.

Tardif, R., & Brodeur, J. (2005). Pharmacokinetics/Toxicokinetics. In W. P (Ed.), *Encyclopedia of Toxicology* (Second Edition ed., pp. 383-390). Elsevier. Retrieved from https://doi.org/10.1016/B0-12-369400-0/00746-8

Tytgat, K., Bovelander, F., Opdam, F., Einerhand, A., Büller, H., & Dekker, J. (1995). Biosynthesis of rat MUC2 in colon and its analogy with human MUC2. *Biochem J, 309*, 221-9. doi:10.1042/bj3090221

Van der Sluis, M., De Koning, B., De Bruijn, A., Velcich, A., Meijerink, J., Van Goudoever, J.,. Einerhand, A. (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology, 131*(1), 117-29. doi:10.1053/j.gastro.2006.04.020

Van der Velden, V. (1998). Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma. *Mediators of Inflammation, 7*(4), 229-237.

Viladomiu, M., Hontecillas, R., Yuan, L., Lu, P., & Bassaganya-Riera, J. (2013). Nutritional protective mechanisms against gut inflammation. *The Journal of nutritional biochemistry, 24*(6), 929-39. doi:10.1016/j.jnutbio.2013.01.006

Wang J, Z. G. (2020). TAK-242 ameliorates DSS-induced colitis by regulating the gut microbiota and the JAK2/STAT3 signaling pathway. *Microb Cell Fact, 19*(1), 158. doi:10.1186/s12934-020-01417-x

Wang, C. L., Abuhammad, A., Lowe, E., Westwood, I., Ryan, A., & Sim, E. (2010). Role of tyrosine 131 in the active site of paAzoR1, an azoreductase with specificity for the inflammatory bowel disease prodrug balsalazide. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications, 66*(1), 2-7. doi:10.1107/S1744309109044741

Wang, C., Hagemeier, C., Rahman, N., Lowe, E., Noble, M., Coughtrie, M., . . . Westwood, I. (2007). Molecular cloning, characterisation and ligand-bound structure of an azoreductase from Pseudomonas aeruginosa. *J Mol Biol, 373*(5), 1213-28. doi:10.1016/j.jmb.2007

Widemann, B., Balis, F., Murphy, R., Sorensen, J., Montello, M., O'Brien, M., & Adamson, P. (1997). Carboxypeptidase-G2, thymidine, and leucovorin rescue in cancer patients with methotrexate-induced renal dysfunction. *Journal of Clinical Oncology, 15*, 2125–2134. doi:10.1200/JCO.1997.15.5.2125

Widemann, B., Sung, E., Anderson, L., Salzer, W., Balis, F., Monitjo, K., . . . Adamson, P. (2000). Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N 10-methylpteroic acid. *Journal of Pharmacology and Experimental Therapeutics, 294*(3), 894-901.

Wilson, I., & Nicholson, J. (2017). Gut microbiome interactions with drug metabolism, efficacy, and toxicity. *Translational research, 179*, 204-222.

Wu T, Y. L. (2018). Chronic glucocorticoid treatment induced circadian clock disorder leads to lipid metabolism and gut microbiota alterations in rats. *Life Sci, 192*, 173-182. doi:10.1016/j.lfs.2017

Yost, J., & Gudjonsson, J. (2009). The role of TNF inhibitors in psoriasis therapy: new implications for associated comorbidities. *F1000 Med Rep, 1*(30). doi:10.3410/M1-30

Zhang, L., Lee, H., Grimm, M., Riordan, S., Day, A., & Lemberg, D. (2014). Campylobacter concisus and inflammatory bowel diseases. *World J. Gastroenterol, 20*, 1259–1267. doi:10.3748/WJG.v20.i5.1259

Zheng, H., Chen, M., Li, Y., Wang, Y., Wei, L., Liao, Z., Xie, Z. (2017). Modulation of gut microbiome composition and function in experimental colitis treated with sulfasalazine. *Frontiers in microbiology, 8*, 1703. doi:10.3389/fmicb.2017.01703

Chapter 2

Encapsulated Cyclosporine does not change the composition of the human microbiota when assessed *ex vivo* and *in vivo*.

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Abstract

Introduction. Management of steroid-refractory ulcerative colitis has predominantly involved treatment with systemic Cyclosporine A (CyA) and infliximab.

Aim. The purpose of this study was to assess the effect of using a colon-targeted delivery system CyA formulation on the composition and functionality of the gut microbiota.

Methodology: *Ex vivo* faecal fermentations from six healthy control subjects were treated with coated minispheres (SmPill®) with (+) or without (-) CyA and compared with a non-treated control in a model colon system. In addition, the *in vivo* effect of the SmPill® + CyA formulation was investigated by analysing the gut microbiota in faecal samples collected before administration of SmPill® + CyA and after seven consecutive days of administration from eight healthy subjects who participated in a pilot study.

Results. Analysis of faecal samples by 16S rRNA gene sequencing indicated little variation in diversity nor relative abundance of the microbiota composition before or after treatment with SmPill® minispheres with or without CyA *ex vivo* or with CyA *in vivo*. Short chain fatty acid profiles were evaluated using gas chromatography; with an increase in the concentration of n-butyrate ($p = 0.02$) and acetate ($p = 0.32$) in the faecal fermented samples incubated in the presence of SmPill® minispheres with or without CyA. This indicated that increased acetate and butyrate production was attributed to a component of the coated minispheres rather than an effect of CyA on the microbiota. Butyrate and acetate levels also increased significantly ($p = 0.05$ for both) in the faecal samples of

healthy individuals following seven days treatment with SmPill® + CyA in the pilot study.

Conclusion. SmPill® minispheres with or without CyA at the clinically relevant doses tested here has negligible direct effects on the gut microbiota composition. Butyrate and Acetate production increased however in the presence of the beads in an *ex vivo* model system as well as *in vivo* in healthy subjects. Importantly, this study also demonstrates the relevance and value of using *ex vivo* colon models to predict the *in vivo* impact of colon-targeted drugs directly on the gut microbiota.

Introduction

Perturbations in the microbial balance and host immune response in the gastrointestinal (GI) tract have been associated with the onset of inflammatory bowel disease (IBD), including ulcerative colitis (UC). Genetic predisposition in patients with IBD has been shown to cause a change in the immune response involved in the host's interaction with bacteria in the gut (Knights, Lassen and Xavier 2013). Cyclosporine A (CyA) is a potent immunosuppressant approved for use in solid organ transplants to prevent rejection. It is a poorly soluble cyclic peptide and is subject to metabolism by various cytochrome P450 enzymes that are expressed mainly in the small intestine and liver (Guada, et al. 2016). It has been used off-label as a treatment for steroid refractory ulcerative colitis for the past twenty years (Sharkey, et al. 2011). Other uses for CyA include treatment of autoimmune disorders such as rheumatoid arthritis, psoriasis and Crohn's disease. The therapeutic guidelines recommend continuous i.v infusion of 2-4 mg/kg/day CyA followed by oral administration 4-8 mg/kg/day for up to three months (Ordás, et al. 2012). Recent studies comparing CyA and infliximab in patients with severe, i.v. steroid-refractory UC demonstrated that CyA efficacy is comparable to infliximab (Laharie 2012), (Williams, et al. 2016). The clear efficacy is counter-balanced by the risk of severe adverse events related to systemic exposure.

As systemic cyclosporine absorption from the colon is negligible (Drewe, Beglinger and Kissel 1992), Sublimity Therapeutics developed a SmPill®-enabled, colon-targeted, multiple minisphere formulation to deliver cyclosporine in solubilised, therefore active, form. An initial randomised, controlled study in

patients with mild-moderate UC demonstrated that 75 mg SmPill® + CyA administered once-daily for four weeks resulted in superior efficacy and comparable safety and tolerability when compared to placebo (O'Donoghue, Bloom and Coulter 2013). A follow-on study in pigs which were orally gavaged with ethylcellulose:pectin-based, SmPill®-enabled CyA formulations, demonstrated high levels of CyA in the colon accompanied with low systemic levels when compared to other available CyA formulations (Keohane, et al. 2016). The pig model was used to monitor the release of CyA from the coated minispheres in the GI tract and to optimise the encapsulation design for increased efficiency. Based on this study an, ethylcellulose-based, SmPill®-enabled CyA formulation was developed. In a pilot study in 8 healthy subjects in which a range of optimised SmPill®+ CYA doses was evaluated for blood, colon luminal content and colon tissue concentrations, it was demonstrated that 75 mg ethylcellulose-coated, SmPill®+CyA administered twice-daily for seven days resulted in colon luminal concentrations that translated to colon tissue concentrations comparable to that achieved following continuous i.v. infusion at the known to be efficacious 2 mg/kg/day, equivalent to 1 µM CyA. Importantly, systemic exposure following administration of the optimised SmPill®+CyA across all doses tested was negligible. The CyA concentration in colon luminal contents sampled using flexible sigmoidoscopy in unprepared bowels was approximately 0.75 mg ml^{-1} (Colombel, et al. 2019). With such low systemic exposure, it is not expected that SmPill®+CyA will be associated with systemic adverse events, thus permitting long term use of cyclosporine as a treatment for patients with ulcerative colitis. The purpose of this study was to evaluate the effect, if any, directly on the host microbiota within the human gut microbiota.

There is increasing interest in the effect of orally ingested drugs on the human gut microbiota. Drugs used for the treatment of IBD include Sulphasasalazine (SASP), 5-aminosalicyclic acid (5-ASA), corticosteroids, immunomodulatory drugs such as methotrexate (MTX), immunosuppressants such as calcineurin inhibitors (including cyclosporine (CyA) and tacrolimus) and anti-TNF-alpha antibodies (such as infliximab). Sulphasalazine is a classic example of the interaction of the gut microbiota with orally administered drugs. There is little known about the interaction of most of these drugs with the gut microbiota. One of these drugs however, Sulphasalazine is a well known example of how bacteria can interact with the drug and change its function. Sulfasalazine is a prodrug made of sulfapyridine linked to 5-aminosalicyclic acid (5-ASA) through an azo bond. Sulfasalazine is partially absorbed in the jejunum after oral ingestion. The remaining drug passes to the colon. It is here that it is reduced by coliform bacteria by the bacterial azoreductase enzyme yielding sulfapyridine and 5-Aminosalicyclic acid. Aminosalicyclic acid is the active component which must be released from the compound in order to treat the inflammation (Peppercorn and Goldman 1972;).

Drugs like CyA, particularly when designed to be released in the colon, which have low permeability and poor solubility, have, by design, most contact with the host microbiota in the large intestine as this is the site where both the human microbiota and the colon-targeted drug payload is most concentrated. It is estimated that approximately 2,000 bacterial species populate the lower region of the GI tract and it is here that the interplay with encapsulated drugs, pro-drugs and their conjugated metabolites largely occurs (Wilson and Nicholson 2017). The gut microbiota and the metabolites they produce significantly affect the

health of the host. For instance, short chain fatty acids (SCFAs) are end-products of the fermentation of undigested carbohydrates and protein by the gut microbiota in the large intestine and play a fundamental role in human health and disease (Tan, et al. 2014). The most dominant SCFAs (90-95%) in the colon are butyric acid (Butyrate), propionic acid and acetic acid. Butyrate is a major energy source for intestinal epithelial cells and plays a role in the maintenance of gut barrier function (Ríos-Covián, et al. 2016).

The aim of this study was to assess the effect of encapsulated CyA in SmPill® minispheres on the diversity and relative abundance of the gut microbiota in an *ex vivo* colon model and in healthy subjects who participated in a seven-day pilot study. As such, we wanted to know whether there could be a microbiota effect which contributed to the *in vivo* efficacy of the treatment. We also investigated the production of SCFAs butyrate, propionate and acetate by the gut microbiota in the *ex vivo* model system in the presence of coated minispheres (SmPill®) with (+) and without (-) CyA. We then assessed the effect of encapsulated CyA, using coated minispheres (SmPill®) + CyA on the levels of various metabolites and SCFAs in faecal samples from healthy human subjects, before and after participating in a seven-day pilot study. *Ex vivo* and *in vivo* results were compared to determine the efficacy of using an *ex vivo* colon model to study the effect of orally ingested drugs on the microbial communities of the GI tract.

Methods

Subject recruitment for ex vivo faecal fermentation study

Six healthy volunteers of varying ages (23-62 year) and gender (2 male and 4 female) provided fresh faecal samples on the day of the experiment. Subjects were in good general health and had not taken antibiotics in the previous 6 months.

Ethics approval and consent to participate

The *ex vivo* studies were carried out in compliance with APC Ethics Protocol 055. The *in vivo* study was performed in compliance with the protocol CYC 102. Ethical approval was obtained by Sigmoid Pharma Ltd. Written informed consent was obtained from all participants.

Fermentation protocol

Fermentations were carried out using the MultiFors fermentation system (Infors UK), as follows: 20% faecal slurry was prepared from faecal samples from the individual donors under anaerobic conditions, by suspending the faecal samples in pre-boiled and anaerobically cooled 50 mM phosphate buffer, pH 7.0 containing 0.05% cysteine (Sigma Aldrich, Vale Road, Arklow, Co. Wicklow, Ireland). One hundred and sixty millilitres of the faecal medium, prepared according to Fooks *et al*. (Fooks and Gibson 2003), was added to each of three fermentation vessels. Anaerobic conditions were established by sparging the fermenters with oxygen-free nitrogen for at least 3 h prior to inoculation with 40 ml of the faecal slurry. The vessels contained (i) faecal medium, faecal slurry and 1.5 g coated SmPill® minispheres (Sublimity Therapeutics Ltd, DCU Alpha Innovation Campus, Old Finglas Road, Dublin) without CyA (minispheres - CyA), (ii) faecal medium, slurry and 1.5 g coated SmPill® minispheres with CyA

(minispheres + CyA [10% w/w]) and (iii) faecal medium and slurry only (non-treatment control, NTC). Fermentations were conducted anaerobically at 37°C for 24 h at pH 6.8, stirring at 200 rpm. Samples were taken at 0, 5, 9, 20 and 24 h for CyA quantification and at 0 and 24 h for Miseq compositional sequencing.

Quantification of CyA by High Performance Liquid Chromatography (HPLC)

Samples from the faecal fermentations (2 mL) were centrifuged for 60 sec at 21,000 x g, filtered using a 0.45 µM filter and a 0.2 µM filter and quantified using HPLC as detailed below. CyA (0.375 mg ml^{-1}) in 70% acetonitrile was diluted ten-fold with 70% acetonitrile to generate a working standard of 0.037 mg ml^{-1}. The standard (50 µl) was injected onto a Proteo Jupiter (250 x 4.6 mm, 90Å. 4 µm) RP-HPLC column (Phenomenex, Macclesfield, UK) with an 88-93% acetonitrile 0.1% Triflouroacetic acid (TFA) gradient where buffer A is 0.1% TFA and buffer B is 90% acetonitrile, 0.1% TFA. CyA elutes at 10 min or 90.5% acetonitrile, which equates with 81.5% acetonitrile. Each sample (50 µl) was run in triplicate and the CyA concentration (mg ml^{-1}) calculated from the area of the standard.

Bacteroides strains and growth conditions

The growth of three *Bacteroides* sp. was tested *in vitro* in the presence of uncoated minispheres with (+) and without (-) CyA (0.75 mg ml^{-1}); (equivalent to colon luminal concentration following administration of 75mg SmPill® + CyA twice-daily for seven days) and in growth medium alone. Minispheres which lacked the ethylcellulose and pectin polymer coating (referred to here as uncoated minispheres) were used to enable rapid release of the CyA to the growth medium.

The *Bacteroides* strains (*Bacteroides distasonis, B. dorei and B. fragilis*) were maintained long-term at -80°C in 30% glycerol/Brain Heart Infusion (BHI) medium (Oxoid LTD, Hampshire, UK) with 0.5% (w/v) yeast extract (Oxoid LTD, Hampshire, UK) (BHIYE). Prior to use, the strains were streaked onto Trypticase Soy Agar (TSA, Becton, Dickinson and Company; Sparks, MD 21152 USA) and incubated anaerobically at 37°C for 48 h. Subsequently, 1 colony was inoculated into BHIYE broth and grown overnight anaerobically at 37°C. SmPill® uncoated minispheres +/- CyA were added to the BHIYE, such that the final concentration of CyA was 0.75 mg ml^{-1}. BHIYE without uncoated minispheres or CyA served as the negative controls. The medium was inoculated at 1% with an overnight culture of each strain and incubated shaking at 200 rpm under anaerobic conditions at 37°C for 48 h. After incubation a sample from the media was serially diluted and spread plated onto Bile Esculin Agar (BEA) prior to incubation for 48 h anaerobically at 37°C. After incubation the colonies were counted, and results expressed as colony forming units/ml (CFU ml^{-1}).

Participant recruitment for pilot study

Faecal samples were obtained from eight healthy volunteers (Male/22-55) who participated in a pilot clinical study. The participants were administered CyA encapsulated in coated Smpill® minispheres and faecal samples taken at the beginning (Day 0; before treatment) and at the end (Day 7) of the trial. Samples were stored at -80°C until required. Ethical approval was obtained by Sigmoid Pharma (SP); Protocol no: CYC 102.

Microbial DNA extraction, 16s rRNA gene amplification and Illumina MiSeq sequencing

In the *ex vivo* study, samples (1 mL) were taken from each fermentation vessel at 0 h and 24 h and snap frozen at -80°C for DNA extraction. In the pilot study, 300-400 mg of each frozen faecal sample was taken for DNA extraction from samples taken before treatment (Day 0) and after (Day 7) treatment. The faecal DNA MiniPrep kit (Zymo Research, Cambridge Bioscience, UK) was used for DNA extraction from all samples. The V3-V4 variable region of the 16S rRNA gene was amplified from faecal DNA using the 16S metagenomic sequencing library protocol (Illumina, Saffron Walden, UK). Samples were quantified and libraries prepared for sequencing as described previously (Fouhy, et al. 2015). In brief, for the 16SrRNA amplicon sequencing, two PCR reactions were completed on the template DNA. Initially the DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). For the second PCR, two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden.) were added to allow for demultiplexing. Samples were run on the Illumina Miseq sequencer at the Teagasc Sequencing Centre, Moorepark, Fermoy, Co. Cork, Ireland using the Miseq 500 cycle V2 kit, following standard Illumina sequencing protocols.

Bioinformatic analysis of compositional sequencing data

Raw Illumina 250 bp paired-end sequence reads were merged using FLASH (fast length adjustment of short reads to improve genome assemblies) (Magoč and Salzberg 2011) and quality checked using the split_libraries script from the QIIME package (Caporaso, et al. 2010). Reads were then clustered into operational taxonomic units (OTUs) and chimeras removed with the 64-bit version of USEARCH (Edgar 2010). Subsequently OTUs were aligned and a phylogenetic tree generated within QIIME (Caporaso, et al. 2010). The minimum level of sequence overlap used when merging the paired reads was 10bp. Reads were filtered at at >Q20 Phred score. 97% similarity threshold was used for clustering OTUs. Singletons and rare sequences were removed. Taxonomical assignments were reached using the SILVA 16S specific database (version 128) (Quast, et al. 2013). Alpha and beta diversity analysis was also implemented within QIIME, based on weighted and unweighted Unifrac distance matrices. Principal coordinate analysis (PCoA) plots were visualised using EMPeror - 0.9.51 dev (Vázquez-Baeza, et al. 2013).

SCFA analysis of faecal fermentation samples

Gas chromatography with flame ionisation detector (GC-FID) was used to determine SCFA content. A 100 mM SCFA stock solution (butyric acid, iso-butyric (ibutyric) acid, propionic acid and acetic acid) were prepared from reagents of the highest purity available (Sigma) with fresh deionized water. Two-ethyl-butyric acid (2-ETBA, Sigma) was used as the internal standard (IS). A 100 mM stock solution of 2-ETBA was prepared with formic acid. A 10 mM working

solution of 2-ETBA and a 20 mM working solution of SCFA mix was made up with deionized milliQ water. Standard solutions containing 70.0, 60.0, 50.0, 40.0, 30.0, 20.0, 10·0, 8·0, 6·0, 4·0, 2·0, 1·0 and 0·5 mM of acetic acid, propionic acid, isobutyric acid and butyric acid, were used for calibration of the instrument. Faecal fermentation samples which had been snap frozen at -80°C were thawed and 2 mL of each sample was centrifuged at 16,000 x g for 5 min. The supernatant was then filtered through a 0.2 µM filter. Thirty microliters of working IS (10 mM) was added in triplicate to 1.5 mL eppendorf tubes and 270 µL of filtered sample supernatant was then added to each tube. Tubes were vortexed and centrifuged at 16,000 x g for 3 min. The supernatant was transferred to GC vials containing an insert and sealed. SCFA concentration was measured using a Varian 8400 GC flame ionisation system, fitted with a ZB-FFAP column (30 m x 0·32 mm x 0·25 mm) (Phenomenex, Macclesfield, UK) and Restek Hydroguard deactivation guard column (5 m, 0.32 mm ID). Helium was used as the carrier gas at a flow rate of 1.3 ml/min. An initial oven temperature of 50^{o}C was held for 0.5 min, raised to 140^{o}C at a rate of 10^{o}C/min, held for 0.5 min for a total of 10 min and finally raised to 240^{o}C at a rate of 20^{o}C/min and held for 12 min to give a total run time of 27 min. The temperature of the detector and injector were set at 300^{o}C. Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain calibration.

Metabolite analysis of faecal samples from the human pilot study

Preparation of faecal water

Four hundred milligrams of frozen faecal material was placed into a sterile 2 mL microcentrifuge tube. Eight hundred microlitres of sterile water was added to the 2 mL microcentrifuge tube, on ice. The sample was vortexed vigorously for 30 seconds to generate a slurry. Samples were then centrifuged at 12,000 x g for 30 min at 4°C. The supernatant was removed and transferred to a new 2 mL microcentrifuge tube. This step was then repeated. The supernatant was then centrifuged at 12,000 x g for 30 min at 4°C. The supernatant was removed again and transferred to a new 2 mL microcentrifuge tube. The supernatant was then centrifuged again at 12,000 x g for 30 min at 4°C. This supernatant was transferred to a 0.2 µM costar® spin-x® centrifuge tube (Sigma) and filtered by centrifugation at 12,000 x g for 10 min at 4°C. The filter was removed from the tube and the faecal water was stored at –20°C for metabolomics testing. Samples were sent to MS-Omics ApS, Thorvaldsensvej 40, 6th floor, T644, DK-1871 Frederiksberg C, Denmark for analysis of metabolites including the SCFA.

Statistical analysis

Non-parametric Kruskal–Wallis and Mann–Whitney U tests were performed in SPSS (version 24) to identify significantly different bacterial taxa in the different groups. Statistical significance was accepted as $p \leq 0.05$, with adjusted measures. To control for multiple testing, false discovery rate (FDR) adjusted q-values ($q \leq 0.05$) were calculated for comparisons of taxa using the Benjamini–Hochberg method (Benjamini and Hochberg 1995) in GraphPad Prism 6.04. To minimize the number of null hypotheses that had to be corrected for, OTUs which were present in at least 50% of samples with a minimum of 0.1% relative abundance were included in the analysis. Univariate analysis of variance (ANOVA) and

post-hoc Tukey tests were performed using SPSS to determine the significant differences between the groups for Alpha diversity, SCFA and metabolites. Statistical significance was accepted as $p \leq 0.05$. Box plots were created using GraphPad Prism 6.04.

Results

The overall aim of this study was to investigate if cyclosporine had any effect on the human microbiota. To achieve this encapsulated cyclosporine was tested both *ex vivo* and *in vivo*.

Measurement of CyA release from coated minispheres during the ex vivo fermentation

The concentration of CyA released from the coated minispheres, in the presence of a faecal inoculum prepared from each of 6 healthy donors, was measured over 24 h in the fermentation system (Figure 1). The results show that the release of CyA from the coated minispheres varied with the donor inoculum however the peak detection time across the 6 donors ranged between 5 and 9 hours.

The effect of CyA and coated minispheres on the faecal microbiota in the ex vivo model

Following total DNA extraction from faecal samples taken at 0 h and 24 h of the *ex vivo* fermentation process, the V3-V4 16S rRNA gene amplicons were generated and sequenced using the Illumina MiSeq. A total of 9,747,625 reads

passing the quality filter were obtained with an average of 270,767 reads per sample.To determine if alterations in microbial diversity occurred in the presence of coated minispheres +/- CyA, diversity, richness and coverage estimations were calculated for each data set (alpha diversity) (Supplementary Table S1). No significant difference in alpha diversity occurred between the three groups across 6 subjects. This was determined using Chao 1 ($p = 0.56$), Simpson's diversity index ($p = 0.23$) and Shannon index measurements ($p = 0.38$). Beta-diversity, a term for the comparison of samples based on the similarity of their OTUs was also measured. Beta-diversity was estimated using distance matrices built from weighted and unweighted Unifrac distances. Principal co-ordinate analysis (PCoA) was performed on the distance matrices from the unweighted UniFrac distance matrices. The data show that the samples cluster by subject, not by treatment (Supplementary Figure S1). Sequence analysis revealed that Firmicutes (62%), Bacteroidetes (30%), Actinobacteria (4%) Proteobacteria (3%) and Cyanobacteria (2%) were the dominant phyla across the six subjects at 0 h. Following 24 h fermentation the treatment groups +/- CyA and no treatment control samples shared common phyla. There was no significant effect of minispheres +/- CyA on the composition of the gut microbiota observed after 24 h incubation (Supplementary Figure S2).

There were 76 family level taxa identified, of which 44 were present in at least one subject, with a minimum relative abundance of 0.1% or greater after 24 h across the three treatment groups. The most dominant families detected across all subjects and treatment groups at 24 h fermentation were *Lachnospiraceae, Ruminococcaceae* and *Bacteroidaceae* (Figure 3). Treatment with minispheres +/- CyA did not have any effect on the relative abundance of any of the families

present, compared to the NTC, and there were no significant differences observed between the three treatment groups. There were 242 genera identified, of which 133 were present in at least one subject with a minimum relative abundance of 0.1% or greater after 24 h across the three treatment groups. The most dominant genera detected across all subjects were *Bacteroides, Blautia, Faecalibacterium* and *Lachnospiraceae* (Supplementary Figure S3). While there was a slight decrease in the relative abundance of *Bacteroides* after treatment with minispheres +/- CyA, this was not significant. There was, however, a slight decrease in Bacteroidetes phyla (not significant) in 4 out of 6 subjects in the groups with minispheres +/- CyA (median 16%) compared to the no-treatment control (NTC, median 22%) (Figure 2a). Since Bacteroidetes was the only Phylum which was decreased in the presence of the minispheres +/- CyA compared to the NTC, the growth of three pure cultures *Bacteroides* sp. representative of the Bacteroidetes phyla were tested *in vitro* in the presence of uncoated minispheres +/- CyA (0.75 mg ml^{-1}) and growth medium alone. The impact on cell viability of the minispheres +/- CyA varied between species. While there was no impact on cell viability of *B. fragilis* after 48 h incubation, there was a 2-log reduction in cell viability for *B. dorei* in the presence of minispheres +/- CyA indicating that it was the minispheres that were impacting the viability of *B. dorea* and not the CyA. In contrast, there was a 1-log reduction in *B. distasonis* when incubated at 37°C for 48 h in the presence of minispheres + CyA but the minispheres alone did not have any additional effect (Supplementary table 6).

The effect of CyA and coated minispheres on SCFA production in an ex-vivo colon model

SCFA production is an important beneficial function of the microbiota and therefore we investigated whether CyA and minispheres had an impact on SCFA levels. Butyrate, propionate and acetate levels were assessed as they are the most abundant SCFAs found in the colon. The concentration of n-butyrate was significantly higher in samples with coated minispheres +/- CyA compared to the non-treatment control group ($p = 0.018$) as shown in Figure 2b. The data indicate that increased n-butyrate production is due to a component of the coated minisphere rather than the effect of CyA on the microbiota. There were increased acetate levels in the samples with minispheres (+/- CyA); however, the increase was not statistically significant. There was no significant change in the levels of isobutyrate or propionate between the three treatment groups (Supplementary Table S2).

The effect of CyA encapsulated in coated minispheres (SmPill®) on the faecal microbiota in healthy subjects participating in a seven-day pilot trial

Faecal samples were collected from 8 healthy subjects who participated in the 7-day pilot study before and after SmPill® + CyA administration. Following total metagenomic DNA extraction from the 16 faecal samples, the V3-V4 16S rRNA gene amplicons were generated and sequenced using the Illumina MiSeq. A total of 4,481,418 reads passing the quality filter were obtained with an average of 280,089 reads per sample. Diversity, richness and coverage estimations were calculated for each data set to determine if alterations in microbial diversity occurred in the presence of SmPill® + CyA. No significant difference in alpha

diversity occurred between the before and after treatment samples across 8 subjects (Supplementary Table S3). This was determined using Chao 1 ($p = 0.99$), Simpson's diversity index ($p = 0.84$) and Shannon index measurements ($p = 0.96$). There was no significant difference in beta diversity on the PCoA plots before and after treatment with SmPill® + CyA ($p = 0.95$). The data show that the samples clustered by subject and not by treatment. As in the faecal ferment samples the most dominant phyla were Firmicutes, Bacteroidetes and Actinobacteria across all 8 subjects before and after treatment. There was no significant difference in the distribution of any of the phyla before and after treatment with SmPill® + CyA after seven days (Supplementary Figure S4). There were 79 family level taxa identified of which 39 were present in at least 1 sample with a minimum relative abundance of 0.1% or greater. The most prevalent families detected across all subjects were *Lachnospiraceae, Ruminococcaceae*, *Bacteroidaceae Bifidobacteriaceae,* and *Erysipelotrichaceae* (Figure 4). There was no significant difference in the relative abundance observed before and after treatment in any of the families present. There were 134 genus level taxa identified out of a total of 257 genera which were present in at least 1 sample with a minimum relative abundance of 0.1% or greater. The most dominant genera across all subjects were *Blautia*, *Faecalibacterium, Lachnospiraceae other, Bifidobacterium*, *Agathobacter*, *Romboutsia* and *Bacteroides* (Supplementary Figure S5). There were no significant differences in the relative abundance of any of these before and after seven days treatment with SmPill® + CyA.

Effect of CyA encapsulated in coated minispheres (SmPill®) on the levels of metabolites in faecal samples from healthy subjects participating in a seven-day pilot study

In vivo, the levels of n-butyrate, i-butyrate, propionate and acetate in the faecal samples of 8 subjects were assessed before the start of the trial and 7 days after treatment. The levels of i-valerate, hexanoate and pentanoate were also measured (Supplementary Table S4). Butyrate ($p = 0.05$) and acetate ($p = 0.048$) were shown to significantly increase after seven days treatment with SmPill® + CyA (See Figure 5a and 5b). Thirty-three additional metabolites and amino acids commonly found in human faecal samples were identified (Supplementary Table S5). There was a significant increase in the concentration of linoleic acid after seven days treatment ($p= 0.048$) (Figure 5c).

Discussion

The liver is the primary site of xenobiotic metabolism and biotransformation, but the method in which drugs are metabolised can vary between individuals. Drugs with narrow therapeutic windows and subject to extensive and variable enzymatic metabolism, such as CyA, often require careful systemic drug concentration monitoring to ensure that patient-specific dosing regimens are implemented (Jorga, Holt and Johnston 2004). Metabolism of the drug starts at the intestinal level and can have a significant impact on drug bioavailability, efficacy and safety, given that the intestinal microbiota can contribute to xenobiotic metabolism (Wilson and Nicholson 2017). In addition, xenobiotic exposure has

the potential to alter the composition of the gut microbiota (Wilson and Nicholson 2017), (Maurice, Haiser and Turnbaugh 2013). The interaction between drugs and the gut microbiota in the intestine can affect the rate of release of the drug and absorption or uptake in the body.

There is an increasing demand to develop novel methods to formulate poorly soluble drugs such that they withstand release in the upper GI tract and are released in solubilised form within specific regions of the intestine, such as the colon. This is particularly relevant for drugs which specifically target IBD. Therefore, the preferred outcome for a drug destined for release in the lower GI tract is to successfully treat the condition whilst having a beneficial effect or at the least a minimal impact on the gut microbes. This study therefore concentrated on determining the effect of SmPill® +/- CyA on the gut microbiota in an *ex vivo* model of the distal colon which had been inoculated with stool samples from healthy adults. Previous work by our lab using the same *ex vivo* colon model using a similar number of subjects has shown the impact that antimicrobials such as vancomycin and metronidazole have on the gut microbiota diversity and abundance (Rea, et al. 2010). In addition, we measured the effect of a treatment-appropriate SmPill® + CyA dosing regimen (75mg BID) on the gut microbiota in 8 healthy subjects who participated in a seven-day pilot study. While we were limited by the number of subjects in both the *in-vivo* and *ex-vivo* studies we believe that this may be more representative of that observed in clinical situation which takes into account the differences in microbial composition between individuals and results in a good diversity of microbiota. Indeed the results from the *in vivo* study attested the findings of the *ex-vivo* work.

The rate of CyA release over time between donor inoculums in the fermentation vessels was found to vary. This may be a consequence of CyA adhering to or entering the bacteria in the biomass which is removed before HPLC quantification, or may be due to CyA being metabolized differently depending on the bacterial composition of the inoculum. CyA has been shown to be highly stable in a colonic model (Wang, et al. 2015) due to its unique cyclic structure, N-methylation and high lipophilicity (Biron, et al. 2008), (Haviv, et al. 1993) making it less prone to metabolism by colonic bacteria. The CyA concentration in colon luminal contents sampled using flexible sigmoidoscopy in unprepared bowels of healthy subjects who were administered 75 mg twice daily for seven consecutive days was approximately 0.75 mg ml^{-1}. The concentration range which we anticipated to measure therefore was between 0.05 and 0.75 mg ml^{-1} representing levels which the bacteria would be exposed to in the human gut following administration of SmPill® + CyA. Although the concentration of CyA released varied between subjects, sufficient levels of CyA were measured across all vessels to obtain clinically relevant colonic tissue concentrations.

CyA encapsulated in SmPill® did not affect the alpha or beta diversity of faecal microbiota *in vivo* in healthy human subjects administered the drug for seven days or in the *ex vivo* fermentation conditions. In the *ex vivo* study, sequencing analysis revealed that the phylum Bacteroidetes was slightly decreased in the presence of minispheres +/- CyA when compared to the non-treatment control. Exposure of three pure cultures of *Bacteroides* sp. to the minispheres +/- CyA revealed variable responses in that the minispheres reduced cell viability of *B. dorei* while CyA decreased the viability of *B. distasonis* but neither minispheres nor drug impacted viability of *B. fragilis*. In addition, CyA had no inhibitory

effect on pure cultures of *Lactobacillus* or *Bifidobacterium* species which were tested previously in our laboratory (data not shown). Therefore, the impact of SmPill® + CyA on Bacteroidetes abundance levels is presumably influenced by the base levels and types of Bacteroidetes members in the microbiota of each individual prior to treatment.

Concentrations of the SCFAs butyrate, propionate and acetate were measured at 0 and 24 hours in the *ex vivo* model. Levels of n-butyrate and acetate were shown to increase in the presence of the SmPill® minispheres +/- CyA indicating that a bead component, yet to be identified, was having an influence on the gut microbiota.

The coating on the minispheres is ethylcellulose based. Ethylcellulose is a plant derived polysaccharide which can be broken down by microbial carbohydrate-degrading enzymes such as cellulases. Some cellulolytic strains which have been isolated and characterized from human faeces include *Ruminococcus sp*, *Clostridium* sp, *Bacteroides sp* and *Eubacterium sp*. (Flint, et al. 2012). SCFAs are a product of microbial fermentation of such indigestible carbohydrates in the large intestine. A high proportion of these SCFAs are absorbed by the host and are therefore an important energy source to the host. It is estimated that these SCFAs account for around 10% of the calories obtained from the diet that otherwise would be excreted as undegraded substrate in the faeces (Flint, et al. 2012), (McNeil 1984).

Other health benefits of SCFAs include maintenance of gut barrier function (Ríos-Covián, et al. 2016), protection against colorectal cancer (CRC) (Canani, et al. 2011), (Keku, et al. 2014) and protection against obesity and insulin resistance (Gao, et al. 2009). Butyrate reduces inflammation, inhibits tumor cell

progression, induces apoptosis, promotes colon motility and increases visceral irrigation (Canani, et al. 2011), (Keku, et al. 2014). The relative abundance of other butyrate producing bacteria which have been suggested to be implicated in Ulcerative Colitis such as *Faecalibacterium prausnitzii* and *Roseburia* sp. was also investigated in this study, however, no significant changes were observed either in the *ex vivo* model or *in vivo* (data not shown). The increase in butyrate concentration could be viewed as a favourable outcome in terms of human health.

Conclusions

We observed that the concentration of encapsulated CyA in the SmPill® formulation (likely to be encountered by the gut microbiota *in vivo*) did not impact gut microbiota composition when tested in both an *ex vivo* colon model and an *in vivo* pilot study in healthy volunteers. CyA is an immunosuppressant and we cannot rule out that its impact on the host immune response could indirectly influence the gut microbiota. However, our study revealed that some component of the SmPill® formulation (described previously) significantly increased the concentration of n-butyrate, a microbial metabolite with known positive attributes for host health.

An initial randomised, controlled study in patients with mild-moderate UC demonstrated that 75 mg SmPill® + CyA administered once-daily for four weeks resulted in superior efficacy and comparable safety and tolerability when compared to placebo.

The seven-day time period of the pilot study should have been sufficient to see changes in the macro-composition of the gut microbiota in this study (as we do

observe with different antibiotics) but it does appear to be sufficient to alter the function of the microbes. We observed that the levels of acetate, butyrate and linoleic acid were all increased (approximately doubled) following SmPill® treatment. The results from the compositional and SCFA analysis of the healthy human subjects correspond to the *ex vivo* faecal fermentation findings suggesting that the *ex vivo* model is effective for studying the impact of orally ingested drugs on the gut microbiota. Moving forward with SmPill® encapsulated CyA, future studies with a larger cohort will investigate the efficacy, safety and tolerability across a range of doses in Ulcerative Colitis patients in clinical trials.

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Chapter 2. Figures

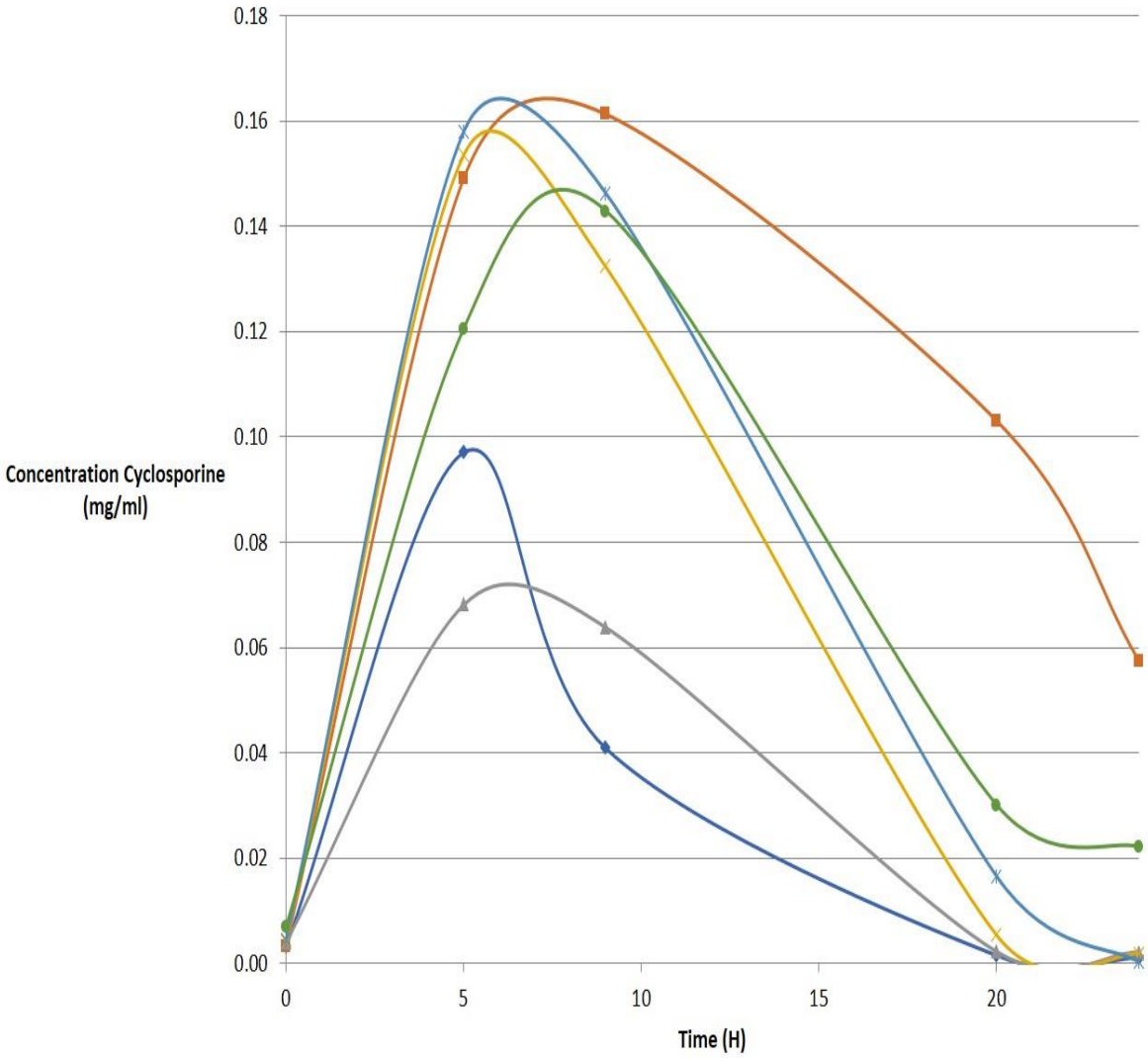


Figure 1. Release of Cyclosporine A (CyA) from SmPill® minispheres over time during 24 h fermentation in an *ex vivo* model. Each colour represents an individual faecal sample.

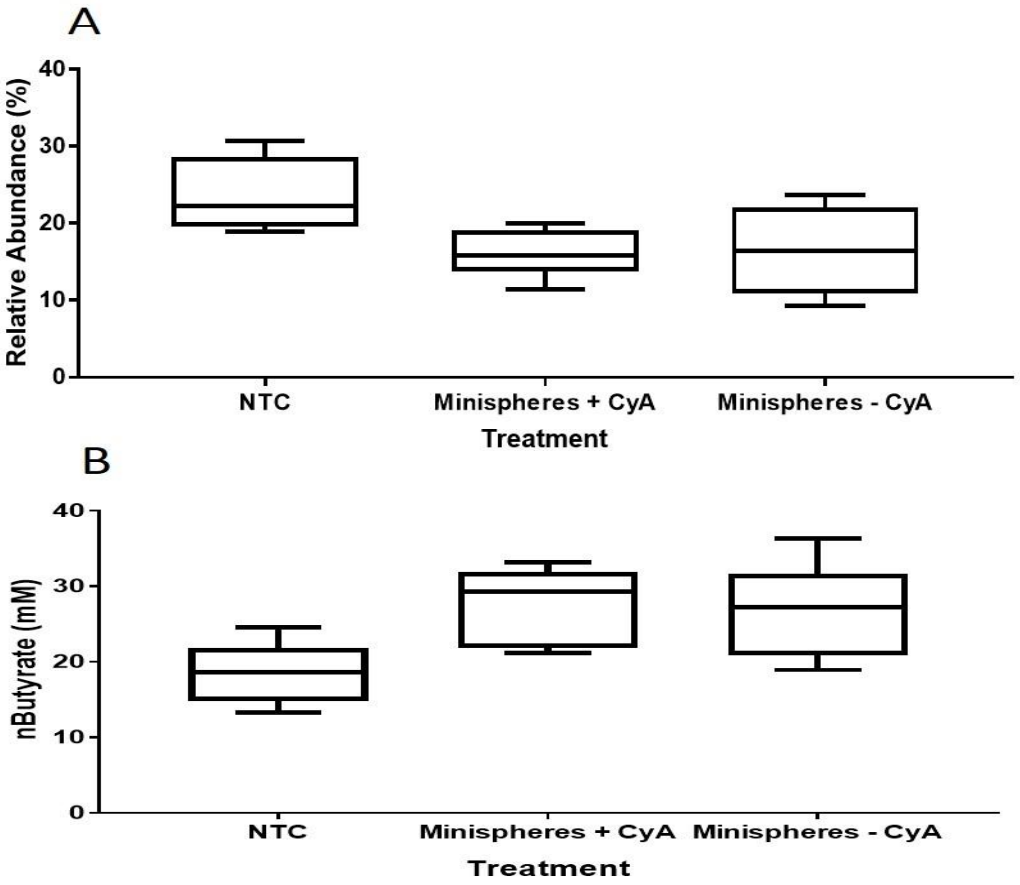


Figure 2. (A) Relative abundance in Operational Taxonomic Units (OTU) (%) of the *Bacteroidetes* phylum from each treatment group following 24 hour fermentation in an *ex vivo* model. Groups: non-treatment control (NTC); Minispheres with (+) Cyclosporine A (CyA), Minispheres without (-) CyA. **(B)** Butyrate analysis in samples from the treatment groups following 24 hour fermentation in an *ex vivo* colon model. Groups: non-treatment control (NTC); minispheres + CyA and minispheres – CyA (P = 0.018).

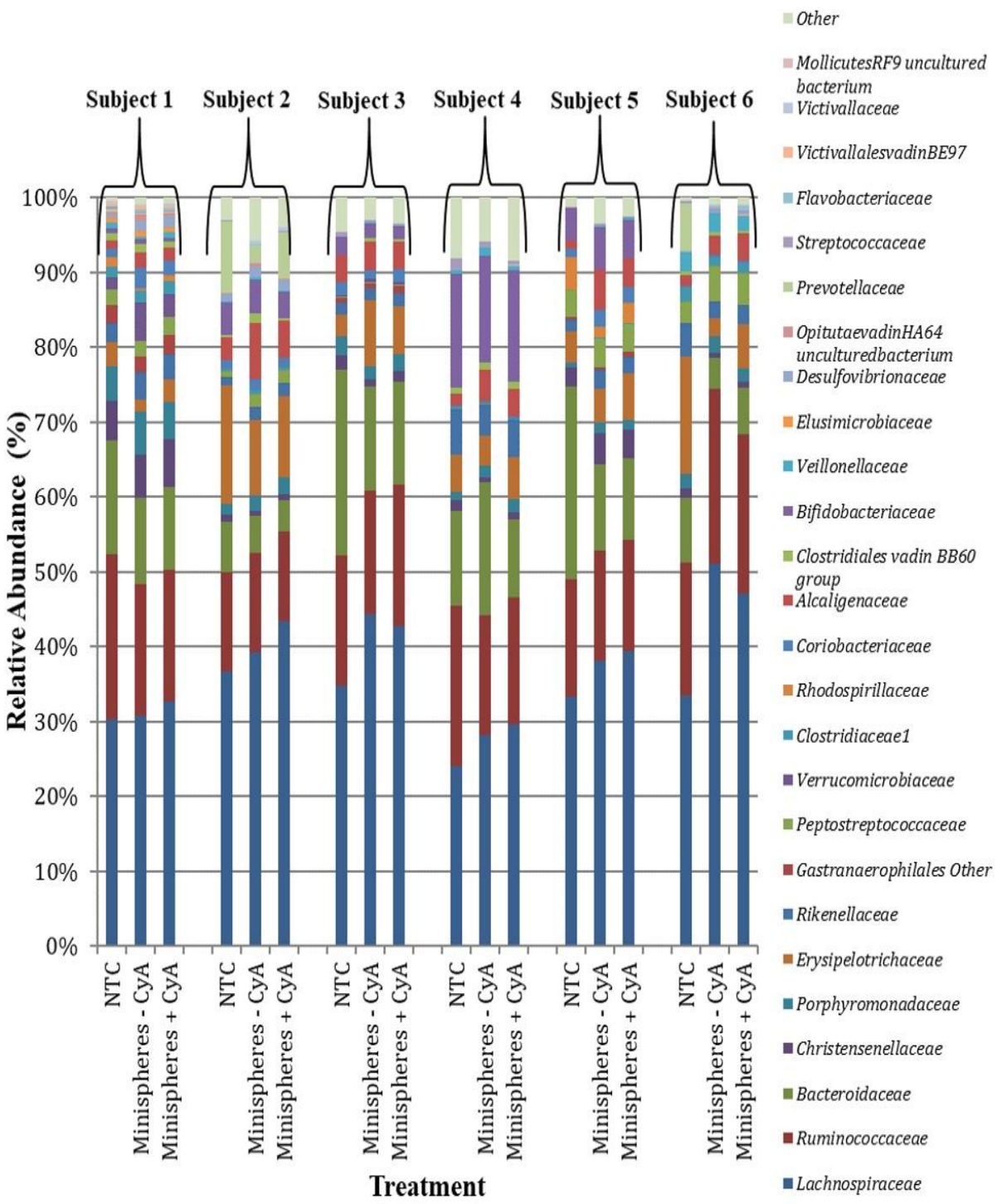


Figure 3. Relative abundance (%) of the bacterial families across treatment groups in faecal samples from six individual subjects (Subjects 1-6) following 24 h fermentation in an *ex vivo* colon model. Groups: non-treatment control (NTC); Minispheres - CyA, Minispheres without (-) CyA, Minispheres with (+) CyA,

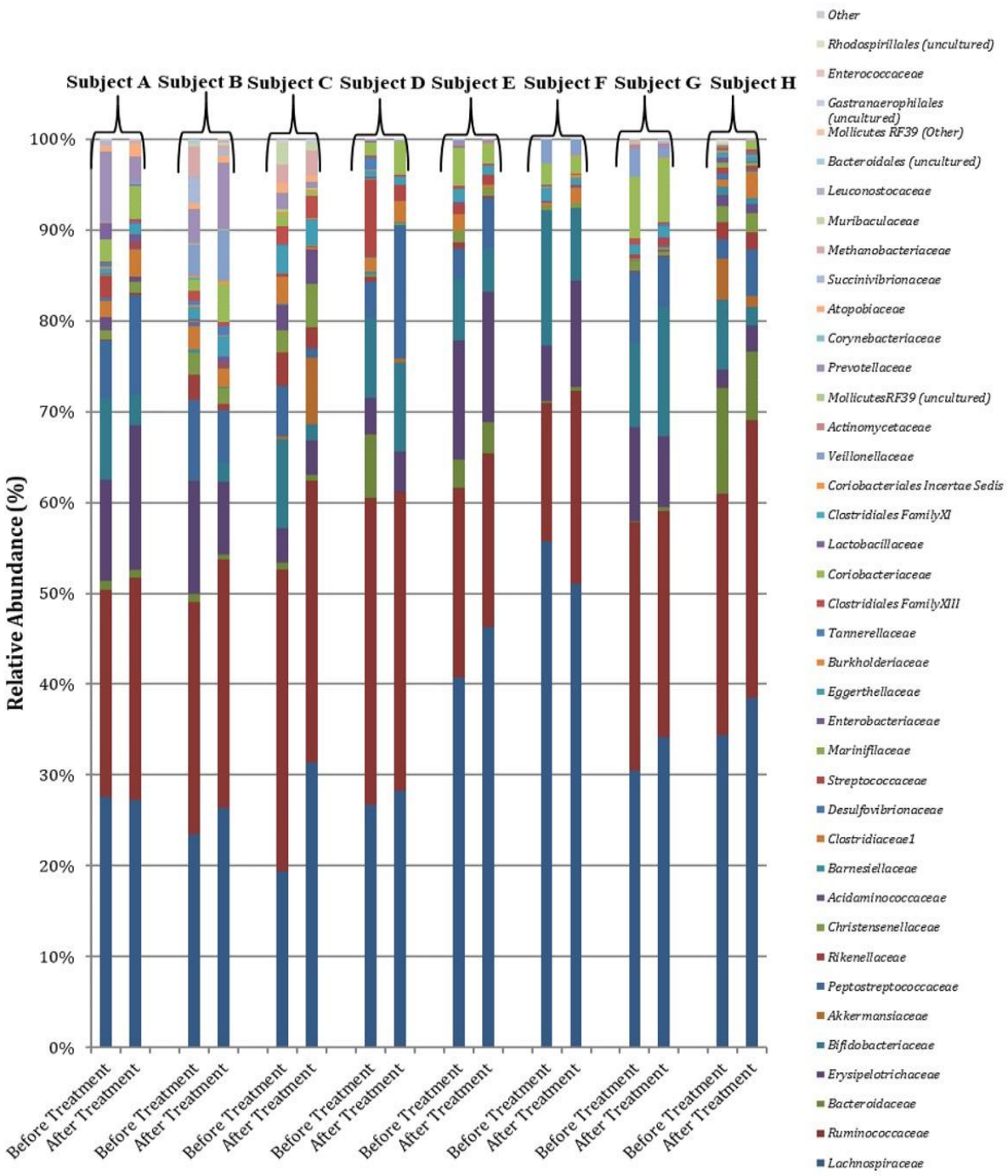


Figure 4. Relative abundance (%) of the bacterial families identified in human faecal samples from eight subjects (A-H), before and after treatment with Cyclosporine A (CyA) encapsulated into Smpill® minispheres.

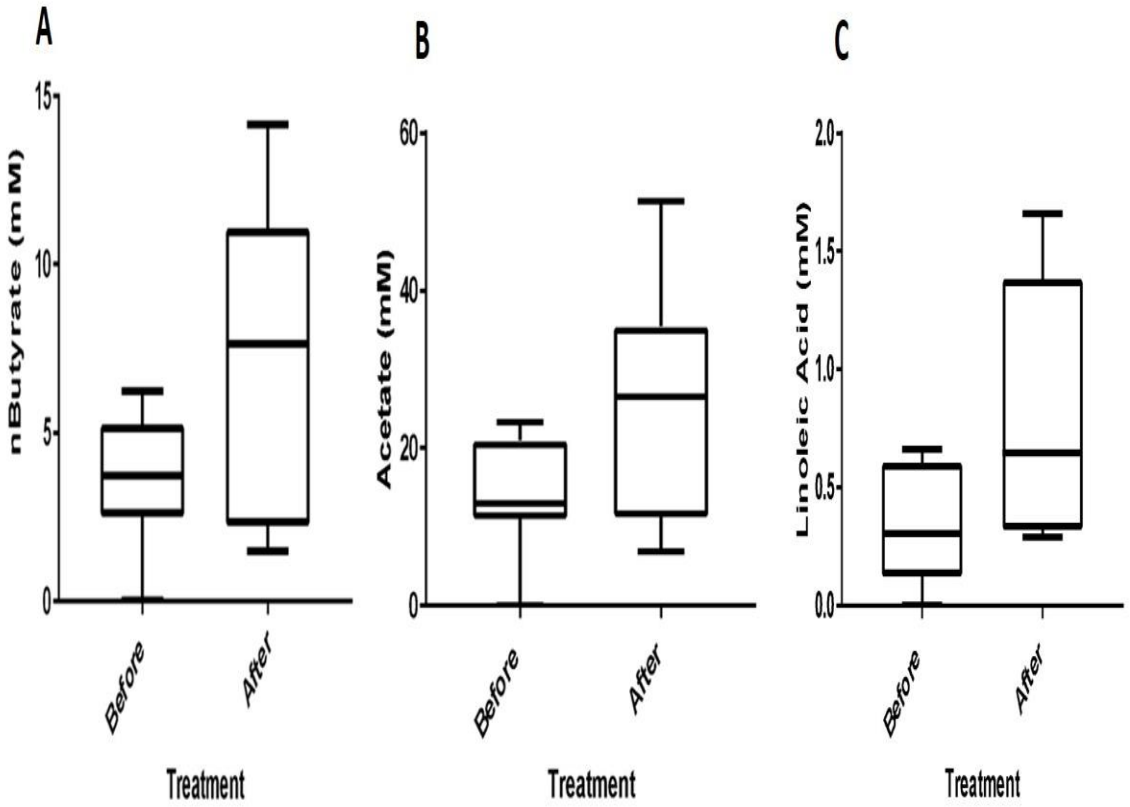
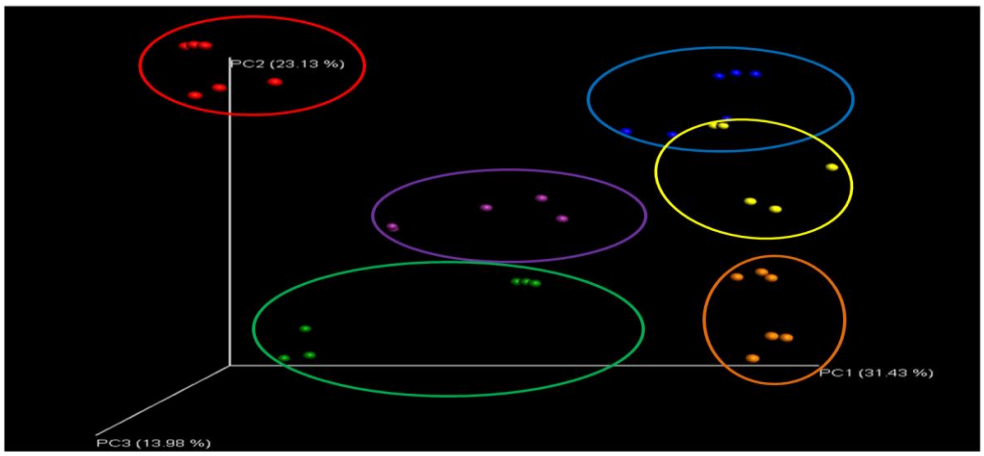
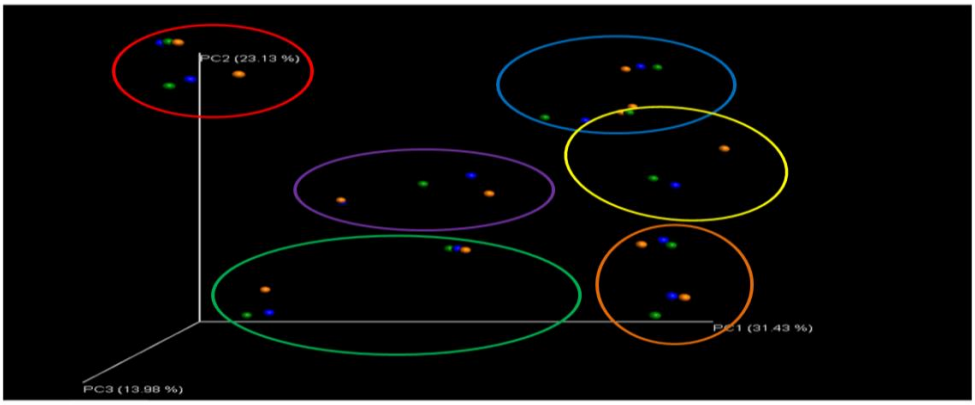


Figure 5. Effect of SmPill® + Cyclosporine A (CyA) on (A) butyrate levels P = 0.05 (B) acetate levels P = 0.05 and (C) linoleic acid levels P = 0.048 in human faecal samples from subjects participating in a pilot study in which they were administered SmPill® + CyA for seven days. Samples were taken before and after treatment.

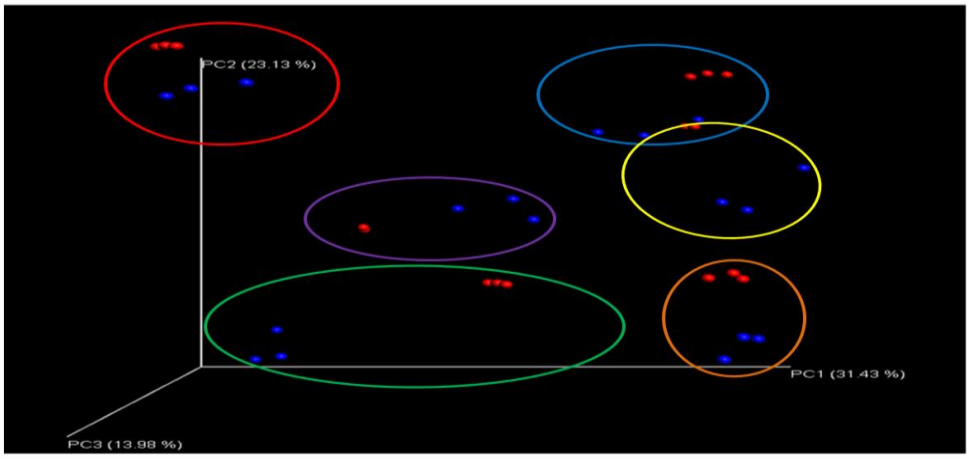
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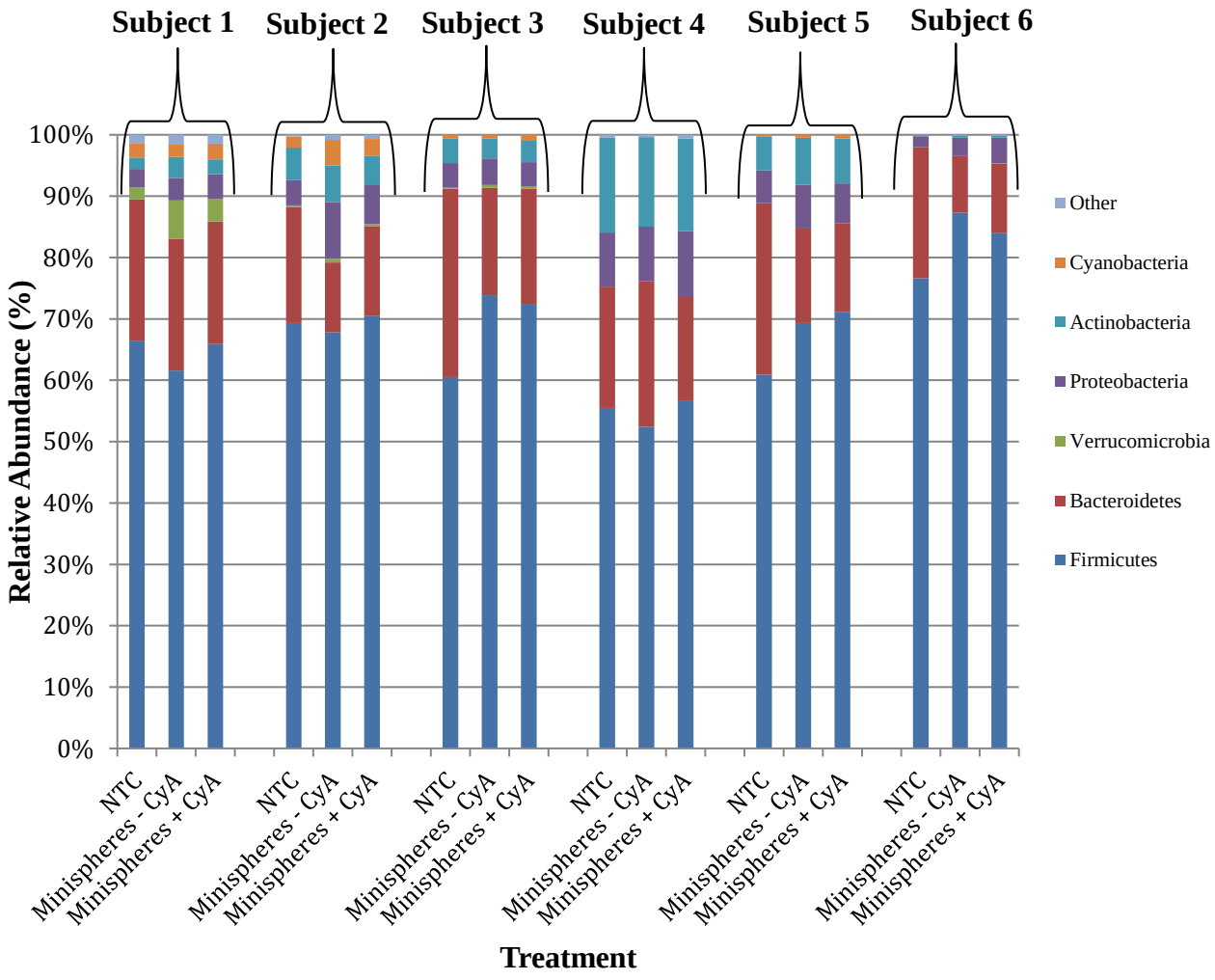
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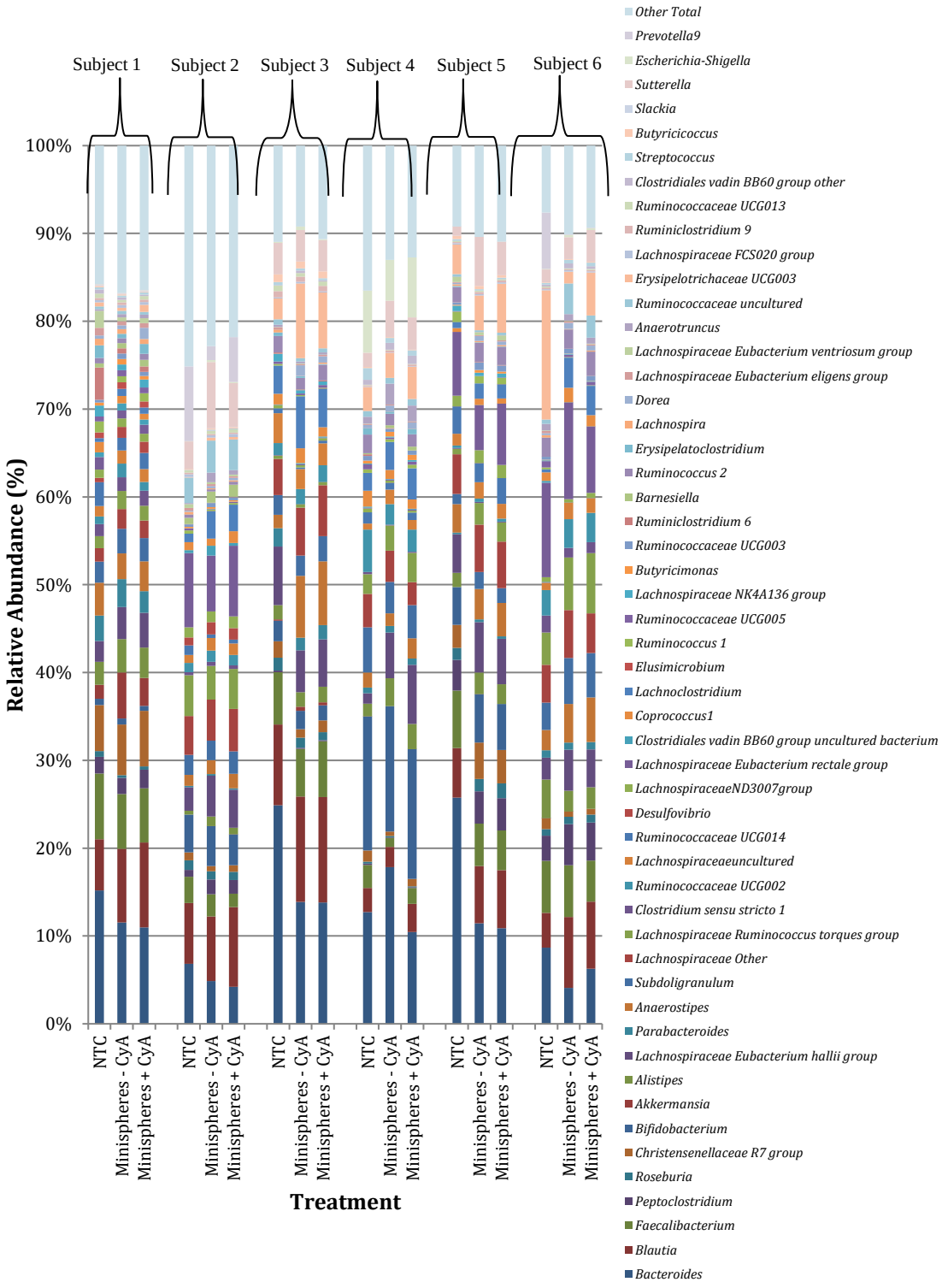
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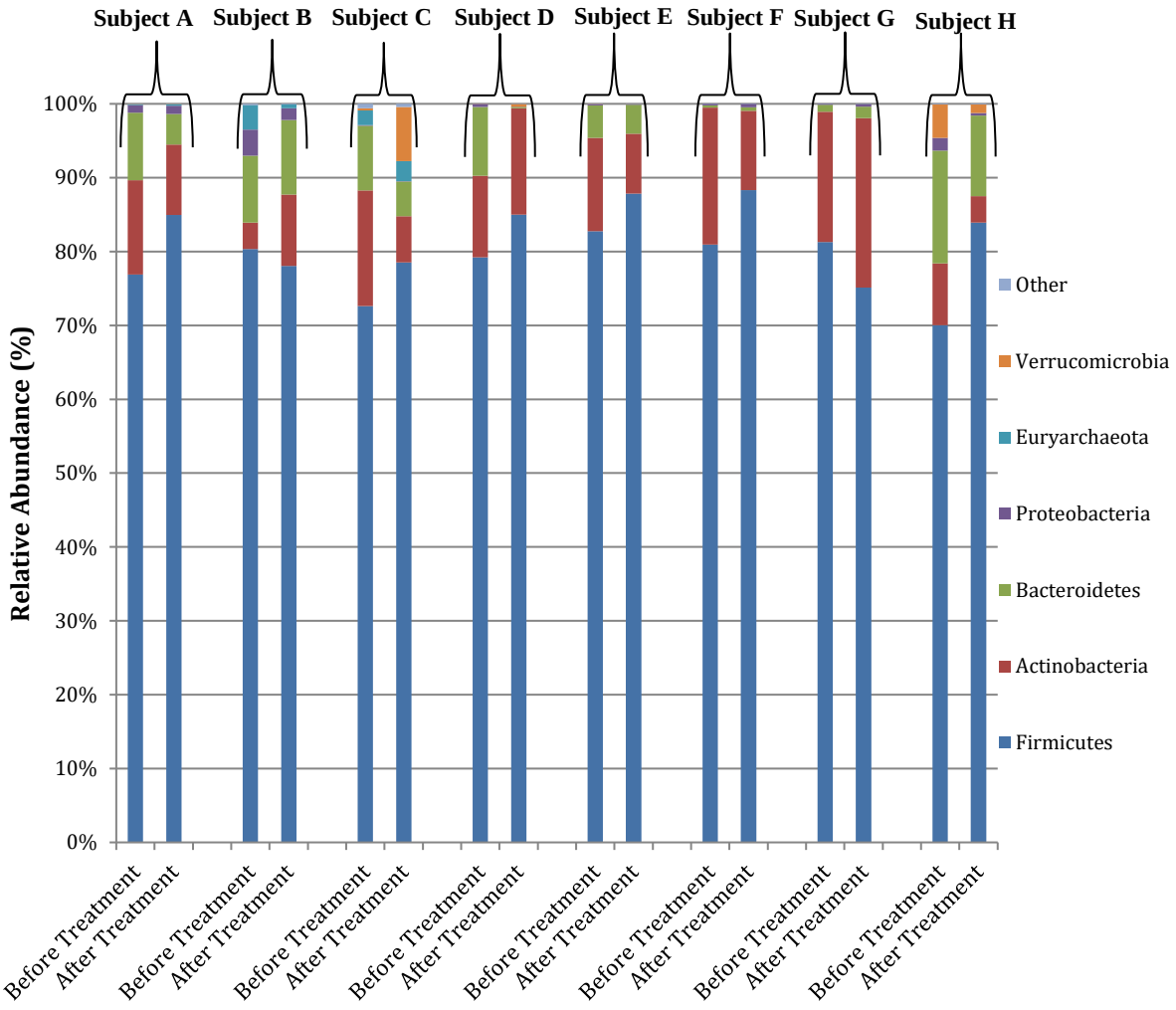
Supplementary Figure S1. PCoA plots based on unweighted UniFrac distance matrices. Each large circle represents an individual fecal donor. **(A)** samples presented by subject. Each sphere colour within the large circle represents an individual fecal donor sample at 0 and 24 h. **(B)** samples by treatment. Each large circle represents a subject and each sphere colour represents a treatment: orange: no treatment; blue: treated with minispheres with Cyclosporine A (CyA); green: treated with minispheres without CyA. **(C)** samples by time-point: red spheres, 0 hour; blue spheres, 24 hours.



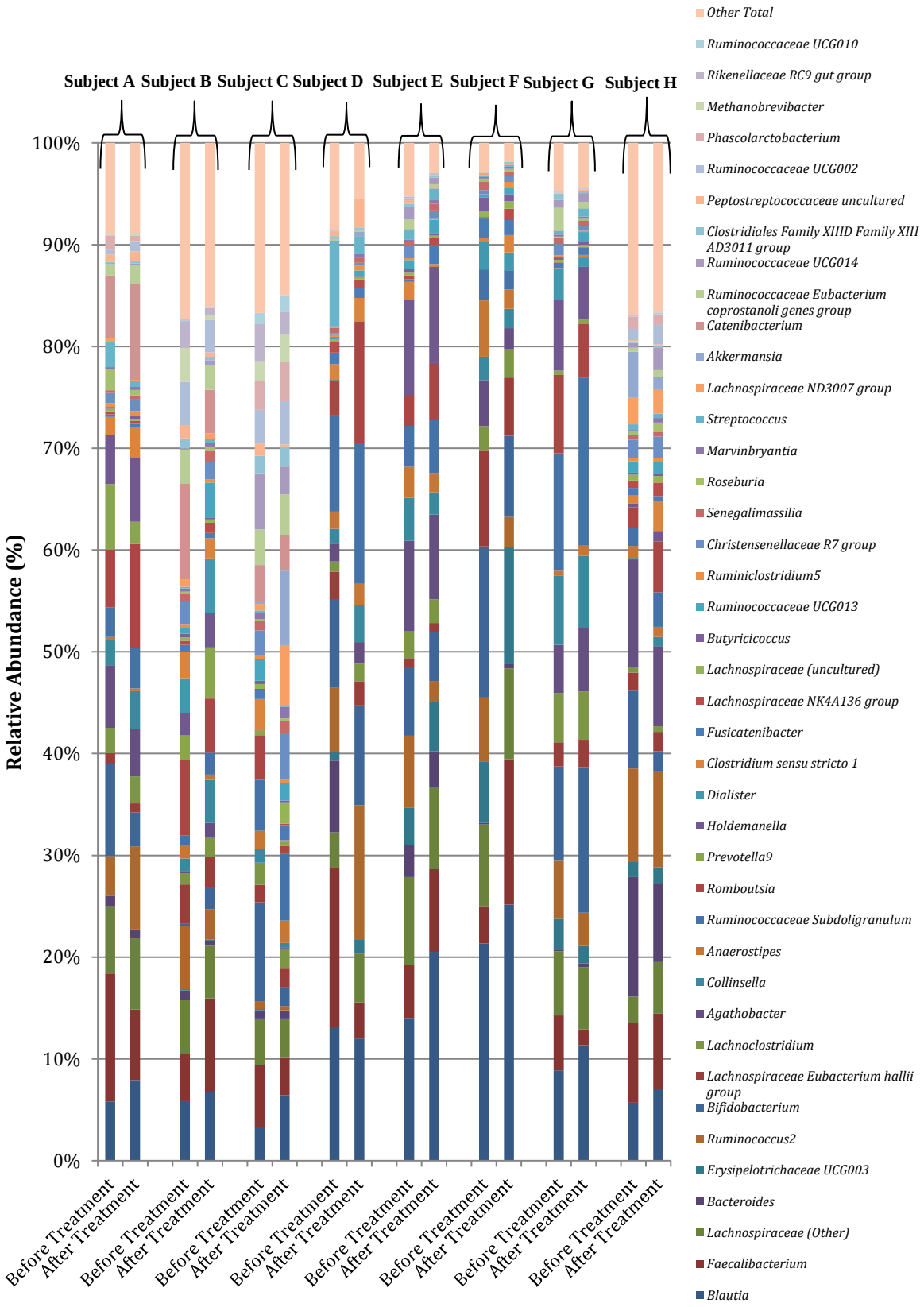
Supplementary Figure S2. Relative abundance (%) of the phyla across treatment groups in fecal samples from six subjects (1-6) following 24 hour fermentation in an *ex vivo* model. Groups: non-treatment control (NTC); Minispheres without (-) Cyclosporine A (CyA), Minispheres with (+) CyA.



Supplementary Figure S3. Relative abundance (%) of the bacterial genera identified in fecal samples in treatment groups from six individual subjects (Subjects 1-6) following 24h fermentation.Groups: non-treatment control; Minispheres without (-) Cyclosporine A (CyA); Minispheres with (+) CyA



Supplementary Figure S4. Relative abundance (%) of the bacterial phyla identified in fecal samples from human subjects (n=8) participating in the pilot study with SmPill® + CyA, before (Day 0) and after (Day 7) treatment.



Supplementary Figure S5. Relative abundance (%) of the bacterial genera identified in human fecal samples from eight subjects (A-H), before and after treatment with SmPill® + Cyclosporine A (CyA)

Supplementary Table S1

Alpha Diversity Indices (mean) for 6 fecal ferment samples across three treatment groups following 24 hour fermentation in an *ex vivo* model.

Index	No Minispheres	Minispheres with CyA	Minispheres without CyA	p value
Chao1 richness estimate	449.1	436.4	430.9	0.56
Simpson's Diversity Index	0.96	0.96	0.96	0.23
Shannon Index	5.8	5.9	5.8	0.38
Number of observed species	420.7	412.3	398.5	0.51

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Supplementary Table S2. Concentration of SCFAs (mM) in fecal samples following 24 h fermentation in an *ex vivo* model.

SCFAs (mM)	**nButyrate**			**iButyrate**			**Propionate**			**Acetate**		
Treatment	MS* - CyA	MS + CyA	No MS	MS - CyA	MS + CyA	No MS	MS - CyA	MS + CyA	No MS	MS - CyA	MS + CyA	No MS
Subject												
1	30.1	31.4	24.5	1.8	1.7	1.7	16	16	13.6	61.9	60	40.8
2	21.5	22.1	15.3	1.3	1.3	1.3	8.9	8.9	8.1	25.1	25.6	22.6
3	25	27.2	16.4	1.9	1.8	2	13.3	12.9	11.9	27.8	29.2	26.7
4	18.9	21.2	13.3	1.4	1.3	1.02	14.3	12.5	9.4	68.5	63.4	46.8
5	36.3	33.2	20.9	2.2	2.2	1.5	20.5	16.1	12.5	53	38	37.4
6	29.4	31.3	21	1.57	1.41	1.22	15.2	15.2	10.9	47.4	49.6	33
Mean	26.9	27.7	18.6	1.7	1.6	1.5	14.7	13.6	11.1	47.3	44.3	34.6
SD	6.4	5.1	4.2	0.3	0.4	0.4	3.8	2.8	2.0	17.7	15.9	9.0
P-Value	0.02			0.50			0.13			0.32		

*MS=Minispheres; CyA = Cyclosporine A; SD = Standard Deviation

Supplementary Table S3. Alpha diversity of gut microbiota in fecal samples from 8 subjects who participated in the pilot study before (Day 0) and after treatment (Day 7) with SmPill® + CyA.

Alpha diversity	Before treatment (Day 0)	After treatment (Day 7)	P-Value
Chao1 richness estimate	431.30	430.61	0.99
Simpson's Diversity Index	0.96	0.96	0.84
Shannon Index	5.57	5.56	0.96
Number of observed species	404.88	394.75	0.88

Supplementary Table S4. Concentration of SCFAs (mM) in fecal samples from healthy subjects before (Day 0) and after (Day 7) participating in the pilot study with SmPill® + Cyclosporine A (CyA).

SCFAs (mM)		nButyrate	iButyrate	Propionate	Acetate	IsoValerate	Hexanoate	Pentanoate
Subject	**Treatment**							
1	**Before**	2.5	0.89	4.8	13.0	0.71	0.06	1.17
	After	1.5	0.43	3.0	9.1	0.30	0.06	0.64
2	**Before**	4.3	0.82	4.9	10.9	0.62	0.18	0.81
	After	7.7	0.84	17.5	36.4	0.58	0.62	1.73
3	**Before**	5.5	0.31	11.7	23.3	0.28	0.31	0.89
	After	14.1	0.75	38.5	51.4	0.49	0.59	2.04
4	**Before**	3.2	0.30	2.8	11.2	0.24	0.00	0.42
	After	4.5	0.37	4.5	21.6	0.25	0.00	0.36
5	**Before**	4.2	0.42	4.0	17.4	0.28	0.00	0.51
	After	7.6	0.29	5.7	22.3	0.20	0.00	0.52
6	**Before**	2.5	0.41	5.5	13.0	0.26	0.00	0.48
	After	10.7	0.46	8.5	31.4	0.38	0.00	0.91
7	**Before**	6.2	0.44	9.1	22.1	0.28	0.00	0.60
	After	11.2	0.41	16.0	32.3	0.26	0.00	0.83
8	**Before**	0.0	0.00	0.0	0.0	0.00	0.00	0.00
	After	1.5	0.18	1.7	6.8	0.15	0.11	0.26
Mean	**Before**	3.6	0.45	5.35	13.87	0.33	0.07	0.61
	After	7.35	0.47	11.93	26.41	0.33	0.17	0.91
SD	**Before**	1.96	0.29	3.61	7.35	0.23	0.11	0.35
	After	4.63	0.22	12.19	14.67	0.15	0.27	0.65
P value		0.05	0.90	0.17	0.05	0.97	0.28	0.27

Supplementary Table S5. Effect of SmPill® + CyA on amino acids and other metabolites detected in fecal samples from healthy subjects (A-H) participating in a seven day pilot study.

Subject	A		B		C		D		E		F		G		H	
Treatment	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Compound (mM)																
Alanine	0.756	0.510	0.367	3.063	0.827	2.705	1.114	4.417	0.959	0.884	1.358	1.219	1.956	1.386	0.034	1.064
Glycine	0.370	0.258	0.146	1.314	0.411	1.288	0.615	1.899	0.566	0.344	0.764	0.727	0.753	0.671	0.010	0.647
Valine	0.345	0.247	0.143	1.019	0.276	0.992	0.512	1.440	0.429	0.311	0.711	0.540	0.594	0.370	0.017	0.478
Leucine	0.370	0.256	0.152	1.022	0.284	0.974	0.554	1.473	0.386	0.348	0.736	0.655	0.698	0.475	0.017	0.465
Isoleucine	0.232	0.162	0.100	0.727	0.202	0.690	0.353	1.021	0.269	0.190	0.534	0.285	0.433	0.271	0.009	0.310
Threonine	0.197	0.132	0.065	0.660	0.178	0.573	0.262	0.500	0.146	0.167	0.417	0.309	0.373	0.260	<LOD	0.150
Proline	0.211	0.161	0.084	0.093	0.132	0.385	0.212	0.913	0.135	0.045	0.326	0.158	0.251	0.123	0.007	0.263
Asparagine	0.013	0.020	0.012	0.025	0.016	0.028	0.011	0.102	0.008	0.009	0.013	0.019	0.012	0.011	0.001	0.013
Glutamine	0.327	0.262	0.257	1.040	0.288	0.934	0.417	1.476	0.565	0.390	0.471	0.545	0.818	0.510	<LOD	0.357
Serine	0.244	0.146	0.086	0.430	0.139	0.319	0.176	0.341	0.108	0.163	0.445	0.389	0.281	0.220	<LOD	0.120
Glutamic acid	1.040	0.655	0.415	2.557	0.779	2.249	1.821	4.253	1.682	1.207	1.569	1.543	1.793	1.290	0.018	1.462
Methionine	0.055	0.052	0.067	0.338	0.111	0.365	0.203	0.504	0.122	0.138	0.246	0.332	0.252	0.184	0.033	0.120
Phenylalanine	0.148	0.100	0.072	0.371	0.123	0.393	0.250	0.562	0.156	0.176	0.322	0.494	0.300	0.223	0.018	0.183
Ornithine	0.081	0.051	0.062	0.416	0.115	0.371	0.219	0.271	0.124	0.168	0.154	0.099	0.188	0.177	0.030	0.213
Lysine	0.393	0.237	0.161	1.445	0.313	1.159	0.624	1.845	0.530	0.405	0.900	0.951	0.986	0.766	0.019	0.563
Histidine	0.323	0.189	<LOD	0.462	<LOD	0.338	0.197	3.294	<LOD	<LOD	0.344	1.545	<LOD	0.407	<LOD	<LOD
Tyrosine	0.121	0.083	0.060	0.278	0.100	0.305	0.242	0.576	0.139	0.159	0.274	0.363	0.277	0.201	0.014	0.154
Tryptophan	0.058	0.054	0.052	0.098	0.059	0.104	0.078	0.143	0.061	0.073	0.083	0.091	0.097	0.090	<LOD	0.062
Aspartate	0.311	0.220	0.096	0.724	0.092	0.640	0.208	1.234	0.123	0.134	0.730	0.967	0.220	0.243	0.017	0.148
Succinic acid	0.135	0.148	0.061	2.303	1.214	2.855	1.934	2.593	2.487	2.911	0.452	14.459	2.025	1.838	0.011	1.663
Lactic acid	0.069	0.032	0.100	2.299	3.489	2.253	0.311	0.326	0.875	1.661	0.157	0.719	0.710	0.925	0.041	0.394
Fumaric acid	0.015	0.009	<LOD	0.025	0.011	0.027	0.007	0.063	0.012	<LOD	0.008	0.077	0.029	0.018	<LOD	0.019
Malic acid	0.419	0.265	0.177	0.322	0.245	0.423	0.201	0.352	0.275	0.112	0.196	0.607	0.600	0.569	0.016	0.237
2-oxoglutarate	0.080	0.058	0.021	0.083	0.028	0.060	0.028	0.089	0.050	0.032	0.041	0.046	0.063	0.047	<LOD	0.041
cis-Aconitic acid	0.019	0.022	0.020	0.031	0.023	0.036	0.016	0.024	0.017	0.018	0.020	0.023	0.021	0.020	0.011	0.015
Isocitric acid	0.037	0.039	0.032	0.055	0.035	0.050	0.043	0.045	0.037	0.038	0.040	0.044	0.050	0.042	0.028	0.038
Citric acid	0.055	0.053	0.045	0.067	0.036	0.060	0.046	0.069	0.040	0.043	0.059	0.049	0.047	0.053	0.026	0.044
Heptanoic acid (C7:0)	0.743	0.403	0.915	4.357	1.262	2.974	<LOD	0.101	<LOD	0.098	0.168	0.227	0.110	0.104	<LOD	0.884
Octanoic acid (C8:0)	<LOD	<LOD	0.305	0.739	<LOD	0.569	<LOD	0.769	<LOD	0.900	<LOD	6.133	<LOD	<LOD	<LOD	0.525
Linoleic acid (C12:2)	0.665	0.415	0.119	1.421	0.134	0.411	0.649	0.868	0.254	1.260	0.457	1.662	0.362	0.292	<LOD	0.293
Phenylacetic acid	2.195	1.078	1.562	1.830	0.229	1.082	0.675	0.640	0.700	0.529	0.881	0.510	0.889	0.552	0.032	0.422
4-Hydroxyphenylacetic acid	0.320	0.599	0.806	1.887	0.228	1.131	0.106	1.422	0.331	0.119	3.039	1.098	0.719	0.492	<LOD	0.129
Indole-3-acetic acid	0.233	0.202	0.046	4.414	<LOD	0.861	0.273	0.392	0.250	1.542	0.123	0.558	1.836	1.075	<LOD	0.126

Supplementary Table S6. The effect of uncoated minispheres with (+) and without (-) Cyclosporine A (CyA) (0.75 mg ml^{-1}) on the growth of *Bacteroides* sp *in vitro* (cfu/ml)

Bacteroides Strain	Treatment	TBC (cfu/ml)
		T48
Bacteroides distasonis	No Treatment control	6.6×10^9
	Minispheres without cyclosporine (uncoated)	2.2×10^9
	Minispheres with cyclosporine (uncoated)	2.6×10^8
Bacteroides dorei	No Treatment control	4.86×10^8
	Minispheres without cyclosporine (uncoated)	3.78×10^6
	Minispheres with cyclosporine (uncoated)	1.78×10^6
Bacteroides fragilis	No Treatment control	2.58×10^6
	Minispheres without cyclosporine (uncoated)	4.04×10^6
	Minispheres with Cyclosporine (uncoated) beads	3.12×10^6

References

Benjamini, Y, and Y Hochberg. 1995. “Controlling the false discovery rate: a practical and powerful approach to multiple testing.” *Journal of the Royal statistical society: series B (Methodological)* 57 (1): 289–300. doi:10.1111/j.2517-6161.1995.tb02031.x.

Biron, E, J Ovadia, O Chatterjee, D Langenegger, J Brueggen, D Hoyer, HA Schmid, R Jelinek, C Gilon, A Hoffman, and H Kessler. 2008. “Improving oral bioavailability of peptides by multiple N-methylation: somatostatin analogues.” *Angewandte Chemie International Edition* 47 (14): 2595-9. doi:10.1002/anie.200705797.

Canani, RB, MD Costanzo, L Leone, M Pedata, R Meli, and A Calignano. 2011. “Potential beneficial effects of butyrate in intestinal and extraintestinal diseases.” *World J Gastroenterol* 17 (12): 1519-28. doi:10.3748/wjg.v17.i12.1519.

Caporaso, JG, J Kuczynski, J Stombaugh, K Bittinger, FD Bushman, EK Costello, N Fierer, et al. 2010. “QIIME allows analysis of high-throughput community sequencing data.” *Nature methods* 7 (5): 335-336. doi:10.1038/nmeth.f.303.

Colombel, JF, IS Coulter, J Hall, M Rosa, B McDonald, and A Cullen. 2019. “P053 Targeted release oral cyclosporine formulations as a potential new therapy for Ulcerative Colitis.” *Gastroenterology* 25 (Supplement_1): S26-S26. doi:10.1053/j.gastro.2019.01.109.

Drewe, J, C Beglinger, and T Kissel. 1992. “The absorption site of cyclosporin in the human gastrointestinal tract.” *Br J Clin Pharmacol* 33 (1): 39–43. doi:10.1111/j.1365-2125.1992.tb03998.x.

Edgar, RC. 2010. “Search and clustering orders of magnitude faster than BLAST.” *Bioinformatics* 26 (19): 2460-2461. doi:10.1093/bioinformatics/btq461.

Flint, HJ, KP Scott, SH Duncan, P Louis, and E Forano. 2012. “Microbial degradation of complex carbohydrates in the gut.” *Gut Microbes* 3 (4): 289–306. doi:10.4161/gmic.19897.

Fooks, LJ, and GR Gibson. 2003. “Mixed culture fermentation studies on the effects of synbiotics on the human intestinal pathogens Campylobacter jejuni and Escherichia coli.” *Anaerobe* 9: 231–242. doi:10.1016/S1075-9964(03)00043-X.

Fouhy, F, J Deane, MC Rea, Ó O’Sullivan, RP Ross, G O’Callaghan, BJ Plant, and C Stanton. 2015. “The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations.” *PLoSONE* 10 (3): e0119355. doi:10.1371/journal.pone.0119355.

Gao Z, Yin J, Zhang J et al. 2009. “Butyrate improves insulin sensitivity and increases energy expenditure in mice.” *Diabetes* 58 (7): 1509-17. doi:10.2337/db08-1637.

Guada, M, A Beloqui, MNV Ravi Kumar, V Préat, M Dios-Viéitez, and MJ Blanco-Prieto. 2016. “Reformulating cyclosporine A (CsA): More than just a life cycle management.” *Journal of Controlled Release* 225: 269-282. doi:10.1016/j.jconrel.2016.01.056.

Haviv, F, TD Fitzpatrick, RE Swenson, CJ Nichols, NA Mort, EN Bush, G Diaz, G Bammert, and A Nguyen. 1993. “Effect of N-methyl substitution of the peptide bonds in luteinizing hormone-releasing hormone agonists.” *Journal of medicinal chemistry* 36 (3): 363–39. doi:10.1021/jm00055a007.

Jorga, A, DW Holt, and A Johnston. 2004. “Therapeutic drug monitoring of cyclosporine.” *Transplantation proceedings* 36 (2): 396S-403S. doi:10.1016/j.transproceed.2004.01.013.

Keku, TO, S Dulal, A Deveaux, B Jovov, and X Han. 2014. “The gastrointestinal microbiota and colorectal cancer.” *Am J Physiol Gastrointest Liver Physiol* 308 (5): G351-63. doi:10.1152/ajpgi.00360.2012.

Keohane, K, M Rosa, IS Coulter, and BT Griffin. 2016. “Enhanced colonic delivery of cyclosporine A self-emulsifying drug delivery system encapsulated in coated minispheres.” *Drug Development and Industrial Pharmacy* 42 (2): 245-253. doi:10.3109/03639045.2015.1044905.

Knights, D, KG Lassen, and RJ Xavier. 2013. “Advances in inflammatory bowel disease pathogenesis: linking host genetics and the microbiome.” *Gut* 62: 1505-1510. doi:10.1136/gutjnl-2012-303954.

Laharie, D. 2012. “"Groupe d'Etudes Therapeutiques des Affections Inflammatories Digestives: Ciclosporin versusu inflixinmab in patients with severe ulcerative colitis refractory intravenous steroids: a parallel, open-label randomised controlled trial."." *Lancet* 380 (9857): 1909-15. doi:10.1016/S0140-6736(12)61084-8.

Magoč, T, and SL Salzberg. 2011. “FLASH: fast length adjustment of short reads to improve genome assemblies.” *Bioinformatics* 27 (21): 2957–2963. doi:10.1093/bioinformatics/btr507.

Maurice, CF, HJ Haiser, and PJ Turnbaugh. 2013. “Xenobiotics shape the physiology and gene expression of the active human gut microbiome.” *Cell* 152: 39-50. doi:10.1016/j.cell.2012.10.052.

McNeil, NI. 1984. “The contribution of the large intestine to energy supplies in man.” *The American journal of clinical nutrition* 39 (2): 338-342. doi:10.1093/ajcn/39.2.338.

O'Donoghue, DP, S Bloom, and I Coulter. 2013. “Colon targeted low systemic absorption soluble ciclosporin in Ulcerative Colititis.” *Gut* 62 (Suppl 2): A1-A2. doi:10.1136/gutjnl-2013-305143.3.

Ordás, I, L Eckmann, M Talamini, DC Baumgart, and WJ Sandborn. 2012. “Ulcerative Colitis.” *Lancet* 380 (9853): 1606-1619. doi:10.1016/S0140-6736(12)60150-0.

Peppercorn, MA, and P Goldman. 1972;. “The role of intestinal bacteria in the metabolism of salicylazosulfapyridine.” *J Pharmacol Exp Ther* 181 (555).

Quast, C, E Pruesse, P Yilmaz, J Gerken, T Schweer, P Yarza, and Glöckner Peplies J ja FO. 2013. “The SILVA ribosomal RNA gene database project: improved data processing and web-based tools.” *Nucleic Acids Research* 41 ((Database issue)): D590-596. doi:10.1093/nar/gks1219.

Rea, MC, A Dobson, O O'Sullivan, F Crispie, F Fouhy, PD Cotter, F Shanahan, B Kiely, C Hill, and RP Ross. 2010. “Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon.” *Proc Natl Acad Sci U S A* 108 (supplement 1): 4639-44. doi:10.1073/pnas.1001224107.

Ríos-Covián, D, P Ruas-Madiedo, A Margolles, M Gueimonde, CG de Los Reyes-Gavilán, and N Salazar. 2016. “Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health.” *Frontiers in Microbiology* 7 (185): 10-3389. doi:10.3389/fmicb.2016.00185.

Sharkey, L, F Bredin, A Nightingale, and M Parkes. 2011. “The use of Cyclosporine A in acute steroid-refractory ulcerative colitis: long term outcomes.” *J Crohn’s Colitis* 5: 91-94. doi:10.1016/j.crohns.2010.10.004.

Tan, J, C McKenzie, M Potamitis, AN Thorburn, CR Mackay, and L Macia. 2014. “The role of short-chain fatty acids in health and disease.” *Advanced Immunology* 121: 91-119. doi:10.1016/B978-0-12-800100-4.00003-9.

Vázquez-Baeza, Y, M Pirrung, A Gonzalez, and R Knight. 2013. “EMPeror: a tool for visualizing high-throughput microbial community data.” *GigaScience* 2 (1): 2047-217X. doi:10.1186/2047-217X-2-16.

Wang, J, V Yadav, AL Smart, S Tajiri, and AW Basit. 2015. “Stability of peptide drugs in the colon.” *European Journal of Pharmaceutical Sciences* 78: 31–36. doi:10.1016/j.ejps.2015.06.018.

Williams, JG, MF Alam, L Alrubaiy, I Arnott, C Clement, D Cohen, JN Gordon, et al. 2016. “Infliximab versus ciclosporin for steroid-resistant acute severe ulcerative colitis (CONSTRUCT): a mixed methods, open-label, pragmatic randomised trial.” *The lancet Gastroenterology & hepatology* 1 (1): 15-24. doi:10.1016/S2468-1253(16)30003-6.

Wilson, ID, and JK Nicholson. 2017. “Gut microbiome interactions with drug metabolism, efficacy, and toxicity.” *Translational research* 179: 204-222. doi:10.1016/j.trsl.2016.08.002.

Chapter 3

Impact of nisin on *Clostridioides difficile* and microbiota composition in a faecal fermentation model of the human colon

Abstract

Nisin represents the prototypical bacteriocin and has a broad spectrum of activity against Gram-positive bacteria including the gut pathogen *Clostridioides difficile* (*C. difficile*). The aim of this study was to assess the activity of nisin at a range of concentrations from 0-500 µM against *C. difficile* in a complex microbial environment and determine the minimum inhibitory concentration at which *C. difficile* growth is suppressed while having minimal impact on the faecal microbiota. Faecal fermentations were performed using the MicroMatrix® fermenter. Slurries were prepared from fresh faecal samples from six individual volunteers and spiked with *C. difficile* on the day of the experiment (10^6 CFU per ml). Nisin was added to each fermentation at a range of concentrations from 0-500 µM. Following 24 h, samples were taken from each and assessed for the presence of viable *C. difficile* while the community was subjected to 16S rRNA gene sequencing. Both 16S rRNA gene sequencing and plating on solid media revealed no viable *C. difficile* in the presence of 50–500 µM nisin. In addition, there was a decrease in diversity of the microbiota and a relative increase in the phylum Proteobacteria in a nisin dose-dependent manner. Nisin predominantly depleted the relative abundance of the Gram-positive bacteria while the relative abundance of Gram-negative bacteria such as *Escherichia-Shigella* and *Bacteroides* increased. This study opens up the potential to use nisin as a therapeutic for clostridial gut infections.

Importance

Clostridioides difficile is a Gram-positive anaerobic spore-forming bacterium which can cause severe gastrointestinal illness. Antibiotic use can lead to disruption of the normal colonic flora resulting in an increased risk of *C. difficile* infection (CDI). Due to the increase in antibiotic resistance and hospital-acquired infections there is an urgent need to develop new treatments for CDI.

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacterial species. Nisin A (nisin) is produced by *Lactococcus lactis* subsp. *lactis* and has a broad spectrum of activity against most Gram-positive bacteria. Nisin has been shown to effectively kill *C. difficile in vitro* at concentrations comparable to antibiotics. Using an *ex vivo* model of the colon, this study demonstrates the ability of purified nisin to selectively deplete *C. difficile* in a faecal microbial environment and establishes the minimum concentration at which this occurs whilst having a minimal impact on the composition of the microbiota.

Introduction

Clostridioides difficile is a Gram-positive anaerobic spore-forming bacterium which can cause severe gastrointestinal illness in individuals undergoing antibiotic treatment (Kelly and LaMont 2008) (Rupnik, Wilcox and Gerding 2009) (Stanley, et al. 2013). Antibiotic exposure generally leads to disruption of the normal colonic flora. This can result in reduced resistance against intestinal colonization by *C. difficile* and result in increased risk of *C. difficile* infection (CDI). Additional risk factors for CDI are inflammatory bowel disease, immunodeficiency and related illnesses (Kyne, et al. 2002) (Ananthakrishnan, McGinley and Binion 2008). Many antibiotics have been linked with CDI including ampicillin, amoxicillin, clindamycin, cephalosporins and fluoroquinolines (Wiström, et al. 2001) (Stevens, et al. 2011). In 2016, a total of 7,711 reported cases of CDI were recorded in 20 countries by the ECDI (European Centre for Disease Prevention and Control), 5,756 of which (74.6%) were healthcare-associated (HA) CDI and 1,955 (25.4%) of which were either community-associated (CA) or of unknown origin (Annual epidemiological report for 2016. Healthcare-associated infections: *Clostridium difficile* infections). *C. difficile* infection can cause acute diarrhoea and pseudomembranous colitis, which can lead to colonic perforation and, if untreated, ultimately death (Kelly and LaMont 2008; Rupnik, Wilcox and Gerding 2009; Stanley, et al. 2013). The antibiotics vancomycin and metronidazole are well established treatments for CDI (Surowiec, et al. 2006). Their use is controversial, however, due to an increase in the number of reported treatment failures and recurrence of CDI (Aslam; 2005). The use of vancomycin has been associated with development of vancomycin-resistant enterococci for

instance (La grotteria, et al. 2006). Similarly, *C. difficile* isolates that are resistant to metronidazole have been identified (Freeman, et al. 2015). Fidaxomicin is the most recent medication approved for the treatment of CDI. Fidaxomicin is expensive, however, and the cost far exceeds that of metronidazole or vancomycin. It is recommended only to be used in populations who are at a high risk of relapse and those in which Fidaxomicin has proven more effective than other treatments (Mullane 2014). Due to the increase in antibiotic resistance and hospital-acquired infections there is an urgent need to develop new treatments for CDI.

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacterial species which can exhibit either a narrow spectrum of activity (targeting members of the same species) or a broad activity spectrum (targeting other species and genera) (Cotter, Ross and Hill 2013). They are generally produced by bacteria within their ecological niches and serve as a competitive advantage and self-preservation tool. Most of the currently recognized bacteriocins are produced by Gram-positive bacterial species with lesser known producers among Gram-negative bacteria (Meade, Slattery and Garvey 2020; Ghodhbane, et al. 2015). Several bacteriocins have proven activity against *C. difficile* such as lacticin 3147 (Rea, et al. 2007), microbisporin (Castiglione, et al. 2008), thuricin CD (Rea, et al. 2010) and nisin (Le Blay, et al. 2007).

Nisin is of interest for several reasons. Nisin A (nisin) is produced by *Lactococcus lactis* subsp. *lactis* and has a broad spectrum of activity against most Gram-positive bacteria and other lactic acid producing bacteria (Abee and Delves-Broughton; 2003). Nisin A was first discovered in 1928 and is the most intensively studied lantibiotic (Rogers and Whittier; 1928). Nisin has been

approved as safe for use in the food industry (as a natural food preservative, E234) by the US Food and Drug Administration (FDA), World Health Organization (WHO) and by the European Union (EU) (And and Hoover; 2003; Delves-Broughton; 1990; Guinane, et al. 2005). Nisin has been shown to effectively kill C. difficile in vitro at concentrations comparable to antibiotics such as metronidazole and vancomycin (Le Lay, et al. 2016). Because nisin is proteinaceous in nature digestive proteases will break it down (albeit to a lesser extent than less modified bacteriocins in our experience). Delivery to the colon for the treatment of *C. difficile* could be achieved through rectal administration, encapsulation or removal of the proteolytic sites. In this study, we used an ex vivo model of the colon to investigate the ability of purified nisin to selectively deplete *C. difficile* in a faecal microbial environment and establish the minimum concentration at which this occurs whilst having a minimal impact on the composition of the microbiota.

Materials and Methods

Donor Recruitment for Faecal Fermentation Studies

Donor recruitment and enrolment were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (protocol no. APC055). All donors completed and signed a consent form to acknowledge their participation in the study. All human subjects were healthy females and males between 23 and 65 years of age. Participants had no antibiotic use in the 6 months prior to participation in the study.

Micro-Matrix™ Cassette Set-up and parameters and Faecal medium

Six healthy volunteers of varying ages (23-62) and gender (3 male and 3 female) provided fresh faecal samples on the day of the experiment. The micro-Matrix™ (Applikon Biotechnology, Heertjeslaan 2, 2629 JG Delft, Netherlands) mini-fermentation system was used to model the distal colon as described previously by O'Donnell, et al; 2018.

The micro-Matrix™ provides simultaneous individual measurement and control of temperature, pH, and dissolved oxygen concentration (dO2), in 24 parallel micro bioreactors (wells) with working volumes of up to 5 ml each. Each well of the micro-Matrix cassette operates as a stand-alone bioreactor. The pre-calibrated integrated pH and DO sensors remove the need to calibrate each well prior to beginning the fermentation (O'Donnell, et al. 2018). Faecal medium was prepared according to Fooks, et al. 2003. In brief, the fermentations were carried out in three sealed micro-Matrix cassettes (24 wells/cassette) with 2 subjects per run. Each condition was carried out in duplicate wells of the micro-Matrix plate. The nisin A used in this study was a high content powder form (95% content/ultrapure; % weight/weight; hydrous potency ≥38,000 IU/mg) which was purchased from Handary (Brussels, Belgium). Fermentations were carried out using the fermenter as follows: 1 mM stock of nisin A was made on the day of the experiment. The 1 mM stock was added to wells at varying volumes for final concentrations of 25, 50, 100, 250 and 500 µM in a total volume of 5 ml. For 25 µM nisin 125 ul of 1 mM nisin was added to 3.875 mls fecal media. For 50 µM nisin 250 ul of 1 mM nisin was added to 3.75 mls fecal media. For 100 µM nisin 500 ul of 1 mM nisin was added to 3.5 mls fecal media. For 250 µM nisin 1.25 mls of 1 mM nisin was added to 2.75 mls fecal media. For 500 µM nisin 2.50 mls

of 1 mM nisin was added to 1.5 mls fecal media. A control with 0 µM nisin was also included for each donor sample. *C. difficile* (Ribotype 027) was spiked in at 10^6 CFU per ml. 20% faecal slurry was prepared under anaerobic conditions using 50 mM phosphate buffer pH 7.0 containing 0.5% cysteine which had previously been boiled and cooled anaerobically. 1 ml was added to each well of the MicroMatrix® plate.

The Time 0 h (T0) sample containing 20% slurry and 80% faecal medium was taken during the setup of the cassette in the anaerobic chamber and snap frozen at -80°C immediately in 1 ml aliquots until used for DNA extraction. The cassette was then clamped and sealed using the transport clamps and transferred to the micro-Matrix. Samples were incubated anaerobically for 24 h at 37 °C at 250 rpm. Anaerobic conditions were established in the fermenters by sparging the system with Nitrogen which was under direct control in the machine set at 40%. The installation of the filled cassette and the attachment of the parts were performed according to the micro-Matrix manual. CO2 was used for downward pH control, 20% (v/v) sodium hydroxide (NaOH) was used as upward pH control, the orbiter was set to 250 rpm. The control set-points parameters on the micro-Matrix were pH 6.8 (6.6-7.2 low and high alarm points respectively), dO2 0% (high alarm 20%).

DNA Extraction

Samples (1 ml) were taken at T0 and T24 from each well of the micro-Matrix cassette and snap frozen at -80°C until use. The Zymo Research ZR faecal DNA kit (Cambridge Biosciences, Cambridge, United Kingdom) was used to extract

DNA from samples. Extractions were carried out according to the manufacturer's specifications.

Library preparation

The V3-V4 variable region of the 16S rRNA gene was amplified from 30 faecal DNA extracts using the 16S metagenomic sequencing library protocol (Illumina Saffron Walden, UK). Samples were quantified, and libraries were prepared for sequencing as described previously (Fouhy, et al. 2015). In brief, two PCR reactions were completed on the template DNA for the 16SrRNA amplicon sequencing. The DNA template was amplified initially with primers specific to the V3–V4 region of the 16S rRNA gene. These primers also incorporate the Illumina overhang adaptor (forward primer 5′ TCGTCGGCAGCGTCAG ATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5′ GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC). Following this a second PCR reaction with two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were added to allow for demultiplexing. Samples were run on the Illumina Miseq sequencer with a Miseq 500 cycle kit on the Illumina Miseq sequencer at the Teagasc Sequencing facility at the Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. Following sequencing, data was analysed bioinformatically to establish the effect of the antimicrobials on the composition of the gut microbiota.

Bioinformatics and Statistical Analysis of Compositional Sequencing Data

Raw Illumina 250 bp paired-end sequence reads were merged using Flash (FLASH: fast length adjustment of short reads to improve genome assemblies)

(Magoč and Salzberg 2011) and quality checked using the split_libraries script from the Qiime package (Caporaso, et al. 2010). Reads were then clustered into operational taxonomic units (OTUs) and chimeras removed with the 64-bit version of USEARCH (Edgar 2010). Subsequently OTUs were aligned and a phylogenetic tree generated within Qiime (Caporaso, et al. 2010). Taxonomical assignments were reached using the SILVA 16S specific database (version 116) (Quast, et al. 2013).

Statistical Analysis

Calypso online software (version 8.84) was used for statistical analysis (Zakrzewski, et al. 2017) and SPSS V8 (IBM) were appropriate. Cyanobacteria and chloroplast signatures as well as OTUs with a relative frequency below 0.01% were removed. Cumulative Sum scaling (CSS) was used and data was Log2 transformed to account for non-normal distribution of taxonomic count data for alpha and beta diversity testing. Multivariate analysis including both RDA and CCA methods were used to investigate the complex associations between microbiota composition and treatment groups. Alpha diversity was measured using Shannon diversity, Simpson diversity and evenness. Beta diversity was measured based on Bray-Curtis distance matrices. ANCOM was used to study the gut microbiota of subjects in each treatment group. ANCOM compares the log ratio of the abundance of each taxon to the abundance of all remaining taxa at one time and then Mann-Whitney U is calculated on each log ratio. ANCOM accounts for compositional constraints of metagenomic data to reduce false discoveries in detecting differentially abundant taxa (Weiss, et al. 2017). Kruskal

Wallis and Mann-Whitney U tests were performed to identify taxonomical differences between treatment groups at the family and genus level. LEfSe: Linear Discriminant Analysis Effect Size algorithm was used to identify predominant taxa between treatment groups considering biological consistency and effect relevance (Segata, et al. 2011). The threshold for the logarithmic LDA score was set at 3.0 for biomarker discovery. All P values were corrected for multiple testing using the False Discovery Rate (FDR) correction. Significance was accepted as $p < 0.05$ following FDR correction. Barcharts showing average relative abundance across treatment groups were created in Excel (Microsoft, Redmond, WA).

Mass Spectrometry

Samples from the faecal fermentations (1 ml) were centrifuged for 60 s at 21 000 g. The supernatant was filtered using a 0.45 µM filter and was removed for analysis. The Mass Spectrometry in all cases was performed with an Axima TOF2MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10 mg ml−1 in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target and left for 1–2 mins before being removed. The residual solution was then air-dried and the sample solution (resuspended lyophilised powder or CMS supernatant) was positioned onto the precoated sample spot. Matrix solution (0.5 µl) was added to the sample and allowed to air-dry. The sample was subsequently analysed in positive-ion reflectron mode.

Results

In this study we used an *ex vivo* human colonic model with *C. difficile* spiked into faecal slurries at concentrations representative of a colonic infection. Purified nisin A was added at a range of concentrations and fermented anaerobically for 24 h at 37°C and constant pH to assess the impact of nisin on the gut microbiota and *C. difficile* growth.

Diversity decreases with increasing concentrations of nisin

16S rRNA gene sequencing resulted in >49,729 reads per sample. The gut microbial composition of the six donor subjects was assessed using operational taxonomic units (OTUs) at time 0 (baseline) samples and after 24 h fermentation without nisin. These were compared to nisin treated samples from the same donor with varying concentrations of nisin from 25 μM to 500 μM after 24 h fermentation. There was a significant decrease in alpha diversity in the presence of nisin in a dose dependant manner across several measures including Shannon Index ($p < 0.001$), Simpson Index ($p < 0.001$) and Evenness ($p = 0.001$). The groups with the highest alpha diversity were the baseline (time 0) and no treatment control groups after 24 h as shown in Figure 1. Redundancy analysis (RDA) plots were used to explore the association between treatment with increasing nisin concentrations on the compositions of the microbial community compared to no treatment and baseline microbiota composition. There was clear separation between the groups treated with nisin and the untreated groups ($p < 0.05$). Similarly, canonical correspondence analysis (CCA) was used to summarize the data set and to evaluate the expected relationships and differences between groups. As with RDA analysis plots, the CCA analysis showed that nisin treatment had an impact on the microbial composition. Figure 2 shows the RDA and CCA plots at the OTU level where distinct clustering is observed depending

on treatment with nisin or no treatment. The samples clustered together in groups that was dependant on the dose of nisin. Beta diversity measures the differences in diversity between samples. Bray-Curtis distance matrices showed a notable separation based on either treatment with nisin or no treatment in samples from the same donor. The PCoA plot shown in Figure 3 shows each sample from each individual donor. Each treatment group is represented by a different colour. The untreated baseline samples taken at time 0 and the no treatment control group after 24 h fermentation cluster together while the nisin treated samples cluster together.

Gut microbiota composition changes in the presence of nisin

At the phylum level 10 out of 15 taxa were included in the analysis. At the family level 72 taxa were identified, 33 of which were included in the analysis. At the genus level 188 taxa were identified, 103 of which were included in the analysis. After 24 hours of fermentation, the non-treatment control (NTC) group was most similar to the baseline group (samples taken at time 0 h). The dominant phyla present in the baseline and NTC groups were the Firmicutes (Average (Av) relative abundance 60.15% and 58.75%, respectively) and Bacteroidetes (28.75% and 27.75%, respectively), followed by Actinobacteria (4.4% and 9.1%, respectively) and Proteobacteria (4.76% and 3.53%, respectively) (See Figure 4). The dominant bacteria present in the nisin treated groups were the Bacteroidetes, Firmicutes and Proteobacteria. The Actinobacteria phyla had decreased to ~ 1% in all nisin treated groups. There was a significant increase in the relative abundance of the Gram negative Proteobacteria and Bacteroidetes groups

between the NTC and nisin treated groups ($p < 0.05$ ANCOM). There was a significant decrease in the Actinobacteria and Firmicutes between the NTC and nisin treated groups ($p < 0.05$ ANCOM). The abundance of Proteobacteria increased from 3.5% in the NTC group after 24 h to 24.2% and 23.9% in the group treated with 25 µM and 50 µM nisin respectively after 24 h incubation. There was a further increase in Proteobacteria to 31.6% in the group treated with 100 µM nisin, 33.3% in the group with 250 µM nisin and then up to 40.8% in the group treated with 500 µM nisin. Following 24 h, the Actinobacteria decreased in the no treatment control from 9.1% to 1% in the nisin treated groups. The average relative abundance of the Firmicutes phyla decreased as the concentration of nisin increased. Firmicutes represented 58.8% of all phyla in the untreated control group after 24 hours. This decreased 2-fold to 27.5% in the group treated with 25 µM nisin. The Firmicutes decreased steadily to 18.6% in the group treated with 500 µM nisin.

The most prevalent families present in the baseline and the NTC group after 24 h fermentation were *Lachnospiraceae, Bacteroidaceae* and *Ruminococcaceae* followed by *Erysipelotrichaceae, Peptostreptococcaceae* and *Bifidobacteriaceae*. *Bacteroidaceae* and *Enterobacteriaceae* were the most abundant families present in the nisin treated groups followed by *Lachnospiraceae* (See Figure 5). Of the thirty-three taxa included in the analysis, there was a significant increase in the relative abundance of *Enterobacteriaceae* and *Bacteroidaceae* between the NTC and the nisin treated groups ($p < 0.05$). In contrast, there was a significant decrease in the relative abundance of *Ruminococcaceae, Lachnospiraceae* and *Clostridoides Family X III* between the NTC and the nisin treated groups ($p < 0.05$). There was also an observed reduction in the relative abundance of

Erysipelotrichaceae, *Bifidobacteriaceae*, *Coriobacteriaceae* and *Peptostreptococcaceae* and *Rikencellaceae* in the nisin treated groups which increased as the concentration of nisin increased compared to the NTC and non treatment control group (not significant after FDR adjustment). The relative abundance of *Enterobacteriaceae* in the baseline samples and after 24 h in the NTC group was negligible and increased as the nisin concentration increased. In the group treated with 25 µM nisin the abundance of *Enterobacteriaceae* was 20%. This increased steadily to 36% in the group treated with 500 µM nisin (See figure 6). The average relative abundance of *Bacteroidaeceae* was 20.1% at time 0 h across the six subjects. After 24 h the relative abundance of *Bacteroideaceae* was 18% in the NTC group. This increased to 40% (2-fold) in the groups treated with 25-250 µM nisin after 24 h. In the nisin group treated with 500 µM, the abundance of *Bacteroideaceae* was 30%. The *Peptostreptococcaceae* (which include *C. difficile*) decreased from 7 to 1% when nisin was added. The abundance of *Ruminococcaceae* decreased from 13.5% in the NTC after 24 h to 2.9% or below in the nisin treated groups. *Lachnospiraceae* decreased from 24.6% in the NTC group after 24 h to 9.7% in the group treated with 25 µM and decreased in a dose dependant manner further to 4.05% in the group treated with 500 µM of nisin. The *Erysipelotrichaceae* decreased from 7.64% in the NTC group to negligible levels in the nisin treated with groups. The *Bifidobacteriaceae* decreased from 6.94% in the NTC group after 24 h to below 1% in the nisin treated groups.

Following 24 hours the most dominant genera in the no treatment control group were the *Bacteroides* (18.4%) followed by *Bifidobacterium* (6.94%), *Blautia* (6.75%) and *Peptoclostridium* (6.75%). Other genera present were *Lachnospira*

anaerostipes (4.2%), *Holdemanella* (4%), *Alistipes* (3.6%), *Faecalibacterium* (3.11%) *Ruminococcaceae Subdoligranulum* (2.97%) and *Barnisella* (2.3%) (Figure 6). The no treatment control group and the baseline group were most similar compared to the nisin treated groups. At the genus level there was a significant difference in 36 out of the 103 genera included in the analysis between the nisin treated groups and the NTC. The predominant genera in the nisin treated groups were *Bacteroides* and *Escherichia-Shigella*. There was a notable decrease in diversity in the nisin treated groups with the loss in relative abundance in groups such as *Bifidobacterium* and *Blautia* and also *Peptoclostridium* (which includes the spiked in *C. difficile* spp.). There was an increase in relative abundance of *Escherichia Shigella* in a dose dependent manner from negligible levels (below 1%) in the NTC group and baseline to 16.8% in the group treated with 25 µM up to 34.1% in the group treated with 500 µM nisin. The *Bacteroides* genera, which is Gram-negative, increased over two-fold from the no treatment control and baseline group to approximately 40% in the groups treated with 25-250 µM nisin and 30% in the group treated with 500 µM nisin. The lower abundance of *Bacteroides* in the group with the highest concentration of nisin may be reflective of the *Escherichia Shigella* being the most abundant genera in that group.

To determine whether bacterial composition in the faecal fermentates was different between nisin and non-nisin treatments after 24 hours we used a feature selection analysis (LDA Effect Size (LEfSe)) approach which determines features (either phylum, family or genus in our study) most likely to explain differences between the treatment groups. The LDA score was set to 3.0. There were four phyla, 12 families and 50 genera that discriminated between nisin treatment and

no nisin treatment after 24 hours (See Fig 7). The Proteobacteria and Bacteroides phyla were the discriminative taxa in the nisin treated groups while the Firmicutes and the Actinobacteria were the discriminative phyla in the no treatment control samples. The discriminative genera in the nisin treated groups were *Escherichia Shigella, Bacteroides, Phascolarctobacterium, Parabacteroides, Prevotella 7, Klebsiella* and *Eubacterium fissicatena* group. The discriminative genera in the no treatment control group were the *Blautia, Faecalibacterium* and *Barnsiella* and 40 other genera (Shown in figure 7).

Effect of nisin on C. difficile growth

The 16S rRNA gene sequencing revealed that the relative abundance of *Peptostreptococcaceae* (which includes *C. difficile*) was 6.75% in the NTC after 24 h fermentation. This decreased to 4.4% in the group treated with 25 µM of nisin. There was a decrease in the RA of the family of between 1-2% in the groups treated with 50µM nisin or above. Plating for viable *C. difficile* on CCEY agar revealed that there was no viable *C. difficile* present in any of the samples following 24 h fermentation in the groups treated with 50 µM nisin or above.

Detection of nisin in the faecal fermentate after 24 h fermentation

MALDI TOF MS of the faecal fermentate showed masses at (3354.46 Da) that correlated with intact nisin after 24 h as shown in figure 8.

Discussion

The aim of this study was to assess the ability of nisin A to kill *C. difficile* in a complex faecal environment and to establish the concentration of nisin at which no viable *C. difficile* was present whilst having the least impact on overall gut bacterial diversity. To our knowledge, this is the only study which has used compositional sequencing to assess the impact of pure nisin on the gut microbiota in a colon model. After 24 h fermentation, no viable *C. difficile* were detected at concentrations of 50 µM nisin or above across all subjects. A previous study used a colon model to assess the efficacy of nisin (in the form of Nisaplin®) to inhibit *C. difficile* and the impact on the composition of the gut microbiota. The study used qPCR coupled with propidium monoazide (PMA) treatment to enumerate six bacterial populations representative of gut commensals (Le Lay, et al. 2015). These included *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidetes*, *Bifidobacteria*, *Enterobacteriaceae* and *Lactobacillaceae*/*Leuconostocaceae* groups. Although the colon model and the experimental set up differed in several ways compared to our study, we can draw some parallels. They found that nisin at 76 µMol/L concentration (20x MIC) was effective at killing *C. difficile*. In our study, nisin caused a decrease in the relative abundance of *Lachnospiraceae*, *Ruminococcaceae* and *Bifidobacteriaceae* while the *Bacteroidetes* and *Enterobacteriaceae* increased, which is consistent with previous findings. 16s rRNA gene sequencing revealed that *Lactobacillaceae* were present in samples from 5 out of 6 subjects at very low abundance (between 1 and 0.001%) and decreased with nisin treatment in a manner which was dose dependent. This is consistent with previous studies where nisin treatment caused a decrease in *Lactobacillaceae*. Previous studies have demonstrated the susceptibility of other

Gram-positive bacteria including pathogens to nisin (Le Blay, et al. 2007). Several other bacteriocins such as lacticin 3147 (Rea, et al. 2007), microbisporicin (Castiglione, et al. 2008) and thuricin CD (Rea et al. 2010) have been shown to effectively kill *C. difficile*. Nisin is the only bacteriocin which has been given approval by the American Food and Drug Administration, the World Health Organization and the European Union as a natural food additive (Delves-Broughton 1990). Le Lay et al 2016 compared the susceptibility of 20 clinical isolates of *C. difficile* to nisin A and Z and compared that with six antibiotics (Erythromycin, tetracycline hydrochloride, chloramphenicol, ciprofloxacin, vancomycin and metronidazole). They found that nisin A MICs were 0.5–4.0 mg ml-1 which were comparable with the MICs for vancomycin or metronidazole which were reported previously by (Drummond, Smith and Poxton 2003). This suggests that nisin is just as effective as the antibiotics such as metronidazole and vancomycin used commonly to treat *C. difficile* infection (Le Lay, et al. 2016). Bacteriocins like nisin are ribosomally synthesized and due to their proteinaceous nature are sensitive to proteolytic digestion in the digestive tract and so may require encapsulation for oral administration. This differs from most antibiotics which are secondary metabolites (Yang, et al. 2014) and not protease sensitive. Bacteriocins have attracted much attention in recent years due to their unique mechanism of action and low propensity to generate resistance and hence could be a solution to some of the most challenging issues of multi-drug resistant pathogens caused by overuse of such antibiotics which cause diseases such as MRSA and VRE.

A previous ex vivo study by Rea et al used faecal fermentations to investigate the effect of a broad spectrum bacteriocin lacticin 3147 and a narrow spectrum

bacteriocin thuricin CD on the composition of the gut microbiota and *C. difficile* (Rea, et al. 2011). They compared the effects of the bacteriocins to metronidazole and vancomycin and reported a dramatic impact on the composition of the bacteria present upon exposure to metronidazole, vancomycin and lacticin 3147. They also recorded a decrease in Firmicutes and an increase in Proteobacteria in the metronidazole, vancomycin and lacticin 3147 treated groups and a dramatic decrease in overall diversity and an increase in *Enterobactericaeae*. In our study we found that nisin also caused a decrease in the Firmicutes and an increase in Proteobacteria similar to another lantibiotic, lacticin 3147. An increase in Proteobacteria is associated with antibiotic use; and has been proposed as a precedent for disease and an indication of an unstable microbial community (Shin, Whon and Bae, 2015). Proteobacteria blooms in humans have been observed in patients with low grade inflammatory conditions such as irritable bowel syndrome (Carroll, et al. 2012). It has also been linked to diseases such as inflammatory bowel disease (Morgan, et al. 2012), necrotizing enterocolitis (Normann, et al. 2013) and colon cancer (Wang, et al. 2012). For this reason, it can be seen as an unfavourable side effect of antibiotic use. Additionally, a reduction in health promoting bacteria such as *Lactobacillus* and *Bifidobacterium* spp. can also be regarded as a negative outcome of antimicrobial treatments. We have observed a similar effect with nisin treatment in a dose dependent manner which is comparable to that seen with antibiotic treatment. The ability of nisin to kill *C. difficile* in a colon model would suggest that this bacteriocin could be a potential alternative to traditional antibiotics.

Although antibiotics have revolutionized modern medicine, their over-use has become a problem due to increased incidence of antibiotic resistance. Antibiotic

overuse has also been linked with autoimmune and allergic diseases. An example of this is the increase in asthma in children in developing countries which has been linked to antibiotics (Iizumi, et al. 2017). One such study conducted in Denmark found that there was an increased risk of childhood asthma and maternal use of antibiotics before pregnancy and post- partum (Stokholm, et al. 2014). Other studies have linked prenatal exposure to antibiotics with an increased risk of obesity and weight gain in babies (Vidal, et al. 2013; Jepsen, et al. 2003). In contrast there is increasing evidence to suggest that nisin may have an immunomodulatory role and has been shown to inhibit cancer cell growth by inducing apoptosis in cancer cell lines such as head and neck squamous cell carcinoma (HNSCC) (Joo, et al. 2012) and colon cancer (Ahmadi, Ghollasi and Hosseini, 2017).

Oral administration of bacteriocins as a treatment for gastrointestinal ailments is a promising area of research and elucidating the optimum dose and delivery mode is necessary. Previous studies using animal models such as mice, rats and chicken with bacteriocins have shown promising results such as a reduction in the number of pathogens or an impact on the composition of intestinal microbiota. There are varying results, however, depending on whether the bacteriocin is delivered as a purified form or in prebiotic or probiotic form. Other influencing factors impacting the variable results include the animal model used and the dosage of the peptide. An example of this is a study by Bernbom, et al. 2006 which investigated the effect of nisin-producing *Lactococcus lactis* strain CHCC5826, non-nisin-producing *L. lactis* strain CHCH2862 and pure nisin on the composition of intestinal microbiota in human flora-associated rats. They found that in the groups fed nisin-producing and non-nisin-producing *L. lactis* for eight

consecutive days that there was a significant increase in *Bifidobacterium* in the faecal samples and a reduction in *enterococci*/*streptococci* in the duodenum, ileum, cecum and colon. This was not the case in the group fed purified nisin. *L. lactis* strains may affect the intestinal microbiota by competing for nutrients or adhesion sites. Therefore, the effect of changes in the composition of intestinal microbiota may be not related to the presence of nisin in *L. lactis* (Yang, et al. 2014).

This study has shown the effectiveness of purified nisin to kill *C. difficile* in a model colon system. In addition, an optimum concentration at which there is minimum collateral damage using 16s rRNA gene sequencing has been established in this system and will be useful for future *in vivo* work with nisin. The use of purified bacterial peptide or protein may be a preferred method of delivery, however, as nisin is subject to digestion it may not reach the lower GI tract intact. Encapsulating purified nisin may overcome this issue and future *in vivo* studies will be necessary to determine this. A previous study in mice by Gough et al; 2018 delivered nisin orally using starch matrices and found that this mode of delivery conferred some degree of protection on the nisin from digestion whilst having an impact on the gut microbiota (Gough, et al. 2018).

A limitation of the 16S rRNA gene sequencing is that it can only reveal alterations in the abundance of OTUs relative to genus level relative to others present and we are unable to ascertain the absolute quantity of a particular bacterial strain in a sample. For instance, whilst we observed an increase in the relative abundance of *Enterobacteriaceae* with nisin treatment and a decrease in *Bifidobacteriaceae* we are unable to deduce of the actual numbers in CFU/ml.

Future studies using larger animal models such as a porcine model which have a digestive system more similar to humans would be beneficial to study this further. It is also not known if the impact of nisin treatment on the diversity of the gut microbiota causes lasting or temporary changes compared to antibiotics and future in vivo work is necessary to determine the efficacy of using nisin as a treatment for *C. difficile* infection.

Chapter 3 Figures

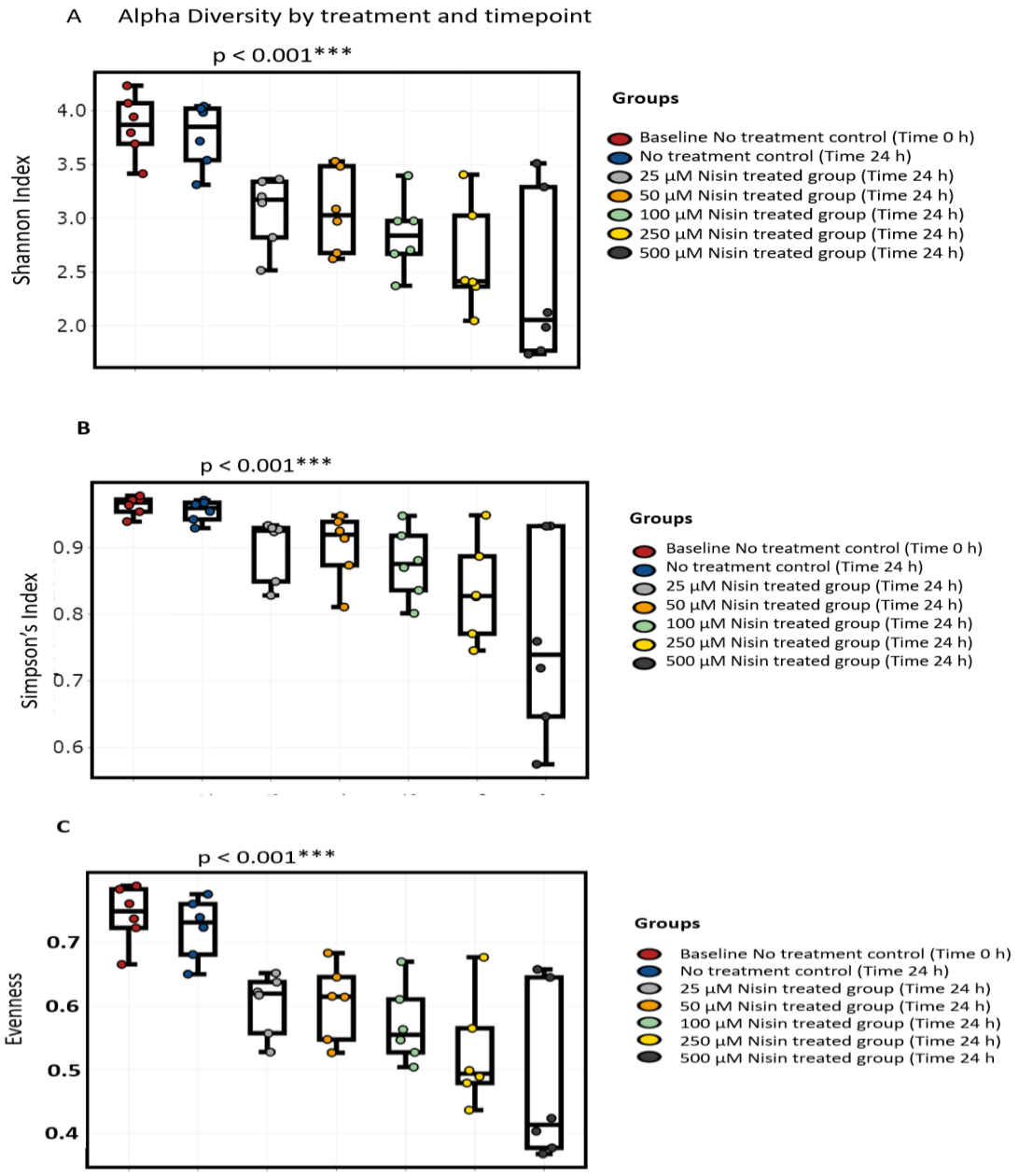


Figure 1 Alpha diversity shown as (a) Shannon and (b) Simpson's Index and (c) Evenness ($p < 0.001$). The Alpha diversity of the baseline no treatment control and the no treatment control after 24 h are more similar to each other. Alpha diversity decreases with increasing nisin concentration. Samples separate based on nisin treatment or no nisin treatment with the no treatment at 24 hours most similar to the baseline microbiota. Red = Baseline no treatment control (Time 0 h), Blue = No treatment control (Time 24 h), Grey = 25 µM nisin treated group (Time 24 h), Orange = 50 µM nisin treated group (Time 24 h), Green = 100 µM nisin treated group (Time 24 h), Yellow = 250µM nisin treated group (Time 24 h), Black = 500µM nisin treated group (Time 24 h)

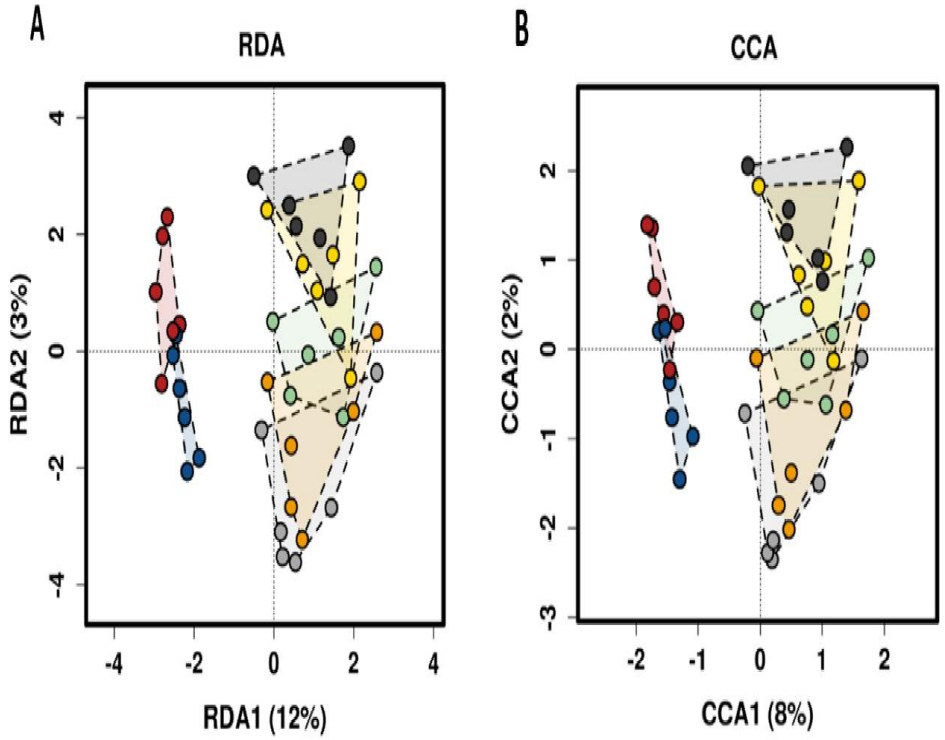


Figure 2 a) Redundancy analysis (RDA) of operational taxonomic units (OTUs) showing separation of samples by presence or absence of nisin. B) Canonical correspondence analysis (CCA) on OTUs. Samples separate based on nisin treatment or no nisin treatment with the no treatment at 24 hours most similar to the baseline microbiota

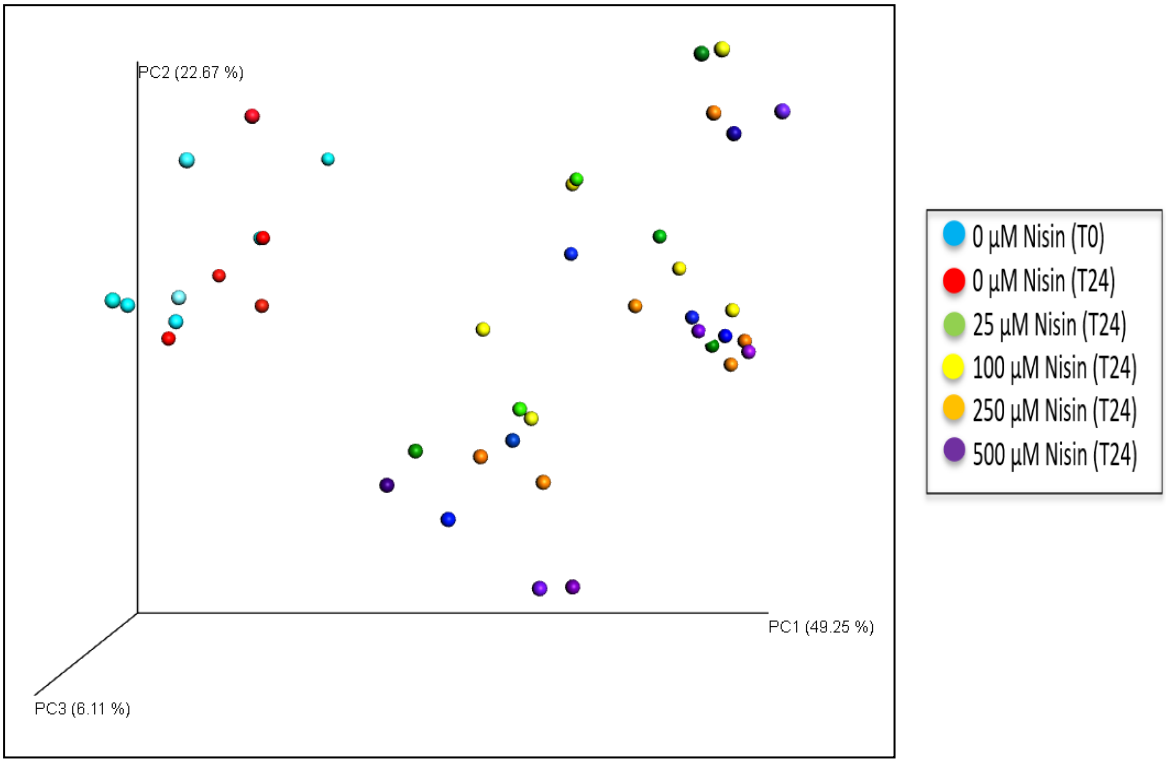


Figure 3 Principle coordinates of analysis (PCoA) plot showing separation of samples based on nisin treatment. Samples separate based on nisin treatment or no nisin treatment with the no treatment at 24 hours most similar to the baseline microbiota ($p < 0.05$).

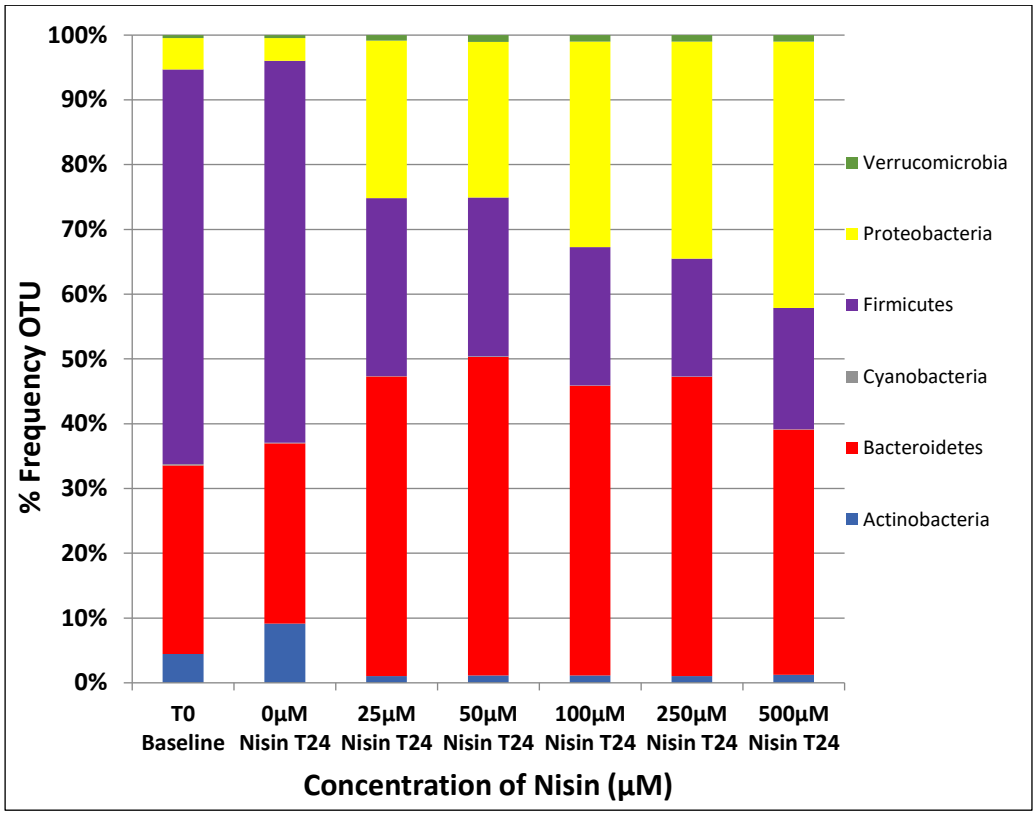


Figure 4 Relative abundance at the phylum level across treatment groups (average n=6 subjects). The baseline no treatment control (Time 0 h) and the no treatment control (Time 24 h) are more similar to each other compared to the nisin treated groups. In the nisin treated groups the is an increase in proteobacteria in a nisin dose dependant manner.

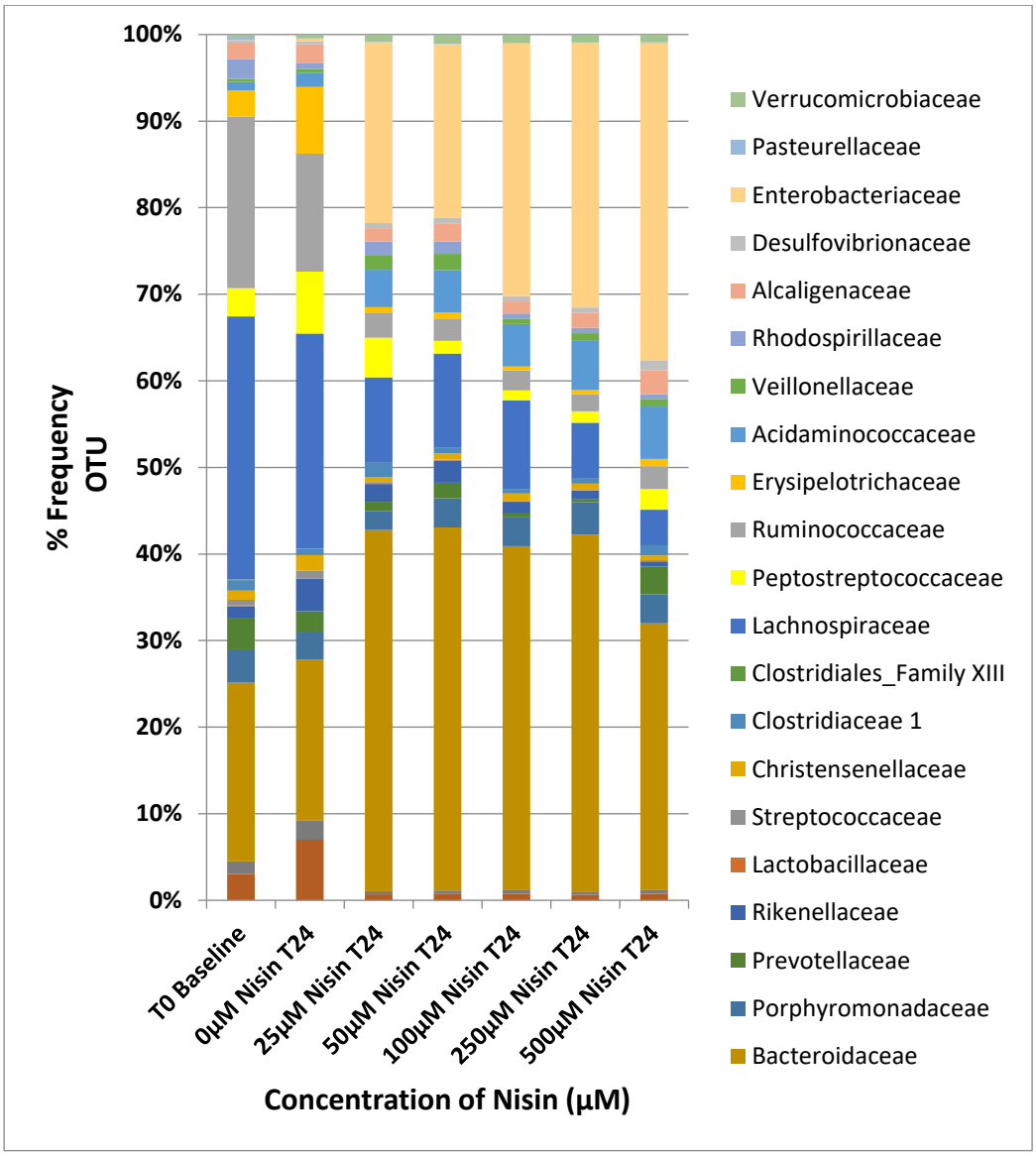


Figure 5 Relative abundance at the family level across treatment groups (average n=6 subjects). The baseline no treatment control (Time 0 h) and the no treatment control (Time 24 h) are more similar to each other compared to the nisin treated groups. In the nisin treated groups the is an increase in enterobacteriaceae in a nisin dose dependant manner. There is a decrease in diversity in the nisin treated groups particularly in the relative abundance of the Gram-positive bacteria

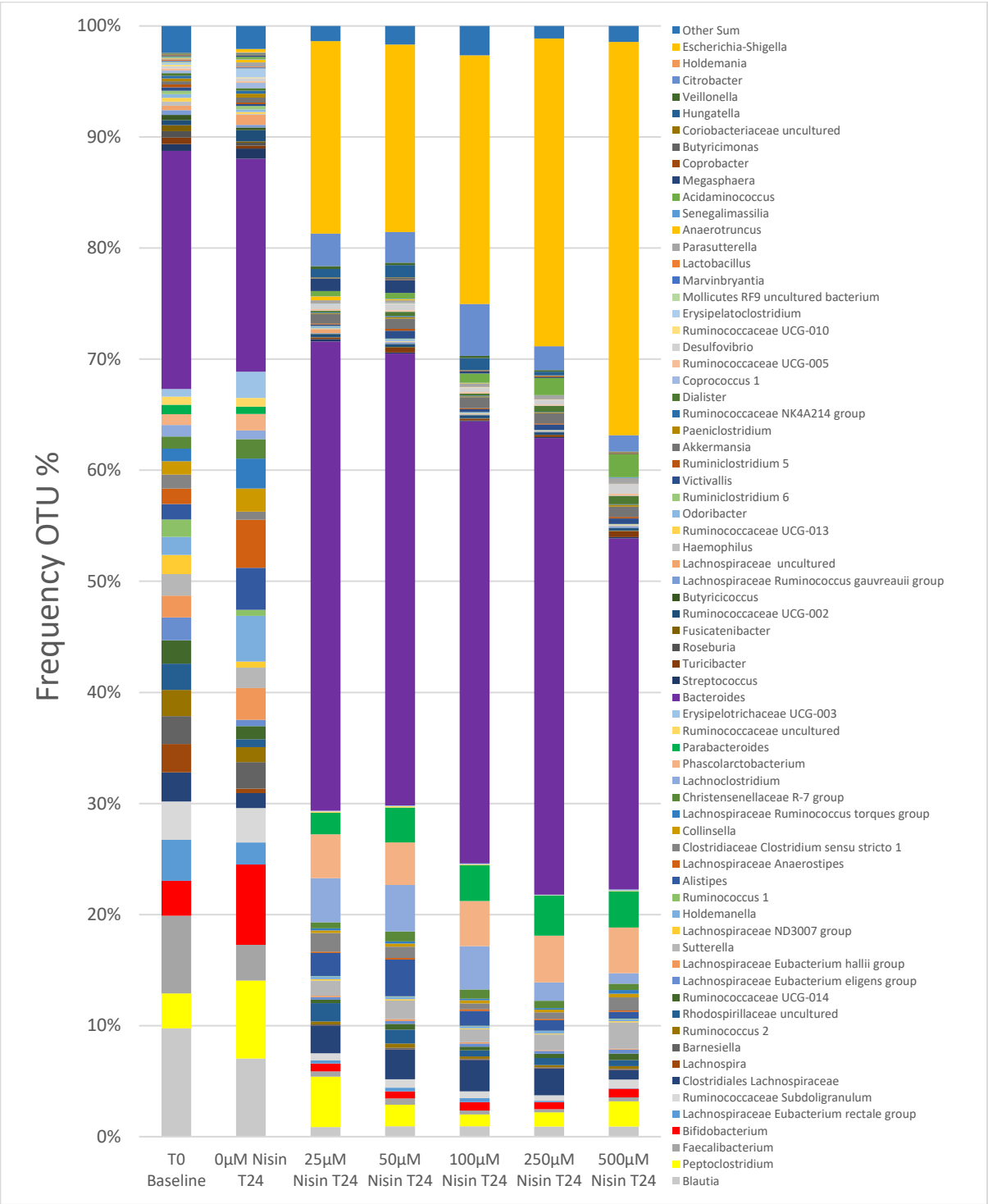


Figure 6 Relative Abundance at the genus level across treatment groups (average n=6 subjects). In the nisin treated groups the is an increase in *Escherchia-Shigella* in a nisin dose dependant manner. There is a decrease in diversity in the nisin treated groups particularly in the relative abundance of the Gram-positive bacteria

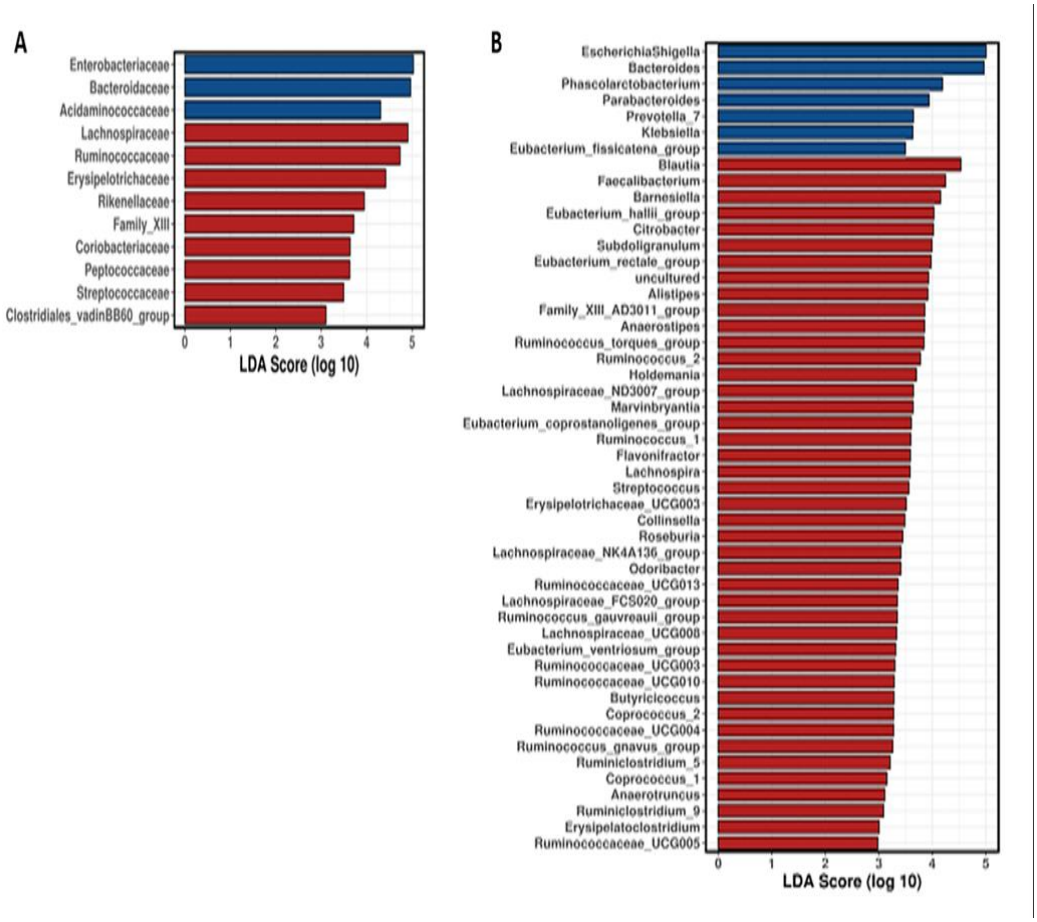


Figure 7 LEfSe (Linear discriminant analysis Effect Size) analysis shows differentially abundant a) family and b) genera as biomarkers for Nisin treated samples (blue) and no Nisin treated samples (red) determined using Kruskal Wallis test ($P < 0.05$) with LDA score >3. LEfSe determines the features (for example operational taxonomic units) most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect. Only taxa meeting a Linear discriminant analysis (LDA) score threshold of 3.0 or greater were considered for analysis.

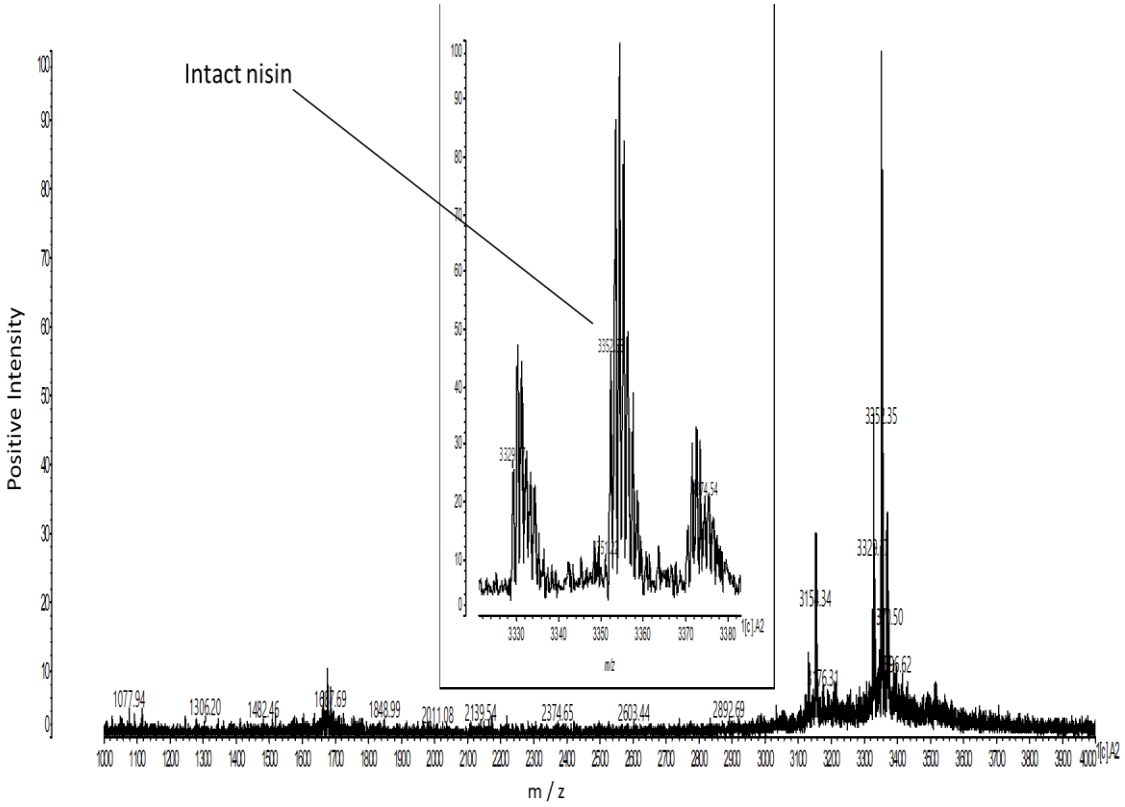


Figure 8 Detection of intact nisin (indicated in figure) with a mass of 3352.35 ± 2 Da by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) in faecal fermentate after 24h incubation

References

Annual epidemiological report for 2016. Healthcare-associated infections: Clostridium difficile infections.

Abee, T., & Delves-Broughton, J. (2003). Bacteriocins - nisin in Food Preservatives. 146-178.

Ahmadi, S., Ghollasi, M., & Hosseini, H. (2017). The apoptotic impact of nisin as a potent bacteriocin on the colon cancer cells. *Microbial Pathogenesis, 111*, 193-197. doi:10.1016/j.micpath.2017.08.037

Ananthakrishnan, A., McGinley, E., & Binion, D. (2008). Excess hospitalisation burden associated with Clostridium difficile in patients with inflammatory bowel disease. *Gut, 57*(2), 205-210. doi:10.1136/gut.2007.128231

And, HC & Hoover, D. (2003). Bacteriocins and their food applications. *Comprehensive Rev Food Sci Food Safety, 2*(3), 82–100. doi:10.1111/j.1541-4337.2003.tb00016.x.

Aslam, S. H. (2005). Treatment of Clostridium difficile-associated disease: old therapies and new strategies. *Lancet, 5*, 549–557.

Bernbom, N., Licht, T., Brogren, C., Jelle, B., Johansen, A., Badiola, I., & al., e. (2006). Effects of Lactococcus lactis on composition of intestinal microbiota: role of nisin. *Appl. Environ. Microbiol, 72*, 239–244. doi: 10.1128/AEM.72.1.239-244.2

Caporaso, J., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F., Costello, E., Huttley, G. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods, 7*(5), 335-336. doi:10.1038/nmeth.f.303.

Carroll, I., Ringel-Kulka, T., Siddle, J., & Ringel, Y. (2012). Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome.

Neurogastroenterology & Motility, *24*, 521-e248. doi:10.1111/j.1365-2982.2012.01891.x

Castiglione, F., Lazzarini, A., Carrano, L., Corti, E., Ciciliato, I., Gastaldo, L., Parenti, F. (2008). Determining the structure and mode of action of microbisporicin, a potent lantibiotic active against multiresistant pathogens. *Chem Biol, 15*(1), 22–31. doi:10.1016/j.chembiol.2007.11.009

Cotter, P., Ross, R., & Hill, C. (2013). Bacteriocins - a viable alternative to antibiotics? *Nat. Rev. Microbiol, 11*(2), 95–105. doi:10.1038/nrmicro2937

Delves-Broughton, J. (1990). Nisin and its uses as a food preservative. *Food Technol, 44*(3), 100-117. doi:doi.org/10.1111/j.1471-0307.1990.tb02449.x

Drummond, L., Smith, D., & Poxton, I. (2003). Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by Clostridium difficile. *J Med Microbiol, 52*, 1033–1038. doi:10.1099/jmm.0.05387-0

Edgar, R. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461. doi:10.1093/bioinformatics

Fooks, L., & Gibson, G. (2003). Mixed culture fermentation studies on the effects of synbiotics on the human intestinal pathogens Campylobacter jejuni and Escherichia coli. *Anaerobe, 9*, 231–242. doi:10.1016/S1075-9964(03)00043-X

Fouhy, F., Deane, J., Rea, M., O'Sullivan, Ó., Ross, R., O'Callaghan, G., & al, e. (2015). The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations. *PLoS ONE, 10*(3), e0119355. doi:10.1371/journal.pone.0119355

Freeman, J., Vernon, J., Morris, K., Nicholson, S., Todhunter, S., Longshaw, C., & Wilcox, M. (2015). Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent Clostridium difficile Ribotypes' Study Group. *Clin Microbiol Infect, 21*(248), 9-16. doi:doi:10.1016/j.cmi.2014.09.017

Ghodhbane, H., Elaidi, S., Sabatier, J., Achour, S., Benhmida, J., & Regaya, I. (2015). Bacteriocins Active Against Multi-Resistant Gram-Negative Bacteria Implicated in Nosocomial Infections. *Infect Disord Drug Targets, 15*(1), 2–12. doi:10.2174/1871526514666140522113337

Gough, R., Cabrera Rubio, R., O'Connor, P., & al, e. (2018). Oral Delivery of Nisin in Resistant Starch Based Matrices Alters the Gut Microbiota in Mice. *Front Microbiol, 9*, 1186. doi:10.3389/fmicb.2018.01186

Guinane, C., Cotter, P., Hill, C., & Ross, R. (2005). Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. *J Appl Microbiol, 98*(6), 1316–1325. doi:10.1111/j.1365-2672.2005.02552.x

Iizumi, T., Battaglia, T., Ruiz, V., & GI, P. (2017). Gut Microbiome and Antibiotics. *Arch Med Res, 48*(8), 727–734. doi:10.1016/j.arcmed.2017.11.004

Jepsen, P., Skriver, M., Floyd, A., Lipworth, L., Schønheyder, H., & Sørensen, H. (2003). A population-based study of maternal use of amoxicillin and pregnancy outcome in Denmark. *Br J Clin Pharmacol, 55*(2), 216-221. doi:10.1046/j.1365-2125.2003.01750.x

Joo, N., Ritchie, K., Kamarajan, P., Miao, D., & Kapila, Y. (2012). Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis via CHAC1. *Cancer Med, 1*, 295–305. doi:10.1002/cam4.35

Kelly, C., & LaMont, J. (2008). Clostridium Difficile – more difficult than ever. *N Engl J Med, 359*(18), 1932 – 1940. doi:10.1056/NEJMra0707500

Kyne, L., Sougioultzis, S., McFarland, L., & Kelly, C. (2002). Underlying disease severity as a major risk factor for nosocomial Clostridium difficile diarrhea. *Infect Control Hosp Epidemiol, 23*(11), 653-659. doi:10.1086/501989

Lagrotteria, D., Holmes, S., Smieja, M., Smaill, F., & Lee, C. (2006). Prospective, randomized inpatient study of oral metronidazole versus oral metronidazole and rifampin for treatment of primary episode of Clostridium difficile–associated diarrhea. *Clinical Infectious Diseases, 43*(5), 547–552. doi:https://doi.org/10.1086/506354

Le Blay, G., Lacroix, C., Zihler, A., & Fliss, I. (2007). In vitro inhibition activity of nisin A, nisin Z, pediocin PA-1 and antibiotics against common intestinal bacteria. *Lett Appl Microbiol, 45*(3), 252-257. doi:10.1111/j.1472-765X.2007.02178.x.

Le Lay, C., Dridi, L., Bergeron, M., Ouellette, M., & Fliss, I. (2016). Nisin is an effective inhibitor of Clostridium difficile vegetative cells and spore germination. *Journal of Medical Microbiology, 65*(2), 169-175. doi:10.1099/jmm.0.000202

Le Lay, C., Fernandez, B., Hammami, R., Ouellette, M., & Fliss, I. (2015). On Lactococcus lactis UL719 competitivity and nisin (Nisaplin(®)) capacity to inhibit Clostridium difficile in a model of human colon. *Front Microbiol, 6*, 1020. doi:10.3389/fmicb.2015.01020

Magoč, T., & Salzberg, S. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963. doi:10.1093/bioinformatics/btr507

Meade, E., Slattery, M., & Garvey, M. (2020, Jan). Bacteriocins, Potent Antimicrobial Peptides and the Fight against Multi Drug Resistant

Species: Resistance Is Futile? *Antibiotics , 9*(1), 32. doi:10.3390/antibiotics9010032

Morgan, X., Tickle, T., Sokol, H., Gevers, D., Devaney, K., Ward, D., . . . Huttenhower, C. (2012). Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biology, 13*(9), R79. doi:10.1186/gb-2012-13-9-r79

Mullane, K. (2014). Fidoximicin in Clostridium difficile infection: latest evidence and clinical guidance. *Ther Adv Chronic Dis, 5*(2), 68-84. doi:10.1177/2040622313511285

Normann, E., Fahlén, A., Engstrand, L., & Lilja, H. (2013). Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. *Acta Paediatr, 102*, 129-136. doi:10.1111/apa.12059

O'Donnell, M., Rea, M., Shanhan, F., & Ross, R. (2018). The Use of a Mini-Bioreactor Fermentation System as a Reproducible, High-Throughput ex vivo Batch Model of the Distal Colon. *Frontiers in Microbiology, 9*, 1822. doi:10.3389/fmicb.2018.01844

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glöckner, F. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research, 41*(D1), D590-D596. doi:10.1093/nar/gks1219.

Rea, M., Clayton, E., O'Connor, P., Shanahan, F., Kiely, B., Ross, R., & Hill, C. (2007). Antimicrobial activity of lacticin 3147 against clinical Clostridium difficile strains. *J Med Microbiol, 56*((Pt 7)), 940–946. doi:10.1099/jmm.0.47085-0

Rea, M., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P.,Ross, R. (2010). Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proc Natl Acad Sci U S A, 108*(supplement 1), 4639-44. doi:10.1073/pnas.1001224107

Rea, M., Sit, C., Clayton, E., O'Connor, P., Whittal, R., Zheng, J., . . . Hill, C. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proceedings of the National Academy of Sciences, 107*(20), 9352-7.

Rogers, L., & Whittier, E. (1928). Limiting factors in the lactic fermentation. *J Bacteriol, 16*, 211–229. doi:10.1128/JB.16.4.211-229.1928

Rupnik, M., Wilcox, M., & Gerding, D. (2009). Clostridium Difficile infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol, 7*(7), 526-536. doi:10.1038/nrmicro2164

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome biology, 12*(R60), 1-8. doi:10.1186/gb-2011-12-6-r60

Shin, N., Whon, T., & Bae, J. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends in Biotechnology, 33*(9), 496–503. doi:10.1016/j.tibtech.2015.06.011

Stanley, J., Bartlett, J., Dart, B. 4., & Ashcraft, J. (2013). Clostridium difficile infection. *Curr Probl Surg, 50*(7), 302 – 337. doi:10.1067/j.cpsurg.2013.02.004

Stevens, V., Dumyati, G., Fine, L., Fisher, S., & van Wijngaarden, E. (2011). Cumulative Antibiotic Exposures Over Time and the Risk of Clostridium difficile Infection. *Clinical Infectious Diseases, 52*(1), 42-48. doi:https://doi.org/10.1093/cid/cir301

Stokholm, J., Sevelsted, A., Bonnelykke, K., & H, B. (2014). Maternal propensity for infections and risk of childhood asthma: a registry-based cohort study. *Lancet Respir Med, 2*(8), 631-637. doi:10.1016/S2213-2600(14)70152-3.

Surowiec, D., Kuyumjian, A., Wynd, M., & Cicogna, C. (2006). Past, present, and future therapies for Clostridium difficile-associated

disease. *Annals of Pharmacotherapy*, *40*(12), 2155–2163. doi:10.1345/aph.1H332

Vidal, A., Murphy, S., Murtha, A., Schildkraut, J., Soubry, A., Huang, Z., & Neelon, S. (2013). Associations between antibiotic exposure during pregnancy, birth weight and aberrant methylation at imprinted genes among offspring. *Int J Obes (Lond)*, *37*(7), 907-913. doi:10.1038/ijo.2013.47

Wang, T., Cai, G., Qiu, Y., Fei, N., Zhang, M., Pang, X., . . . Zhao, L. (2012). Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *The ISME Journal*, 320–329. doi:10.1038/ismej.2011.109

Weiss, S., Xu, Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., . . . Hyde, E. (2017). Normalisation and microbial differential abundance strategies depend upon data characteristics. *Microbiome, 5*, 1-8. doi:10.1186/s40168-017-0237-y

Wiström, J., Norrby, S., Myhre, E., Eriksson, S., Granström, G., Lagergren, L.,. Svenungsson, B. (2001). Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. *Journal of Antimicrobial Chemotherapy, 47*(1), 43-50. doi:10.1093/jac/47.1.43

Yang, S., Lin, C., Sung, C., & Fang, J. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology, 5*, 241. doi:10.3389/fmicb.2014.00241

Zakrzewski, M., Proietti, C., Ellis, J., Hasan, S., Brion, M., Berger, B., & Krause, L. (2017). Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. *Bioinformatics, 33*(5), 782-783. doi:10.1093/bioinformatics/btw725

Chapter 4

Comparison of the effect of fidaxomicin, thuricin CD and nisin on the composition of the human gut microbiota in an *ex vivo* model of the distal colon

Abstract

The treatment of *Clostridioides difficile* (formerly *Clostridium difficile*) has depended heavily on the antibiotics metronidazole and vancomycin. Fidaxomicin is a commercially-available macrocyclic antibiotic which is reported to have a narrow spectrum of activity against the pathogen. It is an alternative, albeit expensive, treatment for *C.difficile*–mainly used in patients that do not respond to traditional treatment. Thuricin CD and nisin are bacteriocins, bacterially-produced antimicrobial peptides, with activity against *C.difficile*. Thuricin CD has a narrow spectrum of activity whereas nisin exhibits a broad spectrum of activity against most Gram-positive organisms. The aim of this study was to investigate the impact of fidaxomicin on the human gut microbiota in a model colon system and to compare the effect with that of nisin, thuricin CD and a no treatment control at equimolar concentrations. Individual faecal slurries were prepared from six donors and incubated for 24 hours with 100µM of each antimicrobial and a no treatment control in a mini-fermentation system. Following 24 h incubation, the groups treated with 100µM thuricin CD and the no treatment control were most similar in their microbial composition across the six subjects. In the fidaxomicin and nisin treated groups there was a decrease in alpha diversity after 24 h. At the family and genus levels there was a change in microbial composition in the fidaxomicin and nisin-treated groups compared to the control and thuricin CD treated groups. We conclude that fidaxomicin has a broader spectrum of activity that is not just selective for *C. difficile* and related species, while thuricin CD exhibited a largely selective spectrum of activity limited primarily to *C. difficile.*

This is the first time a mini-fermentation system has been used to compare the effect of nisin, thuricin CD and fidaxomicin in a complex community.

Introduction

Clostridioides (formerly *Clostridium*) *difficile* is a Gram-positive, rod-shaped anaerobic bacterium which is a major causative agent of hospital-acquired infections (Koo, Garey and Dupont 2010; Shah, et al. 2010). In 2016, the European Centre for Disease Prevention and Control (ECDC) reported 7,711 cases of *C.difficile* infections (CDI) in 20 countries, 5,756 of which (74.6%) were healthcare-associated (HA) CDI and 1,955 CDI cases (25.4%) that were either community-associated (CA) or of unknown origin (Healthcare-associated infections: Clostridium difficile infections). Antibiotic therapy is a major predisposing factor for CDI. Treatment with antibiotics is associated with collateral damage to the microbiota, including the depletion of beneficial gut commensals in a manner that gives *C. difficile* an opportunity to flourish. Many commonly used antibiotics such as clindamycin, amoxicillin, ampicillin, cephalosporins and flouroquinolones have been associated with CDI (Aronsson, Möllby and Nord 1985; Bartlett 2006; Wiström, et al. 2001). Furthermore, antibiotic overuse has selected for multi-drug resistant bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococcus (VRE) and *C. difficile*. CDI is life-threatening for immuno-compromised patients in a hospital setting where antibiotic treatment is ineffective against resistant bacteria (Herzig, et al. 2015). Oral metronidazole and oral vancomycin have been the primary treatment options in the management of CDI for the past 30 years. While vancomycin is effective against *C.difficile*, its use has been associated with VRE and the

development of resistant strains of clostridia and their spread within the hospital environment (George, et al. 1978; Cramer, Burchard and Lohse 2008). For the initial treatment of CDI, Fidaxomicin and vancomycin are equivalent but the recurrence rate is higher with vancomycin than with fidaxomicin (Cornely, et al. 2012; Crook, et al. 2012; Louie, et al. 2011). To reduce the spread of VRE, the use of metronidazole over vancomycin was advocated in the 1995 HICPAC guidelines. However, VRE colonization was shown to arise from exposure to either agent (Centers for Disease Control and Prevention Recommendations for preventing the spread of vancomycin resistance: recommendations of the Hospital Infection Control Practices Advisory Committee (HIC-PAC). 1995), (Al-Nassir, et al. 2008). Despite this, vancomycin is now the most commonly prescribed antibiotic for CDI while Metronidozole is no longer recommended as a first line of therapy for adults.

Fidaxomicin is a macrolide antibiotic which is an alternative to traditional antibiotics. In May 2011, the United States Food and Drug Administration (FDA) approved fidaxomicin for the treatment of CDI. It is the second drug, after vancomycin, to be FDA approved for CDI treatment. In December 2011, it was approved for CDI treatment in the EU by the European Medicines Agency (EMA) (Mullane 2014). Fidaxomicin has been shown to be as effective as vancomycin and is reported to have a narrow spectrum of activity (Zhanel, Walkty and Karlowsky, 2015).

Due to the increase in antibiotic resistance and hospital-acquired infections there is an urgent need to develop new treatments for CDI infection. Bacteriocins, antimicrobial peptides produced by bacteria, are one such

example of alternatives to antibiotics. Nisin is produced by *Lactococcus lactis* subsp. *Lactis* and is the most characterised bacteriocin. It has a broad spectrum of activity against most Gram-positive species and has been approved by the FDA for food use (Bartoloni, et al. 2004). Nisin has been shown to effectively kill *C. difficile in vitro* at concentrations comparable to antibiotics such as metronidazole and vancomycin (Bartoloni, et al. 2004). Previous work in our group has also shown that nisin is capable of effectively killing *C. difficile* in a microbial faecal based environment (in draft). Thuricin CD is an antimicrobial peptide produced by *Bacillus thuringiensis* DPC 6431 and is a member of the sactibiotic class of bacteriocins. This two component bacteriocin effectively kills *C. difficile* and has a narrow spectrum of activity compared to other antimicrobials such as nisin and the antibiotics vancomycin and metronidazole (Rea, et al. 2010).

The aim of the study was to compare the effects of thuricin CD, fidaxomicin and nisin at equimolar concentrations on the diversity and composition of the gut microbiota using a model colon system. We used fresh faecal samples from six individual donors each with unique microbial compositions for this study and looked for trends across the subjects. Individual slurries were created from the faecal samples from each subject and incubated with 100 μM of either nisin, thuricin CD or fidaxomicin for 24 h in a micro-Matrix mini-fermentation system. To assess the effect of each treatment on the composition of the gut microbiota we used 16S rRNA gene sequencing analysis compare the differences in the relative abundances across the groups.

Materials and Methods

Donor Recruitment for Faecal Fermentation Studies

Donor recruitment and enrolment were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (protocol no: APC055). All donors completed and signed a consent form to acknowledge their participation in the study. All human subjects were healthy females and males between 23 and 65 years of age. Participants had not taken antibiotics in the 6 months prior to participation in the study.

Faecal medium,micro-Matrix™Cassette set-up and parameters

The micro-Matrix™ (Applikon Biotechnology, Heertjeslaan 2, 2629 JG Delft, Netherlands) mini-fermentation system was used to model the distal colon (O'Donnell, et al. 2018). The micro-Matrix™ provides simultaneous individual measurement and control of temperature, pH, and dissolved oxygen concentration (dO_2), in 24 parallel micro bioreactors (wells) with working volumes of up to 5 ml each. Each well of the micro-Matrix cassette operates as a stand-alone bioreactor. The pre-calibrated integrated pH anddO_2 sensors remove the need to calibrate each individual well prior to beginning the fermentation. In brief, the fermentations were carried out in three sealed micro-Matrix cassettes (24 wells/cassette) with 2 subjects per run. Each test condition (fidaxomicin, thuricin CD, nisin and control) was assayed in duplicate wells of the micro-Matrix cassette. The experiment was performed as follows:20% faecal slurry w/v from individual donors

was prepared under anaerobic conditions by suspending the faecal sample in 50 mM phosphate buffer pH 7.0 containing 0.05% cysteine (Sigma Aldrich, Ireland) which had previously been boiled and cooled anaerobically. In an anaerobic chamber, faecal medium, prepared according to Fooks, et al. 2003 and 20% slurry was added to each well to make up a final volume of 5 ml. Fidaxomicin, thuricin CD and nisin were added to the appropriate wells at 100 µM concentrations. Each well/subject contained faecal medium, faecal slurry and 100 µM of fidaxomicin, thuricin CD or nisin. Nisin and fidaxomicin were obtained in powder form. 1 mM nisin was prepared in fecal media. For 100 µM nisin, 500 ul of 1 mM nisin was added to 3.5 mls faecal media. The molecular weight of fidaxomicin is 1058.04 g/mol. To make a 100 µM solution 5.29 mgs of fidaxomicin was added to faecal media in a 40 ml volume. 10 mls of faecal media was added to make a total volume of 50ml. 5 ml of this was added to each well. (Note a larger volume was made to accurately weigh out the small volume of fidaxomicin). Thuricin CD was in liquid form and came as separate alpha and beta peptides. The stock concentration of alpha peptide was 12.161 mM. The concentration of beta peptide stock was 9.5 mM

For 100 µM, 50 µM of alpha peptide (20.8 ul) and 50 µM of beta peptide (26.9 ul) were added to 3952.3 ul of fecal media and 1ml fecal slurry for a volume of 5ml. The non-treatment control contained faecal medium and slurry only. A baseline subsample Time 0h (T0) was taken by addition of 1 ml of 20% slurry from each subject to 4 ml of faecal media. This was aliquoted into 1 ml aliquots and snap frozen at -80°C prior to DNA extraction. Once the wells were filled the cassette was clamped and sealed

using the transport clamps and transferred to the micro-Matrix. Samples were incubated anaerobically for 24 h at 37°C at 250 rpm. Anaerobic conditions were established in the fermenters by sparging the system with nitrogen. The installation of the filled cassette and the attachment of the parts were performed according to the micro-Matrix manual. CO_2 was used for downward pH control, 20% (v/v) liquid ammonia was used as upward pH control and the orbiter was set to 250rpm. The control set-point parameters on the micro-Matrix were pH 6.8 (6.6 -7.2 low and high alarm points, respectively), dO_2 0% (high alarm 20%). One ml samples were taken for DNA extraction at time 24h (T24).

DNA Extraction

Samples (1 ml) were taken at T0 for each subject and also after 24 h (T24) incubation for each subject/treatment of the micro-Matrix cassette and snap frozen at -80°C until use. The Zymo Research ZR faecal DNA kit (Cambridge Biosciences, Cambridge, United Kingdom) was used to extract DNA from samples. There were 48 samples (2 from each subject (n = 6) and treatment condition (n = 4). Twenty-four samples (one from each subject and treatment group) were used for the analysis and 6 additional samples chosen at random from the duplicates were also extracted and sequenced for QC purposes (data not shown). Extractions were carried out according to the manufacturer's specifications.

Library preparation

The V3-V4 variable region of the 16S rRNA gene was amplified from the faecal DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). Samples were quantified, and libraries were prepared for sequencing as described previously (Fouhy, et al. 2015). Samples were run on the Illumina Miseq sequencer at the Teagasc Sequencing facility at the Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. Following sequencing, data was analysed bioinformatically to establish the effect of the antimicrobials on the composition of the gut microbiota.

Bioinformatics and Statistical Analysis of Compositional Sequencing Data

Raw Illumina 250 bp paired-end sequence reads were merged using Flash (FLASH: fast length adjustment of short reads to improve genome assemblies) (Magoč and Salzberg 2011) and quality checked using the split_libraries script from the Qiime package (Caporaso, et al. 2010). Reads were then clustered into operational taxonomic units (OTUs) and chimeras removed with the 64-bit version of USEARCH (Edgar 2010). Subsequently OTUs were aligned and a phylogenetic tree generated within Qiime (Caporaso, et al. 2010). Taxonomical assignments were reached using the SILVA 16S specific database (version 116) (Quast, et al. 2013). Statistical analysis was performed using Calypso software version 8.84 (Zakrzewski, et al. 2017). Cumulative Sum scaling (CSS) was performed and data was log2 transformed to account for non-normal distribution of taxonomic count data. Cyanobacteria and chloroplast signatures as well as OTUs with a relative frequency below 0.01 were removed. Alpha diversity (Simpson and Shannon indexes) and multivariate Redundancy Analysis

(RDA) was used to investigate the complex associations between treatment and microbial composition.

Student's t-test and Tukey’s post hoc test were used to detect the significance of the differences in relative abundance between the groups. Differences in relative abundance were evaluated and considered statistically significant at $P < 0.05$ following FDR correction. Abundance of individual taxa across sample groups were visualised by violin plots. Taxonomy bar charts were created in excel.

Results

Fidaxomicin, thuricin CD and nisin are all active against *C.difficile*. However, the effect that each antimicrobial treatment has on the gut microbiota in a complex microbial environment has not been directly compared. Our aim, therefore, was to compare the effects of equimolar concentrations of thuricin CD, fidaxomicin and nisin on the composition and relative abundance of the human gut microbiota. To achieve this, 100 µM of each antimicrobial was added individually to six human faecal slurries and incubated for 24 h in separate wells of a micro-Matrix mini-fermentation system. A previous study by O’Donnell, et al. 2018 using the same system revealed its ability to differentiate the starting microbiota composition from one that has undergone fermentation of a prebiotic carbohydrate. The main benefit of using this mini-fermentation systemin

this study was that it enabled us to compare a number of treatments side by side from faecal slurries from multiple human subjects.

Sequencing Results

Following sequencing on the Miseq; 6,307,417 post quality reads were obtained with an average of 210,247 reads per individual sample.

Firstly, we compared the effect of each antimicrobial to each other and to a no treatment control group on the faecal microbiota of each individual donor sample for the purposes of diversity analysis and assessment of the taxonomic microbial content of samples from each individual donor sample. Although the microbial composition varied between individual donor samples, we found that there was high diversity over all across the six subjects which should be at least more representative of the human gut microbial content within the general population than in any single individual.

In terms of alpha diversity there was a non-significant decrease in the Shannon and Simpson diversity indices in the nisin and the fidaxomicin treated groups compared to the no treatment control (NTC) and the thuricin CD groups (Figure 1). Redundancy analysis (RDA) is an ordination approach which is a useful tool to study variations in a population due to environmental changes (in this case exposure of the gut microbiota to antimicrobials). RDA revealed a trend towards separation between the nisin and fidoxamicin treated groups regardless of the microbial variation

between samples from different subjects. Contrastingly the thuricin group and the no treatment control were more similar to each other (Figure 2).

Following diversity analysis and assessment of statistical differences, the data of the six individuals was combined in order to obtain an overall average to compare the relative abundances and taxonomic content between treatment groups.

Nine of 12 phyla were included in the analysis after filtering for low abundance taxa, unclassified taxa and those that were not present across all subjects. There were no significant differences between the treatment groups at the phylum level. There was, however, an observed increase in the relative abundance of Proteobacteria at the phylum level observed in both the fidaxomicin and the nisin treated group compared to the control and the thuricin CD treated group. Overall, the average (n = 6) relative abundance of Proteobacteria increased almost 2-fold after 24h fermentation. The average relative abundance (%) of Proteobacteria was 12.6% in the NTC and 7.2% in the thuricin CD treated groups and 19.8% in the fidaxomicin and 17.7% in the nisin treated group (Figure 11). There was also a change in the composition of the microbiota at the family and genus level after 24 h fermentation in the fidaxomicin and nisin-treated groups compared to the control and the Thuricin CD treated group. Of 61 family level taxa, 38 were included in the analysis after filtering for low abundance taxa and taxa that were not present across all subjects (Figure 3). The groups treated with 100µM thuricin CD and the NTC for 24 h were most similar in terms of their microbial composition across the six subjects. At the family level there was a reduction in the relative abundance of

Clostridiales Family XI and *Enterococcaceae* in the fidaxomicin treated group compared to the control, Thuricin CD and the nisin treated groups (Figure 4). The most notable difference in relative abundance was in the family *Clostridiales Family XI* between the fidaxomicin and Thuricin CD group ($p = 0.04$). There were no other significant differences between any of the treatment groups at the family level after correcting for multiple testing. After 24h we were unable to detect *Clostridiales Family XI* which decreased from 2.1% in the thuricin treated group and 1.3 % in the control group at 24 h to undetectable levels in the fidaxomicin and nisin treated groups (Figure 3). The relative abundance of *Enterococcaceae* was 7.4% in the thuricin CD treated group and 3.5% in the NTC group at 24 h. At the same timepoint (after 24 h) this decreased to1% in the nisin group and there were no detectable *Enterococcaceae* in the fidaxomicin treated group. There was an average two-fold decrease in *Bifidobacteriaceae* in the nisin and fidaxomicin treated groups compared to the no treatment control and thuricin groups (Figure 3). There was an almost two-fold increase in the relative abundance of *Enterobacteriaceae* from 5.8% in NTC and 6.3% in the thuricin treated groups to 10.2% in the fidaxomicin treated group and 11.7% in the nisin treated group (Figure 3). The relative abundance of *Eubacteriaceae* decreased in the fidaxomicin and nisin treated groups compared to the thuricin and no treatment control group ($p=0.06$) (Figure 5). There was a decrease in the relative abundance of *Rikenellaceae* ($p=0.42$) and *Erysipelotrichacaeae* ($p = 0.25$) (Figure 6) and an almost three-fold increase in *Prevotellaceae* in the nisin treated groups compared to the control, thuricin CD and fidaxomicin treated groups. There also was

an increase in *Erysipelotrichacaeae* in the fidaxomicin treated group compared to the three other groups.

At the genus level 199 taxa were identified, 116 of which were included in the analysis after filtering for low abundance taxa and taxa that were not present across all subjects. There were no significant changes after correcting for multiple testing except between the *Anaerococcus* genus between the fidaxomicin and thuricin treated group ($p = 0.04$). At the genus level there was an observed decrease in the relative abundance of *Anaerococcus*, *Peptoniphilus* and *Enterococcus* in the fidaxomicin group compared to the NTC, thuricin CD and nisin group across the six subjects (Figure 7). The most noticeable change observed at the genus level between groups was a decrease in the relative abundance of *Enterococcus* from 3.5% in the NTC and 7.3% in the thuricin CD groups to undetectable in the fidaxomicin group and below 1% in the nisin treated group. There was a two-fold increase in the relative abundance of the genus *Enterobacter* between the control (2.1%) and Thuricin CD (2.4%) groups and the nisin (5.5%) and fidaxomicin (4.4%) treated groups. There was a two-fold decrease in *Bifidobacterium* from 4.4% in the control and 4.8% in the Thuricin CD treated group to 2.0% in the fidaxomicin treated group and 2.6% in the nisin treated groups. There was also a decrease in the relative abundance of *Anaerofustis*, *Finegoldia*, and the *Eubacterium fissicatena* group in the fidaxomicin and nisin treated group compared to the control and thuricin treated groups (Figure 8). There was a decrease in the relative abundance of the *Erysipelotrichaceae UCG 003* from 6.6% in the control group and 3.8% in the thuricin CD group to undetectable in the nisin group

while the relative abundance increased to 9.8% in the fidaxomicin treated group. There was also an observed decrease of *Eubacterium xylanophilum* group in the nisin treated group compared to the other three groups (Figure 9). There was a slight increase in *Lachnospiracaeae UCG 004* in the nisin treated group compared to the non-treatment control and the thuricin CD treated groups (Figure 9). There was an increase in *Prevotella 9* from 3.0%, 2.5% and 3.6% in the control, thuricin CD and fidaxomicin treated groups to 7.1% in the nisin treated group.

Discussion

Current treatments for CDI include oral vancomycin and metronidazole. Recurrence of infections and treatment failures have been reported to range from 8 to 50% and 2 to 38%, respectively (Rea, et al. 2001; Aslam, Hamill and Musher 2005; Bricker, et al. 2005; Rea, et al. 2011). Alternative treatments need to be developed to treat this infection. Fidaxomicin is one such example of an alternative antibiotic. Fidaxomicin has proven to be superior to vancomycin in successfully sustaining clinical response to therapy (Mullane 2014); it has also shown to be more effective than metronidazole (Zhanel, Walkty and Karlowsky, 2015). The cost of fidaxomicin far exceeds that of metronidazole or vancomycin and therefore it is recommended for use in populations who are at a high risk of relapse or those in which fidaxomicin has proven to be superior over other treatments

(Mullane 2014). While fidaxomicin has been reported to have a narrow spectrum of activity compared to vancomycin and metronidazole (Louie, et al. 2011), (Zhanel, Walkty and Karlowsky, 2015) our aim was to assess the effect of fidaxomicin on gut microbial composition in a complex microbial environment and compare it to the broad and narrow peptide antimicrobials, nisin and thuricin CD, respectively. Our group had previously investigated the effects of vancomycin, metronidazole and thuricin CD on microbial diversity in a model colon system (Rea, et al. 2010) and the results revealed that thuricin CD caused less collateral damage when compared to either vancomycin or metronidazole.

Nisin is a potent bacteriocin produced by *Lactococcus lactis* subsp. *lactis* which has a broad spectrum of activity against gram-positive bacteria including bacterial pathogens (Klaenhammer 1993). It has been shown to be active against *C. difficile* at concentrations similar to that of vancomycin or metronidazole (Rea, et al. 2001), (Bartoloni, et al. 2004). Similarly, thuricin CD is a bacteriocin produced by *Bacillus thuringiensis* DPC 6431 that has been found to be active against *C. difficile* with a narrow spectrum of activity (Rea, et al. 2010). The results from this faecal fermentation study revealed that there was a slight decrease in alpha diversity (not significant) in the nisin and fidaxomicin treated groups compared to thuricin CD and control groups after 24 h.

There was an increase in the relative abundance of both Proteobacteria and *Enterobacteriaceae* (specifically the genus *Enterobacter)* associated with both nisin and fidaxomicin compared to thuricin CD and the control. Since nisin has a broad spectrum of activity it is not surprising to see a decrease in

diversity in the nisin treated group. However, as fidaxomicin is reported to have a minimal impact on the gut microbiota (Tannock, et al. 2010; Louie, et al. 2011) it is interesting to note that the fidaxomicin-treated group showed a similar decrease in diversity to that of the broad spectrum nisin.

The most dramatic reduction in relative abundance at the family level in the fidaxomicin group was in the *Enterococcaeae* and *Clostridiales Family XI*. Fidaxomicin has been shown previously to be more potent at suppressing clostridial RNA polymerase compared to any other bacterial species. It is active against many species of *Clostridium* with moderate to good activity against gram-positive rods and cocci and modest activity against *Staphylococcus* spp. and *enterococcus*, including VRE (Biedenbach, et al. 2010).

A loss in beneficial microbes such as bifidobacteria and lactobacilli is seen as an unfavourable consequence of antibiotic treatment. The relative abundance of bifidobacterial was reduced in the both the fidaxomicin- and nisin- treated groups when compared to the control or thuricin CD-treated groups. It has been well established that bifidobacteria confer positive health benefits to their host via their metabolic activities (O'Callaghan and van Sinderen 2016). The relative abundance of *Lactobacillus* in these faecal fermentation studies was low across most subjects and varied between subjects, making it difficult to determine the effect of the antimicrobials on members of the *Lactobacillus* genus. However, altered relative abundance of *Lactobacillus* species has been observed in disease states such as IBD and as a consequence of antibiotic use.

Zhanel et al; 2015 assessed the antimicrobial effect of fidaxomicin on certain strains of bacteria using MIC data to and reported that *Finegoldia magna, Peptococcus (finegoldia) magna, Aerococcus* and *Enterococcus faecalis* are all sensitive to fidaxomicin treatment. We also observed a decrease in the relative abundance of seven genera in the fidaxomicin-treated vessels (*Anaerococcus, Finegoldia, Peptoniphilus, Anaerofustis, Enterococcus, Bifidobacterium* and *Eubacterium fissicatena* group). Four genera (*Anaerofustis*, *Finegoldia*, *Eubacterium fissicatena* and *Bifidobacterium)* were also decreased in the nisin treated group.

In summary, thuricin CD does not cause major disruption of the gut microbial composition and is largely selective against *C. difficile*. However, fidaxomicin has a broader spectrum of activity compared to thuricin CD and appears to be more similar to nisin, usually described as a broad spectrum antimicrobial against gram positive species. Fidaxomicin is excreted mainly in the faeces along with high concentrations of its major metabolite, OP-1118. OP-1118 has been shown to be highly active against *C. difficile* with a 32-fold higher MIC90 (Babakhani et al., 2007; O'Gorman, et al. 2018). Both fidaxomicin and OP-1118 exhibit a prolonged post-antibiotic effect (approximately 10 h) (Zhanel, Walkty and Karlowsky 2015). Antimicrobial peptides however tend to be broken down by enzymes in the gut at a faster rate than other antibiotics. This could be viewed as favourable in that the gut microbes are not exposed to the peptides for a length of time, a factor that can select for resistance. In addition, the apparent selectivity of thuricin CD for *C. difficile* and inactivity against other gut bacteria could be advantageous in treating *CDI* in the future.

Limitations of the study

A limitation of this study was the number of samples used. The differences observed between treatment groups did not reach statistical significance after correction for multiple tests. This could be due to the limited number of donor samples available combined with the variation in microbial composition between subjects. It would be beneficial in future studies to increase the number of samples from different donors to increase the significance of the findings.

Chapter 4 Tables and Figures

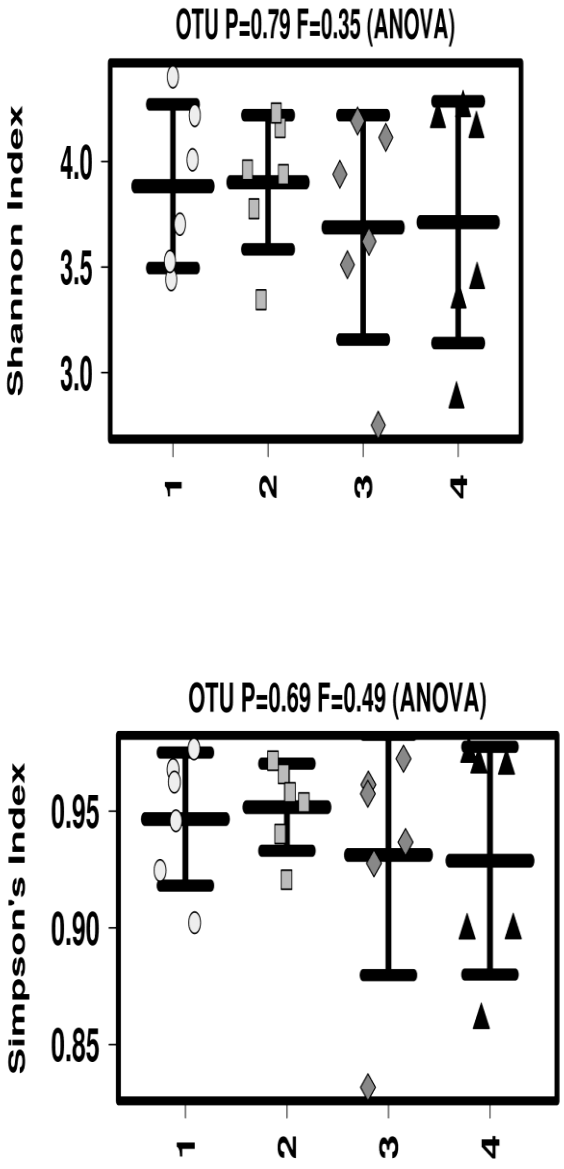


Figure 1. Alpha Diversity shown as Shannon and Simpson Indices after 24 h across treatment groups.1 = No treatment control (NTC) @ 24 h, 2 = Thuricin 100µM@ 24 h, 3 = Fidaxomicin 100µM@ 24 h and 4 = Nisin 100µM.@ 24 h

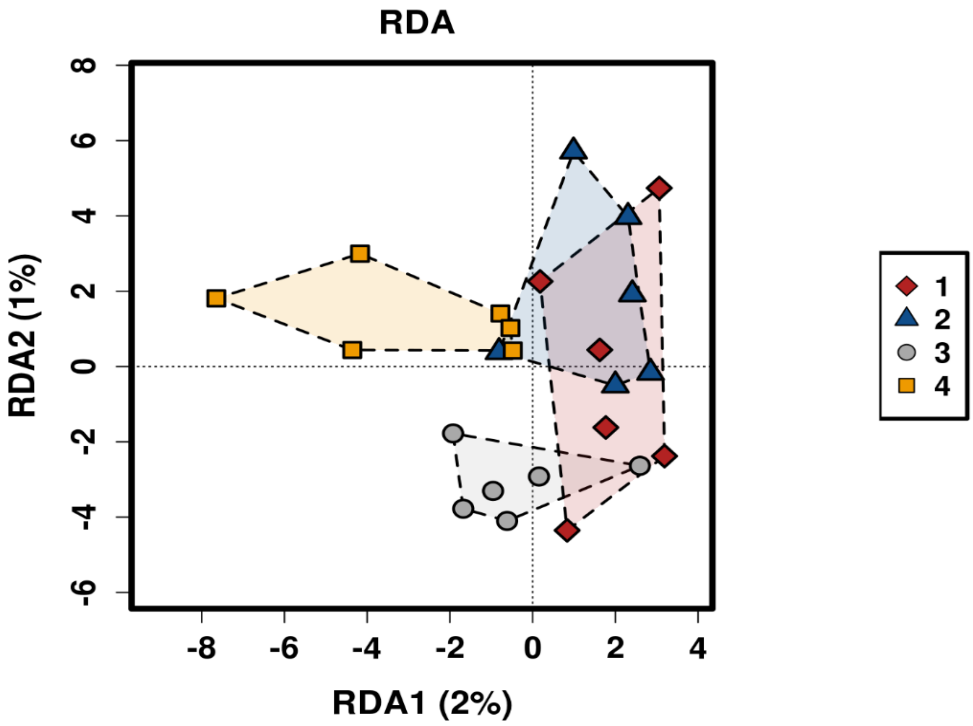


Figure 2. Redundancy Analysis (RDA) plot of operational taxonomic units (OTUs) showing separation of samples by treatment after 24 hours incubation. Group 1 = No treatment control. Group 2 = Thuricin. Group 3 = Fidaxomicin. Group 4 = Nisin (P = 0.27)

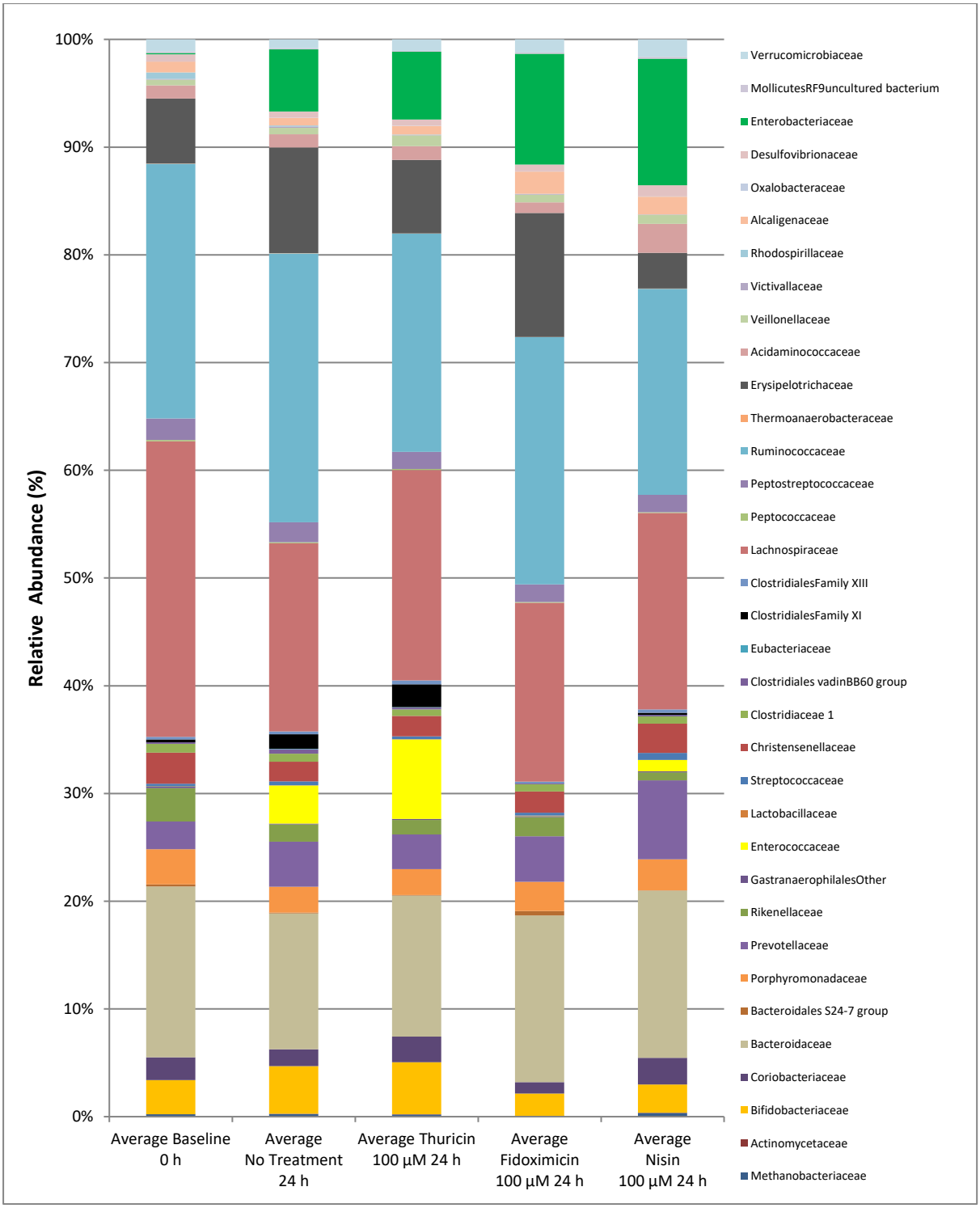


Figure 3. Relative abundance at the family level across treatment groups average of n=6 subjects. The no treatment control (Time 24 h) and the thuricin group are more similar to each other compared to the nisin and fidaxomicin treated groups at 24 h.

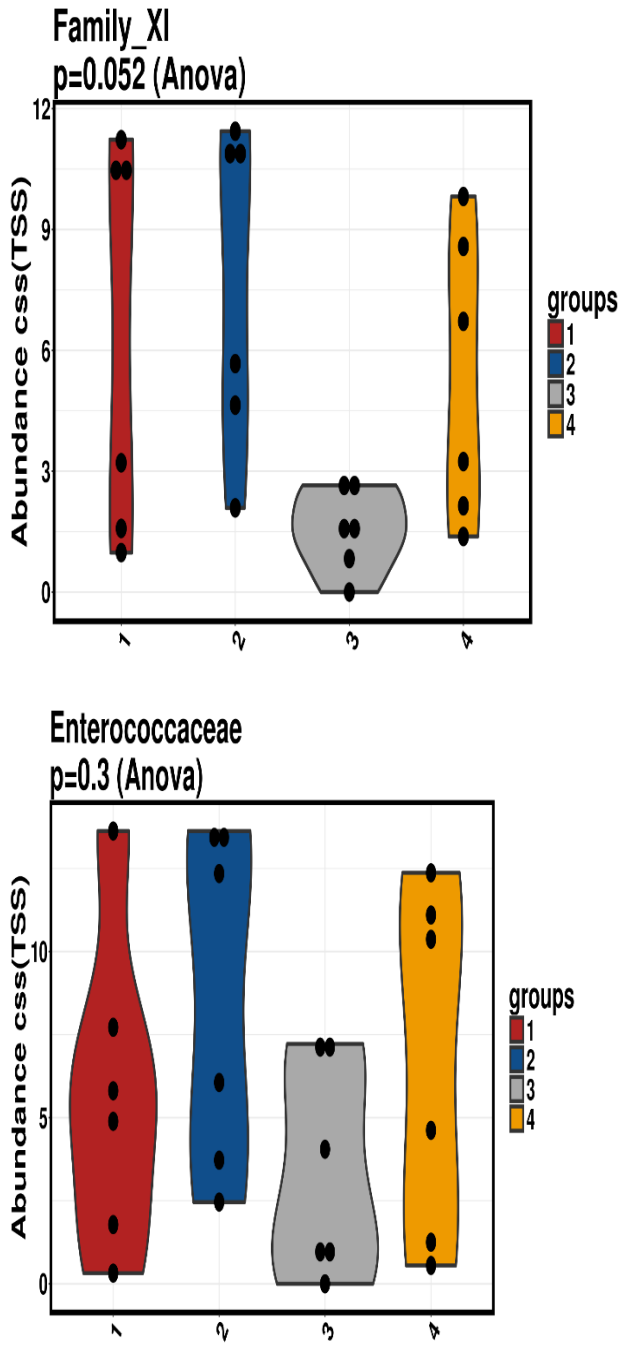


Figure 4. Violin Plot showing the relative abundance at the family level which were decreased in the fidaxomicin treated group (N =6 subjects) compared to the other treatment groups. Group 1 = No treatment control, Group 2 = Thuricin, Group 3 = Fidaxomicin and Group 4 = Nisin

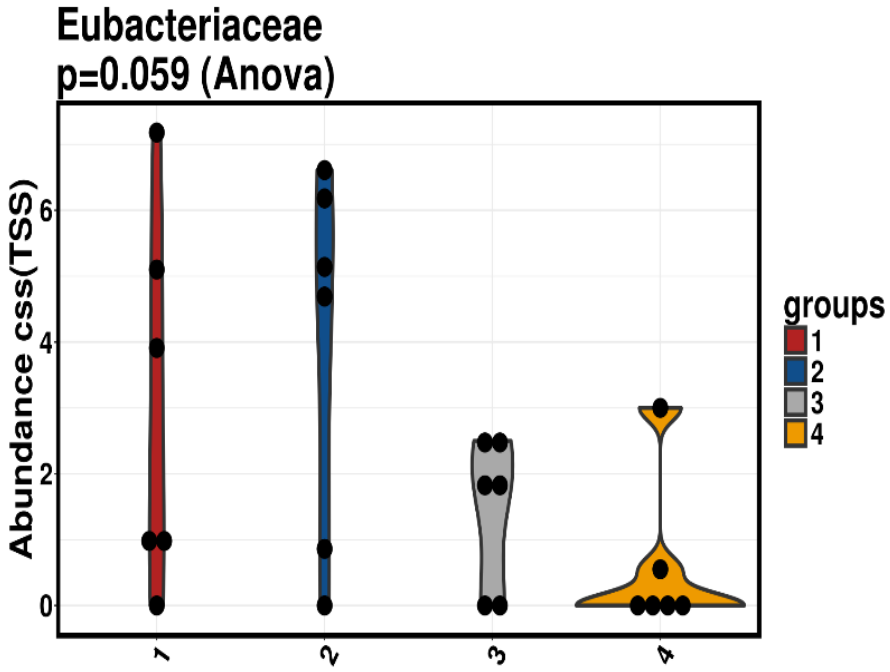


Figure 5. Violin Plot showing the relative abundance at the family level which were decreased in the Fidaxomicin and Nisin treated groups (n=6 subjects). Group 1 = No treatment control, Group 2 = Thuricin, Group 3 = Fidaxomicin and Group 4 = Nisin.

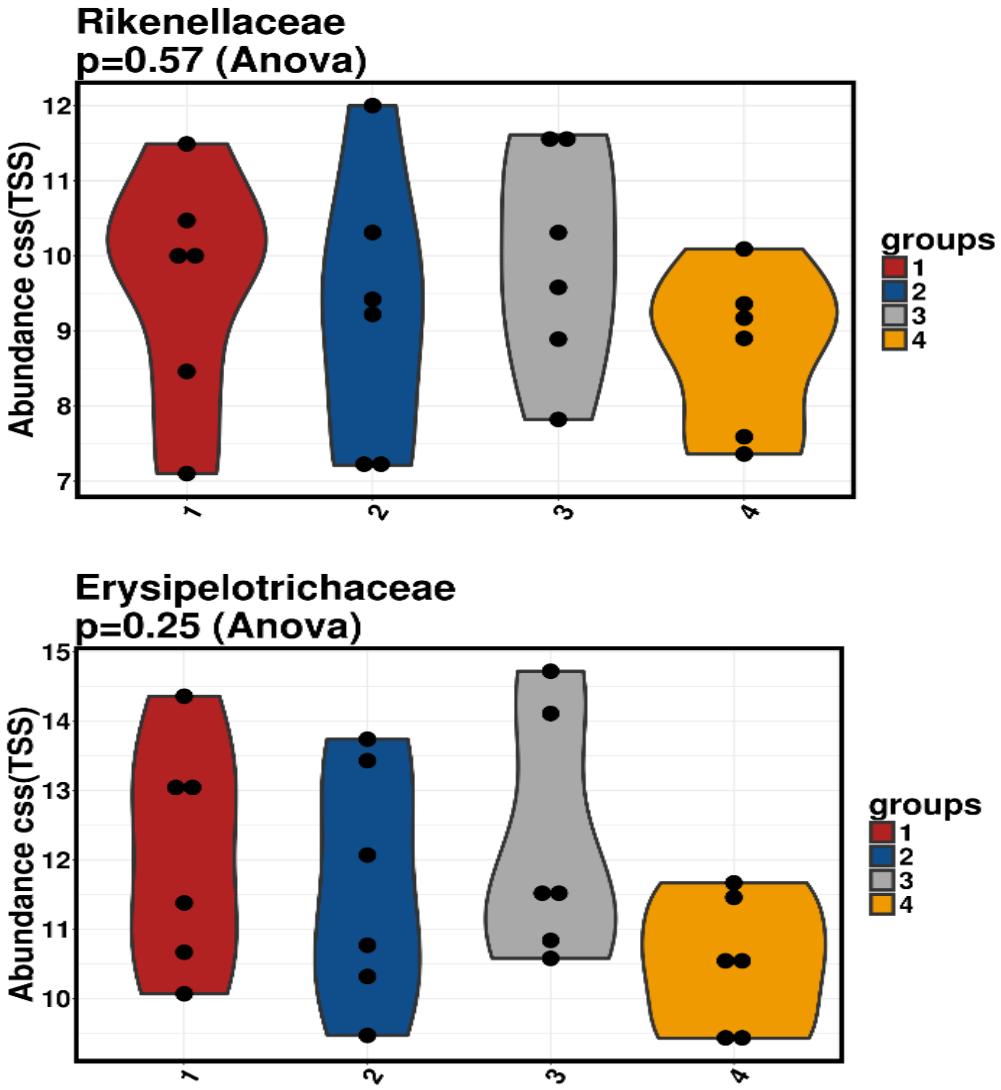


Figure 6. Violin Plot showing the relative abundance at the Family level which were decreased in the Nisin treated groups compared to the other three groups (N =6 subjects). Group 1 = No treatment control, Group 2 = Thuricin, Group 3 = Fidaxomicin and Group 4 = Nisin

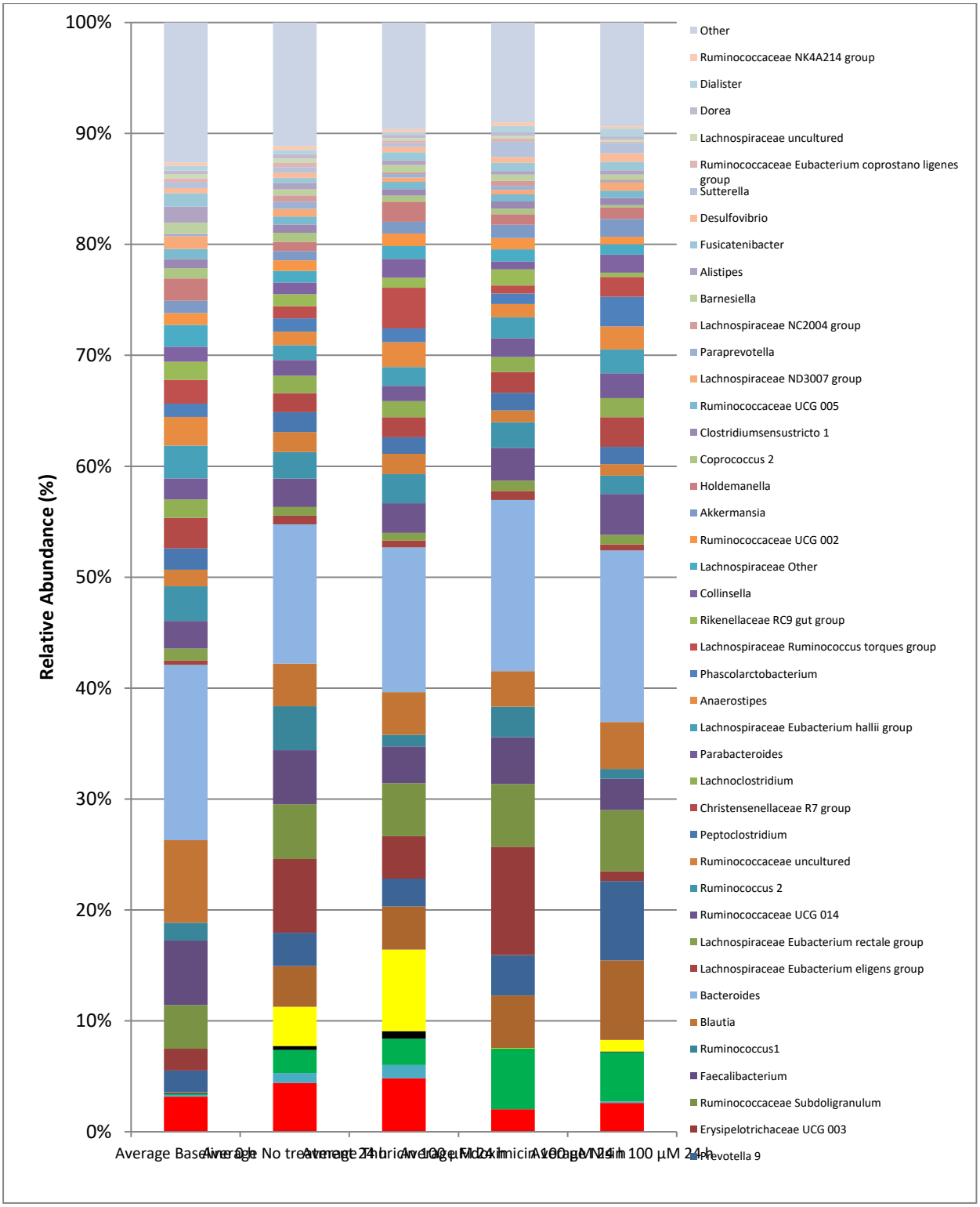


Figure 7. Relative abundance at the genus level across treatment groups average of N =6 subjects. The no treatment control (Time 24 h) and the thuricin treated group (24 h) are more similar to each other compared to the nisin and fidaxomicin treated groups at 24 h.

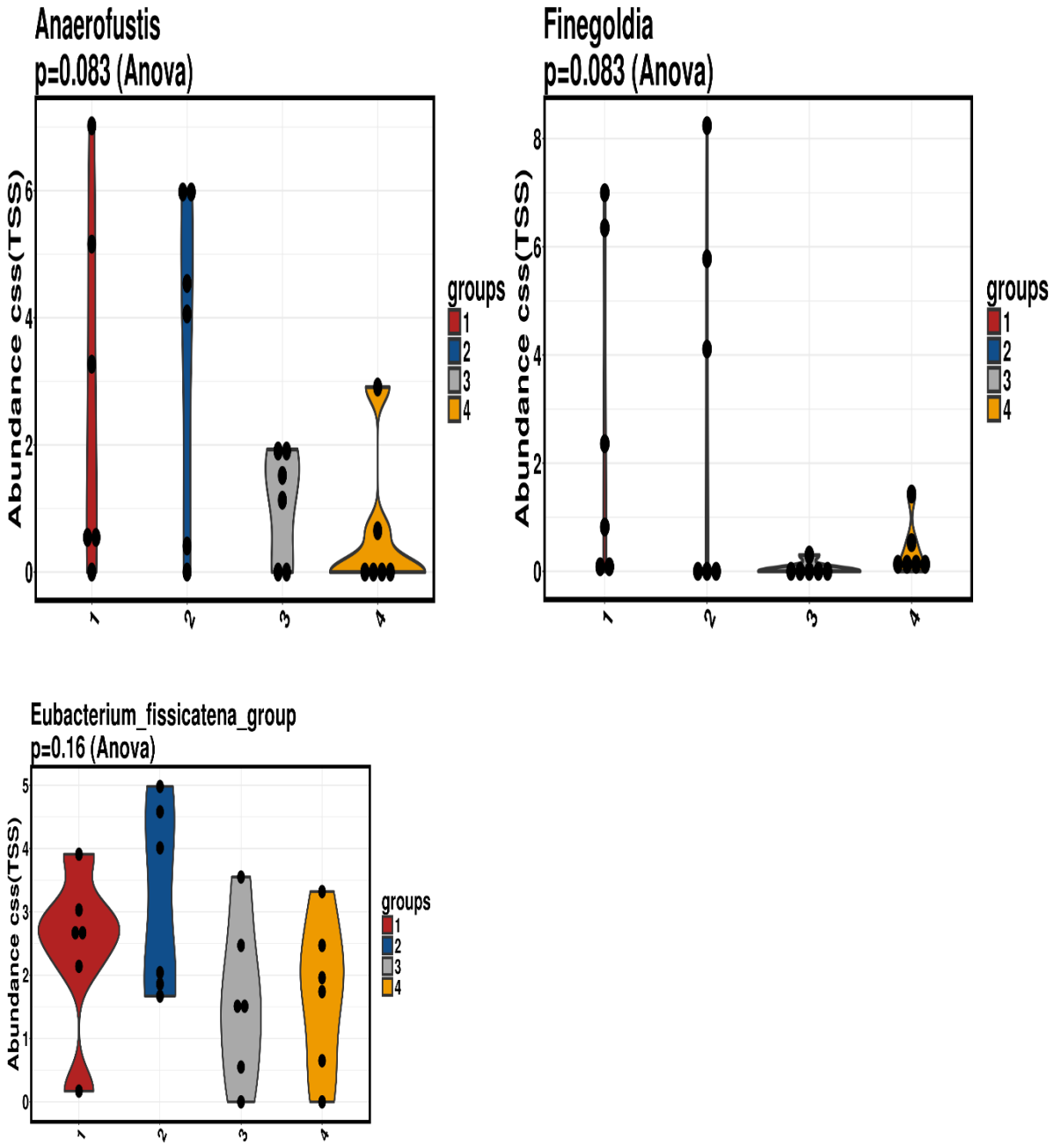


Figure 8. Violin Plot showing the Relative Abundance at the Genus level which were decreased in the Fidaxomicin and Nisin treated groups (N =6 subjects). Group 1 = No treatment control, Group 2 = Thuricin, Group 3 = Fidaxomicin and Group 4 = Nisin.

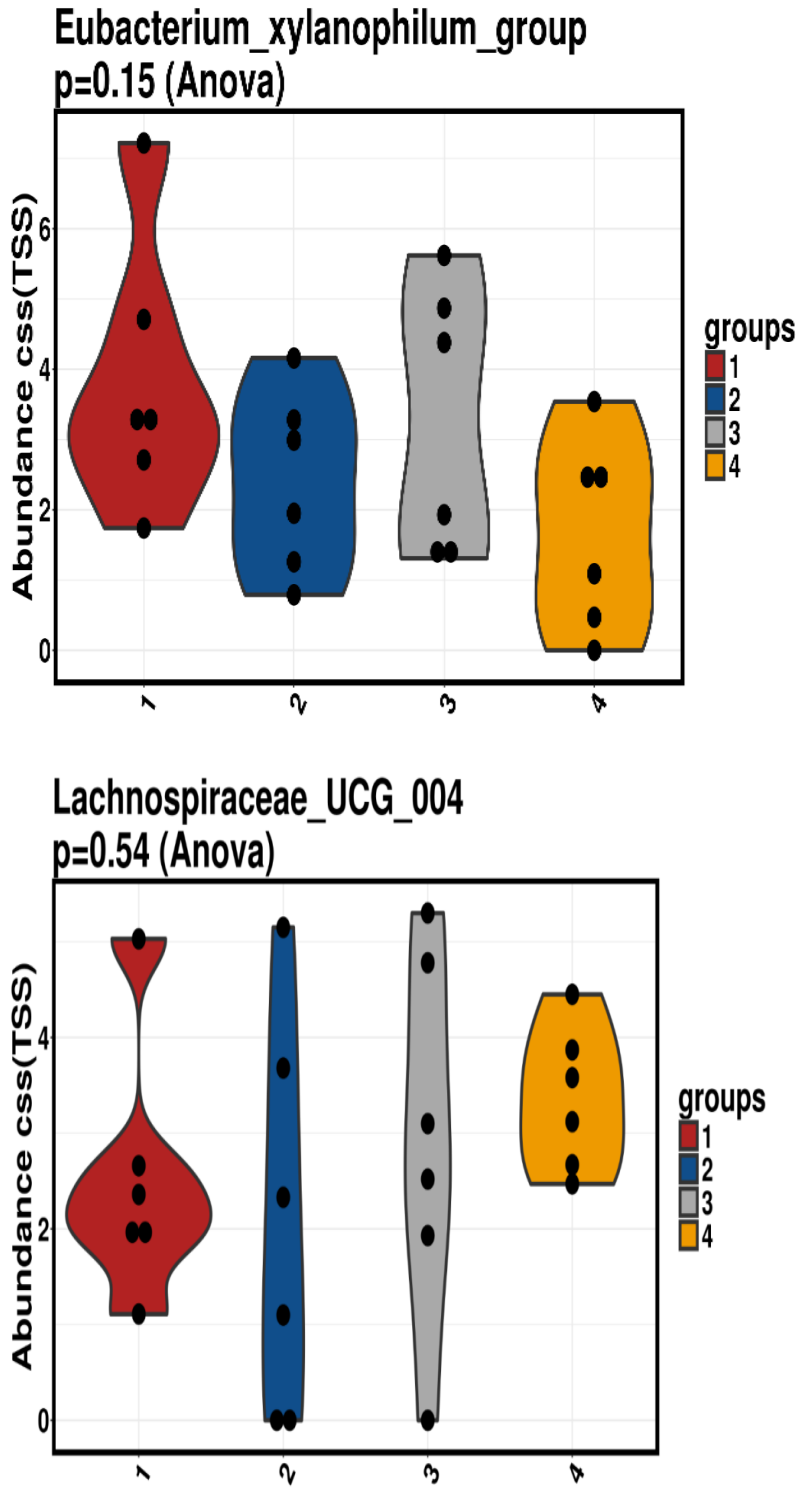


Figure 9. Violin Plot showing the Relative Abundance at the Genus level which were decreased in the Nisin treated groups n=6 subjects. Group 1 = No treatment control. Group 2 = Thuricin Group 3 = Fidaxomicin Group 4 = Nisin

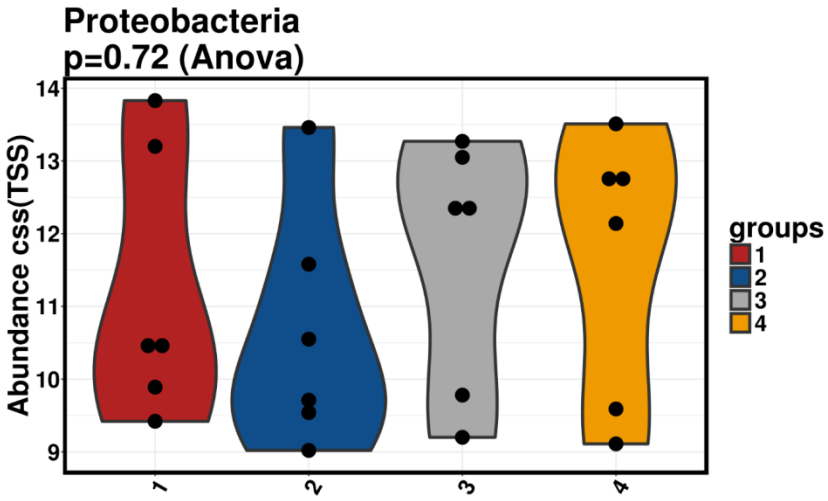


Figure 11. Increase in Relative Abundance of Proteobacteria

Group 1 = No treatment control. Group 2 = Thuricin Group 3 = Fidaxomicin Group 4 = Nisin

References

H.L. Koo, K.W. Garey, H.L. Dupont, Future novel therapeutic agents for Clostridium.

Healthcare-associated infections: Clostridium difficile infections. *Annual epidemiological report for 2016.*

Al-Nassir, W., Sethi, A., Li, Y., Pultz, M., Riggs, M., & Donskey, C. (2008). Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of Clostridium difficile-associated disease. *Antimicrobial agents and chemotherapy, 52*(7), 2403–2406. doi:10.1128/AAC.00090-08

Aronsson, B., Möllby, R., & Nord, C. (1985). Antimicrobial agents and Clostridium difficile in acute enteric disease: epidemiological data from Sweden, 1980-1982. *The Journal of infectious diseases, 151*(3), 476–481. doi:10.1093/infdis/151.3

Aslam, S., Hamill, R., & Musher, D. (2005). Treatment of Clostridium difficile-associated disease: old therapies and new strategies. *The Lancet infectious diseases, 5*(9), 549-57. doi:10.1016/S1473-3099(05)70215-2.

Bartlett, J. (2006). Narrative review: the new epidemic of Clostridium difficile-associated enteric disease. *Annals of internal medicine, 145*(10), 758–764. doi:10.7326/0003-4819-145-10-200611210-00008

Bartoloni, A., Mantella, A., Goldstein, B., Dei, R., Benedetti, M., Sbaragli, S., & Paradisi, F. (2004). In-Vitro Activity of Nisin against clinical isolates of Clostridium difficile. *Journal of Chemotherapy, 16*(2), 119-121. doi:10.1179/joc.2004.16.2.119

Biedenbach, D., Ross, J., Putnam, S., & Jones, R. (2010). In vitro activity of fidaxomicin (OPT-80) tested against contemporary clinical isolates of Staphylococcus spp. and Enterococcus spp. *Antimicrob Agents Chemother, 54*(5), 2273–2275. doi:10.1128/AAC.00090-10

Bricker, E., Garg, R., Nelson, R., Loza, A., Novak, T., & Hansen, J. (2005). Antibiotic treatment for Clostridium difficile-associated diarrhea in adults. *Cochrane Database of Systematic Reviews*(1), CD004610. doi:10.1002/14651858.CD004610.pub2.

Caporaso, J., Kuczynski, J., Stombaugh, J., & al, e. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods, 7*(5), 335-336. doi:10.1038/nmeth.f.303.

Centers for Disease Control and Prevention Recommendations for preventing the spread of vancomycin resistance: recommendations of the Hospital Infection Control Practices Advisory Committee (HIC-PAC). (1995). *Am J Infect Contro, 23*, 87–94.

Cornely, O., Crook, D., Esposito, R., & al., e. (2012). Fidaxomicin versus vancomycin for infection with Clostridium difficile in Europe, Canada, and the USA: a double-blind, noninferiority, randomised controlled trial. *Lancet Infect Dis, 12*(4), 281-289. doi:10.1016/S1473-3099(11)70374-7

Cornely, O., Miller, M., Louie, T., Crook, D., & Gorbach, S. (2012). Treatment of first recurrence of Clostridium difficile infection: fidaxomicin versus vancomycin. *Clin Infect Dis, 55*((Supp 2)), S154–S161. doi:10.1093/cid/cis462

Cramer, J., Burchard, G., & Lohse, A. (2008). Old dogmas and new perspectives in antibiotic-associated diarrhea. *Medizinische Klinik, 103*(5), 325-328. doi:10.1007/s00063-008-1040-0.

Crook, D., Walker, A., Kean, Y., & al, e. (2012). Fidaxomicin versus vancomycin for Clostridium difficile infection: metaanalysis of pivotal randomized controlled trials. *Clin Infect Dis, 55*(Suppl 2), S93–S103. doi:10.1093/cid/cis499

Edgar, R. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461. doi:10.1093/bioinformatics/btq461

Fooks, L., & Gibson, G. (2003). Mixed culture fermentation studies on the effects of synbiotics on the human intestinal pathogens Campylobacter jejuni and Escherichia coli. *Anaerobe, 9*, 231–242. doi:10.1016/S1075-9964(03)00043-X

Fouhy, F., Deane, J., Rea, M., O'Sullivan, Ó., Ross, R., O'Callaghan, G., & al, e. (2015). The Effects of Freezing on Fecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations. *PLoS ONE, 10*(3), e0119355. doi:10.1371/journal.pone.0119355

George, R., Symonds, J., Dimock, F., Brown, J., Arabi, Y., Shinagawa, N., . . . Burdon, D. (1978). Identification of Clostridium difficile as a cause of pseudomembranous colitis. *British medical journal, 1*(6114), 695. doi:10.1136/bmj.1.6114.695

Herzig, C., Reagan, J., Pogorzelska-Maziarz, M., Srinath, D., & Stone, P. (2015). State-mandated reporting of health care-associated infections in the United States: trends over time. *American journal of medical quality : the official journal of thethe official journal of the American College of Medical Quality, 30*(5), 417-424. doi:10.1177/1062860614540200

Klaenhammer, R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS microbiology reviews, 12*(1-3), 39–85. doi:10.1111/j.1574-6976.1993.tb00012.x

Koo, H., Garey, K., & Dupont, H. (2010). Future novel therapeutic agents for Clostridium difficile infection. *Expert Opinion on Investigational Drugs, 19*(7), 825-836. doi:10.1517/13543784.2010.495386

Louie, Miller, M., Mullane, K., Weiss, K., & Lentnek, A. e. (2011). Fidaxomicin versus vancomycin for Clostridium difficile infection. *N Engl J Med, 364*, 422-431. doi:10.1056/NEJMoa0910812.

Magoč, T., & Salzberg, S. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957–2963. doi:10.1093/bioinformatics/btr507

Mullane, K. (2014). Fidaxomicin in Clostridium difficile infection: latest evidence and clinical guidance. *Ther Adv Chronic Dis, 5*(2), 69-84. doi:10.1177/2040622313511285.

O'Donnell, M., Rea, M., Shanahan, F., & Ross, R. (2018). The use of a mini-Bioreactor Fermentation System as a reproducible, High Throughput ex vivo Batch Model of the Distal Colon. *Front. Microbiol, 9,* 1844. doi:10.3389/fnicb.2018.01844

O'Callaghan, A., & van Sinderen, D. (2016). Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Frontiers in microbiology, 7*, 925. doi:10.3389/fmicb.2016.00925

O'Gorman, M. M., Kaplan, S., Otley, A., Kociolek, L., Hoffenberg, E., Kim, K., . . . Sears, P. (2018). Safety and Pharmacokinetic Study of Fidaxomicin in Children With Clostridium difficile-Associated Diarrhea: A Phase 2a Multicenter Clinical Trial. *Journal of the Pediatric Infectious Diseases Society, 7*(3), 210–218. doi:10.1093/jpids/pix037

Quast, C., Pruesse, E., Yilmaz, P., & al, e. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research. *41*((Database issue)), D590-D596. doi:10.1093/nar/gks1219.

Rea, M., Clayton, E., O'Connor, P., Shanahan, F., Kiely, B., Ross, R., & Hill, C. (2001). Antimicrobial activity of lacticin 3147 against clinical Clostridium difficile strains. *Journal of Medical Microbiology, 56*, 940-946. doi:10.1099/JMM.0.47085-0

Rea, M., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P., . . . Ross, R. (2011). Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proceedings of the National*

Academy of Sciences of the United States of America, 108(Suppl 1), 4639–4644. doi:10.1073/pnas.1001224107

Rea, M., Sit, C., Clayton, E., O'Connor, P., Whittal, R., Zheng, J., . . . Hill, C. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proc Natl Acad Sci USA, 107*(20), 9352–9357. doi:10.1073/pnas.0913554107

Shah, D., Dang, M., Hasbun, R., Koo, H., Jiang, Z., DuPont, H., & Garey, K. (2010). Clostridium difficile infection: update on emerging antibiotic treatment options and antibiotic resistance. *Expert Rev Anti Infect Ther, 8*(5), 555-564. doi:10.1586/eri.10.28

Tannock, G., Munro, K., Taylor, C., Lawley, B., Young, W., Byrne, B., . . . Louie, T. (2010). A new macrocyclic antibiotic, fidaxomicin (OPT-80), causes less alteration to the bowel microbiota of Clostridium difficile-infected patients than does vancomycin. *Microbiology, 156*, 3354–3359. doi:10.1099/mic.0.042010-0

Wiström, J., Norrby, S., Myhre, E., Eriksson, S., Granström, G., Lagergren, L., . . . Svenungsson, B. (2001). Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. *The Journal of antimicrobial chemotherapy., 47*(1), 43-50. doi:10.1093/jac/47.1.43

Zakrzewski, M., Proietti, C., Ellis, J., & al, e. (2017). Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics, 33*(5), 782–783. doi:10.1093/bioinformatics/btw725

Zhanel, G., Walkty, A., & Karlowsky, J. (2015). Fidaxomicin: A novel agent for the treatment of Clostridium difficile infection. *Can J Infect Dis Med Microbiol, 26*(6), 305-312. doi:10.1155/2015/934594

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Chapter 5

Orally ingested nisin impacts on gut microbiome composition and functionality in young domestic pigs (*Sus domesticus*)

Abstract

Nisin is a bacteriocin with potential for use as an alternative to traditional antibiotics. In this study we assessed the ability of nisin (encapsulated and unencapsulated) to reach the lower porcine gastrointestinal tract (GIT) in an intact form and to impact the gut microbiota of post-weaning piglets following four consecutive days of feeding. The impact of the initial nisin treatment on the gut microbiota was monitored for another ten days following cessation of the treatment. In addition to observing the effects of the two forms of nisin (encapsulated and unencapsulated), the encapsulant material (ethyl cellulose) was included as a control. Intact biologically active nisin was detected in the faeces of all animals in the nisin-treated groups when the nisin was being fed and also for three days following cessation of treatment. The gut microbiota was substantially altered by nisin treatment, but rapidly recovered when the nisin treatment stopped. During nisin treatment there was a significant relative increase in Proteobacteria and Bacteroides ($p<0.01$) and a concomitant decrease in Firmicutes in the nisin-treated groups as compared to the control groups (encapsulant alone and no treatment control). This is consistent with nisin having greater antimicrobial activity against Gram-positive bacteria. When nisin treatment ceased the relative abundance of these microbial populations returned to pre-treatment levels. Three days after the initial treatments were fed, there was a significant decrease in three of six short chain fatty acids (SCFAs) in the nisin treated groups compared to the no treatment control group. Shotgun metagenomic sequencing revealed significant

correlations between SCFA concentrations and species level changes and between species and metabolic pathways. Our study confirms that orally ingested nisin reaches the lower GIT in an intact form and alters the gut microbiota of post-weaning piglets.

Introduction

Antibiotic overuse and antibiotic resistance in food-producing animals poses a health risk to animals and humans. Antibiotics are administered to animals to improve growth rates and to prevent infections by reducing the burden of pathogens. Approximately 80% of antibiotic sales in the United States are for use in animal husbandry; about 70% of which belong to antibiotic classes of importance in human medicine (Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals 2014). This practice is projected to increase dramatically worldwide over the next 15 years (Van Boeckel, et al. 2015). Antimicrobials have been a key tool used in animal production to enhance performance and to treat and prevent disease (Food and Drug Administration Veterinary feed directive 2015) (Maki, et al. 2019). Nisin is an antimicrobial peptide produced by *Lactococcus lactis* that has a broad spectrum of activity against Gram-positive bacteria (Lubelski, et al. 2008; De Arauz, et al. 2009). Nisin has been approved for use as a food preservative by the US Food and Drug Administration (FDA) (US Food and Drug Administration, 1988) and the European Food Safety Authority (EFSA; E number E234) and is widely used as a food preservative and consequently consumed by humans (Younes, et al. 2017). Nisin (in the form of the commercially available Nisaplin) has been safely consumed by rats without adverse effects at doses of up to 239 mg per kg body weight (Hagiwara et al., 2010).

There are numerous variants of nisin, with nisin A being the most widely studied (Gross and Morell 1971). Another variant, nisin Z, differs from nisin A by a single amino acid residue at position 27, with asparagine replacing histidine (Mulders, et

al. 1991). Nisin A and Z have similar properties as antimicrobial agents, but nisin Z is more soluble at neutral pH and also has a superior rate of diffusion (De Vos, et al. 1993). Due to its proteinaceous nature, nisin has been reported to be subject to proteolytic digestion within the gastrointestinal tract (GIT) by enzymes such as pancreatin, trypsin and chymotrypsin (Heinemann and Williams 1966). Thus, it has been suggested that it may not reach the lower GIT intact (Jarvis and Mahoney 1969; Chan, et al. 1996).

Research into the influence of the gut microbiome on human health continues to reveal its essential physiological function, including nutrient uptake and metabolism, the promotion of immune response to antigens and strengthening the integrity of the gut (Postler and Ghosh 2017; Nicholson, et al. 2012; Power, et al. 2014). Metagenomic approaches have provided deeper insights into the bacteria involved in short chain fatty acid (SCFA) production (Morrison and Preston 2016). Despite this, our understanding of the link between the gut microbiota and SCFAs and the role of these bioactive metabolites in host metabolism and growth is still unfolding.

Nisin would be expected to be broken down to some extent in the upper intestine due to exposure to proteolytic enzymes, as has been shown previously for other bacteriocins, namely lacticin 3147 (Gardiner, et al. 2007) and thuricin CD (Rea, et al. 2014). The porcine GIT closely resembles the human GIT and is an ideal model to assess the likely impact of nisin on the human gut microbiome. Nisin has *in vitro* efficacy against gut pathogens such as *Clostridium difficile* (Le Lay, et al. 2016) and in combination with cinnamaldehyde and ethylene diamine tetra acetic acid (EDTA) has been shown to control the growth of porcine strains of Enterotoxigenic *Escherichia coli* (Field, et al. 2017).

In this study we fed nisin Z (nisin ZP) to post-weaning piglets to determine its impact on the composition and function of the gut microbiota. Nisin ZP is an ultrapure nisin Z powder, produced by a *Lactococcus lactis* strain that was originally isolated from sauerkraut. We encapsulated the nisin preparation to protect it against possible degradation in the upper GIT and to enable it to reach the colon intact. Ethylcellulose SmPill®-enabled formulations have previously been shown to be a successful vehicle to deliver active agents to their targeted region in the GI tract and to then release them (Keohane, et al. 2016). In that study the porcine model was used to monitor the release of cyclosporine (an immunosuppressant for use for the treatment of Ulcerative Colitis or Crohn's disease) from coated minispheres in the GI tract and to optimise the encapsulation design for increased release efficiency (Keohane, et al. 2016). Based on this work an ethylcellulose-based, nisin containing formulation was developed for this study. Prior to this study, there have been a small number of *in vivo* studies investigating the ability of nisin to reach the lower GI tract and to impact the gut microbiota of chickens, mice and rabbits (Bernbom, et al. 2006; Józefiak, et al. 2013; Lauková, et al. 2014; Kieronczyk, et al. 2016). These studies varied widely in their findings for several reasons, including the form and dose of nisin used and the methods used to assess the impact of nisin on the gut microbes. To our knowledge, this is the first study assessing the impact of pure nisin Z in an unencapsulated and encapsulated form on the porcine gut microbiota. One previous study used rRNA 16S gene sequencing to assess the impact of enriched nisin A (from purified Nisaplin with 57.7% purity), fed in starch-based matrices, on the murine gut microbiota. The study found there was an impact on the gut microbial composition and that the nisin was broken down in the GIT (Gough, et al. 2018).

Here, we demonstrate the delivery of nisin to the lower GIT of pigs where it causes shifts in the microbial composition and changes in SCFA levels and metabolic pathways.

Material and Methods

Ethical Approval

This study was approved by the Teagasc Animal Ethics Committee (TAEC185-2018) and an experimental licence number (AE19132/P083) was obtained from the Irish Health Products Regulatory Authority (HPRA). All procedures were performed according to the European Union Directive 2010/63/EU on the protection of animals for scientific purposes.

Dietary Treatments

There were four dietary treatment groups as follows (1) a standard non-medicated link diet (control group; Ctl); (2) the link diet supplemented with 150 mg/kg body weight of nisin powder (Nis-pdr) (Handary, Brussels, Belgium; high content powder nisin ZP (95 % content/ultrapure; % weight/weight; hydrous potency ≥38,000 IU/mg)); (3) the link diet supplemented with 850 mg/kg body weight of encapsulated nisin ZP (Nis-en) (Sublimity Therapeutics, Dublin, Ireland) and (4) the link diet supplemented with 110 mg/kg body weight of the encapsulant material (Encap). The encapsulant material was ethylcellulose (98 %) (DOW Chemical Company Limited, Staines, England; gifted by Sublimity Therapeutics). The dose of nisin administered in the encapsulated nisin was equivalent to that of the nisin powder treated group (Treatment 2). The encapsulant administered (Treatment 4)

was equivalent to the amount in the encapsulated nisin formulation in Treatment 3. Treatments in powder form were top-dressed to a small amount of feed with 5-10 ml water in the morning to ensure that all the nisin was consumed each day.

Animal Management

This study was conducted in the Pig Development Department, Teagasc, Moorepark, Fermoy, Cork, Ireland. A schematic depicting the trial structure is presented in Figure 1. Piglets (Large White X Landrace) were weaned at 28 (±3) days, housed in groups of intact litters and fed a starter diet for 6 days with each pig ear tagged with a unique number for identification purposes. From this group of 150 pigs, 40 males were selected and blocked on body weight and litter origin following which they were randomly assigned, to one of the four dietary treatments detailed above (N=10 pigs/treatment). The pigs were individually housed in fully slatted pens (1.2 m x 0.9 m) and were each provided with a non-medicated link diet for a seven-day acclimatisation period. The room temperature was maintained at 28-30°C during the first week post-weaning and then reduced by 2°C each week until a temperature of 22°C was achieved in the room. Feed and water were provided on an *ad libitum* basis, lighting was ensured for ≥8 h/day and environmental enrichment was provided. Following the seven-day acclimatisation period, dietary treatment commenced. Dietary treatment was provided for four days following which pigs were again fed the common un-medicated link diet for ten days until the end of the trial.

Proximate analysis and amino acid composition of the diets used are presented in Supplementary Table 1. All pigs were individually weighed on D0 (Figure 1) prior

to the start of the trial (baseline (BL)). Growth performance was assessed by weighing all pigs on two further occasions (1) mid-way through the trial (Day 6; two days after treatment had ceased) and (2) at the end of the trial (Day 14; ten days after treatment had ceased). Voluntary feed intake was recorded daily between the start and end of the trial and this divided by the average daily gain was used to calculate feed conversion ratio (FCR).

Treatment groups, Sampling time points and Sample Collection

Faecal samples were taken before the treatment commenced to determine the baseline (D0; BL) microbiota. Further faecal samples were taken 24 hours after initial treatment (T24) was fed, 48 hours after initial treatment was fed (T48) and 72 hours after initial treatment was fed (T72). These samples were taken to assess the immediate impact of treatment on the porcine gut microbiota. Faecal samples were also taken towards the end of the trial, three days after treatment ceased (3d PT) and ten days after treatment ceased (10d PT). These samples were taken to observe the recovery of the gut microbiota when treatment ceased. The fate of the ingested nisin was also determined from these samples.

Faecal samples were from freshly voided when available, otherwise they were collected following rectal stimulation. Samples were collected into sterile pots and stored on ice briefly (< 2 h) and sub-sampled into sterile 2 ml eppendorf tubes. Samples were then immediately snap-frozen in liquid nitrogen and stored at -80^{o}C for DNA extraction and SCFA analysis or at -20^{o}C for screening for the presence and activity of nisin; for well diffusion assays (WDAs) and mass spectrometry (MS).

DNA Extraction

DNA was extracted from 200 mg faeces from each pig at BL, T24, T48, T72, 3d PT and 10d PT using the Zymo Research ZR faecal DNA kit (Cambridge Biosciences, Cambridge, United Kingdom), according to manufacturer's specifications.

16S rRNA Library Preparation

The V3-V4 variable region of the 16S rRNA gene was amplified from 240 faecal DNA samples using the 16S metagenomic sequencing library protocol (Illumina San Diego, CA). Samples were quantified and libraries were prepared for sequencing as described previously (Fouhy, et al., 2015). In brief, the 16S rRNA amplicon sequencing libraries were prepared as follows: two PCR reactions were completed on the template DNA. DNA was firstly amplified with primers specific to the V3–V4 region of the 16S rRNA gene, which also incorporate the Illumina overhang adaptor (forward primer 5′TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGCAG; reverse primer 5′GTCT CGTG GGCT CGGA GATG TGTA TAAG AGAC AGGA CTAC HVGG GTAT CTAATCC). Following this a second PCR reaction was performed with two indexing primers (Illumina Nextera XT indexing primers) added to allow for demultiplexing. Samples were analysed on the Illumina MiSeq at the Teagasc Sequencing Centre, Moorepark, Fermoy, Co. Cork, Ireland using the Miseq 600 cycle v2 kit and following standard Illumina sequencing protocols. Following sequencing, the data was analysed to establish the effect of nisin treatment on the composition of the gut microbiota.

Shotgun Metagenomic Library Preparation

Paired-end sequencing libraries were prepared from the extracted DNA from 120 samples (BL, T72 and 10d PT) using the Illumina Nextera XT library preparation kit (Illumina) followed by sequencing on the Illumina NextSeq 500 platform using high-output chemistry (2 × 150 bp) according to the manufacturer's instructions at the Teagasc Sequencing Centre.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF MS)

Nisin was extracted from the faecal samples as follows: samples were suspended in 1 mL of 0.1 % trifluoroacetic acid (TFA) and 70% isopropyl alcohol (IPA), vortexed thoroughly and allowed to stand at room temperature for 30 min and centrifuged for 5 min at 16,000 *g* and the supernatant retained. The centrifugation step was repeated a further three times with the supernatant being retained each time. The molecular mass corresponding to the nisin peak was observed using MALDI TOF MS with an Axima TOF^2 (Shimadzu Biotech, Kyoto, Japan), as previously described (Field, et al. 2012).

Faecal Water preparation and SCFA Analysis

Four hundred milligrams of frozen faecal material was placed in a sterile 2 ml microcentrifuge tube. Eight hundred microlitres of sterile water was added to the 2 ml microcentrifuge tube. The sample was vortexed vigorously for 30 s to generate a faecal slurry. Samples were then centrifuged at 12,000 g for 30 min at 4°C. The supernatant was removed and transferred to a new 2 ml microcentrifuge tube. This

step was repeated. The supernatant was then centrifuged again at 12,000 g for 30 min at 4°C. This supernatant was transferred to a 0.2 µM costar spin-x centrifuge tube (Sigma-Aldrich, UK) and filtered by centrifugation at 12,000 g for 10 min at 4°C. The filter was removed from the tube and the faecal water was stored at –20°C prior to metabolomic analysis. Samples were analysed by MS-Omics ApS, Frederiksberg, Denmark to determine metabolite levels, including SCFAs.

Antibacterial Activity Assay

Antibacterial activity in the porcine faecal samples was estimated using well diffusion assays (WDAs) (Ryan, et al. 1996) in agar plates seeded with *Lactobacillus delbrueckii* ssp *bulgaricus* LMG6901 as detailed previously (Gough, et al. 2017). Faecal water, prepared as above, was used in the assays. The samples were dispensed into the wells of the seeded agar in 50 µL aliquots and the agar plates incubated anaerobically overnight at 37°C. Antibacterial activity resulted in zones of inhibition surrounding the wells. Samples from animals that had not consumed nisin were used as negative controls to ensure that zones of inhibition were due to nisin inhibition.

Processing of 16S Illumina Sequencing

Paired-end reads were trimmed and quality filtered using PRINSEQ version 0.20.4 (Schmieder and Edwards 2011). Resulting sequences had a mean quality score greater than 20. Forward and reverse paired-end sequences were then joined with a minimum overlap of 10 bp using fastq-join (Aronesty 2013). Sequences were clustered into Operational Taxonomic Units (OTU) using closed-reference

algorithm in USEARCH version 7.0 (Edgar RC 2010) and chimeras were removed with the RDP Gold reference database.

The Bray-Curtis distance matrix was used to calculate beta diversity of 16S ribosomal gene amplicon counts. In the vegan R package (version 2.5-6), the betadisper function assessed homoscedasticity (equal distribution of variance).

Shotgun Metagenomic Pipeline

Shotgun metagenomic sequence files (BCL, base calls) were converted to fastq format using bcl2fastq version 2.19. Forward and reverse fastq files were processed using KneadData version 0.7.2 from the Huttenhower bioBakery suite (McIver, et al. 2018). A reference library was created to remove host DNA in Bowtie2 version 2.3.4 from the NCBI rat genome (*Rattus norgegicus,* GCF 000001895.5). Quality filtering was performed using the default setting (ex., phred=33) and trimming with Trimmomatic version 0.38-1. Resulting high quality paired-end reads for each sample were then concatenated in KneadData. Kraken2 version 2.0.7-beta was used for taxonomic classification with the standard database. Report files of taxonomic counts for each sample were merged into a single count file using a custom R script (Barton, et al. 2018) and ran in R version 3.5.2. Humann2 version 0.11.2 from the Huttenhower lab (Franzosa, et al. 2018) was used for functional predictions of shot-gun metagenomic samples. Briefly, Humann2 was ran with the following setting: i) Metaphlan2 version 2.7.5 with options set to database version 20 for taxonomic assignments used in assessing microbiome functional pathways; ii) uniref90 protein database with sequence clustering by 90% sequence identity; iii) Diamond version 0.9.24.

Shotgun metagenomic sequences were correlated to SCFA concentrations by (i) predicted metabolic pathways from shotgun sequences to SCFA; (2) shotgun sequence taxonomic designation at the species level to SCFA and (3) shotgun sequence taxonomic designation to metabolic pathway predictions. The Humman2 database (McIver, et al. 2018) was used for microbiome pathway predictions. All correlations were performed with Spearman and corrected for multiple comparisons using the q value package. Correlations between SCFA and metabolomic prediction were plotted for rho>0.05.

Statistical Analysis

Differences in SCFA levels across treatment days and treatment groups

To test for differences related to the treatment of the subjects t-tests were performed comparing control and treatment groups on the individual time points. The Benjamini-Hochberg correction was performed with an accepted false positive rate of 10%.

16S Analysis

Alpha diversity and taxonomic composition were assessed using the Kruskal-Wallis test followed by pairwise Mann-Whitney post-hoc analysis. False Discovery Rate (FDR) corrected for multiple comparisons using the q value version 2.14.1 R package (Dabney, Storey and Warnes 2011) with a q value threshold of 0.05.

Significant difference at species level from metagenomic shotgun data

Significant differences at the species level from metagenomic shotgun sequencing data

Analysis of taxonomic tables was completed in R (R Foundation for Statistical Computing, Vienna, 2012) (version 3.5.1). Statistical analysis was completed using the Kruskal-Wallis test, with a Mann Whitney U test performed for significant results to determine the groups between which this difference applied and q value corrections were completed. The significance threshold was set at 0.05. Data visualisations were completed with ggplot2 (version 2.2.1) in R.

Results

The aim of this study was to evaluate the effect of orally ingested nisin on the composition and function of the gut microbiota of pigs. We used both unencapsulated and encapsulated nisin, which were directly added to the pig feed. This study also contained a no treatment control group and a group that was fed the material that was used to encapsulate the nisin.

Growth performance of pigs in response to treatment

Feed conversion ratio (FCR) is a common index used to measure feed efficiency in pigs. Average daily weight gain and average daily feed intake increased across the trial period. There was no difference in pig live weight between the treatment groups (Figure 2a). This was not unexpected given the short duration of the study. The growth performance (FCE) of the pigs is shown in Figure 2b.

Detection of nisin in the faeces

Activity assays showed antibacterial activity in the faeces of all pigs in both the encapsulated nisin (Nis-en) and nisin powder (Nis-pdr) treated groups throughout

the period in which the animals were fed nisin (treatment days). MALDI TOF MS was performed on the faecal samples to determine the presence of intact nisin. A peak corresponding to the molecular mass of intact nisin (3331.05 Da) was detected in the porcine faeces on the treatment days in the two nisin-treated groups (Nis-en and Nis-pdr). Bioactivity, measured by a well diffusion assay, was also detected in the faeces of all pigs in the encapsulated nisin and nisin powder group for three days after feeding of the treatments ceased (3d PT). The zone sizes were smaller than those observed on the nisin treatment days however see Table 1 and Figure 3. This indicated that bioactive nisin was still present in the GIT post feeding. Intact nisin was also detected in all the faecal samples from the pigs in the nisin-treated groups (after a ten-fold dilution of the sample for Mass Spec detection purposes), indicating that the nisin was still present in the GIT. Intact and active nisin was not detected in any of the baseline samples (BL), the encapsulant group (Encap) or in the no treatment control group (Ctl). Intact and active nisin was not detected in any of the samples taken ten days after treatment ceased (10d PT).

Microbial diversity and 16S composition in the faeces of piglets by treatment group

DNA was extracted from the faecal samples taken at BL, T24, T48, T72, 3d PT and 10d PT. 16S rRNA gene amplicons (V3–V4 region) were generated and sequenced using the Illumina MiSeq™ platform. The average number of OTUs per sample was 123,814 +/- 34,649 with a range between 24,578 and 231,778. The BL gut microbiota was similar across treatment groups (See Figure 5).

Treatment with nisin, either in an encapsulated or unencapsulated form, decreased the alpha diversity ($p<0.001$) compared to the encapsulant or no treatment control

groups. Three days after treatment had ceased the alpha diversity increased in the nisin-treated groups and ten days after treatment had ceased the alpha diversity was similar to the control groups. Beta dispersion analysis revealed a shift in the diversity in the groups fed nisin over the days of treatment ($p<0.001$). The most notable shift was in the nisin powder treated group on the treatment days. The groups fed the encapsulant material and the no treatment control group were most similar to each other and no significant differences were observed between these groups. After treatment had ceased the beta diversity increased in the nisin-treated groups and ten days after treatment, as with the alpha diversity, the beta diversity had increased to levels comparable to the non nisin-treated groups (See Figure 4). The most abundant phyla across all treatment groups in the baseline samples were the Firmicutes followed by the Bacteroidetes and the Proteobacteria. There were changes at the phylum level across treatment groups throughout the study (Figure 5). In total there were 81 significant differences ($q < 0.05$) at phylum level between treatment groups at the same time point (Table 2). The microbiota composition at the baseline was similar across treatment groups. The microbiota composition remained relatively stable in the no treatment control and the encapsulant group across the entire trial. There were no significant differences observed at the phylum, family or genus level for the baseline microbiota across treatment groups or in the no treatment control group or encapsulant material group across all time points. Of the most abundant phyla, there was a significant increase in the relative abundance ($p<0.001$) in Proteobacteria in the nisin powder group compared to the no treatment control and encapsulant material groups at T24, T48, T72, and also three days after treatment (3d PT). Likewise, there was a significant increase in the relative abundance of Proteobacteria in the encapsulated nisin group compared to

the control and encapsulant material group at T48 and T72. There was a significant decrease the relative abundance of Firmicutes in the encapsulated nisin group compared to the control group and the encapsulant material group at T48 and T72 after treatment. A significant increase in the relative abundance of Bacteroidetes was observed in the nisin powder group compared to the control group and encapsulant material group at T24, T48 and T72. There was also a significant decrease in the relative abundance of the Bacteroidetes phyla in the encapsulated nisin group compared to the control and the encapsulant material group at T48 and T72. After treatment had ceased the changes observed at phylum level reverted to being more similar to the non nisin-treated groups by 3d PT and 10 PT days the levels returned to resemble those of the untreated groups.

At the family level, forty families were included in the analysis. The most abundant families across all treatment groups in the baseline samples were *Prevotellaceae, Ruminococcaceae, Lachnospiraceae, Clostridiaceae 1, Lactobacillaceae, Erysipelo-trichaceae, Streptococcaceae, Spirochaetaceae, Bacteroidales S24.7 group*, *Rikenellaceae* and *Veillonellaceae*. There was a decrease ($P < 0.001$) in the relative abundance of *Lachnospiraceae, Ruminococcaceae, Streptococcaceae* and *Veillonellaceae* in the nisin powder group compared to the no treatment control group and the encapsulant group 24 h after treatment commenced (T24). At T48 and T72 there was a significant decrease in the relative abundance of *Clostridiaceae 1, Streptococcaceae, Veillonellaceae, Lachnospiraceae* and *Lactobacillaceae* in the nisin powder compared to the no treatment control and the encapsulant group. Additionally, there was a significant increase in the relative abundance of *Prevotellaceae* in the nisin powder group compared to the encapsulant group at T48 and by T72 *Erysipelotrichaceae* and *Rikenellaceae* were

also significantly increased. Three days after treatment ceased (3d PT) there was a significantly lower level of *Clostridiaceae 1*, *Streptococcaceae* and *Erysipelotrichaceae* in the nisin powder (Nis-pdr) group compared to the control group (Ctl). There was also a significantly lower level of *Clostridiaceae 1* in the nisin powder group when compared to the encapsulant material group. There was a significant difference in the relative abundance of *Clostridiaceae 1*, *Streptococcaceae*, *Veillonellaceae* in the control group compared to the encapsulated nisin group at T24. There was also a significant difference in the relative abundance of *Ruminococcaceae* at T24 between the encapsulated nisin group and the encapsulant material group. There was a significant difference in the relative abundance of *Clostridiaceae 1* and *Streptococcaceae* at T48 for the encapsulated nisin group and the encapsulant material group. The relative abundance of the *Veillonellaceae* family significantly increased at T72 while the abundance of *Rikenellaceae*, *Clostridiaceae 1* and *Streptococcaceae* decreased. Three days after treatment ceased (3d PT), there was still a significant decrease in the relative abundance of *Streptococcaceae* family in the nisin powder group compared to the control group. Overall, there were 205 significant differences between the treatment groups across the treatment periods at the family level. Ten days after treatment ceased (10d PT), the most abundant families across all treatment groups were *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Lactobacillaceae*, *Veillonellaceae*, *Erysipelotrichaceae*, *Spirochaetaceae*, *Clostridiaceae 1*, *Bacteroidales S247* group and the *Rikenellaceae*. This was similar to the baseline microbial composition. The abundance of *Streptococcaceae* remained stable over the no treatment control group and the encapsulant material group throughout the trial until the last sampling time point where the relative

abundance decreased 1-fold. Ten days after treatment ceased (10 PT) the relative abundance of *Streptococcaceae* in the nisin-treated groups was still negligible.

There were 130 genera included in the analysis, with the most prevalent in the baseline samples across all treatment groups including *Prevotella 9, Lactobacillus, Streptococcus, Clostridium Senso Stricto 1, uncultured, Prevotella 1, Treponema 2, Lachnospiraceae other, Bacteriodiales S247 group uncultured* and *Blautia* (Figure 6). At the genus level there were 1,017 significant differences between the treatment groups over the treatment periods. At T72 the most abundant genera in the no treatment control (Ctl) were *Lactobacillus, Prevotella 9, Streptococcus, Prevotella 1, uncultured, Clostridium Senso Stricto 1, Bacteriodiales S247 group uncultured, Lachnospiraceae other, Megasphaera* and *Blautia.* In the encapsulant material alone (Encap) the most abundant genera were similar to the no treatment control group with the exception of *Catenibacterium* and *Rikenellaceae RC9 gut group*. In the nisin powder treated group (Nis-pdr) at T72, the most abundant genera were the *Prevotella 1, Prevotella 9, Megasphaera, Mitsuokella, Succinivibrio, Bacteroidales S247 group uncultured bacterium, Lachnospiraceae other, Phascolarctobacterium, Faecalibacterium* and *Escherichia/Shigella*. There was a significant loss in diversity with mainly Gram-negative genera being dominant. In the encapsulated nisin group the most abundant genera were the *Prevotella 9, Lactobacillus, Catenibacterium, Faecalibacterium, Bacteroidales S247 group uncultured, Megasphaera, Lachnospiraceae other, Prevotella 1, Mitsuokella* and *Blautia*.

Interestingly, three days after feeding treatments ceased (3d PT) there was a notable shift in the composition of the microbiota in the nisin-treated groups to compositions more similar to that of the non-nisin fed groups (the Ctl and Encap

groups). By 3d PT the abundance of *Lactobacillus* was equivalent across all treatment groups including the nisin-treated groups (~9% from negligible levels). Ten days after treatment ceased (10d PT) the *Prevotella 9*, *Lactobacillus*, *Clostridium Senso Stricto 1*, *uncultured*, *Bacteriodiales S247 group uncultured* and *Megasphaera* were the most relatively abundant genera across all treatment groups.

SCFA levels by treatment

Faecal samples were taken at the baseline (BL), at T72 and ten days after treatment ceased (10d PT). The SCFA method used is a targeted method which analyses the sample for 10 different compounds (acetate, formic acid, propionic acid, butyrate, isobutyric acid, isovaleric acid, valeric acid, 4-methylpentatoic acid, hexanoic acid and heptanoic acid). With the exception of formic acid all the compounds were found in some samples. 4-methyl-pentanoic acid was only found in 5 samples total, and in all cases in levels very close to the limit of detection. Hexanoic acid and heptanoic acid were excluded from further analysis because the levels in the baseline (BL) samples were below the limit of detection for 50% or more of samples. Hence, six samples were included in the statistical analysis; butyrate, acetate, isovaleric acid; isobutyric acid, valeric acid and propionic acid. At baseline, the levels of SCFAs were similar across the treatment groups. At T72 the SCFA levels were depleted in the nisin-treated groups with the nisin powder group being the most affected; in particular concentrations of butyrate, acetate, isovaleric acid and isobutyric acid were reduced. To test for differences related to the treatment of the subjects, t-tests were performed comparing control and treatment groups at the different time points. After correction for false discovery using Benjamini-Hochberg correction the t-test

showed a significant decrease in the level of acetate (p=0.003), butyrate (p=0.004) and isobutyric acid (p=0.044) in the nisin powder group compared to the control group at T72. There was also a significant decrease in butyrate (p=0.030), isobutyric acid (p=0.048) and isovaleric acid (p=0.039) in the encapsulated nisin group compared to the no treatment control group at T72. No other significant differences were found across treatment groups. Ten days after cessation of treatment the concentration of SCFA levels increased in the nisin treated groups but did not recover to the concentrations measured in the no treatment control or encapsulant groups. The highest variation in SCFA levels across samples was observed at 10d PT. The concentration of SCFA levels (mM) across the time points is presented in Table 3, while Figure 7 shows changes in the levels of butyrate between the groups across time points.

Species level changes across time

Differential abundance analysis of the species-level taxonomic assignments revealed significant differences between the nisin-treated and non-nisin-treated pigs. Many taxa exhibited decreased relative abundances with antimicrobial administration, predominantly those from the phylum Firmicutes. For example, there was a significant decrease in the relative abundance of *Clostridioides difficile* (a known gut pathogen) at T72 in the nisin treated groups compared to the non-nisin treated groups (Figure 8). There was also a significant decrease in many *Lactobacillus* species such as *L. salivarius, L. reuteri*, *L. johnsonii* and *L. acidophilus* in the nisin treated groups at T72 compared to the non-nisin treated groups. There were 484 significant differences between the treatment groups at T72 (200 differences between the encapsulant and nisin powder treated groups, 189

differences between the control and nisin powder group, 40 differences between the encapsulant material and encapsulated nisin group, 44 differences between the control and encapsulated nisin, 9 differences between encapsulated nisin and the nisin powder group and only 1 significant difference between the control and encapsulant (*Bovine alpha herpes virus 1*)). There were no significant differences at baseline or 10d PT between the treatment groups, indicating the recovery of the microbiota by 10 days post treatment.

Associations between species and SCFA levels

We determined whether there was a relationship between the gut microbiota at species level and SCFA levels in the porcine faecal samples. There was a total of 4,783 significant correlations passing FDR between SCFA level and species level changes ($r > 0.5$ or < -0.5, $P < 0.05$). Most of these were negative correlations involving mainly lowly abundant microbes. The most strongly significant negative correlations were found between *Mycoplasma cloacal, Mycoplasma californium, Leptospira interogans, Leptospira biflexa, Heliobacter mustelal, Campylobacter* sp. and SCFA levels. Overall, a strong negative correlation was observed between *Cellulosilyticum lentocellum* and pentanoic acid (valeric acid) (rho -0.41 q = 0.0001) using Spearman's rank correlation coefficient. The top twenty most significant correlations are detailed in Figure 9.

Metabolic Pathways versus SCFA

There were 803 significant correlations passing FDR between changes in SCFA levels and metabolic pathways. The twenty most strongly significant correlations

between changes in metabolic pathways and SCFA levels are shown in Figure 10 (rho>0.5). The most notable was the correlation between pathways in *Megasphaera* sp. Since butyrate and acetate were the most abundant of the SCFA, we focused on the pathways associated with changes in levels of these. There was a strong positive correlation between increasing butyrate levels and certain pathways in butyrate-producing species, such as the chorismite biosynthesis I pathway by the species *Dorea longicatena* (R=0.42, q=0.0007), *Eubacterium rectale* (R=0.37, q=0.004), *Megasphaera elsdenii* (R=0.33, q=0.01) and *Roseburia inulinivorans* (R=0.31, q=0.02). Other pathways which were significantly positively correlated with increases in butyrate were the UMP biosynthesis pathway, coenzyme A biosynthesis II mammalian, superpathway of aromatic amino acid biosynthesis, peptidoglycan biosynthesis I (meso diaminopimelate containing), L lysine biosynthesis, CDP diacylglycerol biosynthesis, 5 aminoimidazole ribonucleotide biosynthesis I, urate biosynthesis inosine 5 phosphate degradation, S.adenosyl L methionine cycle I, starch degradation V, pyruvate fermentation to isobutanol, adenosine ribonucleotides de novo biosynthesis, purine ribonucleosides degradation, D galactose degradation V Leloir pathway.

There were also significant negative correlations between butyrate levels and methanogenesis from H_2 and CO_2, the urea cycle, NADH phosphorylation and dephosphorylation, tetrahydrofolate biosynthesis unclassified, thiazole biosynthesis I (*E. coli*) and guanosine deoxyribonucleotides *de novo* biosynthesis II.

There was a negative association between acetate levels and pathways including the superpathway of glycolysis pyruvate dehydrogenase, TCA and glyoxylate bypass, methylcitrate cycle, ubiquinol 7 biosynthesis prokaryotic, superpathway of

tetrahydrofolate biosynthesis, the glyoxylate cycle and the superpathway of menaquinol 11 biosynthesis. Other pathways specifically related to *Eubacterium limosum* which negatively correlated to SCFA levels were the peptidoglycan biosynthesis I (meso diaminopimelate containing) and 5 aminoimidazole ribonucleotide biosynthesis I (hexitol fermentation to lactate, formate ethanol and acetate) linked to *E. coli*.

Discussion

An important question in microbiome research relates to the extent to which the environment and animal production practices affect microbiota transmission, acquisition and function. Experimental manipulation of GIT systems is one approach which can be used to measure the impact of such systems such as the effect of diet or antibiotic use on the microbiome (Koskella, et al. 2017). In the current study, we employed 16S rRNA sequencing and shotgun metagenomics to explore the effect of nisin feeding on the dynamics of the faecal microbiota composition and function of post-weaning piglets over a 15-day trial period. Nisin is the most studied and most commercially produced bacteriocin.

Our results showed that treatment with nisin powder resulted in a depletion of many Gram-positive bacteria such as *Lactobacillus*. Also, many species from the Lachnospiraceae, Lactobacillaceae and Ruminococcacaeae families decreased in the nisin treated groups on the days of treatment. These species are known to hydrolyze sugars and starch to produce SCFAs such as butyrate (Biddle, et al. 2013; Devillard, et al. 2007; Wong, et al. 2014). There was a significant decrease in SCFA

production in the nisin treated groups; most notably the nisin powder treated group 72 h after initial treatment was fed. The levels of SCFA increased ten days after treatment had ceased in the nisin treated groups, albeit returning to levels lower than the group fed the encapsulant material or the no treatment control group. In the current study, we observed mostly negative correlations between SCFA levels and certain bacterial species.

We also observed an increase in the relative abundance of Gram-negative bacteria such as *Prevotella, Megasphaera, Mutsuokella, Succinivibrio* and *Esherichia/Shigella* among others in the nisin treated groups on the days that the treatments were fed. *Catenibacterium* are Gram-positive, obligatory anaerobes that utilize glucose to produce acetic, lactic, butyric and iso-butyric acids (Yan, et al. 2013). High levels of *Catenibacterium* were found in both the encapsulant material groups and encapsulated nisin group compared to the control group and nisin powder treated group. This suggests that the *Catenibacterium* are potentially degrading the cellulose encapsulant material and using it as an energy source.

Most interestingly, we observed that the gut microbiota diversity and composition was depleted in the nisin treated groups on the treatment days but returned to levels similar to the non-treatment groups as soon as three days after treatment ceased. Additionally, lactobacilli have been shown to metabolize carbohydrates including oligosaccharides and starch, which are fermented in the large intestine to SCFAs and can then be utilized by the pigs. *Bifidobacterium* and *Bacteroides* are two microbial genera reported to have a significant impact on substrate fermentation in the hind gut. In this study, we observed an upregulation in pathways related to methanogenesis from hydrogen and carbon dioxide and the urea cycle when the SCFA levels and the SCFA-producing bacteria decreased. This would suggest that

in the absence of SCFA production that bacteria present may revert to alternate pathways for energy production. It is reasonable then that fluctuations in the abundance of these bacteria influence the energy homeostasis in the host albeit for a short period until they returned to levels similar to pre nisin-treatment levels.

There is some evidence that butyrate-producing bacteria and butyrate itself are protective against Type 2 Diabetes (Gao, et al. 2009) (Vrieze, et al. 2012). Furthermore, by affecting luminal SCFAs, intestinal microbiota can modulate intestinal incretin excretion, and hence, apply another influence on glucose regulation (Wichmann, et al. 2013). From our results we can see that when acetate-producing bacteria are depleted, and the levels of acetate decrease there is a negative correlation with pathways such as the TCA cycle and other pathways that utilise acetate to produce energy. The TCA cycle connects carbohydrate, fat and protein metabolism. It is reasonable that this change in energy production is temporary and once the butyrate-producing bacteria return to pre-treatment levels, this is reversed.

Of particular interest was the observed decrease in gut pathogens such as *C. difficile* for example in the nisin treatment groups in the treatment period. These gut pathogens subsequently increased in relative abundance ten days after treatment ceased. The only family of note for which the relative abundance did not start to increase again was the *Streptococcaceae* in the nisin powder treated group. Certain species in this family are notable pathogens.

In summary, treatment with nisin showed that the decrease in SCFA production was transitory only occurring while treatment was being provided Due to nisin's proteinaceous nature it is most likely degraded by proteolytic enzymes more easily over time compared to other antibiotics that usually have a longer lasting impact

on the gut microbiota. This is potentially an advantage to using nisin as a dietary antimicrobial treatment.

Feeding nisin for 3 days did not affect weight gain, however, a study in which nisin is fed for a longer duration is warranted to determine the long term impact of feeding nisin on weight gain.

In summary, this study highlights the impact of diet and the cross talk that exists between the gut microbiota and the host. These findings suggest that the pig gut microbial community structure and functional capacities can deal with stresses caused by changes in diet which in this case was short-term exposure to nisin, at an early age. Additionally, this study highlights the potential of nisin as an alternative treatment against pathogens.

Chapter 5 Figures and Tables

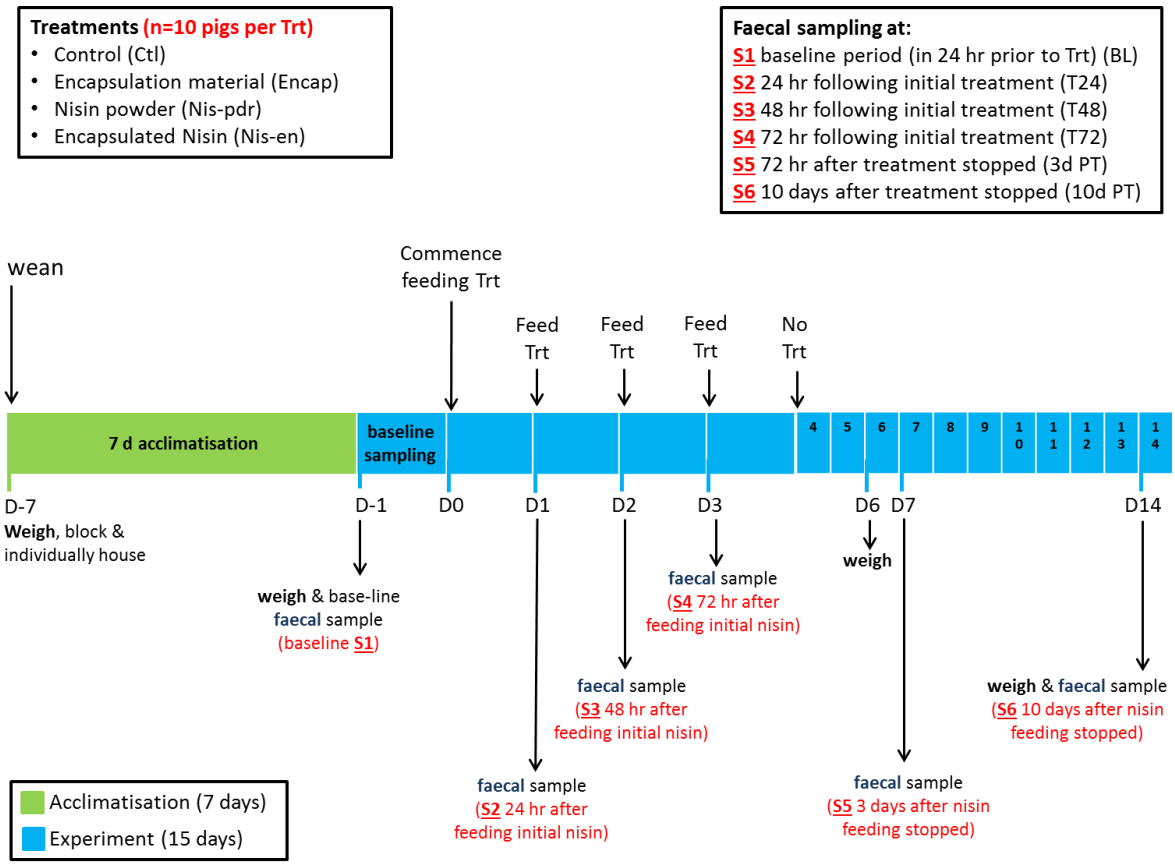
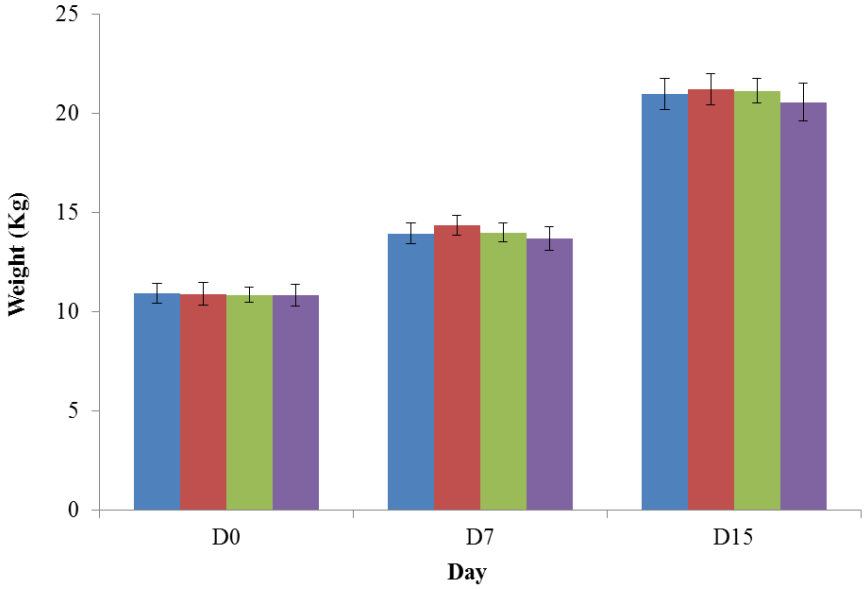


Figure 1: Overview of the treatment groups and the sampling time-line.

(a)



(b)

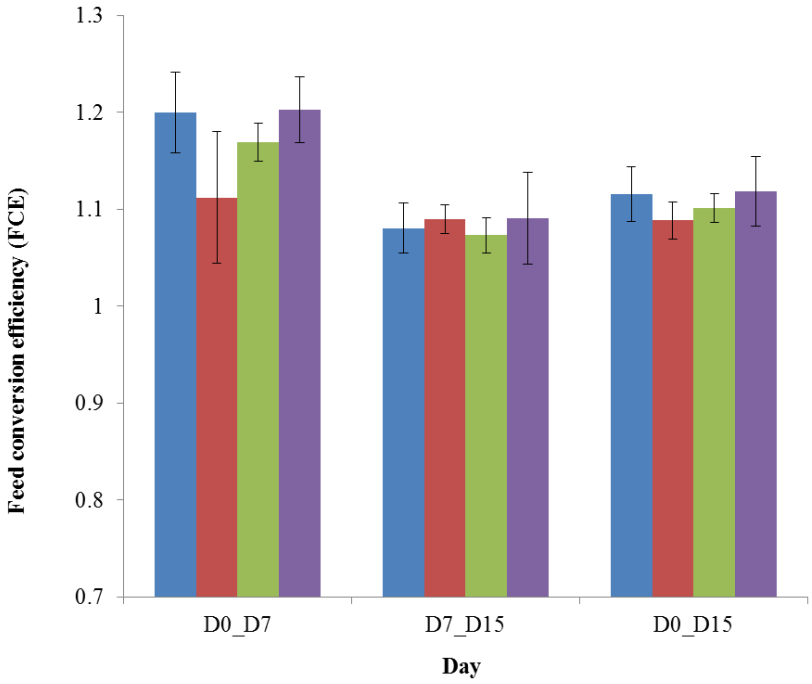


Figure 2. Mean live weight (a) and feed conversion efficiency (FCE) (b) of post-weaning piglets by across the days of weighing over the trial period. Treatments; ■ Ctl, ■ Encap, ■ Nis-pdr and ■ Nis-en.

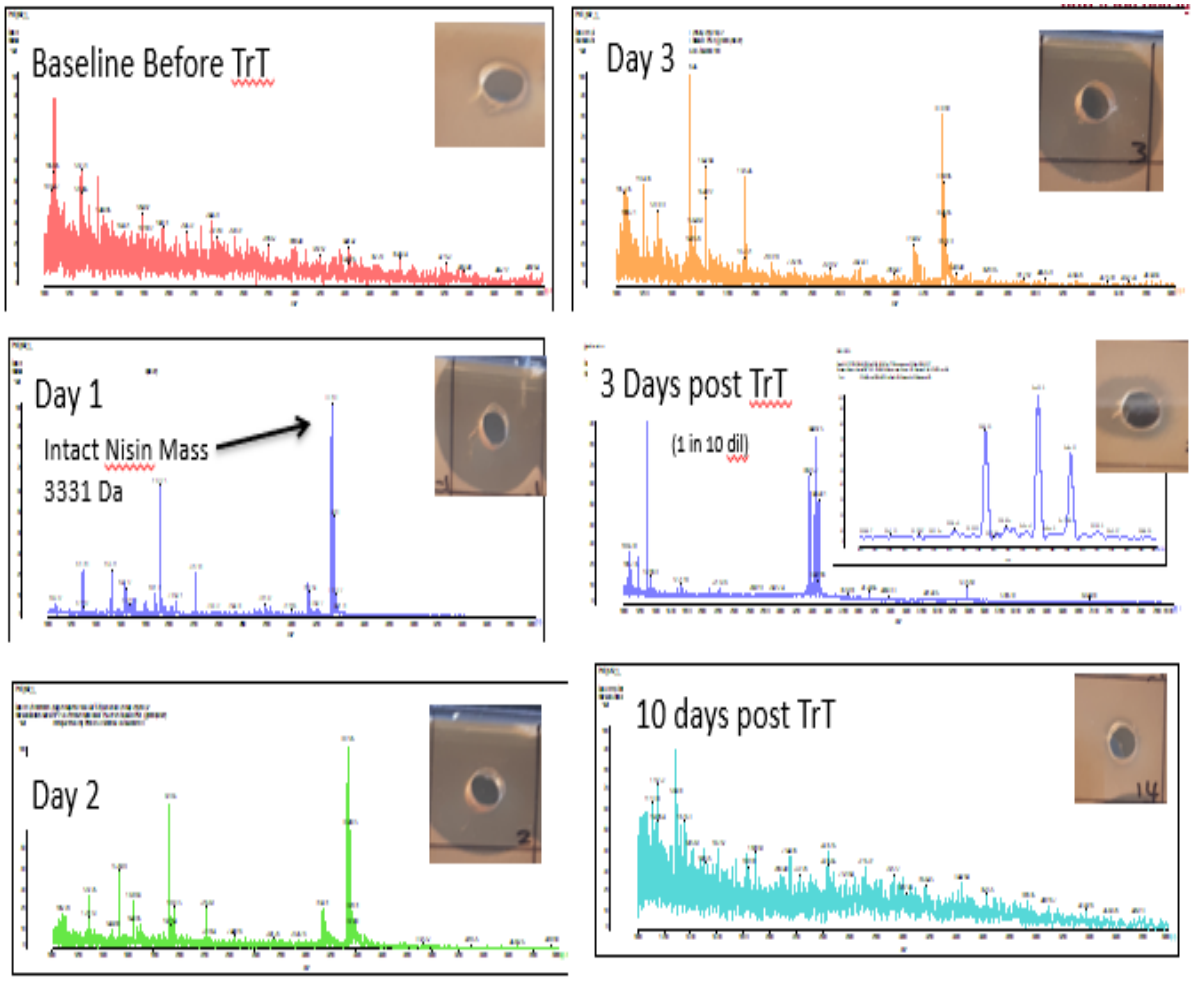


Figure 3. MALDI TOF Mass spectrophotometry analysis and activity assays to detect nisin. Detection of intact nisin in the faeces of pig in the nisin powder treated group. (A) Baseline (BL); (B) 24 hr following initial treatment (T24); (C) 48 hr following initial treatment (T48); (D) 72 hr following initial treatment (T72); (E) 72 hr after initial treatment stopped (3d PT) and (F) 10 days after initial treatment stopped (10d PT).

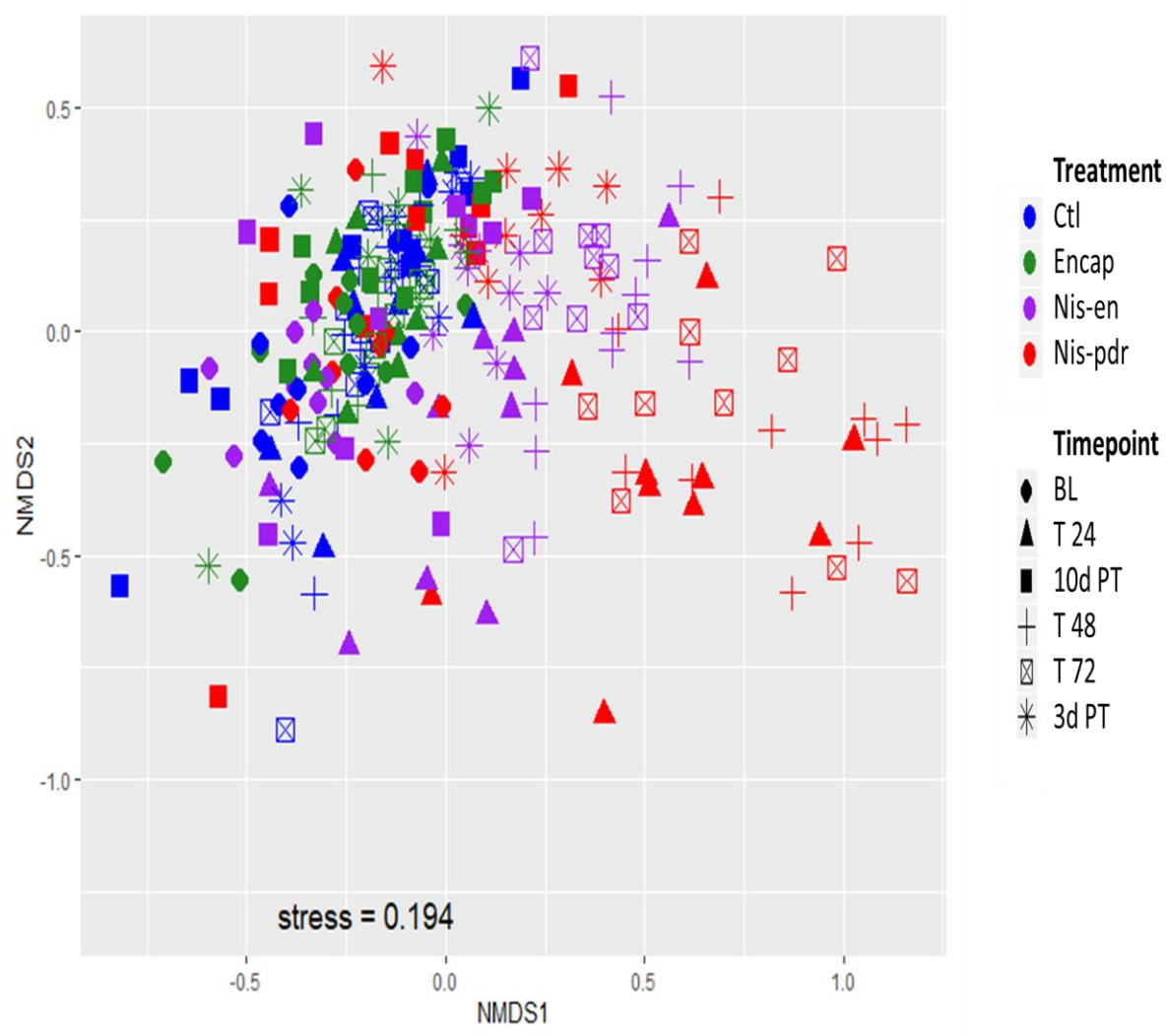


Figure 4. Beta diversity as revealed by 16S rRNA gene sequencing in the form of a non-metric multidimensional scaling (NMDS) plot. The four treatment diets are represented by different colours: no treatment control (Ctl) = blue; encapsulant material (Encap) = green; encapsulated Nisin (Nis-en) = purple and Nisin powder (Nis-pdr) = red. Time points: Baseline (BL), 24 h after initial treatment fed (T24), 48 h after initial treatment fed (T48), 72 h after initial treatment fed (T72), 3 days after feeding treatments ceased (3d PT) and 10 days after feeding treatments ceased (10d PT).

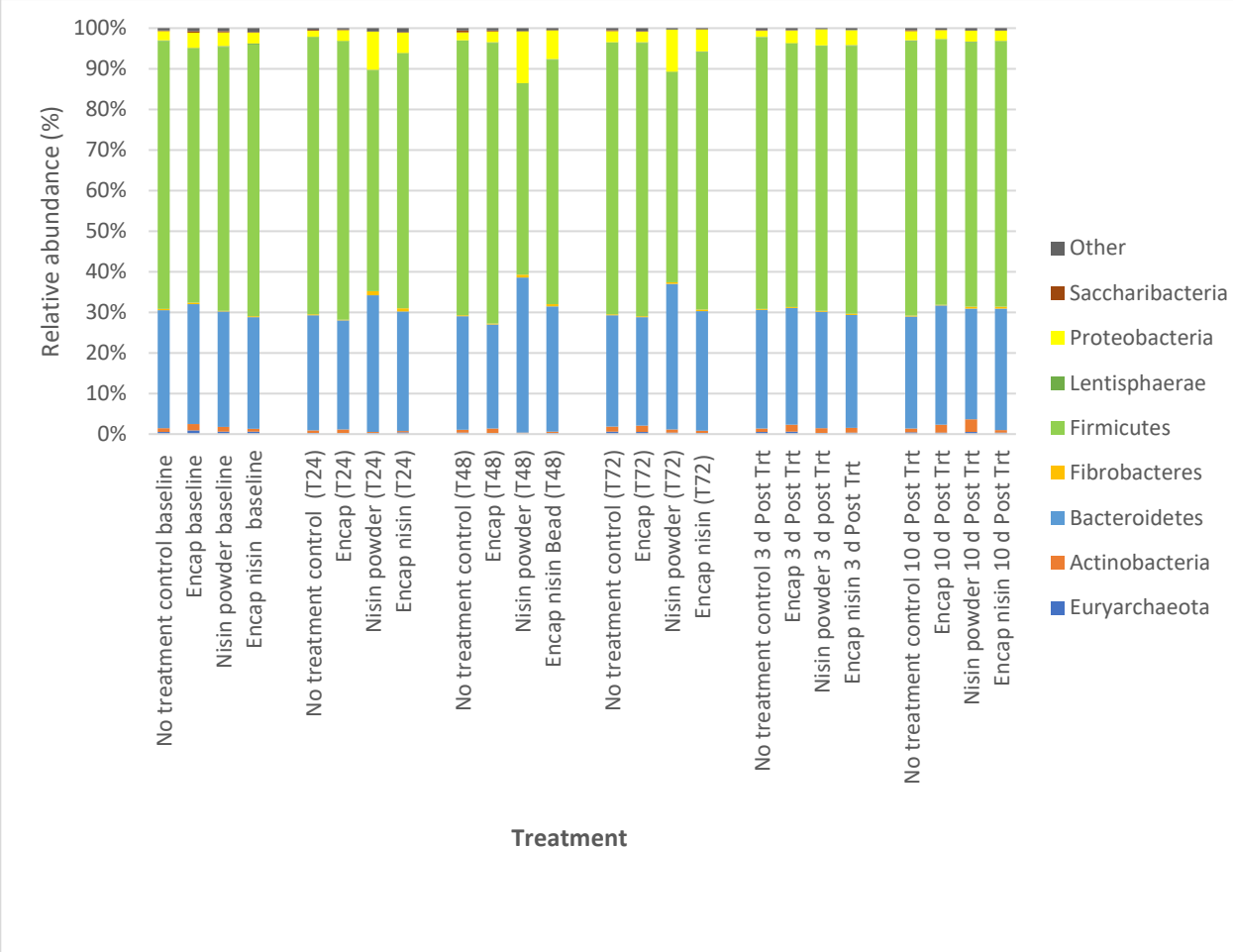


Figure 5. Relative abundance (%) at the phylum level across time points and treatment groups. The four treatment diets are represented: no treatment control (Ctl); encapsulant (Encap); encapsulated nisin (Nis-en) and nisin powder (Nis-pdr). Time points: Baseline (BL), 24 h after initial treatment fed (T24), 48 h after initial treatment fed (T48), 72 h after initial treatment fed (T72), 3 days after feeding treatments ceased (3d PT) and 10 days after feeding treatments ceased (10d PT).

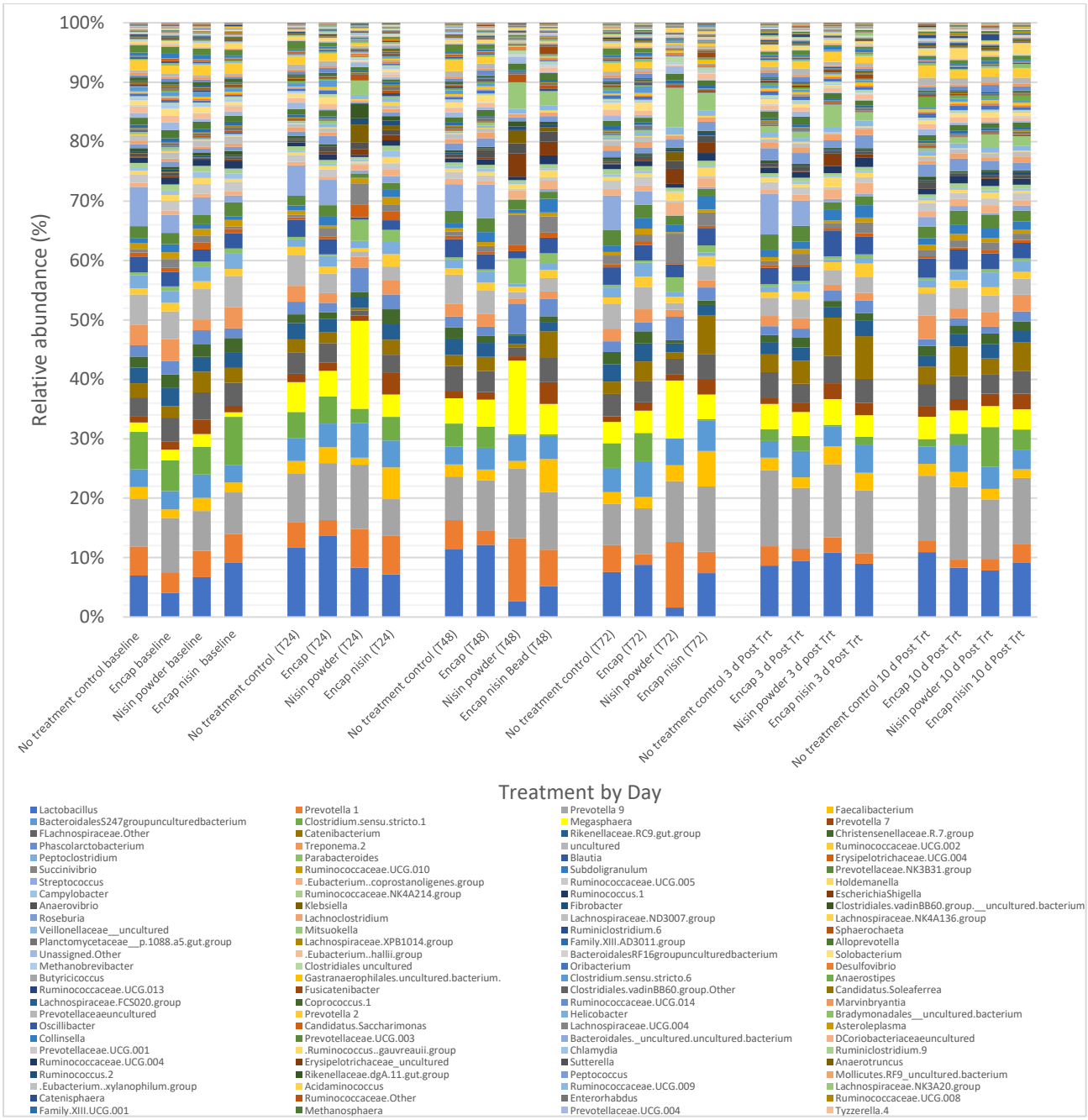


Figure 6. Relative abundance (%) at the genus level across time points and treatment groups. The four treatment diets are shown here: no treatment control (Ctl); encapsulant material (Encap); encapsulated nisin (Nis-en) and nisin powder (Nis-pdr). Time points: Baseline (BL), 24 h after initial treatment fed (T24), 48 h after initial treatment fed (T48), 72 h after initial treatment fed (T72), 3 days after feeding treatments ceased (3d PT) and 10 days after feeding treatments ceased (10d PT).

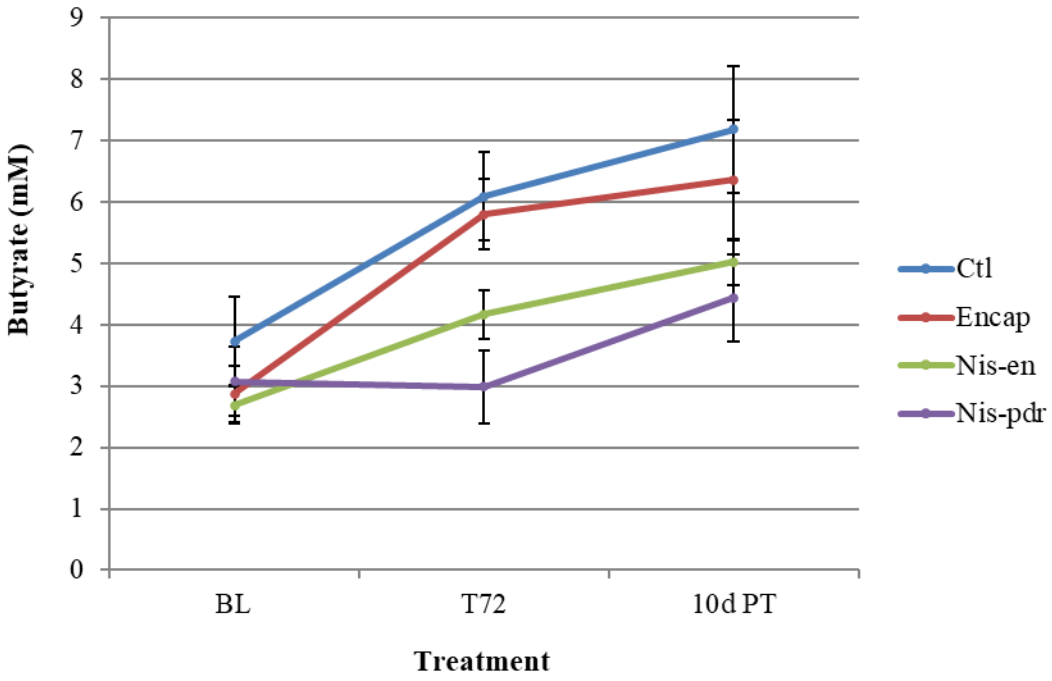


Figure 7. Butyrate concentration (mM) over time across treatment groups. Time points: Baseline (BL), 72 h after initial treatment fed (T72) and 10 days after treatment stopped (10d PT). Treatment groups: No treatment control group (Ctl); encapsulant material (Encap); encapsulated nisin (Nis-en) and nisin powder (Nis-pdr).

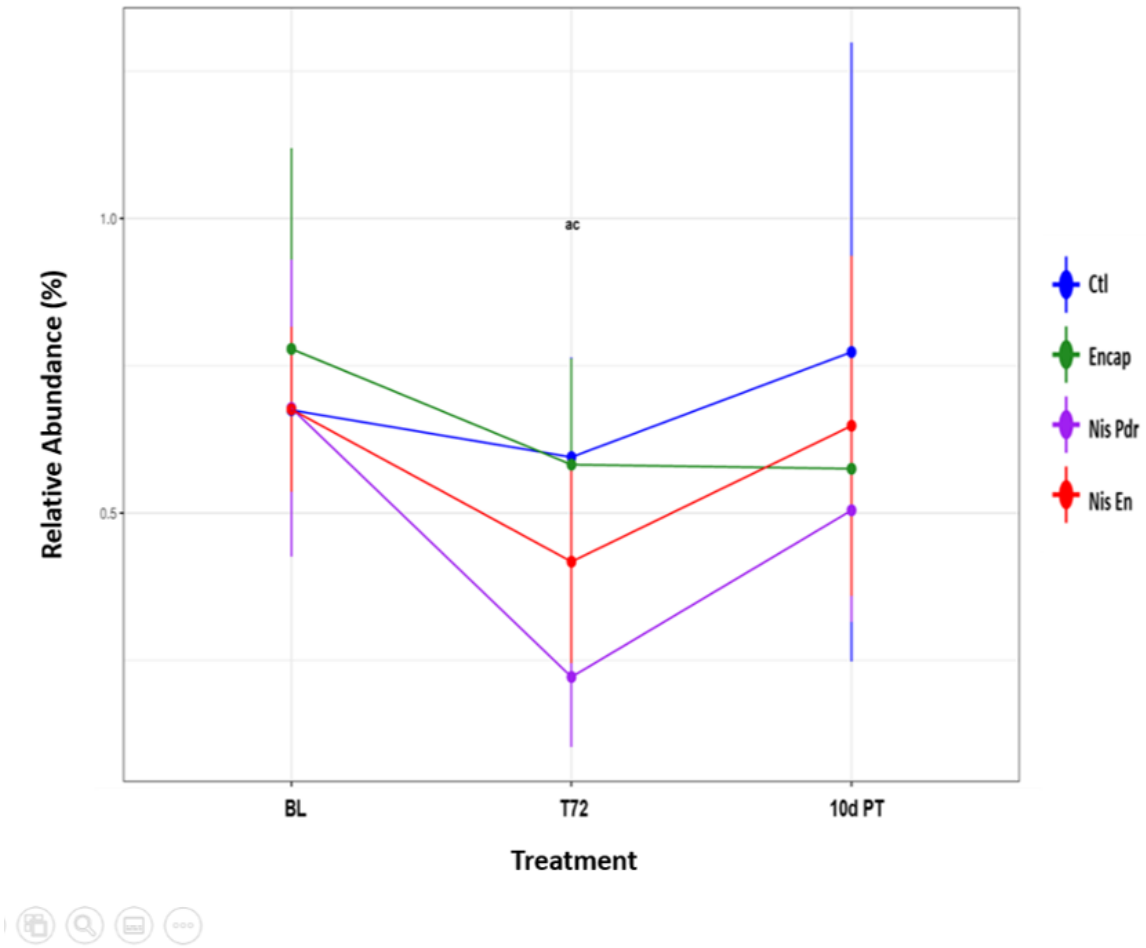


Figure 8. Relative abundance (%) of *Clostridioides difficile* in porcine faecal samples over time. Time points: Baseline (BL), 72 h after initial treatment fed (T72) and 10 days after treatment stopped (10d PT). Treatment groups: No treatment control group (Ctl), encapsulant material (Encap), encapsulated nisin (Nis-en) and nisin powder (Nis-pdr).

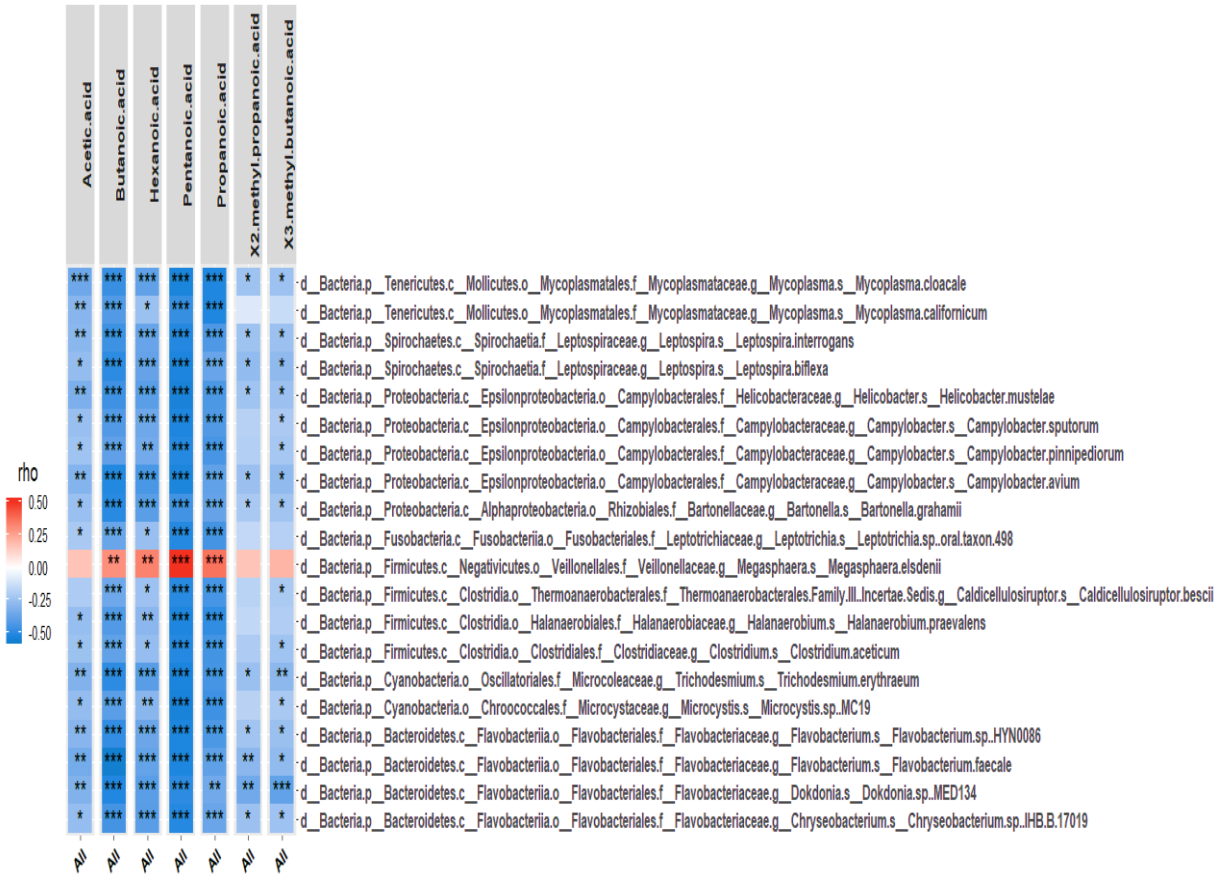


Figure 9. Correlation between SCFA levels and bacterial species across time points and treatments (*** p = 0.001, ** p = 0.01, * p = 0.05).

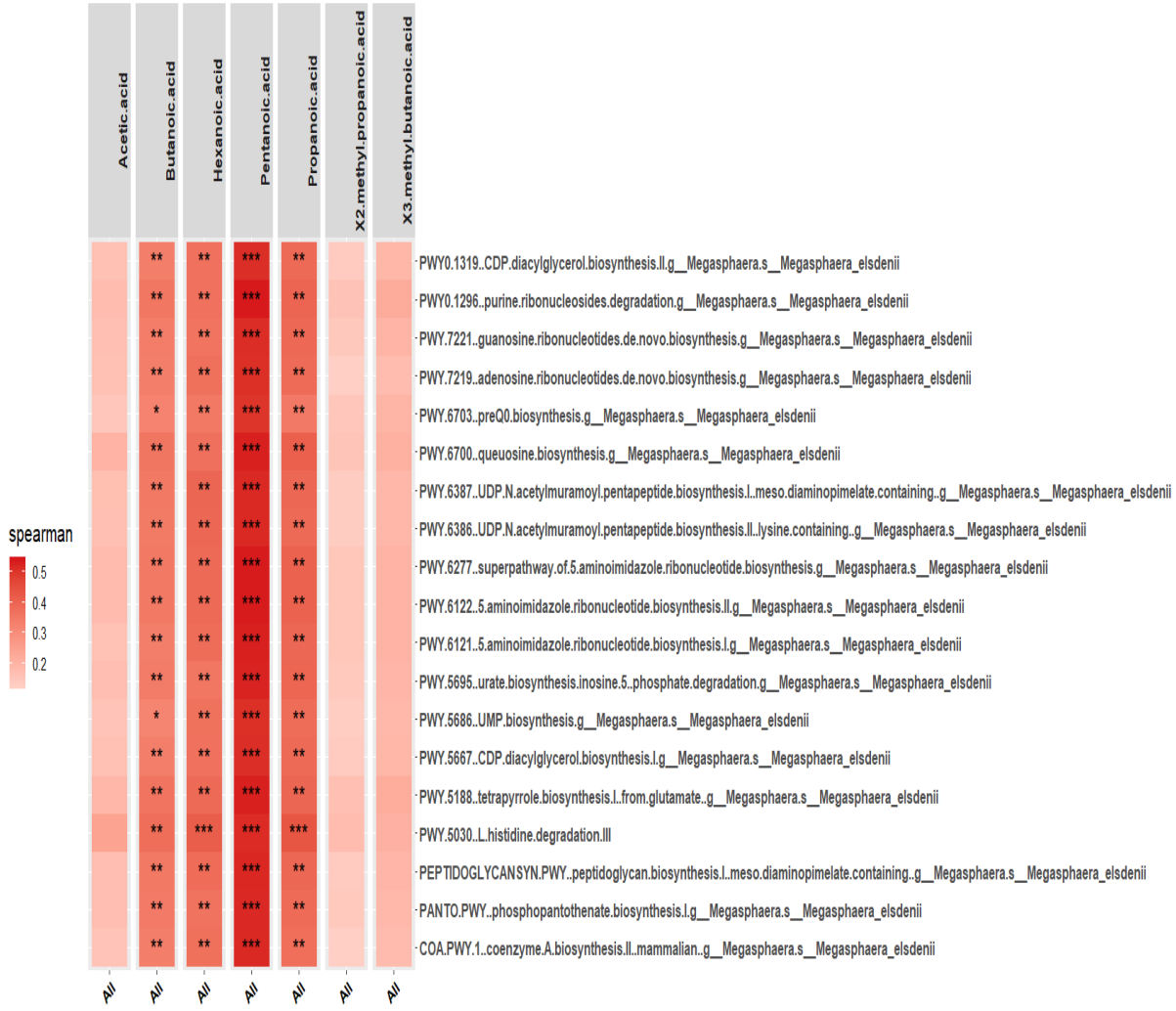


Figure 10. Correlations between metabolic pathways and SCFA levels, as revealed by shotgun metagenomic sequencing (*** p = 0.001, ** p = 0.01, * p = 0.05).

Table 1: Biological activity of nisin and nisin peak detection in porcine faecal samples, using Maldi TOF mass spectrophotometry.

Time point	BL	T24	T48	T72	3d PT	10d PT
No. of samples	18	20	20	20	20	20
Nisin peak detected with mass spectroscopy	no	yes	yes	yes	yes	no
Zone present in WDA	no	yes	yes	yes	yes	no
Average zone size (mm):						
Encapsulated nisin group	0	17.3	17.1	17.3	5.1	0
Nisin powder group	0	16.6	17.1	16.9	7.4	0

Time points: Baseline (BL), 24 h, 48 h and 72 h after initial treatment fed (T24, T48 and T72, respectively) and 3 days and 10 days after treatment stopped (3d PT and 10d PT, respectively). WDA; well diffusion assay.

Table 2: Phylum level differences between treatment groups across sampling time points (lowest 20 of 81 p values).

Treatment Groups	Phylum	p Value	q value
Ctl (T72) v Nis-pdr (T72)	Saccharibacteria	0.000054	0.005048
Encap (T72) v Nis-pdr (T72)	Saccharibacteria	0.000054	0.005048
Ctl (TCtl (T72) v Nis-En (T72)	Saccharibacteria	0.000130	0.005048
Encap (T72) v Nis-En (T72)	Saccharibacteria	0.000130	0.005048
Encap (T48) v Nis-pdr (T48)	Firmicutes	0.000157	0.005048
Ctl (3d PT) v Nis-En (3d PT)	Saccharibacteria	0.000196	0.005048
Ctl (3d PT) v Nis-pdr (3d PT)	Saccharibacteria	0.000196	0.005048
Encap (3d PT) v Nis-en(3d PT)	Saccharibacteria	0.000196	0.005048
Encap (3d PT) v Nis-pdr (3d PT)	Saccharibacteria	0.000196	0.005048
Encap (T72) v Nis-pdr (T72)	Proteobacteria	0.000212	0.005048
Encap (BL) v Nis-En (T24)	Actinobacteria	0.000285	0.005677
Encap (T48) v Nis-pdr (T48)	Bacteroidetes	0.000285	0.005677
Encap (T48) v Nis-pdr (T48)	Proteobacteria	0.000285	0.005677
Encap (T24) v Nis-pdr (T24)	Firmicutes	0.000507	0.007483
Ctl (T48) v Encap (T48)	Saccharibacteria	0.000680	0.007839
Ctl (T48) v Nis-pdr (T48)	Proteobacteria	0.001499	0.012095
Ctl (T48) v Nis-pdr (T48)	Firmicutes	0.004072	0.020604

Table 3: Levels of SCFA (mM) detected across treatment groups and time points (average of 10 samples).

Time point	Treatment	Acetate	Isobutyric acid	Isovaleric acid	Propionic acid	Butyrate	Valeric acid
		mM (average of 10 samples)					
Baseline	Control	19.280	0.939	0.605	7.644	3.731	1.018
	Encapsulant	13.756	0.685	0.486	5.841	2.871	0.658
	Encapsulated Nisin	14.137	0.721	0.473	5.272	2.685	0.624
	Nisin Powder	16.763	0.954	0.646	6.766	3.298	1.002
72 h after fed initial treatment	Control	23.257	1.260	0.903	10.130	6.089	1.637
	Encapsulant	18.708	1.054	0.748	7.924	5.798	1.467
	Encapsulated Nisin	16.134	0.852	0.588	8.836	4.166	1.373
	Nisin Powder	13.816	0.840	0.609	8.515	2.987	1.266
10 days after treatment stopped	Control	23.025	1.080	0.769	11.174	7.183	1.750
	Encapsulant	20.627	0.999	0.711	9.741	6.355	1.734
	Encapsulated Nisin	19.806	0.874	0.625	9.369	5.023	1.240
	Nisin Powder	16.383	0.893	0.659	7.140	4.493	1.176

Supplementary Information

Supplementary Table 1: Proximate analysis, chemical composition and amino acid composition of the basal diets used in this study (as-fed basis).

Analysis:	**Units[a]**	**Starter Feed**	**Link Feed**
Dry Matter	MJ/kg	18.4	17.7
Chemical Analysis:			
Neutral Detergent Fibre	%	4.8	5.5
Crude Protein (N x 6.25) Dumas	%	19.6	18.9
Crude Fibre	%	1.6	2.3
Dry Matter	%	91.6	90.3
Ash	%	5.8	5.3
Total Oil (Oil B)	%	11.8	8.0
Amino Acids Composition*:			
Alanine	%	0.72	0.71
Arginine	%	1.06	1.08
Aspartic	%	1.77	1.70
Cysteine	%	0.22	0.25
Glutamic	%	3.28	3.31
Glycine	%	0.63	0.66
Histidine	%	0.47	0.45
Iso-Leucine	%	0.81	0.75
Leucine	%	1.49	1.38
Lysine	%	1.56	1.43
Methionine	%	0.63	0.53
Phenylalanine	%	0.88	0.83
Proline	%	1.10	1.08
Serine	%	0.91	0.85
Threonine	%	1.01	0.96
Tyrosine	%	0.41	0.33
Valine	%	0.99	0.94
Tryptophan	%	0.32	0.29

[a]MJ per kilogram and percentage (%); *all amino acids are expressed as total e.g. alanine (total).

References

Aronesty, E. (2013). Comparison of Sequencing Utility Programs. *The Open Bioinformatics Journal, 7*, 1-8. doi:10.2174/1875036201307010001

Barton, W., Penney, N., Cronin, O., Garcia-Perez, I., Molloy, M., Holmes, E., . . . O'Sullivan, O. (2018, Apr). The microbiome of professional athletes differs from that of more sedentary subjects in composition and particularly at the functional metabolic level. *Gut, 67*(4), 625-633. doi:10.1136/gutjnl-2016-313627

Bernbom, N., Licht, T., Brogren, C., Jelle, B., Johansen, A., Badiola, I., . . . Nørrung, B. (2006). Effects of Lactococcus lactis on composition of intestinal microbiota: role of nisin. *Applied and Environmental Microbiology*, *72*(1), 239–244. doi:10.1128/AEM.72.1.239-244.2006

Biddle, A., Stewart, L., Blanchard, J., & Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity, 5*(3), 627–640. doi:10.3390/d5030627

Chan, W., Leyland, M., Clark, J., Dodd, H., Lian, L., Gasson, M.,. Roberts, G. (1996). Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin. *FEBS Lett, 390*(2), 129–132. doi:10.1016/0014-5793(96)00638-2

Chen H, H. D. (2003). Bacteriocins and their food applications. *Comprehensive Rev Food Sci Food Safety, 2*, 82–100.

Dabney, A., Storey, J., & Warnes, G. (2011). qvalue: Q-value estimation for false discovery rate control. R package version 1.26.0.

De Arauz, L., Jozala, A., Mazzola, P., & Penna, T. (2009). Nisin biotechnological production and application:a review. *Trends Food Sci Technol, 20*(3-4), 146–154. doi:10.1016/j.tifs.2009.01.056

De Vos, W., Mulders, J., Siezen, R., Hugenholtz, J., & Kuipers, O. (1993). Properties of nisin Z and distribution of its gene, nisZ, in Lactococcus lactis. *Appl Environ Microbiol, 59*(1), 213-218. doi:10.1128/AEM.59.1.213-218.1993.

Delves-Broughton, J. (1990). Nisin and its uses as a food preservative. *Food Technol, 44*, 100-117.

Devillard, E., McIntosh, F., Duncan, S., & Wallace, R. (2007). Metabolism of Linoleic Acid by Human Gut Bacteria: Different Routes for Biosynthesis of Conjugated Linoleic Acid. *Journal of Bacteriology, 189*(6), 2566-2570. doi:10.1128/JB.01359-06

Edgar RC. (2010). Bioinformatics. *Search and clustering orders of magnitude faster than BLAST, 26*(19), 2460-2461. doi:10.1093/bioinformatics/btq461

Field, D., Baghou, I., Rea, M., Gardiner, G., Ross, R., & Hill, C. (2017). Nisin in Combination with Cinnamaldehyde and EDTA to Control Growth of Escherichia coli Strains of Swine Origin. *Antibiotics, 6*(4), 35. doi:10.3390/antibiotics6040035

Field, D., Begley, M., O'Connor, P., Daly, K., Hugenholtz, F., Cotter, P., & al., e. (2012). Bioengineered nisin A derivatives with enhanced activity against both gram positive and gram negative pathogens. *PLOS ONE, 7*, e46884. doi:10.1371/journal.pon

Food and Drug Administration Veterinary feed directive. (2015). *Federal Register, 80*, 31708–31735. Retrieved from https://www.govinfo.gov/content/pkg/FR-2015-06-03/pdf/2015-13393.pdf.

Fouhy, F., Deane, J., Rea, M., O'Sullivan, Ó., Ross, R., & O'Callaghan G, e. a. (2015). The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations. *PLoS ONE, 10*(3), e0119355. doi:10.1371/journal.pone.0119355

Franzosa, E., McIver, L., Rahnavard, G., Thompson, L., Schirmer, M., Weingart, G., . . . Huttenhower, C. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nat Methods., 15*(11), 962-968. doi:10.1038/s41592-018-0176-y

Gao, Z., Yin, J., Zhang, J., Ward, R., Martin, R., Lefevre, M., . . . Ye, J. (2009). Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes, 58*(7), 1509–1517. doi:10.2337/db08-1637

Gardiner, G., Rea, M., O'Riordan, B., O'Connor, P., Morgan, S., Lawlor, P., . . . C, H. (2007). Fate of the two-component lantibiotic lacticin 3147 in the gastrointestinal tract. *Applied and environmental microbiology, 73*(21), 7103-9. doi:10.1128/AEM.01117-07

Gough, R., Cabrera Rubio, R., O'Connor, P., Crispie, F., Brodkorb, A., Miao, S., . . . MC, R. (2018). Oral Delivery of Nisin in Resistant Starch Based Matrices Alters the Gut Microbiota in Mice. *Front Microbiol, 9*(1), 1186. doi:10.3389/fmicb.2018.01186

Gough, R., Gómez-Sala, B., O'Connor, P., Rea, M., Miao, S., Hill, C., & Brodkorb, A. (2017). A simple method for the purification of nisin. *Probiotics and antimicrobial proteins., 9*(3), 363–369. doi:10.1007/s12602-017-9287-5

Gross, E., & Morell, J. (1971). Structure of nisin. *J Am Chem Soc, 93*(18), 4634–4635. doi:10.1021/ja00747a073

Guinane CM, C. P. (2005). Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. *J Appl Microbiol , 98*, 1316–1325.

Heinemann, B., & Williams, R. (1966). Inactivation of nisin by pancreatin. *J. Dairy Sci, 49*(3), 312-314. doi:10.3168/jds.S0022-0302(66)87854-2

Ivarsson E, R. S. (2014). Fermentable non-starch polysaccharides increases the abundance of Bacteroides-Prevotella-Porphyromonas in ileal microbial community of growing pigs. *Anim Int J Anim Biosci 8:1777–87*.

Jarvis, B., & Mahoney, R. (1969). Inactivation of nisin by alpha-chymotrypsin. *J Dairy Sci, 52*(9), 1448–1450. doi:10.3168/jds.S0022-0302(69)86771-8

Józefiak, D., Kieronczyk, B., Juśkiewicz, J., Zdunczyk, Z., Rawski, M., Długosz, J., . . . Højberg, O. a. (2013). Dietary nisin modulates the gastrointestinal microbial ecology and enhances growth performance of the broiler chickens. *PLoS ONE, 8*(12), e85347. doi:10.1371/journal.pone.0085347

Keohane, K., Rosa, M., Coulter, I., & Griffin, B. (2016). Enhanced colonic delivery of ciclosporin a self- emulsifying drug delivery system encapsulated in coated

minispheres. *Drug Dev Ind Pharm, 42*(2), 245-253. doi:10.3109/03639045.2015.1044905

Kieronczyk, B. P.-O., Długosz, J., Engberg, R., & Józefiak, D. (2016). The nisin improves broiler chicken growth performance and interacts with salinomycin in terms of gastrointestinal tract microbiota composition. *J. Anim. Feed Sci, 25*(4), 309–316. doi:10.22358/jafs/67802/2016

Koskella, B, Hall, L., & Metcalf, C. (2017, Nov). The microbiome beyond the horizon of ecological and evolutionary theory. *Nat Ecol Evol, 1*(11), 1606-1615. doi:0.1038/s41559-017-0340-2. Epub 2017

Lauková, A., Chrastinová, L., Plachá, I., Kandricáková, A., Szabóová, R., Strompfová, V., . . . Zitňan, R. (2014). Beneficial effect of lantibiotic nisin in rabbit husbandry. *Probiotics Antimicrob Proteins, 6*(1), 41-46. doi:10.1007/s12602-014-9156-4

Le Lay, C., Dridi, L., Bergeron, M., Ouellette, M., & Fliss, I. (2016). Nisin is an effective inhibitor of Clostridium difficile vegetative cells and spore germination. *J Med Microbiol, 65*(2), 169-175. doi:10.1099/jmm.0.000202

Lubelski, J., Rink, R., Khusainov, R., Moll, G., & Kuipers, O. (2008). Biosynthesis, immunity, regulation, mode ofaction and engineering of the model lantibiotic nisin. *Cell Mol Life Sci, 65*(3), 455–476. doi:10.1007/s00018-007-7171-2

Maki, J., Klima, C., Sylte, M., & Looft, T. (2019). The Microbial Pecking Order: Utilization of Intestinal Microbiota for Poultry Health. *Microorganisms, 7*(10), 376. doi:10.3390/microorganisms7100376

McIver, L., Abu-Ali, G., Franzosa, E., Schwager, R., Morgan, X., Waldron, L., . . . Huttenhower, C. (2018). bioBakery: a meta'omic analysis environment. *Bioinformatics, 34*(7), 1235–1237. doi:https://doi.org/10.1093/bioinformatics/btx754

Morrison, D., & Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes, 7*(3), 189-200. doi:10.1080/19490976.2015.1134082

Mulders, J., Boerrigter, I., Rollema, H., Siezen, R., & de Vos, W. (1991). Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *European Journal of Biochemistry, 201*(3), 581–584. doi:10.1111/j.1432-1033.1991.tb16317.x

Nicholson, J., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., & Jia, W. P. (2012). Host-gut microbiota metabolic interactions. *Science, 336*(6086), 1262–1267. doi:10.1126/science.1223813

Postler, T., & Ghosh, S. (2017). Understanding the Holobiont: how microbial metabolites affect human health and shape the immune system. *Cell Metabolism, 26*(1), 110-130. doi:10.1016/j.cmet.2017.05.008

Power, S., O'Toole, P., Stanton, C., Ross, R., & Fitzgerald, G. (2014). Intestinal microbiota, diet and health. *British Journal of Nutrition, 111*(3), 387-402. doi:10.1017/S0007114513002560

Rea, M., Alemayehu, D., Casey, P., O'Connor, P., Lawlor, P., Walsh, M., . . . Hill, C. (2014). Bioavailability of the anti-clostridial bacteriocin thuricin CD in gastrointestinal tract. *Microbiology, 160*(pt 2), 439-445. doi:10.1099/mic.0.068767-0

Ryan, M., Rea, M., Hill, C., & Ross, R. (1996). An application in cheddar cheese manufacture for a strain of Lactococcus lactis producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl. Environ. Microbiol, 62*(2), 612-619. doi:10.1128/aem.62.2.612-619.1996

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics, 27*(6), 863-864. doi:10.1093/bioinformatics/btr026

Tennyson CA, F. G. (2008). . *Microecology, obesity, and probiotics*.

Van Boeckel, T., Brower, C., Gilbert, M., Grenfell, B., Levin, S., Robinson, T., . . . Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences, 112*, 5649–5654. doi:10.1073/pnas.1503141112

Vrieze, A., Van Nood, E., Holleman, F., Salojärvi, J., Kootte, R., Bartelsman, J., . . . al, e. (2012). Transfer of intestinal microbiota from lean donors increases insulin

sensitivity in individuals with metabolic syndrome. *Gastroenterology, 143*(4), 913–916.e7. doi:10.1053/j.gastro.2012.06.031

Wichmann, A., Allahyar, A., Greiner, T., Plovier, H., Lundén, G., Larsson, T., . . . Bäckhed, F. (2013). Microbial modulation of energy availability in the colon regulates intestinal transit. *Cell Host Microbe, 14*(5), 582–590.

Wong, J., Piceno, Y., DeSantis, T., Pahl, M., Andersen, G., & Vaziri, N. (2014). Expansion of urease- and uricase-containing, indole- and p-cresol-forming and contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. *American journal of nephrology, 39*(3), 230–237. doi:10.1159/000360010

Yan, H., Potu, R., Lu, H., Vezzoni de Almeida, V., Stewart, T., Ragland, D., . . . Nakatsu, C. A. (2013). Dietary fat content and fiber type modulate hind gut microbial community and metabolic markers in the pig. *PLoS One, 8*(4), e59581. doi:10.1371/journal.pone.0059581

Younes, M., Aggett, P., Aguilar, F., Crebelli, R., Dusemund, B., & Filipič, M. e. (2017). Safety of nisin (E 234) as a food additive in the light of new toxicological data and the proposed extension of use. *EFSA J, 15*, 5063. doi:10.2903/j.efsa.2017.506

Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals. (2014). Retrieved from http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM338170.pdf

Chapter 6

General Discussion

Inflammatory Bowel Diseases (IBD) are chronic, inflammatory disorders of the gastrointestinal tract and include Crohn's disease (CD) and ulcerative colitis (UC). The gut microbiota plays a crucial role in human health, and is increasingly being associated with diseases including IBD, obesity and diabetes (Guinane and Cotter 2013). The intestinal microbiota of healthy individuals is known to confer many health benefits including protection against pathogens, host metabolism, nutrition, and modulation of the immune system (O'Hara and Shanahan 2006) (Sekirov, et al. 2010). Historically, the main treatment for UC has been aminosalicylates, short courses of steroids for severe flares, and immunomodulators. Additionally, anti-tumour necrosis factor alpha (anti-TNFα) inhibitors if remission is not maintained (Hazel and O'Connor 2020). Aminosalicylates are largely ineffective in CD. Remission may be induced using corticosteroids in some patients. If remission is not maintained anti-TNFα inhibitors and immunomodulators may be used either as a monotherapy or in combination (Hazel and O'Connor 2020). Cyclosporine A (CyA) is a potent immunosuppressant approved for use in solid organ transplants to prevent rejection. It has been used as a treatment for steroid refractory UC for the past twenty years (Sharkey, et al. 2011). Other uses for CyA include treatment of autoimmune disorders such as rheumatoid arthritis, psoriasis and CD. CyA is a poorly soluble cyclic peptide and is subject to metabolism by various cytochrome P450 enzymes that are expressed mainly in the small intestine and liver (Guada, et al. 2016).

High-dose CyA confers a significant advantage compared to other immunosuppressive drugs due to the fact that it is a very potent, relatively

selective inhibitor of T lymphocyte activation whilst having minimal effects on already activated cytotoxic/suppressor CD8 T cells, granulocytes and macrophages (Shevach 1985). Low-dose CyA can activate the cell-mediated immune response (Bretscher and Havele 1992). Adverse effects of CyA include nephrotoxicity, neurotoxicity, hepatotoxicity and cardiovascular toxicity amongst others. These serious side effects emerged simultaneously with CyA's immunosuppressive benefits. When used initially, CyA was used at high doses, up to 25 mg/kg per day following transplant (Kahan 1989), (Calne, et al. 1978). These unacceptable toxicities then led to a necessary dose reduction (Flores, et al. 2019). Due to its poor solubility and toxicity at high systemic doses there is an increasing demand to develop novel methods to withstand drug release in the upper gastrointestinal tract to reach the lower GIT where the intestine is the target site. This is particularly relevant for drugs like CyA targeting gastrointestinal illnesses such as IBD. Research in this thesis focused initially on assessing the effect of CyA encapsulated into a colon-targeted delivery system on the composition and functionality of the gut microbiota. While the exact etiology of IBD is unknown, a combination of genetic susceptibility, environmental factors, an altered immune response (which could be triggered by re-exposure to a pathogen), shifts in microbial composition or loss of tolerance to resident gut microbes are thought to be involved. Whether the microbial composition of a person affected by IBD is the cause or the result of the disease itself and how the treatments help to ameliorate shifts in microbial composition are yet to be fully understood. Additionally, studies have shown that the gut microbiota can alter the metabolism of certain orally administered drugs and in turn affect their efficacy and toxicity. This interaction between the gut microbiota and xenobiotics can

change the way drugs are metabolised by the host (Maurice, Haiser and Turnbaugh 2013). Treatment with antibiotics or disruption of the gut microbiota can change the composition of the bile acid pool and thereby alter drug absorption and bioavailability (Enright, et al. 2017). This is particularly relevant for poorly water soluble drugs like CyA. Whether the gut microbiota can influence the efficacy and release of CyA in these formulations has not been studied previously. In addition, it is not known if CyA in these formulations or CyA alone impacts the gut microbiota composition. In chapter 2 we assessed the effect of encapsulated CyA in SmPill® minispheres on the diversity and relative abundance of the gut microbiota in an *ex vivo* colon model and also in healthy subjects who participated in a seven-day pilot study. We also investigated the production of SCFAs butyrate, propionate and acetate by the gut microbiota in the *ex vivo* model system in the presence of coated minispheres (SmPill®) withand without CyA. We then assessed the effect of encapsulated CyA, using coated minispheres (SmPill®) + CyA on the levels of various metabolites and SCFAs in faecal samples from healthy human subjects, before and after participating in a seven-day pilot study. *Ex vivo* and *in vivo* results were compared to determine the efficacy of using an *ex vivo* colon model to study the effect of orallyingested drugs on the microbial communities of the GI tract. We observed that the concentration of encapsulated CyA in the SmPill® formulation did not impact the gut microbiota composition when tested in both an *ex vivo* colon model and an *in vivo* pilot study in healthy volunteers. The concentrations used are comparable to that encountered by the gut microbiota *in vivo*. CyA is an immunosuppressant and we could not rule out that its impact on the host immune response could indirectly influence the gut microbiota. However, our study

revealed that some component of the SmPill® formulation significantly increased the concentration of n-butyrate, a microbial metabolite with known positive attributes for host health both *in vivo* and *ex vivo*. The results from the compositional and SCFA analyses of the healthy human subjects supported the *ex vivo* faecal fermentation findings suggesting that the *ex vivo* model was an effective tool for studying the impact of orally ingested drugs on the gut microbiota.

Following on from this study we wanted to use a faecal fermentation model to study the effect of other bioactive compounds on the gut microbiota. Nisin is a bacteriocin with a broad spectrum of activity with potential for use as an alternative to traditional antibiotics. Although antibiotics have revolutionized modern medicine, their over-use has become a problem due to increased incidence of antibiotic resistance. Treatment with antibiotics is associated with collateral damage to the microbiota, including the depletion of beneficial gut commensals in a manner that gives *Clostridioides difficile* (*C. difficile*) an opportunity to flourish. *C. difficile* is a Gram-positive anaerobic spore-forming bacterium which can cause severe gastrointestinal illness in individuals undergoing antibiotic treatment (Ananthakrishnan, McGinley and Binion 2008; Wiström, et al. 2001; Normann, et al. 2013). Many commonly used antibiotics such as clindamycin, amoxicillin, ampicillin, cephalosporins and flouroquinolones have been associated with CDI (Aronsson, Möllby and Nord 1985; Bartlett 2006; Wiström, Norrby, et al.2001). Furthermore, antibiotic overuse has selected for multi-drug resistant bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococcus (VRE) and *C. difficile*.

In Chapter 3 we assessed the activity of nisin at a range of concentrations from 0-500 µM against *C. difficile* in a micro fermentation model colon system and determined the minimum inhibitory concentration at which *C. difficile* growth is suppressed (50 µM) while having minimal impact on the faecal microbiota. A previous *ex vivo* study by Rea et al used faecal fermentations to investigate the effect of a broad spectrum bacteriocin lacticin 3147 (McAuliffe, et al. 1998) and a narrow spectrum bacteriocin thuricin CD (Rea, et al. 2010) on the composition of the gut microbiota and *C. difficile* (Rea, et al. 2011). They compared the effects of the bacteriocins to metronidazole and vancomycin and reported a dramatic impact on the composition of the bacteria present upon exposure to metronidazole, vancomycin and lacticin 3147.

Here, we demonstrated the effectiveness of purified nisin at suspending *C. difficile* growth in a model colon system. Additionally, the optimum concentration at which there was minimum collateral damage using 16s rRNA gene sequencing was established in this system. There was a decrease in relative abundance and microbial diversity in a nisin dose dependent manner which is comparable to that seen with antibiotic treatment. We hypothesized that these findings would be useful for future *in vivo* work with nisin. The ability of nisin to kill *C. difficile* in a colon model would suggest that this bacteriocin could be a potential alternative to traditional antibiotics. This study opens up the potential to use nisin as a therapeutic for clostridial gut infections. Because nisin is a bacterial peptide and is subject to digestion however it may not reach the lower GI tract intact unless protected in some way.

In Chapter 4 we compared the effect of nisin, thuricin CD and fidaxomicin on the human gut microbiota composition in the same model colon system as in chapter

3. Fidaxomicin is a commercially-available antibiotic which has been reported as having a narrow spectrum of activity against *C. difficile* and is mainly used in patients that do not respond to traditional treatment. Thuricin CD has a narrow spectrum of activity whereas nisin exhibits a broad spectrum of activity against most Gram-positive organisms. The aim of this study was to investigate the impact of fidaxomicin on the human gut microbiota in a model colon system and to compare the effect with that of nisin, thuricin CD and a no treatment control at equimolar concentrations. In May 2011, the United States Food and Drug Administration (FDA) approved fidaxomicin for the treatment of CDI. It is the second drug, after vancomycin, to be FDA approved for CDI treatment. In December 2011, it was approved for CDI treatment in the EU by the European Medicines Agency (EMA) (Mullane 2014). Fidaxomicin has been shown to be as effective as vancomycin and is reported to have a narrow spectrum of activity (Zhanel, Walkty and Karlowsky 2015). This is the first time a mini-fermentation system has been used to compare the effect of nisin, thuricin CD and fidaxomicin in a complex community. In summary, thuricin CD does not cause major disruption of the gut microbial comunity and is largely selective against *C. difficile*. However, fidaxomicin has a broader spectrum of activity compared to thuricin CD and appears to be more similar to nisin, usually described as a broad spectrum antimicrobial against gram positive species. Fidaxomicin is excreted mainly in the faeces along with high concentrations of its major metabolite, OP-1118. OP-1118 has been shown to be highly active against *C. difficile* with a 32-fold higher MIC90 (Babakhani et al., 2007; O'Gorman, et al. 2018). Both fidaxomicin and OP-1118 exhibit a prolonged post-antibiotic effect (approximately 10 h) (Zhanel, Walkty and Karlowsky 2015). Antimicrobial

peptides, however, tend to be broken down by enzymes in the gut at a faster rate than other antibiotics. This could be viewed as favourable in that the gut microbes are not exposed to the peptides for a length of time, a factor that can select for resistance. In addition, the apparent selectivity of thuricin CD for *C. difficile* and inactivity against other gut bacteria could be advantageous in treating CDI in the future.

An important question in microbiome research relates to the extent to which the environment and animal production practices affect microbiota transmission, acquisition and function. Experimental manipulation of GIT systems is one approach which can be used to measure the impact of such systems such as the effect of diet or antibiotic use on the microbiome (Koskella, Hall and Metcalf 2017). In Chapter 5 we conducted a large animal trial with formulations containing encapsulated nisin and nisin in a purified powder form (unencapsulated). The aim of the study was to assess if nisin (unencapsulated) and also the encapsulated form could reach the lower porcine GIT in an intact form. We also assessed what impact these had on the gut microbiota following four consecutive days of feeding. The ability of the gut microbiota to recover after cessation of treatment was also determined. We found that there was a significant decrease in the SCFAs levels of acetic acid, hexanoic acid, butanoic acid, 3-methyl butanoic acid, heptanoic acid, and 2-methyl-propanoic acid in the nisin-treated groups nisin compared to the no treatment control group three days after initial treatments were fed. Shotgun metagenomic sequencing revealed significant correlations between SCFA concentrations and species level changes and between species and metabolic pathways. Our study confirms that orally ingested nisin reaches the lower GIT in an intact form and alters the gut microbiota of post-weaning piglets. This finding

was very surprising given the dogma that nisin is broken down in the intestine by proteases – this is clearly not the case. Nisin has a complicated structure which makes it may make it more resistant to protease than naked peptides. This may be an evolutionary mechanism. It is worth emphasizing that the quantities of nisin used in the animal feeding study were far greater than that which would result from ingestion in food.

Treatment with nisin showed that the decrease in SCFA production was transitory only occurring while treatment was being provided. This is potentially an advantage to using the potential use of nisin as a dietary antimicrobial treatment, compared to other antibiotics that usually have a longer lasting impact on the gut microbiota.

Taken together this thesis outlines the benefits of novel encapsulation technologies for the delivery of bioactive compounds to the GIT. We have also demonstrated that nisin is able to be delivered intact to the GIT and it can impact the gut microbiota. These findings pave the way for future studies into the use of bacteriocins as alternative treatments to currently available antibiotics.

References

Ananthakrishnan, A., McGinley, E., & Binion, D. (2008). Excess hospitalisation burden associated with Clostridium difficile in patients with inflammatory bowel disease. *Gut, 57*(2), 205-210.

Ardizzone, S., Cassinotti, A., Manes, G., & Bianchi Porro, G. (2010). Review: Immunomodulators for all patients with inflammatory bowel disease? *Therapeutic Advances in Gastroenterology, 3*(1), 31-42. doi:10.1177/1756283X09354136

Aronsson, B. M. (1985). Antimicrobial agents and Clostridium difficile in acute enteric disease: epidemiological data from Sweden, 1980-1982. . *The Journal of infectious diseases, , 151*(3), 476–481. Retrieved from https://doi.org/10.1093/infdis/151.3

Bartlett, J. G. (2006). Narrative review: the new epidemic of Clostridium difficile-associated enteric disease. *Annals of internal medicine, 145*(10), 758–764. Retrieved from https://doi.org/10.7326/0003-4819-145-10-200611210-00008

Bretscher, P., & Havele, C. (1992). Cyclosporin A can switch the immune response induced by antigen from a humoral to a cell-mediated mode. *European journal of immunology, 22*(2), 349-355.

Calne, R., Thiru, S., McMaster, P., Craddock, G., White, D., Evans, D., . . . Rolles, K. (1978). Cyclosporin A in patients receiving renal allografts from cadaver donors. *The Lancet, 312*(8104), 1323-7.

Carroll, I. R.-K. (2012). Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterology & Motility, 24*, 521-e248. doi:10.1111/j.1365-2982.2012.01891.x

Enright, E., Joyce, S., Gahan, C., & Griffin, B. (2017). Impact of gut microbiota-mediated bile acid metabolism on the solubilization capacity of bile salt micelles and drug solubility. *Molecular pharmaceutics, 14*(4), 1251-1263.

Flores, C., Fouquet, G., Moura, I., Maciel, T., & Hermine, O. (2019). Lessons to learn from low-dose Cyclosporin-a: a new approach for unexpected clinical applications. *Frontiers in immunology., 28*(10), 588.

Guada, M., Beloqui, A., Ravi Kumar, M., Préat, V., Dios-Viéitez, M., & Blanco-Prieto, M. (2016). Reformulating cyclosporine A (CsA): More than just a life cycle management. *Journal of Controlled Release, 225*, 269-282. doi:10.1016/j.jconrel.2016.01.056

Guinane, C., & Cotter, P. (2013). Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Therapeutic advances in gastroenterology. *6*(4), 295–308. Retrieved from https://doi.org/10.1177/1756283X13482996

Hazel, K., & O'Connor, A. (2020). Emerging treatments for inflammatory bowel disease. *Ther Adv Chronic Dis, 11*, 2040622319899297. doi:10.1177/2040622319899297

Kahan, B. (1989). Cyclosporine. *New England Journal of Medicine, 321*(25), 1725-38.

Kelly, C., & LaMont, J. (2008). Clostridium Difficile – more difficult than ever. *N Engl J Med, 359*, 1932 – 1940.

Koskella, B., Hall, L., & Metcalf, C. (2017). The microbiome beyond the horizon of ecological and evolutionary theory. *Nature ecology & evolution, 11*(1), 1606-15.

Kyne L, S. S. (2002). Underlying disease severity as a major risk factor for nosocomial Clostridium difficile diarrhea. *Infect Control Hosp Epidemiol, 23*(11), 653-659.

Lipinski CA, L. F. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev, 46*(1-3), 3-26. doi:10.1016/s0169-409x(00)00129-0

Lozupone C, S. J. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, 220–230. doi:10.1038/nature11550

Maurice, C., Haiser, H., & Turnbaugh, P. (2013). Xenobiotics shape the physiology and gene expression of the active human gut microbiome. *Cell, 152*, 39-50.

McAuliffe, O., Ryan, M., Ross, R., Hill, C., Breeuwer, P., & Abee, T. (1998). Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane

potential. *Appl Environ Microbiol, 64*(2), 439-45. doi:10.1128/AEM.64.2.439-445.1998

Mullane, K. (2014). Fidaxomicin in Clostridium difficile infection: latest evidence and clinical guidance. *Ther Adv Chronic Dis, 5*(2), 69-84. doi:10.1177/2040622313511285.

Normann, E., Fahlén, A., Engstrand, L., & Lilja, H. (2013). Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. *Acta Paediatr, 102*, 129-136. doi:10.1111/apa.12059

O'Gorman, M., Michaels, M., Kaplan, S., Otley, A., Kociolek, L., Hoffenberg, E., . . . Sears, P. (2018). Safety and Pharmacokinetic Study of Fidaxomicin in Children With Clostridium difficile-Associated Diarrhea: A Phase 2a Multicenter Clinical Trial. *Journal of the Pediatric Infectious Diseases Society, 7*(3), 210–218. Retrieved from https://doi.org/10.1093/jpids/pix037

O'Hara, A., & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO reports, 7*(7), 688-93.

Rea, M., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P., Ross, R. (2011). Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proc Natl Acad Sci U S A, 109*(Suppl 1(Suppl 1)), 4639–4644. doi:10.1073/pnas.1001224107

Rea, M., Sit, C., Clayton, E., O'Connor, P., Whittal, R., Zheng, J., . . . Hill, C. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proceedings of the National Academy of Sciences, 107*(20), 9352-7.

Rupnik, M., Wilcox, M., & Gerding, D. (2009). Clostridium Difficile infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol, 7*, 526-536.

Sekirov, I., Russell, S., Antunes, L., & Finlay, B. (2010). Gut microbiota in health and disease. *Physiological reviews*.

Sharkey, L., Bredin, F., Nightingale, A., & Parkes, M. (2011). The use of Cyclosporine A in acute steroid-refractory ulcerative colitis: long term outcomes. *J Crohn's Colitis., 5*, 91-94.

Shevach, E. (1985). The effects of cyclosporin A on the immune system. *Annual review of immunology, 3*(1), 397-423.

Stanley, J. D. (2013). Clostridium difficile infection. *Curr Probl Surg, 50*, 302 – 337.

Stanley, J., Bartlett, J., Dart, B., & Ashcraft, J. (2013). Clostridium difficile infection. *Curr Probl Surg, 50*, 302 – 337.

Wiström, J. N. (2001). Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. *The Journal of antimicrobial chemotherapy. , 47*(1), 43-50. Retrieved from

Wiström, J., Norrby, S., Myhre, E., Eriksson, S., Granström, G., Lagergren, L., .Svenungsson, B. (2001). Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. *Journal of Antimicrobial Chemotherapy, 47*(1), 43-50. doi:10.1093/jac/47.1.43

Zhanel, G., Walkty, A., & Karlowsky, J. (2015). Fidaxomicin: A novel agent for the treatment of Clostridium difficile infection. *Can J Infect Dis Med Microbiol, 26*(6), 305-312. doi:10.1155/2015/934594

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