UNIVERSITY OF CALGARY

Biofilms of Gastrointestinal Microflora

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JANUARY, 2010

UNIVERSITY OF
CALGARY

Abstract

The human gastrointestinal tract hosts a complex community of microorganisms that grow as biofilms on the intestinal mucosa. These bacterial communities are not well characterized, although they are known to play an important role in human health. This project aimed to develop an in vitro model to study these adherent communities. Initially, simple communities were used to develop a biofilm model of bacterial interactions. Bacterial communities were then obtained from human colon biopsies and grown as anaerobic biofilms. These biofilms were similar in community structure to the biopsy communities from which they were formed, including all the major phyla present in the human intestine. These biofilm communities are proposed as a "representative microflora", an in vitro model of bacteria in the colon. This model can be used in future studies of commensal-pathogen interactions, and can be expanded to examine interactions between commensal bacteria and host cells.

Acknowledgements

First and foremost, thanks to Dr. Howard Ceri for the inspiration and guidance he has offered me throughout my undergraduate and graduate education. The time I have spent in his lab has challenged me and helped me grow into the scientist I am today. Thank-you Dr. Ceri! I am very fortunate to have Dr. Buret and Dr. MacNaughton as committee members, thanks to both of them for their guidance and support. Special thanks to Dr. Armstrong for creative advice on method development and for acting as my external examiner.

Thanks to all the members of the Ceri lab, past and present, for their friendship, support and advice. I would especially like to thank Carol Stremick for all her help and encouragement over the years. Thanks to Mark Stanton for his excellent technical assistance, and to Dr. Joe Harrison and Aaron Hirschfeld for teaching me so much during my summers in the lab. To Lisa Nelson and Michelle Stan, thanks for the laughter and coffee breaks.

Thanks to Dr. Kevin Rioux, Ida Rabbani and the Intestinal Inflammation Tissue Bank for providing the biopsy samples for my study. I am indebted to Dr. Jaime Kauffman and Kris Cannon for their help with methods and data analysis. Thanks to Dr. Morck and the members of his lab for allowing me to use their anaerobic chamber. Also, thanks to Dr. Morck for advice on statistical analysis. A huge thank-you to Dr. Erika Lutter for great ideas and for proof-reading this thesis.

Finally, a big thanks to all of my family, the Sproules and Willoughbys, for their love and support. I am especially grateful to my husband, Scott, for his patience and for being my personal chef while I was writing.

Dedication

This thesis is dedicated to my parents, Rod and Karen Sproule, and my grandparents, Russ and Jean Sproule, for their continual support and encouragement, and for inspiring me to pursue higher education.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AHL	acyl-homoserine-lactone
AI	autoinducer
BBE	*Bacteroides* bile esculin agar
BHI	brain heart infusion broth
BHIA	brain heart infusion agar
bio	biofilm
bp	base pair(s)
bx	biopsy
CBA	Columbia blood agar
CBD	Calgary Biofilm Device
cfu	colony forming units
Ct	threshold cycle
dATP	deoxyadenosine triphosphate
dCTP	deoxycytodine triphosphate
ddH_2O	double-distilled water
DGGE	denaturing gradient gel electrophoresis
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
Dunn's	Dunn's multiple comparison test
EDTA	(ethylenedinitrilo)-tetraacetic acid
EHEC	enterohemorrhagic *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
EPS	exopolysaccharide
FAA	Fastidious Anaerobe Agar
FACS	Fluorescence activated cell sorting
FCS	fetal calf serum
FISH	fluoresent in situ hybridization
g	gram(s)
GALT	gut-associated lymphoid tissue
h	hour(s)
IBD	inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KW	Kruskal-Wallis test

L	litre(s)
LB	Luria-Bertani broth
LBA	Luria-Bertani agar
Lp	*Lactobacillus plantarum* 299v
LPS	lipopolysaccharide
MBC_{100}	minimal bactericidal concentration
MBEC	minimal biofilm eradication concentration
mEA	mEnterococcus agar
MiCA	Microbial Community Analysis program
mL	millilitre(s)
mM	milimolar
mm	millimeter(s)
mMRS	modified deMan, Rogosa and Sharpe
mol	mole(s)
mRNA	messenger RNA
MRS	de Man, Rogosa and Sharpe
mTSB	modified tryptic soy broth
ng	nanogram(s)
nm	nanometer(s)
OD	optical density
p	probability value
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEA	phenylethanol agar
pmol	picomole(s)
qPCR	quantitative polymerase chain reaction
rcf	relative centrifugal force
rfu	relative fluorescence units
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse-transcriptase PCR
SD	standard deviation
SEM	scanning electron microscopy
SIgA	secretory immunoglobulin A
sTSY	supplemented tryptic soy with yeast broth
TBE	Tris-borate-EDTA
TGGE	temperature gradient gel electrophoresis
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism

Tris	tris(hydroxymethyl)aminomethane
TSB	tryptic soy broth
UPEC	uropathogenic *Escherichia coli*
UV	ultraviolet
X	fold concentration
x	repetitions (cycles)
α	alpha
β	beta
%	percent
°C	degrees Celsius
µg	microgram
µl	microlitre(s)
µm	micrometer(s)
µM	micromolar

Epigraph

"We and our bacteria have evolved together. Humans are the ones messing with the system - in the brief time since the Stone Age, we humans have profoundly changed our diet, exercise habits and hygiene, leaving the poor microbes to cope with all the new things we're throwing at them. Yet, we continue to point fingers at bacteria when something goes amiss. It's an outdated human-centric mindset that [studies of human microflora] with help change."

-Dr. William Parker, Duke University Medical Center, on the significance of the Human Microbiome Project.

Chapter One: Overview

1.1 Problems and Importance

The gastrointestinal tract harbours a symbiotic population of microbes which outnumbers the eukaryotic cells in the human body by tenfold (Tancrède, 1992). These organisms play an important role in maintaining our health; they help us digest our food, develop our immune systems, and protect us from pathogens (Macfarlane & Macfarlane, 2006; Moreau & Corthier, 1988; Rolfe, 2000). Despite the importance of these organisms, relatively little is known about them. A major challenge in studying these communities is that approximately half of the species have never been cultured in vitro (Flint, 2006). Therefore, most studies cataloguing the organisms that inhabit our gastrointestinal tract are limited to deoxyribonucleic acid (DNA)-based techniques, which do not give information about viability, metabolism, or other behaviours (such as interactions with other bacteria or host cells).

In vitro models of intestinal bacterial populations are limited to those organisms that can be cultured using current techniques. Fermentation systems are able to model intestinal bacteria to an extent, but the seed communities for these experiments are generally fecal samples (Mäkivuokko & Nurminen, 2006). This is problematic because the bacterial populations that interact with human cells most closely are those communities that colonize the gastrointestinal mucosa (Probert & Gibson, 2002). These populations have a unique structure that is not well represented by fecal samples (Eckburg *et al.*, 2005). Another unique feature of these mucosal communities is that they exist in a sessile, biofilm mode of growth, rather than a non-adherent planktonic state. Biofilms are known to behave differently than planktonic cells, and biofilms in the

gastrointestinal tract have different metabolic activities than non-adherent populations (Macfarlane & Macfarlane, 2006).

In vitro models of interactions between commensal flora and host cells generally use a single lab strain of *Escherichia coli* to represent the intestinal microflora (Lewis *et al.*, 2008; Nazli *et al.*, 2004; Nazli *et al.*, 2006). Not only is a single strain a poor representation of a complex community, but *E. coli*, a facultative anaerobe, is very different from the obligate anaerobic species that make up the majority of intestinal bacteria.

In vivo models of intestinal bacteria are also lacking. Animal model systems do not closely represent humans for a few reasons. Firstly, intestinal bacterial populations are relatively species specific (Henriksson, 2006). Some studies have tried to get around this problem by colonizing mice with a "human microflora", however structural and physiological differences between the mouse and human gastrointestinal tract limit the usefulness of these studies (Henriksson, 2006). Secondly, it is normal for rodents to have commensal bacteria directly adherent to the intestinal epithelium, whereas in humans this is generally only the case in diseased individuals (Tannock, 2005). The best representations of human mucosal bacteria come from mucosal biopsy specimens obtained from colonoscopy. However, due to its invasive nature, it is unethical to perform the colonoscopy procedure on healthy volunteers for the sole purpose of biopsy collection. Fortunately, it is acceptable to study biopsies donated for research by patients undergoing colonoscopy for other reasons, such as colon cancer screening. These biopsies contain the best available representation of human mucosal bacterial communities.

In order to gain a greater understanding of the complex ecosystem of our intestines, new methods for studying these bacterial communities are necessary. Descriptive studies using DNA have given us the majority of the knowledge we have to date, but in order to truly understand these communities we must be able to grow the organisms in vitro. An in vitro model is advantageous because it can be easily manipulated and sampled, and the behaviours of the organisms within the communities can be evaluated. In addition, in vitro models allow for the study of interactions between normal intestinal bacteria and pathogens. An ideal in vitro model would also allow for studies of bacterial interactions in the presence of host cells, for this would give the best representation of the gastrointestinal tract without moving into an in vivo study, which is currently not a viable option due to the lack of a suitable animal model.

1.2 Purpose

As demonstrated above, there is a lack of an appropriate model for the study of mucosal bacteria and their interactions with other bacteria and with host cells. The purpose of this study was to create such a model, which would have the following characteristics:

- Be composed of a mucosal, multispecies population from a human subject
- Represent the biofilm mode of growth
- Allow for the study of interactions between the mucosal bacteria and other bacteria (commensal or pathogenic)

We used an adaption of the Calgary Biofilm Device (CBD) (Ceri *et al.*, 1999) to develop a model that would fulfill these requirements. The first step was to design a means of studying interactions between two biofilm populations (for example, one commensal strain and one pathogenic strain). Once the system was established, we sought to create a more representative commensal community by growing multispecies, biofilm communities of bacteria derived from human mucosal colon biopsies. These communities were studied by culture- and non-culture-based techniques and the model was assessed for feasibility of use for additional studies (including commensal-pathogen and commensal-host interaction studies).

1.3 Hypotheses

Multispecies biofilms can be formed in vitro that can serve as a "representative microflora" of the indigenous commensal biofilm communities of the human colon.

The community structure of this "representative microflora" can be quantitatively defined, and therefore can be used as part of a standardized assay system, which will be useful in further characterizing the role of bacteria in health and disease.

1.4 Objectives

- **Develop an in vitro model for the study of interactions between human commensal flora and pathogen biofilms (Chapter 4).**
- **Isolate a diverse bacterial population from the human colon that can serve as a representative community of mucosal bacteria (Chapter 5).**
- **Establish and characterize biofilms from this "representative microflora" (Chapter 5).**
- **Determine the reproducibility of biofilm community structure based on using fresh and frozen samples of the "representative microflora" (Chapter 5).**

Chapter Two: Introduction

2.1 Intestinal Microflora

The majority of the mucosal surfaces of the body are inhabited by bacteria. These non-pathogenic bacteria are referred to as normal flora, commensal flora, microflora, microbiota or autochthonous flora (Tannock, 2005). These bacterial communities participate in a symbiotic relationship with their host, from which both parties benefit (Bengmark, 1998). The gastrointestinal tract, particularly the large intestine, houses the most diverse and dense microbial populations in the human body.

The stomach and small intestine have a relatively small number of bacteria, likely due to the acid, bile and pancreatic secretions, which make conditions inhospitable for most microorganisms (Guarner & Malagelada, 2003). These secretions also act as defences against pathogenic organisms which may have been ingested. The majority of the bacterial diversity in the gastrointestinal tract is found in the large intestine.

The large intestine contains approximately 10^{11}-10^{12} bacterial cells per gram of luminal contents, which accounts for approximately 60% of the mass of fecal solids (Simon & Gorbach, 1984; Stephen & Cummings, 1980). The total population of the gastrointestinal microbiota is estimated at 10^{14} organisms (Young & Schmidt, 2008). In fact, the bacterial community in the large intestine is comprised of approximately 500 species of Eubacteria, Archaea and Eukarya (yeasts) (Eckburg *et al.*, 2005; Moore & Holdeman, 1974). Once the intestinal microflora is established, it is usually stable throughout life. Minor fluctuations can occur due to dietary changes, infection and antibiotic use, but an individual's microflora generally reverts back to the community acquired during his or her youth, even if that individual moves across the world and

drastically changes his or her lifestyle (Bornside, 1978; Dethlefsen *et al.*, 2008; Simon & Gorbach, 1984). This is thought to be partially due to the ability of "pioneering" bacteria to create an ideal habitat for themselves by modulating host cells, while simultaneously excluding colonization by other organisms (Ducluzeau, 1993; Hooper *et al.*, 2001). The appendix has been suggested as a reservoir for microflora, which would allow for replenishment of the native flora after disruptions such as infection (Bollinger *et al.*, 2007b).

Despite the diversity of the intestinal microflora, not all organisms detected in the intestine are part of the microflora. Bacteria that are ingested and appear transiently in the gut (for example, lactobacilli from dairy products), are considered "allochthonous", as opposed to the "autochtonous" nature of the microbiota (Tannock, 2005).

2.1.1 Establishment of the Intestinal Microflora

During the birthing process an infant acquires microorganisms from the vaginal and perianal flora of his or her mother (Mackie *et al.*, 1999). After birth, the intestine continues to be colonized by environmental organisms, as well as organisms passed on to the infant through breast-feeding (Mackie *et al.*, 1999; Martin *et al.*, 2004b). Although an exact mechanism has not been determined, it appears that some bacteria have the ability to cross the placental barrier and colonize the fetus before birth (Martin *et al.*, 2004b). By ten days after birth, lactobacilli, enterococci and *E. coli* begin to replace environmental microbes. Infant microflora have a high proportion of facultative anaerobes and bifidobacteria compared to adult microflora. *Bifidobacteria* species are particularly dominant, making up 60-91% of microflora in breast-fed infants and 28-75%

of microflora in formula-fed infants (Harmsen *et al.*, 2000). Obligate anaerobes appear when solid foods are added to the diet, resulting in the increase of short-chain fatty acid levels as the microbiota begin to take on an adult composition (Tannock, 1994; Tannock, 2005). Adult microflora are dominated by Firmicutes, with high levels of Bacteroidetes and small numbers of Actinobacteria, Proteobacteria and Verrucomicrobia contributing to the population (Eckburg *et al.*, 2005; Gill *et al.*, 2006; Li *et al.*, 2008; Manichanh *et al.*, 2006; Tap *et al.*, 2009).

2.1.2 The Role of Intestinal Microflora in Health

Intestinal microflora play many important roles in contributing to the health of the host. These organisms act as a "first line of defence" against enteric pathogens, enhance host nutrition, and play a vital role in the development of a functional epithelial barrier and immune system.

Commensal flora play a key role in the development of gut-associated lymphoid tissue (GALT), ultimately influencing the function of the immune system. Studies on gnotobiotic (germfree) rodents have been particularly helpful in revealing the importance of bacteria in the development of the host immune system. Gnotobiotic animals have poorly organized GALT, they are lacking intraepithelial T lymphocytes and do not develop Peyer's patches or other lymphoid follicles (Helgeland *et al.*, 1996; Shroff *et al.*, 1995). They also exhibit low levels of immunoglobulin (Ig) A secretion and systemic antibody production (Moreau *et al.*, 1982; Umesaki & Setoyama, 2000).

Commensal bacteria are required for development of systemic immune tolerance to ingested organisms (Moreau & Corthier, 1988; Moreau & Gaboriau-Routhiau, 1996;

Sudo *et al.*, 1997). Having an appropriate intestinal microflora can prevent atopic disease. Kalliomäki *et al.* (2001) found that infants with atopic diseases had different fecal bacteria communities and fatty acid profiles than healthy infants. This is supported by a study linking antibiotic use in the first year of life to the development of asthma (Wickens *et al.*, 1999).

Intestinal microflora are not only required for the development of a functional immune system, but also the development of a functional epithelial barrier. Gnotobiotic animals have altered intestinal permeability, which may be attributed to abnormal expression of zonulin and other tight junction proteins (El Asmar *et al.*, 2002; Mehrazar *et al.*, 1993). Hooper *et al.* (2001) found that *Bacteroides thetaiotaomicron* induced expression of several genes in intestinal epithelial cells in gnotobiotic mice. These experiments offered insights into the function of commensal microbes in enhancing host nutrient absorption, as *B. thetaiotaomicron* increased the messenger ribonucleic acid (mRNA) levels of a sodium-glucose co-transporter. Increases in transcription were also seen for genes associated with lipid metabolism and angiogenesis (Hooper *et al.*, 2001).

Not only do intestinal microflora aid in host nutrient absorption, but they also actively contribute to nutrition. The microflora produce vitamin K and the B group vitamins, and short-chain fatty acids produced by commensal bacteria stimulate epithelial cell growth and are used by the host as an energy source (Hill, 1997; Macfarlane & McBain, 1999).

Another important role of the microbiota is protection against infection. Colonization of the intestinal mucosa blocks the establishment of pathogenic bacteria. This is especially well demonstrated in patients who develop *Clostridium difficile*

infections associated with a loss of microflora due to antibiotic treatment (Baxter *et al.*, 2008). Commensal bacteria may also compete for nutrients with the incoming pathogens (Nataro, 2005). In addition, commensal bacteria have been shown to excrete bacteriocins or biosurfactants with antimicrobial activity (Velraeds *et al.*, 1996; Verellen *et al.*, 1998).

2.1.2.1 Probiotics and Bacterial Interference

The idea of using bacteria to improve health has been around since the early 1900's (Metchnikoff, 1907), but the use of commensal organisms for medicinal purposes has become more popular in recent years. The emergence of antibiotic resistant pathogens has created a need for new treatments, and people are looking toward commensal flora to fill this role (Huovinen, 2001). The medicinal use of commensal flora is generally referred to as probiotics. Probiotics are defined by the World Health Organization as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). Of all the bacteria showing promise as probiotics, the lactic acid bacteria, particularly *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species, have been studied to the greatest extent. These organisms are natural commensals of human mucosal surfaces and have been used to treat a variety of mucosal diseases.

The term "bacterial interference" has been employed to describe the phenomenon by which commensal flora prevent pathogens from colonizing and infecting a host (Reid *et al.*, 2001). Bacterial interference can be as simple as a competitive exclusion event, where a commensal organism is able to out-compete a pathogen for nutrients and adhesion sites on the mucosal surface (Rolfe, 2000). Other interference events are

thought to be mediated by the secretion of signalling molecules which activate the host's immune system (Macpherson & Harris, 2004), or the release of inhibitory substances that kill other organisms (Silva *et al.*, 1987). It is likely that each species utilizes a different strategy or combination of strategies to interfere with other species, and these effects may be pathogen-specific (Rolfe, 2000).

Clinical studies have shown potential for the use of commensal organisms in treating many mucosal infections, including recurrent ear infections and tonsillitis in children (Roos *et al.*, 2001), *C. difficile* infections of the gut (Tvede & Rask-Madsen, 1989), treatment of *Helicobacter pylori*-associated diseases (including peptic ulcer disease) (Canducci *et al.*, 2000), and prevention of recurrent urinary tract infections (Canducci *et al.*, 2000; Darouiche *et al.*, 2001). A variety of organisms have been used in these clinical trials. Alpha-hemolytic streptococci (*Streptococcus sanguis*, *S. mitis*, and *S. oralis*) have been used to treat otitis media and tonsillitis in children (Roos *et al.*, 2001). Non-pathogenic *E. coli* (*E. coli* Nissle 1917 and *E. coli* 83972) have been used in the treatment and prevention of urinary tract infections (Darouiche *et al.*, 2001; Darouiche *et al.*, 2005; Hull *et al.*, 2000; Rembacken *et al.*, 1999; Trautner *et al.*, 2003). *Lactobacillus* species have been examined for their ability to treat and prevent several types of mucosal infections. Patient groups treated with lyophilized *L. acidophilus* in addition to antibiotic therapy had higher eradication rates of *H. pylori* infection than patients groups treated solely with antibiotics (Canducci *et al.*, 2000). Recurrent diarrhea caused by *C. difficile* was successfully treated by displacement with a mixture of bacteria, including *Bacteriodes* species (*B. ovatus*, *B. vulgatus*, and *B. thetaiotaomicron*), which are present

in most healthy humans but were absent from patients afflicted with *C. difficile* prior to treatment (Tvede & Rask-Madsen, 1989).

Prebiotics are substances which encourage the growth of beneficial microorganisms. Generally, these take the form of carbohydrates that may not have any nutritional value to the host, but act as excellent nutrient sources for commensal organisms and can be used to encourage the growth of specific organisms in the gut (Gibson & Roberfroid, 1995; Guarner, 2007). Prebiotics have shown potential as a treatment for ulcerative colitis and Crohn's disease (Casellas *et al.*, 2007; Lindsay *et al.*, 2006). The combination of probiotic and prebiotic therapies is referred to as synbiotics. The strategy of combining a probiotic organism with its preferred substrate may allow for more extensive colonization (Collins & Gibson, 1999).

2.1.3 The Role of Intestinal Microflora in Disease

While the intestinal microflora generally contribute to the health of the host, in rare cases they also contribute to disease. Some members of the intestinal microflora can act as opportunistic pathogens (Gibson & Roberfroid, 1995). For example, *E. coli* and *Bacteroides* species have been implicated in the pathogenesis of appendicitis (Pieper *et al.*, 1982). Other commensal organisms secrete harmful factors. For example, bacteria produce several inflammatory products, such as lipopolysaccharide (LPS), peptidoglycan polysaccharide complexes, muramyl peptides and *N*-formymethionyl oligopeptides (Chadwick & Anderson, 1992; Linskens *et al.*, 2001).

Intestinal microflora are thought to play a role in the development of colon cancer, possibly due to the secretion of inflammatory or carcinogenic products (Moore & Holdeman, 1974). In a study of patients presenting with intestinal polyps, high levels of *Bacteroides* and *Bifidobacterium* species were found to be associated with a high risk of developing colon cancer, however lactic acid producing bacteria (*Lactobacillus* species and *Eubacterium aerofaciens*) were associated with a low risk of developing colon cancer (Moore & Moore, 1995). In addition, butyrate produced by normal flora may have a protective effect against colon cancer (McIntyre *et al.*, 1993). The most prominent disease associated with intestinal flora is inflammatory bowel disease (IBD), which is reviewed below.

2.1.3.1 Inflammatory Bowel Disease

IBD encompasses two diseases, Crohn's disease and ulcerative colitis, which manifest as chronic inflammation and ulceration of the gastrointestinal tract (Huda-Faujan *et al.*, 1997). Crohn's disease is characterized by granulomatous inflammation at any location in the gastrointestinal tract, although it is most common in the ileocecal area (Carty & Rampton, 2003; Huda-Faujan *et al.*, 1997). Ulcerative colitis is characterized by superficial inflammation of the colon and rectum (Carty & Rampton, 2003). The etiology of IBD is unknown, although it appears that a wide variety of factors may predispose one to developing IBD. Several genetic loci have been linked to IBD susceptibility, including NOD2 (a cytosolic pattern recognition receptor expressed in dendritic cells, intestinal epithelial cells and Paneth cells) and the cytokine receptor IL23R (Duerr *et al.*, 2006; Hugot *et al.*, 2001; Ogura *et al.*, 2001; Xavier & Podolsky,

2007). Environmental factors are also thought to play a role in IBD pathogenesis. Over the past twenty years several countries with previously low rates of IBD have experienced rising rates of Crohn's disease (Xavier & Podolsky, 2007).

The literature demonstrates that bacteria are involved in IBD pathogenesis, but the exact role that bacteria play is still unclear. It is well documented that IBD patients have an "altered" commensal flora compared to healthy controls. IBD patients appear to have higher numbers of mucosal bacteria, but these populations demonstrate less diversity than healthy patients (Manichanh *et al.*, 2006; Sepehri *et al.*, 2007; Sokol *et al.*, 2006; Swidsinski *et al.*, 2005). In addition to having more mucosal bacteria, IBD patients were shown to have more bacteria embedded deep in the mucous layer, suggesting an altered mucous layer offering less protection from luminal contents (Schultsz *et al.*, 1999).

Studies have implicated a wide range of specific bacterial species in IBD pathogenesis. Adherent *E. coli* have been detected on the gastrointestinal epithelium of Crohn's disease and ulcerative colitis patients at a much higher frequency than healthy individuals (Burke & Axon, 1988; Darfeuille-Michaud *et al.*, 1998; Martin *et al.*, 2004a). Sulfate reducing bacteria such as *Desulfovibrio* produce hydrogen sulphide, which is known to damage the epithelial barrier and cause irritation in the colon (Huda-Faujan *et al.*, 1997; Roediger *et al.*, 1997). A study by Zinkevich and Beech (2000) detected sulphate reducing bacteria in 92% of ulcerative colitis patients and only 52% of healthy controls. *Bacteroides* species have been shown to induce transmural inflammatory lesions in IBD patients (Guarner & Malagelada, 2003). Ulcerative colitis patients were shown to have higher numbers of *Bacteroides vulgatus*, and also had higher levels of IgG directed against *B. vulgatus* than healthy controls (54% agglutination activity of serum

from ulcerative colitis patients verses 9% in healthy controls) (Matsuda *et al.*, 2000). Swidsinksi *et al.* (2005) used fluorescent probes to demonstrate that *B. fragilis* made up greater than 60% of the mucosal bacterial biofilm mass in patients with IBD, compared to 16% in controls. In addition to its role in gastric cancer, *H. pylori* has also been implicated in IBD and has been found in a larger portion of rectum, cecum and colon biopsies from IBD patients than healthy controls (Streutker *et al.*, 2004). *Listeria monocytogenes* may also play a role in IBD pathogenesis, *L. monocytogenes* antigens were detected in 75% of Crohn's disease patients and 13% of ulcerative colitis patients, but were undetected in healthy controls (Liu *et al.*, 1995).

Although it is clear that IBD patients have altered commensal flora, it unknown if this is a causative factor, or simply a result of the mucosal inflammation associated with IBD. A heightened immune response to the commensal flora of the gut has been observed in IBD patients, resulting in inflammation and tissue injury (Cobrin & Abreu, 2005; Macpherson *et al.*, 1996). Duchmann and colleagues (1995) demonstrated a lack of tolerance to commensal flora in IBD patients. Monocytes from the lamina propria of IBD patients proliferated upon exposure to each patient's own intestinal microflora. In contrast, monocytes from healthy patients only proliferated upon exposure to other patient's intestinal microflora (Duchmann *et al.*, 1995). Commensal organisms are required for the development of IBD in mouse models. For example, interleukin (IL)-10 deficient mice develop spontaneous enterocolitis unless kept in under gnotobiotic conditions, where they do not develop colitis. It only took a single strain of bacteria, *B. vulgatus,* to induce enterocolitis in previously germfree IL-10 deficient mice (Sellon *et al.*, 1998).

Currently, IBD is most commonly treated with 5-aminosalicylic acid (mesalamine) (Stein & Hanauer, 1999). Corticosteroids are used in the treatment of patients with moderate-to-severe disease activity, and patients who do not respond well to steroids may be treated with immunomodulators (Stein & Hanauer, 1999). Antibiotics successfully treat symptoms in a subgroup of IBD patients, but they are ineffective for other patients and are often unsuccessful at preventing relapse (Linskens *et al.*, 2001; Spirt, 1994). Clinical trial data on antibiotic use has not proven them as an effective treatment for IBD (Gionchetti *et al.*, 2006; Guslandi, 2005; Isaacs & Sartor, 2004). An alternative to antibiotic therapy is the use of probiotics, prebiotics or synbiotics to treat IBD. Pilot trials have demonstrated the ability of synbiotic treatment (a combination of probiotic and prebiotic therapy) to decrease intestinal inflammation in both Crohn's disease and ulcerative colitis patients (Fujimori *et al.*, 2007; Furrie *et al.*, 2005).

2.1.4 Methods for Defining the Intestinal Microflora

Many different methods have been used to assess the composition of the intestinal microflora. Because an estimated 40-90% of the intestinal microflora is considered unculturable (Amann *et al.*, 1995; Eckburg *et al.*, 2005; Flint, 2006; Guarner & Malagelada, 2003; Suau *et al.*, 1999; Wilson & Blitchington, 1996), the majority of studies have utilized non-culture-based methods. However, studies on DNA (such as detection of 16S ribosomal RNA (rRNA) genes) give limited information on the behaviour of this complex community, and therefore culture-based approaches are becoming more important. The realization that in vitro culture is essential for more in-

depth analysis of the intestinal microflora has resulted in several successful attempts at culturing novel organisms from the human gastrointestinal tract.

2.1.4.1 Molecular Approaches for Defining the Intestinal Microflora

Almost all molecular studies are based on polymerase chain reaction (PCR) amplification of DNA, usually the 16S rRNA operon. These approaches can be powerful, but there are also drawbacks. DNA extraction protocols must be carefully designed to allow for complete recovery. For example, Li *et al.* (2007) found that a bead-beating step allowed for the detection of two additional peaks during terminal restriction fragment length polymorphism (T-RFLP) analysis of human fecal samples, although extended bead-beating led to extensively sheared genomic DNA. PCR techniques introduce two biases, PCR selection and PCR drift (Wagner *et al.*, 1994). PCR drift results from stochastic events during the early cycles of PCR amplification. Primer choice and PCR reaction conditions can skew results by under- or over-representing species (PCR selection) (Polz & Cavanaugh, 1998). In complex communities, the random binding of primers to the genomic DNA may lead to differences in amplification of each member of the community.

Fingerprinting techniques such as T-RFLP, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) offer a semi-quantitative overview of community diversity. They are quick and relatively low-cost, and are especially helpful in comparing communities from a broad perspective (Li *et al.*, 2007; Marsh, 2005; Schutte *et al.*, 2008). These fingerprinting techniques all begin with PCR using universal or group-specific primers. PCR drift can be avoided by

pooling several PCR replicates (Li *et al.*, 2007). After PCR amplification, fragments are run on gels with a denaturing (DGGE) or temperature (TGGE) gradient, which allows for the separation of the fragments, even though they are all of similar lengths (Lukow *et al.*, 2000). Banding patterns can be compared between communities and bands can be recovered from the gel for sequencing. For T-RFLP, a fluorescent-labelled primer and restriction enzyme digest are used to separate the PCR products based on sequence before running on a gel to separate labelled, digested products. One disadvantage of DGGE and TGGE is that bands making up a small portion of a complex community are difficult to distinguish from background (Heuer & Smalla, 1997). T-RFLP is more sensitive, and able to detect organisms that make up only 1% of a fecal microflora community (Li *et al.*, 2007). In addition, multiple T-RFLP profiles can be generated for each sample by using multiple restriction enzymes (Osborne *et al.*, 2006). This gives T-RFLP additional resolving power in comparison to DGGE and TGGE, and allows resolution of organisms that have the same fragment size.

The techniques used to assign fragments generated from T-RFLP (termed terminal restriction fragments, or T-RFs) to organisms are varied. A "virtual digest" of an online database of DNA sequences can be performed using the ribosomal database project (RDP) (Cole *et al.*, 2007; Cole *et al.*, 2009) and the Microbial Community Analysis (MiCA) III program (Shyu *et al.*, 2007). This is a relatively quick and simple technique for assigning T-RFs at the phylum level, and thus gives an overview of community structure. However, one of the challenges of this method is that peak sizes predicted by a "virtual digest" may not align with experimentally-determined values due

to slight variation in T-RF rates of migration through the sequencing gel (Osborn *et al.*, 2000).

Quantitative PCR (qPCR) gives a more quantitative assessment of communities than T-RFLP and is not as time-consuming as cloning-based sequencing methods. The SYBR green method has been used with group-specific primer sets to enumerate bacterial groups in fecal samples and mucosal biopsies (Bartosch *et al.*, 2004; Conte *et al.*, 2006; Furet *et al.*, 2009). Another qPCR system, the 5' fluorogenic exonuclease assay (TaqMan® assay; Applied Biosystems), has also been used to study intestinal bacteria of humans (Huijsdens *et al.*, 2002; Ott *et al.*, 2004). Both qPCR systems require the use of a standard curve to estimate numbers of bacteria. Calculating colony forming unit (cfu) equivalents from a standard curve only gives estimated values, especially when looking at broad groups, which may have wide variations in 16S rRNA operon copy numbers and genome sizes (Fogel *et al.*, 1999).

Clone libraries offer a very high level of resolution when studying communities, but these studies can be extremely time consuming and comparing communities can be difficult if clone libraries have incomplete coverage (Kent *et al.*, 2003). Tap *et al.* (2009) found that qPCR and cloning-based sequencing techniques gave comparable assessments of human intestinal microbiota. However, next-generation sequencing techniques such as 454/pyrosequencing (Roche, Branford, CT, USA) (Margulies *et al.*, 2005), Solexa (Illumina, San Diego, CA, USA) (Bentley, 2006) and SOLiD (Applied Biosystems) are quickly becoming affordable and accessible to researchers. These powerful techniques are expected to make sequencing a more common technique in community analysis studies (Mardis, 2008).

DNA-based molecular approaches do not distinguish between dead and viable organisms. In order to learn more about the metabolic activities of a community, techniques such as reverse-transcriptase PCR (RT-PCR) and metabolomics can be employed. RT-PCR can be used to follow mRNA levels in a community as a means of studying gene expression. Metabolomic analysis is just beginning to be used to study the role of microflora in health and disease (Marchesi *et al.*, 2007). For example, Dumas *et al.* (2006) used metabolic profiling to suggest a link between gut microflora and the development of insulin resistance in a mouse model.

2.1.4.2 Culture-Based Approaches for Defining the Intestinal Microflora

While culture-independent techniques are useful in describing communities, only so much can be learned from studying DNA. In order to learn about the behaviour of the intestinal microflora, it must be cultured in vitro or studied in vivo. Because in vivo studies are difficult to conduct (especially in humans), an in vitro model of intestinal microflora is a powerful tool for expanding our knowledge of host-microbe and microbe-microbe interactions in the gastrointestinal tract.

It is likely that the portion of "culturable" microbes in the gastrointestinal tract can be greatly expanded by exploring new culture conditions and the use of co-culture to encourage the growth of organisms that are dependent on another species. Sibley *et al.* (2008) detected large numbers of *Streptococcus milleri* group bacteria in sputum samples of cystic fibrosis patients using culture-based methods. These bacteria had been overlooked using routine culture analysis of sputum samples. This is one example of

how the development of novel culture media can lead to the culture of important organisms.

Recently, there has been renewed interest in culturing new species from the colon (Duncan *et al.*, 2007). Several members of the *Clostridium leptum* subgroup of Firmicutes, including *Faecalibacterium prausnitzii, Subdoligranulum variable* and *Anaerotruncus colihominis* have been cultured using carbohydrate substrates (Duncan *et al.*, 2007; Holmstrøm *et al.*, 2004; Lawson *et al.*, 2004). Several species from the genus *Roseburia* (*C. coccoides* subgroup of Firmicutes) were found to grow on a variety of substrates, including starch, xylan, inulin and fucose (Duncan *et al.*, 2006). Novel Gram-negative isolates have also been cultured, including three bile resistant species from the Bacteroidetes phylum. *Cetobacterium somerae* utilized peptides and carbohydrates as substrates, while *Bacteroides goldsteinii* was found to be saccharolytic (Finegold *et al.*, 2003; Song *et al.*, 2005). *Alistipes finegoldii* used an undetermined substrate (Rautio *et al.*, 2003). Novel *Verrucomicrobia* species, as well as novel Archaea have also recently been cultured from the human colon (Derrien *et al.*, 2004; Fricke *et al.*, 2006; Zoetendal *et al.*, 2003).

Molecular approaches have allowed for the identification and enumeration of bacterial groups in the intestine. This knowledge can be used to focus culture-based studies on what are likely to be the most important microbes in the complex ecosystem of the human gastrointestinal tract. The ability to culture even a few members of each bacterial group from the colon will help us to understand the complex metabolism of each species, which is an important step toward understanding the metabolism of the

microbiota as a whole. Isolating pure cultures of organisms also enables whole-genome sequencing, which would provide a girth of information about these microbes.

2.2 Biofilms

Biofilms are multicellular, surface-adherent communities of microorganisms encased in a matrix of extracellular polysaccharide (Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004). Although most studies characterize microorganisms in a planktonic state it is now generally accepted that, in most environments, biofilms are the preferred mode of growth for unicellular prokaryotes and eukaryotes (Costerton *et al.*, 1995; Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004). Because of their surface-adherent nature and multicellular composition biofilms behave differently than planktonic (free-swimming) microorganisms (Costerton *et al.*, 1995). Their community structure and polysaccharide matrix encasement provide protection for cells within the biofilm and allow microorganisms to withstand exposure to numerous hardships, including antibiotic and metal exposure (Ceri *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Harrison *et al.*, 2004; Harrison *et al.*, 2005a; Harrison *et al.*, 2006).

Biofilm formation begins with planktonic cells adhering to a surface. As these cells take on a sessile lifestyle, microcolonies form, eventually resulting in a mature, three-dimensional biofilm, which often consists of mushroom-shaped protrusions surrounded by channels for the diffusion of oxygen and nutrients (Hall-Stoodley *et al.*, 2004). Shear stress created by fluid movement favours biofilm formation (Donlan & Costerton, 2002).

Biofilms play an important role in many human infections. Chronic *P. aeruginosa* infections of the lungs of cystic fibrosis patients are the result of biofilm colonization (Donlan & Costerton, 2002; Govan & Deretic, 1996; Harrison *et al.*, 2005b). Periodontitis and gingivitis are the result of the build-up of a biofilm, or "plaque" on the teeth and surrounding tissue (Kolenbrander & London, 1993). Recurring infections of medical devices, ranging from prosthetic joints and heart valves to catheters and contact lenses, are all due to the ability of biofilms to form on these surfaces and protect themselves from antibiotic treatment and the host immune system (Donlan & Costerton, 2002; Patel, 2005). In the gastrointestinal tract, as with many other organs, pathogens must adhere in order to avoid being flushed out of the system. For example, *Enterococcus faecalis* appears to have a signaling system that initiates biofilm formation, and possibly virulence factor expression, in nutrient-rich environments like the gut (Creti *et al.*, 2006).

Medically, the largest challenge of dealing with biofilm infections is their ability to withstand antibiotic treatment (Patel, 2005). Even after extended treatment plans biofilms are often not fully eradicated and relapsing infections occur. This is due to a number of possible mechanisms of antimicrobial resistance in biofilms, including inhibited diffusion of antibiotics into the biofilm, expression of biofilm-specific resistance genes, and increased resistance due to the emergence of a persister cell population. The impeded diffusion hypothesis has recently fallen out of favour since several antibiotics were shown to diffuse through biofilms (Anderl *et al.*, 2000; Coquet *et al.*, 1998; Patel, 2005). The *ndvB* gene in *P. aeruginosa* is one example of a biofilm-specific gene that may be involved in antimicrobial resistance. *ndvB* mutant strains form

biofilms but the biofilms do not display antibiotic resistance (Mah *et al.*, 2003). Persister cells are phenotypic variants of a population specialized for tolerance of harsh conditions, including antimicrobial exposure (Spoering & Lewis, 2001). While not technically resistant to antimicrobials, these cells are able to survive (tolerate) high concentrations of antimicrobials by entering a somewhat dormant state (Harrison *et al.*, 2005b; Patel, 2005). Persisters are not genetically different from the rest of a population, and therefore can repopulate a community following the removal of an environmental challenge (Harrison *et al.*, 2005a).

Pathogens are not the only microorganisms that live in biofilms. Although the study of commensal biofilms is in its early stages, preliminary work demonstrates that attachment is important in persistence at mucosal surfaces, especially in the gastrointestinal tract (Wang *et al.*, 2006). While biofilm formation was once considered a virulence factor (Parsek & Singh, 2003), studies of the ability of pathogenic and commensal human isolates of *E. coli* to form biofilms showed no correlation between biofilm-forming ability and pathogenicity (Reisner *et al.*, 2006). *Lactobacillus rhamnosus* GG, an organism showing great potential as a probiotic, has been shown to form biofilms *in vitro* on a polystyrene surface (Lebeer *et al.*, 2007b). Additional studies are required to further characterize the role of biofilm formation in both pathogenic and commensal organisms.

Although it is recognized that most biofilms are comprised of multiple species, very few studies have examined multispecies biofilms (Parsek & Singh, 2003). This is largely due to the complicated nature of these communities, but this area must be pursued in order to study biofilms in their natural form. In particular, the role of biofilm

formation in interactions between commensal and pathogenic species has not been determined. When entering the body through a mucosal surface, a pathogen is in planktonic form and must contend with the established commensal flora before it can adhere and begin biofilm formation. While clinical trials demonstrate the ability of probiotic treatment to prevent and eradicate biofilm-associated infections (Darouiche *et al.*, 2005; Hull *et al.*, 2000), the mechanisms by which this occurs are largely undefined.

2.2.1 Biofilms of Mucosal Intestinal Microflora

Most studies of the intestinal microflora have focused on planktonic cells in the lumen (present in fecal samples), but it is recognized that the sessile bacterial growth on the intestinal mucosa has the most intimate interaction with the host (Cheng *et al.*, 1981; Croucher *et al.*, 1983; Probert & Gibson, 2002). The mucous layer provides an ideal environment for the growth of biofilms, and the continuous shedding of mucus in the lumen is proposed to work as a population control mechanism to keep commensal biofilms in check (Probert & Gibson, 2002). A study of *E. coli* growth rates in the mouse intestine identified a replicating subset of bacteria on the mucous layer and a "static" population in the lumen (Poulsen *et al.*, 1995). It is thought that the luminal bacteria were shed from the mucosal populations, although it is unknown if all mucosal bacterial populations behave in this way.

Microscopy has been an important tool in confirming biofilm growth on the gastrointestinal mucosa. Scanning electron microscopy (SEM) studies visualized bacteria colonizing the mucosa of the gastrointestinal tract (Bayliss & Turner, 1982; Croucher *et al.*, 1983), but images of commensal biofilm growth were absent from the literature until

recently, likely due to the fact that biofilms on tissue samples are easily disrupted by standard fixing and staining techniques (Bollinger *et al.*, 2007a; Palestrant *et al.*, 2004; Swidsinski *et al.*, 2005). Once appropriate processing techniques were developed, several groups were able to image biofilms on mucosal biopsies from the human gastrointestinal tract (Macfarlane & Dillon, 2007; Palestrant *et al.*, 2004; Swidsinski *et al.*, 2005). Palestrant *et al.* (2004) visualized distinct layers of bacterial growth on baboon cecum epithelium using transmission electron microscopy (TEM), with smaller bacterial cells along the surface of the mucus and larger cells forming an outer layer toward the lumen. These layers of bacteria were not found in samples from human appendices (only one size of bacteria was seen), but TEM images of both baboon and human samples showed an extracellular matrix surrounding the bacteria (Palestrant *et al.*, 2004). The same study used acridine orange-stained tissue sections to demonstrate that bacteria growing on rat large intestine epithelium were oriented parallel to the direction of flow (Palestrant *et al.*, 2004). The layered community structure, presence of an extracellular matrix, and the orientation of the bacteria in relation to flow strongly support that the observed bacterial communities represent biofilms.

Fluorescent in situ hybridization (FISH) has proven to be a powerful technique for visualizing these commensal biofilms in the intestine. The use of group- or species-specific probes enables the enumeration of various bacteria within the biofilms. Swidsinski *et al.* (2008) used FISH to study the effects of antibiotic treatment on intestinal biofilms in IBD patients. Interestingly, although biofilms were significantly reduced during antibiotic treatment, within one week of the conclusion of antibiotic therapy biofilms rebounded to a level higher than patients who were untreated and

remaining high for at least five months (Swidsinski *et al.*, 2008). Alexander Swidsinski's group has also used FISH to observe differences in mucosal biofilm composition in IBD patients compared to healthy controls. Biofilms in IBD patients were dominated by *Bacteroides fragilis*, which was not the case in control subjects (Swidsinski *et al.*, 2005). George Macfarlane's group has used FISH to visualize multispecies biofilms on both the colonic mucosa and food residues from the intestine (Macfarlane & Macfarlane, 2006; Macfarlane & Dillon, 2007).

There is mounting evidence that biofilms play an important role in the digestion of food particles in the intestine. Macfarlane and Macfarlane (2006) used SEM and FISH combined with confocal microscopy to visualize biofilm communities inhabiting the surfaces of luminal particles. Approximately 5% of bacteria in the lumen of the colon was found to be strongly adherent to food residues. Whereas mucosal biofilms differ in community structure from luminal bacteria, biofilms on food particles appear to resemble the community structure of luminal populations, with high levels of *Bacteroides* and *Bifidobacteria* (Macfarlane & Macfarlane, 2006). The biofilm communities were metabolically distinct from the non-adherent bacteria, and were more efficient at digesting polysaccharides and produced acetate as their main fermentation product, while non-adherent communities more readily fermented oligosaccharides and produced high levels of butyrate (Macfarlane & Macfarlane, 2006). In a similar study, biofilms on food residues were shown to secrete higher levels of digestive enzymes than non-particulate associated bacteria, including xylanase, β-xylosidase, arabinogalactanase, α-arabinofuranosidase and β-galacturonidase (Macfarlane *et al.*, 1997). These studies suggest that there may be a unique role for biofilm bacteria in the digestion process.

The appendix has been proposed as a "safe house" for commensal flora. Biofilms in the human appendix have been visualized, and these communities are predicted to play a role in pathogen exclusion, as well as providing a favourable substratum for adhesion by additional commensal organisms (Bollinger *et al.*, 2007b). In addition, shedding of bacteria from these biofilms may serve as an "inoculum" for the large intestine in the event that the normal flora are disrupted by antibiotic use or infection (Bollinger *et al.*, 2007b).

In 2004, the Bollinger, Parker and Miller laboratories at Duke University put forward a new theory, suggesting that the host immune system plays a role in encouraging biofilm formation in the intestine (Bollinger *et al.*, 2003; Everett *et al.*, 2004; Palestrant *et al.*, 2004). The theory centres on evidence that secretory IgA (SIgA) and mucin increase adherent growth in vitro. Using an in vitro model of biofilm formation on saliva-coated synthetic hydroxapatite, Molisher and colleages (1996) showed that both SIgA and mucin improved *E. coli* adhesion. Studies by the Bollinger, Parker and Miller group found similar results when studying fecal bacteria and *E. coli* adhesion to Caco-2 cells (Bollinger *et al.*, 2003). Bacteria from fecal samples and *E. coli* both demonstrated an increase in adherent growth on fixed epithelial cells in the presence of SIgA. Further studies with *E. coli* showed increased adherent growth on fixed epithelial cells in the presence of mucin, and also an increase in adherence to live epithelial cells in the presence of SIgA (Bollinger *et al.*, 2003). These in vitro studies were supported by the detection of SIgA associated with commensal biofilms in healthy rat colon and human appendix tissue sections (Palestrant *et al.*, 2004). These findings conflict with the traditional "immune exclusion" theory, where SIgA aggregates bacteria,

making them less likely to adhere to and translocate across the epithelial barrier (Williams & Gibbons, 1972). Instead, the Bollinger group suggests an "immune exclusion/inclusion" theory, where the aggregation of commensal flora by SIgA facilitates biofilm formation while still preventing translocation across the epithelium (Everett *et al.*, 2004). They argue that, by encouraging commensal biofilm formation, the SIgA promotes the protective effects of the commensal flora.

2.3 Current In Vitro Models of Intestinal Microflora

The majority of in vitro systems for modeling the microbiota of the gastrointestinal tract are anaerobic fermentation chambers. Several types of model systems exist, including batch cultures, chemostat-type simulators, and non-chemostat-type simulators. Batch cultures are generally composed of fecal or cecal samples sealed inside an anaerobic jar or bottle (Mäkivuokko & Nurminen, 2006). Batch simulators work well for short simulations, but during longer fermentations the build-up of metabolic products significantly changes the conditions (Mäkivuokko & Nurminen, 2006).

Chemostat-type simulators are the most commonly used model because they allow for the continuous flow of nutrients. The first semi-continuous in vitro colon simulator was designed by Miller and Wolin (1981) and contained a single fermentation vessel. The Reading model, designed by Gibson, Cummings and Macfarlane (1988) contains three vessels, simulating the proximal, transverse and distal colon, with continuous flow from one vessel to the next. The Reading model was modified in 1998 and shown to maintain a diverse, stable population of fecal bacteria that was similar to

bacterial populations in intestinal samples from sudden death victims (Macfarlane *et al.*, 1998). The Macfarlane group has also used a two-stage continuous culture system to study the colonization of mucin gels by fecal bacteria, and demonstrated that the biofilms formed on the mucin gels differed in community composition and metabolic activity from non-adherent bacterial populations (Macfarlane & Macfarlane, 2001; Macfarlane *et al.*, 2005).

Another system used to model the microbiota of the gastrointestinal tract was developed by Minekus and colleagues (Minekus *et al.*, 1995; Minekus *et al.*, 1999). They developed two complementary computer-controlled models, one representing the stomach and small intestine (composed of eight modules) and the other representing the proximal colon (composed of four modules). This system is different from chemostat-type systems in that fluids are pushed from one vessel to another by peristaltic valve-pumps, and water and fermentation products are continually exchanged through dialysis membranes (Minekus *et al.*, 1995; Minekus *et al.*, 1999).

In general, current in vitro models of intestinal microflora are representative of luminal, non-adherent bacterial populations, rather than mucosal ones. Although biofilms may form on the surfaces of the fermentation vessels, they are not generally the populations that are sampled. The exception to this is the two-vessel continuous flow system used by Macfarlane and colleagues (Macfarlane & Macfarlane, 2001; Macfarlane *et al.*, 2005), which was used to study the intestinal bacteria colonization of mucin gels. This is perhaps the best in vitro model of mucosal intestinal microflora to date, since it allows for the growth of biofilms on a mucin substratum. However, the system is still inoculated with fecal, rather than mucosal, bacterial populations.

This project aimed to fulfill the need for an in vitro model of mucosal bacterial populations growing as biofilms. The model utilizes a complex bacterial community from human mucosal colon biopsies. The system allows for the study of interactions between biofilm communities, such as interactions between commensal and pathogenic biofilms.

Chapter Three: Methods

3.1 Bacterial Strains

Bacterial strains used in this study are described in Table 1. Bacterial strains were stored at -70°C in MicroBank™ vials (vials contain treated beads and cryopreservative solution; Pro-Lab Diagnostics, Toronto, ON, Canada).

3.2 Growth Media

3.2.1 Solid Growth Media

E. coli strains were routinely cultured on Luria Bertani agar (LBA; EMD Chemicals, Gibbstown, NJ, USA). Commensal strains were cultured on de Man, Rogosa and Sharpe (MRS) agar (BD Biosciences, Mississauga, ON, Canada). Aerobic bacteria isolated from the human colon were cultured on Brain Heart Infusion Agar (BHIA, BD Biosciences) or MacConkey agar (BD Biosciences). Anaerobic bacteria were cultured on Columbia blood agar (CBA, Oxoid, Nepean, ON, Canada), Fastidious Anaerobe Agar (FAA, Lab M, Bury, Lancashire, UK), or Schaedler agar (Oxoid, Nepean, ON, Canada) supplemented with 1 mL per litre of 1% vitamin K_1 (Sigma Aldrich) solution in absolute ethanol. All solid media for anaerobes were supplemented with 5% sterile defibrinated sheep blood (Dalynn Biologicals, Calgary, AB, Canada) and placed in an anaerobic environment at least 24 hours prior to use.

Phenylethanol agar (PEA), *Bacteroides* bile esculin agar (BBE) and mEnterococcus agar (mEA), selective media used to identify bacteria from the human colon, were purchased from Dalynn Biologicals (Calgary, AB, Canada).

Table 1. Bacterial strains and communities used in this study

Strain or Community	Description	Reference or Source
Escherichia coli		
HB101	F-, *supE44*, *lacY1*, *ara-14*, *galK2*, *xyl-5*, *mtl-1*, *leuB6*, Δ*(mcrC-mrr*), *recA13*, *rpsL20*, *thi-1*, Δ(gpt-proA)62, *hsdSB20*, λ-	(Boyer & Roulland-Dussoix, 1969)
E2348/69 (EPEC)	O127:H6, Enteropathogenic, infant gastroenteritis isolate	(Knutton *et al.*, 1987)
86/24 (EHEC)	O157:H7, Enterohemorrhagic, hemorrhagic colitis isolate	(Griffin *et al.*, 1988)
CFT073 (UPEC)	Uropathogenic, acute pyelonephritis isolate	(Mobley *et al.*, 1990)
Commensals/Probiotics		
Lactobacillus plantarum 299v	Probiotic strain, commercially available as TuZen™ (Ferring Inc., Toronto, ON, Canada)	Institut Rosell, Lallemand Inc. (Montreal, QC, Canada)
VSL#3	Mixture of 8 probiotic strains: *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus plantarum*, *Bifidobacterium. infantis*, *Lactobacillus paracasei*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*	VSL Pharmaceuticals Inc. (Toronto, ON, Canada)

3.2.2 Liquid Growth Media

Growth of strains in the CBD (Ceri *et al.*, 1999) was performed in a number of different liquid media. Luria-Bertani broth (LB) was purchased from EMD Chemicals and MRS broth and Brain Heart Infusion broth (BHI) were purchased from BD Biosciences. Modified MRS (mMRS) was prepared from MRS broth plus 0.05% cysteine-HCl (Sigma Aldrich, Oakville, ON, Canada) (Simpson *et al.*, 2004). Modified tryptic soy broth (mTSB) was prepared with 15 g/L tryptic soy broth (TSB) base (EMD Chemicals) and 20 g/L Bacto proteose peptone no. 3 (BD Biosciences) (Lebeer *et al.*, 2007b). For optimization experiments, media were also supplemented with 0.08% agar (EMD Chemicals).

Anaerobes were cultured in supplemented tryptic soy broth with yeast (sTSY). The base consisted of TSB (EMD Chemicals) prepared to manufacturer's specifications supplemented with 4 g/L yeast extract (BD Biosciences). After autoclaving the following was added per 100 mL of base: 1 mL of 5% L-cysteine-HCl (Sigma Aldrich) solution in sterile, double distilled water (ddH_2O) and 1 mL of hemin-menadione solution (50 mg bovine hemin (Sigma Aldrich), 1.74 g K_2HPO_4, 0.4 g NaOH, and 1 mL of a 1 mg/mL menadione (Sigma Aldrich) solution in 95% ethanol, all dissolved in 99 mL ddH_2O). sTSY was placed in an anaerobic environment at least 24 hours before use.

3.3 Biofilm Growth Curves and Optimization

3.3.1 Forming Biofilms

All biofilms were grown in the CBD (commercially available as the MBEC™ P&G device, Innovotech Inc., http://www.innovotech.ca, Edmonton, AB, Canada), which

allows for the simultaneous growth of 96 equivalent biofilms (Ceri *et al.*, 1999). The CBD consists of a lid with 96 polystyrene pegs which extend into the wells of a standard microtitre plate. The pegs generally serve as the growth surface for biofilms, although the wells of the microtitre plate are also a suitable environment for biofilm formation.

Inocula for biofilms of all *E. coli* and commensal strains were started from overnight secondary subcultures on solid media (LBA for *E. coli* strains, MRS agar for commensals). Colonies were picked off the solid media and suspended in approximately 2 mL 0.9% saline until an optical density (OD) equivalent to a 1.0 McFarland standard was reached (approximately $3x10^8$ cfu per mL). This cell suspension was diluted to $1x10^7$ cfu per mL in the appropriate growth medium to make the final inoculum. One hundred and fifty µl of this inoculum was placed in each well of the CBD, and the device was incubated at 37°C on a gyrotary shaker (Model G2; New Brunswick Scientific Company, Edison, NJ, USA) set at the appropriate speed (50 rotations per minute (rpm) or 150 rpm). Alternatively, the CBD was incubated under static conditions. This procedure was used to grow biofilms on both the pegs and the wells of the CBD. Cfu per mL of inoculum was verified by serial dilution and spot-plating on appropriate agar media.

3.3.2 Growth Curves

For growth curve experiments, biofilms were formed on the pegs of the CBD as described above (Section 3.3.1). At time intervals over a period of up to 50 hours, growth was sampled by removing pegs from the lid of the CBD with sterile pliers. These pegs were rinsed in 0.9% saline and sonicated on high for 10 minutes in 200 µl 0.9%

saline in an Aquasonic sonicanor (Model 250HT; VWR Scientific, Edmonton, AB, Canada). A low-output sonicator was used to allow from dispersal of bacteria from the surface without affecting cell viability (Ceri *et al.*, 1999; Morck *et al.*, 1994). Biofilm formation on each peg was enumerated by viable cell counting: sonicates were serially diluted and spot plated on the appropriate agar media. After incubation at 37°C overnight colonies were counted on the agar plates to determine biofilm size (cfu per peg). Biofilm formation on coated pegs was also studied; pegs were incubated in 200 µl of fetal calf serum (FCS; Sigma Aldrich) or 0.03% hog gastric mucin (Sigma Aldrich) for one hour, then allowed to dry for one hour in a biological safety cabinet.

3.3.3 Biofilm Recovery from the CBD

To determine conditions for optimal biofilm recovery from the CBD, sonication times of 6, 10, 15, 20, 30 and 45 minutes were used on 24 hour biofilms of EPEC and *E. coli* HB101 grown in LB with shaking at 150 rpm. Ten minutes was chosen as the sonication time for all other experiments. Twenty-four hour biofilms of EPEC and *E. coli* HB101 were also sonicated for 10 minutes in 0.9% saline or 0.9% saline with 1% Tween-20 (Sigma Aldrich). Saline (0.9%) was used as the sonication solution for all other experiments. Recovery from the peg was assessed using viable cell counting (as described in Section 3.3.2).

3.3.4 Biofilm Optimization

Biofilm growth in the CBD was optimized on both the peg and well surfaces. Strains were grown in a variety of media (LB, MRS, mMRS, TSB and mTSB) with or

without 0.08% agar (used to increase media viscosity). During biofilm growth, strains were shaken at 50 rpm or 150 rpm, or were incubated under static conditions. Generally, growth was optimized at 24 hours, although VSL#3 was grown for up to 72 hours (growth medium in the wells of the CBD was changed at 48 hours). All growth experiments were carried out at 37°C. Biofilms were assessed by viable cell counting. The viable cell counting procedure for peg biofilms is outlined above (Section 3.3.2). For well biofilms, growth medium was removed using a pipette, and then the wells were filled with 200 µl 0.9% saline and emptied two times. Finally, 200 µl of 0.9% saline were added and the wells were sonicated for 10 minutes. Serial dilutions and spot plating were carried out as with peg biofilms (see Section 3.3.2).

3.4 Multispecies Biofilm Assays

3.4.1 Biofilm Co-Culture Experiments

Two-species biofilms were formed by EPEC and *L. plantarum*. These biofilms were grown as usual in the CBD, except the inocula consisted of $1x10^7$ cfu per mL of EPEC and either an equal amount ($1x10^7$ cfu per mL) or a half dose ($5x10^6$ cfu per mL) of *L. plantarum*. The different doses of *L. plantarum* were used to determine the effect of different cell densities of the commensal on EPEC biofilm formation. Biofilms were grown in mTSB with shaking at 150 rpm. LBA and MRS were used for viable cell counting of EPEC and *L. plantarum* peg biofilms, respectively.

3.4.2 Biofilm-Biofilm Exposures

Biofilms of EPEC and *L. plantarum* were grown in separate CBDs in mTSB with shaking at 150 rpm. After 24 hours of growth, the lids of the two CBDs were switched, exposing EPEC and *L. plantarum* biofilms to each other. After 24 hours of exposure, the following biofilms were enumerated: EPEC peg biofilms (exposed to *L. plantarum* well biofilms), EPEC well biofilms (exposed to *L. plantarum* peg biofilms), *L. plantarum* peg biofilms (exposed to EPEC well biofilms), and *L. plantarum* well biofilms (exposed to EPEC peg biofilms). Viable cell counting was conducted using LBA (EPEC) and MRS (*L. plantarum*) as spot-plating media. Controls consisted of exposure of two biofilms of the same species, as well as exposure of biofilms to sterile media (no biofilm).

3.5 Susceptibility Testing of Biofilms

3.5.1 Antibiotic Preparation

Antibiotics were purchased from Sigma Aldrich unless otherwise specified. Ampicillin (MP Biomedicals, Solon, OH, USA), cefotaxime, cefazolin, ciprofloxacin (MP Biomedicals), gentamicin, kanamycin (MP Biomedicals), levofloxacin, and rifampicin were prepared in stock solutions of 5120 µg/mL based on purity and potency information provided by each manufacturer. Stock solutions were stored at -70°C. Working solutions of 16 µg/mL (ampicillin, cefazolin, gentamicin, kanamycin and rifampicin) and 1 µg/mL (cefotaxime, ciprofloxacin and levofloxacin) were prepared in mTSB. Serial two-fold dilutions of antibiotic working solutions were made in mTSB in a 96-well microtitre plate (Nunc, Rochester, NY, USA).

3.5.2 Antibiotic Susceptibility Testing

EPEC biofilms or EPEC and *L. plantarum* biofilm co-cultures were grown on the pegs of the CBD for 24 hours in mTSB with shaking at 150 rpm. The biofilms were rinsed in 0.9% saline and then transferred to a 96-well plate containing serial dilutions of antibiotics. Biofilms were challenged with antibiotic at 37°C for 24 hours, after which time the biofilms were rinsed twice in 0.9% saline and then removed from the pegs by sonication into 200 µl mTSB. Bacterial growth was determined by plus/minus plating. Twenty µl aliquots of biofilm sonicate were spot-plated on agar media. After 24-48 hour incubation at 37°C, the agar plates were examined for presence or absence of bacterial growth. LBA and MRS agar were used to enumerate EPEC and *L. plantarum*, respectively. The minimal biofilm eradication concentration (MBEC) was determined to be the lowest concentration of antibiotic required to kill 100% of the biofilm after the 24 hour exposure period (no growth on the plus/minus plates) (Ceri *et al.*, 1999). The lowest concentration of antibiotic required to kill 100% of the planktonic cells (no growth on the plus/minus plates) after antibiotic exposure was designated as the MBC_{100} (Harrison *et al.*, 2005b). MBC_{100} values were determined by plus/minus plating of aliquots from the antibiotic dilution plate (cells shed from the biofilm served as the planktonic population).

3.5.3 EPEC Susceptibility to Anaerobic Bacteria Spent Media

A bacterial community isolated from a human descending colon biopsy was cultured anaerobically in sTSY for 24 hours. This culture was put through a 0.20 µm syringe filter (Sarstedt, Montreal, QC, Canada) to remove bacteria. Similarly, an EPEC planktonic culture was grown for 24 hours in LB in aerobic conditions and was filtered to

remove bacteria. Anaerobic bacteria spent media, EPEC spent media, and fresh sTSY media were placed in the wells of a 96-well plate (200 µl of the challenge media per well).

EPEC peg biofilms were formed in the CBD in LB for 24 hours with shaking at 150 rpm. These biofilms were rinsed in 0.9% saline and then placed in the 96-well plate containing the challenge media. The EPEC biofilms were challenged for 24 hours at 37°C and biofilms were rinsed two times in 0.9% saline. Biofilms were sonicated into 200 µl saline and enumerated by viable cell counting (described above; Section 3.3.2) using LBA.

3.6 Bacterial Communities from the Human Colon

3.6.1 Biopsy Collection and Processing

Mucosal biopsies of the left (descending) and right (ascending) colon were collected from healthy volunteers undergoing routine colon cancer screening. Patients who had taken antibiotics or probiotics in the three weeks prior to testing were excluded. Biopsies were obtained through the Intestinal Inflammation Tissue Bank at the University of Calgary. The Intestinal Inflammation Tissue Bank Initiative (Grant-ID 18142) has obtained ethics approval from the Conjoint Health Research Ethics Board (University of Calgary) for the collection of biopsies from volunteers, including approval of the collection protocol and informed consent form. Additionally, the specific protocols used for this study underwent ethics approval. Researchers did not have access to patient names or any other patient information, and study findings were not used to modify patient care.

Biopsies were collected in BBL Port-A-Cul tubes (specifically designed to keep samples anaerobic; BD Biosciences) or 1.5 mL tubes with 200 µl phosphate-buffered saline (PBS) containing 0.016% dithiothreitol (DTT) for transport. Samples used for culture experiments were placed in an anaerobic chamber (Bactron Anaerobic/Environmental Chamber; Sheldon Manufacturing Inc., Cornelius, OR, USA) as quickly as possible. The atmospheric composition of the anaerobic chamber was 90% nitrogen, 5% hydrogen and 5% carbon dioxide gases.

Processing was carried out using a protocol adapted from Conte *et al.* (2006). Biopsies were handled with tweezers sterilized with ethanol. Biopsies were incubated in 200 µl PBS with 0.016% DTT for two minutes and then rinsed three times with gentle shaking in PBS. Biopsies to be used for molecular studies were frozen in 80 µl PBS at -70°C until DNA extraction could be performed (see Section 3.6.4). Biopsies to be used for biofilm formation were homogenized in 200 µl sTSY using a sterile mini-tissue homogenizer (Pellet Pestle® Microgrinder System; Kimble-Kontes, Vineland, NJ, USA). The homogenate was used immediately for biofilm formation or 200 µl sterile anaerobic 50% glycerol was added to the samples and they were frozen in 90 µl aliquots at -70°C.

Three separate biopsy collections were carried out; Table 2 lists the samples from each collection and the experiments they were used for.

3.6.2 Biofilm Formation from Colon Biopsy Samples

Several methods were used for seeding biofilm formation in the CBD with colon biopsy bacteria. For aerobic biofilms, processed biopsy samples were serially diluted and

Table 2. Biopsies collected for biofilm study

Collection date	Biopsies for culture[a]	Biopsies for molecular analysis[b]
June 2008	Biopsies W1, W2 (Patient W), Biopsies X1, X2, X3 (Patient X) All biopsies from descending colon	none
October 2008	Biopsies Y1, Y2 (Patient Y), Biopsies Z1, Z2 (Patient Z) All biopsies from descending colon	Biopsies Y3, Y4 (Patient Y), Biopsies Z3, Z4 (Patient Z) All biopsies from descending colon
July 2009	Immediate culture: Biopsies A1, A2, B1, B2, C1, C2 (Patient A, B or C, descending colon) Biopsies A9, A10, B9, B10, C9, C10 (Patient A, B or C, ascending colon) Frozen before culture: Biopsies A3, A4, B3, B4, C3, C4 (Patient A, B or C, descending colon) Biopsies A11, A12, B11, B12, C11, C12 (Patient A, B or C, ascending colon)	Biopsies A5-A8, B5-B8, C5-C8 (Patient A, B or C, descending colon) Biopsies A13-A16, B13-B16, C13-C16 (Patient A, B or C, ascending colon)

[a] Biopsies were used to form biofilms, from which DNA was extracted
[b] DNA was extracted directly from biopsies

plated on BHIA (biopsies W1-2 and X1-3; Table 2). These plate counts were used to determine cfu per biopsy for aerobic bacteria, and bacteria from the agar plates were stored at -70°C in MicroBank™ vials. Then, biofilms were formed and enumerated using the usual CBD protocol (Sections 3.3.1 and 3.3.2) with BHI as the growth medium and BHIA for viable cell counting. Biofilms were grown under static conditions or with shaking at 150 rpm for 24 hours. In a second aerobic biofilm experiment (using biopsies W1 and W2; Table 2), the inocula consisted of 5 µl of biofilm homogenate and 190 µl BHI in each well of the CBD. Biofilms were grown statically for 48 hours, with sampling and 24 and 48 hours.

For anaerobic biofilms with biopsies Y1-2 and Z1-2 (Table 2), biofilms were formed immediately following biopsy collection by adding 10 µl of biopsy homogenate to 195 µl sTSY for each well of the CBD. Biofilms were incubated in the anaerobic chamber at 37°C under static conditions for 143 hours. In a second biofilm experiment, frozen biopsy homogenates from biopsies Y1-2 and Z1-2 were used to start biofilms. A sterile loop was used to transfer a small amount of frozen biopsy homogenate into 5 mL sTSY broth. Overnight cultures of the biopsy homogenate samples were grown at 37°C in an anaerobic hood and diluted to an OD approximately equal to a 1.0 McFarland standard, and then diluted a further 30 fold in sTSY. These diluted cultures served as the inocula for biofilms, which were grown for 48 hours. The CBDs were sealed inside anaerobic bags (AnaeroGen™ Compact System, Oxoid, Nepean, ON, Canada; the anaerobic sachet was not used since the bags were sealed in an anaerobic environment), removed from the anaerobic hood, and placed on a gyrotary shaker at 150 rpm.

After several methods for anaerobic biofilm formation were tested, a standard protocol was put together to examine biofilms formed by biopsy samples from three different patients (July 2009 biopsy collection; Table 2). Immediately following biopsy processing, biopsy homogenates were diluted 50 fold in sTSY and 150 µl aliquots were placed in the wells of the CBD. Alternatively, frozen aliquots of biopsy homogenate were thawed in an anaerobic chamber and diluted in 10 mL sTSY. One hundred and fifty µl of this suspension were used to inoculate each well of the CBD. For biofilms from both fresh and frozen samples, the CBD was sealed in an anaerobic bag and placed on a shaker (150 rpm) at 37°C. Biofilms were grown for 144 hours, with sampling at 2, 24, 48, 96 and 144 hours.

3.6.3 Sampling Anaerobic Biofilms

In order to sample the anaerobic biofilms, they were brought into an anaerobic chamber and removed from the anaerobic bag. Using ethanol-sterilized pliers, pegs were broken from the CBD (five to eight pegs per condition and timepoint). For viable cell counts, pegs were rinsed in 200 µl of 0.9% saline and then each peg was sealed in a 1.5 mL tube containing 200 µl saline. These tubes were removed from the anaerobic hood, sonicated on high for 10 minutes, and returned to the anaerobic hood. Samples were serially diluted in 0.9% saline and spot-plated on FAA, CBA, or Schaedler agar for viable cell counting. Colonies on the agar plates were counted after at least 4 days of anaerobic incubation at 37°C. Samples to be used for DNA extraction were rinsed in 0.9% saline and then two pegs of the same sample were placed in a 2 mL screw-cap tube and frozen at -20°C until DNA extraction could be performed.

3.6.4 DNA Extraction

DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Mississauga, ON, Canada) and protocols adapted from Conte *et al.* (2006).

3.6.4.1 DNA Extraction for qPCR Analysis of Biopsies and Biofilms

Four CBD pegs were collected for each sample at each timepoint, and these samples were pooled during DNA extraction. The 2 mL screw-cap tubes containing 2 pegs each were thawed at room temperature. For DNA extraction from biopsies, each biopsy was placed in its own screw-cap tube. Two hundred µl of lysozyme solution was added to each tube (20 mg/mL lysozyme (Sigma Aldrich) in 20 mM Tris-HCl (tris(hydroxymethyl)aminomethane hydrochloride), pH 8.0, 2 mM (ethylenedinitrilo)-tetraacetic acid (EDTA) and 1.2% Triton X-100). The tubes were placed on their sides (to allow for rolling) on a shaker at 150 rotations per minute (rpm) and incubated at 37°C for at least 30 minutes. Buffer ATL (200 µl; Qiagen) and Proteinase K (20 µl; Qiagen) were added to each tube, along with 0.5 g of sterile 0.1 mm glass beads (BioSpec Products Inc., Bartlesville, OK, USA). Samples underwent bead beating in a Precellys® 24 lysis homogenization system (Bertin Technologies, Montigny le Bretonneux, France) at 6500 rpm with three cycles of 30 seconds beating and 45 seconds rest. Samples were then incubated at 56°C for a minimum of 30 minutes and then at 95°C for 10 minutes. Two hundred µl of Buffer AL (Qiagen) were added and samples were mixed by pulse vortexing for 15 seconds followed by incubation at 70°C for 10 minutes. To precipitate the DNA, 200 µl of absolute ethanol were added and the samples were mixed by pulse vortexing for 15 seconds.

Samples were applied to QIAamp® Mini Spin Columns (Qiagen) using a micropipettor, avoiding transfer of glass beads into the column. Biofilm samples from four pegs were combined at this point (samples from two 2 mL tubes with two pegs each were pooled). Samples were added to columns in 2 to 3 aliquots, followed by centrifugation in a 5415D microcentrifuge (Eppendorf, Mississauga, ON, Canada). All centrifugation steps were carried out at 16 000 relative centrifugal force (rcf). Columns were then washed with 500 µl of Buffer AW1 (Qiagen), centrifuged for 1 minute, washed with 500 µl of Buffer AW2 and centrifuged for 3 minutes. Columns were transferred into new collection tubes (Qiagen) and centrifuged for an additional minute. DNA was eluted in 60 µl of Buffer AE for 30 minutes at room temperature and DNA was collected by centrifugation for 1 minute. Samples were quantified by absorbance at 260 nm using an Ultrospec 200 UV (ultraviolet)/visible spectrophotometer (BioChrom, Cambridge, UK).

3.6.4.2 DNA Extraction for T-RFLP Analysis of Biopsies or Biofilms

DNA extractions for T-RFLP analysis were performed on single biopsies or biofilms using the procedure described in Section 3.6.4.1 without the bead-beating step. When DNA was extracted from colonies of agar plates a swab was used to transfer the bacteria in the 2 mL screw-cap tubes. Biofilm samples were not pooled when DNA was extracted for T-RFLP analysis.

3.6.5 T-RFLP

T-RFLP is a PCR-based technique for semi-quantitative analysis of community structure. Figure 1 describes the steps involved in T-RFLP analysis. Genomic DNA from a bacterial community is used as template DNA in a PCR reaction using universal primers for 16S rRNA genes. The forward primer is labelled on the 5' end with a fluorescent probe, resulting in 5'-labelled products. Products are digested with a restriction enzyme and digests are run on a sequencing gel, from which an electropherogram is produced. An electropherogram is a graph with T-RF size in base pairs (bp) on the x-axis and relative fluorescence units (rfu) on the y-axis. Different T-RF sizes are produced by restriction enzymes cutting in different locations of the PCR product (due to 16S rRNA gene sequence variability). Peak area (in rfu) is correlated to abundance of a particular T-RF within the community.

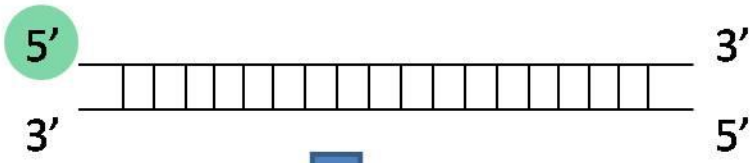
T-RFLP analysis was carried out on DNA extracted from biopsies and biofilms. Reaction mixtures for PCR (50 µl each) consisted of 1 µl template DNA (5 ng/µl), 5 µl 10x PCR Reaction Buffer (without $MgCl_2$; Invitrogen, Burlington, ON, Canada), 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTP mix (10 mM), 1 µl forward primer (10 pmol/µl), 1 µl reverse primer (10 pmol/µl), 0.25 µl recombinant *Taq* polymerase and 39.25 µl ddH_2O. VIC-labelled universal Eubacterial forward primer 8f (5'-VIC®-AGA GTT TGA TCC TGG CTC AG-3') was purchased from Applied Biosystems (Streetsville, ON, Canada). VIC® is a green fluorescent dye which exhibits maximum absorption at 538 nm and maximum emission at 552 nm. Universal Eubacterial reverse primer 926r (5'-CCG TCA ATT CCT TTG AGT TT-3') was purchased from Sigma Aldrich. Recombinant *Taq* polymerase

Figure 1. Steps involved in T-RFLP analysis.

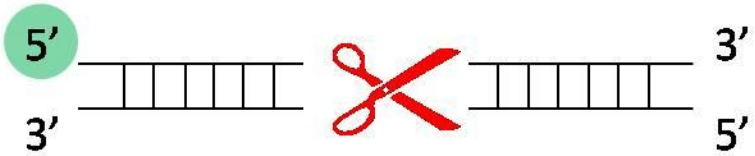
DNA is extracted from the bacterial community. PCR is conducted using universal primers for 16S rRNA genes. The forward primer is labelled on the 5' end with a fluorescent probe, resulting in 5'-labelled products. Products are digested with a restriction enzyme and digests are run on a sequencing gel, from which an electropherogram is produced. Each peak on the electropherogram is representative of a different sized labelled fragment (size in bp is represented along the x-axis of the electropherogram). Peak area (in rfu) is indicative of the relative abundance of that fragment.

DNA Extraction

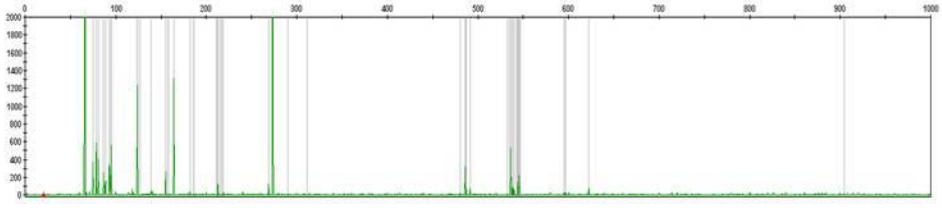
PCR Amplification
with fluorescent primers



Restriction Digest



Electropherogram



and $MgCl_2$ were acquired from Invitrogen and all other PCR reagents were purchased from Qiagen.

PCR reactions were carried out in a GeneAmp 2400 PCR System thermocycler (Applied Biosystems). The reaction conditions were as follows: 94°C for 2 minutes, 25 cycles of 94°C, 56°C and 72°C for 1 minute each, and a final extension at 72°C for 10 minutes. PCR reactions were run in triplicate, pooled and purified using the QIAquick® PCR purification kit (Qiagen). Samples were eluted in 30 µl elution buffer. Purified PCR products were digested with *Hpa*II (Invitrogen) overnight at 37°C. Reaction mixtures (20 µl total) consisted of the following: 5 µl PCR product, 2 µl 10x RE^{ACT}® 8 buffer (Invitrogen), 1 µl *Hpa*II (20 units/µl; Invitrogen) and 12 µl ddH_2O. Digests were stopped by incubation at 65°C for 15 minutes. Digested products were then purified with the QIAquick® PCR purification kit and eluted in 40 µl elution buffer. Five µl of the purified product were submitted to the University of Calgary Core DNA Services (http://www.ucalgary.ca/dnalab/; Calgary, AB, Canada) for fragment analysis using an 3730xl (96 capillary) genetic analyzer (Applied Biosystems). The LIZ1200 size standard (Applied Biosystems) was used to size fragments along with the G5 filter set to detect the VIC®-labelled fragments. T-RFLP data was analyzed using GeneMapper 3.0 Software (Applied Biosystems).

A virtual digest of the "H.Q. 16S Gut Organisms" Database (Sepehri *et al.*, 2007) of the RDP (http://rdp.cme.msu.edu/) (Cole *et al.*, 2007; Cole *et al.*, 2009) was conducted using the Microbial Community Analysis (MiCA) III program (Department of Biological Sciences, University of Idaho, http://mica.ibest.uidaho.edu/) (Shyu *et al.*, 2007). MiCA sensitivity was set to allow 3 mismatches within 15 bp from the 5' end of the primer.

Terminal restriction fragment (T-RF) sizes obtained from T-RFLP analysis were matched to fragment sizes in the virtual digest with *Hpa*II. If fragments could not be assigned to a phylum with at least 90% confidence they were labelled as "unknown".

3.6.6 Group-Specific PCR

DNA extracted from biopsies and biofilms was analyzed using PCR with group-specific primers. Table 3 summarizes the primers used for group-specific PCR as well as qPCR. Reactions mixtures (50 µl total) consisted of 0.5 µl DNA template, 5 µl 10X PCR Reaction buffer (Invitrogen), 3 µl 50 mM $MgCl_2$ (Invitrogen), 1 µl dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP); Qiagen), 1 µl forward primer (50 pmol/µl), 1 µl reverse primer (50 pmol/µl), 0.25 µl recombinant *Taq* polymerase (Invitrogen) and 38.25 µl ddH_2O. PCR reaction conditions are summarized in Table 4.

3.6.7 Electrophoresis

PCR products were separated on 0.7% to 1.0% agarose gels (made with Ultrapure Agarose; Invitrogen) in 1X Tris-borate-EDTA (TBE) buffer (10.9 g Tris, 5.5 g H_3BO_3 and 0.465 g EDTA per L). One µl loading dye (4 g $C_{12}H_{22}O_{11}$ and 25 mg bromophenol blue (Bio-Rad, Mississauga, ON, Canada, Mississauga, ON, Canada) in 10 mL ddH_2O) was added to 5 µl product and the samples were applied to the wells of an agarose gel, along with the appropriate ladder (1 Kb^+ DNA ladder or 50 bp DNA ladder; Invitrogen). Gels were run in a Sub-Cell GT MINI gel electrophoresis unit (Bio-Rad, Mississauga, ON, Canada, Mississauga, ON, Canada) at 70-100 volts for 30 to 50 minutes. Products were visualized with a UV transilluminator (Bio-Rad, Mississauga, ON, Canada) after

Table 3. Primers used for PCR and qPCR studies

Target bacteria	Primer[a]	Sequence (5'→3')	Reference
All Eubacteria	Uni331F Uni797R	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTATCCTGTT	(Nadkarni *et al.*, 2002)
Bacteroides distasonis	BD-1 BD-2	GTCGGACTAATACCGCATGAA TTACGATCCATAGAACCTTCAT	(Wang *et al.*, 1996)
Bacteroides fragilis group	g-BfraF g-BfraR	CACR[b]GTAAACGATGGATGCC GGTCGGGTTGCAGACC	(Matsuki *et al.*, 2002)
Bacteroides-Prevotella species	Bac303F Bac708R	GAAGGTCCCCCACATTG CAATCGGAGTTCTTCGTG	(Bartosch *et al.*, 2004; Bernhard & Field, 2000)
Bacteroides thetaiotaomicron	BT-1 BT-2	GGCAGCATTTCAGTTTGCTTG GGTACATACAAAATTCCACACGT	(Wang *et al.*, 1996)
Bacteroides vulgatus	BV-1 BV-2	GCATCATGAGTCCGCATGTTC TCCATACCCGACTTTATTCCTT	(Wang *et al.*, 1996)
Bifidobacterium adolescentis	BIA-1 BIA-2	GGAAAGATTCTATCGGTATGG CTCCCAGTCAAAAGCGGTT	(Wang *et al.*, 1996)
Bifidobacterium longum	BIL-1 BIL-2	GTTCCCGACGGTCGTAGAG GTGAGTTCCCGGCATAATCC	(Wang *et al.*, 1996)
Clostridium clostridiiforme	CC-1 CC-2	CCGCATGGCAGTGTGTGAAA CTGCTGATAGAGCTTTACATA	(Wang *et al.*, 1996)
Clostridium coccoides group	g-Ccoc-F g-Ccoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	(Matsuki *et al.*, 2002)
Eubacterium biforme	EBI-1 EBI-2	GCTAAGGCCATGAACATGGA GCCGTCCTCTTCTGTTCTC	(Wang *et al.*, 1996)
Faecalibacterium prausnitzii	FPR-1 FPR-2	AGATGGCCTCGCGTCCGA CCGAAGACCTTCTTCCTCC	(Wang *et al.*, 1996)
Lactobacillus acidophilus	LAA-1 LAA-2	CATCCAGTGCAAACCTAAGAG GATCCGCTTGCCTTCGCA	(Wang *et al.*, 1996)
Peptostreptococcus productus	PSP-1 PSP-2	AACTCCGGTGGTATCAGATG GGGGCTTCTGAGTCAGGTA	(Wang *et al.*, 1996)
Prevotella species	g-Prevo-F g-Prevo-R	CACR[b]GTAAACGATGGATGCC GGTCGGGTTGCAGACC	(Matsuki *et al.*, 2002)

[a]Primers were purchased from the University of Calgary Core DNA Services (http://www.ucalgary.ca/dnalab/; Calgary, AB, Canada).
[b]R = purine.

Table 4. PCR reaction conditions

Primer set	Product size (bp)	Reaction conditions	Reference
g-BfraF, g-Bfra-R	527-529	94°C for 7 minutes, 40x [94°C for 20 seconds, 55°C for 20 seconds, 70°C for 30 seconds], 72°C for 5 minutes	(Matsuki *et al.*, 2002)
g-Ccoc-F, g-Ccoc-R	438-441		
g-Prevo-F, g-Prevo-R	527-529		
CC-1, CC-2	255	95°C for 7 minutes, 35x [94°C for 30 seconds, 55°C for 10 seconds, 74°C for 33 seconds], 74°C for 2 minutes, 45°C for 2 seconds	(Wang *et al.*, 1996)
BD-1, BD-2	273		
BT-1, BT-2	423		
BV-1, BV-2	287		
BIA-1, BIA-2	244		
BIL-1, BIL-2	153		
EBI-1, EBI-2	46		
FPR-1, FPR-2	199		
LAA-1, LAA-2	286		
PSP-1, PSP-2	268		

staining for 15-20 minutes with 0.5 µg/mL ethidium bromide in TBE buffer. Gels were photographed using a Gel Doc 2000 and visualized using Quantity One Software (Bio-Rad, Mississauga, ON, Canada).

3.6.8 Quantitative PCR

Quantitative PCR (qPCR) was used to measure specific bacterial groups in biopsy and biofilm samples. The groups that were quantified were total Eubacteria, *Bacteroides-Prevotella* species and the *C. coccoides* group (primers are listed in Table 3). qPCR reactions were carried out using the iQ5 Real-Time PCR Detection System and iQ5 software (Bio-Rad, Mississauga, ON, Canada). Reaction mixtures (25 µl total) were made up of 4 µl template DNA (5 ng/µl), 1 µl each primer (50 pmol/µl), 12.5 µl 2X iQ SYBR Supermix (Bio-Rad, Mississauga, ON, Canada) and 6.5 µl ddH_2O. Reaction conditions are listed in Table 5. All qPCR reactions were followed by melt curve analysis from 60°C to 99°C with a temperature increase of 1°C per second.

qPCR reactions were performed in duplicate, and if threshold cycle (Ct) values for the replicates differed by more than 1.0 cycles the experiment was repeated. Positive and negative controls were used to confirm primer targets, and product melt curves were examined to confirm product specificity. Standard curves (Ct verses $\log_{10}$ cfu equivalents) were created for each experiment using serial dilutions of a known quantity of DNA from a single species. Cfu equivalents were calculated from ng of DNA using the following equation (Harrow *et al.*, 2007): [ng of DNA x $6.02x10^{11}$ molecules/pmol x 1000 pmol/nmol] / [genome size (bp) x 650 ng/nmol (average molecular weight of a bp) x average rRNA operon copy number]. Cfu equivalents for each sample were calculated

Table 5. qPCR reaction conditions

Target	Primers	Reaction conditions	Reference
All Eubacteria	Uni331F Uni797R	95°C for 3 minutes, 35x [95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds]	(Nadkarni *et al.*, 2002)
Bacteroides-Prevotella species	Bac303F Bac708R	95°C for 3 minutes, 35x [95°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds]	(Bartosch *et al.*, 2004; Bernhard & Field, 2000)
C.coccoides group	g-Ccoc-F g-Ccoc-R	95°C for 3 minutes, 40x [94°C for 20 seconds, 55°C for 20 seconds and 72°C for 30 seconds]	(Wang *et al.*, 1996)

using Ct values and the standard curve.

Average genome sizes were determined from a review by Fogel *et al.* (1999) and 16S rRNA gene copy number was determined from the Ribosomal RNA Operon Copy Number Database (http://ribosome.mmg.msu.edu/rrndb/index.php) (Klappenbach *et al.*, 2001; Lee *et al.*, 2008). Table 6 lists average genome sizes and rRNA operon copy numbers used in calculating cfu equivalents for qPCR Ct values. For Eubacteria, average genome size and rRNA operon copy number for gastrointestinal bacteria was calculated based on the following phylum composition: 79.5% Firmicutes, 17% Bacteroidetes, 2.5% Actinobacteria, 1% Proteobacteria (Tap *et al.*, 2009).

3.6.9 Confocal Scanning Laser Microscopy (CSLM)

To visualize biofilms grown in the CBD, pegs with adherent biofilm growth were broken from the CBD lid with sterile pliers. Pegs were rinsed in 200 µl 0.9% saline and stained using the Live/Dead® BacLight™ Kit (Molecular Probes, Burlington, ON, Canada), which consists of Syto-9 (488 nm excitation, green emission, labels live and dead cells due to its freely diffusible nature) and propidium iodide (543 nm excitation, red emission, only permeates cells with compromised membranes). One and one half µl of each dye (Syto-9 and propidium iodide) were added to 1 mL of PBS. Pegs were stained in 200 µl of the dye solution for 30 minutes at room temperature in the dark. Pegs were then rinsed in PBS and visualised using a Leica Microsystems (Bannockburn, IL, USA) DM IRE2 spectral confocal and multiphoton microscope with a Leica TCS SP2 acoustic optical beam splitter (AOBS). The live/dead stain images were processed and quantified using Imaris^{TM} (Bitplane Scientific Software, Saint Paul, MN, USA).

Table 6. Average genome sizes and rRNA operon copy numbers used in calculating cfu equivalents from qPCR values.

Target group	Average genome size[a]	Average rRNA operon copy number[b]
All Eubacteria	3 461 483 bp	5.62
Bacteroides-Prevotella species	4 104 000 bp	6.25
C. coccoides group	3 469 000 bp	9.43

[a]as published by Fogel *et al.* (1999)
[b]as listed in the Ribosomal RNA Operon Copy Number Database (http://ribosome.mmg.msu.edu/rrndb/index.php)(Klappenbach *et al.*, 2001; Lee *et al.*, 2008).

3.6.10 Data Analysis and Statistics

Graphs were generated using Prism 5 Software (GraphPad Inc., La Jolla, CA, USA) and Excel 2007 (Microsoft Canada Co., Mississauga, ON, Canada). Mann-Whitney U tests and Kruskal-Wallis tests (KW) with Dunn's multiple comparison post-tests (Dunn's) were performed using Prism 5 Software. Statistical significance was defined as $p<0.05$. Error bars on graphs represent standard deviation (SD). Sample numbers and replicates are indicated for each experiment. Where possible, at least four samples were taken per treatment group.

Chapter Four: Developing a Model for Commensal-Pathogen Biofilm Interactions

4.1 Introduction

The study of interactions between groups of bacteria is a vast field. For example, bacterial interference, a phenomenon by which commensal flora prevent pathogens from colonizing and infecting a host, has been widely studied in a number of models (Reid *et al.*, 2001; Valdéz *et al.*, 2005). Mechanisms of bacterial interference are beginning to be elucidated (Macpherson & Harris, 2004; Silva *et al.*, 1987), but the biofilm mode of growth has not yet been studied as a factor in interactions between commensal and pathogenic organisms. Recently, Macfarlane and colleagues (2006; 2007) visualized multispecies biofilms in the human colon and on food particles in the human digestive tract. These studies indicate that the biofilm mode of growth is utilized by commensal flora and may be an important factor in bacterial interference.

4.2 Aims

In order to study interactions between commensal and pathogenic biofilms, an *in vitro* model was developed using the CBD. This chapter describes the development of the model, which required three steps:

1. Assess and optimize biofilm formation by representative commensal and pathogenic bacteria in the CBD.
2. Form and characterize biofilms of two species in the CBD (biofilm co-cultures).
3. Develop a system to study interactions between two biofilms that are formed independently then brought together (biofilm-biofilm interactions).

To accomplish the first step (assess and optimize biofilm formation in the CBD), representative commensal and pathogenic species were chosen. EPEC, EHEC and UPEC were chosen as representative pathogens because they have been well characterized. EPEC and EHEC have been used as pathogens in model systems of gastrointestinal infection (Ceponis *et al.*, 2003; Humphries *et al.*, 2009). *E. coli* HB101 is commonly used to represent commensal flora for in vitro studies of host-microbe interactions (Lewis *et al.*, 2008; Nazli *et al.*, 2004; Nazli *et al.*, 2006). *L. plantarum* and VSL#3 are commercially available probiotics but are also part of the normal flora of the colon (Young & Schmidt, 2008), and therefore were chosen as representative commensals. Biofilm formation by the representative commensal and probiotic species was optimized on both the peg and well surfaces of the CBD. The ability for biofilms to form on both the peg and well surfaces of the device was crucial to the third step of model development, which involved bringing two independently established biofilms together.

Biofilm co-culture (formation of biofilms in the CBD using two or more strains of bacteria) was the second step involved in developing a model to study biofilm interactions in vitro. Biofilms of one pathogenic strain (EPEC) and one commensal strain (*L. plantarum*) were formed and studied on the pegs of the CBD. In addition, the antibiotic susceptibility of EPEC within these biofilm co-cultures was examined.

The final step involved in model development was to establish interactions between two independently formed biofilms (biofilm-biofilm interactions). The CBD was used to form biofilms of two species separately and then the two biofilm communities were brought into contact with one another. Therefore, biofilms of one species on the pegs of the CBD were exposed to biofilms of another species in the wells

of the device. The sizes of the interacting biofilm communities were monitored to determine the impact the two communities had on each other.

The last experiment in this chapter studied the impact of spent media from a mixed population of bacteria from the human colon on EPEC biofilms. This experiment is an example of work which can be conducted using this model system. Eventually, the commensal biofilms will not be made up of a single laboratory strain, but rather will be composed of complex bacterial communities from the human colon. In the experiment described here, biofilms of colon bacteria were not used, but the secreted factors from these communities were examined for their ability to impact EPEC biofilm size.

4.3 Results

4.3.1 Assessment and Optimization of Representative Commensal and Pathogenic Bacteria in the CBD

The first step in the development of an in vitro model for the study of interactions between commensal and pathogenic biofilms was to optimize growth by representative commensal and pathogenic species in the CBD. Biofilm growth on the pegs of the CBD was observed under numerous conditions and biofilm recovery from the CBD for enumeration was also optimized. Finally, biofilm growth on the well surfaces of the CBD was optimized. These were key steps in model development because they allowed for two biofilms to be grown independently and then brought together to study interactions.

4.3.1.1 Biofilm Growth Curves

In order to evaluate biofilm growth by gastrointestinal bacteria, growth curve experiments using representative pathogenic and commensal bacteria were performed using the CBD. Biofilms were sampled every two hours (starting at time zero) for the first ten hours and then periodically for a period of up to two days. EPEC was used as a representative pathogen, while *E. coli* HB101 and *L. plantarum* served as representative commensal organisms. EPEC and *E. coli* HB101 were grown on uncoated, mucin-coated, and FCS-coated pegs, while *L. plantarm* was only grown on uncoated pegs. EPEC grew the largest biofilms, with populations as large as $1x10^6$ cfu per peg forming on the uncoated pegs at 12 hours (Figure 2A). Beyond 12 hours EPEC growth was highest on the mucin-coated pegs, with growth peaking at 24 hours on both the mucin-coated and FCS-coated pegs. *E. coli* HB101, on the other hand, had very poor growth on mucin-coated pegs, with populations of approximately $1x10^3$ cfu per peg on uncoated and FCS-coated pegs (Figure 2B). *E. coli* HB101 growth did not increase past 24 hours. *L. plantarum* also formed small biofilms, peaking just above $1x10^3$ cfu per peg on uncoated pegs at 4 hours (Figure 3).

4.3.1.2 Recovering the Biofilms for Enumeration

Sonication time and Tween-20 content in media during sonication were investigated in terms of their ability to impact biofilm recovery from the pegs of the CBD. In some cases, use of a detergent such as Tween-20 has been shown to improve recovery of bacteria from the pegs of the CBD (Davies *et al.*, 2007). None of the sonication times tested (between 6 and 45 minutes) caused a difference in bacterial

Figure 2. EPEC and *E. coli* HB101 biofilm growth curves in the CBD.

EPEC (A) and *E. coli* HB101 (B) were grown in the CBD in LB on uncoated (red circles), FCS-coated (blue squares), and mucin-coated (black triangles) pegs. Biofilm growth was sampled periodically. A minimum of four samples were taken per timepoint. Error bars represent SD.

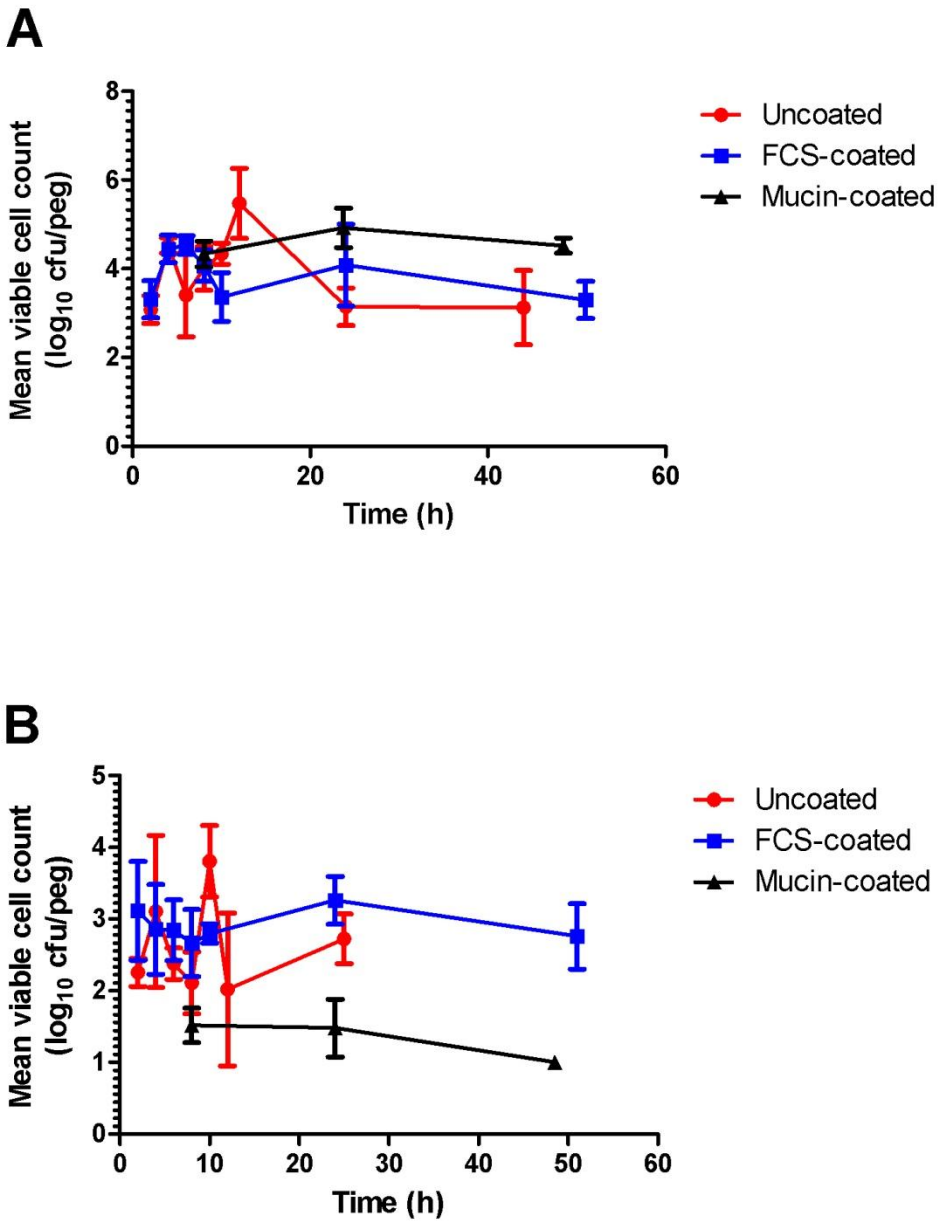
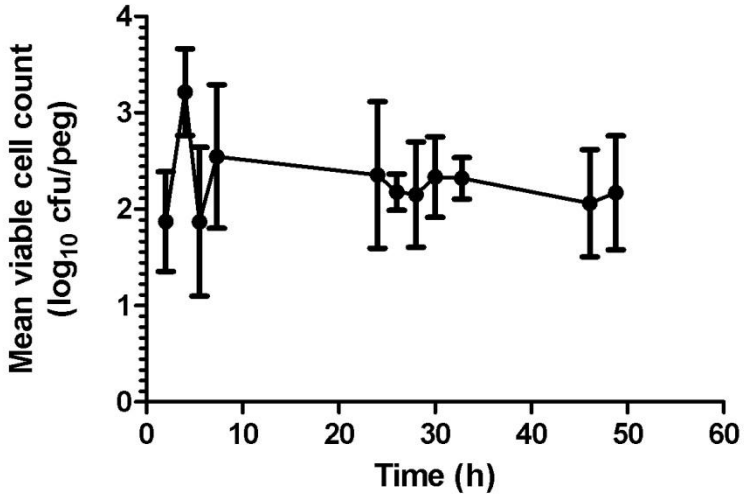

A
Mean viable cell count
(log10 cfu/peg)
Time (h)
Uncoated
FCS-coated
Mucin-coated
B
Mean viable cell count
(log10 cfu/peg)
Time (h)
Uncoated
FCS-coated
Mucin-coated

Figure 3. *L. plantarum* biofilm growth in the Calgary Biofilm Device.

L. plantarum was grown in the Calgary Biofilm Device in MRS broth on uncoated pegs. Biofilm growth was sampled periodically. Data is representative of two replicates (with a minimum of four data points per replicate). Error bars represent SD.



recovery from the CBD for EPEC (p=0.3890; Figure 4A). *E. coli* HB101 recovery was highest at 10 minutes (Kruskal-Wallis (KW) p=0.0111, significantly greater than 15 minutes, Dunn's $p<0.05$), but was also high at 6 and 30 minutes (Figure 4B). Ten minutes was chosen as the sonication time for subsequent experiments. The addition of 1% Tween-20 to the saline used for biofilm recovery during sonication did not significantly alter cfu per peg recovered for EPEC (p=0.0571) or *E. coli* HB101 (p=0.4000) (Figure 5). Therefore, Tween-20 was not used in recovery media for subsequent experiments.

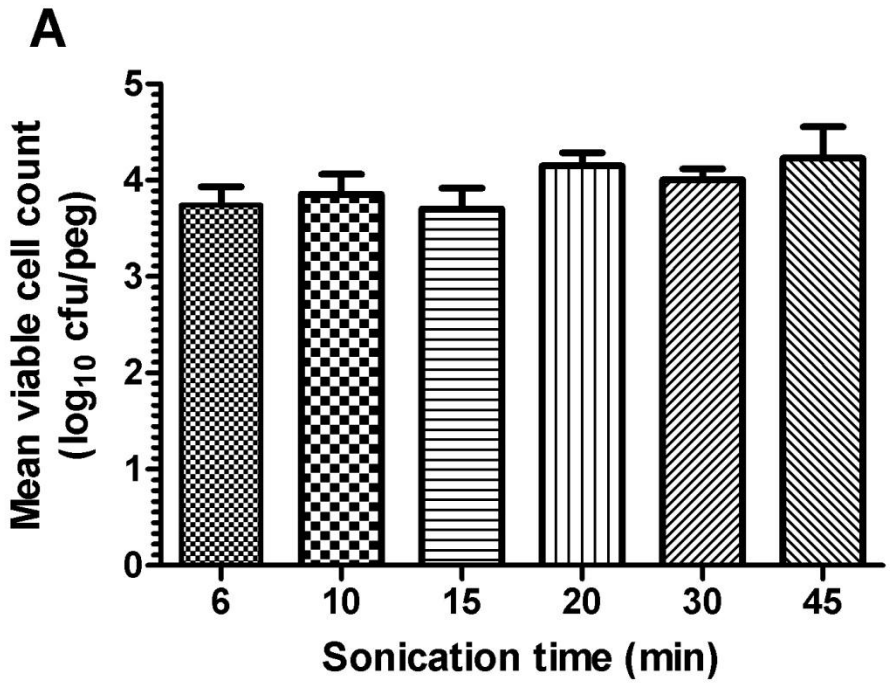
4.3.1.3 Biofilm Optimization

In order to optimize biofilm formation in the CBD, growth assays were performed under various conditions. Factors that were tested during optimization included growth medium type (LB, MRS, mMRS, TSB, mTSB), viscosity of growth medium (with or without 0.08% agar) and shaking speed (static, 50 rpm, or 150 rpm).

Three pathogenic *E. coli* strains, EPEC, EHEC and UPEC, were optimized for biofilm formation in the CBD. All three strains formed substantial biofilms under all conditions tested, although optimal conditions for biofilm formation differed between strains (Figure 6). For EPEC, growth in mTSB plus 0.08% agar with shaking was significantly higher than LB with shaking (KW p=0.0075, Dunn's $p<0.05$), but all other conditions were significantly equivalent (Figure 6A). EHEC growth was significantly equivalent under all conditions tested except mTSB plus 0.08% agar with shaking at 150 rpm verses LB shaking at 150 rpm (KW p=0.0077, Dunn's $p<0.05$) (Figure 6B). UPEC biofilm growth was significantly lower than mTSB plus 0.08% agar with (KW p=0.0011,

Figure 4. Effect of sonication time on recovery of EPEC and *E. coli* HB101 from biofilms on the CBD.

EPEC (A) and *E. coli* HB101 (B) were grown in the CBD in LB on uncoated pegs. Cfu per peg were assessed after 6, 10, 15, 20, 30 and 45 minutes of sonication in saline. A minimum of four samples were taken for each sonication time. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (* denotes $p<0.05$ verses indicated group). For EPEC, no difference was found between sonication times ($p=0.3890$). *E. coli* HB101 recovery differed based on sonication time (KW $p=0.0111$), with Dunn's multiple comparison test finding a difference between 10 and 15 minutes of sonication ($p<0.05$).


A
Mean viable cell count
(log10 cfu/peg)
0
1
2
3
4
5
6
10
15
20
30
45
Sonication time (min)

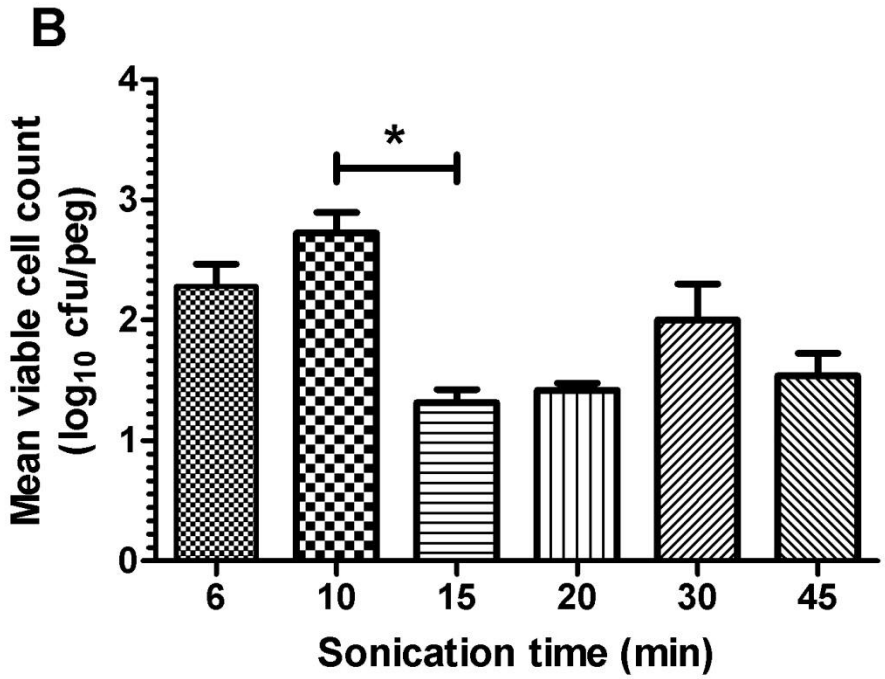
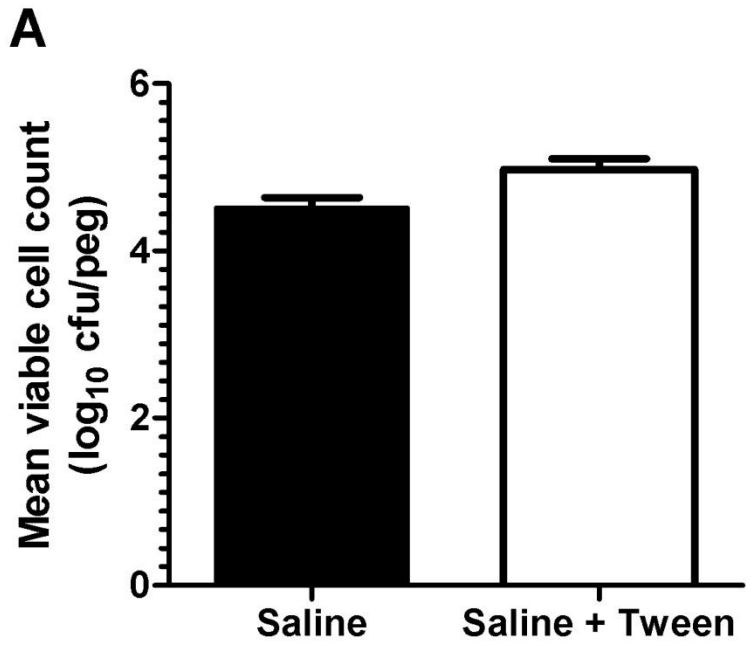

B
Mean viable cell count
(log10 cfu/peg)
0
1
2
3
4
*
6
10
15
20
30
45
Sonication time (min)

Figure 5. Effect of Tween-20 on recovery of EPEC and *E. coli* HB101 from biofilms on the CBD.

EPEC (A) and *E. coli* HB101 (B) were grown in the CBD in LB on uncoated pegs. Cfu per peg were assessed after sonication in saline or saline plus 1% Tween-20. A minimum of four samples were taken for each condition. Error bars represent SD. Data were analyzed using a Mann-Whitney U test. Tween-20 did not significantly improve recovery of EPEC ($p=0.0571$) or *E. coli* HB101 ($p=0.4000$).


A
Mean viable cell count
(log10 cfu/peg)
6
4
2
0
Saline
Saline + Tween

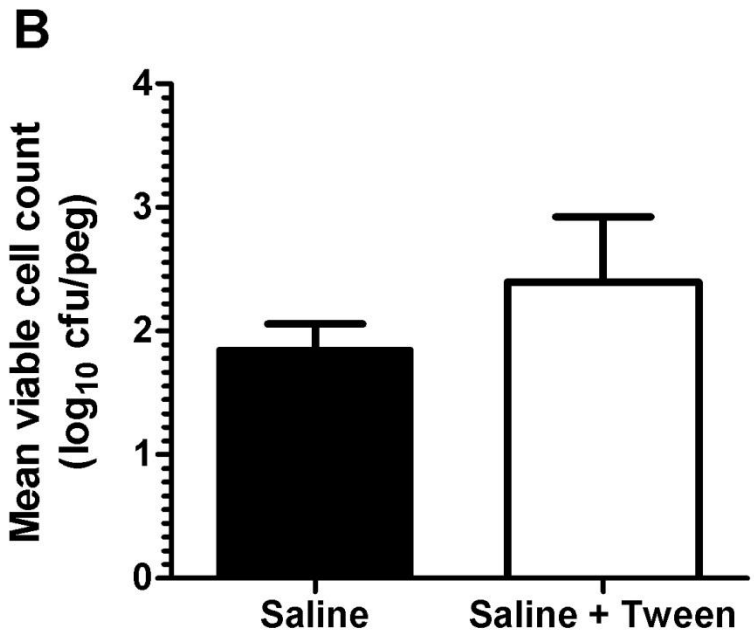
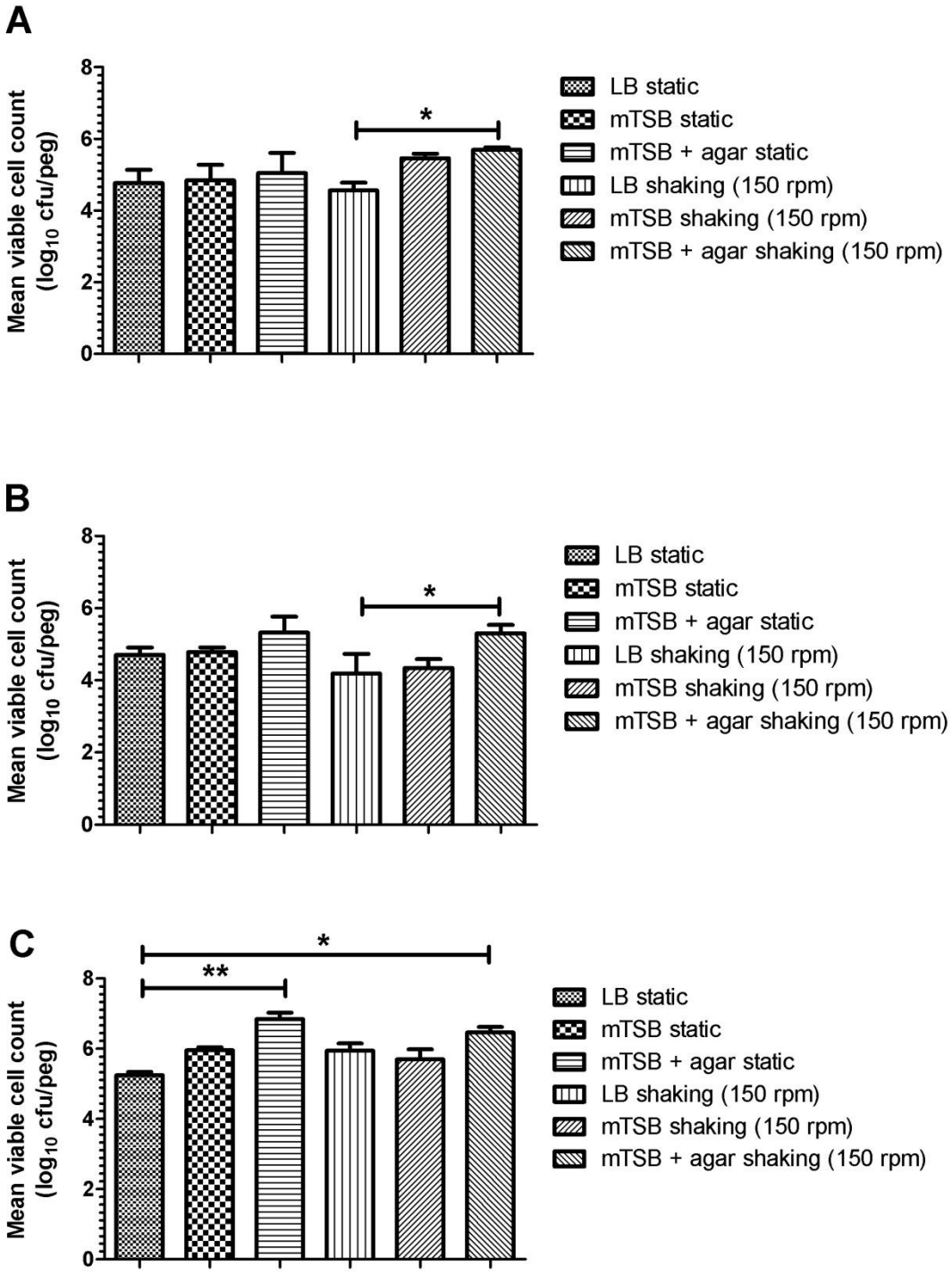

B
Mean viable cell count
(log10 cfu/peg)
4
3
2
1
0
Saline
Saline + Tween

Figure 6. Optimization of biofilm growth by EPEC, EHEC and UPEC in the CBD.

Biofilm formation by EPEC (A), EHEC (B) and UPEC (C) was measured in three broths (LB, mTSB and mTSB with 0.08% agar) in both shaking (150 rpm) and static conditions. A minimum of four samples were taken for each condition. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (* denotes $p<0.05$ verses indicated groups, ** denotes $p<0.01$ verses indicated groups). For EPEC, growth in mTSB plus 0.08% agar with shaking was significantly higher than LB with shaking (KW $p=0.0075$, Dunn's $p<0.05$), but all other conditions were significantly equivalent. EHEC growth was significantly equivalent under all conditions tested except mTSB plus 0.08% agar with shaking at 150 rpm verses LB shaking at 150 rpm (KW $p=0.0077$, Dunn's $p<0.05$). UPEC biofilm growth was significantly lower than mTSB plus 0.08% agar with (KW $p=0.0011$, Dunn's $p<0.01$) and without shaking (KW $p=0.0011$, Dunn's $p<0.05$).


A
Mean viable cell count (log_{10} cfu/peg)
0
2
4
6
8
*
LB static
mTSB static
mTSB + agar static
LB shaking (150 rpm)
mTSB shaking (150 rpm)
mTSB + agar shaking (150 rpm)
B
Mean viable cell count (log_{10} cfu/peg)
0
2
4
6
8
*
LB static
mTSB static
mTSB + agar static
LB shaking (150 rpm)
mTSB shaking (150 rpm)
mTSB + agar shaking (150 rpm)
C
Mean viable cell count (log_{10} cfu/peg)
0
2
4
6
8
**
*
LB static
mTSB static
mTSB + agar static
LB shaking (150 rpm)
mTSB shaking (150 rpm)
mTSB + agar shaking (150 rpm)

Dunn's $p<0.01$) and without shaking (KW $p=0.0011$, Dunn's $p<0.05$) (Figure 6C). While differences between the groups were indicated (KW $p=0.0077$), the only significant difference was between mTSB plus 0.08% agar with shaking at 150 rpm and LB shaking at 150 rpm (Dunn's $p<0.05$). UPEC biofilm formation was statistically equivalent under most conditions tested, with the exception of LB static, which yielded significantly lower growth than mTSB plus 0.08% agar with or without shaking (KW $p=0.0011$, Dunn's $p<0.01$ for mTSB plus 0.08% agar static and $p<0.05$ for mTSB plus 0.08% agar shaking).

Biofilm formation by commensal organisms was also measured in the CBD. Like the pathogenic *E. coli* strains, mTSB-based broths yielded the best biofilm formation by *E. coli* HB101, with optimal biofilm formation in mTSB plus 0.08% agar after 24 hours of static incubation (significantly higher than LB shaking, KW $p=0.0169$, Dunn's $p<0.05$) (Figure 7). *L. plantarum* formed the largest biofilms in MRS (a media formulated specifically for lactobacilli (de Man *et al.*, 1960)) with 0.08% agar and static incubation conditions after 24 hours (significantly higher than mTSB with shaking, KW $p=0.0087$, Dunn's $p<0.01$) (Figure 8). VSL#3, a mixture of eight commensal species, grew biofilms of between $5x10^2$ and $5x10^3$ cfu per peg under all conditions tested, with no condition yielding statistically larger biofilms than the others (KW $p=0.0354$, with no differences indicated by Dunn's multiple comparison test) (Figure 9A). VSL#3 biofilms were studied over a longer growth period (72 hours) to determine if these strains required a longer period to establish biofilms. Biofilms were placed in fresh media at 48 hours. Biofilm growth at 48 hours was similar to growth at 24 hours, whereas 72 hour biofilms were much larger, possibly due to the addition of fresh media at 48 hours (Figure 9B). There were no significant differences between the different growth conditions at 24 hours

Figure 7. Optimization of biofilm growth by *E. coli* HB101 in the CBD.

Biofilm formation was measured in three broths (mTSB, mTSB plus 0.08% agar, and LB) in static, 50 rpm, or 150 rpm shaking incubation conditions at 24 hours. At least four samples were taken for each growth condition. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (* denotes $p<0.05$ verses indicated group). Growth in mTSB plus 0.08% agar static was significantly greater than growth in LB with shaking (KW $p=0.0169$, Dunn's $p<0.05$).

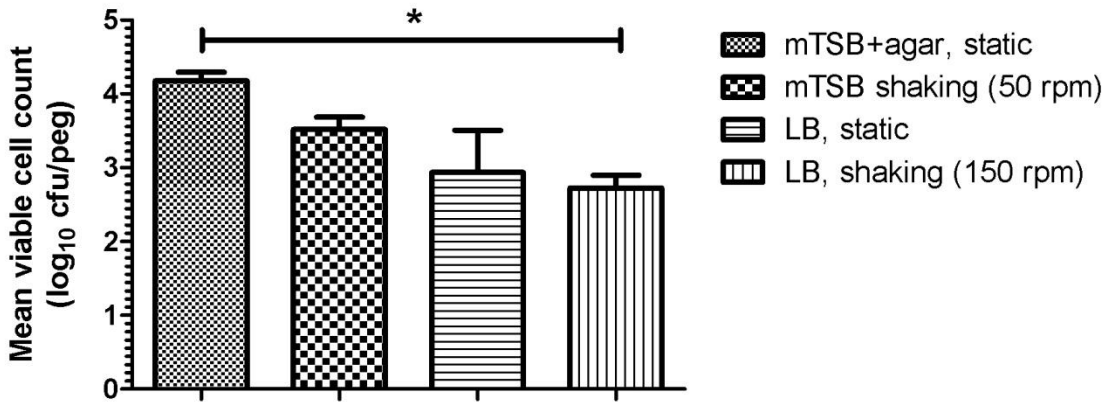


Figure 8. Optimization of biofilm growth by *L. plantarum* in the CBD.

Biofilm formation was measured in two broths (mTSB and MRS) with or without 0.08% agar after 24 hours of growth. Cultures in broths without agar were incubated with shaking at 150 rpm, cultures with agar in the media were incubated statically. Biofilm formation was conducted in an incubator with 5% CO_2. At least four samples were taken for each growth condition. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (** denotes $p<0.01$ verses indicated group). Growth in MRS plus 0.08% agar static was significantly greater than growth in MRS with shaking (KW $p=0.0087$, Dunn's $p<0.01$).

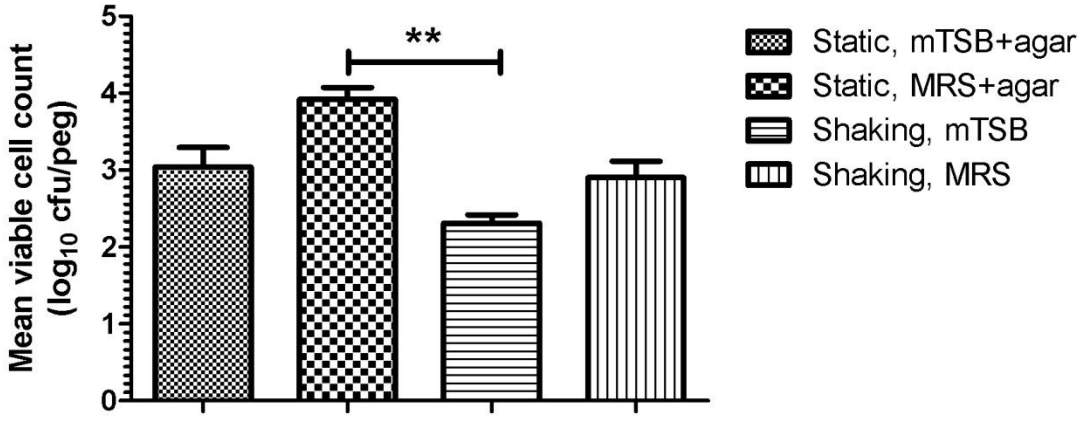
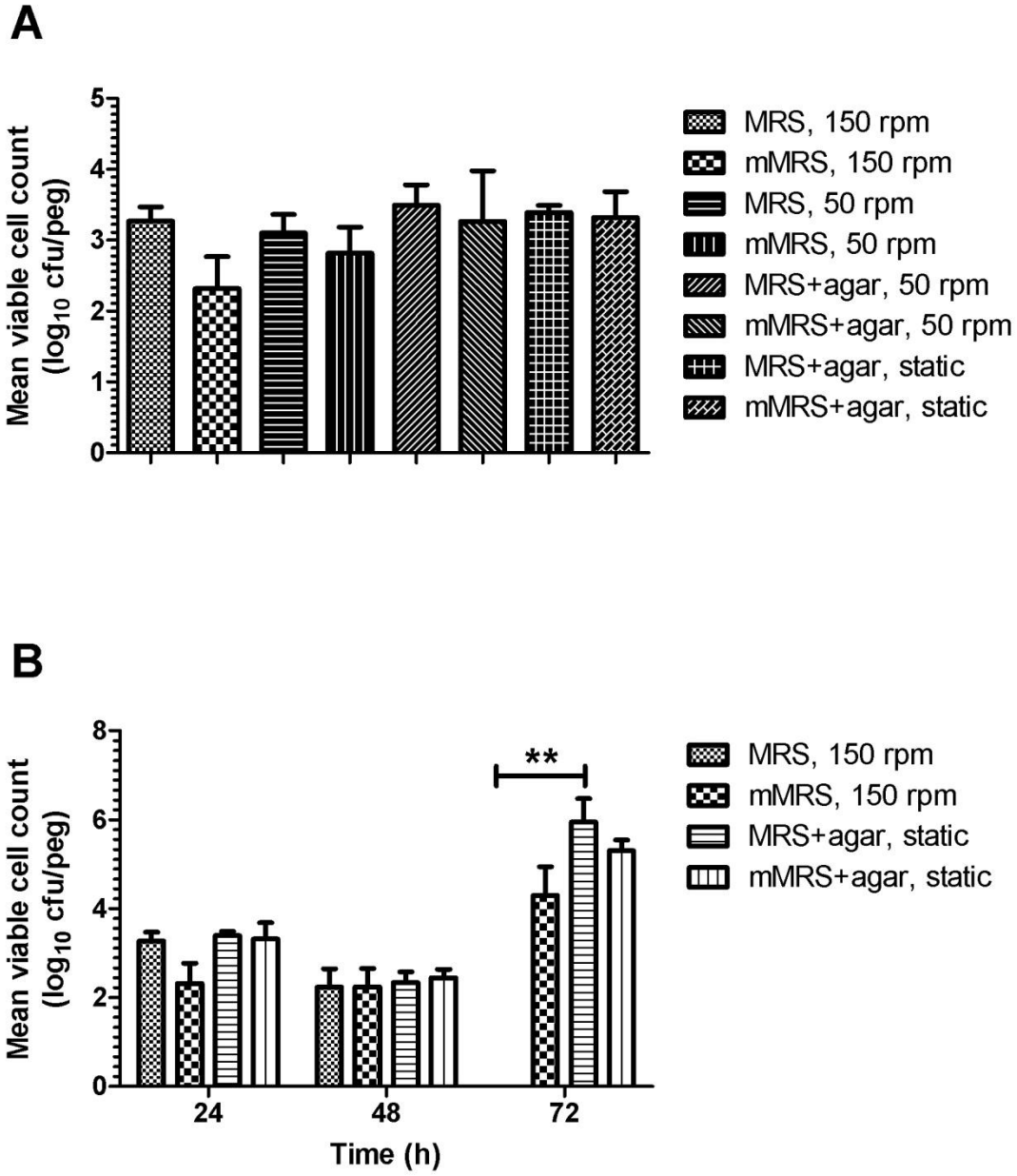


Figure 9. Optimization of biofilm growth by VSL#3 in the CBD.

Biofilm formation was measured at 24 hours (A) or over a 72 hour period (B). At 24 hours, biofilm formation was tested in both MRS and mMRS media, with or without 0.08% agar, and with shaking at 150 rpm (media without agar), 50 rpm (all media) and under static conditions (media with agar). During the 72 hour growth period, 150 rpm incubation (MRS and mMRS) and static incubation (MRS or mMRS plus 0.08% agar) were tested at 24, 48 and 72 hours. Growth medium was changed at the 48 hour timepoint. At least four samples were taken for each growth condition. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (** denotes $p<0.01$ verses indicated group). At 24 hours, biofilm formation did not differ significantly under any of the conditions tested (KW $p=0.0354$, Dunn's failed to find differences). At 48 hours, all conditions tested were also equivalent ($p=0.8505$). At 72 hours, growth in MRS with shaking was significantly lower than growth in MRS with 0.08% agar in static conditions (KW $p=0.0030$, Dunn's $p<0.01$).


A
Mean viable cell count
(log10 cfu/peg)
0
1
2
3
4
5
MRS, 150 rpm
mMRS, 150 rpm
MRS, 50 rpm
mMRS, 50 rpm
MRS+agar, 50 rpm
mMRS+agar, 50 rpm
MRS+agar, static
mMRS+agar, static
B
Mean viable cell count
(log10 cfu/peg)
0
2
4
6
8
**
24
48
72
Time (h)
MRS, 150 rpm
mMRS, 150 rpm
MRS+agar, static
mMRS+agar, static

(p=0.0354, Dunn's failed to find differences) or 48 hours (p=0.8505). At 72 hours, MRS plus 0.08% agar (static incubation) produced the largest biofilms, while biofilms in MRS with shaking at 150 rpm disappeared by 72 hours (KW p=0.0030, significantly lower than MRS with 0.08% agar in static conditions, Dunn's $p<0.01$). It is possible that these biofilms were loosely adherent and cells were dislodged from the substratum during the media change.

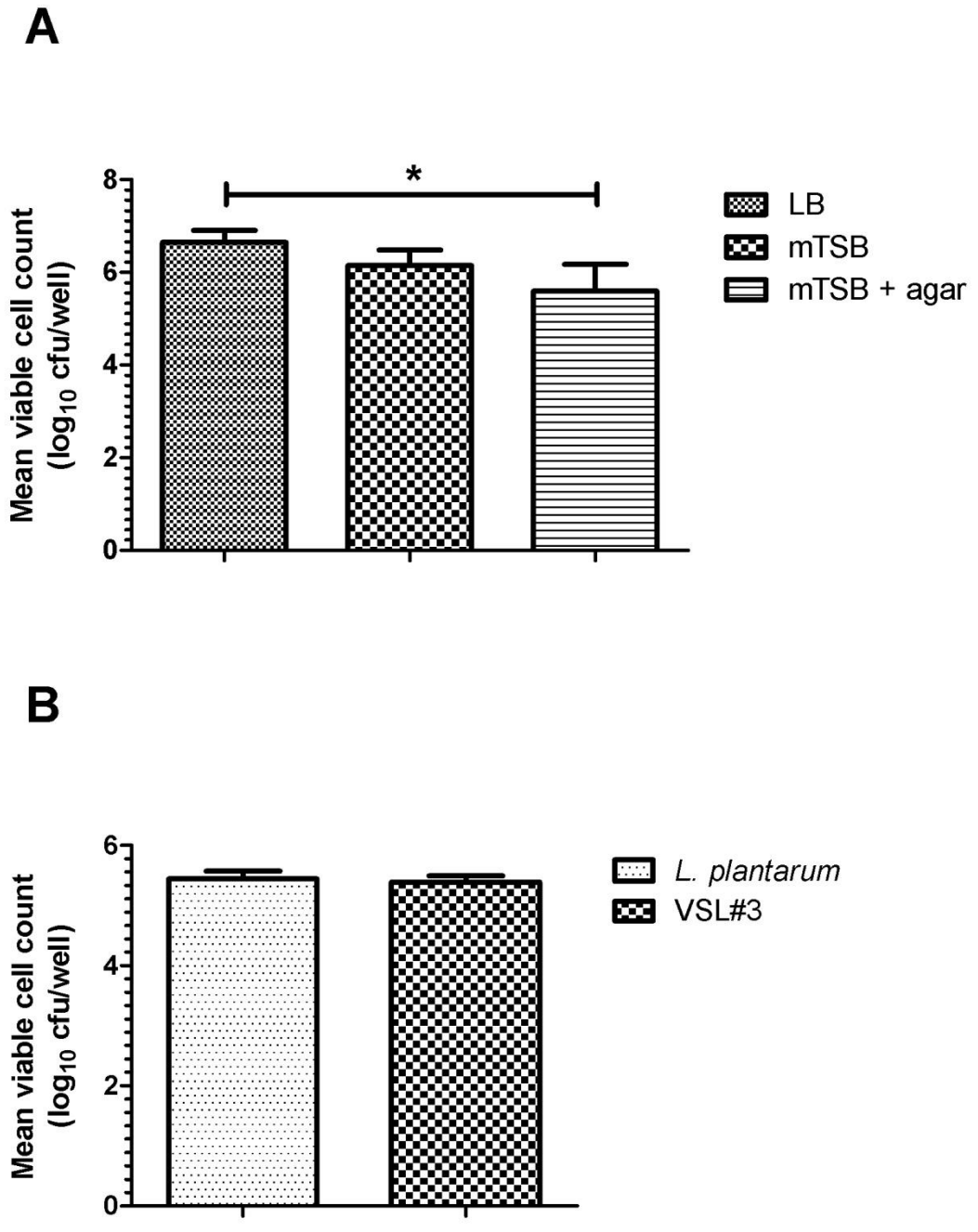
In general, the increased shear force generated by shaking conditions did not improve biofilm formation by the pathogenic and commensal strains examined here. This contradicts the general thought that fluid shear favours biofilm formation (Donlan & Costerton, 2002). Although bacteria in the gastrointestinal tract are certainly subject to shear force due to peristalsis and the flow of fecal material, the amplitude of these forces is unknown. The 50 and 150 rpm shaking conditions may not have mimicked the shear forces in the gastrointestinal tract and therefore did not improve biofilm formation. Alternatively, shear force may not be necessary for biofilm formation by these strains in this particular model system.

4.3.1.4 Growing Biofilms on the Wells of the CBD

Before biofilm-biofilm exposure experiments could be performed, biofilm formation had to be optimized in the wells of the CBD. Twenty-four hour biofilms of both pathogenic (EPEC) and commensal (*L. plantarum* and VSL#3) bacteria were larger in the wells than on the pegs of the device (Figure 10), likely due to increased surface area for growth. EPEC grew biofilms above $1x10^6$ cfu per well in all three media tested, with LB yielding the largest biofilms, which were statistically larger than mTSB plus

Figure 10. Optimization of biofilm growth in the wells of the CBD.

EPEC, *L. plantarum* or VSL#3 biofilms were grown in the wells of the CBD under static conditions for 24 hours. EPEC was grown in LB, mTSB and mTSB with 0.08% agar (A). *L. plantarum* and VSL#3 were grown in mTSB with 0.08% agar (B). A minimum of four samples were taken for each growth condition. Error bars represent SD. EPEC data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (* denotes $p<0.05$ verses indicated group). EPEC biofilm growth was statistically higher in LB than in mTSB plus 0.08% agar (KW $p=0.0170$, Dunn's $p<0.05$).


A
Mean viable cell count
(log10 cfu/well)
8
6
4
2
0
*
LB
mTSB
mTSB + agar
B
Mean viable cell count
(log10 cfu/well)
6
4
2
0
L. plantarum
VSL#3

0.08% agar (KW p=0.0170, Dun's p<0.05) (Figure 10A). *L. plantarum* and VSL#3 each grew biofilms of greater than $1x10^5$ cfu per well (Figure 10B).

4.3.2 Biofilm Co-Culture

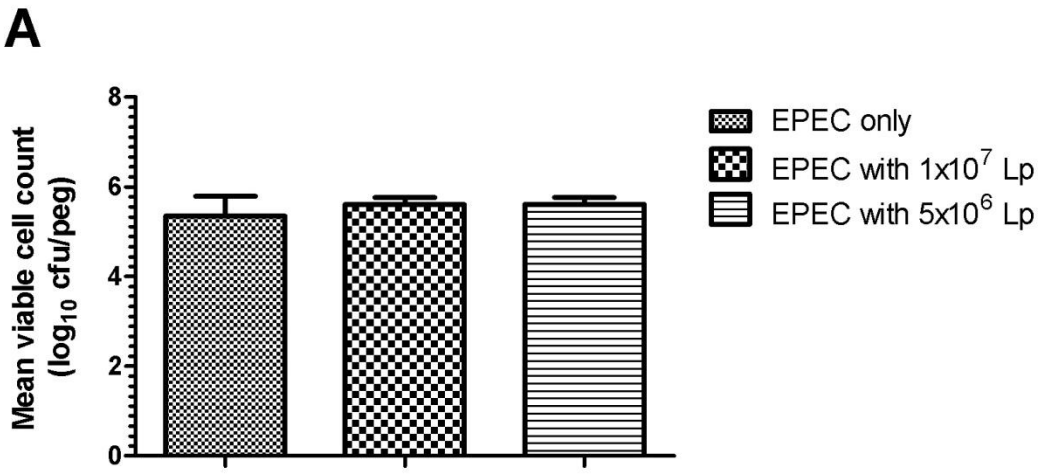
It can be argued that most biofilms in nature are composed of multiple species, rather than just one. This is certainly the case for the gastrointestinal tract, where hundreds of species are present (Mahowald *et al.*, 2009). Therefore, biofilm co-culture (formation of a biofilm by two or more species) was studied in the CBD using EPEC and *L. plantarum*. In addition, the antibiotic susceptibility of EPEC was studied in a monospecies biofilm verses in co-culture with a commensal (*L. plantarum*).

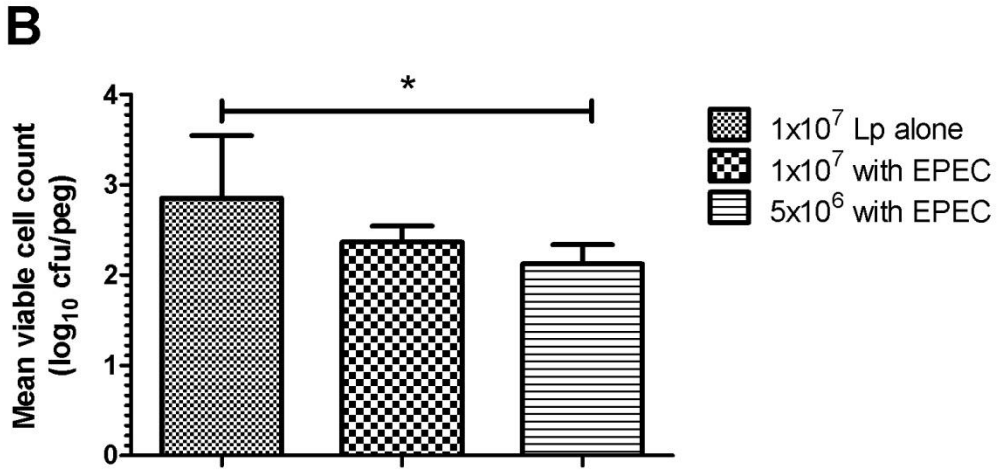
4.3.2.1 Biofilm Co-Culture of EPEC and L. plantarum

Biofilm co-cultures of EPEC and *L. plantarum* were formed to determine the ability of the two species to grow together in a biofilm. Biofilms of mixed inocula (containing both EPEC and *L. plantarum*) were grown for 24 hours in the CBD. The impact of *L. plantarum* on EPEC biofilm formation was studied (Figure 11A), as well as the impact of EPEC on *L. plantarum* (Figure 11B). The number of EPEC cfu per peg was not affected by the presence of *L. plantarum* in the biofilm inocula at $1x10^7$ cfu per mL or $5x10^6$ cfu per mL (p=3.691) (Figure 11A). This may be partially due to the small size of *L. plantarum* biofilms, although biofilm formation by the commensal strain was not significantly lower in the presence of EPEC when inoculated at a full dose, only when inoculated at a half dose (KW p=0.0222, Dunn's p<0.05 for *L. plantarum* monospecies

Figure 11. Enumeration of EPEC and *L. plantarum* within biofilm co-cultures.

EPEC and *L. plantarum* (Lp) were used to seed co-culture biofilms in the CBD. Inocula for the device consisted of $1x10^7$ cfu per mL of EPEC or *L. plantarum* alone, $1x10^7$ cfu per mL of each species, or $1x10^7$ cfu per mL EPEC and $5x10^6$ cfu per mL *L. plantarum*. Biofilms were formed for 24 hours. Graphs represent either EPEC (A) or *L. plantarum* (B) growth within the biofilm co-cultures (species were enumerated using selective media, LBA for EPEC and MRS agar for *L. plantarum*). Four to six samples were taken for each group, and the experiment was repeated two times. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (* denotes $p<0.05$ verses indicated group). EPEC biofilm size was the same whether *L. plantarum* was present or not ($p=0.3691$), while *L. plantarum* biofilms were the same size when the inoculum was $1x10^7$ cfu per mL (regardless of the presence of EPEC), but were significantly smaller when a smaller inoculum ($5x10^6$ cfu per mL) was used (KW $p=0.0222$, Dunn's $p<0.05$).


A
Mean viable cell count
(log10 cfu/peg)
8
6
4
2
0
EPEC only
EPEC with 1x10^7 Lp
EPEC with 5x10^6 Lp


B
Mean viable cell count
(log10 cfu/peg)
4
3
2
1
0
*
1x10^7 Lp alone
1x10^7 with EPEC
5x10^6 with EPEC

biofilms with $1x10^7$ cfu per mL verses $5x10^6$ cfu per mL of *L. plantarum* with $1x10^7$ cfu per mL of EPEC) (Figure 11B).

4.3.2.2 *Antibiotic Tolerance of EPEC in Biofilm Co-Cultures with* L. plantarum

To determine the effect of *L. plantarum* on the antibiotic susceptibility of pathogenic bacteria, EPEC antibiotic susceptibility was determined in EPEC biofilms and EPEC-*L. plantarum* biofilm co-cultures. MBC_{100}, the minimum concentration of antibiotic required to kill 100% of planktonic cells, was measured to determine planktonic susceptibility. MBEC, the minimum concentration of antibiotic required to eradicate biofilm growth, was measured to determine biofilm susceptibility. Twenty-four hour biofilms of EPEC alone or EPEC and *L. plantarum* were challenged with serial dilutions of antibiotics for 24 hours. After antibiotic exposure, planktonic and biofilm cultures were assessed for viability using plus/minus plating on LBA to determine MBC_{100} and MBEC values (as described in Section 3.5.2). Cultures were also plated on MRS agar, which confirmed that *L. plantarum* survived antibiotic exposure to a minimum concentration of the MBC_{100} and MBEC values of EPEC (data not shown). Antibiotics were chosen from several classes: aminoglycosides (kanamycin and gentamicin), β-lactams (ampicillin), cephalosporins (cefotaxime and cefazolin), fluoroquinolones (ciprofloxacin and levofloxacin) and rifamycins (rifampicin).

As shown in Table 7, EPEC antibiotic susceptibility in both planktonic and biofilm modes of growth was the similar whether EPEC was grown as a monoculture or in co-culture with *L. plantarum*. Although *L. plantarum* was found to have no effect on EPEC antibiotic susceptibility, these data demonstrate that this model can be used to

Table 7. Antibiotic susceptibility of EPEC in monoculture and in co-culture with *L. plantarum*.

Antibiotic	MBC_{100}* (µg/mL)		MBEC** (µg/mL)	
	EPEC alone	EPEC-Lp# co-culture	EPEC alone	EPEC-Lp co-culture
Ampicillin	4 - 16	4	2 - >16	4
Cefotaxime	0.06 - >1	0.06 - 0.5	0.06 - >1	0.06 - 1
Cefazolin	2 - >16	2 - 8	2- >16	2 - >16
Ciprofloxacin	0.03 - 0.06	0.03 - 0.06	0.03 - >1	0.03
Gentamicin	2 - 4	4 - 8	2 - 4	2 - 4
Kanamycin	8 - 16	16	8 - >16	≥16
Levofloxacin	0.06 - 0.125	0.06 - 0.125	0.06 - >1	0.06 - 0.125
Rifampicin	8 - >16	8 - >16	8 - >16	8 - >16

*MBC_{100} = minimum bactericidal concentration, a measurement of planktonic susceptibility to antibiotics. Ranges indicate data from experiments performed three times.

**MBEC = minimum biofilm eradication concentration, a measurement of biofilm susceptibility to antibiotics. Ranges indicate data from experiments performed three times.

#Lp=*Lactobacillus plantarum*.

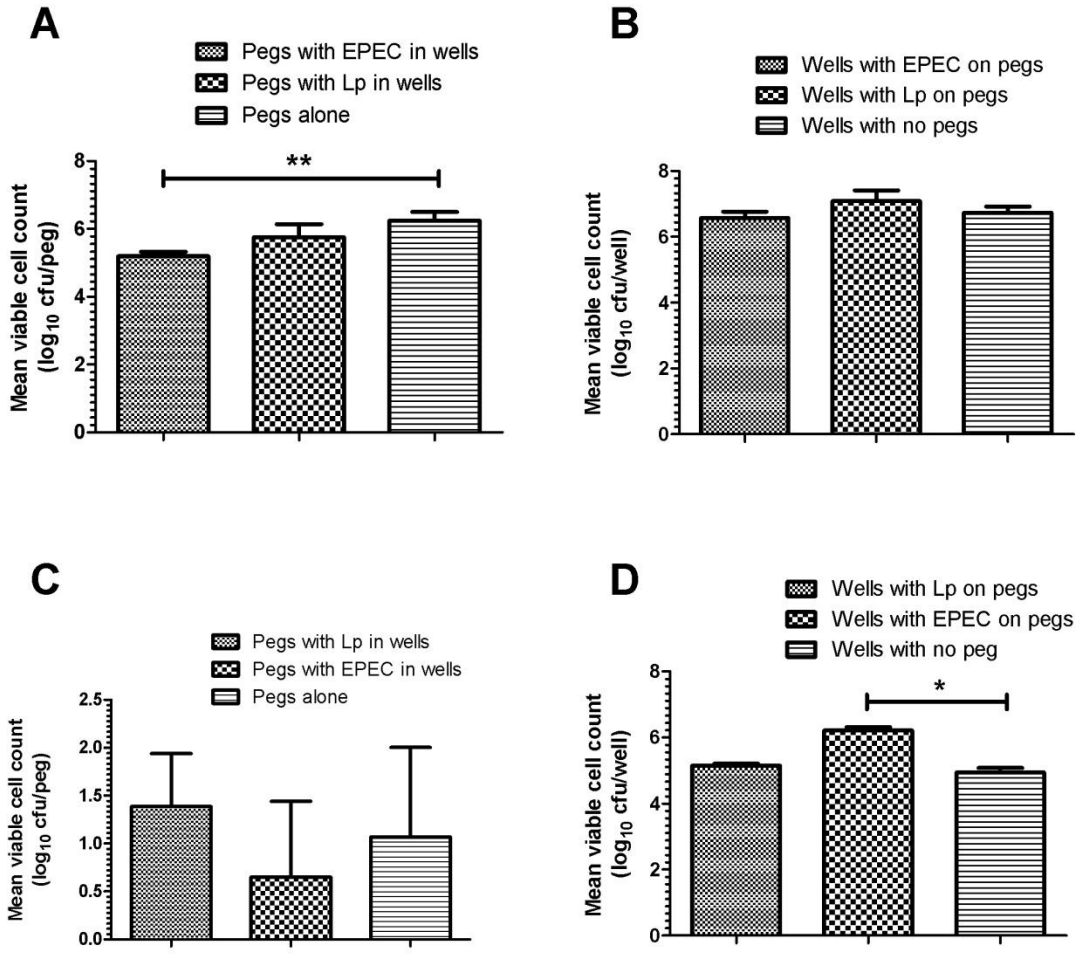
study the impact of one on organism on another organism's antibiotic susceptibility in a biofilm co-culture environment.

4.3.3 Biofilm-Biofilm Exposures

The final step in developing a model for the study of biofilm interactions was to grow two biofilms independently, then bring them together (biofilm-biofilm exposure). To further study interactions between EPEC and *L. plantarum*, biofilms of the two strains were formed separately for 24 hours and then brought together for a 24 hour challenge. Biofilm formation on both the pegs and wells of the CBD were studied (Figure 12). EPEC formation on both pegs (Figure 12A) and wells (Figure 12B) was not statistically different in the presence of *L. plantarum* biofilms on the wells or pegs of the CBD, respectively (KW $p=0.0125$ with Dunn's $p>0.05$ and $p=0.0528$, respectively). Similarly, *L. plantarum* peg and well biofilms were unchanged in the presence of EPEC biofilms ($p=0.5058$ and KW $p=0.0153$ with Dunn's $p>0.05$, respectively) (Figure 12C and D). As an additional control, after 24 hours of growth EPEC peg biofilms were placed in a well with sterile media (no biofilm) and EPEC well biofilms had their associated pegs removed. EPEC peg biofilms grew larger when another biofilm of the same species was not present (KW $p=0.0125$, Dunn's $p<0.01$) (Figure 12A), suggesting that peg and well biofilms of the same species do not contribute to the growth of one another (it is possible that lower total cell densities resulting from removing a second EPEC biofilm allowed EPEC peg biofilms to grow larger). EPEC well biofilms were similar in size whether or not another biofilm of EPEC was present ($p=0.0528$) (Figure 12B). The same control was performed for the *L. plantarum* biofilms. Growth of *L. plantarum* on the pegs

Figure 12. Exposure of EPEC and *L. plantarum* biofilms using the pegs and wells of the CBD as growth surfaces.

Biofilms of EPEC and *L. plantarum* (Lp) were formed on the pegs and wells of the CBD. After 24 hours, EPEC biofilms on pegs (A) and wells (B) were exposed to *L. plantarum* on wells (D) and pegs (C), respectively, for an additional 24 hours. At least four samples were taken for each condition. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (* denotes $p<0.05$ verses indicated group, ** denotes $p<0.01$ verses indicated group). EPEC peg biofilms were not affected by the presence of *L. plantarum*, but were smaller when a second biofilm of EPEC was present (KW $p=0.0125$, Dunn's $p<0.01$). EPEC well biofilms and *L. plantarum* peg biofilms did not differ under any of the conditions test ($p=0.0528$ and $p=0.5058$, respectively). *L. plantarum* growth peg growth was poor under all conditions tested, and the small numbers of adherent cells resulted in large standard deviations between replicates. *L. plantarum* well biofilms grew larger in the presence of EPEC (KW $p=0.0153$, Dunn's $p<0.05$ for *L. plantarum* in wells with no peg verses EPEC on peg).


A
Pegs with EPEC in wells
Pegs with Lp in wells
Pegs alone
**
Mean viable cell count
(log10 cfu/peg)
0
2
4
6
8
B
Wells with EPEC on pegs
Wells with Lp on pegs
Wells with no pegs
Mean viable cell count
(log10 cfu/well)
0
2
4
6
8
C
Pegs with Lp in wells
Pegs with EPEC in wells
Pegs alone
Mean viable cell count
(log10 cfu/peg)
0.0
0.5
1.0
1.5
2.0
2.5
D
Wells with Lp on pegs
Wells with EPEC on pegs
Wells with no peg
*
Mean viable cell count
(log10 cfu/well)
0
2
4
6
8

(p=0.5058) and wells (KW p=0.0153, Dunn's p>0.05) of the CBD was similar whether or not another biofilm of *L. plantarum* was present (Figure 12C and D). However, *L. plantarum* well biofilms with no *L. plantarum* peg biofilms were significantly smaller than *L. plantarum* well biofilms in the presence of an EPEC peg biofilm (KW p=0.0153, Dunn's p<0.05). Although one would expect *L. plantarum* biofilms to be smaller in the presence of a competing species, it is possible that *L. plantarum* was able to take advantage of the fact that EPEC forms larger biofilms, and make use of extracellular matrix material laid down by EPEC in order to form biofilms faster.

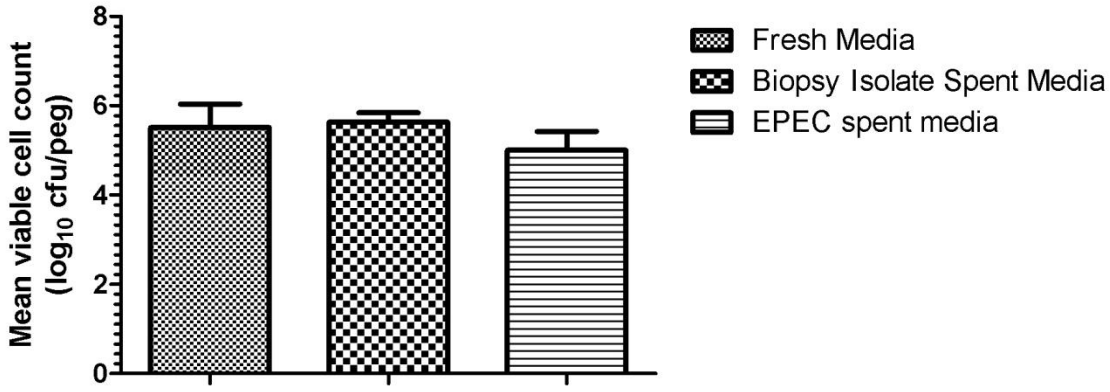
4.3.4 Impact of Secreted Factors from Colon Bacteria on EPEC Biofilms

In order to more closely represent the types of commensal bacteria a pathogen would be exposed to when in the gastrointestinal tract, EPEC biofilms were exposed to secreted factors from a multispecies community of human gastrointestinal bacteria Figure 13). This experiment is a prelude to the work in the following chapter, where biofilms of gastrointestinal microflora were formed. These microflora biofilms can be used to replace single commensal species (*L. plantarum* and *E. coli* HB101) in the model of biofilm interactions to more closely represent conditions in the gastrointestinal tract.

A bacterial community isolated from a human descending colon biopsy was cultured anaerobically for 24 hours, after which the culture was put through a 0.2 µm filter to remove bacteria (filtrate was spot-plated on Schaedler agar and incubated anaerobically to confirm that bacteria were removed). Unfiltered cultures were also spot-plated on Schaedler agar and incubated anaerobically to confirm bacterial growth. Twenty-four hour-old EPEC biofilm cultures (formed on the pegs of the CBD) were

Figure 13. EPEC biofilms challenged with spent media from anaerobic cultures of bacteria from the human colon.

EPEC biofilms (24 hours old) were exposed to spent media from an anaerobic culture of bacteria from the human colon for 24 hours. At least four samples were taken from each group, and the experiment was repeated twice. Error bars represent SD. Data were analyzed using a Kruskal-Wallis test. Anaerobic spent media did not significantly impact the size of EPEC biofilms after a 24 hour challenge (p=0.3519).



exposed to the anaerobic spent media or spent media from a planktonic EPEC culture. Anaerobic spent media did not significantly impact the size of EPEC biofilms after a 24 hour challenge (p=0.3519) (Figure 13).

4.4 Summary

This chapter recounts the process of developing a model for studying the interactions between two biofilms and within a biofilm co-culture. Several pathogenic and commensal strains were optimized for biofilm growth on the pegs and wells of the CBD. The pathogenic strains (EPEC, EHEC and UPEC) formed larger biofilms than the commensal strains (*E. coli* HB101, *L. plantarum* and VSL#3) under the conditions tested, supporting studies that have shown biofilm formation to be an important factor in pathogen persistence in the host (Parsek & Singh, 2003). However, biofilms need not be large to express biofilm behaviours, for example *Staphylococcus epidermidis* and *E. coli* exhibited a two-fold increase in antibiotic tolerance immediately upon attachment to a surface (Das *et al.*, 1998). The antibiotic susceptibility data presented in Table 7 supports this observation, since *L. plantarum* was able to increase EPEC antibiotic susceptibility, even though it was present in very small numbers within the biofilm co-cultures.

The small nature of commensal biofilms may be indicative of how biofilms of microflora form in the gastrointestinal tract, since a dense collection of cells may be viewed as a threat by the host immune system. In addition, it is unlikely commensal organisms form single-species biofilms in vivo. Rather, each species takes up a small niche in a diverse biofilm community, composed of organisms with different metabolic pathways and modes of growth (Macfarlane *et al.*, 1997). This may explain the biofilm

co-culture and biofilm-biofilm interaction experiments in this chapter, where *L. plantarum* and EPEC biofilms did not significantly alter each other. A more complex community is required in order for this model to truly represent biofilm interactions in the gastrointestinal tract. The following chapter attempts to address this problem by studying complex biofilm communities of bacteria from the human colon.

Chapter Five: Biofilm Formation by Gastointestinal Bacteria

5.1 Introduction

The previous chapter described the development of a model for studying interactions between two independently-formed biofilms using the CBD. This model could be useful for the study of many different bacterial interactions in the gastrointestinal tract and on other mucosal surfaces (for example, modeling interactions between normal flora and pathogens in the urogenital tract). A major weakness of the model, however, is that it employs a single model species (for example, *E. coli* HB101) or a simple, defined community (for example, VSL#3) to represent the whole intestinal microflora. This chapter details the process of establishing multispecies biofilms of bacteria from a specific mucosal site: the human colon. These complex biofilm communities can be used as a "representative microflora" in future experiments modeling bacterial biofilm interactions in the gastrointestinal tract and on other mucosal surfaces.

While it is recognized that the in vitro communities grown in this study will not exactly duplicate the diversity of bacterial communities in the colon, this model is the first attempt to create an in vitro "representative microflora" that is grown as a biofilm and seeded by mucosal bacterial communities (rather than fecal bacteria, which may differ from mucosal bacteria). With mounting evidence in the literature for biofilms being the normal mode of growth for microflora in the colon (Macfarlane *et al.*, 1997; Macfarlane & Dillon, 2007), it is felt that an in vitro model of microflora biofilms will be useful in learning more about the many diseases which involve a change in the

gastrointestinal microflora, such as Crohn's disease and ulcerative colitis (Kleessen *et al.*, 2002; Macfarlane & Dillon, 2007).

5.2 Aims

In order to establish a "representative microflora", the following steps were necessary:

1. Isolate a diverse bacterial population from the human colon that can serve as a representative community of mucosal bacteria.
2. Establish biofilms from this complex community (aiming to maintain its diversity).
3. Characterize the biofilms formed in terms of diversity.
4. Determine the feasibility of using these communities in biofilm interaction studies, including:
    a. Quantifying differences in biopsy communities and the biofilms they form:
        i. Within patients: left (descending) colon verses right (ascending) colon.
        ii. Between patients.
    b. The ability to freeze communities for future use while maintaining diversity.

5.3 Results

5.3.1 Colon Biopsy Collection

The first step in establishing an in vitro "representative microflora" was to isolate mucosal bacterial communities from the human colon. Mucosal biopsies of the left (descending) or right (ascending) colon were collected from healthy volunteers undergoing screening colonoscopy. Biopsies from three different collection events were used to generate the data in this chapter. The first two sets of biopsies consisted of five and eight descending colon biopsies, respectively, and were used to develop the methods for examining the third set of biopsies, which consisted of 48 samples from three different patients. Table 8 summarizes the biopsy sets and the experiments each biopsy set was used for. Biopsies were processed to extract bacteria for analysis (see Section 3.6.1).

5.3.2 Biofilm Formation by Bacteria from the Human Colon

Once bacteria were extracted from the colon biopsies, it was necessary to determine if they could be grown as biofilms in vitro. Biofilm growth of the biopsy isolates was studied under both aerobic and anaerobic conditions.

5.3.2.1 Biofilm Formation by Aerobic Bacteria from the Human Colon

Mucosal biopsies of the colon were collected from two individuals (patients W and X) undergoing screening colonoscopy (Table 8). Biopsies W1 and W2 were from patient W, biopsies X1, X2 and X3 were from patient X. The biopsies were processed, serially diluted and spot-plated on BHIA for viable cell counting (see Section 3.3.2).

Table 8. Biopsies used for culture and molecular studies

Collection date	Biopsies for culture[a]	Biopsies for molecular analysis[b]	Data
June 2008	Biopsies W1, W2 (Patient W), Biopsies X1, X2, X3 (Patient X) All biopsies from the left colon	none	Aerobic bacteria counts (Figure 14), Aerobic biofilm formation (Figure 15)
October 2008	Biopsies Y1, Y2 (Patient Y), Biopsies Z1, Z2 (Patient Z) All biopsies from the left colon	Biopsies Y3, Y4 (Patient Y), Biopsies Z3, Z4 (Patient Z) All biopsies from the left colon	Aerobic biofilm formation (Figure 16, Figure 17), Anaerobic biofilm formation (Figure 18, Table 9) T-RFLP (Figure 20, Figure 21, Figure 22), Group-specific PCR (Table 10)
July 2009	Immediate culture: Biopsies A1, A2, B1, B2, C1, C2 (Patient A, B or C, left colon) Biopsies A9, A10, B9, B10, C9, C10 (Patient A, B or C, right colon) Frozen before culture: Biopsies A3, A4, B3, B4, C3, C4 (Patient A, B or C, left colon) Biopsies A11, A12, B11, B12, C11, C12 (Patient A, B or C, right colon)	Biopsies A5-A8, B5-B8, C5-C8 (Patient A, B or C, left colon) Biopsies A13-A16, B13-B16, C13-C16 (Patient A, B or C, right colon)	Biopsy community structure (Figure 24, Figure 25, Figure 26), Biofilm imaging (Figure 27), Biofilm community structure (Figure 28, Figure 29, Figure 30, Figure 31), Community structure of biofilms from frozen colon bacteria (Figure 32, Figure 33, Figure 34)

[a] Biopsies were used to form biofilms, from which DNA was extracted.
[b] DNA was extracted directly from biopsies.

The bacteria that grew on the BHIA plates represented the aerobic bacteria present in the biopsies. Not all biopsies yielded aerobic bacteria, but biopsies W1, W2, and X2 had approximately $1x10^3$ cfu per biopsy (Figure 14). These samples were frozen at -70°C for future experiments.

Biofilms were formed from the frozen aerobic bacteria in the CBD. Samples were cultured overnight on BHIA and then used to prepare the inocula (see Section 3.3.1). Biofilms were grown for 24 hours under static conditions or with shaking at 150 rpm. All three samples formed biofilms in the CBD (biofilm sizes ranged from $2.8x10^4$ to $3.5x10^5$ viable cells per peg), with biopsy samples W1 and X2 forming biofilms of similar size under static and shaking conditions ($p=0.6423$ and 0.2454, respectively). Biopsy W2 formed larger biofilms under static conditions compared to shaking at 150 rpm ($p=0.0294$) (Figure 15).

Biopsies were collected from a second set of two patients in order to more closely examine bacteria from the human colon (patients Y and Z; Table 8). Biopsies Y1-4 were from patient Y, while biopsies Z1-4 were from patient Z. Aerobic biofilms were started directly from the biopsies, rather than from frozen bacteria extracted from the biopsies (as in Figure 15), and were allowed to grow for 48 hours. At 24 hours, aerobic bacteria from patient Y (biopsies Y1 and Y2) formed large biofilms (similar in cfu counts to those formed from frozen bacteria; Figure 15), while biopsies Z1 and Z2 (from patient Z) failed to form aerobic biofilms (Figure 16). The quantity of bacteria in the biofilms produced from biopsies Y1 and Y2 increased only a slight amount between 24 and 48 hours of growth (Figure 16). The aerobic bacteria that participated in biofilm formation were examined by plating on MacConkey agar. All colonies grew reddish-pink with a bile

Figure 14. Aerobic bacteria recovered from human colon biopsies.

Bacterial counts represent aerobic bacteria isolated from left (descending) colon biopsies. Biopsies W1 and W2 were from one patient, while biopsies X1-3 were from a second patient. A single sample was taken for each biopsy.

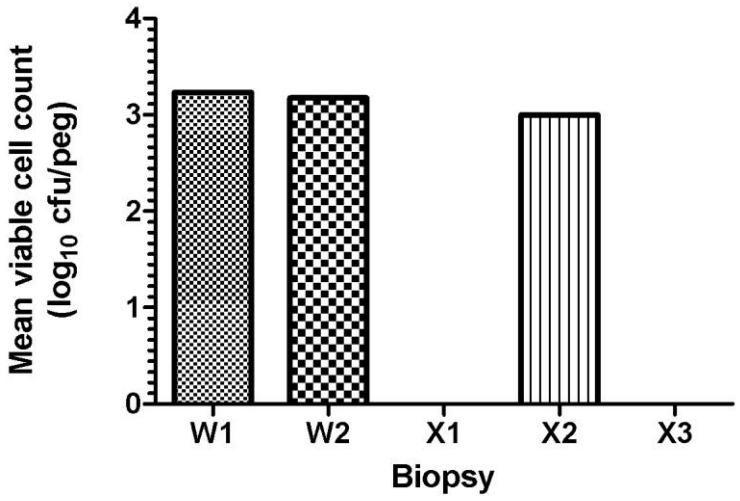


Figure 15. Bacteria recovered from biofilms formed from frozen stocks of aerobic bacteria isolated from colon biopsies.

Frozen biopsy isolates were used to form biofilms in the CBD under static conditions or shaking at 150 rpm for 24 hours in BHI. Four samples were taken under each condition. Error bars represent SD. Growth under shaking verses static conditions for each sample was analyzed using a Mann-Whitney U test (* denotes $p<0.05$ verses indicated group). Biofilm growth by bacteria from biopsies W1 and X2 did not differ under shaking or static conditions ($p=0.6423$ and 0.2454, respectively), but growth by bacteria from biopsy W2 was significantly higher under static conditions ($p=0.0294$).

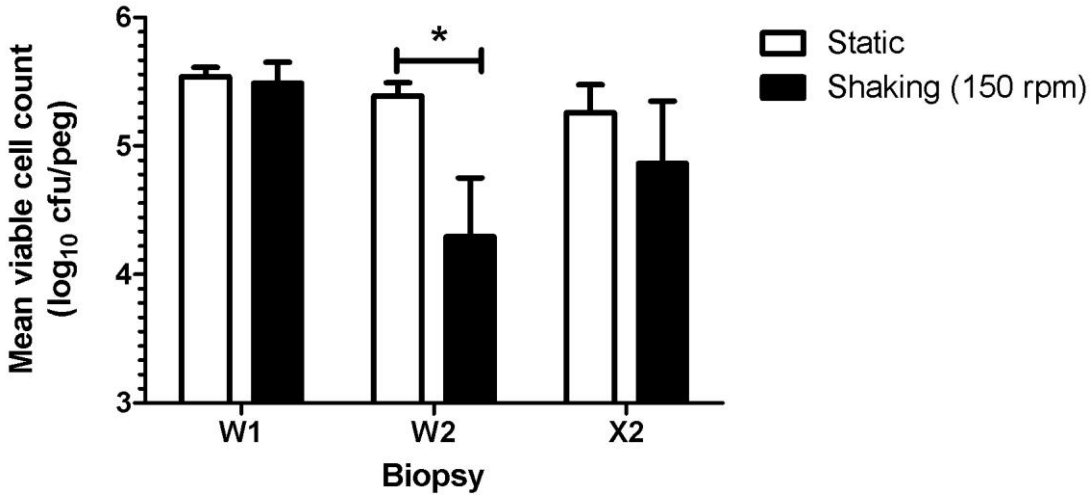
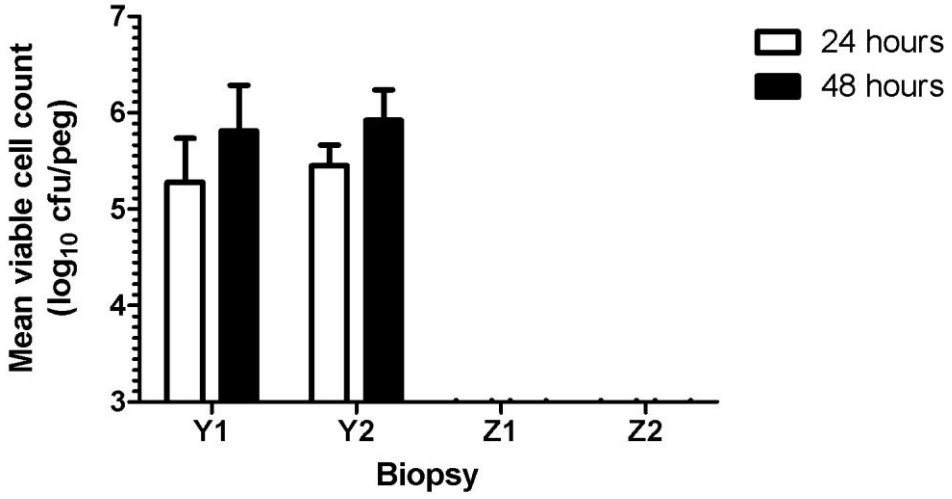


Figure 16. Bacteria recovered from biofilms of aerobic bacteria isolated from colon biopsies.

Immediately after biopsy collection biopsies were processed and used to form biofilms in the CBD under static conditions for 24 or 48 hours in BHI. Two samples were taken under each condition. Error bars represent SD.



precipitate (Figure 17), suggesting that the biofilms were predominately or solely made up of *E. coli* (Difco, 1985). Due to the lack of diversity found in the aerobic biofilms and the knowledge that the majority of bacterial species in the colon are anaerobes (Young & Schmidt, 2008), the remainder of the experiments characterizing bacteria from colon biopsies focused on the populations of anaerobic bacteria.

5.3.2.2 Biofilm Formation by Anaerobic Bacteria from the Human Colon

Biopsies from the second collection (biopsies Y1, Y2, Z1 and Z2; Table 8) were examined for anaerobic bacteria. The biopsies were processed in an anaerobic chamber and used immediately to seed biofilm formation in the CBD. Biopsy homogenates were added to sTSY to create the inocula. The CBD was incubated statically in the anaerobic chamber at 37°C. After 143 hours, biofilm growth was measured by viable cell counting on FAA and CBA. It should be noted that this process only enumerated bacteria that are culturable as pure colonies on agar media. This is likely an underestimation of the total size of the biofilms, since some bacteria may have grown in the complex community of the biofilm but could not be grown in pure culture. This phenomenon has been demonstrated in the dental microflora, where *Tannerella forsynthesis* (formerly *Bacteroides forsynthesis*) cannot be grown in pure culture because it lacks the ability to synthesize *N*-acetylmuramic acid, but thrives in co-culture with *Fusobacterium nucleatum* (Wade, 2002; Wyss, 1989). The anaerobic bacteria formed dense biofilms ranging between $2x10^3$ and $2x10^5$ cfu of culturable bacteria per peg, with the bacteria from patient Y (biopsies Y1 and Y2) forming larger biofilms than the bacteria from patient Z (biopsies Z1 and Z2) (Figure 18). Colony counts on FAA and CBA were

Figure 17. Bacteria recovered from aerobic biofilms formed from mucosal colon bacterial communities.

Bacterial communities isolated from the human colon were used to seed biofilm formation in the CBD under aerobic conditions. Biofilms were grown for 24 hours and then plated onto MacConkey agar. The pink colour of the colonies and precipitated bile suggest that *E. coli* was the most prevalent species in the biofilm community.

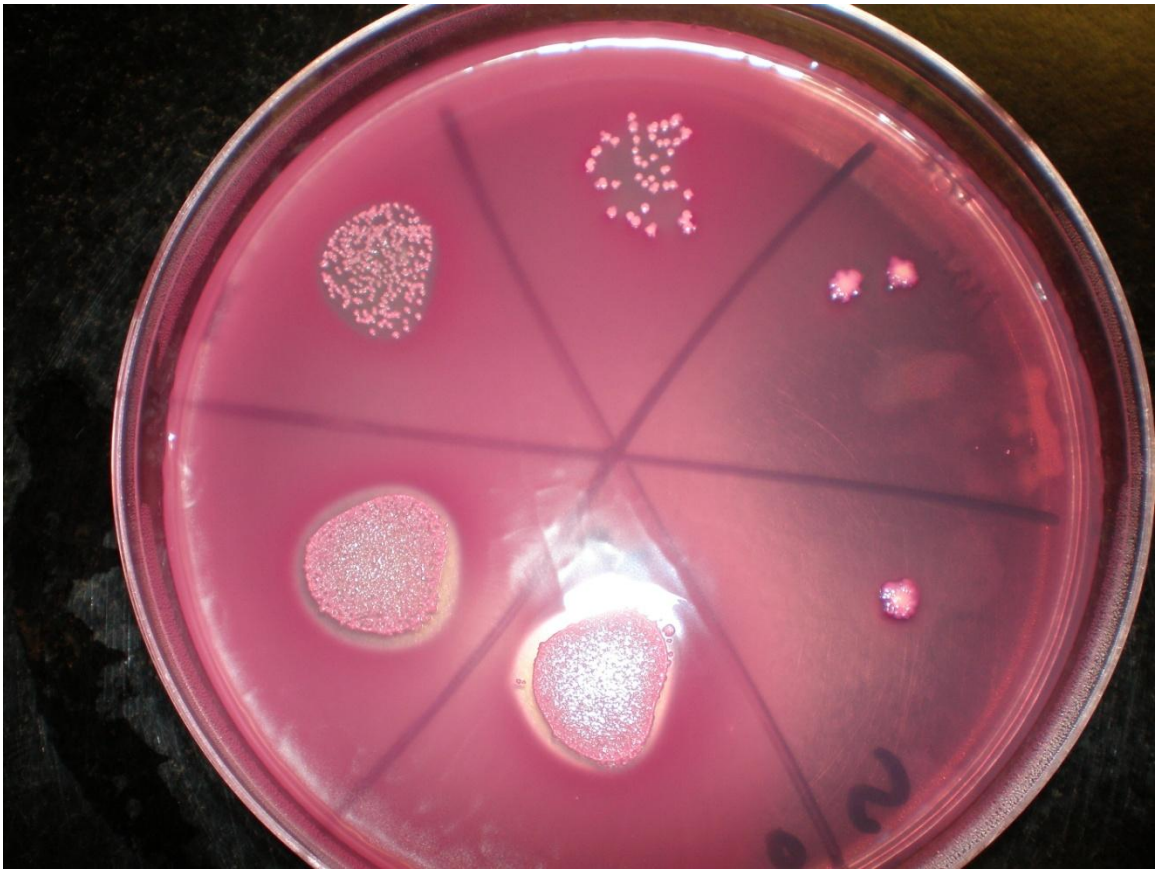
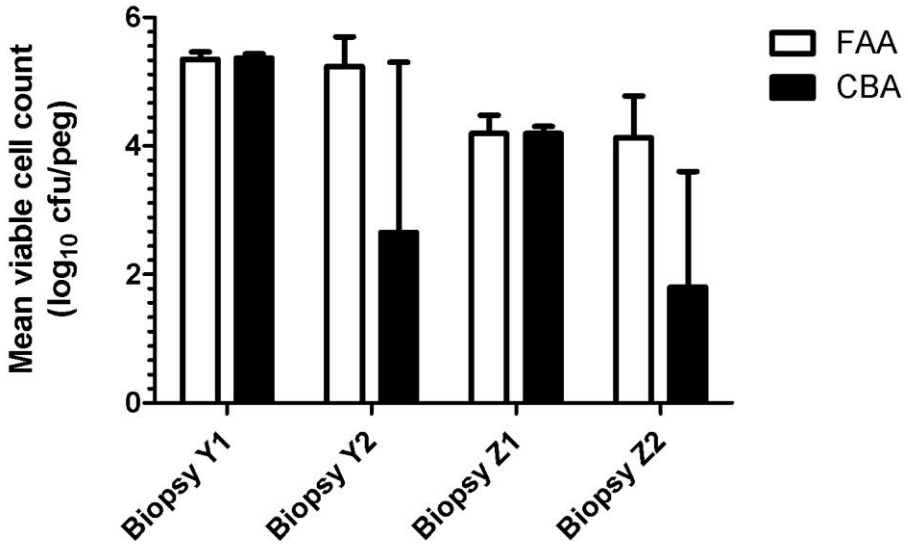


Figure 18. Bacteria recovered from anaerobic biofilms of colon bacteria.

Biopsies from the left (descending) colon were processed and used to seed biofilm formation (143 hours) in the CBD. Bacteria in the biofilms were enumerated by plating on two agar media, FAA (white bars) and CBA (black bars). Two samples were taken for each condition tested. Error bars represent range (upper/lower limits).



similar for biopsies Y1 and Z1. For biopsies Y2 and Z2, colony counts were larger on average on FAA than CBA, although colony counts on CBA showed a wide variance. The colonies from these plates were streaked onto differential media to determine what bacteria were present; the results are presented below (Section 5.3.3.1).

5.3.3 Characterizing Biofilms Formed by Anaerobic Bacteria from the Human Colon

Once in vitro biofilms of colon bacterial communities were established, it was necessary to characterize the bacteria living within the biofilm populations. This was accomplished through numerous methods, including culture on selective media and molecular profiling techniques based on 16S rRNA-encoding genes. Bacterial communities extracted from the biopsies were compared to the in vitro biofilm communities to determine if diversity was lost during in vitro biofilm culture.

5.3.3.1 Culture on Selective Media

Anaerobic biofilms grown from biopsies Y1, Y2, Z1 and Z2 for 143 hours under static conditions were assessed for size (in culturable cfus) by viable cell counting on FAA and CBA (Section 5.3.2.2, Figure 18). In order to determine what groups of bacteria were present in these biofilms, bacteria from the viable cell count plates were streaked onto selective agar plates. As shown in Table 9, bacteria recovered from biofilms of all four biopsy samples displayed growth on PEA, which is selective for *Streptococcus* and *Staphylococcus* species (Difco, 1985). Additionally, all four samples grew on BBE and produced a black colour (indicative of members of the *Bacteroides fragilis* group; *B. fragilis, B. vulgatus*, *B. thetaiotaomicron* and *B. distasonis*

Table 9. Growth of Biofilm Bacteria on Selective Media

Medium	Biopsy Y1 Biofilm	Biopsy Y2 Biofilm	Biopsy Z1 Biofilm	Biopsy Z2 Biofilm
MacConkey agar[a]	+ (pink and white)	+ (pink)	-	+ (pink)
PEA[b]	+	+	+	+
BBE[c]	+ (black)	+ (black)	+ (black)	+ (black)
MRS agar[d]	+	+	+	+
mEA[e]	+ (purple)	+ (purple)	+ (purple)	+ (purple)

[a]Bacterial strains that ferment lactose grow as pink colonies (*E. coli* or other Enterobacteriaceae) on MacConkey agar. *E. coli* also produces a bile precipitate. Non-lactose fermenting Enterobacteriaceae grow as white colonies.
[b]Phenylethanol agar, selective or staphylococci and streptococci.
[c]Bacteroides bile esculin agar, members of the *Bacteroides fragilis* group turn BBE black.
[d]deMan, Rogosa and Sharpe agar, selective for lactobacilli.
[e]mEnterococcus agar, selective for enterococci (*E. faecalis* grows as dark purple colonies).

(Livingston *et al.*, 1978)). Growth on MRS (selective for *Lactobacillus* (Difco, 1985)) and mEA (selective for *Enterococcus*, with *E. faecalis* forming purple colonies (Difco, 1985)) indicated the presence of *Lactobacillus* and *E. faecalis* in biofilms from all four biopsies. The biofilm from biopsy Y1 yielded both pink and white colonies on MacConkey agar. The pink colonies caused the precipitation of bile (indicative of *E. coli*) and the white colonies were likely non-lactose fermenting Enterobacteriaceae (Difco, 1985). It is unknown why these white colonies did not appear on MacConkey plates of in the aerobic biofilm bacteria from the same patient. It is possible that these bacteria were outcompeted by the fast growing *E. coli* in the aerobic biofilms, but were able to persist amongst the slower growing bacteria in the anaerobic biofilms. The bacteria from biofilms formed from biopsies Y2 and Z2 had only *E. coli*-type colonies on MacConkey agar, while the biofilm from biopsy Z1 had no growth on MacConkey agar (Table 9). Figure 19 displays representative images of growth by anaerobic bacteria, which participated in biofilm formation, on selective media.

5.3.3.2 T-RFLP

In order to better characterize the biofilm communities formed by the anaerobic bacteria, molecular techniques were employed. DNA was extracted from the agar plates that were used to enumerate recovered bacteria in the biopsy homogenates (biopsies Y1-2 and Z1-2; Table 8) and in the biofilms formed from these homogenates. T-RFLP analysis was performed on the samples to give a semi-quantitative estimation of community distribution at the phylum level. Figure 20 shows the electropherograms generated from T-RFLP analysis of biopsy Y1 and the biofilm formed from this

Figure 19. Growth of isolates from biofilms of colon bacteria on selective media.

Biopsies from the left (descending) colon were processed and used to seed biofilm formation (143 hours) in the CBD. Bacteria recovered from the biofilms were plated on selective media: MacConkey agar (selective for Enterobacteriaceae), phenylethanol agar (PEA, selective for staphylococci and streptococci), *Bacteroides* bile esculin agar (BBE, members of the *Bacteroides fragilis* group turn the media black), deMan, Rogosa and Sharpe agar (MRS agar, selective for lactobacilli), and mEnterococcus agar (mEA, selective for enterococci, *E. faecalis* grows as dark purple colonies). Pictures are representative of anaerobic biofilm samples grown on MacConkey agar (A), PEA (B), BBE (C), MRS agar (D), and mEA (E).

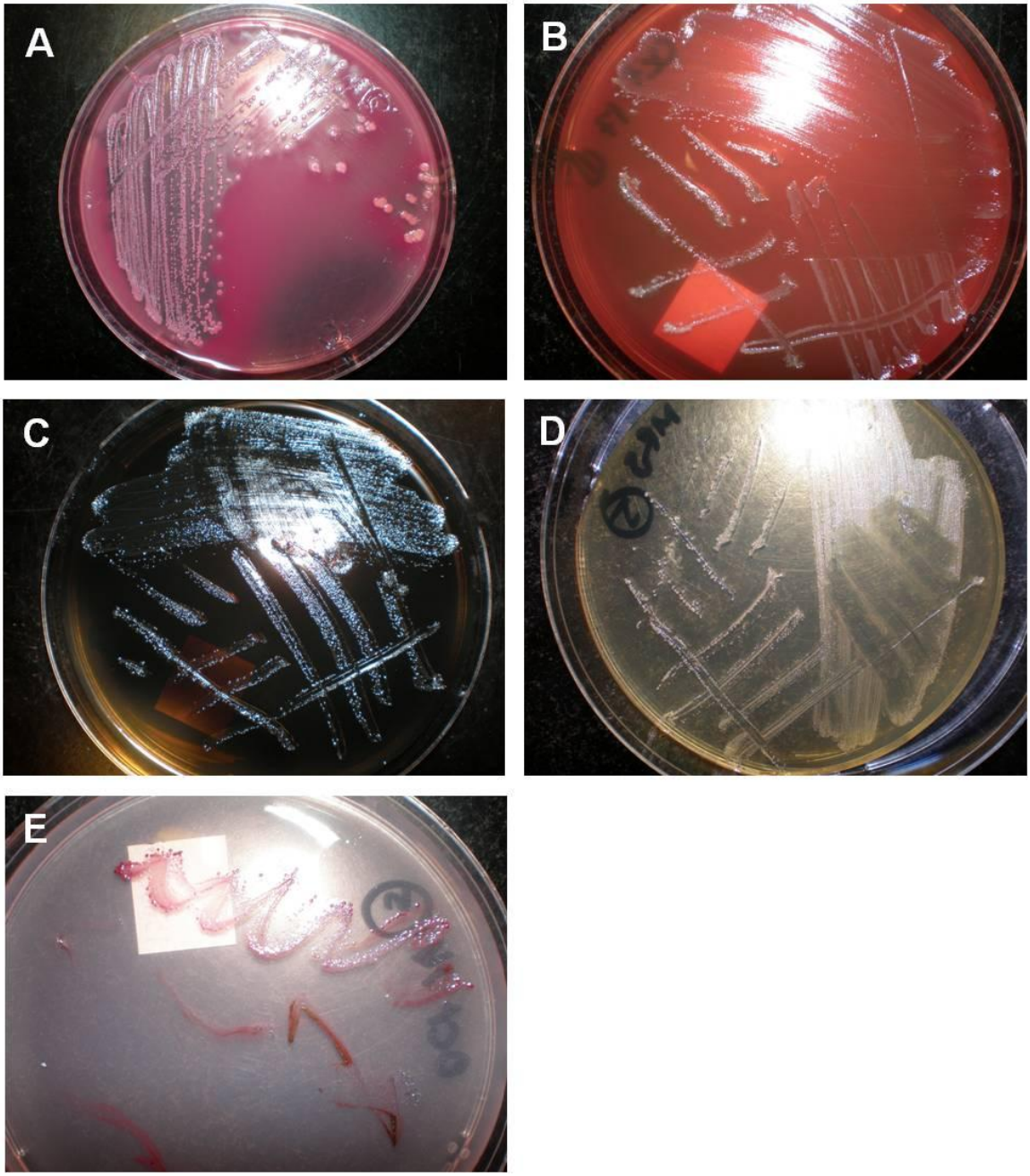
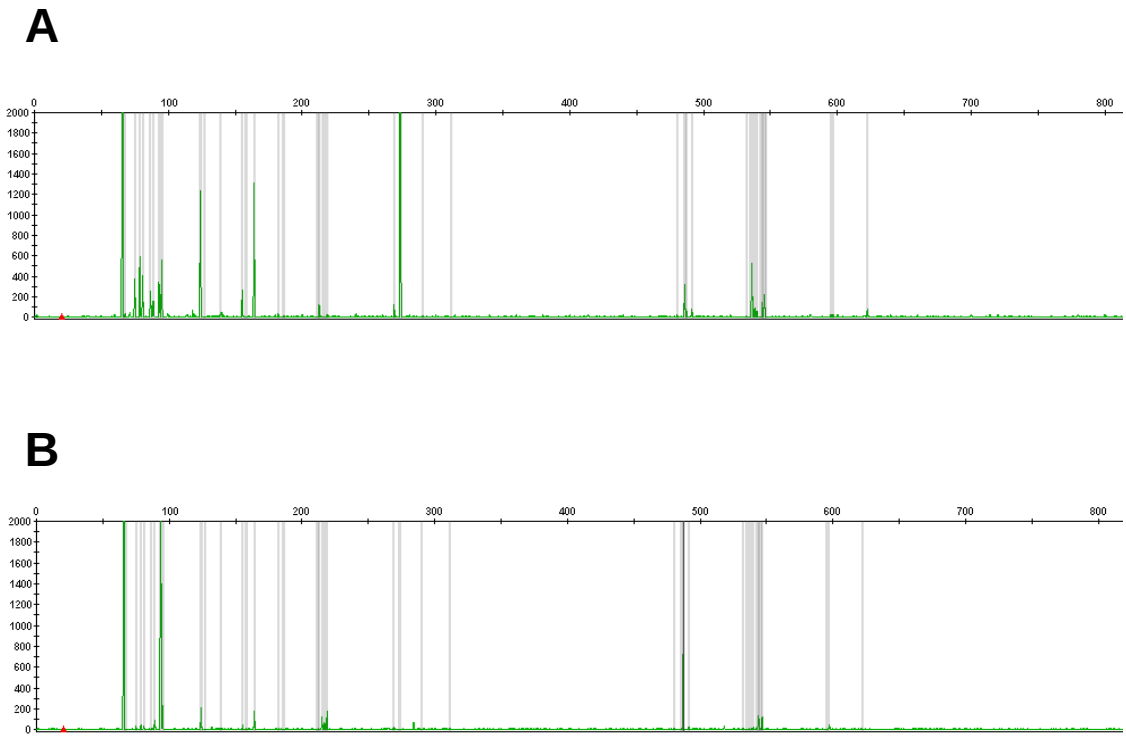

A
B
C
D
E

Figure 20. Electropherograms resulting from T-RFLP analysis.

Electropherograms generated from T-RFLP analysis of the bacterial community from biopsy sample Y1 (A) and the biofilm formed by this community (B). T-RFLP analysis was conducted on DNA extracted from bacterial communities grown anaerobically on agar plates. The x-axis of the electropherograms represents size of the T-RFs in bp, the y-axis represents the fluorescene (in rfu) of each peak, which corresponds to relative abundance of the peaks within the community.



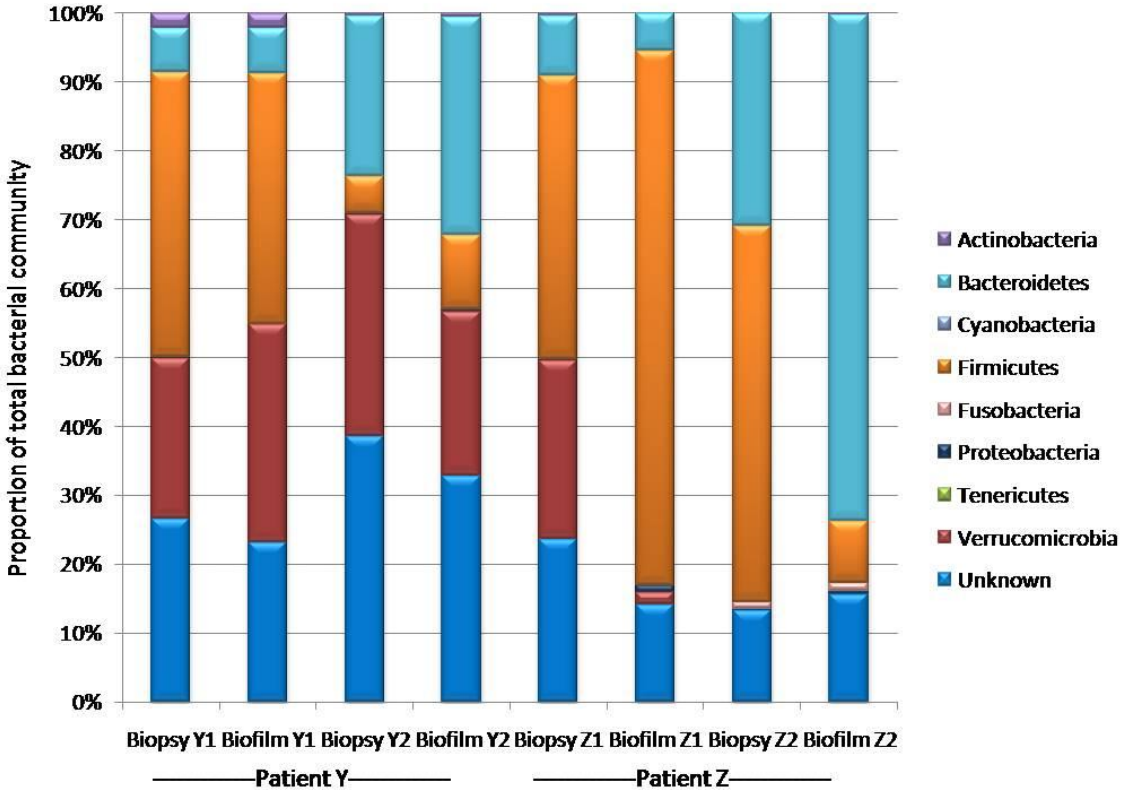
community. These electropherograms give an example of the similarities seen between biopsy and biofilm communities, where many peaks are shared but vary in abundance between the biopsy and biofilm. Community profiles were generated based on the electropherogram data for each biopsy/biofilm pair.

In some cases (biopsies Y1 and Y2), biopsy and biofilm community profiles were very similar to each other, whereas in biopsies Z1 and Z2 the biofilm profiles showed more differences. The biofilm formed from biopsy Z1 bacteria had a much larger proportion of Firmicutes than the biopsy itself, while the biofilm formed from biopsy Z2 had a much greater proportion of Bacteroidetes than its seed community (Figure 21). Although biofilm communities did differ from their seed communities (biopsies), the three major bacterial phyla in the human colon, Firmicutes, Bacteroidetes, and Actinobacteria (Mahowald *et al.*, 2009; Young & Schmidt, 2008), were the predominant phyla in both the biopsies and biofilms. It is not known why biopsies from patient Y yielded biofilms with more similar community structures to the biopsies than patient Z. It is possible that the nature of the bacteria strains from patient Y enabled them to remain in similar ratios within the biopsy and biofilm, whereas patient Z had strains that "overgrew" in the biofilms. The manner in which these strains grew on the agar plates prior to DNA extraction may also have impacted the distribution of strains within the community profiles.

The first T-RFLP experiment was performed on DNA extracted from agar plates used to enumerate bacteria in biofilms and biopsies. In order to get a more accurate representation of the distribution of phyla in the samples, and to avoid losing bacteria due to media selection, bacteria were extracted directly from biopsies and biofilms. Biopsies

Figure 21. Community profiles of cultured bacteria from colon biopsies and biofilms based on DNA extracted from agar plates.

T-RFLP was used to generate a community profile (at the phylum level) of biopsies and biofilms based on bacteria plated on agar media. Biopsies Y1 and Y2 were from patient Y, while biopsies Z1 and Z2 were from patient Z. Data presented represent T-RFs assigned using the RDP with at least 90% confidence.

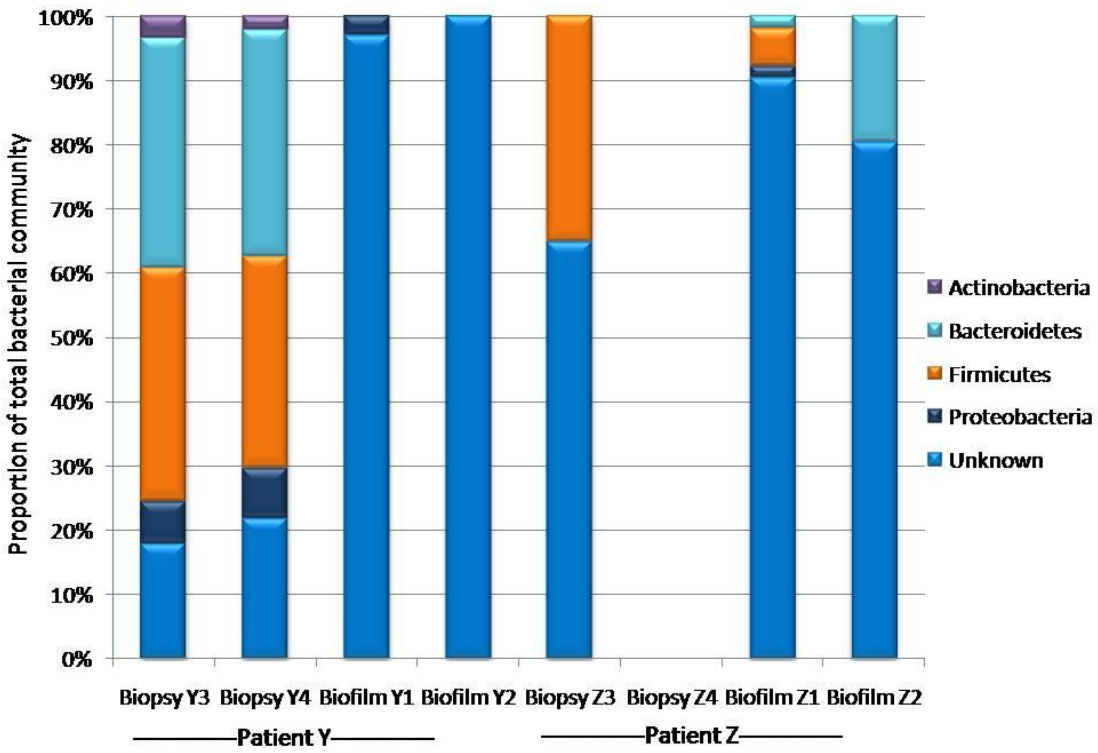


Y3-4 and Z3-4 were used for DNA extraction to determine biopsy community composition (Table 8), while frozen samples from biopsies Y1-2 and Z1-2 (Table 8) were used to form biofilms (from which DNA was extracted). T-RFLP analysis of biopsy Z4 yielded no T-RFs, possibly due to a low yield of genomic DNA extracted from the biopsy. Anaerobic biofilms (incubated with shaking at 150 rpm) were formed from planktonic cultures of frozen biopsy samples. DNA was extracted directly from the biofilms after 48 hours of growth. These samples were used for both T-RFLP and group-specific PCR experiments.

T-RFLP showed a much larger proportion of unknown organisms in these "direct" samples than samples based on plate cultures (Figure 22). It is possible that organisms in the biopsies and biofilms were unculturable as single colonies on agar media, and therefore these unknown species were missed in the previous study. Firmicutes, Bacteroides and Actinobacteria were the major identifiable phyla in these samples (as they were in the first T-RFLP experiment), but no Cyanobacteria, Fusobacteria, Tenericutes or Verrucomicrobia were detected. In addition, biofilm samples had a greater percentage of unknown bacteria than biopsy samples (Figure 19). Because entire biopsy samples were required for DNA extraction from the biopsies, it was not possible to also grow biofilms from these samples. Therefore, a direct comparison of T-RFLP profiles from biopsies and the biofilms from those biopsies could not be conducted. Overall, a less diverse community structure was detected in the samples with direct DNA extraction than with samples from plate cultures, although it is not known whether the different sources of DNA template (biopsies and biofilms verses agar plates) affected the sensitivity of the T-RFLP technique. Direct DNA extractions

Figure 22. Community profiles of colon biopsies and biofilms based on DNA extracted directly from the samples.

T-RFLP was used to generate a community profile (at the phylum level) of biopsies and biofilms (DNA was extracted directly from the biopsies or biofilms). Biopsies Y3 and Y4 were from patient Y, while biopsies Z3 and Z4 were from a patient Z. Biofilms were formed from frozen samples of biopsies Y1-2 and Z1-2. No T-RFs were detected during analysis of biopsy Z4, possibly due to a low yield of extracted DNA. Data presented represent T-RFs assigned using the RDP with at least 90% confidence.



from biopsies and biofilms tended to yield less genomic DNA, which may have resulted in the T-RFLP analysis not detecting less abundant species. This is a limitation of the direct DNA extraction technique, which may be overcome in the future by pooling several biopsy or biofilm samples to increase the DNA yield. This would also give a broader overview of biopsy and biofilm communities by decreasing sampling error caused by variations in individual biopsies and biofilms.

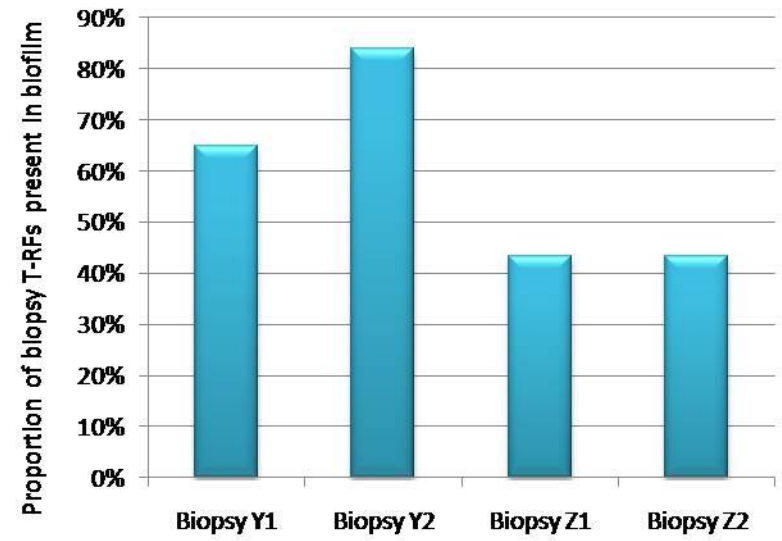
T-RFLP data from biopsies and biofilms were compared to determine the proportion of T-RFs present in biopsies that were also present in biofilms. Figure 23A shows the proportion of T-RFs from biopsies Y1, Y2, Z1 and Z2 that participated in biofilm formation. This data is based on DNA extracted from agar plate cultures, and therefore is skewed toward bacteria that could be cultured. Eighty-three percent of culturable T-RFs present in biopsy Y2 were also present in biofilms of biopsy Y2 bacteria. Biopsy Y1 had 65% of culturable biopsy T-RFs participate in biofilms, and biopsies Z1 and Z2 each had 43% culturable biopsy T-RF participation in biofilm formation.

Figure 23B represents T-RF participation in biofilm formation from both T-RFLP experiments, that is T-RFLP performed on DNA extracted from cultures on agar plates, and DNA extracted directly from biopsies and biofilms. T-RFs for all biopsies within an experiment were pooled and compared against T-RFs for all biofilms. Of the T-RFs detected in all biopsies from the T-RFLP experiment using agar plate cultures, 83% were also detected in biofilms formed from the biopsy bacteria. Therefore, 83% of bacteria that were culturable on agar plates participated in biofilm formation. In contrast, 31% of T-RFs detected using DNA samples extracted directly from biopsies were also found in

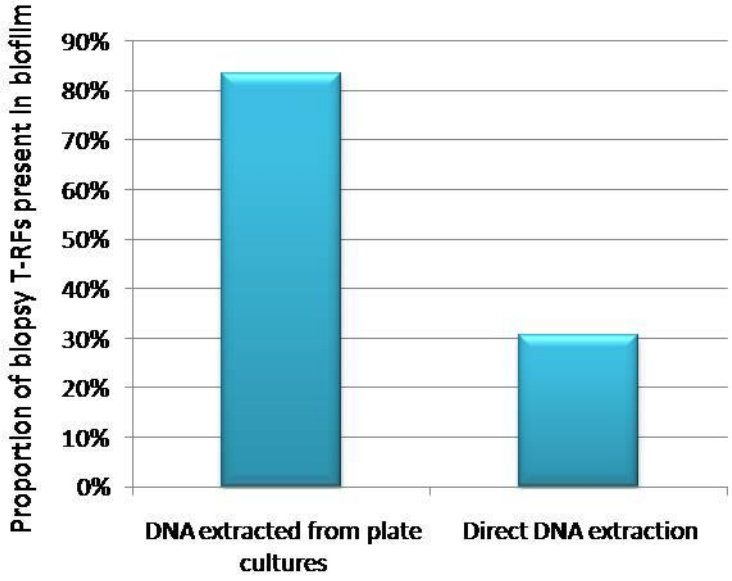
Figure 23. Proportion of biopsy bacteria participating in biofilm formation.

T-RFLP data from biopsies and biofilms were compared to determine the proportion of T-RFs present in biopsies that were also present in biofilms. In (A), T-RFLP data from colonies on agar plates were compared for biopsies and the biofilms formed using bacteria from the corresponding biopsy. In (B), T-RFs from all biopsies were pooled and the percent of T-RFs present in at least one biofilm was determined for T-RFLP conducted on DNA extracted from agar plate cultures as well as a T-RFLP conducted on DNA extracted directly from biopsies and biofilms (with no culturing step). Percentages indicate the portion of bacteria present in the biopsy that participated in biofilm formation.

A


Proportion of biopsy T-RFs present in biofilm
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Biopsy Y1
Biopsy Y2
Biopsy Z1
Biopsy Z2

B


Proportion of biopsy T-RFs present in biofilm
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
DNA extracted from plate cultures
Direct DNA extraction

biofilms of the biopsy bacteria. Therefore, 31% of the total bacterial population of the biopsy was successfully recovered as viable cells and participated in biofilm formation in vitro. These values are to be expected, since all T-RFs in the first experiment were from culturable bacteria (since DNA was extracted from plate cultures). On the other hand, many T-RFs from DNA extracted directly from biopsies would be expected to come from bacteria that are unculturable under the experimental conditions. Since at least 500 species live in the colon (Young & Schmidt, 2008), 31% recovery of the total bacterial population of a biopsy sample represents a very diverse community.

5.3.3.3 Group-Specific PCR

To confirm T-RFLP data and further investigate the biofilms formed by anaerobic bacteria, PCR was used to detect the presence of specific groups of organisms in the direct DNA extraction samples (DNA was extracted from biopsies Y3-4 and Z3-4, and biofilms were formed from frozen samples of biopsies Y1-2 and Z1-2; Table 8). Primer sets (specific for a target organism or group of organisms) were tested with positive and negative controls. PCR results confirmed that both biopsies and biofilms contained a diverse community of organisms (Table 10). The *B. fragilis* group, a prevalent group in the colon from the phylum Bacteroidetes, was present in all biopsy and biofilm samples. Three members of this group were tested for specifically, and while each sample contained *B. thetaiotaomicron* and *B. vulgatus*, none of the samples contained *B. distasonis* (primer specificity and appropriate PCR reaction conditions were confirmed using *B. distasonis* ATCC 8503 genomic DNA). *Prevotella* species (also of the Bacteroidetes phylum) were only identified in patient Y, and were present in the biopsies

Table 10. Presence of bacterial groups or species in colon biopsy samples and biofilms

	Patient Y				Patient Z			
Target organism or group	**Bx Y3**	**Bx Y4**	**Bio Y1**	**Bio Y2**	**Bx Z3**	**Bx Z4**	**Bio Z1**	**Bio Z2**
Bacteroides distasonis	-	-	-	-	-	-	-	-
Bacteroides fragilis group	+	+	+	+	+	+	+	+
Bacteroides thetaiotaomicron	+	+	+	+	+	+	+	+
Bacteroides vulgatus	+	+	+	+	+	+	+	+
Bifidobacterium adolescentis	+	-	-	-	-	-	-	+
Bifidobacterium longum	+	+	+	+	+	+	+	+
Clostridium clostridiiforme	+	+	-	-	+	+	+	+
Clostridium coccoides group	+	+	+	-	+	+	-	+
Eubacterium biforme	+	+	-	-	-	-	-	-
Faecalibacterium prausnitzii	-	-	-	-	+	-	-	+
Lactobacillus acidophilus	+	+	+	+	+	+	+	+
Peptostreptococcus productus	+	+	+	-	+	+	+	+
Prevotella species	+	+	-	-	-	-	-	-

Bx = biopsy sample
Bio = biofilm sample

but not the biofilms. Two members of the phylum Actinobacteria were tested for, *Bifidobacterium adolescentis* and *B. longum*. While *B. longum* was present in all samples, *B. adolescentis* was only detected in one biopsy from patient Y and one biofilm from patient Z.

Several organisms from the phylum Firmicutes were tested for. *C. clostridiiforme* was found in all samples except the two biofilms from patient Y. Surprisingly, the *Clostridium coccoides* group, a very common group in the human colon, was only found in one of the two biofilms from each of patients Y and Z. Other members of the *C. coccoides* group that were tested for included *Eubacterium biforme* and *Peptostreptococcus productus*. *E. biforme* was present in both biopsies from patient Y, while *P. productus* was present in all samples except one biofilm sample from patient Y. *Faecalibacterium prausnitzii*, a member of another prominent group of bacterial microflora, the *Clostridium leptum* group, was present in only one biopsy and one biofilm of patient Z. *Lactobacillus acidophilus*, another member of Firmicutes, was present in all samples.

5.3.4 An in vitro Model of Colon Microflora

In order to further characterize the bacterial communities in the human colon and the biofilms formed by these communities in vitro, 48 biopsies were collected from three different patients (16 biopsies per patient; Table 8). qPCR was used to quantify total Eubacteria, *Bacteroides-Prevotella* species, and members of the *C. coccoides* group in these biopsies and the biofilms grown from them. Comparisons were made between the left (descending) and right (ascending) colon, between patients, and between biofilms

formed from fresh verses frozen biopsy samples. This represents the final stage of establishing a "representative microflora" for use in studies of biofilm interactions.

5.3.4.1 Quantification of Bacteria in Colon Biopsies

Biopsy samples from three patients were examined for total Eubacteria, *Bacteroides-Prevotella* species, and the *C. coccoides* group using qPCR. Between $5x10^2$ and $1x10^3$ bacteria were detected using universal Eubacteria primers (Figure 24A). Levels of *Bacteroides-Prevotella* species were also quite constant between biopsies (with the exception of biopsy B15, in which no *Bacteroides-Prevotella* were detected). However, numbers of *Bacteroides-Prevotella* species reached higher than $1x10^5$ cfu equivalents per biopsy (Figure 24B). Clearly, it is not possible to have greater numbers of *Bacteroides-Prevotella* species than total Eubacteria. Differences in primer sensitivity and the average genome size and average rRNA operon copy number values used to calculate cfu equivalents may be responsible for this discrepancy. The calculation of total Eubacteria is especially prone to errors, due to the large variation in genome size and rRNA operon copy number among Eubacteria. Cfu calculations were based on a Eubacteria population expected in the gastrointestinal tract (see Section 3.6.8), but the calculations still only yield an estimation of cfu equivalents. *C. coccoides* group bacteria ranged from $1x10^1$ to $1x10^3$ cfu equivalents per biopsy, with patient A having fewer *C. coccoides* group bacteria than patients B and C (Figure 24C).

qPCR results from colon biopsies were examined for differences between patients and between biopsies from the left and right colon. Patient A had lower numbers of total Eubacteria than the other two patients (Figure 25A), and these values were significantly

Figure 24. Quantification of bacteria in colon biopsies.

Total Eubacteria (A), *Bacteroides-Prevotella* species (B) and *C. coccoides* group (C) were enumerated in colon biopsies from patients A, B and C using qPCR. Left (descending) colon biopsies are represented by white bars, while right (ascending) colon biopsies are represented by black bars. Error bars represent SD.

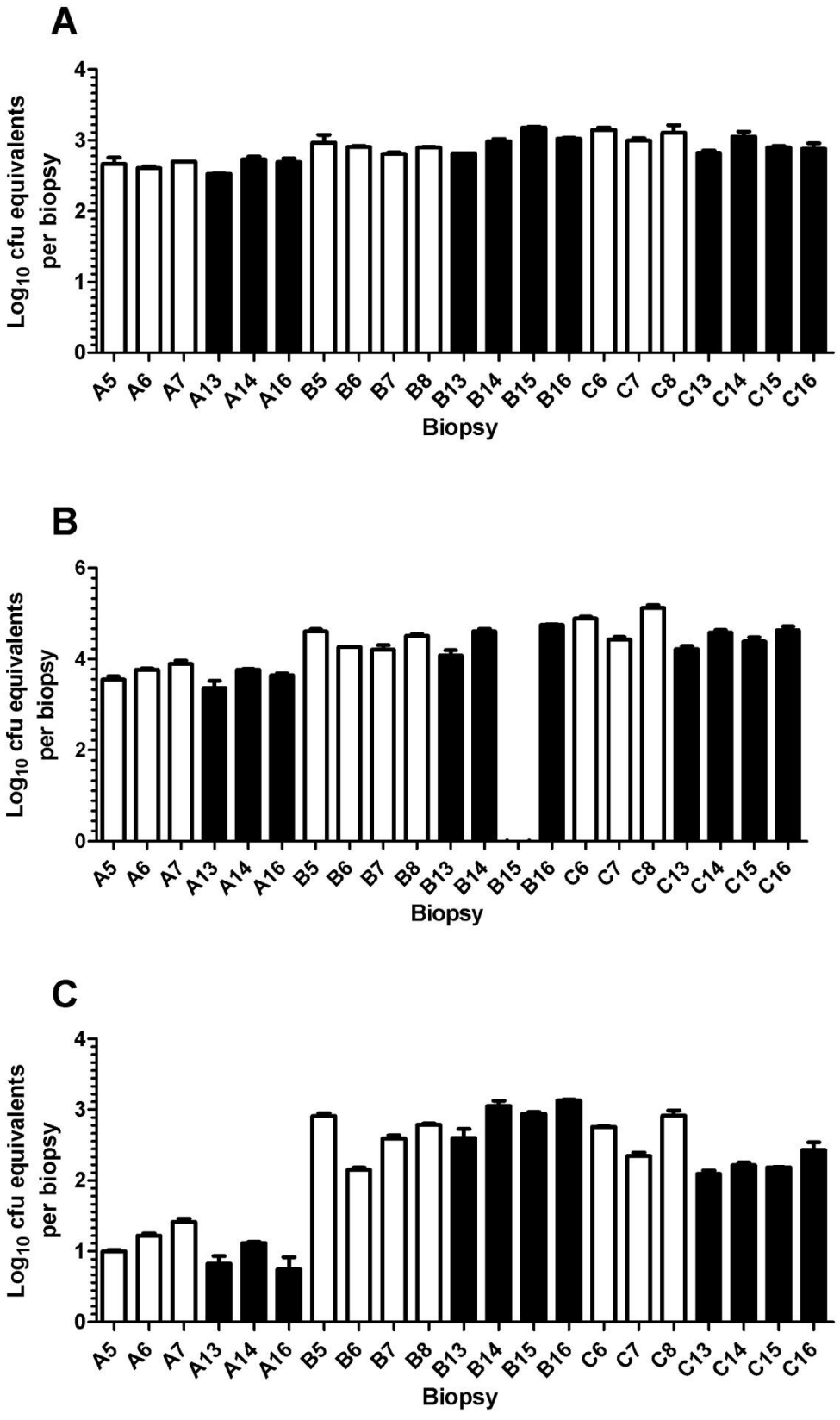
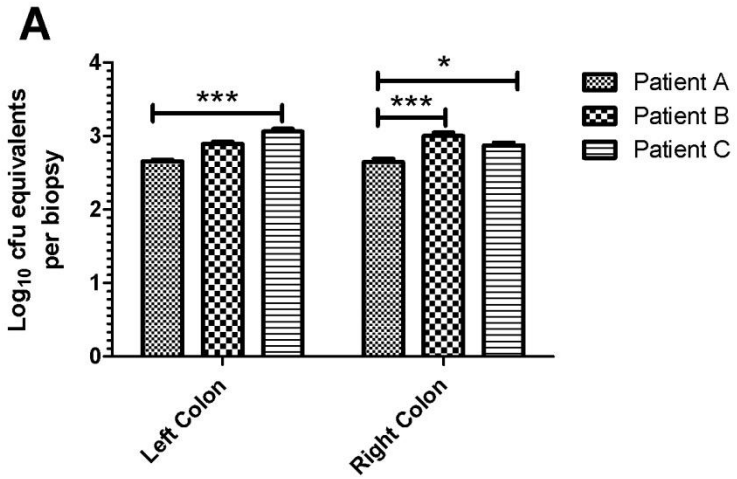
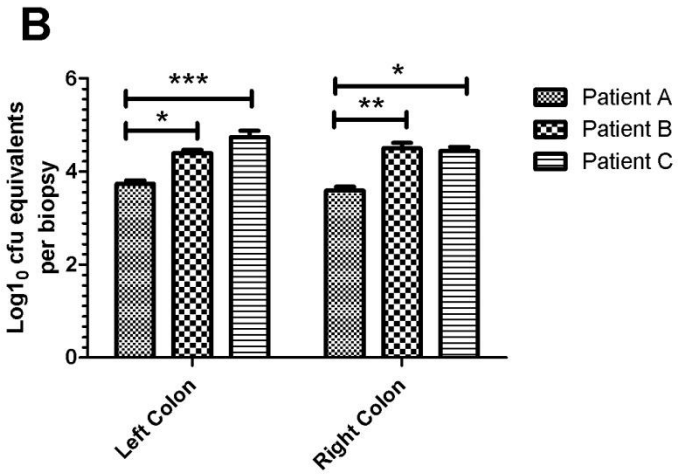
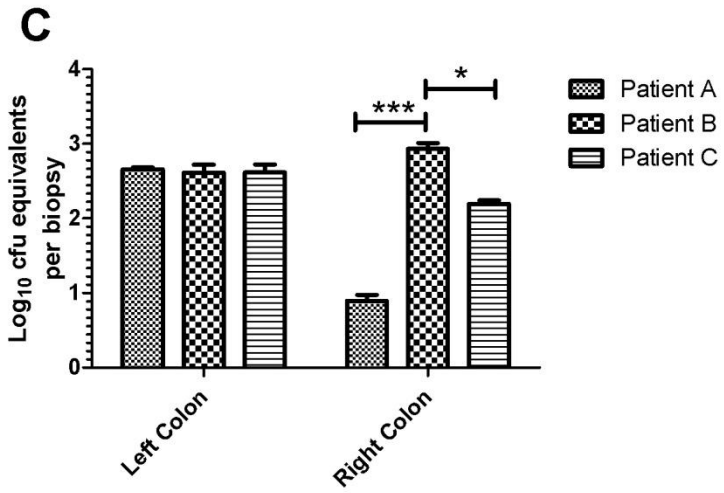

A
Log10 cfu equivalents per biopsy
0
1
2
3
4
A5 A6 A7 A13 A14 A16 B5 B6 B7 B8 B13 B14 B15 B16 C6 C7 C8 C13 C14 C15 C16
Biopsy
B
Log10 cfu equivalents per biopsy
0
2
4
6
A5 A6 A7 A13 A14 A16 B5 B6 B7 B8 B13 B14 B15 B16 C6 C7 C8 C13 C14 C15 C16
Biopsy
C
Log10 cfu equivalents per biopsy
0
1
2
3
4
A5 A6 A7 A13 A14 A16 B5 B6 B7 B8 B13 B14 B15 B16 C6 C7 C8 C13 C14 C15 C16
Biopsy

Figure 25. Bacterial population differences in colon biopsies from three patients.

Total Eubacteria (A), *Bacteroides-Prevotella* species (B) and *C. coccoides* group (C) were enumerated in colon biopsies from patients A, B and C using qPCR. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test (* denotes $p<0.05$ verses indicated groups, ** denotes $p<0.01$ verses indicated groups, *** denotes $p<0.001$ verses indicated groups). For Eubacteria, patients A and C differed significantly in the left colon (KW $p=0.0004$, Dunn's $p<0.001$) and right colon (KW $p=0.0012$, Dunn's $p<0.05$). Patients A and B also differed in the number of Eubacteria in the right colon (KW $p=0.0012$, Dunn's $p<0.001$). *Bacteroides-Prevotella* numbers in patient A biopsies were significantly lower than both patients B and C in the left and right colon (left colon KW $p=0.0009$, Dunn's $p<0.05$ verses patient B and $p<0.001$ verses patient C; right colon KW $p=0.0016$, Dunn's $p<0.01$ verses patient B and $p<0.05$ verses patient C). Numbers of *C. coccoides* group bacteria did not differ in the left colon biopsies ($p=0.7068$), but patient B had significantly higher numbers of *C. coccoides* than patients A and C (KW $p<0.0001$, Dunn's $p<0.001$ verses patient A and $p<0.05$ verses patient C).


A
Log10 cfu equivalents per biopsy
0
1
2
3
4
***
***
*
Left Colon
Right Colon
Patient A
Patient B
Patient C


B
Log10 cfu equivalents per biopsy
0
2
4
6
***
*
**
*
Left Colon
Right Colon
Patient A
Patient B
Patient C

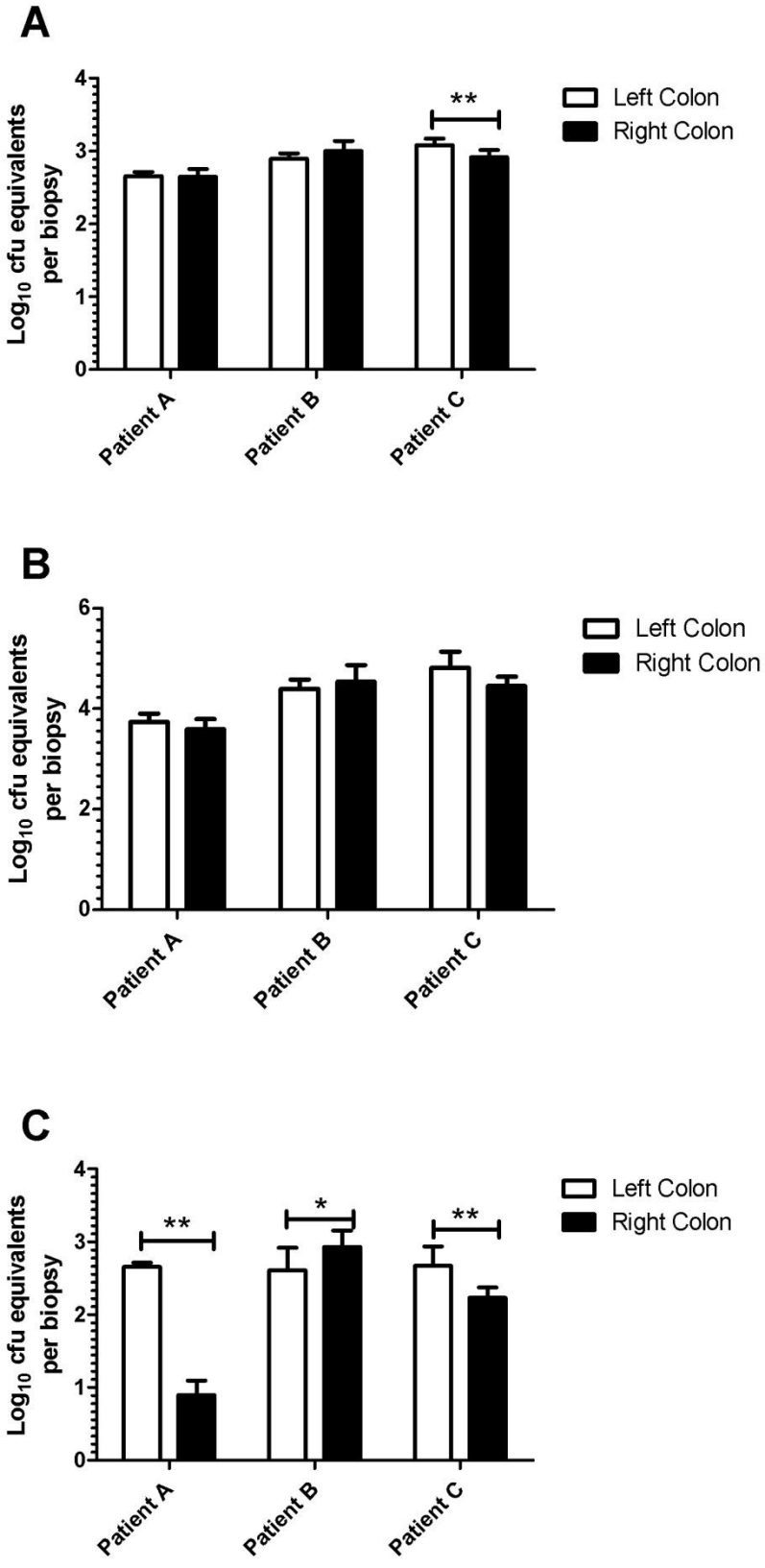

C
Log10 cfu equivalents per biopsy
0
1
2
3
4
***
*
Left Colon
Right Colon
Patient A
Patient B
Patient C

lower than than patient C in the left colon (KW p=0.0004, Dunn's p<0.001) and lower than both patients B and C in the right colon (KW p=0.0012, Dunn's p<0.001 verses patient B, p<0.05 verses patient C). *Bacteroides-Prevotella* numbers in patient A biopsies were significantly lower than both patients B and C in the left and right colon (left colon KW p=0.0009, Dunn's p<0.05 verses patient B and p<0.001 verses patient C; right colon KW p=0.0016, Dunn's p<0.01 verses patient B and p<0.05 verses patient C) (Figure 25B). Numbers of *C. coccoides* group bacteria did not differ between patients in the left colon biopsies (p=0.7068), but patient B had significantly higher numbers of *C. coccoides* than patients A and C in the right colon biopsies (KW p<0.0001, Dunn's p<0.001 verses patient A and p<0.05 verses patient C) **(**Figure 25C).

Comparing within patients, differences between bacteria present in left and right colon biopsies varied depending on the bacterial group assessed. Numbers of Eubacteria did not differ between the left and right colon biopsies for patients A (p=0.9372) and B (p=0.1560), but patient C biopsies from the left colon had significantly more total Eubacteria than biopsies from the right colon (p=0.0080) (Figure 26A). Levels of *Bacteroides-Prevotella* species were not significantly different between left and right colon biopsies for any of the three patients (p=0.2403, 0.1893 and 0.0593 for patients A, B and C, respectively) (Figure 26B). *C. coccoides* populations were higher in the left colon than the right for patients A (p=0.0022) and C (p=0.0047), but were higher in the right colon for patient B (p=0.0281) (Figure 26C).

Figure 26. Bacterial population differences in colon biopsies from the left (descending) and right (ascending) colon.

Total Eubacteria (A), *Bacteroides-Prevotella* species (B) and *C. coccoides* group (C) were enumerated in colon biopsies from patients A, B and C using qPCR. Error bars represent SD. Data were analyzed using a Mann-Whitney U test (* denotes $p<0.05$ verses indicated groups, ** denotes $p<0.01$ verses indicated groups). Numbers of Eubacteria did not differ between the left and right colon biopsies for patients A ($p=0.9372$) and B ($p=0.1560$), but patient C biopsies from the left colon had significantly more total Eubacteria than biopsies from the right colon ($p=0.0080$). Levels of *Bacteroides-Prevotella* species were not significantly different between left and right colon biopsies for any of the three patients ($p=0.2403$, 0.1893 and 0.0593 for patients A, B and C, respectively). *C. coccoides* populations were higher in the left colon than the right for patients A ($p=0.0022$) and C ($p=0.0047$), but were higher in the right colon for patient B ($p=0.0281$).


A
Log10 cfu equivalents per biopsy
0
1
2
3
4
**
Left Colon
Right Colon
Patient A
Patient B
Patient C
B
Log10 cfu equivalents per biopsy
0
2
4
6
Left Colon
Right Colon
Patient A
Patient B
Patient C
C
Log10 cfu equivalents per biopsy
0
1
2
3
4
**
*
**
Left Colon
Right Colon
Patient A
Patient B
Patient C

5.3.4.2 Visualization of Anaerobic Biofilms of Colon Bacteria

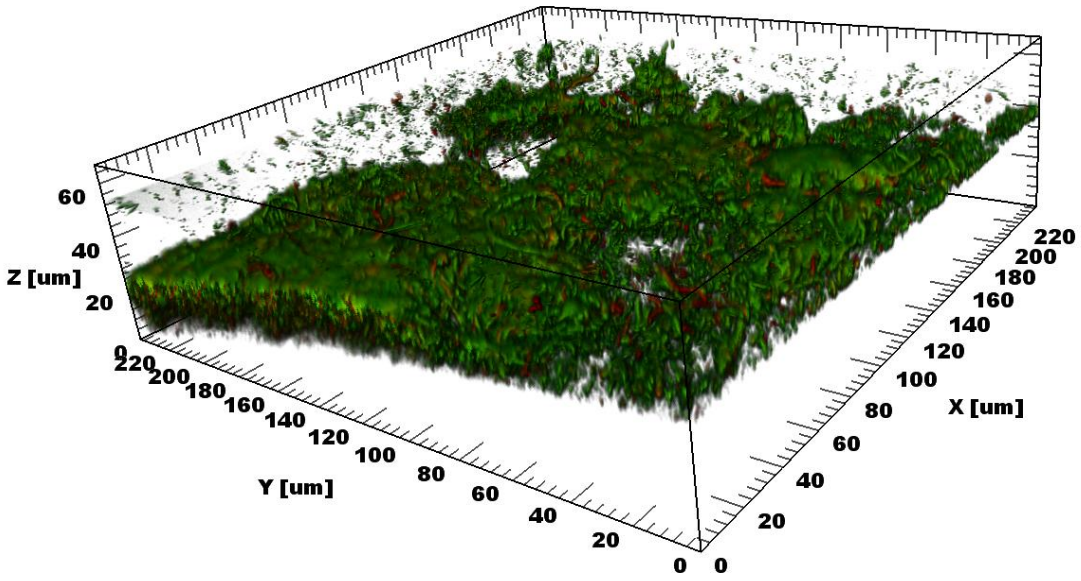
CSLM was used to visualize anaerobic biofilms. Bacteria were stained with a live/dead stain (viable cells stained green, dead cells stained red) and imaged. Images were rendered using Imaris™ (Bitplane Scientific Software, Saint Paul, MN, USA). Images shown are biofilms formed from patient B biopsies, but are representative of biofilms formed by biopsies from all three patients (Figure 27). Images were taken at the atmosphere-liquid interface, where biofilm formation was thickest for all samples examined. Biofilm thicknesses ranged from 20-80 μm. Proportions of live and dead cells are not necessarily representative of live/dead cell ratios during biofilm growth, since biofilms were removed from their anaerobic environments during staining. Interestingly, biofilm formation by the anaerobic bacteria was thickest at the atmosphere-liquid interface. This is typical of biofilm formation by aerobic biofilms because of the accessibility of oxygen at this location (Scher *et al.*, 2005; Spiers *et al.*, 2003). However, the high shear force created by the atmosphere-liquid interface may also play a role in the increased biofilm formation at this location (Kostenko *et al.*, 2008), and therefore shear force may play a role in biofilm formation by anaerobic bacteria.

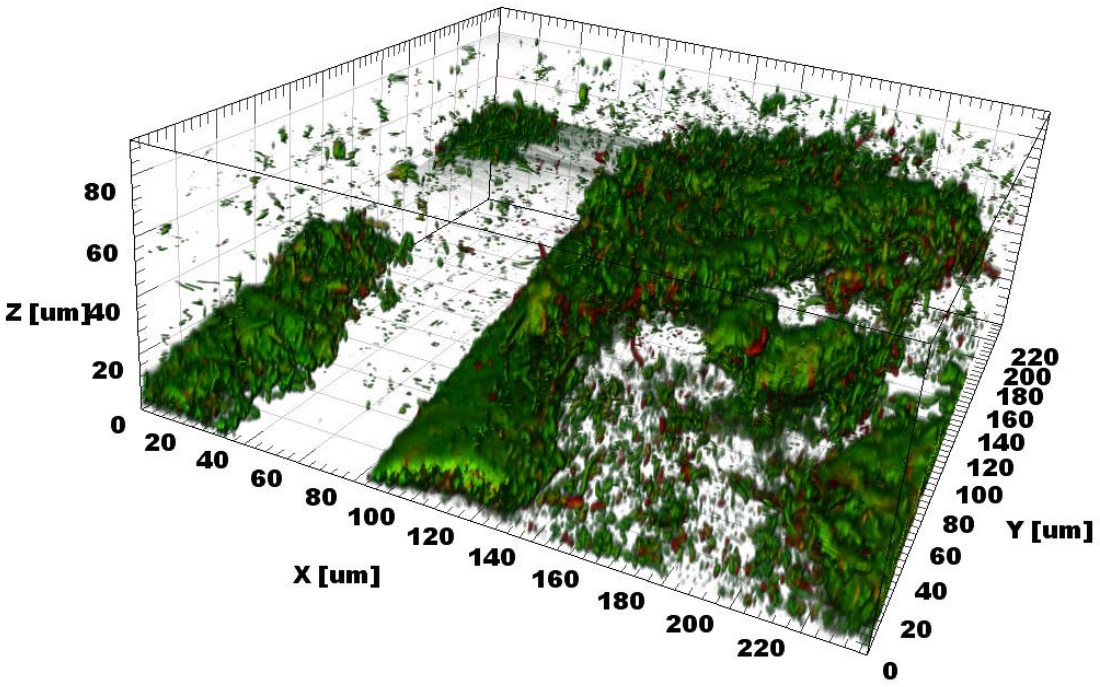
5.3.4.3 Quantification of Bacteria in Anaerobic Biofilms of Colon Bacteria

Once the bacterial community structures of biopsies were determined, it was important to study the community structure of the anaerobic biofilms formed from these communities. Biofilms were studied over a 144 hour period and total Eubacteria, *Bacteroides-Prevotella*, and *C. coccoides* group bacteria were quantified using qPCR. Statistical tests could not be performed on 144 hour data for patients B and C due to low

Figure 27. Confocal microscopy images of anaerobic biofilms of colon bacteria.

Biofilms were grown for 144 hours with shaking at 150 rpm. Pegs were removed from the CBD, stained with a live/dead stain (viable cells stained green, dead cells stained red), and imaged using confocal scanning laser microscopy. Images were produced using Imaris™ (Bitplane Scientific Software).


Z [um]
60
40
20
0
220
200
180
160
140
120
100
80
60
40
20
0
Y [um]
0
20
40
60
80
100
120
140
160
180
200
220
X [um]


Z [um]
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
220
X [um]
0
20
40
60
80
100
120
140
160
180
200
220
Y [um]

samples numbers (DNA extraction from the 144 hour biofilms of biopsies B2, B9, B10, C2, C9 and C10 bacteria failed).

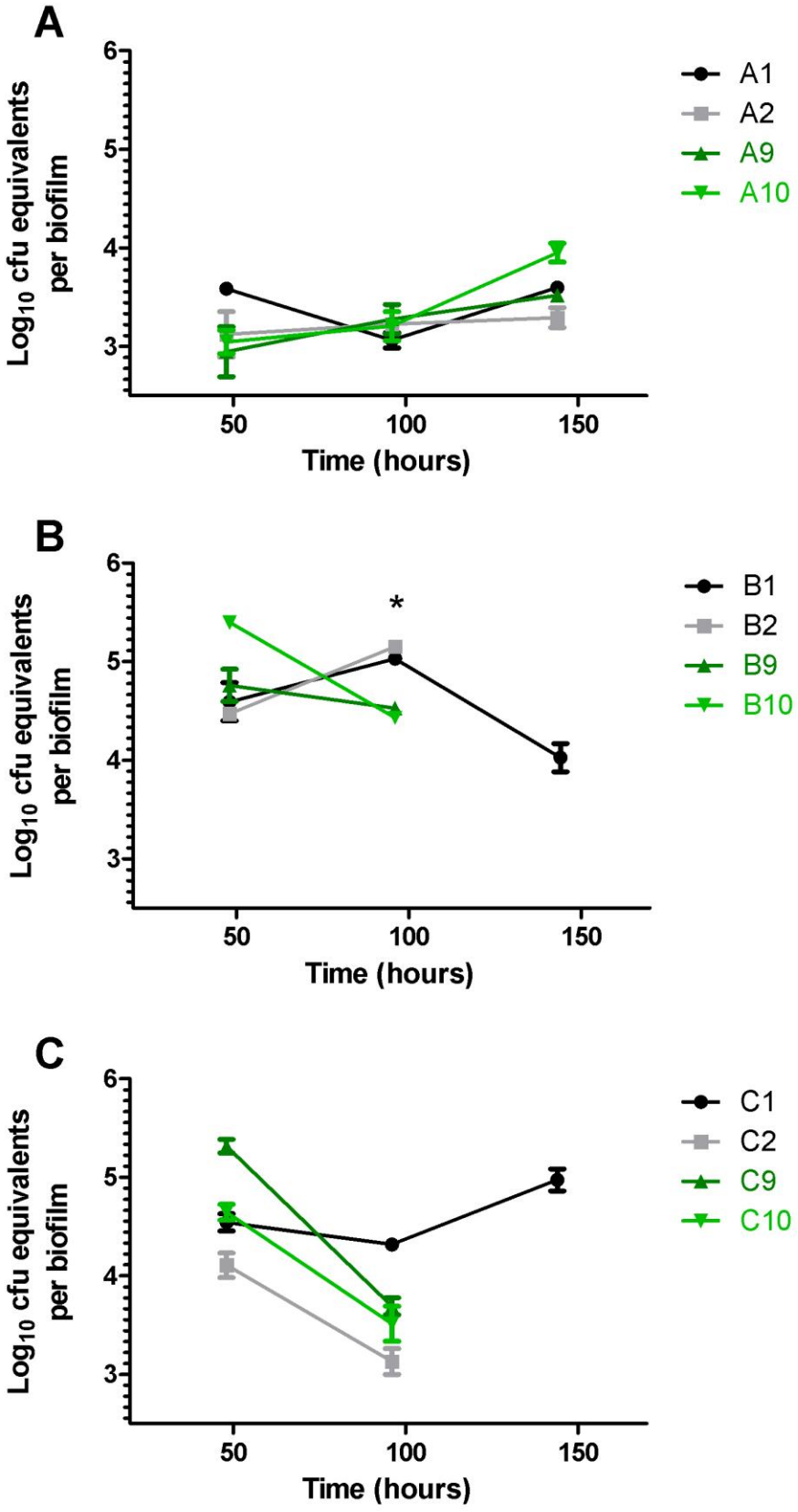
Figure 28 shows levels of total Eubacteria in biofilms formed from biopsy bacteria from three patients (four biofilms per patient). Biofilms from different biopsies of the same patient showed some variance in total Eubacteria numbers, but tended to be within 10 cfu equivalents of each other. Biofilms formed from left and right colon biopsies were compared, and were statistically equivalent in all patients at all timepoints except the 96 hour timepoint for patient B ($p=0.0286$).

Bacteroides-Prevotella in the anaerobic biofilms formed with bacteria from the same patient varied to a greater extent than total Eubacteria. Within a timepoint, biofilm samples varied up to 100 cfu equivalents within patient groups (Figure 29). Differences in *Bacteroides-Prevotella* numbers between left and right colon biopsies were only found at 144 hours in patient A ($p=0.0286$). At all other timepoints, left and right colon biofilms had equivalent numbers of *Bacteriodes-Prevotella*, which agrees with the lack of differences seen in biopsy *Bacteroides-Prevotella* numbers between left and right colon biopsies (Figure 26B).

C. coccoides numbers were found to differ between left and right colon biopsies in all three patients (Figure 26C). However, whereas *C. coccoides* levels were significantly higher in patient A left colon biopsies, the biofilms formed from patient A left colon bacteria had significantly lower levels of *C. coccoides* group bacteria at 48 hours ($p=0.0268$). The *C. coccoides* population dropped below a detectable level in both left and right colon biofilms at 96 hours and 144 hours (Figure 30). Patient B biopsies had greater numbers of *C. coccoides* in the right colon (Figure 26C), and biofilms from

Figure 28. Eubacteria in anaerobic biofilms of colon bacteria.

Total Eubacteria were enumerated in anaerobic biofilms of colon bacteria from patient A (A), patient B (B) and patient C (C) using qPCR. Biopsies 1 and 2 from each patient are from the left (descending) colon (labelled in black and grey, respectively). Biopsies 9 and 10 from each patient are from the right (ascending) colon (labelled in dark green and light green, respectively). Error bars represent SD. Data were analyzed using a Mann-Whitney U test to compare total Eubacteria in left verses right biofilms for each patient (* indicates a difference ($p<0.05$) between pooled left and right colon biofilms). The numbers of Eubacteria in left and right colon biopsies were statistically equivalent in all cases except the 96 hour timepoint for patient B ($p=0.0286$). Biofilms formed from patient A samples had similar amounts of Eubacteria, whether they were from the left or right colon ($p=0.2593$, 0.6200 and 0.6200 for 48, 96 and 144 hours, respectively). Patient B biofilms from left and right colon bacteria were also equivalent at 48 hours ($p=0.0571$), and patient C showed no differences in left and right colon biofilms ($p=0.0571$ for 48 hours and $p=1.000$ for 96 hours). Statistical tests could not be applied to 144 hour data for patients B and C due to small sample size (DNA extraction from the 144 hour biofilms of biopsies B2, B9, B10, C2, C9 and C10 bacteria failed).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Log10 cfu equivalents per biofilm
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Time (hours)
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Time (hours)
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B9
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C
Log10 cfu equivalents per biofilm
6
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Time (hours)
C1
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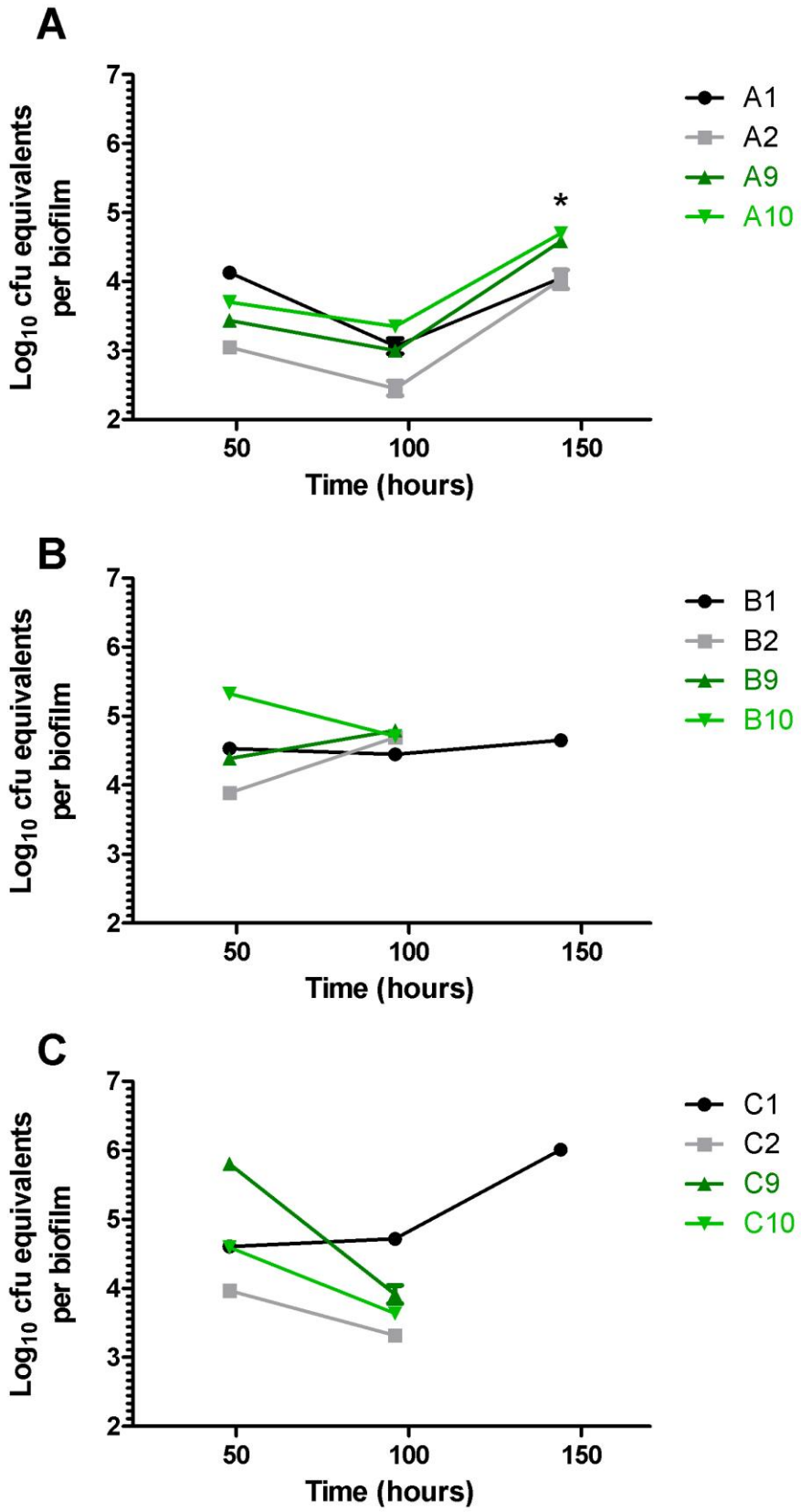
C10

Figure 29. Bacteroides-Prevotella in anaerobic biofilms of colon bacteria.

Bacteroides-Prevotella species bacteria were enumerated in anaerobic biofilms of colon bacteria from patient A (A), patient B (B) and patient C (C) using qPCR. Biopsies 1 and 2 from each patient are from the left (descending) colon (labelled in black and grey, respectively). Biopsies 9 and 10 from each patient are from the right (ascending) colon (labelled in dark green and light green, respectively). Error bars represent SD. Data were analyzed using a Mann-Whitney U test to compare *Bacteroides-Prevotella* numbers in left verses right colon biofilms for each patient (* indicates a difference ($p<0.05$) between pooled left and right colon biofilms). Patient A biofilms from left and right colon bacteria were equivalent at 48 hours ($p=1.000$) and 96 hours ($p=0.1143$), but right colon biofilms had statistically larger populations of *Bacteroides-Prevotella* at 144 hours ($p=0.0286$). Patients B and C showed no differences between left and right colon biofilms (patient B, $p=0.3429$ for 48 hours and $p=0.1143$ for 96 hours; patient C, $p=0.2000$ for 48 hours and $p=1.000$ for 96 hours). Statistical tests could not be applied to 144 hour data for patients B and C due to small sample size (DNA extraction from the 144 hour biofilms of biopsies B2, B9, B10, C2, C9 and C10 bacteria failed).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Log10 cfu equivalents per biofilm
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Log10 cfu equivalents per biofilm
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Time (hours)
B1
B2
B9
B10
C
Log10 cfu equivalents per biofilm
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5
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3
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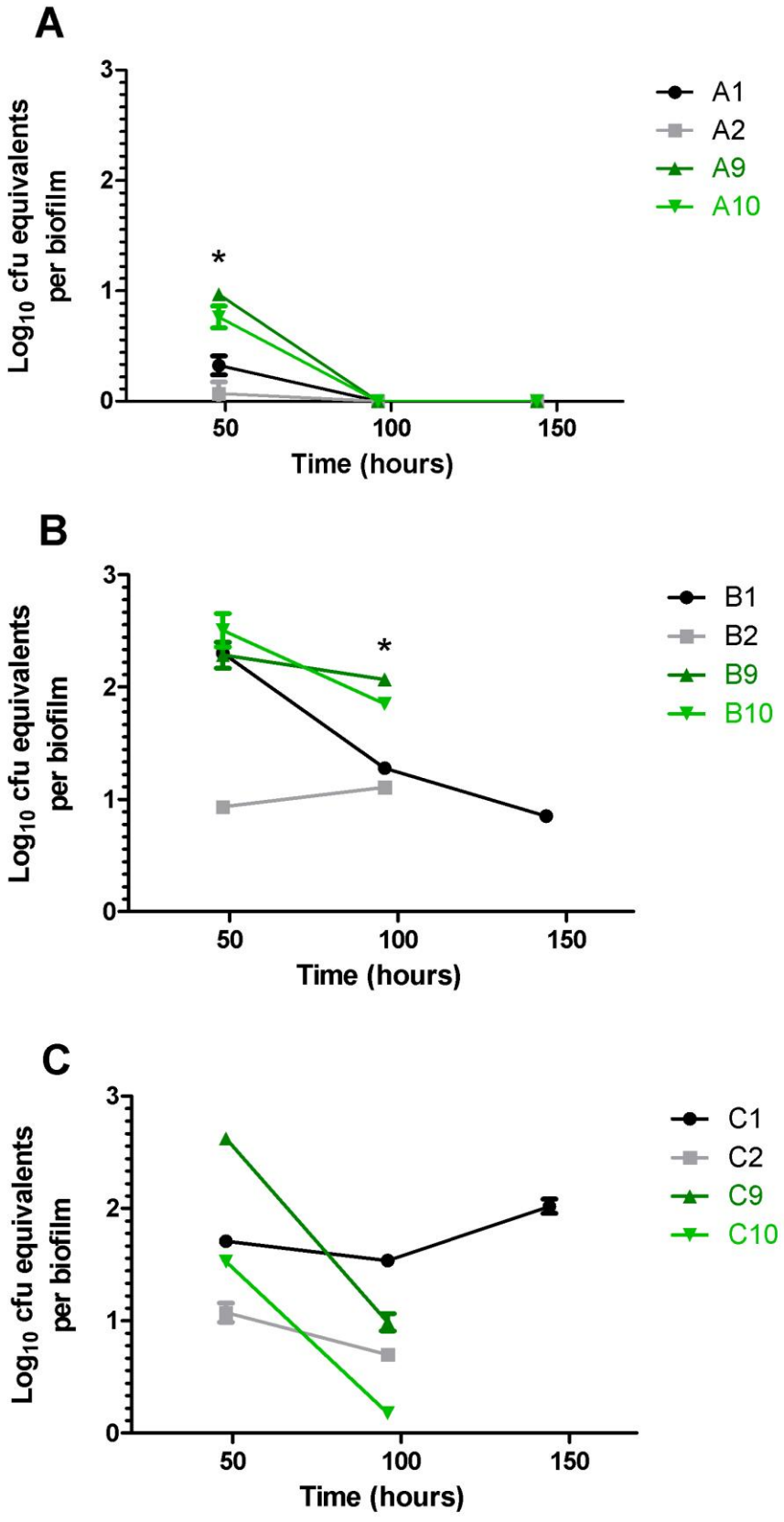
100
150
Time (hours)
C1
C2
C9
C10

Figure 30. *C. coccoides* bacteria in anaerobic biofilms of colon bacteria.

C. coccoides group bacteria were enumerated in anaerobic biofilms of colon bacteria from patient A (A), patient B (B) and patient C (C) using qPCR. Biopsies 1 and 2 from each patient are from the left (descending) colon (labelled in black and grey, respectively). Biopsies 9 and 10 from each patient are from the right (ascending) colon (labelled in dark green and light green, respectively). Error bars represent SD. Data were analyzed using a Mann-Whitney U test to compare *C. coccoides* group numbers in left verses right colon biofilms for each patient (* indicates a difference ($p<0.05$) between pooled left and right colon biofilms). Patient A biofilms had a statistically larger population of *C. coccoides* group bacteria in right colon biofilms than left colon biofilms ($p=0.0268$), but left and right colon biofilms from patient A were equivalent at 96 hours ($p=0.6716$) and 144 hours ($p=0.9063$). Patient B had equivalent levels *of C. coccoides* group bacteria in left and right colon biofilms at 48 hours ($p=0.1143$), but right colon biofilms had a larger *C. coccoides* group population at 96 hours ($p=0.0286$). Patient C had equivalent *C. coccoides* group numbers between left and right colon biofilms at 48 hours ($p=0.3429$) and 96 hours ($p=0.3429$). Statistical tests could not be applied to 144 hour data for patients B and C due to small sample size (DNA extraction from the 144 hour biofilms of biopsies B2, B9, B10, C2, C9 and C10 bacteria failed).


A
Log10 cfu equivalents per biofilm
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Log10 cfu equivalents per biofilm
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Time (hours)
B1
B2
B9
B10
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Log10 cfu equivalents per biofilm
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50
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Time (hours)
C1
C2
C9
C10

right colon bacteria showed higher *C. coccoides* levels at 96 hours (p=0.0286), but not at 48 hours. The largest spread between levels of *C. coccoides* in colon biofilms from the same patient was approximately 100 cfu equivalents (Figure 30).

In addition to examining differences between biofilms of left and right colon bacteria, differences in biofilm community composition between patients were examined. Patient B tended to have the largest numbers of total Eubacteria, *Bacteroides-Prevotella* and *C. coccoides* group bacteria at 48 and 96 hours, however patient C had an increase in all three groups between 96 and 144 hours (Figure 31). Patient A had significantly lower levels of total Eubacteria than both patients B and C at 48 hours (KW p=0.0004, Dunn's $p<0.001$ verses patient B, $p<0.01$ verses patient C). At 96 hours, patient B had significantly higher levels of total Eubacteria than both patients A and C (KW p=0.0003, Dunn's $p<0.001$ verses patient A, $p<0.05$ verses patient C). No significant differences in levels of total Eubacteria were found at 144 hours (KW p=0.0455, Dunn's failed to find any differences between groups).

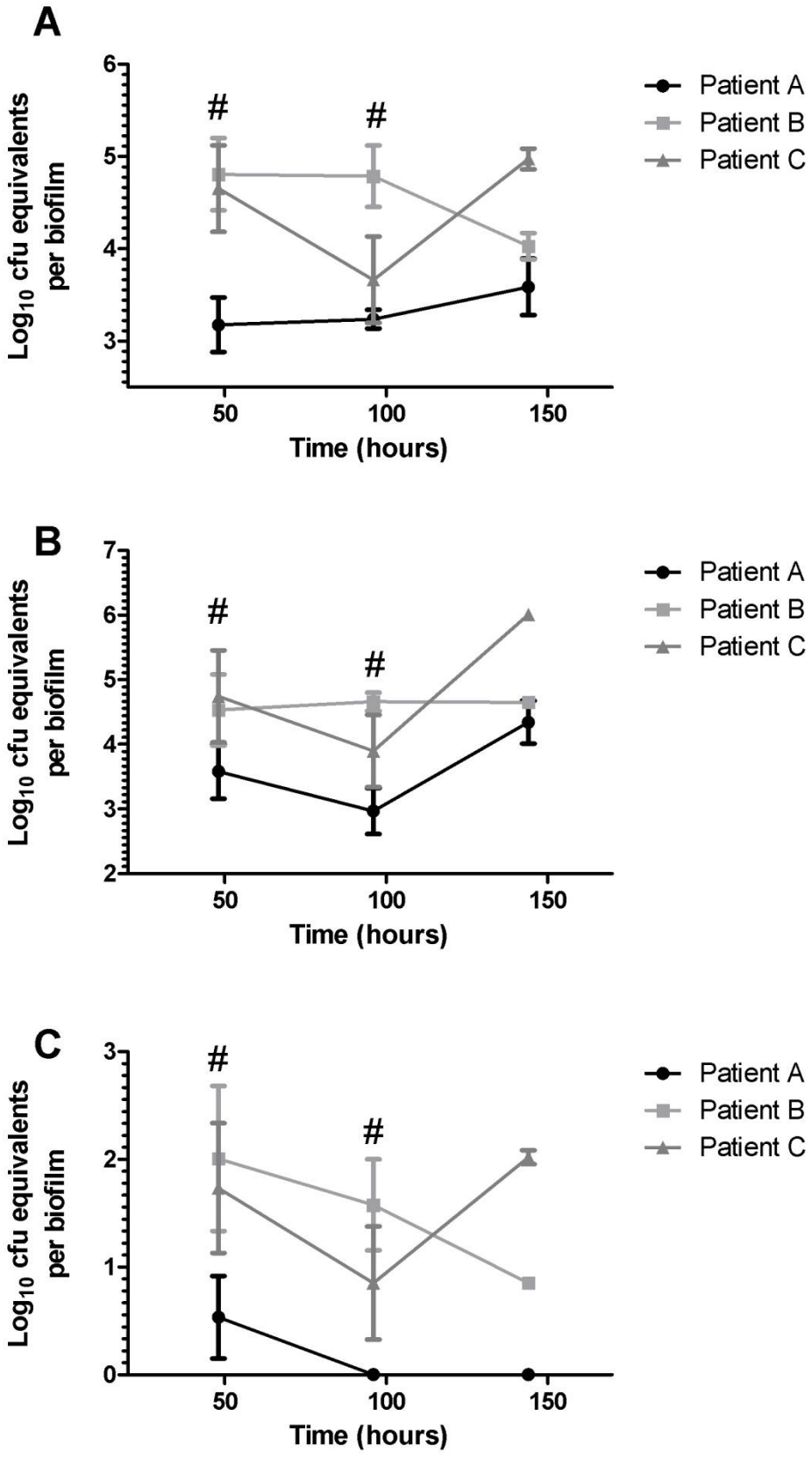
At 24 hours, levels of *Bacteroides-Prevotella* showed the same trend as total Eubacteria, in that Patient A had significantly lower levels than both patients B and C (KW p=0.0019, Dunn's $p<0.05$ verses patient B, $p<0.01$ verses patient C). At 96 hours, patient B had significantly higher levels of *Bacteroides-Prevotella* than patient A (KW p=0.0002, Dunn's $p<0.001$), but not patient C. The three patients were not found to have significantly different levels of *Bacteroides-Prevotella* at 144 hours (p=0.0545).

Patient A had significantly lower numbers of *C. coccoides* group bacteria than both patients B and C at 48 hours (KW p=0.0012, Dunn's $p<0.01$ verses patient B and C). *C. coccoides* group bacteria were not detected in patient A at 96 and 144 hours. Since

Figure 31. Bacterial community composition in anaerobic biofilms of colon bacteria from three patients.

Total Eubacteria (A), *Bacteroides-Prevotella* species (B) and *C. coccoides* group (C) were enumerated in biofilms of colon bacteria from patients A, B and C using qPCR. Error bars represent SD. Data were analyzed using a Kruskal-Wallis (KW) test and Dunn's multiple comparison test to determine differences between patients (in the case that data was only available for two patients, groups were compared using a Mann-Whitney U test). # indicates statistical differences between patients at the timepoint indicated. Patient A had significantly lower levels of total Eubacteria than both patients B and C at 48 hours (KW $p=0.0004$, Dunn's $p<0.001$ verses patient B, $p<0.01$ verses patient C). At 96 hours, patient B had significantly higher levels of total Eubacteria than both patients A and C (KW $p=0.0003$, Dunn's $p<0.001$ verses patient A, $p<0.05$ verses patient C). No significant differences in levels of total Eubacteria were found at 144 hours (KW $p=0.0455$, Dunn's failed to find any differences between groups). At 24 hours, levels of *Bacteroides-Prevotella* showed the same trend as total Eubacteria, in that Patient A had significantly lower levels of *Bacteroides-Prevotella* than both patients B and C (KW $p=0.0019$, Dunn's $p<0.05$ verses patient B, $p<0.01$ verses patient C). At 96 hours, patient B had significantly higher levels of *Bacteroides-Prevotella* than patient A (KW $p=0.0002$, Dunn's $p<0.001$), but not patient C (Dunn's $p>0.05$). No significant differences in levels of *Bacteroides-Prevotella* were found at 144 hours ($p=0.0545$). Patient A had significantly lower numbers of *C. coccoides* group bacteria than both patients B and C at 48 hours (KW $p=0.0012$, Dunn's $p<0.01$ verses patient B and C). *C. coccoides* group bacteria were not detected in patient A at 96 and 144 hours. Since it is

unknown whether these bacteria were absent or were present at levels below the threshold of detection, these data were not included in statistical analyses. Therefore, patient B and C were compared using a Mann-Whitney U test. Patients B and C had equivalent numbers of *C. coccoides* group bacteria at 48 (KW $p=0.0012$, Dunn's $p>0.05$) and 144 hours ($p=0.8360$), but patient B had higher numbers at 96 hours ($p=0.0104$).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Log10 cfu equivalents per biofilm
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Time (hours)
Patient A
Patient B
Patient C
B
Log10 cfu equivalents per biofilm
7
6
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4
3
2
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50
100
150
Time (hours)
Patient A
Patient B
Patient C
C
Log10 cfu equivalents per biofilm
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150
Time (hours)
Patient A
Patient B
Patient C

it is unknown whether these bacteria were absent or were present at levels below the threshold of detection, these data were not included in statistical analyses. Therefore, patient B and C were compared using a Mann-Whitney U test. Patients B and C had equivalent numbers of *C. coccoides* group bacteria at 48 (KW $p=0.0012$, Dunn's $p>0.05$) and 144 hours ($p=0.8360$), but patient B had higher numbers at 96 hours ($p=0.0104$).

5.3.4.4 Composition of Anaerobic Biofilms Formed from Frozen Colon Bacteria Communities

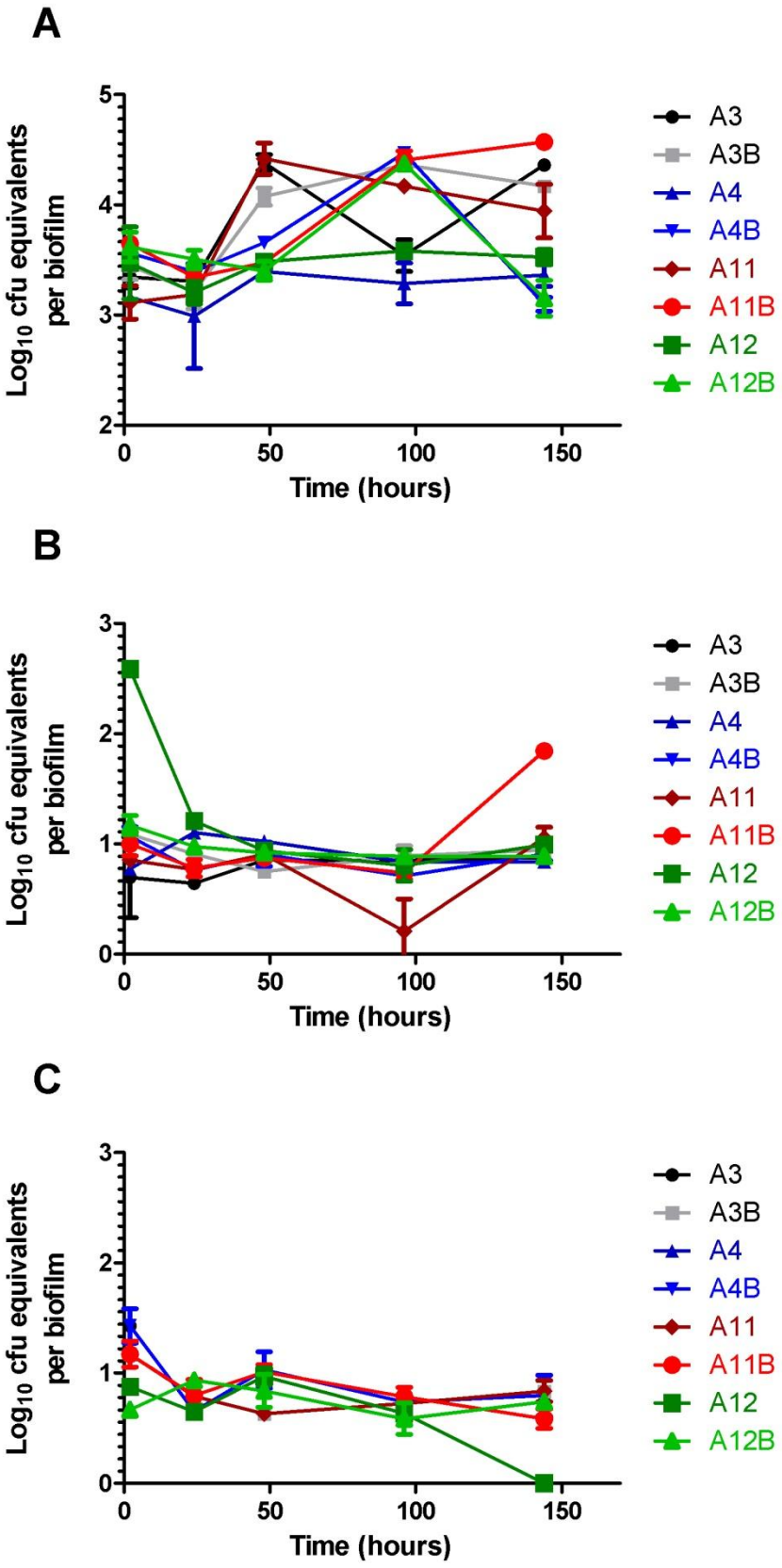
Biopsy bacteria were frozen and used to seed anaerobic biofilm experiments. These experiments were repeated twice, and replicates were compared to determine the feasibility of using frozen bacterial communities as a "representative microflora" in biofilm interaction experiments.

Anaerobic biofilms were started from frozen biopsy samples from patient A (A3, A4, A11 and A12) and grown for 144 hours with sampling at 2, 24, 48, 96 and 144 hours. Total Eubacteria, *Bacteroides-Prevotella* and *C. coccoides* group bacteria numbers were examined using qPCR. Replicate data sets are indicated with a "B" (for example A3B is the repeat of a biofilm from frozen biopsy sample A3). Levels of Eubacteria varied by a maximum of 10 cfu equivalents between replicates (Figure 32A), with lower variation between replicates for *Bacteroides-Prevotella* and *C. coccoides* (Figure 32B and C).

Figure 33 shows variation in replicates of biofilms from frozen samples divided for each biopsy. Biofilms from frozen samples of biopsies A3, A4 and A12 had very similar numbers of Eubacteria between replicates except at the 96 hour timepoint at

Figure 32. Quantification of bacterial groups in anaerobic biofilms formed from frozen colon bacteria communities.

Total Eubacteria (A), *Bacteroides-Prevotella* species (B) and *C. coccoides* group (C) were enumerated in biofilms formed from frozen colon bacteria communities of patient A using qPCR. The experiment was repeated twice (second replicate is indicated with a "B", for example A3B is the repeat of a biofilm from frozen biopsy sample A3, and these two experiments are colour-coded on the graph in black/grey). Replicates are represented in similar colours on the graph; biofilms from biopsy A3 are in black/grey, A4 data is displayed in blue, A11 in red and A12 in green. Error bars represent SD.


A
B
C
Log10 cfu equivalents per biofilm
Time (hours)
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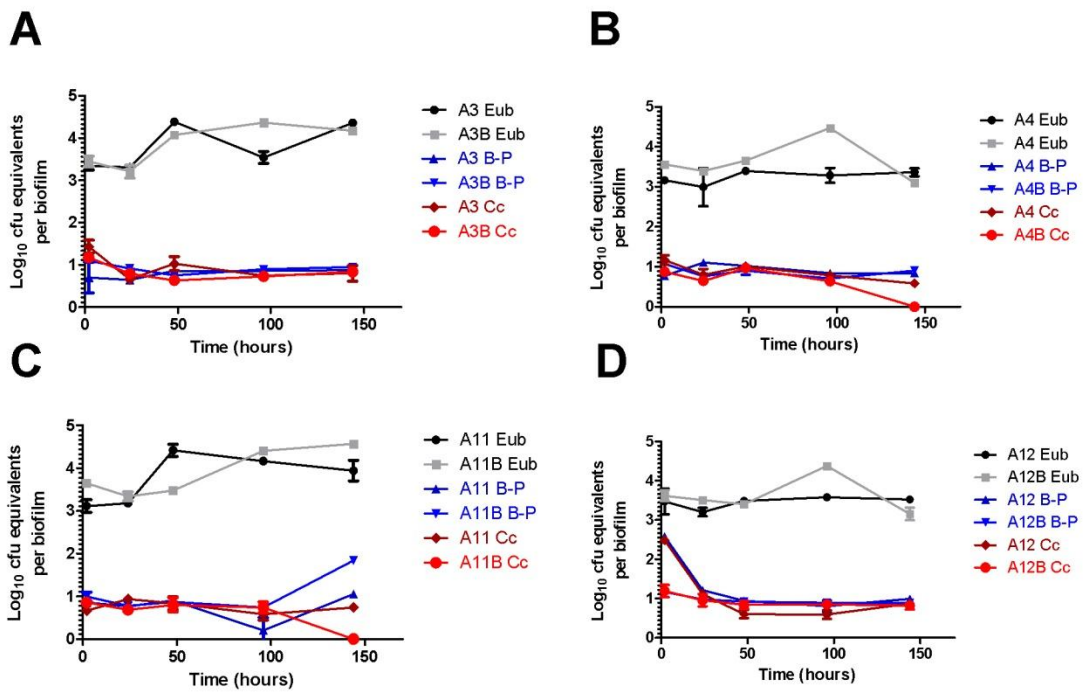
1
A3
A3B
A4
A4B
A11
A11B
A12
A12B

Figure 33. Bacterial composition of biofilms formed from frozen colon bacteria.

Biofilm experiments were repeated twice to determine if biofilm community composition was consistent between replicates. Frozen bacterial communities were from patient A, and the experiment was repeated twice (second replicate is indicated with a "B", for example A3B is the repeat of a biofilm from frozen biopsy sample A3). Total Eubacteria (Eub, black/grey), *Bacteroides-Prevotella* species (B-P, blue) and *C. coccoides* group bacteria (Cc, red) were enumerated in biofilm samples using qPCR. Frozen biopsies A3 (A), A4 (B), A11 (C) and A12 (D) were used to seed biofilm formation. Error bars represent SD.

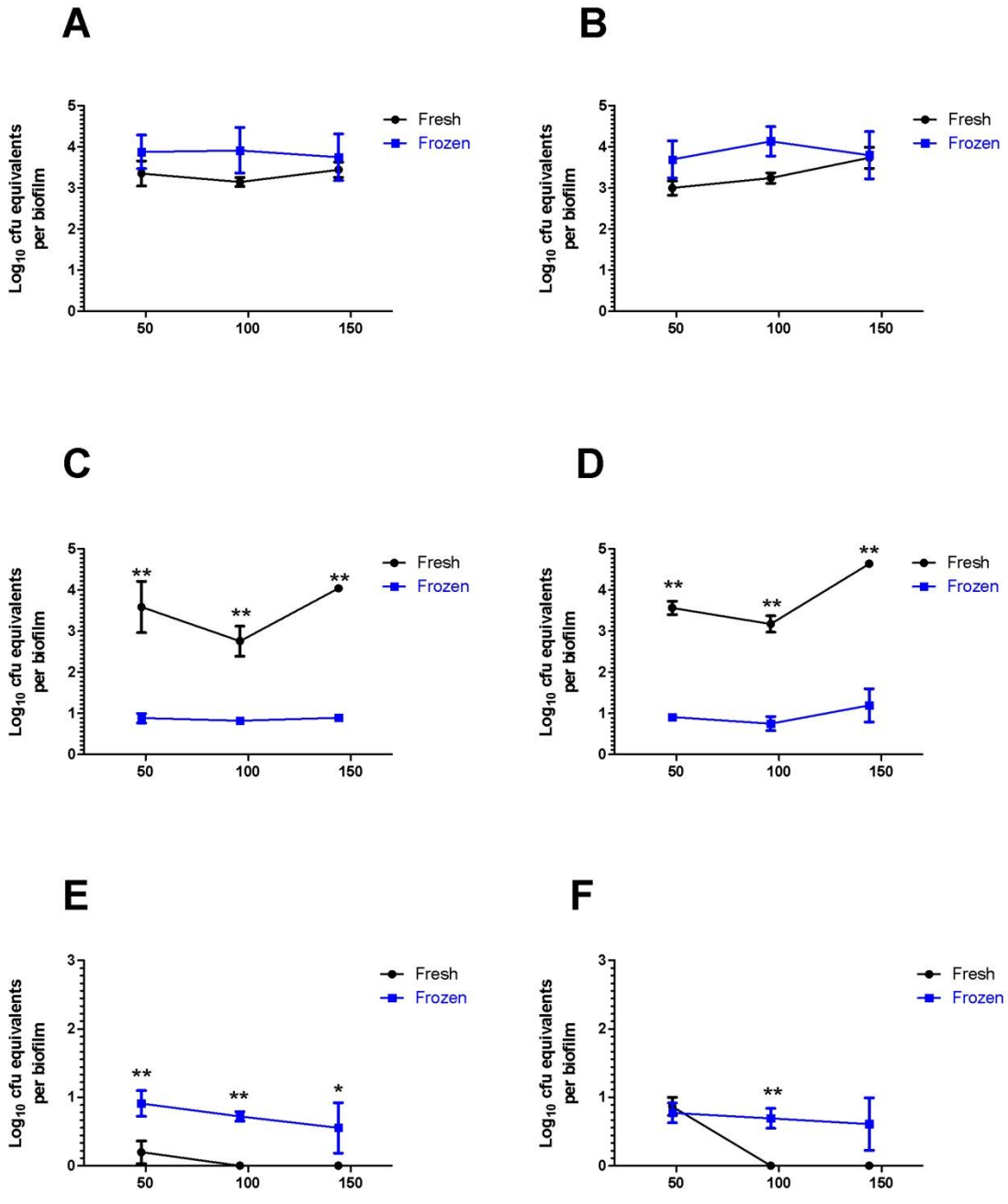


which there was a difference of up to 10 cfu equivalents. For biofilms from frozen biopsy A11 the largest difference between replicates was at 48 hours. In the second replicates of the A4 and A11 samples, *C. coccoides* levels dropped at the 144 hour timepoint (Figure 33B and C). This drop was not observed in the first replicate of these biofilms, and the source of variation is unclear since a drop in *C. coccoides* levels was not seen in the second replicate of A3 and A12 biofilms, which were grown in the same conditions as the second replicate of the A4 and A11 biofilms.

Biofilms from fresh and frozen colon biopsy samples were compared to determine changes in biofilm composition caused by freezing biopsies. Total levels of Eubacteria did not differ between fresh and frozen biofilms (left colon p=0.3357, 0.2319 and 1.000 for 48, 96 and 144 hours, respectively; right colon p=0.1520, 0.1520 and 0.8665, for 48, 96 and 144 hours, respectively) (Figure 34). Freezing colon bacterial communities significantly decreased the amount of *Bacteroides-Prevotella* in biofilms at all three timepoints tested (left colon p=0.0040 for each of 48, 96 and 144 hours; right colon p=0.0040, 0.0061 and 0.0084 for 48, 96 and 144 hours, respectively). *C. coccoides* group bacteria showed the opposite trend, and had significantly higher levels in biofilms from frozen bacteria at all timepoints in the left colon biofilms (p=0.0040, 0.0080 and 0.0131 for 48, 96 and 144 hours, respectively). In right colon biofilms, *C. coccoides* populations were statistically higher in frozen samples than frozen samples at 96 hours (p=0.0084), but not at 48 hours (p=0.2141) and 144 hours (p=0.1988). It is possible that the decrease in *Bacteroides-Prevotella* bacteria in the biofilms from frozen samples allowed for the expansion of the *C. coccoides* populations.

Figure 34. Bacterial community composition of biofilms formed from fresh and frozen colon biopsy samples.

Total Eubacteria (A-B), *Bacteroides-Prevotella* species (C-D) and *C. coccoides* group bacteria (F-G) were enumerated in biofilms formed from colon bacteria communities from the left colon (descending colon, A, C and E) and right colon (ascending colon, B, D and F) using qPCR. Seed communities for the biofilms were either bacteria from fresh biopsy samples (represented by black circles) or biopsies that had been stored at -70°C (frozen, blue squares). Data were analyzed using a Mann-Whitney U test (stars indicate significant differences between fresh and frozen samples; * denotes $p<0.05$, ** denotes $p<0.01$). Left and right colon biofilms did not differ in total Eubacteria numbers at any timepoint (left colon p=0.3357, 0.2319 and 1.000 for 48, 96 and 144 hours, respectively; right colon p=0.1520, 0.1520 and 0.8665, for 48, 96 and 144 hours, respectively). *Bacteroides-Prevotella* numbers were higher in fresh samples at all timepoints for both left and right colon biopsies (left colon p=0.0040 for each of 48, 96 and 144 hours; right colon p=0.0040, 0.0061 and 0.0084 for 48, 96 and 144 hours, respectively). *C. coccoides* numbers were statistically lower in biofilms seeded with fresh samples than frozen samples at all timepoints in the left colon biofilms (p=0.0040, 0.0080 and 0.0131 for 48, 96 and 144 hours, respectively). In the right colon biofilms, *C. coccoides* populations were statistically higher in frozen samples at 96 hours (p=0.0084), but not 48 hours (p=0.2141) and 144 hours (p=0.1988).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Log10 cfu equivalents per biofilm
Fresh
Frozen
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5.4 Summary

This chapter describes the process of establishing an in vitro "representative microflora". Communities of bacteria were isolated from human colon biopsies and were shown to be rich in diversity. Bacterial communities in the colon biopsies varied between patients, supporting previous observations that each person has a unique microflora (Eckburg *et al.*, 2005; Tap *et al.*, 2009). Because of variation between individuals, it was important to grow communities from more than one patient to fully explore the diversity of organisms that can be cultured as part of in vitro biofilms. In general, biopsies from the left (descending) and right (ascending) colon did not differ in total bacterial numbers and the numbers of *Bacteroides-Prevotella* bacteria present, but numbers of *C. coccoides* group bacteria were different between left and right colon biopsies.

Biofilm experiments demonstrated that complex communities of colon bacteria formed in vitro, with T-RFLP indicating that 31% of bacteria detected in biopsies participated in biofilm formation. The number of bacterial species in the human gastrointestinal tract is estimated to be at least 500 (Eckburg *et al.*, 2005; Young & Schmidt, 2008), and although only a portion of those are currently viewed as culturable, the T-RFLP data points to the in vitro biofilm communities being quite rich in diversity.

Biofilms formed from frozen biopsy samples were found to have decreased *Bacteroides-Prevotella* populations compared to biofilms from fresh biopsies. However, biofilms from frozen biopsies maintained populations of *C. coccoides* group bacteria and *Bacteroides-Prevotella*, and replicates of the frozen biofilm experiments were reasonably similar. Therefore, forming biofilms from frozen biofilms remains a possible technique

for producing a constant “representative microflora”. In contrast, biofilms from fresh biopsy samples were more diverse, but the same community cannot be used in multiple experiments (a possible drawback).

In summary, the biofilms formed in these experiments represent the first in vitro biofilm communities of a complex bacterial community (a “representative microflora”) from mucosal colon biopsies. This “representative microflora” may be used in future in vitro studies of interactions between pathogens and the gastrointestinal microflora, as well as interactions between host cells and the microflora.

Chapter Six: Discussion and Future Directions

6.1 Discussion

Gastrointestinal microflora are a very important part of the human body. They enhance digestion, contribute to nutrition, prevent infection and aid in the development of the immune system (Tannock, 2005). However, considering their importance, very little is known about the behaviours of the gastrointestinal microflora and their interactions with host cells and foreign bacteria. This is mostly due to the fact that current in vitro models for studying host-microflora interactions do not accurately represent gastrointestinal microflora. For example, many models use a single species to represent the intestinal microflora (Lewis *et al.*, 2008; Lewis & McKay, 2009; Nazli *et al.*, 2004; Nazli *et al.*, 2006). Other studies use fecal samples to represent the mucosal microflora, and while fecal samples are an improvement over a single species, studies have shown that luminal and mucosal bacterial populations differ in the colon (Eckburg *et al.*, 2005; Macfarlane & Macfarlane, 2001; Macfarlane & Dillon, 2007; Probert & Gibson, 2004; Zoetendal *et al.*, 2002). In addition, it is important to consider that mucosal bacteria in the intestine most likely exist in a biofilm state (Macfarlane & Dillon, 2007). Therefore, there is a need for an in vitro system for the study of bacterial interactions in the biofilm mode of growth using mucosal bacteria from the human colon. This project aimed to develop such a model.

6.1.1 A Biofilm Model of Bacterial Interactions

The first stage of model development involved studying bacterial interactions with well-defined organisms in a biofilm mode of growth. We determined the ability of

model pathogenic and commensal organisms to form biofilms independently and in co-culture. Interactions between biofilms of commensal and pathogenic organisms were also studied; biofilms were studied for altered growth in the presence of another biofilm and the antibiotic susceptibility of EPEC was studied in the presence of commensal biofilms.

6.1.1.1 Biofilm Formation by Commensal and Probiotic Bacteria

Biofilm formation by commensal and probiotic bacteria has not been characterized to the same extent as biofilm formation by pathogenic species. However, it is important to study biofilm formation by commensal bacteria since these communities live as biofilms in the gastrointestinal tract (Macfarlane & Dillon, 2007). Reisner and colleagues (2006) studied biofilm formation by 331 strains of pathogenic and non-pathogenic *E. coli* collected from patients using a 96-well plate and peg-lid system (similar to the CBD) with static incubation. They found a wide variety of biofilm-forming capabilities within both the pathogenic and non-pathogenic groups, and also a poor correlation between type of growth medium and biofilm formation. Our data showed larger biofilm formation in pathogenic strains of *E. coli* (EPEC, EHEC and UPEC) than in the "commensal" strain *E. coli* HB101, but supported Reisner's work in that the four *E. coli* strains we tested exhibited different degrees of biofilm formation in the CBD under the different growth conditions tested.

It is important to note that *E. coli* HB101 is a variant of *E. coli* K12, which has been in a laboratory environment for decades (Boyer & Roulland-Dussoix, 1969). *E. coli* isolated from swine feces have been shown to lose virulence genes in a matter of weeks

in manure holding tanks (Duriez *et al.*, 2008). It is therefore possible that *E. coli* HB101 has lost characteristics, such as the ability to form biofilms, which are favourable in the environment but not in the laboratory. In summary, our studies of *E. coli* biofilm formation support the current literature demonstrating that optimal conditions for biofilm formation and overall biofilm forming capabilities are highly strain-specific. It is likely that this phenomenon extends beyond *E. coli*, and therefore it is important to explore a variety of environmental factors when optimizing biofilm formation in vitro.

There have been a few previous studies of biofilm formation by *Lactobacillus* species in vitro. *L. rhamnosus* GG formed large biofilms in a static CBD-type biofilm assay compared to three other *L. rhamnosus* strains and four *L. casei* strains (Lebeer *et al.*, 2007b). It should be noted, however, that biofilm formation in this case was measured by crystal violet staining, and therefore biofilm measurements were based on populations of live and dead cells as well as bacterial exopolysaccharide (EPS), rather than the viable cell counting technique we used. *L. rhamnosus* GG is known to persist in the intestinal mucosa of healthy humans for more than one week after oral intake (Alander *et al.*, 1999), and this may be partially due to its ability to form biofilms. However, the majority of probiotic bacteria do not persist in the intestine unless taken daily (Mättö *et al.*, 2006). The need for these probiotics to be taken daily makes them highly appealing to pharmaceutical companies because of the potential for larger sales, but also because of increased safety. It is difficult to account for the potential risks of permanently colonizing patients with bacteria, whereas probiotics that cannot colonize carry fewer long-term risks. Therefore, pharmaceutical companies may not be interested in selecting for improved biofilm formation in their probiotic strains. The *L. plantarum*

strain used in our studies (commercially available as TuZen^{TM}, Ferring, Inc., Toronto, ON, Canada) formed very small biofilms in monoculture, which supports the theory that commercially available probiotics are not robust biofilm formers.

In the gastrointestinal tract (and at other mucosal surfaces) it would be rare for a monospecies biofilm to form. Perhaps probiotics are able to form biofilms in vivo by integrating into established biofilms of commensal organisms. In support of this, biofilm formation by *L. plantarum* has been shown to improve upon co-culture with *Actinomyces* species and *Streptococcus mutans* in the multiplaque artificial mouth model (Filoche *et al.*, 2004). In some of our studies, we observed a small increase in *L. plantarum* biofilms when they were grown in co-culture with EPEC. These results stress the importance of studying biofilm formation from a multispecies community perspective, rather than in monoculture.

To address this issue, we used VSL#3, a mixture of eight probiotic bacteria (4 *Lactobacillus* species, 3 *Bifidobacteria* species and *Streptococcus thermophilus*) as an example of a simple multispecies community that may cooperate to improve overall biofilm formation. In our hands, VSL#3 biofilm growth on the pegs and wells of the CBD was similar to monospecies biofilms of *L. plantarum* at 24 hours. However, at 72 hours VSL#3 biofilms were relatively large (averaging $1x10^6$ cfu per peg). Additional experiments are needed to determine if this differs from *L. plantarum* growth at 72 hours, but it is possible that the eight strains work together to establish biofilms. It is also important to further investigate the VSL#3 biofilms to determine the contribution of each of the eight strains to the biofilms, as we did not differentiate between strains when

enumerating biofilms. qPCR with strain-specific primers could be used to shed light on this issue.

The growth of biofilm communities of not only single strains (EPEC, EHEC, UPEC, *E. coli* HB101 and *L. plantarum*), but also a model multispecies community (VSL#3), demonstrated our ability to form biofilms in the CBD model. This accomplishment set the stage for use of the model to study bacterial interactions in the biofilm mode of growth, an area of the literature which has been lacking.

6.1.1.2 Biofilm Co-Culture

The ability to co-culture biofilms is a powerful tool for studying bacterial interactions. The *L. plantarum* and EPEC co-culture experiments performed using our model are the first example of bacterial interference studies where both strains (commensal/probiotic and the pathogen) were grown as biofilms. Previously, commensal bacteria have been shown to affect biofilm formation by pathogens, for example *L. plantarum* secreted factors were shown to inhibit acyl-homoserine-lactone (AHL) secretion, biofilm formation and elastase production by *Pseudomonas aeruginosa* (Valdéz *et al.*, 2005). In contrast, we did not observe an effect of *L. plantarum* on EPEC biofilm formation. It is possible that *L. plantarum* secreted factors are active against a specific subset of bacteria. *P. aeruginosa* is ubiquitous in the environment (Stover *et al.*, 2000), whereas humans and other mammals (such as cattle, rabbits, dogs and swine) are thought to be the primary reservoir for EPEC, although individual humans to not tend to remain colonized for long periods of time (Nataro & Kaper, 1998; Stephan *et al.*, 2004).

It is possible that *L. plantarum* would therefore have more opportunities to evolve defences against *P. aeruginosa* than EPEC.

Because EPEC forms larger biofilms than *L. plantarum* in co-culture, it might have been expected that EPEC would outgrow and exclude *L. plantarum* in these mixed species biofilms. When inoculated in equal amounts, *L. plantarum* and EPEC each contributed the same number of cfu to the biofilm as they did in monoculture. However, when *L. plantarum* was inoculated at a half dose and EPEC at a full dose the *L. plantarum* content of the co-biofilm dropped, but did not fully disappear. Longer co-culture experiments should be conducted to determine if EPEC is able to out-compete *L. plantarum* over time, or if the slower growing *L. plantarum* makes a "comeback" over an extended growth period.

There may be a threshold inoculum beneath which *L. plantarum* can no longer establish itself within the mixed species biofilm. This threshold may also apply to EPEC. In vivo, pathogens such as EPEC encounter a pre-established biofilm of microflora, and must be present in sufficient numbers to colonize. This scenario could be studied in vitro using the model created in this project, where a pre-established microflora biofilm is exposed to a pathogen. It is also important to study the impact of pathogenic biofilms on biofilm formation by microflora. This is especially important in *C. difficile* infection, where *C. difficile* exploits the clearance of microflora biofilms due to antibiotic use (Baxter *et al.*, 2008; Stephan *et al.*, 2004). Once *C. difficile* is established it is exceedingly difficult to clear. *C. difficile* may use biofilm formation on the mucosa as a strategy to prevent recolonization of the normal microflora.

Although our results did not demonstrate the ability of *L. plantarum* to affect EPEC biofilm formation or survival, *L. plantarum* was observed to increase EPEC antibiotic susceptibility in both planktonic and biofilm co-cultures for several of the antibiotics tested, including a β-lactam, cephalosporins and fluoroquinolones. Aminoglycoside and rifampicin activity was not affected by *L. plantarum*. The mechanism by which *L. plantarum* increased the efficacy of antibiotics against EPEC in our in vitro experiments is unknown. However, current research points to the effect of secreted factors, such as bacteriocins and biosurfactants, on biofilm formation and antibiotic susceptibility of pathogens. At least 15 bacteriocins are produced by *L. plantarum* strains (Verellen *et al.*, 1998), and *Lactobacillus* species also produce biosurfactant molecules that display antibacterial properties and were found to inhibit adhesion (and therefore biofilm formation) of *E. faecalis* in an in vitro model (Cameotra & Makkar, 2004; Velraeds *et al.*, 1996). In the case of the data presented here, any products secreted by *L. plantarum* were insufficient to inhibit EPEC growth alone, but perhaps worked synergistically with the antibiotics to inhibit EPEC biofilm and planktonic growth. Our observations are supported by other studies, in which bacterial biosurfactants were shown to exhibit antimicrobial activity against biofilms (Rivardo *et al.*, 2009; Rodrigues *et al.*, 2004), and to act synergistically with antibiotics to enhance antibiotic susceptibility of biofilms (Rivardo, Turner and Ceri, unpublished data). Therefore, it is likely that *L. plantarum* secretes a factor that is capable of acting synergistically with some antibiotics to enhance EPEC susceptibility to antibiotics.

Antibiotics and probiotics are often viewed as opposing choices in the treatment of gastrointestinal conditions such as irritable bowel syndrome (Rioux & Fedorak, 2006).

However, many probiotic organisms have been found to display resistance to several antibiotics (Temmerman *et al.*, 2003). While this is concerning and should be carefully monitored, it may also offer an opportunity for the use of probiotics in combination with antibiotics under carefully controlled conditions. Probiotics have already demonstrated promise in preventing colonization by *C. difficile* following antibiotic use, and probiotics such as *Saccharomyces boulardii* and *L. rhamnosus GG* have been administered during antibiotic treatment (Gorbach *et al.*, 1987; McFarland *et al.*, 1994; Surawicz *et al.*, 1989). These studies, in combination with our data, support the use of probiotics in combination with antibiotic treatment for conditions such as irritable bowel syndrome and IBD.

Probiotic benefits go well beyond bacteriocin and biosurfactant secretion. Many probiotics have the ability to modulate the immune response by promoting host defence mechanisms, including increasing epithelial barrier function (Ewaschuk *et al.*, 2008). The model of biofilm co-culture for the study of bacterial interactions presented here could be altered to include a human epithelial cell component. By studying bacterial biofilm interactions in the presence of host cells, the complex mechanisms of action of probiotic and commensal bacteria could be further elucidated.

In summary, we developed a model of biofilm co-culture that allows for the study of interactions between commensal and pathogenic organisms. Using this model, we demonstrated that *L. plantarum* increased the antibiotic susceptibility of EPEC when the two organisms were grown in a biofilm together. Additionally, we found that VSL#3, a community of eight different strains, formed substantial biofilms. This aligns with other studies that have demonstrated increased biofilm formation by probiotic bacteria when co-cultured with other organisms.

6.1.1.3 The CBD as a Model for Biofilm Interaction Studies

An objective of this project was to develop a model for the study of interactions between two biofilms, for example the commensal microflora and a pathogen in the gastrointestinal tract. Biofilm growth and antibiotic susceptibility assays using the pegs of the CBD are common (Harrison *et al.*, 2004; Lebeer *et al.*, 2007b; Moskowitz *et al.*, 2004; Spoering & Lewis, 2001), as are assays of biofilms grown in the wells of 96-well microtitre plates (Amorena *et al.*, 1999; Davey *et al.*, 2003; O'Toole & Kolter, 1998). However, this work represents the first attempt at using both the peg and well surfaces to bring two independently formed biofilms together to study biofilm-biofilm interactions.

The CBD model of biofilm-biofilm interactions has a number of advantages. It is relatively simple to set up, high-throughput (96 peg and well interactions per plate), and can be adapted for anaerobic culture. Growth conditions can be tailored to each biofilm during their independent growth periods. The pegs and/or wells of the CBD can also be coated or modified to create an ideal surface for biofilm formation.

In biofilm-biofilm exposure experiments conducted with EPEC and *L. plantarum* in the CBD, the two strains did not appear to impact each other significantly in terms of biofilm size. This is consistent with data from the biofilm co-culture experiments, where the two bacteria did not significantly impact one another's growth even though they were colonizing the same space. One result of interest is that *L. plantarum* biofilm growth in the wells of the CBD was significantly increased in the presence of an independently-formed EPEC biofilm (grown on the peg of the CBD). EPEC may have provided a resource for the *L. plantarum* that assisted it with biofilm formation, such as EPS (a coating that promotes biofilm formation) or a quorum-sensing signal that encourages the

biofilm phenotype. *L. rhamnosus* GG biofilms have been shown to be EPS-dependent in some growth media (Lebeer *et al.*, 2007b), and extra EPS from an outside source may decrease energy expenditure requirements for the *Lactobacillus* and allow for faster biofilm growth. EPEC and *L. plantarum* may also communicate through quorum sensing in co-culture. *E. coli* strains and *L. rhamnosus* GG have been shown to produce autoinducer 2 (AI-2)-type molecules (De Keersmaecker *et al.*, 2003; Surette *et al.*, 1999). In addition, an *L. rhamnosus* GG *luxS* mutant was found to be defective in biofilm formation unless co-cultured with a wild-type strain (Lebeer *et al.*, 2007a). This suggests a role for AI-2 in biofilm formation by *Lactobacillus*.

The biofilm-biofilm interaction studies presented here represent a novel approach to studying bacterial interference in the biofilm mode of growth. The increased growth of *L. plantarum* in the presence of an EPEC biofilm may offer insights into biofilm formation in multispecies communities, such as the intestinal microflora. When combined with other studies, our results suggest a role for interspecies signalling through quorum sensing and a shared foundation for biofilm architecture (EPS).

6.1.2 Working Toward a "Representative Microflora"

A main objective of this project was to improve upon current models of gastrointestinal microflora. For example, many studies of the effect of commensal flora on intestinal barrier function use single organisms, such as *E. coli* HB101 or *E. coli* C25, to represent commensal flora (Lewis *et al.*, 2008; Lewis & McKay, 2009; Nazli *et al.*, 2004; Nazli *et al.*, 2006). Proteobacteria is not one of the most prevalent bacterial phyla in the healthy human intestine, and therefore if a single organism is to be used, perhaps a

more representative choice would be a member of Firmicutes or Bacteroidetes. However, the use of anaerobic organisms does create new challenges in terms of experimental design (discussed later in this section).

Bacterial communities extracted from fecal material are also commonly used to represent intestinal microflora (Macfarlane & Macfarlane, 2001; Macfarlane *et al.*, 2005; Probert & Gibson, 2004). Fecal samples are relatively easy to obtain and do not require an invasive procedure to collect. However, although these multispecies communities are a much truer representation of intestinal microflora than a single strain, fecal bacteria have different community compositions than mucosal bacteria (Eckburg *et al.*, 2005; Zoetendal *et al.*, 2002). While mucosal samples represent bacterial biofilms living in the mucous lining of the intestines, fecal samples represent the contents of the intestinal lumen, and contain ingested bacteria and bacteria growing on food particles, as well as bacteria shed from mucosal biofilms. Mucosal colon biopsies, although requiring invasive procedures to collect, give a much more accurate representation of the mucosal microflora than fecal samples, although one must consider the possibility of fecal contamination during collection through the colonoscope (Tannock, 2005). Another factor to consider when interpreting studies based on biopsies is that colonoscopy patients undergo an intestinal purging routine prior to sample collection. Although this may be advantageous in that it removes luminal bacteria, it is unknown if mucosal bacterial populations are altered during the flushing process.

6.1.2.1 Biofilm Formation by Intestinal Microflora

The first step in creating our model of "representative microflora" was to establish biofilms of mucosal colon bacteria in vitro. Macfarlane and colleagues (2007) have imaged biofilms on the mucosa of the intestine using FISH. To our knowledge, we are the first group to establish biofilms of these mucosal bacterial communities in vitro, although biofilms of fecal bacteria have been established in vitro (Macfarlane & Macfarlane, 2001; Macfarlane *et al.*, 2005; Probert & Gibson, 2004). Confocal microscopy confirmed the formation of mature microcolonies in our CBD model, with several cell morphologies present. The images of complex biofilm communities we obtained from our in vitro model have similarities to the images of biofilms on mucosal intestinal biopsies, in that they were three dimensional with clusters of similar cell morphologies distributed throughout the biofilm (Macfarlane & Dillon, 2007).

The goal of growing biofilms of "representative microflora" in vitro was to create a bacterial community as similar as possible in composition to the mucosal biofilms in the intestine. Previous studies have characterized biofilm communities in fermenter systems inoculated with human feces (Macfarlane *et al.*, 2005; Probert & Gibson, 2004), and found that the biofilms contained the major groups of colonic bacteria, including Firmicutes, Bacteroidetes and Actinobacteria (Tap *et al.*, 2009).

The data collected during this project suggest that our in vitro biofilm communities resemble the bacterial composition of intestinal mucosal microflora to the extent that it is characterized in the literature (Eckburg *et al.*, 2005). T-RFLP data indicated that Firmicutes, Bacteriodetes and Actinobacteria were also the most prevalent bacteria in our biofilms. In addition, several key species were identified within our

biofilms, including members of the *C. coccoides* and *C. leptum* groups, several *Bacteroides* species, *Bifidobacteria* species and *L. acidophilus*.

Previous studies, in combination with our results, suggest that it is possible to grow in vitro biofilms that maintain at least a portion of the diversity of the intestinal microflora. In addition, our studies demonstrate that it is possible to grow these biofilms from mucosal bacterial populations rather than feces. This is an important step in modeling mucosal biofilms, since mucosal bacterial communities are known to differ from fecal communities in the intestine.

The advantages of growing our "representative microflora" as biofilms are many. Firstly, evidence supports biofilms as the normal mode of growth of the microflora in the intestine (Macfarlane & Dillon, 2007; Moran & Annuk, 2003). Secondly, the biofilm community is a perfect environment for the culture of fastidious organisms. T-RFLP data indicated that we were able to grow 31% of bacteria from mucosal colon biopsies in our in vitro biofilms. This is a staggering number considering only 10-60% of the hundreds of species that make up the intestinal microflora are considered culturable (Amann *et al.*, 1995; Ley *et al.*, 2006; Zoetendal *et al.*, 2004). Modification of our model may allow us to culture an even greater portion of the microflora, perhaps including some bacteria that were previously considered "unculturable". There are several examples of species that are "unculturable" in monoculture, but can be grown in vitro in co-culture with another organism. *Tannerella forsynthesis* (formerly *Bacteroides forsynthesis*) cannot be grown in pure culture because it lacks the ability to synthesize *N*-acetylmuramic acid, but it thrives in co-culture with *Fusobacterium nucleatum* (Wade, 2002; Wyss, 1989). Kaeberlein *et al.* (2002) used a "simulated natural environment" to grow previously

uncultured bacteria from a beach environment. One of the recovered strains was subsequently found to grow in Petri dishes, but only in the presence of one of several other strains isolated from the same environment. These studies support the likelihood that we were able to grow previously "unculturable" bacteria in our in vitro biofilm system, since biofilms provide a rich environment for bacteria that are dependent on products from other organisms. Additional community analyses are required to confirm this hypothesis.

The next step for this model is to chemically crosslink human mucin to the pegs and/or wells of the CBD to create a more mucosal surface-like environment for biofilm formation. In addition, the growth media should be optimized to encourage growth of fastidious (and previously "unculturable") organisms. Adding a human intestinal epithelial monolayer to our model will be a major step in advancing this model of microflora biofilms. Ideally, a mucin-secreting cell line, such as HT-29-FU cells (Lesuffleur *et al.*, 1991) can be used to allow for biofilms to form in a layer of mucin secretions rather than directly adherent to the monolayer. The integration of human cells into this model will require some reconfiguration of the set-up, since human cells cannot be grown in an anaerobic environment. One possibility for getting around this problem is to grow anaerobic biofilms on top of epithelial cell monolayers in transwells. The top of the transwells could be sealed with tape or mineral oil, but the monolayers would still receive nutrients and oxygen from the basolateral side. Alternatively, a pre-grown biofilm of commensal flora could be grown on a "peg" (such as those used in the CBD) and this peg could be lowered into a well or transwell apparatus containing the epithelial cell monolayer.

6.1.3 Reproducibility of the "Representative Microflora"

A challenge of creating a "representative microflora" is that each individual has a different intestinal "normal" flora. Our data support this, in that microbial communities within the biopsies and biofilms showed differences between patients. Differences between individuals were also found in in vitro biofilms of fecal bacteria (Probert & Gibson, 2002; Probert & Gibson, 2004). In addition, we found variation in biopsy bacterial communities from the same patient, suggesting that microenvironments may exist within the intestine. It is already recognized that microflora communities differ along the length of the gastrointestinal tract (Eckburg *et al.*, 2005), but our data suggest that biopsies taken from adjacent locations may differ in bacterial composition. Biofilms on the intestinal mucosa are therefore not likely to be uniform, but instead exist as a series of microniches, each with a unique composition of microflora.

Another aspect to consider is the reproducibility of the biofilms formed from biopsy bacteria. As discussed above, the biofilms we formed included approximately 31% of bacteria in the biopsies (based on T-RFLP analysis). While working to improve the proportion of species present in the biofilms by encouraging growth of fastidious organisms, it is also important to observe the effect of altered growth conditions on the relative proportion of bacterial groups within the community. For example, the Firmicutes and Bacteriodetes, which should be most prevalent within the biofilms, may be overgrown by faster growing organisms such as the Enterobacteriaceae. Based on the qPCR data gathered to date, it does not seem that either the *C. coccodies* group (Firmicutes) or *Bacteroides-Prevotella* species (Bacteroidetes) populations decreased

during a 144 hour growth period. These data support our observations made using T-RFLP, which suggested that we were able to grow representative, complex and stable biofilm communities in vitro.

In order for the "representative microflora" developed here to be used in studies of interactions of the intestinal microflora with host cells and pathogenic bacteria, the differences between successive "representative microflora" communities must be accounted for so as not to affect reproducibility of the experiments. This can be accomplished from two perspectives: 1) choosing a single "representative microflora", which can be frozen and used repeatedly, or 2) developing an ecological perspective or strategy to account for the differences in "representative microflora" between experiments.

6.1.3.1 The Feasibility of Freezing a "Representative Microflora"

Additional experiments with larger sample sizes must be conducted before a conclusion can be drawn, but replicates of biofilms formed from the same frozen stock bacterial communities did have some differences in community structure based on qPCR analysis. At this point, it is difficult to determine if these differences would significantly affect the results of successive experimental replicates. Additionally, we discovered that the use of frozen samples, rather than freshly collected biopsies, resulted in an altered biofilm community structure. For example, qPCR indicated significant changes in the levels of *Bacteroides-Prevotella* species and *C. coccodies* group bacteria in biofilms from frozen samples compared to biofilms from fresh samples. T-RFLP analysis would be

useful in determining if a loss in diversity occurred, or if the relative proportion of bacterial groups changed without the loss of strains.

6.1.3.2 Accounting for Differences in Samples of "Representative Microflora"

Another approach to dealing with differences between samples of "representative microflora" is to take on a more ecological perspective. It is unlikely that we will ever be able to control such a vastly diverse community without simplifying it, which would defeat the purpose of having a "representative microflora". The "representative microflora" we developed is a vast improvement over the use of a single species such as *E. coli* HB101. It could be that slight variations in the microflora communities do not change the function of the overall community. In fact, it has been suggested that a core set of niches or metabolic roles exist for intestinal microflora, with several different bacteria able to fulfill the requirements for many of the niches (Tannock, 2005). However, metagenomic studies of fecal bacteria from patients all over the world do point to an "intestinal microbiota phylogenetic core", with the approximate makeup of: 79.4% Firmicutes, 16.9% Bacteroidetes, 2.5% Actinobacteria 1% Proteobacteria, 0.1% Verrucomicrobia and 0.1% other phyla (Eckburg *et al.*, 2005; Gill *et al.*, 2006; Li *et al.*, 2008; Manichanh *et al.*, 2006; Tap *et al.*, 2009).

Additional analysis is required to determine if our "representative microflora" biofilms are exactly representative of the "intestinal microbiota phylogenetic core", but slight fluctuations in our biofilm communities may not cause any greater variation than what is seen between study subjects. The qPCR analysis performed on in vitro biofilms supported this, as inter-patient variation tended to be larger than variation between

samples of the same patient. In addition, inter-patient variation was larger than the community fluctuations observed over the 144 hour growth period. Altogether, this suggests that the differences we observed in community structure between biopsy and biofilm samples fall well within the limits of the "intestinal microbiota phylogenetic core" suggested by Tap *et al.* (2009).

If necessary, differences in key groups of bacteria could be monitored using qPCR, or T-RFLP could be used to compare overall microflora community structure between experiments. This could also be an opportunity to explore the function of various groups within the "representative microflora", where the loss of a specific group of bacteria may have a specific effect on experimental outcomes. This may be most effectively studied by monitoring metabolic activities of the biofilms in the presence or absence of key bacterial groups. For example, RT-qPCR could be used to monitor expression of genes involved in specific metabolic pathways, or the entire metabolomic profiles of two different communities could be compared.

6.1.4 Limitations of Profiling Methods

The community profiles produced during this project are approximations. A combined approach using both T-RFLP and qPCR profiling techniques was used to increase the accuracy of findings. It is important to note that the majority of molecular techniques lack the sensitivity to detect less abundant organisms within a community (Li *et al.*, 2007). In addition, the databases used for indentifying species are incomplete (Schutte *et al.*, 2008).

The techniques used to assign T-RFs to organisms are varied. For this project, we performed a "virtual digest" of the H.Q. database of T-RFs from gut organisms using the RDP and MiCA (Cole *et al.*, 2007; Cole *et al.*, 2009; Sepehri *et al.*, 2007). This technique gives a fast, coarse overview of community structure at the phylum level. However, one of the challenges of this method is that estimated peak sizes can vary based on T-RF migration through the gel (Osborn *et al.*, 2000). The fluorescent dye used to label the forward primer may cause variation in the migration rates of the T-RFs (Schutte *et al.*, 2008). Therefore, additional T-RFLP analysis must be conducted for each sample using other restriction enzymes, so that the T-RF assignments from multiple experiments can be corroborated.

Although the 8f and 926r primers used in these studies were determined to be one of the best primer sets for community analysis, these primers are not suited to amplification of 16S rRNA genes from some *Bifidobacterium* and *Aeromonas* species (Liu *et al.*, 1997). These bacteria can be detected by running additional experiments using specific primers for these genera, but this does not allow for the same degree of community analysis because it is difficult to analyze data collected from different T-RFLP experiments. Differences in rRNA operon copy numbers can also skew species representation by PCR-based techniques (Farrelly *et al.*, 1995).

The qPCR method used in these studies is limited by the calculations used to convert Ct values into cfu equivalents. When enumerating a group such as total Eubacteria, which has a wide range of 16S rRNA operon copy numbers and a large variation in genome sizes, one needs to interpret results with caution (Fogel *et al.*, 1999).

Despite the limitations of the T-RFLP and qPCR techniques, this study was successful in demonstrating the diversity and complexity of mucosal bacteria and the in vitro biofilms formed from these communities. Although the cfu counts determined using the molecular techniques were approximations, the trends they represent offer new insights into the community structure of commensal bacteria and their ability to form biofilms in vitro.

6.1.5 How Representative is the "Representative Microflora"?

The model described here incorporates a "representative microflora", which serves to mimick the colonic microflora in the in vitro model. As previously discussed, this community contained approximately 31% of bacteria present in mucosal colon biopsies based on T-RFLP analysis. While almost one third of biopsy bacteria were represented in the "representative microflora", they were not necessarily present in the same abundance. Therefore, the "representative microflora" acts as an in vitro approximation of the adherent colonic microflora, rather than being an exact replication. We strived to make the "representative microflora" as close to in vivo conditions as possible but, as with other in vitro model systems, it is unlikely this model will ever completely duplicate the conditions of the human gastrointestinal tract.

Although the "representative microflora" is not an exact replication of biopsy bacterial communities, it is an improvement over current in vitro models for studying colonic microflora. The "representative microflora" is grown in a biofilm mode of growth, and contains a population of mucosal bacteria. These two characteristics have never been combined in an in vitro model before. Therefore, the "representative

microflora" developed here should be viewed as a work in progress, a community that can be improved upon in future studies. As anaerobic biofilm culture techniques progress it is likely the "representative microflora" will become closer in composition to human colonic microflora. However, the current "representative microflora" is the closest in vitro approximation of human mucosal colonic microflora available, and therefore is the most representative microflora available for in vitro studies.

As alluded to in Section 6.1.3.2, another difficulty associated with developing a "representative microflora" is the fact that each individual has a unique microflora. Therefore, even if an in vitro model was able to exactly replicate one individual's microflora, that model microflora may not be representative for another individual. Rather than focusing on replicating the phylogenetic composition of the mucosal microflora, it may be more effective to focus on establishing an in vitro "representative microflora" that duplicates the core functions carried out by the intestinal microflora of healthy humans. Although each human has a unique microflora, these diverse microflora communities appear to perform the same functions in each individual (Tannock, 2005). Therefore, it is possible that an in vitro model community could model the functionality of the mucosal microflora without being an exact replica of any one individual's colonic microflora.

6.2 Future Directions

This work describes the development of a model for growing biofilms of "representative microflora" in vitro. The model allows for the study of interactions

between this "representative microflora" and other bacteria, but the model has not yet been fully utilized to study biofilm interactions.

6.2.1.1 Microflora-Pathogen Interactions

The intestinal microflora is known to protect the host from numerous enteric pathogens. The "representative microflora" biofilms should be exposed to pathogens such as *Campylobacter jejuni*, EPEC, and *C. difficile* to determine the ability of microflora to prevent biofilm formation and colonization of the pathogens. Studying the minimal amount of pathogenic bacteria required to establish growth in the presence of the "representative microflora" may also give a better understanding of infectious doses.

In the early stages of model development we studied EPEC biofilm formation in the presence of *L. plantarum*. In addition to this experiment, a study of EPEC biofilm formation on a surface already colonized by a mature *L. plantarum* biofilm should be conducted. This more accurately represents most cases in the gastrointestinal tract, where a pathogen enters an environment already colonized by intestinal microflora. For example, *C. difficile* readily colonizes the intestines when microflora have been displaced by antibiotic use (Baxter *et al.*, 2008), but it is likely that *C. difficile* cannot grow large biofilms in an environment where it has to compete with normal flora.

In addition to studying competitive exclusion, cell-free supernatants of gastrointestinal microflora could be tested for antibacterial activity against a range of pathogens. Exposure of microflora supernatant to EPEC biofilms did not result in an alteration of EPEC biofilm size, but it is possible that these supernatants may be capable of altering EPEC in other ways, such as virulence factor expression or antibiotic

susceptibility (as *L. plantarum* was shown to alter antibiotic susceptibility of EPEC in our model). In addition, microflora supernatants may be active against other pathogens.

6.2.1.2 Expanding the Model

To simulate the human intestine in the most accurate way possible human cells, for example mucin-secreting HT-29-FU cells (Lesuffleur *et al.*, 1991), should be incorporated into the model so that host-microbe interactions can be studied. Many of the protective effects of intestinal microflora involve immunomodulation, rather than interactions with the pathogen. For example, probiotic bacteria have been shown to enhance epithelial barrier function in IL-10 deficient mice (Madsen *et al.*, 2001) and stimulate the production of cytokines (including interferon (IFN)-γ, IL-6 and IL-12) in human and rodent cell lines (Erickson & Hubbard, 2000). The logistics of adding a human cell monolayer to the existing model were previously discussed (Section 6.1.2).

By growing biofilms of "representative microflora" on top of an epithelial cell layer, pathogen colonization could be studied from a new angle. The ability of pathogens to penetrate the commensal biofilm to make contact with host cells could be assessed. In addition, treatments such as antibiotics could be examined for their ability to prevent infection by pathogens, but also the impact they have on the commensal community.

6.2.1.3 Additional Techniques for Assessing Biofilm Community Diversity

In order to further characterize the biofilms of "representative microflora" additional community analysis should be conducted. To date, only total Eubacteria, *Bacteroides-Prevotella* and *C. coccoides* group bacteria have been enumerated using

qPCR. Many of the primers used for group-specific PCR can be adapted for qPCR (Table 3), and further qPCR experiments with these primers would give a more complete picture of the "representative microflora" communities.

Difficulties with assigning T-RFs can be overcome by conducting T-RFLP experiments with group-specific primers (Blackwood *et al.*, 2005; Li *et al.*, 2007). This narrows the possible species identities for T-RFs, thus making data analysis simpler and more accurate. This would also help to overcome PCR selection difficulties, since universal primers are not required. The use of more specific primer sets decreases the number of degeneracies expected between the primers and template DNA sequences, therefore avoiding unequal binding of primers to template DNA. T-RFLP and qPCR analysis should also be expanded to look at microbes beyond the Eubacteria, since Archaea and Eukarya (yeasts), as well as viruses also contribute to the intestinal microflora (Young & Schmidt, 2008).

To explore the "representative microflora" biofilms beyond cataloguing the species that make up the communities, additional microscopic analysis should be undertaken. FISH would be a powerful tool for visualizing the distribution of various bacterial groups throughout "representative microflora" biofilms, and has been successfully used to examine intestinal bacterial biofilms in vivo (Macfarlane & Dillon, 2007). With access to a flow cytometer, FISH could be used to label bacteria that would then be counted. The capacity for fluorescence activated cell sorting (FACS) would be particularly useful because it can sort cells into groups. This technique could be used to isolate bacteria of interest, which could then be studied further with more specific

molecular analyses. For example, FISH has been used in combination with FACS to enumerate members of the *C. leptum* group in human fecal samples (Lay *et al.*, 2005).

6.3 Conclusions

The intestinal microflora make up a complex community which is difficult to simulate in vitro. This study endeavoured to create a model that improved upon current methods used in the literature. The CBD-based model that resulted allowed for the study of interactions in biofilm co-cultures, and interactions between two independently-formed biofilms. In addition, the model successfully incorporated a "representative microflora", which is an improvement over current models in that it is:

- A multispecies community from the human colon
- Composed of mucosal bacteria, rather than luminal (fecal) bacteria
- Composed of the major phyla present in the intestine
- A model of biofilm growth (thought to be the primary mode of growth for mucosal bacteria)

In addition, the "representative microflora" was shown to maintain its diversity (at least at a coarse level) for a minimum of one a week. Freezing the "representative microflora" resulted in a change in community structure compared to freshly collected communities, but replicate biofilms formed from the same frozen bacterial communities were similar.

Overall, this study was successful in developing a novel approach to the characterization of intestinal microflora and their interactions with pathogenic organisms.

The model demonstrated the complexity of the biofilms formed by mucosal intestinal microflora and provided a novel approach to growing in vitro anaerobic biofilms. This work lays the foundation for future studies which will further elucidate the role of the intestinal microflora in human health.

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