A Novel *in vitro* Mucosal Gut Bacterial Adhesion Model

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TO MY GRANDFATHER
AND HIS ETERNALLY CURIOUS MIND

Abstract

The gut bacterial system is truly a dynamic, complex organ. Although there is a constant flux of activity, the microflora can be considered as a bioreactor at a quasi-steady state throughout a person's life. In fact, the relative composition of different bacterial genera can lead directly to health or disease. By understanding how the gut is colonized and what can be administered to alter overall composition, one would be able to use the gut as a legitimate target for drug delivery. *In vitro* gut adhesion models have been developed exactly for this purpose however have several limitations.

In this thesis, an attempt has been made to develop a new gut adhesion model that included several key components associated with bacterial adhesion to the gut mucosal lining. For this, mucus-coated beads were used to simulate the mucosal lining. As well, beads were incubated with intestinal bacteria from a fresh human fecal sample. In this way, one would be study to observe the interactions between different bacteria within the gut, and the interaction between these commensal bacteria and any potential therapeutic design. This new model is a continuous model, allowing for real-time analysis of the mucosal-associated flora. This will allow its use to understand the effect of different external factors over an indefinite experimental period on gut bacterial adhesion.

Results demonstrated that this model was highly effective in providing a stable microbial ecosystem for a single bacterial strain or for a large number of aerobic or anaerobic bacteria. The model was also shown to perform very well over long-term studies. This model has numerous applications and includes the investigation of probiotics, prebiotics, and antibiotics on altering a mucosal-associated microflora.

Resume

La flore intestinale est un organe dynamique et complexe qui se trouve à l'intérieur du tube digestif. Bien qu'il y ait un flux d'activité constant, la microflore peut être considérée comme un bioréacteur ayant un quasi état d'équilibre durant toute la vie d'une personne. En fait, la composition relative des différents types de bactéries est directement reliée à la santé de l'individu. En comprenant comment l'intestin est colonisé et ce qui peut être administré pour modifier sa composition générale, on serait en mesure de l'utiliser comme une cible légitime pour la livraison de médicaments. Des modèles in-vitro d'adhérence intestinale ont été mis au point exactement à cette fin.

Un modèle comprenant plusieurs éléments clés associés à l'adhésion bactérienne sur la muqueuse intestinale a été développé pour cette thèse. Tout d'abord, des capsules d'alginates recouvertes de mucus ont été utilisées afin de simuler la muqueuse intestinale. En outre, ces capsules ont été incubés avec les bactéries intestinales à partir d'un échantillon frais provenant des fécales humaines. De cette façon, on serait en mesure d'observer les interactions entre les différentes bactéries dans l'intestin, et l'interaction que ces bactéries ont avec tout autre traitement. Finalement, ce modèle est continu, permettant une analyse en temps réel de la muqueuse associée à la flore et nous permet de comprendre l'éffet de différents facteurs environnementaux sur de longues périodes de temps.

Les résultats ont démontré que la plate-forme a été très efficace dans la fourniture d'un écosystème stable microbien pour une seule souche bactérienne ou pour un grand nombre de bactéries aérobies ou anaérobies. Le modèle a également montré de très bonnes performances au cours des études à long terme en utilisant plusieurs échantillons pendant l'expérience. Les applications de ce modèle sont pratiquement infinies, et permettent notamment d'enquêter sur l'effet que les probiotiques, les prébiotiques, et les antibiotiques ont sur la modification d'une microflore associée à la muqueuse.

Acknowledgment

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Finally, I would like to acknowledge the unconditional support for my family - my mother and father, sister and grandparents, from which my fascination of medicine and all things science has stemmed. Without them, I would be nothing.

Preface

In accordance with the McGill thesis preparation and submission guidelines, I have decided to write this thesis as a compilation of original papers. This section is provided in the McGill University Thesis Preparation and Submission Guidelines, which reads as follows:

"As an alternative to the traditional thesis style, the research may be presented as a collection of papers of which the student is the author or co-author (i.e., the text of one or more manuscripts, submitted or to be submitted for publication, and/or published articles (not as reprints) but reformatted according to thesis requirements as described below). These papers must have a cohesive, unitary character making them a report of a single program of research."

The research articles presented in this thesis (chapters 3, 4 and 5) are divided into the following sections: Abstract, Introduction, Materials and Methods, Results and Discussion, and Conclusions. This thesis also includes a common Abstract, General Introduction, Literature Review, Summary of Results, Discussion, Conclusions and future recommendations and thesis cited references.

List of Abbreviations

ANOVA - analysis of variance

APA - alginate-poly-l-lysine-alginate

BSH - bile salt hydrolaze

GI - gastrointestinal

MAF – mucosal associated flora

MRS - de Man, Rogosa and Sharpe media

SCFA – short chain fatty acids

SD - standard deviation

SEM - scanning electron microscopy

Units

CFU - colony forming units

cm - centimeters

mm - millimeter

ml - milliliters

µl - microliters

µM - micromolar

mM - millimolar

g - grams

mg - milligrams

µg - micrograms

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Chapter I: General Introduction

The gut microbiota is a complex microbial ecosystem within the gastrointestinal system of humans containing trillions of living, metabolically active bacteria that play a significant role in the overall health of the host. In fact, bacterial cells in the gut outnumber mammalian cells in the body by a factor of 10, while at the same time, contain 100 times more genetic information than mammalian cells [1,2]. The vast metabolic, immunological and structural functions that can be attributed to the bacteria living in the gut directly lead to health consequences, whether they are positive or negative. In fact, one can consider the gut microbiota to be at a quasi-steady state throughout the life of an adult, with relative proportions of different bacterial phyla remaining constant. In the event of an altered state of this quasi-steady state by relative increases of one phylum, genus or even species can lead to an altered metabolite profile and could lead to disease[3-8]. This altered state can be a consequence of lifestyle [9,10], diet [11,12] or genetic factors [13]. As such, one can consider the gut microbiota as a legitimate target for disease prevention and treatment.

In order to effectuate changes in the gut, it is essential to understand the mechanism by which bacteria colonize the mucosal lining and the challenges they must endure in order to become permanent inhabitants. The best way to investigate this is through *in vitro* models of the gut. When developing these models, there are several key criteria that must be met. Firstly, it is important to

recognize that the mucosal associated microbiota are the more permanent members of the gastrointestinal system as opposed to those living in the lumen [14,15]. Therefore, one design criterion for an *in vitro* assay would be to investigate strictly bacteria that adhere to a simulated mucosal surface. Secondly, it would be important to have a fully representative sample of normal gut bacteria growing in a competitive microenvironment. Finally, real-time sampling would be essential for a model in order to investigate how a population changes as a function of time. With these design criteria in mind, a novel mucosal-based *in vitro* adhesion model was the main research goal of the presented thesis.

1.1 Research Objectives

The main objective of the presented thesis is to develop a novel mucosal-based *in vitro* model of the gut microbiota in which a number of key aforementioned design criteria would be fulfilled, namely: (i) a model that allows for the investigation of the interaction between bacteria and with the gut mucosal layer, (ii) a model that incorporates a large spectra of intestinal bacteria, (iii) a model that allow gut bacteria interaction studies over a long time period in real-time. The specific research objectives are:

1) To perform an extensive literature review on current *in vitro* gut-mucosal adhesion models and summarize their respective strengths and weakness and develop a new model which addresses several key criteria
2) To design an *in vitro* gut bacterial cell mucosal adhesion model

3) To investigate the feasibility of an *in vitro* gut adhesion model to establish a stable microbial ecosystem.
4) To study the model suitability in probiotic studies by investigating the effect of the administration of a probiotic on
    a. A potential pathogen
    b. The intestinal microbiota immobilized on an *in vitro* gut adhesion model in real-time.

Chapter II: Literature Review

2.1 The Gut Microbiota as an organ within an organ

The gastrointestinal (GI) system is one of the most complex systems in the human body. It consists of several different organs, all of which perform essential functions for the health state of the host. The large intestine has garnered significant interest over the last decade due to the magnitude of genetic information stored in the living species that thrive in that microenvironment. These members consist mainly of bacteria, however, there are also significant amounts of representatives from the domain Archaea and Eukarya, along with different viruses and bacteriophage [16]. Their importance is based on the shear quantity and variability in species and genetic information. In fact, the bacteria living in the gut outnumber all mammalian cells in the body by a factor of 10 [2] and they contain 100 times as much genetic information as those cells as well [1]. This bank of genetic information contains essential information on the metabolism of different substances, which flow through the GI system. The gut microbiota performs a variety of different functions, which can be broken into three main categories.

2.1.1 The gut microbiota executes essential metabolic functions

The most obvious functions carried out by the gut microbiota are its metabolic functions. This highly active system of bacteria found in the GI system has evolved into an efficient bioreactor that is critical in vitamin production, amino acid synthesis, bile acid biotransformation and the fermentation of non-digestible

substrates and endogenous mucus[17,18]. These fermentation processes stimulate the production of short-chain fatty acids (SCFAs), including acetate, butyrate, propionate, lactate, ethanol, succinate, formate, valerate, caproate, isobutyrate, 2-methyl-butyrate and isovalerate.

2.1.2 The gut microbiota ensures protection

In the highly competitive ecosystem found in the gut, commensal organisms can provide a protective role in preventing against any foreign pathogenic colonization. Nutrient competition, attachment site competition and production of anti-microbial peptides are all key ways in which the gut microbiota can act as an immunological barrier. The microbiota is also an important player in immune system development, as demonstrated in experiments with germ-free mice that displayed underdeveloped lymphatic systems [19]. The microbiota has also been shown to regulate the production of pro-inflammatory cytokines, including interleukin-17[19].

2.1.3 The gut microbiota performs structural and histological functions

The gut microbiota also plays an important role in overall intestinal structure through many different ways. Firstly, it has been shown that different SCFAs, a metabolic by-product of bacterial metabolism, particularly butyrate, reinforce the colonic defense barrier by inducing the secretion of mucins, the major protein component to mucus, trefoil factors and antimicrobial peptides [20]. A second proposed mechanism for how the gut microbiota affects the structural integrity of

the intestine is their effect at the level of the tight junctions, protein clusters that form a barrier between the lumen and the lamina propria. Finally, SCFAs have also been shown to stimulate epithelial cell and tissue development.

All the described functions are listed in the figure below.

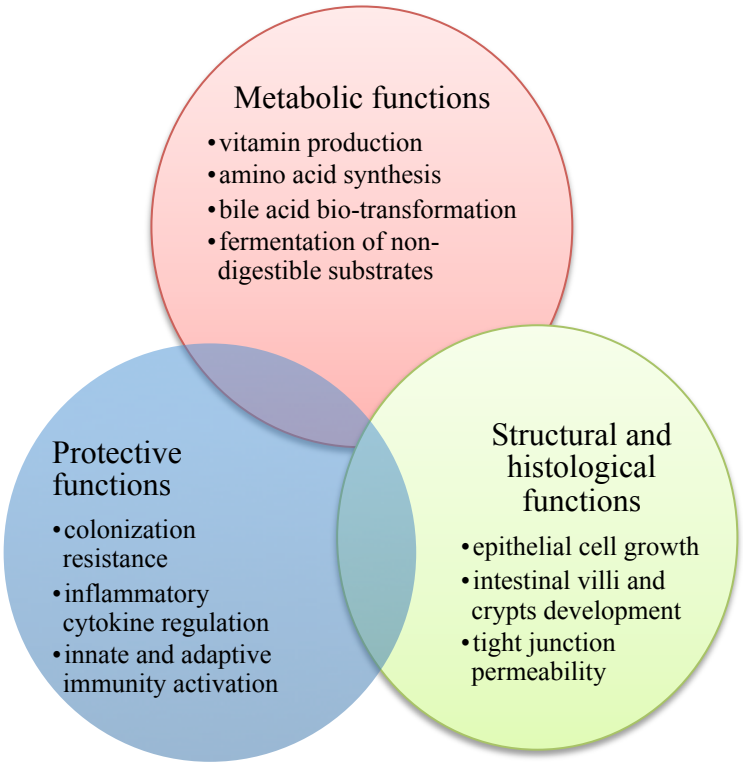

Figure 2.1: The main beneficial functions of the human gut microbiota [17,18,21,22]

As such, the flora that live in the gut are critical in maintaining a healthy state for the host, and on the flip side, can contribute directly to different disease states. In fact, one can consider the gut microbiota to be at a quasi-steady state throughout the life of an adult, with relative proportions of different bacterial phyla remaining

constant. In the event of an altered state of this quasi-steady state by relative increases of one phylum, genus or even species can lead to an altered metabolite profile and could lead to disease[3-8]. This altered state can be a consequence of lifestyle [9,10], diet [11,12] or genetic factors [13].

2.2 Modulation of gut microbiota for human health

As stated above, it has been well established that an altered state of the gut microbiota can lead to a disease state. By determining which genus or species is associated with a disease state, researchers can monitor disease development, and more importantly, can modulate the gut microbiota from an unhealthy status into one associated with health and wellbeing. The gut microbiota can be modulated in several different ways:

i. Probiotics: Probiotics have been described as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host [23]. The way in which probiotics confer health benefits to the host can be explained with several hypotheses presented here: remodeling of microbial communities and suppression of pathogens, pro-inflammatory factors, and affecting gut epithelial cells directly in improving the intestinal barrier [24]

ii. Prebiotics: Prebiotics are non-digestible carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon [25]. In

doing so, they alter the microbial ecosystem by promoting the selective proliferation of ‘health-promoting bacteria’.

iii. Synbiotics: Synbiotics are the combined treatment of probiotics and prebiotics with the hope that the effect of both treatments will be synergistic when implemented together[26]. As of yet, there is no clear evidence that a combinatorial treatment of probiotics and prebiotics will provide any added benefit to the overall health of the host[27].

iv. Antibiotics: Antibiotics can be used to modulate the gut microbiota by targeting and killing a particular set of ‘bad bacteria’ typically associated with a specific disease state[28].

Currently, probiotics are the most commonly used and researched methods for modulating the gut microbiota in order to promote health. Although they are widely used across Europe, clinical skepticism is a significant obstacle for widespread use in North America. In order to understand this skepticism, one must understand the significant challenges facing these orally ingested probiotics throughout their transit in the gastrointestinal system.

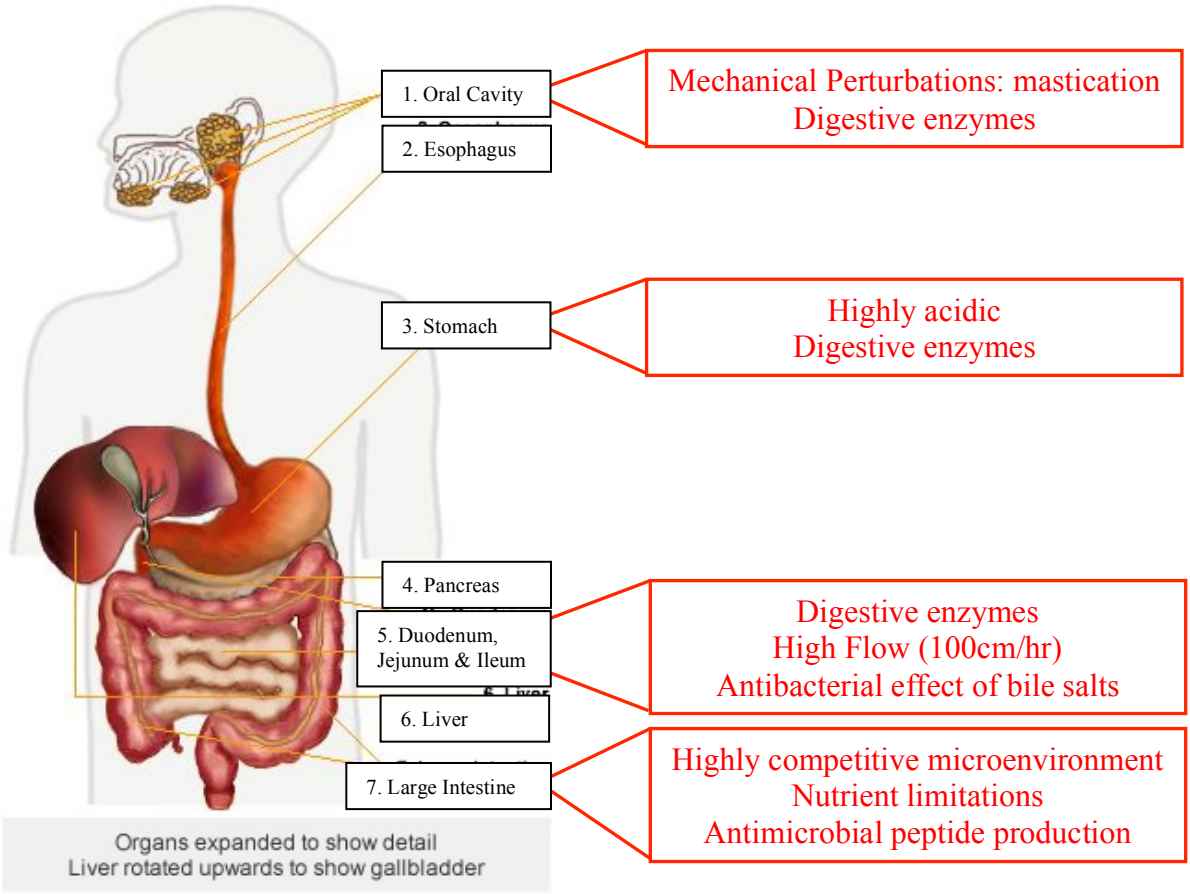

Figure 2.2: Challenges associated with oral delivery of probiotics during gastrointestinal transit

Each obstacle has garnered new research interests, and required the development of new technologies in the field of biomedical engineering and biotherapeutics. One major innovation developed at McGill University was the microencapsulation of live cells in order to create a physical barrier protecting the encapsulated substance from its surrounding environment [29].

2.3 Microencapsulation for increased gastrointestinal viability

In order to enhance bacterial cell numbers in the targeted region of the gastrointestinal tract, the large intestine, probiotics can be microencapsulated into

micron-dimension particles. These microcapsules are selectively permeable to small metabolites and substrates to ensure the survival of the probiotics while at the same time protecting them from the host's immune system. The membrane typically used is alginate-poly-lysine-alginate[30-36]. All three layers interact with one another through electrostatic interactions and so are responsive to pH changes. As such, the microcapsules enable a longer-term viability for the microencapsulated cells throughout the harsh conditions of the gastrointestinal transit [37].

2.4 Adherence Paradigm

It has been well established that there are two main ecosystems within the gut: bacteria free-living within the lumen and mucosal associated flora (MAF) [38]. Research has also shown that MAF are significantly more resistant to GI transit washout and in fact show altered transcriptional profiles [14,15]. These observations are critical in understanding why one orally ingested probiotic could potentially have a longer lasting and more observed effect over another. In other words, if a foreign bacterial cell can manage to transition from the lumen ecosystem into a mucosal associated ecosystem, one could potentially label that foreign substance as resilient, and thus more likely to have an effect on the overall health of the host. The research question can therefore become how to measure such transition or adherence into an ecosystem already well inhabited by permanent residents of the gut. In order to answer such a question, one must well

understand the anatomy of the gut lining and then create an *in vitro* model that can accurately represent different facets of this lining.

2.5 Anatomy and Physiology of the Gut Lining

The epithelium is the main lining of the internal tissues of the whole human body, and has as a main goal to protect the body from any external factors such as microbes that could cause infection. There are two types of epithelial surfaces – the dry epithelium (our skin) and the moist epithelium (eg the gastrointestinal tract) [39]. I will focus my discussion on the moist epithelium, as it is more relevant for the gastrointestinal system. It consists of a layer of specialized cells invariably coated by a layer of mucus (making it 'moist') that consists mainly of glycoprotein's known as mucins whose molecular weights are typically in the range of 10^5 to 10^6 Da [39]. Maintaining the structure of the gut epithelial cell layer are several different mechanical structures that serve different purposes. Tight junctions seal cells just below the mucosal layer and selectively permit only the smallest molecules to diffuse through. Gap junctions consist of channels between epithelial cells that again selectively allow the passage of medium sized molecules (less than 1000 Da). Adherens junctions serve more as a mechanical tool to attach cells together. They act by linking adjacent transmembrane proteins known as cadherins [39]. Any disruption to typical junction structure and function could lead to the passage of unwanted molecules into the blood stream and subsequently, different diseases[11,40,41].

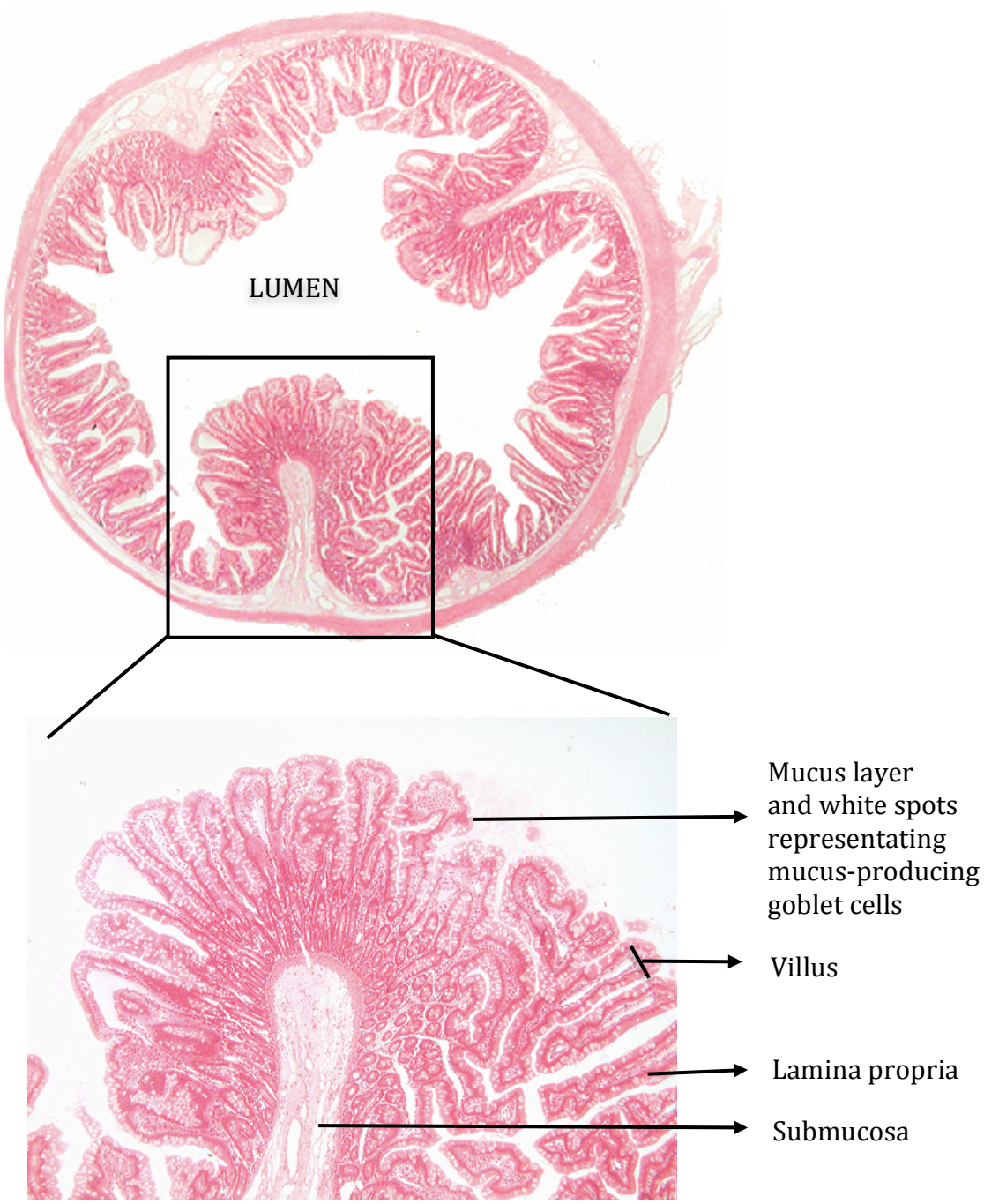

Figure 2.3: A histological cross-section through the jejunum at low magnification (1x – top) and higher magnification (4x – bottom) to highlight the different layers associated with the lining of the GI system. The layer making contact with the lumen is the mucosal layer, which is constantly being replenished by mucus-producing goblet cells seen as white dots scattered throughout the lining. Villi are the finger-like folds in the lining of the gut and serve to increase the surface area of the GI lining [42]

The mucus layer lining the moist epithelia has many key functions. One important function is its role in adhesion of enteric and foreign microbes [43]. Adhesion is often a result of complex interactions between receptors in the mucus layer and the bacteria. In the case of lactobacilli, it has been shown that adhesion is mediated by different secretory proteins[44-47]. These mucus binding proteins have been shown to have a mucin-binding domain and a domain attaching to the peptidoglycan of the bacterial cell wall [48]. Furthermore, mucosal recognition by the probiotic can upregulate the translation of mucin binding (Mub) proteins in order to transition into the mucosa associated ecosystem. A second critical function of the mucus layer is its regulatory capacity for the microbial ecosystem that grows on it. It has been shown to act as a delivery system for the numerous antimicrobial compounds produced by epithelial cells. The mucus layer also is as a form of protection from physical and chemical damage caused by a number of different agents typically found in the GI system [39]. Finally, it also has lubricating and moisturizing properties that allow for the propulsion of solution throughout the GI [39].

2.6 *In vitro* models of the gastrointestinal system

Following the transit throughout the stomach and small intestine, in which the ingested foreign bacteria have been bombarded with very harsh conditions; very low pH, presence of bile acids and other digestive enzymes, the next challenge is the probiotic's viability and permanency within the large intestine. In order to

analyze this, several different *in vitro* models of the gastrointestinal system approaches have been used.

2.6.1 Summary of lumen-associated gut microbiota models

The simplest and most crude approach is a static batch system in which fresh feces or colonic contents are incubated in a beaker over a short term, and metabolic or enzymatic activity can be monitored [49-51]. The major limitations are the short time scales of analysis and the rapid change in ecosystem composition. Semi-continuous batch systems have also been used where there is a semi-continuous flow of nutrients into and out of the system. These types of models can in fact be used for longer-term studies [52,53]. Finally, continuous systems modeling the gastrointestinal system have also been used[54-56]. The best documented of these systems is called the Simulated Human Intestinal Microbial Ecosystem (SHIME) developed by Molly et al in 1993[37,56]. The SHIME system contains five distinct ecosystems representing five different stages of the gastrointestinal transit, the stomach, the small intestine, the colon ascendans, transversum and descendans. Each stage is pH controlled and residence times for the distinctive stages are also closely monitored, as shown in the table below. There is daily administration of a nutrient rich solution and nitrogen flushes also occur on a daily basis to maintain anaerobic conditions. Jacketed beakers on all the vessels maintain the operating temperature to be at a physiologically relevant 37^{o}C. Monitoring of bacterial concentrations, metabolite

concentrations and other relevant data can be done using sampling ports on all the vessels.

Vessel	Intestinal Segment	Volume (L)	Retention time (hours)	pH
1	Stomach	0.2	2	2.0 - 2.5
2	Small intestine	0.3	6	5.0 - 6.0
3	Colon ascendans	0.35	9	5.5 – 6.0
4	Colon transversum	0.75	18	6.0 – 6.4
5	Colon descendans	0.4	11	6.6 – 6.9

Table 2.1: Properties of the different vessels contained in the SHIME system

Although these previously described *in vitro* models of the intestinal microbiota provide important information regarding the competitive nature of the microbial ecosystem within the gut, none actually address the bacteria adhering directly to the mucosal lining of the gut because all of these models strictly analyze for bacteria living in suspension, or in the lumen of the gut.

2.6.2 Summary of mucosal-associated gut microbiota models

2.6.2.1 *In vitro* cell line models

Bacterial adhesion to the mucosa has long been considered a multifactorial process involving the crosstalk between the microflora in the gut and the epithelial cells which make up the gut lining [57]. The most critical aspect of these in vitro models is the selection of gut epithelial cell lines. Typically, three cell lines (all of which are colon adenocarcinoma) are used in *in vitro* studies to

represent the gut epithelium: Caco-2 cells, HT-29 cells and SW-480. The most standard protocol for bacterial adhesion investigation using cell line models is described. Monolayers of cell lines were prepared in well plates, at which point a solution of the experimental strain of bacteria is added to the wells. Incubation times with bacteria are in the range between 0.5 hours and 1.5 hours. Wells with cells and bacteria are then washed and rinsed well in order to remove any non-specifically adhered bacteria.

In determining the functionality, significance and impact of a specific *in vitro* adhesion model, one of the most important parameters to analyze is the reproducibility of the measurement technique. Based on a comprehensive literature review, there is a wide variation in how bacterial adhesion is determined. Microscopy is a useful, albeit crude tool for bacterial enumeration on fixed surfaces. Once the sample has been fixed on a glass slide and stained with Gram stain, a specific number of random microscopic areas are selected and bacteria are counted in those regions, thereby getting a surface concentration of bacteria (cell/unit area). This method is very approximate; however, if counting is done by the same experimenter and in the same way, relative proportions of bacterial adhesion can be determined from the set of experiments. This method's reproducibility on a larger and more significant scale is less convincing. Fluorescence and radioactive tagging of bacteria [45] can also be employed to simplify quantification. Samples were subsequently homogenized, and a multiscan fluorometer or liquid scintillation counting was used to determine overall fluorescence or radioactivity, respectively[58]. This reading would

directly relate to bacterial viability adhered on different cell lines. This method would seem to be more promising as whole sample use reduces the subjectivity of the readings and thus increases the reproducibility of the measurement.

The following table demonstrates the variability in key experimental parameters of in vitro adhesion assays, namely the ratio of bacteria to gut epithelial cells and the incubation time.

Cells (cell/cm^2) used in *in vitro* models	**Bacteria (CFU/ml)**	**Ratio (Bacteria: Gut Epithelial cells)**	**Incubation time (hours)**	**References**
2 x10^4 cells/cm^2 HT-29/HT-29-MTX 1.4 x 10^4 Caco-2	2x10^8	10,000:1 14,286:1	1	Coconnier et al [44]
6.3 x 10^4 Caco-2	0.5x10^8	793:1	0.5	Elo et al [59]
1.5 x 10^6 Caco-2	2x10^8 - 10^{10}	142:1 – 6,667:1	1	Forestier et al [60]
5 x 10^4 Caco-2/HT29-MTX/90:10 Caco-2:HT29-MTX	8.2 x 10^8	16,400:1	1	Laparra et al [58]
5x10^5 Caco-2	4x10^8	800:1	1.5	Tuomola et al [61]
5x10^5	5x10^8 - 10^9	1,000:1 – 5,000:1	1	Zarate et al [62]

Table 2.2: Comparison of key experimental parameters of *in vitro* gut adhesion models using cell lines

By extension, the lack of consistency in bacterial quantification makes it almost impossible to combine data and gain widespread insight on which strains of

probiotic adhere best to gut epithelial cells. In order to demonstrate investigate relative adhesion values, one particular set of experiments will be elaborated on and data from this study will be shown.

This particular study [58] investigated the adhesion of two probiotic bacteria (*Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *Lactis* Bb12), two commensal bacteria (*B. animalis* IATA-A2 and *B. bifidum* IATA-ES2) and pathogenic bacteria (*E. coli* and *L. monocytogenes*) on a simulated *in vitro* epithelial lining. These simulated epithelial linings consist of two different gut epithelial cell lines (Caco-2 and HT29-MTX) and type II crude mucin in combination with one another or separate as summarized in the table below.

Simulated lining	Preparation
Polycarbonate	Control
Mucin	0.5 mg/ml solution diluted in PBS (pH 7.2) added to well plates in 0.5ml aliquots and incubated for 1h at which point wells were rinsed with PBS
Caco-2	Cell density of 50,000 cells/cm^2 added to well plates
HT29-MTX	Cell density of 50,000 cells/cm^2 added to well plates
Caco-2/HT29-MTX	90:10 ratio of Caco-2:HT29-MTX at a cell density of 50,000 cells/cm^2 added to well plates
Caco-2/Mucin	Cell density of 50,000 cells/cm^2 added to well plates + previously stated treatment for mucin

Table 2.3: Experimental design of bacterial adhesion experiment

In order to quantify bacterial adhesion, carboxyfluorescein diacetate (CFDA) was used as a fluorescent tag, by incubating bacteria with 75 μmol/l CFDA at 37^{o}C for 30 minutes. After the incubation period with cell lines, media was removed and 1% (w/v) sodium dodecyl sulphate in 0.1 mol/l NaOH and incubated for 1 hour at 37^{o}C. This treatment would remove any bacteria from the cell monolayer and fluorescence was read. Adhesion was then expressed as a percent fluorescence recovered after binding.

Results demonstrated the expected variability amongst different strains of commensal, probiotic and pathogenic bacteria and their respective interactions with different gut lining models. One important conclusion is the important contribution of mucus glycoproteins that do partly explain the adhesion ability of probiotic, commensal and pathogenic strains[58].

There are several downfalls to the current *in vitro* models for adhesion using cell lines. The first, and most significant is that the initial contact point between bacteria and the lining of the gut is not with gut epithelial cell lines but with a mucosal lining [58,63]. Although the crosstalk between gut epithelial cells and the gut microflora, whether they are commensal or foreign, play an important role in bacterial colonization, there is no physical adhesion of bacteria onto cell lines. There are certain cell lines that do produce a mucosal covering and so those could provide a more accurate depiction of *in vivo* condition.

A second issue with these *in vitro* adhesion models is the time scale over which a typical experiment would take place. Normally, bacteria are incubated for approximately 1 hour to 90 minutes on the cell lines. These short-term studies don't investigate longer-term residency of probiotic, commensal and pathogenic bacteria, as they experience the wash out effect due to the shear stresses of solid matter and liquid passing through the colon. Longer-term studies cannot be performed on cell lines because of their viability in the presence of the bacteria therefore this presents a significant challenge for the use of this model.

2.6.2.2 Immobilized mucus models

Immobilized mucus models have been very popular in order to study bacterial adhesion because of their simplicity and significant physiological resemblance. Mucus is the main lining protecting gut epithelial cells from the complex ecosystem called the gut microbiota and is the first point of interaction for bacteria that end up in the gut [64]. It is made up mainly of water, and the main protein component is mucin [64]. Mucins are a family of high molecular weight, heavily glycosylated proteins produced by epithelial tissues [64].

There is a well-defined procedure for mucus isolation and purification from fresh faecal samples that will be described here. Fecal mucus samples are initially suspended in ice-cold phosphate buffered saline (pH 7.2) containing 0.5 g/l NaN3, 1mM phenylmethylsulfonyl fluoride, 2mM iodoacetamide, and 10 mM EDTA. The suspension is to be thoroughly mixed and centrifuged 30 minutes at 15,000g.

At this point, the supernatant is kept and is precipitated twice with ice-cold ethanol and dissolved in a solution of PBS. The crude mucus can be further purified by applying it to a Sepharose column [64]. Another option would be to purchase mucin in a powder form and dissolve it into solution.

Typically, bacterial adhesion experiments using immobilized mucus as a platform for growth are performed in the following way. The first step is in immobilizing the mucus onto a surface, typically polystyrene microtiter plates. This task is done by incubation for between 12 and 24 hours at 4^{o}C at which point excess mucus is washed away.

Once mucus is immobilized onto microtiter plates, bacteria is introduced and incubated for a range of times between 60 and 90 minutes. Similar methods for bacterial quantification can be performed as described above.

A second type of mucus-immobilization based gut model was found in the literature[65]. Instead of immobilizing mucus onto microtiter plates, mucin-alginate beads were prepared, and packed into tubing. The tubing was porous with a molecular weight cut off point of 1000 daltons in order to allow the diffusion of different metabolites into and out of the simulated gut model. Fresh faecal slurry was used to inoculate a full spectrum of bacteria onto the beads. Analysis was done by fluorescence *in situ* hybridization at the end of the experiment. There were several disadvantages to this presented model. Firstly, the system was very complex with no regard to the advantages held by having a dialysis membrane for

diffusion of different metabolites. Secondly, the closed design of the system eliminated one major advantage to having a continuous system, namely, that sampling could be done in real-time. Bacterial enumeration was only done at the end of the experiment. In doing so, there was no indication of stable microbial communities, and subsequently no chance for treatment-effect experiments, which are the main purpose of *in vitro* models of the gut.

2.6.3 Summary of adhesion models

In vitro gut adhesion models are critical in better understanding how bacteria populate the large intestine and their relative fluctuations over time. As described earlier, the microflora in the large intestine play a crucial role in health of the host and therefore it would be important to understand what makes specific bacteria adhere to the mucosal lining in the gut rendering them more important players in the overall health of the individual. It would be critical to know the adhesive capacity of different probiotic or pathogenic bacteria in order to render treatment of different diseases more effective (whether it be treatment using probiotics or treatment using antibiotics to eliminate specific pathogenic bacteria in the gut).

Currently, there are numerous models present in the scientific community, all slightly different from one another. An important note to mention when discussing these models is their comparability and consistency on a larger scale across the scientific community. It is essential that there is a certain amount of uniformity when dealing with *in vitro* models in order to have a basis of

comparison over a large spectrum of scientific papers. By creating a standard protocol, scientists would be able to determine the relative adhesion of different bacteria to an *in vitro* gut-lining model. By the same token, it would be difficult if not impossible to combine, all in one model, the multitude of different features associated with bacterial adhesion to a model of the gut lining. Therefore, an alternative approach would be to create standard protocols for several different *in vitro* adhesion models, each considering a unique aspect of the challenges associated with bacterial adhesion in the gut. For example, adhesion models using cell lines would have clearly defined experimental parameters for incubation times, ratios of bacteria to cells and analytical techniques.

There remain significant issues, however, that need to be addressed when considering a model representative enough to have consequences outside of the lab setting and into potential clinical settings. A.C. Ouwehand and S. Salminen provided a handful of recommendations for improved *in vitro* analysis of bacterial adhesion [63] when attempting to develop one's own model. These include the influence of the normal intestinal microbiota on foreign bacteria adhering, appropriate growth conditions, adhesion incubation time, intestinal flux, appropriate probiotic concentration, use of physiologically relevant buffers, and the exposure of probiotics to digestive enzymes and conditions.

2.7 Introduction to current approach: novel *in vitro* adhesion model

In order to address the downfalls and shortcomings of the currently available *in vitro* models investigating bacterial adhesion, an attempt has been made in this

thesis to develop a novel system. The key characteristics of this system are its simplicity in design, efficacy in addressing the major obstacles for commensal, probiotic and pathogenic bacterial adhesion and ease of sampling and quantification of a wide range of different parameters.

The idea of packed bed bioreactors has been around for decades [66]. The concept has expanded from one research field to another, ranging from environmental engineering, water purification [67], waste treatment [68], biotechnology and different biomedical applications [69]. Bioreactors are a simple and effective platform best served to utilize the processing and machining abilities of bacteria to produce or deplete different compounds, whether they are enzymes, or substrates or products of enzymatic activities. A substrate or nutrient rich solution is circulated into the contained location where the bacteria can be found, and the flow of solution leaving the bioreactor generally has the product of interest. Packed-bed bioreactors were introduced in order to maximize on the potential of these bacteria by immobilizing them on packing material, typically beads, thereby increasing the surface area to volume ratio of the given bioreactor. By maximizing this critical ratio, diffusion of different compounds to the micromachines, which have been immobilized, increases greatly and therefore increases the effectiveness of the bioreactor.

After analysis of all the relevant parameters, a packed bed bioreactor will be designed for use as an *in vitro* model of adhesion in the gut. The reason for this is that by using a column filled with bacteria-coated beads, sampling can be done at

any time, by simply removing a bead, and performing analysis. As well, the inlet and outlet ports of the bioreactor can easily be reconnected to a sampling chamber that would enable the analysis of the supernatant at any given time. The system will be maintained at body temperature (37^{o}C) by placing the whole setup in an incubator. Feasibility experiments will be performed using a single strain of bacteria, and following this, in order to achieve an accurate representation of the gut mucosal lining, intestinal flora isolated from fresh human feces will be immobilized on the beads.

The following sections will demonstrate the applications of this *in vitro* bacterial gut adhesion model in determining the efficacy of a specific probiotic strain of bacteria to adhere to a simulated model of the gut mucosa with a normal intestinal flora already present.

Research articles presented in Chapters III, IV and V:

1. **Michael Coussa-Charley**, Laetitia Rodes, Arghya Paul, Marc Fakhoury, Hani Al-Salami, Sana Abbasi, Afshan Afsar Khan and Satya Prakash. A novel continuous gut adhesion model using a packed bed bioreactor. To be submitted to *Biotechnology Research International*.

2. **Michael Coussa-Charley,** Daniel Marinescu, Arghya Paul, Afshan Afsar Khan and Satya Prakash*. *Lactobacillus* probiotic can efficiently hydrolyze bile salts and displace potential pathogen in a simulated gut model. To be submitted to *Journal of Biologics: Targets and Therapy*.

3. **Michael Coussa-Charley**, Laetitia Rodes, Arghya Paul, Marc Fakhoury, Hani Al-Salami, Sana Abbasi, Afshan Afsar Khan and Satya Prakash. Investigation of the effect of *Lactobacillus* probiotic strain addition on the intestinal microflora immobilized on a novel continuous gut adhesion model. To be submitted to *Journal of Biologics: Targets and Therapy*.

N.B.: All the manuscripts presented in this thesis are under preparation

Published Contributions Not Included in Thesis

Original research articles

1. Marc Fakhoury, **Michael Coussa-Charley**, Hani Al-Salami and Satya Prakash*. Articifial cell microcapsule for oral delivery of thalidomide: design, preparation, and in-vitro characterization for Crohn's disease application. Submitted to *Journal of Drug Delivery*.
2. Marc Fakhoury, **Michael Coussa-Charley**, Arghya Paul, Laetitia Rodes, Wei Shao, Hani Al-Salami, Imen Kahouli and Satya Prakash*. Anti-inflammatory potential of artificial microcapsules containing thalidomide for use in treating Crohn's disease. To be submitted to *Journal of Drug Delivery*.
3. Marc Fakhoury, **Michael Coussa-Charley**, Hani Al-Salami, Imen Kahouli and Satya Prakash*. Effect of different doses of artificial microcapsules containing thalidomide in TNBS-induced Crohn's disease in mice. To be submitted to *Journal of Drug Delivery*.
4. Catherine Tomaro-Duscheneau, Shyamali Saha, Meenakshi Malhotra, **Michael Coussa-Charley**, Hani Al-Salami, Mitchell L. Jones, Alain Labbé and Satya Prakash*. Lactobacillus fermentum NCIMB 5221 has a greater potential for the production of ferulic acid when compared to other ferulic acid esterase active Lactobacilli. *International Journal of Probiotics and Prebiotics*.
5. Laetitia Rodes, Arghya Paul, **Michael Coussa-Charley**, Hani Al-Salami, Catherine Tomaro-Duschescneau, Marc Fakhoury and Satya Prakash*.

Transit time affects the community stability of *Lactobacillus* and *Bifidobacterium* species in an *in vitro* model of human colonic microbiota. *Artificial Cells, Blood Substitutes, and Biotechnology*, submitted.

Review articles

1. Satya Prakash*, Laetitia Rodes, **Michael Coussa-Charley** and Catherine Tomaro-Duschesneau. Gut microbiota: next frontier in understanding human health and development of biotherapeutics. *Biologics: Targets and Therapy*, 2011: 5 1-16.

Contributions of Authors

Subsequent chapters are original research articles and I am the first author in each. I was responsible for designing studies, conducting experiments, analyzing data and finally, preparing manuscripts. Dr Satya Prakash, was reported as the last author in all my articles, is my supervisor, research advisor and corresponding author. Laetitia Rodes, Mark Fakhoury, Arghya Paul, Sana Abbasi, Daniel Marinescu, Afshan Khan and Hani Al-Salami have all provided suggestions and assistance in designing and performing experiments. Details of authors' contributions are described at the beginning of each chapter.

Chapter III:

A novel continuous gut bacterial adhesion model using a packed bed bioreactor

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Preface:

The present study presents the development of a novel continuous gut adhesion model using a packed bed bioreactor design. It involves the selection of packing material based on highest bacterial adhesion and the physiological conditions associated with the gut lining. A feasibility experiment was performed using a probiotic and determining its adherence over a 14-day test period. Co-authors have contributed in experimental execution (Laetitia Rodes, Marc Fakhoury), experimental design and statistics (Arghya Paul, Hani Al-Salami) and special technique training for SEM (Sana Abbasi, Afshan Afsar Khan).

3.1 Abstract

The mucosal associated microflora is a complex microbial ecosystem requiring *in vitro* investigation to determine how it is colonized. In this study, we developed a novel continuous gut adhesion model to investigate the adhesion of the common probiotic bacterial strain *Lactobacillus fermentum*. Results showed that mucin-alginate beads provided a superior platform for selective bacterial immobilization (6.23 ± 0.15 log(CFU/cm^2)) when compared to glass (4.52 ± 0.077 log(CFU/cm^2)), polystyrene (5.24 ± 0.11 log(CFU/cm^2)) and alginate (4.84 ± 0.22 log(CFU/cm^2)). Various bacteria (*Lactobacilli, Enterococci and Bifidobacteria*) were tested to determine the specificity of the surface used. A stable bacterial immobilization of 6.464 ± 0.572 log(CFU/cm^2) was obtained throughout the 14 day treatment period. Results indicate that this continuous *in vitro* adhesion model is a viable, easy to use and versatile tool to investigate the effect of probiotics, prebiotics and antibiotics on microbial attachment in real-time on a physiologically relevant surface.

3.2 Introduction

Gut adhesion models are very useful tools to investigate the adherence capacity of different probiotics to a simulated model of the gut lining. In order to develop appropriate models, it is important to understand the challenges these probiotics face when travelling through the gastrointestinal system. Probiotics are defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" [23]. These health-promoting bacteria have been used as biotherapeutics in numerous animal trials and clinical trials for the treatment of many disease states, namely: metabolic syndromes [70-75], colon cancer [8,36,76], and inflammatory diseases such as irritable bowel disease (IBD) [28,77,78]. Although probiotics used as biotherapeutics have great promise, one major concern is whether these allochtonous bacteria can colonize the microflora living in the gut ensuring long lasting effects. The majority of the trillions of bacteria called the gut microflora are attached to a mucus layer that protects the gut epithelial cells lining the gastrointestinal system [79]. When a host orally ingests a probiotic, they must first survive gastrointestinal transit through the stomach and small intestine. Once they reach the gut, the probiotics must adhere to the mucus layer in order for them to permanently affect the composition of the gut microflora hence the important role of gut adhesion models.

The most typical of these models are static and involve the adhesion of different bacterial strains on microtiter plates using either intestinal epithelial cell lines [44,58,59,63,80-84] or immobilized mucus [58,63-64,115]. Although there are several disadvantages, the most significant is the short duration of adhesion experiments that can be performed on these models, which is typically less than 2

hours. Continuous flow systems hold promise for longer studies but there are currently few that have investigated for bacterial adhesion [65,85,86].

An *in vitro* gut adhesion model was developed in our laboratory with the aim of addressing the above-mentioned shortcomings. Bacterial adhesion was obtained on a physiologically relevant surface for experimentation over long time periods with an open design allowed for intermittent sampling.

3.3 Materials and methods

3.3.1 Chemicals

Beef extract, calcium chloride, low-viscosity sodium alginate, mucin from porcine stomach (type 2), peptone, sodium chloride and yeast extract were all purchased from Sigma-Aldrich Canada (Oakville, Ontario).

3.3.2 Bacteria and bacterial growth media

Lactobacillus fermentum NCIMB 5221 was purchased from the National Collection of Industrial, Marine and food Bacteria (Aberdeen, Scotland, UK), *Escherichia coli* ATCC 8739 and *Bifidobacterium longum* ATCC 15707 were purchased from Cedarlane Laboratories (Burlington, Ontario, Canada). De Man-Rogosa-Sharpe (MRS) broth was obtained from Difco (Sparks, Md, USA).

3.3.3 Preparation of alginate-based beads

Solutions of 2% (w/v) sodium alginate were dissolved in physiological saline and autoclaved. Mucin-sodium alginate solutions were made by adding 3% mucin in sterile conditions. The sodium alginate/mucin-sodium alginate solutions were

added dropwise from a height of 15cm into a 0.2M $CaCl_2$ bath [87]. At contact, beads were formed due to ionoirotpic gelation [88]. Beads were left overnight in 0.2M $CaCl_2$ bath and then transferred to physiological saline for storage at 4^{o}C.

3.3.4 Investigation of bacterial immobilization on four different types of packing material for a continuous gut adhesion model

Bacterial adhesion to four surfaces was investigated: glass beads (3 mm, Fisher Scientific, USA), polystyrene beads (Polyballs, 1/8" diameter, etched; Polysciences, USA), alginate beads (~3mm, protocol described above) and mucin-alginate beads (~3mm, protocol described above). *L. fermentum* NCIMB 5221 was added as a 1% inoculum to 30 ml MRS supplemented with 20 mM $CaCl_2$ in a 100 x 15mm sterile petri dish. Sterile beads were added to the petri dish and incubated for 16 hours at 37^{o}C with a shaker on at 50 rpm. Beads were then removed from the petri dishes and rinsed with physiological saline to remove any bacteria non-specifically attached to the bead. Beads were then placed in 1ml of physiological saline and vortexed thoroughly. Serial dilutions were carried out and 100μl of solution was plated on MRS-agar plates.

3.3.5 Investigation of bacterial viability on mucin-alginate surfaces using fluorescence microscopy

A confirmation of the viability of bacteria immobilized on the four previously mentioned materials (glass, polystyrene, alginate, mucin-alginate) was performed using a Viability Assay Kit for Bacteria Live & Dead Cells live/dead fluorescence

assay (Catalogue number 30027, Biotium, Inc., California). The beads that had already been coated with bacteria from the previous experiment were used and stained using the following protocol: (i) 1 volume of DMAO and 2 volumes of EtD-III were added to a microcentrifuge tube and mixed well, (ii) 7 volumes of physiological saline was added and again mixed well, (iii) 10 μl of fluorescent solution was added to the surfaces and it was left for 15 minutes at room temperature, (iv) under a fluorescent microscope (TE2000-U, Nikon, USA), live bacteria appear green and dead bacteria fluoresce red.

3.3.6 Investigation of the adhesion of L. fermentum as a function of bacterial incubation time using microscopy and standard bacterial quantification techniques

In order to visualize the immobilized bacteria on a mucin-alginate surface, scanning electron microscopy (SEM), using a Hitachi S-4700 FE scanning microscope was performed. MRS supplemented with 20mM $CaCl_2$ was added to the different wells, each well corresponding to a specific time point (0h, 4h, 8h, 24h). Mucin-alginate beads were then placed in each well and *L. fermentum* NCIMB 5221 was inoculated at 1%. The 6-well plate was then incubated at 37^{o}C. At specific times (0h, 4h, 8h, 24h), the beads were removed from the well, rinsed with physiological saline. Beads were then placed in a microcentrifuge tube with 1ml of physiological saline to quantify bacterial immobilization using plate counting and the other beads were air-dried overnight. Air-dried beads were then coated with gold-palladium for SEM.

3.3.7 Investigation of the immobilization of three bacteria on mucin-alginate beads

The immobilization of three different bacteria on mucin-alginate beads was compared to determine the selectivity of the surface used. The bacteria used were *L. fermentum* NCIMB 5221, *E. coli* ATCC 8739 and *B. longum* ATCC 15707 and their growth conditions are outlined below. *L. fermentum* NCIMB 5221 was grown in MRS broth at 37^{o}C at 5% CO_2, *E. coli* ATCC 8739 was grown in a nutrient broth comprising peptone (5 g/L), beef extract (1 g/L), yeast extract (2 g/L) and NaCl (5 g/L) dissolved in deionized water and incubated at 37^{o}C in aerobic conditions, and *B. longum* ATCC 15707 was grown in MRS at 37^{o}C in anaerobic conditions using anaerobic jars, with anaerobe atmosphere generating bags (Oxoid, Canada). Briefly, 6-well plates were used and media was pipetted into each well supplemented with 0.2 M $CaCl_2$. Beads were added to the first well, and incubated with bacteria at a 1% inoculum. The 6-well plates were kept in an incubator at 37^{o}C with a shaker on at 50 rpm. Each day, beads were removed from the well with bacteria and placed in a fresh media solution. One bead was rinsed well with physiological saline, and put in 1 ml of physiological saline in a microcentrifuge tube. Microcentrifuge tubes were vortexed well and solutions were plated on MRS agar and a nutrient agar (aforementioned nutrient rich media and agar (15 g/L)) for *E. coli*.

3.3.8 Investigation of the immobilization of L. fermentum in an in vitro continuous gut adhesion model over a two-week period

In order to investigate the feasability of an *in vitro* continuous gut adhesion model, experiments were carried out with *L. fermentum* NCIMB 5221 as a test probiotic. Beads were first incubated overnight in a 1% inoculum of the probiotic (MRS media supplemented with 20 mM $CaCl_2$) at 37^{o}C, in an EnvironShaker at 50 rpm. Bacteria-coated beads were transferred to column in sterile conditions. This was considered day 0. Beads were kept on the side to allow for adhered bacterial enumeration. MRS supplemented with 20 mM $CaCl_2$ began circulating through the column on day 1 at a flowrate of 0.5 ml per minute using a Watson-Marlow peristaltic pump 323 E/D (Watson Marlow Pumps Group, MA). The whole system was kept at 37^{o}C. At a given time point, beads were extracted from the column (alternating from the top of the column and bottom of the column in order to ensure homogeneity of bacterial growth of beads) and bacterial enumeration was performed. Media was replaced daily with a fresh supply of nutrients.

3.3.9 Study design and statistical analysis

Statistical analysis was performed using Prism software (Prism, Version 5.0 for Mac). Values are expressed as means ± SD. Statistical comparisons between different time points were carried out using unpaired student's t-test. Experiments were conducted in triplicates and statistical significance was set at $p < 0.05$.

3.4 Results and Discussion

3.4.1 Design and characterization of continuous gut adhesion model

3.4.1.1 Overall design of continuous gut adhesion model

A gut adhesion model was developed with the intentions of running long-term experiments with daily sampling. In order to accomplish this, a glass column (10 x 1 cm) was filled with mucin-alginate beads (approximate diameter of beads is 3 mm) that would represent the gut mucosal lining. The surface of the beads was the point of contact for bacterial attachment. A nutrient rich media (MRS) was circulated throughout the system at a flowrate of 0.5 ml per minute using a Watson Marlow peristaltic pump 323 E/D (Watson Marlow Pumps Group, MA) and the entire system was kept at 37^{o}C. Daily sampling was performed by unscrewing the top of the column, and using a pair of sterile tweezers to extract beads. A schematic of the experimental setup is shown in figure 3.1.

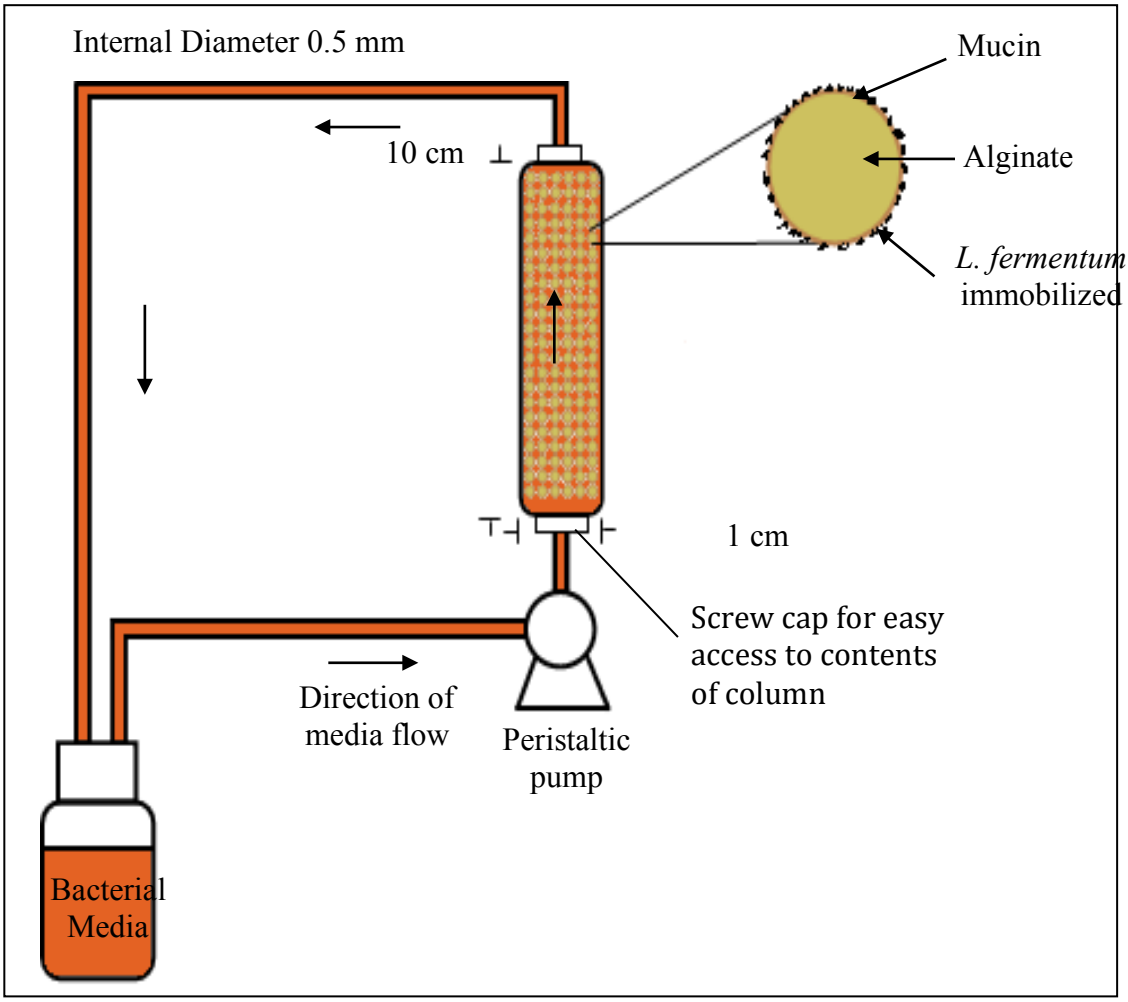

Figure 3.1: Schematic and mode of function of *in vitro* gut adhesion model. Media was pumped out of a beaker using a Watson Marlow peristaltic pump 323 S/D into the bottom of a column (glass column, 1 x 10 cm) packed with mucin-alginate beads. Mucin-alginate beads had *L. fermentum* NCIMB 5221 immobilized on them. Media continued out of the top of the column and back into the beaker containing media. The whole system was kept at 37^{o}C. Immobilized bacteria are represented in black on the mucin-alginate bead.

3.4.1.2 Investigation of bacterial immobilization on four different types of packing material for a continuous gut adhesion model

In designing a continuous gut adhesion model, the first step was to investigate different materials in order to pack a column. Beads were used as a packing material so that sampling could be done throughout the length of an experiment

without disturbing the overall setup, and to increase the surface area for bacterial immobilization. Glass, polystyrene, alginate, and mucin-alginate were all investigated and the bacterial immobilization on the different surfaces were compared. Bacterial immobilization on glass was lowest at 4.52 ± 0.077 log(CFU/cm^2), on polystyrene it was 5.24 ± 0.11 log(CFU/cm^2), on alginate beads it was 4.84 ± 0.22 log(CFU/cm^2) and on mucin-alginate beads, bacterial immobilization was highest at 6.23 ± 0.15 log(CFU/cm^2). Results are shown in figure 3.2a. Statistical analysis was carried out to determine whether the different materials yielded significantly different results. The mucin-alginate beads demonstrated the highest bacterial adhesion in comparison to all the other surfaces used in the investigation ($p<0.05$). In order to confirm that the immobilized bacteria were viable, a second viability assay based on fluorescence was performed. The assay uses two fluorescent nucleic acid dyes that stain both live and dead bacteria differently (green for live cells and red for dead cells). Fluorescence images (figure 3.2b – e) demonstrated a higher density of viable bacteria corresponding with the mucin-alginate surface compared to the other materials. The fact that the mucin-alginate beads represent a more accurate depiction of the intestinal mucosal lining and are also very simple and inexpensive to produce for a large set of experiments further highlights the advantages of using these surfaces in this system. Based on all of these findings, mucin-alginate beads were used as packing material in a column to develop an *in vitro* gut adhesion model.

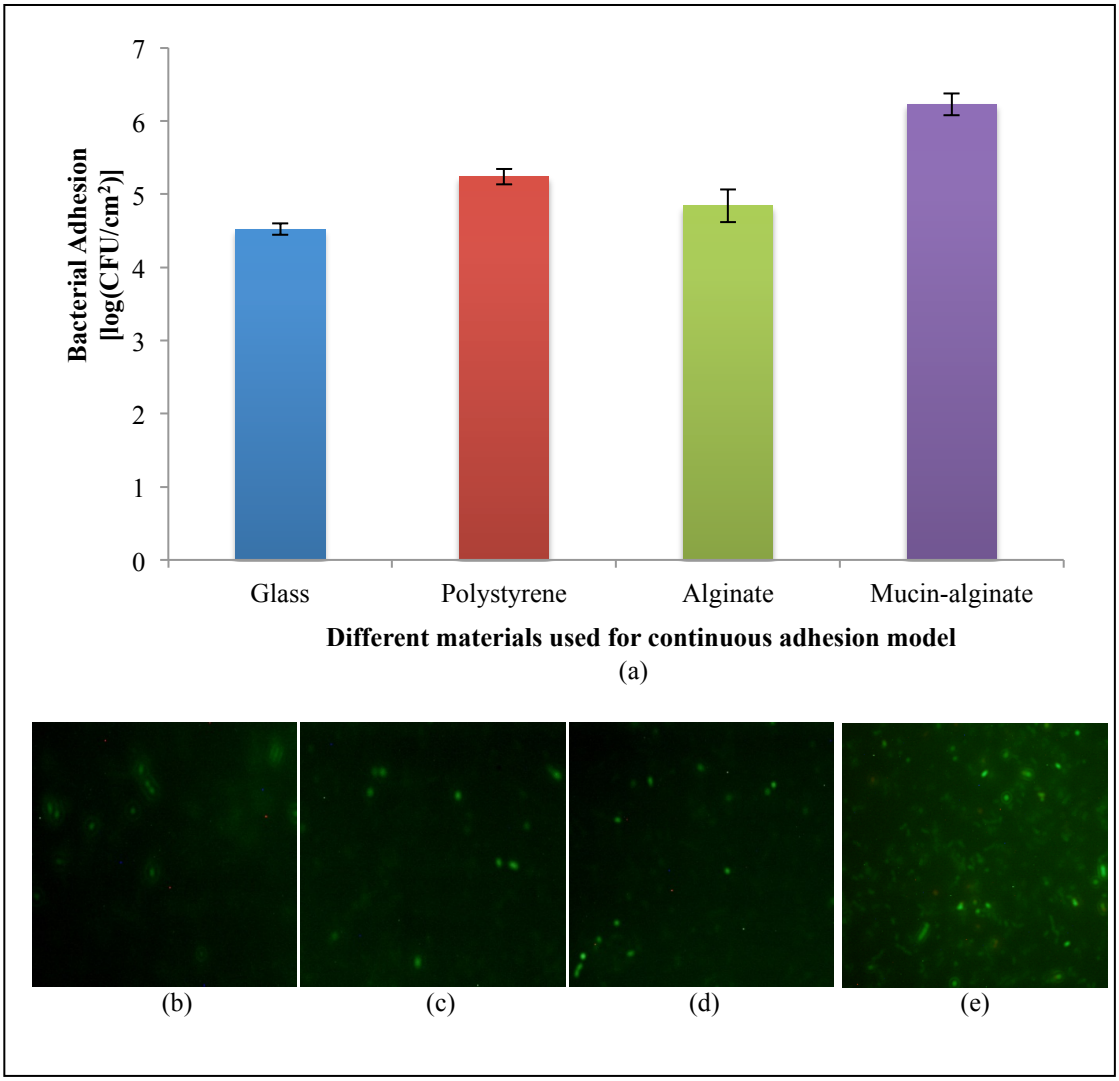

Figure 3.2: (a) The effect of four surfaces (glass, polystyrene, alginate, mucin-alginate) of beads on the immobilization of *L. fermentum* NCIMB 5221 as measured by the log(CFU/cm^2) in order to determine the best packing material for an *in vitro* gut adhesion model (error bars included ± SD, n=3). Bacteria was incubated on the different surfaces for 16 hours at 37^{o}C at which point the surface was rinsed well with physiological saline to remove any non-tightly bound bacteria. A one-way ANOVA test was performed along with Tukey's Multiple Comparison Test to compare the immobilization of bacteria on mucin-alginate beads with the three other materials. Immobilization of bacteria was significantly higher on mucin-alginate than on any other material ($p<0.05$). Figures 2b-2e demonstrate the effect of four different surfaces (b) glass, c) polystyrene, d) alginate, e) mucin-alginate on the viability of immobilized bacteria (*L. fermentum* NCIMB 5221) after 16 hours of incubation at 37^{o}C using a live/dead fluorescence assay (Biotium, Inc. Viability/Cytotoxicity Assay Kit for Bacteria Live & Dead Cells). Green fluorescent cells were viable while red fluorescent cells were dead. Magnification was 600x

3.4.1.3 Investigation of the adhesion of *L. fermentum* as a function of bacterial incubation time using microscopy and standard bacterial quantification techniques

These experiments were performed in order to better understand the progression of bacterial adhesion over the first 24 hours of contact between bacteria and a mucin-alginate surface. Results from colony counting experiments and SEM images (figure 3.3a – e) illustrated the time dependent nature of bacteria adhering onto a mucin-alginate surface. Within 4 hours of incubation, bacteria began to grow in clusters with very minimal coverage over the entire surface and low cell numbers (2.754 ± 0.186 log(CFU/cm^2)). 4 hours later, the mucin-alginate surface was fully colonized, as can be seen in figure 3c and the bacterial concentration doubled to 5.626 ± 0.0634 log(CFU/cm^2)). After 24 hours, there was a slight increase in bacterial adhesion (6.26 ± 0.10 log(CFU/cm^2)). These results imply that within the first 8 hours, bacteria interact directly with the mucin-alginate matrix; however, after 8 hours, immobilization becomes dependent on the interaction between mucosal-associated bacteria that are immobilized and bacteria still growing in suspension.

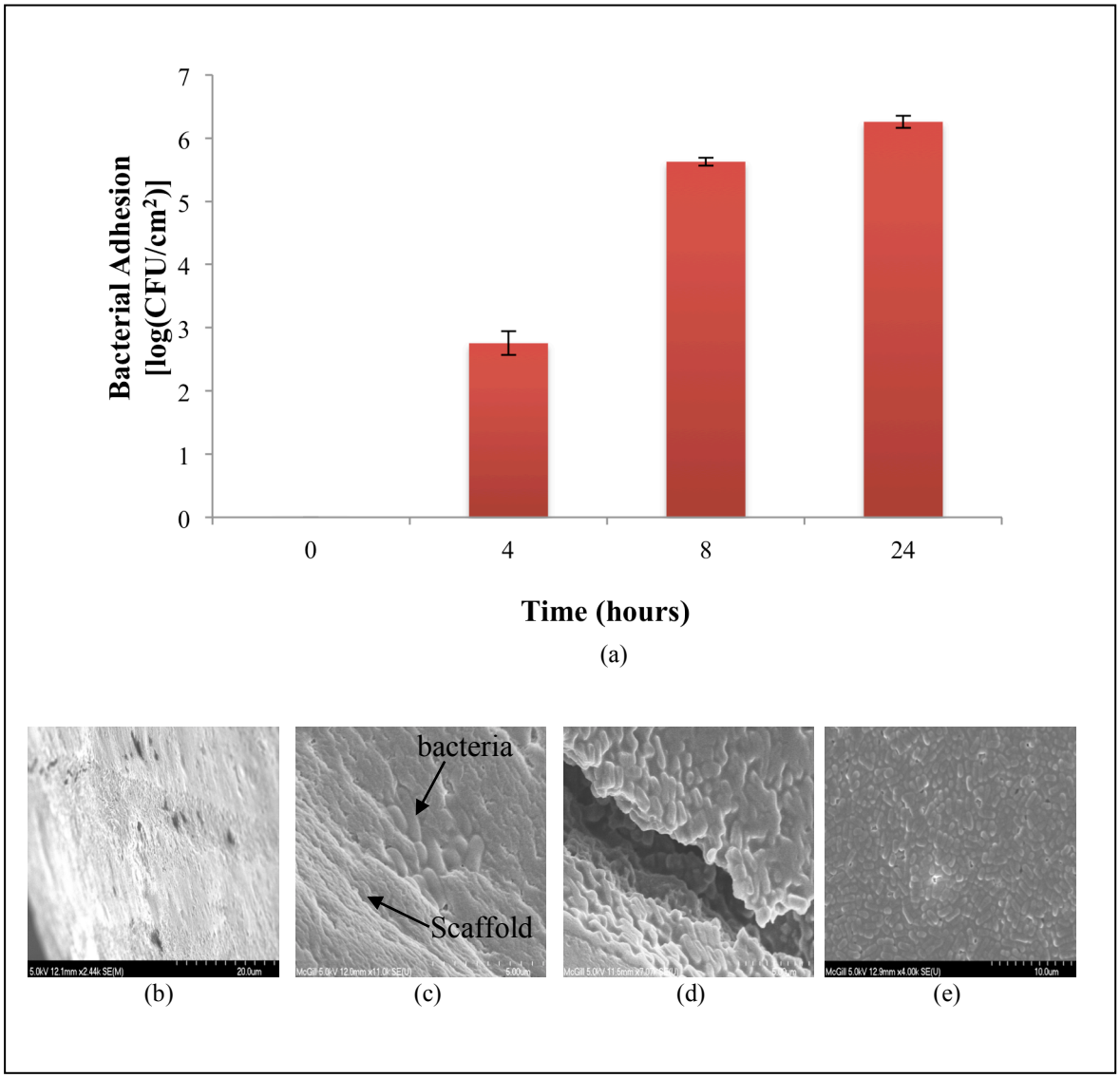

Figure 3.3: a) Time profile of *L. fermentum* NCIMB 5221 immobilization on mucin-alginate surface (error bars included ± SD, n=3) along with Scanning Electron Microscope images after (b) no incubation of bacteria, (c) 4 hours of incubation of *L. fermentum* NCIMB 5221 at 37°C (d) 8 hours of incubation of *L. fermentum* NCIMB 5221 at 37°C and (e) 24 hours of incubation of *L. fermentum* NCIMB 5221 at 37°C.

3.4.1.4 Investigation of the immobilization of three bacteria on mucin-alginate beads over a 7-day period

In order to validate the selected packing material, the adhesion of different bacteria on mucin-alginate surfaces were investigated and then compared with data found using other gut adhesion models. These results shown in figure 3.4 demonstrated that bacteria showed selective immobilization onto mucin-alginate surfaces. There was a significantly higher average concentration of *L. fermentum* NCIMB 5221 over the 7 day period (6.616 ± 0.117 log(CFU/cm^2)) compared to *B. longum* ATCC 15707 (5.651 ± 0.029 log(CFU/cm^2)) and *E. coli* ATCC 8739 (4.133 ± 0.050 log(CFU/cm^2)). Although comparing results from different *in vitro* gut adhesion models is not a foolproof method for validating our model, there are general trends shown by other models. Several publications have shown that *Escherichia* adherence was significantly lower than *Lactobacillus sp.* and/or *Bifidobacterium sp.* [58,63]. As far as comparing the results of *L. fermentum* NCIMB 5221 immobilization to *B. longum* ATCC 15707, there is no general consensus on which would adhere better to a simulated lining of the gut.

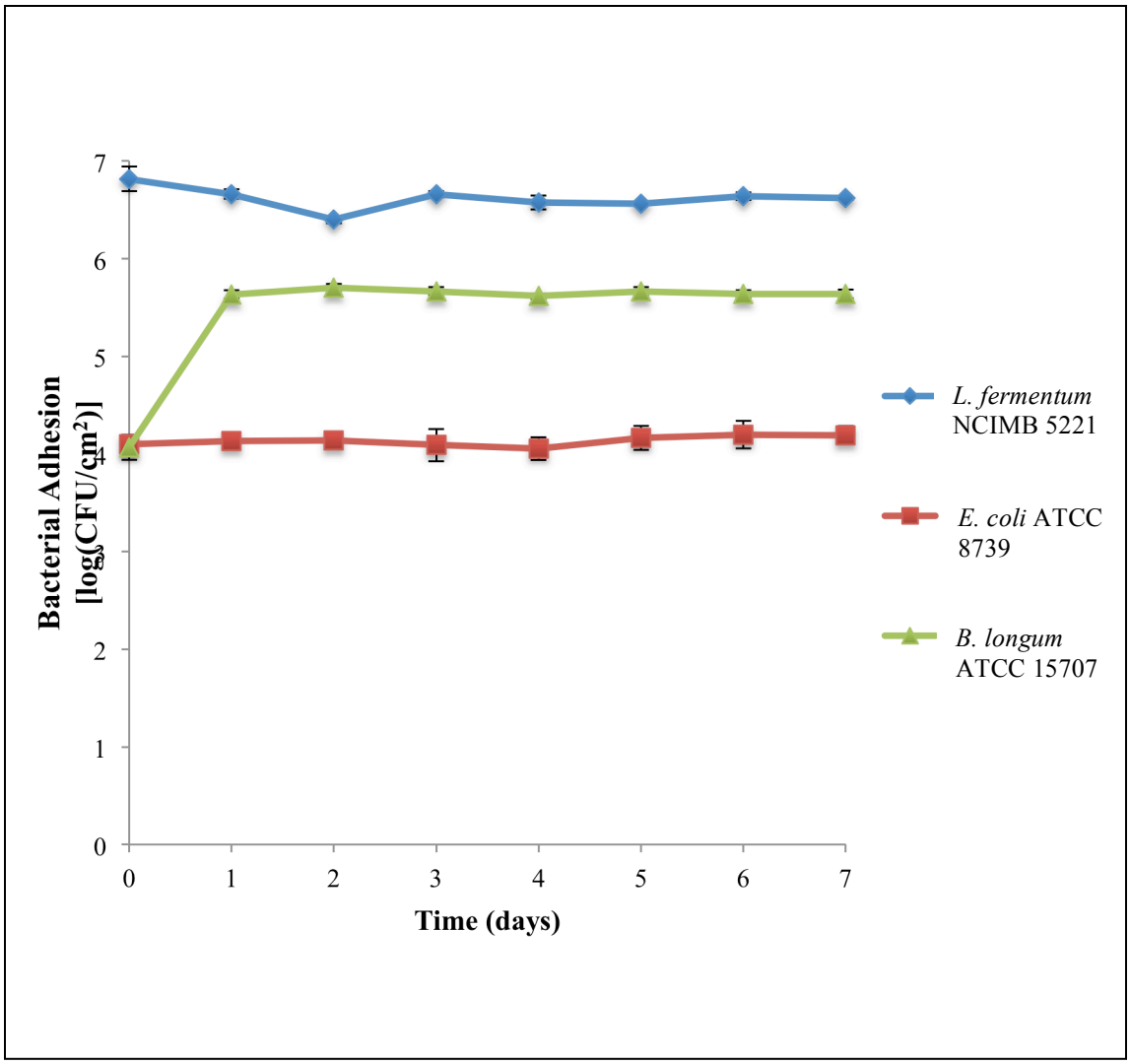

Figure 3.4: Investigation of the immobilization of three different bacteria on mucin-alginate beads over a 7-day period (error bars included ± SD, n=3). *L. fermentum* NCIMB 5221 and *B. longum* ATCC 15707 were grown in MRS media (*B. longum* was grown in anaerobic conditions). *E. coli* ATCC 8739 grew in a nutrient media described in the Methods section. A one-way ANOVA test was performed along with Tukey's Multiple Comparison Test to compare the immobilization of different bacteria on mucin-alginate beads. NCIMB 5221 had the highest immobilization ($p<0.05$, 6.616 ± 0.117 log(CFU/cm^2)) as compared to *E. coli* ATCC 8739 (4.133 ± 0.050 log(CFU/cm^2) or *B. longum* ATCC 15707 (5.651 ± 0.029 log(CFU/cm^2). Briefly, beads were loaded into 6-well plates filled with media supplemented with $CaCl_2$ and placed in an EnvironShaker (50 rpm). Each day, beads were transferred to a well with fresh media and one was kept on the side for quantification.

3.4.2 Investigation of the feasability of an in vitro continuous gut adhesion model using L. fermentum

The feasibility of running a continuous gut adhesion model over the course of a two-week period was conducted using *L. fermentum* as a test probiotic. Over the course of 14 days, bacterial populations remained stable on the mucin-alginate beads with an average concentration of 6.464 ± 0.572 log(CFU/cm^2) as shown in figure 3.5. At day 0, the bacterial concentration was 4.881 +/- 0.067 log(CFU/cm^2) and at the end of the experimental study period, bacterial viability had increased to 6.779 ± 0.053. Results indicate that this particular strain of bacteria could maintain immobilization to a simulated intestinal model of the mucosal lining over a two week time period while experiencing shear stress associated with the flow of a nutrient rich solution past it.

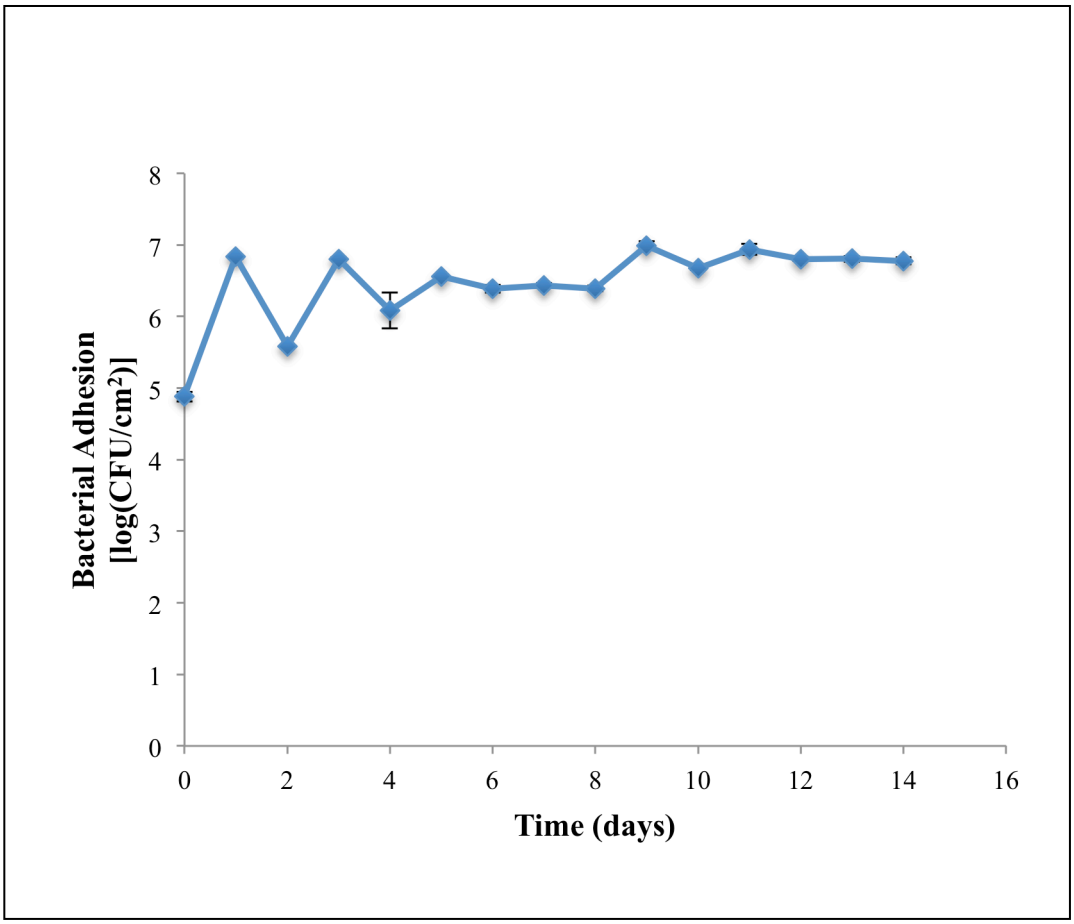

Figure 3.5: The effect of time on the immobilization of *L. fermentum* NCIMB 5221 (as measured by log(CFU/cm^2)) in the continuous gut adhesion model kept at 37^{o}C (as shown in figure 3.1) over a 14 day period (error bars included ± SD, n=3). On a daily basis, one mucin-alginate bead was removed from the system, rinsed with physiological saline to remove any non-attached bacteria and then vortexed well in 1 ml of physiological saline to remove remaining immobilized bacteria off of the bead for quantification. Media was also replaced daily. The average bacterial concentration of the probiotic on mucin-alginate beads was 6.46 ± 0.572.

3.4.3 Advantages and limitations of the presented continuous gut adhesion model to investigate probiotic adhesion

There are several advantages to the model presented in this study. The first is the fact that a mucus layer is used as a platform for bacterial attachment. Results

showed that it yielded the highest bacteria attachment and was physiologically comparable to the gut lining. In addition, sampling of bacterial concentrations can be done in real time over long periods of time using this model. As well, the concentrations of different metabolites can be monitored throughout the duration of an experiment by sampling the solution passing through the system at different time points. As opposed to models with a closed design that don't allow intermittent sampling, this continuous model allow for analysis of different parameters at several time points throughout the experiment.

The limitations of the model presented in this chapter are primarily that the ecosystem present in the column over the course of an experiment is simplified with respect to what occurs in reality. The lack of a full microbial ecosystem interacting on the mucosal-lining which can affect bacterial adhesion has not been addressed here however will be in a subsequent chapter. The main purpose of these experiments was to demonstrate the feasibility of this model.

3.5 Conclusion

The intestine is an extremely complex system in which there is constant shift and equilibration of trillions of bacteria that can contribute to human health in either a beneficial or harmful way. It has been demonstrated that the administration of probiotics, or bacteria that are known to have beneficial effects on human health, can prevent, alter or slow down the progression of a plethora of diseases. In order for probiotics to have a long lasting effect on the host, it is essential that they survive in a competitive harsh microenvironment - the gut microflora. The first

step in probiotic research is to provide an accurate *in vitro* model that could best represent physiological conditions. The proposed model represents specific properties of the different challenges in gastrointestinal transit, namely, the point of surface contact and the shear stress associated with mucosal adhered bacteria, which is often ignored. It has also been designed in a way so as to allow ease of use, as sampling can be done in real-time during an experiment by the extraction of beads or circulating media. Experiments performed for the development of this model have indicated that mucin-alginate beads yield a high and selective bacterial immobilization, and provide a stable microbial ecosystem within 14 days of the system startup. Finally, the system is very versatile. Applications ranging include *in vitro* modeling of complex microbial ecosystems such as those found in the gut, lungs or mouth to purification systems where immobilized bacteria can extract toxic chemicals from the bulk phase and convert them into harmless by-products [67,68]. Its essence is simply the immobilization of viable bacteria on beads packed into a column. Beads are used to maximize the diffusion of chemicals by increasing the surface area to volume ratio. By using mucin-alginate beads, we were able to target the bacterial adhesion of mucin-binding bacteria, in this case, our probiotic of interest. Different surfaces and different surface modifications can yield the selective adhesion of different bacteria performing different metabolic tasks.

Acknowledgements

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Chapter IV:

Lactobacillus probiotic can efficiently hydrolyze bile salts and displace potential pathogen in a simulated gut bacterial adhesion model

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Preface:

The present study demonstrates the application of the previously described (in chapter 3) gut adhesion model to investigate the biotherapeutic potential of a probiotic through two potential mechanisms. Firstly, the bacterial production of a potential cholesterol-lowering enzyme to hydrolyze bile salts was monitored over a 5-day period. Secondly, the pathogen displacement potential of the probiotic was determined in real-time in the gut adhesion model. Following this study, it would be important to apply a representative sample of the gut microflora to the presented gut adhesion model in order to determine the effect of a treatment on a

variety of different microbial communities. Co-authors have contributed in experimental execution (Marc Fakhoury), experimental design and statistics (Arghya Paul), special technique training for SEM (Afshan Afsar Khan) and use of a BSH spectrophotometric assay (Daniel Marinescu).

4.1 Abstract

Probiotics as biotherapeutics have huge potential but mechanisms of action need to be investigated *in vitro*. In this study, the production of a potential cholesterol-lowering enzyme, bile salt hydrolase (BSH), and the pathogen displacement of a probiotic were investigated in a novel continuous gut adhesion model. Results showed a stable bacterial community of a BSH-active *Lactobacillus reuteri* ATCC 701359 growing in the column (6.32 ± 0.44 log(CFU/cm^2) for *L. reuteri* ATCC 701359) producing BSH at a rate of 2.70 ± 0.34 (umol/hr/CFU x 10^6). As well, the treatment of *L. reuteri* on mucin-alginate beads pre-coated with *E. coli* ATCC 8739 significantly reduced the adhesion of the potential pathogen and its viability (reduction from 4.60 ± 0.06 log(CFU/cm^2) to 3.82 ± 0.18 log(CFU/cm^2)). In conclusion, the presented gut adhesion model was used to show the pathogen displacement potential of a probiotic as well as the production of a cholesterol-lowering enzyme, BSH.

4.2 Introduction

Probiotics are defined as live microorganisms, which, when administered in significant amounts, confer health benefits to the host [23]. Lactic acid bacteria are an important class of probiotics and are found frequently in pharmaceutical products and in different food products [89]. There are numerous studies that have shown potential health benefits of these probiotics in a variety of different disease states, namely, cancer [36,90-93], inflammatory diseases [94-97] and different metabolic diseases [98-100]. Most recently, there has been significant interest in their role to lower cholesterol levels [101,102]. One potential hypothesis for this is through the enzymatic deconjugation of bile salts performed by bile salt hydrolase (BSH) enzymes produced by different probiotic bacteria [34]. Probiotics have also garnered significant interest in their ability to prevent and treat gastrointestinal infections [103-106]. The major challenge for demonstrating probiotic effect in humans would be to determine how 'resilient' these probiotics are. Resilience can be considered the ability of an orally administered probiotic to maintain a permanent status within the highly complex and competitive microenvironment within the gut [16]. In order to investigate probiotic 'resilience' *in vitro*, gut adhesion models have been developed.

Until now, there are no studies that have demonstrated, firstly, the adhesion of a genetically engineered BSH-active bacteria, secondly, that these immobilized bacteria are able to maintain the production of the useful, potentially therapeutic BSH enzyme, and thirdly that these bacteria would be able to displace any pathogenic bacteria that may colonize the gut. A novel *in vitro* model of the gut

mucosal lining described in previous work will be used to investigate the adhesion capacity of this probiotic.

4.3 Materials and methods

4.3.1 Chemicals

Arabinogalactan, calcium chloride, low-viscosity sodium alginate, mucin from porcine stomach (type 2), peptone, D-sorbitol, sodium chloride, starch, xylan and yeast extract were all purchased from Sigma-Aldrich Canada (Oakville, Ontario). Glucose and pectin were purchased from Acros Organics (New Jersey, US) and cystein was purchased from Fisher Scientific (USA).

4.3.2 Bacteria and bacterial growth media

Lactobacillus fermentum NCIMB 5221 was purchased from the National Collection of Industrial, Marine and food Bacteria (Aberdeen, Scotland, UK). *Lactobacillus reuteri* ATCC 701359 and *Escherichia coli* ATCC 8739 were purchased from Cedarlane Laboratories (Burlington, Ontario, Canada). De Man-Rogosa-Sharpe (MRS) broth was obtained from Difco (Sparks, Md, USA) and MacConkey broth was obtained from Sigma-Aldrich (Oakville, Ontario). *E. coli* ATCC 8739 was grown in a nutrient broth comprising peptone (5 g/L), beef extract (1 g/L), yeast extract (2 g/L) and NaCl (5 g/L) dissolved in deionized water.

4.3.3 Preparation of alginate-based beads

A solution of 2% (w/v) sodium alginate was dissolved in physiological saline and autoclaved. 3% mucin was then added in sterile conditions. The mucin-sodium alginate solutions were added dropwise from a height of 15cm into a 0.2M $CaCl_2$ bath [107]. At contact, beads were formed due to ionoirotpic gelation [108]. Beads were left overnight in 0.2M $CaCl_2$ bath and then transferred to physiological saline for storage at 4^{o}C.

4.3.4 Investigation of the immobilization of a BSH-active L. reuteri in an in vitro continuous gut adhesion model

In order to investigate the production of BSH in an *in vitro* continuous gut adhesion model previously described (chapter 3), *L. reuteri* ATCC 701359 and a non-BSH active bacteria, *L. fermentum* NCIMB 5221, were immobilized on mucin-alginate beads. Beads were first incubated overnight in a 1% inoculum of the probiotic (MRS media supplemented with 20 mM $CaCl_2$) at 37^{o}C, in an EnvironShaker at 50 rpm. Bacteria-coated beads were transferred to column in sterile conditions. This was considered day 0. Beads were kept on the side to allow for adhered bacterial enumeration. MRS supplemented with 20 mM $CaCl_2$ began circulating through the column on day 1 at a flowrate of 0.5 ml per minute using a Watson-Marlow peristaltic pump 323 E/D (Watson Marlow Pumps Group, MA). The whole system was kept at 37^{o}C. At a given time point, one bead was extracted from the column (alternating from the top of the column and bottom of the column in order to ensure homogeneity of bacterial growth of beads) and

bacterial enumeration was performed. Media was replaced daily with a fresh supply of nutrients.

4.3.5 Investigation of the production of BSH in an in vitro continuous gut adhesion model

Bile salt hydrolase activity was determined using a modified version of the spectrophotometric method, described from [109]. Bacteria were removed from mucin-alginate beads by vortexing in 1 ml of saline and this was supplemented with 30 µl protease inhibitor solution (Protease Inhibitor Cocktail Tablets, 25X conc. stock solution, Sigma Aldrich). While maintained on ice, the sample solution was sonicated for 7 pulses of 10 seconds, at 50% power (Misonix XL2000 Ultrasonication System) while on ice. This lysate was then centrifuged at 20000xg for 10 minutes at 4^{o}C. Following centrifugation, 20mM GDCA (Sodium gluconodeoxycholic acid, Sigma Aldrich), 10 mM dithiothreitol (DTT) and protease inhibitors were added to the mixture and its pH was reduced to 4.2 drop-wise with 0.5M hydrochloric acid (HCl). The protease inhibitors block both endogenous protease activities from the bacterial lysate. The cell-free bile salt hydrolysis reaction tube is incubated at 37^{o}C. At time points of 0 min, 30 min, 60 min and 90 min, 50 µl of the reaction mixture was removed and added to 50 µl of 15% (w/v) TCA solution to precipitate all proteins and cease the hydrolysis. The sample was centrifuged at 10 000xg for 10 minutes at 4^{o}C. From the supernatant, 50 µl were removed and 950 µl of ninhydrin solution (5:12:2 ratio by volume of 2% (v/v) ninhydrin: glycerol: 0.5M sodium citrate pH 5.5) was added. The sample was boiled for 14 minutes, and let to cool at room temperature for 10 minutes.

Absorbance was read at 570 nm using a PerkinElmer 1420 Multilabel Counter. The concentration was determined against the optical densities of serial diluted glycine standards that were similarly incubated. Glycine standards were prepared with concentrations of 2.25, 2, 1.75, 1.5, 1.25, 1, 0.75, 0.4, 0.3 and 0 mM in identical buffer. From each of these preparations, 50 µl was removed and added to 50 µl of 10% (v/v) TCA solution. To 50 µl of this mixture, 950 µl of ninhydrin reagent were added. The samples are boiled 14 minutes and allowed to cool at room temperature for 10 minutes, before the absorbance is read at 570 nm. Using viability measurements, BSH-activity can be calculated as a rate of glycine release per CFU (standardized to workable values by multiplying by 10^6).

4.3.6 Investigation of the interaction between a probiotic bacteria and potentially pathogenic bacteria in a continuous gut adhesion model within the first 24 hours using microscopy

In order to understand the interaction between different bacteria on a mucin-alginate surface within the first 24 hours of incubation, scanning electron microscopy (SEM), using a Hitachi S-4700 FE scanning microscope, and selective agar quantification was performed. Initially, a 1% inoculum of *E. coli* ATCC 8739 was incubated in a nutrient rich solution supplemented with 20 mM $CaCl_2$ at 37^oC on mucin-alginate beads that had previously been prepared. Following this, bacteria-coated beads were transferred to the previously described column setup. At time = 0 hours, meda which have been inoculated with a 1% inoculum of *L. reuteri* ATCC 701359 began circulating through the system.

Mucin-alginate beads were then removed from the column at given time points for bacterial quantification and SEM preparation.

4.3.7 The effect of daily-administered probiotics on an immobilized potential pathogen in a continuous gut adhesion model

In order to investigate the pathogen displacement potential of *L. reuteri* in a continuous gut adhesion model, the following experiment has been performed. Initially, the potential pathogen, *E. coli* ATCC 8739 was immobilized on mucin-alginate beads by overnight incubation as a 1% inoculum in the previously described nutrient solution supplemented with 20 mM $CaCl_2$ at 37^{o}C, in an EnvironShaker at 50 rpm. Bacteria-coated beads were then transferred to the column in sterile conditions. This was considered day 0 of the experiment. Beads were transferred to microcentrifuge tubes to perform bacterial enumeration. The nutrient solution supplemented with 20 mM $CaCl_2$ began circulating through the column on day 1 at a flowrate of 0.5 ml per minute. The whole system was kept at 37^{o}C. At a given time point, one bead was extracted from the column and bacterial enumeration was performed. Media was replaced daily. On day 1, the probiotic was added. *L. reuteri* ATCC 8739 was subcultured as a 1% inoculum in MRS media throughout the experiment. After 16 hours of incubation, bacteria reached a concentration of 10^8 CFU/ml. 1 ml of MRS was pipetted into microcentrifuge tubes and centrifuged for 10 minutes at 7000 rpm. The supernatant was disposed of and the pellet was resuspended in physiological saline and centrifuged again for 10 minutes at 7000 rpm. The supernatant was once again disposed of and the pellet was resuspended at which point, the solution

that contained the appropriate amount of bacteria (10^8 CFU) was added to the nutrient rich solution that circulated through the system. Selective agar was used to quantify the gram-positive bacteria (*L. reuteri* ATCC 701359) from the gram-negative bacteria (*E. coli* ATCC 8739). Sorbitol-MacConkey agar was used to quantify gram negative bacteria [110], and LAMVAB was used to quantify *L. reuteri* [111].

4.3.8 Study design and statistical analysis

Statistical analysis was performed using Prism software (Prism, Version 5.0 for Mac). Values are expressed as means ± SD. Statistical comparisons between different time points were carried out using unpaired student's t-test. All experiments were conducted in triplicates. Statistical significance was set at $p < 0.05$.

4.4 Results

4.4.1 Investigation of the immobilization of a BSH-active L. reuteri in an in vitro continuous gut adhesion model

In order to determine the adhesion capacity of a BSH-active *Lactobacillus* probiotic was incubated on mucin-alginate beads and packed in a continuous gut adhesion model. Over a five day investigation period, the average viability on beads was 6.32 ± 0.44 log(CFU/cm^2) and was stable from day 2 – day 5($p<0.05$) as shown in figure 4.1. A non-active BSH-active probiotic, *L. fermentum* NCIMB 5221, was also grown on mucin-alginate beads as a control for BSH activity. The

average viability of *L. fermentum* was 6.58 ± 0.11 log(CFU/cm^2) over the 5 day investigation period as shown in figure 4.1.

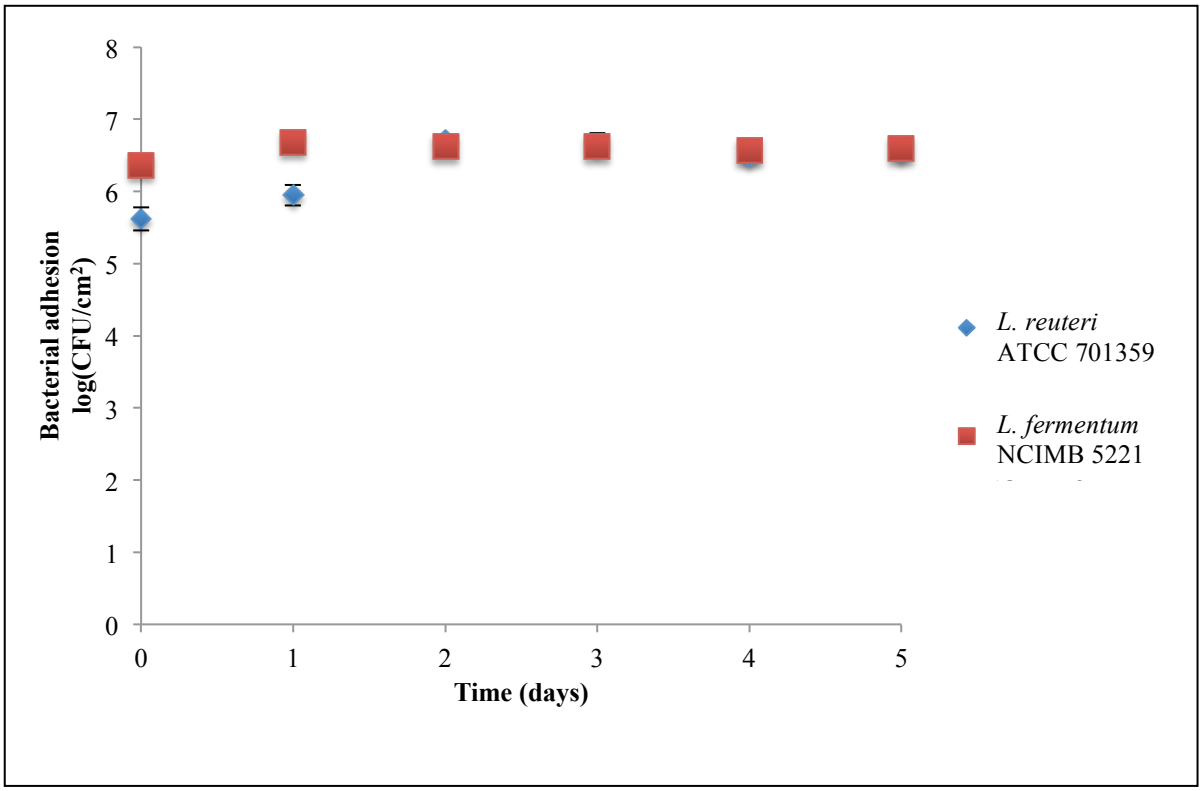

Figure 4.1: Investigation of the immobilization of a BSH-active probiotic, *L. reuteri* ATCC 701359 and a control probiotic which did not produce BSH, *L. fermentum* NCIMB 5221 on mucin-alginate beads in a continuous gut adhesion model over a 5 day period (error bars included ± SD, n=3). *L. reuteri* ATCC 701359 and *L. fermentum* NCIMB 5221 were grown in MRS media. Bacterial adhesion was comparable between the two strains of *Lactobacillus* (adhesion for *L. reuteri* ATCC 70139 was 6.32 ± 0.44 log(CFU/cm^2) and 6.58 ± 0.11 log(CFU/cm^2) for *L. fermentum* NCIMB 5221). Briefly, on a daily basis, mucin-alginate beads were removed from the system, rinsed with physiological saline to remove any non-attached bacteria and then vortexed well in 1 ml of physiological saline to remove remaining immobilized bacteria off of the bead for quantification. Media was also replaced daily.

4.4.2 Investigation of the production of BSH in an in vitro continuous gut adhesion model

BSH activity was determined using an adapted spectrophotometric method as described previously [109]. *L. reuteri* ATCC 701359 immobilized on mucin-alginate beads was shown to continuously produce bile salt hydrolase at a rate of 2.70 ± 0.34 (µmol/hr/CFU x 10^6) as compared to the control that was 0.28 ± 0.16 (µmol/hr/CFU x 10^6) ($p<0.01$) shown in figure 4.2.

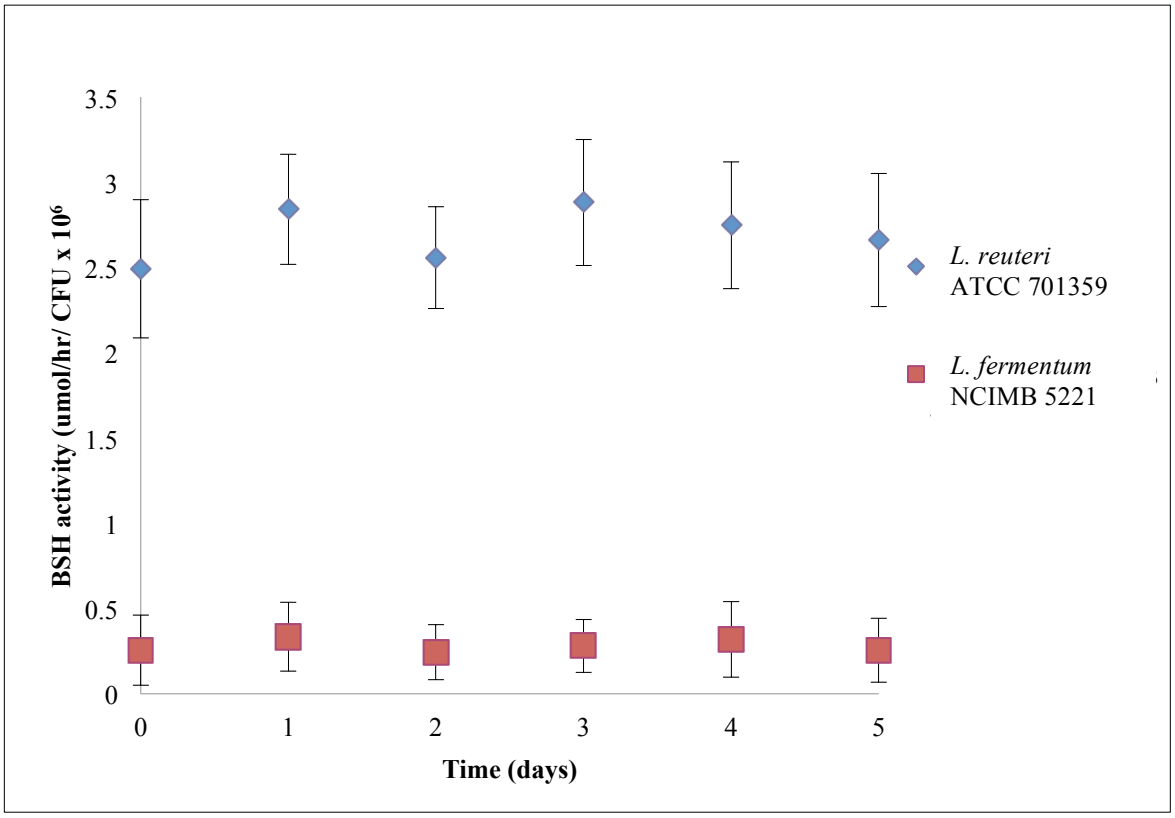

Figure 4.2: Investigation of the production of BSH in a continuous gut adhesion model in which a BSH-active probiotic, *L. reuteri* ATCC 701359 and a non-active BSH probiotic, *L. fermentum* NCIMB 5221 have been immobilized (error bars included ± SD, n=3). The average production of BSH was significantly higher with *L. reuteri* ATCC 701359 over a 5 day period was 2.70 ± 0.34 (µmol/hr/CFU x 10^6) as compared to the control which was 0.28 ± 0.16 (µmol/hr/CFU x 10^6) ($p<0.01$).

4.4.3 Investigation of the interaction between a probiotic bacteria and potentially pathogenic bacteria in a continuous gut adhesion model within the first 24 hours using microscopy

As demonstrated by plate counting and SEM images, there was an immediate effect of the addition of the probiotic on *E. coli* viability. Initially, *E. coli* adhesion on beads was 4.86 ± 0.31 log(CFU/cm^2) and dropped by 1 log unit within four hours and after 24 hours exposure to *L. reuteri*, *E. coli* adhesion decreased to 3.57 ± 0.14 log(CFU/cm^2). Bacterial viability of *L. reuteri* on mucin-alginate beads was not affected by *E. coli* presence (increased to 5.20 ± 0.08 log(CFU/cm^2) within 24 hours). Figure 4.3b is the control image where a pure culture of *E. coli* was grown on mucin-alginate beads and the subsequent images (4.3c through 4.3e) demonstrate the effect of the added probiotic on the viability of *E. coli*. As shown in figure 4.3c, within four hours of incubation, *L. reuteri* surrounded the more elongated bacteria, *E. coli*, and prevented their growth. Subsequent images demonstrated that *L. reuteri* adhesion increased whereas *E. coli* adhesion stagnated.

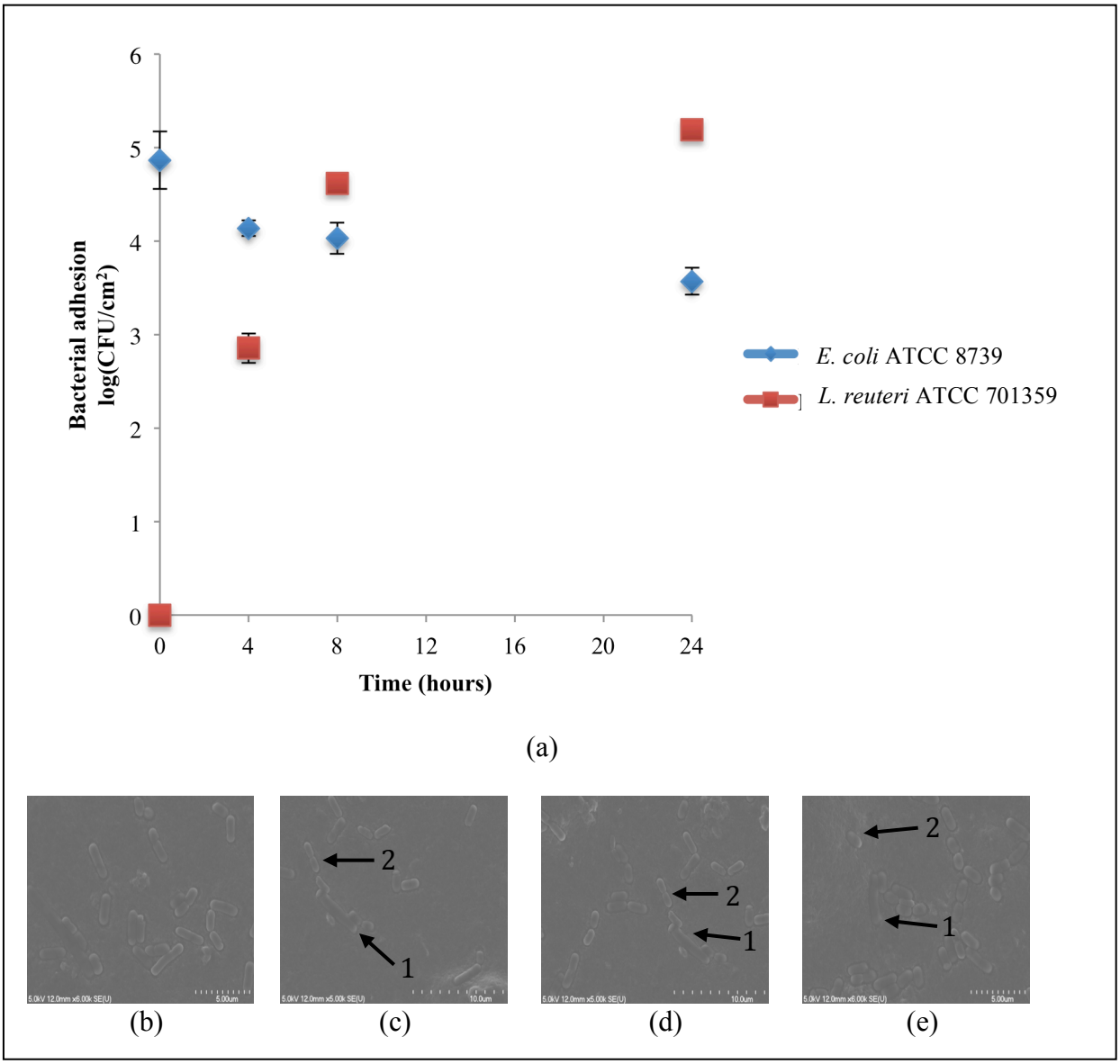

Figure 4.3: Time profile of the interaction between a potential pathogen (*E. coli* ATCC 8739) and a probiotic (*L. reuteri* ATCC 701359) in gut adhesion model (error bars included ± SD, n=3) along with Scanning Electron Microscope images after (b) 24 hour incubation with *E. coli* on mucin-alginate (control) (c) 4 hours after administration of probiotic in gut adhesion model containing *E. coli*, (d) 8 hours after administration of probiotic in gut adhesion model containing *E. coli* (e) 24 hours after administration of probiotic in gut adhesion model containing *E. coli*. Elongated bacteria is *E. coli* (labeled as 1 in image) and the other bacteria are the probiotics (labeled as 2 in image). As demonstrated in the images, *E. coli* was surrounded by *L. reuteri* within the first four hours of treatment, and *L. reuteri* adhesion increased whereas *E. coli* adhesion stagnated.

4.4.4 The effect of daily-administered probiotics on an immobilized potential pathogen in a continuous gut adhesion model

E. coli ATCC 8739 was immobilized in the continuous gut adhesion model over a 24 hour incubation period. The viability on beads was 4.60 ± 0.09 log(CFU/cm^2). On day 1, 10^8 CFU of *L. reuteri* ATCC 701359 was added to the nutrient solution circulating through the system. *E. coli* concentrations on the beads showed a significant decrease after treatment started (to an average 3.82 ± 0.18 log(CFU/cm^2) adhesion), and *L. reuteri* concentrations increased to an average adhesion of 5.65 ± 0.07 log(CFU/cm^2).

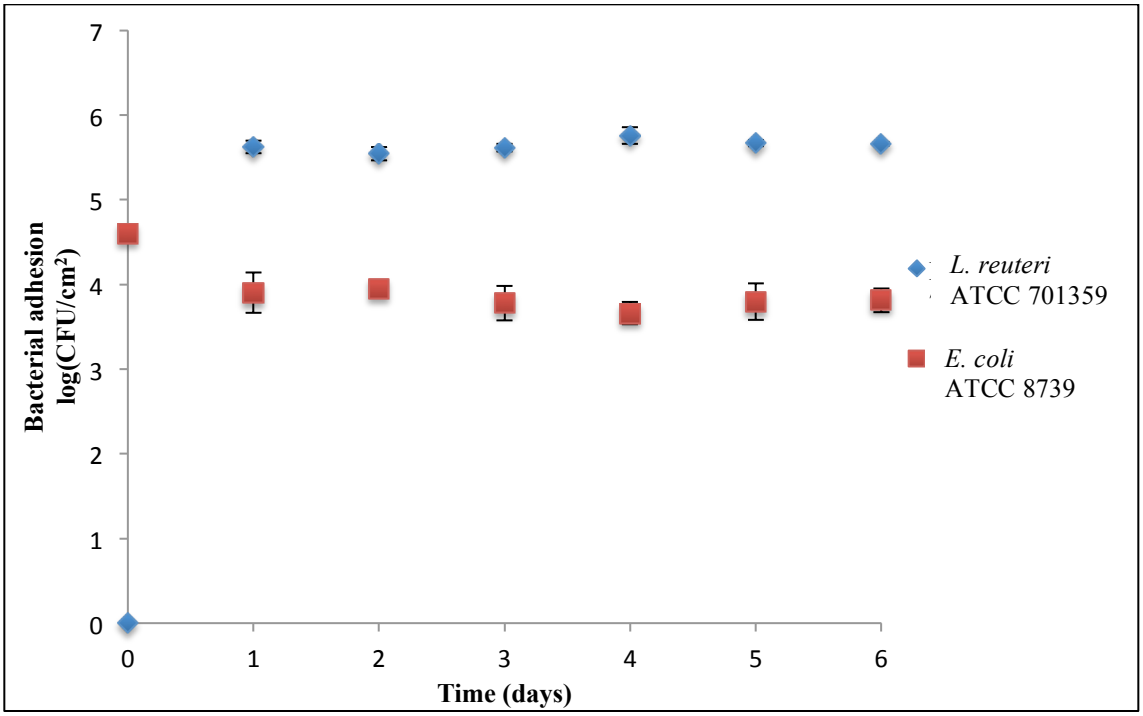

Figure 4.4: The effect of the daily administration of a probiotic (*L. reuteri* ATCC 701359) on the viability of immobilized *E. coli* ATCC 8739 in a continuous gut adhesion model (error bars included ± SD, n=3). Briefly, on day 0, mucin-alginate beads coated with *E. coli* were added to the column after being incubated with the beads overnight. On day 1, 10^8 CFU of the probiotic was administered by adding it to the circulating solution, and this was repeated on a daily basis.

4.5 Discussion

The current study demonstrates the adhesion capacity of BSH-active probiotic bacteria in a novel continuous gut adhesion model over a 5-day period. Immobilized bacteria were also able to produce bile salt hydrolase. This is an important finding to understand the 'resilience' of a given probiotic and how well it can maintain its viability and metabolic activity in a gut adhesion model. As well, this probiotic was shown to be able to affect the viability of a potential pathogen that had already been immobilized on a simulated model of the gut. There have been several studies that have demonstrated this same effect in different gut adhesion models; however, these models were undertaken over very short incubation times, typically in the range of 1 to 1.5 hours [112-114]. This study is the first of its kind to show that probiotic bacteria can inhibit potential pathogens over a long time period.

Gut adhesion models are very important tools to understand the interaction of different gut bacteria on a simulated model of the gut lining. Consequently, it is critical to develop models that are simple, reproducible, physiologically accurate and versatile. The majority of gut adhesion models typically occur in 96-well plates coated with either mucus [58,115-117] or different gut epithelial cell lines [44,58,59,80,83,118]. The main disadvantages of these models are that they are not continuous, and that samples cannot be taken throughout the experiment. The unique design of this presented gut adhesion model allows for long-term studies with a continuous flow of a nutrient rich solution that can contain a potential

biotherapeutic and intermittent sampling to witness the dynamics of a complex microbial ecosystem.

The question remains, however, whether this reduction in pathogen viability within the gut has clinical significance. In a clinical setting, at the end of an antibiotic treatment, there would be no detectable remnants of the pathogenic bacteria. In this case, the probiotic treatment reduced the viability of the pathogen, however, did not eliminate it altogether over the course of the treatment period. This does not necessarily indicate that this particular probiotic is not an effective treatment, as there are important differences to note. Firstly, typical pathogen infections can be classified as such with concentrations of bacteria (CFUs) in the hundreds and even less (133) whereas the initial concentration of the pathogen in these experiments was significantly higher. Secondly, the immune response to a foreign body in the human body is extremely complicated and multifaceted. The highly competitive microbial ecosystem residing in the gut and the immune response initiated by neutrophils and lymphocytes are all factors that were not accounted for here. These experiments isolate the probiotic-pathogen interaction in a controlled environment. The complexities that are lacking in the presented model, by the same token, yield results that underestimate the treatment capacity of a probiotic, and thus are conservative. These estimates can be compared from one probiotic strain to another through massive screening efforts, isolating probiotic strains that can have the highest effect on pathogen viability. These results can, in turn, simplify and reduce experiments carried over to animal studies and finally clinical studies.

4.6 Conclusion

Probiotics have demonstrated significant potential as biotherapeutics in a variety of different disease states. One potential application of their use is for their inhibition and displacement of previously ingested pathogens to prevent infection. Although probiotic research has steadily increased, this has not directly translated into commercially available products. One major challenge for probiotic use is how well they can adhere to the gut mucosal lining and effectuate permanent changes to the gut microbiota. In order to investigate this *in vitro*, gut adhesion models have been developed. The presented gut adhesion model is a versatile and useful tool to firstly determine how well a probiotic can adhere to a simulated model of the gut and secondly to determine its capacity to displace potential pathogens and prevent infections. A *lactobacillus* probiotic was shown to adhere well to mucin-alginate beads and maintain their metabolic activity, as demonstrated by their production of bile salt hydrolase, an enzyme that has been linked with the cholesterol-lowering effects of probiotics [34]. As well, this study was the first of its kind to demonstrate that this probiotic can displace and inhibit the growth of a potential pathogen in a continuous gut adhesion model over a 24-hour period.

Acknowledgements

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Chapter V:

Investigation of the effect of *Lactobacillus* probiotic strain addition on the intestinal microflora immobilized on a novel continuous gut bacterial adhesion model

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Running header: Effect of a probiotic in a novel gut adhesion model

Preface:

Chapter 3 and 4 demonstrated the feasibility of a continuous gut adhesion model using the concept of a packed-bed bioreactor to investigate the adhesion, metabolic activity and dynamic interaction between gut bacteria. As a follow-up study, the previously mentioned continuous gut adhesion model will be used to simulate the intestinal microflora associated to the gut mucosal lining by using fresh human fecal samples. The microbial communities living in the model were

characterized and once stability was reached, the treatment was administered. The effect of this probiotic was determined by analyzing the changes in the microbial communities after the treatment. Co-authors have contributed in experimental execution (Laetitia Rodes, Marc Fakhoury), experimental design and statistics (Arghya Paul, Hani Al-Salami) and special technique training for SEM (Sana Abbasi, Afshan Afsar Khan).

5.1 Abstract

The gut microbiota is an extremely complex system and it is necessary for the development of *in vitro* models to determine the effect of different treatments on its composition. We have developed a continuous gut adhesion model and investigated the effects of the daily administration of a *Lactobacillus* bacteria on intestinal flora immobilized in the model. Results demonstrated that the mucin-alginate beads provided a stable microbial ecosystem for bacterial growth and that the administered probiotic significantly increased *Lactobacilli* concentration on the beads (prior to treatment: 5.811 ± 0.0438 log(CFU/cm^2) and after treatment: 6.866 ± 0.122 log(CFU/cm^2), $p<0.05$) and also increased the viability of *Bifidobacteria* (prior to treatment: 4.51 ± 0.0397 log(CFU/cm^2) and after treatment: 6.09 ± 0.0968 log(CFU/cm^2), $p<0.05$). Bacterial communities of *Clostridia* and *Enterococci* were not affected by the addition of the probiotic (average *Enterococci* concentration was 5.348 ± 0.173 log(CFU/cm^2) and average *Clostridia* concentration was 5.377 ± 0.137 log(CFU/cm^2)). This model was an effective, physiologically relevant and versatile platform to determine the effect of different treatments on the gut microflora in real-time.

Keywords: beneficial bacteria, gut microflora, gastrointestinal model, bifidobacteria, packed bed bioreactor

5.2 Introduction

Gut adhesion models are very useful tools to investigate the colonization of allochtonous and commensal bacteria on the gut microflora. This dynamic, complex microbial ecosystem living within our gastrointestinal is very important in the overall health of the host [17,119]. The oral ingestion of different allochtonous bacteria can help to modulate the microflora and result in positive, in the case of probiotics, or negative effect, for pathogenic bacteria, on overall health [3,120,121]. Probiotics are defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" [23]. Although very relevant in laboratory scale research, there remains a certain amount of skepticism for use of probiotics in humans for the treatment of different diseases. One major challenge for the use of probiotics in humans is the question of how a small amount of allochtonous bacteria can lead to a permanent change in a highly competitive microenvironment found in the gut. For this reason, *in vitro* gut adhesion models have been developed. Their main objective would be to investigate how well a specific bacterial strain can survive and ‘stick’ to the lining of the gut, and consequently, maintain a more significant, longer lasting effect on the overall health of the host.

The most common *in vitro* adhesion models used involves bacterial adhesion to different intestinal epithelial cell lines[44,58,59,63,80-84]. Although cross-talk between gut epithelial cells and bacteria are critical in establishing bacterial population equilibrium, they do not interact directly with one another [58]. A mucus layer normally covers these cells and is key in establishing this rapport

[79]. As well, incubation times for these types of experiments are typically very short, in the range of 1 hour. These short incubation times are due to the reduced viability of gut epithelial cells in the presence of the bacteria. In order to provide a more physiologically relevant *in vitro* adhesion model, researchers have used immobilized intestinal mucus as a platform for bacterial growth and attachment [58,63-64,115]. Although this approach addresses several of the issues present in cell line-based models, it also compromises the key aspect of the cross-communication between bacterial cells and epithelial cells.

A continuous *in vitro* adhesion model of the gut was developed in our laboratory with the aim of addressing the above-mentioned shortcomings. The model was subsequently used to determine the effect of daily-administered probiotic bacteria on the intestinal flora immobilized on the *in vitro* gut adhesion model where microbial competition, wash out factors and mucosal-associated microflora.

5.3 Materials and methods

5.3.1 Chemicals

Arabinogalactan, low-viscosity sodium alginate, mucin from porcine stomach (type 2), pepton, starch, yeast extract, and xylan were purchased from Sigma-Aldrich Canada (Oakville, Ontario). Glucose and pectin were purchased from Acros Organics (New Jersey, US) and cystein was purchased from Fisher Scientific (USA).

5.3.2 Bacterial and bacterial growth media

Lactobacillus fermentum NCIMB 5221 was purchased from the National Collection of Industrial, Marine and food Bacteria (Aberdeen, Scotland, UK). De Man-Rogosa-Sharpe (MRS) broth and Enterococcus Selective Agar (ESA) was obtained from Difco (Sparks, Md, USA). Tryptose Sulphite Cycloserin Agar (TSCA) was obtained from Sigma-Aldrich Canada (Oakville, Ontario).

5.3.3 Preparation of mucin-alginate beads as packing material for continuous gut adhesion model

Mucin-alginate beads were used as packing material for the continuous gut adhesion model due to unpublished studies previously carried out regarding its specific adherence properties for probiotics and its physiological relevance. A solution of 2% (w/v) sodium alginate was dissolved in physiological saline and subsequently autoclaved for 5 minutes at 121^{o}C. 3% mucin was then added in sterile conditions. The mucin-sodium alginate solution was added dropwise from a height of 15cm into a 0.2M $CaCl_2$ bath [87]. At contact, gel beads were formed due to an ion exchange of sodium and calcium [88] with an approximate diameter of 3 mm. Beads were left overnight in 0.2M $CaCl_2$ bath and then transferred to a beaker filled with physiological saline for storage at 4^{o}C.

5.3.4 Investigation of the adhesion of L. fermentum as a function of bacterial incubation time using microscopy and standard bacterial quantification techniques

In order to understand the immobilization of the gut microflora bacteria on a mucin-alginate surface within the first 24 hours of incubation, scanning electron microscopy (SEM), using a Hitachi S-4700 FE scanning microscope, and selective agar quantification was performed. Diluted human fecal samples supplemented with 20 mM $CaCl_2$ were added to the different wells along with mucin-alginate beads, each well corresponding to four different time points: namely, 0 hours of incubation, 4 hours of incubation, 8 hours of incubation and 24 hours of incubation. The 6-well plate was incubated at 37^{o}C. At specific times (0h, 4h, 8h, 24h), the beads were removed from the well and rinsed with physiological saline. Beads were placed in a microcentrifuge tube with 1ml of physiological saline to quantify bacterial immobilization using plate counting, and the other beads were air-dried overnight. Air-dried beads were then coated with gold-palladium for SEM.

5.3.5 Investigation of the stability of adhered intestinal microflora during a 16-day period on an in vitro continuous gut adhesion model

The adhesion of the intestinal microflora on mucin-alginate beads over a 16-day stabilization period was investigated in order to evaluate the feasibility of the presented model. The first step was to inoculate beads with a representative sample of the human gut microflora. Fresh human fecal samples were taken from a healthy 23-year-old male with no recent history of antibiotic use. Samples were

homogenized and diluted to 15% in physiological saline. Mucin-alginate beads were then incubated with this solution for 24 hours at 37^{o}C. Preparation of the column was as follows: initially, 70% ethyl alcohol was pumped through the column and tubing and soaked for 24 hours. Sterilized physiological saline was subsequently pumped through the system and soaked for another 24 hours. Bacteria-coated beads that had been incubated with the intestinal microflora were then transferred to the column aseptically. This marked the beginning of the experiment. Beads were kept on the side to allow for the enumeration of immobilized bacteria. A nutrient rich media (table 5.1) supplemented with 20 mM $CaCl_2$ was continuously passed through the column at a flowrate 0.5 ml/min using a Watson Marlow peristaltic pump 323 E/D (Watson Marlow Pumps, MA). The system was kept at 37^{o}C. At each time point, one bead was extracted from the column (alternating from the top of the column and bottom of the column in order to ensure homogeneity of bacterial growth of beads) and selective agar bacterial quantification was performed. Each day, the nutrient rich media was replaced with a fresh solution.

Nutrient	Concentration (grams/liter)
Arabinogalactan	1
Pectin	2
Xylan	1
Starch	3
Glucose	0.4
Yeast extract	3
Pepton	1
Mucin	4
Cystein	0.5

Table 5.1: Composition of nutrient rich media circulating through the gut adhesion model [126]

5.3.6 Investigation of the effect of the addition of a probiotic on a stable microbial community immobilized in the continuous gut adhesion model

Once the stabilization of the microbial ecosystem was reached, we investigated the effect of the addition of a probiotic on the intestinal microflora growing in the gut adhesion model. The addition of a probiotic was performed as follows. *L. fermentum* NCIMB 5221 was subcultured as a 1% inoculum in MRS media throughout the experiment. After 16 hours of incubation, bacteria reached a concentration of 10^8 CFU/ml. 1 ml of MRS was pipetted into microcentrifuge tubes and centrifuged for 10 minutes at 7000 rpm. The supernatant was disposed of and the pellet was resuspended in physiological saline and centrifuged again for 10 minutes at 7000 rpm. The supernatant was once again disposed of and the pellet was resuspended at which point, the solution that contained the appropriate amount of bacteria (10^8 CFU) was added to the nutrient rich solution that circulated through the system.

5.3.7 The use of selective agar for the quantification of four gut bacteria

In order to quantify the adhesion of the intestinal microflora, four major intestinal bacterial genera (two major anaerobic genera - *Clostridia* and *Bifidobacteria*) and two major aerobic genera - *Lactobacilli* and *Enterococci*) were selected and colony counting was performed using selective agar. Table 5.2 describes the different agar media used to selectively quantify different genera. *Clostridia* and *Bifidobacteria* were incubated in anaerobic conditions using anaerobic jars, with anaerobe atmosphere generating bags (Oxoid, Cambridge, UK), for 48 hours at 37^{o}C. *Enterococci* and *Lactobacilli* were incubated in aerobic conditions (with 5% CO_2) at 37^{o}C.

Bacterial Genus	Agar used	References
Enterococci	Enterococcus Selective Agar	[127]
Lactobacilli	LAMVAB	[128]
Bifidobacteria	Raffinose Bifidobacterium	[129]
Clostridia	Tryptose Sulphite Cycloserin Agar	[130]

Table 5.2: Selective agar used for the quantification of four bacterial genera found in the gut
Abbreviation: LAMVAB, Lactobacillus Anaerobic MRS with Vancomycin and Bromocresol green

5.3.8 Study design and statistical analysis

Statistical analysis was performed using Minitab software (Minitab, Version 16; Minitab Inc, Pennsylvania, USA). Values are expressed as means ± SD. Study was considered a randomized block design. Statistical comparisons between different groups were carried out using the general linear model (GLM). All

experiments were conducted in triplicates. Statistical significance was set at $p < 0.05$.

5.4 Results

5.4.1 Overall design of continuous gut adhesion model

A gut adhesion model was developed to determine the effect of probiotic addition on an intestinal microflora during long-term experiments with daily sampling. In order to accomplish this, a glass column (10 x 1 cm) was filled with mucin-alginate beads (approximate diameter is 3 mm) that would represent the gut mucosal lining. These mucin-alginate beads were previously incubated with a diluted sample of fresh human feces to immobilize a representative sample of intestinal microflora. A nutrient rich media (with or without treatment) was circulated throughout the system at a flowrate of 0.5 ml per minute using a Watson-Marlow 323 E/D peristaltic pump and the entire system was kept at 37^{o}C. An image of the experimental setup is shown in figure 5.

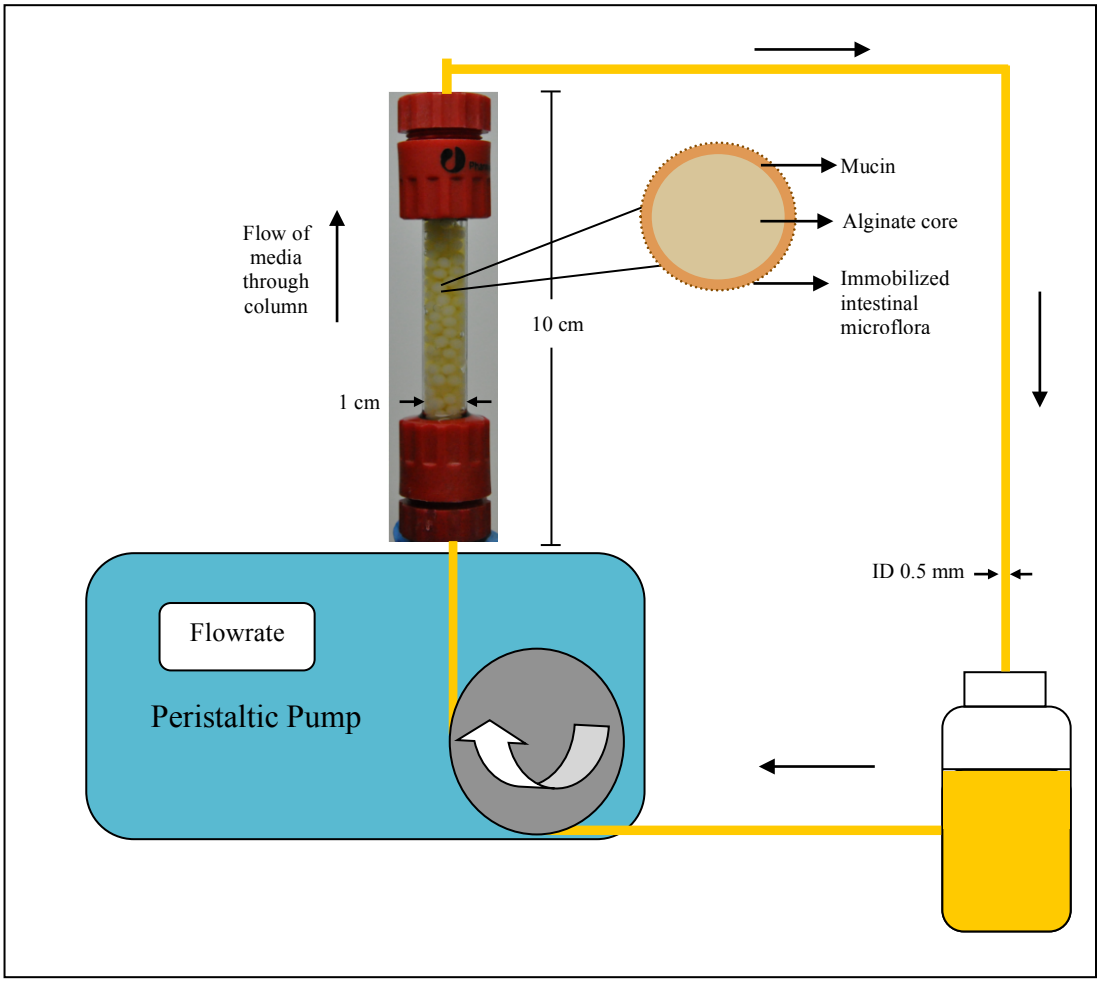

Figure 5.1: Experimental setup of continuous *in vitro* gut adhesion model. Media (whose composition is described in table 1) was pumped out of a beaker using a Watson Marlow Peristaltic pump 323 S/D into the bottom of a column (glass column, 1 x 10 cm) packed with mucin-alginate beads. Mucin-alginate beads had intestinal microflora immobilized on them by using homogenized fresh human fecal samples from a healthy 23 year old male. Media continued out of the top of the column and back into the beaker containing media. The whole system was kept at 37^{o}C. In order to investigate the effect of a probiotic on the intestinal microflora immobilized on mucin-alginate beads, *L. fermentum* NCIMB 5221 was added at a concentration of 10^{8} CFU in the nutrient rich media. ID: internal diameter of tubing

5.4.2 Investigation of the adhesion of L. fermentum as a function of bacterial incubation time using microscopy and standard bacterial quantification techniques

SEM images and plate counting was performed in order to determine the progression of a complex microbial ecosystem adhering to a mucin-alginate surface during the first 24 hours of bacterial inoculation. Figure 5.2a – 5.2e and table 5.3 provide the images and data found during these experiments. Four hours after microbial incubation, small pockets of bacteria began adhering to the surface and bacterial concentration was low for all genera (ranging between 2.77 ± 0.23 to 3.09 ± 0.075 log(CFU/cm^2) for *Lactobacilli* and *Clostridia* respectively). There was no statistically significant difference in bacterial concentration between genera after four hours of incubation on mucin-alginate ($p<0.05$). After eight hours, bacterial immobilization had significantly increased except in the case of *Bifidobacteria* that remained relatively low (3.28 ± 0.091 log(CFU/cm^2) for *Bifidobacteria* versus 4.56 ± 0.066 log(CFU/cm^2) for *Enterococci*, 4.62 ± 0.055 log(CFU/cm^2) for *Lactobacilli* and 4.66 ± 0.033 log(CFU/cm^2) for *Clostridia*). SEM images of the mucin-alginate surface after eight hours of bacterial incubation were very similar to those for twenty-four hour incubation. In this case, bacteria fully covered the mucin-alginate scaffold. The main difference however in terms of cell number between 8 hours of incubation and 24 hours of incubation was a large increase in *Bifidobacteria* (concentration increased to 4.69 ± 0.022 log(CFU/cm^2)).

Time	Concentration of *Enterococci* (log(CFU/cm^2)	Concentration of *Lactobacilli* (log(CFU/cm^2)	Concentration of *Clostridia* (log(CFU/cm^2)	Concentration of *Bifidobacteria* (log(CFU/cm^2)
0	0	0	0	0
4	2.85 ± 0.086	2.77 ± 0.23	3.09 ± 0.075	2.85 ± 0.11
8	4.56 ± 0.066	4.62 ± 0.055	4.66 ± 0.033	3.28 ± 0.091
24	5.14 ± 0.035	5.28 ± 0.079	5.33 ± 0.044	4.69 ± 0.022

Table 5.3: Investigation of the concentration of immobilized bacteria on a mucin-alginate scaffold during the first 24 hours of incubation
Abbreviation: CFU, colony forming units

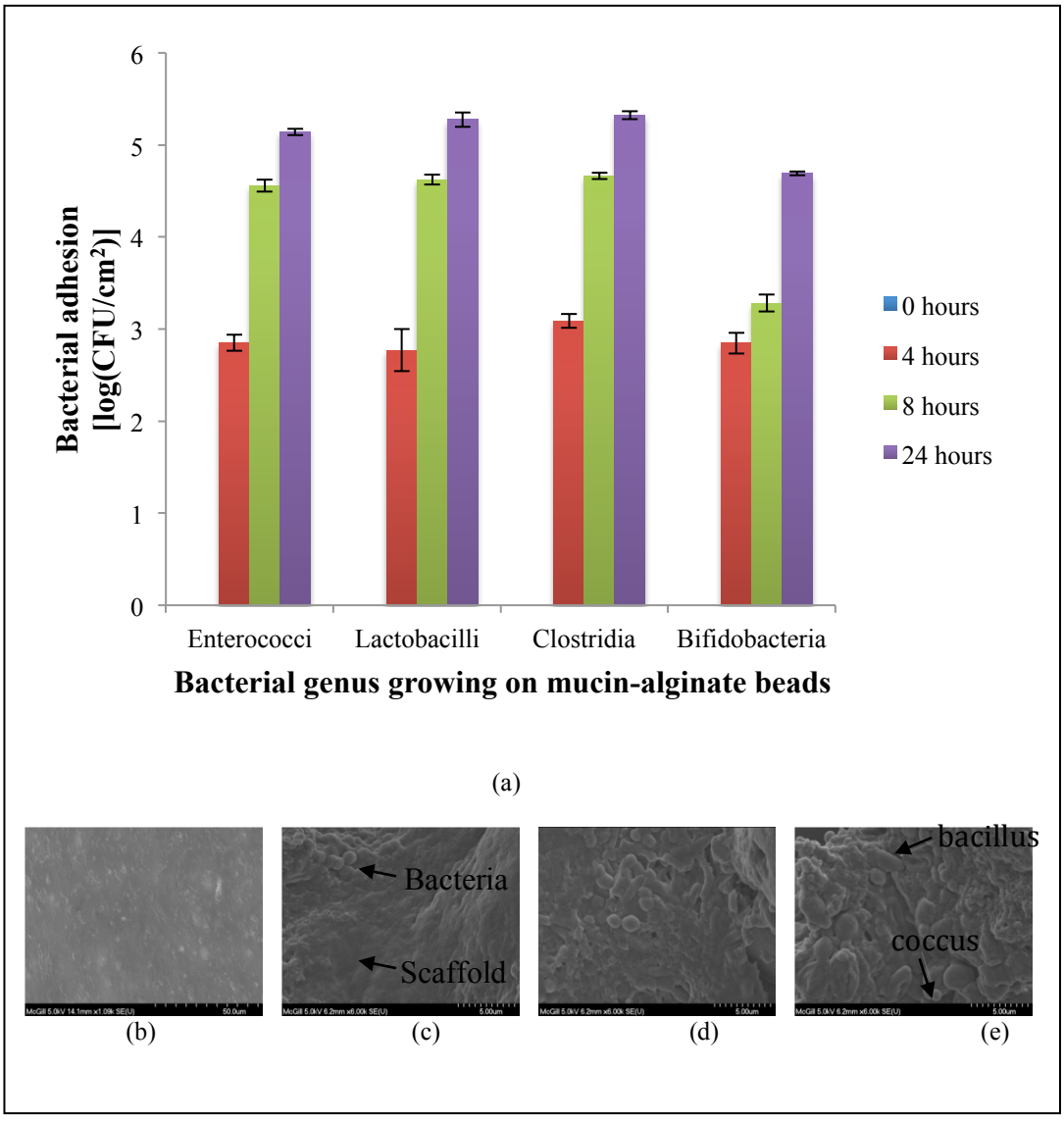

Figure 5.2: Time profile of intestinal microflora (taken from fresh human fecal samples diluted in physiological saline) immobilization on mucin-

alginate surface at different time points (error bars included ± SD, n=3) along with SEM images after (b) 0 hours of incubation (c) 4 hours of bacterial incubation at 37^{o}C (d) 8 hours of incubation at 37^{o}C (e) 24 hours of incubation at 37^{o}C. As outlined by arrows in figure 2e), there is clearly a diverse bacterial community with different morphologies.

5.4.3 Investigation of the adhesion of the intestinal microflora during a 16-day stabilization period on an in vitro continuous gut adhesion model

In order to investigate the effect of different treatments on a complex microbial ecosystem, resident bacteria must have reached stable numbers. Analysis was done over a 16-day observational period on four key gut bacteria species and results were presented in figure 5.3. The average concentration of *Enterococci* over the 16 day period was 5.229 ± 0.192 log(CFU/cm^2), *Lactobacilli* concentration was 5.513 ± 0.262 log(CFU/cm^2), *Clostridia* concentration was 5.367 ± 0.099452 log(CFU/cm^2) and *Bifidobacteria* concentration was 4.638 ± 0.188 log(CFU/cm^2). Stabilization was reached for all bacterial genera ($p>0.05$).

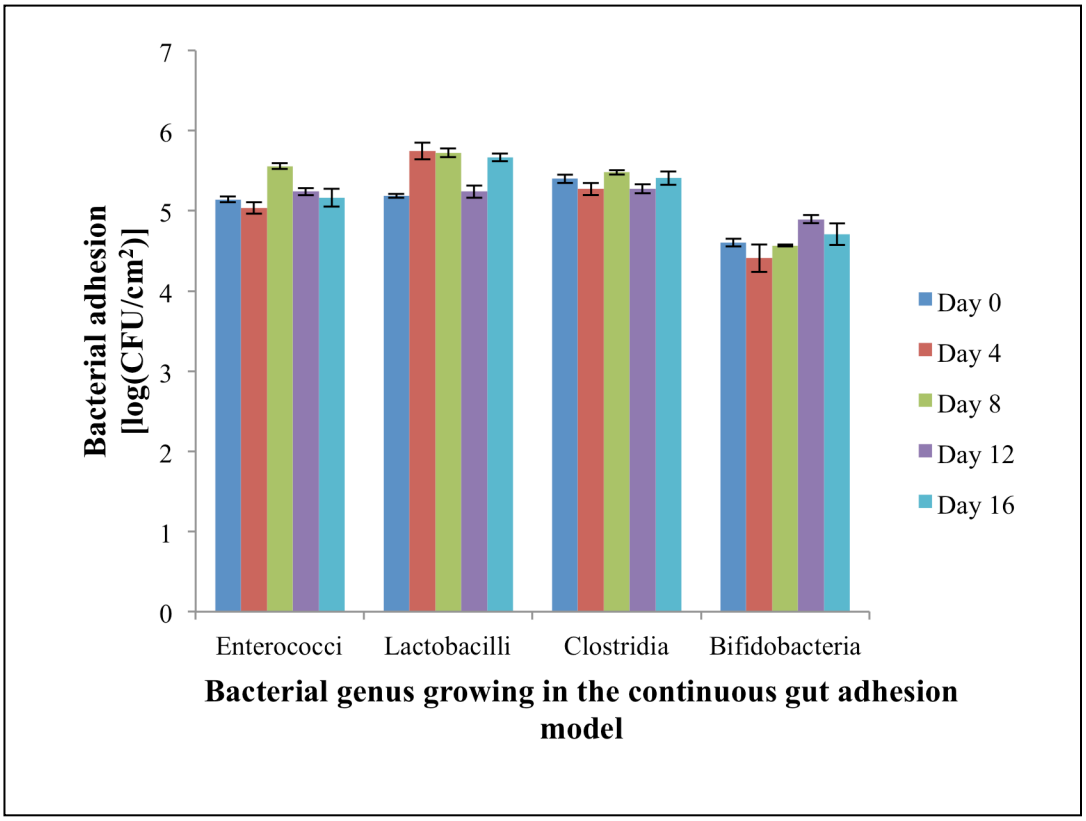

Figure 5.3: The effect of time on the immobilization of four different bacterial genera found in the intestinal microflora in the continuous gut adhesion model kept at 37°C (as shown in figure 5.1) over a 16-day period (error bars included ± SD, n=3). On a daily basis, one mucin-alginate bead was removed from the system, rinsed with physiological saline to remove any non-attached bacteria and then vortexed well in 1 ml of physiological saline to remove any remaining immobilized bacteria off the bead for quantification. Media was replaced daily. Statistical comparisons between different time points for each bacterial genus using the general linear model were performed. It was found that the $p>0.05$ for all bacterial genera, indicating that time had no effect on bacterial concentration, indicating stability.

5.4.4 Investigation of the effect of the addition of a probiotic on a stable microbial community immobilized in the continuous gut adhesion model

The addition of a daily dose of 10^8 CFU of *L. fermentum* NCIMB 5221 was done to a packed-bed bioreactor model of the human gut (which had already reached stability) in order to determine the effect of this probiotic on the system. As

shown in figure 5.4, there was a significant increase in *Lactobacilli* concentration over time with the addition of the probiotic strain (5.81 ± 0.044 prior to treatment vs 6.99 ± 0.14 log(CFU/cm^2), $p < 0.01$) and there was significant increase in *Bifidobacteria* (4.52 ± 0.040 prior to treatment vs 6.09 ± 0.10 log(CFU/cm^2), $p<0.05$). *Enterococci* and *clostridia* concentrations were stable throughout the experiment (5.35 ± 0.17 log(CFU/cm^2) and 5.38 ± 0.14 log(CFU/cm^2), respectively, $p>0.05$).

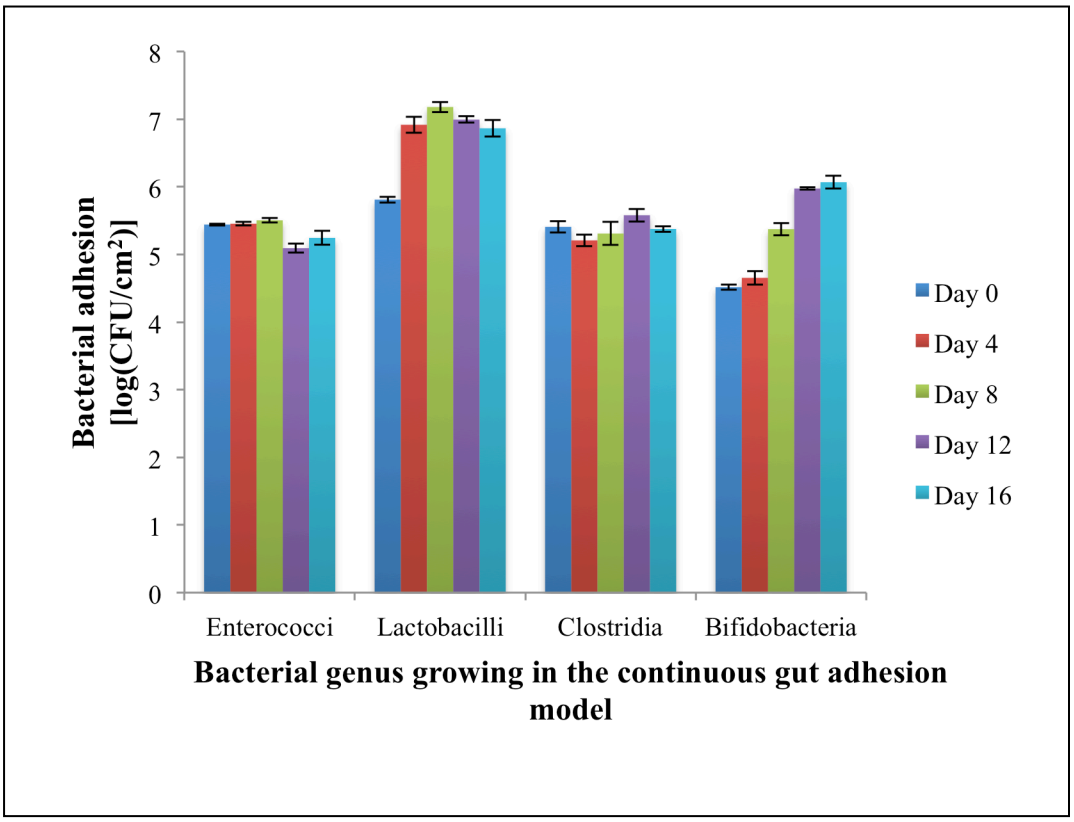

Figure 5.4: The effect of the addition of a probiotic (*L. fermentum* NCIMB 5221) on a stable intestinal microflora growing in the continuous gut adhesion model shown in figure 1 over a 16 day period (error bars included ± SD, n=3). Day 0 is the first day of probiotic treatment after a 16-day stabilization period (data shown in figure 5.3). Four different bacterial genera were quantified: a – *enterococci*, b – *lactobacilli*, c – *clostridia*, d – *bifidobacteria*. On a daily basis, 10^8 CFU of *L. fermentum* NCIMB 5221 was added to the freshly added media. Quantification was performed in the following way: one mucin-alginate

bead was removed from the system, rinsed with physiological saline to remove any non-attached bacteria and then vortexed well in 1 ml of physiological saline to remove any remaining immobilized bacteria off the bead for quantification. Statistical comparisons between different time points for each bacterial genus using the general linear model were performed. *Lactobacillus* and *Bifidobacteria* populations significantly increased ($p<0.05$) over the 16-day treatment period whereas *Enterococcus* and *Clostridia* populations remained stable ($p>0.05$).

5.5 Discussion

A representative sample of intestinal microflora adhered on mucin-alginate beads during the first 24 hours of incubation as shown in figure 5.2. Once these bacteria adhered to mucin-alginate beads, stabilization of bacterial communities was reached over a 16-day experimental period where a nutrient rich media circulated throughout the packed bed bioreactor ($p>0.05$). Finally, the addition of a probiotic to a stable microbial community significantly increased the concentration of *Lactobacilli* and *Bifidobacteria* ($p<0.01$ and $p<0.05$, respectively). This would indicate that the administered bacteria transitioned from being in solution to living in the complex microbial ecosystem immobilized on the simulated gut mucosal lining. In other words, the administered probiotic was able to permanently affect the microbial ecosystem thriving in the continuous gut adhesion model by actually adhering to the mucin-alginate beads themselves. This is one proposed mechanism of action by which probiotics can affect the health of the individual – by directly colonizing the gut mucosal lining and remaining in the ecosystem for an indefinite time period [122]. A second key finding was that the administration of a *Lactobacillus* probiotic had a positive effect on the overall concentration of *Bifidobacteria* ($p < 0.05$). This potential synergistic effect of two well-known health-conferring gut bacteria could provide another explanation by which

probiotics provide health benefits to its host. Through what could be described as 'paracrine' effects, competing bacterial communities could provide, in a selective manner, favorable ecosystems for other bacterial genera to thrive in. This could potentially work in a beneficial fashion, in the case of 'good' bacteria helping other 'good' bacteria, or in a negative snowball effect in the case of 'disease-associated' bacteria. The exact mechanism by which this occurs requires further investigation. One approach to understanding this would be to systematically analyze metabolites associated to different gut bacteria and determine their functionality. In fact, the rapidly growing field of metabolomics has been often applied to gut metabolism in the hopes of answering these questions [123,124]. Metabolomics is the systematic identification and quantification of all metabolites in a given organism or biological system [125]. The metabolic footprints of different probiotic, commensal or pathogenic bacteria would likely further our understanding of how they interact with one another. Consequently, the effect of orally administered probiotics can be improved by combining different metabolic factors to improve their viability and adhesion.

5.6 Conclusion

In attempting to understand whether orally administered probiotics can in fact have a long lasting effect on the overall health of the host, it has become critical to develop *in vitro* models that can investigate how well these probiotics stick to the gut mucosal lining. These *in vitro* adhesion models must provide an accurate depiction of the challenges faced by allochtonous bacteria throughout the gastrointestinal system. The gut adhesion model developed in our laboratory has

combined many of those challenges associated with gastrointestinal transit including an exposed mucosal layer on which bacteria interact directly (as shown in a schematic of the anatomy of the lining of the gut in figure 5), a harsh microenvironment consisting of trillions of commensal bacteria and is a continuous flow system. By administering probiotic bacteria to this system, we were able to determine the effect of this treatment over time on the different bacterial communities within the human gut. Not only did the results seem to demonstrate that the administered probiotic itself was able to adhere to the mucosal lining, but also that it was affecting the viability of another bacterial community, namely *Bifidobacteria*.

The simplicity and versatility of this model allows it to be useful for numerous other types of experiments. Further investigation on the effect of different probiotics or combined probiotics should be carried out and compared to one another. As well, the effect of prebiotics on a mucosal associated microflora could be witnessed in real-time. Finally, the effectiveness of different antibiotics can be determined by their ability to target mucosal associated pathogens. By considering the gut microbiota as one single bioreactor, one can determine the effect of a treatment on several competing factors, whether those effects may result in changing bacterial communities or metabolite concentrations. In this way, researchers can analyze how the gut microbiota self-regulates itself and what can send it into dysbiosis.

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CHAPTER VI: Summary of results

To design a novel gut adhesion model, the following results were obtained:

1. The first step was to design a gut adhesion model that satisfied the following design criteria: the model should be easy to use, inexpensive to run, and should allow for intermittent sampling throughout an experimental protocol. A new model was developed using a glass column (10 x 1 cm) filled with mucin-alginate beads. Media was pumped throughout the system using a peristaltic pump. This model can be used to determine probiotic adhesion, enzymatic conversion kinetics of immobilized bacteria, and complex microbial interactions in real-time.
2. In order to determine the feasibility of a novel gut adhesion model, a probiotic was selected and its adherence on four potential materials (glass, polystyrene, alginate and mucin-alginate) was determined that would be used as packing material for a column. Mucin-alginate beads were selected as the most representative packing material with the highest bacterial adhesion (bacterial adhesion on glass was lowest at 4.52 ± 0.077 log(CFU/cm^2), on polystyrene it was 5.24 ± 0.11 log(CFU/cm^2), on alginate beads it was 4.84 ± 0.22 log(CFU/cm^2) and on mucin-alginate beads, it was 6.23 ± 0.15 log(CFU/cm^2)).
3. Mucin-alginate beads were packed into a column (1 x 10 cm) and the adhesion of *Lactobacillus fermentum* NCIMB 5221 was investigated over a 14-day period to determine if this model was truly suitable for long-term studies with intermittent sampling. Over the course of 14 days, the

bacterial population remained stable on the mucin-alginate beads with an average concentration of 6.464 ± 0.572 log(CFU/cm^2).

4. The gut adhesion model was then used to determine the adhesion capacity of three different gut bacteria (*L. fermentum* NCIMB 5221, *E. coli* ATCC 8739 and *B. longum* ATCC 15707) over a 7-day period. Results showed that there was a significantly higher average concentration of *L. fermentum* NCIMB 5221 over the 7 day period (6.616 ± 0.117 log(CFU/cm^2)) compared to *B. longum* ATCC 15707 (5.651 ± 0.029 log(CFU/cm^2)) and *E. coli* ATCC 8739 (4.133 ± 0.050 log(CFU/cm^2)).
5. A second probiotic that was BSH-active, *Lactobacillus reuteri* ATCC 701359, was tested in this gut model to demonstrate that adhered bacteria were able to maintain their metabolic activity and that the platform could allow for easy sampling of metabolites. Results showed a stable bacterial community of *Lactobacillus reuteri* ATCC 701359 growing in the column (6.32 ± 0.44 log(CFU/cm^2)) producing BSH at a rate of 2.70 ± 0.34 (umol/hr/CFU x 10^6).
6. This gut adhesion model was also used to investigate the use of *Lactobacillus reuteri* ATCC 701359 as a potential biotherapeutic for the inhibition and displacement of a mucosal-associated pathogen, *Escherichia coli* ATCC 8739. *E. coli* concentrations on the beads showed a significant decrease after 5 days of probiotic treatment (10^8 CFU) (from an initial value of 4.60 ± 0.09 log(CFU/cm^2) to an average 3.82 ± 0.18 log(CFU/cm^2) adhesion). The presence of *E. coli* did not inhibit the

growth and adhesion of *L. reuteri* as it increased to an average adhesion of 5.65 ± 0.07 log(CFU/cm^2).

7. Once the model was shown to provide a stable microbial ecosystem for one bacterial species, the same experiments were performed and validated using intestinal flora isolated from fresh human fecal samples over a 16-day period. The average concentration of *Enterococci* over the 16 day period was 5.229 ± 0.192 log(CFU/cm^2), *Lactobacilli* concentration was 5.513 ± 0.262 log(CFU/cm^2), *Clostridia* concentration was 5.367 ± 0.099452 log(CFU/cm^2) and *Bifidobacteria* concentration was 4.638 ± 0.188 log(CFU/cm^2). Stabilization was reached for all bacterial genera ($p>0.05$).

8. To test the feasibility of the mucosal gut bacterial adhesion model to immobilize bacteria, probiotics were delivered on a daily basis and the results demonstrated that

   a. The administered probiotic increased *Lactobacilli* cell numbers within the system (5.81 ± 0.044 prior to treatment vs 6.99 ± 0.14 log(CFU/cm^2), $p < 0.01$)

   b. The administered probiotic also increased the cell number of *Bifidobacteria* (4.52 ± 0.040 prior to treatment vs 6.09 ± 0.10 log(CFU/cm^2), $p<0.05$)

   c. Concentrations of both *Enterococci* and *Clostridia* remained constant throughout the treatment period (5.35 ± 0.17

log(CFU/cm^2) and 5.38 ± 0.14 log(CFU/cm^2), respectively, $p>0.05$).

CHAPTER VII: General discussion

The gut microbiota is a veritable living entity within the gastrointestinal system of humans that contributes directly to the overall health of the host. Its responsibilities are numerous, varied and essential. They include the metabolism of key nutrients [17,18], immunological protection against foreign pathogens [22], and structural functions [20]. A shift in the relative composition of the microflora in the gut, can, by consequence, lead to altered states of health, and potentially disease states [4-8,98]. In this way, the gut microbiota is a legitimate target for disease prevention and treatment.

Probiotics are defined as living bacteria, which, when administered in significant number, confer potential health benefits [36,70-76]. In order to confer these potential benefits, probiotics must endure a treacherous transit throughout the gastrointestinal system, and remain metabolically active in the highly competitive microenvironment found in the gut. In order to investigate the effectiveness of a given strain of probiotic, it is essential to develop *in vitro* models that can accurately depict the complex *in vivo* situation. *In vitro* gut adhesion models are effective tools designed to understand the mechanisms by which different bacteria can adhere to the lining of the gut and thereby permanently affect the health of the host.

Through an extensive literature review on gut adhesion models, there are to two main types of models used with slight variations amongst them. Cell-line based

models involve the adhesion of different bacteria to a gut epithelial cell line (typically HT-29 and/or Caco-2 cells) [44,58-61,82]. Adhesion data of different probiotic, pathogenic and commensal bacteria can be compared and conclusions on how these bacteria will react *in vivo* can be deduced. A major concern with these types of experiments is that there are no direct interactions between gut epithelial cells and the microflora in the gut and therefore, the model's physiological relevance is not as accurate. As well, incubation time of bacteria and cell lines is typically very short, between 1-2 hours and so these models cannot accurately investigate how well probiotics adhere over long periods of time. Mucus-based gut adhesion models have also been used to investigate probiotic adhesion [58,64,65] in order to more accurately mimic *in vivo* conditions. Currently there are very few continuous gut adhesion models [65,65] that can be used to investigate the effect of a probiotic on the complex micro-ecosystem found in the gut mucosal lining, however, there are significant design issues that render these models less practical.

With the limitations and downfalls of the previously mentioned gut adhesion models under consideration, the main design criteria used to develop a model for this thesis are presented here:

i. Simple and inexpensive to design
ii. Continuous model with non-invasive sampling in order to design long-term experiments with time points throughout.
iii. Representative of *in vivo* conditions;

a) Initial contact of bacteria to the gut lining being the mucus layer covering epithelial cells
b) Provide a model that has a microbial ecosystem similar to that found in the large intestine
c) Shear stresses associated with the flow of solution passing through the gastrointestinal system

1) Design of a continuous gut adhesion model

A model was designed using mucin-alginate beads that were packed into a column. A nutrient rich solution circulated throughout the system using a pump and the media were replaced daily. A peristaltic pump was used to ensure sterility of the system so that no part of the pump would come into contact with the circulating solution. Sampling was done very easily in a non-invasive way, which allowed for experimental time points to be within the time frame of an experiment. Beads were removed from the column, rinsed with physiological saline to remove any non-specifically attached bacteria, and then vortexed well in physiological saline to remove all remaining bacteria.

The advantages of this system include: easy, simple and rapid sampling which allows for a continuous experiment lasting several days or weeks, a representative model of the gut lining with immobilized mucus as the point of bacterial contact and flexibility in terms of the types of experiments outlined in the previous chapters. The system also addresses several of the recommendations presented by A.C. Ouwehand and S. Salminen [63], namely the use of fresh faecal matter to

provide a normal intestinal microbiota, appropriate growth conditions, relevant adhesion incubation times – experiments can run for over the span of many days or weeks and intestinal flux – there is a continuous flow of nutrient rich solution passing through the column. Finally, as opposed to other continuous models that have a closed design, this novel model can be stopped and restarted within the framework of a single experiment for intermittent sampling.

2) Investigation of a novel gut microbial adhesion model for bacterial immobilization

The first step was to determine the suitability of the new model for bacterial cell adhesion, by determining the adhesion of a strain of bacteria, *Lactobacillus fermentum* NCIMB 5221 over a 14-day period. Data from these experiments demonstrated that there was selective bacterial adhesion for different strains and provide evidence that this model is comparable to current established models with published data. Experiments were also carried out to determine the adhesion of these bacteria on mucin-alginate beads in the first 24 hours of incubation. These time-scale adhesion experiments are the first of their kind to be done *in vitro*. The interest in these experiments is in understanding how quickly a bacterial biofilm forms over the mucus layer in the intestine and that although the mucus layer provides an anchor for the bacterial communities growing in the gut, most of the adhesion occurring is between bacteria in the lumen of the gut and the bacteria that are mucosal-associated.

Once it was confirmed that attached bacteria were able to remain viable, the next step in experimentation was to incorporate a large sample of bacteria typically found in the gut. This model was shown to provide a stable microbial ecosystem for a normal intestinal flora. The implications of having such a tool are, as will be shown in the following discussion, that one can determine the effect of a given therapeutic on an *in vitro* gut adhesion model having many important physiologically relevant features.

3) The effect of daily administration of probiotics on the viability of a pathogen immobilized in an *in vitro* gut adhesion model

A common indication for probiotic use as a biotherapeutic is their potential to displace and inhibit gut pathogens to prevent further infection [112-114]. Potential mechanisms by which probiotic bacteria can affect the survivability of pathogenic bacteria in the gut include the previously described mechanisms for immune modulation activation, the inherent competition for binding sites and nutrients in the gut mucosal lining, and secretion of antimicrobial substances [131]. The inhibitory effect of probiotics has been shown in a number of gut adhesion models; however, they have all been performed over very short time periods (typically between 1 and 1.5 hours). Using the aforementioned gut adhesion model, the inhibitory effect of probiotics on mucosal-associated pathogens was demonstrated in real-time, over a 5-day period. There was a decline in *E. coli* population as soon as the treatment period began and this decline was maintained throughout the experiment. These findings demonstrate that not only do

administered probiotics have the capability to displace and inhibit pathogenic bacteria but also that they can maintain their activity over a long time.

4) The effect of daily administration of probiotics on a complete *in vitro* gut adhesion model inoculated with fresh human feces

The logical next step was to introduce a treatment into the system to determine its effect on the overall composition of the microbial communities living in gut model. In order to do so, a probiotic, *Lactobacillus fermentum* NCIMB 5221, was introduced daily at a concentration of 10^8 CFU. Results demonstrated firstly that bacteria in the bulk mobile phase in the column were able to adhere onto mucin-alginate beads already inhabited by a full spectrum of intestinal bacteria. This would indicate that administered probiotics could have a direct effect on the health of the host by increasing the number of healthy bacteria attached to the gut mucosal lining. The second finding of increased *Bifidobacteria* concentrations could potentially indicate that the administered probiotic has synergistic effects on other bacterial genera in the gut. Through different metabolic factors secreted by the ingested bacteria, other bacteria can selectively benefit from the increased presence of the administered bacteria and, therefore, can increase in cell number. In order to truly understand the mechanisms by which this occurs, one would have to undertake a metabolomic analysis of the system, ie the analysis of all metabolites produced in real-time and their relative effects.

Not only do the findings from these experiments provide important information on how a complex microbial ecosystem can behave in real time, but also, the

model used can be used in a variety of very different applications. The first case demonstrates the capability of the model to be applied to a biotechnology setting, where, through the use of different materials and surface modifications, one can selectively adhere a type of bacteria and be useful for different biochemical reactions. By using beads, surface area can be maximized and therefore can dramatically increase the yield of a specific product. The second case is geared more towards biomedical applications, where the model can be used as a drug-screening platform for orally administered therapeutics. By initially adhering bacteria typically found in the gut, one can consider the mucus and bacteria coated beads to be an accurate depiction of the gut mucosal lining. The addition of probiotics, prebiotics and/or antibiotics can be administered and their effect on the gut mucosal lining can be investigated simply, efficiently and in real time.

5) Usefulness of this model for future scientific experimentation

The versatility and simplicity of the system is certainly the model's most impressive asset. Its applications in probiotic screening is the tip of the iceberg, as one would easily be able to examine the effect of any orally administered therapeutic whether it be prebiotics, antibiotics or any combination of the former, on a single bacterial species or metabolite of interest. The effect can be determined by previously identified bacterial quantification techniques, such as plating or qPCR, or metabolite analysis given the appropriate assay. Inlet and outlet streams can be sampled and analyzed in order to determine the metabolic activity of the bacteria packed in the column. This proposed approach to testing

the effectiveness of different probiotics can open up a huge door of new biotherapeutics based on targeting the microflora found in the gut.

6) Limitations of the current model

The gut is an extremely complex, highly diverse, dynamic ecosystem that varies tremendously from person to person and more so, population to population. It is therefore a huge challenge to develop *in vitro* models that can accurately represent the gut *in vivo*. The main components missing from this model are the contributions of gut epithelial cells and goblet cells in bacterial adhesion, which cannot be overlooked. In deciding on a direction to take when designing a gut adhesion model, my priority was placed on the previously stated objectives, notably on having a continuous experimental model that would allow for intermittent sampling. The initial point of contact is consistent in both the model developed and *in vivo* situations; however, the signaling and adhesion molecules produced by epithelial cells are not taken into account. The added complexity of combining mammalian cells and bacterial cells would have significantly limited the design of the model and made it less user-friendly, more labor intensive and less appropriate for various different applications. For example, one potential design for the gut adhesion model that had been considered will be described here. A flow cell device with an inlet and outlet stream would have been customized to contain two chambers within the cell, separated by a semi-permeable membrane coated in mucus. Each chamber would have separate inlets and

outlets for bacterial and mammalian culture media respectively. As such, the model would allow for the cohabitation of gut epithelial cells and bacteria. This potential model has several advantages; namely, that any effects of gut epithelial cells on microbial adhesion could be witnessed over a long time period. However, the major disadvantage and reason why development wasn't advanced on this model, was that there would have been no way to have intermittent sampling using this. When sampling would have been required, the system would have had to have been taken apart at which point bacterial viability could be determined. This creates a major restriction on what can and can't be achieved using this model. As well, the model would have required specialized, potentially costly equipment that would have, yet again, limited its versatility and wide-spread applicability. The model presented in this thesis requires no special or customized equipment; simply a glass tube and a peristaltic pump.

CHAPTER VIII: Conclusion and future recommendations

The gut microbiota is an exceptionally complex and important biological system within the human body that has been ignored for far too long. It has been well documented that an altered state of the microflora in the gut due to some external factors such as diet, stress, exercise of different genetic factors, can lead directly to disease. By selectively modulating the gut microflora, one can potentially reverse the progression of a disease or prevent it from occurring at all by targeting the root cause of that disease. In order to modulate the gut, one potential strategy would be to administer some health promoting bacteria, or probiotics, and hope that these probiotics can remain in the gut permanently or, more specifically, have a permanent effect in the gut. Due to the nature of the system, and the difficulty and discomfort in accessing the gut *in vivo* in humans or animals, *in vitro* models of the gut have been developed to determine the effect of probiotics. The purpose of this thesis was to develop an *in vitro* gut adhesion model that would accurately represent the most important feature of probiotic resilience in the gut – that being the adherence of these probiotics to the gut mucosal lining. The developed model is continuous and has an open design that allows for real-time monitoring of the system and more direct results. As well, the model includes several important features relevant to the physiological reality of the gut mucosal lining, namely, that a mucus layer is the first line of contact that the lumen-associated bacteria face, and that there is a complex and significantly more numerous microbial

ecosystem that already reside in the gut with the sole purpose of destroying any foreign species from colonizing the gut.

Not only do the findings from these experiments provide important information on how a complex microbial ecosystem can behave in real time, but also, the model can be used in a variety of different applications. The different chapters of this thesis demonstrate the drug-screening capacity of this model for orally administered therapeutics. This can be done through the comparative analysis of adhesion of different probiotics, the analysis of metabolic activity of adhered bacteria, the ability to suppress and inhibit gut pathogens, and their effect on a complex microbial ecosystem comparable to that of the gut. By initially adhering bacteria typically found in the gut, one can consider the mucus and bacteria-coated beads to be an accurate depiction of the gut mucosal lining. The effectiveness of probiotics and prebiotics to colonize the gut and modulate the gut microflora can be determined simply, efficiently and in real-time. As well, it has been well established that pathogenic bacteria respond differently to antibiotics when living in suspension or when embedded in a biofilm [132] and so this model can be a very important tool to investigate antibiotic-pathogenic bacteria dynamics in a model of the gut mucosal biofilm. This model is therefore extremely useful and can be used for a huge number of applications.

It is important to note that the model presented here is not and should not be the only tool for measuring probiotic efficiency within the gastrointestinal system. It would be important to corroborate results from this presented *in*

vitro model with bacterial adhesion experiments conducted in animals. A proposed experiment for future use could be the continuous monitoring of bacterial adhesion within the *in vitro* gut adhesion model presented here and within the gut of rodents, through intermittent sacrifice of the animal and quantification of bacterial species in the gut through PCR analysis. These experiments could provide the *in vitro* model with a calibration to be able to extract clinical significance of the results obtained. Eventually, it could also be interesting to include different molecules associated with gastric/pancreatic and liver secretions in the circulating fluid to simulate the complexity of the solution that passes through the gut. Finally, different factors, such as signaling and adhesion molecules could be included in the circulating fluid to be able to selectively control the adhesion of different bacteria, potentially at the species level, within a complex microbial ecosystem.

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