#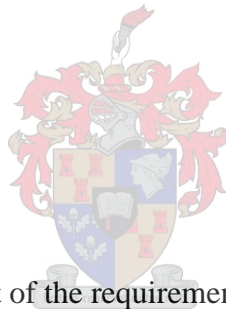 Isolation and characterization of novel *Lactobacillus* spp. with promising gastro-intestinal survival and adhesion properties

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in the Faculty of Science at Stellenbosch University

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Summary

The human gastrointestinal tract (GIT) is a complex organ system, and is closely associated with immunological and hormonal functions. A delicate balance needs to be maintained between the selective and beneficial colonization of allochthonous and autochthonous microorganisms, which contribute to the preservation of gut homeostasis and protect the host against infections. *Lactobacillus reuteri* HFI-LD5 and *Lactobacillus rhamnosus* HFI-K2, isolated from the feces of healthy humans, formed biofilms on a hydrophobic abiotic surface (polystyrene) under static conditions and were selected for further studies. Meaningful differences in cell surface properties were observed between the isolates, with strain HFI-K2 exhibiting a significantly greater basic surface property, in addition to a significantly higher surface hydrophobicity (37.71 %, $p < 0.05$) compared to that recorded for strain HFI-LD5 (8.82 %, $p < 0.05$). The hydrophobic nature of *L. rhamnosus* HFI-K2 in conjunction with better biofilm formation, may contribute to a greater GIT colonization ability. Neither of the two strains isolated degraded mucus, and their growth was not irreversibly inhibited when exposed to acidic conditions (pH 2.5) and bile salts (0.5 % and 1.0 % w/v), suggesting that they may survive conditions in the GIT.

To confirm planktonic and sessile survival of *L. rhamnosus* HFI-K2 and *L. reuteri* HFI-LD5 in the human GIT, the effect of three simulated, fasting-state gastrointestinal fluids (gastric fluid, pH 2, 2 h exposure; intestinal fluid, pH 7.5, 6 h exposure and colonic fluid pH 7, 24h exposure) on both free-living and attached cell viability of the strains was assessed. Exposure to simulated gastric juice had the greatest effect on both planktonic cell viability and biofilm metabolic activity. The sequential introduction of the simulated gastrointestinal fluids initiated the detachment of biofilm biomass, accompanied by a decrease in the metabolic activity, as recorded by changes in CO_2 production, by the use of the carbon dioxide measurement system (CEMS). However, as soon as the exposure was halted and sterile culture medium was reintroduced, the remaining biofilm biomass responded by producing CO_2, followed by the recovery of biofilm biomass and re-establishment of pre-exposure activity within 24 h. In contrast to the complete loss of planktonic *L. rhamnosus* HF1-K2 viability after exposure to gastric juice, biofilms of this strain not only recovered within 24 h after exposure, but also exhibited increased metabolic activity after recovery. To our knowledge, this is the first study to assess the effect of simulated, fasting-state gastrointestinal fluids on lactobacilli biofilms.

Monitoring of CO_2 production as a real-time indicator of metabolic activity in a biofilm provided insight to the differential survival responses of lactic acid bacteria under fasting-state gastrointestinal conditions. The ability of *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 to survive acid, bile and simulated gastrointestinal fluid induced stresses, coupled with biofilm formation under dynamic flow conditions, may contribute to improved survival and persistence of these strains within the human GIT. These characteristics, especially those exhibited by *L. rhamnosus* HFI-K2, are promising indicators for the application of these isolates as probiotic supplements.

Opsomming

Die mens se spysverteringskanaal (SVK) is 'n komplekse orgaanstelsel en is nou verbind met immunologiese en hormonale funksies. 'n Delikate balans moet gehandhaaf word tussen die selektiewe en voordelige kolonisasie deur inheemse en indringer mikroörganismes, wat bydra tot die instandhouding van derm homeostase en die beskerming teen mikrobiese infeksies. *Lactobacillus reuteri* HFI-LD5 en *Lactobacillus rhamnosus* HFI-K2, geïsoleer uit gesonde menslike feces, vorm biofilms onder statiese toestande op 'n hidrofobiese, abiotiese oppervlak (polistireen), en gekies vir verdere studies. Betekenisvolle verskille in seloppervlak eienskappe is tussen die isolate waargeneem, waar *L. rhamnosus* HFI-K2 'n aansienlik hoër basiese, tesame met 'n aansienlik hoër hidrofobiese, seloppervlak (37.71 % vs 8.82 %, $p < 0.05$) getoon het. Die hidrofobiese aard van *L. rhamnosus* HFI-K2 asook die beter vermoë om biofilms onder statiese groei te vorm, kan bydra tot beter kolonisasie van die SVK. Nie een van die twee isolate was daartoe instaat om slym af te breek nie. Blootstelling aan suurtoestande (pH 2.5) en galsoute (0.5 % and 1.0 % w/v) het nie 'n noemenswaardige effek op enige van die isolate gehad nie, wat verder dui op die potensiaal om oorlewing onder SVK toestande.

Om oorlewing in die SVK te bevestig, is die effek van drie gesimuleerde, vastende staat SVK vloeistowwe (maagvloeistof, pH 2, 2 h blootstelling; dermvloeistof, pH 7.5, 6 h blootstelling en kolonvloeistof pH 7, blootstelling 24 h) op die lewensvatbaarheid van beide vrydrywende en oppervlak-geassosieerde selle van beide isolate bepaal. Blootstelling aan gesimuleerde maagvloeistof het die grootste invloed op die lewensvatbaarheid van beide vrydrywende en biofilm-geassosieerde metaboliese aktiwiteit getoon. Blootstelling van *L. reuteri* HFI-LD5 en *L. rhamnosus* HFI-K2 biofilms aan hierdie toestande het die verlies van biofilm biomassa geïnisieer, en was vergesel deur 'n afname in metaboliese aktiwiteit, soos bepaal deur veranderinge in CO_2-produksie deur die gebruik van die CO_2 meting stelsel (CEMS). Sodra blootstelling gestaak is deur die invloei van steriele groeimedium, het die oorblywende biofilm biomassa gereageer met produksie van CO_2, gevolg deur die herstel van biofilm biomassa en hervestiging van voorblootstelling aktiwiteit binne 24 uur. In teenstelling met die volledige verlies van vrydrywende *L. rhamnosus* HF1-K2 lewensvatbaarheid na blootstelling aan maagsap, het oppervlak geassosieerde selle van hierdie stam nie net herstel binne 24 uur na blootstelling nie, maar verhoogde metaboliese aktiwiteit na herstel getoon. Sover ons kennis strek, is hierdie die eerste studie om die invloed van gesimuleerde, vastende staat SVK

vloeistowwe op melksuurbakterie biofilms te evalueer. Die gebruik van CO_2 produksie as 'n riële aanwyser van biofilm metaboliese aktiwiteit bied insig in die differensiële oorlewingsreaksie van melksuurbakterieë onder gesimuleerde vastende staat maag kondisies. Die vermoë van beide *L. reuteri* HFI-LD5 en *L. rhamnosus* HFI-K2 om suur, gal en gesimuleerde maagvloeistof-geïnduseerde spanning te oorleef, tesame met die vermoë om biofilms te vorm onder dinamiese vloeitoestande, kan bydra tot verbeterde oorlewing en voortbestaan van hierdie stamme in die menslike SVK. Hierdie eienskappe, veral dié van *L. rhamnosus* HFI-K2, is belowende aanwysers vir die inkorporering van hierdie isolate in probiotiese aanvullings.

Biographical Sketch

Kyle Brent Klopper was born in Bloemfontein on the 6th of August 1990. He matriculated at Fairmont High School, Cape Town, in 2009. He enrolled for a B.Sc. degree in Human Life Sciences at Stellenbosch University in 2010 and obtained the degree in 2012, majoring in Microbiology, Biochemistry, Genetics and Physiology. In 2013 he obtained his B. Sc. (Hons) in Microbiology at the Department of Microbiology, at Stellenbosch University. In 2014 he enrolled as a M.Sc. student in Microbiology at the Department of Microbiology, at Stellenbosch University.

"To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science."

Albert Einstein

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Introduction

This study involved two distinct fields in microbiology, i.e. probiotics and biofilms. These two distinct research fields were combined to allow the investigation of *in vitro* biofilms formed by probiotic bacteria. The intent was to establish a novel and improved understanding of biofilms formed by probiotic bacteria, since studies involving this topic are sparse and the overall comprehension of potential biofilm formation by probiotics lags behind that of other biofilm fields.

A thorough scientific investigation into the ability of different probiotic, lactobacilli strains to form biofilms, and the consequent potential for improved GIT persistence associated with this surface-attached form of microbial growth, is lacking. This knowledge gap, therefore, provided valuable research questions that were addressed in this study. The ability of different lactobacilli strains to form biofilms under static conditions has to some degree been evaluated, using the widely-accepted microtiter screening assay. However, this does not provide a realistic simulation of the environmental conditions that gastrointestinal-associated biofilms experience or resolve the question whether probiotic microorganisms can form biofilms under conditions of flow.

The isolation of novel and distinctly different species of lactobacilli, originating from human luminal content, was critical for this study. Previous static, microtiter lactobacilli biofilm studies have alluded to species and even strain specific variations. The differences identified between these species provided insight into the potential variation in biofilm forming abilities of probiotic lactobacilli. The characterization of these isolates with respect to cell surface hydrophobicity and auto-aggregation ability led to further understanding of the potential relevance of these routine probiotic-screening criteria to biofilm formation under static and dynamic conditions. Therefore, this study sought to investigate not only static biofilm formation by lactobacilli, but moreover the ability to form biofilms under dynamic flow conditions, such as found in the GIT. This is more pertinent to human gastrointestinal conditions where probiotics exert their beneficial effects.

The differential response of planktonic and sessile microbial populations has been well established in the field of biofilm research and cannot be ignored. This is especially relevant to probiotics with respect to the general stress response induced by detrimental environmental conditions, such as

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those experienced within the human gastrointestinal tract (GIT). The secondary focus of this study was to determine whether this documented differential survival response is also relevant to the probiotic field. To circumvent the ethical considerations required in obtaining human gastrointestinal fluids, simulated fasting-state gastrointestinal fluids were utilized to realistically mimic the harsh environmental conditions that are prevalent in the human GIT (pH changes, bile and enzymatic damage). The use of these biologically relevant fluids facilitated investigating the *in vitro* survival response of both planktonic and sessile populations of lactobacilli following exposure to GIT-relevant conditions.

Significance and motivation for this study

Probiotic supplements are a multi-billion-dollar industry, with the field of probiotic research at the forefront of scientific research and development owing to recent insights into importance of gastrointestinal health. Despite the advances made to date, most probiotic researchers and by extension the probiotic industry, have ignored a critical aspect of microorganisms, namely the propensity of microbes to persist as attached or sessile populations. The vast majority of past and present probiotic research has been conducted on planktonic microbial populations, despite the fact that it has been established that free-floating suspensions are not the dominant mode of microbial growth. This deficiency in knowledge and understanding with respect to probiotic biofilms is made evident by the limited number of original scientific articles addressing this topic. Therefore, it is of critical importance that probiotic microorganisms be studied not only planktonically, as it relates to the preparation and packaging of probiotic supplements, but also as biofilms, since probiotics are expected to associate with surfaces in the human GIT.

Classical screening methods to identify strains with potential probiotic properties involve subjecting the planktonic cells to acidic and bile-enriched conditions. Although this technique may detect putative probiotic strains, it is entirely synthetic and lacks the biological relevance that is required to adequately simulate the gut. Conversely, the use of human subjects and/or their gastrointestinal fluids create significant ethical considerations and provide a notable barrier to probiotic research. A middle-ground approach thus involves the use of simulated human gastrointestinal fluids consisting of biologically relevant compounds at appropriate concentrations. In this study, the use of simulated fasting-state gastrointestinal fluids to mimic the stresses induced on bacterial biofilms allowed for greater insight into potential probiotic survival and persistence within the human GIT.

The ability to assess the response of bacterial biofilms to metabolic stress is critical and provides insight into how these communities may function within the natural environment. The use of carbon dioxide as an indicator of biofilm metabolic activity allowed for the real-time analysis of

bacterial biofilm formation and response to adverse conditions. The combined usage of metabolic activity monitoring and simulated gastrointestinal fluids permitted the investigation of the survival and potential persistence of probiotic biofilms *in vitro*. To our knowledge, this is the first study to assess the effects of simulated fasting-state gastrointestinal fluids on biofilms formed by *Lactobacillus* species.

Research questions

Several research questions were addressed in this study, namely:

Do planktonic lactobacilli cells exposed to low pH and bile salt stress (classical probiotic screening techniques) differ in survival compared to planktonic cells exposed to biologically relevant gastrointestinal fluids?

Does static biofilm formation by different lactobacilli, isolated from human luminal contents, differ?

Does surface hydrophobicity and auto-aggregation contribute to the ability of bacteria to form biofilms?

Do planktonically cultured populations of lactobacilli differ in survival after exposure to simulated fasting-state gastrointestinal fluid compared to sessile populations?

If simulated fasting-state gastrointestinal fluids disturb lactobacilli biofilms cultured under flow conditions and induced severe metabolic stress, would these biofilms recover upon amelioration of the unfavorable environmental conditions induced by the fluids?

Overview of chapters

The human body is a complex system comprised of multiple organs, with the gastrointestinal tract (GIT) being solely responsible for the acquisition of nutrients critical to the maintenance of overall homeostasis. The first chapter provides an overview of the underlying complexity of the human GIT by providing insight into the anatomical, physiological and microbial parameters that contribute to the complex nature and function thereof. A brief summary of probiotic supplements and its history is included, along with probiotic selection criteria and tolerance of probiotic bacteria to GIT conditions. The relevance and occurrence of bacterial biofilms in the human GIT is also discussed. The chapter is concluded with a summary of various model systems used to simulate the human GIT, with specific focus on simulated gastrointestinal fluids.

The physiological processes that govern the human body are controlled by homeostatic regulation and the human body must maintain a fine balance between the prevention infection and beneficial colonization by allochthonous and autochthonous microorganisms. In the second chapter, two novel lactobacilli, namely *Lactobacillus reuteri* HFI-LD5 and *Lactobacillus rhamnosus* HFI-K2, isolated from human feces, were examined for their suitability as probiotic supplements. Classical probiotic screening methods were employed to compare the isolates with respect to predefined criteria, including bile tolerance, mucinolytic activity, auto-aggregative ability and cell surface hydrophobicity. The performance of the novel isolates was benchmarked against that of two commercially available probiotic supplements. In addition to these desirable probiotic characteristics, the ability of the two lactobacilli isolates and commercial strains to form static biofilms on hydrophobic abiotic surfaces, under various nutrient conditions, was also evaluated. Complex and diverse intra- and inter-species interactions take place within the human GIT. Gut microbiota research, including that involving probiotics, predominately focus on microbes in suspended or planktonic growth rather than sessile or biofilm-associated cells. In the third chapter, the effects of three simulated, fasting-state gastrointestinal fluids on the viability of both planktonic and sessile cells of the two lactobacilli isolates (*L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2) were assessed. Real-time monitoring of biofilm metabolic activity provided insight into whether any differential survival responses exist between planktonic and sessile populations of the respective lactobacilli strains under simulated fasting-state gastrointestinal conditions.

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CHAPTER 1

CHAPTER 1: Literature Review

1.1. The gastrointestinal tract

The human gastrointestinal tract (GIT) is an intricate body system, fulfilling a critical role with respect to immunological and metabolic functions (1, 2). The GIT is more than just a collection of tissues but rather represents a fine balance between eukaryotic and prokaryotic interactions. It is the most heavily colonized body system, with approximately 70 % of all microorganisms found in and on the human body residing within the colon (3). The human GIT provides a diverse variety of environmental niches, governing the selective colonization of these anatomically and physiologically distinct sites. Microbial colonization at birth has a significant impact on shaping the development of the gut microbiota and has long-term effects on human development and the maintenance of homeostasis.

The human gastrointestinal tract has a surface area in excess of 32 m^2 which, combined with the physiological parameters present, creates an ideal environment for microbial colonization (4). The human body is colonized by 10 to 100 trillion microbial cells, representing 2 to 3 % of total body weight and out numbering human cells by 10-fold (5–7). The two major sections of the GIT, namely the upper and lower GIT, represent anatomically and physiologically distinct environments. These two sections contain 4 environmental niches: the oral cavity, stomach, small intestine and colon.

The fetal and infant GIT was thought to be essentially devoid of microorganisms and is immediately colonized at birth by various microorganisms (6, 8). It is generally believed that an infant's GIT is naturally colonized with microorganisms from the uterus and vagina during birth (9). However, more recent studies have shown the presence of bacteria in the placenta (10) and amniotic fluid (11–14) before birth, in blood sampled from the umbilical cord (15) and fetal membranes of healthy newborns (13, 14, 16), suggesting that the intestinal tract of the fetus is colonized when still in the womb. This represents the first major gut microbial community succession, which is dependent on factors such as mode of birth (cesarean section or vaginal birth), diet (formula or breastfed) and environmental conditions (hospital or home birth) (6, 8). It has been

scientifically shown that this initial colonization is critical and influences the final, stable adult microbiota (17). If initial colonization is examined, you begins to understand the importance and influence of environmental factors on microbial populations within the GIT. This is exemplified by the contrast that exists between modes of birth. Birth by Cesarean section prevents the contact that the infant would have had with the birth canal and the associated vaginal microbiota and instead exposes the infant to the mother's skin microbiota. This is evident in the increased prevalence of skin-associated staphylococci in the GIT of cesarean-delivered infants (8). A decrease in *Bifidobacterium* and *Bacteroides* numbers, an increase in clostridial species, along with smaller numbers of *Escherichia coli* have also been observed, in conjunction with an overall reduction in bacterial diversity (8, 18). In contrast, infants delivered via the birth canal are colonized by the vaginal and intestinal microbiotas, which contain strict anaerobes largely absent in cesarean born infants (19).

The second major gut microbial community succession occurs when infants are weaned. The microbial population increases in diversity and starts to resemble the population common in adults (8, 20). A resilient and stable adult-like gut microbiota is achieved at 3 to 5 years of age and tends to be stable throughout adulthood (17, 21). Colonization of the infant gut plays a pivotal role in immunological and metabolic functionality and also affect disease susceptibility later in life (6, 8, 17, 20).

1.2. Anatomical, physiological and microbial parameters of the GIT

The GIT is broadly defined as a hollow, muscular tube extending from the oral cavity to the anus (22, 23). It can be divided into 4 distinct sections, with the inclusion of accessory organs (gall bladder etc.) further complicating the physiology and microbiota of this system.

1.2.1. Upper GIT

The upper GIT is comprised of the oral cavity, esophagus and stomach with the exact anatomical distinction between the upper and lower GIT being at the suspensory muscle of the duodenum (24). This region of the GIT is responsible for the initial digestion of dietary components, starting

within the oral cavity and ending with stomach. The upper GIT provides numerous environmental niches. It is dominated by three genera, *Streptococcus*, *Gemella* and *Bacteroides* (25), while phyla such as Firmicutes, Proteobacteria and Fusobacteria constitute a smaller fraction of the microbiota. The dramatic changes in pH and enzyme concentration within the upper GIT provide a significant hurdle to the survival of microorganisms.

Oral Cavity and Esophagus

Although the primary purpose of the oral cavity is the ingestion and mastication of food, secondary functions such as limited digestion of carbohydrates through amylase activity and the absorption of small molecules (e.g. glucose) through the mucosal wall also take place (23, 26). The oral cavity extends externally from the lips and cheeks to the anterior pillars of the fauces internally, and extends downwards from the hard and soft palates to the tongue (23). The oral cavity is lined with stratified, keratinised squamous epithelial cells, to protect against microbial adhesion during mastication (27). Mastication is achieved through the mechanical action of the 32 teeth present in the adult human mouth, and the masticated food mixture leads to insalivation once combined with saliva (23). Saliva is a complex, highly viscoelastic fluid comprising 99 % water, with the remaining constituents primarily consisting of proteins (mucin, amylases and antimicrobial agents) and ionic components (23, 28). The pH of saliva is near neutral (6.2 to 7.6), and the flow rate is governed by stimulated and unstimulated states to 2.0 ml.min^{-1} and 0.3 ml.min^{-1}, respectively (23, 29). Upon completion of mastication, the partially digested and homogenized food bolus enters the esophagus via the pharynx. The esophagus moves the bolus from the oral cavity to the gastric compartment via peristalsis (23, 28).

The human oral cavity provides a distinct niche, containing various microenvironments (30, 31). The near neutral pH and nutrient rich environment encourages microbial colonization by endogenous and exogenous microbes. The oral microbiota consists of in excess of 700 species, with a high degree of diversity (25, 30, 31). It is home to six phyla, namely Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteria and TM7 (30, 31). Even though the Firmicutes phylum is one of the smallest, it is extremely diverse. For example, the genus *Lactobacillus* shows a large degree of species diversity within the oral cavity, with the reported

isolation of *Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus salivarius* and *Lactobacillus vaginalis*. These species are considered to be autochthonous to the oral cavity (32), with *L. acidophilus* being the most dominant *Lactobacillus* sp. in the oral cavity (33). The rapid transit time from mouth to esophagus limits proliferation of planktonic microbes within the oral cavity and provides a continuous inoculum for the rest of the GIT.

The rapid transit of the bolus through the relatively short esophagus, ensures minimal colonization. The esophageal microbiota is similar in composition to that of the oral microbiota (34). The same six phyla dominate (Firmicutes, Bacteroides Actinobacteria, Proteobacteria, Fusobacteria, and TM7) with *Streptococcus* being the numerically dominant genus (39 %) (35). The similarities between the oral and esophageal microbiota may be attributable to the periodic inoculation of the esophagus by the bolus.

The secondary phase of digestion takes place within the confines of the stomach or gastric compartment.

Stomach

The human stomach is an impressive and intricate organ, facilitating complex processes such as nutrient absorption, partial digestion and partial exclusion of some microbial pathogens within the GIT. The stomach, with its acidic gastric fluid, facilitates the partial digestion of complex food into various dietary components and the absorption of select components including some medication and ethanol (36). The human stomach is a hollow, muscular, expanding organ, comprised of 4 regions (fundus, gastric body, pyloric antrum and pylorus) (23, 28). The stomach provides a surface area of approximately 0.05 m^2 when fully distended, holding about 1 000 ml of gastric contents (4). The gastric wall is complex in structure and is comprised of mucosa (surface epithelium, lamina propria and muscularis mucosae), which contains various gastric glands (secretory epithelial cells) (23).

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The gastric glands are responsible for the secretion of mucin, pepsinogen, hydrochloric acid, intrinsic factor, bicarbonate and gastrin (23, 28, 37, 38). The gastric fluid is an acidic cocktail of hydrochloric acid, enzymes and mucus, which facilitates partial digestion of complex foods. The pH of gastric fluid varies between pH 1.0 and pH 5.0, depending on the nature of the stomach content (38, 39). During the fasting-state the pH of the stomach contents decreases to below 2. The introduction of the bolus into the stomach causes a temporary increase in gastric pH, to within the range of pH 4.0 to 7.0 (40). The buffering capacity of the bolus during fed-state provides a temporary reprieve from the highly acidic conditions, followed by a steady decrease to fasting-state pH values within 2 hours post feeding (41). The gastric enzyme, pepsin, is one of the major digestive enzymes, and is tailored specifically for the hydrolysis of exogenous proteins. While it is inactive at near neutral pH, it functions optimally at pH 2.0 (42, 43). Lingual lipase is another digestive enzyme present in the stomach, and it is responsible for the hydrolysis of medium- and long-chain triglycerides (44, 45). In contrast to pepsin, lingual lipases function optimally at a pH range of between 3.0 and 6.0 (45).

The near-constant acidic condition and presence of gastric enzymes within the stomach, is critical for partial digestion of complex food matrixes. This stringent environment necessitates the protection of the secretory epithelial layer containing the gastric glands. The surface of the secretory epithelial cells are coated with a protective 200 µm-thick viscous, polymeric-gel layer, comprised of two layers of mucus (46, 47). The dense inner layer is firmly attached to the secretory epithelium and gives rise to the loose, thicker outer mucus layer (4 to 5-fold thicker) through the activity of endogenous proteases (47). The gastric mucosal surfaces are protected by two main mechanisms; high cell/mucus turnover rates and acid neutralization. Turn-over rates within the stomach are fast, with the entire gastric mucosa being renewed every 3 to 54 days (dependent on cell types) (48). The inner gastric mucus layer is renewed hourly by the goblet cells, allowing for the constant expansion of this layer to replenish the outer mucus layer to ensure a protective barrier (49). Acid-neutralization at the luminal surface of the epithelial cells is achieved through the secretion of bicarbonate by the gastric mucosa (50). The bicarbonate is encased in the mucus layers, and although it provides limited buffering capacity, it maintains the surface of the epithelium at pH 7.0 (50, 51). These mechanisms maintain a balance between digestion and auto-digestion under homeostatic conditions.

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Mixing within the stomach and the movement of food from the stomach to lower GIT is achieved through peristaltic muscular contraction of stomach walls (52). This rhythmic peristaltic movement gives rise to laminar flow conditions with a low Reynolds number, i.e. smooth and gentle movement (52, 53). Gastric transit times are unique to a specific individual and also dependent on the nature of the stomach contents. The interval from the time of entry of the bolus through the esophageal sphincter, to the exiting of the chyme (mixture of digestive enzymes and partially digested food) through the pyloric sphincter varies from 15 min to in excess of 120 min (54–58).

The stomach is a harsh environment to colonize, with constant acidic conditions and enzymatic activity. As a result, microbial diversity in the stomach is relatively low compared to the rest of the GIT (59, 60). The stomach microbiota is mainly comprised of three phyla; Firmicutes, Proteobacteria and Bacteroidetes, in descending order of dominance (61). The presence of the mucus bilayer creates two distinct microenvironments within the stomach. The outer thick, loose mucus layer is colonized by non-acidophilic bacteria, e.g. *Helicobacter* spp. (60). The presence of *Helicobacter* spp. decreases overall diversity within the stomach (60). Acid-tolerant species of *Lactobacillus*, *Staphylococcus*, *Streptococcus* and *Neisseria* are frequently present (60). The human stomach is generally devoid of large numbers of lactobacilli, however studies of the stomach microbiota have revealed the presence of various species of lactobacilli such as *L. fermentum, gasseri, reuteri, salivarius* and *vaginalis* (32, 62, 63). Roos et al. (62) isolated four novel *Lactobacillus* spp. from gastric biopsies, *Lactobacillus antri, Lactobacillus gastricus, Lactobacillus kalixensis* and *Lactobacillus ultunesis,* which alludes to the adaptation of some *Lactobacillus* spp. to the harsh conditions prevalent in the stomach. The gastric microbiota more closely resemble the lower GIT microbiota than that of the oral microbiota (61).

1.2.2. Lower GIT

Starting at the pyloric sphincter and terminating with the anus (64), the small intestine and colon constitute the lower GIT in humans. It is the region where the majority of digestion and absorption of nutrients occurs. The lower GIT environment is physiologically more conducive to microbial colonization and persistence. This is primarily due to the near-neutral pH, slower transit time, and presence of microenvironments in the crypts and mucus layers. However, although it is relatively favorable for microbial colonization, significant chemical barriers such as the presence of bile salts and digestive enzymes need to be overcome.

Small intestinal tract

The small intestine is the site where digestion is completed and adequate nutrient absorption starts. The anatomical start point of the small intestine is below the pyloric sphincter and it terminates at the ileo-caecal valve (23). The small intestine can be defined as a hollow, invaginated, muscular tube and is comprised of 3 anatomical regions; the duodenum, jejunum and ileum (22, 23). In contrast to the stomach mucus layer, the small intestinal mucus layer is singular in nature and lacks the dense, firmly attached inner layer present in the stomach (47). The single mucus layer is not permanently attached to the epithelial layer but rather loosely associated with the surface (47). The mucus layer lubricates the transit of chyme, traps microorganisms and neutralizes the chyme exiting from the stomach. Bicarbonate, produced by the Brunner glands located in the mucosa, is responsible for neutralization (65). The quantity of bicarbonate produced by the Brunner glands is more than 6 times the amount that is produced within the stomach, and increases the chyme pH from 2.0 to 3.0 to pH 6.5 to 7.5 (51). A rapid increase in chyme pH to $\pm$ 6.0 is facilitated upon entry into the duodenum and is further elevated during transit through the jejunum and ileum to a final pH of 7.5 (40, 66).

Owing to its function, the intestinal mucosa is thicker and more vascular than the gastric mucosa (23). Circular folds, or plicae circulares, protrude into the lumen due to the underlying submucosa forming ridges (23, 67). The intestinal surface area is further increased by intestinal-villi, microvilli and glands (67). The presence of plicae circulares, villi and microvilli facilitate the bulk

absorption of nutrients within the GIT, even though the small intestine is only 6 m in length (67). The surface area of the entire small intestine is 400 times greater than that of the stomach (30 m^2 versus 0.05 m^2). This significant difference in surface area can be attributed to the intestinal villi and microvilli increasing the surface area by 6.5 and 13 times respectively (4). This vast surface area is critical for absorption and secretion, in addition to interactions with eukaryotic and prokaryotic organisms.

The small intestinal secretions include enzymes and surfactants. Upon entry into the duodenum, the chyme is combined with pancreatic fluid originating from the pancreas and bile produced by the liver (67). Pancreatic fluid possesses a dual functionality of neutralizing chyme to provide the optimal environment for pancreatic enzyme function and reducing acid damage to intestinal mucosa (67). Pancreatic amylases complete the digestion of carbohydrates started by salivary amylase in the upper GIT (67). Large protein fragments generated in the stomach through gastric enzymatic activity, are further degraded to smaller peptides by pancreatic proteinases (trypsin and chymotrypsin) (67). Bile is a complex secretion with both excretory and digestive functions (68). Bile is constantly produced within the liver and stored within the gallbladder, prior to secretion into the duodenum (67, 68) and its concentration within the small intestinal lumen ranges from 0.2 to 2.0 % (w/v) (69). As a digestive aid, bile acts as a surfactant and emulsifier for subsequent absorption of fats and lipophilic vitamins from the chyme (70). Bile also has a tertiary function as an antimicrobial agent, due to its bacteriostatic action which reduces the colonization ability of allochthonous microorganisms (68).

The chyme is propelled through the small intestine by a combination of segmentation (bidirectional movement) and peristalsis (unidirectional movement) (67). Segmentation functions predominantly to homogenize the chyme and intestinal secretions, and achieves only a small amount of forward propulsion. Conversely, peristalsis (wave-like muscular contractions) has limited homogenizing activity but is the primary mode of propulsion of intestinal content (23, 67). The combination of segmentation and peristalsis yields a low Reynolds number, indicative of laminar flow conditions, and a transit time specific to intestinal conditions. The transit of chyme, upon entering duodenum to entering ascending colon, is between 0.5 and 6 h (54, 56, 71).

The transit of the microbial-laced bolus through the pyloric sphincter into the duodenum introduces the upper GIT microbial communities into the lower GIT. The composition of the communities entering the small intestine is largely shaped by the gastric acid and duodenal secretions containing bile and pancreatic enzymes (72). Chyme digestion within the small intestine causes increased enzymatic stress on the microbial communities present. These stresses shape the communities and as a result a relatively small indigenous population is prevalent in the small intestine (72). The dominant phyla within the small intestinal tract are Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria*,* with the following genera being prominent: *Bacteroides, Clostridium, Eubacterium, Lactobacillus, Prevotella* and *Ruminococcus* (59–61, 72)*.* The diversity of *Lactobacillus* spp. within the small intestine includes some species that are not prevalent in the upper GIT, namely *Lactobacillus delbrueckii, Lactobacillus reuteri* and *Lactobacillus ruminus* (72)*.* As would be expected, various *Lactobacillus* spp. present within the upper GIT are found in the small intestine (chyme-associated), including, *L. acidophilus, casei, gasseri, paracasei, plantarum* and *rhamnosus* (32, 33, 72).

Colon

The colon mainly ensures osmotic homeostasis and provides a microenvironment for the gut microbiota. It is responsible for water, electrolyte and energy recovery from digested food entering from the small intestine. The colon extends from the ileo-caecal valve to the anus, and is a hollow, muscular tube with haustrations or wall invaginations. It consists of 3 sections, termed the ascending, transverse and sigmoidal colon (23, 73). The colon has a similar microstructure to that found within the small intestine, however it lacks villi and the characteristic plicae circulares (circular folds) of the small intestine (23). These circular folds are replaced by semi-circular haustrations, small pouches caused by sacculation (sac formation), giving rise to the characteristic segmental form of the colon (23, 74). The luminal surface of the colon is lined with columnar epithelial cells, goblet (mucus) cells and microfold cells (23). These cells enable the dual functions of the colon as an absorptive and secretory organ. The replacement of villi with blunt microfold cells, as well as the relatively short length (1.5 m) of the colon, reduces the surface area to 2 m^2, which is 15-fold lower than the surface area of the small intestine (4).

The colon has a mucus bi-layer that resembles that of the stomach, rather than the single layer present in the small intestine (75). The inner mucus layer is dense, stratified and firmly attached to the colonic mucosa and is impermeable to gut microbiota (75). The proteolytic degradation of the inner layer, causes a 4-fold expansion in thickness and the generation of the loose outer layer (75). The mucus bi-layer protects the underlying mucosa from damage caused by organic acid production due to bacterial fermentation, provides lubrication to limit abrasion by passing food particles, and creates an environmental niche for gut microbiota (67, 76).

The colonic environment is neutral to slightly alkaline, with pH values ranging from 5.5 to 8.5, dependent on the section of the colon and dietary composition (37, 77). The colon is heavily colonized by microorganisms and the neutral pH can be temporarily decreased by the production of organic acids. Bacterial fermentation of the previously undigested food fraction produces short-chain fatty acids which are used as an energy source by colonic cells (78, 79). The bicarbonate-containing mucus bi-layer ensures that homeostatic pH conditions are maintained, thereby mitigating any detrimental effects of bacterial metabolites (organic acids) on the epithelial cells (67). The colon also recoups bile salts that escape reabsorption in the distal ileum, thus reducing the bile concentration within the colon (80, 81).

Transit time through the colon, achieved through haustral contractions, is significantly longer than the transit through the rest of the GIT (67, 82). Haustral contractions fulfill the combined function of segmentation and peristalsis that occur in the small intestine, i.e. mixing and forward propulsion. Colonic transit time is typically in the range from 7 h to in excess of 24 h (67, 83).

The colon is the section of the GIT best suited to microbial colonization and coincidently also the best-studied part of the GIT in terms of its microbial community. The microbial community contained within the colon is largely (90 %) comprised of obligatory anaerobic bacteria (60, 84, 85). The colonic environment is the least hostile environment in the entire GIT, which is corroborated by the high microbial diversity observed here (60). Culture-based methods only identified a small fraction of the microbial community within the colon, with 40 species comprising an estimated 90 % of the culturable population (60). In stark contrast, culture-independent methods estimate that more than 800 species are represented in the colon (60). The

dominant phyla within the colon, in descending order of abundance are, *Bacteroidetes, Firmicutes, Proteobacteria* and *Fusobacteria* (59)*.* The abundance and diversity of lactobacilli are the highest within the colon, with species such as, *L. casei, fermentum, paracasei, reuteri, ruminus* and *salivarius* being present (32, 85). The relative microbial abundance and diversity within the colon remains stable over time and is less prone to community fluctuations when compared to the small intestine and stomach.

1.3.Probiotics in a Human Context

The global probiotic market size is in excess of $ 34 Billion as of 2015, and it is estimated that in a mere six years the market size may nearly double (86). The renewed interest in GI health and functional foods has driven investigations into host-microbe interactions and the effects on human health and disease states (87). The microbial community contained within the human GIT forms a complex "organ within an organ", sometimes referred to as the "forgotten organ" (88, 89). This "forgotten organ" plays a critical role in human metabolism, immunological functionality and overall maintenance of gut homeostasis (88, 89).

The large surface area of the human GIT is favorable for microbial colonization and provides a large and critical target for pharmaceutical and probiotic supplementation effects on the human body (4).

1.3.1. History of probiotics

Microorganisms have colonized and been an integral part of the human GIT from before the beginning of modern civilization (90). Although humans and microorganisms have coexisted for many years, scientists only took notice of the inherent health benefits of fermented dairy products in the 1800's (91). The link between general human wellbeing and microorganisms was not made until the early 1900's after two scientists identified two genera that were associated with the observed health benefits. In 1905, Eli Metchnikoff determined that the health benefits associated with the consumption of yogurt was not due to the yogurt, but rather the *Lactobacilli* that fermented the milk (92). A year later Henry Tissier isolated *Bifidobacterium* from an infant and claimed that

health benefits could be attributed to the bacterium (91). The three decades preceding the 1950's heralded an era of probiotic discovery and screening, in which numerous potential probiotic strains were isolated (91). By the early 2000's the total number of publications concerning probiotics was in excess of 200 a year, with an exponential increase observed over the subsequent decade, in conjunction with evidence-based clinical trials. (91). Since the identification of microorganisms as the primary source of health benefits associated with fermented foods, the probiotic field has been dominated by the two genera *Bifidobacterium* and *Lactobacillus*, with a few other microbes being identified as probiotics (e.g. *Escherichia coli* Nissle 1917 and *Saccharomyces boulardii* spp.) (91, 93, 94).

1.3.2. Selection of Probiotic Microorganisms

As previously stated, the human GIT is host to multiple species of microorganisms, however not all of these microorganisms are considered to be conducive to human health. Therefore, the selection of beneficial microorganisms is critical for the development of probiotics. Globally, no legislative definition exists for what constitutes a probiotic or what functions it must perform (87). A consensus was reached in 2001 with regards to a definition, whereby probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (95). Three major criteria are contained within this definition; namely the potential probiotic needs to be metabolically active within the GIT ("live"), the number of viable microorganisms needs to be of significant proportion to elicit a response ("adequate amounts") and needs to contribute to host's health ("health benefit").

The ability of potential probiotic microorganisms to contribute to human health is dependent on them being viable within the human GIT (96–98). Probiotic candidates are intrinsically required to survive the rigors of the human GIT, governed mainly by the chemical conditions that are prevalent. Alternating acidic and the alkaline conditions, coupled with bile acids, provide a significant hurdle for exogenously introduced microbes to overcome (96–98). Therefore, any microorganism that is considered a candidate in probiotic supplementation needs to be screened and individually evaluated for tolerance to acidic conditions and bile-induced stress.

Even if the microorganism is resistant to the adverse environmental conditions within the GIT, the number of viable cells may be low, and the concentration of probiotic microbes has to be adequate to exert health benefits (99). In general, the concentration of viable cells in the supplement correlates with the survival rate through the GIT. Controversy around the minimal dose as well as frequency of administration of probiotic supplements exists, and there is no established minimal dose to ensure health benefits (99). As a general rule, 10^7 to 10^9 CFU.g^{-1} is recommended for clinical relevance in humans (99). This general rule however fails to account for species and strain variability with respect to viability, as well as the proportion of cells retained in the GIT versus those washed out. Therefore, further research into the dose-dependent nature of probiotics, such as minimal dose required for health benefits and viability purposes, and the frequency of dosage for each probiotic supplement is required.

The health benefits associated with endogenous and/or exogenous microorganisms are vast, ranging from inhibition of enteric pathogens, to immunomodulation and overall maintenance of GIT homeostasis. The ability of microorganisms to modulate the immune system has become a recent focus area within probiotic research.

Probiotic microorganisms have the ability to influence the host's immune system by regulating and modulating the immune response (100–102). This modulation can occur on a mucosal and systemic level, through either interaction of the probiotic with other microorganisms contained within the microbiota (commensal and pathogenic) or cross-talk mediated communication between the probiotic and host cells (100, 101). The latter communication is mediated through Microbe-Associated Molecular Patterns (MAMPs), which are essential, conserved structural components of microbial cells, such as lipoteichoic acids, nucleic acids, peptidoglycan and other cell surface proteins (101, 103). These MAMPs are recognized and interact with receptors on antigen presenting host cell surfaces, known as Pattern Recognition Receptors (PRRs), which form part of the innate immune system (101, 104). The MAMPs-PRRs interactions initialize a signaling cascade within the host, either triggering a pro- or anti-inflammatory immune response (105). The exact immunological influences and modulations caused by microbes, and specifically probiotic microorganisms, are comprehensively covered in various reviews (105–108). Through these mechanisms, probiotic supplements have been shown to have a beneficial effect on allergies

(allergic rhinitis and eczema) and asthma, which are immune-mediated diseases (100, 109). It must however be noted that probiotic supplementation should not be used as a primary treatment or prevention of immune-mediated diseases (109).

Probiotic supplementation has been positively associated with the prevention and reduction of Antibiotic-Associated Diarrhea (AAD) (110). AAD occurs due to the ecological imbalances caused by the administration of antibiotic regimes, which render the entire GIT in a state of dysbiosis (111). AAD accounts for nearly a third of all cases of diarrhea and is broadly defined as unexplained incidences of diarrhea associated with the administration of antibiotics (112, 113). Furthermore, in excess of 20 % of AAD cases are found to be caused by the out-growth of *Clostridium difficile* within the GIT (111, 113). The recalcitrant nature of *C. difficile* infections (CDIs) are coupled with adverse health effects such as pseudomembranous colitis and sepsis (114). This makes the effective treatment of CDIs critical for both healthcare professionals and patients. Probiotic supplementation before and during antibiotic administration significantly reduces AAD and CDIs (113). Probiotics may achieve this through the amelioration of GIT dysbiosis, by blocking of attachment sites for pathogens and pathogen-derived metabolites, and inhibiting pathogens through antimicrobial production (111). It is notable that the mechanisms behind this reduction are varied and probiotic strain specific (111). Although not the perfect treatment for AAD, its strength lies in its capacity to be co-administered with the antibiotic regime, giving credence to the idiomatic phrase, "prevention is better than cure".

1.3.3. Probiotic Tolerance to GIT Conditions

As previously discussed, the conditions within the human GIT are generally unfavorable for microbial colonization. The first significant hurdle for any microbial cell, and therefore probiotic, is the need to overcome the stress induced by gastric fluid conditions (low pH, rapid transit etc.) (37, 39). LAB are considered to be aciduric in nature, although exposure to acidic conditions such as those prevalent within the human stomach have a significant effect on viability (115, 116). The low pH of gastric fluids induces stress responses in various lactobacilli strains (i.e. acid shock response) with concurrent decrease in survivability (116, 117).

Various probiotic studies have shown that although microbial cells withstand the low pH and rapid transit through the stomach, it comes at a significant cost in terms of survival. A rapid decline in probiotic viability is seen upon exposure to gastric fluid, with some studies reporting an adverse response within five minutes (116). The dramatic influence of the acidic gastric fluid is clearly illustrated through the observation made by van Bokhorst-van de Veen et al. where a decrease in gastric fluid pH by as little as 0.1 units significantly influenced survival (118). Pre-exposure of probiotic strains to acidic conditions during growth primes cells and enhances survival upon exposure to the gastric environment (118). Alterations to cell wall composition (decreasing proton permeability), down-regulation of genes involved in basic cellular processes and up-regulation of proteins (chaperones) are all mechanisms employed by lactobacilli strains, such as *L. casei, L. rhamnosus* and *L. reuteri* to overcome acid stress (118–120). The effect of food matrices and the encapsulation of probiotic supplements (gelatin capsules and fillers) should be taken into account, since the buffering capacity of these compounds also contributes to the survival of probiotics within the stomach (119).

Although the near-neutral pH within the small intestine provides environmental conditions more suited to colonization by probiotic microorganisms, the presence of bile and digestive enzymes may affect viability. Some studies involving *L. casei,* have shown that a loss in viability occurs only after 45 minutes of exposure to intestinal fluid (115). Furthermore, interspecies variation occurs among lactobacilli with respect to bile tolerance which alludes to some species being better equipped for survival within the small intestinal environment (32, 121, 122). The survival and persistence of *L. reuteri* strains within the GIT of mammals, including humans, has led this species of lactobacilli to be considered allochthonous (indigenous) to the human intestinal environment (32, 122). In contrast, *L. rhamnosus* is considered to be autochthonous, since it only seems to be a transient GIT resident originating instead from the oral cavity (32, 122). However, *L. rhamnosus* strains are known to rapidly transit through the upper GIT, hereby only allowing for the colonization of the colon (122).

The colonic environment is a finely-tuned ecosystem, colonized by a stable microbial population (122, 123). The abundant availability of undigested and complex carbohydrates, in addition to the presence of deep colonic crypts provide a microenvironment for bacterial attachment and

protection from the flowing luminal content (124). An increase in pH, in conjunction with lower bile concentrations and slow transit times further facilitate colonization (39).

In conclusion, the human GIT provides numerous environmental niches for the colonization and subsequent proliferation of probiotic microorganisms that are considered to be transient in the GIT. It is however to be noted that the survival of supplemented probiotics within the GIT is species and strain specific; it is thus essential to evaluate the survival and colonization characteristics of each potential probiotic within a GIT context.

1.3.4. The genus *Lactobacillus*

The order *Lactobacillales or* Lactic Acid Bacteria (LAB) comprises Gram-positive, catalase negative, non-sporulating, low G + C content bacteria (125, 126). LAB consist of 13 genera, namely *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Sporolactobacillus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (127). The most well studied of all LAB is the genus *Lactobacillus,* which is a group of facultative anaerobic, fermentative, rod-shaped bacteria, characterized by the ability to ferment sugars into lactic acid (127, 128). This specific genera exhibits immense species diversity, with in excess of 100 species that are known to occur in various ecological niches, including the human GIT, fermented foods and milk (126, 127, 129). Commonly occurring lactobacilli species in the GIT include *L. acidophilus, L. brevis, L. casei, L. fermentum, L. plantarum, L. reuteri, L. rhamnosus* and *L. salivarius* (122, 125, 130).

Lactobacilli can be classified according to their ability to ferment hexose sugars into various metabolic end products such as lactic acid, carbon dioxide, ethanol, acetic acid and various other minor products. Three fermentation classifications exist; namely obligatory homofermentative, facultative heterofermentative and obligatory heterofermentative (126, 129–131). Obligatory homofermentative lactobacilli ferment hexose sugars to lactic acid via the Embden-Meyerhof-Parnas metabolic pathway (126, 129). These LAB are unable to ferment pentoses or gluconate due to the lack of the enzyme phosphoketolase (126, 129). Homofermentative pathways yield 1.8 mol lactic acid for every 1 mol glucose (129). Facultative heterofermentative LAB utilize the same

Embden-Meyerhof-Parnas pathway as obligatory heterofermentative lactobacilli, however the presence of inducible aldolases and phosphoketolases allows for the effective fermentation of both pentose, hexose sugars and gluconate (126, 129). Lactobacilli exhibiting obligatory heterofermentative metabolism, ferment both hexose and pentose sugars utilizing a completely different metabolic pathway, namely the phosphogluconate pathway (126, 129). This allows obligatory heterofermentative lactobacilli to ferment 1 mol glucose or gluconate to lactic acid, carbon dioxide and acetic acid/ethanol in a 1:1:1 ratio (129).

Lactobacillus reuteri **and** *Lactobacillus rhamnosus* **as probiotics**

L. reuteri is obligatory heterofermentative and is predominantly found within the GIT of various animals and humans (122, 126, 132). It is considered to be one of the rare lactobacilli that are autochthonous inhabitants of the human GIT and other body sites (132). *L. reuteri* was first isolated in the 20th century, but was initially considered to be a *L. fermentum* sp. until the 1960's when Gerhard Reuter classified *L. reuteri* as a subspecies of *L. fermentum*, called *L. fermentum* Biotype II (133). This classification persisted until the 1980's, when Kandler et al. (134) found that the two organisms were in fact distinctive species and subsequently renamed *L. fermentum* biotype II as *L. reuteri* in honor of Gerhard Reuter.

L. reuteri DSM 17938 is the best studied of all the *L. reuteri* spp. It was initially isolated from Peruvian breast milk in the 1990's as *L. reuteri* ATCC 55730, but was later renamed to *L. reuteri* DSM 17938 after two antibiotic resistance plasmids were cured from the strain (135). This allowed *L. reuteri* DSM 17938 to become the first *L. reuteri* strain applied to human use and it subsequently become a popular probiotic supplement (135–137). The origin of this strain is not atypical as recent work conducted by Sinkiewicz and Ljunggren (138) found that 50 % of breast milk samples taken from women living in rural areas contained *L. reuteri,* whilst samples originating from urban environments had little or no detectable *L. reuteri*.

L. reuteri strains are often used in probiotic supplements, owing to the health benefits they confer on the host. The species is well adapted to the survival and persistence within the human GIT, with excellent tolerance to stress induced by the low pH and presence of bile (139–142). Persistence in

the GIT has been postulated to be the result of their ability to adhere to both intestinal epithelial cells and the mucus layer covering these cells (143–146). The antimicrobial capability of this species of lactobacilli is considered to be excellent owing to the production of reuterin, reutericyclin, lactic acid and hydrogen peroxide (147–150). The immunological effect of *L. reuteri* strains are well established, with various strains, including DSM 17938, exhibiting anti-inflammatory effects and aiding in the overall regulation of immune responses within the human body (136, 151, 152).

L. rhamnosus is a facultative heterofermentative species and is found in close association with the human body (153). While this species is considered to be allochthonous to the human GIT, it is still considered to be able to colonize this body system (154–157). The species name *L. rhamnosus* was only suggested by Collins *et al*. in 1989, since *L. rhamnosus* was considered to be a subspecies of *L. casei* (*L. casei* subsp. *rhamnosus*) up until that point (153). This lack of species delineation applied to even the most well-known probiotic, namely *L. rhamnosus* GG. *Lactobacillus rhamnosus* GG was isolated in 1983 by Sherwood Gorbach and Barry Goldin from healthy human gut lumen contents and submitted as *L. acidophilus* (ATCC 53103) prior to being reassigned to the species *L. rhamnosus* (154–156, 158–160).

Although it is considered to be a transient colonizer of the human GIT, colonic biopsies indicate the presence of *L. rhamnosus* cells even when at or below detectable levels in fecal matter (154, 156, 157). *L. rhamnosus* strains have been documented as having superior tolerance to acid induced stress conditions and bile salts (154, 155). This may allude to its potential to colonize and persist within the GIT, which is further enhanced by the strong adherence of *L. rhamnosus* strains to gut epithelial cells and mucus (156, 157). Probiotic supplements containing *L. rhamnosus* strains have been shown to exhibit immunomodulatory effects, in addition to preventing and alleviating antibiotic associated diarrhea and aiding the treatment of allergic reactions (154, 156).

Antimicrobial substances produced by *L. rhamnosus* strains include lactic acid and hydrogen peroxide (154). Although the production of bacteriocins has yet to be detected under standard laboratory conditions, it is to be noted that screening of the *L. rhamnosus* GG genome has revealed the presence of numerous bacteriocin-related genes (154).

It is well established that use of either *L. reuteri* or *L. rhamnosus* independently as probiotic supplements confers substantial health benefits to the host (32, 132, 154, 156, 161). The established efficacy and safety of these two lactobacilli species make them well suited for this purpose. The potential synergistic effect of dual supplementation of these two species of lactobacilli has also been documented, with specific emphasis on the use of this combination for the treatment and prevention of vulvovaginal candidiasis and urinary tract infections in humans (162–164).

1.4. Bacterial Biofilms

Free-living, or planktonic bacterial cells only represent a small fraction of a bacterial population, with the majority of cells forming close associations with surfaces to form adherent, sessile populations known as biofilms (165, 166). Biofilms are broadly defined as a community of microorganisms that assemble at an interface and encase themselves within a protective extracellular polymeric substance (EPS) matrix (167). Microbial biofilms are as old as microorganisms, although it was not until the 17th century when Anton Von Leeuwenhoek, first observed microbial aggregates (biofilms) whilst studying dental plaque (166, 168). The term biofilm was first used in the late 1970's by J.W. (Bill) Costerton, thereby formalizing and naming the propensity of all microorganisms to form biofilms on substrata under the appropriate environmental conditions (168). The difference between planktonic and sessile cells is exemplified by the greater tolerance of bacterial biofilms to deleterious environmental factors, i.e. low pH conditions (169, 170). This increased tolerance and tenacious survival ability is best observed and understood with respect to antibiotic resistance. Bacterial resistance to antibiotics is greater within biofilm-associated bacterial populations than within planktonic populations (169, 170). The administration of antibiotic concentrations lethal to planktonic bacterial populations has been shown to be sub-lethal to biofilm-associated bacterial cells and this is correlated to a 10 to 100-fold higher minimum biofilm inhibitory concentration than the corresponding planktonic concentration (170). This discrepancy in tolerance and survival has been partially attributed to the differential expression of genotypes and subsequent phenotypes between adherent and non-adherent bacterial populations (165, 166, 169, 171).

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The phenotypic differentiation between planktonic and sessile populations does not account for the existence of a second planktonic phenotype (172). The production and release of planktonic cells from sessile biofilms is hypothesized to represent a third phenotype (172). A number of authors have confirmed that a distinction between the batch culture-derived planktonic population and the biofilm-derived planktonic population exists, with the observation of differences with respect to adhesion, antimicrobial susceptibility and virulence (172–175). Therefore, it is of critical importance to take the origin of planktonic cells into account. This emphasizes the importance of studying microbial populations in a holistic manner, rather than in isolated populations, since factors such as survival, virulence and phenotypic changes may be determined by the origin of the population in question.

1.4.1. Human GIT-associated Biofilms

The most well characterized biofilms within a human context are those contained in the oral cavity (176, 177). These biofilms colonize tooth surfaces and the interfaces between the gums and teeth, in the form of dental plaque (178). A fine balance exists between these biofilms and the host; on the one hand it forms an integral part of the healthy host's defense mechanisms, and on the other hand it leads to dental caries and periodontitis (177). It is well established that oral biofilms are comprised of diverse and complex microbial communities, with a reported diversity in excess of 800 species (177, 178).

The luminal surface of the human GIT is lined with either a single layer (oral cavity and small intestine) or a dual-layer (stomach and colon) of protective mucus as discussed previously (165, 179). The mucosal layer also provides an adherent surface and creates a distinct microenvironment within the larger GIT (169, 180). This microenvironment is important for the formation of biofilms within mammalian GIT (181). Bacterial biofilm formation in the mammalian GIT has been studied extensively in animals, with only a few studies addressing the presence of biofilms within the human GIT (169, 181). Mucosal micro-colony formation has been observed in the GIT of various mammals, but the rapid turn-over of the mucus layers may hinder long-term persistence (169, 180). However, since the biofilm itself is in a perpetual cycle of detachment and regrowth, it overcomes

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the hindrance posed by mucus shedding (181). This constant state of biofilm shedding and regeneration may therefore account for the survival and persistence of bacteria within the human GIT. Recently, Bollinger et al. (181) observed epithelial-associated bacterial biofilms on the mucosa of the human appendix. This observation, coupled with the cyclic nature of biofilm detachment and regeneration, led to the postulation that the appendix may serve as a reservoir for commensal microorganisms in the human GIT (181). Due to the regular sloughing off of parts of the appendix-associated biofilms, the colon is reinoculated with commensal microbes thereby facilitating the persistence of these microbes within the human GIT.

The presence of micro-colonies or biofilms within the healthy human GIT has been an area of contention in recent years. This is mainly due to the limited number of new fundamental studies on the presence or absence of biofilms within the GIT, in contrast to a large number of reviews on the subject (76, 166, 169, 180, 182). A large number of reviews on the subject exist, but there is a lack of fundamental research on biofilm-associated microbes within the intestines of humans, leading to limited understanding of intestinal-associated biofilms (166, 180). As mentioned previously (Section 1.2.1), the esophagus and stomach are not considered to be microbial reservoirs in healthy humans, since the adverse conditions and rapid transit times through this GIT region may limit biofilm formation. Although a healthy esophagus may be sparsely colonized by aciduric, Gram positive bacteria, the presence of more extensive micro-colony and biofilm formation has been observed in diseased individuals, such as those suffering from Barret's esophagus (180, 183, 184).

Since the microbial composition of the mucosal and fecal microbiota are dissimilar, greater in-depth investigation of these two communities is critical (166, 180). The intestinal region of the GIT consists of complex ecosystems and is a site of intense metabolic activity (165). As previously mentioned, the human appendix may serve as a source of inoculum for the colon; this is supported by the progressive decrease in biofilm formation from the appendix to the distal end of the colon (181). Complex plant-based polymers are degraded in the colon through the synthrophic conversion by multispecies biofilms within this region (166). While it is generally accepted that biofilms are formed within the healthy intestines, it is thought that more extensive biofilm formation occurs in diseased individuals (185).

Although the presence of biofilms has been observed within the GIT of diseased individuals, the apparent lack of similar observations within healthy humans still remains an unresolved matter. (181). Limited progress has been made in the last decade and the overall lack of clarity with respect to the definitive absence or presence of biofilms within the human GIT has not allowed for better understanding. The significant impact that GIT-associated biofilms may have on the intestinal microbiome and host interactions is however recognized and agreed to be critical to our understanding of the human gut microbiome (166).

The ability of lactobacilli strains from probiotic supplements or fermented foods to form biofilms *in vitro* has only been assessed in the last decade, with a limited number of studies focused on this aspect (156, 186–190). The ability of *L. fermentum, L. plantarum, L. reuteri* and *L. rhamnosus* strains to form static biofilms on abiotic surfaces to varying degrees has been shown (156, 186, 187, 189, 190). Despite the fact that these studies have provided critical insight with respect to species and moreover, strain variation, among lactobacilli, a notable deficiency is the use of static, rather than dynamic flow conditions. The studies fail to take critical environmental conditions into account that may influence microbial persistence and propagation. Specific GIT conditions, such as flow-induced shear (GI fluids), abrasion-related detachment (digested food), gas and nutrient gradients cannot be taken into account using static cultivation techniques (191–194). Therefore, it is of critical importance that microbial biofilm studies use static biofilm cultivation as the screening tool for which it was intended, followed by continuous-flow biofilm studies to better mimic the natural microbial environment.

The essential and complex role of the GIT in human health has, in recent years, been highlighted. Probiotic supplementation, although prescribed in an attempt to improve GIT health, remains semi-understood, and there is a lack of clarity regarding probiotic interactions with commensal microbiota, pathogens and the human host, as well as the role of microbial biofilms in the GIT.

1.5. Simulating the Human GIT

The ability to simulate the human GIT is important for various industries, as well as pharmaceutical, food and academic research initiatives. The global pharmaceutical industry is worth an estimated 1 trillion US dollars as of 2014, with more than 7 000 pharmaceuticals in development globally (195, 196). However, less than 12 % of these pharmaceuticals will pass clinical trials and be approved by the regulatory bodies (196, 197). This, coupled with the estimated pharmaceutical development costs of 500 – 2 000 million dollars, makes it critical to ensure that adequate and extensive *in vitro* studies are done prior to the transition to *in vivo* studies, involving animals and humans (197).

The simulation of the anatomical and physiological parameters within the GIT is complicated through the requirement for integrated and coordinated synergism of the multiple tissue and cell types. The development of techniques that simulate the GIT can be divided into two distinct model systems, which are either static or dynamic in nature.

1.5.1. Static model

The static model for GIT simulation is the oldest, simplest and most widely used model of the GIT (198, 199). The static model fundamentally compartmentalizes the GIT into its three main regions; the stomach, small intestine and colon (198, 199). This is achieved through individual, closed systems each representing one of the three regions. Each system functions independently with respect to the others, or as part of a multi-phase system. While multi-phase systems do exist, these do not function in a fully integrated and coordinated manner.

The static model allows for the simulation of biochemical parameters within the GIT, at specific time points and phases of digestion. The closed nature of this model facilitates reproducibility with respect to standardized environmental conditions (198, 199). The simplicity of this model allows for rapid experimental set-up and runs, with experimental modifications easily achieved without long lead-times or complicated parameter and equipment changes (198, 199). All of these factors contribute to this model being cost-effective and easily implemented (198, 199).

However, this model is unable to adequately simulate the physiological changes and states that exist within the GIT (198, 199). This can be attributed to the lack of peristaltic movement and overall flow conditions (i.e. static condition) and the absence of a mucosal barrier, with its absorption and secretory functions (198, 199). This, together with the absence of standardized protocols and the requirement to adjust the various parameters to suit every objective, renders data obtained from this model difficult to compare between experiments and studies (198, 199).

Despite its drawbacks, this model is a critical tool for generating valuable preliminary data prior to progressing to more complex models, such as cell lines and complete artificial GIT systems or *in vivo* trials. However, the experimental parameters need to be closely aligned with experimental objectives and the physiological parameters under investigation. For example, in acid tolerance studies, special focus must be given to maintaining the correct pH parameters. To conclude, a thorough understanding of the advantages and limitations of this model is critical for the generation of reliable and reproducible data.

1.5.2. Dynamic Model Systems

The GIT is dynamic in nature, with few parameters remaining constant for long periods. Static model systems, though useful and indispensable as preliminary or screening tools, are not realistic and lack the flexibility required to simulate the dynamic nature of the GIT (200, 201). Dynamic model systems allow for the study of potential effects of GIT conditions on food digestion, pharmaceuticals and probiotic supplements (200–202). They incorporate the realistic physiological parameters of the GIT with the control and reproducibility obtained through computer controlled systems (201). Dynamic model systems consist of the following models; namely *in vitro* fermentation models, artificial GIT systems, human cell line models, and *in vivo* GIT studies involving animal models and human trials.

In vitro Fermentation Models

In vitro fermentation models are generally used as a substitute for the colonic region of the GIT (203, 204). These models rely on the use of single or multiplexed chemostats to simulate the fecal/colonic microbiota (203–205). These models have evolved from simple batch systems (static model system) to complex systems using continuous flow conditions and narrower parameter controls to facilitate the stable cultivation of the colonic microbiota (203, 206). *In vitro* fermentation models circumvent the ethical concerns that arise when conducting microbiota studies involving human or animal subjects (203).

This model system allows for the finer regulation of physiochemical parameters through the use of complex computational and fluid management systems (206). This finer regulation, coupled with the ability to multiplex various chemostats, allows for the simulation of either the average colonic environment or of the individual regions of the colon (ascending, transverse and proximal colon) within a multiplexed system (203). Planktonic and sessile microbial populations are reproducible within these systems. Sessile populations are established through the cultivation of microbiota on porous polysaccharide matrixes prior to inoculation of the chemostat (203, 207).

Although *in vitro* fermentation models are capable of simulating both the planktonic and sessile microbial populations within the GIT, the model is limited as it cannot account for transitional and compositional changes that occur within the GIT (203, 208). These models furthermore only assess microbial functions and not important host functionalities, such as immunological and cellular parameters, that are characteristic of the GIT (203, 209). As is the case with the static model systems, careful consideration of the experimental objectives is required to ensure that the optimal model is used (i.e. single versus multiplex systems), to represent singular or multiple regions of the GIT (203, 209).

The functionality of this model extends further than mimicking only the biochemical parameters of the GIT, but also simulates the microbial population within the GIT (203, 208, 209). The collective attributes of this model system allow for the stable and reproducible cultivation of a microbial population *ex vivo*, thereby facilitating a greater understanding of the interactions

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between microbial populations, ingested food and pharmaceuticals. Improved insight into these interactions may promote a smoother transition from *in vitro* to *in vivo* studies and are generally accepted on both scientific and ethical grounds.

Artificial GIT Models

To adequately simulate the GIT, a single model is required that will incorporate the movement, mixing, absorption and secretory parameters present within the GIT. However, until the early 1990's this objective was only partially fulfilled by other models, such as those simulating simple gastric functions (210). The advent of an artificial GIT model by Minekus et al. allowed for the most realistic simulation of the monogastric human GIT to date (211). The system allowed for GIT-relevant physiological parameters such as pH, bile concentration and enzymatic actions to be matched *ex vivo* with a high degree of accuracy and reproducibility (201, 212). These artificial systems are based on computer-controlled simulation using *in vivo* data to direct a multi-compartmental system consisting of a series of peristaltic valve pumps, membranes, filters, pH and temperature sensors (201, 211).

The ability to simulate the luminal enzymatic, absorption and secretion parameters within these models allows for better *in vitro-in vivo* correlation and predictions (201, 212). It promotes a better understanding of food, pharmaceutical and nutraceutical behavior and efficacy within the human GIT, prior to the transition to *in vivo* trials (211), and is, therefore, cost effective and minimizes ethical considerations.

Despite achieving good reproducibility and *in vitro-in vivo* correlations, factors such as host functions are only partially accounted for through the removal and secretion of metabolites and enzymes, whereas mucosal parameters are not assessed due to their complexity (201, 213, 214). Other host functions, such as induced inflammatory responses (immunological) or cellular interactions (cross-talk between cells) are not assessed, leaving a deficiency in the complete simulation of digestion. The complexity of these models means that specialized training and equipment is required for successful use of these systems (201, 211, 213).

Artificial GIT models provide scientists and industries with a powerful tool for understanding the interaction between exogenous substances and the GIT, and provide the most accurate, though incomplete, systems for simulating the human GIT *in vitro*.

Human Cell Line Models

The use of human cell lines as a proxy for human body systems has allowed a greater depth of understanding with respect to human health and disease states. In the 65 years since the first human cell line was isolated and used (HeLa cells in 1953), their availability has increased to close to 4,000 cell lines, maintained by the American Type Culture Collection repository (215, 216). Human cell lines circumvent many ethical concerns associated with human and animal trials, in addition to mitigating inter-individual variations, which provide a distinct variable in human and animal studies (217). Human cell line models are generally characterized by a monolayer of viable, polarized and fully differentiated cell types and can be co-cultured with other monolayer cell lines to produce more complex systems, such as combining two intestinal epithelial cell lines with different functions (i.e. Caco-2 and HT29-MTX cells) (218, 219).

Due to the clonal nature of cell lines, these models allow for consistency and reproducibility between experiments (203, 220). These models successfully simulate mucosal surfaces and have allowed researchers to elucidate the mechanistic, immunological and physiological effects of food, pharmaceutical, nutraceutical and toxic compounds (220–223). The use of living cells not only provides greater insight into the potential cytotoxicity (concentration required to be cytotoxic) of compounds, but also allows the effect of these compounds to be assessed in real time as it would occur within the human body (223).

The vast number and variation in cell lines leads to a large number of models, which necessitates the careful selection of specific models and cell lines in accordance with experimental objectives (203). A reductionist approach is practiced within cell culturing research, whereby complex body systems or tissues are reduced to mono- or bi-layers of cells (220, 224). The physiological complexity observed *in vivo* is lacking, and although advances have been made with respect to complex *in vitro* models, *in vivo* models cannot be replaced completely by current cell line models

(203). The process of eukaryotic cell culturing furthermore strongly relies on the maintenance of aseptic conditions, since cell lines are prone to microbial contamination (217, 219, 224).

Human cell line models provide valuable insight into the immunological and cellular responses that human tissues might experience when subjected to certain conditions or compounds. The requirement for specialized equipment and strict aseptic techniques limit the feasibility of some studies. The limited complexity of current models may also restrict the types of studies conducted or may not provide complete insight into effects on tissues consisting of multiple cell types.

In vivo GIT model systems

In vivo model systems provide the most realistic simulation of the human GIT, with all its complexity and functions. Since the anatomical and physiological structures and processes are functionally incorporated in these systems, these models are capable of achieving that which other models cannot, namely the simulation of all host functions. As a result, these models are powerful tools to elucidate effects and functions within the GIT.

In vivo Animal Models

Animal models, although not identical to the human body, provide a significantly better analog to the human body than the model systems previously discussed. The processes of digestion and absorption within specific, monogastric animal models are similar to those in the human GIT (225, 226). The presence of both luminal and mucosal surfaces allows for a more holistic scenario, rather than the simulation of either one or the other surface as seen in other models (227).

Animal models have often been used in place of human subjects, since there are fewer ethical considerations that need to be taken into account (227). The sample size and by default the statistical power is dramatically increased when using animal models compared with human subjects (227). This, in conjunction with decreased inter-individual variation within animal models, provides a better representative sample of effects or response within a broader population (227). Both the adaptive and innate immunological responses are better studied using animal

models than human cell lines (225, 227). Owing to the moderate to high degree of similarity between the human GIT and that of other mammals (pigs, rodents and non-human primates), animal models allow reasonable predictions to be made regarding the interaction between compounds and the human GIT (226, 227).

Although animal models provide great insight into GIT functionality (absorption and secretion) and responses (immune) due to exogenous compounds, limitations do exist. Despite the anatomical and physiological homology that exists between human and other mammalian GIT systems, there is also a large degree of variation. These include variations in food transit times, digestive enzyme composition and concentrations, as well as metabolism, and therefore animal trials can never fully replace human trials (225–227). Since each model has its own advantages and limitations, careful matching of the model with the experimental objectives prior to starting the trial, in addition to scrutinizing the experimental outcome, is required (226, 227). The other limitations that these models suffer from is the high cost required to establish and maintain specialized handling facilities for the animals (226, 227), and the ethical concerns involving animal experimentation.

Even though no single animal model is a perfectly complete analog for human subjects, these models do provide a "perfectly-imperfect" system for the vital screening and evaluation of objectives prior to transitioning into human trials, which may ensure better performance in and successful transitioning through human trial phases.

Human Trials

The culmination of all medical research is the transition from proxy models into human studies. Human trials provide the final and often most critical data pertaining to scientific research (228). The first semi-formalized human trial, was conducted in 1747 by Dr James Lind pertaining to scurvy (228, 229). Human trials have evolved significantly in the last 250 years, with most of the progress occurring in the last 100 years. The first randomized controlled human trial involving streptomycin was only conducted half-way through the 20th century (1947), and since that time human trials have become a completely standardized procedure (229). The use of human subjects

has provided the medical and scientific communities with great insights into the effects of food, pharmaceuticals and microorganisms on the human body and GIT specifically.

The use of human subjects is inevitably the only way of determining effects, influences and interactions within the human body. The generation of "real-world" safety and efficiency data is critical for the generation of knowledge and development of new compounds (228). The ability of human trials to examine any potential adverse effects that the introduction of an exogenous substance or organism may have, make them a powerful and indispensable tool (228).

The complexity of the human body and inter-individual variability has a dramatic effect on the outcomes of human trials, which is further complicated by the selection criteria of the study (228). The selection criteria are often stringent and thus select for a sub-population of individuals which fit these criteria (228). This complicates the extrapolation of trial outcomes to the broader population. Human trials are lengthy and the time lapse from the start of a clinical trial to approval by regulatory bodies can span years. The ethical considerations supporting these types of studies further restrict the types of objectives that can be examined (230), and as a result these trials are limited to cause-and-effect studies of dietary components, lifestyle preferences and pharmaceutical interventions (230, 231).

Human trials have over the course of the last century fundamentally shaped our outlook and understanding of the human body and interactions within it. Invaluable information has been garnered with respect to our understanding of human metabolism, immunological functionality, pathogenesis and human-microbial interactions (228, 230, 231). The use of randomized, controlled studies has become the gold standard in understanding the causality between dietary, lifestyle, medical and pharmaceutical interventions in a human context (228, 232).

1.5.3. Simulated Gastrointestinal Fluids

The complexity and ethical considerations behind the use of *in vivo* models, makes the use of *in vitro* GIT models more common and easier to implement. However, the use of non-*in vivo* models requires special focus on the fluids used to simulate gastrointestinal (GI) fluids (40). GI fluids can be separated into four major, compositionally distinct fluids; saliva, gastric fluid, small intestinal fluid and colonic fluid. In order for correlations to be made between *in vitro* and *in vivo* studies, the fluids used to simulate GI conditions need to be biologically relevant, such as those available from Biorelevant Media, which was founded on research into the development of biologically appropriate simulated GI fluids. The standardization and commercial availability of these simulated gastric fluids have allowed for greater experimental reproducibility and better physiological correlation.

Biorelevant media are a varied group of fluids that seek to realistically simulate GI conditions, based on ever-evolving physiological data (233, 234). These media are used during early stages of drug discovery and development, to provide better understanding of effects GI fluids may have on these compounds and allow for better *in vitro-in vivo* correlations, which may reduce the number of animal of human subjects required during *in vivo* trials (233).

The human GI system is made more complex through the presence of two distinct physiological states; namely fed- and fasting-state (40, 233, 234). At any given time, the stomach and small intestine are considered to be in one of these two states, whilst the oral cavity and colonic regions have temporal fluctuations, lacking distinct fed- and fasting-states (40, 233).

The biorelevant media differ with respect to three main factors; pH, osmolarity (electrolyte concentration) and enzymatic composition. The composition of the four biorelevant media will be discussed in brief, in view of the use of simulated fasting-state GI fluids in Chapter 3.

Simulated Saliva

Human saliva is difficult to simulate owing to its complex composition and exogenous effects of diet, fluid intake and so forth (40, 235). The pH of saliva is critical for the functioning of the lingual lipases and salivary amylases, and is therefore maintained in the range of 5.3 to 7.8 by bicarbonate ions (40, 235). No standardized or recommended composition for simulated saliva exists, and various formulations with distinct buffer composition are available (40, 236).

Simulated Gastric Fluid

The ability to simulate gastric fluid is critical to the pharmaceutical industry and medical sciences. Simple aqueous buffers lack the ability to appropriately represent the physiological parameters of gastric fluid (77). Various attempts have been made to formulate and standardize the composition of both fed- and fasting-state gastric fluids, many of which have used synthetic surfactants and components, in addition to lower concentrations of components than that present at normal physiological states (233, 237, 238). The emphasis of functional biorelevance over mere compositional biorelevance (239) led to the development of a biorelevant gastric medium, a term coined by Vertzoni et al. in 2005, which realistically represented both fed- and fasting-states (238). The physiological accuracy of this biorelevant medium is evident by the lack of major differences between gastric aspirate and the simulated gastric fluid (77).

Fasting-State Gastric Fluid

The composition of fasting-state simulated gastric fluids (FaSSGFs) generally involves a sodium buffer combined with surfactants, with a pH ranging from 1.2 to 2.0 (40, 233, 238). The first FaSSGFs made use of synthetic or non-physiological surfactants such as triton-X or sodium lauryl sulfate in order to reduce surface tension (233, 240). However, significant inconsistencies in composition occurred due to instability as a result of the hydrolysis of sodium lauryl sulfate at pH values lower than 4 (233). Obtaining the correct physiological surface tension with the use of natural/biorelevant surfactants was achieved by Vertzoni et al. (238). Replacing triton-X and

sodium lauryl sulfate with physiologically concentrations of bile and lecithin allowed for good correlation between FaSSGF and fasting-state fluids obtained during human studies (77, 233, 238).

Fed-State Gastric Fluid

Simulating a fed-state gastric fluid is not as easily achieved, since the dynamic and heterogeneous compositional changes in the fed stomach over time need to be considered (40, 233). Homogenized, long-life milk has been used in dissolution media, due to a similar carbohydrate:fat:protein ratio to the stomach contents of fed, healthy individuals (40, 233). The changing intragastric composition that occurs during fed-state can be simulated by using "snapshot" media which simulate early, middle and late fed-state phases (40, 233). Each phase differs with respect to buffer composition, buffer to milk ratio and pH. The middle phase is considered to be representative of the entire process and has led to the development of a representative fed-state simulated gastric fluid (FeSSGF) (40, 238).

Simulated Small Intestinal Fluid

Small intestinal fluid is mainly comprised of water, pancreatic enzymes, bile and mucus (67). For many years simple Simulated Intestinal Fluid (SIF) was used, consisting of a phosphate buffer and pancreatin at a pH of 6.8 (241). However, as in the case of the first simulated gastric fluids, this simple buffered solution did not accurately represent the physiological conditions due to a lack of natural surfactants (233, 240). The subsequent addition of bile salts and phospholipids achieved a biologically relevant surface tension (240). Similar to the stomach, the small intestine has a fed- and fasting-state, which both need to be simulated in order to fully represent conditions within this environment.

Simulated Fasting-State Small Intestinal Fluid

The original Fasting-State Simulated Intestinal Fluid (FaSSIF) developed by Dressman et al. (240) in the late 1990's was based on a phosphate buffer, similar to the original SIF (240, 241). The addition of sodium taurocholate and lecithin to the buffer took into consideration the solubilizing ability of small intestinal fluids (233). The pH of FaSSIF is slightly lower than that of SIF (pH 6.5 versus 6.8). The composition of FaSSIF was recently modified based on additional physiological data obtained from human trials (40, 77, 233). These minor changes included the reduction of lecithin concentration (0.75 mM to 0.2 mM), changes in buffer species from phosphate to maleate buffer, and changes in osmolarity (77). The modifications to FaSSIF to produce FaSSIF-V2 has further standardized and contributed to a reproducible and physiologically relevant media (77, 233, 240).

Simulated Fed-State Small Intestinal Fluid

The development of Fed-State Simulated Intestinal Fluid (FeSSIF) occurred concurrently with that of FaSSIF, with the major difference being the higher concentration of sodium taurocholate and phospholipids (4:1 ratio maintained), with an accompanying decrease in pH to 5.0 from 6.5 (233, 240). The buffer capacity and osmolarity of FeSSIF were also adjusted to physiologically relevant conditions. As with FaSSIF, Jantratid et al. (77) revisited the composition and physiological relevance of FeSSIF, since it was determined that the pH in the duodenum and jejunum decreased slowly. This lead to the development of a "snapshot" media as with FeSSGF, which took into account the gradual changes in pH, bile and lipid composition within the small intestine (77). Glyceryl monooleate and sodium oleate (lipolysis products) were incorporated into the updated media. FeSSIF-V2 is representative of postprandial conditions within the small intestine (233). The development and use of a single "snapshot" medium for the simulation of fed-state in the small intestine, has eliminated the need for three separate formulations and allowed for better standardization.

Simulated Colonic Fluid

Colonic fluid is mainly comprised of water, electrolytes and short-chain fatty acids (67). Two basic media formulations were developed to simulate colonic fluid (SCoF1 and SCoF2); the first made use of a phosphate buffer adjusted to pH 7, while the latter consisted of an acetate buffer with a significantly lower pH of 5.8 (40, 233). SCoF1 and SCoF2 simulate the colonic environment as a complete system and do not discriminate between the three regions of the colon or between fed- and fasting-states. The development of fed- and fasting-state colonic fluids was only achieved in 2010 (40, 242).

Fasting-State Colonic Fluid

Vertzoni et al. (242) developed a physiologically relevant fasting-state colonic fluid that simulates the fluid composition of the ascending colon (242). Fasting-State Simulated Colonic Fluid (FaSSCoF) replaced the acetate and phosphate buffers with a Tris/maleate buffer with a final pH of approximately 7.8 (40, 242). The major difference between FaSSCoF and both SCoF1 and SCoF2 is the presence of bile salts, phospholipids and short-chain fatty acids in FaSSCoF (40, 242).

Fed-State Colonic Fluid

While the Fed-State Simulated Colonic Fluid (FeSSCoF) utilizes the same buffer as FaSSCoF, it has a lower osmolarity as well as a significantly lower pH (6.0 versus 7.8) (40, 242). In addition, it contains a four-fold higher bile salt concentration in conjunction with an approximate doubling in phospholipid and short-chain fatty acid concentrations (40, 242).

The relatively recent introduction of simulated fed- and fasting-state colonic fluids has brought *in vitro* experimental conditions slightly closer to conditions present in the human colon. However, the presence of a large microbial population within the colon may potentially alter the colonic fluid composition through metabolite production, thus additional developments are required to improve simulated colonic fluids.

The need for biologically relevant GI media as a surrogate for GI fluids obtained from human subjects is critical for the pharmaceutical and medical research industries. The ethical and cost-effective advantages of *in vitro* testing using appropriate simulated gastric fluids incentivizes the continued development and refinement of these systems and fluids. Each improvement with respect to better simulation of the *in vivo* physiological conditions allows for better prediction of *in vivo* performance of pharmaceuticals and nutraceuticals.

Researchers and commercial probiotic manufacturers assume that their strains and formulations persist within the human GIT, however as previous stated probiotic supplementation is thought to be only a transient process. However, since research conducted on probiotic supplementations only examine planktonic populations and ignore the sessile populations. Therefore, the need to experimental assess survival of sessile populations of probiotics under the prevailing conditions within the human GIT are required to complete the scientific knowledge behind probiotic supplementation. To circumvent the ethical considerations required in obtaining human gastrointestinal fluids, simulated fasting-state gastrointestinal fluids need to be utilized to realistically mimic the harsh environmental conditions that are prevalent in the human GIT (pH changes, bile and enzymatic damage). The use biologically relevant fluids could facilitate the investigation of the *in vitro* survival response of both planktonic and sessile populations of bacteria following exposure to GIT-relevant conditions.

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CHAPTER 2

CHAPTER 2: Lactobacillus rhamnosus HFI-K2 and Lactobacillus reuteri HFI-LD5, isolated from human feces, exhibit promising adhesion characteristics

2.1. Abstract

Lactobacillus reuteri HFI-LD5 and *Lactobacillus rhamnosus* HFI-K2, isolated from human feces, formed biofilms on hydrophobic abiotic surfaces. The two strains displayed different cell surface properties, with *L. rhamnosus* HFI-K2 having a significantly higher electron donating or basic surface, in addition to a higher degree of cell surface hydrophobicity. Neither of the two strains possesing *in vitro* mucinolytic activity. Furthermore, no atypical antibiotic resistance was observed for either strain. The survival of both isolates following exposure to acidic conditions (pH 2.5) and bile salt stress (0.5% and 1.0% w/v), the ability to adhere, may contribute to persistence of these strains within the human GIT. The significantly higher surface hydrophobicity of *L. rhamnosus* HFI-K2, compared to *L. reuteri* HFI-LD5 (37.71 % vs 8.82 %, $p < 0.05$), more extensive *in vitro* biofilm formation by *L. rhamnosus* HFI-K2, is especially promising with regards to potential application as a probiotic.

2.2. Introduction

The gastrointestinal tract (GIT) is one of the most heavily colonized organs within the human body, with an estimated 70 % of microorganisms residing in the colon (1). The Human Gut Microbiota (HGM) plays a significant role in human development, nutrition and physiology (1) and is often referred to as a "virtual organ within an organ" (2). Continuous use of antibiotics, long-term drug treatments, chemotherapy, abnormal stress and sudden changes in hormone levels are a few of the causes leading to an imbalanced HGM and, ultimately, a dysfunctional GIT (2).

The use of probiotics to ameliorate gut dysfunction and maintain gut homeostasis is the corner stone of the probiotic field. Probiotic bacteria may regulate gut dysfunction through the production of metabolic compounds such as antimicrobial peptides, organic acids and hydrogen peroxide (3). This may reduce enteric pathogen levels and alleviate antibiotic-associated diarrhea. Recent developments have, however, revealed that probiotics may also exert a larger systemic effect on the host by regulating immunomodulation, nutrition, metabolism and providing an overall maintenance of gut homeostasis.

A probiotic is defined as an organism that is resistant to bile salts, susceptible to antibiotics, lacks pathogenicity, is able to adhere to cell surfaces and modulate the immune system, while eliciting positive health benefits (4). The selection of probiotic microorganisms is based on fulfilling these criteria. Probiotic supplements are dominated by species from the two genera *Lactobacillus* and *Bifidobacterium*, with both genera having been awarded GRAS status (5, 6). Lactobacilli are estimated to comprise 7 % of the total HGM (7) and members of this genus are considered to be both autochthonous (*L. gasseri, L. mucosae, L. salivarius* and *L. reuteri*) (8, 9) and allochthonous (*L. acidophilus, L. fermentum, L. plantarum* and *L. rhamnosus*) (9) to the GIT.

Few lactobacilli spp. are considered to be autochthonous to the human body and specifically the human GIT (10), except for *L. reuteri* spp. which are not only able to colonize the human GIT but also persist within this harsh environment (10, 11). This species of lactobacilli has been shown to adhere to both mucin and epithelial cells, whilst also being able to form biofilms within the GIT of some animals (11–13).

Although *L. rhamnosus* spp. are considered to be transient colonizers of the GIT, numerous studies focused on *L. rhamnosus* GG have provided evidence for its successful survival and persistence within the human GIT (14–18). *L. rhamnosus* GG has shown excellent *in vitro* and *in vivo* adherence to epithelial cells and mucus, thus providing an explanation for its persistence for weeks after cessation of supplementation (19). This however fails to take into account potential biofilm formation, which may better explain persistence within the human GIT (20).

Therefore the potential for successful GIT survival and colonization should be evaluated on a case-by-case basis, rather than basing it on species classification. This study therefore aimed to compare two novel *Lactobacillus* spp. isolated from healthy human GIT and classified as *L. reuteri* and *L. rhamnosus,* with respect to classical/ideal probiotic criteria as well as biofilm formation abilities.

2.3. Materials and Methods

2.3.1. Isolation of bacteria

Fecal samples of healthy individuals were homogenized in sterile maximum recovery diluent (MRD, Oxoid Ltd., Basingstoke, Hampshire, UK) and serially diluted. Dilutions were plated onto MRS Agar (Biolab, Biolab Diagnostics, Midrand, SA), supplemented with 1.0 % (w/v) bile salts (Oxoid), 0.25 % (w/v) $CaCO_3$ and 0.1 % (w/v) cycloheximide (Sigma-Aldrich, St. Louis, MO, USA). Acid production, observed as colonies with clear halos, were streaked to purity on MRS Agar (Biolab). Plates were incubated at 37°C for 48 h. Two reference strains, *Lactobacillus reuteri* DSM 17938 and *Lactobacillus rhamnosus* R11, were isolated from commercially available probiotic supplements and cultured at 37°C on MRS Agar (Biolab).

2.3.2. Growth at low pH

Acid-producing isolates were streaked onto MRS agar, prepared by adding 1.0 % (w/v) Phytagel (Sigma-Aldrich) to MRS Broth (Biolab) that had been adjusted to pH 2.5 and 3.0, respectively, with 1 M HCl. The medium was sterilized by autoclaving. Plates were incubated at 37°C for 48 h.

2.3.3. Hemolytic- and mucinolytic activity

Colonies selected from MRS Phytagel plates were streaked onto blood agar plates (National Health Laboratory, Cape Town, South Africa) and incubated at 37°C for 48 h. Isolates with no hemolytic activity (no clear zones surrounding the colonies) were selected and streaked onto Plantarum Minimal Medium 5 (21), supplemented with either 1.0 % (w/v) glucose or 0.5 % (w/v) mucin. Both media were solidified by adding 1.8 % (w/v) agarose. Mucin from porcine stomach (type II, Sigma-Aldrich) was purified according to the method of Zhou *et al*. (22) and lyophilized. All plates were incubated at 37°C for 72 to 96 h and then flooded with 0.1 % (w/v) amido black (Sigma-Aldrich), dissolved in 3.5 M acetic acid. After 30 min, the plates were destained with 1.2 M acetic acid. The hydrolysis of mucin was observed as clear zones surrounding the colonies.

2.3.4. Identification of isolates

Selected isolates were Gram-stained and tested for catalase activity by covering 24 h-old colonies on MRS Agar (Biolab) plates with 5 % (v/v) H_2O_2. Genomic DNA was extracted from each of the isolates by using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research Corporation, Irvine, CA, U.S.A.), according to the manufacturer's instructions. The *16S rRNA* gene was amplified using primers 8F: (5'-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and 1512R: (5'-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3') and Q5 High-fidelity DNA polymerase (Inqaba Biotechnical Industries, Pretoria, South Africa). The following thermal cycling conditions were used: Initial denaturing at 98°C for 1 min, followed by 30 cycles of denaturing (98°C for 10 s), annealing (58°C for 30 s) and elongation (72°C for 1 min), with a final elongation step at 72°C for 2 min. The recombinase A gene *(recA)* was amplified using primers AmpF:(5'-GCC CTA AAA AAR ATY GAA AAG AAH TTY GGT AAA GG-3') and AmpR; (5'-AAT GGT GGC GCY ACY TTG TTT TTH ACA ACT TT-3') and Q5 High-fidelity DNA polymerase (Inqaba Biotechnical Industries). Initial denaturing was at 98°C for 5 min, followed by 35 cycles of denaturing (98°C for 1 min), annealing (55°C for 30 s), elongation (72°C for 1 min) and a final elongation step at 72°C for 2 min. Amplicons were analyzed by electrophoresis on an agarose gel (0.8 % w/v) stained with ethidium bromide. DNA fragments corresponding to the size of *16S rRNA* and *recA* were recovered from the gel and purified using the QIAquick PCR purification kit (Qiagen). DNA fragments were cloned using the CloneJET PCR cloning kit® (Inqaba Biotechnical Industries) and sequenced. Sequences were checked using Vector NTI Advanced 11.0 (Invitrogen, Carlsbad, CA) and compared with known sequences in GenBank (National Center for Biotechnology Information). Sequence alignment was done using the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov.ez.sun.ac.za/Blast.cgi).

2.3.5. Antibiotic susceptibility

Antibiotic susceptibility was assessed using the disk diffusion method, according to the British Society for Antimicrobial Chemotherapy (23). Paper disks, impregnated with antibiotics, were placed onto Mueller Hinton agar (Oxoid) plates, each seeded with 500 µl (10^8 CFU.ml^{-1}) of the test strain. *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 2592 served as

controls. The antibiotics tested were Trimethoprim (1.25 µg), Sulfamethoxazole (100 µg), Sulfamethoxazole-Trimethoprim (25 µg), Sulphonamides compound (300 µg), Oxacillin (1 µg), Methicillin (25 µg), Dicloxacillin (25 µg), Gentamicin (10 µg), Tetracycline (30 µg), Kanamycin (30 µg), Nitrofurantoin (300 µg), Ampicillin (10 µg), Meropenem (10 µg), Penicillin (10 µg), Rifampicin (5 µg), Tobramycin (10 µg), Chloramphenicol (30 µg) and Clindamycin (2 µg). Plates were incubated for 24 to 48 h at 37°C and the diameter of inhibition zones measured. Susceptibility was recorded based on guidelines of the Clinical and Laboratory Standards Institute (24).

2.3.6. Microbial adhesion to solvents (MATS) and auto-aggregation properties

Non-hemolytic isolates that were resistant to 1.0% (w/v) bile, able to grow at pH 2.5 and unable to hydrolyze mucin, were selected for further studies. Cell surface properties of the strains were studied by determining their Lewis acid-base properties, according to the microbial adhesion to solvents (MATS) test used by Bellon-Fontaine *et al*. (25). In short, isolates were cultured in MRS Broth (Biolab) for 18 h at 37°C, harvested (2000 x *g*, 10 min, 4°C), washed twice with sterile phosphate buffered saline (PBS, pH 7.2) and resuspended in sterile PBS to an optical density (OD$_{600nm}$) of 0.70 ± 0.05 (A_0 at *t*=0). Suspended cells (4.25 ml) were added to 750 µl chloroform (acidic monopolar solvent), and in a separate experiment to hexadecane (non-polar solvent). The experiments were repeated, but with ethyl acetate (basic monopolar solvent) and decane (non-polar solvent). All chemicals were of the highest purity and from Sigma-Aldrich. Cell suspensions were vortexed for 90 s and then stored at 37°C for 15 min to allow separation of the two phases. From each of the cell suspensions, 1 ml of the aqueous phase was carefully removed and optical density determined at OD$_{600nm}$. The percentage adherence (affinity of cells for non-polar and monopolar solvents) was calculated using the following equation:

$$\text{Percentage adherence} = \left[1 - \left(\frac{A_F}{A_0} \right) X \ 100 \right]$$

Where A_0 represents the initial absorbance ($t = 0$) and A_F the absorbance after 15 min.

Auto-aggregation properties of the isolates were determined according to the method described by Kos *et al*. (26), with a few modifications. The isolates were each cultured in MRS Broth (Biolab)

at 37°C for 18 h, harvested (2 000 x *g*, 10 min, 4°C), washed twice with sterile PBS (pH 7.2) and resuspended in sterile PBS to an OD_{600nm} of 0.50 ± 0.05 (A_0 at *t*=0). Fifteen milliliters of the cell suspension were transferred to sterile test tubes and incubated at 37°C for a further 20, 60, 180, 360 and 720 min. At each of these time points, 1 ml of the upper part of the cell suspension was transferred to a disposable cuvette and the OD_{600nm} reading recorded (A_t at each time point). The percentage auto-aggregation was determined as follows:

$$\text{Percentage auto} - \text{aggregation} = \left[1 - \left(\frac{A_t}{A_0}\right)\right] X \ 100$$

Where A_0 represents the initial absorbance ($t = 0$) and A_t the absorbance readings recorded at each of the time points.

2.3.7. Screening for bacteriocin production

Strains that adhered to polar solvents (chloroform or ethyl acetate) and with auto-aggregation properties were inoculated into MRS Broth (Biolab) and incubated at 37°C for 24 h. Cells were then harvested (15 000 x g, 10 min, 4°C), the cell-free supernatant of each strain adjusted to pH 6.5-7.5 with 1 M NaOH and heated at 80°C for 10 min to kill viable cells. The agar well-diffusion method of Tagg and McGiven (27) was used to test for bacteriocin activity. Twenty milliliters of MRS Agar (Biolab) was inoculated with 200 µl (10^8 CFU.ml^{-1}) *Lactobacillus sakei* LMG 13558 (received from Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium). Another plate, prepared with 20 ml of Listeria Enrichment Agar (Oxoid), was inoculated with 200 µl (10^8 CFU.ml^{-1}) *Listeria monocytogenes* EGD-e (received from Colin Hill, University College Cork, Cork, Ireland). The agar concentration in both plates was 0.8 %, w/v). Wells prepared in the agar were filled with 50 µl of the heat-inactivated cell-free supernatant. Plates seeded with *L. sakei* were incubated at 30°C and those seeded with *L. monocytogenes* at 37°C for 24-48 h.

2.3.8. *In vitro* biofilm assay

Biofilm formation was assayed according to Lebeer *et al*. (19), with minor modifications. Strains were inoculated into the following growth media, undiluted and 1:10 diluted: MRS Broth (Biolab), modified MRS (mMRS) with glucose replaced by 20 g.l^{-1} gluconate, and modified Tryptone Soy Broth (mTSB) consisting of 15 g.l^{-1} TSB (Biolab) enriched with 20 g.l^{-1} peptone from meat (Sigma-Aldrich). All growth media were adjusted to pH 6.5 with HCl before autoclaving. Incubation was at 37 °C for 18 h. Cells were harvested (2 000 x g, 4 °C, 10 min) and resuspended to 10^8 CFU.ml^{-1} in the respective growth media (undiluted and diluted). Two-hundred microliters of each cell suspension was transferred to wells in a 96-well U-shaped polystyrene microtiter plate (Greiner Bio-One GmbH, Kremsmünster, Austria). Cells of *L. reuteri* DSM 17938 and *L. rhamnosus* R-11, prepared in the same way, served as controls. All plates were incubated at 37 °C for 72 h, without shaking. Wells were washed three times with sterile PBS to remove loosely adhered cells. Adhered cells were stained for 30 min at 25˚C with 200 µl 0.1 % (w/v) crystal violet, dissolved in isopropanol:methanol:PBS (1:1:18 v/v). Excess staining solution was aspirated and the wells rinsed three times with distilled water. The rinsed plates were air-dried for 30 min and the adherent dye solubilized through the addition of 200 µl 30 % (v/v) glacial acetic acid (Sigma-Aldrich). After 15 min, 125 µl of each suspension was transferred to clean wells of a 96-well flat bottom polystyrene microtiter plate and absorbance measured at 595 nm using xMark^{TM} (Bio-Rad). Nine biological replicates in two independent experiments were performed for each of the strains or set of conditions. Readings were presented as means ± standard deviation. Sterile medium was used as negative control.

2.3.9. Statistical analyses

All experiments were conducted in triplicate. Results of growth under bile stress, auto-aggregation and MATS are expressed as the mean and standard deviation of three independent experiments. One-way ANOVA, followed by Tukey's post-test was used to determine statistically significant differences. *P* values < 0.05 were considered significant. Pearson Correlation analysis was conducted for auto-aggregation versus hydrophobicity with statistical significance determined at

p < 0.05. Statistical analysis was performed on the data using SPSS 22.0 (IBM, SPSS, Chicago, Ill., U.S.A.).

2.4. Results

Nine Gram-positive, catalase negative, acid-producing strains were isolated from feces. Five of these isolates tested positive for alpha hemolytic activity and were excluded from further analysis. None of the remaining four isolates hydrolyzed mucin (results not shown). Three of the four isolates had identical 16S rDNA and recA sequences and displayed greater than 98% DNA homology with both genes of *L. reuteri* DSM 20016 (NR 075036 and CP000705.1 respectively). Isolate HFI-LD5 was selected as the most representative. Isolate HFI-K2 shared greater than 98% 16S rDNA and recA sequence homology with *L. rhamnosus* JCM 1136 (NR_043408.1) and *L. rhamnosus* GG (FM179322.1) respectively. Nucleotide sequences of the *16s rRNA* and *recA* genes of the two isolates were deposited in the NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore/) 16S rDNA and recA sequences for *L. reuteri* HFI-LD5 (KT803960 and KT803962) and *L. rhamnosus* HFI-K2 (KT803961 and KT803963) respectively.

L. reuteri HFI-LD5 and *L. rhamnosus* HFI-K2 were resistant to Trimethoprim (1.25 µg), Sulfamethoxazole (100 µg), Sulfamethoxazole-Trimethoprim (25 µg), Sulphonamides compound (300 µg), Oxacillin (1 µg), Methicillin (25 µg), Dicloxacillin (25 µg) and Gentamycin (10 µg). *L. rhamnosus* HFI-K2 displayed intermediate resistance to Tetracycline. The reference strain, *L. rhamnosus* R-11, on the other hand, was completely resistant to Tetracycline. Both strains of *L. rhamnosus* were susceptible to Penicillin (10 µg), Chloramphenicol (30 µg) and Clindamycin (2 µg), but only one of them (strain HFI-K2) was susceptible to Ampicillin (10µg) and Meropenem (10 µg). *L. reuteri* HFI-LD5 displayed intermediate resistance to Kanamycin (30 µg), Nitrofurantoin (300 µg) and Chloramphenicol (30 µg). *L. reuteri* DSM 17938, on the other hand, was completely resistant to all three of these antibiotics. Both strains of *L. reuteri* were susceptible to Rifampicin (5 µg) and Tobramycin (10 µg). Strain HFI-LD5 differed from strain DSM 17938 in being susceptible to Ampicillin (10 µg) and Clindamycin (2 µg).

L. reuteri DSM17938, *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 showed a significantly higher affinity for chloroform and a much lesser affinity for hexadecane (Table 2.1). The basic properties of *L. reuteri* HFI-LD5 and DSM 17938 were further supported by the significantly higher affinity for the non-polar solvent decane over basic ethyl acetate (Table 1). Of the four strains tested, *L. rhamnosus* HFI-K2 had the highest affinity for hexadecane (37.71 % ± 8.02 %), suggesting that the cell surface of the strain is hydrophobic. Cells of *L. rhamnosus* R-11 had a much lower affinity (6.24 % ± 2.1 %) for hexadecane and was more-or-less similar to results obtained for *L. reuteri* HFI-LD5 and *L. reuteri* DSM 17938 (4.91 % ± 2.21 %).

Table 2.1. Affinity of *Lactobacilli* isolates and reference strains for non-polar and monopolar solvents (MATS analysis).

Organism	% Affinity			
	Chloroform	**Hexadecane**	**Ethyl acetate**	**Decane**
L. reuteri HFI-LD5	30.73 (6.17)[1]	8.82 (4.89)[3]	0.63 (0)[2]	24.37 (0)
L. reuteri DSM 17938	15.82 (0.80)[1]	4.91 (2.21)	3.46 (1.95)[2]	14.96 (2.90)
L. rhamnosus HFI-K2	94.18 (3.61)[1]	37.71 (8.02)[3,4]	60.59 (5.40)	75.15 (2.52)
L. rhamnosus R11	11.29 (1.36)	6.24 (2.11)[4]	16.54 (2.34)	15.04 (2.58)

Standard deviation is given in parentheses.

[1] Significant differences in chloroform affinity between lactobacilli.

[2] Significant differences in ethyl acetate affinity between lactobacilli.

[3] Significant differences in affinity for hexadecane between *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 (P = 0.002).

[4] Significant differences in affinity for hexadecane between *L. rhamnosus* HFI-K2 and *L. rhamnosus* R11 (P = 0.001).

Of the four strains studied, *L. rhamnosus* HFI-K2 displayed the highest percentage auto-aggregation (Figure 2.1 D), with cells aggregated to the extent that a distinct clearing of the upper part of the cell suspension was visible after 3 h of incubation. This correlated with the high affinity strain HFI-K2 had for hexadecane (Figure 2.2).
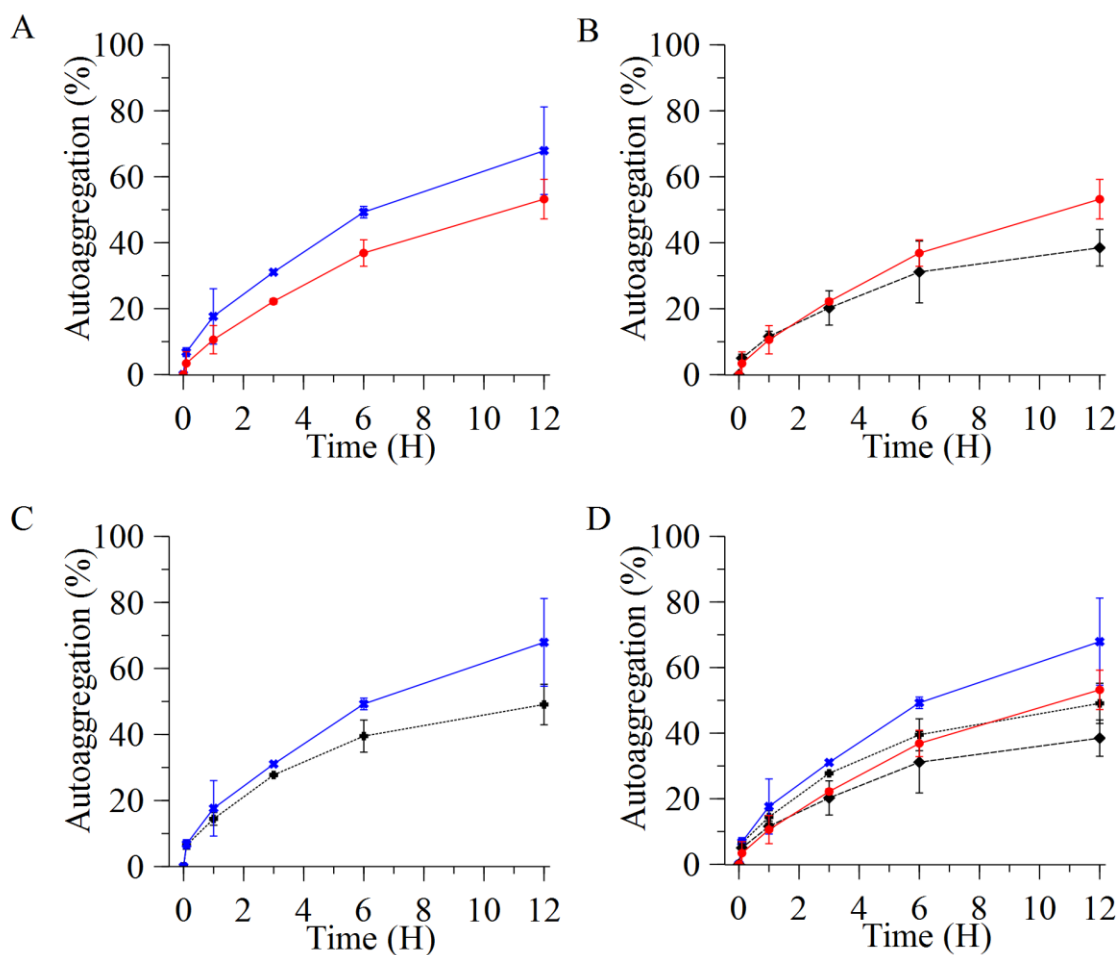
Figure 2.1. Comparison of the autoaggregating ability of *L. reuteri* HFI-LD5, *L. rhamnosus* HFI-K2 and reference strains, *L. reuteri* DSM 17938 and *L.rhamnosus* R-11. Each of the four graphs represent the following comparisons: (A) *L. reuteri* HFI-LD5 (●, Red line) and *L. rhamnosus* HFI-K2 (✖, Blue line). (B) *L. reuteri* HFI-LD5 (●, Red line) and the reference strain *L. reuteri* DSM 17938 (◆, dotted line). (C) *L. rhamnosus* HFI-K2 (✖, Blue line) and *L. rhamnosus* R-11 (✚, Dotted line). (D) Comparison of both isolates and reference strains, with the same keys as above. The error bars represent standard deviations of 3 independent experiments.
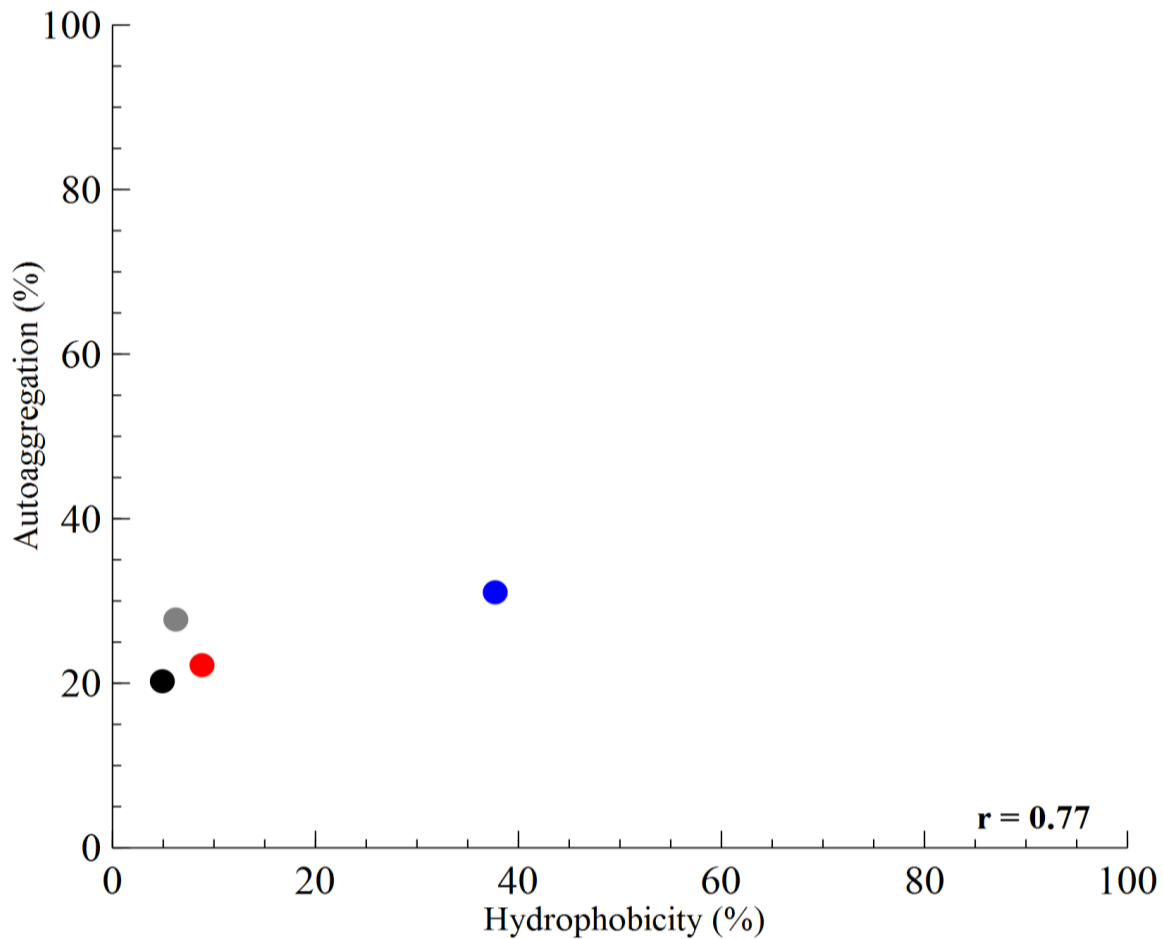
Figure 2.2. The relationship between autoaggregation and hydrophobicity of Lactobacillus strains. (●) *L. reuteri* HFI-LD5 (●) *L. reuteri* DSM 17938 (●) *L. rhamnosus* HFI-K2 (●) *L. rhamnosus* R-11. Cell surface hydrophobicity is expressed as the percentage bacteria adsorbed by hexadecane.

Cell-free supernatants of all four strains inhibited the growth of *L. monocytogenes* EDG-e, but not *L. sakei* LMG 13558. However, neutralized cell-free supernatant (pH 6.5 to 7.5) had no effect on the growth of the two-target species.

Lactobacillus reuteri HFI-LD5 formed a slight biofilm after 72 h of incubation at 37°C in the presence of MRS (Figure 2.3). Substitution of glucose with gluconate (mMRS medium) increased biofilm formation (Figure 2.3). MRS media allowed for only slight biofilm formation under the culturing conditions, however the substitution of glucose for gluconate resulted in a marked increase in biofilm formation. All lactobacilli strains exhibited biofilm formation when cultured in mTSB, irrespective of the concentration. When cultured in mTSB, *L. rhamnosus* HFI-K2 exhibited the best biofilm formation properties on a hydrophobic surface (polystyrene) of all the strains and various media compositions used. Interestingly, minimal biofilm formation was observed for either *L. reuteri* DSM 17938 or *L. rhamnosus* R-11, irrespective of the media or concentration (Figure 2.3), clearly illustrating the extensive strain variability with respect to biofilm formation on polystyrene.
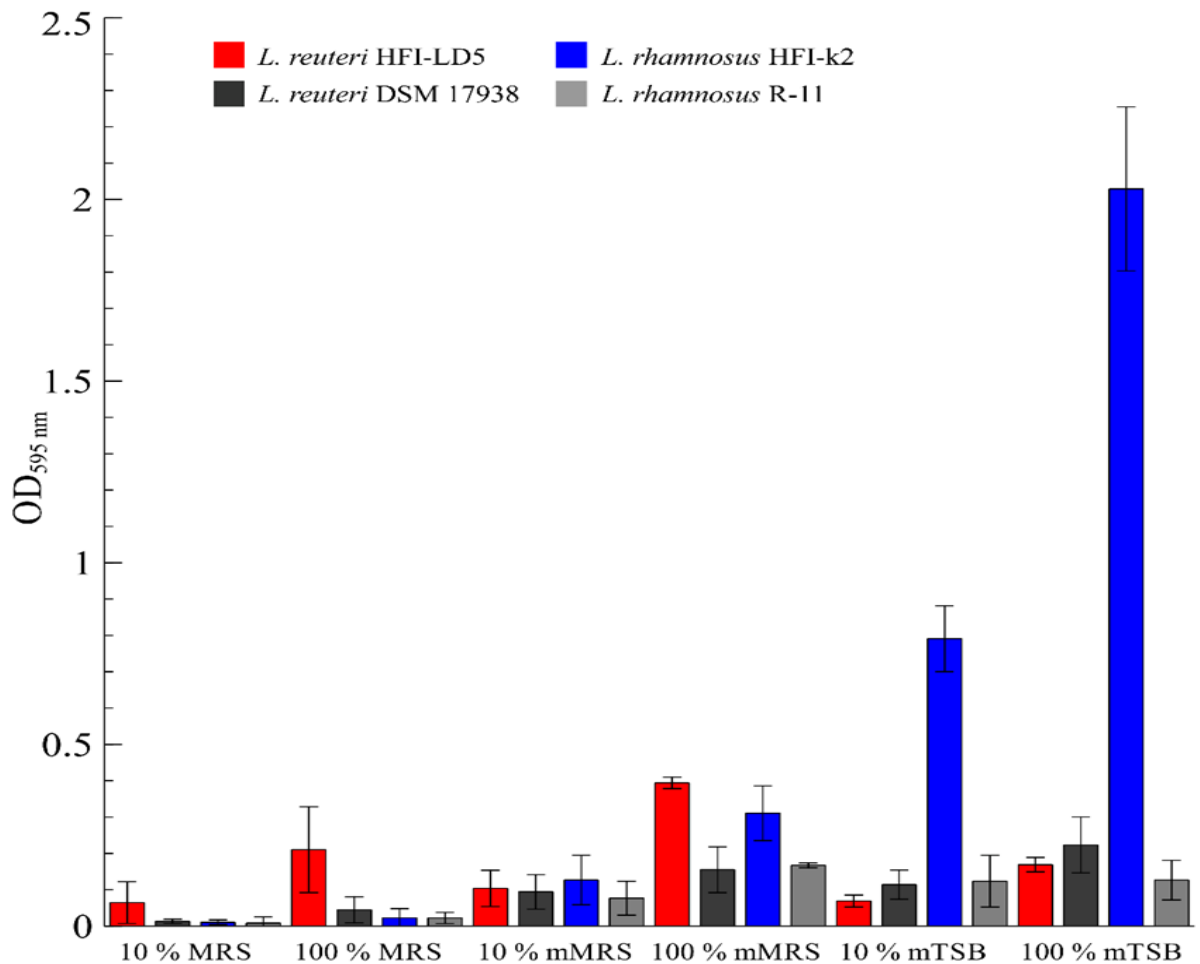
Figure 2.3. Comparison of biofilm formation capacity by *Lactobacillus reuteri* and *Lactobacillus rhamnosus* spp. Capacity of the two human fecal isolates (*L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2) were compared to *L. reuteri* DSM 17938 and *L. rhamnosus* R-11 under different culture conditions (Nutrient rich, different carbon source and low C/N ratio). The results are expressed as the mean of two independent experiments, with nine biological repeats each. Standard deviation represented by error bars.

2.5. Discussion

A juxtaposition exists between the requirement of the human body to protect against infection and the selective colonization by beneficial allochthonous and autochthonous microorganisms, which contribute to the maintenance of gastrointestinal tract (GIT) homeostasis. The ability of lactobacilli to survive the harsh conditions within the human GIT is well documented, with pH values in the stomach ranging from pH 1.5 to 3.0 and bile concentrations within the intestines reaching 0.5 % - 2.0 % (28–30). Although *Lactobacillus* spp. are able to withstand these stresses, these species only represent 7 % of the total HGM (7). This underrepresentation was evident during the isolation of lactobacilli in this study, in which a limited number of acid producing, Gram positive, catalase negative and hemolytic negative bacilli were isolated (n = 4) from the human fecal matter.

The genus Lactobacillus is the largest and most diverse genus amongst lactic acid bacteria (LAB). However, on a taxonomical front there is still a lack of clarity, with large numbers of lactobacilli having been classified based on phenotypic characteristics and a single gene, namely the *16S rRNA* gene (31). This leads to limited species delineation, and there is thus a need for multiple other gene sequences or whole genome sequencing to be used for adequate *Lactobacillus* spp. identification. Therefore the *16S rRNA* gene and *recA* gene were independently used for identification of the isolated strains (31, 32).

Mucus, comprised of a thick loose, outer layer and a dense, stratified inner layer, is considered to be an evolutionary defense mechanism within the human GIT (33). Mucus consists of glycoprotein mucins, which can be degraded and utilized as a complex carbohydrate by certain lumen bacteria (34, 35). A select few commensals within the human GIT are capable of metabolizing and utilizing mucin although never to the same degree as pathogenic microorganisms (34, 36, 37). Both *L. reuteri* and *L. rhamnosus* spp. are known to form close associations with the mucosal surface within the GIT (38). Mucinolytic activity is not characteristic for either *L. reuteri* or *L. rhamnosus* spp. which is in agreement with results obtained in this study (22, 39). This implies the lack in both *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 of the enzymes that are required to degrade and utilize mucin as a potential carbon source (35–37).

Both *L. reuteri* and *L. rhamnosus* spp. have been assigned GRAS status owing to their general inherent health benefits and low prevalence of infections. A few isolated cases of Lactobacilli-associated infections have, however, been documented. These cases are rare and generally a result of a complication of other medical disorders and diseases (40). Lactobacilli susceptibility to antibiotics is species-specific and even strain-specific (41). *L. reuteri* and *L. rhamnosus* spp. have been documented as having intrinsic resistance to tetracycline, which matches the results seen in this study (42–44). The effective use of β-lactams against lactobacilli is variable. However, inhibitory activity is seen, as found in this study (45, 46).

The interaction of microbes with abiotic and biotic surfaces has been studied for decades, however, the interaction of microbes with one another and with human cells has only come to the fore in recent years (47, 48). Microbial persistence within the human GIT relies on their ability to interact not only with one another but also with the host's mucosal layers and epithelial cells. Numerous groups have listed factors such as cell surface proteins, cell surface hydrophobicity, autoaggregation ability and surface appendages (pili) as having an influence on the interaction and adhesion of microbes to various surfaces (26, 49, 50).

The adhesion of microbial cells to abiotic and biotic surfaces is governed by complex interactions between van der Waals, Lewis acid-base and electrostatic forces (51, 52). Our results indicate that the cell surfaces of both *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 are basic or electron donating, with *L. rhamnosus* HFI-K2 being a stronger electron donor and weaker electron acceptor than the other *Lactobacillus* spp. in this study (Table 2.1). This is due to the physiochemical properties of the microbial cell surfaces varying at a species level (53). The high chloroform affinity observed for *L. rhamnosus* HFI-K2 (Table 2.1) is characteristic of the species (53). The basic properties of *L. rhamnosus* HFI-K2 may be due to the abundance of carboxylic and phosphate groups over amino groups. This increased basic and negative charge on the surface may benefit adhesion to biotic surfaces such as tissue (54).

Cell surface hydrophobicity has been associated with the first steps involved in colonization and interaction with host mucosal layers, owing to its effect on the adhesion behavior of microbial cells (55, 56). Cell surface hydrophobicity of lactobacilli can be classified according to one of three

categories, namely low (0-35 %), moderate (36-70 %) and high (70-100 %) (57). The results of the hydrophobicity assay indicate that *L. reuteri* HFI-LD5 and both reference strains are weakly hydrophobic in nature (< 35 %), with *L. rhamnosus* HFI-K2 exhibiting a moderate degree of hydrophobicity (Table 2.1 and Figure 2.2). Similar results have been reported by other groups, with various lactobacilli (strain independent) possessing weakly hydrophobic cell surface properties and a few lactobacilli strains being hydrophobic in nature (53, 57, 58).

The formation of microbial aggregates is important in several ecological niches, such as the human GIT, and has been identified as a desirable property for probiotics to possess for the initial colonization of the host (50, 59). All of the strains indicated a moderate degree of autoaggregate formation, with *L. rhamnosus* HFI-K2 presenting the highest autoaggregative capacity among the strains tested (Figure 2.1). The aggregation of the isolates may provide a protective shield against the harsh conditions within the human GIT and furthermore allow for transient GIT colonization (50, 60). A positive correlation was observed between autoaggregation ability and cell surface hydrophobicity of the lactobacilli strains. Although *L. rhamnosus* HFI-K2 demonstrates a moderate degree of autoaggregate formation and cell surface hydrophobicity, it did not cluster with *L. reuteri* HFI-LD5 or the reference strains (Figure 2.2). These findings are corroborated by numerous other studies both within and outside of LAB research, that show a positive correlation between microbial cell autoaggregation and microbial cell surface hydrophobicity (26, 50, 60, 61).

The attachment and persistence of probiotics in the human GIT is a critical factor, since the ability to colonize is required to ensure that their beneficial effects are exerted. The study of probiotic persistence within the GIT generally involves *in vitro* cell line models or animal models. However, little attention is given to the potential for bacterial biofilm on the surface of the epithelial cells. Here we show that both *L. reuteri* HFI-LD5 and *L.rhamnosus* HFI-K2 are capable of forming *in vitro* biofilms on abiotic surfaces, in contrast to the respective control strains tested (Figure 2.3). This strain dependent variation with respect to *in vitro* biofilm formation is well established (19, 62, 63). The lack of adequate biofilm formation at both concentrations of MRS (Figure 2.3) is corroborated by various other studies, wherein it was found that the high glucose concentration and presence of the surfactant, Tween 80, repressed biofilm formation (19, 63).

The ability of *L. rhamnosus* HFI-K2 to form biofilms on the hydrophobic polystyrene surface when cultured in mMRS and mTSB was clearly illustrated in this study, and is supported by the significantly higher cell surface hydrophobicity exhibited by this strain (Figure 2.3 and Table 2.1). It is well established that cell surface properties, especially hydrophobicity, are positively correlated to biofilm formation (64, 65). Biofilm formation by lactobacilli spp. is dependent on the strain and environmental conditions, as can be seen from this study and others (19, 62, 63). The intrinsic ability of the two novel isolates to form biofilms under *in vitro* conditions may contribute to the colonization ability of these two strains *in vivo*.

It was established that *L. rhamnosus* HFI-K2 exhibits similar intrinsic tolerance to the stresses experienced within the human GIT, as *L. reuteri* HFI-LD5. The two strains share similar characteristics with their corresponding reference strains, *L. reuteri* DSM 17938 and *L. rhamnosus* R-11, both of which are commercially available probiotics. *L. rhamnosus* HFI-K2 exhibited a higher degree of hydrophobicity in conjunction with a better ability to form biofilms on hydrophobic abiotic surfaces. The ability to survive acid and bile stresses coupled with biofilm formation may contribute to persistence of these strains within the human gastrointestinal tract, especially *L. rhamnosus* HFI-K2. Further characterization is required to understand the mechanisms contributing to *in vitro* biofilm formation by *L. rhamnosus* HFI-K2, with expansion into formation of *in vivo* biofilms.

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CHAPTER 3

CHAPTER 3: Differential survival response of planktonic and sessile *Lactobacillus rhamnosus* HFI-K2 and *Lactobacillus reuteri* HFI-LD5 to fasting-state human gastrointestinal conditions

3.1. Abstract

Most research on gut microbiota, including probiotic bacteria, focus on planktonic cells, even though evidence exists in support of microbial biofilm formation in sections of the GIT. The effect of three simulated, fasting-state gastrointestinal fluids (gastric fluid, pH 2, 2 h exposure; intestinal fluid, pH 7.5, 6 h exposure and colonic fluid pH 7, 24 h exposure) on planktonic and sessile cell viability of *Lactobacillus reuteri* (strain HFI-LD5) and *Lactobacillus rhamnosus* (strain HFI-K2), isolated from the human GIT, is described. The viability of batch-cultivated planktonic cells after exposure to gastric, small intestinal and colonic fluids could only be assessed individually, whereas biofilms were exposed sequentially to each of the gastrointestinal fluids while monitoring CO_2 production as an indicator of metabolic activity. Simulated gastric juice exposure had the greatest effect on planktonic cell viability and biofilm metabolic activity. A decline in planktonic cell numbers of *L. reuteri* HFI-LD5 from 3.8 x 10^6 CFU.ml^{-1} to 2.8 x 10^3 CFU.ml^{-1} was observed during the 2 h exposure period to gastric fluid, whereas cell numbers of *L. rhamnosus* HFI-K2 showed a decline from 4.3 x 10^6 CFU.ml^{-1} to 0 CFU.ml^{-1}. Exposure of *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 biofilms to similar conditions initiated the detachment of biofilm biomass, accompanied by a decrease in metabolic activity, as recorded by changes in CO_2 production. However, as soon as the exposure was halted and the simulated colonic fluid was replaced with sterile culture medium, the remaining biofilm biomass responded by producing CO_2, followed by recovery and re-establishment of pre-exposure activity within 24 h. In contrast to the complete loss of planktonic *L. rhamnosus* HFI-K2 cell viability after exposure to gastric juice, biofilms of this strain not only recovered within 24 h after exposure, but exhibited increased metabolic activity after recovery. To the best of our knowledge, this is the first study to assess the effect of simulated, fasting-state gastrointestinal fluids on biofilms of *L. reuteri* and *L. rhamnosus.* The use of CO_2 production as a real-time indicator of biofilm metabolic activity provides insight into the

differential survival responses of lactic acid bacteria under human simulated fasting-state gastrointestinal conditions.

3.2. Introduction

Renewed interest in functional foods and probiotics is predominantly driven by the increase in public awareness of critical functions fulfilled by the human GIT and the importance of maintaining GIT homeostasis (1–3). This led to the development of several new probiotic formulations with strains other than *Lactobacillus* and *Bifidobacterium* ssp. Probiotic bacteria need to survive conditions in the GIT to be of benefit to the host, thereby fulfilling the definition of a probiotic as being "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (4–7).

The human GIT is an intricate organ system that hosts various immunological and metabolic functions (8–10) and contains the greatest microbial diversity and density associated with the human body (11). The diverse GIT ecosystem supports a well-balanced symbiosis between eukaryotic and prokaryotic cells, regulated by changes in chemical and physical conditions (12). Microbial survival and growth in the GIT is mainly influenced by changes in pH, bile concentrations and transit times. This gives rise to the development of distinct environmental niches in the three major regions of the GIT, i.e. the stomach, small intestine and colon (13, 14). Microorganisms in the stomach are challenged by the high acidity of gastric fluid, digestive enzymes and often rapid luminal flow rates, depending on the nature of the stomach content (15). Stomach pH varies between 1.0 and pH 5.0, with the lowest values recorded during fasting (16–21). Transit times from entering through the esophageal sphincter to leaving through the pyloric sphincter may be as rapid as 15 min or in excess of 130 min (16–18, 22). These harsh conditions are thought to largely prevent microbial colonization of the stomach, which puts a limit on the number of viable cells reaching the lower GIT. Microbes that do survive conditions in the stomach do not necessarily survive the high bile concentrations and sudden changes in pH in the first section of the small intestine, which varies between pH 4.0 and 8.0 (15, 16, 23). Transit time through the small intestine varies from 30 min to 6 h (16). However, the colonic region of the GIT is the most favorable to microbial colonization due to the relative pH stability, a near-neutral pH (pH 5.5 to 7.5), lower bile concentrations and slow transit time (approximately 24 h), thus allowing for microbial establishment and proliferation (23–25).

The microbial diversity, activity and its association with the host are postulated to be enhanced by the presence of biofilms. Microbial biofilms associated with intestinal epithelial cells would facilitate metabolic interaction by shortening the distance across which metabolites must diffuse and prolonging the period of association, in contrast to free-floating planktonic cells in the GIT lumen. Biofilms are broadly defined as a community of microorganisms that assemble at an interface and encase themselves within a protective extracellular polymeric substance (EPS) matrix (29). Planktonic or suspended microbial growth is atypical in natural environments, with sessile biofilm formation thought to be the dominant mode of growth (27).

Most studies on microbial biofilm formation in humans focus on the oral cavity (oral caries and periodontitis), specific diseases, such as cystic fibrosis, and soft tissue infections, including burn wounds, prosthetic devices and stents (28, 29). The study of biofilms within the context of the human GIT has been problematic, owing to the lack of non-destructive sample preparation techniques and the invasive nature of sample acquisition from human subjects (11, 30, 31). These difficulties may explain why more is known about the biofilm-epithelial cell associations observed in the upper GITs of animals, including pigs, rodents and chickens (11, 30, 32). During the course of the last two decades very few novel studies relating to microbial biofilms within the human GIT have been conducted, with instead a larger number of reviews on this topic being published (11, 31, 33). The role of biofilms in microbial survival in the GIT of humans is thus largely unknown. Probiotic research has focused on the study of planktonic microorganisms, and this narrow focus has most likely caused an underestimation in the survival ability of probiotic microorganisms within the human GIT, owing to increased tolerance to stress conditions observed within microbial biofilms (34, 35). Therefore, the potential role of biofilm formation in the survival and persistence of probiotic and other microbes within the GIT remains unresolved to a significant extent.

In this study, we report on the differential survival of batch-derived planktonic and biofilm cells of *Lactobacillus reuteri* HFI-LD5 and *Lactobacillus rhamnosus* HFI-K2, isolated from the human GIT, in the presence of gastrointestinal fluids that simulate a fasting condition. The ideal scenario for the evaluation of survivability would involve sequential exposure of the same microbes to the simulated gastric fluids (gastric, intestinal, and lastly colonic fluid), followed by recovery in

growth medium. The free-floating nature of planktonic cells complicates this ideal testing scenario, since the recovery of cells with centrifugation, followed by washing steps, prior to exposure to the next simulated gastric fluid may introduce unknown variability and lead to the loss of biomass. Instead of sequential exposure, the survival of planktonic cells, cultivated in batch, was evaluated after separate exposure to each simulated fluid. Biofilm survival and recovery during sequential exposure to the simulated gastric fluids was monitored in real-time by recording the production of CO_2 as an indicator of metabolic activity.

3.3. Materials and Methods

3.3.1. Strains and growth conditions

Lactobacillus reuteri HFI-LD5 and *Lactobacillus rhamnosus* HFI-K2, isolated from stool samples of healthy individuals, were cultured in MRS broth (36), but with glucose substituted by 20 g.l^{-1} K-gluconate (mMRS). Incubation was at 37°C in anaerobic jars (Oxoid Ltd., Hampshire, UK), equipped with Anaero Pack-MicroAero gas-generating sachets (Mitsubishi Gas Chemical Co., Inc, Tokyo, Japan). Planktonic and biofilm survival experiments were performed at 37°C, in mMRS (pH 5.5).

3.3.2. Preparation of simulated fasting-state gastrointestinal fluids

Simulated intestinal fluid powder (Biorelevant Media, London, UK) was used to prepare fasting-state simulated gastric fluid (SGF) and fasting-state simulated intestinal fluid (SIF), as prescribed by the manufacturer. The pH of SGF and SIF was adjusted to 2.0 and 7.5, respectively, with 1 M HCl and NaOH respectively. Simulated colonic fluid (SCoF) was prepared in phosphate-buffered saline (PBS, pH 7.0), according to Marques *et al*. (39).

3.3.3. Survival of batch-cultured planktonic cells of *L. rhamnosus* HFI-K2 and *L. reuteri* HFI-LD5 in the presence of SGF, SIF and SCoF

L. reuteri HFI-LD5 and *L. rhamnosus* HFI-K2 were cultured separately in mMRS broth for 18 h at 37 ˚C, the cells harvested (2 000 x *g*, 10 min, 4°C) hereby minimizing damage to cells, washed twice with PBS (pH 7.0) and resuspended to an OD$_{600nm}$ of 0.8. One milliliter of each cell suspension was added to 9 ml SGF, SIF and SCoF, respectively, briefly vortexed and incubated at 37°C in anaerobic jars (Oxoid Ltd., Hampshire, UK), equipped with Anaero Pack-MicroAero gas-generating sachets (Mitsubishi Gas Chemical Co., Inc, Tokyo, Japan) on a shaker. Samples were

taken at specific time intervals (Table 3.1), serially diluted in Maximum Recovery Diluent (MRD, Oxoid) and plated onto MRS Agar (Biolab, Biolab Diagnostics, Midrand, SA). Plates were incubated at 37°C and colonies counted after 48 h.

3.3.4. Survival of *L. rhamnosus* HFI-K2 and *L. reuteri* HFI-LD5 biofilms in the presence of SGF, SIF and SCoF

The GIT environment was simulated using the CO_2 evolution measurement system (CEMS) described by Kroukamp *et al*. (39). The system, schematically presented in Figure 3.1, consisted of 1.5 m long, thin-walled, silicon tubing (Freudenberg Medical, Carpinteria, CA, USA) with an inner diameter of 1.58 mm (outer diameter = 2.4 mm), encased in E-3603 Tygon tubing (VWR International, Mississauga, ON, Canada) with an inner diameter of 4.8 mm (outer diameter = 7.9 mm). The volume of the inner thin-walled silicon tubing was 2.94 ml. Each end of the tubing was sealed with a 17 x 10 mm custom-made connector, fitted with separate gas and liquid ports. The gas port of the upstream connector connected the annular space between the outer silicone tubing surface and the inner Tygon tubing surface to a compressed gas cylinder containing high purity N_2 (Afrox, Cape Town, South Africa). The downstream gas port was connected to a non-dispersive, infrared LI-820 CO_2 gas analyzer (LI-COR Biosciences, Lincoln, NE, USA). The flow rate of N_2 through the annular space was controlled at 20 ml.min^{-1} by using a GFM17 thermal gas mass flow controller (Aalborg Instruments and Controls, Inc., Orangeburg, NY, USA).

Culture medium and fasting-state simulated gastrointestinal fluid reservoirs were connected upstream of the CEMS via the liquid port of the custom connector to the inner, thin-walled, silicone tubing. A custom-made, airtight, 6-port borosilicate glass manifold, equipped with a bubble trap, was inserted upstream of CEMS to allow sequential switching between medium and simulated gastric fluids without introducing air into the system. The manifold was connected to a 205U multichannel peristaltic pump (Watson-Marlow Fluid Technology Group, Falmouth, Cornwall, UK) with E-3603 Tygon tubing (Saint-Gobain Performance Plastics, Charny, France) of 1.6 mm inner diameter and 3.2 mm outer diameter. Changes in effluent pH were recorded downstream of

CEMS with a Vernier pH sensor using LabPro and Logger Pro 3.10.1 software (Trac, Stellenbosch, South Africa), housed in custom borosilicate glass T-pieces. Changes in CO_2 production and effluent pH were logged continuously at 1 min intervals for the duration of the experiment.
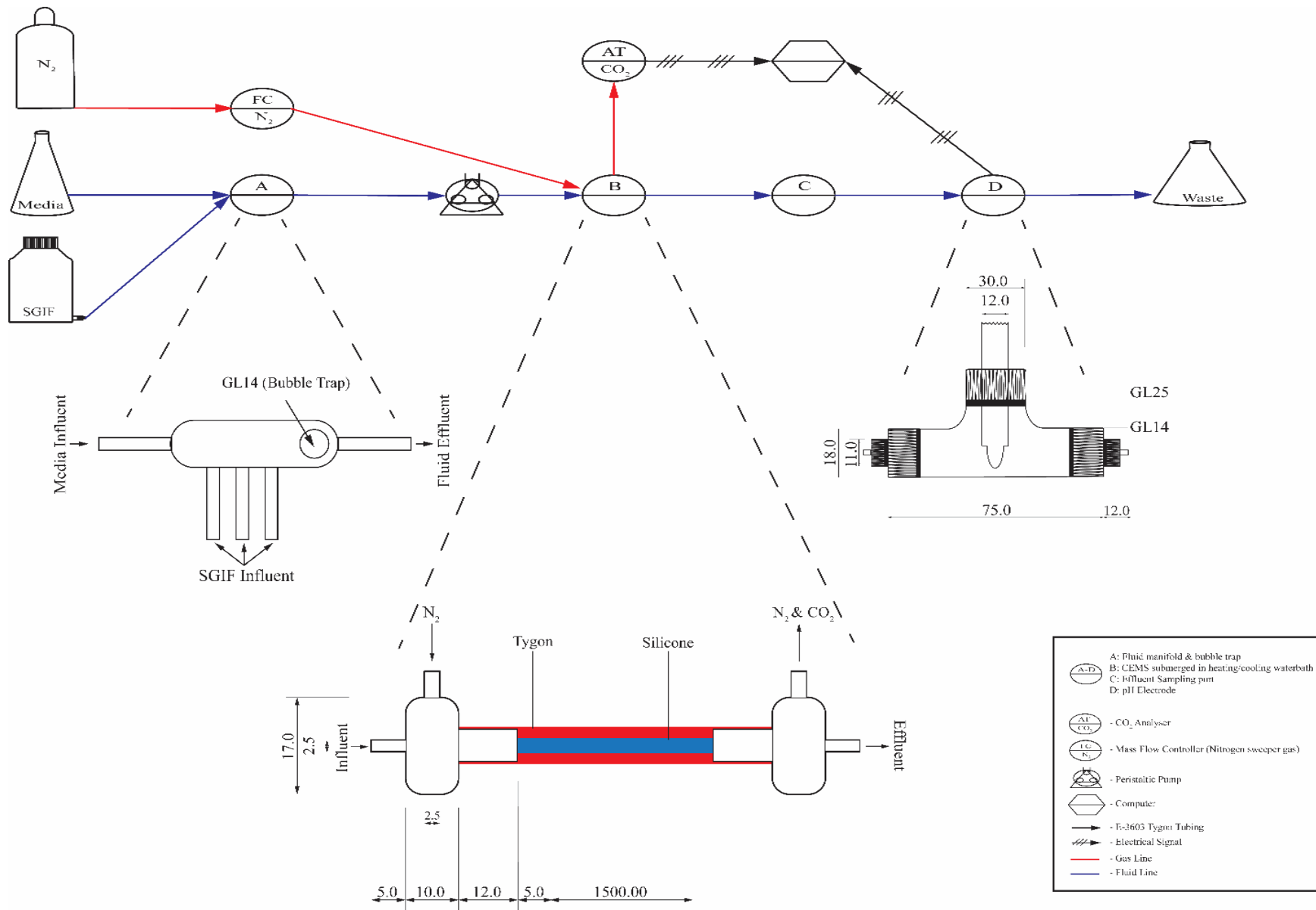
Figure 3.1. Schematic diagram of the carbon dioxide evolution measurement system (CEMS). Medium and fasting-state simulated gastrointestinal fluids were smoothly and sequentially introduced into CEMS via the fluid manifold/bubble trap (**A**) from the respective reservoirs at a flow rate of 12.5 ml.h^{-1} provided by the peristaltic pump. CEMS was contained in a heating/cooling water bath set at 37°C (**B**). CEMS consists of thin-walled silicone tubing, encased within Tygon tubing with a larger inner diameter. CO_2 produced by the biofilm growing on the inner surface of the silicone tubing diffuses across the CO_2-permeable silicone into the annular space where it is contained by the Tygon tubing. The sweeper gas (high purity nitrogen) was introduced into the annular space of CEMS (**expanded B**, red shaded region) via a thermal gas mass flow controller at a rate of 20 ml.min^{-1}, hereby collecting biofilm-derived CO_2 prior to the gas mixture being analyzed by the CO_2 analyzer. Effluent samples for the enumeration of biofilm-derived planktonic cells were collected downstream of CEMS via a sampling port (**C**) prior to effluent entering the pH manifold (housing the pH electrode) thereby facilitating real-time pH measurements. Fluid lines and flow (—) and Gas lines and flow (—). All dimensions are to nearest mm.

3.3.5. Survival of L. reuteri HFI-LD5 and L. rhamnosus HFI-K2 in biofilms exposed to SGF, SIF and SCoF

The CEMS was sterilized by flushing with 3.5 % (vol/vol) sodium hypochlorite for 2 h and then rinsed overnight with a constant flow of sterile distilled water. Culture medium (mMRS) was pumped through the inner silicone tubing for 1 h at a flow rate of 12.5 ml.h^{-1} to record baseline values for CO_2 and pH. Thereafter, medium flow was stopped and the CEMS inoculated with 1 ml of an actively growing culture (OD600 = 0.8 in mMRS) of *L. reuteri* HFI-LD5, followed by 1.5 h of stagnant conditions to allow adhesion of the bacteria to the inner wall of the thin-walled silicon tube. The CEMS tubing was then submersed in a temperature-controlled water bath set at 37°C and the flow of mMRS resumed at 12.5 ml.h^{-1} (0.21 ml.min^{-1}). At this flow rate, the retention time in the system was 14 min, thereby selecting for biofilm formation and restricting planktonic cell replication in the CEMS to a minimum. The flow of mMRS was maintained for up to 120 h to allow biofilm formation and stable CO_2 production, indicative of a culture in steady-state metabolic activity. The medium was replaced by SGF, at a flow rate of 12.5 ml.h^{-1} for 2 h, followed

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by SIF for 6 h and SCoF for 24 h at the same flow rate. The experiment was performed in duplicate for *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2.

Effluent from the CEMS was collected at specific time points (Table 3.1) at the sampling port upstream of the pH sensor. Serial dilutions were made in sterile saline and plated onto MRS Agar (Biolab). Viable cell numbers were determined after 48 h of incubation at 37˚C. These cell numbers represented biofilm-derived planktonic cells produced during biofilm establishment and recovery, as well as detaching biofilm biomass during exposure to simulated gastrointestinal fluids.

In a separate experiment, the effect of 2 h SGF exposure, followed by recovery growth medium on *L. reuteri* HFI-LD5 biofilms was evaluated with respect to CO_2 production, effluent pH and biofilm-derived cell numbers, as described above. To account for the effect pH on the solubility of CO_2, sterile mMRS, SGF, SIF and SCoF were injected into the CEMS and readings recorded as described.

3.3.6. Statistical analysis

Significant differences in bacterial cell numbers were determined by conducting unpaired *t*-tests in IBM SPSS Statistics 22 (IBM Corp. Armonk, NY). A *p*-value of < 0.05 was considered statistically significant. Batch-derived planktonic cell experiments were conducted in triplicate, whilst biofilm studies were performed in duplicate.

3.4. Results

Batch-derived planktonic cell numbers of *L. rhamnosus* HFI-K2 declined significantly from 4.3 x 10^6 CFU.ml^{-1} to no detectable cell numbers within 40 min of exposure to SGF (pH 2.0) (Figure 3.2A). In contrast, the number of viable planktonic cells of *L. reuteri* HFI-LD5 declined from 3.8 x 10^6 CFU.ml^{-1} to 2.9 x 10^4 CFU.ml^{-1} within the first 40 min of exposure to the same conditions, followed by a gradual decline to 2.8 x 10^3 CFU.ml^{-1} over the next 60 min (Figure 3.2A).

The viability of batch-derived planktonic *L. rhamnosus* HFI-K2 cells remained unchanged at 5.0 x 10^5 CFU.ml^{-1} during 6 h exposure to SIF (pH 7.5), whereas *L. reuteri* HFI-LD5 cell numbers declined rapidly from 4.1 x 10^6 CFU.ml^{-1} to 3.4 x 10^5 CFU.ml^{-1} during the first 2 h of exposure (Figure 3.2B). This was followed by a 2-h stabilization period and further significant reduction to 3.7 x 10^4 CFU.ml^{-1} during the last 2 h (Figure 3.2B). Both strains showed similar survival trends during exposure to SCoF (pH 7.0), with the only significant difference in survival observed at 6 h ($p < 0.05$). After 24 h exposure both *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 exhibited only a marginal reduction in overall viability (Figure 3.2C).

During the first 80 h after inoculation of CEMS with *L. rhamnosus* HFI-K2, a steady decrease in effluent pH due to the metabolism of gluconate to lactic acid was observed (pH 5.5 to 4.9). This observation, accompanied by a slight increase in overall biofilm metabolic activity (CO_2 production) and a two-fold increase in the number of biofilm-derived planktonic cells released into the effluent, indicated that biofilm development had commenced (Figure 3.3 and 3.4). The rate of CO_2 production by the biofilm rapidly increased once the pH dropped below 5.0. An initial steady-state biofilm was reached at approximately 110 h with an effluent pH of 4.9 and the production and release of 1.5 x 10^{10} CFU.ml^{-1} of planktonic cells into the effluent (Figure 3.4).
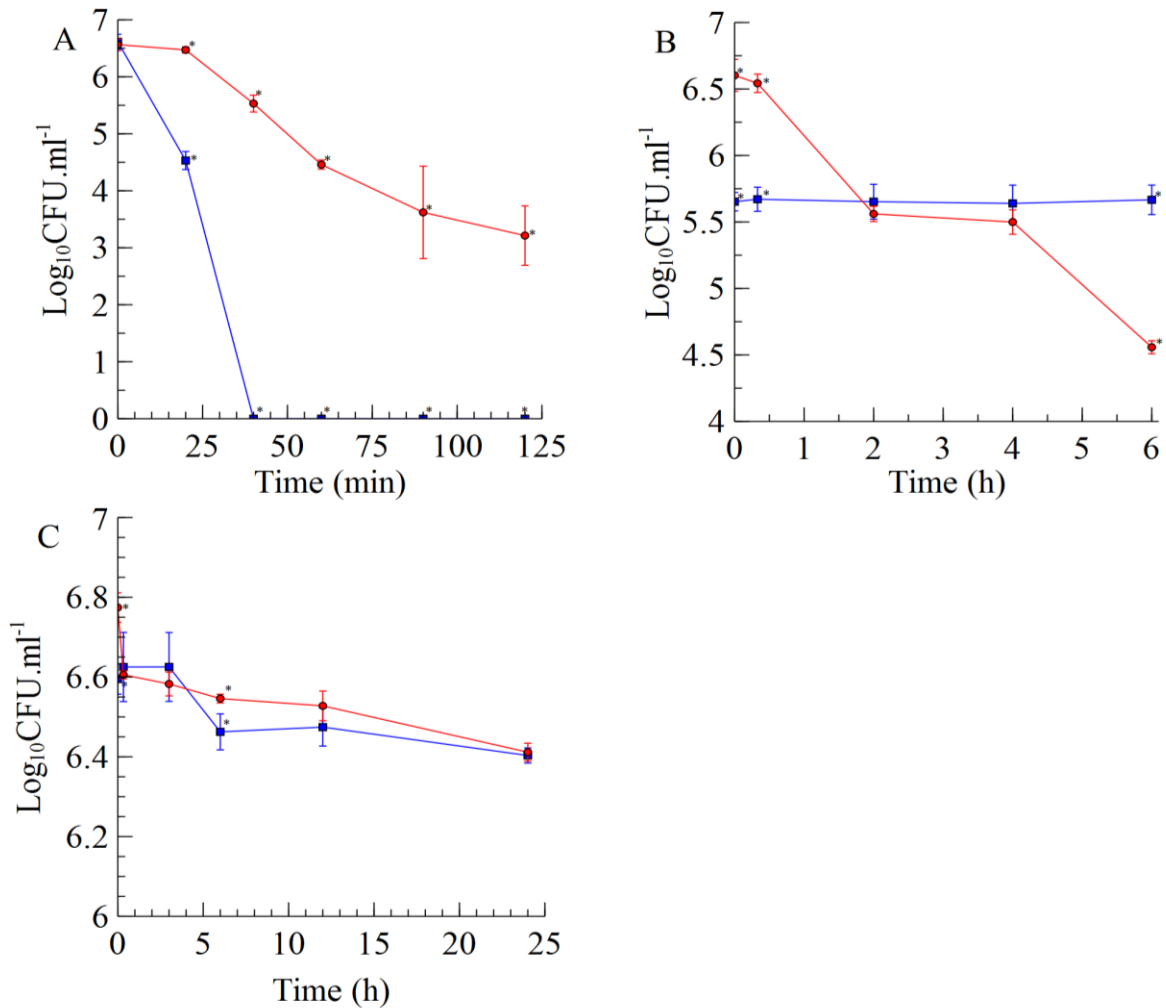
Figure 3.2. S**urvival of planktonic** *Lactobacillus reuteri* **HFI-LD5 (●, red line) and** *Lactobacillus rhamnosus* **HFI-K2 (■, blue line) in fasting-state simulated gastrointestinal fluids.** **(A)** Fasting state simulated gastric fluid, pH 2.0 for 120 min; **(B)** Fasting state simulated intestinal fluid, pH 7.5 for 6 h; **(C)** Simulated colonic fluid, pH 7.0 for 24 h. The data represent the means of triplicate experiments and the error bars indicate standard deviation. Significant differences between the two strains ($p < 0.05$) are indicated by *.

The subsequent introduction of SGF (pH 2.0) into the system at 123 h resulted in a notable and rapid decrease in biofilm metabolic activity (Figure 3.3). The metabolic stress induced by the acidic gastric fluid (pH 2.0) and the cessation of nutrient supply to the biofilm is clearly illustrated by the reduction in biofilm metabolic activity to approximately the same basal levels displayed by uninoculated growth medium (Figure 3.3). This curtailing of metabolic activity was accompanied by a sustained decline in pH and the loss of large amounts of biomass from the system, as was evident from the increasing effluent cell counts. Despite the loss of biofilm biomass, the effluent pH value never reached that of the SGF (pH 2.0), indicating that sufficient biomass remained within the system to confer buffering capacity (Figure 3.3).

The subsequent switch to SIF (pH 7.5) at 125 h (Figure 3.3) had no detectable effect on biofilm activity, while the number of viable cells in the effluent decreased, further corroborating the lack of biofilm metabolic activity observed. The introduction of SCoF (pH 7.0) at 131 h exhibited no effect on the metabolic activity of the biofilm, potentially reflecting a state of metabolic dormancy (Figure 3.4 and 3.5). The decrease in biofilm biomass as a result of the previous two treatments resulted in a rapid decrease in from pH 7.5 to 7.0, which was maintained until the introduction of recovery growth medium at 155 h. Initiation of the recovery phase elicited a prompt metabolic response from the surviving biofilm biomass within the system (Figure 3.3). The reintroduction of growth medium facilitated the regrowth of the lost biofilm biomass to the extent that the steady-state activity of the recovered biofilm exceeded the initial steady-state by 1.5-fold (Figure 3.3). The establishment of a higher steady-state was further corroborated by the effluent pH reaching pretreatment levels (pH 4.9) within 24 h of the reintroduction of growth medium (pH 5.5) and the sustained release of biofilm-derived planktonic cells to the effluent from 3 h post-treatment onwards (Figure 3.4 and 3.5).
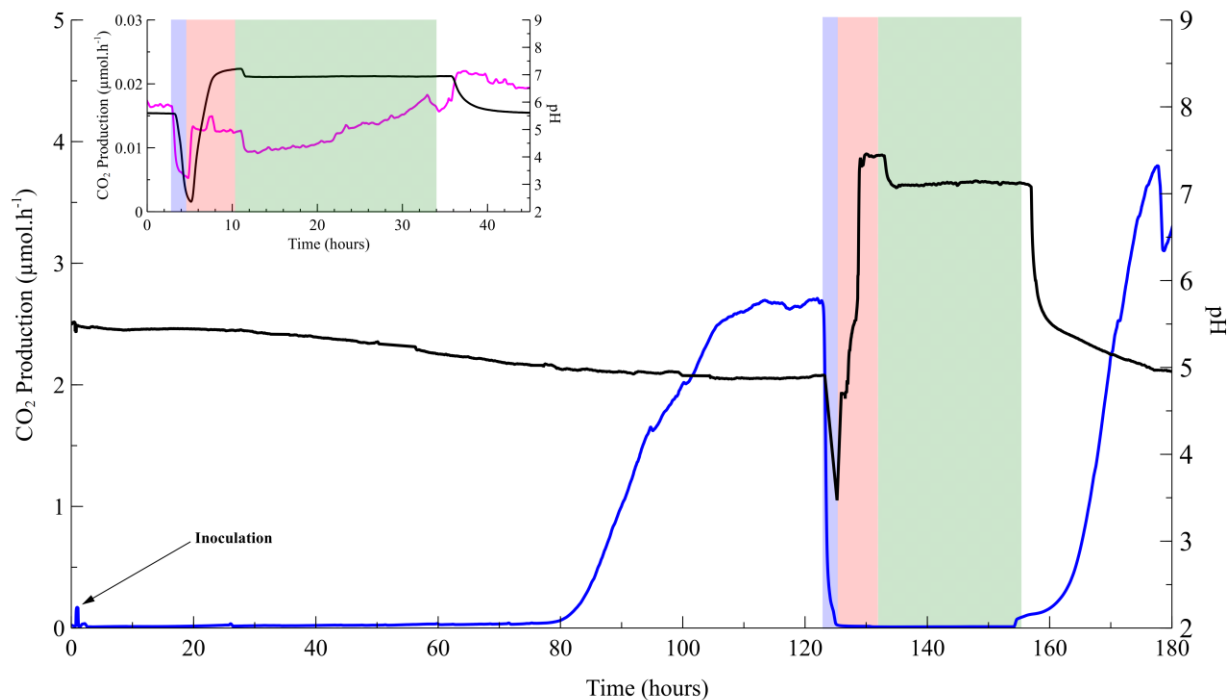
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Figure 3.3. Representative CO$_2$ production rates, changes in effluent pH and biofilm-derived cell numbers of *Lactobacillus rhamnosus* HFI-K2 biofilms in response to sequential exposure to simulated gastrointestinal fluids. The introduction of fasting-state simulated gastric fluid at 123 h (pH 2.0, 2 h in duration, purple shade), followed by fasting-state simulated intestinal fluid at 125 h (pH 7.5, 6 h in duration, pink shade); simulated colonic fluid at 131 h (pH 7.0, 24 h exposure interval, green shade); and the reintroduction of growth medium (pH 5.5, white shade). The solid black line indicates effluent pH, the blues lines represent *L. rhamnosus* HFI-K2 biofilm metabolic activity and the purple line indicates baseline CO$_2$ values for the uninoculated growth medium and various SGF. The resilience of *L. rhamnosus* HFI-K2 biofilms to perturbations in pH, osmolarity and enzyme concentrations after exposure to simulated gastrointestinal fluids is evident, with anew steady-state achieved after reintroduction of medium into the system. The nested graph illustrates that abiotic influences (evolution of CO$_2$ due to pH perturbations) are negligible.
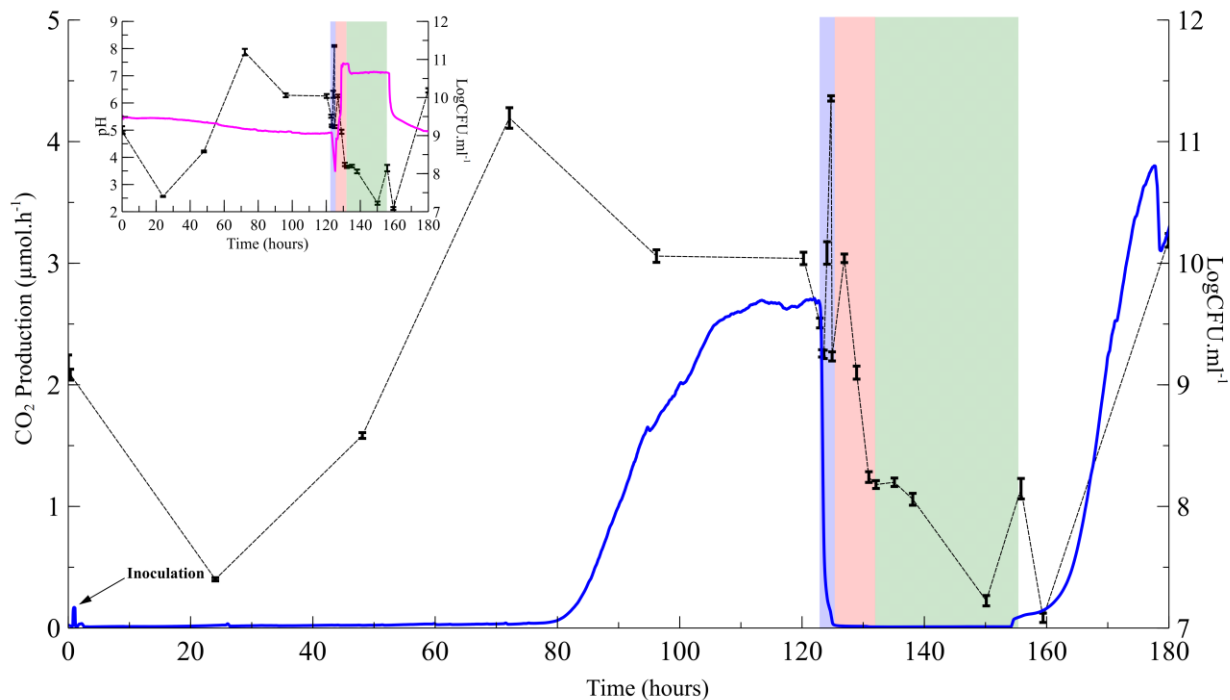
Figure 3.4. Representative CO₂ production rates and changes biofilm-derived cell numbers of *Lactobacillus rhamnosus* HFI-K2 biofilms in response to sequential exposure to simulated gastrointestinal fluids. The introduction of fasting-state simulated gastric fluid at 123 h (pH 2.0, 2 h in duration, purple shade), followed by fasting-state simulated intestinal fluid at 125 h (pH 7.5, 6 h in duration, pink shade); simulated colonic fluid at 131 h (pH 7.0, 24 h exposure interval, green shade); and the reintroduction of growth medium (pH 5.5, white shade). The dotted, black line indicates biofilm-derived cell, the blues lines represent *L. rhamnosus* HFI-K2 biofilm metabolic activity and the purple line in nested graph indicates pH changes. Biofilm-derived planktonic cell numbers enumerated from the CEMS effluent. The nested graph illustrates effluent cell number compared to perturbations in pH.

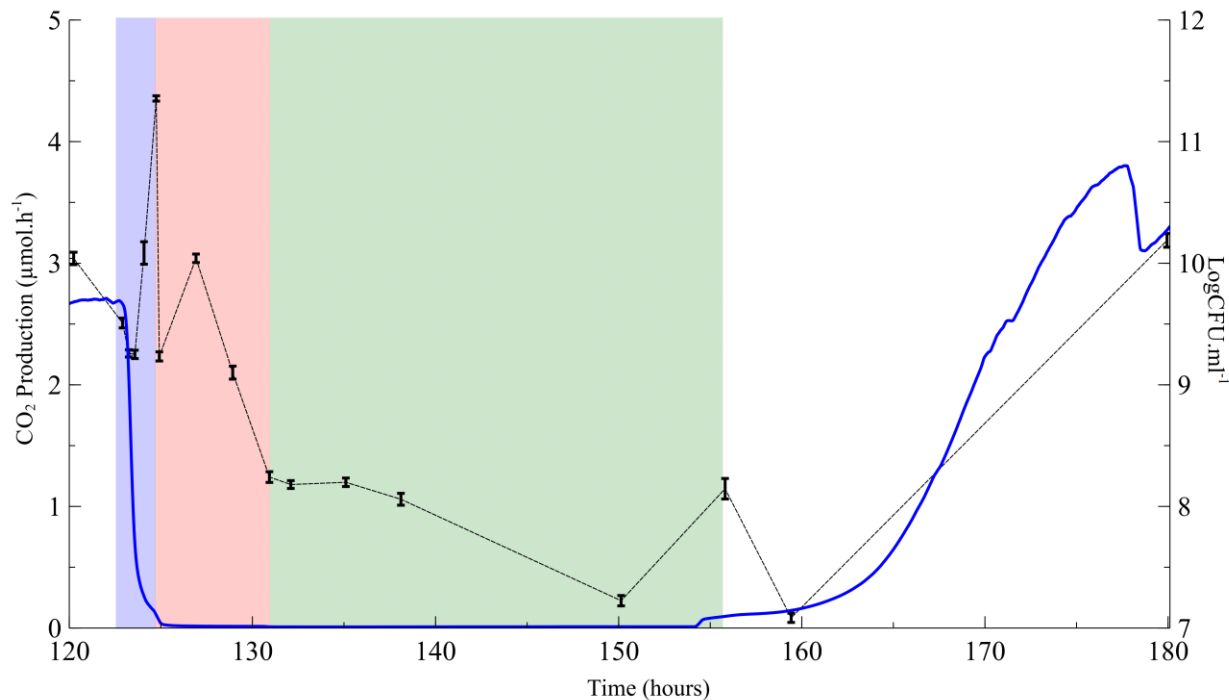Stellenbosch University https://scholar.sun.ac.za

Figure 3.5. Focused representative CO₂ production rates and changes biofilm-derived cell numbers of *Lactobacillus rhamnosus* HFI-K2 biofilms in response to sequential exposure to simulated gastrointestinal fluids during treatment phase. The introduction of fasting-state simulated gastric fluid at 123 h (pH 2.0, 2 h in duration, purple shade), followed by fasting-state simulated intestinal fluid at 125 h (pH 7.5, 6 h in duration, pink shade); simulated colonic fluid at 131 h (pH 7.0, 24 h exposure interval, green shade); and the reintroduction of growth medium (pH 5.5, white shade). The blues lines represent *L. rhamnosus* HFI-K2 biofilm metabolic activity and the dotted, black line represents cell numbers. A clear illustration of changes in cell numbers and metabolic activity during the treatment and recovery phase of *L. rhamnosus* HFI-K2.

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No detectable CO_2 was produced by *L. reuteri* HFI-LD5 during the initial 40 h post-inoculation period (Figure 3.6). A slight decrease in effluent pH as well as a two order of magnitude increase in biofilm-derived planktonic cell numbers released into the effluent (from 10^7 CFU.ml^{-1} to 10^9 CFU.ml^{-1}) (Figure 3.7 and 3.8) indicated that surface colonization had started. Thereafter, an exponential increase in biofilm CO_2 production rate was observed, which was associated with a further decrease in effluent pH. The biofilm reached an initial metabolic steady-state with 5.5 µmol CO_2 produced per hour after 60 h of incubation with an effluent pH of 5.0 (0.5 units lower than influent medium pH of 5.5).

Similar to the response of *L. rhamnosus* HFI-K2 biofilms, the introduction of SGF at 69 h resulted in an initial spike in metabolic activity, prior to a rapid and notable decrease owing to the metabolic stress induced by the SGF (Figure 3.6). A significant increase in the cell numbers released into the effluent was observed at 40 min after commencement of treatment (up to 10^{10} CFU.ml^{-1}), likely due to the detachment of biofilm biomass from within the system owing to the significant change of influent medium pH and the subsequent acid stress induced on the biofilm (Figure 3.7 and 3.8).

The ensuing introduction of SIF at 71 h, followed by SCoF at 77 h resulted in CO_2 detection rates similar to that observed for uninoculated growth medium. The effluent pH decreased and reached a minimum value of 2.7 after the 2 h of SGF exposure. The 0.7 pH unit difference observed compared to the influent SGF (pH 2.0) is attributable to the buffering capacity of the biomass within the system (Figure 3.6). During the subsequent exposure to SIF (pH 7.5) the effluent pH values detected downstream of the biofilm increased from pH 2.7 to 7.1. The decrease in pH during SIF treatment may again be credited to the remaining biomass within the system buffering the effect of the treatment (Figure 3.6). Despite not being able to detect biofilm activity throughout the treatment period (from 69 h to 101 h), viable biomass continued to detach from the biofilm (Figure 3.7 and 3.8). These viable cells are most likely the result of biomass detachment and not active biofilm-derived release of planktonic cells due to the absence of nutrients. Reintroduction of growth medium (pH 5.5) into the system subsequent to SGIF exposure at 101 h resulted in a prompt increase of biofilm metabolic activity and correlated with the release of more biofilm-derived planktonic cells into the effluent (Figure 3.7 and 3.8), with the re-establishment of the pre-

treatment steady-state metabolic activity within 10 h (Figure 3.7). This steady-state was disrupted by a sloughing event, followed by recovery and establishment of a new steady-state, with the new state being 1.2-fold higher than initial state (data not shown). The rapid response and recovery after the metabolic stresses induced by SGIF was accompanied by a decrease in pH from 5.5 to 5.0 within 5 h of resumption of growth medium, which occurred nearly 10-fold faster than during the establishment of the biofilm (pre-40 h). An increase in biofilm-derived planktonic cell production to a log unit higher than the initial steady-state value corroborated the rapid recovery of biofilm biomass after exposure to SGIF and the establishment of a higher steady-state metabolic activity.
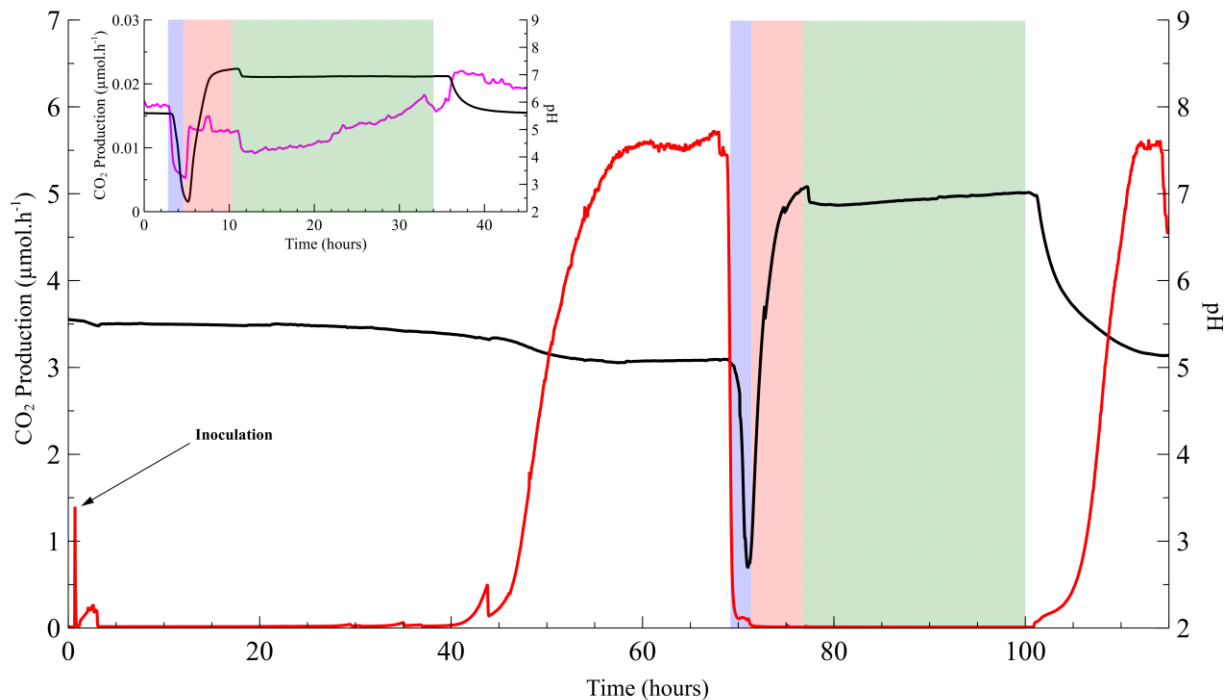
Figure 3.6. Representative CO_2 production rates by *Lactobacillus reuteri* HFI-LD5 biofilms and accompanying changes in effluent pH and culturable biofilm-derived cell numbers during biofilm establishment and subsequent exposure to simulated fasting-state gastrointestinal fluids (SGIF). The introduction of simulated gastric fluid at 69 h (pH 2.0, 2 h in duration, purple); followed by simulated intestinal fluid at 71 h (pH 7.5, 6 h in duration, pink); simulated colonic fluid at 77 h (pH 7.0, 24 h exposure interval, green); and growth medium (pH 5.5, white). The solid black lines indicate effluent pH, the red line represents the CO_2 production rate of the *L. reuteri* HFI-LD5 biofilm and the purple line indicates baseline CO_2 values for the uninoculated growth medium and various SGF. The results of one of the duplicate biofilm experiments are shown and are representative of the duplicate biofilm experiment. The response of the *L. reuteri* HFI-LD5 biofilm to the sequential introduction of SGIF is clearly visible in the main graph, with a new steady-state reached after recovery. The nested graph illustrates that pH perturbations had a negligible influence on CO_2 evolution in comparison to the biotic response.
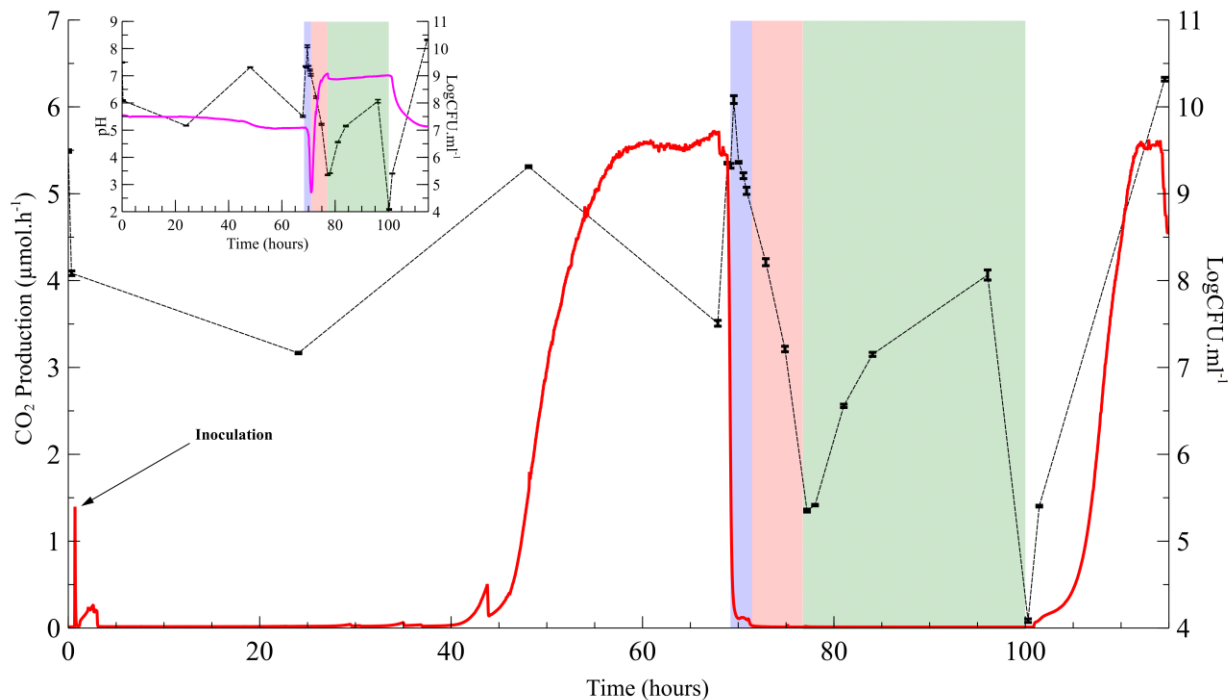
Figure 3.7. Representative CO₂ production rates by *Lactobacillus reuteri* HFI-LD5 biofilms and accompanying changes in effluent pH and culturable biofilm-derived cell numbers during biofilm establishment and subsequent exposure to simulated fasting-state gastrointestinal fluids (SGIF). The introduction of simulated gastric fluid at 69 h (pH 2.0, 2 h in duration, purple); followed by simulated intestinal fluid at 71 h (pH 7.5, 6 h in duration, pink); simulated colonic fluid at 77 h (pH 7.0, 24 h exposure interval, green); and growth medium (pH 5.5, white). The solid black lines indicate effluent pH, the red line represents the CO₂ production rate of the *L. reuteri* HFI-LD5 biofilm and the purple line indicates baseline CO₂ values for the uninoculated growth medium and various SGF. The results of one of the duplicate biofilm experiments are shown and are representative of the duplicate biofilm experiment. The correlation between biofilm development and an increase in biofilm-derived cells released from the system.
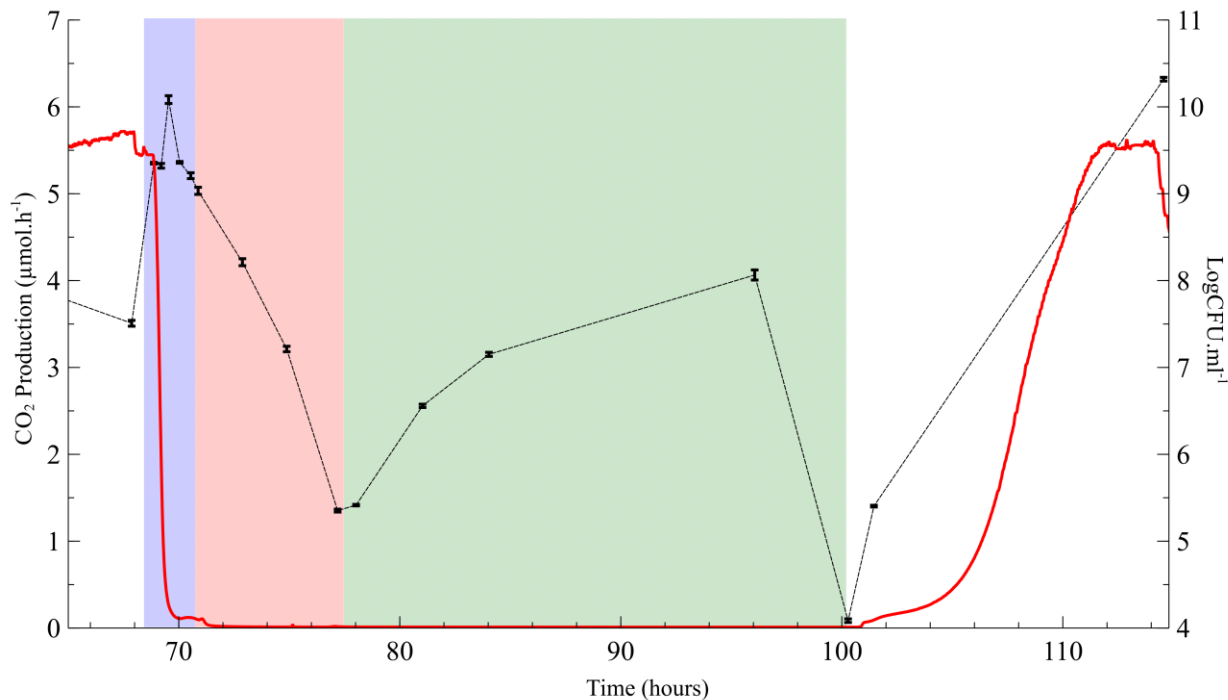
Figure 3.8. Focused representative CO$_2$ production rates by *Lactobacillus reuteri* HFI-LD5 biofilms and accompanying changes in effluent pH and culturable biofilm-derived cell numbers during biofilm establishment and subsequent exposure to simulated fasting-state gastrointestinal fluids (SGIF). The introduction of simulated gastric fluid at 69 h (pH 2.0, 2 h in duration, purple); followed by simulated intestinal fluid at 71 h (pH 7.5, 6 h in duration, pink); simulated colonic fluid at 77 h (pH 7.0, 24 h exposure interval, green); and growth medium (pH 5.5, white). The solid black lines indicate effluent pH, the red line represents the CO$_2$ production rate of the *L. reuteri* HFI-LD5 biofilm and the purple line indicates baseline CO$_2$ values for the uninoculated growth medium and various SGF. The results of one of the duplicate biofilm experiments are shown and are representative of the duplicate biofilm experiment. An expansion of the treatment and recovery phases provides a clear illustration of changes in effluent cell numbers and *L. reuteri* HFI-LD5 biofilm metabolic activity.

Similar to previous results, the effect of the highly acidic SGF on a *L. reuteri* HFI-LD5 biofilm was evident (Figure 3.9). A rapid decrease in metabolic activity from 7 µmol CO_2 .h^{-1} to 3.5 x 10^{-4} µmol CO_2.h^{-1} was detected within 5 min after the introduction of SGF to a 50-h old steady-state biofilm. A rapid and sustained increase in metabolic activity was observed upon the reintroduction of growth medium (pH 5.5) after the 2 h treatment period, with the establishment of a new steady-state within 6 h of cessation of SGF treatment (Figure 3.9). Biofilm-derived effluent cell numbers decreased from 1.4 x 10^{11} CFU.ml^{-1} pretreatment to 4.1 x 10^9 CFU.ml^{-1} post-treatment, followed by the recovery to pre-treatment values (10^{11} CFU.ml^{-1}) within 15 h (Figure 3.10 and 3.11).

Figure 3.9. Representative CO_2 production rates by a *L. reuteri* HFI-LD5 biofilm and accompanying changes in effluent pH and culturable biofilm-derived cell numbers during biofilm establishment and subsequent exposure to simulated fasting-state gastric fluid (SGF) for 2 h. Fasting-state simulated gastric fluid was introduced at 50 h (pH 2.0, 2 h in duration, purple) followed the reintroduction of growth medium (pH 5.5, white). Solid black lines indicate effluent pH, the red line represents the CO_2 production rate. The response of the *L. reuteri* HFI-LD5 biofilm to acidic SGF introduced at 50 h, followed by a recovery of the pre-exposure steady-state within 6 h after exposure to simulated gastric fluid.
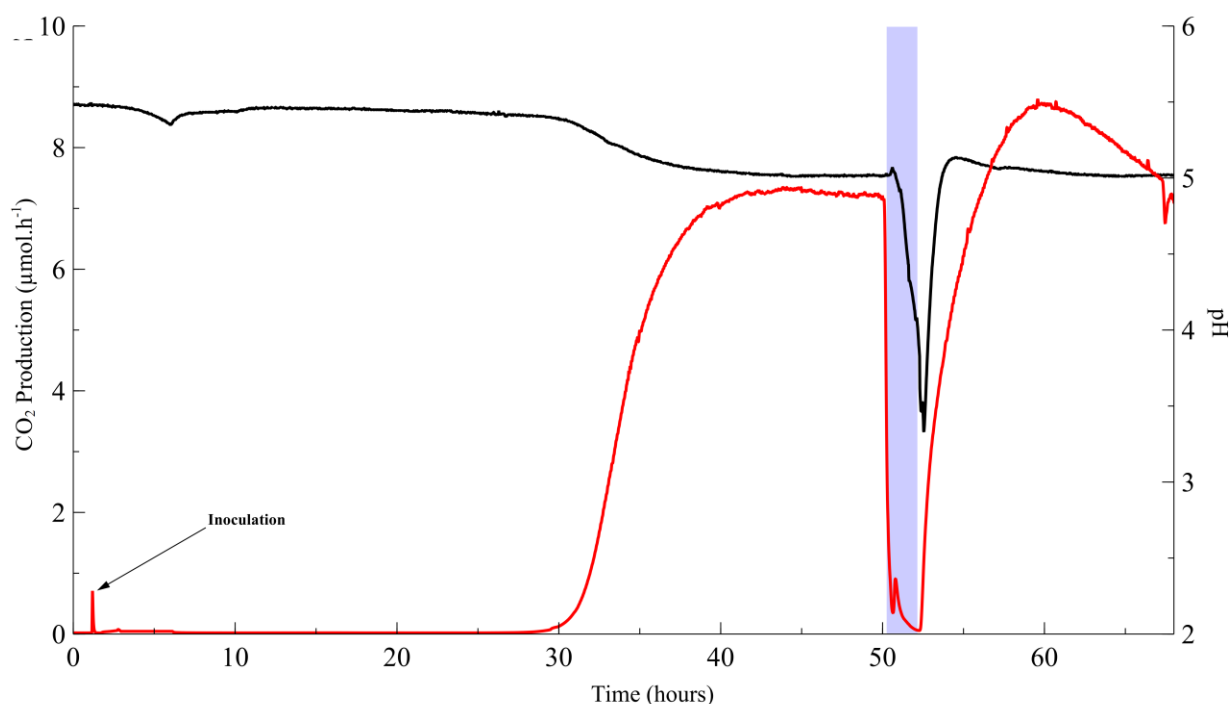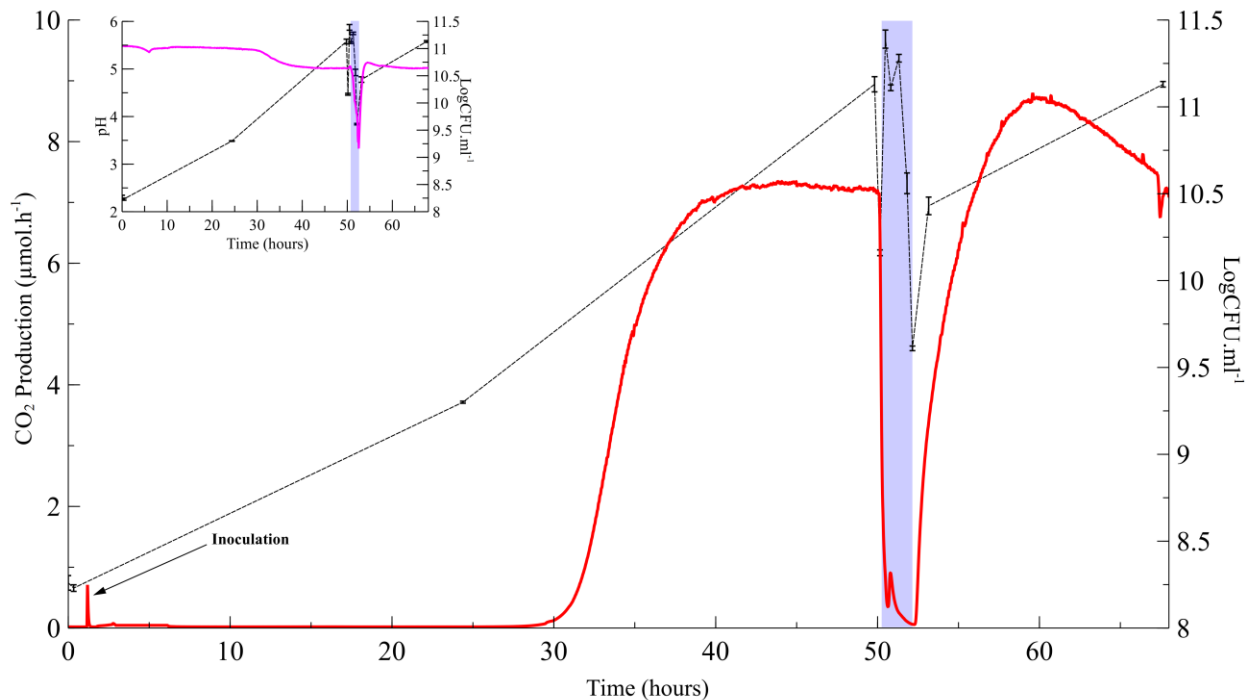
Figure 3.10. Representative CO$_2$ production rates by a *L. reuteri* HFI-LD5 biofilm and accompanying changes in effluent pH and culturable biofilm-derived cell numbers during biofilm establishment and subsequent exposure to simulated fasting-state gastric fluid (SGF) for 2 h. Fasting-state simulated gastric fluid was introduced at 50 h (pH 2.0, 2 h in duration, purple) followed the reintroduction of growth medium (pH 5.5, white). The red line represents the CO$_2$ production rate and the dotted, black line represents the biofilm derived cell numbers. Solid magenta line in nested graph indicate effluent pH. The correlation between biofilm development and an increase in biofilm-derived cells released from CEMS. During the treatment period, effluent cell numbers initially increased, most likely due to biomass detachment. After 60 min of exposure the cell numbers decreased prior to the reintroduction of growth medium (pH 5.5) whereupon a rapid increase in cell numbers was observed, which is further corroborated by the observed recovery of biofilm metabolic activity. The nested graph illustrates cell number compared to perturbations in pH.
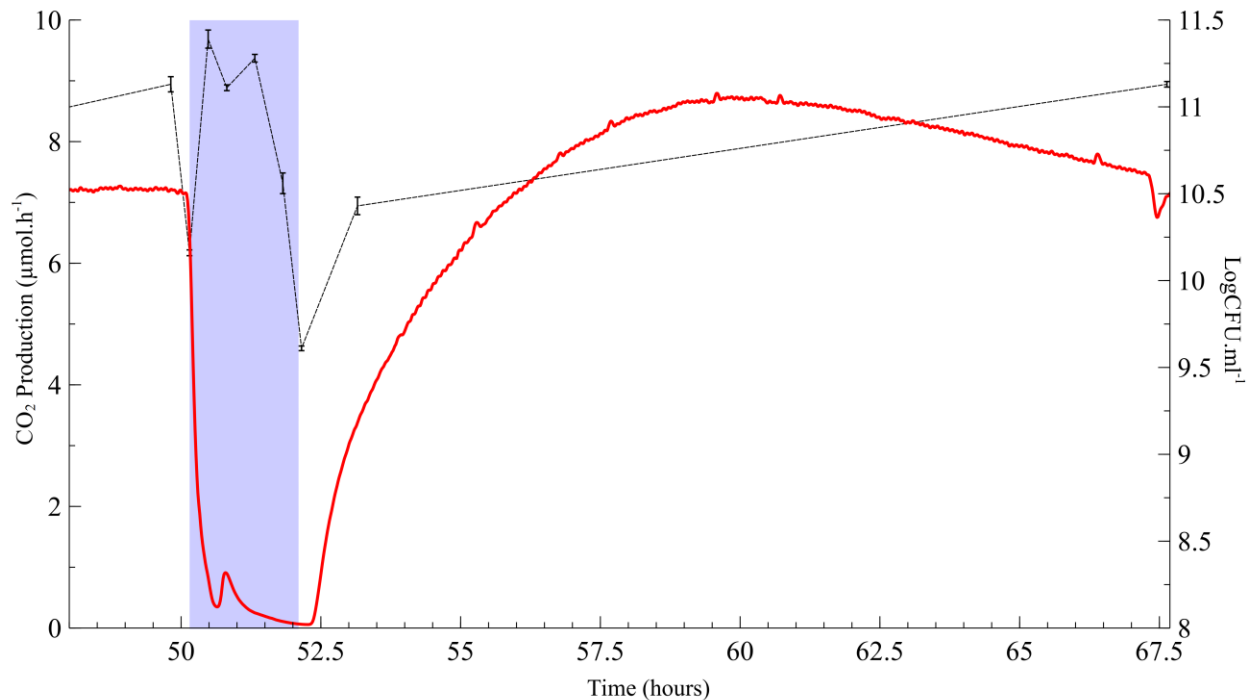
Figure 3.11. Focused representative CO_2 production rates by a *L. reuteri* HFI-LD5 biofilm and accompanying changes in culturable biofilm-derived cell numbers during biofilm establishment and subsequent exposure to simulated fasting-state gastric fluid (SGF) for 2 h. An expanded view of the treatment and recovery phases as seen in Figures 3.9 and 3.10. Fasting-state simulated gastric fluid was introduced at 50 h (pH 2.0, 2 h in duration, purple) followed the reintroduction of growth medium (pH 5.5, white). The red line represents the CO_2 production rate and the dotted, black line represents the biofilm derived cell numbers.

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3.5. Discussion

The capability of facultative heterofermentative lactic acid bacteria (LAB) to metabolize hexoses to lactic acid, CO_2 and ethanol/acetic acid in equimolar amounts is well-documented, whereas obligatory heterofermentative LAB metabolize both glucose and gluconate to the same metabolic end-products. This principle was exploited by monitoring CO_2 production as an indicator of biofilm metabolic activity (40). In addition to quantifying the CO_2 production rate, the in-line monitoring of effluent pH changes were indicative of organic acid production by the biofilm, thereby leaving only ethanol production unassessed (Figure 3.3, 3.6 and 3.9) (40).

A number of *Lactobacillus* spp. is considered intrinsically resistant to the harsh environmental conditions, notably the perturbations in pH, enzyme concentrations and osmolarity, that occur within the human GIT (41–43). These bacteria employ various adaptations to circumvent and mitigate the metabolic and physiological stresses induced by acidic conditions. The ability of lactobacilli to tolerate acidified environmental conditions is due to the maintenance of a constant pH gradient between the intra- and extra-cellular environment (41). This constant pH gradient is established and maintained through various processes such as expulsion of protons through the F1F0-ATPase, glutamate decarboxylase systems, alkalization of the external environment, generalized stress response proteins, alterations to cell surface composition and repair of proteins (5, 44, 45).

Although intrinsic resistance to GIT conditions may be present, the degree of such resistance varies among *Lactobacillus* spp., as is evident from this study. During exposure to SGF (pH 2.0, 2 h), planktonic cells of *L. reuteri* HFI-LD5 cultivated in batch exhibited significantly better tolerance to the prevailing acidic conditions and enzymatic stresses over the entire duration of exposure compared to the complete loss of viability exhibited by *L. rhamnosus* HFI-K2 within 40 min of treatment (Figure 3.2). The ability of *L. reuteri* strains to survive acidic conditions, and specifically gastric fluid, has been well documented (43, 45, 46). The survival and rapid recovery of *L. reuteri* HFI-LD5 biofilms following SGF exposure further illustrated its resilience to gastric fluid (Figure

3.6) and the potential for biofilm-association to enhance this persistence. *L. reuteri* strains are not only autochthonous to, but also the numerically dominant Lactobacillus species in the GIT of humans (47, 48). The autochthonous nature of L. reuteri strains, may therefore explain the significantly better survival of *L. reuteri* HFI-LD5 compared to *L. rhamnosus* HFI-K2.

The complete loss of planktonic *L. rhamnosus* HFI-K2 culturability in SGF is most likely due to the prevailing acidic conditions (Figure 3.2). *L. rhamnosus* strains exhibit a greater sensitivity to acid stress than to bile stress, with drastic reduction or complete loss of viability when exposed to pH values lower than 3.0 (49). *L. rhamnosus* strains are indigenous to the oral cavity (48) and considered to be allochthonous to the human GIT. The reduced tolerance of this *Lactobacillus* species to acidic conditions may thus be indicative of a lack of the appropriate environmental adaptations required for stomach colonization (50, 51). The human stomach is considered to be largely devoid of microorganisms during the fasting period between meals, with those microorganisms that are present being Gram-positive, aciduric bacteria (31, 52). It is therefore not surprising that bacterial biofilms within the healthy human stomach have yet to be described, although microcolonies (biofilms) have been noted in individuals suffering from Barrett's esophagus (52). Several studies relating to biofilms within the upper GITs of mammals and birds have been published (51, 53), which may allude to the presence of biofilms in the human stomach. This hypothesis is supported by the findings in this study where *L. rhamnosus* HFI-K2 was able to survive the full duration of SGF exposure when cultivated as a biofilm, in contrast to its inability to survive short periods of exposure when grown planktonically (Figure 3.2 and 3.3).

The near neutral pH and longer transit time within the small intestine renders this compartment more conducive to microbial colonization and proliferation. However, elevated bile levels in the small intestine may have detrimental effects on the bacteria entering this compartment, owing to its bacteriostatic action (15, 54). As previously mentioned, *L. reuteri* strains are considered to be autochthonous to the human GIT, specifically the intestinal tract (47, 51, 55), rendering it able to survive, proliferate and thus form stable populations under these environmental conditions. This accounts for the moderate reduction in planktonic *L. reuteri* HFI-LD5 viability during exposure to SIF (pH 7.5, 6 h) (Figure 3.2). The presence of bile and moderate duration of exposure to SIF had

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a minimal effect on the viability of free-floating *L. rhamnosus* HFI-K2 (Figure 3.2). This maintenance of viability is corroborated by other studies, wherein *L. rhamnosus* strains exhibited elevated tolerances to bile induced stress, even in the presence of 2 % bile (56, 57). Although *L. rhamnosus* is considered to be allochthonous in the human GIT, the pH of SIF is close to that of the oral cavity (pH 6.8) where *L. rhamnosus* is a resident species, thereby potentially explaining the stability with respect to viability observed (50, 58).

The human colon is considered to be the largest single reservoir of bacteria, both within the GIT and within the rest of the human body. This can be attributed to the properties inherent to the colonic environment, such as a near neutral pH (5.5 to 7.5), reduced bile concentrations, increased retention time, deep colonic crypts and abundance of energy sources in the form of non-digestible carbohydrates (37, 38, 59, 60). Autochthonous and allochthonous *Lactobacillus* species that survive the harsh conditions which prevail within the upper GIT are rapidly introduced into the ascending colon where these bacteria may colonize and subsequently proliferate (15, 47, 61). This is corroborated by the current observation regarding the survivability of planktonic *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 in SCoF (pH 7.5, 24 h) where a slight decrease in viability was observed (Figure 3.2). The lack of active proliferation within the SCoF can be attributed to the deficiency of nutrients owing to fasting-state experimental conditions being utilized.

Although biofilms in the human intestinal tract have been described, a significant number of questions remain, owing to the difficulty of sample acquisition and inadequacy of preparation methods (11, 30, 31, 52). It has long been thought that the rapid transit time of digesta through the upper GIT limits permanent colonization, and although this may be partially true for the stomach, this assumption may be disputed in the case of the small intestinal tract. Two distinctive microbial communities exist within the small intestine and the colon, namely those associated with either the mucosa or the lumen (11, 52). The former community is the most challenging to investigate and therefore least studied, as this is the interface between the mucosal surface and luminal contents where biofilm formation can occur. Various studies, although not all involving the human GIT, have described the formation of microcolonies and complex heterogeneous biofilm communities on and in the mucosa of the intestinal tract (30, 46, 52, 62). Swidsinski et al. (63) studied mucosal

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biopsies obtained from individuals with and without inflammatory gut disorders, and observed that individuals with inflammatory bowel disease had a mucosal biofilm mainly comprised of the Bacteroides/Prevotella group, whilst Gram-positive anaerobes dominated the mucosal biofilms in the non-inflamed group of individuals. Both *L. reuteri* and *L. rhamnosus* species have been observed to form microcolonies (biofilms) on the mucosa and in the mucus layers of the GIT, with biofilm formation and production of exopolysaccharides by *L. rhamnosus* GG being considered important attributes aiding GIT persistence (11, 32, 46, 64). These findings corroborate those presented here, wherein both Lactobacilli sp. were able to form *in vitro* biofilms that not only persisted, but also recovered rapidly after exposure to SGF.

Microbes contained within a biofilm express a phenotype notably different from their planktonic counterparts and are protected from harsh environmental conditions due to the EPS matrix that encases the cells and facilitates the establishment of microenvironments (29, 31). The preference for sessile growth by microbes may be due to the inherent structural nature of biofilms provided by a protective matrix consisting of EPS, cellular proteins and other cells, which renders a suitable environment for microbial proliferation and persistence (27). Cells growing in a multicellular environment would garner protection afforded by a sessile biofilm lifestyle, in addition to being exposed to symbiotic or competitive intra- or inter-species interactions (27, 65, 66). Antibiotic tolerance within biofilms is the most well studied example of the remarkable differences that exist between biofilm-associated cells and planktonic cells (29). In vitro biofilm susceptibility studies have shown that biofilms tolerate antibiotic concentrations 100 to 1000 times higher than planktonic cells (29). These biofilm-related adaptations allow microbes to persist and survive under harsh environmental conditions, including pH changes and nutrient deprivation, which are experienced within compartments of the human GIT (29, 31, 62). Biofilm formation is thus especially important for survival in harsh environments such as the GIT where cells are exposed to a variety of chemicals, and fluctuation in pH and shear forces (67). This preference for sessile growth is, however, at variance with planktonic growth, since the latter is considered important for colonizing new niches, despite potentially being at the cost of survival and persistence (27).

Although the CEMS has allowed for the non-destructive, real-time monitoring and quantification of LAB biofilm metabolic activity, it is limited in some respects. The requirement of employing thin-walled silicone tubing (hydrophobic) as adhesion surface for both biofilm formation and gas exchange, may influence or preclude adhesion of hydrophobic LAB (Figure 3.1). The small diameter of the tubing combined with the invasive nature of pH measurement probes furthermore renders in situ pH monitoring impractical, thus preventing monitoring of pH changes within the biofilm structure, and limiting measurement to the effluent. While CEMS as an analytical technique provides valuable insight into whole-biofilm metabolism, it does not lend itself to the direct on-line quantification of biofilm biomass but rather indirect monitoring of, for example, the number of cultivable cells released or detached from the biofilm.

To the best of our knowledge this is the first study to assess the effects of fasting-state gastrointestinal fluid on microbial biofilms rather than only planktonic cells. In accordance with existing literature, the robustness of bacterial biofilms compared to planktonically cultured bacteria was clearly observed in this study. Despite being subjected to nutrient deprived conditions simulating the fasting state between meals, significant pH fluctuations and enzymatic stresses, the respective lactobacilli biofilms were able to persist and recover, providing a unique insight into the differential survival response of these two LAB species compared to planktonic cells. These findings may lend credence to claims made by commercial probiotic manufacturers that their formulations persist within the human GIT, due to these formulations potentially giving rise to biofilms or integrating into existing biofilms within the GIT.

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Concluding Remarks

The rapid expansion of the probiotic field in the last two decades has provided some insight into the principles underpinning human gastrointestinal health. Although this understanding with respect to the immunological and physiological effects of probiotic supplementation on human health has ameliorated gastrointestinal dysfunction in many individuals, many unanswered questions remain.

As a proxy for survivability under human GIT conditions, classical probiotic screening methods test the ability to tolerate stresses induced by both the acidic nature and the presence of bile under planktonic growth conditions. This study has clearly shown that although classical planktonic screening parameters may identify preferential probiotic candidates for further study, the survival and persistence potential of these candidates under GIT conditions require more in-depth evaluation.

Despite the fact that the novel isolates identified in this study fulfilled the classical probiotic selection criteria, i.e. acid and bile tolerance, further examination of both *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 highlighted the need to study probiotics in a manner that best incorporates all of the physiological factors within the GIT, including physiologically relevant fluid composition and concentrations, as well as mode of growth. This was evident when planktonic suspensions of both isolates were exposed to simulated fasting-state gastrointestinal fluids; even though both strains were selected for tolerance to low pH environments, a differential survival response was observed. Based on this observation, it was expected that the exposure of biofilms of both strains to the simulated gastric fluids would result in the same response. However, in contrast to the planktonic response, biofilms of both strains survived and persisted despite exposure to the same harsh environmental conditions. This emphasizes the importance firstly of examining microorganisms under biologically relevant conditions, and secondly that the differential responses to stress conditions observed between planktonic and sessile bacterial populations extends to probiotic microorganisms.

The ability of probiotic microbes to tolerate and proliferate in the presence of bile is critical for survival and persistence within the human GIT, owing to its bacteriostatic properties. In this study the inhibitory effects of bile are illustrated, since the addition of bile salts to standard culture media (MRS) differentially affected the rate and extent of planktonic growth of both *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2, although only minor detrimental effects were observed. *L. rhamnosus* HFI-K2 was more susceptible to bile compared to *L. reuteri* HFI-LD5, which is characteristic of the *L. rhamnosus* spp. This observed susceptibility of *L. rhamnosus* HFI-K2 was however contradicted when the two strains were exposed to simulated fasting-state gastrointestinal fluids, specifically the simulated fasting-state intestinal fluid. The physiologically relevant concentration of bile present in the simulated fluid had a distinct effect on planktonic *L. reuteri* HFI-LD5 viability, with no observable effect on *L. rhamnosus* HFI-K2. However, when the two strains were allowed to form biofilms prior to simulated fasting-state gastrointestinal fluid exposure, the bacteriostatic effect of bile on these microorganisms appeared to be mitigated entirely. These contradictions may be due to the influence of media components, pH or to the actions of digestive enzymes. Regardless of the cause, this observation underlines the importance of examining probiotic response to stress conditions under different growth conditions and with specific experimental objectives in mind.

Probiotic research has always kept pace with the ever-evolving scientific research field, with the progression from classical microbiology to "omics" orientated microbiology in recent years. This constant evolution has allowed for the expansion of our knowledge in the fields of gastroenterology and highlighted the critical role gut homeostasis plays in the maintenance of human health. However, although probiotic research has been at the forefront of scientific research, some fundamental concepts have been overlooked and require investigation to complete our understanding of probiotic-host interactions. One of these fundamental concepts was partially addressed within this study, namely probiotic biofilm formation. The species and strain variability with regard to static lactobacilli biofilm formation was clearly illustrated. This, coupled to the differential effect of media composition on biofilm accumulation, emphasizes the requirement for strain and media specific evaluation of probiotic lactobacilli biofilm formation.

The transition from static lactobacilli biofilm cultivation to biofilm formation in a dynamic system, highlighted the lack of understanding with respect to lactobacilli biofilm development. The formation of static biofilms by both *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 in modified MRS (glucose replaced with gluconate) was minimal. However, during biofilm studies conducted under dynamic flow conditions (CEMS) using modified MRS, notable biofilm accumulation was observed and rapid recovery in biofilm activity was recorded after exposure to detrimental environmental conditions. This alludes to the requirement for probiotic (lactobacilli) biofilm studies to be conducted not only under static conditions, but also under dynamic flow conditions that better simulate the biotic environment.

One of the major outcomes derived from this study was the differential survival response observed between planktonic and sessile lactobacilli populations to stress conditions. The vast majority of probiotic gastrointestinal fluid survival studies is conducted on planktonic microbial cells and ignores the importance and prevalence of sessile microbial populations. The difference in survival in response to gastrointestinal fluid induced stress between planktonic and sessile populations of both *L. reuteri* HFI-LD5 and *L. rhamnosus* HFI-K2 was clearly observed in this study. This illustrates the fact that populations derived from genetically identical microorganisms are capable of exhibiting differential responses. It is well established that distinct epigenetic, phenotypic and population variations exist between biofilm and free-living populations of microbial cells, which may explain the dissimilar responses. Based on this and the understanding garnered in this study, the case for examining probiotic microorganisms as biofilms under dynamic flow conditions is made clear.

Despite the progress made with respect to our understanding of probiotics and their interaction with humans, a vast knowledge gap still exists. Probiotic microorganisms can no longer be seen as isolated, free-living cells but should rather be viewed in the context of multispecies biofilms, working in synergism to ensure survival and persistence within broader environments, and specifically within the human GIT. The need to reevaluate our experimental approaches to generate an understanding of probiotics in the context of sessile populations is clear, instead of simply extrapolating or forming assumptions based on planktonic studies.

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Expansion based on current study

To our knowledge, the current study was the first to examine probiotic (lactobacilli) biofilms under dynamic flow conditions and to assess the effects of simulated gastrointestinal fluids on the survival of biofilm-associated lactobacilli cells. A lack of original research studies in the field of probiotic biofilms has left many unanswered questions and as with any scientific study, this study has generated more questions than answers. It is however to be noted that the generation of new scientific questions and hypotheses, based on existing or newly generated scientific knowledge, is the cornerstone of science.

This study has laid the foundation for future investigations of probiotic biofilms and the mechanisms underlying lactobacilli biofilm formation and development. The strain-specific nature of static biofilm formation as seen in this study and in others, clearly indicates the need to understand the fundamentals of lactobacilli biofilm formation. This aspect could be expanded to investigate lactobacilli biofilm formation on different surfaces (abiotic and biotic), with different properties (hydrophobic versus hydrophilic) and further determining whether it correlates to cell surface hydrophobicity. This has particular relevance to increasing biofilm formation under experimental conditions and reducing biofilm formation in industry, where it may be a significant problem.

Structural analysis of lactobacilli biofilms should be assessed utilizing confocal laser scanning microscopy in conjunction with fluorescent stains or integrated proteins, for example. This avenue was partially explored during the course of this study, but required extensive optimization owing to a lack of knowledge around lactobacilli biofilms and attention was rather focused on the optimization of the CEMS system to monitor lactobacilli biofilm metabolism. Microscopic analysis of these biofilms could provide greater insight into biofilm form and function, as well as response to simulated gastric fluids.

The presence of oxygen gradients in various GIT regions, along with the ability of most lactobacilli to grow in the absence or presence of oxygen, has led to the question of whether lactobacilli biofilms cultured under anaerobic, microaerophilic and aerobic environmental conditions would

exhibit altered biofilm properties and thus different stress responses. The feasibility of establishing lactobacilli biofilms in CEMS under anaerobic conditions was established during the beginning of this study, however it was decided to focus on the microaerophilic setup to first achieve a reproducible system prior to changing environmental parameters. Therefore with the establishment of a reproducible system, achieved by in this studies these different environmental parameters can be adequately and reproducibly assessed in future studies.

Another aspect that needs to be adequately expanded on is the influence of media composition and individual media components on biofilm formation by lactobacilli. This expansion on the current work and that of others, will include investigating whether standard culture conditions, such as nutrient-rich MRS is conducive to biofilm formation, or whether nutrient deprivation is required. The role of carbon source and concentration and carbon to nitrogen ratio are critical aspects to consider, especially under stress conditions. It is also important to state that all of this fundamental biofilm work cannot be conducted solely using static biofilm systems, but should rather be done in conjunction with dynamic biofilm models, which provide a more realistic simulation of the natural environment.

The human GIT is always in one of two states, either that of being fed or that of being in a state of fasting. This study only evaluated the effects of fasting-state gastrointestinal conditions on planktonic and biofilm-associated lactobacilli, owing to this representing the most extreme environment that lactobacilli in the human GIT might experience. It is therefore necessary that the interactions and stresses induced by fed-state gastrointestinal conditions on lactobacilli strains be assessed both under planktonic and sessile conditions. The simulation of fed-state gastrointestinal conditions would need to be assessed in two manners, namely the increased physiochemical and enzymatic conditions as well as these changes in conjunction with the addition of nutritional components/complete meal such as those contained in parenteral nutrition. The evaluation of the effects of fed-state on lactobacilli survival *in vitro* under both planktonic and sessile conditions may better our understanding as to the survival and persistence of probiotic microorganisms within the human GIT.

The question of biofilm-mucus interaction still remains unanswered with respect to lactobacilli and probiotics in general. It is well established that microorganisms used as probiotic supplements should preferentially interact with mucin but not possess mucinolytic activity. The standard procedure, as was conducted within this study, assesses the mucinolytic activity of planktonic cells. However, this fails to take into account potential mucinolytic activity induced by attachment to mucin. The known differential response of planktonic and biofilm-associated cells underscores the requirement of evaluating mucinolytic activity under both growth conditions. Therefore future studies in the probiotic-biofilm field will need to investigate whether sessile populations of probiotic microbes are capable of degrading and utilizing mucin as a nutrient source and whether the presence of mucin influences biofilm formation by these microorganisms.

Even though a few basic studies relating to the field of probiotic biofilms have been published, these have done little more than to prove that lactobacilli spp. may form biofilms and emphasized the lack of scientific knowledge and understanding in the probiotic-biofilm field. This deficiency provides a novel field of scientific research, wherein classical and modern scientific approaches can be seamlessly integrated for the expansion of the probiotic and biofilm fields of scientific research. In return, a better understanding of the role of probiotics and probiotic biofilms in human health will be generated. It is hoped that this study lays the foundation for further studies into the fundamentals underpinning lactobacilli (probiotic) biofilm formation and to understanding commensal and probiotic interaction, survival and persistence within the human GIT.