Master's thesis

NTNU
Norwegian University of Science and Technology
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Department of Biotechnology and Food Science

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Microbial Therapeutics and Oral Delivery Systems

An Evaluation of Microencapsulation of Microbiota

Master's thesis in Nanotechnology
Supervisor: Berit Løkensgard Strand
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Abstract

The understanding of the role of microorganisms in human health and disease has rapidly expanded in recent years. The emerging field of microbial therapeutics refers to the notion of introducing exogenous microbes to the human body in order to establish a balance in the microbial communities of the host, thereby obtaining beneficial health effects. However, using microbes for therapeutic means has given rise to controversy in the scientific community. In particular, probiotics and its proclaimed health benefits suffer from much debate and questionable study results. Instead, approaches involving whole microbiotas, such as fecal microbiota transplantations (FMTs), are displaying impressive results in the treatment of certain diseases and disorders. Here, the use of *in vitro* cultivated gut microbiota offers a less expensive, more controlled, and possibly more consistent treatment than FMT. However, both FMT and *in vitro* cultivated microbiota rely on cumbersome endoscopic interventions to deliver the product, suggesting that there is a potential for developing novel delivery systems for such microbiota-based approaches, e.g. oral delivery.

In the present work, a critical review of microbial therapeutics is provided, which addresses the controversy in probiotic research in relation to microbiota-based therapies. In addition, the possibilities for oral delivery systems of microorganisms are explored, focusing on microencapsulation systems and delivering an overview of the hurdles for microbial delivery.

Next, an experimental study was conducted on a putative oral delivery microencapsulation system for the *in vitro* cultivated microbiota product 'Anaerobically Cultivated Human Intestinal Microbiota' (ACHIM). The ACHIM bacteria were successfully immobilized in Ca-alginate microbeads, manifesting the first-ever microencapsulation of a microbiota-based product. The alginate-bacteria beads were exposed to simulated gastrointestinal (GI) conditions and a fluorescent viability assay was used to address the viability of the entrapped bacteria. Following sequential exposure to a simulated gastric fluid (SGF) and a simulated intestinal fluid (SIF), the beads were found to stay intact during gastric exposure but rapidly dissolved in a matter of seconds to minutes in SIF, indicating potential targeted delivery to the intestines. The viability data showed signs of bacterial cell loss in the low pH environment of SGF. However, there was variability in the viability data between similar experiments, suggesting that methodological issues such as varying degrees of exposure to oxygen between experiments could potentially have had significant impacts on the bacteria. In conclusion, the experimental study indicates a potential for applying microencapsulation technologies to next-generation microbiota-based products.

Sammendrag

Forståelsen av mikrobenes rolle i sykdom og helse har økt betydelig de siste årene. Mikrobiell terapi er et voksende fagfelt som sikter mot å bruke mikroorganismer for å gi gunstige helseeffekter ved å sørge for balanserte mikrobielle miljøer på og i menneskekroppen. Bruken av mikrober for terapi har ført til mange diskusjoner i det vitenskapelige samfunnet, spesielt når det gjeler effekten av probiotiske produkter. Metoder som benytter seg av fullstendige mikrobiotaer, som for eksempel fekal mikrobiota transplantasjon (FMT), viser derimot sterke indikasjoner på å gi terapeutiske effekter i visse sykdommer og lidelser. Her kan bruken av *in vitro* kultivert tarm-mikrobiota tilby en billigere, mer kontrollert og muligens mer konsekvent behandling enn FMT. Likevel baserer både dagens FMT og mikrobiota-baserte tilnærminger seg på endoskopi, som er en tungvint måte å levere produktene på. Derfor burde det være motivasjon og muligheter for å utvikle nye leveringsmetoder for slike prosedyrer, som for eksempel oral levering.

I det følgende arbeidet presenteres en kritisk gjennomgang av den vitenskapelige litteraturen på mikrobiell terapi, hvor kontroversen rundt probiotika adresseres i forhold til mikrobiota-baserte terapier. I tillegg utforskes mulighetene for orale leveringssystemer av mikrober, og det gis en oversikt over noen av hindringene som finnes for mikrobiell levering.

Det ble også utført en eksperimentell studie i dette arbeidet. Studien undersøker mulighetene for et oralt mikroinnkapslingssystem av et *in vitro* kultivert mikrobiota-produkt kalt «Anaerobically Cultivated Human Intestinal Microbiota» (AHCIM). ACHIM-bakterier ble immobilisert i mikrokuler av Ca-alginat, og det vises til første gangen et mikrobiota-basert produkt har blitt mikroinnkapslet. Alginatkulene med bakterier ble eksponert for simulerte mage- og tarm-omgivelser, og en fluorescens-basert levedyktighetsanalyse ble brukt for å vise til levedyktigheten til de innkapslede bakteriene. Det ble vist at alginatkulene muligens kunne tilby målrettet levering til tynntarmen ettersom at kulene holdt seg intakte i en simulert magesyrevæske (SGF), og at de gikk i oppløsning etterfulgt av eksponering til en simulert tarmvæske (SIF). Levedyktighetsdataene indikerte at noen bakterier døde i SGF. Likevel var det variabilitet i levedyktighetstallene mellom like eksperimenter, noe som kunne antyde at metodiske problemer, som for eksempel ulik eksponering for oksygen mellom eksperimentene, kunne føre til signifikante innvirkninger på bakteriene. Det ble konkludert med at det finnes potensielle bruksområder for mikroinnkapslingssystemer for utviklingen av neste-generasjons mikrobiota-baserte produkter.

Preface

The following work is based on my own idea, including the experimental study presented in this thesis, which was conducted at the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU) in Trondheim. Unfortunately, due to the events of the COVID-19 pandemic, further experimental work was limited into the spring of 2020. However, effort was put into altering the structure and scope of the thesis in order to dive deeper into the literature and provide a thorough review on the research related to the experimental study.

I would like to thank my supervising Professor, Berit Løkensgard Strand, for accepting my proposal and helping me in the development and execution of the project together with her research team. Also, my gratitude extends to NIM Supplement AS (former ACHIM Biotherapeutics AS) who generously provided the cultivated microbiota used in the experimental study and assisted with guidance.

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Abbreviations

ACHIM	Anaerobically Cultivated Human Intestinal Microbiota
B.	*Bifidobacterium*
C.	*Clostridioides*
CAP	Cellulose Acetate Phthalate
CFU	Colony-Forming Unit
CLSM	Confocal Laser Scanning Microscopy
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FMT	Fecal Microbiota Transplantation
GI	Gastrointestinal
GRAS	Generally Recognized As Safe
IBD	Inflammatory Bowel Disorder
IBS	Irritable Bowel Syndrome
ISAPP	International Scientific Association for Probiotics and Prebiotics
L.	*Lactobacillus*
LPS	Lipopolysaccharide
MAMP	Microbe-Associated Molecular Pattern
NLR	NOD-Like Receptor
PRR	Pattern Recognition Receptor
ROS	Reactive Oxygen Species
SCFA	Short-chain fatty acid
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
TLR	Toll-Like Receptor

1 Background

The human gastrointestinal (GI) tract is inhabited by multiple different microorganisms, including bacteria, archaea, viruses and fungi. The gut microbiota encompasses all the microbial products and microorganisms that live in the GI tract, which is known to influence essential host processes, including nutrition, immunity, hormone activity, gut permeability, and neurochemistry (1). The role of the gut microbiota in health and disease is rapidly expanding. As a result, there is no agreed definition of a healthy – often called eubiotic – gut microbiota, but high microbial diversity has been associated with an eubiotic state (2).

Perturbations in the composition of bacteria in the gut (dysbiosis) is associated with a number of disorders and diseases (3). The field of probiotics aims to address dysbiotic microbial environments and achieve beneficial health effects following introduction of exogenous microbes to the body. However, probiotic research is suffering from much controversy due to conflicting clinical results and emerging insights into the immense complexity of the gut microbiota (4). Instead, approaches involving a whole microbiota, e.g. transplantation of human feces to reestablish a beneficial gut environment, have shown promising results in patients with different diseases and disorders (5). Here, the use of *in vitro* cultivated gut microbiota offers a less expensive, more controlled, and possibly more consistent treatment than the traditional fecal microbiota transplantation (FMT) (6).

Various oral delivery systems exist for probiotic bacteria, whose purpose is to improve the viability and possibly efficacy of probiotics in food products and supplements. Here, microencapsulation has been suggested in order to form a controlled microenvironment depending on the encapsulation material and technique, as well as allowing for targeted delivery to specific locations in the GI tract (7). Consequently, microencapsulation technologies might offer improved viability and precise delivery of probiotics for numerous applications within foods or pharmaceutics. Still, probiotics as therapeutic agents remain controversial, implying that improving their efficacy by encapsulation technologies have its limitations. Alternatively, developing similar oral delivery systems for cultivated microbiota is of interest in order to discover next-generation microbial products and permit more frequent deliveries in microbiota-based treatments. In addition, oral delivery of microbiota-based products will avoid the currently used delivery method of endoscopy, which is expensive, time-consuming, and uncomfortable for the patient.

1.1 Specific aims

The goal of this project can be divided into two specific aims:
1) Provide a review on the field of probiotics in relation to microbiota-based approaches and address the controversy in probiotic research. In addition, investigate oral delivery systems, focusing on microencapsulation systems, and provide an overview of the hurdles in microbial delivery and commonly used delivery systems.
2) Perform a preliminary experimental study on microencapsulation of an *in vitro* cultivated human intestinal microbiota in alginate beads and explore the possibilities for a novel oral delivery system for microbiota-based products.

2 Review

2.1 Microbial therapeutics

The concept of introducing live microorganisms as a means of inducing health benefits has intrigued humans for centuries. In the middle of the 20th century, the term 'probiotic' arose as an antonym to 'antibiotic' when studying microorganisms that produced growth-promoting factors for other microorganisms (8). In more recent years, with the rise of the omics era and genetic sequencing technologies, the field of microbiome research has emerged as a potential paradigm shift within biomedicine and elucidated the role of microbes in health and disease (9). For instance, large study cohorts such as the integrative human microbiome project (HMP) has carried out discoveries that link interactions between humans and their microbiomes (10), i.e. the collection of microorganisms in and on the human body. Consequently, the potential of using microbes for therapeutic means has never been more noticeable, yet has it perhaps never been more controversial.

2.1.1 Probiotics

Probiotics are normally defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (11). Despite this seemingly straightforward definition, the term 'probiotics' has been subject to much debate, particularly in the question of its health benefits. Due to a lack of consensus and unfortunate misusages of the term 'probiotics', an expert panel from the nonprofit collaboration of scientists called the International Scientific Association for Probiotics and Prebiotics (ISAPP) suggested in 2014 a more detailed scope of probiotics in order to clarify a probiotic framework and provide guidance for stakeholders (12).

The recommended probiotic framework presented by ISAPP included a number of microbial applications in foods and drugs, and ultimately concluded that labelling a product a 'probiotic' should be substantiated by extensive research on the microorganisms of interest and their ability to confer a health benefit on the host. For this reason, undefined consortia of microbes, such as fecal microbiota transplants (FMTs) and fermented foods with undefined microbial content, were not included in the probiotic framework. Nevertheless, the panel did acknowledge that well-defined mixtures of microbes, such as the stool substitute preparation RePoopulate (later MET) (13), indeed met the criteria of a probiotic. However, to avoid discussing technicalities, FMT and the more general concept of microbiota-based therapy will not be considered as 'probiotics' going forward. In this thesis, 'probiotics' will be regarded as approaches where a narrow selection of well-defined microbes, often only a single bacterial strain, is utilized for prophylactic or therapeutic means (Figure 1).

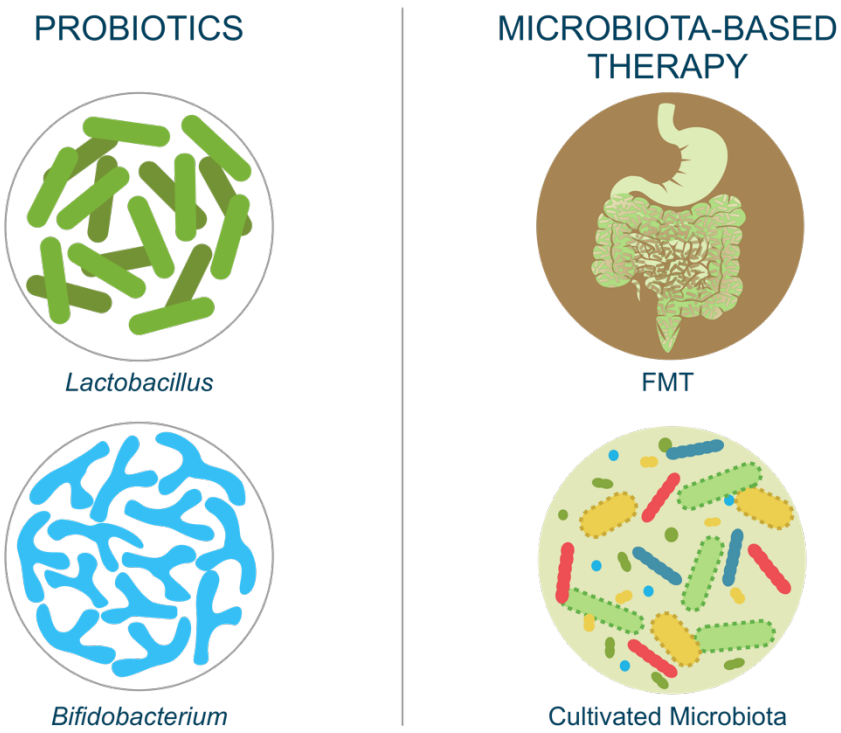

Figure 1. Illustration of the difference between the two main approaches within microbial therapeutics, as regarded in this thesis. 'Probiotics' denotes approaches where a narrow selection of microorganisms (e.g. strains from *Lactobacillus* or *Bifidobacterium*) is utilized for prophylactic or therapeutic means, while 'microbiota-based therapy' refers to all microbial therapies that derive from a microbiota, often with minimal manipulation, such as fecal microbiota transplantation (FMT) or an *in vitro* cultivated microbiota.

2.1.1.1 Conventional probiotics

The probiotic industry is a major international business and a growing market expected to reach a global value of USD 77.09 billion by 2025 (14). There are a number of different probiotic products on the market, many of which are developed for use in animal feed in order to boost growth and improve health. This review, however, will focus on probiotic products that are targeted for human consumption, such as supplements and functional foods. Here, conventional probiotic strategies include a phylogenetically limited diversity of bacteria and a few yeast strains. Commonly used probiotics include strains from the genera of *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Saccharomyces* (yeast), as well as the *E. coli* strain Nissle 1917 (15,16). Among these, *Lactobacillus rhamnosus GG* (LGG®) and *Bifidobacterium animalis ssp. lactis* BB-12 (BB-12®) are the two most studied probiotic strains, with more than 1000 scientific publications between them, of which about 500 are clinical studies (17,18).

Probiotics for human consumption are often included in dairy products like fermented milk, but probiotic supplements in the form of tablets or capsules are also common, especially on the American market (19). In any case, the supply chain of a commercial probiotic product is often complicated and involves multiple companies. First, the specific strain of interest is often trademarked by a supplier who provides preparations (starter-cultures) of the strain to a distributor. Then, the distributor manufactures a finished product containing the probiotics that can be bought by retailers and wholesalers. An overview of the major players who supplies and distributes probiotic products, strains, and technologies for protection and delivery of probiotics can be found in Table 1.

Table 1. Some companies on the probiotic market; examples of products, trademarks, and strains included in the products. (*L. = Lactobacillus*, *B. = Bifidobacterium*)

Company	*Role*	*Product*	*Trademark*	*Species (Strain)*	*Description*
Nestlé	Mainly distributor	Yogurts	ActiPlus®	*L. acidophilus*	Commercial products
		Supplements	ProNourish®	*B. animalis* subsp. *lactis* (BB-12)	Commercial products
Danone	Mainly distributor	Yogurts & smoothies	Activia® Actimel®	*B. animalis* subsp. *lactis* (DN-173 010), *L. casei* (DN-114 001), *L. Bulgaricus*, *Streptococcus Thermophilus*	Commercial products
Valio	Mainly distributor	Yogurts & kefirs	Gefilus®	*L. rhamnosus* (GG)	Commercial products
Yakult Honsha	Mainly distributor	Fermented milk	Yakult®	*L. casei* (Shirota)	Commercial products
Dupont	Mainly supplier	Probiotic cultures	HOWARU® FloraFIT®	Depends on country	Tailored cultures supplied to distributors
Chr. Hansen	Supplier only	Probiotic strains	BB-12® LA-5® LGG®	*B. animalis* subsp. *lactis* (BB-12) *L. acidophilus* (LA-5) *L. rhamnosus* (GG)	Strains supplied to distributors
		Tableting technology	PROBIO-TEC®	–	Tablet capable of including secondary ingredients (e.g. vitamins)
Probi	Supplier only	Probiotic strains	LP299V®	*L. plantarum* (299v)	Strains supplied to distributors
		Tableting technology	Bio-Tract®	–	Bi-layer release tablet for targeted delivery to the intestines

2.1.2 Microbiota-based therapy

The term 'microbiota-based therapy' is not commonly used in the literature. However, it can be defined as microbiota that, when introduced in adequate amounts, can confer a beneficial effect on the microbial ecosystem of the host and, expectantly, provide a health advantage. This definition differs from the definition of probiotics in that it reflects a more holistic view on the composition of microorganisms in the host and does not explicitly appreciate the effects of single strains as the main characteristic but, rather, the collective effect of the microbiota as a whole. In addition, the definition indirectly includes the health benefits of altering a microbiota, which is a question yet to be answered by microbiome research, as the cause and effect relationship of microbiota interventions on health has proven to be difficult to determine (20).

Today, microbiota-based approaches almost exclusively involve some sort of intervention of the gut microbiota, but one should not exclude the possibility of microbiotas in other locations in or on the human body to be the topic for future treatments, such as the vaginal tract, oral cavities or skin. In this review, 'microbiota-based therapy' will refer to all therapeutic approaches that derive from a microbiota, either directly or indirectly. Synonyms of the term 'microbiota-based therapy' that occur in the literature are 'microbiome therapy' and 'microbial ecosystems therapeutics' (21,22). However, neither of these terms explicitly state that the therapy is derived from a microbiota, meaning that probiotics could, in theory, be included under these alternative names. In addition, the 'microbiome' refers to the genes of the microbes of interest, i.e. the composition of the microorganisms, while 'microbiota' broadens the scope by also including microbial products and other components that may be of importance.

As previously discussed, microbiota-based therapy and probiotics are considered separate approaches within microbial therapeutics in this thesis (Figure 1). However, the general concept of altering a microbiota with another microbiota for therapeutic means is a relatively new thought in western medicine. Hence, microbiota-based therapy can be regarded as a constantly changing field. Establishing a consensus between stakeholders on the definition and guidelines of microbiota-based approaches has proven to be challenging, especially in the question of regulation, e.g. should procedures like FMT be considered a drug or a transplantation? The following will therefore be an attempt to provide an objective overview of the field and address the conflicts and some limitations with the different approaches.

2.1.2.1 Fecal microbiota transplantion (FMT)

FMT is the simplest form of microbiota-based therapy involving the gut. The procedure involves straightforward infusion of a healthy donor stool blend into the recipient's intestinal tract, typically performed by endoscopy, with the intent of restoring a normal function of the gut microbiota. FMT differs from defined consortia of microorganisms by utilizing minimally manipulated fecal material in order to achieve the high degree of complexity and functionality in a natural microbiota, which is difficult to replicate *in vitro*.

In many respects, the gut microbiome can be viewed as a microbial organ or tissue composed of complex microbial communities that have co-evolved with their human hosts (23), leading some scientists to deem FMT as a form of tissue transplantation (24). Still, many regulatory agencies in the world have categorized FMT as a drug, which can cause conflicts between researchers and regulators when applying for research funds and executing clinical trials. The regulatory frameworks that exist for drugs are essentially focused on molecules and have specified measures of molecular tests that a new drug

needs to overcome. Naturally, such requirements are not possible to apply on microorganisms living in a complex environment, which has caused the regulatory agencies to struggle in their classifications of procedures like FMT because no suitable regulations exist.

Even so, there have been attempts to establish methodologies that ensures the safety and optimizes the efficacy of an FMT procedure. Here, the so-called stool banks that can readily provide high-quality donor fecal preparations has become a business in some countries, where the US organization OpenBiome is a front-runner (25). However, the development of stool preparations must follow strict regulations and continuously perform thorough testing of their donors, which are even subject to change. For instance, there is some evidence that the SARS-CoV-2 virus causing the ongoing COVID-19 pandemic can be shed and therefore potentially transmitted via stool from both COVID-19 symptomatic and asymptomatic individuals (26,27). As the virus may remain dormant in the gut for up to several months after recovery from the disease (28), developing a stool test for the virus is crucial and will probably need to be standardized as part of the screening process for any future FMT donor samples, at least for the time being (29).

The term 'FMT' is extensively used in the literature, even though the approach of interest may not involve a direct fecal transplant. For example, when using cultivated microbes derived from feces, authors tend to coin the procedure a 'fecal microbiota transplantation', while a more accurate phrasing would be something like 'cultivated gut microbiota supplement'. The reason for these inaccurate phrasings of microbiota-based approaches is probably that FMT has become a popular treatment, thus making 'FMT' a familiar term that will draw the attention of more readers. However, as the field of microbiota-based therapy advances and expands into more complex approaches, accurately distinguishing between FMT and non-FMT approaches will become more important.

2.1.2.2 Cultivated gut microbiota

Although FMT has demonstrated remarkable results in the treatment of certain conditions (30), there are some limitations to the procedure when it comes to consistency and safety. Transferring human stool from healthy donors to sick patients comes with inherent risks. Hence, donors need to be carefully selected and tested to prevent any adverse effects. However, mistakes can occur, as was seen recently with an FMT containing multidrug-resistant bacteria that resulted in a patient death (31). For this reason, finding a substitute for stool mixtures is certainly of interest and has been attempted in various ways (6,13,32,33).

Cultivating the intestinal microbiota *in vitro* seems like an obvious solution to the problem. However, cultivating microbes outside of their natural environment is challenging, especially obligate anaerobes that are intolerant to oxygen and comprise the majority of the human gut microbiome (34). Consequently, the various attempts to create a microbial consortium that resembles the gut microbiota have had varying success. For example, isolating cultivatable species individually before formulating them into one product, as is the case for the aforementioned preparation RePoopulate (13), does not generate an ecosystem of microbes with the same environmental features as FMT. Accordingly, this lack of resemblance to a natural microbiota could explain why such an approach has not been fully implemented, i.e. the successor product to RePoopulate, Microbial Ecosystem Therapeutic (MET), is still undergoing relatively small trials (35,36).

On the other hand, the notion of recreating a microbial ecosystem could be attempted in a more simplistic way by cultivating a fecal sample anaerobically in a suitable culture medium. Here, the favorable outcomes are probably driven by more practical attributes such as the compatibility of the fecal sample with the culture medium and methodological steps of cultivation. Indeed, such a construct may be possible to achieve, as has been demonstrated by researchers in Sweden over the last few decades (6,37,38). Their culture, which has been dubbed 'anaerobically cultivated human intestinal microbiota' (ACHIM), has been confirmed in a number of patients to have a similar clinical effect as FMT (6,38). All in all, successful cultivation of human intestinal microbiota can be regarded as a next-generation FMT approach by exhibiting better overall control and improving treatment consistency.

2.1.3 Microbial therapeutics: mechanisms of acivity

The mechanisms of how microorganisms can assert health benefits are perhaps the most intriguing aspect of microbial therapeutics. Mechanistic studies have become a hot topic for the field in general, and they are crucial for providing better insight and predictability for microbial interventions, as well as enabling next-generation approaches. Currently, there exists a number of claims and theoretical effects that a microorganism or a microbial community can have when introduced to the human body. Some mechanisms are thought to be widespread across multiple microbial genera, while others might be rare and present in only a few strains of a given species (12). Unsurprisingly, most of the studied mechanisms focus on the gut microbial community, yet some effects can have systemic outcomes, including psychological effects through the gut-brain axis (39). It is important to acknowledge that the proposed mechanisms of activity can in theory apply to all microbial interventions regardless of the approach, but some effects are more likely to occur in microbiota-based approaches due to the sheer magnitude and diversity of microbes involved.

The human GI tract is home to some 40 to 100 trillion bacteria (40,41 p. 269), all competing for nutrients and survival but also cooperating and living in a symbiotic relationship with their host. Each part of the human GI tract comprises a distinct microbiome, contributing to the different physiologies of the gut, such as nutrition- and oxygen-profiles (42). Naturally, the current stage of microbiome research is far away from understanding the meticulous complexity of the microbiota, but much progress has been made in understanding some fundamental functions and mechanisms in recent years (43), unlocking new knowledge on how the microbiota is altered following introduction of exogenous microorganisms through approaches like FMT or probiotic supplements.

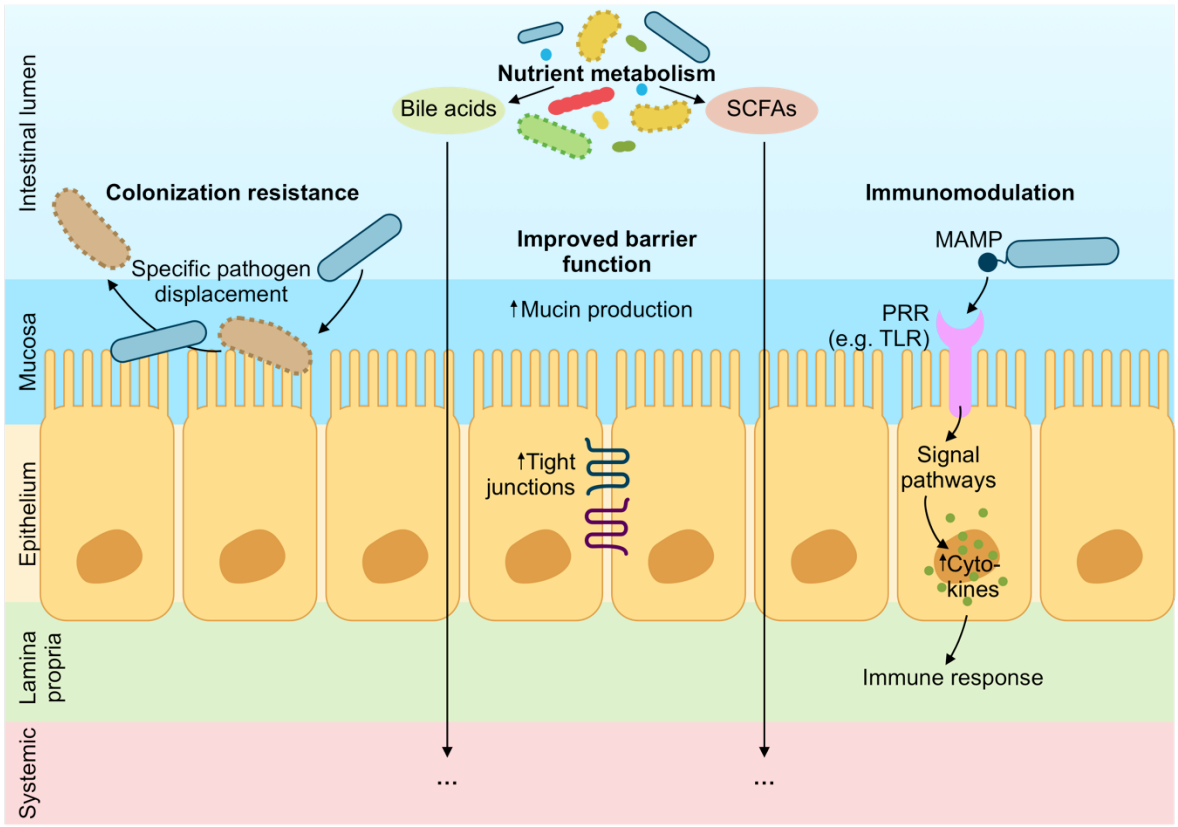

Figure 2. Some mechanistic interactions between microbes in the gut and the host. Colonization resistance is the sum of all factors inhibiting newcomers to be established (e.g. specific pathogen displacement). Improvement of the gut barrier function is mostly linked to signaling pathways upregulating mucin production and tight junctions. Microbial immunomodulation generally works by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) on the host cells, recognizing microbe-associated molecular patterns (MAMPs), which stimulates both innate and adaptive immune responses through an array of cytokine release. In addition, metabolic production of bile acids and short-chain fatty acids (SCFAs) can exert a number of effects on the host, including systemic outcomes.

2.1.3.1 Colonization resistance

One of the most commonly suggested modes of action of a microbial intervention is the competitive interactions of a eubiotic microbiota preventing the growth of any newcomers, including pathogenic bacteria. The effect is referred to as colonization resistance and can, in turn, be elucidated by a number of underlying mechanisms, several of which may act concurrently, making it sometimes difficult to accurately distinguish one underlying mechanism from another. However, specific pathogen displacement and the production of inhibitory substances are two examples that can be considered as separate mechanisms both contributing to the suppression of potential pathogens and maintaining eubiosis in the microbial community of the host.

Specific pathogen displacement describes the attachment of other bacteria to the epithelial cells of the intestinal wall, which will prevent specific pathogens to adhere and colonize in the gut (Figure 2). In other words, commensal bacteria essentially function as a physical barrier against potential pathogens in a normal gut flora. Therefore, introducing commensal bacteria in the case of a pathogenic infection, e.g. with FMT, could regain the effect of specific pathogen displacement, hence detaching and limiting the growth of the infectious bacteria. Thus, from a therapeutic perspective, one can imagine that specific pathogen displacement is a widespread effect across a broad range

of bacteria, i.e. as long as the introduced bacteria is able to colonize the gut properly, it will obstruct the growth of other, possibly pathogenic bacteria. On the other hand, one can also imagine that the effect is heavily dependent on the host, since the microbiota of the host must accept colonization of the infused microbes.

Another underlying mechanism that can explain how the competition between microbes hinder the establishment of other microbes is the secretion of inhibitory substances like bacteriocins. Bacteriocins are bacterially produced antimicrobial peptides that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) (44). For example, *Lactobacillus salivarius* strain UC118 secrete a bacteriocin called Abp118, which has broad spectrum activity against Gram-positive bacteria (45). Interestingly, pretreating mice with the *L. salivarius* strain was shown to protect against infection of the foodborne pathogen *Listeria monocytogene* (46), suggesting that bacteriocin-producing strains could be applicable in the prevention or treatment of certain infections.

Furthermore, other substances worth mentioning are secondary bile acids and short chain fatty acids (SCFAs), which can be produced by certain bacteria as metabolites. These compounds are involved in a number of host cell functions, including gene regulation and immune system function (47), but they can also affect the growth of bacteria. An interesting example is how the composition of bile-acids affect the spore germination of *Clostridioides difficile* (previously *Clostridium difficile*), an opportunistic pathogen in the gut that can become infectious following an antibiotic treatment. Standard treatment against *C. difficile* infection (CDI) involves additional antibiotics, but *C. difficile* is able to form spores that will withstand an antibiotic treatment. Instead, the antibiotics can inhibit other microbes responsible for secondary bile acid metabolism, thus disturbing the bile-acid composition in the gut, resulting in an appropriate environment for the spores of *C. difficile* to germinate and possibly cause recurrence of the infection (20,48). Put differently, the bile-acid metabolic bacteria in the gut are important for limiting the development of *C. difficile* spores and keeping a balanced gut environment. In the case of recurrent CDI, FMT is proving to be a far better alternative treatment than antibiotics (5), which could possibly partly be explained by the restoration of secondary bile-acid metabolic bacteria keeping the *C. difficile* spores at bay (48).

Altogether, the above mechanisms can contribute to normalizing a perturbed microbiota, which is in itself often the goal of microbiota-based treatments like FMT. However, normalizing the gut microbiota with FMT in diseases with more complex dysbiosis than a single pathogen infection, like irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD), has turned out to be more difficult to accomplish. Consequently, FMT studies have had varying success in the treatment of these disorders, which could possibly be explained by the varying degree of similarities in the gut microbiome of patients with the same diagnosis, ultimately affecting their susceptibility to an FMT procedure (49).

2.1.3.2 Improved gut barrier function

In addition to work competitively to limit the growth of newcomers in the gut, microbes can implement a number of signaling pathways to increase the gut's ability to isolate the intestinal environment from the bloodstream and the rest of the body. Translocation of bacteria across the epithelial cell wall frequently occurs even in a healthy gut, but the bacteria quickly dies in the high oxygen-levels of the bloodstream or is taken care of by the immune apparatus. However, a certain containment of the gut environment is

necessary, and it is achieved by well-functioning mucosal surfaces along the intestinal walls, as well as tight junctions (membrane proteins) holding adjacent epithelial cells together. A perturbed gut barrier function is characterized by degradation of mucosal membranes or a leaky epithelial barrier caused by compromised tight junctions. Consequently, microbial signaling pathways that improve the gut barrier function is mostly linked to the production or function of the mucosa and to the reinforcement or upregulation of tight junctions (Figure 2).

There are several studies that supposedly demonstrate improvement of the gut barrier function following a probiotic intervention (15,16). One example is a study by Ahl et al. (50) where a mouse model was used to show that two *Lactobacillus reuteri* strains could decrease intestinal inflammation, which was possibly explained by an increase of the mucosal thickness and upregulation of tight-junction proteins. Another study, also in mice, showed that administration of the *E. coli* strain Nissle 1917 upregulated the gene expression of tight-junction proteins, which resulted in reduced gut leakiness and enhanced mucosal integrity (51). However, there is a lack of evidence in humans on the effects of probiotics on the epithelial barrier function, as is the case for many mechanistic probiotic studies in general.

SCFAs, which are produced by gut microbes from ingested complex carbohydrates, are among the substances that can promote the epithelial barrier function. Butyrate, an abundant SCFA in the gut and important for energy contributions, has been shown to reduce the epithelial permeability by a number of mechanisms, including signaling pathways that reinforce the tight junctions and upregulate mucin glycoprotein production to increase the mucus layer thickness (52,53). In agreement with these findings, the proof-of-concept study by Geirnaert et al. (54) applied six butyrate-producing strains to Crohn's disease patient microbiota *in vitro*, which in turn caused an upregulation of mucus production in the epithelial cells.

It should be noted, however, that the respective amounts of the different SCFAs are decisive for their overall effect. For instance, increased levels of the SCFA propionic acid is associated with various neurological pathologies, including autism spectrum disorders (55). Interestingly, several studies have shown that injection of propionic acid to rats causes temporary autistic-like behavior along with altered biochemical and neurophysiological characteristics (56,57). Accordingly, since propionic acid is mainly produced by enteric microbes in the gut, both probiotic and FMT treatments have been suggested for the improvement of autism symptoms in human subjects (58), which have shown inconclusive results from probiotic studies but positive preliminary results from FMT (59,60). Although the FMT trials are few and in need of refinements, they suggest that beneficially altering the microbiome through microbiota-based approaches in diseased individuals can have systemic outcomes that reach beyond the gut.

2.1.3.3 Immunomodulation

Microbes in the gut play an essential role in stimulating and developing both the innate and adaptive immune system. Moreover, dysbiosis in the gut microbiota can cause immune dysregulation, leading to so-called autoimmune disorders (61), i.e. immune responses against host cells and tissues. In general, microbial immunomodulation works by the host cells recognizing certain traits of the microbiota, which causes a signaling cascade that will result in the production of cytokines and in turn activate the immune system (Figure 2). Microbial traits that can be recognized by host cells are conserved repetitive structures on microbes called microbe-associated molecular patterns (MAMPs),

such as flagellin or lipopolysaccharide (LPS). MAMPs are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) on the cell surface or NOD-like receptors (NLRs) in the cytoplasm, resulting in an array of cytokines to be released, which stimulates inflammation and activates adaptive immune mechanisms (41).

There are numerous downstream effects these signaling pathways can trigger. For instance, the observed link between obesity and intestinal dysbiosis can partly be elucidated by transportation of LPS from Gram-negative bacteria across the mucosal barrier that interact with a PRR called TLR4 (Toll-like receptor 4), which in turn stimulates inflammatory cytokines that can affect liver metabolism and promote weight gain (62). In addition, the SCFAs butyrate, propionic acid, and acetic acid, may all contribute to weight gaining factors by having effects on hunger, fat production, and inflammation, respectively (41).

Furthermore, there are several autoimmune diseases where the microbiome seems to play a significant role in the dysfunction of the immune system (61). Although more research is needed to find the exact molecular mechanisms of how microbes can lead to autoimmunity, preliminary studies are showing promising results on the relief of GI symptoms following microbiota-based treatments in some cases (63). For example, a recent study by Fretheim et al. (64) administered the aforementioned ACHIM microbiota culture to patients with the rheumatic disease systemic sclerosis. The first-in-man pilot study resulted in improvement of intestinal motility functions and altered microbiome compositions. The effects were possibly linked to the triggering of an adaptive immune response demonstrated by alterations in immunoglobulin coating patterns of certain intestinal bacteria.

Taken together, it is important to remember that none of the mechanisms presented above are mutually exclusive, meaning that they can all occur in parallel and even complement one another. For instance, improving the gut barrier function could be achieved by a healthier competitive environment of the microbiome, which in turn can lead to less activation of the immune system, ultimately resulting in a beneficial health effect. As such, understanding and relating the different interactions for specific diseases might be considered one of the biggest challenges for the field of microbial therapeutics going forward. Continued efforts to reveal the mechanistic intricacy of the microbiota is important for understanding the causality relationship between microbiota and disease and unlocking the full potential of microbial interventions.

2.1.4 Limitations of microbial therapeutics

2.1.4.1 Safety concerns

There are different types of theoretical risks related to the use of microbes for therapeutic means. Firstly, microbes may be responsible for infection through translocation, i.e. the passage from the digestive tract to extra-intestinal sites. As a probiotic is likely to die relatively quickly after entering the bloodstream and it is, in fact, often given to prevent translocation of pathogens from happening through various mechanisms (see section 2.1.3.2), the risk of any inflammatory responses as a result of translocation is considered low for most probiotic strains (65). Still, one should not rule out the possibility of certain strains being able to perform translocation into unwanted areas of the body or increase the translocation capability of other bacteria by disrupting the indigenous microbial ecosystem. Indeed, there have been a few reported cases of

widely used probiotic strains, such as *Lactobacillus rhamnosus GG* (LGG) and *Saccharomyces boulardii* (yeast), causing infection (66). However, it should be noted that most of the reported probiotic-related infections occur only in patients with risk factors, suggesting that, overall, the most commonly used probiotics are not likely to cause any acute adverse effects.

A second plausible safety concern of introducing exogenous microbes to the human body is the production of toxic or unwanted metabolites that can lead to a number of undesirable consequences. For example, the production of d-lactate from different lactic acid bacteria may in theory lead to the development of lactic acidosis in the early stages of life, especially in children with short bowel syndrome (66). In addition, d-lactate is essentially a useless compound for the human body as we are not able to digest or harvest any energy from it. Thus, although studies have so far shown little evidence of any unfavorable d-lactate-induced effects from probiotic administration (67), following the precautionary principle, it might be wise to avoid giving d-lactate producing probiotics to children under one year of age.

In addition to introducing toxic compounds, bacteria can also act as vectors for antibiotic resistant genes, which poses a major area of concern as genes can be transferred horizontally to the indigenous microbiome and, in turn, to potential pathogens. Plasmids with antibiotic resistant genes have been found in several probiotic strains belonging to the *Lactobacillus* and *Bifidobacterium* genera, and the transfer of such genes to commensal bacteria have been shown in animal models (66). In addition, the risk of antibiotic resistance transmission occurring in microbiota-based procedures like FMT is of great concern, since FMT holds less control of the exact microbial composition involved, even though extensive testing of the donor stool is required (31). In fact, the donor tests are usually targeted towards multi-drug resistant organisms, which might not detect resistant plasmids present in commensal bacteria capable of transferring to the microbiome of the recipient (68). Nevertheless, FMT is generally considered safe and there are even evidence that the procedure can help eradicate antibiotic-resistant genes in the microbiome of the recipient (69).

The long-term effects of microbial interventions in the gut issues yet another potential risk. The theoretical concern is linked to how a microbial intervention can introduce microbiota traits that can make the individual more susceptible to certain diseases. Such claims are difficult to prove, but there have been a number of studies, both in humans and in animals, suggesting that probiotics can result in a dysbiotic ecosystem configuration in the host, especially following an antibiotic treatment (4). This potential problem could be of lesser concern with FMT, as the procedure uses minimally manipulated microbial communities that are already optimized by co-evolution with their human host. However, there exist only limited long-term follow-up clinical data on FMT (3), hence the precise long-term effects of FMT and similar procedures remain to be established.

2.1.4.2 Conflicting research on probiotics

Interestingly, despite probiotic supplements having been extensively researched and commercially available for consumers for decades, neither the European Food Safety Authority (EFSA) nor the US Food and Drug Administration (FDA) have yet to approve any probiotic products with claims to prevent or treat health problems (16). Certainly, this could in part be explained by the lack of knowledge on the mechanisms of action of probiotics, but even with the seemingly large number of studies with positive results on

the use of probiotics one would come to think that at least a few strains should show clear evidence of being beneficial for human health. However, looking closer at the studies that are conducted with probiotics, several reasons come to mind as of why probiotic products remain controversial for their health benefits.

First of all, there is a high degree of heterogeneity in the design of probiotic studies. The number of patients involved in clinical trials, the types of probiotic strains used, and studies performed *in vitro* or in animal models instead of humans are all factors that differ between studies and ultimately affect the conclusive evidence of probiotics. Furthermore, clinical trials are not always of high quality, often lacking placebo controls and randomization, which decrease the credibility of the results. In any case, even among high-quality, placebo-controlled studies, different trials uncover conflicting putative benefits of probiotics (70,71). So, to counteract these methodological limitations and overcome underpowered findings, systematic reviews and meta-analyses are frequently performed to integrate the results from multiple studies. However, such tools may be susceptible to biases, such as the inclusion of outlier studies that dominate the collective results and obscure actual effects, or grouping studies testing unrelated supplemented microbes under the same umbrella (4). Consequently, even meta-analyses focusing on similar topics may conflict with one another (72,73).

Next, the disparity of studied strains poses yet another problem for probiotic research. The principal microorganisms used in the industry often originate from traditional fermented dairy products, which have long been assumed to have beneficial health effects based on old publications or cultural beliefs (66). With the increasing knowledge on health-associated mechanisms of action of microbial interventions, it becomes clear that many traits are species- or even strain-specific (74). As such, postulating that a single genus or species is exclusively beneficial for human health is an oversimplification. Accordingly, the immense complexity of human microbial ecosystems and the inter-individual variability signify that two subjects can respond differently to the same supplemented probiotic, even if the subjects have the same disease or disorder. In turn, this interpretation may partially explain the inconsistencies observed between probiotic studies.

2.1.5 Concluding remarks

Arguably, it could be that the field of probiotics has failed to fully appreciate the vast complexity of the human gut microbiota, which could in turn explain the inconsistent results observed between probiotic studies. Moreover, there is a high degree of variability in the gut microbiota between individuals, sometimes even for individuals diagnosed with the same disease or disorder. Thus, using a standardized probiotic or microbiota-based formula will affect each individual differently and may, therefore, not yield the same effect across all subjects. As more information about the microbiome and its role in health and disease is being revealed, the field of microbial therapeutics should seek to understand this relationship in order to provide better, more personalized microbial products, which are often referred to as ‘smart’ or ‘precision’ probiotics (75,76). Today, however, the best available microbial therapy approach seems to be microbiota-based treatments where the target microbiota is treated with an ecological perspective and not necessarily on a bacterial strain level.

2.2 Oral delivery systems

Oral delivery is the most preferred route of administration for any active substance or drug due to its convenience, cost-effectiveness, and high patient compliance (77). Pharmaceutical approaches to oral release formulations have been extensively investigated and developed for decades. Depending on the drug and application, oral delivery systems come in a variety of forms, ranging from matrix systems, where the drug is distributed throughout the whole unit, to reservoir (coated) systems, which comprise a drug-containing core enclosed within a distinct barrier coat (Figure 3). The system can consist of a single unit, like a whole tablet or capsule, or it could be a multi-unit (multiparticulate) dosage form where the drug is incorporated into several smaller particles (Figure 3), which are then often filled into capsules. Furthermore, the release profile of the system can be modified to improve the therapeutic efficacy by increasing the bioavailability or offering targeted delivery of the active substance. Here, delayed release refers to the drug being released at some time point after initial administration, while extended or sustained release denotes the drug being released over an extended period of time (78 p. 18).

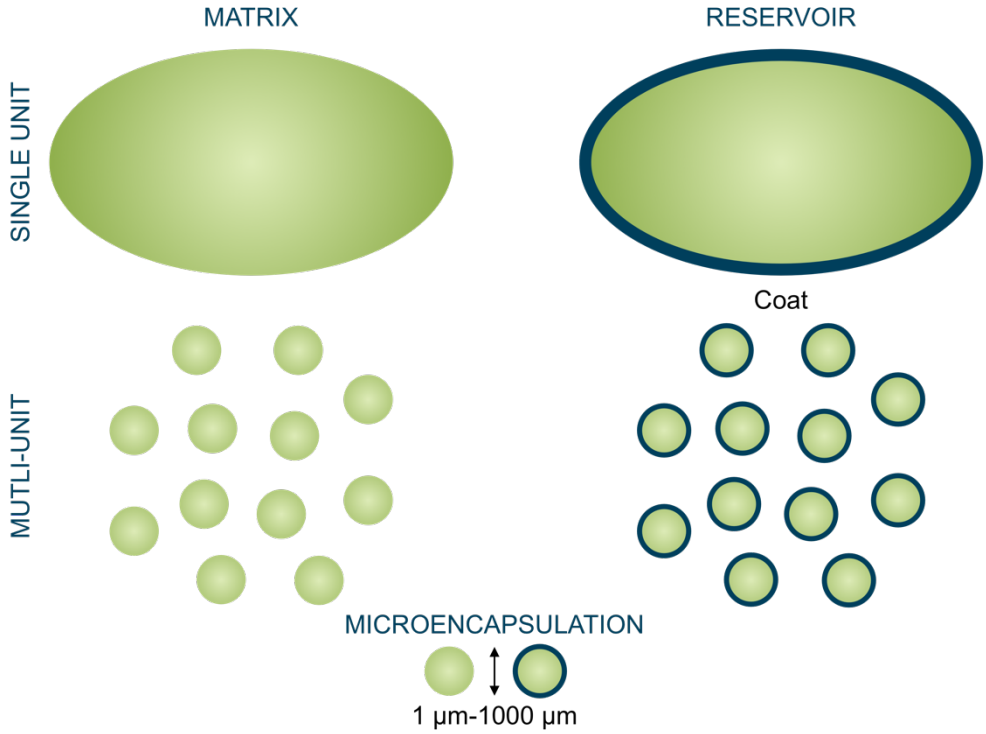

Figure 3. Some oral dosage forms. The active substance (e.g. drug or probiotic) in an oral delivery system can be evenly distributed throughout a matrix system or confined within a reservoir by coating with a polymer barrier coat. Moreover, the system can consist of a single unit or multiple smaller units. Microencapsulation is defined as multi-unit systems on the size order of 1 µm to 1000 µm in diameter.

For targeted delivery to the intestines, polymers are common materials that can exploit the physiological changes throughout the GI tract to offer controlled release of the active substance at specific sites. The release mechanisms of polymers rely on attributes such as swelling, pH sensitivity, physical erosion or even microbial metabolism (79). For example, a delayed release profile is often achieved by an enteric coating of a tablet or capsule, i.e. a resistive layer allowing the contents to unaffectedly pass through the harsh environments of the stomach before being released in the small intestine.

Moreover, polymers can be used to prepare a multi-unit system of smaller particles, such as microcapsules or nanoparticles, functioning as carriers that can create a suitable environment for biological substances like DNA or entire cells (80).

In this thesis, the focus will be on oral delivery systems for the administration of microorganisms, which in reality will center around probiotic delivery systems. Here, the increasingly explored field of microencapsulation will receive additional attention, along with commonly used polymer materials and production techniques applied in microencapsulation technologies.

2.2.1 Microencapsulation

Microencapsulation is normally defined as the process of enclosing small particles, a liquid, or a gas within a layer of coating or within a matrix with a final size of about 1 µm to 1000 µm in diameter (81 p. 3). The concept of microencapsulation roots back to 1957 with a patent on ink containing microcapsules that was used to develop carbonless paper (82). Quickly, it became apparent that a similar technology could be useful for the immunoprotection of transplanted cells, dubbed 'artificial cells', and microencapsulation was soon described for achieving specificity in drug delivery in the 1970s (80). Since then, microencapsulation has been applied within a number of fields, including pharmaceutics, cosmetics, foods, and agriculture.

Throughout the 1990s and early 2000s, extensive research was conducted to develop novel microencapsulation technologies, especially in the food industry. Here, the largest market has been in food flavorings, where microencapsulation has been utilized to manufacture higher quality and more innovative food products, such as chewing gums with improved flavor profiles (83). However, some attention has also been given to the probiotic market, where microencapsulation can offer protection and control the release of probiotics from different food products, e.g. dairy products like yogurt or fermented milk. Still, a wide implementation of the technology has yet to be established, which could partly be explained by reasons such as high cost and long product development times. Moreover, only a few *in vivo* studies have been carried out to test the beneficial effect of encapsulated vs. non-encapsulated probiotics in various pathologies (84), possibly resulting in limited motivation for the development of microbial microencapsulation systems.

Furthermore, as outlined earlier, the effects of probiotics remain controversial as no products have been authorized to claim any therapeutic effects. This has caused the probiotic market to remain within the food industry as opposed to becoming a pharmaceutical area of interest (85). In turn, the attention of probiotic research may have been more directed towards possible food applications and improvements of probiotic-containing products rather than exploring the actual therapeutic potentials of microorganisms, leaving the field overall in stagnation. For instance, the lack of diversity in the strains used in probiotic research might in itself be limiting the success of probiotics, and so applying new technologies to improve the effects of these strains may not yield any considerable upshots. All in all, the field of probiotics may benefit from an altered perspective on the therapeutic ability of microbes towards a more holistic view with microbial ecosystems in focus, similar to that of microbiota-based approaches. Accordingly, applying the same technologies to microbiota-based products might become of interest in the future. The following will therefore be an attempt to motivate for the use of microencapsulation technologies in microbial therapeutics, which is a topic that has yet to be discussed in light of the interpretation of the field given in this thesis.

2.2.2 Viability factors for microbial delivery

In the following, microbial viability will refer to the ability of a cell to grow and possibly generate a colony under defined environmental conditions. Colony-forming units (CFU) is a measure of viability calculated by counting the number of colonies formed from a diluted sample on a solid or semi-solid medium and then multiplied by the dilution factor, usually found to be on the order of 10^6 CFU ml^{-1} to 10^9 CFU ml^{-1} (or CFU g^{-1}) for most bacterial cultures. Traditionally, ensuring the viability of supplemented microbes is considered a prerequisite for the functionality of probiotics and can be assumed to be equally important for microbiota-based approaches like FMT, although there are some approaches that may not require viable cells in order to be effective (86). Nevertheless, to ensure that a product contains enough viable cells, the probiotic industry has adopted a minimum recommended level of 10^6 CFU ml^{-1} at the time of consumption (87), i.e. the 'therapeutic minimum'. However, it is important to note that the actual number of delivered viable cells will also depend on the administration route and the properties of the product. For example, techniques like encapsulation can improve the viability of probiotics during storage and delivery, which may ultimately require a lower dose in order to be effective compared to a non-encapsulated product.

There are a number of challenges with designing delivery systems for microbial cells in order to optimize the viability during manufacturing and storage, as well as during transit through the GI tract. Below is an overview of the most common factors that need to be accounted for, relating to intrinsic properties of the product like additives and oxygen levels, microbiological parameters such as the applied strains, and processing conditions including fermentation and storage. The pros and cons of different materials and techniques for encapsulating microbes are discussed in more detail in the later sections, however, the following are important factors to keep in mind while developing any microbe-based product, whether encapsulated or not.

2.2.2.1 Chemical factors

The properties of a microbial product immensely depend on its type and use case. For instance, probiotic-containing food products often lack the option of containing an ideal environment for microorganisms because additives such as antimicrobials or sugars affect the viability of the cells. On the other hand, an FMT procedure essentially ignores most environmental considerations, since the product (i.e. stool) is either administered immediately or stored frozen. In any case, there are certain factors that need to be carefully considered in order to obtain a product with a favorable environment for the microbes involved.

The amount of oxygen present during manufacturing and storage affect the viability of many microbial cultures, especially those originating from the gut where there are mainly obligate anaerobes (34). Such organisms do not use oxygen as their terminal electron acceptor and have markedly reduced levels of enzymes that destroy reactive oxygen species (ROS). Typical ROS include superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$), which are molecules capable of damaging DNA, RNA, proteins, and lipids, ultimately generating a toxic living environment. Some anaerobes may be tolerant to relatively high oxygen levels either by switching to oxygen as their electron acceptor (facultative anaerobes) or just possessing enzymes to cope with ROS (aerotolerant anaerobes).

For this reason, many of the most commonly used probiotic strains are facultative anaerobes, such as those from the *Lactobacillus* genera, due to their ability to withstand

oxygen, thereby easing the manufacturing process (88). Still, most bacteria in the human gut remain obligate anaerobes. In fact, they are estimated to outnumber facultative anaerobes and aerobes by approximately 2 to 3 orders of magnitudes (89). Moreover, even when obligate anaerobes are used in probiotic products, such as some *Bifidobacterium* strains, the manufacturing and storage conditions are not always well considered to minimize oxygen levels, especially in many food products. In turn, such failures to account for oxygen may explain why some probiotic-containing food products have been found to have low viability counts, sometimes as low as 10^3 CFU ml^{-1} (90).

2.2.2.2 Biological factors

Oral administration of most microorganisms results in a large loss of viability as they pass through the harsh conditions of the upper digestive tract, where gastric acid in the stomach and bile salts in the small intestine can deteriorate the cell membrane of the bacteria (91). Several studies have shown losses of 10^6 CFU g^{-1} to 10^8 CFU g^{-1} of probiotic bacteria during artificial gastric digestion, implying that the remaining probiotic counts are not sufficient to exert health beneficial effects (92). Other than applying encapsulation techniques to protect microorganisms during GI transit, altering the biological properties of the product has been investigated in order to optimize the environment for the microbes in the product (93).

The strain type used in a probiotic product influence the viability, especially in food products where many additional factors play a role, including antagonism with starter cultures or the natural microflora of the product (92). In probiotic supplements where the microorganisms are included in a dried form, the choice of strain type may presumably be of less significance because the microbes remain more or less dormant during storage. However, as the dried supplement enters the body and the bacteria activates, the interplay between the microbes in the product may become hard to predict. Furthermore, the indigenous microbiota of the host can inhabit microbes that will outcompete the administered bacteria through colonization resistance effects (see section 2.1.3.1), leaving the microbes dormant or dead. In fact, no probiotics to date have been shown to colonize the gut over extended periods of time (4), suggesting that the strains used in probiotic products are not fully compatible with the human intestinal ecosystem.

2.2.2.3 Physical factors

The integrity of microorganisms can be damaged during long-term storage as well as from commonly used manufacturing processes such as drying or pasteurization. In particular, spray-drying has been shown to significantly decrease the viability of many heat-sensitive bacteria due to the high outlet-temperatures (83,94), which are often set to around 80 ˚C (95). On the other hand, freeze-drying may cause damage due to the potential formation of ice crystals (81). More details about these processes can be found in section 2.4.2.

For the storage of microbiota-based products like stool samples or cultivated intestinal microbiota, applying such drastic processing methods is not common. Instead, direct freezing at -80 ˚C with or without the addition of cryoprotectants is used. Although such a procedure is unlikely to be optimal for all the microbes initially existing within the product, a minimalistic manipulation of the microbiota is possibly achieved this way. In addition, in the case of a cultivated microbiota, the culture is kept continuously active in controlled conditions in the laboratory and it is likely not possible to re-cultivate the culture once frozen. Accordingly, a microbiota-based product should not be frozen and thawed multiple times, as it is likely damaging to the microbial composition and might

reduce its therapeutic potential. Finding new and more efficient ways to store and protect microbiota-based products is certainly of interest and will probably depend on the type of product and the specific microbiota in use.

2.3 Materials for microencapsulation

A number of materials have been suggested for encapsulating both eukaryotic and prokaryotic cells for different applications (96). Whether the goal is to encapsulate pancreatic islets for the treatment of diabetes or offering protection of probiotics in a food product, polymers are commonly applied to offer a suitable environment for biological components and cells. For microencapsulation of probiotics, spherical polysaccharide or protein gel microcarriers have been reported to offer protection against high oxygen levels, acidic environments, freezing, and during simulated GI transit (97).

In addition to the aforementioned factors for microbial viability, the material used in an encapsulation system is of importance. Thus, the viability of encapsulated cells is often tested and used as an indicator for the performance of a material. However, the encapsulation technique can alter the properties of a material and vastly change the protecting ability of the system. For this reason, the apparent ability of a material to protect microorganisms may differ amid seemingly similar studies, which is important to keep in mind in the following sections. Other aspects of each material, such as physicochemical properties, biocompatibility, and common applications will also be discussed.

2.3.1 Alginate

Alginate occurs as the structural component in marine brown algae (*Phaeophyceae*), such as *Laminaria hyperborea* and *Macrocystis pyrifera* (98). It is located mainly in the intercellular mucilage and algal cell wall, where it constitutes up to 40 % of the dry matter of the cells and provides mechanical strength and flexibility in the form of an insoluble gel in Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}-salts (98). In addition, there are bacteria that produce alginates as protective exocellular polymers, such as the biofilms of the species *Azotobacter* and *Pseudomonas*, where alginate is the main constituent (99).

The biocompatibility of alginate *in vivo* depends on the application. For instance, alginate beads as implantation material for cell therapy are known to induce a fibrotic reaction in tissue (100). However, the use of alginate as food additives is widespread and has acquired GRAS (generally recognized as safe) status (91). Consequently, for GI passage, alginate holds a potential for the development of oral delivery systems and is well-suited for bacterial entrapment due to the mild gelling conditions and being highly biocompatible and biodegradable.

2.3.1.1 Structure and gelling mechanism of alginate

Alginate is a an unbranched polyanion consisting of the two sugar residues β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked by 1→4 glycosidic bonds (101). The monomers are arranged in a pattern of blocks along the chain, with homopolymeric regions known as M-blocks and G-blocks, and regions of alternating structure called MG-blocks, as illustrated in Figure 4.

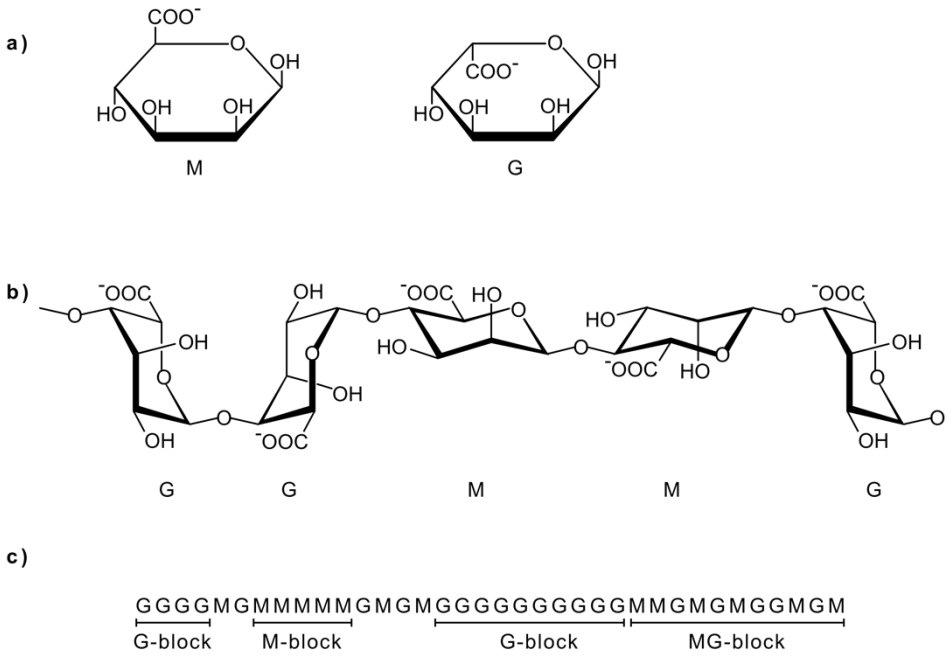

Figure 4. Alginate structures. a) Haworth projection of β-D-mannuronic acid (M) and α-L-guluronic acid (G). b) Chair conformation of the alginate chain. c) Symbolic representation of an example alginate chain showing all the possible block structures.

Gelling of alginate can be achieved by ionic crosslinking of the G-block regions, which generates ordered junction zones in the chain network. As such, the properties of alginate changes with the length of each block and the ratio between the mannuronic (M) and guluronic (G) acids. Longer chains and a higher G content are generally associated with a higher rigidity, mechanical strength, and better tolerance to salts and gelling ion sequestering agents, which is partly explained by the formation of more G-G crosslinks creating a tighter network (102). For this reason, high-G alginate is often preferred in biomedical applications.

Furthermore, the ionic crosslinking is also dependent on the type of cation present in the gelling solution. In general, the affinity of alginate to different crosslinking ions decrease in the following order: trivalent cations > Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+}, Ni^{2+}, Zn^{2+} > Mn^{2+} (103). However, it turns out that different block structures in the alginate bind the ions to a different amount, hence predicting the properties of an alginate material becomes more complicated than by simply considering the gelling ion and the alginate composition separately. For example, a study by Mørch et al. (104) found that the strength and stability of a high-M alginate increased when using Ca^{2+} rather than Sr^{2+}, although Sr^{2+} in general has a higher affinity to alginate. The observation was justified by Sr^{2+} binding solely to G-blocks in the alginate, while Ca^{2+} could bind both G- and MG-blocks, so an alginate with a high-M content would have more MG-blocks for Ca^{2+} to bind and result in a stronger gel.

The reported intricacies in the gelling properties of alginate are interesting because most studies using alginate for encapsulating probiotics do not seem to be concerned with the physicochemical properties of the alginate system they use (105–107). Consequently, this unawareness might be part of the reason why there are conflicting reports on the

success of alginate-based microencapsulation of bacteria in simulated gastric fluid and storage tests (104).

2.3.1.2 Swelling and stability

A microencapsulation system should be stable under physiological conditions over extended periods of time. Alginate gel beads can be viewed as an osmotic swelling system, meaning the osmotic pressure and the elastic reaction of the gel network are opposing effects that are at an equilibrium in a stable gel (108). Since the gelling ion is important for the strength and elasticity of an ionically cross-linked gel, introducing molecules with high affinity for the gelling ion, e.g. phosphate and citrate for Ca^{2+}, will sequester the cross-linking gelling ions and destabilize the gel (102). As such sequestering agents are notably present in intestinal fluid, alginate beads are thought to swell and dissolve when exposed to the physiological conditions of the small intestine (103).

Moreover, the presence of non-gel-inducing ions in the alginate solution, such as Na^{+} and Mg^{2+}, can interfere with the gelling ion and cause swelling. However, if the concentrations of the non-gel-inducing ions are similar in the alginate gel and in the solvent outside the gel, the chemical potential of the non-gel-inducing ions becomes small, resulting in reduced diffusion of the ions into the gel and, hence, less swelling (108).

Lastly, depending on the ionic strength of the solution, the uronic acid residues in alginate have a pK_a value of 3.3 to 4.0, meaning the polymer will be protonated in acidic environments when the pH is lowered below the pK_a value. In turn, protonation of alginate gels in solution will result in shrinkage of the gel caused by a reduction in the electrostatic repulsions between the carboxyl groups of the alginate monomers, as well as the establishment of covalently cross-linked M-block regions (109). Consequently, due to the pH-responsiveness of alginate gels and considering the pH variations along the GI tract, alginate has been investigated for the development of pH-dependent oral delivery systems for targeted delivery to the small and large intestines (103).

2.3.2 Other polysaccharides

Although alginate gels are the most common system for cell immobilization (110), there are plenty of other materials that can be utilized to encapsulate or entrap cells. Note that all of the substances presented below have GRAS status, implying that they should be safe to use in most applications, although there can be some hurdles to overcome when developing a microbial delivery system. Moreover, the following polysaccharides can also be used in combination with alginate in order to improve the physiological stability or targetability of a delivery system during GI transit.

2.3.2.1 Chitosan

Commercially produced chitosan is normally prepared by deacetylation of naturally occurring chitin, a linear polymer isolated from crustacean shells that predominantly consists of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucose (GlcNAc; A-unit) (111). As such, chitosans may be considered as a family of linear binary copolymers composed of GlcNAc (A-units) together with some proportion of its deacetylated form, i.e. 2-amino-2-deoxy-β-D-glucose (GlcN; D-unit). Indeed, the degree of *N*-acetylation, F_A (fraction of A-units), and the distribution of the acetyl groups affect properties such as conformation and chain stiffness of chitosan in solution, as well as determining solubility of the

polymer (112). In addition, increasing the ionic strength of the solution, e.g. by the addition of salts, will lower the solubility of chitosan (113).

Chitosan is frequently used in combination with other polymers, especially alginate, as a coating agent (114). The amino-group of GlcN (D-unit) has a pKa of 6.2 to 7.0 (115), meaning it will be protonated at low pH and possess a high charge density. Consequently, chitosan becomes a polycation that can form a complex and bind to negatively charged alginate. In combination with alginate beads, low molecular weight chitosan is often preferred rather than high molecular weight, since it diffuses faster into the alginate matrix, resulting in the formation of spheres with higher density and strength (84). However, chitosan has inhibitory effects on certain bacteria, which have been reported to affect the viability of encapsulated microbes (116,117). Therefore, from a viability perspective, using higher molecular weight chitosans that sticks more to the surface of an alginate bead may be preferred, rather than having the chitosan penetrate into the alginate matrix and interact more with the encapsulated cells.

2.3.2.2 κ-Carrageenan

Carrageenans are hydrophilic polysaccharides that exist as matrix materials in numerous species of red seaweeds (*Rhodophyta*). κ-Carrageenan is one of the six classes of carrageenans and is commonly used as a food additive to substitute for fat and improve food texture (118). Dissolution of the κ-carrageenan requires elevated temperatures reaching between 60 °C and 90 °C for high concentrations (2 % to 5 %), and gelation occurs when the temperature is lowered down to room temperature (84). In order to achieve immobilization, the cells need to be added to the mixture before gelation occurs, i.e. at a relatively high temperature (around 45 °C), which could be damaging for some bacteria. In addition, K^+ ions (in the form of KCl) are added to the solution after the beads are formed in order to stabilize the gel, which have been reported to have an inhibitory effect on some lactic acid bacteria (119).

Furthermore, although κ-carrageenan is frequently used in foods and has been reported to improve the viability of several probiotic strains in different food products and in acidic environments (120), the consumption of the polymer remains questionable due to some evidence showing potential adverse effects in the gut (121). For instance, studies have shown that several classes of carrageenans, including κ-carrageenan, can induce colitis in animal models (122), which could be linked to the reduction of anti-inflammatory bacteria in the intestinal microbiome (123). Still, the polymer retains its GRAS status for applications in foods as more detailed studies are warranted to clarify the potential harmful effects in humans. Unsurprisingly, the increased usage of carrageenans in food products in recent years along with lacking evidence on their presence in humans have caused controversial discussions in the scientific community (124).

2.3.2.3 Xanthan gum and gellan gum

Xanthan gum, an exopolysaccharide derived from *Xanthomonas campestris*, is the most commonly used gum and consists of repeated pentasaccharide units formed by two glucose units, two mannose units, and one glucuronic acid unit (125). Gellan gum is also a microbial exopolysaccharide. Derived from *Pseudomonas elodea* it is constituted of a repeating unit of the four monomers glucose, glucuronic acid, glucose, and rhamnose. The two polymers can be used in combination with each other (126), or in combination with other polymers, including alginate (127), to form beads with a high resistance to acid, making them suitable for oral delivery applications.

2.3.2.4 Cellulose acetate phthalate (CAP)

Cellulose acetate phthalate (CAP) is a cellulose polymer where some of the hydroxyls on the β-D-glucose units are esterified with acetyls and some with phthalic acid, i.e. it is a synthetic polysaccharide (81). The polymer is widely used in pharmaceutical applications as an enteric coating material for targeted release to the intestines, which is possible because CAP is physiologically inert, as well as being insoluble in acid media (pH < 5) but becomes soluble when the pH rises above its pK_a value at around pH 6 (128). The properties of CAP, including its pH-sensitive solubility, are determined by the degree and type of substitution on the β-D-glucose units (79).

For encapsulation of probiotics, CAP has been used either as the main constituent or in combination with other polymers, yielding seemingly positive results on the viability of certain strains (127,129–132). For example, a study by Fávaro-Trindade et al. (133) showed that using CAP as the wall material in spray-dried microcarriers was able to protect the two probiotic strains *L. acidophilus* La-5 and *B. animalis subsp. lactis* BB-12 against acid and bile solutions. Nevertheless, a widespread use of CAP for encapsulating microbial cells has not been implemented, and the consistency of the existing studies is lacking, meaning parameters such as the probiotic strain, encapsulation technique, and even the viability assay used are highly heterogenous.

2.3.2.5 Starch

Starch is a polysaccharide composed of α-D-glucose units linked by glycosidic bonds and is produced by all green plants. There are various ways to classify different types of starches depending on characteristics such as enzymatic breakdown, nutritional value, or chemical chain structures generating amorphous and crystalline regions (134). Resistant starch is one class of starch that is of interest in pharmaceutical applications due to its resistance to digestion (enzymatic breakdown) in the small intestine, hence useful for targeted delivery to the colon, where it will be fermented by the indigenous microbiota. Moreover, starch is an ideal surface for certain microbial cells to adhere to, possibly enhancing the viability of encapsulated cells and delivering them in a metabolically active state (135), i.e. starch is essentially functioning as a prebiotic.

Using starch in combination with alginate has been tested for microencapsulation of bacteria (136–138). However, as starch is not a charged polymer, it will not be able to strongly bind to alginate and form an enteric coating, meaning that diffusion and stability properties of the beads are likely to remain unimproved. Instead, the starch is mixed with the alginate either homogeneously or as granules. Thus, an alginate-starch system possibly becomes more of an improved environment for the encapsulated microbes, where starch is functioning as a prebiotic, rather than protecting against harsh environments and enabling targeted delivery (137). Here, more research is needed to confirm or disprove such a hypothesis for the use of starch in microencapsulation technologies.

2.3.3 Proteins

In addition to polysaccharides, proteins have been investigated for encapsulating probiotics (139). Gelatin and milk proteins such as whey protein and casein are frequently applied in different industrial delivery systems and food products, such as macro-sized gelatin capsules or spray-dried whey protein powder (140,141). Accordingly, using these proteins in microencapsulation systems in combination with polysaccharides has been explored in order to improve performance (142). For instance, alginate coated with whey protein has been shown to positively affect encapsulated *L. plantarum* survival

at low pH (143). In general, advantages of using protein materials in oral delivery systems are low cost, ease of use, and good biocompatibility. However, it should be noted that a vast number of people have milk protein related allergies, which potentially poses a limitation for the widespread application of milk proteins in encapsulation systems for microbial products.

2.3.3.1 Gelatin

Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides containing between 300 and 4000 amino acid units obtained from hydrolysis of collagen derived from the skin, white connective tissue, and bones of animals (144). The characteristics of gelatin are generally determined by the parent collagen and the irreversible hydrolysis treatment (145). Gelation of gelatin is a thermally reversible temperature-dependent process and happens when the temperature is raised above 40 °C and then lowered back to room temperature. The gel strength depends on the source of the gelatin, its molecular weight, concentration, temperature, and setting time (146). Moreover, like all proteins, gelatin is amphoteric in nature due to the presence of both amine and carboxylic acid groups. Thus, gelatin can be linked to negatively charged polymers when the pH is adjusted below gelatin's isoelectric point because the gelatin becomes net positively charged (118).

In physiological conditions, gelatin is thought to be disintegrated by the presence of pepsin in gastric acid (147). Therefore, in probiotic delivery systems, gelatin is often combined with other substances in order to improve the stability during gastric exposure. For instance, coating gelatin microspheres with alginate was shown to improve survival of a *B. adolescentis* strain during exposure to simulated GI conditions (148). The observed improvement in survival after exposure to simulated gastric conditions was thought to be partly due to a protective pH buffering effect exerted by the gelatin core – an effect also reported by a whey protein-based system (149).

2.4 Techniques for microencapsulation and entrapment

There are many different technologies for encapsulating or entrapping microbial cells. Some of them include performing the immobilization in a hydrocolloid solution, i.e. aqueous dispersions containing gel-forming agents (150). Other techniques utilize drying mechanisms in order to form solid particles that can potentially provide a more stable system for prolonged storage purposes (151). However, drying methods are generally more harmful to microorganisms because water is important in stabilizing biological molecules, while hydrocolloid technologies are often difficult to scale up to industrial standards due to the relatively slow formation of beads (7).

Note that, in the following, the word 'encapsulation' will refer to a system where the active substance resides within a core and is protected by a polymeric membrane, hence generating a 'capsule'. On the other hand, 'entrapment' will denote a uniformly dispersed matrix system with no distinct external wall or membrane, resulting in the formation of a 'bead' or 'sphere'. Although these definitions have been used interchangeably in the preceding sections, they are more important to distinguish when discussing the manufacturing of micro-sized structures.

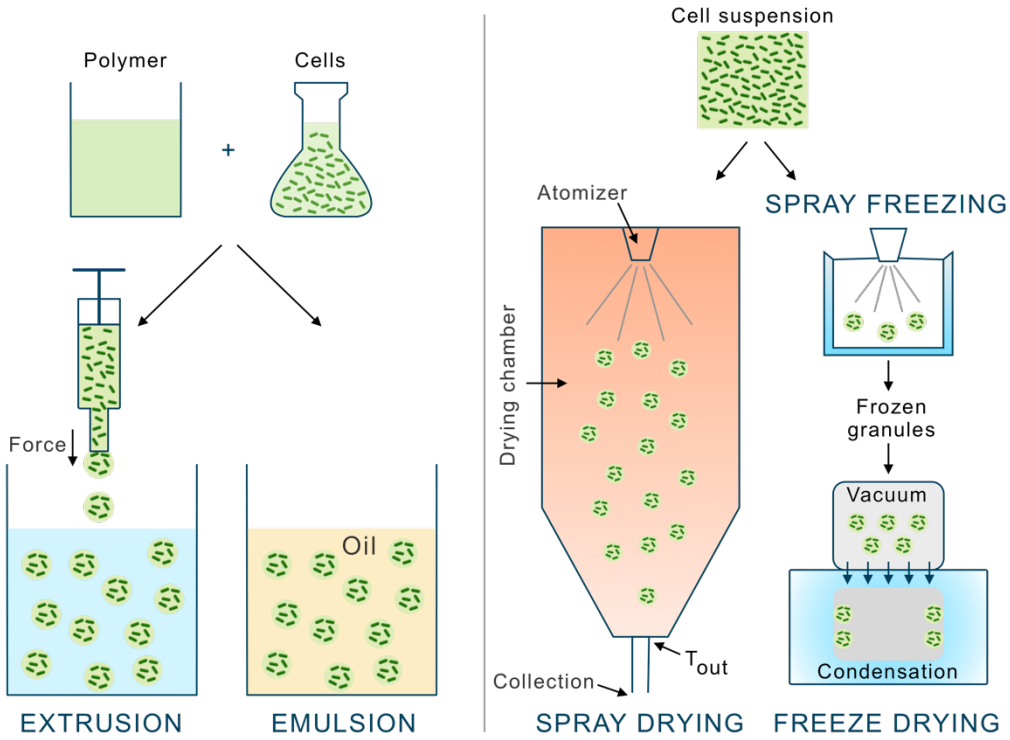

Figure 5. Some techniques for immobilizing microbial cells in microcarrier systems. Hydrocolloid microcarriers can be made by extrusion or emulsion, generally resulting in entrapment of the cells in the polymer matrix. On the other hand, drying techniques result in powders with a low water content. Here, spray drying is performed under elevated temperatures to evaporate residual water from the atomized liquid particles, while spray freeze drying is a combination of rapid freezing (spray freezing) followed by sublimation of water in a vacuum (freeze drying).

2.4.1 Extrusion and emulsion

The extrusion technique is the oldest and most common approach when making hydrocolloid beads. For entrapping microorganisms, extrusion simply involves mixing the hydrocolloid solution with the cells, followed by extrusion of the suspension through a syringe needle in the form of droplets to free-fall into a gelling or setting bath, as depicted in Figure 5. Extrusion typically results in entrapped cells in a matrix bead, although encapsulation can be achieved through co-extrusion by placing the beads in a bath of coating material that react at the droplet surface. To generate beads of a size smaller than 1 mm in diameter, an external force is needed to pull the droplets off the syringe needle before they are large enough to fall due their own weight (152). Some techniques that have been used to apply such force include coaxial airflow to cut the droplets off the needle, sound wave induced vibrations to vibrate the droplets off, and electrostatic force to pull a charged material off the needle, such as negatively charged alginate (84). Accordingly, the size and shape of the beads depend on the diameter of the needle, the viscosity and flow rate of the polymer solution, the distance of the free fall, as well as the magnitude of any external forces used to pull the droplets off the needle, e.g. the strength of an electrostatic field (153).

The emulsion technique is based on the relationship between a discontinuous phase of the cell-polymer suspension and a continuous phase of oil, making it slightly more complex than extrusion. First, the discontinuous phase is mixed homogenously with a large volume of the continuous phase to form a water-in-oil emulsion. Then, the water-soluble polymer is insolubilized (cross-linked) to form the particles within the oil phase, and the beads are harvested by filtration. The size of the beads is controlled by the speed

of agitation or stirring, and can vary between 25 μm and 2000 μm (118). Similar to extrusion, the gel beads can be introduced to a second polymer solution to create a coating layer that provides added protection (154).

Extrusion for microencapsulation purposes are generally considered to be difficult processes to upscale due to the slow formation of beads. However, some authors have expressed opinions that certain extrusion techniques are relatively simple to scale up, like those including vibrational forces to break the beads off the needle, because it is only a matter of developing multi-nozzle devices (145). On the other hand, the emulsion technique is regarded as easier to scale up to industrial standards (83). Still, a key disadvantage of emulsion is that it provides a large variability in the size and shape of the beads, which is not ideal for consistent performance. For this reason, extrusion could be regarded as the preferred method, as it produces uniform beads under mild conditions and can even be performed under anaerobic surroundings to account for oxygen-sensitive microbes (154).

2.4.2 Spray drying and freeze drying

Spray drying is extensively used in pharmaceutical and biotechnology industry because it allows to produce microparticles in large quantities with high reproducibility in a one-step process (103). The technique involves mixing the cells and a polymer solution, followed by homogenization of the cell-polymer suspension. Then, atomization of the mixture is performed into a drying chamber, leading to evaporation of the solvent. The drying process causes the polymer solution to shrink into a pure polymer envelope enclosing the core material, resulting in capsules in the form of free-flowing dry powder (83 p. 84).

Logically, elevated temperatures are required in the drying step, which is the major limitation on the viability of spray-dried probiotics (150). During drying, the temperature of the droplets rises towards the outlet temperature of the drying chamber, T_{out}, which varies between 45 °C and 105 °C (155). As such, both the value of T_{out} and the time of exposure to heat are parameters that will affect the final viability of the microbes. In addition, T_{out} can influence the powder quality, i.e. a high moisture content will lead to high residual water activity which is not preferred for prolonged storage (156). Consequently, the spray-drying process should be optimized for the specific microbial product in order to simultaneously keep T_{out} as low as possible and achieve an acceptable moisture content in the final powder. Optimization strategies include multi-stage drying in order to enable a lower T_{out} over consecutive drying steps, the addition of crystallizers to capture moisture and reduce water activity, as well as the addition of thermoprotectants, such as trehalose which will replace the role of water during dehydration by stabilizing biological membranes and proteins (157).

Next, freeze drying has traditionally been the most widely used method for preserving sensitive bacteria, but for immobilizing microbes in polymer systems it is a less common approach because it is expensive and energy demanding (158). The freeze-drying process typically involves freezing the liquid containing the bacteria, and then reducing the surrounding pressure to allow the frozen water to sublimate directly from the solid phase to the gas phase. When applying the technique for encapsulation purposes it is referred to as spray freeze drying, which is characterized by including an atomization step to generate droplets that are subsequently freeze-dried (154). The problems associated with freeze drying are due to the formation of ice crystals during the freezing step, which is commonly avoided by adding cryoprotectants and carefully controlling the temperature and pressure to obtain glass formation which will minimize molecular

mobility (81 p. 474). Additionally, similar to spray-drying, the final water content affects the viability of the entrapped microbes, i.e. one can expect higher survival rate with higher water content, but that will come at the cost of reduced inactivation upon storage.

Finally, the rehydration capacity refers to the functional recovery of dried probiotics after exposure to moisture. Here, there is evidence that the optimal rehydration temperature differs between certain spray-dried and freeze-dried bacteria (89), suggesting that there is an actual physiological difference between bacteria exposed to the different drying conditions. Moreover, the effect of drying techniques on microorganisms heavily depends on the physiological properties of the specific strains, such as heat-sensitivity and tolerance to low water content (159). For instance, Picot and Lacroix (160) showed that two *Bifidobacterium* strains each encapsulated in the same whey protein system displayed different tolerance to a spray drying technique. Thus, applying drying technologies to more complex microbial communities like a cultivated microbiota may seem unsuitable due to the magnitude of different microbes involved, many of which are likely to be intolerant to the rather unforgiving drying conditions. However, with the many optimization and innovation measures that exist for the two technologies, the use of drying techniques on microbiota products should not be ruled for future applications.

3 Experimental Study

The review presented in this thesis was aimed to address the controversial field of probiotics in relation to the emerging field of microbiota-based therapies. In addition, the review focused on oral delivery systems for microorganisms, focusing on microencapsulation systems, their characteristics, and the research conducted on the ability of such systems to potentially improve the effects of microbial products, essentially probiotics. However, as the review noticeably discusses, microbiota-based approaches may seem to be the better alternative for the general field of microbial therapeutics. Here, little effort has been made into finding new and innovative approaches to replace the rather uncontrolled, though effective, FMT treatment, and the cumbersome administration routes of such procedures (endoscopy). Therefore, the second aim of this thesis was to perform a preliminary experimental study on a putative delivery system for a cultivated microbiota. The previously mentioned ACHIM culture was used as the microbiota-based product. Moreover, a microencapsulation system using extrusion of alginate beads was chosen for the study, due to its promising potential and convenience factors related to a solid knowledgebase at the Department of Biotechnology and Food Science at NTNU, Trondheim.

It should be noted that much of the experimental work was developed during my project thesis in the fall of 2019 and, due to the events of the COVID-19 pandemic, further experimental work was limited into the spring of 2020. Originally, the plan was to develop and optimize a consistent methodology that would generate reproducible results, before continuing with testing alternative delivery systems and characterization experiments. Although no alternative delivery systems or characterizations were tested, a handful optimization experiments were conducted, which possibly generated better-quality results than what was presented in the project thesis. Naturally, these updated results will be presented in the following and discussed in relation to any previous results. For this reason, some of the following parts will be slightly modified segments adapted from the project thesis, in particular the introduction and methods sections. Furthermore, to avoid a high degree of similarity between the following and my project thesis, mostly updated results will be presented while any relevant previous results will be included in Appendix A.

3.1 Introduction

3.1.1 Physiology of the gastrointestinal tract

When designing an oral delivery system based on immobilization of cells with the aim of providing controlled release it is necessary to consider the complex physiology of the gastrointestinal (GI) tract. The GI tract can be thought of as a 9-meter-long tube (in adults) going from mouth to anus divided into different compartments, all with specific functions and microbiota (161).

After ingestion, a microcarrier will pass quickly through the esophagus and reach the stomach, where the gastric acid is constructed to selectively kill off bacteria. Following the stomach are the small and the large intestines, which are responsible for further digestion and absorption of food in a controlled manner. In addition, the intestines are home to a variety of bacteria (at least 800 species) producing many bioactive compounds, acting locally and at distance (162).

Table 2. Transit times and changes in pH throughout the GI tract.

Region	pH	Transit time
Esophagus	~7.0	10 s to 14 s
Stomach	1-2.5 (up to 5 fed)	5 min to 2 h
Small intestine	6.2-7.8	(3.2 ± 1.6) h
Large intestine	5.2-7.0	Highly variable

Along the GI tract there are various possible methods for release of the contents of a microcarrier, e.g. based on pH, time, peristaltic pressures, and even bacterial fermentation (91). Table 2 presents reported pH changes and transit times throughout the GI tract. Although the transit times and pH levels have been found to be highly variable (163–165), they are important factors to take into account when designing a delivery system. In addition, it is well known that oxygen levels decrease gradually throughout the GI tract and is essentially 0 % in the colon.

3.1.2 Microencapsulation system

3.1.2.1 Alginate as matrix material

Biotechnological applications of alginate are mostly linked to its exceptional gelling properties, which allow easy hydrogel formation under mild conditions. Gelation of alginate occurs in the presence of divalent cations like Ca^{2+}, while in its non-gelled state alginate remains a liquid with varying viscosity (98). Alginate hydrogels can therefore be used to immobilize living cells (102).

3.1.2.2 Electrostatic bead generator

An electrostatic bead generator is an extruder that works by establishing an electrostatic potential between the needle where alginate is extruded from and the gelling bath. The voltage of the applied field (typically 4 kV to 20 kV) will be proportional to the pulling force on the alginate solution, hence a higher voltage will exert a stronger pulling force and result in smaller beads. Other parameters that affect the bead size are the needle diameter, flow rate, and molecular weight of the alginate, which all yield smaller beads if they are decreased (166).

3.1.3 Fluorescent viability staining of bacteria

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation. The phenomenon is extensively used in the life sciences generally as a non-invasive way of tracking or analyzing biological molecules (167). The process of fluorescence is a two-step chemical reaction where a fluorophore is excited by absorbing light, followed by emission of light with a longer wavelength as the electrons of the excited fluorophore fall back to a lower energy state. The resulting fluorescence from a fluorophore can be observed using a microscope equipped with optical filters to channel only the emitted light. Here, confocal laser scanning microscopy (CLSM) offers improved resolution and contrast due to a narrow pinhole that blocks out-of-focus light and allows for cross-sectional imaging of the sample (168).

It is possible to use a two-component fluorescent stain to determine bacterial cell viability. Such systems are based on different characteristics between live and dead cells, such as cell metabolism or integrity of the cell membrane (169). An example of cell membrane integrity dependent staining is the use of the nucleic acid stains propidium iodide (PI) and SYTO 9, which differ in their spectral characteristics and in their ability to penetrate healthy bacterial cells. As shown in Figure 6, SYTO 9 is able to penetrate all bacterial cell walls, while PI only penetrates the damaged membranes of dead or dying bacteria, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present (170). Consequently, with an appropriate mixture of the two stains, live bacteria with intact cell membranes stain fluorescent green (SYTO 9), whereas dead bacteria with damaged membranes stain fluorescent red (PI). In turn, such fluorescent stains can be used to obtain quantitative data on live/dead bacteria, e.g. by means of image analysis of CLSM fluorescent images.

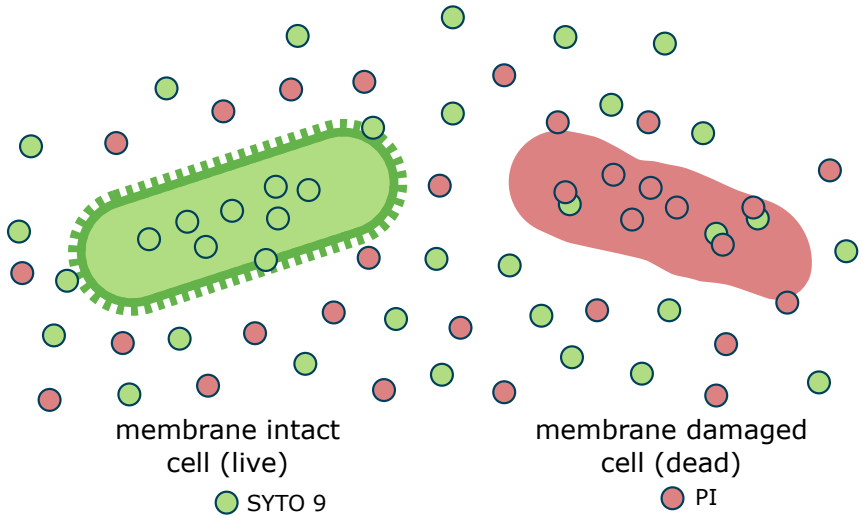

Figure 6. The mechanism for live/dead fluorescent labeling of bacterial cells using nucleic acid stains that penetrate the cells depending on membrane integrity. One fluorophore (SYTO 9) can penetrate healthy cell walls, while another (PI) can only penetrate the damaged membranes of dead or dying cells. As the SYTO9 signal is reduced when PI gains access to the nucleic acids in the cell and is excited, the resulting system emit light with different wavelengths depending on the membrane integrity of the cells, which can be observed using a CLSM instrument and used to assess the viability of bacterial cells.

3.2 Methods

The major limitation of the previous experimental work was the variability in the quantitative viability data on the microbes (Appendix A). Therefore, repeating the experiment and harvesting more data was necessary in order to optimize the analysis and generate more credible results. However, not every methodological step was necessary to repeat in order to elucidate the results from previous work, i.e. only the following steps from my project thesis were required, some of which were slightly altered.

3.2.1 Preparation of solutions

All solutions were made with MQ deionized water (EMD Millipore; USA) with pH 5.5 due to absorption of atmospheric CO_2. The final solutions were bubbled with N_2 gas for 1 min prior to an experiment in order to achieve a deoxygenized environment that would better accommodate for the strictly anaerobic bacteria in the microbiota culture. New batches of each solution were made regularly to enable repetition of the experiments.

3.2.1.1 Simulated gastrointestinal fluids

Two different solutions were prepared to simulate human GI conditions. To mimic stomach conditions, 154 mM NaCl (0.9 wt%; physiological saline) was adjusted from pH 5.5 to pH 2.0 with 0.2 M HCl to form a simulated gastric fluid (SGF). Next, simulated intestinal fluid (SIF) was prepared according to the salt compositions described in the INFOGEST standardized method by the European Cooperation in Science and Technology (COST) (171), and adjusted from pH 8.4 to pH 7.0 with 0.2 M HCl. The salt concentrations are presented in Table 3. Enzymes and bile salts were not included in the simulated fluids. Both the SGF and SIF solutions were stored at room temperature.

Table 3. Salt concentrations in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

	SGF (mM)	SIF (mM)
KCl	–	6.8
$KH_2PO_4(H_2O)_3$	–	0.8
$NaHCO_3$	–	85.0
NaCl	154	38.4
$MgCl_2(H_2O)_6$	–	0.3

3.2.1.2 Alginate and gelling solution

High-G sodium alginate from the *L. hyperboria* stipe (LF10/60) obtained from FMC Biopolymer, Norway, was used to prepare a 1.8 wt% alginate solution in 0.3 M mannitol. The dry alginate powder was mixed with the mannitol solution at room temperature overnight using a magnetic stirrer, and then stored at 4 °C. The gelling solution for alginate consisted of 10 mM HEPES buffer (Sigma Aldrich) and 50 mM $CaCl_2$. The pH was adjusted to pH 7.2 using 1 M NaOH, and the solution was stored at 4 °C.

3.2.1.3 Anaerobically cultivated human intestinal microbiota (ACHIM)

Suspensions of anaerobically cultivated human intestinal microbiota (ACHIM) were kindly donated by the Norwegian company NIM Supplement AS (former ACHIM Biotherapeutics

AS) in the form of frozen 1 ml aliquots, which were immediately stored at -80 °C upon arrival. The culture contained anaerobic human intestinal bacteria that had been cultivated regularly *in vitro* since 1994 – originating from a single stool sample – using a technology developed by the Karolinska Institute in Stockholm, Sweden.

Before each experiment, one 1 ml aliquot of ACHIM was thawed to room temperature and the bacterial cells were harvested by a two-step washing procedure. First, the 1 ml aliquot was centrifuged at 5,000 × g for 10 min at 25 °C, followed by removal of the supernatant and resuspension of the pellet in 10 ml deoxygenized physiological saline (0.9 wt% NaCl; pH 5.5). Then, the cells were centrifuged again under the same conditions and, after discarding the supernatant, the pellet was resuspended in 3 ml of the saline solution before mixing with 4 ml of the alginate solution in order to generate a cell-alginate suspension with a dilution factor of 150 relative to the initial ACHIM culture.

The washing procedure was developed based on literature findings (114,148,169,172) and previous preliminary experiments in order to avoid accumulation of large biological particles thought to be microcolonies of bacteria that would impair the subsequent fluorescent staining procedure (Appendix B).

3.2.2 Bead production and immobilization of bacteria

3.2.2.1 Procedure for bead production using electrostatic bead generator

A 10 ml syringe (BD Plastipak; USA) containing 5 ml of the cell-alginate suspension was mounted on a Graseby 3500 syringe pump (Graseby Medical Ltd.; United Kingdom) with a flow rate set to 30 ml/h. A silicone tube (VWR International; USA) with an inner diameter of 1 mm was connected to the syringe nozzle in one end and to the electrostatic bead generator (NTNU dept. of physics; Norway) in the other. The electrostatic needle had a diameter of 0.35 mm and was placed approximately 3 cm above a gelling bath containing 50 ml deoxygenized gelling solution, which was gently stirred using a magnetic stirrer. The beads were allowed to gel for around 15 min before they were washed in physiological saline. Washing was done by letting the beads sink to the bottom of a 15 ml centrifuge tube in order to remove the gelling solution and replace it with the saline solution.

3.2.2.2 Entrapment of bacteria

Following the bacterial encapsulation method by Krasaekoopt et al. (114), the saline-washed bacteria were mixed with the 1.8 wt% deoxygenized alginate solution in a 1:4 ratio before drawn into the 10 ml bead generator syringe to produce alginate-bacteria beads as described in the section above. The electrostatic potential was set to 6.3 kV in order to generate beads with an approximate diameter of ~500 µm in the final washing solution, which was shown in previous work (173).

3.2.3 Exposure to simulated GI fluids

Prior to a simulation experiment, all solutions were deoxygenized with N_2 gas and pH was tested using a calibrated pH meter. Figure 7 depicts the experimental setup for running the GI simulations. 2 ml alginate-bacteria beads washed in physiological saline were added to 10 ml of deoxygenized SGF kept in a 30 ml flat bottom screw cap container. The container was kept warm in a water bath holding 37 °C and stirred briefly by vortexing every 15 min to simulate GI conditions. The beads were exposed to SGF for 30 min before approximately 6 ml of beads were removed and washed thoroughly in saline, then stored at 4 °C until fluorescent staining. Then, in order to simulate GI transit and assess degradation of the alginate gel, beads exposed to SGF for 30 min were transferred to SIF during continuous observation under a brightfield microscope.

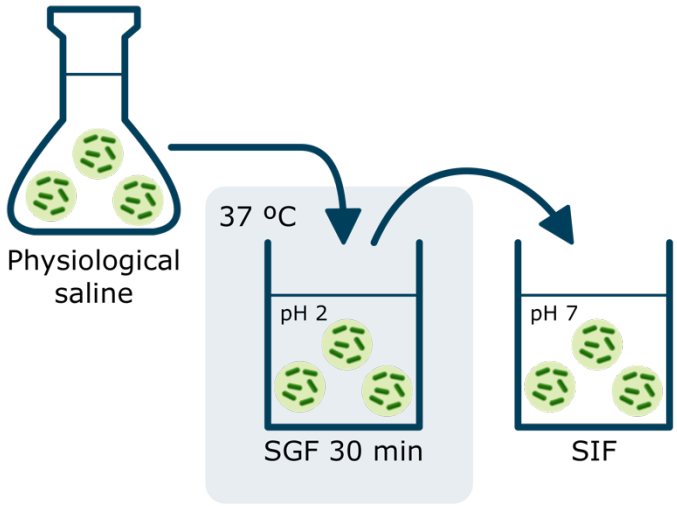

Figure 7. Experimental setup for simulated GI conditions. Bacteria entrapped in alginate beads which had been washed in physiological saline were added to SGF tempered at 37 °C for 30 min, followed by washing of the beads in physiological saline. Beads exposed to the SGF solution for 30 min were also directly transferred to SIF for degradation assessment.

3.2.4 Characterization of bacteria

3.2.4.1 Live/dead fluorescent staining procedure

Staining of immobilized bacteria in alginate beads was performed on beads washed in physiological saline and on beads subsequently exposed to SGF for 30 min and then washed in physiological saline. SYTO9 (S-34854) and propidium iodide (PI; 81845; Sigma) from the commercially available LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen; Thermo Fisher Scientific) was used as the fluorescent viability stains. The kit (L-7012) provided the stains as separate solutions in dimethylsulfoxide (DMSO), with SYTO9 at 3.34 mM and PI at 20 mM. Staining was carried out according to the kit manual by adding 3 µL of a 1:1 mixture of SYTO9 and PI to 1 ml of sample solution. The sample solution was then mixed by vortexing and incubated at room temperature in the dark before observing with a confocal laser scanning microscope.

3.2.4.2 Confocal laser scanning microscopy

A Zeiss LSM800 confocal laser scanning microscope (Carl Zeiss AG; Germany) with the accompanying Zeiss Service software (Carl Zeiss; Germany; Microsoft Corporation; USA) was used to observe the viability of the fluorescently labeled bacteria. Beads containing the cells were placed in a glass bottom dish (Nunc™; Thermo Fisher Scientific) and

magnified using a 10x water immersion objective with 0.45 numerical aperture (C-Apochromat 10x/0.45W). The fluorophores were excited using a 561 nm laser for PI and a 488 nm laser for SYTO9. A filter was applied to each emission channel in accordance with the emission spectra of the fluorophores, i.e. SYTO9 and PI emissions were filtered from 487 nm to 560 nm and from 590 nm to 700 nm, respectively. The channel colors were digitally set to green for SYTO9 emission and red for PI emission.

Image slices of the equatorial cross-sections of the beads in solution were acquired for each emission channel, as well as z-stacks containing multiple image planes separated by a constant vertical distance step set to ~4 µm for overlapping planes and ~10 µm for non-overlapping planes. Each image slice had a horizontal resolution of 0.62 µm in both the x- and y-directions and contained 1024 × 1024 pixels, yielding a final field of view of 639 µm × 639 µm. An optimal pinhole diameter was automatically found by the software in order to get a vertical resolution of 1.0 µm (z-direction) and the laser intensity and master gain of the signal were manually adjusted to form a well-exposed image.

3.2.4.3 Quantitative image analysis

All images were analyzed using the image processing software ImageJ (version 2.0.0-rc-69/1.52r). Bead sizes were measured from brightfield images by using the software to calculate the lengths of manually drawn diameter lines on the images.

The quantitative viability image analysis underwent many iterations in order to produce as consistent results as possible. The final quantification method consisted of multiple image processing steps. First, a composite image of the two fluorescent channels was used to count the total number of cells (#total). Next, each channel was analyzed separately to count the number of alive (#alive) and dead (#dead) cells, respectively. Then, overlapping cells (#overlaps) were accounted for by subtracting the total number of cells from the sum of #alive and #dead cells:

$$\#\text{overlaps} = \#\text{alive} + \#\text{dead} - \#\text{total}$$

The overlapping cells were assumed to be dead cells; hence they were subtracted from #alive in order to compute the true number of alive cells (#trulyAlive):

$$\#\text{trulyAlive} = \#\text{alive} - \#\text{overlaps}$$

Finally, percentages of alive and dead cells were calculated from the following equations:

$$\%\text{alive} = \frac{\#\text{trulyAlive}}{\#\text{total}} \times 100\,\%$$

$$\%\text{dead} = \frac{\#\text{dead}}{\#\text{total}} \times 100\,\%$$

An ImageJ macro script was developed for automatic detection and counting of the bacterial cells in order to minimize inconsistencies in manual counting methods. Further specifications of the ImageJ script can be found in Appendix C.

3.3 Results

3.3.1 Bead properties

The harvested ACHIM bacteria were successfully immobilized in the Ca-alginate beads (Appendix A). Furthermore, shrinkage and swelling of the alginate-bacteria beads occurred in the simulated GI fluids. Specifically, alginate beads shrunk in SGF and swelled in SIF. A time plot of bead size reduction in SGF is shown in Figure 8.

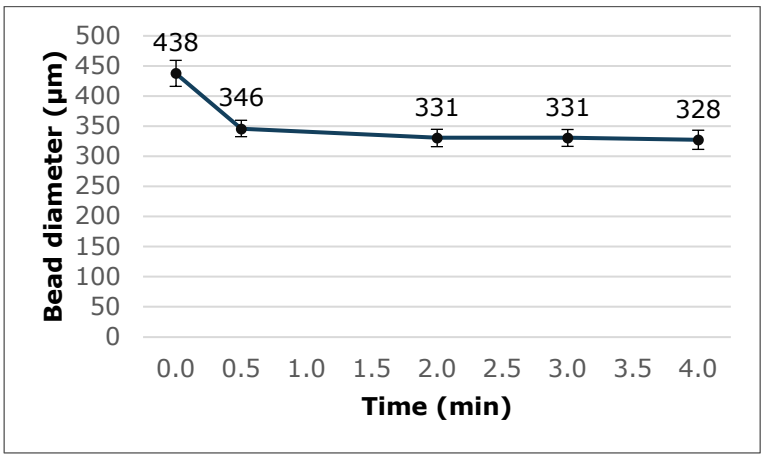

Figure 8. Time plot of alginate-bacteria bead size reduction following exposure to SGF. Beads washed in physiological saline (time-point 0.0) were exposed to SGF and the diameter of the beads were measured from brightfield microscope images. Mean bead diameters were calculated from n = 18 beads. Error bars show standard deviation.

The alginate beads containing bacteria stayed intact under exposure to SGF for 30 min. However, beads that were subsequently exposed to SIF directly after exposure to SGF for 30 min rapidly degraded and dissolved in the matter of seconds to minutes, demonstrating that the system has potential for targeted delivery to the intestines. Figure 9 displays alginate-bacteria beads exposed to SGF for 30 min followed by exposure to SIF for different time intervals.

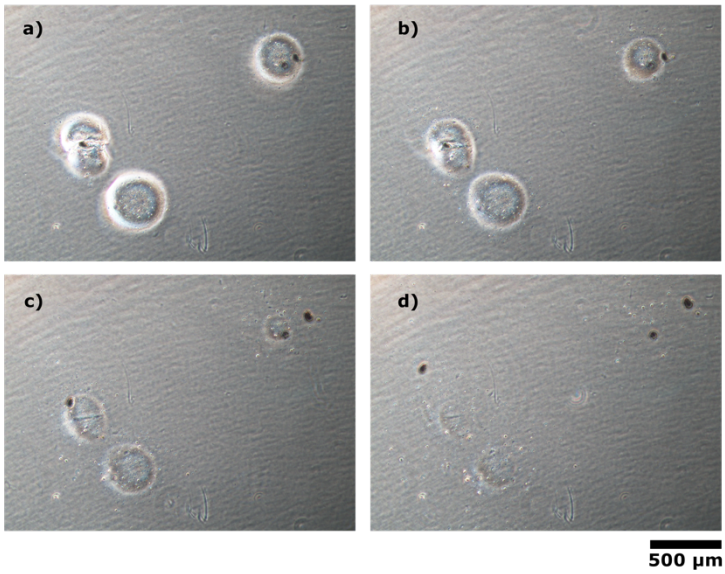

Figure 9. Brightfield images showing dissolution of alginate-bacteria beads during simulated GI transit. Exposure to SGF for 30 min followed by SIF for a) 20 seconds, b) 30 seconds, c) 60 seconds, and d) 80 seconds (completely dissolved).

3.3.2 Quantitative viability analysis

Quantitative viability data were based on viability staining and image analysis of either single slice or z-stack fluorescent images from the most relevant updated experiments. Figure 10 shows percentages of alive bacteria relative to the total cell counts for alginate-bacteria beads washed in physiological saline after gelation and for beads subsequently exposed to the SGF solution for 30 min. Each field of view captured approximately one bead and contained cell counts on the order of 10^2 bacterial cells. For unknown reasons, several of the updated experiments suffered from overexposed red channels in SGF samples possibly due to problems with the staining procedure, which is why there are little SGF data in experiment one and two. The problem was eventually fixed, allowing for one proper experiment (experiment three) to be conducted before the experimental work was abruptly discontinued.

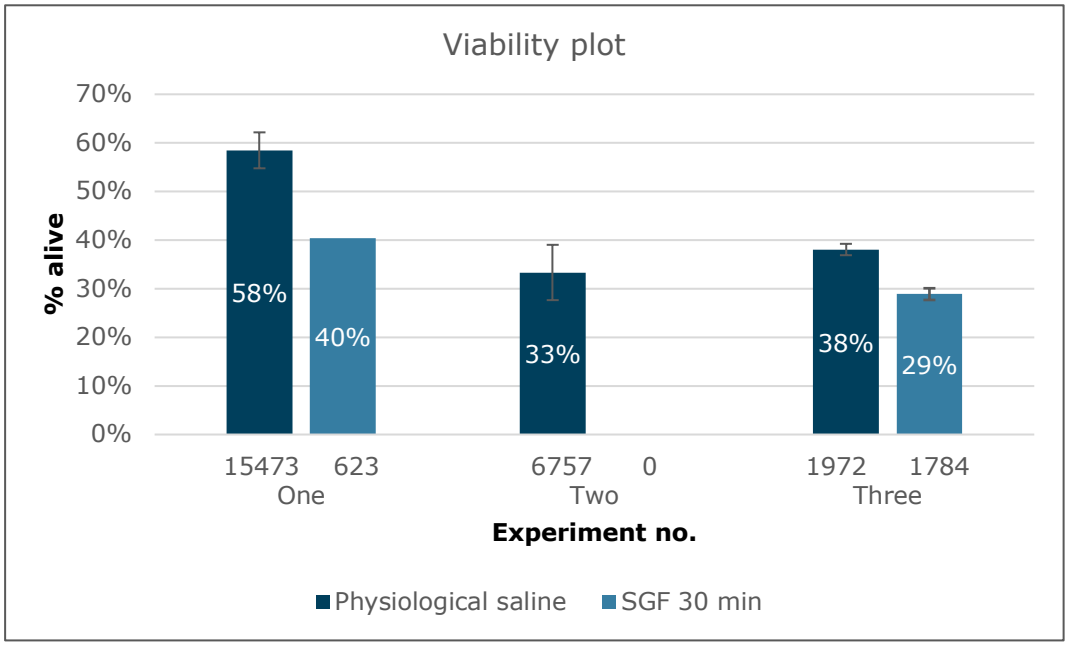

Figure 10. Viability plot of the most relevant updated experiments. Bacterial cell viability was calculated for alginate-bacteria beads in physiological saline and for beads exposed to SGF for 30 min based on image analysis of CLSM fluorescent images (single slices and z-stacks). The percentages of alive (% alive) bacteria in each sample relative to total cell counts are indicated by the bars and numbers within the bars. The numbers under each bar show the total cell count in the corresponding calculation. Error bars indicate standard deviation between multiple images (fields of view).

Noticeably, the updated image analysis process generated consistent results within each experiment, as seen from the relatively low standard deviations in Figure 10. On the contrary, inconsistent results were observed between experiments, such as experiment one and three in Figure 10, indicating that there are other, perhaps more practical methodological steps in need of optimization. Moreover, the beads washed in physiological saline displayed variable bacterial viabilities in the different experiments, further suggesting that the system is sensitive to methodological factors. In any case, there is evidence that the viability of the entrapped bacteria decreased in the low pH SGF solution compared to the samples in physiological saline, which is in agreement with previous results (Appendix A).

As formerly stated, the quantitative viability analysis was thought to be the major weakness from previous work. Looking back at previous analyses it became clear that

many of the utilized images had underexposed red channels, which likely resulted in artificially high viability counts (~71 %; Appendix A). For instance, Figure 11 depicts two fluorescent images from previous work taken from the same physiological saline sample with two different CLSM settings causing varying degree of exposure in the two channels, ultimately resulting in two vastly different viability counts (71 % vs. 40 %). Consequently, in the present work, images were carefully inspected to not have under- or overexposed channels, which likely contributed to generating more precise viability data along with the improved image processing method.

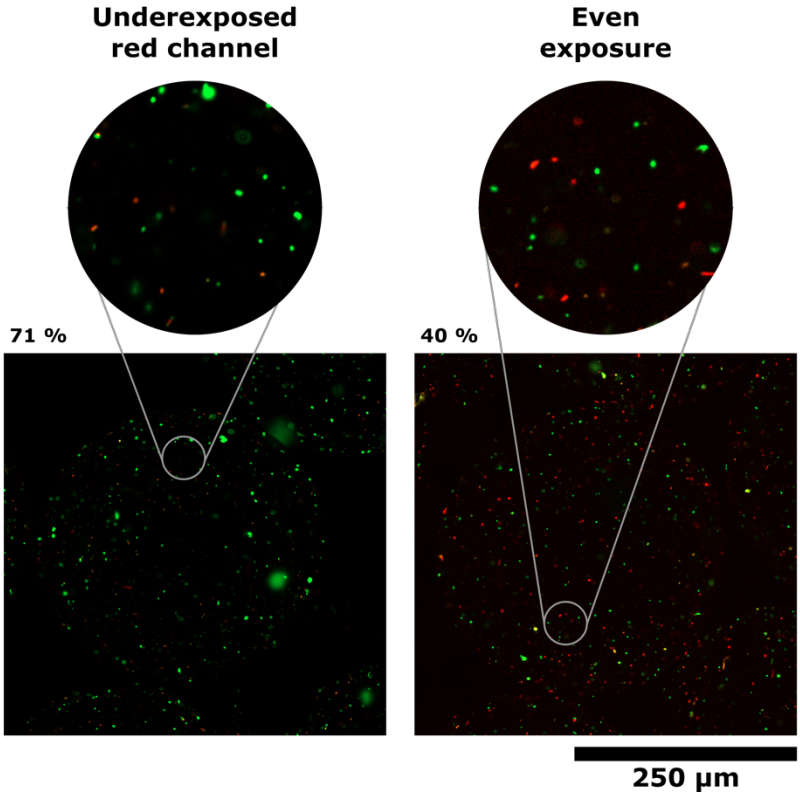

Figure 11. Two CLSM fluorescent images from the same alginate-bacteria beads sample in physiological saline but with different exposures of the red and green channels. Right: Underexposed red channel led to an overestimation of the viability (71 % alive). Left: More even exposure presumably resulted in a more accurate viability count (40 % alive).

3.4 Discussion

3.4.1 Bead properties

Alginate-bacteria beads ionically cross-linked by Ca^{2+} were shown to stay intact under exposure to SGF solution, signifying that the system could offer elevated protection of the entrapped bacteria during gastric transit. Moreover, the dissolution of the beads following sequential exposure to SIF after SGF suggests that release of the bacteria is likely to occur in the small intestine, which is indeed analogous to the current endoscopically delivered ACHIM enema (174). However, the preferred manner of release of a microbiota product is potentially a sustained release that extends to further down the GI tract, which could be made possible by adjusting the system's ability to better withstand intestinal fluid and thereby offer a controlled delivery to the large intestine (see section 3.4.5).

Swelling and dissolution of Ca^{2+} cross-linked alginate beads is well-documented and known to occur in simulated GI conditions due to the presence of non-gel-inducing ions and Ca^{2+}-sequestering agents (100,175). Specifically, Ca^{2+}-sequestering phosphate and the non-gelling ions Na^{+}, Mg^{2+}, and K^{+} were present in the SIF solution from the dissolved salts (Table 3). Consequently, beads exposed to the SIF solution alone would swell and possibly dissolve after a while, which has also been previously shown (173). However, pre-exposure to low pH in the SGF solution seemed to accelerate the swelling and dissolution rates significantly, as the alginate-bacteria beads were dissolved in the matter of seconds to minutes (Figure 9).

The rapid dissolution dynamics observed in the present work can possibly be explained by a combination of an acidic alginate gel appearing in SGF and the introduction of non-gel-inducing ions and Ca^{2+}-sequestering agents from the SIF solution, thereby accelerating swelling and dissolution of the beads. First, the detected size reduction of alginate-bacteria beads in SGF was likely due to shrinkage of the gel following protonation of the alginate polymer. The pH in the SGF solution (pH 2.0) was below the pK_a value of the uronic acid residues (3.3 to 4.0) in the alginate polymer, meaning negative charges on the carboxyl groups would be protonated and neutralized. Consequently, electrostatic repulsions within the gel network would be weakened, resulting in contraction of the gel. The gel stayed intact possibly due to an increase of covalent cross-links between segments of the alginate gel which were not strongly ionically cross-linked with Ca^{2+}, such as M-blocks (109). Moreover, protonation of the carboxyl groups would reduce the number of non-condensed charges inside the gel (108), causing a reduction in the ion concentration difference between the alginate gel beads and the surrounding solution, in turn leading to release of Ca^{2+} ions from the gel. As the shrinkage occurred over a relatively short period of time in the acidic SGF solution (Figure 8), it is likely that protons rapidly diffused into the alginate beads and performed ion exchanges with Ca^{2+}.

Next, when the alginate polymer was subsequently exposed to SIF holding a neutral pH, the carboxyl groups would be deprotonated, causing increased electrostatic repulsions within the gel and break the covalent cross-links formed in the acidic alginate gel. As a result, the gel would expand, and the beads would swell. There would also probably be less Ca^{2+} present in the SGF-exposed alginate beads due to the proton exchange and release of Ca^{2+}, suggesting that there would be few putative ionic cross-links to be formed in the subsequent SIF-exposed deprotonated alginate gel, thus lowering the strength of the beads. In addition, the SIF solution had a relatively high ionic strength

compared to the alginate beads due to the dissolved salts (Table 3), meaning that the non-gel-inducing ions (Na^+, Mg^{2+}, and K^+) and phosphate from SIF would readily diffuse into the gel and exchange or sequester any remaining Ca^{2+} ions, further reducing the ionic cross-linking effect and allow for accelerated swelling. Taken together, these concurrent mechanisms could probably explain the observed swelling and dissolution dynamics in the alginate-bacteria beads exposed to the simulated GI conditions.

3.4.2 Quantitative viability analysis procedure

The overall goal for the quantitative viability analysis in this work was to enable consistent results. Therefore, several measures were taken in order to improve the credibility of the image analysis data. First, the image analysis process in ImageJ was markedly enhanced in order to better account for double staining of the cells with the two fluorescent stains (PI and SYTO9) and provide more consistent counts. The specifics of the upgraded ImageJ analysis can be found in Appendix C.

Next, the use of z-stacks enabled more data to be collected from each alginate-bacteria bead by collecting several image planes. Initially, since the bacteria were scattered across multiple image planes in the beads, it was thought that z-stacks could generate significantly better focus of the bacteria by using a small step size and project a few overlapping image planes into one image. While this was partly true, projecting multiple planes into one image yielded the same results as when compared to analyzing each image plane separately. For this reason, z-stacks were mostly acquired with a step size of ~10 µm in order to not get overlapping image planes that could be projected into one image, but rather collect more data from each alginate-bacteria bead.

Finally, manual adjustment of the CLSM instrument was found to generate better-quality images than by automated methods. Hence, the CLSM instrument was manually adjusted in multiple ways, such as laser intensity and master gain, in order to generate visually good-looking images by accounting for attributes such as exposure, contrast or signal-to-noise ratio, and double staining of the cells with the two fluorophores. Reevaluation of previous work strongly suggested that images had frequently been acquired with an underexposed red channel, which ultimately resulted in artificially high viability counts. Therefore, in the updated experiments, exposure of the two channels (red and green) were carefully inspected and adjusted to yield a more even intensity from the channels in the final fluorescent image. In short, added experience with the CLSM instrument provided improved images for further analysis.

3.4.3 Survival of the ACHIM bacteria

Although the updated quantitative viability analysis generated more consistent results within each experiment, it produced variable results amid analogous experiments, especially for the alginate-bacteria beads washed in physiological saline (Figure 10). In turn, this observation may suggest that there are other methodological steps accounting for the discrepancies in the viability data. For example, small variations in the exposure to oxygen between different experiments could potentially have inflicted significant fluctuations on the viability of obligate anaerobes present in the ACHIM culture. As it is not generally known how quickly many obligate anaerobes die in the presence of oxygen, performing the experiments with minimal or at least a constant time of exposure to air by timing each methodological step carefully and promptly operating the CLSM instrument could have yielded more consistent results overall.

Nevertheless, the quantitative viability analysis of alginate-bacteria beads indicated a difference in the survival of ACHIM bacteria between beads washed in physiological saline and beads subsequently exposed to the SGF solution (Figure 10). These results are in line with results from previous work and suggest that, while the beads stay intact and the bacteria are immobilized during SGF exposure, the bacteria are possibly not well protected against the unfavorable acidic environment. For this reason, adding $CaCO_3$ to the alginate-bacteria beads was tested in previous work in order to generate a buffer system that would counteract changes in pH and thereby enhance the survival of the bacteria in SGF. However, the added $CaCO_3$ caused unexpected cracking of the beads in SGF possibly due to dissolution of $CaCO_3$ in acid, which resulted in unimproved viability counts for the alginate-$CaCO_3$ system (173).

3.4.4 Comparison of quantitative viability data with literature findings

In the literature, plate counting expressed in colony-forming units (CFU) is the most widely used measure of microbial viability. However, the specific microbiota product used in this study, i.e. ACHIM, is a unique anaerobic culture with a patented cultivation technology, hence assessing the viability of the bacteria based on cultivation methods was deemed impractical. Furthermore, there is no standardized method for measuring the viability of a cultivated microbiota as very few such products even exist. For these reasons, the LIVE/DEAD *Bac*Light viability fluorescent stain kit was chosen in order to yield a rapid and direct evaluation of the viability of the ACHIM bacteria.

Fluorescent viability assays are not commonly applied to assess the viability of probiotic delivery systems, but it has been used on numerous microorganisms, including probiotic strains (176). For example, Auty et al. (177) used the LIVE/DEAD *Bac*Light viability kit in conjunction with a CLSM instrument to investigate viability of the two probiotic strains *Lactobacillus paracasei* NFBC 338 and *Bifidobacterium sp.* strain UCC 35612 from fermented milk. In the study, fluorescent viability counts were compared to plate counts (CFU ml^{-1}) by computing the microscopic factor of the CLSM instrument and multiplying by the average number of cells in the microscope images to generate a direct enumeration count. As the plate counts were approximately 20-fold to 10-fold lower relative to the direct microscopic counts for the two strains, the authors concluded that plate counting potentially led to an underestimation of actual bacterial numbers.

In the present work, a similar coarse comparison was attempted by the following line of reasoning. First, the ACHIM bacteria were harvested by centrifugation and a number of dilution steps, resulting in a final dilution factor of about 150 (see section 3.2.1.3). Then, as each CLSM fluorescent image slice displayed dimensions of 639 µm x 639 µm x 1 µm in the x-, y-, and z-directions, respectively, and contained bacterial cells (dead and alive) on the order of 10^2, it was possible to extrapolate that the initial ACHIM culture contained about 10^{10} to 10^{11} bacterial cells per ml. However, these numbers are considerably higher than the counts proclaimed by NIM Supplement AS, the supplier of the ACHIM culture, which are based on cultivation in diluted mediums and are on the order of 10^9 to 10^{10} cells per ml. On the other hand, according to the abovementioned study by Auty et al. (177), a discrepancy between direct enumeration counts and cultivation-based counts is perhaps to be expected, where cultivation-based counts conceivably underestimate the actual number of bacteria in a culture. Consequently, the observed total cell counts from CLSM images could indeed offer a more accurate estimation of the concentration of bacteria in the ACHIM culture.

Moreover, assuming an average bacterial cell size of 1 µm to 2 µm in diameter and a maximum theoretical packing factor of 0.74 for spherical structures (hexagonal close-packed or face-centered cubic), it becomes clear that the theoretical limit for packing spherical bacterial cells in a volume is in fact on the order of 10^{10} to 10^{11} cells per ml. Hence, one could postulate that the ACHIM bacteria were initially very closely packed and possibly not present as single cells, but rather formed more complex microbial communities. This hypothesis would be in agreement with the observed microcolonies in the undiluted ACHIM sample that inhibited single-cell fluorescent staining, as illustrated in Appendix B. As a result, it is likely that assembly of the ACHIM bacteria was significantly modified following the dilution procedure, insinuating that the culture could display an altered therapeutic ability in the final delivery system. This statement is especially true when taking into account that the supernatant containing microbial products and other components that may be of importance was discarded in order to harvest the bacteria for entrapment in alginate beads and subsequent viability assessment.

Cell loading or 'encapsulation efficiency' is another parameter frequently measured in studies on cell delivery systems. The parameter refers to the number of cells that are successfully immobilized within a confined structure. Although cell loading can be addressed in multiple ways, it generally states the number of viable immobilized bacteria in relation to the number of bacteria in the initial culture or solution. Here, alginate-based systems are known to generate a high fraction of immobilized cells, reaching almost 100 % for extrusion approaches (178). In previous and present work, the ACHIM bacterial cells were successfully immobilized in the alginate beads with no observation of cells outside the beads. Hence, one could assume that the cell loading was close to 100 %, which both allows for the above enumeration calculations to be performed and enables calculation of the number of cells in the alginate solution upon mixing, which would simply be 150 times less than the previously stated numbers, i.e. on the order of 10^8 to 10^9. To date, the endoscopically administered amount of the ACHIM enema to patients is usually about 50 ml (6). Therefore, assuming a concentration of bacteria in the ACHIM enema of 10^{10} cells per ml, it would be necessary to administer around 5000 ml of the alginate-bacteria beads to achieve the same number of bacteria, which would, naturally, be impractical to perform in a single administration. However, the preferred dosage of the ACHIM culture is uncertain, suggesting that smaller, more frequent doses could yield the same or even improved results over a longer time span.

3.4.5 Further research

For the future, several interesting paths can be taken in order to provide more information on the possibilities of a microencapsulated oral delivery system for a microbiota-based product. First, improving the viability of the encapsulated bacteria in low pH environments can be enabled by coating of the alginate beads with polycation materials such as chitosan or even multilayer alginate beads by offering improved protection and a more beneficial microenvironment for the bacteria (114,172,179). In addition, such systems could potentially display improved stability during GI transit and extend the release of the bacteria to include colonic delivery (103), which may be the preferred release mechanism of a microbiota-based product.

Next, assessing the impact of exposure to oxygen on the ACHIM bacteria could be made possible by including encapsulation experiments on specific known aerobe and obligate anaerobe strains as controls. For instance, consistently varying the degree of exposure to oxygen for two strains, one aerobe and one obligate anaerobe, during the encapsulation

procedure and followed by fluorescent viability assessment could clarify the effect of oxygen on obligate anaerobes in the time frames and methods of the encapsulation process. Moreover, comparison of encapsulated bacteria vs. non-encapsulated bacteria, which has been previously attempted unsuccessfully (173), could provide more information on the effects of microencapsulation technologies on microbiota-based products in general. Lastly, a more precise measure of the specific bacterial species and strains present before and under the simulated GI conditions can be obtained by using 16S rRNA sequencing before and at each checkpoint in the simulated GI transit. Combined with an overall improved execution of the experiments as discussed in the preceding sections, these proposals will further elucidate the promises of microencapsulation technologies for oral targeted delivery of microbiota-based products.

3.5 Conclusion

In the present work, bacteria from the microbiota-based product ACHIM were harvested and successfully entrapped in Ca-alginate beads. The alginate-bacteria beads were shown to stay intact during simulated gastric transit in a low pH solution (SGF). As the beads were subsequently exposed to a simulated intestinal environment with a neutral pH (SIF), swelling and dissolution of the alginate gel transpired rapidly, resulting in release of the bacteria from the beads, indicating that physiological delivery would occur in the small intestine. Next, the quantitative viability analysis of CLSM fluorescent images was optimized to accurately be able to count the number of alive and dead cells from a well-exposed fluorescent image. Although the quantitative viability data displayed variable results between similar experiments, there were indications that the entrapped bacteria died off in SGF. A rough comparison of the direct microscopic enumeration counts to culture-based counts was attempted by an extrapolation calculation, which indicated that there were in fact 10-fold to 100-fold more bacteria present in the ACHIM sample than as stated by the supplier. The discrepancy in the counting methods was possibly explained by the potential underestimation of cultivation-based counting, as well as the formation of more complex microbial communities in the undiluted ACHIM sample allowing for bacterial packing close to the theoretical limit for spherical bacteria. Furthermore, the variability in the quantitative viability results could potentially be explained by methodological issues such as varying degree of exposure to oxygen between experiments. Therefore, for future studies, it would be interesting to include known aerobe and anaerobe strains as controls in order to assess the effect of exposure to oxygen during the experimental procedures. In addition, improving the encapsulation system could be made possible by coating with polysaccharides or producing multilayer alginate beads in order to offer enhanced protection of the bacteria in SGF and extend the delivery further down the GI tract. All in all, the experimental study conducted here and in previous work signify that there is a potential for applying microencapsulation technologies to next-generation microbiota-based products in the future.

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Appendix

Appendix A: Relevant previous results

Appendix B: Microcolonies of bacteria

Appendix C: ImageJ macro script for automated cell counting

Appendix A: Relevant previous results

The most relevant results from previous work (project thesis) are included below. In Figure 12 is a brightfield image showing the ACHIM bacteria successfully immobilized in a Ca-alginate bead, which was also achieved in the updated experiments. Then, viability data from a previous experiment are presented in Figure 13, which likely suffered from artificially high viability counts due to underexposed red channels in the fluorescent images. Moreover, the viability data displayed large variations depending on the image analysis method and the variable exposures of the two fluorescent channels, suggesting that an overall improved quantitative viability analysis was needed.

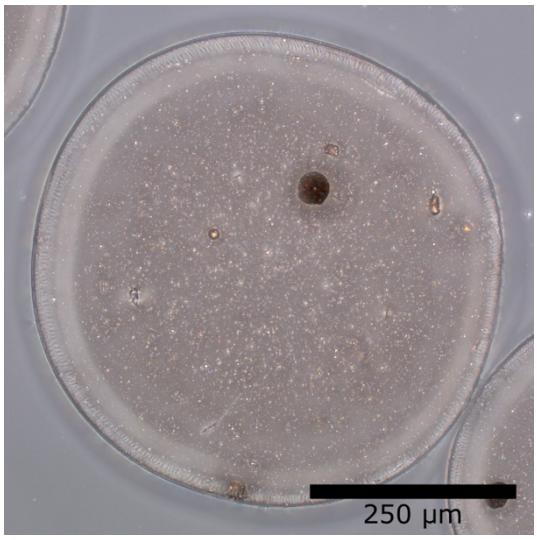


Figure 12. Brightfield image of ACHIM bacteria successfully immobilized in an alginate bead. The image was acquired by projecting multiple cross-sectional planes into one image.

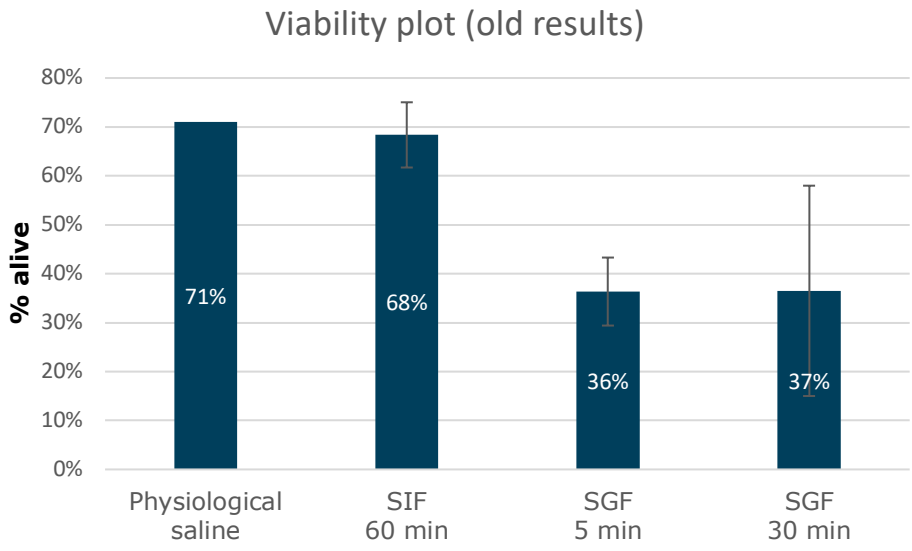


Figure 13. Viability plot from one previous experiment. Bacterial cell viability was calculated for alginate-bacteria beads in physiological saline and for beads exposed to SIF alone for 60 min and SGF for 5 min and 30 min based on image analysis of CLSM fluorescent images (only single slices). The percentages of alive (% alive) bacteria in each sample relative to total cell counts are indicated by the bars and numbers within the bars. Error bars represent standard deviation between triplicate samples in the one experiment.

Appendix B: Microcolonies of bacteria

Microcolony-forming bacteria were packed so tightly *in situ* that several adjacent cells or whole cell clusters were detected as one single object, rendering fluorescent images unusable for image analysis, as demonstrated in Figure 14. Reduction of these microcolonies was achieved by an improved washing procedure of the bacteria before mixing with the alginate polymer solution.

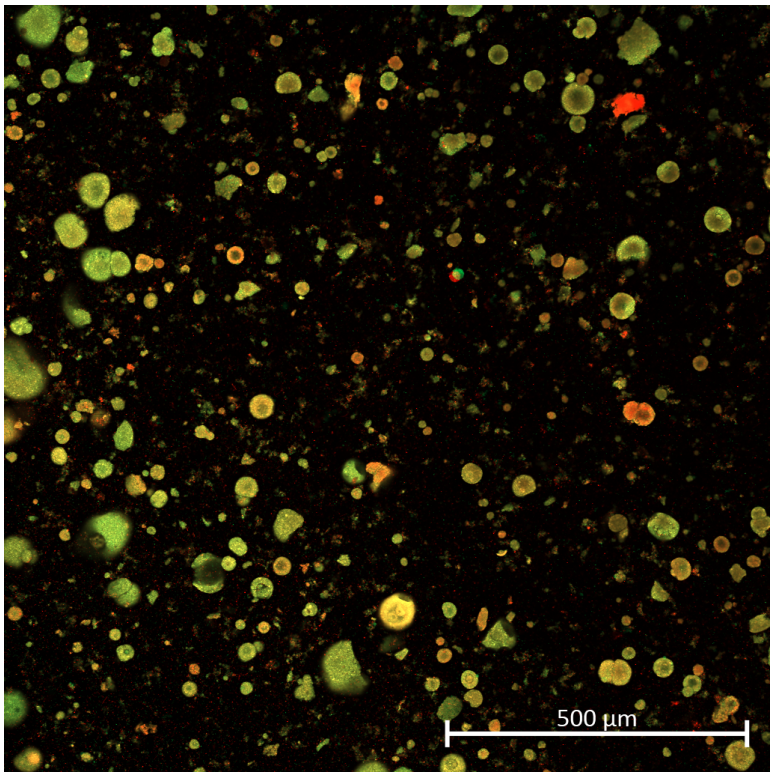
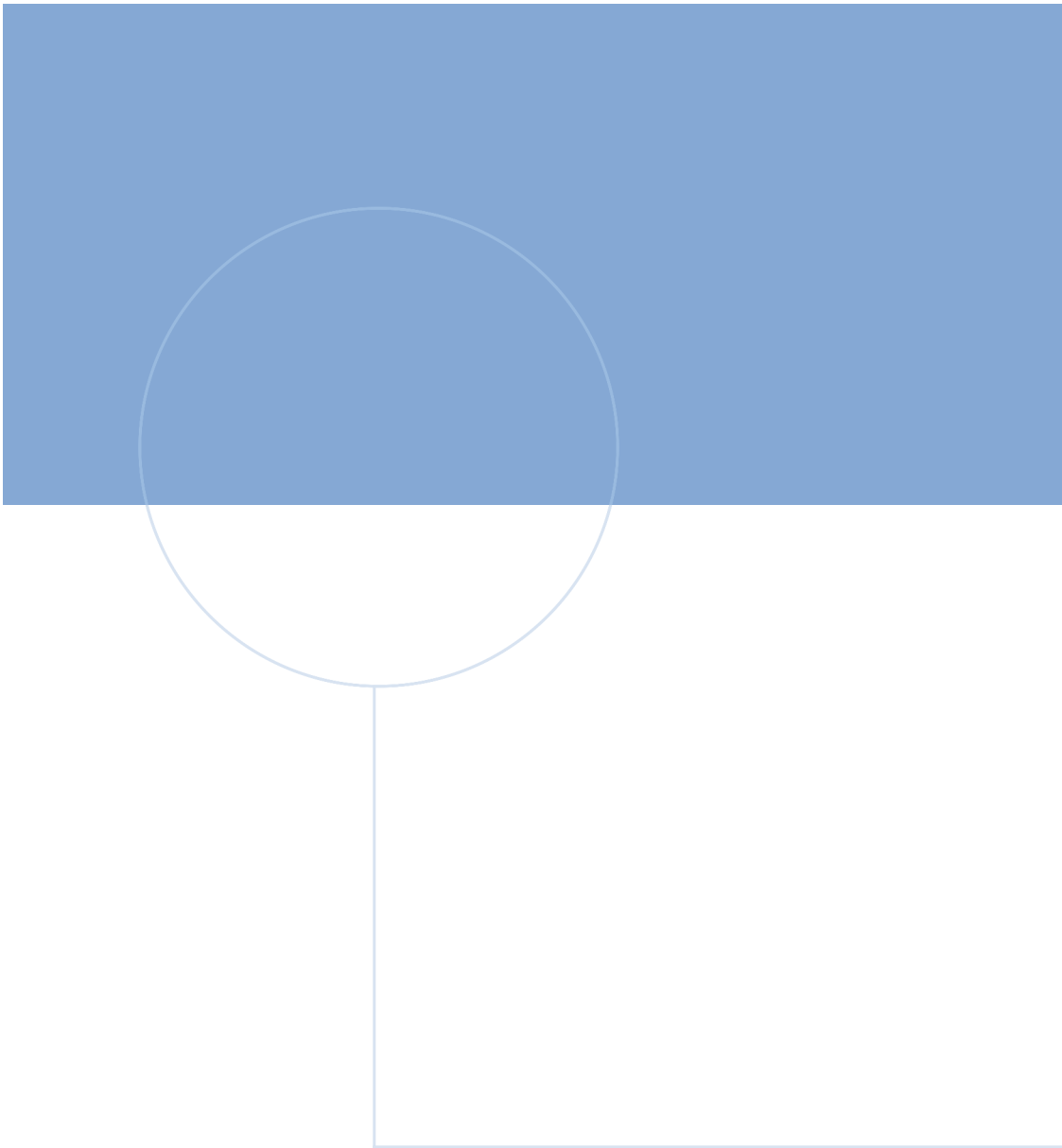


Figure 14. CLSM image of undiluted ACHIM bacteria containing microcolonies which impaired the fluorescent viability analysis.

Appendix C: ImageJ macro script for automated cell counting

The following script was developed in order to provide consistent counts of the total number of cells (composite image) and the number of alive (green channel) and dead (red channel) cells from a two-channel fluorescent image. After performing several manual counting measurements, it was found that applying the same threshold to all images (composite and green and red channels) of 20 times the mean intensity of the RGB composite image generated the most consistent counts.

```
//open a file and get the title...

run("Open...");

originalTitle = getTitle();

//prepare the image for analysis...

run("Despeckle"); //remove speckle noise

run("RGB Color"); //convert to 32-bit RGB color

getStatistics(area, mean, min, max, std, histogram); //retrieve the mean intensity

thresh = 20*mean; //store threshold value (20*mean intensity of the composite RGB)

//count total cells from composite RGB image...

colorThresh(thresh); //apply threshold using the autogenerated colorThresh function

run("Analyze Particles...", "size=1-100 circularity=0.00-1.00 display clear add
      stack"); //size restriction given in um^2

total = roiManager("count"); //store total cell counts

roiManager("reset");

selectWindow(originalTitle);

run("Split Channels"); //split composite image into two channels (red and green)

//red channel...

selectWindow("C1-" + originalTitle);

run("RGB Color"); //convert to 32-bit RGB color

colorThresh(thresh); //apply threshold

run("Analyze Particles...", "size=1-100 circularity=0.00-1.00 display clear add
      stack"); //size restriction given in um^2

dead = roiManager("count"); //store number of dead cell counts

roiManager("reset");

//green channel...

selectWindow("C2-" + originalTitle);

run("RGB Color"); //convert to 32-bit RGB color

colorThresh(thresh); //apply threshold

run("Analyze Particles...", "size=1-100 circularity=0.00-1.00 display clear add
      stack"); //size restriction given in um^2

alive = roiManager("count"); //store number of alive cell counts

print(mean, thresh, total, alive, dead); //output
```



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