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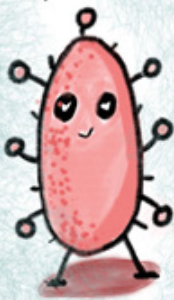
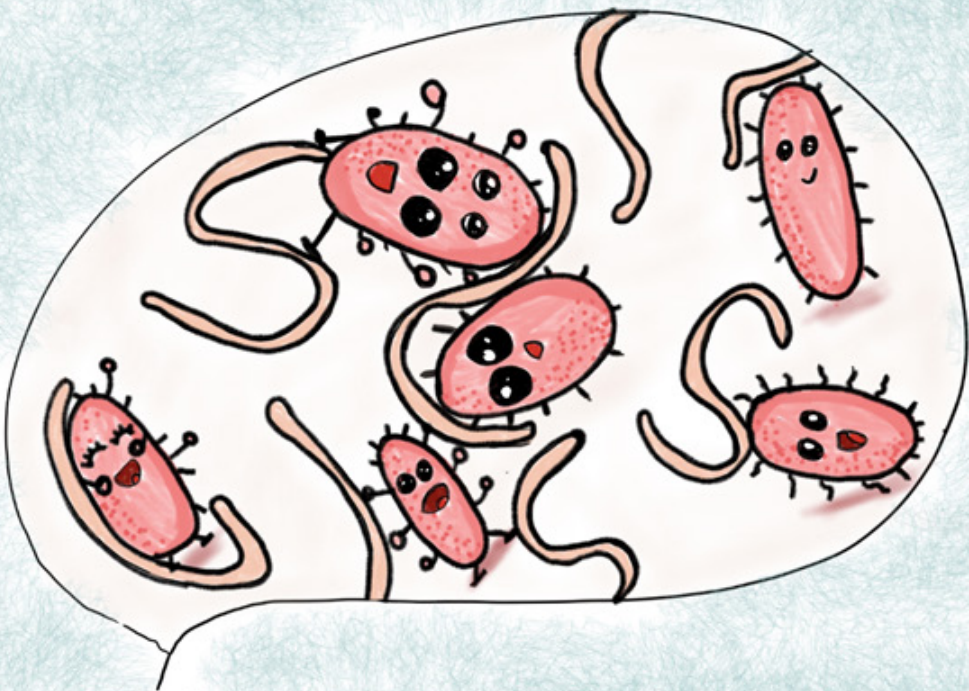
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Oleogels as controlled delivery systems for probiotics

LINGPING ZHANG

DEPARTMENT OF PROCESS AND LIFE SCIENCE ENGINEERING | LUND UNIVERSITY



My name is *L. reuteri*,
I love to stay in oleogel.



Oleogels as controlled delivery systems for probiotics

Oleogels as controlled delivery systems for probiotics

Lingping Zhang



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on 14th of June at 09.15 in lecture hall A, Kemicentrum, Naturvetarvägen 14, Lund.

Faculty opponent

Professor Hanne Mörck Nielsen

University of Copenhagen, Denmark.

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Abstract:

A new encapsulation strategy for probiotics is highly needed to tackle the challenge of significant viability loss of bacteria during storage and passage through the gastrointestinal system. A new controlled delivery system for probiotics based on EC oleogels is proposed in this thesis.

A relatively non-complicated in vitro lipolysis model was established to evaluate the relative conversion from triglycerides to free fatty acids and the retarding effect on lipolysis. Using this in vitro model, EC oleogels show a good retarding effect.

To apply EC oleogels as the controlled delivery system for *Limosilactobacillus reuteri*, a cold-mixing method was developed to tackle the high-temperature requirement. Using the cold-mixing method, probiotics were successfully encapsulated in oleogels. Meanwhile, oleogels are harmless to the bacteria, have protection against a lethal environment at the early part of the intestine (high bile concentrations and various enzymes), and can deliver live bacteria to the target location (ileum and colon). The main limitation is the limited release of encapsulated bacteria within 120 min.

By adding different excipients (lipid excipients and hydrophilic powder excipients) during the cold-mixing step, different release kinetics and release mechanisms can be obtained. Accelerating release (the ideal release kinetic) over time can be obtained by adding disintegrants or the combination of lipid excipients and disintegrants. A full release can also be achieved over a certain time.

The 90-day storage stability study (30°C/75% relative humidity and 40°C/75% relative humidity) shows that EC oleogels remain relatively stable after a short relaxation period (less than a week at room temperature). Furthermore, EC oleogels can keep the high viability of the encapsulated bacteria (less than 1 log₁₀ decrease in viability after 90 days at 40°C), protect the encapsulated bacteria in the in vitro solution, and deliver a high number of live bacteria to the ileum and colon.

To conclude, EC oleogels have good protection during storage and passage through the upper part of the gastrointestinal system. Meanwhile, EC oleogel formulations can deliver a high number of live bacteria to the target location. Hence, EC oleogels serve as a promising controlled-release delivery system for probiotics.

Key words: Ethylcellulose, oleogel, controlled delivery system, ileum and colon, probiotics, *L. reuteri*, lipolysis mechanism, in vitro, release kinetics, release mechanism, disintegrating.

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MADE IN SWEDEN 

*To my family and friends, who supported and loved me
My journey is the sea of the stars*

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Abstract

A new encapsulation strategy for probiotics is highly needed to tackle the challenge of significant viability loss of bacteria during storage and passage through the gastrointestinal system. A new controlled delivery system for probiotics based on EC oleogels is proposed in this thesis.

A relatively non-complicated *in vitro* lipolysis model was established to evaluate the relative conversion from triglycerides to free fatty acids and the retarding effect on lipolysis. Using this *in vitro* model, EC oleogels show a good retarding effect.

To apply EC oleogels as the controlled delivery system for *Limosilactobacillus reuteri*, a cold-mixing method was developed to tackle the high-temperature requirement. Using the cold-mixing method, probiotics were successfully encapsulated in oleogels. Meanwhile, oleogels are harmless to the bacteria, provide protection against a lethal environment at the early part of the intestine (high bile concentrations and various enzymes), and can deliver live bacteria to the target location (ileum and colon). The main limitation is the limited release of encapsulated bacteria within 120 min.

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To conclude, EC oleogels have good protection during storage and passage through the upper part of the gastrointestinal system. Meanwhile, EC oleogel formulations can deliver a high number of live bacteria to the target location. Hence, EC oleogels serve as a promising controlled-release delivery system for probiotics.

Popular scientific summary

Today, the importance of gut health has taken the center stage in the health world. Over 100 trillion microbial cells are living in our gut. Researchers have found that maintaining a healthy and balanced gut microbiota can largely enhance overall body health. Thus, the use of probiotics is becoming increasingly popular and is commonly known as “Probiotics – the superheroes of gut health”.

Probiotics are tiny beneficial bacteria that, when consumed, are thought to improve the balance of gut microbes, thereby promoting better digestion and overall health. For a probiotic supplement to be effective, each dose must contain at least 1 million viable cells. Despite efforts to meet this demand, challenges remain as probiotics can easily lose their viability.

Today's probiotic supplement products are mainly in solid form, e.g., tablets and powders in capsules. These solid-form probiotic products, when exposed to high temperature and high humidity, are not stable. This instability can result in a drastic reduction in the number of live probiotic cells by the time they reach consumers, causing the product to not work as well as advertised. A famous phrase for this is “Only the first spoon is working”.

This problem is compounded by the hostile environment of the gastrointestinal (GI) tract itself. Our GI tract was evolved to prevent the invasion of outsider bacteria to keep the host healthy. Without adequate protection, probiotics have difficulty surviving the acidic conditions of the stomach and the lethal environment at the beginning of the small intestine. Therefore, even if consumers follow dosing instructions carefully, the actual number of functional probiotic cells delivered to the target location is far lower than what is claimed on the package.

Recognizing these challenges, we developed a new oleogel formulation to enhance the stability and efficacy of probiotics. This new oleogel formulation, compared to probiotics without protection, provides around 10 times higher storage stability, as well as 10 times higher survivability in the lethal environment in the upper GI tract. Therefore, a total of almost 100 times higher viability could be achieved by using the new oleogel formulation. Besides protection, oleogel can also provide target delivery. Instead of having bacteria released at any position along the gut, the oleogel can deliver the probiotics to the place where they can function. This further increases the efficacy of probiotics.

This innovative oleogel formulation not only provides a protective matrix for the probiotic bacteria and controlled delivery to the target location, but it also offers several additional advantages. First, oleogels are easy to produce and the procedure does not harm the probiotics. The simple manufacturing process could potentially reduce production costs. Additionally, using the new method we developed, easy customization into various forms to suit different purposes and providing flexibility

in product design is highly possible. Meanwhile, the oleogel is safe for consumption and meets strict regulatory standards. Last but not least, its cost-effectiveness makes it an attractive option for both manufacturers and consumers.

In addition to their role in probiotic delivery, oleogels have the potential to serve as vehicles for delivering nutrients, such as healthy fats and prebiotics. This multi-faceted approach has the potential to revolutionize the probiotic industry, providing consumers with a more reliable, cost-effective, and versatile way to support gut health and overall well-being.

Essentially, while probiotics hold great promise in improving gut health, their effectiveness is often undermined by stability and survival barriers. Still, the emergence of breakthrough formulas like oleogels heralds an era of change. These innovations provide a beacon of hope that, through creative solutions, barriers to probiotic efficacy can be overcome. As a result, we have the potential to unlock the true power of probiotics and usher in a new era of enhanced digestive health and overall health.

Populärvetenskaplig sammanfattning

Idag har vikten av tarmhälsa hamnat i centrum i hälsovärlden. Över 100 biljoner mikrobiella celler lever i våra tarmar. Forskare har funnit att en hälsosam och balanserad tarmmikrobiota till stor del kan förbättra den allmänna hälsan. Användningen av probiotika blir därför allt mer populär och den är allmänt känd som "Probiotika – tarmhälsans superhjältar".

Probiotika är små nyttiga bakterier som, när de konsumeras, tros förbättra balansen mellan tarmmikrober och därigenom främja både bättre matsmältning och allmän hälsa. För att ett probiotiskt tillskott ska vara effektivt måste varje dos innehålla minst 1 miljon livskraftiga celler. Trots ansträngningar för att möta detta krav kvarstår dock utmaningar eftersom probiotika lätt kan förlora sin livskraft.

Dagens probiotiska kosttillskott är huvudsakligen i fast form, t.ex. tabletter och pulver i kapslar. Dessa probiotiska produkter i fast form är inte stabila när de utsätts för hög temperatur och hög luftfuktighet. Denna instabilitet kan resultera i en drastisk minskning av antalet levande probiotiska celler när de når konsumenterna, vilket gör att produkten inte fungerar så bra som annonserats. En berömd fras för detta är "Endast den första skeden fungerar".

Detta problem förvärras av den fientliga miljön i själva mag-tarmkanalen. Vår mag-tarmkanal utvecklades för att förhindra invasion av utomstående bakterier för att hålla värden frisk. Utan tillräckligt skydd har probiotika svårt att överleva de sura förhållandena i magen och den dödliga miljön i början av tunntarmen. Därför, även om konsumenterna följer doseringsinstruktionerna noggrant, är det faktiska antalet funktionella probiotiska celler som levereras till målplatsen mycket lägre än vad som påstås på förpackningen.

Vi insåg dessa utmaningar och utvecklade en ny oleogelformulering för att förbättra stabiliteten och effekten av probiotika. Denna nya oleogelformulering, jämfört med probiotika utan skydd, ger cirka 10 gånger högre lagringsstabilitet, samt 10 gånger högre överlevnadsförmåga i den dödliga miljön i den övre mag-tarmkanalen. Därför kan totalt nästan 100 gånger högre livskraft uppnås genom att använda den nya oleogelformuleringen. Förutom skydd kan oleogelen också ge målleverans. Istället för att bakterier frigörs var som helst längs tarmen kan oleogelen leverera probiotikan till den plats där de kan fungera. Detta ökar effekten av probiotika ytterligare.

Denna innovativa oleogelformulering ger inte bara en skyddande matris för de probiotiska bakterierna och levererar kontrollerat till målplatsen, utan den erbjuder också flera ytterligare fördelar. För det första är oleogeler lätta att producera och proceduren skadar inte probiotikan. Den enkla tillverkningsprocessen kan potentiellt minska produktionskostnaderna. Dessutom, med hjälp av den nya metoden vi utvecklat, är det mycket möjligt att enkelt anpassa oleogelen till olika

former för att passa olika ändamål och ge flexibilitet i produktdesignen. Samtidigt är oleogelen säker för konsumtion och uppfyller strikta regleringsstandarder. Sist men inte minst gör dess kostnadseffektivitet det till ett attraktivt alternativ för både tillverkare och konsumenter.

Förutom sin roll i probiotikatillförseln har oleogeler potential att fungera som fordon för att leverera näringsämnen, såsom hälsosamma fetter och prebiotika. Detta mångfacetterade tillvägagångssätt har potential att revolutionera den probiotiska industrin och ge konsumenterna ett mer pålitligt, kostnadseffektivt och mångsidigt sätt att stödja sin tarmhälsa och allmänt välbefinnande.

I grund och botten, även om probiotika är mycket lovande för att förbättra tarmhälsan, undergrävs deras effektivitet ofta av stabilitets- och överlevnadshinder. Ändå förebådar framväxten av banbrytande formler som oleogeler en era av förändring. Dessa innovationer ger en ledstjärna av hopp om att hinder för probiotisk effekt kan övervinnas genom kreativa lösningar. Som ett resultat har vi potential att låsa upp den sanna kraften i probiotika och inleda en ny era av förbättrad matsmältningshälsa och allmän hälsa.

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To my love **Björn Arvidsson**, I am so glad that you are in my life. Whenever I look at you, I feel happy, calm, and content. With your support, I feel I can do everything.

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List of Papers

Paper I: Oil-Based Delivery Control Release System Targeted to the Later Part of the Gastrointestinal Tract-A Mechanistic Study.

Zhang L., Wahlgren M., and Bergenståhl B. (2022). *Pharmaceutics*, 14(5), 896. <https://doi.org/10.3390/pharmaceutics14050896>

Paper II: Ethylcellulose oleogel as a controlled release delivery system for probiotics. Development and validation of a cold-mixing method.

Zhang L., Wahlgren M., Oscarsson E., and Bergenståhl B. (Manuscript)

Paper III: Ethylcellulose oleogels as controlled release systems for probiotics. The effect of disintegrating excipients on the release.

Zhang L., Wahlgren M., Bergenståhl B., Östsjö M., and Oscarsson E. (Manuscript)

Paper IV: A stability study of ethylcellulose oleogels as the controlled release delivery system for probiotics

Zhang L., Tomasson J., Ishimwe F., Östsjö M., Ekdahl V., Oscarsson E., Wahlgren M., and Bergenståhl B. (Manuscript)

Author's contribution to the papers

Paper I

I designed the study with my supervisors. I performed all the experimental work. Data analysis was discussed with my supervisors. I wrote the first draft of the paper, which was revised by the coauthors.

Paper II

I designed the study with my supervisors. I performed all the experimental work. Data analysis was discussed with my supervisors. I wrote the first draft of the paper, which was revised by the coauthors.

Paper III

I designed the study with my supervisors. I performed all the experimental work. Data analysis was discussed with my supervisors. I wrote the first draft of the paper, which was revised by the co-authors.

Paper IV

I designed the study with my supervisors. I performed most of the experimental work at BioGaia. Flow cytometry was conducted by co-authors. Data analysis was discussed with my supervisors. I wrote the first draft of the paper, which was revised by the co-authors.

Abbreviations

API	Active pharmaceutical ingredients
ATR	Attenuated total reflectance
CFU	Colony-forming unit
C12E5	Pentaethylene glycol monododecyl ether
EC	Ethylcellulose
ESIN	Engineered Stomach and small intestinal multi-compartments
GDO	Glycerol dioleate
GI	Gastrointestinal
GMO	Glycerol monooleate
GMP	Glycerol monopalmitate
GMS	Glycerol monostearate
MCT	Medium chain triglycerides
MD	Maltodextrin
NaTDC	Taurodeoxycholate
NaTC	Sodium taurocholate
NaCDC	Sodium chenodeoxycholate
PGPR	Polyglycerol polyricinoleate
POC	Polyoxyl castor oil
RH	Relative humidity
SCCM	Polyphenol oxidase sodium croscarmellose
SSG	Sodium starch glycolate
SHIME	Simulator of the human intestinal microbial ecosystem
SAXS	Small angle x-ray scattering
TBU	Tributylin units
TIM	TNO intestinal model
USP	US pharmacopeia
USAXS	Ultra-small angle x-ray scattering
WAXS	Wide angle x-ray scattering

Introduction

Microbiota and probiotics

Microorganisms that can coexist peacefully with the host are referred to as microbiota or microflora [1, 2]. It is estimated that human bodies contain up to 10^{14} bacterial cells, which are located on our skin, on the surface of the genitourinary, gastrointestinal (GI) tract, and respiratory tract [3, 4]. The number of microorganisms increases along the GI tract from proximal to distal. Around 70% of these bacteria are located in the colon [3, 5].

In recent years, studies have shown that the microbiota has an impact on immunomodulation, protection, and function of the GI tract and outside of the GI tract such as central and peripheral neural processes [6-10]. Studies have also shown that there exists communication and signaling between the microbiota and the host, microbiota, and pathogens, among microbiota, and between host pathogens [6]. It is thus of high interest and long tradition to consume 'safe' bacteria (i.e., probiotics) to modulate the gut microbiota and to improve gut health [11].

Probiotics are "live micro-organisms, which when consumed in adequate amounts, confer a health effect on the host" [12]. The health benefit can be classified into three modes, I) modulating the host immune system, II) directly effecting other microorganisms, and III) affecting microbial products and host products [13]. Probiotics occupy 65% of the world's functional food market. Those that have shown health benefits among probiotics are *Lactobacillus spp.*, *Bifidobacterium spp.*, *Saccharomyces boulardii*, *Propionibacterium spp.*, *Streptococcus spp.*, *Bacillus spp.*, *Enterococcus spp.*, and some specific strains of *Escherichia coli* [14].

The most used probiotics so far belong to the *Limosilactobacillus* and *Bifidobacterium* genera which are usually presented in fermented dairy products. *Limosilactobacillus* can be found in human milk, the urinary tract, and skin and is the sub-dominant microbiota in the GI tract [15, 16]. It is located in the epithelial surface, mucus layer, and intestinal lumen [6].

In this study, we used *Limosilactobacillus reuteri*. It has been proven that even with a high dosage ($2.9 \cdot 10^9$ CFU), *L. reuteri* is safe and efficacious in the GI tract. Meanwhile, *L. reuteri* has shown various health benefits on the host with secretions of reuteri, vitamins (B12 and B9), histamine and exopolysaccharide, etc. [16]. Clinical trials are using different *L. reuteri* strains to relieve caries, functional

abdominal pain, diarrhea, infant colic, atopic dermatitis, eczema, respiratory allergy, and regurgitation [17-24].

An adequate number of live bacteria (recommended to be 10^6 to 10^8 CFU per dose) is needed to exert health benefits [25]. Hence, the stability of probiotic formulations is crucial. There are reports of poor survivability of probiotics in different products during storage [26, 27]. Furthermore, to be able to induce health benefits after ingestion, probiotics need to pass through tough environments such as the low pH in the stomach, various enzymes, and high bile salts in the duodenum until they reach the ileum and colon [28]. The low pH and high bile salts also decrease the viability to a large extent. Therefore, to meet the requirement of an adequate number of live bacteria at the target delivery location after a certain period of storage, probiotic products usually need to have at least 100 to 1000 times the recommended number. This will significantly increase the cost of the product.

Even though some of the *L. reuteri* strains have shown resistance to low pH and bile salts [29, 30], the strain used in this study (DSM 17938) is highly sensitive to bile salts. A preliminary study (**Paper 2**) shows that 99% of the unprotected *L. reuteri* were dead after 30 min in vitro trials. Therefore, the main goal of this study is to develop a controlled release delivery system that could protect *L. reuteri* from the high bile concentration in the early part of the gastrointestinal tract and release the encapsulated bacteria at the later part of the small intestine (ileum) and colon.

Ileum and colon targeted delivery system for probiotics

Among different administrate routes, oral administration has the highest patient compliance [31]. For the drugs that are acid-sensitive as well as bile- and pancreatic enzyme-sensitive, various oral controlled-release delivery systems have been developed [32, 33]. The most common strategy is to use enteric coating [32].

Different pharmaceutical active ingredients (APIs), e.g., proteins, peptides, and probiotics, need to be delivered further to the ileum and colon to treat local diseases. The reason is that the colon is good for the adsorption of proteins and peptides meanwhile reducing systemic side effects [32].

Current ileum and colon-targeted delivery systems can be grouped into pH-controlled, magnetic/enzyme/bacteria-triggered, time-triggered, osmotic pressure-triggered, and ligand-receptor-based delivery systems by the mechanisms [33, 34]. The main challenges for pH-controlled, time-triggered, and osmotic pressure-triggered systems are the differences in the individual (fasted state/fed state, healthy/sick), and different gastric emptying times [33]. The main materials used in the target delivery to the ileum and colon in the literature contain alginate, k-carrageenan, gellan gum, xanthan gum, chitosan, starch, gelatine, cellulose acetate

phthalate, milk proteins, etc.[35]. Some studies have claimed that a microbiota-activated delivery system is one of the most efficient colon-targeted delivery system [33, 36]. However, these microbiotas are colonized in the colon, whereas *L. reuteri* is mainly colonized in the ileum.

Because of the fragility of probiotics against various conditions, an efficient ileum and colon-targeted delivery system is needed. One of the most common techniques used is encapsulation. The importance of the encapsulation of probiotics has been highlighted in 'Food Encapsulation: Global Market Analysis, Trends, and Forecasts'[37]. Different encapsulation of probiotics has been proven to provide higher stability and higher efficacy of probiotics [38]. Besides the conventional delivery system of food, various technologies have been explored for encapsulating probiotics, such as freeze drying, spray-freeze drying, emulsion process, extrusion process, electrospinning, etc.[34].

For commercially available probiotic products, different techniques are used. These techniques mainly can be divided into three groups, dried powders in tablet/soft capsules, dual/tri-layers of encapsulation, and microcapsule techniques [34]. Unfortunately, the techniques employed often lack clarity in most cases for customers [34]. In general, there are limitations in these current delivery systems for probiotics. For tablets and soft capsules, there usually is no extra protection against lethal environments in the gastrointestinal system. Dual/tri layers of encapsulation techniques are usually water-based systems which means that the viability of the encapsulated bacteria is at risk when the storage time is long. Microencapsulation usually is done with different techniques such as spray drying, extrusion, and spray coating [39]. These techniques involve high temperature which is detrimental to bacteria. Therefore, a new delivery system that is designed for probiotics is needed.

Targeted delivery systems for probiotics can learn from other colon-targeted delivery systems, but there are several parameters to consider. An ideal controlled release delivery system for probiotics should meet several the criteria, such as no harm to the bacteria, provide protection from harmful conditions during storage (high oxygen and high humidity), give protection from the lethal environment in the GI tract (low pH, high bile salts concentration, and different enzymes and other components) [40, 41]. This ideal formulation should also be able to control the release less influenced by different conditions of different groups of people (children, adults, and elderly) or different conditions between sick and healthy people as probiotics can be either a treatment for certain diseases or just as a health supplement. Also, hopefully, the ideal formulation has a relatively easy processing procedure. Furthermore, the release pattern of such ideal formulation aims for less or no release until the later part of the small intestine with a burst/constant release at the ileum and the colon.

In this thesis, we proposed an oil-based controlled delivery system targeting the ileum and colon. Generally, oil is harmless to bacteria or the body. Oil is a good encapsulation medium for probiotics because it could be the isolation barrier between probiotics and the oxygen and humidity in the surrounding environment. Oxygen and humidity are the two major parameters that deprive the viability of bacteria during storage [34]. A low moisture content and anaerobic environment is usually preferred. However, too low moisture content is not recommended. Bacteria viability decreased by 44% when moisture content decreased from 2.8% to 0% [42].

Despite all the potential benefits from the oil-based delivery systems, there are lots of challenges. The main challenge is that the majority of the ingested oil will be digested in the small intestine before reaching the ileum [43]. Therefore, pure oil cannot be the delivery system for target delivery to the ileum and colon. Meanwhile, ingestion of oil will induce the secretion of bile salts in the duodenum. When the oil is digested from the oil-based delivery system, causing the release of bacteria, the released bacteria will soon die due to the high concentrations of bile salts in the environment.

One way to utilize an oil-based delivery system targeting the ileum and the colon is to slow down/retard the lipolysis, thereby creating a delayed release in the later part of the small intestine. In the later part of the small intestine, bile salts and pancreatic lipase are at much lower concentration thus much milder for probiotics. Bile salts are gradually absorbed along the small intestine, and pancreatic enzymes are partially digested and partially absorbed [44, 45]. Therefore, the key modification required for an oil-based delivery system is to limit the lipolysis in the early part of the small intestine and then release along the ileum and the colon.

Objectives

The main objective of this thesis is to formulate a new oil-based controlled release delivery system targeting the later part of the gastrointestinal (GI) tract.

First, modify an *in vitro* model in such a way that it becomes relevant for evaluating the retarding effect on lipolysis and for the release of probiotics.

Then design and evaluate different delivery systems with the potential of delivering probiotics to the later part of the GI tract.

After designing a possible system, load probiotics, and evaluate the releasing and survivability in the GI tract. During this stage, modifications of the formulation will be done to have better control of the release pattern of the payload.

Lastly, perform a storage stability study and investigate the physical stability of the new formulation and the viability and survivability of the encapsulated probiotics.

In vitro lipolysis

In vitro digestion models have been widely used to understand the digestion of food and pharmaceuticals during the GI tract [46, 47]. The main difference between food and pharmaceuticals is the complexity. Compared to pharmaceuticals which usually is much simpler in components, food usually contains different macronutrients that need various enzymes to digest. In this work, we are aiming to design an oil-based delivery system, therefore, the in vitro analysis is focused on in vitro lipolysis. Meanwhile, since it is intended for a pharmaceutical application and drugs can be taken before meals, the physiological parameters should align with those of the fasted state.

Lipid digestion in vivo occurs in both the stomach and the small intestine. In the stomach, the gastric lipase can digest up to 1/3 of the ingested lipid [48]. The majority of lipolysis occurs in the upper part of the small intestine. In vivo, lipolysis includes mainly enzymatic reactions using different lipases, colipases, and bile salts. Lipolysis also involves mechanical movements including those in the stomach, during transport to the small intestine, and in the small intestine. Furthermore, in the gastrointestinal tract, the products of lipolysis (e.g., free fatty acids) and the bile salts could be gradually absorbed along the small intestine. Therefore, it is a very complex procedure to digest and absorb lipids and free fatty acids.

Bio-relevance between in vivo and in vitro is the key to evaluating different in vitro lipolysis systems. Physiological conditions, mechanical movements, and dynamic absorbance are the three key parameters. There are different challenges with these three parameters as they could be different among different age groups (children, adults, and elderly). It also changes if one is sick or healthy or differs among healthy individuals. So, the first challenge is to set the in vivo reference conditions. In this work, we use parameters from a healthy adult as the reference.

The second challenge is deciding between static or dynamic models. In a dynamic model setting, the system can mimic the transportation between the stomach and the small intestine. It can also mimic the absorption of bile salts, removal of free fatty acids, etc. There are several valid dynamic models available including TIM, SHIME, ESIN, etc. [49]. However, the more close-settings toward in vivo, the more complicated adjustment and usage it will bring. The primary goal of developing this in vitro method is to assess whether oil digestion could be modified with different oil formulations. The absorption of free fatty acids or the transportation from

different compartments is thus out of scope. Meanwhile, we mainly focused on lipid digestion in the small intestine. Therefore, a static model will fit the purpose well.

In the static in vitro model, the main challenge is to determine the chemical parameters of the in vitro settings that mimic the physiological condition. In the small intestine, lipolysis usually involves lipase, colipase, and bile salts. Lipase and colipase are used to hydrolyze triglycerides. Bile salts are necessary for lipid digestion as they help emulsify lipids and help locate the colipase and lipase [50-52]. A summary of different in vitro settings is shown in *Table 1*.

The first setting is the lipase sources and concentrations. Studies have shown that oil digestion needs both lipase and colipase [53]. Usually, the pancreatin extract (containing both lipase and colipase) from porcine is used as it is very similar to human pancreatic extract [54]. The concentration is usually calculated only on the activity of the porcine lipase. There are two main measurements used to measure the activity of the lipase, the USP method and the TBU method [55, 56]. Nevertheless, the baseline is that the activity of lipase should be more than needed to digest the lipid that is added into the in vitro system.

Bile salts, on the other hand, are more complicated. Porcine bile extract is one of the common bile salt sources used. It is a crude extract that not only contains different bile salts but also other components from the bile, e.g., bile pigment, lecithin, fatty acids, and protein [57]. This complexity is somewhat closer to the situation in vivo. Bile salt species and concentrations from porcine bile extract differ from those in the human small intestine. The bile acids in human bile are made up of ca. 40% cholic acid, 40% chenodeoxycholic acid, and 20% deoxycholic acid, with traces of ursodeoxycholic acid and lithocholic acid [58]; while the bile acid composition of porcine bile is ca. 30% glycocholic acid, 40% taurocholic acid, 7% taurodeoxycholic acid, 15% glycodeoxycholic acid and 5% hyodeoxycholic acid [59]. However, porcine bile extract is still widely used as the bile source in different in vitro models (*Table 1*). This is because studies have shown that porcine bile extract has the highest lipolysis extent compared to other bile salt sources or combinations [60].

Sometimes, the complexity of bile extract could insert benefits, while other times it makes the system too complicated to study. Meanwhile, there might be batch-to-batch variations among different porcine extract batches. Therefore, it is also common to use a specific bile salt to replace whole bile extracts even though it does not fully represent the extracts and is more expensive. The most commonly used single bile salts are taurodeoxycholate (NaTDC), sodium taurocholate (NaTC), and sodium chenodeoxycholate (NaCDC) (*Table 1*).

Table 1. Some examples of in vitro lipolysis parameters.

Reference	Lipase source	Lipase activity in the digestion medium	Bile species in the digestion medium	Concentration of bile in digestion medium	Phospholipids	Calcium addition	pH (buffer)	Buffer capacity (mmol l ⁻¹ ΔpH)	Initial volume in the digestion medium	Duration
Dahan, A. et al., (2006). [61]	Porcine pancreatin (8x USP)	1000 IU/mL	NaTC	5 mM	1.25 mM Phosphatidylcholine (PC)	5 mM	Tris-maleate (50 mM)	6.8-7.4	40	30
Li, Y. et al., (2010) [62]	Porcine pancreas type II (L3126, Sigma)	100-400 U/mg, 2.4 mg/mL	Porcine bile extract	20 mM		10 mM	Phosphate (5 mM)	7	37.5	20-120
Zangenberg, N.H., et al., (2001). [63]	Porcine pancreatin (3x USP)	300-800 USP units/mL	Porcine bile extract	5-30 mM	1-5 mM PC	Continuous addition (0.045-0.181 mmol/min)	Tris-maleate (2 mM)	6.5	300	40-90
Kaukonen, A.M., et al., (2004). [64]	Porcine pancreatin (8x USP)	1000 TBU/mL	NaTDC	5-20 mM	1.25-5 mM lecithin (60% PC)	5 mM	Tris-maleate (50 mM)	7.5	10-40	30-60
Ali, H., et al., (2008). [65]	Pancreatin extract	8 TBU/mg	Bile salts	5 mM	1.25 mM lecithin	5 mM	Tris-maleate (50 mM)	6.5	100	40
Han, S. F., et al., (2009) [66]	Porcine pancreatic lipase	40000 IU/g, 4,000 TBU/mL	NaTDC	5 mM	1.25 mM lecithin (92% PC)	5 mM	Tris-maleate (50 mM)	7.5	20	35
Larsen, A.T., et al., (2011). [67]	Porcine Pancreatin (3x USP)	300-800 USP units/mL	Porcine Bile Extract containing various bile acids	5-30 mM	1.25-7.5 mM PC	Continuous addition of calcium at 0.045-0.181 mmol/min	Tris maleate (2 mM)	6.5	300 mL	
	Porcine Pancreatin (8x USP)	1000 TBU/mL	Taurodeoxycholic acid	5-20 mM	1.25-7.5 mM Lecithin (60% PC)	Initial addition of 5 mM calcium	Tris maleate (50 mM)	7.5	10-40 mL	
	Porcine Pancreatin (8x USP)	1000 TBU/mL	Taurocholic acid	5 mM	1.25 mM PC	Initial addition of 5 mM calcium	Tris maleate (50 mM)	6.8-7.4	40 mL	

In this thesis, the choice of bile salts contributes to the complexity. In the beginning, porcine bile extract was used as the source of bile salts. At this step, an in vitro system was established to investigate the lipolysis mechanism. Therefore, an in vitro system that could reach as high a lipolysis extent as possible is ideal. With this ideal in vitro system, the retarding effect can be relatively easy to determine. Results show that an in vitro lipolysis system with porcine bile extract could have 100% lipid digestion. In the later part of this thesis, the bile salts source was switched to single bile salt (NaTDC) during the investigation of the development of formulations for probiotics. This modification was necessary due to the presence of numerous unknown ingredients in the porcine bile extract. These unknown ingredients may interact with probiotics in various ways during in vitro release. Furthermore, the impurities would hinder further analysis of the system, particularly when employing techniques like flow cytometry.

Usually, chemicals are added to the same level as in vivo. However, in static in vitro models, calcium ions are usually added at much higher levels than compared to physiological conditions. It is because calcium ions are added to precipitate the free fatty acids to mimic the absorbance of free fatty acids. Literature has shown that free fatty acids could remain on the lipid surface, which would hinder the lipolysis procedure [53].

The pH also needs to be considered. The pH in the small intestine varies from around 6 to around 7 along different parts of the small intestine (duodenum jejunum and ileum). The choice of a pH level set at 7 was informed by prior literature, which has established that at a pH of 7, the C8 and C10 fractions of triglycerides (comprising more than 98% of the MCT oil) can achieve maximum lipolysis while remaining within the physiological pH range [68].

The next important parameter is the stirring because lipolysis happens at the O/W interface [43]. The more the total interfacial area, the faster the lipolysis as lipolysis is a surface-driven reaction. In the beginning, all samples were added directly into the digestion media. A high-shear mixer (Ultra turrax) was used to fully mix the samples and the digestive media before in vitro lipolysis. During lipolysis, only a magnetic stirrer was used (in *paper 1*). The stirring from the magnetic stirrer was not sufficient especially when EC oleogels were added. They tend to flow on the surface of the digestion media. To improve this, a USP basket was added beside the magnetic stirrer (*papers 2, 3, and 4*). The EC oleogel samples were first put into a gelatine or HPMC capsule and added to the basket which was connected to a motor. This motor had a speed of 500 rpm. The usage of a capsule is not only to mimic the real situation when consumers consume the product but also to standardize the protocol and minimize possible errors caused by different surface areas without using capsules.

The last parameter is the experiment time. The experiment time is set to be 120 min trying to mimic the time from entering the duodenum till entering the ileum. After

120 min, the formulation with APIs is highly likely to have arrived in the ileum section [69, 70].

To summarize, modifications were made specifically to our system (formulations containing 0.5 g MCT oil). The setting of our in vitro system can have a lipolysis extent very close to 100%. Therefore, the differences that occurred in the lipolysis are purely coming from different retardants added. Detailed in vitro settings are shown in **Table 2**.

Table 2. The initial compositions of the simulated intestinal fluids. (From paper 1)

Name	Concentration
Porcine bile extract or NaTDC	5 mmol/L
Phosphatidylcholine(soybean origin)	1.25 mmol/L
NaCl	150 mmol/L
CaCl ₂	5 mmol/L
Trizma-maleate	2 mmol/L
Pancreatin	675 TBU
Total volume	100 mL

The basis of this static in vitro lipolysis system lies in titrating the free fatty acids released during lipolysis. By continuously adding NaOH (1 mol/L), the system maintains a pH of 7. Relative conversion is calculated and compared using **Eq. 1**.

$$\Phi_{\text{Relative conversion}} = \frac{V_{\text{formulation}} - V_{\text{background}}}{V_{\text{MCT}} + V_{\text{other degradable}}} \quad \text{Eq. (1)}$$

Where V is the volume of consumed NaOH.

There are limitations to this model. I) The model lacks an absorption step for bile salts. In vivo, the majority of bile salts (95% to 99%) are gradually absorbed in the distal ileum. [71, 72]. Bile concentration is critical as it largely influences the survivability of probiotics. With gradually decreased bile salt concentration, the survivability of released probiotics at the later part of the small intestine will significantly increase. II) The model also lacks an absorption step for lipolysis products (free fatty acids). Extra calcium ions were added to precipitate free fatty acids to compensate for the situation. However, calcium ions can also interfere with biological macromolecules, enzymes, and phospholipids, and, thus, might have some side effects. Nevertheless, added calcium ions in this in vitro setting increased the lipolysis rate. III) A stomach section is missing. As we are mainly interested in the deliver passage in the small intestine, adding an enteric coating can protect the formulation from the low pH. However, probiotics are highly sensitive to low pH in the stomach. Therefore, it might be interesting to see the whole delivery passage including the stomach and small intestine.

Summarized results

Evaluation of different delivery strategies

A mechanistic understanding of lipolysis is needed to explore the possibility of an oil-based delivery system for probiotics. Lipolysis (oil digestion) is an enzymatic reaction in which triglycerides are degraded into free fatty acids [43]. More specifically, because of the poor solubility of oil in water and enzymes are only water-soluble, lipolysis can only happen at the O/W interface. Therefore, materials that can interfere with the O/W interface could largely change the lipolysis. These interferences include the diffusion of the substrate to the O/W interface, the diffusion of hydrolysis products from the O/W interface, and the subsequent diffusion of these into the bulk phase [73, 74]. These steps can be related to agitation, electrolyte concentration, and the presence of surface-active agents (e.g., bile salts, monoglycerides, free fatty acids, and free fatty acids soaps) [73]. Different substrates (different chain lengths of triglycerides) also showed different lipolysis speeds because of pH-dependent solubility and differences in viscosity [75]. The longer the chain length and the higher the viscosity results in lower lipolysis speed. But this aspect is out of scope in this thesis where we use MCT as the oil substrate. The focus is on the diffusion of different components during lipolysis. As discussed above, the diffusion can be divided into three parts, and one of them is the diffusion of hydrolysis products. The design of the *in vitro* method has eliminated further exploration of this where the high concentration of calcium ions precipitates free fatty acids. Thus, the diffusion aspects in this thesis include the diffusion of substrate from the oil phase (MCT oil) to the O/W interface, and the diffusion of substrates from the water phase (pancreatic lipase and bile salts) to the O/W interface.

There are different ways to slow down lipolysis and the most common way is to add surface active components. Fundamentally, adding surfactants is to slow down the diffusion of water-soluble components to reach the O/W interface by competing with pancreatic lipase and bile salts. Usually, more surface-active components (lower interfacial tension compared to bile salts and lipase) are used to successfully occupy the O/W interface. Besides this, surfactants might have a strong influence on lipase structure which would significantly influence the activity of lipase as the activity is largely dependent on the structural combination between lipase and colipase [76]. In this area, discussions are around different types of surfactants, e.g., different molecular masses of surfactants, different structures of surfactants, and

different charges of surfactants. Studies have shown that for small molecular surfactants, the ability to inhibit or promote lipid digestion depends on the concentrations and electrical characteristics [77]. For bigger molecular surfactants, block molecules with several hydrophobic domains and hydrophilic domains seem to easily occupy the O/W interface and create a steric repulsion effect thus slowing down lipolysis [78]. In our study, both small modular surfactants (e.g., monooleate with a molar mass of 356 g/mol) and block molecular surfactants (e.g., poloxamer 407 with a molar mass of 12600 g/mol) were used.

Most of the studies on this topic usually focus on one aspect of modulating lipolysis. While in our study, we tried to focus on the general picture of the retardation of the enzymatic lipolysis reactions. Instead of putting all the parameters into one diffusion concept, we tried to group the mechanism that might influence the diffusion of substrates (including both the oil phase and aqueous phase) to three aspects: the diffusion of the oil molecule towards the interface (in liquid status), the thermodynamic activity of the oil phase, the status of the oil phase (liquid/semi-solid), and the interfacial composition that may prevent the lipase from adsorbing.

It is important to note that thermodynamic activity in this situation is an estimation, where hexadecane in oil is considered to be an ideal solution. Because it is an intrinsic parameter that influences the conversion of MCT oil, the thermodynamic activity of lipolysis can be applied to different retarders regardless of other retarding mechanisms. However, despite we tried to separate different retarding mechanisms, the observation of the lipolysis might be the result of the sums of different parameters.

Three groups of retarders were investigated using the developed in vitro lipolysis method, i.e., bulk retarders (Dimethicon 25 cSt, dimethicon 750 cSt, polyglycerol polyricinoleate (PRPG), and hexadecane), interfacial retarders (monooleate, polysorbate 80, poloxamer 407, Pentaethylene glycol monododecyl ether (C12E5), and cetearyl glucoside) and gelating retarder (ethylcellulose (EC)). All the retarders are soluble in oil under different conditions.

By increasing the viscosity, the diffusion of oil molecules towards the O/W interface might be hindered thus slowing down the lipolysis. The results show that there is a minor retarding effect, with viscosity reaching as high as 5 times that of pure MCT oil. However, when the viscosity increased to a certain level which makes the oil phase semi-solid like, the retarding effect becomes significant. In this case, after adding 10% EC₃₀₀, a stiff oleogel was formed. Less than 50% of the oil digestion was achieved in 120 min for EC oleogel. While for pure MCT oil, 50% of the lipid digestion is achieved in 5 min.

When comparing thermodynamic activities, samples that have lower viscosity than MCT oil were chosen to minimize the influence from viscosity difference. The thermodynamic activity of different samples results from 0.58 to 0.73. Compared to

pure MCT oil, of which the thermodynamic activity is 1, there is a minor retarding effect.

Lastly, using interfacial retarders to hinder access of the lipase to the surface did only give a minor retardation when low molecular surfactants were used. However, a polymeric non-digestive surface-active retarder such as poloxamer 407, was able to form a steric layer that could significantly retard the lipolysis.

Thus, both the change in the consistency of the oil phase and the exclusion of the lipase from the interface are possible mechanisms for retardation of lipolysis. However, the demands on the molecules used are extensive: in the first case the retardation is only seen when the molecules used can form an oleogel; in the latter case, the surface-active component must have strong irreversible adsorption to the interface (**Paper 1**). Summarized mechanisms can be seen in **Figure 1**.

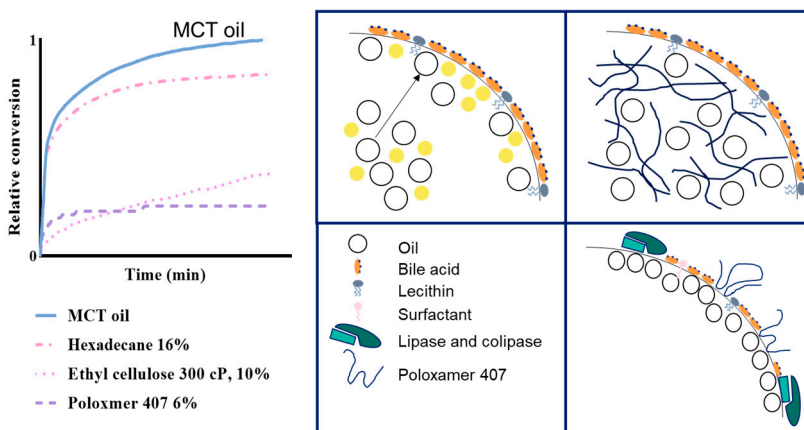


Figure 1. Retardation effect from three representative retarders of three retardation mechanisms. Hexadecane represents the thermodynamic activity parameter, ethylcellulose represents the viscosity parameter, and poloxamer represents the interfacial property parameter. (Summarized from paper 1)

To conclude, results show that it is not easy to significantly slow down the lipolysis or decrease the lipolysis extent. Among all the retarders that have been tried, only EC and poloxamer 407 can efficiently retard the lipolysis. A minor difference could be observed between the digestion curves of EC oleogel and poloxamer 407. For EC oleogels, the relative conversion increased along 120 min. For poloxamer 407, the relative conversion increases more in the beginning and remains the same afterwards. The accelerating release from EC oleogel is more suitable for delivering and releasing the encapsulated probiotic to the later part of the small intestine. Therefore, EC oleogels become the only candidate for a potential new oil-based delivery system for probiotics.

Ethylcellulose and ethylcellulose oleogels

Ethylcellulose is the product of etherification between cellulose and ethyl chloride or sulphate in a high-alkaline solution [79]. The etherification degree can largely influence the properties of EC, such as water solubility and incipient softening points [79]. Commercially available EC products used in this study have 48.0-49.5% (w/w) ethoxyl basis. In other words, it has the equivalent of about 1.4- 2.5 hydroxyl groups etherified [79]. EC can form gels when dissolved in certain hydrophobic solvents, such as triglyceride oils, commonly referred to as oleogels [80-82]. EC oleogels are formed after dissolving the EC powder in the oil using a heating procedure (above the glass transition temperature of EC, around 130-140°C) [83, 84].

Physical characterization has been well explored to compare EC oleogels with natural fats. Studies include rheological properties (rheometer), mechanical properties (texture analyzer, oil entrapment), and thermal properties (differential scanning calorimetry). During these experiments, parameters varied with different EC (different cellulose chain length and different concentrations) with the same oil (e.g., triglycerides) and the same EC with different oil (e.g., different sauces triglycerides, diglycerides, and monoglycerides and waxes) [80, 85-88]. The studies also examined EC oleogels with different plasticizers/surfactants, such as sorbitan monostearate and glycerol monostearate, to investigate the potential alteration of the oleogel's characteristics [89]. The structure of the EC powders and EC oleogels have been studied with crystallographic studies, infra-red spectrometry, and X-ray diffractometry to investigate the molecular interactions and polymer structures [82, 90, 91].

Four main conclusions from the literature mentioned above, regarding physical characterization, are as follows. I) Increasing EC concentrations or increasing EC molecular mass (increasing chain length) would increase the viscosity/hardness of EC oleogels. II) The saturation of the triglycerides will influence the viscosity/hardness of EC oleogels. III) There might be secondary structures in EC oleogels, e.g., hydrogen bonding induced structure. IV) Gelation procedure-related parameters, such as cooling rate and temperature-related structure change are also relevant to EC concentrations and EC polymer length.

Further elaboration on the correlation between EC polymer and solvent is also widely discussed. EC oleogel has EC polymer network formation surrounded by triglycerides, where the junctions of different polymer chains are physical—no chemical bonds between EC polymers or between EC polymers and triglycerides. Nevertheless, there might exist hydrogen bonds (H-bond) either intra- or intermolecularly of EC polymers [92]. These H-bonds primarily happened on the C6 position, which is the position of the remaining unsubstituted OH-group.

Therefore, theoretically, in EC oleogels, there might exist H-bonds which would lead to a secondary structure.

There also exists polymer-solvent interaction, in this case, EC polymer and triglycerides. However, ATR spectroscopy data from the literature states that the H-bond occurs only between polymer strands and is responsible for the formation of the tie points in the EC network [82]. The same literature also states that both the EC polymer backbone and the ethyl groups do not change in different oleogels, it is the oil packing within the polymer network that has changed in different concentrations of EC [82]. Despite a high degree of substitution of EC (2.5 out of 3.0), it appears that the EC polymer chains under solid state still can form a semi-crystalline structure via the remaining H-bonds and possibly via van der Waals interactions [93]. Literature also shows that the cellulose subunit length along the polymer chain of 10.4 Å (dimer length), regardless of the polymer source or type [94].

Different microscopic methods have also been used to explore the microstructure of EC oleogels, e.g., transmission electron microscopy (TEM), scanning electron microscope (SEM), and atomic force microscopy (AFM). These different microscopies were used to reveal the network on the surface of EC oleogels. However, these microscopic techniques have some complications during sample preparation including, removing the oil from oleogels using organic solvent, freezing, sputter, etc., which could, to a different extent, change the microscopic structure of EC oleogels. Therefore, the reliability of structure from these microscopic images is limited.

On the other hand, SAXS being a non-intruding method has shown success in measuring the EC powders. SAXS, USAXS, and WAXS were used to measure the EC powders, where a supramolecular polymer-ordered structure was observed at lower q . At higher q , a fractal aggregation was proposed with a fractal dimension of 2.8 [91].

EC oleogels have been widely studied on different levels from the EC molecules to the physical parameters of the oleogels. However, only one study has been done on the digestion properties of EC oleogels [95]. The digestion property or the retarding effect on lipolysis of EC oleogels is the main interest of this thesis. Therefore, the first task is to investigate the digestion properties of EC oleogels with different EC molecular mass and concentrations.

Meanwhile, another main challenge comes from the heating temperature needed to make oleogels (140-160°C). This high temperature is not applicable for temperature-sensitive compounds such as probiotics. Therefore, a new method is needed to incorporate probiotics into EC oleogels without being detrimental to the probiotics.

Development and validation of a cold-mixing method

EC oleogels are formed after dissolving EC powders in MCT oil at high temperatures (140-160°C). In this part, we explored the possibility of EC oleogels as the controlled release delivery system for probiotics by developing a cold-mixing procedure. The cold-mixing procedure is based on several observations. I) The gel formation is based on the gel temperature instead of the cooling time. This is supported by the observation that the same gel status has similar temperature when using different cooling rates, regardless of different EC oleogels used (different EC chain lengths and different concentrations). II) There are limited differences in gel status with the first heating cycle and re-heating cycle. III) The oleogel is not fully solidified when the temperature is around 40°C. These three observations together indicate that it is possible to reform EC oleogels after disturbing (by adding different components) the EC oleogel around 40°C.

The description of the cold-mixing procedure is as follows. After heating the EC powders and MCT oil to 140°C to 160°C, this oleo-solution mixture is allowed to cool. At around 40 °C, the probiotic pre-mixture or a placebo pre-mixture is added to the oleogel at different ratios. The warm gel and the added pre-mixture are mixed for about one minute or until the mixture appears macroscopically homogenous. The cold-mixed EC oleogels are stored over night before further characterization. The flowchart of the procedure is shown in *Figure 2*.

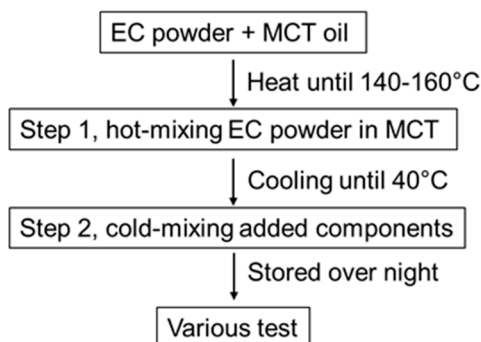


Figure 2. Flow chart of formulations manufacturing process for cold-mixing formulations. (From paper 2)

The main investigation with this cold-mixing method is focused on whether the cold-mixed oleogels could reform a continuous gel structure, and if this cold-mixed oleogel has the same gel characteristic as the hot-mixed oleogel. Furthermore, verify whether these cold-mixed oleogels have the same retardation effect as the oleogels produced without a cold-mixing step. Also, if this retardation effect is as predictable

(e.g., certain trend) as the hot-mixed oleogels. Lastly, measure the viability of the encapsulated probiotics from the cold-mixed EC oleogel.

In *paper 2*, we presented the results. In general, EC oleogels prepared using the cold-mixing method appeared to be continuous oleogels (one-phase system macroscopically). The cold-mixed oleogels were a bit lower in hardness and oil entrapment compared to hot-mixed oleogels.

To further explore the differences in physical properties of cold-mixed and hot-mixed oleogels, storage modules and yield values were measured on 0 week (the second day after making the gel), 1 week, and 2 weeks. As shown in **Figure 3**, the storage modules at week 0 are similar between hot-mixed and cold-mixed oleogel. After 1 week, the storage modules for both oleogels are increased to a similar extent and maintained similarly between 1 week and 2 weeks. The change in the storage modules can be possibly explained by the gel relaxation phenomenon. This relaxation phenomenon happened not only on the cold-mixed oleogels but also on the hot-mixed ones.

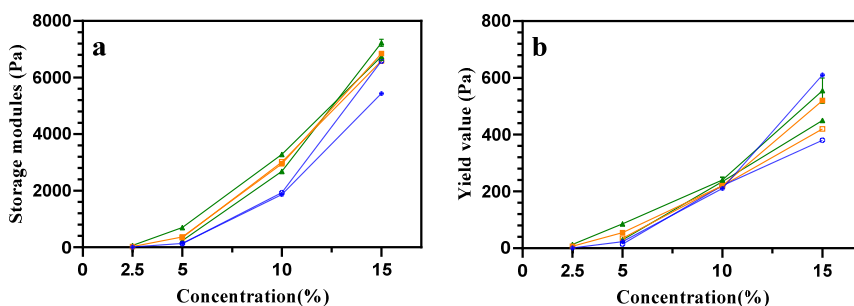


Figure 3. Storage modules (a) and yield value (b) of hot-mixed oleogel using EC₂₂, 0 weeks (● blue), 1 week (■ orange), and 2 weeks (▲ green), and for cold-mixed oleogel 0 weeks (○ blue), 1 week (□ orange), and 2 weeks (△ blue).

For yield modules, it is quite similar for both hot-mixed and cold-mixed oleogels except for 15% EC₂₂. In both storage modules and yield values, a higher concentration of 15% has larger error bars. This is primarily because of the preparation step of the samples using cone-plate geometry. Oleogels have 15% EC₂₂ require extra force (manually) to achieve the ideal gap between the cone and the plate. We also found out that it is almost impossible to measure higher concentrations (e.g., 20%) when oleogels started to slide rather than deform during oscillation.

Combining the results of hardness, storage modules, and yield values, we can conclude that after a short period of gel relaxation (less than 2 weeks), cold-mixed

EC oleogels and hot-mixed oleogels are very similar in physical gel properties. The similarity in microstructure was also shown between hot-mixed and cold-mixed oleogels after 1-week storage at room temperature (**Figure 4**). These two SAXS figures show that after cold-mixing, there is no swelling in dimensions (similar slopes). In other words, after adding cold oil to warm EC oleogel, the oil gradually incorporated into EC polymer networks probably due to gel relaxation.

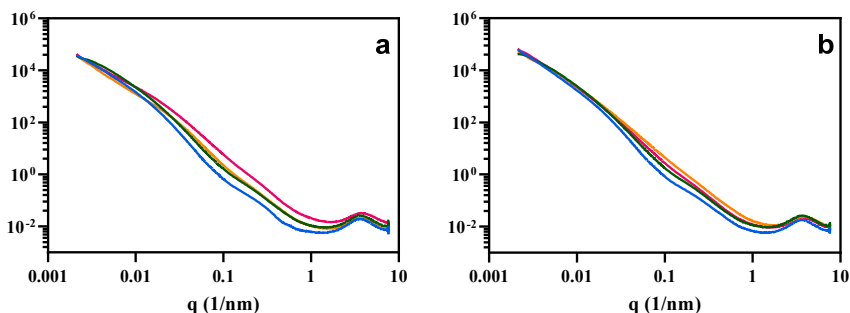


Figure 4. SAXS results after 1 week of storage at room temperature. a) hot-mixed oleogels of EC₂₂ 5% (blue), 10% (green), orange (15%), and red (20%). b) cold-mixed oleogels (only MCT oil added during the cold-mixing step) after 1-week storage at room temperature of EC₂₂ 5% (blue), 10% (green), orange (15%), and red (20%).

Both cold-mixed and hot-mixed oleogels with the same concentration and same EC also have similar relative conversions (one example in **Figure 5**). When the hardness is below a certain level, the harder the EC oleogels, the higher the retardation effect. When the concentration is higher than 10%, and the molecular mass higher is than EC₄₆, the lipid digestions of the oleogels are at a similarly low level indicating a strong retarding effect. For all the harder EC oleogels, there are remaining oleogel lumps after the 120 min in vitro lipolysis.

The significant retarding effect from EC oleogels seems promising for it being the controlled delivery system for probiotics. This might increase the survivability of the encapsulated bacteria in the upper part of GI tract, where bile salts are a major challenge.

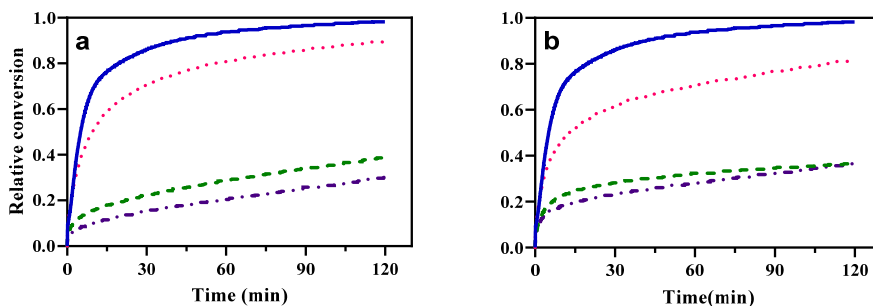


Figure 5. Relative conversions of different EC oleogels during in vitro lipolysis. MCT oil (—, blue) is the reference in both figures. a). Relative conversions of 10% of EC₂₂ (···, pink), EC₄₆ (---, green) and EC₁₀₀ (-·-, purple) hot-mixed EC oleogel. b). Relative conversions of 10% of EC₂₂ (···, pink), EC₄₆ (---, green) and EC₁₀₀ (-·-, purple) cold-mixed EC oleogel. (From paper 2)

The next step is to encapsulate *L. reuteri* into EC oleogels using the cold-mixing method. By comparing the viability from freeze-dried pallets, and the mixture before and after cold-mixing, we can conclude that both EC and the cold-mixing method have harmless character to probiotics (details see *paper 2*). Furthermore, the in vitro release of encapsulated bacteria and the viability of these bacteria in the remaining oleogels also demonstrate that EC oleogels offer effective protection against bile salts and ensure sustained release, leading to enhanced survivability (*Table 3*).

Table 3. Survivability of bacteria in MCT oil, weak oleogel (5% EC₂₂), and strong oleogel (10% EC₄₆) during 120 min in vitro lipolysis. (From paper 2)

Formulations	Freeze-drying matrix dispersed in MCT oil			Freeze-drying matrix dispersed in a weak oleogel (5% EC ₂₂)		Freeze-drying matrix dispersed in a strong oleogel (10% EC ₄₆)		Remaining gel	
	Time (min)	30	60	120	60	120	60		120
Viability (CFU/g freeze-dried bacteria)		0.2 · 10 ⁹	0.2 · 10 ⁹	0.1 · 10 ⁹	1 · 10 ⁹	2 · 10 ⁹	0.8 · 10 ⁹	0.7 · 10 ⁹	1 · 10 ⁹

* Average, n=3, standard error of the mean is 0.08 · 10⁹

While developing the cold-mixing method, several observations might be helpful for further applications of this method. I) If one wants to keep a similar gel property (e.g., hardness) as the hot-mixing oleogels, the concentration of added excipients should be in a certain range. The more added excipients, the harder for the oleogels

to reform the gel structure. Results show that when added below 20% extra MCT oil, the hardness has a minor change. When above 20%, the hardness is decreased more obviously. II) When the EC oleogels are too hard (high EC molecular weight or high concentration), the cold-mixed oleogels might be inhomogeneous, which might finally result in phase separation. Phase separation will cause a detrimental influence on the retarding effect of EC oleogels.

To conclude, the cold-mixing method enables EC oleogels to encapsulate temperature-sensitive compounds, such as probiotics. More importantly, the cold-mixing method also provides a means to tune the EC oleogel properties by adding different concentrations of different excipients at the cold-mixing step.

The effect of disintegrating excipients on the release

Cold-mixed EC oleogels have been proven to be a delivery system for probiotics with no harm to the bacteria, good protection against harmful environment in the upper part of the small intestine, and sustained release of encapsulated bacteria.

However, EC oleogels have quite low digestion of the encapsulated oil and incomplete release of the encapsulated bacteria within 2 hours. Thus, in this part, the main goal is to develop an EC oleogel formulation that has both good protection in the harmful environment (upper part of the small intestine) and a higher release in a safer environment (later part of the small intestine). Obtaining a higher release with EC oleogels, which exhibit a strong retarding effect, is challenging due to their stiffness and resistance to break down. The higher the concentration and molecular mass, the stronger the oleogels, and the more residue remaining after 120 min in vitro lipolysis (*paper 2*). 10% EC₂₂ was selected as the model for formulation development because of its moderate hardness. To be more specific, not too high hardness will result in a more successful cold-mixing step, and not too low hardness will result in a strong enough retarding effect as well as enough space for formulation development.

The results from *paper 2* also show that the differences between the release curves of placebos and formulations loaded with APIs are limited. Therefore, during formulation development, placebo formulations are good representatives. After choosing the model EC oleogel (10% EC₂₂), the next step is to explore the method to tune the release from the oleogel. The strategy is to apply some excipients which would increase the oleogel break down during lipolysis.

Lipolysis is an enzymatic reaction where several factors could influence the speed. First is the total interfacial area as lipolysis happened at the O/W interface [96]. The second is the removal speed of the lipolysis products (free fatty acids) from the O/W interface. Free fatty acids are surface active, which will accumulate at the O/W

interface, thus preventing enzymes from reaching the interface [97]. Therefore, the release profile may be altered through the manipulation of these transport processes. This could involve inducing the disintegration/breakdown of the oleogels or improving the porosity and water permeability within the oleogel matrix.

To systematically investigate the strategy for developing high-release EC oleogels, the critical step is to understand the release kinetics of formulations with different added excipients. Subsequently, the aim is to correlate the release kinetics with the structure, lipolysis, and ultimately the release mechanisms. In general, the release mechanisms can be controlled by various phenomena, such as dissolution, diffusion, partitioning, osmosis, swelling, and erosion [98].

There are five most common release kinetic models, zero-order, first order, Higuchi model, Hixon-Crowell model, and Ritger–Peppas–Kormeyers model. In this thesis, the Ritger–Peppas–Kormeyers model (*Eq. 2*) was used as it was developed specifically for the release of a drug molecule from a polymeric matrix and has been applied to hydrogels [99, 100]. After fitting the experimental release data into the mathematical model, the release constant k and exponent n will be obtained, which determine the release mechanisms.

$$\frac{M(t)}{M_{tot}} = kt^n \quad \text{Eq. (2)}$$

Where $M(t)$ is the mass of material released at time t , M_{tot} is the total mass of releasable material at time 0. k is a rate constant (the released fraction at a one-time unit) describing the magnitude of the release rate. n is the exponent describing the time dependence, which is indicative of the release mechanism.

The application of Peppas model in this thesis is to evaluate the release by evaluating the digestion of oil, assuming that the release of bacteria is proportional to the digestion of oil. Our results (later in the project, fraction of released bacteria versus digested fraction of oil, details in *Figure 9*) show that this hypothesis is correct.

Two groups of excipients were added to explore the possibility of increasing the lipid digestion of EC oleogels, i.e., lipid excipients and hydrophilic powder excipients. Literature shows that by incorporating lipid excipients into EC oleogels, varied mechanical properties could be obtained due to interactions between EC polymer chains and the excipients [89]. Lipid excipients including polyoxyl castor oil (POC), glycerol monooleate (GMO), glycerol dioleate (GDO), glycerol monopalmitate (GMP), and glycerol monostearate (GMS) were selected. Hydrophilic powder excipients including sodium starch glycolate (SSG), maltodextrin (MD), and sodium croscarmellose (SCCM) were studied. These powder excipients could dissolve in water thus increasing the in vitro digestion.

The relative conversions of different added excipients are summarised in *Figure 6*. It shows different relative conversions (from 0.1 to almost 1) could be obtained by

adding different excipients into EC oleogels through the cold-mixing step. In other words, different extents of digestion of EC oleogels can be obtained by adding excipients. This easy-to-change release mechanism will be promising to adapt EC oleogels to different requirements of different APIs.

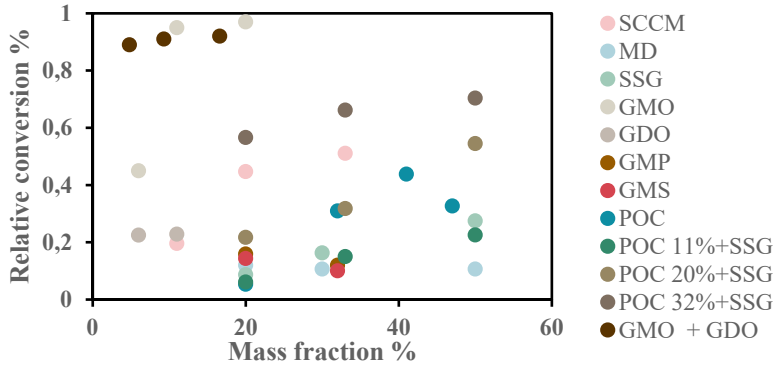
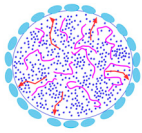
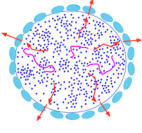
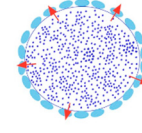
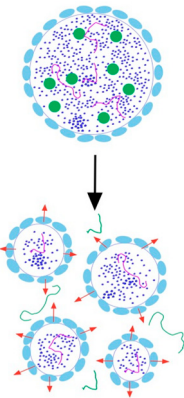


Figure 6. Summarized relative conversions of different excipients in paper 3.

After using the Peppas models to fit release curves of all the formulations, the n value from the Peppas equation and the proposed release mechanisms are summarized in **Table 4**. Notably, the fitting stops at 60% of the relative digestion. This is because particle size becomes a more significant factor influencing the kinetics once the relative conversion surpasses 60%.

Table 4. Release mechanisms from EC oleogels. The structural illustration is a model of enzymatic digestion of an ethylcellulose oleogel (EC). The digestive enzymes (blue ovals) operate at the surface. The medium chain triglyceride (MCT, dark blue dots). The violet molecules inside the gel are diffusion-restricting molecules such as the EC or added non-digestible lipids. The green-filled circles illustrate the swelling disintegrating material dissolved in the aqueous phase. The red arrows indicate the digestion rate controlling step, and n refers to equation 3. (From paper 3)

	Release mechanism	Range of n	Illustration	Samples in the category
a	Diffusion-limited degradation (Fickian release)	0.35-0.65		Oleogel EC ₂₂ 10%, GMO 6%, POC 41%, POC 47%
				MD 20%
b	Transition between diffusion and surface limited digestion	0.65-0.85		MCT oil, GMP 32%, GMS 32% POC 20%
				MD 33%, MD 50%
c	Surface limited digestion combined (0 order reaction rate)	0.85-1		GMP 20%, GMS 20%, GMO + GDO 2, GMO + GDO 1
				SCCM 11%
				POC 11% + SSG 20%, POC 11% + SSG 50%
d	Disintegration limited digestion.	>1		GMO 11%, GMO + GDO 0.5, GMO 20%, GDO 6%, GDO 11%, POC 32%
				SCCM 20%, SCCM 33%, SSG 20%, SSG 33%, SSG 50%
				POC 11% + SSG 33%, POC 20% + SSG 20%, POC 20% + SSG 33%, POC 20% + SSG 50%, POC 32% + SSG 20%, POC 32% + SSG 33%, POC 32% + SSG 50%

When no excipients were added, the release kinetic of EC oleogel is within the diffusion-controlled group (type *a*). It can be well explained as EC oleogel is a relatively hard and sticky material and the diffusion of triglycerides to the O/W interface is the main limiting step.

Formulations with added hydrophilic powder disintegrants are mainly grouped in type d where the disintegration is the limit factor. Usually, these formulations have a low k indicating a lower release at the duodenum and jejunum and a high n indicating a high release at the ileum and colon (details in *paper 3*). These results demonstrate the feasibility of tuning the controlled digestion of the EC oleogel for controlled delivery to the ileum and colon through the incorporation of disintegrating excipients (dry powder of polyelectrolytes). Furthermore, 12-hour in vitro results indicate that up to 100% relative conversion could be achieved with different disintegrants up to 10 hours (*paper 3*).

For lipid excipients, it is notable that more hydrophilic surfactants, like monoglycerides, may degrade the gel and thereby result in a rapid release. More hydrophobic excipients (e.g. diglycerides), as well as larger oil soluble complex surfactants (polyglycerol poly ricinolate), give a limited improvement of the release, possibly by the reduction of the interfacial tension. Meanwhile, some of the lipid excipients have high melting points and remain in solid format when added during the cold-mixing step. These excipients will strengthen the EC oleogel hardness therefore lowering the release. As there are different limiting factors playing, formulations with different concentrations of these lipid excipients result in different release mechanism groups.

Combinations of surface-active lipids (e. g., polyethoxylated castor oil) with swelling hydrophilic polymers (e.g., sodium starch glycolate) all have high n value ($n>1$) compared to other formulations. When n is above 1, the formulation has accelerating release. In other words, a high fraction of the encapsulated bacteria is released at the target time and position in the GI tract (ileum and colon). The combination of surface-active lipids and swelling hydrophilic polymers also enables a versatile, controlled disintegration of the oleogel, allowing for a controlled release over a long time.

To conclude, the application of the Peppas model to the oil digestion of EC oleogels seems reasonable. The different release kinetics (grouped by exponent n) fit well with the actual physical structure of EC oleogels and the release phenomenon. Therefore, the combination of quantitatively descriptive models with release mechanisms can be a potentially useful tool. This tool can be helpful in the development of EC oleogels tailored for targeted release in the GI system.

A storage stability study

The viability of probiotics can be influenced by oxygen level, water activity, temperature, pH, etc. [25, 101, 102]. Different forms of probiotics, e.g., powders, suspensions, and microencapsulation, have different issues of losing viability during process and storage [103]. Studies have shown that during storage, the viability can be lowered to different extents ($0.8 \log_{10}$ to $2.8 \log_{10}$) under accelerated conditions ($37^{\circ}\text{C}/75\% \text{RH}$) and different oxygen levels with *L.reuteri* [104]. Furthermore, the viability of probiotics could be largely decreased during the GI tract when meeting low acidity, bile salts, and other biological secretions [105, 106].

The recommended number of viable bacteria is around 10^6 to 10^8 CFU/g in the product at the time of consumption [61]. As probiotics should be alive when consumed, it is important to examine the viability after storage. The viability of the encapsulated probiotics is also influenced by the physical and chemical stability of the formulation itself. As discussed earlier, there is a relaxation phenomenon existing in EC oleogels within one week under room temperature. This change in the physical structure of EC oleogels might influence the viability of the encapsulated probiotics. Meanwhile, the relaxation might behave differently at accelerated conditions. Therefore, the hardness of EC oleogels is necessary to measure together with the viability of the bacteria. An in vitro digestion method was applied to evaluate the release of probiotics from EC oleogels.

To summarize, in this part, three steps of evaluation of the formulations were applied. I) The physical stability of EC oleogels with and without probiotics was measured using a texture analyzer. II) The viability of the encapsulated probiotics was measured, where a stomacher was used to release the encapsulated bacteria from EC oleogels. III) The release profile and the viability of the released probiotics from the EC formulations were evaluated using in vitro digestion. During in vitro analysis, probiotics were under the physiological conditions of the small intestine (exposure to bile, exposure to pancreatin, removal of free fatty acids, pH 7).

Two formulations were chosen, one added pure MCT during the cold-mixing step represents the simple formulation. Another one added SCCM during cold-mixing represents the controlled-release formulation. Each formulation has two batches, one without bacteria and one with added 2% freeze-dried *L. reuteri*. Detailed information can be seen in *paper 4*. Two storage conditions were selected ($30^{\circ}\text{C}/75\% \text{RH}$, and $40^{\circ}\text{C}/75\% \text{RH}$) with a period of 90 days. All measurements were done at time points of 0, 17, 30, 60, and 90 days. Day 17 was added because of the low reproducibility viability results from day 0. For in vitro analysis, samples were withdrawn from the digestion media at 10, 20, 30, 60, and 120 min.

Results show that there is an increase in both hardness and relative digestion within the first 30 days. This increase is similar between the two storage conditions. After 30 days, results from both measurements are similar regardless of different storage

conditions. This change aligns with the rheology data discussed earlier, where gel relaxation phenomena might be the reason. Gel relaxation occurred and finished within 1 week (room temperature, **Figure 4**).

The relaxation does not appear to affect the viability of the encapsulated bacteria (**Figure 7**). The viability does not decrease the most in the first 30 days compared to the rest of the storage days. Instead, the viability gradually decreased in 90 days. The loss of viability is between 0.2 to 1 \log_{10} among all the batches (both plating and flow cytometry results).

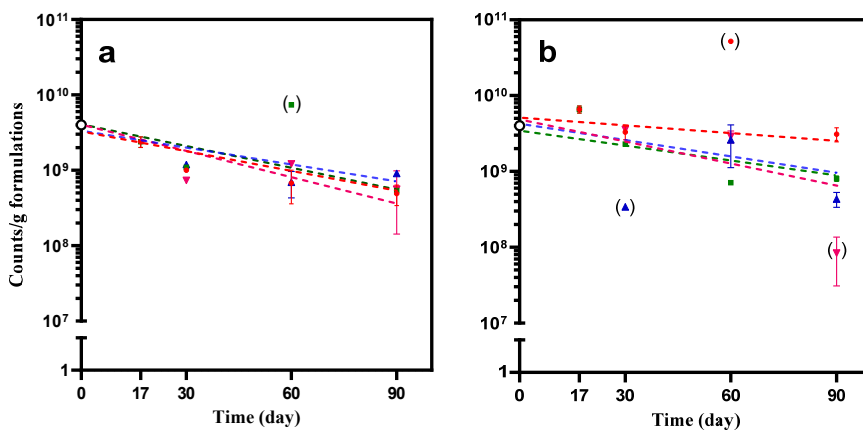


Figure 7. Viability from both plating and flow cytometry measurements of four samples 0, 30, 60, and 90 days. On the y-axis, the unit is CFU/g formulation for plating results and cells/g formulation for flow cytometry results. The guiding line for data sets is not considering outliers (data point in parenthesis). The aimed formulated alive number ($4 \cdot 10^9$ CFU/g, calculated number from percentage) of the bacteria is the aimed formulated alive number ($4 \cdot 10^9$ CFU/g, calculated number from percentage) of the bacteria is the black circle at time point 0. The formulated bacteria number are presented at time 0 using black circle and white as the filling color. (From paper 4)

a) Oleogels without disintegrants: EC₂₂/MCT-B/30-plating (●, red), EC₂₂/MCT-B/40-plating (■, green), EC₂₂/MCT-B/30-FC (▲, blue), EC₂₂/MCT-B/40-FC (▼, purple).

b) Oleogels with disintegrants EC₂₂/SCCM-B/30-plating (●, red), EC₂₂/SCCM-B/40-plating (■, green), EC₂₂/SCCM-B/30-FC (▲, blue), and EC₂₂/SCCM-B/40-FC (▼, purple).

Figure 8 shows the release curves of live bacteria during 120 min in vitro lipolysis. When exposed formulations to a lethal environment during in vitro lipolysis (high bile concentration), EC oleogel formulations showed both protection of the encapsulated bacteria and controlled release of bacteria. For simple formulations (EC₂₂/MCT-B/30 and EC₂₂/MCT-B/40), the number of live bacteria is much lower than the total number of encapsulated bacteria. But for controlled release formulations (EC₂₂/SCCM-B/30 and EC₂₂/SCCM-B/40), EC oleogels keep releasing live bacteria into the in vitro solution and can maintain a high number of live bacteria from 30 to 120 min. A similar trend of results could be seen from FC

data. Both the total and dead numbers of bacteria increased from 60 min to 120 min, and the total number of bacteria is very close to the theoretical value of encapsulated bacteria for the controlled release formulation (formulations added SCCM) (*Paper 4*).

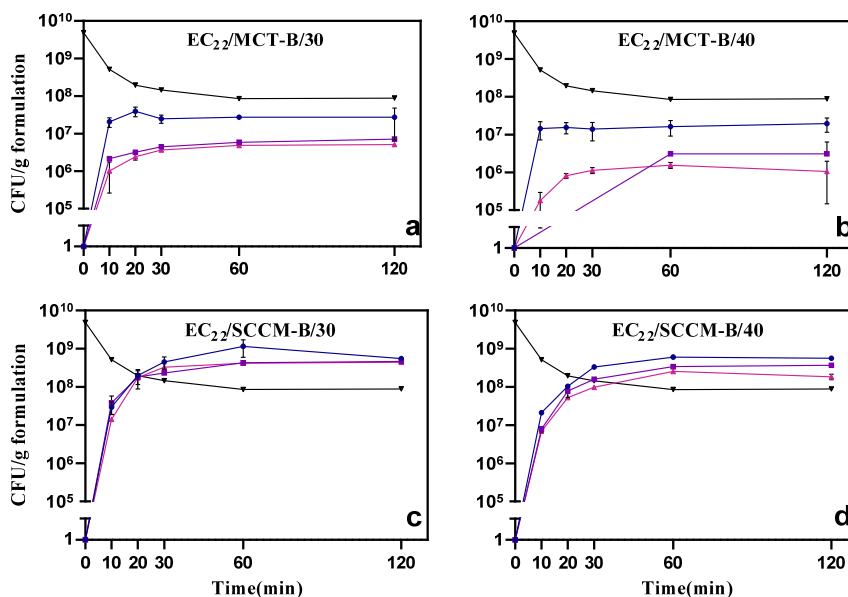


Figure 8. Plating results of EC₂₂/MCT-B/30, EC₂₂/MCT-B/40, EC₂₂/SCCM-B/30, and EC₂₂/SCCM-B/40 from in vitro lipolysis after a storage time of 30(●), 60(■), and 90 days (▲). Bacteria in oil is the reference (▼). The formulated bacteria number is $4 \cdot 10^8$ CFU/g. (From paper 4)

This stability study has demonstrated that EC oleogel formulations are quite stable after a limited time (probably less than 1 week). Limited difference in hardness and viability between 30°C and 40°C further proves the high stability.

Viability data show that the number from controlled release formulations is higher than the simple formulations. This could be explained by the fact that the stomach cannot release all the bacteria from the simple formulation. EC oleogels are sticky materials that also do not dissolve in water. It is possible that some bacteria were still entrapped into the EC oleogels. Nevertheless, the difference between simple formulation and controlled release formulation at time point 0 is not big (around 0.3 log₁₀). Therefore, this systematic error should not largely interfere with the viability results, especially the trend of viability within the sample batch.

Viability, release, and survivability of bacteria from EC oleogels

Bacteria counting was used to evaluate the quality of the EC oleogels as the controlled release delivery system for probiotics. High quality of EC oleogels encapsulating bacteria includes high viability in the formulation, high survivability under biological conditions, and a high number of live bacteria at the target location in the GI tract.

It is a challenge to determine the bacteria number which is caused by different reasons. It is influenced by the intrinsic characteristics of the EC oleogels. EC oleogels are highly viscous and sticky and cannot dissolve in water. Therefore, it is challenging to measure the viability of the encapsulated bacteria as it is hard to get all the bacteria out from EC oleogels. The solution is to use a stomacher, which applies a physical force to the solution, but it might be not sufficient as can be seen a bit lower number of cells in the simple formulations. Whereas with controlled release formulations that contain disintegrants, the detergents can help release bacteria during the stomacher and thus result in a higher number.

Another challenge in bacteria counting can also be caused by the relatively high bile salt concentration during the 120-minute in vitro digestion. During in vitro lipolysis, live cells are gradually released from EC oleogels and gradually become dead because of high bile concentrations. Therefore, the live cell number from plating cannot represent the released number of cells from EC oleogels. Extra measurements that could count both alive and dead bacteria are needed (using flow cytometry). However, flow cytometry by itself is a challenging technique. Literature shows that the difference in the results of plating and flow cytometry can vary depending on the bacteria and stain used [107]. For example, in this experiment, there exists a large number of debris which increases the hardness of collecting and identifying data. Surprisingly, results show that the alive cell numbers from flow cytometry are at a similar magnitude as the plating results, also, it is comparable to the reference number (formulated bacteria number). Therefore, the flow cytometry data are valid.

The combination of released bacteria from flow cytometry and the digestion of oil in EC oleogels also proves the hypothesis brought up during formulation development is correct. The release of bacteria number does increase with the increasing digestion of the encapsulated MCT oil. Furthermore, the correlation between the fraction of the released bacteria and the digested fraction of oil is almost linear (*Figure 9*).

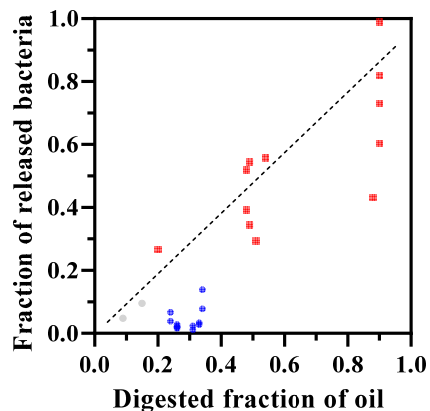


Figure 9. Summarize the fractions of released bacteria versus the digested fraction of oil from the EC oleogels using flow cytometry, EC₂₂/MCT-B (●, grey for 0 days and blue for 30, 60, and 90 days), EC₂₂/SCCM-B (■, green for the odd data of EC₂₂/SCCM-B/30 at 30 days and EC₂₂/SCCM-B/40, and red for 30, 60, and 90 days). The dashed line is the trend line. (From paper 4)

In this part, we have demonstrated that EC oleogels have good physical stability. The storage stability of the encapsulated bacteria is slightly decreased over time. During *in vitro* lipolysis, EC oleogels with disintegrants can continuously release the encapsulated bacteria within 120 min and maintain the high live cell numbers from 30 to 120 min. Furthermore, this release of bacteria correlates reasonably with the degree of oil digestion in the EC oleogels.

Discussion

Ileum and colon targeted delivery system for probiotics

General colon-targeted delivery systems and their trigger mechanisms have been discussed in the introduction. However, not many of them have been applied to probiotics. Some of the most discussed materials in the literature are polymeric carriers in different formats such as microgels and beads, capsules and tablets, and microdevices [108]. Some of these delivery strategies show successful protection during the GI tract and can release encapsulated bacteria in the colon [109, 110]. One drawback for these new delivery strategies is the challenge of sustaining viability during storage. Water-based, double emulsion delivery systems show good protection during the upper part of the small intestine, but a severe decrease in viability was observed during storage just for 3 days at 4°C [111].

Commercially available probiotic products are mainly in dried powder format and stored in either capsule (typically gelatine and HPMC capsule) or sachets or directly compressed into tablets [108]. Probiotics in dry format have low survivability during storage because of oxygen and humidity [104]. Meanwhile, there is low or no protection in the gastrointestinal tract with dry formats. Nevertheless, these dry formats might be suitable for the most common probiotics currently (e.g., *Limosilactobacillus*), which are less sensitive to oxygen (facultative anaerobic) and bile, and mainly colonized in the small intestine. For more sensitive probiotics, such as probiotics that are strictly anaerobic, sensitive to the environment in the GI tract, and target delivery to the colon, a new delivery system is urgently needed [112, 113].

The oil-based delivery system is extra interesting for probiotics as it has low water activity and is a barrier between probiotics and air/oxygen. The main shortage of a pure oil delivery system is the fast digestion and release in the stomach, duodenum, and jejunum resulting in no protection during GI tract and no controlled delivery to the ileum and colon. On the other hand, an oil-based delivery system with retarding effect on lipolysis at the early part of the small intestine might be the ideal candidate delivery system for probiotics.

To evaluate the retarding effect more accurately for different delivery strategies, an *in vitro* lipolysis model was modified. Several parameters such as increasing calcium concentration and using high mixing technology were adjusted to achieve the fast speed of lipolysis (for pure MCT oil) at the beginning (for details see in

vitro method section). Using the modified in vitro lipolysis model, 70% pure oil can be fully converted from triglycerides to free fatty acids in 10 min and reach 100% conversion within 120 min. The fast digestion speed at the beginning and full digestion within the experiment time makes it suitable for evaluating the retarding effect both slowing down the lipolysis and decreasing the lipolysis extent.

The retarding effect of different delivery strategies was evaluated using the in vitro method, from which, EC oleogels show great potential because of their high lipolysis retardation within 120 min. There are several studies of EC oleogel application but mainly on edible applications such as sausages [87], dough products [114], and chocolate [115]. The only application of EC oleogels as the delivery system is for lipophilic bioactive substance delivery (beta-carotene) [95]. In almost all the applications of EC oleogels with other components (including APIs and excipients), the other components were added either before the heating or when the temperature of the EC mixture was higher than 100°C. This high temperature eliminates the possibility of applying EC oleogels to temperature-sensitive components, such as probiotics.

A cold-mixing method was developed to tackle this problem. EC oleogels with cold-mixing method thus can be the delivery system for probiotics. Three aspects of EC oleogels (both hot-mixed and cold-mixed oleogels) were investigated, including physical characteristics, encapsulation quality, and oil digestion and delivery properties of the encapsulated probiotics.

Physical characteristics of EC oleogels

The physical stability of EC oleogels is critical to investigate because the cold-mixing method disturbs the gel structure at a relatively low temperature. At this low temperature, the viscosity is already increased to some extent. This relatively higher viscosity indicates that the gel structure has formed to some extent. Therefore, it is not obvious whether the gel structure will be reformed after the disturbance. Measurements including rheology properties (storage modules and yield value), hardness, oil entrapment, and SAXS were done on both hot-mixed and cold-mixed oleogels.

Results from all these four measurements show that for both oleogels, increasing the concentrations and increasing the molecular mass of EC will result in higher hardness, oil entrapment, storage modules, and yield value.

The disturbance induced during the cold-mixing step results in lower hardness, reduced oil entrapment capacity, and decreased storage modules within 1 week of storage at room temperature, compared to hot-mixed oleogels (without cold-mixing). However, similar storage modules and yield values were observed between

1-week and 2-week samples (room temperature) for both hot-mixed and cold-mixed oleogels. The microstructure gained from SAXS also shows the similarity of the two gels after 1 week of storage under room temperature. These indicate that there might exist gel relaxation in both cold-mixed and hot-mixed oleogels. After relaxation, cold-mixed oleogels are similar to hot-mixed oleogels at both molecular level and at a larger scale (e.g., domains around 50-300 nm). Furthermore, no further changes on hardness for longer storage time (up to 90 days) and under accelerated storage conditions (30 and 40°C). To conclude, cold-mixed EC oleogels are stable after relaxation, of which the time length is less than 1 week at room temperature.

Another important parameter for an effective delivery system is the adjustable characteristics so that the formulation can be easily modified to different demands. The physical characteristics of EC oleogels can be tuned during the cold-mixing step. A higher concentration of oil added can largely decrease the hardness and a higher fraction of solids added can largely increase the hardness. With the possibility to tune the physical characteristics of EC oleogels by cold-mixing method, the potential of developing EC oleogels for different purposes is largely increased.

Encapsulation quality for probiotics of EC oleogels

A high-quality encapsulation formulation should protect probiotics from various stresses, thus maintaining a high viability when reaching the target delivery location. The stress are the oxygen and humidity in the environment during storage, and the low pH and high bile concentration in the GI system before reaching the ileum and colon. Using EC oleogel as the delivery system, the stress can also come from the relatively high temperature (around 40°C) during the encapsulating procedure (cold-mixing procedure), and different materials used in the formulation.

Results show that EC polymer, MCT oil, different lipid excipients and different hydrophilic powders are harmless to the bacteria, the same as the cold-mixing procedure. Meanwhile, because the bacteria were mixed in the EC polymer system, the encapsulation efficiency during the producing process could be as high as 100%.

EC oleogels can also maintain the high viability of the encapsulated probiotics. The viability has less than 1 log₁₀ decrease at 40°C for 90 days. Also, the encapsulation quality does not seem to be influenced by the gel relaxation. The delivery strategies in aqueous solutions (e.g., double emulsions), where good protection in the stomach was achieved, the encapsulation efficiency (live cells in the internal aqueous phase) started at around 96% at time 0 and decreased to 55% after 14-day storage at 4°C (*L. acidophilus*) [116]. For microencapsulation delivery strategies using chitosan and xanthan gum, and incorporated in yogurt, the storage stability of the

encapsulated bacteria decreases between 0.7 log₁₀ to 1.2 log₁₀ at 4°C for 3 weeks (*L. acidophilus*) [117].

The good protection to the encapsulated bacteria in both storage and in vitro analysis could indicate that EC oleogels are a good barrier against oxygen and humidity/water. This characteristic makes it a unique opportunity to further apply EC oleogels to more strict anaerobic bacteria (e.g., next-generation probiotics).

Oil digestion and delivery properties of EC oleogels

Retarding effect of EC oleogels on lipolysis is one of the most critical parameters for target delivery to the ileum and colon. The stronger the retarding effect, the more promising the delivery to the ileum and colon. In both hot-mixed and cold-mixed oleogels, the higher the concentration and higher the molecular mass of EC oleogels in MCT oil will result in a stronger retarding effect (harder oleogels). However, unlike the hardness of EC oleogels, the retarding effect has a limit where similar retarding effects were observed despite increased concentrations or molar mass (details in *paper 2*). A reasonable explanation for this is that at this limit, the hindrance from the gel structure is strong enough to limit encapsulated oil from migrating to the oil-water interface. Therefore, the relative conversion remains similar within 120 min in those solid-like oleogel formulations.

The retarding effect or the digestion fraction of the oil does not directly correspond to the release of the bacteria. One complication can come from the interaction between probiotics and EC oleogels. Freeze-dried probiotics are embedded in cryo-protection materials including various components, such as sucrose and maltodextrins, and then encapsulated in the EC oleogels. Studies have shown that there might be interactions (e.g., hydrogen bonds) between EC polymer and sucrose [118]. Furthermore, as EC polymers remain insoluble in water after lipolysis, there will likely be bacteria remaining in EC polymer aggregate in the digestion media. Nevertheless, results show almost all numbers of the encapsulated bacteria were released. Meanwhile, the digested fraction of oil and released fraction of bacteria seem to be not only positively correlated but are almost linear (*Figure 9*).

The almost linear correlation between the fraction of digested oil and the fraction of released bacteria provided fundamental support for formulation development where a placebo (no bacteria was encapsulated) was used. During formulation development, the release properties of the EC oleogels can be evaluated by the lipid digestion behaviour of EC oleogels. Because of the cold-mixing method, the release properties could also be easily tuned by adding excipients during the cold-mixing step. The added excipients will have less influence from the relatively low temperature (40°C) therefore avoiding possible oxidation or other reactions from the high temperature (above 100°C).

Release property is one of the most important parameters for EC oleogels being the controlled delivery system. We found out that EC oleogels can have different release properties when adding different excipients (lipid excipients and hydrophilic powder excipients). Digested fraction of encapsulated oil can vary from around 10% to almost 100% within 120 min with different release kinetics. Accelerating release can be obtained by adding disintegrants, the combination of disintegrants and lipid excipients. However, the release property of a certain formulation can be complicated as different mechanisms might influence the same time, e.g., diffusion, erosion, and enzymatic reaction. Therefore, the Peppas model was used to analyse and group the release kinetics.

After applying the Peppas model, different release kinetics were obtained with different n values. More importantly, the release kinetics from oil digestion results seem to match very well with the actual release mechanisms (**Table 4**). This well match conversely proves that the Peppas model is a suitable model for the EC oleogel system. It is the first time that the Peppas model was used to analyse the release from EC oleogels. The tool (combination of quantitative model and release mechanisms) proposed during the formulation development built a solid ground for understanding and for helping formulation development of other applications.

The high stability of EC oleogel itself and the accelerating release properties of EC oleogels build the foundation for EC being the possible controlled release system for probiotics. The actual release profile of probiotics from the ideal oleogel formulations under physiological conditions adds to the advantages. **Figure 8** shows that both EC oleogel formulations can gradually release the encapsulated bacteria. For the accelerating release formulation (added disintegrants during the cold-mixing step), the bacteria number is 1 \log_{10} higher than the reference formulation (bacteria in MCT oil at time 0) when the live number is stable. The high number of live bacteria after 120 min exposure to high bile concentration shows both the protection and the controlled release characteristic of the EC oleogels.

For accelerating release formulation (added disintegrants) after 90 days storage time at 40°C, the live bacteria number (under physiological conditions) show an overall only around 1 \log_{10} decrease of live cells compared to the formulated number (**Figure 8**). This is strong evidence that EC oleogels can protect the encapsulated bacteria from various stresses (both at storage and during the GI tract), resulting in overall high viability until reaching the targeted location in the GI tract.

To conclude, the release properties of EC oleogels can be easily tuned with certain levels of control. This controllable manner of the release properties enhances the success of EC oleogel as the controlled delivery system for different APIs and different target locations. Another promising characteristic of EC oleogel is the high stability of EC oleogel formulation itself. Last but most importantly, EC oleogels can protect the encapsulated bacteria from oxygen and bile salts and deliver the live bacteria to the later part of the small intestine.

Experimental limitations and challenges

One experimental limitation is the choice of model bacteria. *L. reuteri* was used instead of other more oxygen-sensitive probiotics. *L. reuteri* is one of the most common probiotics that is commercially available. It is gram-positive and facultatively anaerobic, which means that the bacteria are quite robust and can handle oxygen to some extent. These characteristics make the experiment easier as the main method used to evaluate the viability is conventional plating. Nevertheless, the high viability of *L. reuteri* under accelerated storage conditions shows that EC oleogels are a good barrier toward oxygen and bile concentrations. This gives an insight that EC oleogels might also be a good encapsulation system for restricting anaerobic bacteria (next-generation probiotics).

There are several experimental challenges under the current experiment settings. One improvement that could be made is the gel-making procedure. During the cold-mixing procedure, different mixtures of MCT oil and excipients were added into oleogels at around 40°C, and the mixing was done by using a stainless spatula and with handcraft. A certain degree of standardization based on observation has been applied. For example, during the cold-mixing step, the mixing stops when the gel appears homogeneous. But when different not-soluble excipients were added, the oleogel appeared to be white and thus it was hard to determine when to stop. When a larger amount was needed during stability study, a mixer was applied instead of a spatula but the judge of when to stop the mixing was still based on observation. Therefore, there might exist inhomogeneity in the samples.

The next challenge is the filling of EC oleogels into different containers, i.e., gelatine and HPMC capsules and glass bottles. Especially when making samples for the stability study. There are lots of containers to fill in and accurate mass is needed for certain containers. The temperature-dependent gel characteristic of EC oleogels makes the long filling process extra difficult as the oleogel is gradually cooling down. The lower the temperature, the harder the oleogels are. It might be possible that different gel characteristics could be observed, for example from the first glass bottle to the last glass bottle. Fortunately, a gel relaxation was also observed among both the cold-mixing and hot-mixing oleogels. After relaxation, both gels with the same EC and same concentration have almost the same gel characteristics. Therefore, despite the difference during the filling step, a similar oleogel could be obtained.

Another experimental challenge is the extraction of bacteria from EC oleogels. EC oleogels are highly viscous and sticky, and EC polymers are insoluble in water. The method used for the viability test is by adding physical force to the mixture of the oleogels and MRS agar solution (detailed procedure see *papers 2 and 4*). Surprisingly, the number of extracted live bacteria from EC oleogels was very close to the formulated bacteria number. The same magnitude of live bacteria number was observed between plating results and flow cytometry. All these results prove that the extraction method used was reliable.

Conclusions

This thesis has presented the progress of formulation development, while also providing a certain level of fundamental scientific understanding regarding the developed formulation, such as release kinetics and release mechanisms.

An *in vitro* lipolysis model is modified based on the needs of this project. This modified *in vitro* lipolysis allows for the full digestion of MCT oil in a way that no experimental condition is a hindrance to the digestion speed or digestion extension. It also allows a direct correlation between relative conversion and retarding effect from different retarders. Furthermore, different oil-based delivery strategies were compared using this *in vitro* model. EC oleogel shows a strong retarding effect and continuous digestion characteristics, making it the most promising delivery system for probiotics.

The development of the cold-mixing method solves one of the main challenges (high temperature) and enables the application of EC oleogel as the delivery system for probiotics. Meanwhile, the cold-mixing method provides a way to modify EC oleogels in various aspects. The two most important aspects include the ability to incorporate temperature-sensitive components, as well as the ability to modify the release mechanism. The latter was proven by adding different excipients during the cold-mixing step, resulting in varying release mechanisms and release extent. More importantly, a high level of delivery of live probiotics at the target location (ileum and colon) in the GI tract is obtained by adding disintegrating hydrophilic powder excipients.

Storage stability study shows that EC oleogels are stable over a long time. EC oleogels with the cold-mixing method can protect the encapsulated bacteria during both storage and passage through the upper part of the gastrointestinal system (high bile salts concentrations and various enzymes).

Therefore, EC oleogels show great potential as a controlled delivery system for probiotics.

Outlook of the future

This thesis has so far demonstrated that EC oleogels can be the controlled release delivery system for probiotics with good protection during both storage (temperature, humidity, oxygen) and under physiological conditions (bile salts and enzymes).

An effective *in vitro* method was modified for this thesis. However, it would be interesting to investigate how EC oleogels perform under the whole gastrointestinal system instead of only the small intestine compartment, i.e., including the stomach section with low pH, the small intestine section where the bile concentration can be gradually lowered, the colon section where if the remaining encapsulated bacteria could be fully released, and finally under oxygen-depleted condition.

Adhesion and colonization would also be an interesting topic to investigate in the future. These two are the key steps before a probiotic can insert its function [119]. Some literature shows there is limited adhesion of probiotics that are orally administrated [120]. Furthermore, *in vivo* analysis could be one option for further study.

One interesting application for EC oleogels is the delivery system for biologics (e.g., vaccines, proteins, and monoclonal antibodies). Oral administration of biologics has encountered numerous challenges primarily due to their low stability within the gastrointestinal tract. The low stability is caused partially by their natural characteristics and partially because of the complicated system from the gastrointestinal system. This situation is similar to probiotics. Therefore, there is a possibility that EC oleogels can also be the controlled release delivery system for biologics.

Lastly, there is more that can be done on the commercial aspect. In this thesis, a commercial model probiotic (*L. reuteri*) was applied in a model delivery situation. And there already exist lots of successful commercial products of this probiotic. It would be interesting to apply EC oleogels to new probiotic strains, for example, the ones that have more restrictive protection requirements, e.g., strict anaerobic probiotics. During formulation development, we have shown that the controlled release function can be easily tuned by adding different excipients. This can be very helpful as different bacteria may require different target location, e.g., the stomach, later part of the small intestine, and colon.

References

1. Kunz, C., S. Kuntz, and S. Rudloff, (2009). *Intestinal flora, in breast-feeding: Early influences on later health*, Springer Netherlands: Dordrecht. 67-79.
2. Neish, A.S. (2009). Microbes in Gastrointestinal Health and Disease. *Gastroenterology*. 136(1). 65-80.
3. Ley, R.E., D.A. Peterson, and J.I. Gordon. (2006). Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine. *Cell*. 124(4). 837-848.
4. Savage, D.C. (1977). Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol*. 31. 107-133.
5. Whitman, W.B., D.C. Coleman, and W.J. Wiebe. (1998). Prokaryotes: the unseen majority. *Proc Natl Acad Sci U S A*. 95(12). 6578-6583.
6. Sekirov, I., S.L. Russell, L.C.M. Antunes, and B.B. Finlay. (2010). Gut microbiota in health and disease. *Physiological Reviews*. 90(3). 859-904.
7. Rhee, S.H., C. Pothoulakis, and E.A. Mayer. (2009). Principles and clinical implications of the brain–gut–enteric microbiota axis. *Nature Reviews Gastroenterology & Hepatology*. 6(5). 306-314.
8. Herbrand, H., G. Bernhardt, R. Forster, and O. Pabst. (2008). Dynamics and function of solitary intestinal lymphoid tissue. *Critical Review in Immunology*. 28(1).
9. Cash, H.L., C.V. Whitham, C.L. Behrendt, and L.V. Hooper. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science*. 313(5790). 1126-1130.
10. Vaishnav, S., C.L. Behrendt, A.S. Ismail, L. Eckmann, and L.V. Hooper. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proceedings of the National Academy of Sciences*. 105(52). 20858-20863.
11. Islam, S.U. (2016). Clinical uses of probiotics. *Medicine*. 95(5). e2658.
12. FAO/WHO (2006). Health and nutritional properties and guidelines for evaluation in Report of a joint FAO/WHO expert consultation on Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. WHO, Geneva.
13. Oelschlaeger, T.A. (2010). Mechanisms of probiotic actions – A review. *International Journal of Medical Microbiology*. 300(1). 57-62.
14. Kechagia, M., D. Basoulis, S. Konstantopoulou, D. Dimitriadi, K. Gyftopoulou, N. Skarmoutsou, and E.M. Fakiri. (2013). Health benefits of probiotics: a review. *International Scholarly Research Notices(Hindawi)*. 2013. 481651.

15. Butel, M.J. (2014). Probiotics, gut microbiota and health. *Médecine et Maladies Infectieuses*. 44(1). 1-8.
16. Mu, Q., V.J. Tavella, and X.M. Luo. (2018). Role of *Lactobacillus reuteri* in human health and diseases. *Front Microbiol*. 9. 757.
17. Stensson, M., G. Koch, S. Coric, T. Abrahamsson, M. Jenmalm, D. Birkhed, and L.-K. Wendt. (2014). Oral administration of *Lactobacillus reuteri* during the first year of life reduces caries prevalence in the primary dentition at 9 years of age. *Caries research*. 48(2). 111-117.
18. Weizman, Z., J. Abu-Abed, and M. Binsztok. (2016). *Lactobacillus reuteri* DSM 17938 for the management of functional abdominal pain in childhood: a randomized, double-blind, placebo-controlled trial. *The Journal of pediatrics*. 174. 160-164. e161.
19. Agustina, R., F.J. Kok, O. Van De Rest, U. Fahmida, A. Firmansyah, W. Lukito, E.J. Feskens, E.G. Van Den Heuvel, R. Albers, and I.M. Bovee-Oudenhoven. (2012). Randomized trial of probiotics and calcium on diarrhea and respiratory tract infections in Indonesian children. *Pediatrics*. 129(5). e1155-e1164.
20. Savino, F., S. Fornasero, S. Ceratto, A. De Marco, N. Mandras, J. Roana, V. Tullio, and G. Amisano. (2015). Probiotics and gut health in infants: A preliminary case-control observational study about early treatment with *Lactobacillus reuteri* DSM 17938. *Clinica chimica acta*. 451. 82-87.
21. Rosenfeldt, V., E. Benfeldt, N.H. Valerius, A. Pærregaard, and K.F. Michaelsen. (2004). Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. *The Journal of pediatrics*. 145(5). 612-616.
22. Abrahamsson, T.R., T. Jakobsson, M.F. Böttcher, M. Fredrikson, M.C. Jenmalm, B. Björkstén, and G. Oldaeus. (2007). Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *Journal of Allergy and Clinical Immunology*. 119(5). 1174-1180.
23. Abrahamsson, T.R., T. Jakobsson, B. Björkstén, G. Oldaeus, and M.C. Jenmalm. (2013). No effect of probiotics on respiratory allergies: a seven - year follow - up of a randomized controlled trial in infancy. *Pediatric Allergy and Immunology*. 24(6). 556-561.
24. Indrio, F., A. Di Mauro, G. Riezzo, E. Civardi, C. Intini, L. Corvaglia, E. Ballardini, M. Bisceglia, M. Cinquetti, E. Brazzoduro, A. Del Vecchio, S. Tafuri, and R. Francavilla. (2014). Prophylactic Use of a Probiotic in the Prevention of Colic, Regurgitation, and Functional Constipation: A Randomized Clinical Trial. *JAMA Pediatrics*. 168(3). 228-233.
25. Tripathi, M.K. and S.K. Giri. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*. 9. 225-241.
26. Agrawal, R. (2005). Probiotics: An emerging food supplement with health benefits. *Food Biotechnology*. 19(3). 227-246.
27. de Vos, P., M.M. Faas, M. Spasojevic, and J. Sikkema. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal*. 20(4). 292-302.

28. Morelli, L. (2007). In vitro assessment of probiotic bacteria: From survival to functionality. *International Dairy Journal*. 17(11). 1278-1283.
29. Seo, B.J., M.R. Mun, R.K.V. J, C.J. Kim, I. Lee, Y.H. Chang, and Y.H. Park. (2010). Bile tolerant *Lactobacillus reuteri* isolated from pig feces inhibits enteric bacterial pathogens and porcine rotavirus. *Veterinary Research Communications*. 34(4). 323-333.
30. Krumbeck, J.A., N.L. Marsteller, S.A. Frese, D.A. Peterson, A.E. Ramer Tait, R.W. Hutkins, and J. Walter. (2016). Characterization of the ecological role of genes mediating acid resistance in *Lactobacillus reuteri* during colonization of the gastrointestinal tract. *Environmental Microbiology*. 18(7). 2172-2184.
31. Nagar, P., K. Singh, I. Chauhan, M. Verma, M. Yasir, A. Khan, R. Sharma, and N. Gupta. (2011). Orally disintegrating tablets: formulation, preparation techniques and evaluation. *Journal of Applied Pharmaceutical Science*. 35-45.
32. Luo, Y., C. De Souza, M. Ramachandran, S. Wang, H. Yi, Z. Ma, L. Zhang, and K. Lin. (2022). Precise oral delivery systems for probiotics: A review. *Journal of Controlled Release*. 352. 371-384.
33. Kotla, N.G., S. Rana, G. Sivaraman, O. Sunnapu, P.K. Vemula, A. Pandit, and Y. Rochev. (2019). Bioresponsive drug delivery systems in intestinal inflammation: State-of-the-art and future perspectives. *Advanced drug delivery reviews*. 146. 248-266.
34. Yoha, K.S., S. Nida, S. Dutta, J.A. Moses, and C. Anandharamakrishnan. (2022). Targeted delivery of probiotics: Perspectives on Research and Commercialization. *Probiotics Antimicrob Proteins*. 14(1). 15-48.
35. Sarao, L.K. and M. Arora. (2017). Probiotics, prebiotics, and microencapsulation: A review. *Critical Reviews in Food Science and Nutrition*. 57(2). 344-371.
36. Sinha, V. and R. Kumria. (2003). Microbially triggered drug delivery to the colon. *European Journal of Pharmaceutical Sciences*. 18(1). 3-18.
37. Global Food Encapsulation Market Outlook 2019-2024 *Micro-encapsulation of probiotics to drive growth - ResearchAndMarkets.com*. [cited 2023 July 12]; Available from: <https://www.businesswire.com/news/home/20190613005360/en/Global-Food-Encapsulation-Market-Outlook-2019-2024>.
38. Douillard, F.P. and W.M. de Vos. (2019). Biotechnology of health-promoting bacteria. *Biotechnology Advances*. 37(6). 107369.
39. Burgain, J., C. Gaiani, M. Linder, and J. Scher. (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering*. 104(4). 467-483.
40. Heuman, D.M., R.S. Bajaj, and Q. Lin. (1996). Adsorption of mixtures of bile salt taurine conjugates to lecithin-cholesterol membranes: implications for bile salt toxicity and cytoprotection. *Journal of Lipid Research*. 37(3). 562-573.
41. Begley, M., C.G. Gahan, and C. Hill. (2005). The interaction between bacteria and bile. *FEMS Microbiol Rev*. 29(4). 625-651.

42. Govender, M., Y.E. Choonara, P. Kumar, L.C. du Toit, S. van Vuuren, and V. Pillay. (2014). A review of the advancements in probiotic delivery: Conventional vs. non-conventional formulations for intestinal flora supplementation. *AAPS PharmSciTech*. 15(1). 29-43.
43. Zechner, R., R. Zimmermann, Thomas O. Eichmann, Sepp D. Kohlwein, G. Haemmerle, A. Lass, and F. Madeo. (2012). Fat signals - Lipases and lipolysis in lipid metabolism and signaling. *Cell Metabolism*. 15(3). 279-291.
44. Rothman, S., C. Liebow, and L. Isenman. (2002). Conservation of digestive enzymes. *Physiol Rev*. 82(1). 1-18.
45. Ticho, A.L., P. Malhotra, P.K. Dudeja, R.K. Gill, and W.A. Alrefai. (2019). Intestinal Absorption of Bile Acids in Health and Disease. *Compr Physiol*. 10(1). 21-56.
46. Minekus, M., M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, and D. Dupont. (2014). A standardised static in vitro digestion method suitable for food—an international consensus. *Food & Function*. 5(6). 1113-1124.
47. Berthelsen, R., M. Klitgaard, T. Rades, and A. Müllertz. (2019). In vitro digestion models to evaluate lipid based drug delivery systems; present status and current trends. *Advanced drug delivery reviews*. 142. 35-49.
48. Liao, T.H., P. Hamosh, and M. Hamosh. (1983). Gastric lipolysis in the developing rat ontogeny of the lipases active in the stomach. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*. 754(1). 1-9.
49. Sensoy, I. (2021). A review on the food digestion in the digestive tract and the used in vitro models. *Current Research in Food Science*. 4. 308-319.
50. Borgström, B. (1975). On the interactions between pancreatic lipase and colipase and the substrate, and the importance of bile salts. *Journal of Lipid Research*. 16(6). 411-417.
51. Momsen, W.E. and H.L. Brockman. (1976). Effects of colipase and taurodeoxycholate on the catalytic and physical properties of pancreatic lipase B at an oil water interface. *Journal of Biological Chemistry*. 251(2). 378-383.
52. McClements, D.J., E.A. Decker, and Y. Park. (2009). Controlling lipid bioavailability through physicochemical and structural approaches. *Crit Rev Food Sci Nutr*. 49(1). 48-67.
53. Reis, P., K. Holmberg, H. Watzke, M.E. Leser, and R. Miller. (2009). Lipases at interfaces: A review. *Advances in colloid and interface science*. 147-148. 237-250.
54. Capolino, P., C. Guérin, J. Paume, J. Giallo, J.-M. Ballester, J.-F. Cavalier, and F. Carrière. (2011). In vitro gastrointestinal lipolysis: replacement of human digestive lipases by a combination of rabbit gastric and porcine pancreatic extracts. *Food digestion*. 2. 43-51.
55. Borgström, B. and C. Erlanson. (1973). Pancreatic lipase and co - lipase: Interactions and effects of bile salts and other detergents. *European Journal of Biochemistry*. 37(1). 60-68.

56. Zangenberg, N.H., A. Müllertz, H.G. Kristensen, and L. Hovgaard. (2001). A dynamic in vitro lipolysis model: I. Controlling the rate of lipolysis by continuous addition of calcium. *European Journal of Pharmaceutical Sciences*. 14(2). 115-122.
57. Christensen, J.Ø., K. Schultz, B. Mollgaard, H.G. Kristensen, and A. Mullertz. (2004). Solubilisation of poorly water-soluble drugs during in vitro lipolysis of medium- and long-chain triacylglycerols. *European Journal of Pharmaceutical Sciences*. 23(3). 287-296.
58. Carulli, N., M. Bertolotti, F. Carubbi, M. Concari, P. Martella, L. Carulli, and P. Loria. (2000). Effect of bile salt pool composition on hepatic and biliary functions. *Alimentary Pharmacology & Therapeutics*. 14. 14-18.
59. Okoli, A.S., M.J. Raftery, and G.L. Mendz. (2012). Effects of human and porcine bile on the proteome of *Helicobacter hepaticus*. *Proteome Science*. 10(1). 27.
60. Calvo-Lerma, J., V. Fornés-Ferrer, A. Heredia, and A. Andrés. (2019). In vitro digestion models to assess lipolysis: The impact of the simulated conditions of gastric and intestinal pH, bile salts and digestive fluids. *Food Research International*. 125. 108511.
61. Dahan, A. and A. Hoffman. (2006). Use of a Dynamic in Vitro Lipolysis Model to Rationalize Oral Formulation Development for Poor Water Soluble Drugs: Correlation with in Vivo Data and the Relationship to Intra-Enterocyte Processes in Rats. *Pharmaceutical Research*. 23(9). 2165-2174.
62. Li, Y. and D.J. McClements. (2010). New Mathematical Model for Interpreting pH-Stat Digestion Profiles: Impact of Lipid Droplet Characteristics on in Vitro Digestibility. *Journal of Agricultural and Food Chemistry*. 58(13). 8085-8092.
63. Zangenberg, N.H., A. Müllertz, H. Gjelstrup Kristensen, and L. Hovgaard. (2001). A dynamic in vitro lipolysis model: II: Evaluation of the model. *European Journal of Pharmaceutical Sciences*. 14(3). 237-244.
64. Kaukonen, A.M., B.J. Boyd, W.N. Charman, and C.J.H. Porter. (2004). Drug solubilization behavior during in vitro digestion of suspension formulations of poorly water-soluble drugs in triglyceride lipids. *Pharmaceutical Research*. 21(2). 254-260.
65. Ali, H., M. Nazzal, A.A.A. Zaghoul, and S. Nazzal. (2008). Comparison between lipolysis and compendial dissolution as alternative techniques for the in vitro characterization of α -tocopherol self-emulsified drug delivery systems (SEDDS). *International Journal of Pharmaceutics*. 352(1). 104-114.
66. Han, S.f., T.t. Yao, X.x. Zhang, L. Gan, C. Zhu, H.z. Yu, and Y. Gan. (2009). Lipid-based formulations to enhance oral bioavailability of the poorly water-soluble drug anethol trithione: Effects of lipid composition and formulation. *International Journal of Pharmaceutics*. 379(1). 18-24.
67. Larsen, A.T., P. Sassene, and A. Müllertz. (2011). In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems. *International Journal of Pharmaceutics*. 417(1). 245-255.
68. Benito-Gallo, P., A. Franceschetto, J.C.M. Wong, M. Marlow, V. Zann, P. Scholes, and P. Gershkovich. (2015). Chain length affects pancreatic lipase activity and the extent and pH-time profile of triglyceride lipolysis. *European Journal of Pharmaceutics and Biopharmaceutics*. 93. 353-362.

69. Lee, Y.Y., A. Erdogan, and S.S. Rao. (2014). How to assess regional and whole gut transit time with wireless motility capsule. *J Neurogastroenterol Motil.* 20(2). 265-270.
70. Steinbrück, I., M. Keuchel, F. Hagenmüller, and A. von Herbay. (2014) Normal small intestine, in video capsule endoscopy: A reference guide and atlas. Springer Berlin Heidelberg. 167-181.
71. Ticho, A.L., P. Malhotra, P.K. Dudeja, R.K. Gill, and W.A. Alrefai. (2019). Intestinal absorption of bile acids in health and disease. *Comprehensive Physiology.* 10(1). 21.
72. Roberts, M.S., B.M. Magnusson, F.J. Burczynski, and M. Weiss. (2002). Enterohepatic circulation: physiological, pharmacokinetic and clinical implications. *Clinical pharmacokinetics.* 41. 751-790.
73. Mattson, F. and R. Volpenhein. (1966). Enzymatic hydrolysis at an oil/water interface. *Journal of the American Oil Chemists Society.* 43(5). 286-289.
74. Brockman, H.L. (2000). Kinetic behavior of the pancreatic lipase-colipase-lipid system. *Biochimie.* 82(11). 987-995.
75. Zhang, R., Z. Zhang, H. Zhang, E.A. Decker, and D.J. McClements. (2015). Influence of lipid type on gastrointestinal fate of oil-in-water emulsions: In vitro digestion study. *Food Research International.* 75. 71-78.
76. Delorme, V., R. Dhouib, S. Canaan, F. Fotiadu, F. Carrière, and J.-F. Cavalier. (2011). Effects of Surfactants on Lipase Structure, Activity, and Inhibition. *Pharmaceutical Research.* 28(8). 1831-1842.
77. Li, Y. and D.J. McClements. (2011). Inhibition of lipase-catalyzed hydrolysis of emulsified triglyceride oils by low-molecular weight surfactants under simulated gastrointestinal conditions. *European Journal of Pharmaceutics and Biopharmaceutics.* 79(2). 423-431.
78. Torcello-Gómez, A., M. Wulff-Pérez, M.J. Gálvez-Ruiz, A. Martín-Rodríguez, M. Cabrerizo-Vilchez, and J. Maldonado-Valderrama. (2014). Block copolymers at interfaces: Interactions with physiological media. *Advances in colloid and interface science.* 206. 414-427.
79. Koch, W. (1937). Properties and uses of ethylcellulose. *Industrial & Engineering Chemistry.* 29(6). 687-690.
80. Heng, P.W., L.W. Chan, and K.T. Chow. (2005). Development of novel nonaqueous ethylcellulose gel matrices: rheological and mechanical characterization. *Pharm Res.* 22(4). 676-684.
81. Kolpak, F.J. and J. Blackwell. (1976). Determination of the structure of cellulose II. *Macromolecules.* 9(2). 273-278.
82. Laredo, T., S. Barbut, and A.G. Marangoni. (2011). Molecular interactions of polymer oleogelation. *Soft Matter.* 7(6). 2734-2743.
83. Davidovich-Pinhas, M., S. Barbut, and A.G. Marangoni. (2015). The gelation of oil using ethyl cellulose. *Carbohydrate Polymers.* 117. 869-878.
84. Davidovich-Pinhas, M., S. Barbut, and A. Marangoni. (2014). Physical structure and thermal behavior of ethylcellulose. *Cellulose.* 21. 3243-3255.

85. Gravelle, A.J., S. Barbut, and A.G. Marangoni. (2013). Fractionation of ethylcellulose oleogels during setting. *Food & Function*. 4(1). 153-161.
86. Davidovich-Pinhas, M., A.J. Gravelle, S. Barbut, and A.G. Marangoni. (2015). Temperature effects on the gelation of ethylcellulose oleogels. *Food Hydrocolloids*. 46. 76-83.
87. Zetzl, A.K., A.G. Marangoni, and S. Barbut. (2012). Mechanical properties of ethylcellulose oleogels and their potential for saturated fat reduction in frankfurters. *Food & Function*. 3(3). 327-337.
88. Gravelle, A.J., M. Davidovich-Pinhas, A.K. Zetzl, S. Barbut, and A.G. Marangoni. (2016). Influence of solvent quality on the mechanical strength of ethylcellulose oleogels. *Carbohydrate Polymers*. 135. 169-179.
89. Davidovich-Pinhas, M., S. Barbut, and A.G. Marangoni. (2015). The role of surfactants on ethylcellulose oleogel structure and mechanical properties. *Carbohydrate Polymers*. 127. 355-362.
90. AIACHE, J.-M., P. GAUTHIER, and S. AIACHE. (1992). New gelification method for vegetable oils I: cosmetic application. *International Journal of Cosmetic Science*. 14(5). 228-234.
91. Davidovich-Pinhas, M., S. Barbut, and A.G. Marangoni. (2014). Physical structure and thermal behavior of ethylcellulose. *Cellulose*. 21(5). 3243-3255.
92. Nishiyama, Y., P. Langan, and H. Chanzy. (2002). Crystal Structure and Hydrogen-Bonding System in Cellulose I β from Synchrotron X-ray and Neutron Fiber Diffraction. *Journal of the American Chemical Society*. 124(31). 9074-9082.
93. Kondo, T. (2005). Hydrogen bonds in cellulose and cellulose derivatives. *Polysaccharides: Structural diversity and functional versatility*. 69-98.
94. O'sullivan, A.C. (1997). Cellulose: the structure slowly unravels. *Cellulose*. 4. 173-207.
95. O'Sullivan, C.M., M. Davidovich-Pinhas, A.J. Wright, S. Barbut, and A.G. Marangoni. (2017). Ethylcellulose oleogels for lipophilic bioactive delivery – effect of oleogelation on in vitro bioaccessibility and stability of beta-carotene. *Food & Function*. 8(4). 1438-1451.
96. Verger, R. (1976). Interfacial enzyme kinetics of lipolysis. *Annu Rev Biophys Bioeng*. 5. 77-117.
97. Reis, P., H. Watzke, M. Leser, K. Holmberg, and R. Miller. (2010). Interfacial mechanism of lipolysis as self-regulated process. *Biophysical Chemistry*. 147(3). 93-103.
98. Bruschi, M.L., (2015). 5 - Mathematical models of drug release, in Strategies to Modify the Drug Release from Pharmaceutical Systems. Woodhead Publishing, p. 63-86.
99. Ford, J.L., K. Mitchell, P. Rowe, D.J. Armstrong, P.N. Elliott, C. Rostron, and J.E. Hogan. (1991). Mathematical modelling of drug release from hydroxypropylmethylcellulose matrices: effect of temperature. *International Journal of Pharmaceutics*. 71(1-2). 95-104.

100. Pillay, V. and R. Fassihi. (1999). In vitro release modulation from crosslinked pellets for site-specific drug delivery to the gastrointestinal tract: I. Comparison of pH-responsive drug release and associated kinetics. *Journal of Controlled Release*. 59(2). 229-242.
101. Champagne, C.P. and P. Fustier. (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. *Current Opinion in Biotechnology*. 18(2). 184-190.
102. Corona - Hernandez, R.I., E. Álvarez Parrilla, J. Lizardi Mendoza, A.R. Islas Rubio, L.A. de la Rosa, and A. Wall Medrano. (2013). Structural stability and viability of microencapsulated probiotic bacteria: a review. *Comprehensive Reviews in Food Science and Food Safety*. 12(6). 614-628.
103. Kieps, J. and R. Dembczyński. (2022). Current Trends in the Production of Probiotic Formulations. *Foods*. 11(15).
104. Bai Palmkron, S. (2024). *Enhancing the stability of probiotics: Freeze-drying and encapsulation* (doctoral thesis). Lund University.
105. Riaz, Q.U.A. and T. Masud. (2013). Recent Trends and Applications of Encapsulating Materials for Probiotic Stability. *Critical Reviews in Food Science and Nutrition*. 53(3). 231-244.
106. Prakash, S., C. Tomaro-Duchesneau, S. Saha, and A. Cantor. (2011). The gut microbiota and human health with an emphasis on the use of microencapsulated bacterial cells. *Journal of Biomedicine and Biotechnology*. 2011.
107. Wilkinson, M.G. (2018). Flow cytometry as a potential method of measuring bacterial viability in probiotic products: A review. *Trends in Food Science & Technology*. 78. 1-10.
108. Torp, A.M., M.I. Bahl, A. Boisen, and T.R. Licht. (2022). Optimizing oral delivery of next generation probiotics. *Trends in Food Science & Technology*. 119. 101-109.
109. Asgari, S., A. Pourjavadi, T.R. Licht, A. Boisen, and F. Ajallouei. (2020). Polymeric carriers for enhanced delivery of probiotics. *Advanced drug delivery reviews*. 161. 1-21.
110. Cui, J.H., J.S. Goh, P.H. Kim, S.H. Choi, and B.J. Lee. (2000). Survival and stability of bifidobacteria loaded in alginate poly-l-lysine microparticles. *International Journal of Pharmaceutics*. 210(1-2). 51-59.
111. Van der Ark, K.C., A.D.W. Nugroho, C. Berton-Carabin, C. Wang, C. Belzer, W.M. de Vos, and K. Schroen. (2017). Encapsulation of the therapeutic microbe *Akkermansia muciniphila* in a double emulsion enhances survival in simulated gastric conditions. *Food Research International*. 102. 372-379.
112. Derrien, M., E.E. Vaughan, C.M. Plugge, and W.M. de Vos. (2004). *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *International journal of systematic and evolutionary microbiology*. 54(5). 1469-1476.
113. Foditsch, C., T.M. Santos, A.G. Teixeira, R.V. Pereira, J.M. Dias, N. Gaeta, and R.C. Bicalho. (2014). Isolation and characterization of *Faecalibacterium prausnitzii* from calves and piglets. *PLoS one*. 9(12). e116465.

114. Gravelle, A.J. and A.G. Marangoni, *Ethylcellulose oleogels: structure, functionality, and food applications*, in *Advances in food and nutrition research*. 2018, Elsevier. p. 1-56.
115. Marangoni, A., (2012). Chocolate Compositions Containing Ethylcellulose. Patent US 20120183651 A1.
116. Wang, L., M. Song, Z. Zhao, X. Chen, J. Cai, Y. Cao, and J. Xiao. (2020). *Lactobacillus acidophilus* loaded pickering double emulsion with enhanced viability and colon-adhesion efficiency. *LWT*. 121. 108928.
117. Shu, G., Y. He, L. Chen, Y. Song, J. Meng, and H. Chen. (2017). Microencapsulation of *Lactobacillus Acidophilus* by xanthan-chitosan and its stability in yoghurt. *Polymers*. 9(12). 733.
118. Stortz, T.A., D.C. De Moura, T. Laredo, and A.G. Marangoni. (2014). Molecular interactions of ethylcellulose with sucrose particles. *RSC advances*. 4(98). 55048-55061.
119. Van den Abbeele, P., C. Grootaert, S. Possemiers, W. Verstraete, K. Verbeken, and T. Van de Wiele. (2009). In vitro model to study the modulation of the mucin-adhered bacterial community. *Applied Microbiology and Biotechnology*. 83(2). 349-359.
120. Servin, A.L. and M.-H. Coconnier. (2003). Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Practice & Research Clinical Gastroenterology*. 17(5). 741-754.



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