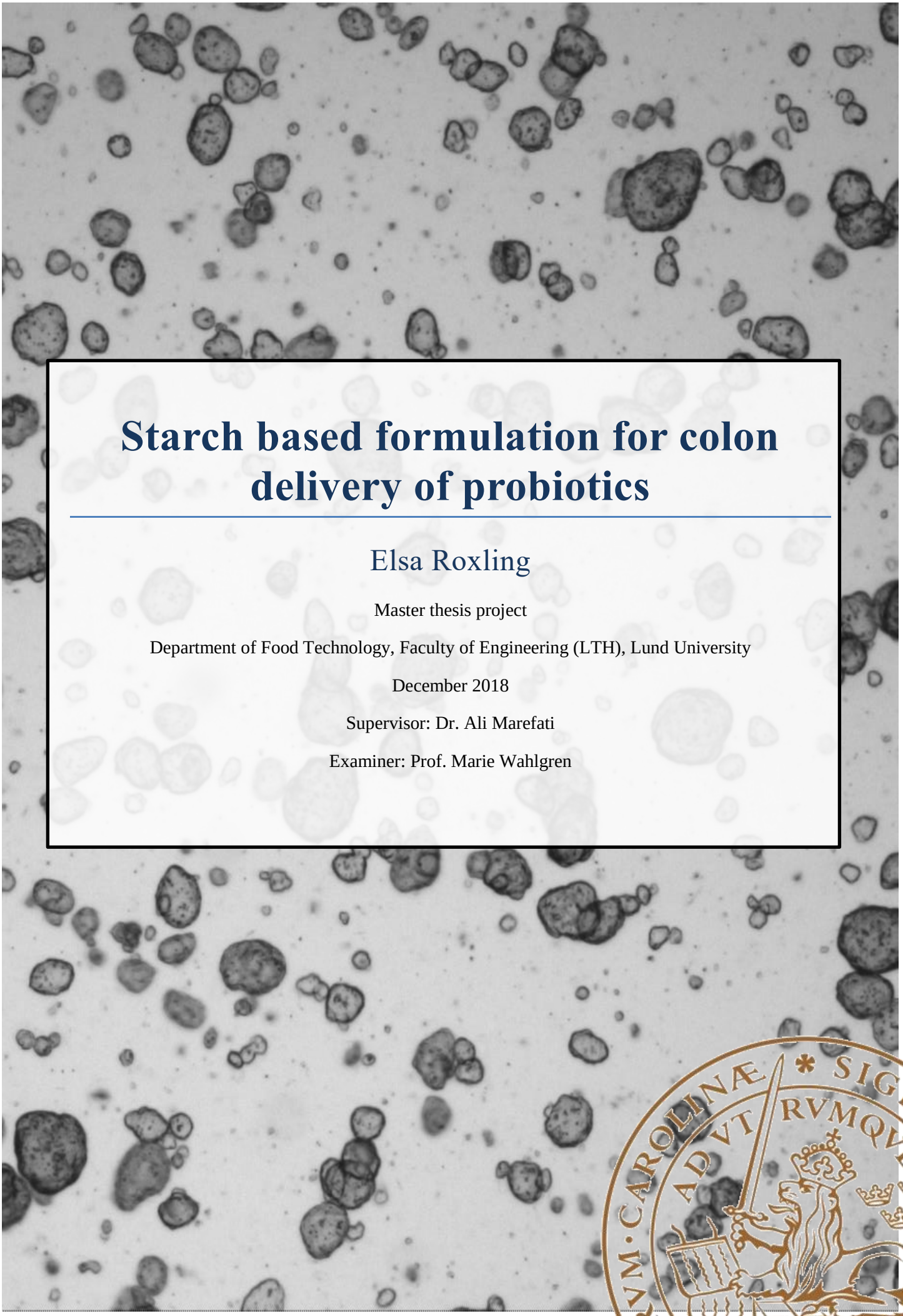

# Starch based formulation for colon delivery of probiotics


Elsa Roxling

Master thesis project

Department of Food Technology, Faculty of Engineering (LTH), Lund University


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Supervisor: Dr. Ali Marefati

Examiner: Prof. Marie Wahlgren

## Abstract

The interest in probiotics has grown in the last few years. In addition to being effective in treating and preventing acute diarrhea, there are indications that it might be possible to use probiotics to prevent other diseases such as colon cancer. The challenge with probiotics is that for it to have any effect, enough viable bacteria must reach the colon. To deliver live probiotics to the colon, it is necessary to protect the bacteria in some manner as most of them cannot survive the harsh environment in the gastrointestinal tract. A formulation consisting of starch microspheres with the bacteria encapsulated in the starch matrix was investigated. Starch microspheres were made by an aqueous two-phase system, with dissolved starch as the internal phase and dissolved polyethylene glycol (PEG) as the external phase. To crystallize the starch, the samples were incubated at 6 °C followed by 37 °C. Two different average molecular weight PEGs and several different PEG concentrations, as well as three different acid hydrolyzed waxy starches were investigated to find the best combination. 13 wt% PEG 2 000 000 and acid hydrolyzed waxy maize starch was chosen to produce microspheres containing microorganisms. Microorganisms of the type strain *Lactobacillus reuteri DSM 20016* were successfully encapsulated in microspheres; however the freeze drying process of the bacteria prior to encapsulation killed most of them. The microspheres were analyzed with a simulated *in vitro* intestinal digestion. The work done in this project was very early explorative phase and a lot of work is needed to optimize the production process, and the freeze drying of the microorganisms.

## Svensk sammanfattning

Intresset för probiotika har vuxit de senaste åren. Förutom att vara ha en förebyggande effekt och vara en effektiv behandling mot akut diarré, finns det forskning som indikerar att probiotika kanske kan användas för att förebygga andra sjukdomar, t.ex. tjocktarmscancer. Utmaningen med probiotika är att för att den ska ha någon effekt, krävs det att tillräckligt många levande bakterier når tjocktarmen. För att göra det, måste bakterierna skyddas på något sätt eftersom de flesta inte överlever de tuffa miljöer som finns i mag- och tarmkanalen. En formulering som består av bakterier inkapslade i stärkelsemikrosfärer undersöktes. Stärkelsemikrosfärer var tillverkade med ett vattenbaserat två-fas-system, med upplöst stärkelse i den inre fasen och upplöst polyetylenglykol (PEG) i den yttre fasen. För att kristallisera stärkelsen inkuberades proverna i 6 °C, följt av 37 °C. Två sorters PEG med olika genomsnittlig molekylvikt och flera olika PEG-koncentrationer, samt tre olika syrahydrolyserade stärkelser med hög andel amylopektin, testades för att hitta den bästa kombinationen. 13 wt% PEG 2 000 000 och syrahydrolyserad hög amylopektin majsstärkelse valdes för att producera mikrosfärer som innehöll bakterier. Mikroorganismer av typstammen *Lactobacillus reuteri DSM 20016* inkapslades framgångsrikt i mikrosfärer; men frystorkningen av bakterierna innan inkapslingen dödade de flesta av dem. Mikrosfärerna var analyserade med hjälp av en *in vitro*-simulering av tunntarmen. Arbetet som utfördes i detta projekt var väldigt initialt och mycket arbete med att optimera produktionsprocessen, och frystorkningen av bakterierna är nödvändigt.

## Populärvetenskaplig sammanfattning

# Inkapsling av probiotika i stärkelsemikrosfärer

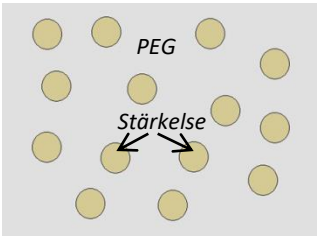
**Intresset för att påverka tarmfloran med hjälp av probiotika växer allt eftersom vi inser tarmflorans betydelse för vårt hälsotillstånd. Bakterierna behöver levereras levande till tjocktarmen, vilket är en utmaning. I det här arbetet har en stärkelsebaserad formulering för inkapsling av bakterier undersökts.**

Människans mag- och tarmkanal är hem till omkring $10^{13}$ bakterier, vilket är lika många som antalet celler i vår kropp. Majoriteten av bakterierna befinner sig i tjocktarmen, eftersom miljön i magen och tunntarmen gör det svårt för bakterier att överleva. Dessa bakterier är en del av vår tarmflora som har flera viktiga funktioner. Till exempel har den en stor inverkan på immunförsvaret, bryter ner fibrer i mat, och producerar vitaminer och andra viktiga ämnen, för att nämna några få. Tarmfloran spelar alltså stor roll när det kommer till vårt hälsotillstånd, vilket gör att det är av intresse att kunna påverka den med t.ex. probiotika.

Probiotika är levande bakterier som vid tillräckliga mängder ger en positiv hälsoeffekt. Utmaningen med probiotika är hur man får bakterierna levande till tjocktarmen, med tanke på miljön i magen och tunntarmen som tidigare nämndes. I ett försök att lösa detta problem kapslades bakterier av typstammen *Lactobacillus reuteri DSM 20016* in i kristallina stärkelsemikrosfärer. ”Vanlig” stärkelse som finns i vår mat bryts ner munhålan och tunntarmen, medan kristallin stärkelse bryts ner först i tjocktarmen.

Försöken att kapsla in bakterier var lyckade, även om det visade sig att de flesta bakterier som kapslades in var döda redan från början vilket gjorde det svårt att säga hur mikrosfärernas tillverkningsprocess påverkade dem. Anledningen till att så många bakterier var döda var att de frystorkades. Det är en process som ofta är väldigt påfrestande för bakterier, men som är en bra metod för att de ska överleva länge i produkter, om bakterierna frystorkas i rätt vätska och rätt inställningar på själva frystorken används. Mycket arbete kring att optimera tillverkningsmetoden är nödvändigt om formuleringen ska kunna användas till en kommersiell produkt. De tillverkade stärkelsemikrosfärerna analyserades genom att utsätta dem för en simulering av miljön i tunntarmen. Mikrosfärerna bröts ner fortare än önskat, men vi tror att detta kan förbättras om tillverkningsprocessen och även simuleringen av tunntarmen optimeras.

Tillverkningsmetoden som användes är baserad på forskning bedriven på Avdelningen för Livsmedelsteknologi, LTH, och bygger på en emulsion med upplöst polyetylenglykol (PEG) i yttre fasen, och upplöst stärkelse i inre fasen.




*Figur 1. Schematisk bild av emulsion med PEG i yttre fasen och stärkelse i inre fasen.*

Bakterier tillsattes till den upplösta stärkelsen innan den blandades med PEG. Stärkelsedropparna fick sedan stelna till mikrosfärer. Därefter tvättades PEGen bort för att extrahera mikrosfärerna. Flera olika formuleringar testades genom att variera PEG-koncentrationen, PEG-sorten, stärkelsesorten, samt tillsatser av andra bionedbrytbara polymerer till mikrosfärerna. En intressant upptäckt som gjordes var att stärkelsen stelnade fortare vid högre PEG koncentrationer jämfört med lägre.

## Acknowledgements

I have many people I would like to thank for their help during my thesis work. First and foremost, my supervisor Dr. Ali Marefati, who has very patiently helped me with my work in the lab, tried his very best to answer all of my questions, made many valuable comments on my report during the writing process. Thank you for the encouragement when the experiments have not gone as planned. I would like to thank Prof. Marie Wahlgren for giving me the opportunity to do this very interesting project and answering any questions I have had about my project. I thank associate professor Åsa Håkansson for helping me with acquiring the bacteria, and PhD student Elin Oskarsson, for helping me cultivate them and also answering any questions that I had regarding the bacteria. I would like to thank senior lecturer Magnus Carlqvist at the Department of Applied Microbiology, Lund University for helping me with running the flow cytometer. I thank everyone else at the Department of Food Technology and Nutrition, Lund University, for all the help, encouragement, and laughs during breaks. Last, but certainly not least, I would like to give a big thank you to all of my friends and family who have supported, encouraged, and been proud of me during the last 5 years. I am so lucky to be surrounded by such a lovely bunch of people, I love you all!

## Abbreviations

CFU – Colony forming unit

CMC – Carboxymethyl cellulose

DNS – 3,5-dinitrosalicylic acid

GOS – Galacto-oligosaccharides

GRAS – Generally regarded as safe

GI tract – Gastrointestinal tract

FDA – Food and Drug Administration (USA)

OD – Optical density

OSA – Octenylsuccinic anhydride

PBS – Phosphate buffered saline

PEG – Polyethylene glycol

SIF – Simulated intestinal fluid

WHO – World health organization

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## Aims

The main aim of this project was to adapt a starch microsphere formulation intended for prolonged release of proteins to a formulation for colon delivery of probiotics. In addition to this, the project had the following more detailed aims:

- To investigate different PEGs and starches and whether they can form microspheres using a water-in-water emulsion technique.
- To investigate a formulation based on starch microspheres with different polymers.
- To encapsulate microorganisms into starch microspheres.
- To compare the survival rate of freeze-dried bacteria in GOS or lactose.
- To see the effect of simulated *in vitro* digestion on the release of bacteria as well as reducing sugars from microspheres with flow cytometry and DNS assay.

# 1. Background

## 1.1. The gut microbiota

The gut microbiota consists of bacteria, fungi, archaea, protozoa, and viruses (Azad et al. 2018). The bacteria are abundant; a total number of microbes in one human's microbiota often mentioned is $10^{13}$-$10^{14}$ bacteria (Gill et al. 2006, Guarner and Malagelada 2003, Derrien and van Hylckama Vlieg 2015, Rosenbaum, Knight, and Leibel 2015), but a recent study suggests that this estimated number might be 10-100 times too high (Sender, Fuchs, and Milo 2016). Still, it is a huge number; about the same as the number of cells in the human body (Sender, Fuchs, and Milo 2016). The bacteria of the gut microbiota reside mainly in the colon, with $10^{7}$-$10^{12}$ colony forming units (CFU)/mL (Lin et al. 2014). The low pH of the stomach and the fast flow of content through the stomach and small intestine prevents bacteria from colonizing and staying alive for long in those parts of the GI tract (Sender, Fuchs, and Milo 2016, Huckle and Zhang 2011). The microbiota has multiple different functions such as maintenance of the epithelial barrier, inhibition of pathogen adhesion to intestinal surfaces, modulation and proper maturation of the immune system, degradation of otherwise non-digestible carbon sources such as plant polysaccharides, and production of different metabolites such as vitamins and short-chain fatty acids (Sánchez et al. 2017). Thus, the gut microbiota plays a major role in human health and prevention of diseases, which is why there is a great interest in promotion of the properties of gut microbiota.

## 1.2. Probiotics

Probiotics are defined as "Live organisms which when administered in adequate amounts confer a health benefit on the host" by FAO and WHO (Food and Agriculture Organization of the United Nations. and World Health Organization. 2006). There are many different products containing probiotics on the market, such as ProbiMage, Lactiplus, BioGaia ProTectis, Bifiform, etc.

Probiotics have shown to be effective in preventing and treating acute diarrhea (Guarner and Malagelada 2003). In addition, probiotics have also been shown to prevent colon cancer in some animal species, but is not confirmed yet for humans (Guarner and Malagelada 2003). It is important to note though that just because one strain has a health benefit; it might not be the same for another strain, even if the probiotic is of the same species (Sanders et al. 2018).

For probiotics to have any effects, a sufficient number of viable bacteria must reach the colon. There is no consensus on what the minimum therapeutic dose is, but a review article mentions numbers between $10^{6}$-$10^{12}$ CFU (Huckle and Zhang 2011). Delivering viable bacteria to the colon is challenging as the gastrointestinal (GI) tract is a very harsh place for probiotics. Most bacteria cannot survive the plethora of enzymes along the GI tract, the high acidity in the stomach, and the bile salts in the intestinal fluids. Thus, there is a need to protect the bacteria in some manner. One way of doing it is enteric coating of tablets or capsules (Huckle and Zhang 2011).

### 1.2.1. Lactobacillus reuteri

*Lactobacillus reuteri (L. reuteri)* is a lactic acid bacteria which can be found in a number of different sites in the human body, including the GI tract, urinary tract, skin, and breast milk (Mu, Tavella, and Luo 2018). There are several *L. reuteri* strains which can form biofilms. Biofilms can aid in preventing colonization by other bacteria, and also benefit colonization of its own species. Thus, biofilms of probiotic bacteria could aid in preventing pathogenic bacteria from colonizing (Salas-Jara et al. 2016).

## 1.3. Starch

Starch is a carbohydrate which consists of amylose and amylopectin, see *Fig. 1*. Amylose is a linear molecule made up of glucose residues bond together by α-D-(1,4)-linkages. Amylopectin is made up of glucose residues as well, but has both α-D-(1,4)- and α-D-(1,6)-linkages, which results in a large, branched molecule. Starches high in amylopectin are called waxy starches (Šárka and Dvořáček 2017).

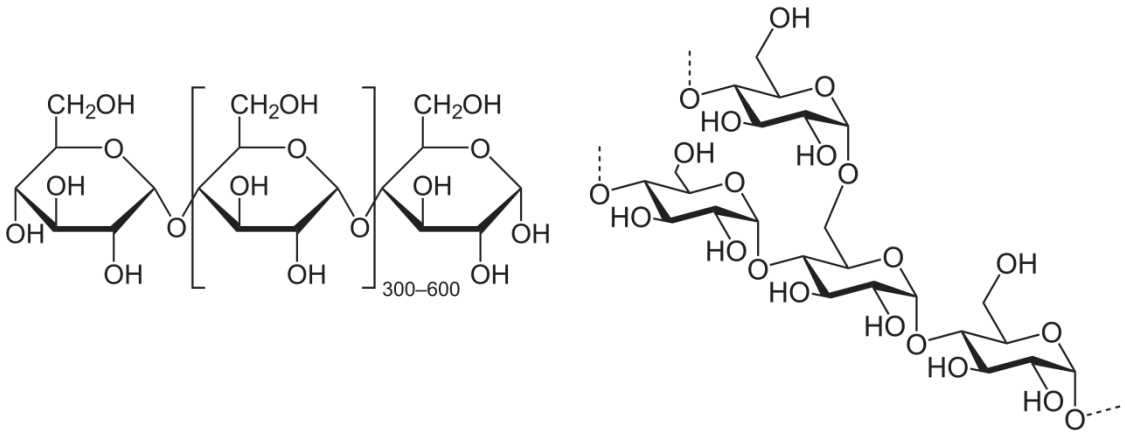



***Fig. 1.*** *Molecular structure of amylose (right) and amylopectin (left). From user NEUROtiker, Wikimedia Commons.*

Starch is digested by enzymes called amylases present in the saliva and pancreatic juices. This is not the case for so-called *resistant starch*, which is not broken down until it reaches the colon where it can be fermented by the microbiota residing there (Sharma, Yadav, and Ritika 2008). The resistance towards enzymatic degradation can be due to many different reasons, for example larger particle size and crystallinity results in lower rate of degradation by enzymes. As the digestion rate of amylopectin and amylose differ, different starches will be broken down differently depending on the amylopectin/amylose ratio (Sharma, Yadav, and Ritika 2008). α-amylase is an enzyme which hydrolyzes the α-(1,4)-glycosidic linkages, but not α-(1,6)-glycosidic linkages in amylose and amylopectin (Priebe et al. 2018).

Starch can be modified in different ways; in this project three different starches which has all been acid hydrolyzed has been used. During acid hydrolysis of starch, the amorphous regions are removed and the crystalline parts are converted to starch crystals of different sizes (Song et al. 2008).

## 1.4. Starch microspheres

Starch microspheres intended for injection have successfully been produced previously using an aqueous two-phase system (Elfstrand, Eliasson, and Wahlgren 2009b, Elfstrand et al. 2004, Elfstrand et al. 2006, Elfstrand, Eliasson, Jönsson, Reslow, et al. 2007, Elfstrand, Eliasson, Jönsson, Larsson, et al. 2007, Elfstrand, Eliasson, and Wahlgren 2009a).

Aqueous two-phase systems can be formed by mixing two water-soluble polymers dissolved in water if the concentration of the polymers is high enough. The interface between the two phases has a very low interfacial tension compared to water-oil systems (Albertsson 1961). Aqueous two-phase systems are used in biotechnology for separation and purification applications. The main cause of the phase separation is the repulsive molecular interactions between the polymers (Gustafsson, Wennerström, and Tjerneld 1986). For this project, the two polymers were starch and polyethylene glycol (PEG), see *Fig. 2*.

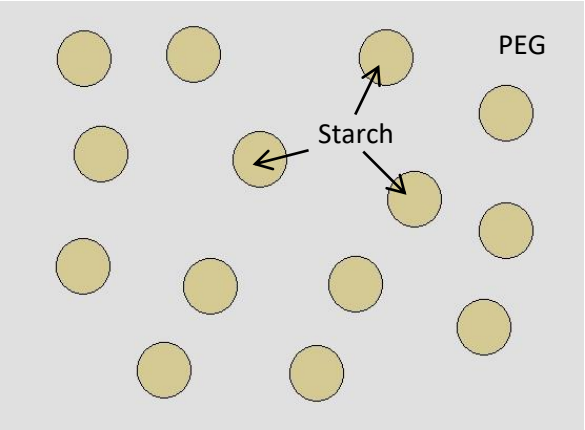



***Fig. 2.*** *Schematic representation of water-in-water emulsion. The external phase (grey) consists of dissolved PEG and the internal phase (beige) dissolved starch.*

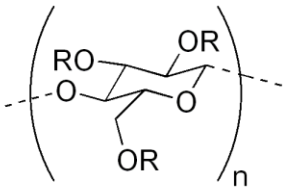
The method used in (Elfstrand et al. 2006) is as follows: an aqueous starch solution is added to an aqueous PEG solution which gives rise to an emulsion. Even though both phases are water-based solutions they do not mix due to the polymers (starch and PEG) not mixing. By incubating the emulsion at 6 °C, nucleation sites for crystallization of the starch can form. The emulsion is then incubated at 37 °C to promote crystal growth, until the starch has fully solidified into microspheres, which will be extracted by centrifugation followed by drying in a freeze dryer. The production process for the microspheres in this project is based on the above mentioned articles. The first incubation (at 6 °C) affects the amount of ordered structure, and the second incubation (at 37 °C) increases the thermal stability of the microspheres (Elfstrand, Eliasson, Jönsson, Reslow, et al. 2007). High quality microspheres are solid, round-shaped, and well-defined sharp contours (Elfstrand et al. 2006).

## 1.5. Other polymers

In this project, in order to improve the microspheres' resistance to enzymatic degradation before reaching the colon, carboxymethyl cellulose, dextran, or pullulan was added to the starch. They are all carbohydrates that are not thought to be digested by α-Amylase.

### 1.5.1. Carboxymethyl cellulose

Carboxymethyl cellulose (CMC) is a polymer used in a wide range of industries, including the food and pharmaceutical industry. It is cheap and easily available. A number of carboxymethyl groups have been added to the cellulose molecule in order to increase the water solubility (Biswal and Singh 2004). The Food and Drug Administration (FDA) has given CMC generally regarded as safe (GRAS) status (FDA 2018a). The molecular structure of CMC can be seen in *Fig. 3*.




R = H or $CH_2CO_2H$

***Fig. 3.*** *Molecular structure of carboxymethyl cellulose. From user Edgar181, Wikimedia Commons.*

### 1.5.2. Dextran

Dextrans are polysaccharides which can be produced by many different bacterial species. The structure consists of glucose units bond by α(1,6)- and α(1,3)-linkages, as can be seen in *Fig. 4*. The exact structure differs depending on the species producing it. Some applications for dextran is for restoring blood volume and in formulations for eye and skin care (Naessens et al. 2005). The FDA has given dextrans with an average $M_w$ < 100 000, GRAS status (FDA 2018a).

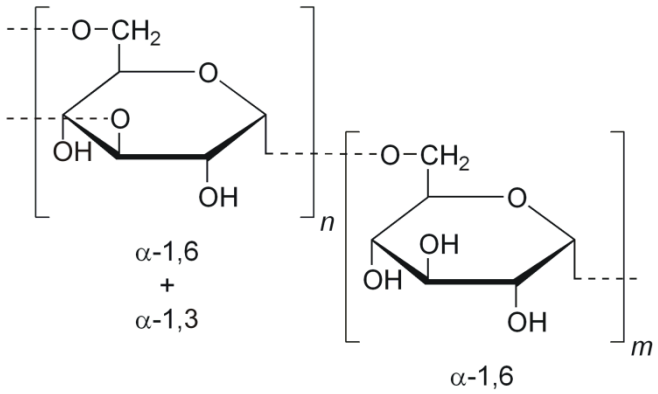



***Fig. 4.*** *Molecular structure of dextran. From user Zeldaoot23, Wikimedia Commons.*

### 1.5.3 Pullulan

Pullulan is a linear polysaccharide produced from starch by fermentation. The structure can be seen in *Fig. 5.* below and consists of maltotriose units with α(1,6)- and α(1,4)-linkages in regular alternation. It is water-soluble and even at relatively low concentrations it gives high-viscosity solutions. It is used in many industries, including food, pharmaceuticals, and cosmetics. Pullulan is used for tablet coatings, as a film-forming agent, as a texturizer, as a binder for flavors, etc. It is resistant to mammalian amylases (Park and Khan 2009). The FDA has given pullulan GRAS status (FDA 2018b).

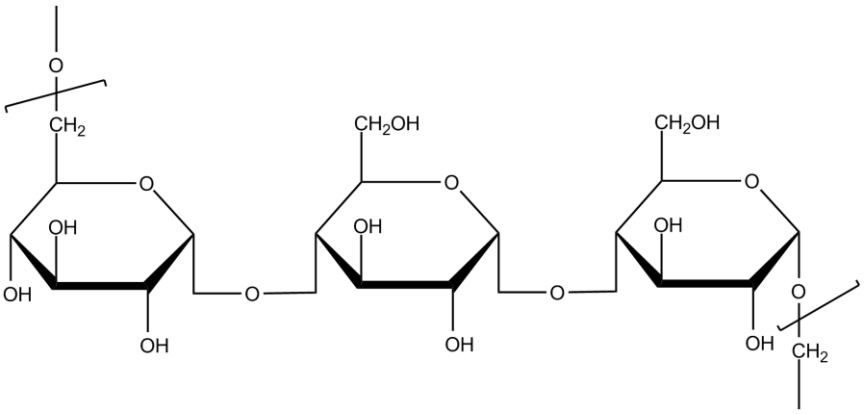



***Fig. 5.*** *Molecular structure of pullulan. From user Klever, Wikimedia Commons.*

## 1.6. Freeze drying

Dehydration is widely used within the pharmaceutical industry to increase the shelf life of products. The longer shelf life and possibility to store products such as probiotics at room temperature is convenient for everyone in the supply chain, from manufacturers to consumers (Huckle and Zhang 2011). The reason for dehydration increasing the shelf life is a decreased water activity, $a_w$, (Adams, Cook, and Ward 2015), because deteriorative reactions such as lipid oxidation are limited at low $a_w$ (Broeckx et al. 2017).

There are several techniques which can be used for dehydration of material. One of them is freeze drying, or lyophilization as it is also known.

A freeze dryer consists of:

- A chamber, which is where the material is placed during the freeze drying process
- Cooling system
- A process condenser, which traps water during the drying process
- A vacuum pump, to decrease the pressure

Before beginning the freeze drying process, the material has to be completely frozen. The freeze drying process can be divided into two parts; primary drying during which the frozen water sublimates and secondary drying during which any moisture adsorbed to the material is removed by desorption. For the sublimation to be effective it is important to establish a pressure gradient from the sample to the condenser and vacuum pump, to make sure the water migrates from the sample. For the condenser to trap any sublimated water, its temperature has to be lower than the temperature of the material which is being freeze dried. The freeze dried material needs to be stored in a dry environment (like a desiccator) or it will quickly absorb moisture from the air (Adams, Cook, and Ward 2015).

For the freeze drying to work properly it is important that the samples are completely frozen when placed in the freeze dryer. If there is liquid water, it will start to boil as the pressure drops in the freeze dryer. Boiling will damage the structure of the sample.

Freeze drying is used for dehydrating sensitive materials such as enzymes, hormones, bacteria, vaccines, etc. (Adams, Cook, and Ward 2015). The disadvantage is the loss of viability of the microorganisms due to reasons such as temperature changes and damage of cell components due to formation of ice crystals (Huckle and Zhang 2011).

## 1.7. *In vitro* digestion

The digestion of food and pharmaceuticals by humans is a highly complex process. To simulate this process, *in vitro* methods of varying complexity are used in the pharmaceutical and food industry. The methods can include the whole GI tract (oral, gastric, small intestinal, colon digestion) or parts of it, depending on what the aim of the study is. To mimic the *in vivo* environment many variables has to be taken into account, some examples are digestive enzymes, pH, digestion time, and ion concentrations (Minekus et al. 2014).

## 1.8. Flow cytometry

Flow cytometry is a technique which has a multitude of uses within microbiology, such as cell counting, cell sorting, determining cell characteristics and function, detecting microorganisms, etc. A liquid sample containing cells or particles is analyzed by the instrument by running the sample through a channel, ideally one cell at a time. A laser is pointed said channel and the light scattering caused by passing cells is detected. Two different types of scattering are detected; forward scatter (FSC) and side scatter (SSC) which relates to the cell size and granularity of the cell respectively (Macey 2007). A schematic image of a flow cytometer can be seen in *Fig. 6.*

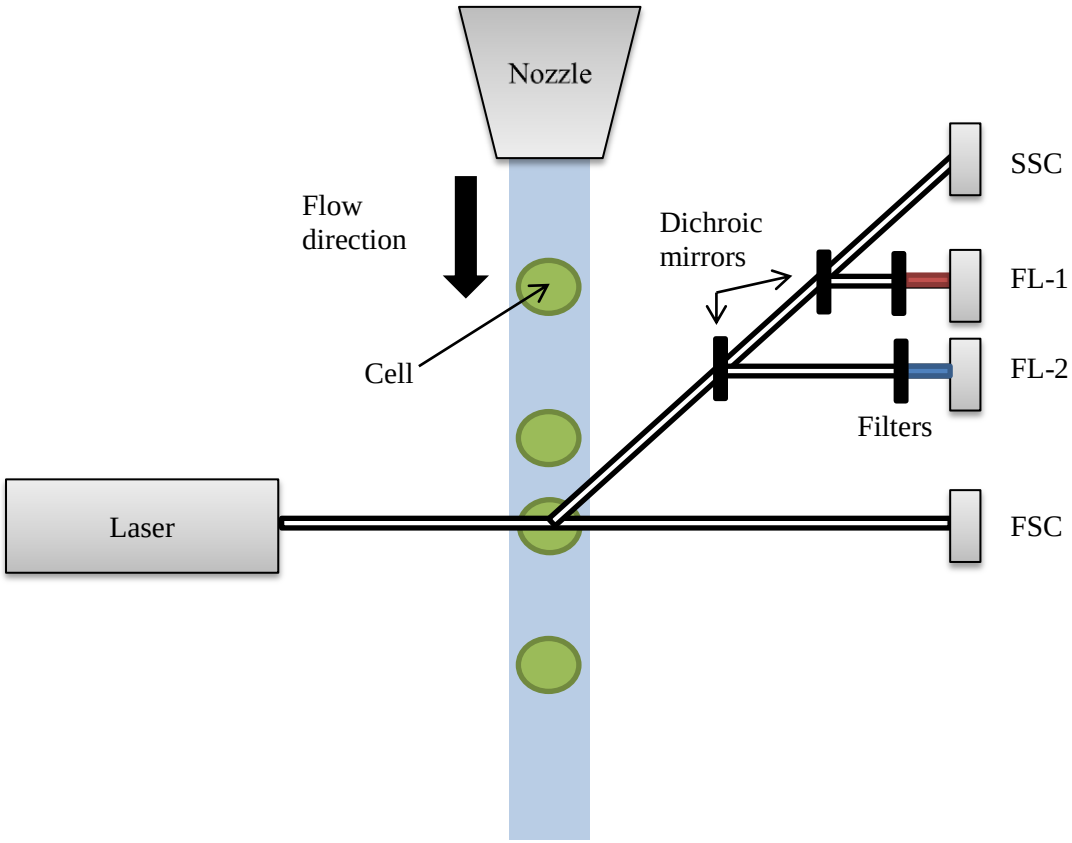



***Fig. 6.*** *A schematic image of a flow cytometer. The sample containing cells flows through the channel at which a laser is pointed. Several light detectors are used; FSC and SSC detects forward scattered and side scattered light respectively. If the cells have been stained with a fluorescent dye, those can be detected by FL-1 and FL-2 which detects light of a certain wavelength.*

Before injecting the sample into the flow cytometer, it can be treated with dyes which stain certain intracellular cell components, or fluorescent molecules conjugated to antibodies specific for certain molecules present on or in the cell. This enables the possibility to tell different cells apart, and even cell sorting (FACS – Fluorescence-activated cell sorting) if one so desires. Fluorescent molecules emit light at certain wave lengths which can be detected, giving information about what kind of cell it is (Macey 2007). For this project, the samples were stained with SYBR Green and propidium iodide (PI). SYBR Green stains all cells by binding to the DNA. PI binds to the DNA as well, but as it cannot pass the cell membrane of living cells, it only stains dead cells (as dead cells' cell membrane is damaged PI is able to access the DNA) (Gregori et al. 2001).

## 1.9. DNS assay

The 3,5-dinitrosalicylic acid (DNS) assay is used to quantitatively determine the amount of reducing sugars in a sample. Maltose, as well as other reducing sugars, reduces DNS to 3-amino-5-nitrosalicylic acid. The amount of reduced DNS can be detected as 3-amino-5-nitrosalicylic acid which absorbs light with a wavelength of 540 nm (Miller 1959).

# 2. Methods and materials

## 2.1. Materials

To produce the microspheres, three different starches were used; waxy barley starch (gift from the Department of Food Technology, Lund University) which had been acid hydrolyzed for 24 h, Lyckeby PU 92-000 (Culinar, Sweden) which is an acid hydrolyzed waxy maize starch dried with a roller compactor, and Lyckeby Keep 92001 (Culinar, Sweden) which is an acid hydrolyzed octenyl succinic anhydride(OSA)-modified waxy maize starch dried with a roller compactor. For the external phase of the W/W emulsion, PEG 20 000 (ThermoFisher GmbH, Kandel, Germany) and PEG 2 000 000 (Serva Feinbiochemica, Heidelberg, Germany) were used, where the difference is the average molecular weight of the polymer chains which are 20 000 and 2 000 000 respectively. Other polymers added to the starch microspheres were carboxymethyl cellulose (CMC) sodium salt (200-800 cP) (ICN Biomedicals, Inc., Ohio, USA), pullulan (Sigma-Aldrich, St. Louis, USA), and dextran ($M_w$ 9 000-11 000) (Sigma-Aldrich, St. Louis, USA). During the production process two different buffers were used; carbonate buffer (50 mM, pH 8.00) and phosphate buffer (5 mM, pH 5.00).

The microorganisms used were freeze-dried bacteria of the type strain *L. reuteri DSM 20016* (BioGaia AB, Sweden). The bacteria were cultivated and subsequently freeze dried using MRS broth (Merck KGaA, Darmstadt, Germany), 0.85 % sterile NaCl solution, lactose (Merck KGaA, Darmstadt, Germany), and galacto-oligosaccharides (GOS) of the brand *Cup Oligo P* (gift from Department of Applied Microbiology, Lund University), which consists of a mix of oligosaccharides including 4'-galactosyllactose. For the colony counting, Petri dishes with MRS agar (Merck KGaA, Darmstadt, Germany) were used.

For the *in vitro* digestion, simulated intestinal fluid (SIF) (0.05 M $KH_2PO_4$ (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), pH 6.8) was made based on the United States Pharmacopeia. Bile salts (80 mM sodium cholate (Merck KGaA, Darmstadt, Germany) and sodium deoxycholate (Sigma-Aldrich, St. Louis, USA) (Ifeduba and Akoh 2015)) were added to the SIF. For the release test the enzyme α-Amylase from porcine pancreas (Sigma-Aldrich, St. Louis, USA) was added to the SIF to get an activity of 200 U/mL (Minekus et al. 2014).

For the flow cytometry, SYBR Green (gift from the Department of Applied Microbiology, Lund University) and PI (gift from the Department of Applied Microbiology, Lund University) were used to stain the cells.

For the DNS assay, the DNS reagent was made with 1 % (w/v) DNS (Sigma-Aldrich, St. Louis, USA), 30 % (w/v) sodium potassium tartrate (VWR International, Leuven, Belgium), and 0.4 M NaOH. A standard calibration curve was made by dissolving maltose (Merck, KGaA, Darmstadt, Germany) in SIF.

## 2.2. Methods

### 2.2.1. Producing microspheres

The method for producing the starch microspheres is based on the work of Lidia Elfstrand (Elfstrand, Eliasson, and Wahlgren 2009b, Elfstrand et al. 2004, Elfstrand et al. 2006, Elfstrand, Eliasson, Jönsson, Reslow, et al. 2007, Elfstrand, Eliasson, Jönsson, Larsson, et al. 2007, Elfstrand, Eliasson, and Wahlgren 2009a). It is based on a water-in-water emulsion (W/W). The continuous phase consists of dissolved PEG (polyethylene glycol) and the dispersed phase of dissolved starch. The articles

mentioned above used a solution of 38 wt% PEG 20 000 dissolved in carbonate buffer (50 mM, pH 8.00).

### 2.2.2. The effect of different PEGs and PEG concentrations

#### *2.2.2.1. PEG 2 000 000*

Five different PEG 2 000 000 concentrations were used; 38 wt%, 15 wt%, 13 wt%, 10 wt% and 6.5 wt%, to see the effect of concentration of PEG on microsphere formation. The same amount of starch as in previous work was used, and the samples were left in the cold room for 42 h and in room temperature for 64 h. The crystallization process of the acid hydrolyzed waxy barley starch was compared between the different samples.

The PEG 2 000 000 solution was diluted to 3.3 wt%, 1.6 wt%, 0.8 wt%, and 0.4 wt% with the intent of finding the lowest possible concentration which would still give rise to a two-phase system together with acid hydrolyzed barley starch.

#### *2.2.2.2 PEG 20 000*

Three PEG 20 000 concentrations together with acid hydrolyzed waxy barley starch was used to see if it would be possible to produce microspheres with those; 38 wt%, 13 wt% and 6.5 wt%.

### 2.2.2. The effect of different starches

Three different starches were used to see if there would be a difference in the microspheres produced. Acid hydrolyzed waxy barley starch was used for production of microspheres together with 38 wt%, 13 wt%, and 6.5 wt% PEG 20 000, and 38 wt%, 15 wt%, 13 wt%, 10 wt% and 6.5 wt% PEG 2 000 000. Acid hydrolyzed waxy maize starch and acid hydrolyzed OSA-modified waxy maize starch were used together with 13 wt% PEG 2 000 000.

### 2.2.3. Addition of other polymers to the starch

Three different polymers were added to the acid hydrolyzed waxy barley starch to see how it would affect the formation of microspheres. The intention was to see if said polymers could affect the digestion rate of the microspheres. The polymers tested were carboxymethyl cellulose (CMC) sodium salt, pullulan, and dextran. All were added to the starch before dissolving it. Pullulan and dextran were added with a ratio of 9:1 starch:pullulan/dextran. CMC was added with a ratio of 96:4 starch:CMC as a higher amount would not be soluble in the carbonate buffer. The external phase used was 38 wt% PEG 20 000.

### 2.2.4. Cultivating bacteria

The freeze dried bacteria (*L. reuteri*) donated by BioGaia AB were cultivated in MRS-broth for 3 days at 37 °C. To harvest the bacteria, they were centrifuged at 6000 rpm for 5 min, and washed with sterile 0.85 % NaCl solution.

The centrifuged bacteria were resuspended in autoclaved MilliQ-water and the optical density (OD) was measured using a spectrophotometer at 620 nm. The OD is a measurement of the amount of transmitted light through a sample. A dispersion of a high concentration of bacteria will give a higher OD than a dispersion of a lower concentration as the bacteria will scatter the light. Using the OD, the suspension was diluted to a concentration of about $10^8$ bacteria/ml. Half of the bacteria were freeze dried in lactose and the other half in GOS (galacto-oligosaccharides), with the purpose of acting as cryoprotectants. GOS and lactose were added to get a solution mix with 15 wt% GOS or lactose. The solutions were freeze dried for 3 days using a laboratory freeze dryer (CD 12, Hetosicc, Denmark) with 20 °C in the drying chamber, -50 °C in the cooling unit, and a vacuum of $10^{-2}$ mbar. Once dry, the material was crushed and stored in Duran bottles with the lid wrapped in Parafilm in 6 °C.

The solution containing bacteria and MilliQ-water was also cultivated on MRS agar for 3 days at 37 °C to get a more accurate value than by using OD measurement for the actual concentration.

After freeze drying, the freeze-dried bacteria/cryoprotectant was dissolved in sterile 0.85 % NaCl solution and cultivated on MRS agar plates to see how many bacteria were still alive after the freeze drying.

### 2.2.5. Producing microspheres containing microorganisms and placebos

Microspheres containing microorganisms were produced in a few different variations; see *Table 1*. All samples were made in duplicates. The starch used was Lyckeby PU 92-000, i.e. acid hydrolyzed waxy maize starch.

***Table 1.*** *The following mixtures were added to 13 wt% PEG 2 000 000 solution with the ratio 1:7.4 starch/bacteria mixture:PEG solution.*

| Sample | Carbonate buffer (wt%) | Starch (wt%) | Pullulan (wt%) | Bacteria (wt%) |
|---|---|---|---|---|
| Starch/lactose/bacteria | 52 | 22 | 0 | 26 *freeze dried in lactose |
| Starch/GOS/bacteria | 52 | 22 | 0 | 26 *freeze dried in GOS |
| Starch/pullulan/lactose/bacteria | 52 | 20 | 2 | 26 *freeze dried in lactose |
| Starch/pullulan/GOS/bacteria | 52 | 20 | 2 | 26 *freeze dried in GOS |

The starch, together with GOS and/or pullulan, was dissolved in carbonate buffer by heating it for 4x6 s in a microwave oven. After letting the solution to cool down to 28 °C the freeze dried bacteria was added to the starch mixture (see *Table 1*. for ratios). For the samples that were supposed to contain GOS, bacteria freeze dried in GOS was used, and no extra GOS was added. Bacteria freeze dried in lactose was used for the samples not supposed to contain GOS. The starch/bacteria mixture was added to 13 wt% PEG 2 000 000 solution at a ratio of 1:7.4 starch/bacteria mixture:PEG solution and kept at 28 °C while stirring for 15 min. The samples were then moved to a cold room (6 °C) and kept there for 25 h while stirring. Lastly, the samples were kept at 37 °C for 28 h while stirring. The samples were centrifuged (Allegra X-15R, Beckman Coulter, USA) at 4750 rpm for 30 minutes at room temperature, followed by centrifugation at 3000 rpm for 3x15 min. Between each centrifugation, the samples were washed with phosphate buffer (5 mM, pH 5.00).

The sediment was transferred to stainless steel containers, weighed and covered with aluminum foil. The samples were then stored in a freezer until all samples had been produced, to be able to freeze dry them all at the same time and under the same conditions. Just before freeze drying, the aluminum foil covering the samples was punctured. The samples were freeze dried for 3 days using a laboratory freeze dryer (CD 12, Hetosicc, Denmark) with 20 °C in the drying chamber, -50 °C in the cooling unit, and a vacuum of $10^{-2}$ mbar. After freeze drying, the samples were weighed and stored at room temperature in a desiccator until analysis could be performed. The whole production process is described in the flow chart in *Fig. 7*.

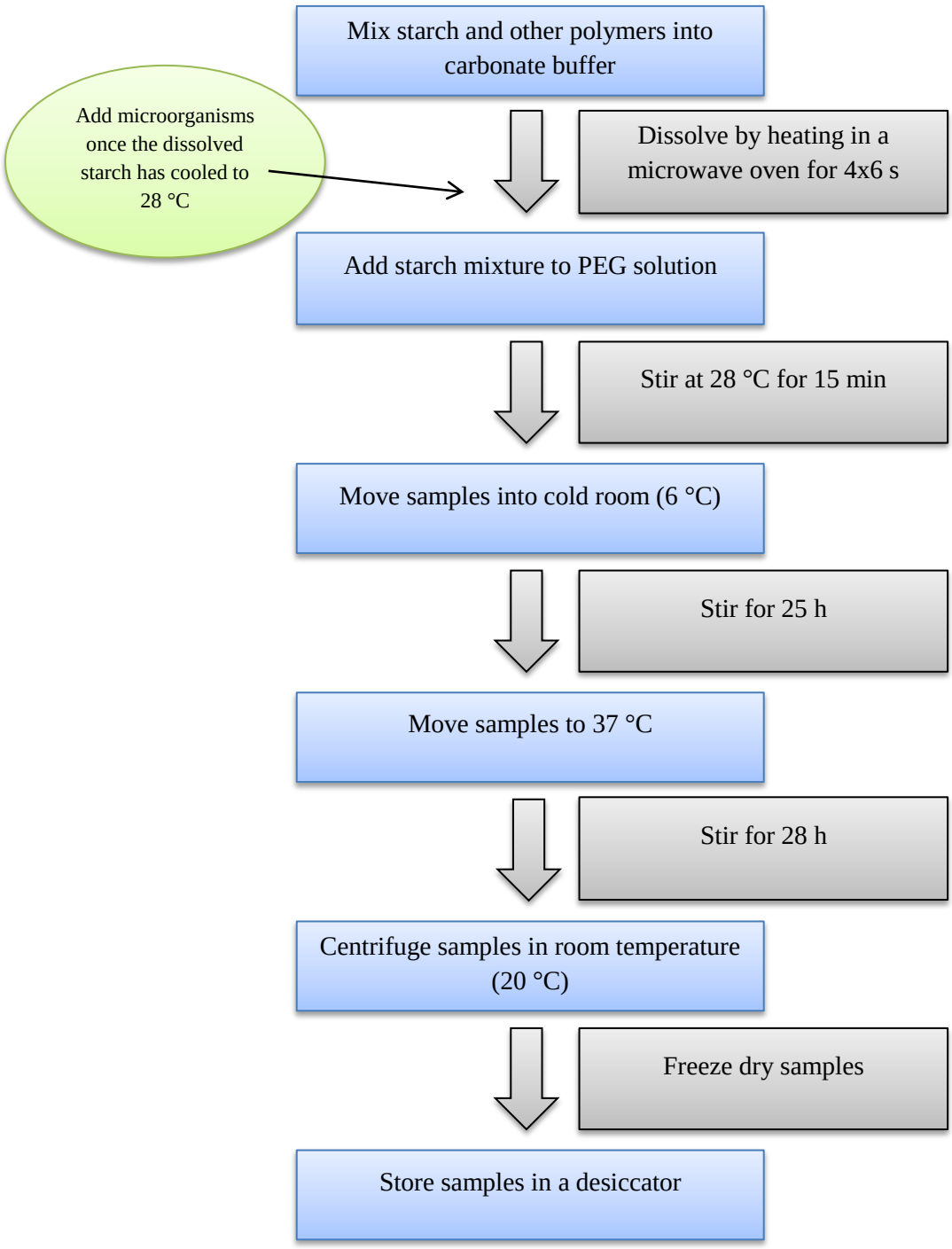



***Fig. 7.*** *Flow chart for microsphere production process based on (Elfstrand et al. 2006).*

### *2.2.5.1. Producing placebo microspheres for the ones containing bacteria*

The production process for the placebo microspheres followed the same process as described above for the microspheres containing bacteria, with one modification to not increase the amount of starch in the samples as there was no bacteria added; the starch mixture (see *Table 2.*) was added to the PEG solution with the ratio 1:10 starch mixture:PEG solution instead.

***Table 2.*** *The following mixtures were added to 13 wt% PEG 2 000 000 solution with the ratio 1:10 starch mixture:PEG solution.*

| Sample | Carbonate buffer (wt%) | Starch (wt%) | Pullulan (wt%) | GOS (wt%) |
|---|---|---|---|---|
| Starch | 70 | 30 | 0 | 0 |
| Starch/GOS | 67 | 29 | 0 | 4 |
| Starch/pullulan | 70 | 27 | 3 | 0 |
| Starch/pullulan/GOS | 67 | 26 | 3 | 4 |

### 2.2.6. Characterization

#### *2.2.6.1. Water activity measurements*

Water activity measurements were done on solutions of PEG 2 000 000 at concentrations of 6.5 wt%, 10 wt%, 13 wt% and 15 wt% using an AquaLab (Aqualab Series 3TE, Decagon Devices Inc, USA). The purpose was to investigate whether the different concentrations had different water activities, which in that case would be a possible reason for the varying crystallization times for the starch. Before doing any measurements, the machine was calibrated with deionized water and a solution with water activity 0.760.

#### *2.2.6.2. Release test of microspheres containing microorganisms and placebos*

The samples containing microorganisms which had successfully formed microspheres and the corresponding placebos were used in the release test. The test was performed by simulating the small intestine. 150 mg of each sample was added to 9.90 mL of SIF at 37 °C while stirring. α-Amylase from porcine pancreas was added and a timer was started. Samples were taken at time intervals (see *Table 3*.) to be analyzed with flow cytometry, a DNS assay, and light microscopy. According to (Minekus et al. 2014), the small intestinal phase is usually only simulated for 2 h, but as the aim of the analysis was to find out how many bacteria were encapsulated, the test was conducted for a longer amount of time. When all of the starch has been digested, all bacteria have been released.

***Table 3.*** *The table describes when samples were taken for the different analyses methods. The last sample was taken after 6 h the first round of experiments, but was changed to 24 h due to practical reasons.*

| Time point | DNS assay (50 µL) | Flow cytometry (1 mL) | Light microscopy (50 µL) |
|---|---|---|---|
| 1 min | Yes | | |
| 5 min | Yes | | |
| 10 min | Yes | Yes | Yes |
| 30 min | Yes | Yes | Yes |
| 1 h | Yes | Yes | Yes |
| 2 h | Yes | Yes | Yes |
| 3 h | Yes | Yes | Yes |
| 4 h | Yes | Yes | Yes |
| 6 h/24 h | Yes | Yes | Yes |

#### *2.2.6.3. Flow cytometry of microspheres and freeze dried bacteria*

Flow cytometry was performed on the samples taken during the release test with the purpose of finding out at what rate the bacteria were released from the microspheres, and whether they were dead or alive.

Samples of 1000 µL were taken after 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, and 6 h or 24 h and stored on ice. The OD was measured using a spectrophotometer (Cary 50 Bio UV-Vis, Varian, USA) at 620 nm. When needed the samples were then diluted with SIF to get an OD of 0.1-0.2. Before running the samples through the flow cytometer (BD Accuri C6 Plus, BD Life Sciences, USA) they had to be filtered with a 40 µm filter as particles larger than that would clog the instrument. 500 µL of each sample at the right OD was stained with 6 µL of a mixture of SYBR Green and PI and then incubated at room temperature for 10 min before it was run through the flow cytometer.

The freeze dried bacteria were also analyzed in the flow cytometer. The freeze dried material was dissolved in PBS (phosphate buffered saline) as well as SIF. PBS is the medium normally used to suspend cells. SIF was used to investigate how the bacteria were affected by it as it was the medium used for the release test. The freeze dried material was added to the respective liquids and then analyzed in the same way as described above for the digested microspheres.

#### *2.2.6.4. DNS assay*

A DNS assay was performed on the samples taken during the release test with the purpose of finding out the rate at which the starch in the microspheres was digested.

Samples of 50 µL were taken at time intervals which can be seen in *Table 3* above, and put in Eppendorf tubes containing 200 µL Milli Q water and 300 µL DNS reagent. The Eppendorf tubes were put in the freezer until they could be analyzed.

The Eppendorf tubes containing the frozen samples were put in a boiling water bath for 7 min, cooled on ice for 2 min, and centrifuged at 13400 rpm for 2 min. 20 µL of each sample plus 180 µL of SIF was added to the same plate (for the absorbance to be within the range the instrument can accurately read). The absorbance was measured at 540 nm with an absorbance plate reader (SPECTROstar Nano, BMG Labtech, Germany).

#### *2.2.6.5. Light microscopy*

Light microscopy (BX50, Olympus, Japan) was performed to visually get an idea of the breakdown of the microspheres in SIF and α-amylase over time. The samples of 50 µL were taken during the release test at intervals which can be seen in *Table 3* and put in Eppendorf tubes on ice until they could be analyzed.

Additionally, samples of the freeze dried microspheres were dispersed in PBS and analyzed with the light microscope as well.

# 3. Results and discussion

## 3.1 The effect of different PEGs and PEG concentrations

### 3.1.1. PEG 2 000 000

The solution with 38 wt% PEG 2 000 000 was too viscous to work with, and was not used for further experiments. The solution was diluted to 15wt%, 13 wt%, 10 wt% and 6.5 wt%. Waxy barley starch was added according to the protocol. All dilutions gave rise to a phase separation between the starch and PEG. After 20 h in the cold room, the microspheres in 15 wt% and 13 wt% PEG had crystallized. The starch in the sample with 10 wt% PEG had formed aggregates and was not used for further experiments. The reason behind the starch aggregation in that particular sample is unknown. The starch in 6.5 wt% PEG was still liquid and no trace of crystallites could be seen using a light microscope. Pictured below are the samples with 13 wt% and 6.5 wt% PEG 2 000 000 (*Fig. 8*. below). The sample with 15 wt% PEG 2 000 000 was very similar to the 13 wt%-sample.

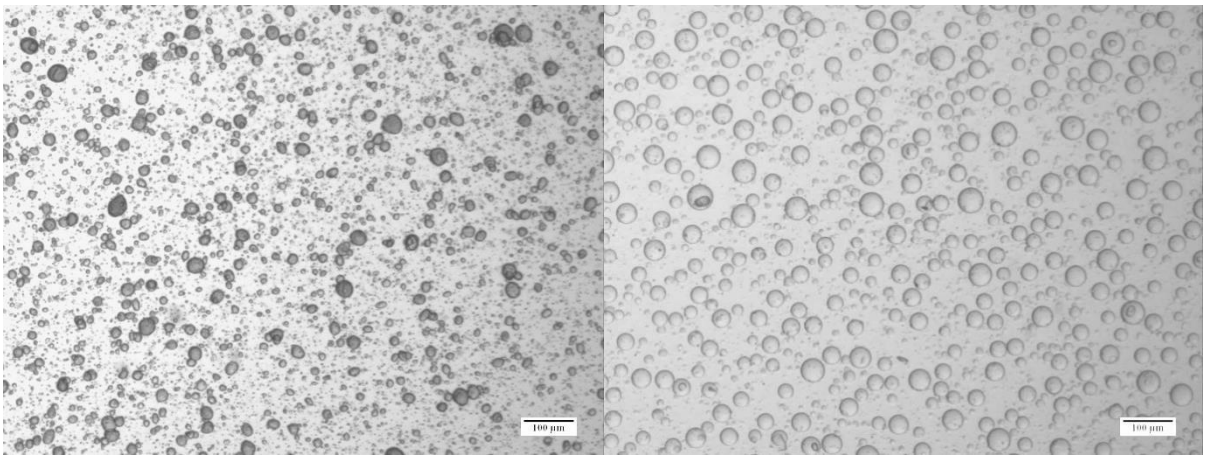


***Fig. 8.*** *Samples with 13 wt% PEG 2 000 000 (left) and 6.5 wt% PEG 2 000 000 (right) after 20 h in 6 °C. The left image shows microspheres of crystallized starch while the sample in the right image has droplets of starch which is still in liquid form.*

The difference in crystallization between the different samples indicates that a higher PEG concentration gives a faster crystallization of the starch. The samples were left in the cold room for another 22 h. The sample with 13 wt% and 15 wt% PEG 2 000 000 had not changed visually. The starch in 6.5 wt% PEG had started crystallizing, which can be seen in *Fig. 9. (left)* as the surface of the droplets looks uneven. They were not fully crystallized though, which became obvious when pressing on the cover glass as the starch containing droplets collapsed. The samples were then placed in room temperature and stirred for 64 h. No visual change of the microspheres in the sample with 15 wt% and 13 wt% PEG 2 000 000 could be seen.

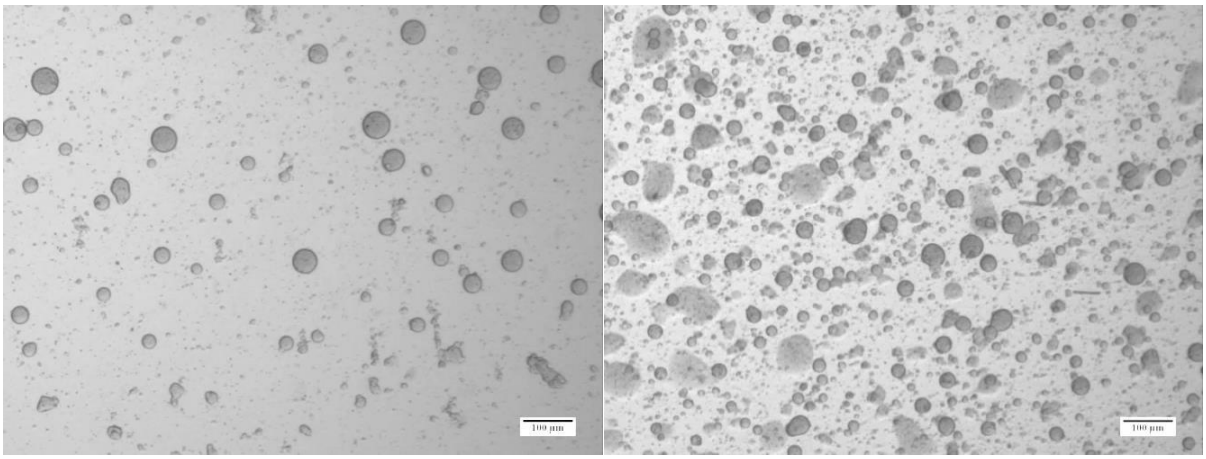


***Fig. 9.*** *6.5 wt% PEG 2 000 000 after 42 h in 6 °C (left) and another 64 h in room temperature (right). The microspheres had started crystallizing but were not solid yet. This is obvious especially in the right image, as there are droplets which have collapsed.*

In *Fig. 9. (right)* the sample with 6.5 wt% PEG after the 64 h in room temperature can be seen. The starch was not fully crystallized, which can be seen by the many droplets which have collapsed, probably when diluting the sample with some water. The samples were very thick as a lot of water had evaporated. Due to this no further work was done with these samples. With longer time, it might have been possible to achieve microspheres with 6.5 wt% PEG 2 000 000.

The solution was diluted even further to see how low the PEG 2 000 000 concentration could be while still giving rise to a phase separation with waxy barley starch. 3.3 % and 2.6 % PEG 2 000 000 gave a two-phase system but the starch droplets were smaller in the lower concentration compared to the higher one. 1.6 wt% PEG 2 000 000 did not give rise to a two-phase system. The limit for the creation of a two-phase system with starch therefore is somewhere between 1.6 wt% and 2.6 wt% PEG 2 000 000.

For the production of microspheres containing bacteria 13 wt% PEG 2 000 000 was chosen, since it did not take too long (which 6.5 wt% did). Both 13 wt% and 15 wt% worked, but 13 wt% was chosen because it is desirable with a lower PEG concentration. 10 wt% was not considered as it formed clusters of starch.

#### *3.1.1.1 Water activity measurements*

*Table 4.* below shows the water activity for four different PEG concentrations. There is only a very slight difference in water activity between the different PEG concentrations. Thus, the reason for the varying starch crystallization rates in different PEG concentrations is probably not due to the water activity of the PEG solutions. Further research is needed to explain this phenomenon.

***Table 4.*** *Water activity for 6.5 wt%, 10 wt%, 13 wt%, and 15 wt% PEG 2 000 000.*

| **Sample** | **Temperature (°C)** | **Water activity, $a_w$** |
|---|---|---|
| Salt solution 0.760 $a_w$ | 21.0 | 0.762 |
| Deionized water | 20.2 | 0.999 |
| 6.5% PEG 2 000 000 | 20.2 | 1.000 |
| 10% PEG 2 000 000 | 20.3 | 1.002 |
| 13% PEG 2 000 000 | 20.3 | 1.003 |
| 15% PEG 2 000 000 | 20.3 | 1.002 |

### 3.1.2. PEG 20 000

All of the PEG 20 000 concentrations (38 wt%, 13 wt%, and 6.5 wt%) created W/W emulsions with acid hydrolysed waxy barley starch. However, after only a few hours in 6 °C clusters of starch formed for all concentrations.

We are not sure of the reason for PEG 20 000 not working, especially not for the samples containing only starch. It might just be that the starch used (acid hydrolyzed waxy barley starch) is not the same as what was used in (Elfstrand et al. 2004) (acid hydrolyzed waxy maize starch). Different starches do not necessarily behave the same way. Two more samples with only starch and 38 wt% PEG 20 000 were made, with the same result. Due to these results, PEG 20 000 was not used for producing microspheres containing microorganisms.

## 3.2 Addition of other polymers to the starch

Starch, starch/CMC, Starch/pullulan, and starch/dextran gave rise to a two-phase system using 38 wt% PEG 20 000 and acid hydrolyzed waxy barley starch. After 15 min of stirring in 28 °C, the samples containing CMC and dextran had begun to form clusters of droplets. The droplets seem to be sticking together a little bit for the starch only as well, but the clusters were not as obvious as for the samples containing CMC and dextran. The droplets in the sample containing pullulan did not stick together. See *Fig. 10*. below.

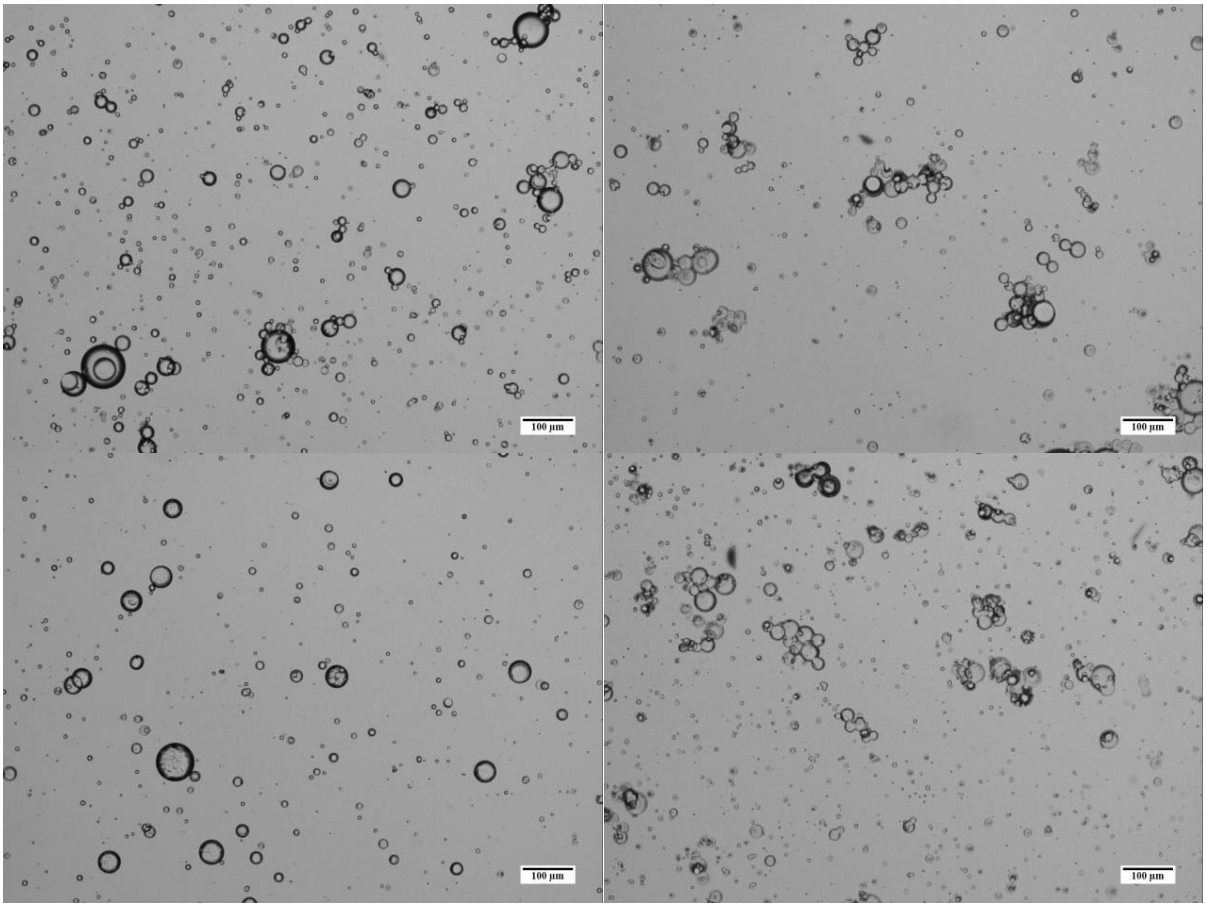



***Fig. 10.*** *Starch (top left), starch/dextran (top right), starch/pullulan (bottom left), and starch/CMC (bottom right) in 38 wt% PEG 20 000 after 15 minutes of stirring in 28 °C.*

After 2.5 h in the cold room, the starch aggregated and formed clusters in all samples, see *Fig. 11*. below. Due to this the experiment was not continued. CMC and dextran were abandoned but we wanted to try with pullulan again as the emulsion looked good (the starch/pullulan droplets did not stick together) initially (*Fig. 10., bottom left*).

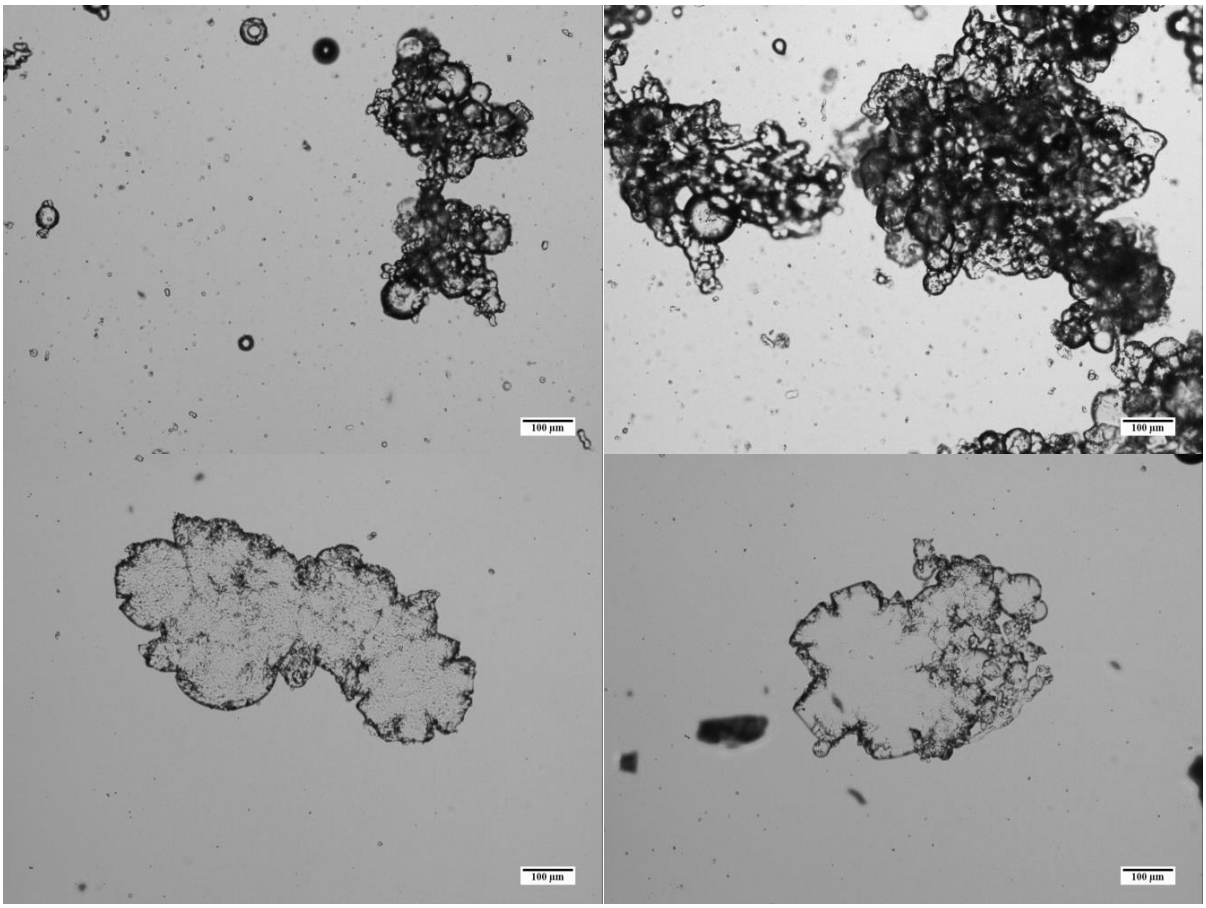



***Fig. 11.*** *Starch (top left), starch/dextran (top right), starch/pullulan (bottom left), and starch/CMC (bottom right) in 38 wt % PEG 20 000 by weight after 2.5 h of stirring in 6 °C. Starch and starch/dextran looks similar, and starch/pullulan and starch/CMC looks similar.*

## 3.3. Different starches

As can be seen in section "*3.1.1. PEG 2 000 000"*, the acid hydrolyzed waxy barley starch produces microspheres with 13 wt% PEG 2 000 000.

The two other starches, acid hydrolyzed waxy maize starch and acid hydrolyzed OSA-modified waxy maize starch both produces very similar two-phase systems with 13 wt% PEG 2 000 000, see *Fig. 12.* below.

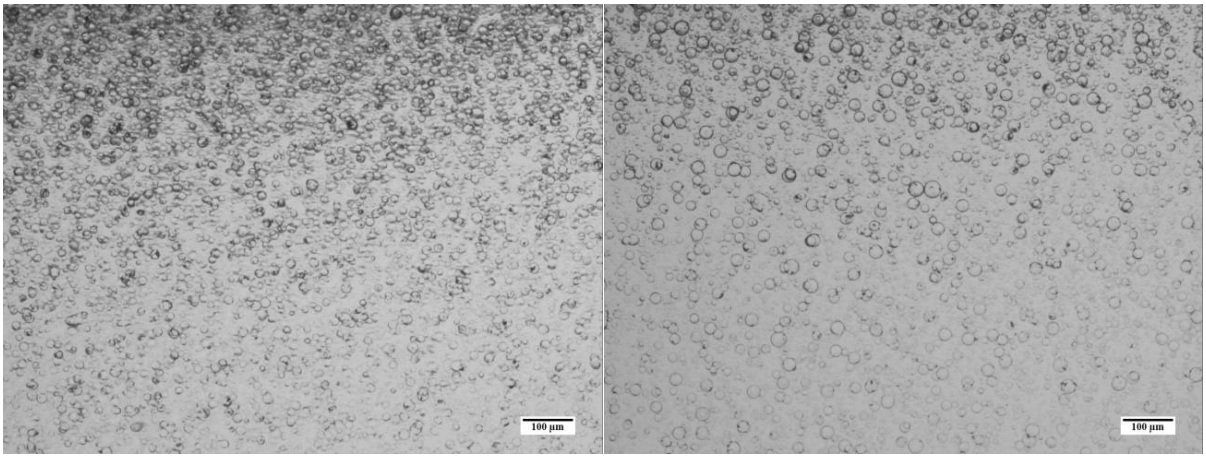



***Fig. 12.*** *Acid hydrolyzed waxy maize starch (left) and acid hydrolyzed OSA-modified waxy maize starch (right) in 13 wt% PEG 2 000 000 after 15 min of stirring in 28 °C.*

After 25 h in 6 °C, acid hydrolyzed waxy maize starch had crystallized, which can be seen by the irregular shapes and uneven surfaces of the particles (*Fig. 13., left*). Acid hydrolyzed OSA-modified waxy maize starch had started crystallizing but was still mostly liquid, which can be seen by the black dots and irregular shapes of some of the droplets (*Fig. 13., right*). This difference also became very obvious when pressing down on the cover glass, as the droplets of acid hydrolyzed OSA-modified waxy maize starch changed their shape, or even burst, while the microspheres of acid hydrolyzed waxy maize starch kept their shape and did not break.

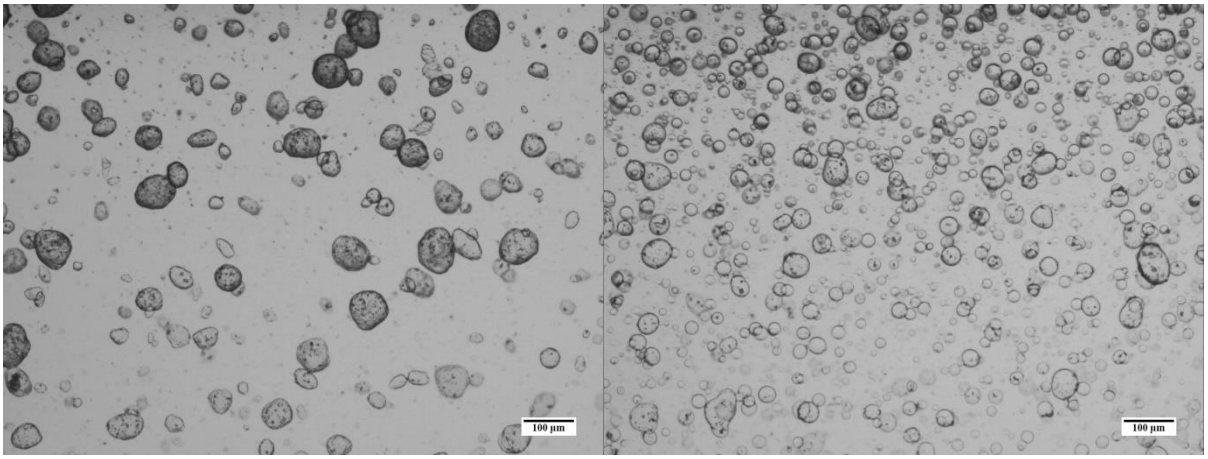



***Fig. 13.*** *Acid hydrolyzed waxy maize starch (left) and acid hydrolyzed OSA-modified waxy maize starch (right) in 13 wt% PEG 2 000 000 by weight after 25 h of stirring in 6 °C.*

After the 25 h in 6 °C the samples were kept in 37 °C for 28 h while stirring. According to what could be detected in light microscopy no substantial changes was seen for acid hydrolyzed waxy maize

starch, see *Fig. 14. (left*). Acid hydrolyzed OSA-modified waxy maize starch however, had changed. The droplets had decreased substantially in size, see *Fig. 14. (right)*, and looked like they did before the 25 h in 6 °C (*Fig 12, right*). The droplets had not crystallized, and they had been broken apart into smaller ones. The reason for this could be the OSA-modification of the starch, as the hydrophobic side chains could be repelling the carbohydrate chains, making crystallization difficult. It could also be that the nucleation sites that had formed during the incubation in 6 °C were so small that they dissolved again when placed in 37 °C before any crystal growth could occur. If that is the case, it might be interesting to see if microspheres would form when increasing the time in 6 °C or letting the second incubation be at a lower temperature than 37 °C.

Due to these results, only acid hydrolyzed waxy maize starch was used for further experiments.

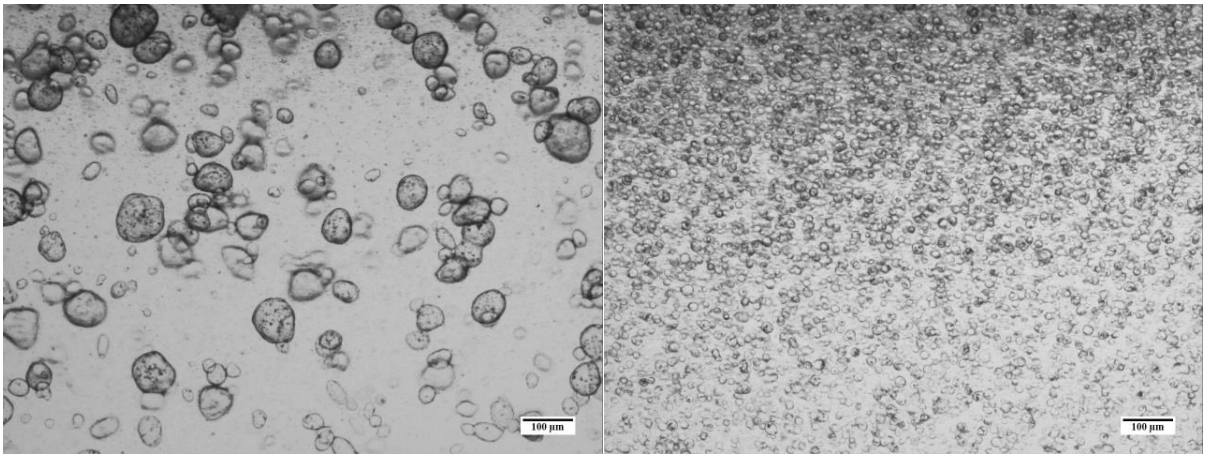



***Fig. 14.*** *Acid hydrolyzed waxy maize starch (left) and acid hydrolyzed OSA-modified waxy maize starch (right) after 25 h in 6 °C followed by 28 h in 37 °C.*

## 3.4 Cultivating bacteria

It proved to be difficult to disperse the pellet again after the centrifugation, which might be due to the biofilms that *L. reuteri* produces. It required quite vigorous mixing by vortex for the pellet to finally disperse.

The resuspended bacteria were cultivated on MRS agar plates. Unfortunately, the dilutions chosen to cultivate were a bit too low, meaning the plates we could base the number of bacteria on only had 2 and 7 colonies respectively, giving a concentration of $4.5 \cdot 10^7$ bacteria/mL. Since the colonies were so few (normally you want between 30-300 colonies/plate for statistical reasons), this value should be seen as a rough estimate. Additionally, as the bacteria were stored in water only overnight before cultivating them on agar plates, some might have died, which means the final concentration after the cultivation might have been higher.

The dispersions were weighed before and after freeze drying to calculate the relative amounts of bacteria and GOS or lactose in the freeze dried material. In the GOS/bacteria there was 2.5 wt% bacteria and 97.5 wt% GOS. However, for the lactose/bacteria, the calculations of the amount of lactose in the freeze dried material led to a higher lactose weight than what all of the freeze dried material weighed in total. The reason for this is unknown, but most probably all of the lactose added to the solution before freeze drying was not fully dissolved, and the part of the solution that was placed in the freeze drier did in fact not consist of 13 % lactose.

The freeze dried material was then cultured on MRS agar in Petri dishes 4 days after freeze drying to find out how many bacteria had survived. GOS seems to have better freeze protecting abilities than lactose, as the bacteria/GOS mix had 5 200 000 CFU/g freeze dried material, while the bacteria/lactose mix only had 1 150 000 CFU/g freeze dried material.

## 3.5. Producing microspheres containing microorganisms and placebos

All systems showed a phase separation after the initial 15 min of stirring in 28 °C. After 6 h in a cold room (6 °C) the samples were still emulsions. Until this moment the emulsions looked very similar to the placebos. After 25 h in 6°C, the starch had crystallized. The samples starch/lactose/bacteria as well as starch/GOS/bacteria still looked like the placebos. The samples containing starch/pullulan/lactose/bacteria looked similar to the placebo, but the microspheres had started aggregating, see *Fig. 15. (left)*. After another 28 h in 37 °C the aggregated microspheres seemed to have merged giving rise to highly irregular elongated particles, see *Fig. 15. (right)*.

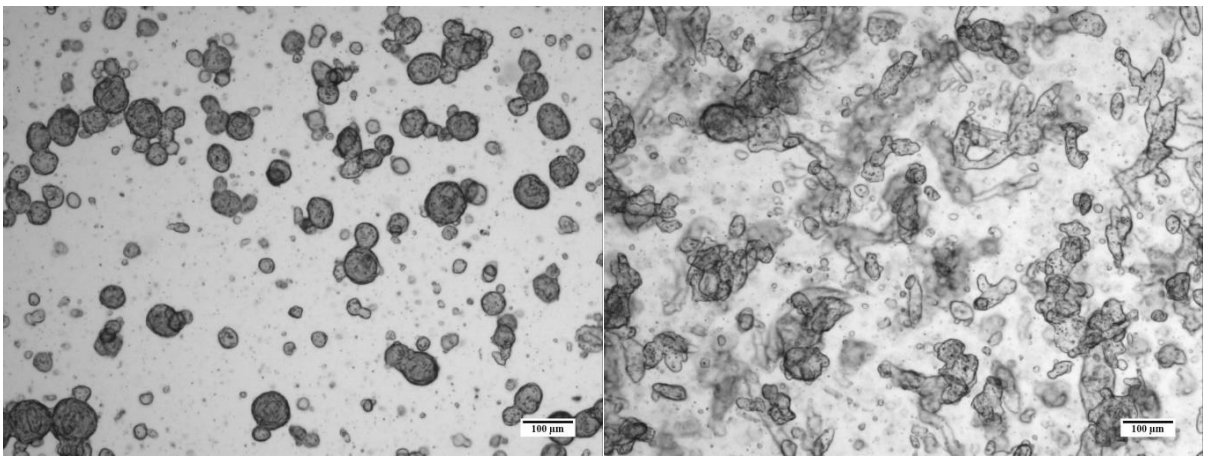



***Fig. 15.*** *Sample containing starch/pullulan/lactose/bacteria after 25 h of stirring in 6 °C (left). The microspheres are distinct and similar to the placebos but have started aggregating. In the same sample but after another 28 h of stirring in 37 °C (right) the aggregated microspheres seems to have merged into irregular, elongated particles.*

The samples containing starch/pullulan/GOS/bacteria looked very different compared to the placebo after 25 h of stirring in 6 °C, see *Fig. 16. (left)*. The droplets/microspheres had not grown much in size, however they had formed clusters. After 28 h of stirring in 37 °C, the clusters seem to have merged and elongated (*Fig. 16., right*), though not to the same extent as the starch/pullulan/lactose/bacteria (*Fig. 15., right*).

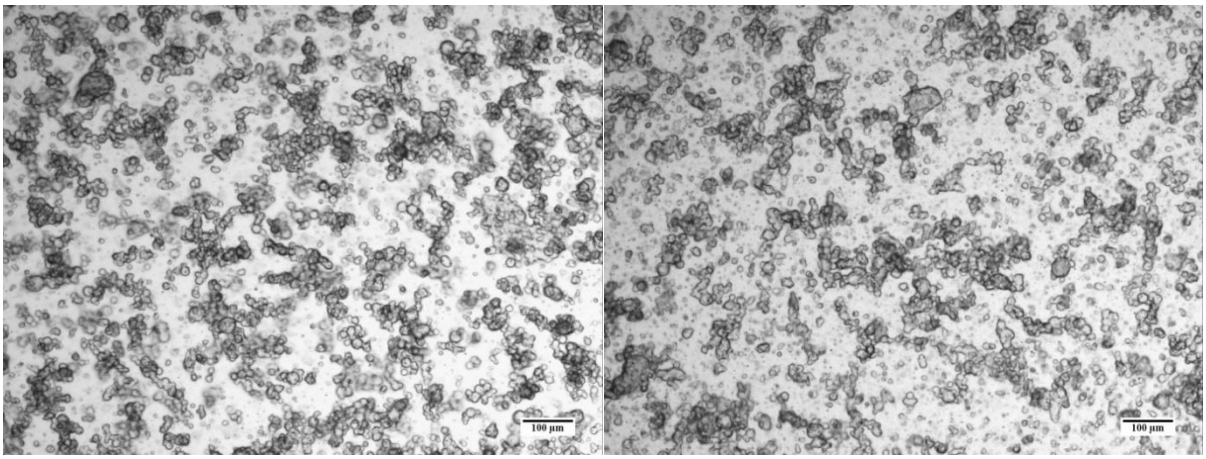


***Fig. 16.*** *Sample containing starch/pullulan/GOS/bacteria after 25 h of stirring in 6 °C (left), clusters have formed. After another 28 h of stirring in 37 °C (right), the clusters seem to have merged into more elongated, irregular particles.*

Initially starch/pullulan/GOS/bacteria and starch/pullulan/lactose/bacteria were both similar to the placebos, but after 25 h in 6 °C they appear different from each other. In both samples the microspheres had started aggregating, but starch/pullulan/lactose/bacteria had microspheres very similar to placebo, while the microspheres in starch/pullulan/GOS/bacteria were much smaller. After 28 h in 37 °C, the aggregates had merged into irregular, elongated particles in both samples, but to a greater extent in the starch/pullulan/lactose/bacteria sample it seems like.

This difference might be due to the fact that lactose is a small molecule while GOS is a larger oligomer. Lactose has a molecular weight of 342.3 g/mol. The GOS used is a mixture of oligosaccharides of different sizes, including 4'galactosyllactose which has a molecular weight of 504.4 g/mol. Maybe the larger molecules of GOS interfered more with the crystallization process, resulting in the much smaller particles (*Fig. 16.)* compared to the ones in *Fig. 15.*

### 3.5.1. Placebo microspheres for ones containing bacteria

All systems showed a very similar phase separation after the initial 15 min of stirring in 28 °C. Below is an image of starch/GOS (*Fig. 17.)*

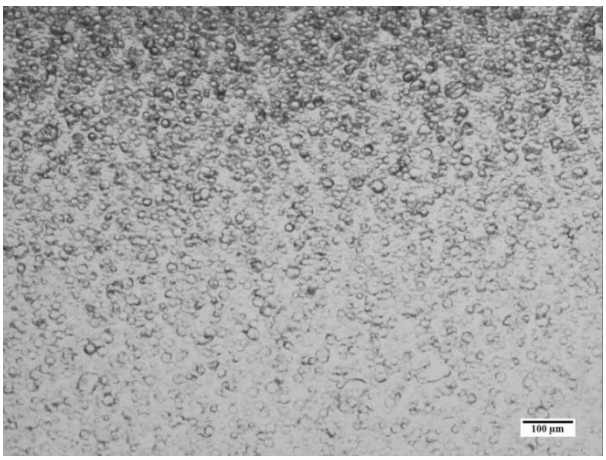


***Fig. 17.*** *Placebo starch/GOS in 13 wt% PEG 2 000 000 after 15 min of stirring in 28 °C. All other placebos looked had very similar looking emulsions.*

After 6 h in a cold room (6 °C) the samples were still emulsions. After 25 h in 6°C, the starch had crystallized. All samples looked good with very similar, solid starch microspheres for all samples. *Fig. 18*. below shows an image of starch/GOS as an example.

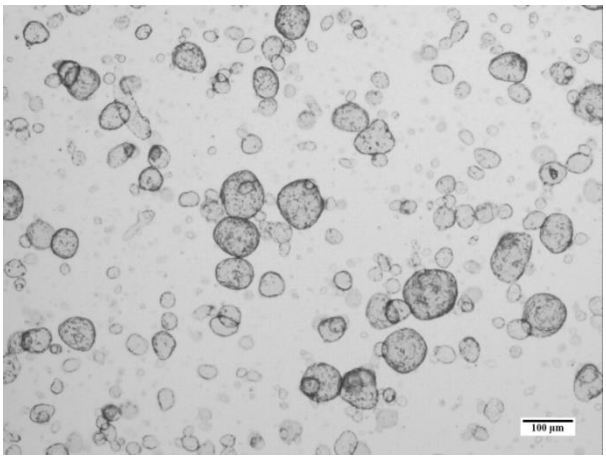



***Fig. 18.*** *Placebo starch/GOS in 13 wt% PEG 2 000 000 after 25 h of stirring in 6 °C. All other placebos had very similar looking, solid microspheres.*

Even though the starch had crystallized already in the cold room, the samples were then placed in 37 °C for 28 h as is described in (Elfstrand et al. 2004), followed by freezing and freeze drying. Exceptions are the placebos consisting of starch/pullulan/GOS, as the water bath had turned off during the night. When analyzed with a light microscope the microspheres looked fine, most probably because they were already crystallized when they were moved to 37 °C. Thus, already 22 h after they were moved to 37 °C the samples were centrifuged and placed in a freezer. An image of the placebo starch/GOS can be seen below in *Fig. 19*. All placebo versions had similar looking microspheres.

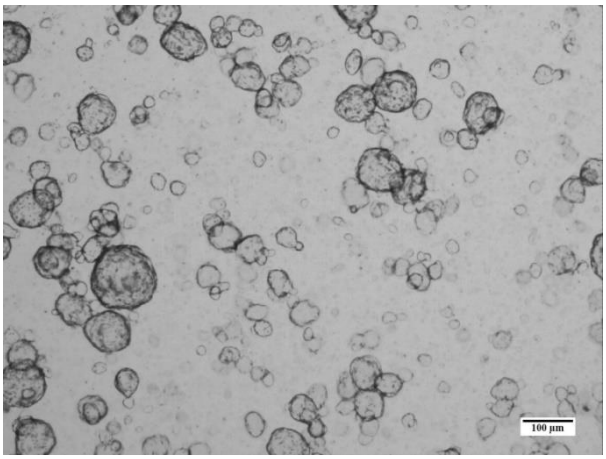



***Fig. 19.*** *Placebo starch/GOS in 13 wt% PEG 2 000 000 after 25 h of stirring in 6 °C followed by 28 h of stirring in 37 °C. All other placebos had very similar looking, solid microspheres.*

There is room for optimization of the process, as the reason for placing the samples in the cold room is for the formation of nucleation sites for crystal growth to form in the starch. The main part of the crystal growth can occur in 37 °C as it will be quicker at a higher temperature. Even though the starch had already solidified after 25 h in 6 °C, being placed in 37 °C might not be without merit. According to (Elfstrand, Eliasson, Jönsson, Reslow, et al. 2007), the incubation at 37 °C increases the thermal stability of the crystalline structure of the microspheres. Thus, the incubation in 37 °C should not be

removed, but the incubation in 6 °C could be shortened considerably and let most of the starch crystallization occur in the higher temperature.

## 3.6. Characterization

### 3.6.1. Release test of microspheres containing microorganisms and placebos

Only the samples which formed microspheres and the corresponding placebos were analyzed, see *Table 5*. below. The last time point for Sample A-D was 6 h. Due to practical reasons, for Sample E-L the last time point was at 24 h instead (no sample was taken after 6 h).

***Table 5.*** *All samples used for the release test.*

| Name | Sample | Contents |
|---|---|---|
| A | Starch 1 | Starch |
| B | Starch 1 | Starch |
| C | Sb 1 | Starch/lactose/bacteria |
| D | Sb 1 | Starch/lactose/bacteria |
| E | Sb 2 | Starch/lactose/bacteria |
| F | GOSb1 | Starch/GOS/bacteria |
| G | GOSb1 | Starch/GOS/bacteria |
| H | GOSb2 | Starch/GOS/bacteria |
| I | Starch 1 | Starch |
| J | Starch 2 | Starch |
| K | GOS 1 | Starch/GOS |
| L | GOS 2 | Starch/GOS |

### 3.6.2. Flow cytometry of microspheres and freeze dried bacteria

According to the results of the flow cytometry of the freeze-dried bacteria, pretty much all bacteria were dead before even adding them to the dissolved starch. Thus, it is not surprising that no live bacteria could be detected for any of the samples. There are dead bacteria which have been released from the microspheres, see *Fig. 20*. Any live bacteria would have shown up in the area marked as "Live cells" as that indicates cells which are stained with SYBR Green but not PI. We are unable to say anything about how many and whether the amount released is changing over time as there is too much background coming from the starch (compare *Fig 20, left and right*).

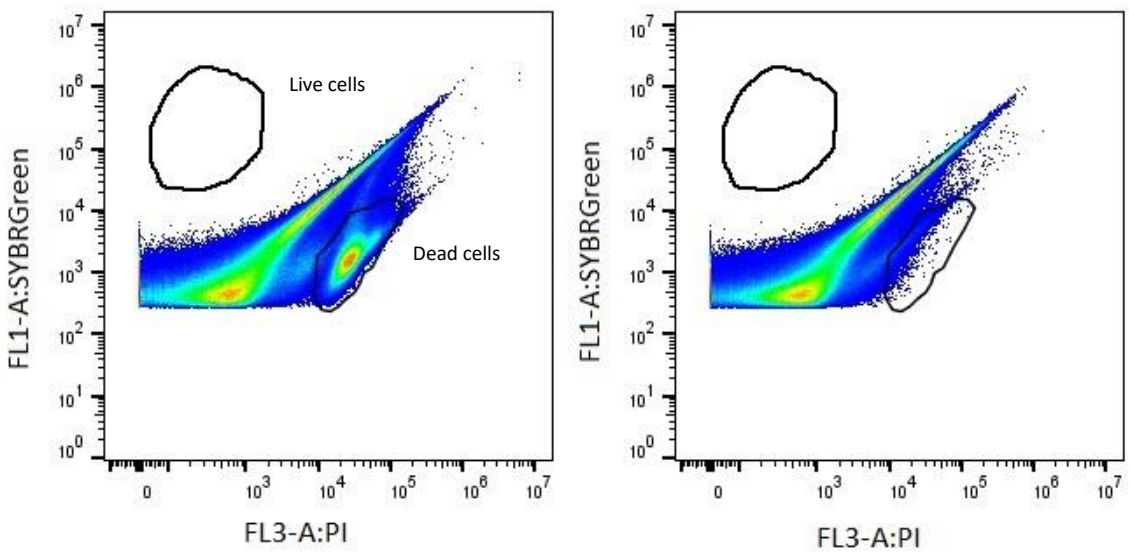



***Fig. 20.*** *Sample containing bacteria (left) and Starch placebo (right). Ideally nothing should show up on the placebo, but as can be seen the starch causes a lot of background. The circled area marked with "Dead cells" is where the dead cells should show up in the dot plot. Any live cells would have shown up in the circled area marked "Live cells".*

For further research, some other type of analysis would have to be used. If one really wants to use flow cytometry, maybe it would be possible if the samples were either diluted further or filtered through a finer filter than 40 µm to remove more starch. If the bacteria incorporated were alive, they might end up in a part of the plot which makes them distinguishable from the starch, which would enable analysis of release of live bacteria. Other possible analysis methods would be plating and counting colonies, or fluorescence microscopy.

#### *3.6.2.1. Flow cytometry of freeze dried bacteria*

1 g of freeze dried bacteria was dissolved in 11 mL of PBS to see the amount of viable bacteria. To see the effect of SIF on the bacteria, 1 g of freeze dried bacteria was dissolved in 11 mL SIF. The ODs of the dissolved samples can be seen below in *Table 6* in column OD 1. The OD of bacteria/GOS dissolved in both SIF and GOS was over 0.2 and had to be diluted slightly. For simplicity, all samples were diluted with the ratio 9:1 solution of bacteria:PBS/SIF. The OD was measured again; the results can be seen in column OD 2 in *Table 6*. As all ODs were between 0.1-0.2, these samples were used for flow cytometry.

***Table 6.*** *OD 1 is of 1 g dissolved freeze-dried bacteria in 11 mL liquid. OD 2 is after diluting the solutions measured in OD 1 9:1.*

| Sample | Liquid | OD 1 | OD 2 |
|---|---|---|---|
| Bacteria/lactose | PBS | 0.18 | 0.16 |
| Bacteria/lactose | SIF | 0.20 | 0.18 |
| Bacteria/GOS | PBS | 0.21 | 0.19 |
| Bacteria/GOS | SIF | 0.22 | 0.20 |

The results of the flow cytometry of the freeze dried bacteria can be seen in *Table 7*. For the bacteria freeze dried in GOS dissolved in PBS there is an average of 0.51% viable bacteria, which is higher than the bacteria freeze dried in lactose dissolved in PBS, which has an average of 0.075 % of viable

bacteria. This is in line with the results achieved from the colony counting on agar plates. The bacteria in SIF showed an even lower viability.

There were live bacteria just after freeze drying as proved by the colony counting. The flow cytometry was performed about 5 weeks after the freeze drying. It seems like a lot of bacteria died during the storage. The reason for this is not known, but it could be the storage conditions. Lactic acid bacteria are sensitive to humidity (Huckle and Zhang 2011), maybe they should have been stored in a desiccator instead of just a Duran bottle wrapped in Parafilm in 6 °C. Other possible causes are the freezing, as the formation of ice crystals might damage cell walls, and freeze drying, which might have damaged some of the cells. The damaged cells might have been able to grow colonies but could not handle storage for any extended periods of time.

***Table 7.*** *Results of flow cytometry of freeze dried bacteria.*

| Sample | Liquid | Viable bacteria (%) | Injured bacteria (%) | Dead bacteria (%) |
|---|---|---|---|---|
| Bacteria/lactose | PBS | 0.08 | 0.37 | 98.80 |
| Bacteria/lactose | PBS | 0.07 | 0.38 | 98.80 |
| Bacteria/lactose | SIF | 0.00 | 0.15 | 98.50 |
| Bacteria/lactose | SIF | 0.00 | 0.13 | 98.60 |
| Bacteria/GOS | PBS | 0.50 | 1.92 | 94.00 |
| Bacteria/GOS | PBS | 0.52 | 2.02 | 93.70 |
| Bacteria/GOS | SIF | 0.01 | 0.11 | 97.90 |
| Bacteria/GOS | SIF | 0.00 | 0.14 | 99.10 |

### 3.6.3. DNS assay

As DNS is reduced by reducing sugars, it is important to keep in mind that the GOS as well as the lactose might affect the results of this assay. For Samples E-L a last sample was taken after 24 hours, but the values registered for these were unreasonably high, and thus discarded. Most probably water had evaporated during the 24 hours, resulting in concentration of the samples. For future experiments, this could be prevented by slightly changing the setup. Instead of withdrawing samples from a beaker at the chosen time points, the same number of test tubes as the number of samples one wants to take could be prepared. At each time point one test tube is removed from the 37 °C and placed on ice until further analysis can be performed. *Fig. 21*. below shows the standard calibration curve for the DNS assay using maltose.

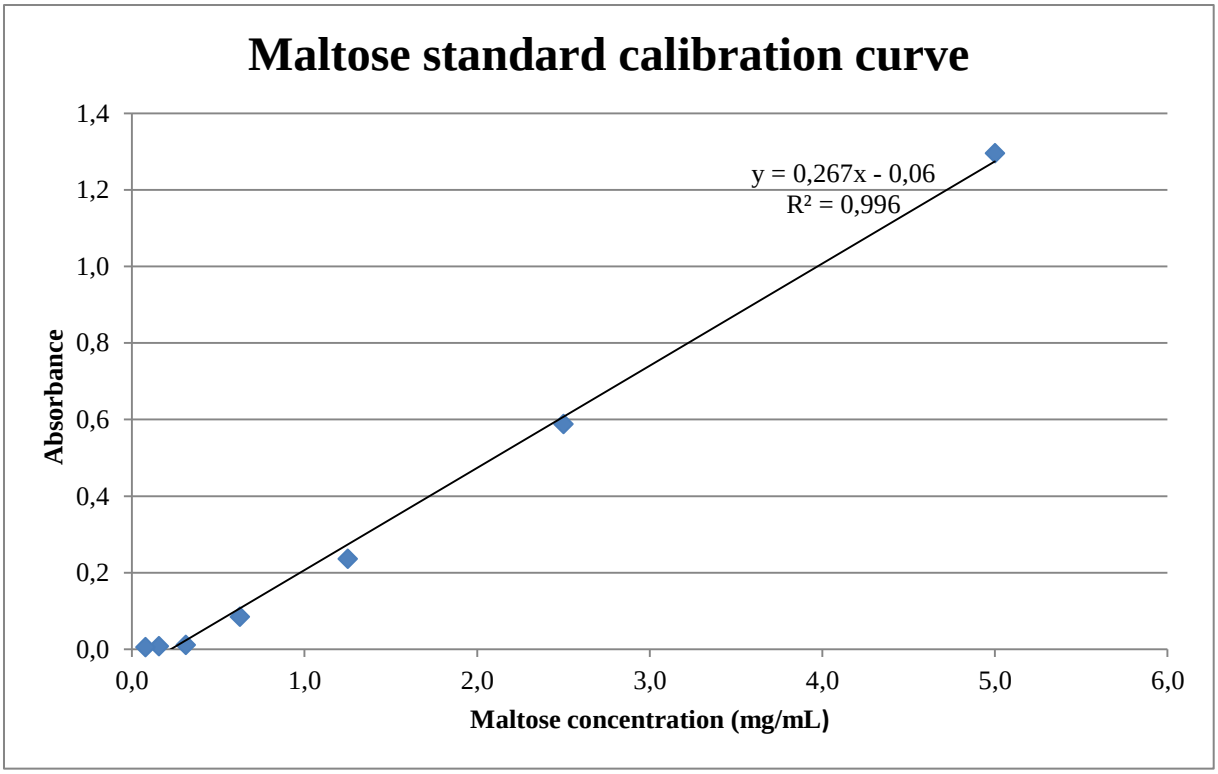



***Fig. 21.*** *Standard calibration curve with maltose for the DNS assay.*

*Fig. 22.* shows the reducing sugar concentration as percentage of the reducing sugar concentration after 240 min. This was done to account for the fact that there is a different amount of starch in the different variations of microspheres. The 6 h time point for sample A-D was not used as we wanted the time point at which the concentration was set to 100 % to be the same for all samples. All different samples seem to follow the same pattern of a quick initial digestion, and then evening out after about an hour.

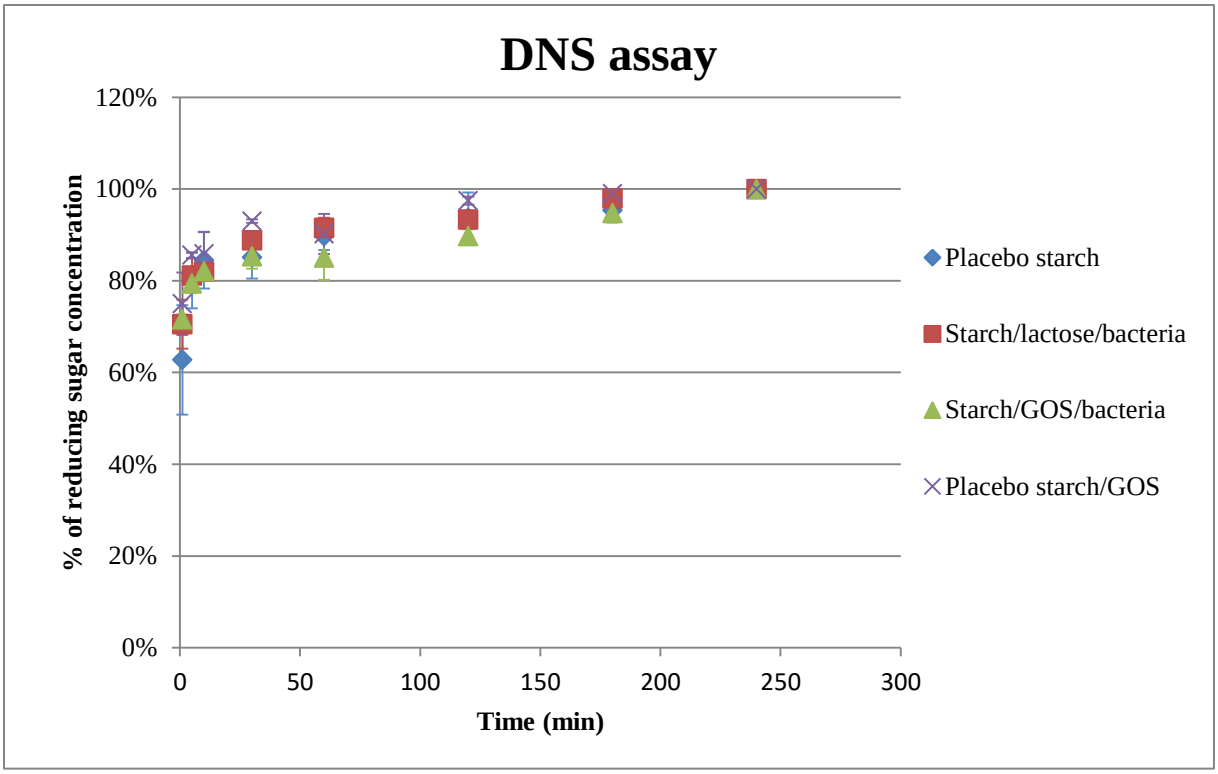



***Fig. 22.*** *Reducing sugar concentration over time as percentage of the reducing sugar concentration of the last time point (240 min).*

### 3.6.4. Light microscopy

The freeze dried microspheres looked fine for the most part when dispersing them in PBS, in *Fig. 23. (left)* microspheres consisting of starch/bacteria/lactose can be seen. Some samples had microspheres that were a bit damaged but not consistently for both duplicates, one of the duplicates of starch/GOS/bacteria can be seen in *Fig. 23. (right)*. Most probably the damages occurred during the freezing of the samples, due to formation and growth of ice crystals. Another possible cause is the freeze-drying. It is very important that the samples are completely frozen when placed in the freeze dryer, for the water to sublimate. If the sample is not completely frozen, it will begin to boil once the pressure decreases. Boiling will destroy the structure of the sample. As the samples were very small, they might very well had started to defrost during the time from being removed from the freezer to being placed in the freeze dryer. Also, the freeze dryer that was used is a very simple one. There is no cooling of the shelves which the samples are placed on; which might have caused the samples to melt.

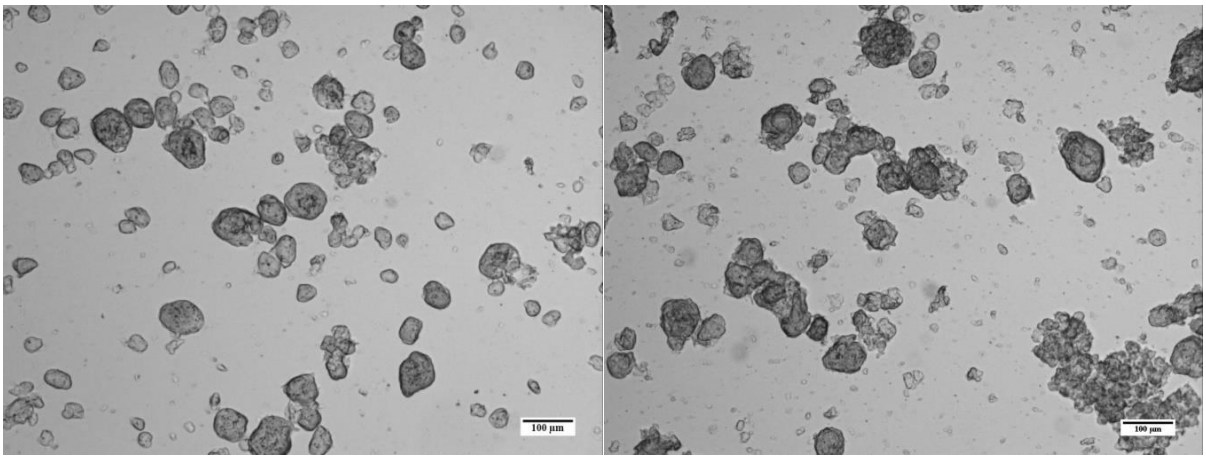



***Fig. 23.*** *Freeze dried microspheres consisting of starch/lactose/bacteria (left) and starch/GOS/bacteria (right) dissolved in PBS. Most microspheres look intact, but some almost looks like they have been crushed, especially in the starch/bacteria/GOS sample.*

All samples of the digested microspheres were very similar. Already after 10 min it was not possible to see any microspheres, below is an image of Sample E after 10 min (*Fig. 24, left*) and 24 h (*Fig. 24., right*) of digestion. This is in line with the results of the DNS assay, where up to 90 % of the starches, regardless of the formulation, were digested to maltose.

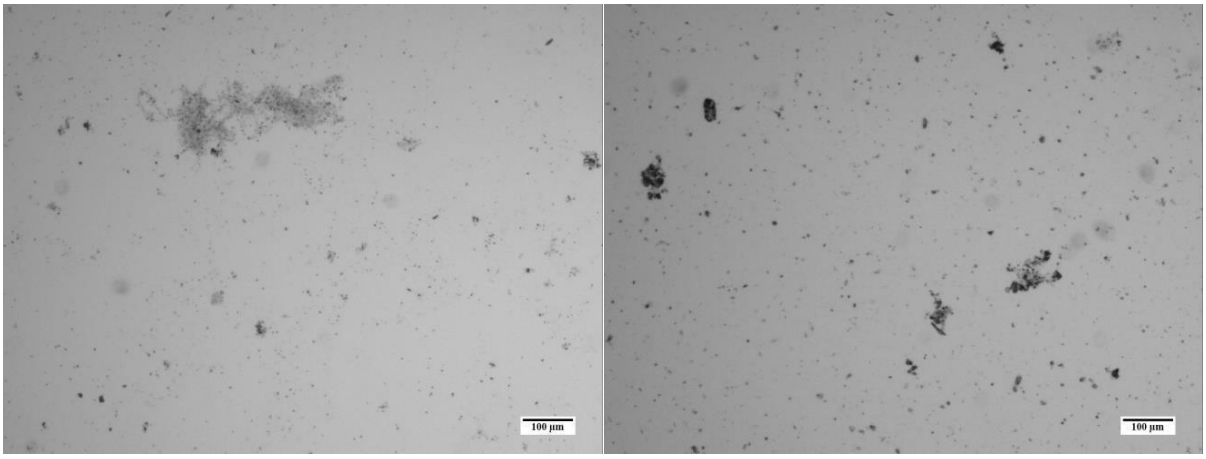



***Fig. 24.*** *Sample E (starch/lactose/bacteria) after 10 min (left) and 24 h (right) of digestion. All samples look very similar.*

It is difficult to tell from the images in *Fig 24.* but during the analysis at the microscope, the samples seemed to "clear up" when comparing early time points to later ones. The "clearing up" could be the starch being digested. It is concerning that the microspheres broke down this quickly. There are many possible explanations, and further experiments are needed to determine the cause. Most probably, the amount of microspheres added to the SIF and α-amylase was too small and the starch was broken down very quickly. It could also be that the stirrers broke the microspheres, but some sort of stirring/mixing is needed during the experiment as the environment in the GI tract is not static. It could also have been the bile salts, or something with the structure of the microspheres.

# 4. Conclusions

No microspheres were successfully made with PEG 20 000 and acid hydrolyzed waxy barley starch. PEG 2 000 000 however worked for producing microspheres with both acid hydrolyzed waxy barley starch and acid hydrolyzed waxy maize starch.

The starch crystallization rate seems to be affected by the PEG concentration; a higher concentration gives a faster crystallization.

The addition of other polymers to the starch proved difficult. It worked making microspheres containing pullulan (the placebo microspheres) with acid hydrolyzed waxy maize starch and 13 wt% PEG 2 000 000, but the microspheres containing pullulan *and* bacteria aggregated and fused into elongated, irregularly shaped particles.

A commercial starch, acid hydrolyzed waxy maize starch, worked for making microspheres with 13 wt% PEG 2 000 000. Acid hydrolyzed OSA-modified waxy maize starch created a two-phase system with 13 wt% PEG 2 000 000, but did not form microspheres, at least not with the incubation times used.

GOS seemed to work as a better cryoprotectant than lactose for the bacteria. However, most of the bacteria that survived the freeze drying died after a few weeks of storage in 6 °C.

In the release test, the microspheres were digested very quickly; no microspheres could be distinguished using the light microscope even after only 10 min of digestion. All different samples seemed to follow the same pattern of a quick initial digestion, and then evening out after about an hour according to the DNS assay. We were not able to analyze the result of the flow cytometry of the microspheres as there was too much background from the starch in the samples. Thus we were unable to say anything about the total number as well as the release rate of the bacteria.

# 5. Future work

For further research, we suggest scaling up the method, as the yield of microspheres per batch as it is now is only a couple of 100 mg. It is not much to work with when it comes to analyzing, especially for the digestion test. The process also has to be up-scaled if it is ever to be used industrially. With scaling up come larger volumes, which might make it possible to use an autoclave to dissolve the starch instead of a microwave oven. An autoclave makes it possible to control the temperature, which increases the reproducibility of the process.

Along with scaling up the process, the incubation times should be optimized. In 13 wt% PEG 2 00 000, acid hydrolyzed waxy maize starch had solidified completely after 25 hours in the cold room. Thus this time could most probably be shortened considerably. It only has to be long enough for crystallites to form, the rest of the crystallization can occur in 37 °C instead. To optimize the process one could analyze the crystallinity, particle size, and shape of the microspheres.

There was too much background in the data from the flow cytometry of the digested microspheres. To use this analysis method, it would be necessary to change something, maybe filter the samples with a finer filter than the 40 μm that was used. Another solution is to use other techniques for evaluation of the viability of microorganisms including fluorescence microscopy or colony counting on agar plates. The disadvantage of using agar plates is that it only gives the number of live bacteria; it does not say anything about the number of dead bacteria. It is also a much more time consuming process than flow cytometry. With fluorescence microscopy might make it possible to see the microorganisms that have been incorporated in the microspheres, if the bacteria are stained (before encapsulation) with fluorescent molecules that are stable enough to handle the whole microsphere production process.

The freeze dryer used during the project is quite simple and does not allow for much control of the process. A freeze dryer with the possibility to control cooling rates etc. would enable optimizing the freeze drying to maybe increase the number of surviving bacteria. Additionally, to get a higher yield of viable bacteria the freezing solution should be optimized, by adding glycerol for example.

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