Does growth of *Limosilactobacillus reuteri* DSM 17938 in infant formula have an impact on its probiotic features?

Kan fermentering i bröstmjölksersättning ha en påverkan på Limosilactobacillus reuteri´s probiotiska egenskaper?

Astrid Hägg & Carolin Marklund

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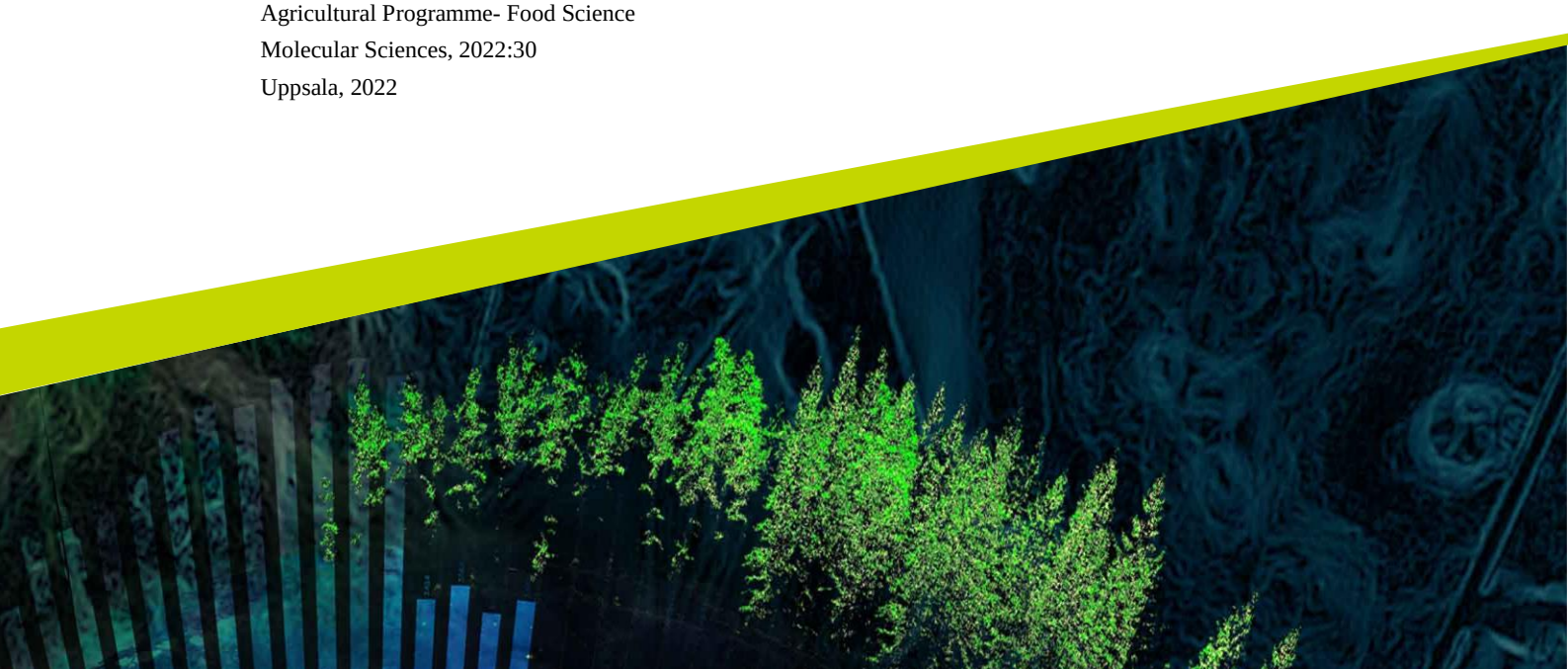

Does growth of *Limosilactobacillus reuteri* DSM17938 in infant formula have an impact on its probiotic features?

Astrid Hägg and Carolin Marklund

Supervisor:	**Stefan Roos, SLU, Department of Molecular Sciences**
Assistant supervisor:	Ludwig Lundberg, SLU, Department of Molecular Sciences
Examiner:	Hans Jonsson, SLU, Department of Molecular Sciences

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Swedish University of Agricultural Sciences
NJ Faculty
Department of Molecular Sciences

Abstract

The human gut microbiota establishes at birth and forms a life-long relationship with its host. A rich diversity in the microbiota has been suggested as a key association for healthy individuals. To ensure a microbial equilibrium, clinically proven beneficial bacteria in the form of probiotics can be applied. *Limosilactobacillus reuteri* DSM 17938 is one of the most studied and documented probiotic strains. A prerequisite for a probiotic product today is that it contains live microorganisms, and probiotics are therefore assessed on their viability. However, whether a probiotic ought to contain solely live bacteria or a mix of dead and live bacteria including their metabolites is an ongoing discussion. Postbiotics are on the contrary non-viable by definition, instead, their efficiency is based on properties of different bacterial bioactive components and metabolites. Both probiotics and postbiotics have been applied in the production of infant formula with the aim to mimic human breast milk with its complex composition. Interaction studies of bacteria grown in infant formula are of interest as the final product can contain both live probiotics and postbiotics such as short chain fatty acids (SCFA) and membrane vesicles (MV) with reported beneficial effects. The present study intended to evaluate if fermentation of DSM 17938 in different infant formulas could affect its probiotic features. Two different infant formulas (formula A& B) were used as fermentation media and after lyophilization the bacterial tolerance to bile salts and low pH, as well as ability to adhere to mucus membranes was assessed. In addition, HPLC was performed on the supernatants to examine presence of metabolites. First of all, the bacteria both grew better and survived drying better in formula A than in formula B. Furthermore, it was shown that bacteria cultivated in formula A had a better tolerance for both gastric acid and bile than the bacteria grown in formula B. Interestingly, the HPLC results showed that only the bacteria grown in formula B had produced glycerol, whereas propionate was found in both formula A and B after fermentation. Although the reason for the different bacterial activity between the two formulas is most likely multifactorial, it can be concluded that fermenting DSM 17938 in infant formula *does* affect its probiotic features. Despite the fact that neither of the infant formulas in this experiment are optimized for bacterial growth, *L. reuteri* DSM 17938 showed a relatively good growth. The demonstrated bacterial growth in formula A and B entails that this type of fermentation media can be considered for DSM 17938 in prospective probiotic products like fermented infant formulas (FIFs). Future studies may include further investigation of the composition of infant formula A and B to untangle their potential as fermentation medium in a FIF product. In addition, this study suggests a more comprehensive definition of products such as FIFs which goes beyond the information obtained by viability.

Keywords: probiotics, postbiotics, Limosilactobacillus reuteri, gastrointestinal tract, infant formula

Sammanfattning

Människans mikrobiota etableras vid födseln och utgör sedan en livslång symbios med sin värd. En stor mångfald i mikrobiotan har föreslagits vara avgörande för friska individer. För att säkerställa en mikrobiell jämvikt kan kliniskt bevisat goda bakterier, i form av probiotika, användas som tillskott. *Limosilactobacillus reuteri* DSM 17938 är en av de mest studerade och dokumenterade probiotiska stammarna i världen. En förutsättning för att en produkt ska få kallas probiotika idag är att den innehåller levande mikroorganismer. Probiotika är därför värderad efter sin viabilitet. Huruvida probiotika ska innehålla endast levande bakterier eller en blandning av döda och levande med producerade metaboliter, är en pågående diskussion. Postbiotika är till skillnad från probiotika inte levande per definition, dess effektivitet grundar sig istället på egenskaperna hos de bioaktiva metaboliter de producerat. Med målet att efterlikna den komplexa sammansättningen av bröstmjölk kan både pro- och postbiotika användas i produktionen av bröstmjölksersättning. Interaktionsstudier av bakterier fermenterade i bröstmjölksersättning är intressanta eftersom slutprodukten kan innehålla likväl probiotika som postbiotika, så som kortkedjiga fettsyror och membran-vesiklar. Dessa kan bidra med dokumenterad probiotisk effekt. Den här studien avsåg att undersöka om fermentering av DSM 17938 i olika bröstmjölksersättningar kunde påverka dess probiotiska egenskaper. Två olika bröstmjölksersättningar (formula A & B) användes som fermentationsmedier och bakteriell tolerans mot gallsalter och lågt pH, samt förmåga att fästa till slemhinna testades. Även HPLC utfördes på supernatant för att undersöka eventuellt närvarande metaboliter. Bakterierna fermenterade i formula A både växte och överlevde torkning bättre än bakterierna i formula B. Dessutom visade det sig att bakterierna fermenterade i formula A hade en bättre tolerans mot både galla och lågt pH. Intressant nog visade resultaten från HPLC att endast bakterierna som vuxit i formula B hade producerat glycerol medan propionat hittades i både formula A och B efter fermentering. Även om orsaken bakom de skilda resultaten mellan fermentering i formula A och B är multifaktoriell, så bekräftar den här studien att fermentering av DSM 17938 påverkar dess probiotiska egenskaper. Med tanke på att ingen av bröstmjölksersättningarna som användes i den här studien är optimerade för bakteriell tillväxt, så visade *L. reuteri* DSM 17938 relativt bra tillväxt. Den påvisade tillväxten i formula A och B innbebär att den här typen av fermentationsmedium kan övervägas för DSM 17939 i framtida probiotiska produkter som fermenterad bröstmjölksersättning. Framtida studier kan innefatta vidare undersökning av kompositionen av formula A och B för att kartlägga deras fermentationsegenskaper. Dessutom, föreslår den här studien en mer omfattande definition av produkter som fermenterad bröstmjölksersättning vilken omfattar mer än den information som viabilitet erhåller.

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Abbreviations

BAs	Bile acids
BHI	Brain Heart Infusion
BSH	Bile Salt Hydrolyse
CFU	Colony forming unit
EVs	Extracellular vesicles
FIF	Fermented Infant Formula
GI	Gastrointestinal
LAB	Lactic acid bacteria
L. reuteri	*Limosilactobacillus reuteri*
MVs	Membrane Vesicles
MRS	De Man, Rogosa and Sharpe agar
NDCs	Non digestible carbohydrates
SCFAs	Short Chain Fatty Acids
SLU	Swedish University of Agricultural Sciences
TFTC	Too Few to Count

1. Introduction

The inherent bacterial microbiota of the human digestive tract (gut) was for a long time most considered from a pathogenic view based on the knowledge that some pathogenic bacteria had the capacity to cause local or systemic infections. However, studies show that the gut microbiota form a fundamental and life-long relationship with its host where the interaction is considered as mutually beneficial (Gomaa 2020). In fact, it is now clear that the complex gut microbiota, with trillions of microorganisms, plays an important role in human health and disease (ISAPP 2021).

Most of the microbes that reside in the human gut are found in the colon while the stomach and small intestine are more sparsely colonized (ISAPP 2017). The community of microbes is highly diverse among both populations and individuals. This diversity is mainly due to genetic, environmental, and nutritional factors and the establishment of the microbiota begins at birth (Gomaa 2020). It has been suggested that a rich diversity of the intestinal microbiota is important for a microbial equilibrium (Sommer et al. 2017). A stable homeostatic interaction between the microbiota and its host has been suggested as a key requirement for staying healthy (Sommer et al. 2017). The symbiotic relationship implies that the energy harvested from food and fibres by the bacteria in the gut enables various bacterial activities such as production of metabolites and enzymes and interaction with host microbiota and cells (ISAPP 2017).

Dysbiosis is the term for any disturbance of the microbiota homeostasis. To date, mounting evidence shows that this imbalance can be related to several human infections and diseases such as inflammatory bowel disease, cardiovascular disease, obesity and diabetes, autoimmune diseases and more (Hou et al. 2022). Further research is required to untangle the interplay between the gut microbiota, health, and disease. However, the bacterial composition in the human gut has become an increasingly important therapy target for improved health (Skelly et al. 2019).

1.1 Probiotics

As a way of preventing, or even curing diseases, there has been a raise of interest in modulating the microbiota composition and function (Hou et al. 2022). The application of pre-, pro-, syn- and postbiotics are all strategies to modulate both bacterial composition and activity towards a more favourable state (Skelly et al.

2019). Prebiotics are nondigestible fibre compounds in food which are well fermented by seemingly beneficial bacteria of the microbiota (Gomaa 2020).

Probiotics are live bacteria and are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” by the World Health Organization (Hill et al. 2014). Synbiotics consist of a combination of pre- and pro- biotics (Skelly et al. 2019). Postbiotics, which are the newest member in the -biotics family, refers to various bioactive compounds produced by live bacteria. They are produced during fermentation (Skelly et al. 2019) or released after bacterial lysis (Cuevas-González et al. 2020) and will be discussed more in depth later.

In general, live bacteria are present in most foods. Yoghurt, sauerkraut, and kimchi are long-familiar examples of fermented foods where lactic acid bacteria (LAB) is the main bacteria used in the production. However, not all bacteria in fermented foods are considered probiotic. To be considered a probiotic, there are certain criteria. Firstly, the bacterial strain must reach its site of action in the gut, thus, it must survive the physical stress in the gastrointestinal (GI) tract during ingestion. The human stomach is highly acidic, and the intestine contain bile which can be stressful for the bacteria. The major functional components of bile are bile acids (BA). These are synthesized in the liver and stored in the gall bladder before being transported and released into the intestine (Long et al. 2017). Secondly, the probiotic strain should not carry any transferable antibiotic resistance genes. Third, the characterized strain should hold scientific evidence for a beneficial effect on health in its host (Mu et al. 2018). The administration of probiotics is usually in the form of strain specific, or mixed strain dietary supplements with controlled amounts of live bacteria. Many probiotics include LAB, such as *Lactobacillus* sp. and *Bifidobacterium* sp. (Hou et al. 2022).

Within the mentioned criteria, probiotics can provide a wide range of health effects, although many of these effects are strain specific and influenced by the interaction with the host. However, frequently reported, and concluded probiotic effects are for example reduced colic symptoms in breastfed babies and decreasing the risk of gut infections. Although health benefits have been demonstrated, the exact mechanisms behind probiotic benefits remain challenging to confirm (ISAPP 2017).

1.2 Probiotic production

1.2.1 Fermentation and lyophilization

In addition to the previously probiotic features, the probiotic bacteria must sustain its properties through product processing as well as when formulated in the food supplement matrix. As mentioned, the definition of probiotics is based on *live* organisms, thus, the thoroughness of the manufacturing process is essential for the final product. Steps generally include a scaled-up fermentation followed by a drying step, such as spray-drying or lyophilization (=freeze drying). Lyophilization is a convenient way of putting the cells in a non-metabolic, resting state for long-term

preservation (Wendel 2022). In lyophilization, the bacteria cells are quickly frozen (-40 to -70°C, or less), using liquid nitrogen for example, after which the pressure is decreased, and frozen water is removed through sublimation. Before freeze drying, a protecting agent, cryo/lyoprotectant, can be added to the bacterial solution to maintain a high viability. Common cryoprotectants are disaccharides e.g., sucrose. The sugars function as protective molecules by forming a high viscosity matrix which limits diffusion-regulated degradation of the bacterial cells (Fonseca et al. 2015). Although lyophilization provides an efficient protection of the bacterial cells, it also implies multiple physical stressors that may damage the different cells, their properties and ultimately reduce viability (Wendel 2022).

1.2.2 Probiotic viability

To ensure probiotic quality for consumers, producers are legally required to state minimum numbers of viable microorganisms at the end of shelf life expressed as colony-forming units (CFUs) on the label of their product. The CFUs are based on traditional plate counting through serial dilutions. Shelf life of a probiotic foods supplement is typically between 12 and 24 months, and during this time, some bacteria will inevitably die (Fiore 2020). However, there is an ongoing debate on the complexity of determining if a probiotic is suitable for beneficial consumption, based on the definition of what counts as bacterial viability. Wendel (2022) discussed the complex concept of assessing viability and argues that it is much more to the picture than the strains' ability to replicate. Defining viability exclusively on enumeration can result in a comprehensive underestimation of active cells, thus a highly misleading result. In fact, it has been stated that not all probiotic effects rely on bacterial viability (Cuevas-González et al. 2020).

1.3 Postbiotics

In 2021, the definition of postbiotics was determined as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" by the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Salminen et al. 2021). Unlike probiotics, postbiotics are not viable by definition nor is viability a prerequisite for their suggested health benefits. Instead, the efficacy of postbiotics is based on the properties of the different bioactive metabolites. Examples of postbiotic compounds are short-chain fatty acids, (SCFAs), functional proteins, vitamins, extracellular polysaccharides (EPS) and extracellular vesicles (EVs) (Skelly et al. 2019). Both *in vitro* and *in vivo* studies have demonstrated that some postbiotics may be responsible for health promoting effects observed in humans. Suggested bioactivities behind these effects are anti-inflammatory, anti-proliferative, immunomodulatory, antioxidant, and antimicrobial. Although postbiotics hold valuable potentials for the development in food science and human health and nutrition, the field of postbiotics is still relatively new and several aspects remain unexplored (Cuevas-González et al. 2020).

1.3.1 Fermented infant formula

Even though postbiotics is a fairly new field of research, various products containing postbiotics are available on the market today. One type of product which is available is fermented infant formulas (FIFs). LAB is commonly used in the production process in the interest of their ability to produce bioactive molecules during fermentation, although, the final product does not contain many live bacteria (Agostoni et al. 2007). In contrast to probiotics, the production of postbiotics focus on the production of metabolites and the loss of viable microorganisms (Cuevas-González et al. 2020). For example, the production of FIFs normally includes physical treatments such as homogenization, pasteurization, sterilization and/or spray-drying to kill the viable bacteria (Agostoni et al. 2007). As postbiotics circumvents the challenges of viability, postbiotic products are not limited in terms of requirements for the final product, transport approach as well as length of shelf life.

The remaining postbiotic compounds may participate in the modulation of the gut microbiota of the host, which can promote the growth of favourable microbes and limit the growth of potenital pathogens (Wegh et al. 2019). Some studies show that an addition of fermented formula in the diet of infants can lower the pH of the stool (Indrio et al. 2007). This can be an indication of higher SCFA production and a beneficial change in the composition of the microbiota. In other words, postbiotics can have a positive effect on the gut microbiota by promoting the growth of profitable microbes and inhibiting the growth and function of potential pathogens (Wegh et al. 2019).

1.4 Limosilactobacillus reuteri

Limosilactobacillus reuteri is a species that is believed to have evolved together with mammals (e.g., humans) for millions of years (Frese et al. 2011). *L. reuteri* DSM 17938 was originally isolated from human breast milk by the scientist Ivan Casas. It is classified as one of the most studied and documented probiotic strains in the world. *L. reuteri* DSM 17938 is safe for all ages to use and has shown strong probiotic effects in a wide range of indications. *L. reuteri* DSM 17938 cannot only be found in breast milk, but also in the mouth, stomach, intestine, colon, and vagina (Mu et al. 2018, Biogaia 2022).

1.4.1 Probiotic features of *L. reuteri*

L. reuteri possess all the qualifications required to be ranked a probiotic bacterium; it is not only safe for humans to consume; it can also survive at low pH and in environments filled with digestive enzymes (Mu et al. 2018). Moreover, it holds biofilm forming abilities (Salas-Jara et al. 2016) and can conquer pathogenic microbes and attach to the mucus layer of the epithelial cells. *L. reuteri´*s capacity to adhere to mucus is important for a probiotic effect to occur (Mu et al. 2018, Roos et al. 2002).

Reuterin production

As the name insinuates, *L. reuteri* can produce reuterin, a metabolite with antibacterial activity. The fact that *L. reuteri* is more resistant to reuterin than most other bacteria found in the intestine, suggests that reuterin production is an evolutionary favourable characteristic for *L. reuteri*. Production of reuterin requires glycerol (Zhang et al. 2020).

Biofilm production

The ability to form biofilm is one of the reasons believed to be important for the resistance of *L. reuteri* to the harsh environment in the gut. In addition, the biofilm can promote colonization and longer persistence in the host mucosa, which might allow it to impede colonization of pathogenic bacteria (Salas-Jara et al. 2016). Biofilm are gathered microbial cells, forming a protecting film by attaching to either a substratum or each other. The microbes are surrounded by a matrix of extracellular polymeric substances produced by themselves. Multiple factors affect the formation of the biofilm, for example, the environmental factors, the bacterial strain, and the qualities of the surface. The microbes e.g., bacteria can communicate and thus cooperate by quorum sensing, a process that may regulate gene expression. (Salas-Jara et al. 2016). Another protective characteristic of a biofilm is its ability to form an EPS matrix. These polysaccharides additionally play an important role in *L. reuteri´*s capacity to attach to epithelium (Sala-Jara et al. 2016). Studies show that biofilm grown *in vitro* on microsphere, intend to promote the adherence of the bacteria to the epithelium cells of the host GI, and therefore increase the opportunity for interaction and subsequently also the probiotic potential of *L. reuteri* (Walter et al. 2008, Olson et al. 2016).

Production of SCFAs

Some probiotic microorganisms, including *Lactobacillus*, may participate in several important functions in the human intestinal microbiome, including the production of SCFAs (Markowiak-Kopeć et al. 2020). SCFAs in the microbiota are the result of bacterial fermentation of non-digestible carbohydrates (NDCs). The SCFAs produced are mainly acetic acid, propionic acid, and butyric acid. (Chaia et al. 2003). SCFAs are not solely important for maintaining a normal function of the intestines, they are also involved in pH regulation, absorption of calcium, iron, and magnesium. In addition, SCFAs play an important role in inhibiting pathogens from invading the intestinal epithelium (Markowiak-Kopeć et al. 2020).

1.4.2 Early life nutrition and *L. reuteri*

As mentioned, humans establish their microbiota at birth and then continuously during their first years of life. The number of bacteria increases until the age of three and thereafter remains the same throughout life (Biogaia 2022). Therefore, the early life nutrition is of great importance, to initiate a good foundation and establish the possibility to a continued healthy gut.

Studies show that when *L. reuteri* is used to treat infantile colic, crying time can be reduced by 50% (Sung 2018, Savino et al. 2010, Indrio et al. 2014) and the frequency of bowel movements increased (Coccorullo et al. 2010). There are several additional studies on the effect of *L. reuteri* in infants, where it e.g. has proven to have a positive effect on traveller diarrhoea and constipation (Mu et al. 2018). These results increase the interest in using *L. reuteri* in infants. It also builds an eagerness to continue study its effects and further develop products containing *L. reuteri*.

1.5 Probiotic assessment methods

1.5.1 Fermentation media

Fermentation media is designed to contribute all nutrients required for microorganisms to ferment. A common media used for LAB is De Man, Rogosa and Sharpe agar (MRS). The main carbon source in this medium is glucose. For the ability to synthesize proteins the bacteria need a source of nitrogen. In MRS, peptone, Lab-Lemco powder and yeast extract is used for this purpose. The yeast extract also contributes as a source of micronutrients and vitamins. As an inorganic nutrient, manganese sulphate and magnesium are used. Additionally, MRS also contains sorbitan mono-oelate, dipotassium hydrogen phosphate, sodium acetate, triammonium citrate. The pH of the media is 6.2 (Oxoid 2022).

According to a study made to compare the impact of different pH in fermentation media, *L. reuteri* DSM 17938, grown at higher pH (5.5-6.5) had a higher survival to freeze-drying than cells grown at lower pH (4.5-5.5). However, the bacteria grown at a lower pH had a better survivability against GI tract conditions than those grown at a higher pH (Hernández et al. 2019). Consequently, the MRS with a pH at 6.2 could be a preferable medium when it comes to lyophilization, although less suitable regarding tolerance testing of the GI tract conditions.

1.5.2 Gastric acid and bile salt tolerance

In the aim of developing a probiotic product, it is essential to investigate the bacteria's ability to survive the passage through the human GI tract. This is usually done through simulating the conditions of the GI tract *in vitro*. Gastric acid/low pH and bile salts are the main stress factors to consider when examining the tolerance of a strain. Design and performance of these *in vitro* tolerance assays can vary, likewise the methods for analysing the tolerance results. However, plate enumeration of CFUs before and after the exposure for stress is a common method. Assay components which may differ are state of the investigated bacterial culture (fresh cells, freeze-dried cells etc.); type of stressor (artificial, biological) and exposure times (Wendel 2022). This calls for diligence when comparing results from assays of various form. It should also be mentioned that *in vitro* assays could

be less or more stressful and damaging to the cells compared to *in vivo* environments. The former situation only exposes the bacteria for one stressor at the time, while the latter compose a complex mixture of different stress factors simultaneously. In addition, the *in vivo* conditions might vary over time, unlike the commonly static conditions in an *in vitro* assay (Wendel 2022).

1.5.3 Mucus adhesion

In addition to examine a probiotic's ability to survive the conditions of the GI tract, it is also significant to assess its capability to interact with its host. Mucus membranes in the GI tract is the most prominent point of interaction between probiotics and humans. This entails mucus adhesion as a prime property for an effective probiotic. A general way of assessing mucus adhesion *in vitro* involves quantification of fractional residual bacteria bound to mucus-coated surfaces after washing. Mucus material is commonly extracted from gut epithelial cells or organic tissues and washing is performed with fluids of ranging pH values. Mayes et al. have shown that some bacteria strains might show altering adhesion characteristics depending on the organic mucus material, which should be considered when analysing a result. The quantifying method can vary, however enumeration of CFUs on agar plates is widely applied (Mayes et al. 2020).

1.6 Aim

As discussed, a well-functioning microbiota is essential for general health and proper development of an infant. Human milk has been proven to greatly contribute to establishing a balanced microbiota, as it contains many bioactive compounds such as various bacteria and their metabolites, immune cells, and human milk oligosaccharides (Salminen et al. 2020). Thus, breastfeeding is very important for the infant. The World Health Organization recommends breastfeeding exclusively for the first six months of life (WHO 2021) however, this is not an option for everyone. When breastfeeding isn't an option, parents relay on alternatives such as various infant formulas for feeding their baby. In the aim of replacing the complex human milk composition while meeting the nutritional needs of an infant, infant formulas continue to develop. In an article from 2020, Salminen et al. discussed the application of pro-, pre-, syn-, and post-biotics in infant formulas as an approach to mimic human milk as close as possible. Some products, such as probiotic-containing formulas and FIFs are already on the market (Salminen et al. 2020), even so, there is room for improvement. As stated, *L. reuteri* DSM 17938 has been proven to have several positive effects in infant gut-health, furthermore, many properties and prerequisites of the strain are known (BioGaia 2020). However, the impact of various fermentation factors on strain DSM 17938 has not yet been fully explored (Hernández et al. 2019).

This study aimed to investigate the hypothesis if fermentation conditions could affect probiotic features of rehydrated freeze-dried *L. reuteri* DSM 17938 cells. The bacterial tolerance for bile salts and low pH, as well as ability to adhere to mucus

was assessed. In addition, HPLC was performed on the supernatants to examine metabolites. Two different infant formulas were used as a fermentation media for 24 hours. Formula A and B are produced and distributed by two separate companies but have similar, yet not identical, ingredients. Both formulas are hypoallergic, free amino-based powders, enriched with iron.

2. Method

Web of Science, Google Scholar, Scopus, Science Direct, and PubMed databases were used for the systematic search of relevant literature up to the year 2022.

2.1 Fermentation and lyophilization

Previously established and controlled frozen stocks of *L. reuteri* DSM 17938, kindly provided by BioGaia AB, was used as a primary inoculum in this study.

Frozen stocks of *L. reuteri* DSM 17938 were inoculated into Man Rogosa Sharpe (MRS) broth (Oxoid, Thermo Scientific, Hampshire, England) and anaerobically incubated (Anaerocult, Merck, Darmstadt, Germany) overnight at 37 °C. Infant formulas A and B were prepared according to the producer included instructions. Thereafter, the culture was inoculated into formula A and B in the proportion of 1%. The same procedure was done in MRS broth as a control. The samples were anaerobically incubated for 22.5 h at 37 °C. After incubation the pH was measured, and samples were serially diluted and plated on MRS agar (Merck, Darmstadt, Germany) for viable count before lyophilization.

Samples A and B were supplemented with sucrose powder (10%) following the fermentation. The control (C) was centrifuged (4000 rpm, 20 °C, 15 min) and the supernatant was discarded. The remaining bacterial pellet was resuspended in sucrose solution (10%). All samples were aliquoted into vials of two ml and stored at –50 °C overnight. The vials were freeze-dried for 48 h (Martin CHRIST, GmBH, Epsilon 2-6D LSCplus, programme 50 h). A graph with the drying program can be found in appendix I. Thereafter the vials were capped under pressure, taken out and stored in –20 °C. Dilution series were made from each sample (A, B, C) and plated on MRS agar plates. This was done to examine how lyophilization had affected the bacteria.

2.2 Assays

Two tolerance assays and one binding assay were performed to simulate the path of the bacteria through the human gut; a gastric acid tolerance test; a bile salt tolerance test; and a mucus adhesion test. The purpose was to investigate the tolerance and adhesion properties of *L. reuteri*, DSM 17938, and if these had been

affected depending on the growth media. All assays were performed in biological triplets as well as technical duplicates.

2.2.1 Gastric acid tolerance assay

The method for gastric acid tolerance was based on two publications (Rosander 2008, Wall et al. 2007). To prepare the synthetic gastric juice, lacking enzymes and bile, the composition from Wall et al. 2007 was used; 2.075 g Bacterial peptone (Oxoid), 0.875 g D-glucose (Sigma, S:t Louis, MO, USA), 0.5125 g NaCl (VWR, Pennsylvania, USA), 0.275 g $CaCl_2$ (Fluka Chemicals), 0.0925 g KCl (Merck, Darmstadt, Germany) was dissolved and mixed in 250 ml water. The solution was adjusted to pH 2.0 with HCl prior to autoclavation. At the day of experiments, freeze dried bacteria (sample A, B, and C) were resuspended in 2 ml phosphate buffer saline, PBS (Sigma). A reference sample was taken at t_0. 100 µl of each bacterial suspension were inoculated into 10 ml synthetic gastric juice, incubated in a water bath at 37°C. 100 µl were extracted from sample A, B and C at timepoints 20, 50 and 90 minutes. Relevant dilution series were made followed by viable counts on MRS agar, where standard plate colony counting as well as the drop method (Jett et al. 1997) was used. Plates were anaerobically incubated for 48h at 37°C after which colonies were counted.

2.2.2 Bile tolerance assay

The method for bile tolerance was based on one publication (Pallin, 2018) and further developed in advice by the supervisors. The assay was divided into two parts: bacterial survival in bile and bacterial growth in bile. For the survival test, porcine gall bile (ChemCruz, Dallas, USA) was added to MRS broth (Oxoid) (0.5%). For the growth test, bovine bile extracted from ox (Sigma) was added to MRS broth (Oxoid), preparing suspensions of 0.1%, 0.2% and 0.3%.

Survival assay

Freeze dried bacteria (sample A, B, and C) were resuspended in 2 ml PBS (Sigma). A reference sample at t_0 was taken. Plates were anaerobically incubated (Anaerocult, Merck) at 37°C for 48 hours. 100 µl of each bacterial suspension were inoculated into 10 ml of MRS broth with porcine bile, (0.5%). The samples were incubated in 37°C. From each sample (A, B, C), 100 µl were taken at timepoints 30, 60 and 90 minutes. Dilution series were made followed by viable counts on MRS agar using standard plate colony counting as well as the drop method (Jett et al. 1997). The former method was used for sample A and C, while the latter was applied to sample B, to ensure a broad detection range as this sample was known to have a lower CFU/ml than the others. Plates were anaerobically incubated for 48h at 37°C.

Growth assessment in plate reader

Freeze dried bacteria (sample A, B, C) were resuspended in 2 ml PBS. 10 µl of each bacterial suspension were inoculated into 990 µl of MRS broth with bovine bile,

(0.1%, 0.2%, 0.3%). 300 µl of each sample were added into wells and placed in the plate reader for 24 h (Thermo Labsystems Bioscreen C Automated Microbiology Growth Curve Analysis System Type FP-1100-C, Raisio, Finland).

2.2.3 Mucus adhesion assay

Serial dilutions and viable counts were taken for reference. For coating, 32 µl mucus material (extracted from pig intestine) was diluted in 3968 µl PBS Tween 20, (pH 6), (PBST6) making a total of 4 ml. The solution was then incubated overnight in microtiter wells (150 µl in each well) at 4°C with slow rotation. After incubation, the wells were washed with PBS (pH 7.4) with 0.05% Tween 20 (PBST). Thereafter the wells were blocked with 0.2 ml PBS (pH 7.4) with 1% Tween 20 for 1 h and then washed twice with PBST. Freeze dried bacteria samples of A, B and C were resuspended in 2 ml PBS. The control was diluted (x100) to better match initial CFUs/ml in sample A and B. Bacterial suspensions were centrifuged (11 000 rpm, 2 min), washed with PBST twice and finally resuspended in PBST6. Thereafter, 150 µl bacterial suspension from each vial was added to each well and anaerobically incubated for binding at 37 °C for 4 h with slow rotation. After incubation, the wells were washed with PBST four times and thereafter treated with 150 µl trypsin EDTA for release of the bacteria and incubated anaerobically at 37°C for 30 min. The bacterial suspensions were diluted, then plated on MRS agar plates and anaerobically incubated for 48 h at 37°C.

This assay was performed twice due to ambiguous results in the first run. One control was diluted (x100) to better match initial CFUs/ml in sample A and B, while another control was left undiluted to assess the method.

2.3 HPLC

Freeze dried bacteria (sample A, B, C) were resuspended in 2 ml PBS (Sigma) and centrifuged for 15 minutes at 11 000 rpm. Thereafter the clear segment of the supernatant was collected, while the fat layer and remaining pellet was discarded. The same procedure was repeated once again. From each tube, 700 µl of the clear supernatant was transferred to new 2 ml Eppendorf tubes to which 70 µl of 5 M H_2SO_4 was added. Thereafter, the samples were centrifuged once again at 11 000 rpm for 15 min. All samples were filtrated (NYLON 0.2 µl, AGILENT) into HPLC vials and ran in the HPLC with an Agilent 1100 Series with a refractive index detector and an ion exclusion column (Rezex ROA - Organic Acid H^+, 300 x 7.80 mm, Phenomenex). The mobile phase used was 5 mM H_2SO_4 with a flow rate of 0.6 ml min^{-1}.

HPLC results were analysed with help of a column specific in-house compound list.

2.4 Infant formula assessment

To ensure the results of all assays, both infant formulas were investigated and tested for other possible microbes. Rehydrated powder of formula A and B were plated on MRS (GranuCult) and BHI (GranuCult, VWR Chemicals) agar plates. The plates were incubated both anaerobically and aerobically for 24 h at 37° C. Also, pH measurements were made on untreated samples of both formulas.

2.5 Data analysis

A two-way ANOVA was used to estimate statistics on the obtained data from the gastric acid and bile tolerance assay.

3. Results

3.1 Detection limit

The detection limit in this study was $1x10^2$ CFU/ml, enumeration under this limit was designated too few to count (TFTC).

3.2 Freeze-drying survival

Freeze drying survivability is defined as the ratio between intact cells before and after freeze drying. Viability of bacteria after lyophilization in sample A was calculated to $1.1x10^7$ CFU/ml, which equals a survival rate of 38 % from $2.9x10^7$ CFU/ml before freeze drying. In sample B, the survival bacteria were counted to $7.6x10^4$ CFU/ml, which equals a survival value of 15 % compared to $5.1x10^5$ CFU/ml, that was counted before lyophilization. The control had a survival rate of 55 % after freeze drying, from $1.2x10^9$ CFU/ml to $6.7x10^8$ CFU/ml.

3.3 Gastric acid tolerance

The untreated reference average for this assay was $2.5x10^7$ CFU/ml in sample A, $1.5x10^5$ CFU/ml in sample B and $5.0x10^8$ CFU/ml in the control. Average viable counts for sample A, B, and the control after exposure to gastric acid were calculated and are shown in figure 1.

After 20 minutes of exposure to gastric acid, a significant difference between sample A and B ($p=0.0052$), as well as between sample A and the control ($p=0.0010$), and between sample B and the control ($p=<0.0001$) was observed. Furthermore, a survival difference between sample A and B was observed at timepoint 50 minutes and 90 minutes which was also distinguished from the results of the control.

Sample A showed a population decrease of 4-log at t_{20}, from $2.5x10^7$ to $6.5x10^3$ CFU/ml. The sample showed another 1-log decrease at t_{50}, from $6.5x10^3$ to $3.3x10^2$ CFU/ml. At t_{90}, bacterial colonies were TFTC. Sample B showed high sensitivity to the treatment, whereas no growth was observed at any of the measured timepoints. The control displayed a population decrease by 2-log at t_{20}, from

$5.0x10^8$ to $3.3x10^6$ CFU/ml and then further 1-log reduction at t_{50}, from $3.3x10^6$ to $2.6x10^5$ CFU/ml. At t_{90} the population had decreased another 2-log, from $2.6x10^5$ to $7.8x10^3$ CFU/ml.

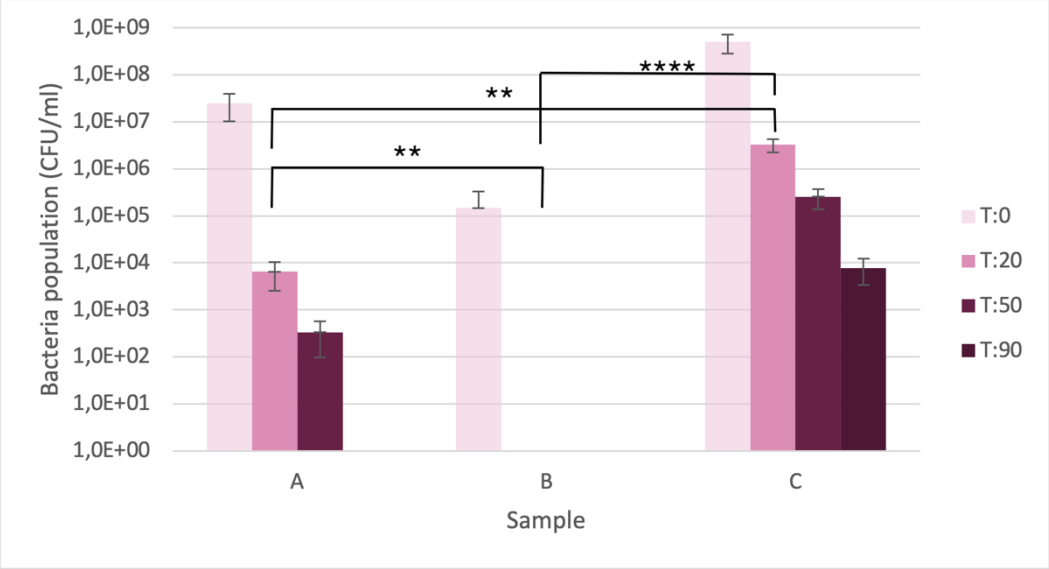

*Figure 1. Average of viable count results for L. reuteri in gastric acid tolerance assay (CFU/ml), pH 2. Viable counts were performed at each timepoint and counted after 48 h incubation anaerobically. Error bars represent standard deviations. ** Indicates a significant difference (p<0.01), **** Indicates a significant difference (p<0.0001). Copyright Astrid Hägg, Carolin Marklund.*

3.4 Bile salt exposure

The untreated (t_0) average for this assay was $4.0x10^6$ CFU/ml in sample A, $1.8x10^5$ CFU/ml in sample B, and $6.1x10^8$ CFU/ml in the control. Average viable counts for sample A, B, and the control after exposure to bile salts were calculated and are shown in figure 2.

No significant difference could be identified between sample A, B, and the control after 30 minutes exposure to bile. However, there was a significant difference between sample A and B (p=0.0035) and between sample B and the control (p=0.0108) after 60 minutes of exposure to bile. After 90 minutes of exposure to bile, a significant difference between sample A and B (p=0.0059) as well as between sample B and the control (p=0.0358) could be identified. Sample A showed a population decrease of almost 3-log at t_{30}, from $4.0x10^6$ to $9.5x10^3$ CFU/ml. At t_{60}, the growth in sample A had increased, from $9.5x10^3$ to $2.3x10^4$ CFU/ml. The sample showed another rise at t_{90}, from $2.3x10^4$ to $3.9x10^4$ CFU/ml. Sample B displayed 1-log decrease at t_{30}, from $1.8x10^5$ to $2.0x10^4$ CFU/ml. At t_{60} sample B showed another 1-log reduction, from $2.0x10^4$ to $1.7x10^3$ CFU/ml and then further 1-log decrease at t_{90}, from $1.7x10^3$ to $1.5x10^2$ CFU/ml. The control (C) displayed a population decrease of almost 5-log at t_{30}, from $6.1x10^8$ to $4.5x10^3$ CFU/ml. At t_{60} the sample showed an increase from $4.5x10^3$ to $1.2x10^4$ CFU/ml. The control then showed a population reduction from $1.2x10^4$ to $6.2x10^3$ CFU/ml.

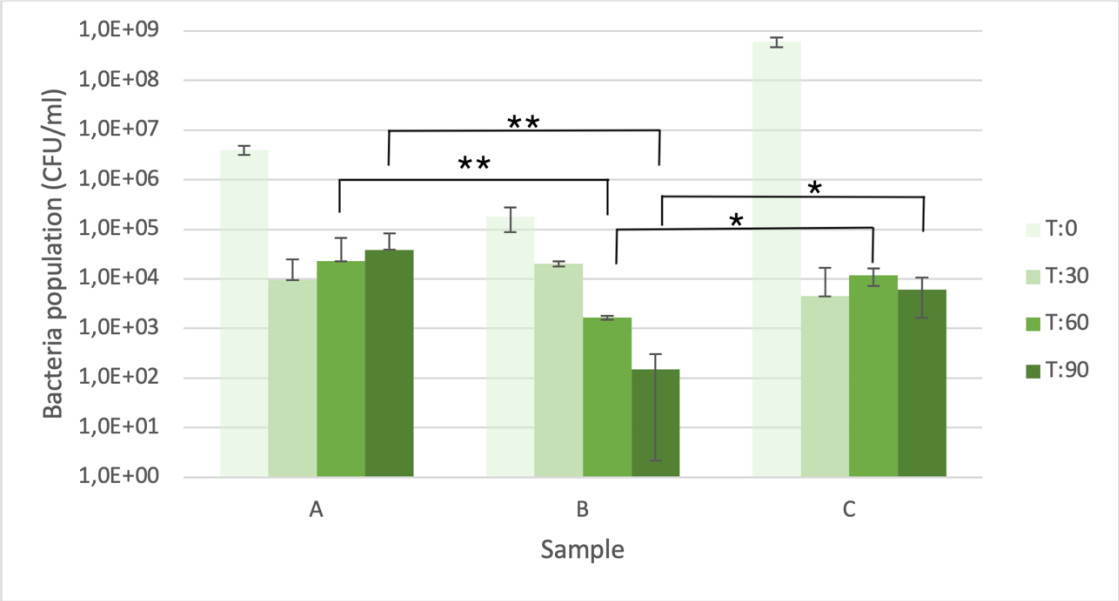

*Figure 2. Average of viable count results for L. reuteri in bile salt (0.5%) tolerance assay (CFU/ml. Viable counts were performed at each timepoint and counted after 48 h incubation anaerobically. Error bars represent standard deviations. *Indicates a significant difference ($p<0.05$); ** Indicates a significant difference ($p<0.01$). Copyright Astrid Hägg, Carolin Marklund.*

3.5 Growth in bile

Sample A showed the highest final OD in 0.1% bile, with an OD value of 1.83, after 20 hours and 15 minutes. The highest maximal OD in sample B is measured to 1.70 in 0.3% bile, after 24 hours. The control showed the highest maximal OD in 0.3% bile, with an OD value of 1.95 after 24 hours.

Table 1. OD measurements from growth in bile (0.1%, 0.2%, 0.3%) assessment in plate reader. Copyright: Astrid Hägg, Carolin Marklund

	Sample	Initial OD	Max OD	Time of max OD (h)	Lag phase (h)
Reference	A	0.50	1.89	19:45	6
	B	0.90	1.90	23:45	12
	C	0.30	1.82	17:45	3
0.1%	A	0.41	1.83	20:15	6
	B	0.76	1.63	21:00	8
	C	0.22	1.76	15:15	3
0.2%	A	0.46	1.82	23:30	6
	B	0.83	1.55	18:00	11
	C	0.22	1.91	15:45	4
0.3%	A	0.41	1.71	24:00	6
	B	0.87	1.70	24:00	13
	C	0.23	1.96	22.15	4

A constant trend in this assay was that sample B had the longest lag phase in all bile concentrations (0.1%, 0.2%, 0.3%), followed by sample A while the control (C) had the shortest lag phase (figure 3-6). Sample A showed a lag phase of around six hours in all concentrations, similar to the reference sample, with a slightly reduced rising curve in 0.3% bile. Sample B showed a lag phase of around twelve hours in the reference sample, around eight hours in 0.1% bile, and approximately eleven hours in 0.2% bile. In 0.3% bile, sample B displayed a lag phase of around thirteen hours. The control showed a lag phase of three hours in the reference and in 0.1% bile. Thereafter, the lag phase was around four hours in 0.2% and 0.3% bile, although the rising curve of 0.3% is slightly declined.

In the 0.1% and 0.2% bile test, sample A shows a similar growth curve to the control. However, the samples distinguished after maximum OD, as the control declines while sample A stays around the same OD. Sample B shows a less dramatic curve with a slight decline after maximum OD in 0.1% bile and a larger decline in 0.2% bile. In 0.3% bile, all samples have a less dramatic curve, compared to the tests with lower concentration.

It is important to clarify that the media used for sample A and B was more turbid than the control. This is due to ingredients of the infant formulas. Furthermore, formula B displayed an even more turbid suspension than formula A. This explains the different initial OD value of the samples, and consequently also the final OD value (Table 1). However, final OD was not always equivalent to the maximum OD.

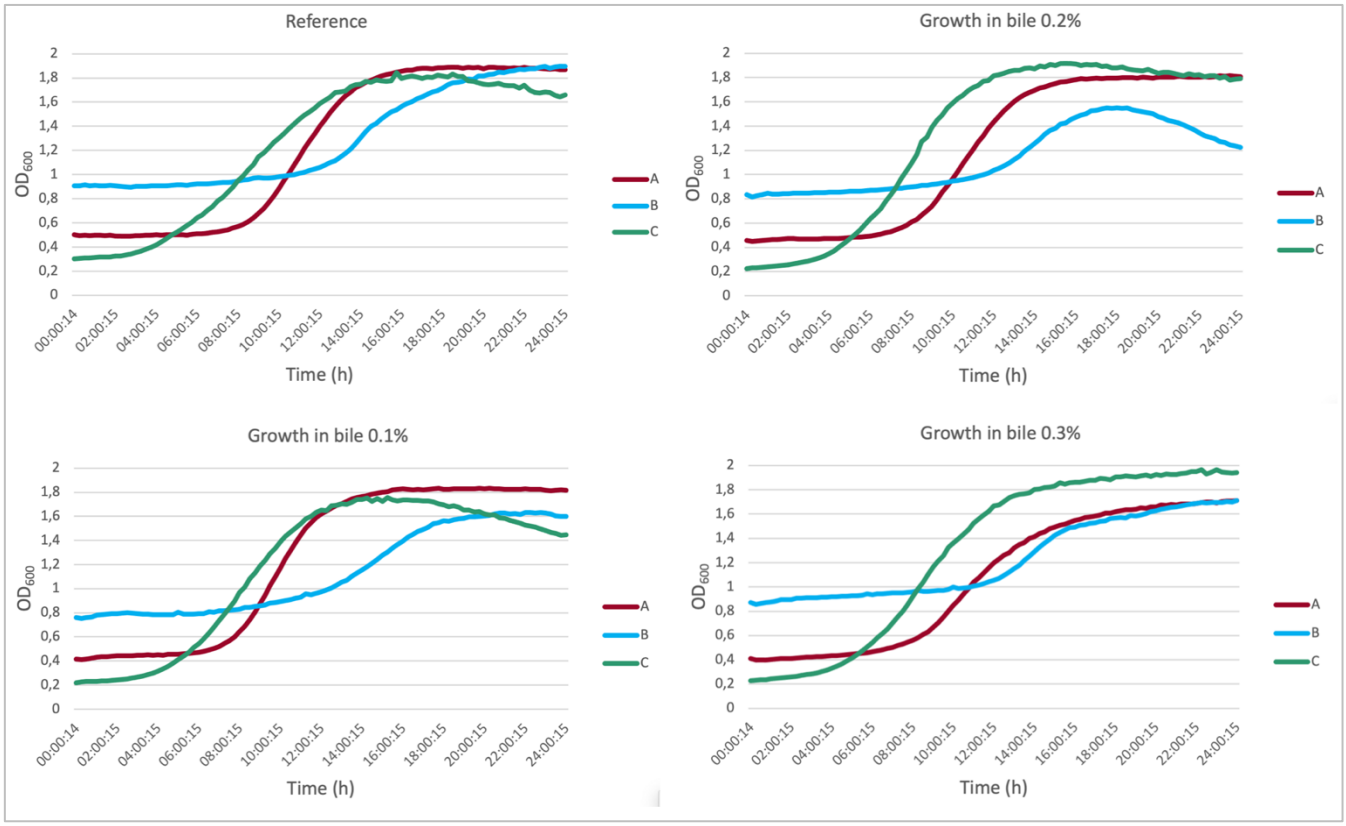

Figure 3. Average OD measurement of growth of L. reuteri in bile salt (0.1%, 0.2%, 0.3%) growth assay in sample A, B, C. OD was measured every 15 minutes in the plate reader and plotted. Reference has no bile salts. Copyright: Astrid Hägg, Carolin Marklund.

3.6 Mucus adhesion

The viable count results from the first mucus adhesion assay showed low numbers. 1/6 plates from wells with sample A displayed growth ($1x10^4$ CFU/ml). The untreated reference from vial A had a viable count of $1.2x10^8$ CFU/ml ($\bar{x}$). 2/6 plates from wells with sample B showed growth ($1x10^2$ CFU/ml, $1x10^3$ CFU/ml). The viable count of the untreated reference from vial B was $1.4x10^6$ CFU/ml ($\bar{x}$). The plates from vial C had a viable count of $3x10^3$, $1x10^4$, $1x10^3$ and $1x10^3$ CFU/ml. The untreated reference from vial C showed a viable count of $1.4x10^9$ CFU/ml ($\bar{x}$).

In the second mucus adhesion assay, the wells from sample A and B showed no visible growth in any of the performed dilutions. The untreated reference from sample A had an average viable count of $2.3x10^6$ CFU/ml and the untreated reference from sample B showed a viable count of $7.3x10^4$ CFU/ml ($\bar{x}$). The undiluted control had an average viable count of $6.4x10^3$ CFU/ml. The untreated reference from wells with the control showed a viable count of $2.4x10^7$ CFU/ml ($\bar{x}$).

3.7 Infant formula assessment

The results from the infant formula assessment, both on the MRS and BHI plates, showed no other microbial growth under neither aerobic nor anaerobic conditions. The pH of formula A was measured to 7.36 and the pH of formula B was 7.45. The pH after fermentation was 5.75 in formula A and 6.94 in formula B.

3.8 HPLC

The HPLC results displayed eleven peaks for sample A and B while the control only displayed 6-7 peaks on the chromatogram (appendix IV). From the identified peaks, following substances could be assumed to be present in all samples (A, B, C), galactose, fructose, lactic acid, and acetate. Sample A and B both showed peaks for maltotetraose, maltose and propionate. Furthermore, only sample A showed peaks for ethanol while only sample B showed peaks for glycerol and fumaric acid.

Discussion

This study investigated if fermentation and lyophilization in infant formula have an impact on tolerance for bile salts and low pH, as well as on mucus adhesion in *L. reuteri* DSM 17938 cells.

In conclusion, given that neither of the infant formulas in this experiment are optimized for bacterial growth, it is exciting to see that *L. reuteri* DSM 17938 is growing relatively well. The demonstrated bacterial growth in formula A and B entails that this type of fermentation media can be considered for prospective consideration probiotic products.

As previously mentioned, glucose is the primary carbon source for the bacteria in MRS. Infant formula A and B contain a high amount of carbohydrates. However, as the specific carbohydrates are not stated on the label, it is possible that these might be in present in a form which is hard for DSM 17938 to break down and use as energy source. Consequently, this could imply that infant formula A and especially B serve as poor growth substrates for DSM 17938. As the survival rate of lyophilization (15% in B; 38 % in A; 55% in the control) shows, all bacteria in this study were damaged during this process. In particularly the bacteria in formula B, but also in formula A and the control. Cell damage can happen due to dehydration, freeze stress, shear stress and osmotic stress during lyophilization. Additionally, the removal of water during this method can result in instability of DNA, proteins and lipids which ultimately leads to changes in cell structure (Santivarangkna, C. 2008). The ranging survival rate indicates that DSM 17938 has a better tolerance to freeze-drying stress when fermented in MRS compared to infant formula A and B. Most likely, this is partly due to the fact that the bacteria in formula A and B are struggling to obtain energy from their fermentation media.

In addition, the lyophilization method for the bacteria in formula A and B in this study was different from the standard method which was performed on the control. Prior to freeze-drying, the control was centrifuged, and supernatant was discarded after which the bacteria pellet was resuspended in sucrose solution. Whereas sample A and B were freeze-dried directly in their fermentation media (infant formula) with an addition of sucrose powder. This method disparity could also be a contributing factor to the different survival rates.

Tolerance to gastric acid

The 4-log reduction in sample A is significantly separated, from the 2-log reduction of the control after 20 minutes for exposure to gastric acid. Whereas the bacteria in

sample B does not seem to be vital at all at this point, indicating that the two formulas impact the pH tolerance differently. After 50 minutes exposure, sample A showed a number of live bacteria about 2-log lower than the control. After 90 minutes, the bacteria in the control were the only survivors. Although, it should be mentioned that 90 minutes is a top edged time frame, to capture any extreme viability, as the food normally only stays in the stomach for around 40 minutes (Shaikh et al. 2021).

The immense drop in viable counts in all samples, including the control, indicates that the bacteria does not tolerate the low pH of the synthetic gastric acid. The sharp difference in result between sample A and B could be due to several factors. First of all, sample B has a shorter interval between its initial CFU/ml and the detection limit. As sample B already had a low number of viable counts prior to this assay, one could suspect that the bacteria cells fermented in infant formula B were more stressed and therefore less able to tolerate the low pH stress. Although, the fact that the control also drops greatly in viable counts indicates that this assay was harsh for DSM 17938 in general. Possibly, the bacteria were damaged due to to dehydration, freeze stress, shear stress and osmotic stress during lyophilization. Additionally, the removal of water during this method can result in instability of DNA, proteins and lipids which ultimately leads to changes in cell structure (Santivarangkna, C. 2008). In an already damaged state, the DSM 17938 cells were most likely less prone to survive and proliferate. Another reason for the low viable counts could be the fact that the pH value (2.0) of the synthetic gastric acid was in the lower range and resemble a type of stress that doesn't quite mimic physiological conditions in the gut. The pH in the human gut varies a lot, depending on e.g., diet and hour of the day. It rarely drops below 3.0 when food is present, even though pure gastric acid has a pH of 1.8 (Howard et al. 2022). As reported, DSM 17938 cells grown at a lower pH (4.5-5.5) had a better survivability against GI tract conditions than those grown at a higher pH (5.5-6.5) (Hernández et al. 2019). The measured pH of formula A (5.75) and formula B (6.94) after fermentation was higher compared to the MRS (3.67) after fermentation. Thus, excluding the compositional changes between formula and MRS, the results in this study endorse that a lower pH of the fermentation media might be favourable when the target is better tolerance to the conditions of the GI tract.

Bile exposure

To conclude this assay, the significant differences identified between sample A and B as well as sample B and the control at t_{60} and t_{90} (figure 2) is a clear indication that formula B appears to be a less suitable fermentation media for *L. reuteri* compared to formula A, when the aim is to improve its tolerance and growth in bile salt.

The bile salt exposure assay revealed a large drop in number of bacteria in both sample A and the control after 30 minutes. In fact, the drop of bacteria in the control is even more dramatic than in sample A, indicating a possible protection from other ingredients in the formula. Thereafter, the numbers are more or less stabilized throughout the analysis. However, it is interesting to note that the number of

bacteria in sample A stays higher than the control throughout the whole experiment, even though it had a lower initial CFU/ml. The bacteria grown in formula B successively reduces through the assay, not reaching a stable state as the other two, appearing as if the stress was continuously detrimental. The number of bacteria in formula B is lower compared to formula A and the control at all timepoints. This indicates that formula B is a less favourable choice of medium for fermentation of *L. reuteri*, at least when aiming to select a suitable medium that allows for persistence in the human intestinal tract. Formula A on the other hand, shows to be at least equivalent to the control, if not preferred, for *L. reuteri* to ferment in, when the aim is improved bile tolerance.

After the population decrease of almost 3-log at $t_{30,}$ sample A surprisingly seem to increase in viable counts at t_{60} and $t_{90.}$ However, this result is somewhat misleading due suspected method errors. Neither of the duplicates in test 1 showed growth at $t_{30,}$ for sample A, while both duplicates in tests 2 and 3 did (appendix III). This entails a lower mean CFU/ml for sample A at t_{30}, consequently making the comparison to viable counts at$_{60}$ look like an increase while it presumably was closer to unchanged. Additionally, the duplicates of sample A at t_{90} in tests 2 and 3 are close in CFU numbers, while the duplicates in test 1 diverge. One of the duplicates displayed about 1-log higher CFUs/ml compared to the other, which increases the mean. In summary, one can suspect that there were several errors in the method of test 1, implying a population increase.

Bile growth

As mentioned, all samples have different initial concentrations. With this in mind, the bacteria in sample A seems most vital, according to its sharp upward curve in all three bile concentrations. It also keeps a stable OD throughout the measurement, without decreasing. The bacteria in sample B, however, does not show the same alertness, on the contrary, which display a distinct difference between the bacteria grown in the two formulas.

Both sample A and B had a longer lag phase than the control. Sample B had the longest lag phase in all bile concentrations. The lag phase of sample A was shorter than in sample B, although longer than in the control. However, as all samples have different bacterial concentrations at t_0 which could impact the lag phase length. A possible explanation for the longer lag phase in sample B could be due to the fact that the bacteria weren't able to break down and utilize molecules in formula B for energy. In addition, it is interesting to address the fact that the OD in sample B at 0.3% bile, as well as in the reference, does not flatten after 24 hours. Is this only caused by the delay of the lag phase or is it an indication that the bacteria in sample B have a better tolerance to bile at higher concentrations and also have the ability to grow in these conditions (Gray 2019). Additional incubation time in the plate reader would provide a more comprehensive picture of how long the curve continues and thus shed some light on this hypothesis.

Interestingly, sample B and the control reach their maximum OD in 0.3% bile salt. This result is not expected, as a higher bile concentration supposedly would entail a rougher environment for the bacteria. In a review from 2017, Long et al. discussed

the interactions between gut bacteria and bile. As mentioned, bile found in the host intestine is mainly consisting of BAs. The review highlights how microbial enzymes, such as bile salt hydrolase (BSH) contribute significantly to BA metabolism which transforms conjugated BAs into unconjugated BAs and primary BAs into secondary BAs. Further, these BSH enzymes has been reported found in *Lactobaciullus spp.* Additionally, the review emphasizes how conjugated BAs in the intestine are known to be toxic to bacteria, thus influence their ability to grow in the gut. Consequently, making the presence of BSH advantageous and protective in bacteria. However, BSH activity also liberates amino acids which some bacteria can use as a source of energy (Long et al. 2017). However, it has not been found that DSM 17938 is capable of this type of bile metabolism.

Mucus binding

This assay was slightly modified due to the high turbidity in our samples. In the original method, OD is measured at 600nm to ensure that the samples have an initial recommended OD around 0.5, corresponding to approximately $1x10^7$ CFU/ml. The first mucus adhesion assay showed surprisingly low numbers, even in the control. Therefore, a second assay was performed where another undiluted control was added to the method as a way of assessing the process. In the second assay, the undiluted control showed an average viable count of $6.4x10^3$ CFU/ml, indicating that the assay did work. A possible explanation for the general low results in this assay could be the fact that the initial concentration of bacteria in sample A and B were much lower compared to the control and moreover the recommended initial CFU/ml for this method.

It should also be mentioned that the formulas hold many components which could complicate this assay since there is a possibility that the bacteria could bind to these instead of the mucus cells.

HPLC

To measure which potential metabolites that were produced during fermentation, HPLC were run on the samples. Propionate was found in fermented sample A and B, but not in the fermented control. In sample A, propionate displayed a peak area of 36 510 to 40 379 milli-Absorbance Units (mAU) while sample B displayed almost the double area, 72 954 to 73 304 mAU. Additionally, glycerol was exclusively found in sample B, verified in the content description of the provider. Together, these results are interesting since glycerol is needed for reuterin production (Zhang et al. 2020) and propionate is an intermediate in the production chain of reuterin (Amin 2013). As mentioned, reuterin is an antimicrobial compound and an intermediary in mediating the probiotic health benefits of *L. reuteri* (Zhang et al. 2020). The fact that sample B contained both glycerol and propionate thus indicate that reuterin may have been produced in sample B. If this is the case, formula B might be advantageous in manufacturing products used for individuals with dysbiosis, as reuterin acts as an inhibitor of pathogens. While formula A is having a more general area of use. Sample A also contained propionate, implying that reuterin has been produced there as well, even though there was no peak for glycerol. The same cannot be said about the control, as it

showed no peak for propionate. Consequently, a possible future study should include an inhibition plate assay, investigating the actual presence of reuterin in the samples. In a study from 2020, Zhang et al. reported that DSM 17938 showed clear growth enhancement with addition of glycerol as well as greater capacity for reuterin production on maltose and glucose. This corresponds to the results of our study, as both maltose and glucose can be found in sample A and B (originating from the infant formula) along with propionate. Maltose and glucose are not present in the control.

Infant formula

The clear divergent results from the two formulas used in this study arouses curiosity regarding what the reason for the difference may be. Therefore, it is interesting to examine what distinguishes the two formulas in the ingredient lists. As previously mentioned, the two formulas consist of similar ingredients, with some differences in quantity. In addition, some substances are only included in one of the formulas and vice versa. Two interesting ingredients were taurine and L-carnitine which were found exclusively in formula A.

Considering more tangible factors, taurine is an example of a substance which is only found in formula A. Taurine is a sulphur-containing organic acid that is included in breast milk and is found to have several biological functions, for example, cell volume regulation, membrane stabilization, anti-oxidative activity, mitochondrial protein translocation, and modulation of intracellular calcium levels in human cells (Tochitani 2017). Interestingly, it is the most common free amino acid in human breast milk, after glutamic acid (Chuang et al. 2005). Since infants does not have the ability to synthesize taurine, transportation from the mother is important, both through the placenta, and through the breast milk, during perinatal life (Tochitani 2022). This taurine-transfer is not only an important part in the process of creating a bond between the mother and her offspring, but also to have a positive impact on the infant´s future health (Tochitani 2022). Due to this, it is of great interest to companies that manufacture infant formulas to add taurine to their product, to improve the nutritional properties and make it more similar to breast milk. However, there is no scientific evidence that the addition of taurine to infant formula have the same benefits regarding the development and health of the infant as breast milk, and therefore, more scientific research is needed (Almeida et al. 2021). Yet, what is relevant to know in relation to this project, is whether the presence of taurine in formula A, contributed to better bacterial growth. According to a new clinical study, taurine has useful properties for the body to use in its defence against pathogens. For instance, one metabolic by-product of taurine contributes to inhibit colonization of damaging bacteria and therefore promote the growth of probiotic bacteria. Taurine also serves as a microbial nutrient, and this could be one contributory factor to the bacteria in sample A growing better than in sample B (Stacy et al. 2021).

Another compound only found in formula A is L- carnitine. It is an amino acid that works as a transport molecule, important in fatty acid metabolism. L-carnitine is

also classed as a nonvitamin micronutrient. Humans can synthesize carnitine from lysine and methionine or receive it from the diet. However, a reduction of L-carnitine has been detected in infants, resulting in a common addition of the compound in infant formulas (Sleator et al. 2009).

It was noted early in this study that formula B was visually more turbid than formula A. As turbidity is an indication of presence of larger molecules in a solution, it could be suspected that one factor for the general low vitality and growth in formula B is that the bacteria are not able to break down and use these larger molecules. In addition, percentages of carbohydrates, fats and proteins are stated on the label of the formulas, however the exact composition of these is not specified. This gap of information implies a wide range of possible inequalities between formula A and B which could be important in terms of fermentation conditions. In summary, the reason for the differences in bacterial growth between formula A and B is most likely multifactorial and therefore hard to clearly identify within this study.

Conclusion

In conclusion, given that the infant formulas used as growth medium in this study are not optimized for bacterial growth, it is exciting to see that *L. reuteri* DSM 17938 is growing relatively well, since this is a prerequisite for considering infant formula as a future growth medium for probiotic products.

As discussed, viable counts as assessment method for the efficacy of a probiotic product could be questioned, as it measures viability. Perhaps even more so if the fermentation media is a part of the final product, as in the case of fermented infant formula. A product of this kind might not need the same high amount of living bacteria per dose (CFU/dose) as a lower number could be offset by present postbiotics. Although sample A and B both showed lower CFU/ml compared to the control, they might hold inactive bacterial cells and postbiotic components. In the light of assessing these compounds, which can't be measured in viable counts, future studies should focus on quantifying not only viable, but also dead cells as well as other components in the supernatant. HPLC assessment could be expanded and perhaps, for example, complemented by flow cytometry to assess multiple cellular properties and other present bioactive molecules. In the aim of further understanding and fairly assessing the composition of probiotic products, a redefinition of these might be useful. Today, probiotic products commonly contain pro- and post-biotics all at the same time, although the only thing being stated on the label is the viable counts of the probiotic bacteria. Products such as fermented infant formulas would probably benefit from a more extensive clarification of its actual content. The redefinition should not change the status of probiotics but rather include a more characterized "containing -biotic" products. Within this definition, it is important to also assess the safety and proven beneficial effects of all present strains and components.

On the other hand, additional components in probiotic products like fermented infant formula should also be investigated. It could be speculated that prebiotics or other additional substances in the product, such as various carbohydrates in the infant formulas, also could promote growth of pathogens. Although these

compounds ideally should promote growth of DSM 17938 and/or benefit its probiotic features, they could simultaneously have another impact on other bacteria present in the human gut. This *in vitro* study did not assess complications of this matter, but it should be considered to add a method for such when designing a "multi-containing -biotic" product.

The viability inequity between samples and the control when applied in assays of this study was addressed through primary comparing each sample to itself in the results of the assays. On this basis, the results of cultivating in formula A and B were later compared. Although this issue was partly addressed, it could still imply a variety of unequal prerequisite factors for the bacterial cells.

In conclusion, this study shows that fermenting DSM 17938 in infant formula *does* affect its probiotic features. Future studies should include further investigation of the composition of infant formula A and B to untangle their impact on the fermentation process. In addition, a more profound examination of the supernatant should be carried out to further assess postbiotic compounds in the fermented infant formula.

References

Agostoni, C., Goulet, O., Kolacek, S., Koletzko, B., Moreno, L., Puntis, J., Rigo, J., Shamir, R., Szajewska, H., Turck, D., & ESPGHAN Committee on Nutrition (2007). Fermented infant formulae without live bacteria. *Journal of Pediatric Gastroenterology and Nutrition*, 44 (3), 392–397. https://doi.org/10.1097/01.mpg.0000258887.93866.69 [2022-03-31]

Almeida, C.C., Mendonça Pereira, B.F., Leandro, K.C., Costa, M.P., Spisso, B.F. & Conte-Junior, C.A. (2021). Bioactive Compounds in Infant Formula and Their Effects on Infant Nutrition and Health: A Systematic Literature Review. *International Journal of Food Science*, 2021, e8850080. https://doi.org/10.1155/2021/8850080 [2022-04-05]

Amin, H.M., Hashem, A.M., Ashour, M.S. & Hatti-Kaul, R. (2013). 1,2 Propanediol utilization by Lactobacillus reuteri DSM 20016, role in bioconversion of glycerol to 1,3 propanediol, 3-hydroxypropionaldehyde and 3-hydroxypropionic acid. Journal of Genetic Engineering and Biotechnology, 11 (1), 53–59. https://doi.org/10.1016/j.jgeb.2012.12.002 [2022-03-30]

Biogaia – Mjölksyrabakterien Lactobacillus reuteri (2020). https://sv.biogaia.com/var-forskning/limosilactobacillus-reuteri-stammar/ [2022-03-30]

Chaia, A.P. & Oliver, G. (2003). Intestinal Microflora and Metabolic Activity. Gut Flora, Nutrition, Immunity and Health. Oxford, UK: Blackwell Publishing Ltd, 77–98. https://doi.org/10.1002/9780470774595.ch4 [2022-03-10]

Chuang, C.-K., Lin, S.-P., Lee, H.-C., Wang, T.-J., Shih, Y.-S., Huang, F.-Y. & Yeung, C.-Y. (2005). Free Amino Acids in Full-Term and Pre-Term Human Milk and Infant Formula. Journal of Pediatric Gastroenterology and Nutrition, 40 (4), 496–500. https://doi.org/10.1097/01.MPG.0000150407.30058.47 [2022-04-12]

Coccorullo, P., Strisciuglio, C., Martinelli, M., Miele, E., Greco, L. & Staiano, A. (2010). Lactobacillus reuteri (DSM 17938) in Infants with Functional Chronic Constipation: A Double-Blind, Randomized, Placebo-Controlled Study. The Journal of Pediatrics, 157 (4), 598–602. https://doi.org/10.1016/j.jpeds.2010.04.066 [2022-04-12]

Cuevas-González, P.F., Liceaga, A.M. & Aguilar-Toalá, J.E. (2020). Postbiotics and paraprobiotics: From concepts to applications. Food Research International, 136, 109502. https://doi.org/10.1016/j.foodres.2020.109502 [2022-04-12]

Fiore, W., Arioli, S. & Guglielmetti, S. (2020). The Neglected Microbial Components of Commercial Probiotic Formulations. Microorganisms, 8 (8), 1177. https://doi.org/10.3390/microorganisms8081177 [2022-04-03]

Fonseca, F., Cenard, S. & Passot, S. (2015). Freeze-Drying of Lactic Acid Bacteria. I: Wolkers, W.F. & Oldenhof, H. (red.) Cryopreservation and Freeze-Drying Protocols. New York, NY: Springer, 477–488. https://doi.org/10.1007/978-1-4939-2193-5_24 [2022-05-03]

Frese, S.A., Benson, A.K., Tannock, G.W., Loach, D.M., Kim, J., Zhang, M., Oh, P.L., Heng, N.C.K., Patil, P.B., Juge, N., MacKenzie, D.A., Pearson, B.M., Lapidus, A., Dalin, E., Tice, H., Goltsman, E., Land, M., Hauser, L., Ivanova, N., Kyrpides, N.C. & Walter, J. (2011). The Evolution of Host Specialization in the Vertebrate Gut Symbiont Lactobacillus reuteri. PLOS Genetics, 7 (2), e1001314. https://doi.org/10.1371/journal.pgen.1001314 [2022-03-30]

Gomaa, E.Z. (2020). Human gut microbiota/microbiome in health and diseases: a review. Antonie van Leeuwenhoek, 113 (12), 2019–2040. https://doi.org/10.1007/s10482-020-01474-7[2022-03-30]

Gray, D.A., Dugar, G., Gamba, P., Strahl, H., Jonker, M.J. & Hamoen, L.W. (2019). Extreme slow growth as alternative strategy to survive deep starvation in bacteria. *Nature Communications*, 10 (1), 890. https://doi.org/10.1038/s41467-019-08719-8 [2022-04-20]

Amin, H.M., Hashem, A.M., Ashour, M.S. & Hatti-Kaul, R. (2013). 1,2 Propanediol utilization by Lactobacillus reuteri DSM 20016, role in bioconversion of glycerol to 1,3 propanediol, 3-hydroxypropionaldehyde and 3-hydroxypropionic acid. Journal of Genetic Engineering and Biotechnology, 11 (1), 53–59. https://doi.org/10.1016/j.jgeb.2012.12.002 [2022-04-20]

Hernández, A., Larsson, C.U., Sawicki, R., van Niel, E.W.J., Roos, S. & Håkansson, S. (2019). Impact of the fermentation parameters pH and temperature on stress resilience of Lactobacillus reuteri DSM 17938. AMB Express, 9 (1), 66. https://doi.org/10.1186/s13568-019-0789-2 [2022-03-27]

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C. & Sanders, M.E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology, 11 (8), 506–514. https://doi.org/10.1038/nrgastro.2014.66 [2022-05-03]

Hou, K., Wu, Z.-X., Chen, X.-Y., Wang, J.-Q., Zhang, D., Xiao, C., Zhu, D., Koya, J.B., Wei, L., Li, J. & Chen, Z.-S. (2022). Microbiota in health and diseases. Signal Transduction and Targeted Therapy, 7 (1), 1–28. https://doi.org/10.1038/s41392-022-00974-4 [2022-03-27]

Howard F., Loomis Jr., D.C. (2022). Digestion in the Stomach - Food Enzyme Institute (u.å.). https://www.foodenzymeinstitute.com/content/Digestion-in-the-Stomach.aspx [2022-05-19]

Indrio, F., Ladisa, G., Mautone, A. & Montagna, O. (2007). Effect of a fermented formula on thymus size and stool pH in healthy term infants. Pediatric Research, 62 (1), 98–100. https://doi.org/10.1203/pdr.0b013e31806772d3 [2022-03-27]

Indrio, F., Di Mauro, A., Riezzo, G., Civardi, E., Intini, C., Corvaglia, L., Ballardini, E., Bisceglia, M., Cinquetti, M., Brazzoduro, E., Del Vecchio, A., Tafuri, S. & Francavilla, R. (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. https://doi.org/10.1001/jamapediatrics.2013.4367 [2022-03-27]

Jett, B.D., Hatter, K.L., Huycke, M.M. & Gilmore, M.S. (1997). Simplified Agar Plate Method for Quantifying Viable Bacteria. BioTechniques, 23 (4), 648–650. https://doi.org/10.2144/97234bm22 [2022-03-27]

Long, S.L., Gahan, C.G.M. & Joyce, S.A. (2017). Interactions between gut bacteria and bile in health and disease. Molecular Aspects of Medicine, 56, 54–65. https://doi.org/10.1016/j.mam.2017.06.002 [2022-03-31]

Mays, Z.J.S., Chappell, T.C. & Nair, N.U. (2020). Quantifying and engineering mucus adhesion of probiotics. ACS synthetic biology, 9 (2), 356–367. https://doi.org/10.1021/acssynbio.9b00356 [2022-03-31]

Microbiome (2022). International Scientific Association for Probiotics and Prebiotics1 (ISAPP). https://isappscience.org/for-scientists/resources/microbiome/ [2022-03-31]

Mu, Q., Tavella, V.J. & Luo, X.M. (2018). Role of Lactobacillus reuteri in Human Health and Diseases. *Frontiers in Microbiology*, 9, 757. https://doi.org/10.3389/fmicb.2018.00757 [2022-05-19]

Olson, J.K., Rager, T.M., Navarro, J.B., Mashburn-Warren, L., Goodman, S.D. & Besner, G.E. (2016). Harvesting the benefits of biofilms: A novel probiotic delivery system for the prevention of necrotizing enterocolitis. Journal of Pediatric Surgery, 51 (6), 936–941. https://doi.org/10.1016/j.jpedsurg.2016.02.062' [2022-05-19]

Oxoid - Product Detail (2022). http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0361&c=UK&lang=EN [2022-05-06]

Pallin, A. (2018). Improving the functional properties of Lactobacillus uteri. Faculty of Natural Resources and Agricultural Sciences Department of Molecular Sciences

Uppsala. Available: https://pub.epsilon.slu.se/15635/7/pallin_a_180803.pdf [2022-05-01]

Probiotics (2022). International Scientific Association for Probiotics and Prebiotics2 (ISAPP). https://isappscience.org/for-scientists/resources/probiotics/ [2022-03-31]

Roos, S. & Jonsson, H. 2002 (u.å.). A high-molecular-mass cell-surface protein from Lactobacillus reuteri 1063 adheres to mucus componentsThe GenBank accession number for the sequence reported in this paper is AF120104. Microbiology, 148 (2), 433–442. https://doi.org/10.1099/00221287-148-2-433 [2022-05-19]

Rosander, A., Connolly, E. & Roos, S. (2008). Removal of Antibiotic Resistance Gene-Carrying Plasmids from Lactobacillus reuteri ATCC 55730 and Characterization of the Resulting Daughter Strain, L. reuteri DSM 17938. Applied and Environmental Microbiology, 74 (19), 6032–6040. https://doi.org/10.1128/AEM.00991-08 [2022-05-03]

Salas-Jara, M.J., Ilabaca, A., Vega, M. & García, A. (2016). Biofilm Forming Lactobacillus: New Challenges for the Development of Probiotics. Microorganisms, 4 (3), 35. https://doi.org/10.3390/microorganisms4030035 [2022-05-19]

Salminen, S., Collado, M.C., Endo, A., Hill, C., Lebeer, S., Quigley, E.M.M., Sanders, M.E., Shamir, R., Swann, J.R., Szajewska, H. & Vinderola, G. (2021). The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nature Reviews Gastroenterology & Hepatology, 18 (9), 649–667. https://doi.org/10.1038/s41575-021-00440-6 [2022-05-19]

Santivarangkna, C., Kulozik, U. & Foerst, P. (2008). Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. Journal of Applied Microbiology, 105 (1), 1–13. https://doi.org/10.1111/j.1365-2672.2008.03744.x

Salminen, S., Stahl, B., Vinderola, G. & Szajewska, H. (2020). Infant Formula Supplemented with Biotics: Current Knowledge and Future Perspectives. *Nutrients*, 12 (7), 1952. https://doi.org/10.3390/nu12071952 [2022-05-19]

Savino, F., Cordisco, L., Tarasco, V., Palumeri, E., Calabrese, R., Oggero, R., Roos, S. & Matteuzzi, D. (2010). Lactobacillus reuteri DSM 17938 in Infantile Colic: A Randomized, Double-Blind, Placebo-Controlled Trial. Pediatrics, 126 (3), e526–e533. https://doi.org/10.1542/peds.2010-0433 [2022-05-19]

Shaikh J., Uttekar P. S. (2021) Digestive System: Does It Take 30 Minutes to Digest Food? MedicineNet. https://www.medicinenet.com/does_it_take_30_minutes_to_digest_food/article.htm [2022-05-19]

Skelly, A.N., Sato, Y., Kearney, S. & Honda, K. (2019). Mining the microbiota for microbial and metabolite-based immunotherapies. Nature Reviews Immunology, 19 (5), 305–323. https://doi.org/10.1038/s41577-019-0144-5 [2022-05-01]

Sleator, R.D., Banville, N. & Hill, C. (2009). Carnitine Enhances the Growth of Listeria monocytogenes in Infant Formula at 7°C. Journal of Food Protection, 72 (6), 1293–1295. https://doi.org/10.4315/0362-028X-72.6.1293 [2022-05-01]

Sommer, F., Anderson, J.M., Bharti, R., Raes, J. & Rosenstiel, P. (2017). The resilience of the intestinal microbiota influences health and disease. Nature Reviews. Microbiology, 15 (10), 630–638. https://doi.org/10.1038/nrmicro.2017.58 [2022-05-01]

Stacy, A., Andrade-Oliveira, V., McCulloch, J.A., Hild, B., Oh, J.H., Perez-Chaparro, P.J., Sim, C.K., Lim, A.I., Link, V.M., Enamorado, M., Trinchieri, G., Segre, J.A., Rehermann, B. & Belkaid, Y. (2021). Infection trains the host for microbiota-enhanced resistance to pathogens. Cell, 184 (3), 615-627.e17. https://doi.org/10.1016/j.cell.2020.12.011 [2022-05-01]

Sung, V., D'Amico, F., Cabana, M.D., Chau, K., Koren, G., Savino, F., Szajewska, H., Deshpande, G., Dupont, C., Indrio, F., Mentula, S., Partty, A. & Tancredi, D. (2018). Lactobacillus reuteri to Treat Infant Colic: A Meta-analysis. Pediatrics, 141 (1), e20171811. https://doi.org/10.1542/peds.2017-1811 [2022-05-01]

Tochitani, S. (2017). Functions of Maternally-Derived Taurine in Fetal and Neonatal Brain Development. I: Lee, D.-H., Schaffer, S.W., Park, E., & Kim, H.W. (red.), Dordrecht, 2017. 17–25. Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-024-1079-2_2 [2022-04-30]

Tochitani, S. (2022). Taurine: A Maternally Derived Nutrient Linking Mother and Offspring. *Metabolites*, 12 (3), 228. https://doi.org/10.3390/metabo12030228 [2022-04-30]

Zhang, Z., Wang, K., Oh, J.-H., Zhang, S., van Pijkeren, J.-P., Cheng, C.C., Ren, D., Wei, H., Gänzle, M.G. & Walter, J. (2020). A Phylogenetic View on the Role of Glycerol for Growth Enhancement and Reuterin Formation in Limosilactobacillus reuteri. Frontiers in Microbiology, 11. https://www.frontiersin.org/article/10.3389/fmicb.2020.601422 [2022-05-12]

THE GUT MICROBIOTA: Our Microbial Partners (2017). International Scientific Association for Probiotics and Prebiotics (ISAPP). http://4cau4jsaler1zglkq3wnmje1-wpengine.netdna-ssl.com/wp-content/uploads/2019/04/Gut_Microbiota_rev1029.pdf [2022-03-31]

Wall, T., Båth, K., Britton, R.A., Jonsson, H., Versalovic, J. & Roos, S. (2007). The Early Response to Acid Shock in Lactobacillus reuteri Involves the ClpL Chaperone and a Putative Cell Wall-Altering Esterase. Applied and

Environmental Microbiology, 73 (12), 3924–3935. https://doi.org/10.1128/AEM.01502-06 [2022-05-03]

Walter, J., Schwab, C., Loach, D.M., Gänzle, M.G. & Tannock, G.W.Y. 2008 (u.å.). Glucosyltransferase A (GtfA) and inulosucrase (Inu) of Lactobacillus reuteri TMW1.106 contribute to cell aggregation, in vitro biofilm formation, and colonization of the mouse gastrointestinal tract. Microbiology, 154 (1), 72–80. https://doi.org/10.1099/mic.0.2007/010637-0 [2022-04-30]

Wegh, C.A.M., Geerlings, S.Y., Knol, J., Roeselers, G. & Belzer, C. (2019). Postbiotics and Their Potential Applications in Early Life Nutrition and Beyond. International Journal of Molecular Sciences, 20 (19), 4673. https://doi.org/10.3390/ijms20194673 [2022-04-30]

Wendel, U. (2022). Assessing Viability and Stress Tolerance of Probiotics—A Review. Frontiers in Microbiology, 12, 818468. https://doi.org/10.3389/fmicb.2021.818468 [2022-04-30]

World Health Organization, WHO (2021), Infant and young child feeding. Available: https://www.who.int/news-room/fact-sheets/detail/infant-and-young-child-feeding[2022-05-03]

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Appendix I

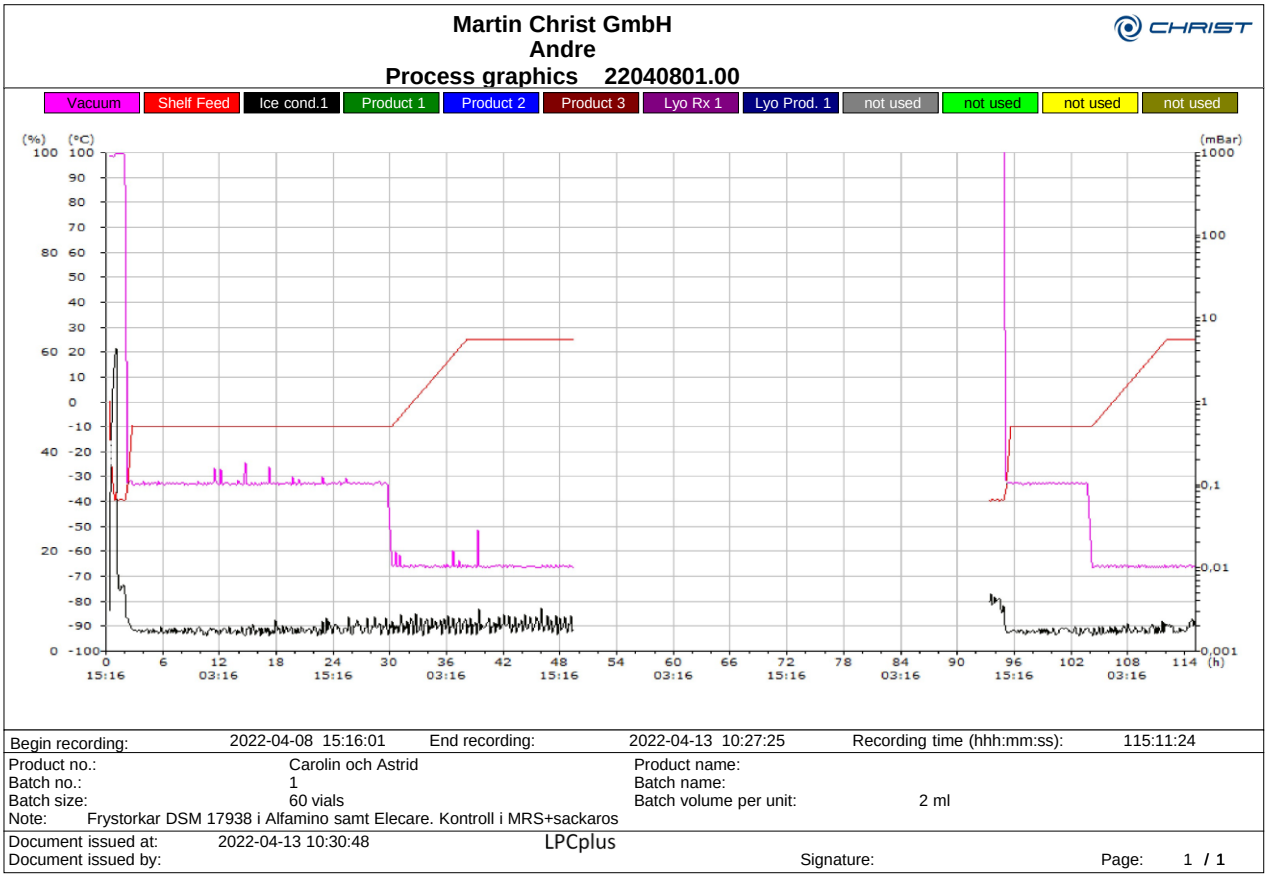

Figure 4. Programme graph of lyophilization process (48h) for L. reuteri DSM 17938.

Appendix II

*Table 2. Technical duplicate average of viable count results for L. reuteri in gastric acid tolerance assay (CFU/ml). *TFTC: Too few to count. Copyright: Astrid Hägg, Carolin Marklund*

Timepoint	Sample	Test 1	Test 2	Test 3
Reference,	A	$3.0x10^7$	$4.0x10^7$	$5.0x10^6$
T_0	B	$4.0x10^5$	$3.0x10^4$	$1.4x10^4$
	C	$4.0x10^8$	$8.0x10^8$	$3.0x10^8$
T_{20}	A	$4.5x10^3$	$1.2x10^4$	$3.0x10^3$
	B	TFTC*	TFTC*	TFTC*
	C	$4.5x10^6$	$2.0x10^6$	$3.3x10^6$
T_{50}	A	$5.0x10^2$	$5x10^2$	TFTC*
	B	TFTC*	TFTC*	TFTC*
	C	$1.2x10^5$	$4.0x10^5$	$2.5x10^5$
T_{90}	A	TFTC*	TFTC*	TFTC*
	B	TFTC*	TFTC*	TFTC*
	C	$2.5x10^3$	$7.5x10^3$	$1.4x10^4$

Appendix III

*Table 3. Technical duplicate average viable count (mean of duplicates) results for L. reuteri in bile salt tolerance assay (CFU/ml). *TFTC: Too few to count. Copyright: Astrid Hägg, Carolin Marklund.*

Timepoint	Sample	Test 1	Test 2	Test 3
Reference, T_0	A	$5.0x10^6$	$4.0x10^6$	$3.0x10^6$
	B	$8.0x10^4$	$1.5x10^5$	$3.0x10^5$
	C	$8.0x10^8$	$5.3x10^8$	$5.0x10^8$
T_{30}	A	TFTC*	$4.0x10^3$	$2.5x10^4$
	B	$6.0x10^4$	$1.5x10^2$	TFTC*
	C	$1.0x10^4$	$2.0x10^3$	$1.5x10^3$
T_{60}	A	$4.1x10^4$	$3.0x10^3$	$2.5x10^4$
	B	$5.0x10^3$	TFTC*	TFTC*
	C	$2.9x10^4$	$4.5x10^3$	$1.5x10^3$
T_{90}	A	$1.0x10^5$	$6.0x10^3$	$1.0x10^4$
	B	$3.6x10^2$	$1.0x10^2$	TFTC*
	C	$1.2x10^4$	$1.0x10^3$	$5.5x10^3$

Appendix IV

Results from HPLC.

Table 4. Retention time and peak area for sample A (first replicate).

#	Time	Area	Height	Width	Symmetry
1	7.515	301419.1	16788.3	0.2992	0.8
2	8.307	17658.6	1044.5	0.2818	0.821
3	8.938	59847.1	3421.2	0.2915	0.892
4	9.372	2222.4	166.8	0.2221	1.042
5	9.957	2604319	121766.6	0.3565	0.959
6	10.757	2668053.3	111627.3	0.3984	1.066
7	12.715	28254.1	1002	0.47	0.931
8	13.645	82545.7	2782	0.4945	1.142
9	15.871	41114.2	1189	0.5763	0.995
10	18.448	40379.3	829.7	0.8111	1.047
11	22.124	9468	214.4	0.714	1.115

Table 5. Retention time and peak area for sample A (second replicate).

#	Time	Area	Height	Width	Symmetry
1	7.517	283637.2	15871.8	0.2978	0.8
2	8.309	17149.3	1016.5	0.2812	0.831
3	8.938	57977.9	3310.2	0.2919	0.888
4	9.364	2107.5	161.2	0.2179	0.888
5	9.958	2452070.3	114905.1	0.3557	0.953
6	10.757	2517915.5	105274.1	0.3986	1.062
7	12.708	26487.3	941	0.4692	0.958
8	13.644	78012.8	2628.1	0.4947	1.099
9	15.869	36930.9	1067.7	0.5765	0.989
10	18.443	37510.7	782	0.7995	1.059
11	22.139	4119.8	95.9	0.6782	1.362

Table 6. Retention time and peak area for sample B (first replicate).

#	Time	Area	Height	Width	Symmetry
1	7.544	807331.6	47117.6	0.2856	0.871
2	8.323	669927.6	37018.8	0.3016	0.827
3	8.936	124089.5	6657.7	0.3106	0.849
4	9.966	2676568.5	124826.3	0.3574	1.003
5	10.764	2566820.8	107047.7	0.3996	1.026
6	13.111	1008.3	49.4	0.3403	0.365
7	13.644	10686.6	394.6	0.4513	1.226
8	14.048	987.4	39.7	0.2934	0
9	14.731	456.3	22.9	0.3322	0
10	15.87	32202	954.5	0.5623	1.032
11	18.473	72954.3	1781	0.6827	1.116

Table 7.Retention time and peak area for sample B (second replicate).

#	Time	Area	Height	Width	Symmetry
1	7.538	798159.9	46609.4	0.2854	0.87
2	8.317	658567.1	36428.8	0.3013	0.828
3	8.931	124281.9	6657.3	0.3111	0.85
4	9.961	2674425.8	125216.6	0.356	1.003
5	10.758	2587078	107306.7	0.4018	1.03
6	13.118	1068.9	53.5	0.3327	0.474
7	13.651	10290.2	380	0.4514	1.197
8	14.314	1053.4	42.4	0.3872	0.515
9	14.688	517	27.8	0.3099	0
10	15.869	31544.7	940.5	0.559	1.05
11	18.47	73304.9	1789.5	0.6827	1.115

Table 8. Retention time and peak area for sample C (first replicate).

#	Time	Area	Height	Width	Symmetry
1	8.286	38039.6	1849.1	0.3429	0.848
2	8.931	48119.1	2517.9	0.3185	0.871
3	9.961	3154525.8	148568.9	0.3539	0.994
4	10.762	3254398.8	136054.2	0.3987	1.006
5	11.908	15213.6	422.1	0.6007	0.244
6	13.628	6613.6	234.9	0.4693	1.026
7	15.901	4548.9	96.9	0.6171	0.711

Table 9. Retention time and peak area for sample C (second replicate).

#	Time	Area	Height	Width	Symmetry
1	8.287	45954	2209.3	0.3467	0.836
2	8.924	48533.6	2503	0.3232	0.903
3	9.96	3203014.5	150140.4	0.3556	1.006
4	10.762	3332199	138043.7	0.4023	1.002
5	13.632	7570.7	260.9	0.4837	1.078
6	15.891	5186.2	99.9	0.7567	0.635

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