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Abstract

The probiotic bacteria *Lactobacillus reuteri* DSM17938 was evaluated as a starter culture in cheese production, with the intention to produce a new interesting product within the functional food market. Growth and acidification rate in milk subjected to different heat treatments, as well as supplementation with growth promoting factors or support cultures was measured during controlled fermentations. The results showed that the weak proteolytic activity of *L. reuteri* could be compensated for by supplementation with 1% casamino acids or 5% MRS broth. Furthermore, a decrease in growth and acidification rate could be seen when using yoghurt culture or *Lactobacillus delbrueckii* as adjunct cultures. *L. reuteri* DSM 17938 was successfully incorporated into a fresh cheese with high viability during the storage period, suggesting that fresh cheese is an excellent carrier for the bacteria. The inhibitory activity of *L. reuteri* on a non-pathogenic mutant of *Escherichia coli* O157:H7 (EHEC) and *Staphylococcus aureus* was also tested. Both pathogens were inhibited in cheese with *L. reuteri* and the control, opening for further investigations in the field. A limited population of *L. reuteri* DSM 17938 was also successful incorporated in a hard cheese produced at Skogsbackens dairy. However, the survival was excellent during the storage period of 83 days, suggesting that the cheese could be a good carrier for *L. reuteri*.

Key words: Probiotic cheese, Lactobacillus reuteri, milk supplementation, adjunct culture, inhibitory activity

Sammanfattning

Den probiotiska bakterien *Lactobacillus reuteri* DSM 17938 utvärderades som startkultur i ostproduktion, med avsikt att producera en ny intressant produkt med mervärden. Tillväxt och syraproduktion i mjölk utsatt för värmebehandlingar av varierande grad, eller tillsats av tillväxtstimulerande faktorer eller stödkulturer, mättes under kontrollerade omständigheter. Resultatet indikerar att ett svagt proteolytiskt system hos *L. reuteri* kan kompenseras med en tillsats av 1% casamino syra eller 5% MRS buljong. En ökning av tillväxt och syraproduktion sågs även vid tillsats av yoghurtkultur eller *Lactobacillus delbrueckii* som stödkultur. *L. reuteri* DSM17938 visade en god överlevnad under hela lagringsprerioden när den användes som starterkultur till färskost. Detta tyder att denna typ av produkt är att bra sätt att få i sig probiotiska bakterier via kosten. Förmågan att inhibera tillväxt och överlevnad av en avpatogeniserad mutant av *Escherichia coli* O157:H7 (EHEC) and *Staphylococcus aureus* testades också. Båda patogenerna blev inhiberade både i ost innehållandes *L. reuteri* och kontrollosten, vilket öppnar upp för fler undersökningar inom detta område. En låg halt av *L. reuteri* DSM17938 fanns även i den hårdost som producerades på Skogsbackens mejeri som en del av projektet. Överlevnaden var emellertid mycket hög under en lagringsperiod på 83 dagar vilket tyder på att även denna ost skulle fungera bra som medium för *L. reuteri*.

Nyckelord: Probiotisk ost, Lactobacillus reuteri, tillsatsämnen i mjölk, stödkultur, inhibering av patogener

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Introduction

Background

Probiotics are subject to intensive research in the food industry due to their claimed health promoting effects as well as their marketing value in so called functional foods. There is no universal definition of the term functional food, but food has been regarded as functional if it can demonstrate a positive effect to one or more functions in the body, beyond adequate nutritional effects, that may either improve the health or reduce the risk of disease (Diplock et al., 1999). Claimed benefits from the intake of probiotic bacteria are many. Some of these effects include improvement of intestinal health by improving the balance of microbiota (Lidbeck et al., 1991), reducing symptoms of lactose intolerance (Blanchette et al., 1995), anti-carcinogenic activity (Kumar et al., 2010), decreased insulin resistance (Andreasen et al., 2010), cholesterol lowering activity (St-Onge et al., 2000) and enhanced immune response (Isolauri, 2001; Valeur et al., 2004). According to FAO, probiotics are defined as 'live microorganisms which when administered in adequate amount confer health benefits to the host' (FAO/WHO, 2001).This means that the food product must contain bacteria with maintained viability and metabolic activity at the time of consumption and also survive the passage through the gastrointestinal (GI) tract.

A popular way to ingest probiotics is by eating or drinking supplemented dairy products, for example fermented milk drinks or yoghurt. Dairy products are suggested to be a good matrix for transferring probiotic bacteria into the GI tract, as milk proteins is thought to function as buffering agents and thereby protect the cells during transit (Carteris et al., 1997). During the past years, cheese has been promoted as good carriers for probiotic bacteria along with other dairy products. This is mainly due to their higher fat content and denser structure that may protect the bacteria against the acidic environment of the GI tract (da Cruz et al., 2009). Another benefit with cheese as a probiotic carrier is the fact that it is included in the long term diet of people all over the world, and has a high nutritional value. Especially some varieties with a lower fat content could be beneficial as they can be consumed in higher volumes. A prerequisite of manufacturing probiotic cheese, is however, that the probiotic bacteria survives the ripening period, which can be between 1 day for a fresh cheese, and 3 years for some parmesan varieties for example (Wastra et al., 1999). This aspect should be taken into account when selecting a probiotic strain to incorporate in cheese. A daily consumption of 10^9 CFU probiotic bacteria has been proven to offer a health benefit when ingested with cheese as a carrier (Ibrahim et al., 2010). It is therefore suggested that a concentration of 10^6-10^7 CFU/g is needed in the final product in order to be claimed as probiotic. This number is recommended in scientific literature and is today the standard adopted by the industry (da Cruz et al., 2009; Karimi et al., 2011).

Probiotics in cheese production

Cheese is the generic name of dairy products made with milk from different animals, such as cow, goat or buffalo (NE, 2012). Acidification or the addition of rennet converts the milk into a solid coagulum, the curd, which contains mainly casein and fat. Most of the water, carbohydrates (lactose) and whey proteins are expelled during a process called syneresis. The special character of different cheese varieties is due to several processing steps such as pressing, salting and ripening.

The cheese consumption in Sweden has grown during the past years, with a present annual consumption of 19 kg per inhabitant, which is higher than the average consumption in the European Union (Svensk Mjök, 2012). Despite large variations in the consumption, cheese products are produced worldwide with a great diversity in shape, textures and sensory attributes. For example, they can be categorized according to the moisture content or age. Hard or semi-hard cheeses have low moisture content and are stored for a longer period. The ripening process develops a unique flavor and character of the cheese. As a contrast, fresh cheese is produced without a period of ripening and can be consumed directly after production. Both hard cheese and fresh cheese has certain advantages as carriers. With a higher fat content and a denser matrix, hard cheese offers an excellent protection during the transition through the GI tract. However, it is important to consider whether or not the probiotic bacteria can survive the prolonged ripening period. The suitability of a fresh cheese as a probiotic carrier is high because of the manufacturing process. The cheese is basically ready to eat instantly and no longer periods of ripening and storage are needed (de Souza et al., 2006). This fact makes the probability of a good bacterial survival higher. The water activity is also higher compared to hard cheese, and the salt content is lower.

Both hard and fresh cheese has been used to deliver probiotic bacteria successfully (da Cruz et al., 2009, Karimi et al. 2011). Gardiner et al. (1998) used *Lactobacillus salivarius* and *Lactobacillus paracasei* as starter culture in cheddar cheese with good results. Further, Bergamini et al. (2004) produced a semi-hard Argentinian cheese containing *Lactobacillus acidophilus* and another containing *L. paracasei*. The bacteria increased a log cycle during cheese manufacturing and remained constant during 60 days of storage, suggesting a good survival in cheese. In another study by de Souza et al. (2006) a fresh cheese was made with *L. acidophilus*. The survival was high during the storage and sensory performance was improved after addition of probiotics. A number of probiotic cheese varieties are available on the market today, mainly in big economies like the United States. In northern Europe, a limited number of probiotic cheeses have been incorporated on the market. One of the early producers in the world to manufacture a probiotic cheese was for example Lillehammer dairy in Norway. At the present, no probiotic cheese is available on the market in Sweden, opening for an interesting new market segment.

A number of health benefits has been claimed to be provided by ingestion of probiotic cheese. In order to use these health benefits in marketing, it most however demonstrate good results in controlled validated clinical trials. Clinical benefits have been reported connected to ingestion of probiotic cheese in a number of studies. For example, a study by Ahola et al. (2002)

studied the effect of probiotic cheese on the occurrence of caries. It suggests that the consumption of probiotic cheese could reduce the risk for caries. Other benefits for the oral health were claimed by Hatakka et al. (2007). They found that the intake of probiotic cheese decreased the prevalence of oral candida in elderly compared with a control group. Moreover, the risk of hyposalivation decreased, suggesting that probiotic cheese could be beneficial for the oral health in general. Medici et al. (2004) showed an increase in phagocytic activity and thus an enhanced immune response in mice fed with probiotic fresh cheese for 7 days. In another study by Ibrahim et al. (2010) it could be shown that intake of probiotic cheese containing *Lactobacillus rhamnosus* and *L. acidophilus* enhanced the immunity of elderly. Considering the numerous studies made on general health aspects connected to probiotics, there is potential for more studies made with probiotic cheese.

Probiotic starters

Production of cheese includes the addition of a starter culture, with the objective to acidify the milk and develop special characteristics during the process of ripening. The starter consist of lactic acid bacteria that converts the lactose of the milk into lactic acid which decrease the pH of the milk, and at a pH of approximately 4.5, the milk starts to thicken. This is the procedure behind the production of yoghurt and other dairy products. In cheese production, the starter also contributes to the texture and sensory profile of the end product by producing acetic acid, ethanol, aroma compounds and several enzymes (Azhari Ali, 2010).

The starters are often classified according to the optimum temperature for growth, of which mesophilic bacteria grow fastest at 20-30°C and thermophilic ones at 35-45°C. Mesophilic DL-starter is frequently used for production of hard cheeses in northern Europe. The starter often consists of a mixture of the following species: *Lactococcus lactis* subsp. *lactis, Lc. lactis* ssp. *cremoris, Lc. lactis* ssp. *lactis biovar diacetylactis* and subspecies of *Leucocnostoc mesenteriodes* (Rehn et al. 2010). Cheese made with termophilic starters normally includes mixtures of *Streptococcus salivarius* ssp. *thermophilus* and different *Lactobacillus* species, such as *Lactobacillus delbrueckii* ssp. b*ulgaricus, Lactobacillus helveticus* and *L. casei.* The proteolytic activity of thermophilic starter bacteria is generally higher compared to mesophilic ones (Walstra et al., 1999). This influence the maturation process as more free amino acids and short peptides in the cheese gives rise to more complex sensory attributes after a shorter period of storage.

Among the many probiotic strains used in the food industry at the present, many belong to the genus *Lactobacillus*. They are regarded as safe in general, and many strains with beneficial health effects of for example *L. acidophilus* and *L. casei* have been isolated. They are gram-positive facultative anaerobic rod or coccus shaped bacteria (Azhari Ali, 2010; Adams & Moss, 2008) and belong to the group of lactic acid producers. Further, they are divided into homo and hetrofermentative bacteria, based on the end product of the glucose metabolism. During hetrofermentation, a mixture of lactic acid and other compounds such as carbon dioxide, acetic acid and ethanol are produced. Homofermentative bacteria, on the other hand,

have lactic acid as major metabolic end product. It is important to note that not all strains of lactobacilli possess the therapeutic properties of potential probiotics.

There are several technical hurdles when it comes to the application of probiotic bacteria in the development of functional foods. A major challenge in production is for example their viability during processing and storage (da Cruz et al., 2009). The maturation of cheese involves an increasing number of nonstarter acid bacteria (NSLAB) which can outgrow the starter lactic acid bacteria (SLAB), including added probiotic cultures. Studies have shown that probiotic dairy products often fail to deliver a bacterial count over 10^6 CFU/g. In a study by Jayamanne and Adams (2005), five of ten probiotic yoghurt tested failed to provide the accurate number of *Bifidobacterium* in the end of the products shelf life. With a prolonged storage period, the challenge is even greater during cheese production compared to yoghurt and fermented milk. The suitability to act as a starter culture depends of which type of probiotic bacteria that is used. It is important to note that different strains of probiotic bacteria have different characteristics as starter culture depending on their ability to resist stress from salt, acidity, temperature among other factors. The presence of oxygen could also pose a threat against the survival, since many probiotics used in food products have an anaerobic metabolism. Lactobacilli are in general less sensitive against oxygen, but this varies between different species and strains. Sometimes, the processing needs to be modified in order to suit the probiotic bacteria employed. This can result in a change of incubation temperature, salt concentration or addition of second starter bacteria for example. The result of this modification can alter the sensory quality and or/texture of the cheese, why it have to be evaluated properly.

Lactobacillus reuteri as starter in cheese production

Among the species of *Lactobacillus* claimed to be of probiotic nature, *Lactobacillus reuteri* is a hetrofermentative, facultative anaerobic rod shaped bacillus. It is reported to inhabit the GI tract of all vertebrates, including humans and other mammals (Xanthopoulos et al, 2000. Casas & Dobrogosz, 2000), and is suggested to be one of few indigenous lactobacillus species of man (Reuter, 2001). A number of characteristics of putative importance for a probiotic activity have been proposed for different strains of *L. reuteri* (Reid, 1999; Jacobsen et al., 1999) including the ability to survive the passage through and colonize the GI tract (Shornikova et al., 1997; Jacobsen et al., 1999; Dommels et al., 2009) and documented health effects in humans (Shornikova et al., 1997; Casas et al., 2000; Connolly, 2004; Sinkiewicz, 2010; Szajewska et al., 2012). They are also able to produce anti-microbial substances whereof the most well documented is reuterin, which is produced during anaerobic growth in the presence of glycerol. Reuterin has been found to be inhibitory against a number of pathogens, for example for *Listeria monocytogenes*, *Escherichia coli* O157:H7 (EHEC) (El-Ziney et al., 1998), *Staphylococcus aureus*, *Salmonella choleraesuis* ssp. *choleraesuis*, *Yersinia enterocolitica*, *Aeromonas hydrophila* ssp. *hydrophila* and *Campylobacter jejuni*. (Arqués et al., 2004). Other mechanisms to inhibit growth of pathogenic bacteria include the extraction of lactic acid and hydrogen peroxide (Connolly, 2004). *S. aureus* and coliform

bacteria such as *E. coli* are associated with milk products as they are naturally occurring in raw-milk and can often be found in food after fecal contamination or inadequate hygiene procedures. One aspect of the incorporation of *L. reuteri* in cheese could be the inhibitory effect on the mentioned bacteria, and therefore improve the hygienic quality of the cheese.

When it comes to different kinds of mold and yeasts, they are the most important sources for microbial defects and spoilage in fermented milk products (Wastra et al., 1999). Both groups are able to grow in pH below 3.8, and may thus be a problem in more acidic products such as fresh cheese. Lactobacilli are known to inhibit the growth of yeast (Narendranath et al., 1997). The production of lactic acid and the competition for nutrition and other growth factors are suggested to be the main factors reducing yeast growth when lactic acid bacteria are present. Reuterin has been found to inhibit some yeast and fungi (Chung et al., 1988). 4 to 5 units/ml of the substance was enough to prevent the growth of tested species completely. Since different kind of yeast and mold are known to be a problem in cheese production, it could be of value to evaluate the inhibitory activity of *L. reuteri*.

The combination of positive effects, including health promoting properties of probiotic bacteria and the antimicrobial activity of reuterin, makes *L. reuteri* an interesting complement to traditional starter cultures in cheese. Although different strains of *L. reuteri* demonstrate a variety of health benefits and shows good viability in different food stuffs, the occurrence in dairy foods is low (Hildago-Morales et al., 2005). One reason for this is probably that many strains of *L. reuteri* together with other probiotic bacteria are unable to ferment milk adequately due to their slow growth and level of acidification (Shah, 2000; Xanthopoulos et al., 2000; da Cruz et al., 2009; Mohammadi el al., 2012). Lack of adequate acidification capacity might lead to a prolonged fermentation time, with economical and hygiene consequences, and also influence the sensory attributes of the cheese (Mohammadi el al., 2012). A number of studies have been published concerning insufficient growth and acidification of *L. reuteri* in milk, and different suggestions have been made concerning the underlying reason for this. Xanthopoulos et al. (2000) showed that *L. reuteri* didn't acidify milk at levels of pH 4.5 after 24 hours, which is important in the production of fermented milk. A later study by Hidalgo-Morales et al. (2004) also confirms that *L. reuteri* have a low acidification ability. Low β-galactosidase activity was suspected as possible cause of low milk acidification in this study. However, results indicated that growth was more related to a weak proteolytic system, rather than weak β-gal. activity, since the addition of 1% casein peptone increased the acidification abilities. Other studies have shown a faster growth of *L. reuteri* (Østlie et al., 2004; Hekmat & Reid, 2006), mainly after addition of growth promoting factors, adding to the theory that regular milk not is an optimum growth medium for this bacteria. In general, probiotic lactobacilli are considered to grow slow in milk due to lack of easily accessible nitrogen such as free amino acids and small peptides (Shah, 2000; Oliveira et al., 2001; Hidalgo-Morales et al., 2004; Mohammadi el al., 2012), and there is reasons to believe that this applies for *L. reuteri* as well, considering the studies mentioned.

Since probiotics grow slowly in milk, different methods are used to compensate for that. Milk can for example be fortified with different growth factors such as casein, whey protein

hydrolysate, yeast extract, glucose, vitamins, minerals and antioxidants (Mohammadi el al 2012). Addition of casein and whey protein is mainly to compensate for the weak proteolytic activity. However, the usual practice is to grow the probiotic bacteria in co-culture with for example yoghurt bacteria in order to reduce fermentation time, which is referred to as "adjunct culture" or "support culture" (Shah, 2000). The traditional flora of yoghurt consists of both *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. In order to achieve the characteristic flavor of yoghurt, the two species are present in equal numbers, approximately. They have a synergistic relationship, as they both grow better together than in pure culture. *L. delbrueckii* provides small peptides and amino acids by its proteolytic activity, of which valine, with a low concentration in milk, is the most important one. *S. termophilus* is weakly proteolytic, and the presence of *L. delbrueckii* will therefore promote its growth in milk. On the contrary, *S. termophilus* produces formic acid and CO_2, which enhances the growth of *L. delbrueckii*. The production of free amino acids could be the foundation of a synergistic relationship with probiotic bacteria, depending on the strain and process conditions (Oliveira et al., 2001). Both *S. thermophilus* and *L. delbrueckii* have formerly been used in probiotic cheese in order to improve technological properties and taste of the final product (Karimi et al., 2005). A drawback worth mentioning is the ability of *L. delbrueckii* to produce acid during cold storage of the cheese, known in the industry as post-acidification. This increase in acidity could influence the survival of some probiotic bacteria. The probiotic strain *L. reuteri* DSM17938 used in this study is, however, known to be acid resistant (Sinkiewicz, 2010).

With health benefits, and the potential to inhibit bacterial growth by the production of reuterin, *L. reuteri* has a good potential as probiotic bacteria in cheese production. The suitability of *L. reuteri* DSM17938 as a starter culture in cheese was tested in cooperation with Skogsbackens ost, a small dairy located outside Örsundsbro. The specialty of this dairy is artisan cheese made from mainly unpasteurized milk. A possible inhibitory activity could give an improved hygienic quality since unpasteurized milk is a source of unwanted organisms such as pathogens and spoilage bacteria. The work was sponsored by an EU-funded SMURF project, which aims to build partnerships between small businesses and the university.

Objectives of project

The objective of this project was to incorporate *L. reuteri* DSM 17938 as a starter culture in a hard cheese produced as a pilot trial at the small-scale dairy Skogsbackens ost. In order to evaluate *L. reuteri* as a starter, and optimize the growth in milk, a number of studies were made concerning growth promoting factors. The factors tested are summarized in table 1. The growth of *L. reuteri* in milk was also tested in co-culture with yogurt culture (mild yoghurt, Arla) and *L. delbrueckii*. The objective was to investigate if the proteolytic activity of *L. delbrueckii* could promote the growth of *L. reuteri* in milk.

A probiotic fresh cheese was also produced at the laboratory, in order to monitor the growth and survival of *L. reuteri* DSM 17938 during processing and the storage period. The desired number of bacteria in both the hard and fresh cheese was at least 10^6 CFU/g at the time of consumption, which is suggested to be the minimum for a probiotic product.

The fresh cheese was used as a growth medium for number of species of mold and yeast, as well for a non-pathogenic mutant of *E. coli* O157:H7 (EHEC) and *S. aureus* (Table 2.). The objective was to evaluate the inhibitory activity by *L. reuteri.*

Table 1. Growth promoting factors tested for *L. reuteri*

Trial	Growth promoting factor	Method
1	Heat treatment of milk	Incubation in pasteurized, sterilized, autoclaved and UHT-treated milk
2	Free amino acids	Addition of casamino acids
3	Reducing activity of cysteine	Addition of cysteine
4	MRS broth	Addition of MRS broth

Methods

Propagation of bacterial strains

L. reuteri DSM 17938 and *L. delbrueckii* ssp. *bulgaricus* isolated from yoghurt (Arla mild) was inoculated into sterilized de Man-Rogosa-Sharpe (MRS) broth (Oxoid) and grown overnight at 37°C. Cultures were collected by centrifugation and diluted in phosphate buffered saline (PBS). *E. coli* O157:H7 (EHEC) and *S. aureus* were cultured in Brain heart infusion (BHI) broth (Merck) overnight, incubated at 37°C.

Growth of *L. reuteri* in milk

Heat treatment

The following heat treatments were performed on milk (Arla pasteurized, 3% fat): (i) high temperature treatment in water bath (90°C) for 30 min (referred to as sterilization in this study), (ii) autoclaving (125°C) for 15 min. Samples were compared with ultrahigh temperature (UHT) treated milk (Arla UHT, 1.5% fat) and a control without heat treatment (Arla past., 3% fat). All cultures were prepared with 1% inoculums of *L. reuteri* DSM17938, which were produced by adding 100µl of bacteria and PBS suspension to each 10 ml tube, and incubated in a 40°C water bath. Growth of *L. reuteri* DSM17938 was monitored trough the rate of acidification, which is positively correlated to lactic acid bacteria growth. This was done by pH measurements after 0, 4, 6, 8, and 24 hours.

Growth promoting supplements

Milk (Arla past., 0.5 or 3% fat) was divided into 10 ml tubes and sterilized by putting them in a 90°C water bath for 30 minutes. Growth promoting supplements was added according to table 2. The rate of acidification was monitored trough pH measurements after 0, 4, 6, 8 and 24 hours. One 10 ml tube was used on each measurement occasion, in order to avoid contamination between samples. All cultures were prepared with 1% inoculums of *L. reuteri* DSM17938. Subsequently, the samples was incubated in a 40°C water bath and enumeration of *L. reuteri* and total lactobacilli was done after 6 hours for all trials.

Table 2. Growth promoting supplements

Supplement	Concentration
Casamino acids	1%
Cysteine	0,2%
MRS broth	5%

Support culture

Milk (Arla past., 0.5 or 3% fat) was divided into 10 ml tubes and sterilized. Essentially, the same procedure as above was used when testing *L. reuteri* with two different support cultures. One consisted of only *L. delbrueckii*, and the other one was yoghurt culture (Arla mild, 3%) with *L. delbrueckii* and *S. termophilis*. The cultures were prepared with 1% inoculums of *L. reuteri* DSM17938 with an addition of 1% of the tested support culture or an additional 1% L. *reuteri* for the control**.** The rate of acidification was monitored by pH measurements after 0, 4 and 8, or 0, 4, 7 and 24 hours, for fermentation with yoghurt culture and *L. delbrueckii*, separately. Samples for enumeration of *L. reuteri* were collected after 8 hours for the trial with yoghurt culture.

Production of hard cheese at Skogsbackens ost

One pilot-scale cheese making trial was performed at Skogsbackens dairy. 100 liters of unpasteurized, non-homogenized milk was heated to 37°C and a 1.5% inoculum of *L. reuteri* DSM17938 was added together with 0.5% mesophilic culture. The milk and culture mixture was left to mature for one hour and 30 ml of rennet was added. In the next step, the milk was allowed to set for 40 minutes until a firm curd was formed. The coagulum was cut into cubes and heated to 40°C in order to promote growth of *L. reuteri*. The coagulum was drained, placed in cheese molds with 1 kg capacity and stored in room temperature overnight. As control, one cheese with 0.5% mesophilic culture and 1.5% yoghurt culture instead of *L. reuteri* was made. Samples for enumeration of *L. reuteri* and total lactobacilli was collected in duplicates from two different cheeses before pressing in molds and after 1, 3, 10, 21 and 83 days of storage at 12°C.

Production of fresh cheese

Based on results from studies of growth of *L. reuteri* in milk with yoghurt culture as adjunct culture, a mixed starter culture was produced. Milk (Arla past., 0.5% fat) was sterilized for 30 min at 90°C in 50 ml tubes and stored overnight. Subsequently, the milk was inoculated with 1% *L. reuteri* DSM17938 and 1% yoghurt culture (Arla mild yoghurt, 3% fat). The inoculum was incubated for 6 hours in a 40 °C water bath and transferred to 2°C for storage.

Four laboratory-scale trials (T1a, T1b, T2a and T2b) were performed at two different occasions. T1a and T1b were inoculated with 2% yoghurt and *L. reuteri* mixed-culture while T2a and T2b acted as controls with 2% yoghurt culture. 3 liters of pasteurized milk (Arla, 3% fat) was heated to 37°C and starter culture was added. The milk and culture mixture was left to mature for three hours at 37°C until the pH reached 6.3-6.4. 30 ml of rennet (Apoteksbolaget) was added and the mixture was left to set overnight at room temperature. The coagulum was drained for 6 hours at room temperature. Samples were collected for enumeration of *L. reuteri* and total lactobacilli after 1, 7, and 14 days, and stored at 2°C. Fresh cheese was also saved in order to perform the subsequent growth inhibition assay.

Growth inhibition assay

Fresh cheese was used to evaluate sensitivity of different yeast, molds and pathogens against reuterin, the anti-microbial compound produced by *L. reuteri*. Species tested are summarized in table 2. Approximately 25g of fresh cheese was spread on plastic petri dishes and 50 µl of yeast/mold suspension (concentration unknown) was added on the surface. A duplicate was made on each plate which was incubated at 15°C for 8 days. Growth was detected at day 1, 2, 5 and 8 by visual inspection. *S. aureus* and a non-pathogenic strain of EHEC (25µl bacteria suspension of unknown concentration) was mixed into duplicates of cheese samples and stored at 2°C for 10 days. Samples were collected after 0, 3 and 10 days and subjected to microbial analysis with the objective to enumerate *S. aureus* and *E. coli*.

Table 2. Species tested for inhibition

No.	Name
1	*Penicillium roqueforti*
2	*Pichia anomala*
3	*Rhodotorula glutinis*
4	*Yarrowia lypolytica*
5	*Geotrichum candidum*
6	*Depariomyces hanseii*
7	*Klyveromyces lactis*
8	*Staphylococcus aureus*
9	*Escherichia coli* O157:H7 (EHEC)[a]

[a] Non-pathogenic mutant

Microbiological analysis

The following microbiological assays were performed: (i) enumeration of total lactobacilli, including SLAB and NSLAB on MRS agar (Oxoid) for 48-72 h at 37°C, (ii) enumeration of *L. reuteri* DSM 17938 on selective Rogosa agar (Merck) containing 2µg/ml ampicillin for 48-72 h at 37°C, (iii) enumeration of *S. aureus* on Mannitol salt agar (MSA) (Oxoid) for 48-72 h at 37°C, (iiii) enumeration of *E. coli* on MacCONKEY (Merck) agar for 48-72 h at 37°C. Assays (i) and (ii) was incubated anaerobically using a Gaspack system (Becton, Dickinson and Company). In order to homogenize the hard cheese, a Stomacher was used. Approximately 5 g of cheese was blended with 45 mL of sterile PBS and submitted to further serial dilutions. All samples, both cheese and acidified milk, was diluted in PBS and submitted to serial dilutions of the order 10^{-1}- 10^{-6}, with the exception of the dilution series for fresh cheese which was made 10^{-1} - 10^{-5}. Enumeration of bacteria was done by viable count of incubated plates.

Confirmation with PCR

Polymerase chain reaction (PCR) was used in order to detect and confirm *L. reuteri* DSM 17938 in samples. The primer pair 1694f (TTAAGGATGCAAACCCGAAC) and r (CCTTGTCACCTGGAACCACT) was used to amplify the *Rib* gene with a size of approximately 177 bp, including primers (Roos, 2011).

Bacteria were suspended by collecting one colony from the growth medium into 100 µl of nuclease free water. Each reaction contained 0.5 µl bacterial suspension, 12,5 µl DreamTaq Green DNA Polymerase (Fermentas), and 12 µl water and primer mix, containing 0,4 µM of each primer, in a total reaction volume of 25 µl. As negative controls, PCR tubes containing only PCR reagents were used and suspension from a plate with confirmed growth of *L. reuteri* DSM17938 was used as positive control. DNA was initially denatured at 95°C for 3 min and then amplified in a thermal cycler (BioRad) for 30 cycles with the following conditions: denaturation at 95°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30s. These cycles were followed by a final extension at 72°C for 10 minutes. PCR products were analyzed by electrophoresis on an agarose gel (1%) stained with EtBr in TBE buffer.

Results and discussion

Growth of *L. reuteri* in milk

During the trial period, *L. reuteri* DSM 17938 repeatedly failed to achieve an acidification rate sufficient to ferment the milk to the desired pH of 4.5 within 24 hours. Generally, the fermentations with only *L. reuteri* added to sterilized milk provided a pH of just below 6 after 24 hours of incubation at 40°C. In some cases, the pH didn't drop under 6 during the same

period. Results confirm those reported by Xanthopoulos et al. (2000) and Hidalgo-Morales et al. (2005) concerning low acidification ability. However, a viable count of approximately 10^7 CFU/m could be monitored after 6 hours, which indicates that *L. reuteri* still grow and is metabolically active in milk. There is limited information concerning the growth of *L. reuteri* DSM17938 in milk, and of the metabolic pathways used by these bacteria during fermentation. The results of this study suggest that milk is not an optimal growth medium for *L. reuteri* DSM 17938, leading to the conclusion that measures need to be taken in order to promote bacterial growth (section 3.1.2 and 3.1.3).

Effect of heat treatment

The acidifying activity of *L. reuteri* DSM 17938 in milk subjected to different heat treatments is summarized in figure 1. Both UHT treatment, autoclaving, sterilization were meant to kill all microorganisms, including bacterial spores in this study. Considering this fact, only *L. reuteri*, which is added after heat treatment, was supposed to acidify the milk. Also, all milk enzymes are inactivated after heat treatment at high temperature. In the pasteurized milk without subsequent heat treatment, on the other hand, the presence of other bacteria as well as milk enzymes cannot be excluded. The initial pH was lower in the autoclaved milk, compared to the rest of the samples. This is related to the heat treatment, as a higher temperature decreases the pH of the milk with 0.2 units (Walstra et al., 1999). In general, the acidification was slow in all samples as they failed to drop to a pH of 4.5 within 24 hours, which is required in the production of a cheese culture. The control milk without heat treatment was close with a final pH of 4.55 after 24 hours. The final pH of the remaining samples was between 5.59-5.81. There is a possibility that the difference in acidification level is due to contamination of other LAB in the control milk. They could easily outgrow *L. reuteri* after a certain period of time, and thus produce more lactic acid. There is also a possibility that the presence of enzymes in the control milk could contribute to a higher *L. reuteri* growth by providing proteolytic activity. The access to more available amino acids could promote the growth of probiotic bacteria (Shah, 2000; Oliveira et al., 2001; Hidalgo-Morales et al., 2004; Mohammadi el al., 2012). This statement is however contradictory to the fact that high temperature treatment also changes the protein composition of the milk. The original idea was that heat treatment would degrade proteins and make the amino acids more accessible for the bacteria through this process. Also, the sterile environment should exclude other bacteria, and thus reduce the competition for nutrients. Nevertheless, the preliminary results of this study suggest that growth and metabolism of *L. reuteri* is independent of prior heat treatment of the milk, as no big difference could be seen between tested samples.

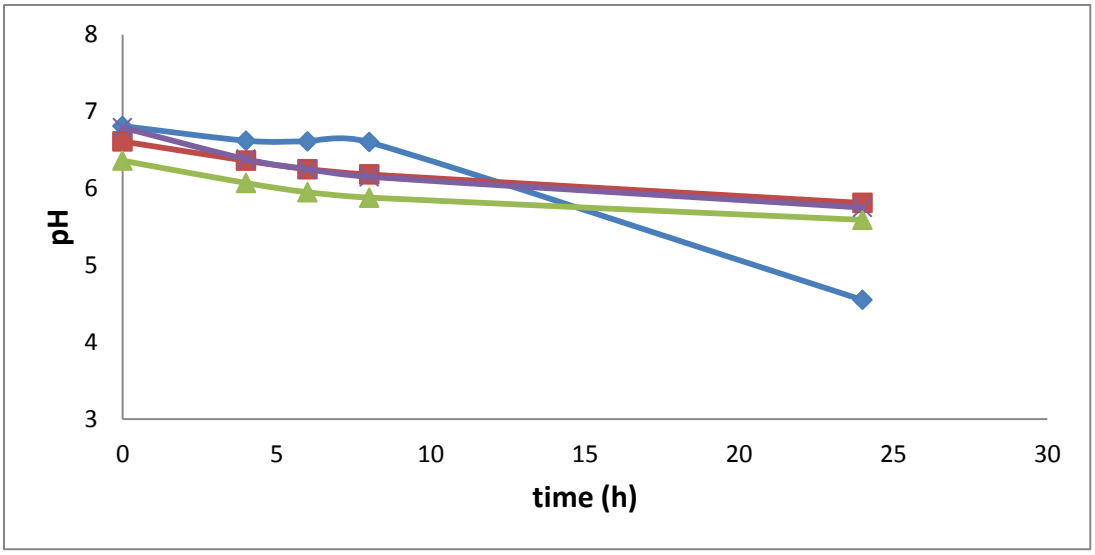

Fig 1. Growth of *L. reuteri* in milk subjected to different heat treatments: pasteurized (control) (◊), UHT (X), autoclaved (▲) and sterilized (■).

Effect of milk supplementation on growth

The following supplements were tested in order to promote growth and acidification of *L. reuteri* in milk: casamino acids, cysteine and MRS broth. Results concerning effect of supplementation are shown in figures 2-4. Supplementation with 1% casamino acids or 5% MRS broth contributed to a more rapid decline of pH, compared to control fermentations in un-supplemented milk. It should yet be noted that since the measurements was performed in different trials, the initial pH varied. The results are in line with previous studies made on different strains of *L. reuteri*. Hekmat and Reid (2006) studied the growth and survival of *L. reuteri* RC-14 in milk with different supplements. They established that *L. reuteri* was able to grow in milk fortified with yeast and/or inulin. The acidification rate was however faster when yeast was provided. Yeast extract is one of the ingredients of MRS broth, and contains nutrition that can be used to promote bacterial growth. This theory is supported by the acidification rate of *L. reuteri* after supplementation with MRS broth (fig. 4). The maximum acidification rate was achieved in milk supplemented with 1% casamino acids. A final pH of 4.34 was reached after 24 hours in the latter case, compared to 4.89 in milk supplemented with 5% MRS. A study made by Hidalgo-Morales et al. (2005) observed higher growth rates and acidification abilities of *L. reuteri* NRRL 14171 after enrichment with casein peptone. This indicates that an efficient proteolytic system is needed for adequate growth in milk. Also Østlie et al. (2004) used the approach to add an extra source of peptides, this time from tryptone, to support the growth of different probiotic bacteria in milk. They found that *L. reuteri* had the fastest growth of all bacteria tested for temperatures between 37°C and 45°C, which is considered as good temperatures for the growth of *L. reuteri* in other mediums. Without the addition of tryptone, the difference in growth rate would probably be too slow to monitor. In the present study, these former observations were confirmed as casamino acids would fill the same purpose as casein peptone and tryptone when it comes to provide easily accessible amino acids.

Cysteine was tested as growth promoting supplement on the basis of its reducing activity. In the trial with cysteine supplementation, no increase in milk acidification could be detected (fig 3). The population was also low, with only $9.1x10^6$ CFU/ml after 6 hours incubation compared with $1.1x10^7$ CFU/ml for the control fermentation. This indicates that the growth of *L. reuteri* is unaffected by the possible reducing power of cysteine. It is nevertheless important to consider that the action of cysteine seems to be concentration dependent. A study was made by Dave and Shah (1998) concerning the effect of milk supplement on growth of probiotic bacteria. They found that the time to reach pH 4.5 increased considerably on addition of 250 and 500 mg cysteine. However, samples with smaller concentrations added showed a drop in pH during the first 24 hours similar to the control. These results indicate that higher concentrations of cysteine could adversely affect the acid production.

Previous studies have suggested that the growth of *L. reuteri* is limited in milk without growth promoting supplements (Xanthopoulos et al., 2000; Hidalgo-Morales et al., 2005), and this is also confirmed in this study since only a limited decrease in pH was achieved in the control milk for all trials. The viable cell count after 6 hours of incubation varied between the trials, and was supposed to be positively correlated to the acidification rate. The population was $2.9x10^8$ CFU/ml and $1.1x10^7$ CFU/ml in milk supplemented with casamino acids and without supplementation, respectively. This result clearly demonstrates that the supplementation of casamino acids enhances the growth and acidification rate for *L. reuteri*. However, in the case of cysteine supplementation, the viable cell count seems to be negatively correlated to the pH as the supplemented milk showed a population of $9.1x10^6$ CFU/ml despite a pH lower than the control fermentation pH after 6 hours. The viable count of control fermentation showed a population of $1.1x10^7$ CFU/ml at the same time. No viable cell count was made on the trial with MRS broth supplementation.

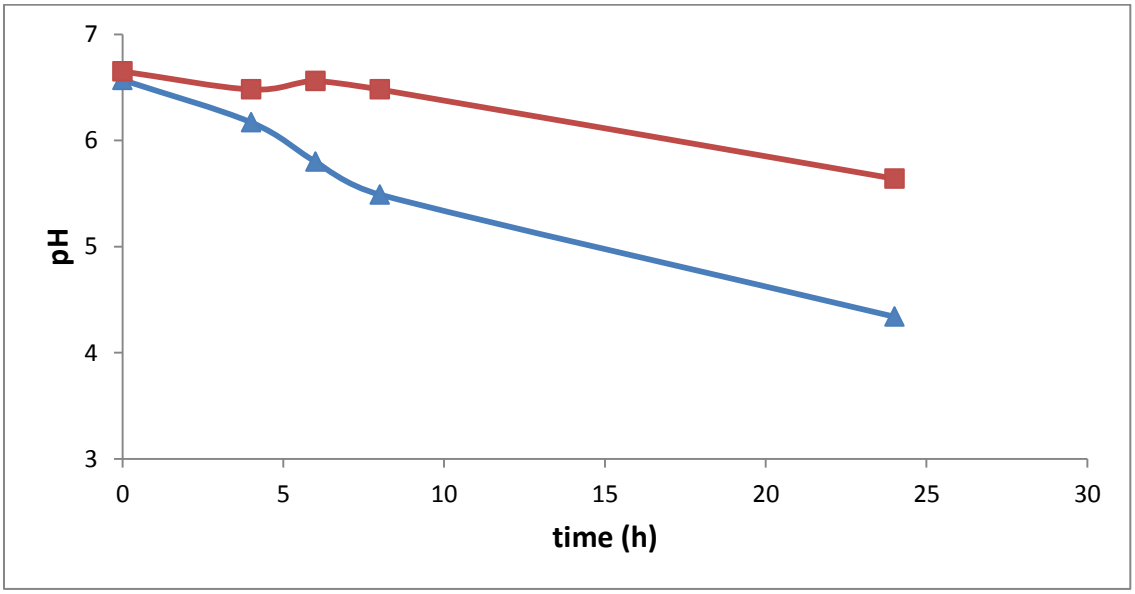

Fig 2. Mean Growth of *L. reuteri* DSM 17938 in milk from duplicate measurements. Supplementation with 1% casamino acids (▲) and without supplementation (■).

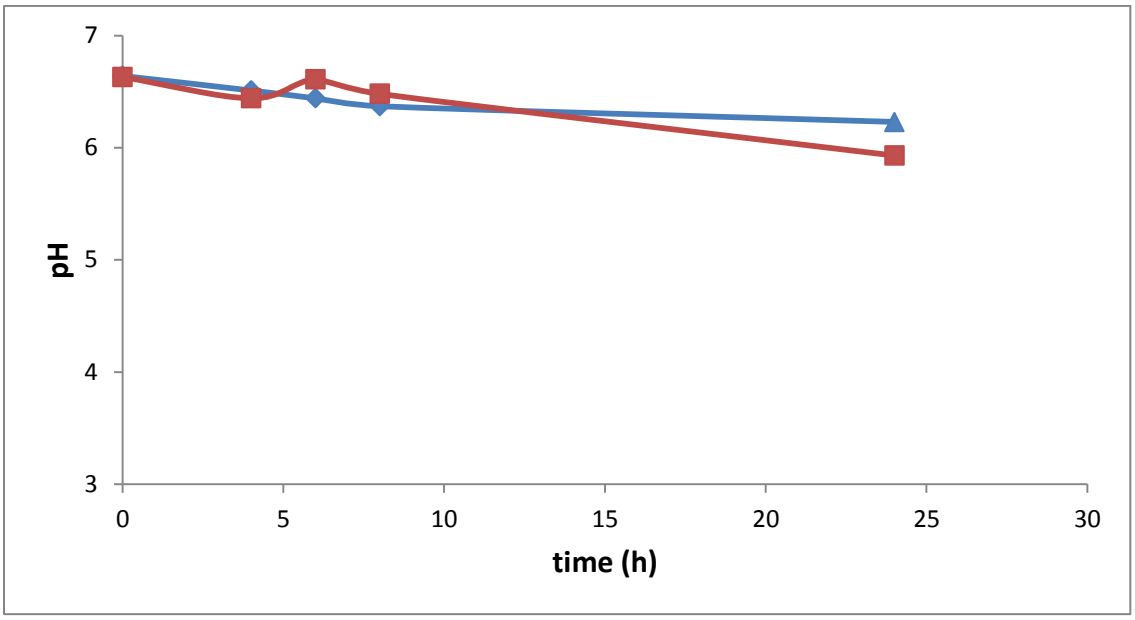

Fig 3. Growth of *L. reuteri* DSM 17938 in milk. Supplementation with 0,2% cysteine (▲) and without supplementation (■).

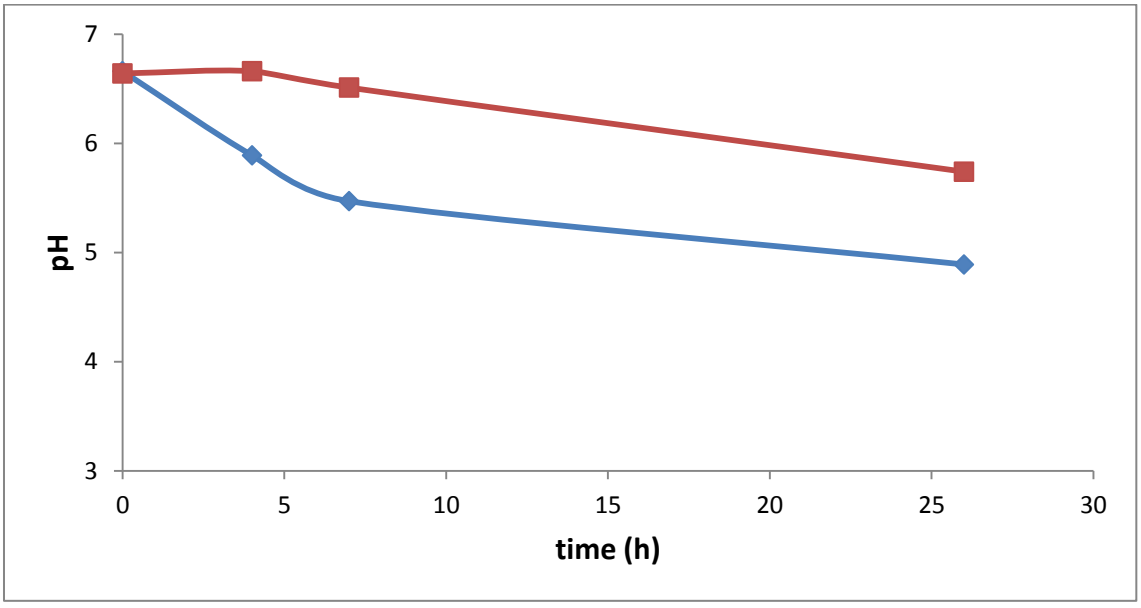

Fig 4. Growth of *L. reuteri* DSM 17938 in milk. Supplementation with 5% MRS (▲) and without supplementation (■).

Effect of support cultures

Acidification rate of *L. reuteri* DSM17938 in pure culture and with yoghurt or *L. delbrueckii* as adjunct culture are compared in figure 5 and 6, respectively. Supplementation with both yoghurt culture and *L. delbrueckii* contributed to a more rapid decrease in pH compared to *L. reuteri* in pure culture. The same pattern could be monitored when comparing with control

fermentation involving simply yoghurt culture or *L. delbrueckii*. This indicates that the increase in acidification rate is not only controlled by the support culture. Supplementation with yoghurt culture provided a final pH of 5.24 after 8 hours, compared with 6.45 and 5.65 for fermentation with *L. reuteri* and yoghurt bacteria, respectively (fig. 5). The viable count of *L. reuteri* was also higher in the former case with $6.5x10^7$ CFU/ ml compared with $1.2x10^7$ CFU/ml for the fermentation in pure culture. This indicates that the more rapid acidification was a consequence of increased growth of *L. reuteri* as well of growth of other LAB in the milk. The specific strains present in the yoghurt culture used in this study (Arla mild, 3%) were not characterized. However, since traditional yoghurt culture usually contains the synergistic bacteria pair *S. thermophilus* and *L. delbrueckii*, the yoghurt was assumed to contain these species. Observations during the present study indicate that *L. delbrueckii* is accessory in the increased acidification during fermentation with yoghurt culture as adjunct culture for *L. reuteri*. The pH of milk fermented with *L. reuteri* and *L. delbrueckii* in co-culture was down to 5.63 after 6 hours, compared to 6.46 and 6.01 for *L. reuteri* and *L. delbrueckii* in pure culture. The results are similar to the evaluation of *L. reuteri* with yoghurt as adjunct culture, indicating that *L. delbrueckii* could be involved in the increase in growth and acidifying activity of *L. reuteri.* This theory is supported by that fact that *L. reuteri*, as well as other probiotic bacteria, have a weak proteolytic system similarly to *S. thermophilus* (Hidalgo-Morales et al., 2005). With this in mind, *L. delbrueckii* should be able to support the growth of *L. reuteri* in the same manner, which is indicated in this study.

Adjunct cultures have formerly been used in order to increase the acidification level of different probiotics. Saxelin et al. (1999) tested a number of probiotic strains with *S. thermophilus* or yoghurt culture as support culture. Acidification time was mainly controlled by the support culture, but in some cases the combination of probiotic and support culture enhanced the acidification rate. The results varied with different probiotic strains, however, and demonstrate the importance in selecting the optimal support culture for specific bacteria. In this study, a higher acidification rate was achieved in fermentations with yoghurt culture, containing both *S. thermophilus* and *L. delbrueckii*. This indicates that *S. thermophilus* could act as a support culture to *L. reuteri* as well. The potential for using adjunct cultures in order to promote growth of *L. reuteri* is good, but needs to be supplemented with further trials concerning other species and strains used in the mixed culture.

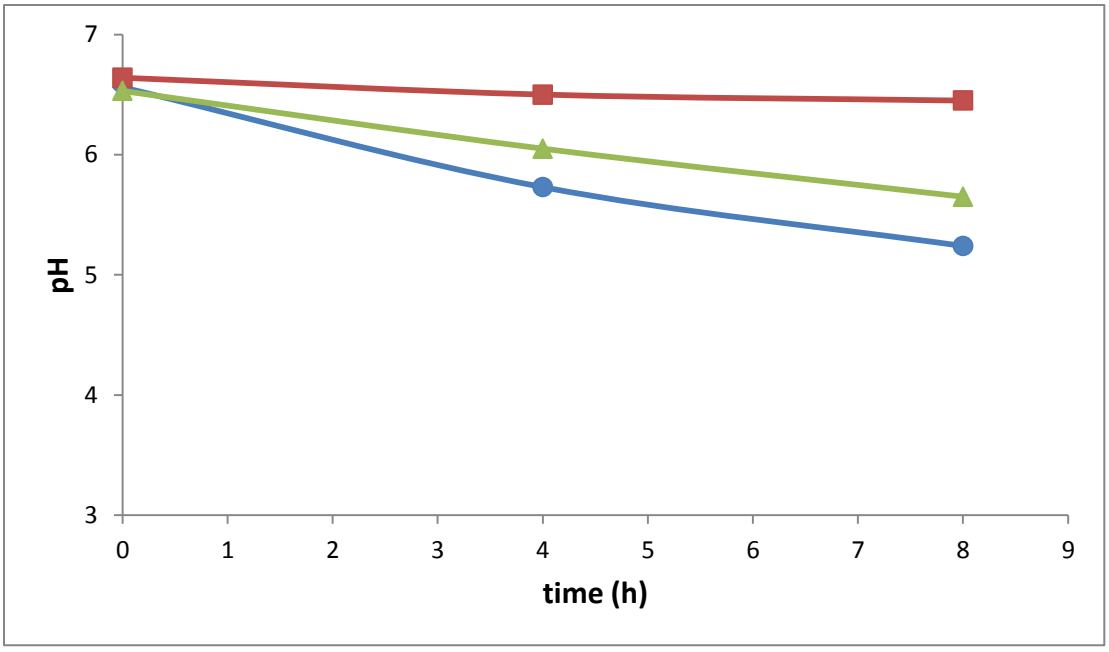

Fig 5. Mean Growth of *L. reuteri* DSM 17938 in milk from duplicate measurements. Growth in pure culture (■), with yoghurt as support culture (●) and control fermentation with only yoghurt culture are shown (▲).

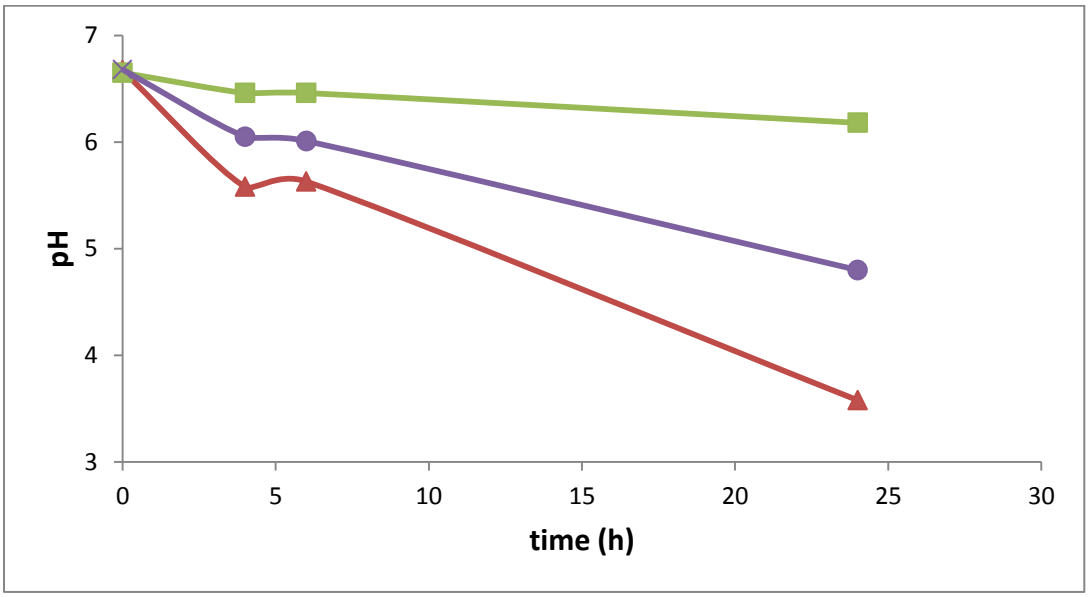

Fig 6. Growth of *L. reuteri* DSM 17938 in milk in pure culture (■) and with L. delbrueckii as support culture (▲). Control fermentation with L. delbrueckii is also shown (●).

Growth and survival of *L. reuteri* in hard cheese

L. reuteri and total lactobacilli counts were monitored during storage of hard cheese for a period of 83 days. Low concentrations of *L. reuteri* were found in both the test-cheese and the control-cheese. Only 10^3 CFU/ml was found in the milk inoculum in the beginning of the cheese manufacturing, and this number remained more or less constant in the curd before

molding. After pressing and 1 day storage in room temperature, the population was one order higher in all cheeses. After 21 days of storage, levels of $2.5x10^3$ and $2x10^4$ CFU/g were found on the selective Rogosa medium for the test and control cheese respectively. The presence of *L. reuteri* could despite growth on the selective medium for both cheeses, only be confirmed with PCR for the test-cheese. During early stages of the project, there were some doubts concerning the reliability of the Rogosa ampicillin medium, as they failed to give consistent results. There is also a possibility that another strain of *L. reuteri* was present in the control cheese. However this is not very likely, since only DSM17938 is resistant to ampicillin, which makes growth on the selective medium possible. There are several possible reasons for the low viable count during cheese production and storage. The low initial count was probably due to insufficient growth of *L. reuteri* in the starter culture. Since inoculation with an old starter was made, there is a possibility that contamination with other bacteria could have taken place. Since the growth of *L. reuteri* in milk is slow, they are easily outgrown by other bacteria if they are favored by the environment. There are also process factors that could have promoted the bacterial growth during cheese making, if optimized properly. For example, the temperature used during cheese making and during storage, as well as the aerobic environment and inoculation method must be evaluated (Karimi et al., 2011). In the production of cheese at Skogsbackens, a cooking temperature of 40°C was used. According to Østlie et al. (2004), *L. reuteri* SD2112 shows a fast growth in milk at both 37°C and 45°C, indicating that the temperature chosen for the cheese production should be sufficient. It is however important to note that the optimum temperature could vary between different strains of a certain species.

There is a lot of potential in supporting the growth of *L. reuteri* in the milk inoculum during starter and cheese production. Results from preliminary tests concerning growth enhancing formulation (section 3.1.2 and 3.1.3) suggest that addition of growth promoting supplements, such as casamino acids and/or adjunct cultures, would promote the growth of *L. reuteri* in cheese. For example, good results were achieved when *L. reuteri* was grown together with a traditional yoghurt culture to produce a starter culture used for cheese production (data not published/shown).

The survival of *L. reuteri* in the cheese was high, since the final bacterial count after 83 days storage was constant and even higher with a population of $6x10^3$ CFU/g. This suggests that a higher initial count in the milk inoculum could provide a cheese with a satisfactory amount of probiotic bacteria at the end of the ripening period. The adjunct lactobacilli from the mesophilic starter culture were found to survive during cheese making with a total count of 10^7 CFU/g in the milk inoculum. At the end of the storage period, the total count of lactobacilli (including NSLAB) was $5x10^8$ CFU/g. However, a characterization of LAB present in the cheese was not made during storage, and there is a possibility that a shift from SLAB to NSLAB that could influence the composition of bacteria present. Proliferation of NSLAB can be a problem as this microbial group competes for nutrients and sometimes creates a problem when it comes to qualitatively determine the viability of probiotic bacteria (da Cruz et al., 2009).

A PCR product of the expected size 177 bp including primers, was obtained for colonies isolated from the cheese stored for 21 days, showing that *L. reuteri* had been successfully incorporated in the test-cheese. The levels were not high enough to meet the standards for probiotic cheese, but adjustments of the procedure could be made in order to increase the initial level of probiotic bacteria in the milk inoculum. However, the high survival of *L. reuteri* DSM 17938 indicates that it could be used as probiotic bacteria in a hard cheese with good results.

Growth and survival of L. reuteri in fresh cheese

The viability of *L. reuteri* and total lactobacilli was monitored during storage of fresh cheese for a period of 14 days. Several scientific papers propose a minimum amount of probiotic bacteria in cheese corresponding to 10^6-10^7 CFU/g (Buriti et al., 2004; Cruz et al., 2009 Karimi et al., 2011). In the present study**,** the population of *L. reuteri* in fresh cheese T1a containing a mixed *L. reuteri* and yoghurt starter was over the minimum level of 10^6 CFU/g during the storage period (table 3). Cheese T1b failed to reach the threshold value after 1 day, but eventually reached a higher population after 7 days of storage. At this point, T1a and T1b containing *L. reuteri* in co-culture with a yoghurt culture, revealed populations of $3.6x10^8$ and $4.0x10^6$ CFU/g, respectively. The final levels were $1.0x10^8$ and $3.9x10^6$ CFU/g after 14 days of storage. The difference in final counts between the batches was due to a lower level of *L. reuteri* in the starter culture for T1b. The explanation for this is probably overgrowth with yoghurt culture, as the drop in pH was faster than normal during production of the starter. The lactobacilli from adjunct culture were found to survive cheese making with a total count of 10^8-10^9 CFU/g in the milk inoculum. The total count of lactobacilli was constant after 14 days of storage with 10^9 CFU/g in all trials. It is therefore suggested that the variation in viable count of *L. reuteri* not is influenced by the activity of the support culture. Similarly to the situation during the study of hard cheese, no characterization of LAB present in the cheese was made during storage, and there is a possibility that a shift from SLAB to NSLAB that could influence the composition of bacteria present.

PCR was used to confirm the presence of *L. reuteri* DSM 17938 in T1a and T1b, and a product of the expected size 177 bp, was obtained from colonies isolated from the fresh cheese, indicating that *L. reuteri* had been successfully incorporated. The results of this study suggest that fresh cheese appears to be well suited to serve as a carrier for *L. reuteri*. This is supported in a number of studies, reporting the development of fresh cheeses containing a satisfactory amount of probiotic bacteria during storage. Souza et al. (2008) produced a fresh cheese supplemented with *L. acidophilus*, added solely or with *S. thermophilus* as support culture. Cheese produced with only *L. acidophilus* presented populations above 10^6 CFU/g after 7 days of storage and 10^7 CFU/g after 14 days. Cardarelli et al. (2008) carried out a study on a symbiotic petit-suisse cheese manufactured with *L. acidophilus* and *Bifidobacterium animalis* ssp. *lactis*, with populations of 10^6 CFU/g and 10^7 CFU/g, respectively. In the present study we found that *L. reuteri* were viable during storage at levels of $1.0x10^8$ and $3.9x10^6$ in the fresh cheese produced. These levels are high enough to meet the required

standard in the final product. The results indicate that *L. reuteri* has potential to be a new probiotic bacteria incorporated in fresh cheese.

Table 3. Viability of *L. reuteri* in fresh cheeses T1a and T1b during storage at 2°C

Storage days	T1a (CFU/g)	T1b(CFU/g)
1	$3x10^7$	-[a]
7	$3.6x10^8$	$4x10^6$
14	$1x10^8$	$3.9x10^6$

[a] Number under the detection level of 10^6 CFU/g

Inhibition of mold and yeast growth

As a consequence of inadequate draining, cheese T1b and T2a had higher moisture content than T1a and T2b. This made the evaluation of mold and yeast growth uncertain as cells and spores was spread with the liquid lost from cheese during storage. A difference in growth could be seen between the different yeasts. The growth of *Yarrowia lypolytica* and *Geotrichum candidum* was high in all trials with a bigger growth diameter than other yeast tested. The only mold tested, *Penicillium roqueforti* showed a limited growth in all trials, with a growth diameter of 1 cm after 8 days storage. However, since the initial concentration of the suspension used was unknown, it is hard to evaluate any significant difference between the species. Further trials are necessary in order to evaluate the inhibitory activity of *L. reuteri* DSM 17938 against molds and yeasts.

Inhibitory activity of *L. reuteri*

Results of the inhibitory activity of *L. reuteri* DSM17938 on *S. aureus* and EHEC are shown in figure 7 and 8 as viable counts after 0, 3 and 10 days**.** The initial counts of pathogens were relatively high in all trials with a population of 10^7-10^6 CFU/g. There is however still a possibility to encounter this number in cheese, according to literature. Khayat (1987) found that a population of 10^2-10^6 or 10^3 CFU/g was normal in hard and fresh cheese, for coliforms and *S. aureus* respectively. The higher count was found in fresh cheese products indicating that contamination and growth may be a bigger problem in the mentioned group.

S. aureus was clearly inhibited in both fresh cheese with mixed *L. reuteri* and yoghurt starter and pure yoghurt starter. The initial count of *S. aureus* was $3.3x10^6$ CFU/g in the cheese with mixed *L. reuteri* and yoghurt starter, which dropped to $2.5x10^4$ CFU/g after 10 days storage. This was compared with a drop from $1.1x10^7$ CFU/g to $3.75x10^5$ CFU/g for the control cheese. When looking at the survival of *S. aureus* in the samples, the figure is 0.76% survival with *L. reuteri* added, compared with 3.4% without. It should be noted that the higher initial count in the control cheese probably is a consequence of uneven distribution of bacteria in the sample, since one of the duplicate samples had a considerable higher population than the other. When looking at the duplicate with lower initial count, the decrease in population was lower compared with the control cheese. Another trial would, however, be needed in order to

confirm this result. The cheese samples were incubated at a low temperature and with the high acidification rate, not offering the optimum growing conditions for *S. aureus*. In order to survive, a pH over 4.2 is necessary depending on acid used. Lactobacilli in general are known to have inhibitory activity on pathogens in food products during food fermentations, as they may produce hydrogen peroxide and other bactericidal compounds (Liu et al., 2012). In order to evaluate the inhibitory effect of reuterin on *S. aureus* from DSM 17938, further investigations are needed.

Initially, the viable count of EHEC in the cheese with *L. reuteri* and yoghurt culture increased from $5.6x10^6$ CFU/ml to $1.1x10^7$ during 3 days storage. A small decrease in survival could be seen after 10 days with a population of $3.2x10^6$ CFU/ml. However, the same pattern could also be monitored in the test cheese. It is suggested that a fast souring starter could prevent the growth of EHEC and other coliforms in cheese, as the pH drops fast enough to inhibit growth (Walstra et al. 1999). Studies made on the inhibitory activity of *L. reuteri* generally suggest a good effect against both *S. aureus* and *E. coli* (including EHEC). El-Ziney et al. (1998) found that *L. reuteri* 12002 was inhibitory against EHEC in cottage cheese. The effect seems to be concentration dependent, as the rate of population reduction was 2, 3 and 6 log cycles after 7 days for reuterin concentrations of 50, 100 and 150 units per gram, respectively. The inhibitory activity of the lowest concentration was still higher than the one monitored during the trial of this present study, indicating that the concentration of reuterin produced in fresh cheese may have been insufficient to inhibit growth of EHEC. Since no detection of reuterin was made in the fresh cheese, it is unclear whether the concentration was high enough to provide a bactericidal effect. Knowledge of the reuterin concentration produced by the starter is important in order to draw any conclusions from the study. In a study by Mohamadi Sani et al. (n.d.), reuterin produced by *L. reuteri* 20015 was tested against pathogens in feta-cheese, including *S. aureus* and *E. coli.* The viable counts of *S. aureus* dropped from 10^7 to 2.5×10^6 CFU/g within 5 hours. The population of *E. coli* dropped even further, below the detection limit. This present study suggests a similar result for *S. aureus*, indicating that *L. reuteri* DSM 17938 may have an inhibitory affect against the bacteria. Since *S. aureus* turned out to be inhibited in the test cheese as well, it is not clear whether or not the inhibitory activity origins from *L. reuteri* or adjunct lactobacilli present in the cheese. Further investigations are necessary in order to evaluate the inhibitory activity of *L. reuteri* DSM 17938 in cheese.

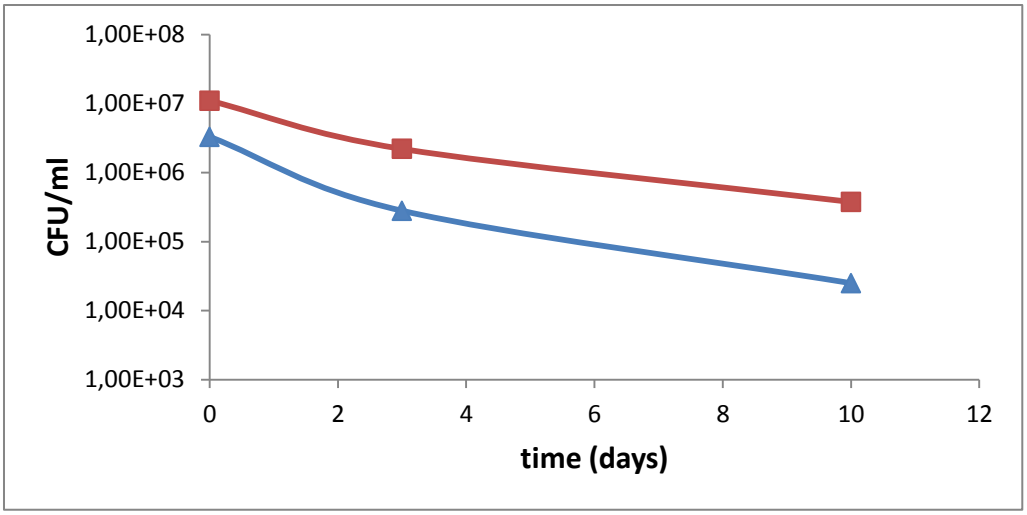

Fig 7. Viable count of *S. aureus* on mannitol salt agar (MSA) after 0, 3 and 10 days. Data are shown for fresh cheese with mixed *L. reuteri* and yoghurt starter (▲) and pure yoghurt starter (■).

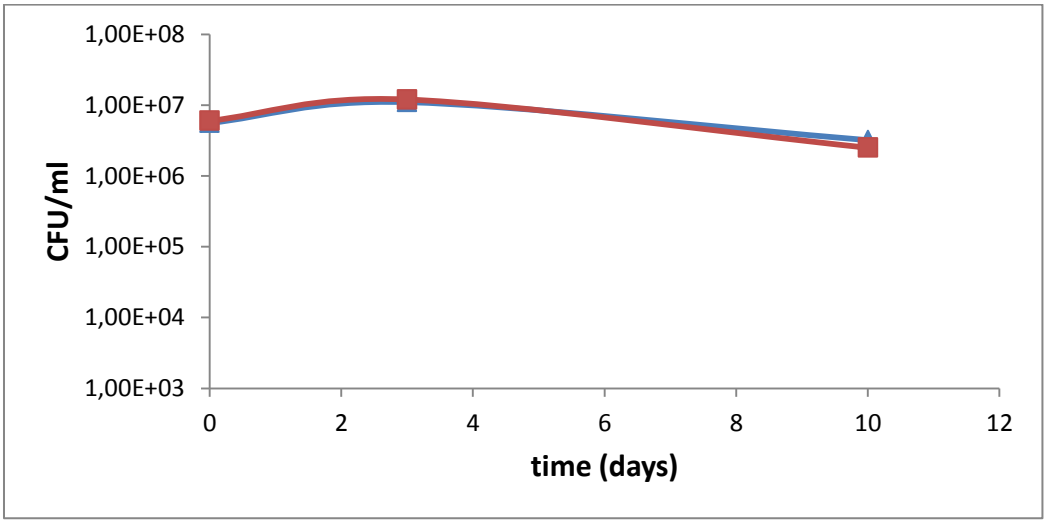

Fig 8. Viable count of EHEC on MacConkey agar (MCA) after 0, 3 and 10 days. Data are shown for fresh cheese with mixed *L. reuteri* and yoghurt starter (▲) and pure yoghurt starter (■).

Conclusions

The results of this study indicate that the probiotic bacteria *L. reuteri* DSM 17938 can grow in milk, but the acidification rate was low during all trials. Addition of casamino acids or MRS broth as supplements resulted in both higher levels of viable organisms and higher acidification rate. Moreover, the addition of an adjunct starter culture resulted in faster growth and metabolism. This indicates that a low proteolytic activity could be the reason for the limited growth in milk, which probably isn't an optimum growth medium for *L. reuteri* DSM17938. This problem can however clearly be encountered by compensating for this with addition of growth enhancing supplements or a proteolytic adjunct culture. Acidified milk with high numbers of viable bacteria can be used as starter in the production of cheese and other fermented dairy products. The present study demonstrates that cheese can be used as a carrier *L. reuteri* DSM 17938. The survival was high in both fresh and hard cheese when *L. reuteri* was incorporated together with yoghurt culture as adjunct starter. Furthermore, both *S. aureus* and EHEC was inhibited in a fresh cheese with both the addition of *L. reuteri* DSM 17938 and a yoghurt culture, respectively. There was an indication of a stronger inhibitory activity on *S. aureus* in the presence of *L. reuteri*, but further trials are needed in order to confirm this. With a number of claimed health benefits and indications of inhibitory activity against pathogens, *L. reuteri* is a good candidate for probiotic starter in cheese production. Results of this study suggest that strain DSM 17938 can be used as a starter in the production of fresh cheese with sufficient viable counts at the time of consumption. The possibility to use the bacteria when manufacturing a hard cheese is probably also good, as survival turned out to be high in this kind of cheese despite a low initial count in the milk inoculum.

Acknowledges

For teaching me a lot about cheese manufacturing and for giving me the opportunity to take part in your production, I wish to thank Marih Jonsson at Skogsbackens ost. Great acknowledges to Stefan Roos for excellent supervison, and for always being very helpful. Further, I also thank Hans Jonsson and the rest of the lab staff for practical support and good companionship.

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Appendix

Populärvetenskaplig sammanfattning

Användandet av probiotiska bakterier i matproduktion är något som under de senaste åren fått en ökad popularitet på grund av dess positiva egenskaper för hälsan. Probiotika definieras som ”levande mikroorganismer som när de intas i tillräcklig mängd ger hälsoeffekter hos individen”, och är idag en viktig del i mångas kost då kosttillskott och berikade livsmedel blivit vanligare de senaste åren. Särskilt populära är de många yoghurtsorter och mjölkdrycker som innehåller probiotika. De bakterier som används som probiotika tillhör de så kallade mjölksyrabakterierna, och vanligast är olika stammar av lactobaciller eller bifidobakterier. Genom att omvandla mjölksocker, eller laktos som det också kallas, till mjölksyra sänker de pH-värdet i produkten, vilket bidrar till såväl smak som mognadsprocessen i livsmedlet. *Lactobacillus reuteri* är en bakterie som är intressant i detta sammanhang. Olika stammar har visat sig ha hälsofrämjande egenskaper i väldokumenterade studier, och har tidigare använts i kosttillskott såväl som i matproduktion. Den producerar dessutom en substans, reuterin, som kan hämna tillväxten av diverse andra bakterier och andra enklare organismer. Denna egenskap har potential att bidra till en bättre hygienisk kvalitét hos ost tillverkad på opastöriserad mjölk, då risken finns att den kan innehålla sjukdomsalstrande bakterier.

I denna studie tillverkades två olika ostar med *L. reuteri* som starterkultur: en hårdost gjord på opastöriserad mjölk, och en färskost gjord på pastöriserad mjölk. I båda ostarna visade bakterien en hög överlevnad under lagringstiden, vilket krävs om halten probiotika ska vara hög nog att kunna ge några hälsovinster i slutprodukten. Eftersom *L. reuteri* har en begränsad tillväxt i mjölk var det dock problematiskt att tillverka en fungerande starterkultur till ostproduktionen. I studien utfördes därför ett antal försök, med syfte att förbättra *L. reuteri´*s förmåga att växa och producera syra i mjölk. Denna egenskap är viktig för att kunna producera en ost av god kvalitet. Det finns en uppfattning om att *L. reuteri* och vissa andra probiotiska bakterier växer dåligt i mjölk eftersom de saknar lättillgängliga kvävekällor och andra näringsämnen i mjölken och har en låg förmåga att tillverka dessa själva. Detta kunde bekräftas då tillsats av aminosyror eller andra näringsrika lösningar bidrog till en ökad tillväxt och syrabildande förmåga då *L. reuteri* användes för att fermentera mjölk.

En annan metod för att berika mjölken med lättillgängliga proteiner är att använda en så kallad stödkultur. Denna kan bidra till en förbättrad tillväxtmiljö genom att producera de ämnen som *L. reuteri* saknar för att fungera optimalt. *L. reuteri* odlades tillsammans med en yoghurtkultur för att kunna producera en startkultur lämplig för osttillverkning. En traditionell yoghurtkultur består av bakterierna *Streptococcus salivarius* ssp. *thermophilus* och *Lactobacillus delbrueckii* ssp. *bulgaricus.* De båda hjälps åt att syra mjölk till yoghurt och kunde i denna studie även bidra till en ökad tillväxt av *L. reuteri* när de odlades tillsammans. Denna metod användes för att tillverka en färskost med ett högt innehåll av både *L. reuteri* och yoghurtbakterier. Till denna ost tillsattes två olika sjukdomsalstrande bakterier, *Escherichia coli* O157:H7 (EHEC) and *Staphylococcus aureus*, vilka båda kan förekomma i

ost, för att se om förekomsten av *L. reuteri* kunde bidra till en minskad tillväxt och överlevnad av dessa bakterier. Resultaten tyder på att ett högt antal *L. reuteri* skulle kunna bidra till en minskning av *S. aureus* i ost. Detta skulle vara fördelaktigt vid tillverkning av ost, då processen är känslig för kontaminering. Vid modern tillverkning är den hygieniska kvaliteten hög. Dock går det inte att utesluta förekomsten av sjukdomsalstrande samt förstörelsebakterier i osten, särskilt när man använder sig av opastöriserad mjölk som råmaterial Det är därför viktigt med en startkultur som kan hindra tillväxten av dessa.

Det finns en stor marknad för nya probiotiska produkter, och då särskilt för probiotiska ostar, som idag är ett begränsat område. Med många rön angående dess hälsofrämjande egenskaper och dess potentiella bidrag till en säker produkt av hög hygienisk kvalitet, finns det höga förhoppningar att kunna använda *L. reuteri* som startkultur i ostproduktion.