Microbial composition of artisan starter cultures used in Swedish dairy products

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Abstract

Food products with a strong connection to their geographical origin have shown to be beneficial on the market. A potential is seen in dairy products with biodiverse autochthonous natural starter cultures. A collaboration between Swedish Farmhouse Dairy Producers (Svenska Gårdsmejerister) and the Swedish University of Agricultural Sciences, SLU (Sveriges lantbruksuniversitet) has started for documentation and preservation of the methods for starter culture production and promotion of the final products. The project is funded by the Swedish Board of Agriculture (Jordbruksverket) and has the journal number 2022-2724.

The aim of the master's project was to identify the microorganisms in starter cultures made by Swedish farmhouse dairy producers. Thermophilic and mesophilic starter cultures from three dairy producers were sent to the laboratory at SLU several times during a time frame of half a year to see how the starter cultures differ between producers and over time. Samples from the cultures were collected, prepared, and cultivated on agar plates before the colony forming units were counted. After recultivation on specific agar plates, the microorganisms were identified by a MALDI-TOF mass spectrometer.

Generally, the number of colony forming units per milliliter increased in the starter cultures with time. Most bacteria species detected in the cultures could be categorized as beneficial microorganisms favorable for the dairy product. The results showed that all three starter culture producers had cultures containing *Lactococcus lactis* and *Leuconostoc pseudomesenterioides*. Two producers had starter cultures which contained *Leuconostoc mesenteroides* and *Micrococcus luteus.*

On the other hand, a few unwished microorganisms were found which could be problematic if present in the dairy product. *Escherichia coli, Straphylococcus haemolyticus, Enterococcus hirae* and *Enterococcus faecium* were found in some of the starter cultures. Otherwise, most starter cultures showed a unique microbial composition with producer B having the least diverse cultures and producer C having the most diverse ones. In summary, the method used for the production as well as the recultivation rate of the starter culture strongly affects the numbers and composition of microorganisms.

Keywords: Lactic acid bacteria, starter culture, cheese, fermentation, geographical indication

Sammanfattning

Livsmedelsprodukter med en stark koppling till dess geografiska ursprung har visat sig vara fördelaktiga på marknaden. Därmed finns det potential hos mejeriprodukter som gjorts på naturliga, ursprungliga och diversifierade startkulturer. Ett samarbete mellan föreningen Sveriges Gårdsmejerister och Sveriges lantbruksuniversitet (SLU) har påbörjats för att dokumentera och bevara metoderna som används till produktionen av startkulturer. Projektet är finansierat av Jordbruksverket och har journalnumret 2022–2724.

Syftet med masterprojektet var att identifiera mikroorganismerna i starkulturerna som har producerats av svenska gårdsmejerister. Termofila och mesofila startkulturer från tre mejerister skickades till laboratoriet på SLU ett flertal gånger under ett halvår. På det sättet gick det att undersöka hur kulturerna skiljer sig åt mellan producenterna och över tid. Prover från kulturerna samlades in, preparerades och odlades upp på agarplattor för att antalet kolonier skulle räknas. Efter renstrykning på specifika agarplattor blev mikroorganismerna identifierade med en MALDI-TOF-masspektrometer.

Överlag ökade antalet kolonier per milliliter i startkulturerna med tiden. Majoriteten av bakteriearterna som upptäcktes i kulturerna kunde kategoriseras som fördelaktiga mikroorganismer som är gynnsamma i mejeriprodukter. Resultaten visade att alla tre av producenterna hade kulturer som innehöll *Lactococcus lactis* och *Leuconostoc pseudomesenterioides*. Två av producenterna hade startkulturer som innehöll *Leuconostoc mesenteroides* och *Micrococcus luteus*.

Några oönskade mikroorganismer, såsom *Escherichia coli, Straphylococcus haemolyticus, Enterococcus hirae* och *Enterococcus faecium*, upptäcktes under identifieringen. De kan orsaka problem om de finns kvar när mejeriprodukterna konsumeras. För övrigt hade majoriteten av startkulturerna en unik mikrobiologisk sammansättning. Producent B hade minst mångfald i sina kulturer medan kulturerna från producent C hade störst mångfald. Sammanfattningsvis kan det fastställas att både metoden för och tillväxthastigheten hos startkulturen i hög grad påverkar antalet och sammansättningen av mikroorganismer.

Nyckelord: Mjölksyrebakterier, startkultur, ost, fermentering, geografiskt ursprung

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Abbreviations

SLU	Swedish University of Agricultural Sciences
LAB	Lactic acid bacteria
NSLAB	Non-starter lactic acid bacteria
PAB	Propionic acid bacteria
DVS	Direct vat starter
GABA	γ-aminobutyric acid
PDO	Protected Designation of Origin
PGI	Protected Geographical Indication

1. Introduction

1.1 The project

1.1.1 The collaboration

Swedish Farmhouse Dairy Producers (Sveriges Gårdsmejerister) have applied for and granted project support from The Swedish Board of Agriculture to stimulate and conserve the cultural heritage within artisan dairy products from Swedish farmhouses. It is a collaboration between Swedish University of Agricultural Sciences (SLU), Swedish Farmhouse Dairy Producers, restaurants and cheese shops for development and marketing of small-scale cheeses and other dairy products (Sveriges Gårdsmejerister n.d.).

SLU was tasked to document the methods used to produce biodiverse autochthonous natural starter cultures for cheese production and characterize the microbial composition over time. Microbial identification can ensure the quality of the cultures and decrease the risk of detrimental microorganisms causing illness and product spoilage. It can also lead to protection of the characteristics connected to the terroir. On top of that, the knowledge about traditional methods for starter cultures is a cultural heritage which is thought to be important to preserve.

1.1.2 Geographical indication

Terroir is a French term first used in the wine industry which refers to the characteristics of a specific location including soil, climate, and other environmental factors. Terroir means literally soil or land. The terroir affects the phenotype of a crop and consequently artisan food products including wine, coffee, tea, single malt whisky or cheese. Some terroirs are famous due to the wine produced in that area, for example the sparkling wine champagne which is produced in Champagne, France. The terroir can work as a link between production area and product quality, and therefore play an important role in trading. That understanding

has led to the phenomenon of geographical indication of food products (Meloni & Swinnen 2018).

Geographical indication can be used in marketing of food products. Names of traditional foods can be protected by being quality schemed by the European Union. Wrångebäcksost is the only Swedish cheese which has the label Protected Designation of Origin (PDO). Svecia and Sörmlands Ädel have the label Protected Geographical Indication (PGI) (European Commission n.d.). There are several Swedish dairy products which have applied for the geographical indication, and which are planned to be promoted in the project. The link between products and territory of production can be strengthened by using biodiverse autochthonous natural starter cultures for cheeses with Protected Designation of Origin (Chessa et al. 2021).

1.2 Starter cultures

A starter culture is a microbiological culture which assists the fermentation process in numerous foods and drinks including sourdough bread, cheese, kombucha and wine. In dairy production, the functions of starter cultures include acid production, proteolytic activity, aroma formation, exopolysaccharide formation, carbon dioxide production and production of inhibitory components. The organic acids have preservative effects. The carbon dioxide is responsible for eye formation. The transition of raw materials and foodstuff contributes to desirable taste, aroma, texture, and viscosity. Mesophilic and thermophilic strains can be used. In dairy production, mesophilic strains refer to a growth optimum temperature between 20 and 32 ºC and thermophilic strains refer to a growth optimum temperature between 37 and 45 ºC (Adams 2016; Tunick 2013).

The microbial activity in cheese production is often divided into two groups: the added starter culture and the secondary microflora. The activity of the added starter culture plays an important role in the beginning of the production while the secondary microflora is more active during cheese maturation (Adams 2016; Tunick 2013).

1.2.1 The secondary microflora

Yeast, mold, and bacteria from the raw milk and the surrounding are included in the secondary microflora. This natural flora has a unique composition for each farm. Therefore, non-starter yeasts and molds from raw milk may be transferred to raw milk cheese and influence the cheese ripening (Lavoie et al. 2012).

Non-starter lactic acid bacteria (NSLAB) either survive pasteurization or get into the milk from the surrounding (Tunick 2013). Many cheese varieties are dependent on NSLAB for development of diversity and typical features. The most important components of the NSLAB are mesophilic lactobacilli including lactococci, pediococci, enterococci and *Leuconostoc* sp. Thermophilic LAB are also part of NSLAB. NSLAB can be considered as adjunct cultures to control and improve cheese ripening (Gobbetti et al. 2015). They dominate the cheese matrix after lactose is consumed and contribute largely to the distinctive flavors in hard cheeses. They grow on other nutrients in an environment of pH 5.0-5.5 at 8-12°C (Shah et al. 2021).

1.2.2 Types of starter cultures

Even though milk contains natural lactic acid bacteria, starter cultures are added to enrich the milk with more and other species. Often, an added starter culture gives a higher number of LAB than the untreated milk. Therefore, the added starter culture can take over the original microflora in the milk. If the original microflora in the milk is wanted, it should be grown instead of being outcompeted. There are several methods for production of both defined and natural undefined starter cultures.

Defined starter cultures

Defined starter cultures are categorized as single-strain starters containing one strain of a certain species, multiple-strain starters containing different known strains of one species and multiple-mixed-strain starters containing different defined strains of different species. Defined starter cultures can have different origin and forms. Commercial fermented dairy products can be used as source of bacteria. Direct Vat Starters (DVS) is the most common type of starter culture used in the industrial dairy production. DVS are produced in laboratories on a large scale by multi-national companies and are available as frozen powders, liquids and frozen liquids (Tunick 2013).

Undefined starter cultures

A biodiverse autochthonous natural starter culture can be produced by souring milk on site. It is done by keeping fresh milk in a warm place for hours to ferment and sour. The bacteria naturally present in milk are multiplied in this suitable environment. The temperature for the incubation depends on the bacteria, generally room temperature for mesophilic cultures and heating cabinets for thermophilic cultures. The culture is incubated for several hours to grow in order to be ready to use. The culture is set when the pH has dropped to a specific interval, the texture is thick, and the aroma and taste is sourish. This type of culture can be placed in a fridge for storage and future use. Other natural sources of microorganisms can also be used in the production of starter culture including whey and plant material.

Multiple factors, such as the environment on the farm, feeding and animal species affect the microbial composition and characteristics of the starter culture (Tunick 2013).

In the same way as a sourdough can be fed, used, and stored for a long time, a starter culture used for dairy products can be used several times by using the back-slopping method. This is done by adding a small amount of the mother culture to the fresh milk creating a daughter culture. The new culture is produced when a stable microbial community is formed. This is controlled by pH-measurement and/or titration, and evaluation of texture, aroma and taste (Tunick 2013).

1.3 Lactic acid bacteria

Lactic acid bacteria (LAB) is a term with no strict taxonomic significance but with several common features. They are a group of gram-positive, non-respiring, non-sporulating bacteria which are useful in food fermentation. LAB include cocci and rods shaped bacteria which are anaerobic but aerotolerant, acid tolerant and lactate producing. They are found naturally in nutrient rich environments such as food products and occur as a normal flora in mammals and on plants in nature (Adams 2016). The genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are considered the principal LAB (de Souza Motta & Da Silva Mesquita Gomes 2015).

LAB are divided into three fermentation groups based on their glucose metabolism. The selection of pathways is determined at family level except for the genus *Lactobacillus* which include three groups with different metabolism. Cellular energy is derived from one of two different pathways used for fermentation of carbohydrate to lactic acid. LAB which use only the glycolysis pathway are obligately homofermentative and produce only lactate from the fermentation of glucose, lactose, sucrose, and other sugars. LAB which use the phosphoketolase pathway for fermentation of glucose, pentose and related compounds to ferment glucose to roughly equimolar amounts of lactate, ethanol/acetate and carbon dioxide are obligately heterofermentative. LAB which use the glycolysis pathway to ferment glucose and also use phosphoketolase pathway to ferment pentoses and related compounds are facultatively heterofermentative (Holzapfel & Wood 2014).

LAB require more than sugar to grow. Nucleotides, vitamins, amino acids, and peptides are needed. Proteolytic activity makes the nitrogen available which is

essential for the function of dairy starters and important for flavor development during ripening or maturation (Adams 2016).

1.3.1 LAB in food products

LAB is found in several fermented food products including sauerkraut, kimchi, sausage, sourdough, soy sauce, cocoa beans and kombucha. Often, the quality is kept, and safety is improved due to LAB´s ability to inhibit other microorganisms. Growth of LAB lowers the pH which inhibits many spoilers and most of the pathogens. Factors contributing to inhibition of other microorganisms by LAB include production of organic acids, bacteriocins, hydrogen peroxide, ethanol, and diacetyl, together with nutrient depletion, and low redox potential. Another effect of the pH drop is the transformation of the components in the foodstuff such as protein denaturation (Adams 2016).

Fermented foods are seen as health-promoting. Beneficial effects claimed for LAB include nutritional improvement of foods, inhibition of enteric pathogens, alleviation of diarrhea/constipation, hypocholesterolemic action, anticancer activity and simulation of the immune system (Adams 2016). On top of that, literature reports that probiotic LAB can restore the normal vaginal microbiota which increases woman wellbeing (Patrignani et al. 2019). Use of autochthonous LAB cultures isolated from milk with its own characteristics and designation of origin may be applied in development of artisan food products (de Souza Motta & Da Silva Mesquita Gomes 2015).

1.4 Microorganisms in milk and cheese

Microorganisms involved in the production and ripening of cheese include LAB, propionic acid bacteria (PAB), micrococci, staphylococci, coryneforms, yeast, and molds (Hayaloglu 2016). Homofermentative together with the heterofermentative LAB are the most important groups for cheese production of dairy foods since they generate lactic acid.

1.4.1 Microorganisms in milk

Milk contains water, fat, protein, lactose, vitamins, and minerals. Variations in composition depend on animal species, breed, stage of lactation, intervals between milking, time of day, time of the year, number of previous lactations, general nutritional state, and health of the cow. Milk is an excellent medium for microbial growth due to its high water activity, moderate pH (6.4-6.6) and ample supply of nutrients (Adams 2016).

The udder interior, the teat exterior and the equipment used in milking and milk-handling affects the microorganisms found in milk. The most commonly isolated microorganisms from the milk include various bacteria, primarily those involved in the fermentation of milk and those associated with spoilage. Examples include micrococci, streptococci and some species of *Corynebacterium* (Adams 2016).

If the milk is chilled and kept at a temperature below 7ºC, the only organisms capable of growing will be psychrotrophs (cold tolerant bacteria or archaea). Gram-negative rods of the genera *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, psychrotrophic coliforms, predominantly *Aerobacter* spp., and Gram-positive *Bacillus* spp. are the most commonly psychrotrophic species found in raw milk (Adams 2016).

Pasteurization was introduced around 1890 by the dairy industry to retard souring and prevent spread of disease. After pasteurization, the milk contains a much smaller number of bacteria and other microorganisms. Even though the heat treatment is an effective way to inhibit growth of unwanted microorganisms, some pathogens survive. Heat treatment can be divided into four groups. Low temperature holding (LTH): 63 ºC for 30 minutes, high temperature short time (HTST): 72 ºC for 15 seconds, ultra-high temperature (UHT): 135 ºC for 1 second and sterilized: >100 ºC typically 20-40 minutes (p. 139, Adams 2016). In general, cheese made from raw milk has higher bacterial counts at the first stage of ripening than cheese made from pasteurized milk (Hayaloglu 2016).

1.4.2 Lactic acid bacteria in milk and cheese

As mentioned, the genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are considered the principal LAB. Some of these genera, including *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Enterococcus*, are highly represented in fermented dairy products (de Souza Motta & Da Silva Mesquita Gomes 2015).

Lactococcus

Lactococcus are homofermentative coccus commonly used in the production of dairy products. Mesophilic starters are used in most cheesemaking and contain strains of *Lactococcus lactis* and its subspecies (Adams 2016). “Domesticated” LAB strains with faster acidification and loss of useless characteristics have come to have an increased specialization due to the intensive selection of LAB on properties in the dairy industry. For example, *L. lactis* has been classified into “domesticated” and “environmental” strains. A larger number of desirable or undesirable compounds are generated by “wild” strains of *L. lactis* (Thierry et al.

2015). Lactobacilli with glutamate dehydrogenase activity have shown ability to convert amino acids to keto and hydroxy acids which subsequently can be converted by *L. lactis* subsp. *cremoris* to carboxylic acids, which are important compounds for cheese aromas (Kieronczyk et al. 2003).

Lactococcus garviae is a facultatively anaerobic bacteria often present as NSLAB in cheese production. It can take part in preservation of the typicality of PDO cheese (Guarcello et al. 2016). Even though L. *garviae* can be involved in fish diseases, cases of human infections are very seldom reported (Casalta & Montel 2008). The LAB *Lactococcus laudensis* has been isolated from raw milk. *L. laudensis* has been tested as starter culture for cheese making and showed production of ethanol, acetic acid, diacetyl, and acetoin. "Wild" *lactococci*, strains isolable from raw milk or non-dairy environments, produce specific flavors (Tidona et al. 2018).

Lactobacillus

The genus *Lactobacillus* has resulted in 25 genera. The novel genera *Lactiplantibacillus, Lacticaseibacillus* and *Levilactobacillus* are included in an emended description. For example, formerly known *Lactobacillus plantarum* has the new name *Lactiplantibacillus plantarum* (Zheng et al. 2020). *Lactobacillus* include three groups with different metabolism: obligate homofermentaters, obligate heterofermenters and facultative heterofermenters. *Lactobacillus helveticus*, *Lactobacillus casei*, *L. lactis* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* are common strains in thermophilic starters. Thermophilic starters are used for Emmentaler and parmesan which are produced in a higher temperature (Adams 2016).

Leuconostoc

Leuconostoc is a genus of mesophilic and psychrotolerant/psychrotrophic bacteria. Leuconostoc spp. are obligately heterofermentative LAB and metabolize glucose via the phosphoketolase pathway. Their production of carbon dioxide and diacetyl from milk citrate makes them important in dairy fermentations (Lonvaud-Funel 1999). Fermentation of citrate to diacetyl gives flavor development and production of carbon dioxide gives formation of eyes inside of the cheese which is required in some cheese varieties. This bacterial group are called DL-cultures since they are diacetyl generating *Leuconostoc* (Hayaloglu 2016). *Leuconostoc* spp. are also a NSLAB which influence the flavor development in many dairy products. *Leuconostoc mesenteroides* isolated from brine cheeses have revealed a high genomic diversity and carried no pathogenic factors or virulence factors (Ruppitsch et al. 2021). *L. mesenteroides* has also been evaluated for its chemical activity and have shown good potential for usage in mixed starter cultures for cheese manufacture (Seixas et al. 2018). *Leuconostoc pseudomesenteroides* is a widely found as a flavor-producing NSLAB in the dairy industry (Meslier et al. 2012). *L.*

pseudomesenteroides can also be found in mesophilic cheese starters (Pedersen et al. 2014).

Enterococcus

Enterococcus species are common in raw milk dairy products and give long-ripened cheeses a specific flavor. They are LAB with proteolytic and lipolytic activities and most enterococci have showed ability to convert citrate to various aromatic compounds. Enterococci can be used as food preservatives due to their production of bacteriocins which are effective against spoilage and pathogenic bacteria. On the other hand, they can have adverse effects on human health. Some strains with undesirable traits may carry antibiotic-resistance genes and various virulence factors. Other strains with desirable traits have shown strong health-promoting effects (Terzić-Vidojević et al. 2021). Some naturally occurring *Enterococcus* strains isolated from cheese have showed probiotic properties and safety and have therefore potential to be used in functional food formulations (Kouhi et al. 2022) (de Castro Santos Melo et al. 2021).

Enterococcus hirae is a potential cheese contaminant (Moe et al. 2012). Both non-starter *Enterococcus faecium* and *E. hirae* isolated from homemade Egyptian dairy products have shown to have potential as safe and useful strains in a starter culture (El-Ghaish et al. 2015). *E. faecium* is a common bacterium in cheeses due to its important role in ripening and development of sensory characteristics. *Enterococcus faecium* is no longer considered a "typical endocarditis pathogen" in the 2023 Duke-ISCVID (International Society for Cardiovascular Infectious Diseases) (Lindberg et al. 2023). *E. faecium* E16 isolated from goat cheese, showed antibacterial activity, in particular against *Listeria monocytogenes* (Hammi et al. 2019). *E. faecium* together with *Enterococcus durans* have shown to improve the sensory properties of Feta cheese (Hayaloglu 2016).

1.4.3 Other wished bacteria in milk and cheese

Propionic acid bacteria

Propionic acid bacteria (PAB) are used in starter cultures for the aromatizing properties and/or the ability to generate holes. These bacteria use lactate fermentation, amino acid catabolism and fat hydrolysis as the three main pathways in the production of the typical flavor compounds. All PAB convert lactic acid to propionic acid, acetic acid, and carbon dioxide, and forms the typical flavor. They also produce succinic acids and other organic acids with antifungal activities. Specific strains are chosen for the properties wished for the final cheese. The flavor compounds include free fatty acids from lipolysis and volatile fatty acids from amino acid catabolism. The formation of holes is done by production of carbon

dioxide. *Propionibacterium freudenreichii* is an example of a PAB present in especially Swiss-type cheeses (Thierry et al. 2015).

Micrococcus and *Staphylococcus*

Micrococcus is a genus in the family Micrococcaceae. It has been found in water, soil, dust and skin of humans and animals, and has been isolated from dairy products. Unlike LAB, *Micrococcus* is aerobic with a strictly respiratory metabolism. *Micrococcus* and *Staphylococcus* are both found on the surface of some cheeses. Even though certain *Staphylococcus* spp. can be pathogenic, other *Staphylococcus* spp. are wished for in cheese. The genera are similar microscopically but phylogenetically unrelated to each other. For example, *Micrococci* produce acid only aerobically whereas *Staphylococci* produce acid aerobically and anaerobically from glucose (Nuñez 2014).

1.4.4 Unwished bacteria in milk and cheese

Staphylococcus

Straphylococcus aureus is a pathogenic bacteria species in fermented food systems and other systems (Charlier et al. 2009). It occurs naturally on humans and animals and can be found in unpasteurized milk (Livsmedelsverket n.d.). Transmission to handmade food from humans in the production can cause sickness among the consumers (Livsmedelsverket n.d.). Other *Staphylococcus* species can be pathogenic. *Staphylococcus haemolyticus* is a pathogen found in dairy cows and small ruminants (Leitner et al. 2009). It has been isolated from soft cheese and shown production of enterotoxin (Rall et al. 2010).

Coliform bacteria

Coliform bacteria are a group of many types of bacteria found throughout the environment. They produce acids and gases using β-galactosidase. Growth of some coliform bacteria and potential pathogenic organisms can be stimulated by changes in cheese acidity. Coliform bacteria can cause early blowing and large gas holes in white-brined cheese which is undesirable (Hayaloglu 2016). *Citrobacter*, *Enterobacter*, *Klebsiella* and *Escherichia* are genera included in the group of coliform bacteria. *Escherichia coli* is a common intestinal bacterium which can be transferred to foods. It can use lactose as energy source and is killed by pasteurization. Most *E. coli* -types are not disease-causing. However, some *E. coli* can be a food hazard (Livsmedelsverket n.d.). Shiga-toxin producing *E. coli* (STEC) strains can survive or grow during cheese production (Perrin et al. 2015). Therefore, there are health risks associated with raw milk cheeses (Vernozy-Rozand et al. 2005).

Other spoilage- and pathogenic bacteria

Spore-forming bacteria in cheese may cause spoilage and foodborne disease outbreaks. *Bacillus* spp. and *Clostridium* spp. are often found in milk, dairy farm environment and processed cheese. They form spores and survive processing conditions (Oliveira et al. 2016). *Clostridium tyrobutyricum* is a bacteria found in milk and cheeses. This microorganism ferments glucose and xylose to butyric acid, acetic acid, and hydrogen gas. It causes late-blowing and other defects in brine-salted, hard, and semihard cheeses (Ivy & Wiedmann 2014). *Salmonella*, *Campylobacter*, *Bacillus cereus* and *Listeria monocytogenes* are other examples of unwished bacteria in dairy products (Livsmedelsverket n.d.). On the other hand, until a sufficient number of strains of each species are tested, general conclusions on the properties of these species should not be drawn (Thierry et al. 2015).

1.4.5 Other microorganisms in cheese

Except for bacteria, several other microorganisms including both wished and unwished mold, yeast and bacteriophages can be found in milk, dairy products and starter cultures.

Molds

Penicillium roqueforti is a well-known mold found in so-called blue-veined cheeses including Roquefort, Gorgonzola, French Bleu, Stilton, Cabrales, and Valdeón. It is responsible for the characteristic texture, blue-green spots, aroma, and synthetization of secondary metabolites (Chávez et al. 2023). *Penicillium camemberti* is an equally known white mold used for the maturation of soft cheeses including Camembert and Brie (Ropars et al. 2020). *Geotrichum candidum* is a fungus which occurs in the human microbiome, in soil and in the dairy production. It is used on wash-rind cheeses and bloomy rind cheeses such as Camembert since it contributes to ripening and flavor formation (Boutrou & Guéguen 2005). Other cheese fungi are not well known but they are found in two classes, Eurotiomycetes and Sordariomycetes. Since cheese is extremely rich in protein and fat, some fungi are probably adapted to that habitat (Ropars et al. 2012). Even though there are extensively used species in the cheese production, molds are the most common cheese spoilage organisms. On the other hand, multiple LAB possess antifungal activity and can be used as biopreservatives in cheese (Cheong et al. 2014).

Yeasts

Yeasts obtain carbon from mostly sugars and are either obligate aerobes or facultative anaerobes. They are present on the surface on for example white brined cheeses and contribute to the flavor development and proteolysis. Several species are found in blue cheeses. On the other hand, its activity can cause gas blowing, unpleasant odor and softening in texture (Hayaloglu 2016). Examples of yeast

species found on the surface of cheese include *Debaryomyces hansenii* and *Yarrowia lipolytica* (Mounier et al. 2005).

Bacteriophages
"A bacteriophage is a bacterial virus which in its virulent state infects the bacterial cell, multiplies within it, eventually causing the cell to burst (lysis)" (Adams 2016:373). During cheese fermentation, the bacteriophages inhibit acidification and might grow. The source of phages can be the organisms in the starter culture, carrying the lysogenic phages within them and by that induce the virulent state. Often, starter cultures are replaced after a few weeks. Starters containing a single strain, or a few strains have a risk of getting phages specific to that organism. The phages grow and increase the risk of fermentation failure if the starter culture is used for a long time (Adams 2016).

1.5 Methodology for identification of microorganisms

1.5.1 Culturing bacteria

Growth of a population of microorganisms can be supported by a growth medium. Liquid media or media solidified with agar can be used for microbiological examination. Individual viable microorganisms multiply, can be seen and their colonies can be counted. Agar is a polysaccharide produced by species of red algae which is used in the media. It has several useful properties including ability to form a gel at low concentrations and stability to microbial hydrolysis. A medium generally contains a source of amino nitrogens, an energy and carbon source, phosphate, yeast extract or other growth promoting factors, mineral salts and metal ions, selective agents for inhibition of unwanted organisms, and indication systems. The formulation of a medium depends on what group of organisms is being studied and the overall purpose of the study (Adams 2016).

Culture media are used for general cultivation and selective media are used for growth of selected microorganisms. Three types of growth media were used within the master's project for bacterial culturing: Plate count agar (PCA), de Man, Rogosa and Sharpe (MRS) and M17 agar. PCA is used for aerobic cell count. It is a general-purpose media containing adequate nutrition for total bacterial growth. It contains enzymatic digest of casein/tryptone, yeast extract, glucose and agar (Corry et al. 2011). MRS agar is used to cultivate the whole group of lactic acid bacteria and primarily for lactobacilli. MRS agar consists of tryptic digest of casein, beef extract, yeast extract, glucose, sorbitan monooleate, di-potassium hydrogen orthophosphate, magnesium sulfate, manganese (II) sulfate, ammonium citrate, sodium acetate, agar, and distilled or deionized water (Corry et al. 2003). M17 agar

is a selective medium for lactic streptococci. Lactose is added for differentiation between lactose-positive streptococci and lactose-negative mutants. M17 agar contains peptone from casein, peptone from meat, peptone from soya, yeast extract, lactose, disodium-β-glycerophosphate magnesium sulfate, ascorbic acid, agar, and distilled or de-ionized water (Corry et al. 1995).

1.5.2 MALDI-TOF mass spectrometry

The identification is based on the unique mass fingerprint of proteins present in the bacterial cells. Each bacterial species has a characteristic mass spectrum due to the presence of specific proteins. In mass spectrometry methods, the mass-to-charge ratio of ions are measured, and a mass spectrum is presented as a result. MALDI-TOF mass spectrometry is a technique with a two-phase procedure used to analyze biomolecules and organic molecules. MALDI stands for Matrix Assisted Laser Desorption Ionization (Bruker n.d.).

In the MALDI-TOF spectrometer, a matrix is a small organic molecule used to facilitate the ionization process by absorption of UV light generated by a laser. The matrix molecules absorb the energy in the form of UV light and blasts the analyte molecules into the gas phase without fragmenting or decomposing them. The analyte molecules vaporize into the vacuum at the same time as they are ionized and application of high voltage accelerate the charged particles. (ARCC Chem 2016)

The second step is the time-of-flight mass spectrometry phase where the particles will impinge upon the detector within a few nano seconds after ionization. Higher mass molecules will arrive later than lighter ones. Each peak in the spectrum corresponds to the specific mass of the particle. Software programs are needed for usage of the instrument and a database is needed for identification. The spectra for the isolates from the colonies are compared with reference spectra in a library which needs to be suitable for the investigated microorganisms. The species that are not in the database cannot be identified (flexControl n.d.; Bruker n.d.).

2. Aim

The aim of this master project was the characterization of the microbiological composition of dairy farmhouse starter cultures by culturing methods and Maldi-TOF mass spectrometry. A compilation of a questionnaire about starter culture methods sent to Swedish farmhouse dairy producers was also included.

3. Method

3.1 Literature review

Scientific books and articles were used to cover the basic knowledge. The databases PUBMED, Google Scholar, ScienceDirect and Web of science were used to search for relevant information in previously published scientific studies. The references used were published between 1992 and 2024, of which the majority were published after 2010. Webpages and personal communication with the artisan dairy producers were used for understanding about the practical perspectives.

3.2 Questionnaire

A questionnaire about starter cultures was sent to artisan dairy producers. The questions covered the methods used to produce starter cultures including type of milk, source of LAB culture, incubation length and temperature, quality control, storage etc. (Appendix 1). The answers were compiled, and diagrams were made.

3.3 Starter culture producers

Cultures from three Swedish artisan cheesemakers were used within the laboratory project. The producers sent two, three or four cultures each per batch. Both thermophilic and mesophilic cultures were included. We denote these producers: starter culture producer A, starter culture producer B and starter culture producer C.

3.3.1 Starter culture producer A

One thermophilic and one mesophilic starter culture were sent from starter culture producer A on four different occasions. Producer A used purchased pasteurized cow’s milk, DVS, and back-slopping.

Even though the milk was pasteurized before it arrived at the production, microorganisms could have entered the milk on the way. Therefore, the milk was pasteurized again at 90ºC in the dairy production area for 20 minutes. The thermophilic and mesophilic culture were back-slopped several times and 4 batches were sent to SLU at different dates. The thermophilic DVS contained only the LAB *Streptococcus salivarus subsp. thermophilus*. The mesophilic DVS of the brand Flora Danica is a common mesophilic culture which produces flavor and carbon dioxide. It contains the four LAB *Lactococcus lactis* subspecies *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Lactococcus lactis* subsp. *lactis* and *Leuconostoc*.

The DVS were added to milk in pots and incubated with a lid on. The thermophilic starter culture was incubated at 43-45ºC for 3 to 4 hours and the mesophilic starter culture at 23ºC for 18 hours or overnight. A pH meter was used to check if the pH reached 4.5 during the incubation. Appearance, smell, and taste was checked by human senses after incubation.

Picture 1: Back-slopping. Stored mother culture is added to cooled pasteurized milk to get a daughter culture.

3.3.2 Starter culture producer B

One thermophilic starter culture from 2018 and two mesophilic starter cultures from 2012 were back-slopped and sent from starter culture producer B on four occasions.

Producer B used own goat's milk, milk souring and back-slopping. All cultures from producer B had pH 4.4-4.5 when they were considered ready for use.

The goats were milked by a milking machine. The thermophilic starter culture was produced by having hay in high pasteurized milk. Back-slopping was done by cooling high pasteurized milk down to 44ºC before addition of the hay enriched stock culture and incubation in the same temperature for 5 hours. The hay was taken out before the culture was back-slopped. Back-slopping was done 5 times with freezing in between before the first batch of starter culture was sent to the lab. Back-slopping was done again for every new batch.

A mesophilic starter culture with pH 4.5 was produced by milk souring 2012. Directly after milking, a container was filled with the fresh milk and placed in a 38ºC heating cabinet for 24 hours. After one day, the milk was visually analyzed to make sure it looked like yoghurt and was without gas formation. Back-slopping was done with high pasteurized milk and incubation in 23ºC for 16 hours.

The mesophilic starter culture from 2012 was divided into two cultures. The first mesophilic culture was done using back-slopping with frozen bacteria culture. The culture from the freezer was thawed in the cooled high pasteurized milk before incubation. When the second mesophilic culture was done, the culture from the freezer was placed in the fridge before it was added to the pasteurized milk. All three cultures, the one thermophilic and the two mesophilic, were sent to the lab at different dates in the same way as producer A´s starter cultures did.

3.3.3 Starter culture producer C

Several freshly made thermophilic and mesophilic starter cultures were sent from Starter culture producer C distributed on three dates. Producer C used own unpasteurized milk, milk souring and back-slopping method for all starter cultures.

The milk originated from cows with different breeds such as bohuskulla, mountain cow (fjällko) and jersey cattle. The milk in the starter cultures come from only one, two or three individual cows. The cows were milked with a machine and the milk was collected in a bucket. In one of the starter cultures, herbs were added to the unpasteurized milk for 30 minutes before incubation. The herbs included red clover, white clover, timothy, dandelion, stinging nettle, and pea. Since different breed give different milk, the incubation time was adjusted to the milk character. After incubation, aroma and appearance were inspected by producer C´s senses. Back-slopping was done several times before the cultures were sent for analysis.

The thermophilic cultures were incubated at 42ºC around 4 hours until the pH dropped. The mesophilic cultures were incubated at 36ºC for 24 hours if the milk was from the cow named Bella and 36 hours if the milk was from the cows named Mumsemor and Filippa. The difference in time is due to different milk qualities. All cultures were considered ready when the pH had dropped to 4.9.

When the batch 1 was done, the cows were mostly outdoors on the field grazing gras on the field or in the forest. During the night, the cows might have been indoors on straw. When the cultures for batch 2 were done, the cows were still partly outdoors. The cows were indoors on peat when batch 3 was produced. The names and breeds of the cows used in the production of the milk for the starter cultures are shown in table I, II and III.

Table I: Description of starter cultures from producer C; batch 1

Batch 1		
Starter culture	**Cow name**	**Breed**
Thermophilic culture 1	Bella	Bohuskulla
Thermophilic culture 2	Mumsemor, Filippa	Bohuskulla
Mesophilic culture 1	Bella	Bohuskulla
Mesophilic culture 2	Mumsemor, Filippa	Bohuskulla

Table II: Description of starter cultures from producer C; batch 2

Batch 2			
Starter culture	**Cow name**	**Breed**	**Comments**
Thermophilic culture 1	Mumsemor, Filippa	Bohuskulla	
Thermophilic culture 2	Bella	Bohuskulla	
Mesophilic culture 1	Bella	Bohuskulla	Older culture
Mesophilic culture 2	Mumsemor, Filippa	Bohuskulla	Older culture
Mesophilic culture 3	Bella	Bohuskulla	Newer culture
Mesophilic culture 4	Bella	Bohuskulla	Added herbs

Table III: Description of starter cultures from producer C; batch 3

Batch 3		
Starter culture	**Cow name**	**Breed**
Mesophilic culture 1	Gullros, Mumsemor	Jersey, bohuskulla
Mesophilic culture 2	Britta, Tea, Filippa	Jersey, mountain cow, bohuskulla

3.4 Laboratory part

3.4.1 Sample collection

The samples were sent by post from the starter culture producers to a private address before transfer to the lab. The cultures were sent in the test tubes surrounded by iced gel in a package. The transport took approximately two days. The cultures were placed in the fridge directly after delivery and taken out just before the sample preparation and analysis in the SLU laboratory, usually the day of arrival.

3.4.2 Sample preparation and inoculation

Peptone water was prepared by mixing 10.0 g peptone powder (Peptone from casein, Sigma-Aldrich, USA) and 5.0 g sodium chloride with 1000 ml deionized water before sterilization in an autoclave. Agar plates with three types of growth medium PCA (Plate Count Agar, Sigma-Aldrich, Spain), MRS (MRS Agar, Sigma-Aldrich, Darmstadt, Germany) and M17 (M-17 Agar, Sigma-Aldrich, Switzerland), were prepared by mixing growth medium powder with deionized water in glass bottles according to the instructions of manufacture, autoclaving and pouring the media into Petri dishes.

The samples were prepared by diluting the starter cultures in peptone water. Different dilutions were used to determine the most suitable ones. After testing, the thermophilic cultures were diluted to 1:100 and 1:1000, and the mesophilic cultures were diluted to 1:1 000 000 and 1:10 000 000. PCA, MRS and M17 agar were used as growth media for all starter cultures and dilutions. All cultivation was done in triplicates resulting in six agar plates of each medium per culture.

A pipette was used to place 100µl of the diluted culture on each agar plate. A plate spreader was used to get an even distribution of the bacteria on the agar. After inoculation, the agar plates were incubated in the heating rooms, the thermophilic in 37°C and the mesophilic culture in 25°C. The MRS and M17 agar plates were placed in an anaerobic chamber to support growth of anaerobic microorganisms.

3.4.3 Recultivation

Four days after inoculation all agar plates were taken out from the heated rooms for colony counting and isolation. The number of colony forming units per ml culture (CFU/ml) was calculated as followed:

(CFU • dilution factor) / V = CFU/ml
CFU = the number of counted colonies on the agar plate.
V = volume (ml) applied on the plate

A T-streak method was used to isolate bacterial colonies. Five individual colonies from each type of medium and culture were recultivated resulting in quintuplicates of all three media. The exceptions were the first thermophilic and mesophilic batches from starter culture producer 1 where 10 new agar plates were T-streaked for only PCA and MRS media. The chosen colonies were as different as possible in morphology. The colonies were spread with loops on the same type of medium and placed in the same temperature during the same time as during their first period of growth.

3.4.4 Identification with MALDI – TOF mass spectrometry

A MALDI-TOF spectrometer (Biotyper, Microflex, Bruker Daltonik GmbH, Germany) was used to identify the microorganisms in the starter cultures. Four days after recultivation the plates were taken out from the heating rooms for screening. Colonies were placed on a target plate, using a toothpick. Five colonies as different as possible in morphology were used from each quintuplicate. Duplicates were done with each colony resulting in 30 spots for each batch, except for the first batches that had 40 spots, that were placed in the instrument. 1 µl matrix containing α-cyano-4-hydroxycinnamic acid mixed with 250µl 50% acetonitrile and 2.5% trifluoroacetic acid was added on each spot. When the matrix dried the target plate was placed in the MALDI-TOF-MS instrument for the acquisition. The software flexControl showed the laser and the curves whilst the software MBT Compass was used for inscribing the numbers for the samples. The spectra for the isolates were compared with the reference spectra in the Maldi Biotyper Compass Library, update Revision K (2022), 11897 msp.

3.4.5 Glycerol stock

Glycerol stock was done by mixing the same proportions of glycerol (Glycerine ≥99.5%, AnalaR NORMAPUR ACS, bidistilled, VWR Chemicals BDH, USA) and deionized water before sterilization in an autoclave. A loop was used to take one colony from each agar plate used for recultivation aimed for the MALDI-TOF to an Eppendorf tube containing one milliliter glycerol stock. A vortex was used to homogenize the suspension before the tubes were placed in -80°C freezer for storage and eventual later usage.

3.4.6 Statistical Analysis

Excel was used to compile the data from both the colony counting and the MALDI-TOF identification results. Minitab (Minitab version 19.2020.1.0, Minitab LLC, USA), a statistical analysis program, was used for statistical analysis of the results

from the colony counting. Mean values, standard deviation and differences between batches were evaluated by one-way ANOVA and Tukey test.

4. Results

4.1 Colony forming units

The means of CFU/ml in the cultivated starter cultures are shown below. Several plates were not analyzed (NA) since there were no colonies on the plate or the number of colonies were too high.

4.1.1 Starter culture producer A

The means of CFU/ml from three of the four batches of thermophilic starter cultures cultivated on PCA and MRS media from producer A are shown in table 1. Comparison between the batches show variation in CFU/ml over time. In both cases, the number of CFU/ml in batch 2 is significantly higher (p=0.001) compared to batch 1 and 4. CFU from M17 media and batch 3 from the thermophilic cultures and all the plates from the mesophilic cultures were not analyzed.

Table 1: Colony forming units per milliliter (CFU/ml) of thermophilic starter cultures from starter culture producer A, cultivated aerobically on PCA and MRS media.

Agar type	Batch 1	Batch 2	Batch 4
PCA	$3.2\times10^3 \pm 2.7\times10^{3(B)}$	$6.1\times10^5 \pm 7.1\times10^{4(A)}$	$5.5\times10^3 \pm 6.4\times10^{3(B)}$
MRS	$2.2\times10^3 \pm 1.1\times10^{2(B)}$	$6.8\times10^5 \pm 1.7\times10^{5(A)}$	$6.0\times10^3 \pm 5.7\times10^{3(B)}$

Differences between batches were evaluated by one-way ANOVA and Tukey test. Mean values and standard deviation are included. ^{A,B} values within rows with different letters are significantly different (P<0,05).

4.1.2 Starter culture producer B

The means of CFU/ml in the thermophilic cultures from starter culture producer B are shown in table 2. Comparison between the batches show variation in CFU/ml over time. Except for the plates which were not analyzed, the number of CFU/ml is increasing for every batch for all media types. The results show no significant difference between batch 2 and 3 for PCA and M17 while batch 4 was significantly higher for both PCA and M17.

Table 2: Colony forming units per milliliter (CFU/ml) of thermophilic starter cultures from starter culture producer B, cultivated aerobically on PCA, MRS and M17 media.

Agar type	Batch 1	Batch 2	Batch 3	Batch 4
PCA	$4.1\times10^{4} \pm 1.0\times10^{4(C)}$	$5.9\times10^{5} \pm 1.6\times10^{5(B)}$	$9.8\times10^{5} \pm 3.6\times10^{4(B)}$	$3.0\times10^{6} \pm 3.6\times10^{5(A)}$
MRS	$3.4\times10^{4} \pm 1.1\times10^{4(C)}$	$6.3\times10^{5} \pm 1.6\times10^{5(B)}$	$1.0\times10^{6} \pm 2.1\times10^{5(A)}$	NA
M17	NA	$6.8\times10^{5} \pm 1.8\times10^{5(B)}$	$8.6\times10^{5} \pm 2.4\times10^{5(B)}$	$3.4\times10^{6} \pm 5.7\times10^{5(A)}$

Differences between batches were evaluated by one-way ANOVA and Tukey test. Mean values and standard deviation are included. ^{A,B,C} values within rows with different letters are significantly different (P<0,05). NA=Not analyzed.

The means of CFU/ml for two mesophilic cultures (meso 1from batch 3 and meso 2 from batch 4) from starter culture producer B are shown in table 3. Plates only from batch 3 and 4 were analyzed due to difficulties to count the number of CFU in batch 1 and 2. Also, meso 2 in batch 3 and meso 1 in batch 4 were also not countable. Therefore, the number of CFU over time cannot be analyzed statistically. However, high numbers and numerical differences are shown from both cultures and all media types.

Table 3: Colony forming units per milliliter (CFU/ml) of two different mesophilic starter cultures from starter culture producer B, cultivated aerobically on PCA, MRS and M17 media.

Agar type	Batch 3	Batch 4
PCA (meso 1)	$2.39\times10^{10} \pm 3.72\times10^{9}$	NA
PCA (meso 2)	NA	$3.96\times10^{9} \pm 1.53\times10^{9}$
MRS (meso 1)	$2.07\times10^{10} \pm 4.09\times10^{9}$	NA
M17 (meso 1)	$3.04\times10^{10} \pm 1.83\times10^{9}$	NA
M17 (meso 2)	NA	$3.63\times10^{9} \pm 1.00\times10^{9}$

Mean values and standard deviation are included. Results were not analyzed statistically due to missing samples. NA=Not analyzed.

4.1.3 Starter culture producer C

The means of CFU/ml of four thermophilic cultures (thermo 1 and thermo 2 from batch 1, thermo 1 and thermo 2 from batch 2) from starter culture producer C are shown in table 4. Since the starter cultures from producer C expired from new mother cultures for batch 1 and batch 2, statistical comparisons between the batches could not be done. However, the number of CFU are higher in batch 2 compared to batch 1.

Table 4: Colony forming units per milliliter (CFU/ml) of different thermophilic starter cultures from starter culture producer C, cultivated aerobically on PCA and MRS media.

Agar type	Batch 1	Batch 2
PCA (thermo 1)	$1.2\times10^{3} \pm 1.1\times10^{3}$	$2.5\times10^{4} \pm 1.3\times10^{4}$
PCA (thermo 2)	$2.6\times10^{3\dagger}$	$4.4\times10^{4} \pm 7.1\times10^{4}$
MRS (thermo 1)	NA	$2.5\times10^{5} \pm 8.6\times10^{4}$
MRS (thermo 2)	NA	$5.9\times10^{4} \pm 5.4\times10^{4}$

Mean values and standard deviation are included. Results were not analyzed statistically since the starter cultures were not comparable. CFU=Colony Forming Units. †Standard deviation was not included due to low number of samples. NA=Not analyzed.

The means of CFU/ml for five mesophilic cultures (meso 1 and meso 2 from batch 1, meso 1 and meso 2 from batch 2, and meso 2 from batch 3) from starter culture producer C are shown in table 5. Since the starter cultures from producer C expired from new mother cultures for batch 1, batch 2 and batch 3, statistical comparisons between the batches could not be done.

Table 5: Colony forming units per milliliter (CFU/ml) of different mesophilic starter cultures from starter culture producer C, cultivated aerobically on PCA, MRS and M17 media.

Agar type	Batch 1	Batch 2	Batch 3
PCA (meso 1)	NA	$1.03\times10^{9} \pm 4.61\times10^{8}$	NA
PCA (meso 2)	NA	$2.04\times10^{10} \pm 5.00\times10^{9}$	NA
MRS (meso 1)	$4.82\times10^{8} \pm 3.68\times10^{7}$	$5.83\times10^{8} \pm 1.95\times10^{8}$	NA
MRS (meso 2)	4.00×10^{8}†	$2.26\times10^{10} \pm 4.81\times10^{9}$	NA
M17 (meso 1)	NA	$7.04\times10^{8} \pm 2.08\times10^{8}$	NA
M17 (meso 2)	NA	$2.69\times10^{10} \pm 6.63\times10^{9}$	$3.09\times10^{9} \pm 1.22\times10^{9}$

Mean values and standard deviation are included. Results were not analyzed statistically since the starter cultures were not comparable. †Standard deviation was not included due to low number of samples. NA=Not analyzed.

4.2 Identification with MALDI-TOF MS

4.2.1 Starter culture producer A

Table 6 shows bacterial composition of thermophilic starter cultures from producer A. The samples were cultivated and re-cultivated on PCA, MRS and M17 agar and further identified using MALDI-TOF MS instrument. A total of 67 different observations (n) were made based on colony morphology and size: Among them, 10 species were identified whereof the most abundant was *Lactococcus lactis*, observed in all batches (Table 6). Some colonies could not be identified due to the lack of peaks or reference spectra.

Table 6: Microbial composition in percentage of thermophilic starter cultures from starter culture producer A, cultivated aerobically on PCA and anaerobically on MRS and M17 media and identified by MALDI-TOF MS. In total, n=67 (batch 1n=34, batch 2 n=14, batch 3 n=8, batch 4 n=11).

Bacteria	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 4**
Lactococcus lactis	62	43	13	64
Staphylococcus borealis	3			
Staphylococcus haemolyticus	3		13	
Leuconostoc pseudomesenteroides	15			

Streptococcus salivarius ssp thermophilus	17			
Escherichia coli		14	25	
Staphylococcus hominis		14		
Enterococcus durans		29		
Micrococcus luteus			25	36
Kurthia gibsonii			25	

Table 7 shows bacterial composition of mesophilic starter cultures from producer A. The samples were cultivated and re-cultivated on PCA, MRS and M17 agar and further identified using MALDI-TOF MS instrument. In total 51 different observations were made: For batch 1 n= 24, batch 2 n=14, batch 3 n=7 and batch 4 n=6. In total, 5 species were identified whereof the most abundant was *Lactococcus lactis*, observed in all batches.

Table 7: Microbial composition in percentage of mesophilic starter cultures from starter culture producer A, cultivated aerobically on PCA and anaerobically on MRS and M17 media and identified by MALDI-TOF MS. In total, n=51 (batch 1 n=24, batch 2 n=14, batch 3 n=7, batch 4 n=6).

Bacteria	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 4**
Lactococcuc lactis	83	93	86	83
Leuconostoc pseudomesenteroides	17			
Lactococcus laudensis		7		
Micrococcus luteus			14	
Pseudomonas viridiflava				17

4.2.2 Starter culture producer B

Table 8 shows bacterial composition of thermophilic starter cultures from producer B. The samples were cultivated and re-cultivated on PCA, MRS and M17 agar and further identified using MALDI-TOF MS instrument. In total 65 observations were made: For batch 1 n= 19, batch 2 n=16, batch 3 n=17 and batch 4 n=13. In total, 3 species were identified whereof the most abundant was *Enterococcus faecium*, observed in all batches.

Table 8: Microbial composition in percentage of thermophilic starter cultures from starter culture producer B, cultivated aerobically on PCA and anaerobically on MRS and M17 media agar and identified by MALDI-TOF MS. In total, n=65 (batch 1 n=19, batch 2 n=16, batch 3 n=17, batch 4 n=13).

Bacteria	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 4**
Enterococcus faecium	42	81	88	100
Enterococcus hirae	58	6	6	
Staphylococcus haemolyticus		13	6	

Table 9 shows bacterial composition of mesophilic starter cultures from producer B. The samples were cultivated and re-cultivated on PCA, MRS and M17 agar and

further identified using MALDI-TOF MS instrument. In total 67 observations were made: For batch 1 n=17, batch 2 n=19, batch 3 n=15 and batch 4 n=16. In total, 3 species were identified whereof the most abundant was *Lactococcus lactis*, observed in all batches.

Table 9: Microbial composition in percentage of mesophilic starter cultures named meso 1 from starter culture producer B, cultivated aerobically on PCA and anaerobically on MRS and M17 media and identified by MALDI-TOF MS. In total, n=67 (batch 1 n=17, batch 2 n=19, batch 3 n=15, batch 4 n=16).

Bacteria	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 4**
Lactococcus lactis	65	89	100	94
Leuconostoc mesenteroides	12	11		6
Leuconostoc pseudomesenterioides	24			

Table 10 shows bacterial composition of mesophilic starter cultures from producer B. The samples were cultivated and re-cultivated on PCA, MRS and M17 agar and further identified using MALDI-TOF MS instrument. In total 43 observations were made: For batch 1 n= 14, batch 2 n=4, batch 3 n=9 and batch 4 n=16. In total, 3 species were identified whereof the most abundant was *Lactococcus lactis*, observed in all batches.

Table 10: Microbial composition in percentage of mesophilic starter cultures named meso 2 from starter culture producer B, cultivated aerobically on PCA, and anaerobically on MRS and M17 media and identified by MALDI-TOF MS. In total, n=43 (batch 1 n=14, batch 2 n=4, batch 3 n=9, batch 4 n=16).

Bacteria	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 4**
Lactococcus lactis	71	100	100	100
Leuconostoc mesenteroides	7			
Leuconostoc pseudomesenteroides	21			

4.2.3 Starter culture producer C

Table 11 shows bacterial composition of thermophilic starter cultures from producer C. The samples were cultivated and re-cultivated on PCA, MRS and M17 agar and further identified using MALDI-TOF MS instrument. In total 68 observations were made: For batch 1 thermo 1 n= 16, batch 1 thermo 2 n=22, batch 2 thermo 1 n=15 and batch 2 thermo 2 n=15. In total, 14 species were identified whereof the most abundant were *Lacticaseibacillus paracasei*, present in all four cultures, *Staphylococcus aureus* and *Lactococcus lactis*, present in three out of four cultures.

Table 11: Microbial composition in percentage of thermophilic starter culture samples from starter culture producer C, cultivated aerobically on PCA and anaerobically on MRS and M17 media and identified by MALDI-TOF MS. In total, n=68 (batch 1 thermo 1 n=16, batch 1 thermo 2 n=22, batch 2 thermo 1 n=15, batch 2 thermo 2 n=15).

Bacteria	**Batch 1**		**Batch 2**	
	Thermo 1	**Thermo 2**	**Thermo 1**	**Termo 2**
Staphylococcus aureus	25	27		13
Enterococcus gallinarum	31			13
Lactobacillus plantarum	13			6
Lacticaseibacillus paracasei	25	5	33	40
Levilactobacillus brevis	6			
Escherichia coli		27	7	
Pediococcus acidilactici		14		
Enterococcus gilvus		5		
Lactococcus lactis		14	20	7
Lactococcus garviae		9	7	
Enterococcus faecalis			7	
Staphylococcus haemolyticus			7	13
Enterococcus durans			20	
Micrococcus luteus				7

Table 12 shows bacterial composition of mesophilic starter cultures from producer C. The samples were cultivated and re-cultivated on PCA, MRS and M17 agar and further identified using MALDI-TOF MS instrument. In total 84 observations were made: For batch 1 meso 1 n=21, batch 1 meso 2 n=18, batch 2 meso 1 n=8, batch 2 meso 2 n=13, batch 2 meso 3 n=12, batch 2 meso 4 n=5, batch 3 meso 1 n=4 and batch 3 meso 2 n=3. In total, 13 species were identified whereof the most abundant were *Lactococcus lactis*, present in six out of seven cultures, and *Leuconostoc mesenteroides*, present in four out of seven cultures.

Table 12: Microbial composition in percentage of mesophilic starter culture samples from starter culture producer C, cultivated aerobically on PCA and anaerobically on MRS and M17 media and identified by MALDI-TOF MS. In total, n=84 (batch 1 meso 1 n=21, batch 1 meso 2 n=18, batch 2 meso 1 n=8, batch 2 meso 2 n=13, batch 2 meso 3 n=12, batch 2 meso 4 n=5, batch 3 meso 1 n=4, batch 3 meso 2 n=3).

Bacteria	**Batch 1**		**Batch 2**				**Batch 3**	
	Meso1	**Meso2**	**Meso1**	**Meso2**	**Meso3**	**Meso4**	**Meso1**	**Meso2**
Lactococcus lactis	86	67	63	77	42	20		67
Leuconostoc pseudomesenteroides	14	6						
Escherichia coli		22						
Leuconostoc mesenteroides		6	13	8				33
Micrococcus luteus			25					
Staphylococcus haemolyticus				15				
Staphylococcus aureus					25			
Enterobacter hormaechei					8			
Lacticaseibacillus paracasei					17			
Klebsiella oxytoca					8	60		
Raoultella ornithinolytica						20	25	

Lactobacillus plantarum	50
Enterobacter bugandensis	25

4.3 Questionnaire

The seven farmhouse dairy producers who filled in the questionnaire produced different types of dairy products. About half of them made only cheese, for example goat cheese, feta, eldost (Swedish halloumi), mozzarella, cream cheese, and Alpine cheese. Some producers made other dairy products including yoghurt and the Swedish dairy products filmjölk, långfil and tjockmjölk. A few made both cheese and other products.

Figure 1 shows the percentage distribution of milk types among the farmhouse dairy producers. 57% used only cow's milk in their dairy production. 43% used goat's milk while 14% used both. None of the producers used sheep's milk.

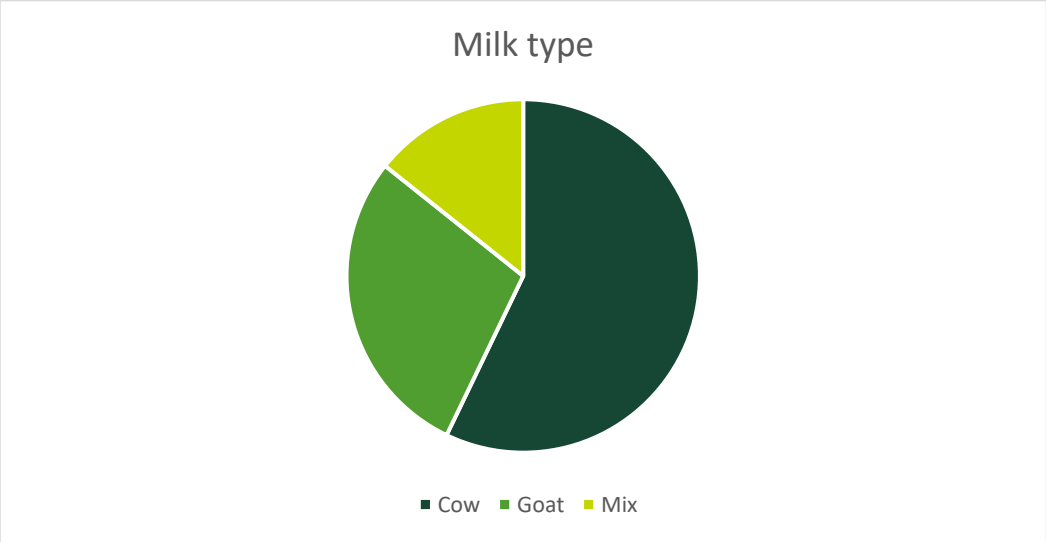

Figure 1: Distribution of milk type. 57% of the producers used only cow's milk, 43% used goat's milk and 14% used a mix.

Figure 2 shows the percentage distribution of milk source used by the producers. 29% used milk from their own animals while 71% used bought milk for their production. Most producers who used goat milk had their own animals whilst the producers who used cow milk often bought the milk.

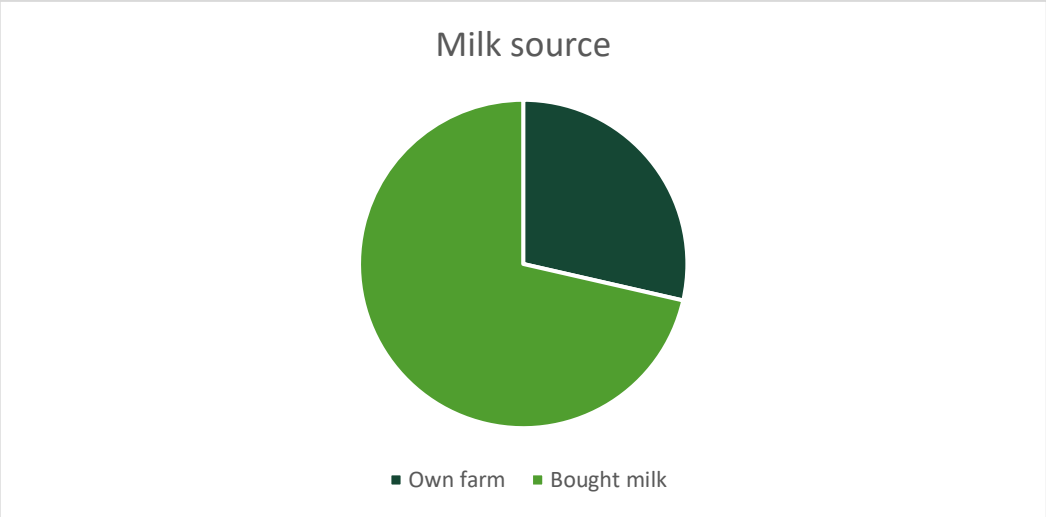

Figure 2: Milk source. 29% of the producers used milk from own animals and 71% bought milk for dairy production.

Figure 3 shows the percentage distribution of the type of starter culture used by the producers. None of the producers used only thermophilic cultures. 57% used mesophilic starter cultures and 43% used a mix of cultures - both thermophilic and mesophilic.

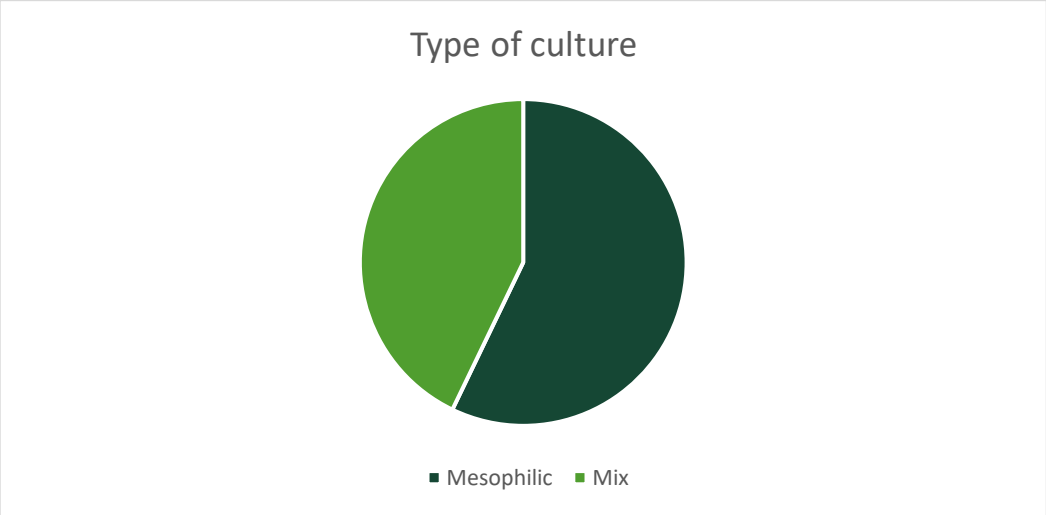

Figure 3: Type of culture. 57% of the producers used only mesophilic starter cultures. 43% used a mix of cultures - both thermophilic and mesophilic.

Figure 4 shows the percentage distribution of treatments used for the milk before production of the starter culture. Freshly milked, warm, unpasteurized milk was used for starter culture production by 43% of the producers. 43% used cooled pasteurized milk while 14% used cooled unpasteurized milk.

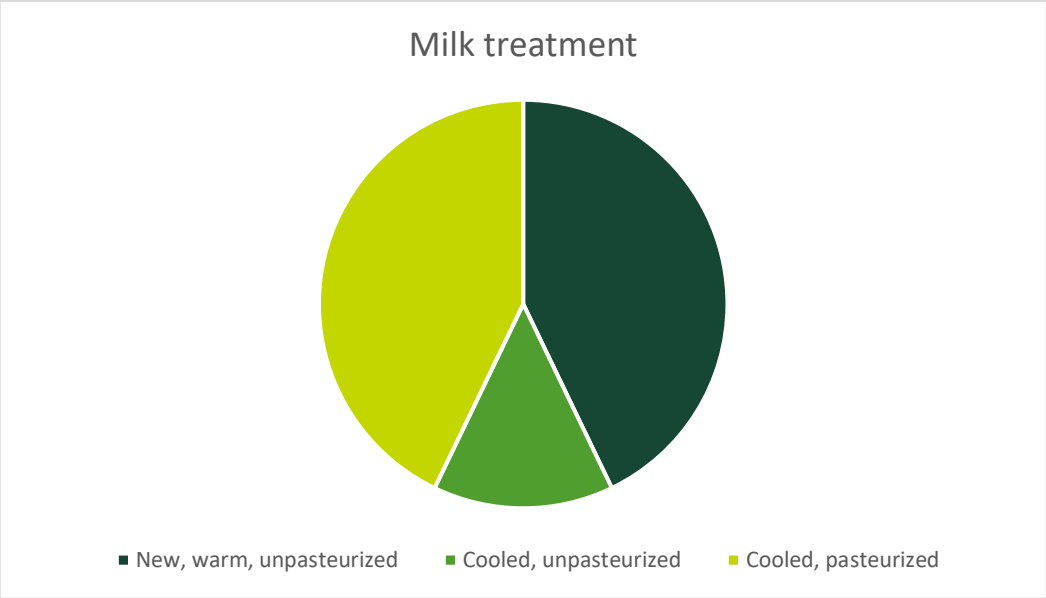

Figure 4: Milk treatment. 43% of the producers used freshly milked (new), warm, unpasteurized milk to produce the starter culture. 43% used cooled pasteurized milk while 14% used cooled unpasteurized milk.

Figure 5 shows the percentage distribution of the source of LAB used by the producers. 14% of the producers used soured milk, 29% used a commercial freeze-dried culture, 14% used soured milk with added plant material, 14% used a commercial fermented dairy product and 29% used more than one method for starter culture production.

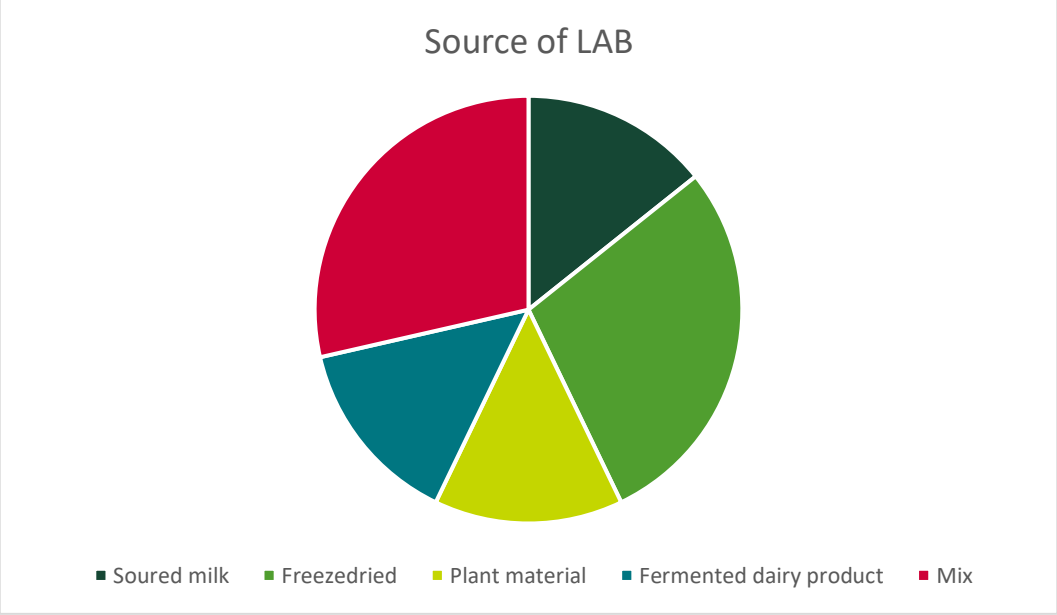

Figure 5: Source of LAB. 14% of the produces had soured milk only as LAB source, 29% used freeze-dried cultures, 14% used soured milk with added plant material, 14% used a commercial fermented dairy product and 29% used a mixture of methods.

Several farmhouse dairy producers used a specific temperature and time for incubation when they were making a new batch of starter culture. The temperature for the mesophilic cultures usually varied between 20 and 23°C. One producer used finger-warm milk. All producers who described their methods for mesophilic culture production, had an 18-hour incubation time in room temperature. When thermophilic starter cultures were produced, the milk was usually placed in 44°C for 3 to 4 hours.

pH, appearance, fresh and sour taste, aroma, and texture are all important indicators for a good starter culture. The producers examined all mentioned factors when they determined if the culture was set and had good quality. Some producers relayed more on the pH measurements while others used their senses to a higher degree. Some producers examined and plotted the pH decrease during the incubation while others waited with the measurements until the end.

Some producers always used a new starter culture when they made dairy products. Some producers stored the culture for later usage in a fridge or a freezer depending on if the culture would be used the coming days or after more than a week. A couple of the producers had stored and used the same culture for months and one of the producers had back-slopped the same culture since 2012.

Several habits were used to protect the starter cultures from contamination of bacteria, yeast, mold, bacteriophages etc. The hygiene in the production area was kept on a high level. All producers prioritized cleaning the tools and washing the hands. At least a couple of the producers who answered the questionnaire had lids on the containers when the cultures were prepared and incubated.

5. Discussion

5.1 Colony Forming Units

More data points are needed to draw conclusions for the CFU. Even though there are results from CFU counting, only the results for MALDI-TOF identification will be discussed since that was the main aim of the master project.

5.2 Starter culture producer A

5.2.1 The thermophilic cultures

The MALDI-TOF results from the thermophilic starter cultures from starter culture producer A showed a high diversity of detected bacteria species in total. The identified microorganisms included *Lactococcuc lactis, Leuconostoc pseudomesenteroides, Streptococcus salivarius_ssp_thermophilus, Enterococcus durans, Kurthia gibsonii, Staphylococcus borealis, Staphylococcus haemolyticus, Staphylococcus hominis, Escherichia coli* and *Micrococcus luteus.*

Lactococcus lactis (*L. lactis*) is the most extensively studied LAB (Neves et al. 2005). As mentioned in the introduction, *L. lactis* is homofermentative and often used in starter cultures. Multiple subspecies can be found in dairy product and the strains are classified into "domesticated" and "environmental" (Thierry et al. 2015). The thermophilic DVS used by producer A did not contain *L. lactis*. The mesophilic DVS used contained *L. lactis* subspecies *cremoris*, *L. lactis* subsp. *lactis* biovar. *Diacetylactis* and *L. lactis* subsp. *Lactis*. The results from the MALDI-TOF MS did not show subspecies. Therefore, the *L. lactis* in the starter cultures could include several strains.

As mentioned in the introduction, *Leuconostoc pseudomesenteroides* is a widely found flavor-producing NSLAB and LAB in mesophilic cheese starters (Pedersen

et al. 2014). It would probably therefore favor the dairy products made by farmhouse dairy producers.

Streptococcus salivarius_ssp_thermophilus is a LAB used in the production of yoghurt and cheese (Amoroso & Manca de Nadra 1992). This strain was a part of the DVS used by Producer A for cheese production.

Enterococcus durans (*E. durans*) is a LAB found in multiple dairy products. *E. durans* has shown to improve the sensory, probiotic and functional properties (Hayaloglu 2016) (Akpinar et al. 2020). The bacterium was only detected in batch 2 which makes it unlikely that it was present in all batches. The culture should therefore not be stored and back-slopped for too long if *E. durans* is preferred in the dairy products.

Kurthia is a strictly aerobic bacterial genus that do not produce acid from glucose. *Kurthia gibsonii (K. gibsonii*) has been isolated from fresh meat, fat, air and more, and has been associated with off-flavors of meat products. It may be one of the *Kurthia* spp involved in spoilage of meat (Stackebrandt et al. 2006). *K. gibsonii* is a proteolytic and lipolytic psychrotrophic bacteria which has been also isolated from raw milk (Ribeiro et al. 2019). However, *Kurthia* strains may not have a spoilage effect on fresh milk (Stackebrandt et al. 2006). Furthermore, *K. gibsonii* has been isolated from fresh acid-set paneer where it showed an antimicrobial activity. Therefore, the bacteriocins (toxins produced to inhibit similar bacterial strains) from *K. gibsonii* may be used as food preservatives (Chauhan & Samant 2022).

As mentioned in the introduction, *Straphylococcus aureus* is not the only pathogenic species among *Staphylococcus (Rall et al. 2010)*. *Staphylococcus haemolyticus* has been isolated from soft cheese and shown production of enterotoxin CNS (Rall et al. 2010). *Staphylococcus borealis* (*S. borealis)* is a facultative anaerobic bacterial species which have been isolated from mammals including human skin and blood, and cow´s milk. *S. borealis* can be involved in intramammary infections in cattle and therefore be a pathogen of bovine mammary glands (Król et al. 2023). *Staphylococcus hominis* has been isolated from cheese and shown presence during the ripening period (Freitas et al. 1996). Multiple subspecies have shown pathogenicity (Zhang et al. 2013).

As mentioned in the introduction, *Escherichia coli* (*E. coli*) is a coliform bacterium with disease-causing strains. Shiga-toxin producing *E. coli* (STEC) strains in raw milk cheeses are associated with health risks. (Perrin et al. 2015) Since the results from the MALDI-TOF do not go deeper than species level, *E. coli* is considered unwished in dairy products including the ones from small-scale producers.

Micrococcus luteus (*M. luteus*) is an oxidase-positive obligate aerobe and a part of the normal microbiota on humans. *M. luteus* has been found at the midpoint of ripening on the surface of smear-ripened cheeses (Mounier et al. 2005). *Micrococci* produce proteinases and lipases but the exact role in cheese is not clear. *M. luteus* strains have been reported to cause infections and may therefore be regarded as pathogens (Nuñez 2014).

The composition varied between the batches and there was a substantial decrease in the number of species from batch 3 to batch 4. *Lactococcus lactis* was the most dominant bacteria species and the only species present in all batches. The LAB *Streptococcus thermophilus* which was added to the milk in freeze-dried form in the beginning, was only detected in batch 1. Some of the species detected in the first batches may have been out-competed by the other species over time in the back-slopping or may have needed a more optimal environment to survive.

In summary, *Lactococcus lactis, Leuconostoc pseudomesenteroides, Streptococcus salivarius_ssp_thermophilus, Enterococcus durans* and *Kurthia gibsonii* may be considered wished bacterial species whereas *Staphylococcus borealis, Staphylococcus haemolyticus, Staphylococcus hominis, Escherichia coli* and *Micrococcus luteus* may be unwished in dairy products.

There are high risks with products on the market containing unwished microorganisms, for example toxin producing *E. coli* or pathogenic *Staphylococcus* species. The consumers might get a disease after eating the food or end up with an uneaten spoiled food product. Farmhouse dairy producers might as well lose too many customers and in the long term have difficulties continuing to operate the business.

5.2.2 The mesophilic cultures

The results from the mesophilic starter cultures from starter culture producer A showed a lower diversity of bacteria compared to the thermophilic culture. The bacterial species *Lactococcuc lactis, Leuconostoc pseudomesenteroides, Lactococcus laudensis, Micrococcus luteus* and *Pseudomonas viridiflava* were detected.

As mentioned in the introduction, *Lactococcus laudensis* (*L. laudensis*) is a LAB found in raw milk. It has been tested as starter culture for cheese making and showed production of wished components (Tidona et al. 2018). *L. laudensis* was however only detected in batch 2 which makes it likely that it was outcompeted before or during the back-slopping for batch 3.

Pseudomonas viridiflava (*P. viridiflava*) is a soil bacterium found on multiple plants, often as pathogen (Lipps & Samac 2022). It has also shown antifungal activity against fungal pathogens of plants (Gerlin et al. 2021). Other *Pseudomonas* strains are responsible for casein hydrolysis and cheese pigmentation and are therefore considered cheese spoilage bacterial strains (Quintieri et al. 2013). Therefore, *P. viridiflava* could be considered an unwished bacterium in a starter culture.

All four batches contained two species each. Interestingly, only *Lactococcus lactis* was present in all cultures. All other bacteria species were found in only one of the batches. On the other hand, the added freeze-dried starter culture contained three subspecies of *Lactococcus lactis* and the proportion of the subspecies are not known from the results. The fourth LAB in the added freeze-dried starter culture, *Leuconostoc,* was found in batch 1 only.

In summary, *Lactococcuc lactis, Leuconostoc pseudomesenteroides* and *Lactococcus laudensis* may be considered wished bacterial species whereas *Micrococcus luteus* and *Pseudomonas viridiflava* may be unwished in dairy products.

5.3 Starter culture producer B

Enterococcus faecium, Enterococcus hirae and *Staphylococcus haemolyticus* were detected in the thermophilic starter cultures from producer B. Both mesophilic cultures contained *Lactococcus lactis, Leuconostoc mesenteroides* and *Leuconostoc pseudomesenterioides.*

The proportion of *Enterococcus faecium* increased for every batch in the thermophilic starter cultures until it was the only species found in batch 4. This may get some attention since *Enterococcus faecium* plays an important role in ripening and sensory development of a cheese (Lindberg et al. 2023). *Enterococcus hirae* may also be considered a whished bacterial strain in a starter culture (El-Ghaish et al. 2015).

As mentioned, *Staphylococcus haemolyticus* has shown production of enterotoxin CNS (Rall et al. 2010). Therefore it may be considered an unwished bacterial species.

Both *Lactococcus lactis* and *Leuconostoc mesenteroides,* are useful in starter cultures. *Leuconostoc pseudomesenterioides* is also found in fermented milks and may be considered wished in dairy products (Alexandraki et al. 2016).

A decrease in the number of bacterial species over time was shown in all cultures from starter culture producer B. Only one or two species were detected in all starter cultures in batch 4. When comparing the two mesophilic cultures from starter culture producer B, it can be observed that the first cultures (table 9) kept diversity for a longer time compared to the second culture (table 10) where only one bacteria species was detected in batch 2, 3 and 4. That is something to consider when the same starter cultures are used for a long period of time in cheese making. The cultures were produced in 2012 and that may give consequences for the cheese production. Long-term freezing might be considered as unfavorable as the bacterial diversity might be affected negatively over time.

In summary, *Enterococcus faecium, Enterococcus hirae, Lactococcus lactis, Leuconostoc mesenteroides* and *Leuconostoc pseudomesenterioides* may be considered wished bacterial species whereas *Staphylococcus haemolyticus* may be unwished in dairy products.

5.4 Starter culture producer C

5.4.1 The thermophilic cultures

Enterococcus gallinarum, Enterococcus gilvus, Enterococcus faecalis, Enterococcus durans, Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Levilactobacillus brevis, Pediococcus acidilactici, Lactococcus lactis, Lactococcus garviae, Escherichia coli, Staphylococcus aureus, Staphylococcus haemolyticus, and *Micrococcus luteus* were detected in the thermophilic starter cultures.

As mentioned in the introduction, *Enterococcus* species are common in raw milk dairy products and give long-ripened cheeses a specific flavor. *Enterococcus gallinarum* has been part of NSLAB and shown contribution to the cheese volatile organic compound composition (Guarrasi et al. 2017). *Enterococcus gilvus* (*E. gilvus*) is a yellow-pigmented facultative anaerobic LAB found in cheese however, not much is known about its role. Though, it is known for its production of carotenoids which can act as quenchers of toxic oxygen radicals. Due to the possible nutritionally advantages, presence of *E. gilvus* in cheese and other fermented foods might be of interest (Zago et al. 2009). *Enterococcus faecalis* is present as NSLAB in raw milk cheeses and has shown antimicrobial activity (Silvetti et al. 2014).

Enterococcus faecium together with *Enterococcus durans* have shown to improve the sensory properties of Feta cheese (Hayaloglu 2016). The *Enterococcus* species detected in the starter cultures from producer C may therefore have an important role regarding the sensory properties in artisan dairy products.

Lactiplantbacillus plantarum (*L. plantarum*) is a widespread LAB found in multiple fermented products. Mesophilic *lactobacilli* including *L. plantarum* are often part of NSLAB in cheddar cheese production for their influence on proteolysis and development of sensory characteristics during ripening (Lynch et al. 1999). The strain *L. plantarum* L10-11 has shown antioxidant activity and γ-aminobutyric acid (GABA) production during fresh cheese processing (Woraratphoka et al. 2022). GABA is an inhibitory neurotransmitter which is thought to have a calming effect. A development is seen regarding GABA enriched foods due to their claimed health benefits and fermented dairy products are included (Sjöblom 2021). GABA-containing dairy products with antioxidant activity may have a unique selling point when marketed.

Lacticaseibacillus paracasei (*L. paracasei*) is a homofermentative LAB commonly found in probiotic cultures and dairy product fermentation. After comparative genomic analysis of strains named *L. casei*, a reclassification of the majority of "*L. casei*" strains as *L. paracasei* has been recommended (Ghosh et al. 2019). *L. paracasei* may be used to efficiently inhibit hazardous bacteria (Aprea et al. 2021). *L. paracasei* produces fatty acids and volatile organic compounds during milk fermentation (Moiseenko et al. 2023).

Levilactobacillus brevis (*L. brevis*) is a heterofermentative LAB present in fermented food products and humans. *L. brevis* has shown fast acidification rate and good techno-functional traits (Margalho et al. 2021). The strain *L. brevis* CGMCC1.5954 has been used to ferment GABA enriched yoghurt which also showed improved aroma and texture (Fan et al. 2023). If the same or comparable strain was present in the starter culture, it could be used to produce a more special product which could increase the demand for a group of customers.

Pediococcus acidilactici (*P. acidilactici*) is a homofermentative bacterium commonly found as NSLAB in cheese and in other fermented foods. It has shown influence in amino acid metabolism and production of volatile compounds in cheese (Eugster et al. 2019). Additionally, *P. acidilactici* strains isolated from cheese have showed inhibitory activity against unwished bacteria including *Listeria monocytogenes* and *Enterococcus* spp. (Todorov et al. 2021). Both the products from the metabolism and inhibitory activity against unwished bacteria are favored qualities in bacteria present in a starter culture.

As mentioned in the introduction, *Lactococcus garviae* often takes part in cheese production as NSLAB (Guarcello et al. 2016).

As mentioned in the introduction, *Staphylococcus aureus* is a pathogenic bacteria in foods and may cause problematic consequences if present in cheese (Charlier et al. 2009).

An association between milk and bacteria composition can be seen in the results from the thermophilic starter cultures from starter culture producer C in table 11. Thermo 1 from batch 1 and thermo 2 from batch 2 were both made from milk from Bella and had partly the same microbial composition specifically regarding *Lacticaseibacillus paracasei* but also *Staphylococcus aureus*. Similarly, the two cultures from Mumsemor´s and Filippa´s milk, thermo 2 from batch 1 and thermo 1 from batch 2, showed similarities in detected numbers of *Lactococcus lactis* and *Lactococcus garviae*. That indicates differences in milk composition between the individual cows even within the same breed on the same farm. The dissimilar microbial compositions of the starter cultures may give different characters of the cheeses produced with these starter cultures.

Starter cultures made of milk from different cows with different microbial composition might be more or less suitable for particular cheese types. If the milk for the starter culture and the cheese has a high variation over time, the cultures will lack stability and the product might change character. Depending on the preferences of the customers, dairy products with variation over time might be unwished.

In summary, *Enterococcus gallinarum, Enterococcus gilvus, Enterococcus faecalis, Enterococcus durans, Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Levilactobacillus brevis, Pediococcus acidilactici, Lactococcus lactis* and *Lactococcus garviae,* may be considered wished bacterial species whereas *Escherichia coli, Staphylococcus aureus, Staphylococcus haemolyticus* and *Micrococcus luteus* may be unwished in dairy products.

5.4.2 The mesophilic cultures

A big variety of bacteria species were detected in the mesophilic starter cultures from producer C. Breed, feeding and time of year may affect the composition. Surprisingly, the starter culture with added herbs, meso 4 from batch 2, did not contain a higher number of species than average. On the other hand, this was the only culture containing *Klebsiella oxytoca*.

The species detected in the mesophilic starter cultures from producer C include *Lactococcus lactis, Leuconostoc pseudomesenteroides, Leuconostoc*

mesenteroides, Lacticaseibacillus paracasei, Lactobacillus plantarum, Enterobacter bugandensis, Escherichia coli, Micrococcus luteus, Staphylococcus haemolyticus, Staphylococcus aureus, Enterobacter hormaechei, Klebsiella oxytoca and *Raoultella ornithinolytica.*

Enterobacter bugandensis (*E. bugandensis*) is a facultatively anaerobic bacterium that has been used as heavy metal-immobilizing bacteria to inhibit the uptake of cadmium and lead by plants (Han et al. 2021). *E. bugandensis* has been identified in artisanal cheese (Erhardt et al. 2023). More research is needed about the role of *E. bugandensis* in cheese.

Enterobacter hormaechei is a pathogenic facultative anaerobic bacterium present in milk (Sadiq et al. 2018). *Enterobacter* spp. are included in the most common contaminants in dairy products (Mohamed et al. 2023).

Klebsiella oxytoca (*K. oxytoca*) is a facultative anaerobic bacterium. It has shown production of acetate, citrate and other compounds in cheese (Delbès-Paus et al. 2012). *K. oxytoca* might be responsible for early blowing in raw goat´s milk cheese. Hole formation might lead to spoilage if unwished in the cheese (Tabla et al. 2018).

Raoultella ornithinolytica (*R. ornithinolytica*) is an aerobic bacterium that belongs to the Enterobacteriaceae family, same family as *Salmonella*, *Escherichia*, *Klebsiella* and *Enterobacter*. *R. ornithinolytica* is found on several places in the environment and is an emerging bacterium in human infections (Hajjar et al. 2020).

A higher number of bacteria species was detected in the starter cultures from starter culture producer C than in the starter cultures from producer B even though both used similar methods. On the other hand, the producers used milk from different animal species and different milking techniques. On top of that, producer B had back-slopped and stored the same starter cultures for years in the freezer while producer C prepared fresh made starter cultures for every batch.

In summary, *Lactococcus lactis, Leuconostoc pseudomesenteroides, Leuconostoc mesenteroides, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum* and *Enterobacter bugandensis* may be considered wished bacterial species whereas *Escherichia coli, Micrococcus luteus, Staphylococcus haemolyticus, Staphylococcus aureus, Enterobacter hormaechei* and *Raoultella ornithinolytica* may be unwished in dairy products. Depending on the type of cheese produced, *Klebsiella oxytoca* may be either wished or unwished since it causes early blowing.

5.5 The questionnaire

We can conclude that the majority of the answering farmers used cow's milk which was bought from other farms. Equal number of producers used either the unpasteurized, warm milk directly after milking or cold pasteurized milk as a base substrate for starters. The mesophilic cultures were the most used ones within the answering group and the source of bacteria were either only freeze-dried stock or a mix of different sorts of starter cultures.

5.6 Improvements of methods

In production, the starter cultures used are often freshly made or back-slopped just before usage. In the project, the transport of the starter cultures took approximately two days which makes the cultures approximately three days when they were cultivated in the lab. This time may have affected the composition of the culture and consequently given other results than if the starter cultures would be cultivated just after production. However, the results can give a good indication about the stability and diversity of farm specific starters.

The colony forming units on the agar plates were often impossible to count since they were way too many despite the high dilution factors. After the first tries, more agar plates could be used for counting but the dilution needed for a suitable amount of CFU was still rather unpredictable since all starter cultures were different. Therefore, the limited number of countable plates may have been a bias. More than two dilutions of the culture would probably have been needed to get presentable results.

The MALDI-TOF instrument identified a minority of the samples on the target plate which limited the results. One explanation for lack of identification is the limited reference library. In some cases, the amount of material from bacteria may have been too small for the instrument. To identify the rest of the samples, the reference library used in the MALDI-TOF instrument may be updated or sequencing of the bacterial 16s rRNA gene can be used. On top of that, only a limited number of randomly selected colonies were used for identification. This means that even if the microorganisms were not detected they still might have been present within the cultures.

Regarding the questionnaire, only seven answers were obtained which is low to draw conclusions. This information can be completed if more producers hand in the questionnaires for evaluation. However, it gives an indication about the procedures the artisan food producers follow.

5.7 Conclusion

In conclusion, the number of CFU was increasing with time in two out of three cases. The number of CFU was higher in the mesophilic cultures. The starter cultures in general were unique for each producer and highly diverse, likely affected by the environment and the production method used for the cultivation. Several unwished microorganisms were detected in the thermophilic cultures from producer A and B while only wished species were detected in their mesophilic cultures. That indicates that more unwanted species are found under elevated temperatures. In contrast, more unwished microorganisms were found in the mesophilic starter cultures compared to corresponding thermophilic cultures from producer C. However, most of the species found can be considered wished, probably contributing to a specific characteristic of final products. Therefore, the farm specific starter cultures might be one of the key factors to create unique, site-specific, delicious dairy products which can meet the demand for traditional, artisan food products.

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Popular science summary

Bacteria cultures have power over food products. They affect texture, flavor and shelf life. Microorganisms have been used since ancient times to produce fermented foods including beverages, bread, and wine. As long as microcultures are fed and kept under the suitable conditions, they can be used again and again. A common example is sourdough used for bread. The sourdough is full of yeast and bacteria species which use their metabolism to release nutrients and produce volatile compounds. The same principle applies to cheese production. A bacteria culture is added to lower the pH and transform the components in the milk. This will inhibit unwished microorganisms and improve the flavor.

Nowadays, only a few, known microbial strains are used in the large-scale production of fermented foods. In this way, the character of the food is consistent, and it is much easier to have control of the process. On the other hand, usage of a unique microculture can form a rich, site-specific food product which can be both more interesting in taste and be connected to a geographical origin. An increased interest in artisan food and nearness to food producers has led to a higher demand for artisan cheese. By discovering the secrets behind methods used since ancient times, starter cultures can be used to produce cheeses with a high sensory experience and deep meaning.

Swedish farmhouse dairy producers are enthusiastic about using self-made starter cultures affected by environmental factors in their dairy products. The challenge is to make sure the cultures are free from unwished microorganisms which can cause both spoilage and food poisoning. Knowledge about traditional methods and identification of starter cultures is a way to preserve cultural heritage and market artisan cheese. In this master project, some of the microorganisms in farm specific starter cultures for cheesemaking were identified. It is a part of a collaboration with the Swedish Farmhouse Dairy Producers (Sveriges Gårdsmejerister) and the Swedish University of Agricultural Sciences (Sveriges lantbruksuniversitet).

4 batches with thermophilic and mesophilic starter cultures from farmhouse dairy producers were sent to SLU. The starter cultures were cultivated and the number of colony forming units were counted. Generally, the number of colony forming units increased in the starter cultures over time. After recultivation, the microorganisms

were identified by a MALDI-TOF mass spectrometry instrument. Most bacteria species found in the cultures can be categorized as beneficial microorganisms favorable for the dairy product. The most abundant bacteria species were *Lactococcus lactis, Leuconostoc pseudomesenterioides* and *Leuconostoc mesenteroides.* On the other hand, a few unwished microorganisms such as *Escherichia coli, Straphylococcus haemolyticus, Enterococcus hirae* and *Enterococcus faecium* were detected in some of the starter cultures. This could lead to unwelcome consequences in the dairy foods.

In summary, the starter cultures in general were unique for each producer, highly diverse and affected by the environment and the production method used for the cultivation. The inimitable composition of the microorganisms might lead to exceptional artisan foods manufactured by small-scale producers.

Appendix 1

Enkät avseende tillverkning och användning av egen starterkultur

***Sveriges Gårdsmejerister – samarbeten för utveckling av småskalig ost och filmjölkskultur* med journalnummer 2022- 2724 inom landsbygdsprogrammet 2014–2020**

- **Vilken typ av mjölk använder du, dvs ko, get, får, annat, för tillverkning av dina produkter?**

- **Är mjölken från egna djur eller köps den in?**
  - **Egna djur**
  - **Köps in**
  - **Annat, specificera**

- **Vilken/ vilka mejeriprodukter tillverkar du i ditt mejeri?**

- **Vilken typ av starterkultur använder du i tillverkningen av dina produkter?**
    - **Mesofil**
    - **Termofil**
    - **Annat, specificera:**

- **Vilken typ av mjölk används för att framställa starterkulturen?**
    - **Nymjölkad och spenvarm, inte pastöriserad**
    - **Tankmjölk, opastöriserad**
    - **Tankmjölk, pastöriserad**
    - **Annan – ange vilken**

- **Vilken källa till mjölksyrabakterier används, dvs var kommer bakterierna ifrån?**
    - **Mjölkens egna, dvs medföljarfloran i mjölken**
    - **Köpt, frystorkad kultur**
    - **Kulturen som man själv tagit fram på ett speciellt sätt; specifiera**
    - **Tillsats av växtmaterial (örter, gräs, hö, löv ...); specifiera**

- **Tillsats av kommersiell filmjölk (om mesofil) eller kommersiell yoghurt (termofil kultur)**
  - **Om ja**
    - **Egentillverkad produkt?**
    - **Köpt mejeriprodukt?**
- **Tillsats av frystorkad kultur (ready-set)**
- **Annat sätt? Om ja, specificera**

- **Hur inkuberas kulturen för att ”växa till”?**
  - **Temperatur**
  - **Tid för inkubering**
    - **Hur länge står kulturen innan den tas i bruk, dvs när den bedöms vara färdig?**
    - **Vad är slutpunkten?**
      - **När kulturens konsistens är tjock**
      - **När kulturen smakar syrligt och friskt**
      - **När ph är ca 4,5 *gläntar på locket* luktar bra om syrligt och friskt**

- **Hur kontrolleras att kulturen är av god kvalitet?**
  - **pH mäts vid slutet**
  - **pH mäts flera gånger och ”plottas” mot tid**
  - **Smak, doft och utseende**
    - **Specificera**

- **Kontroll av kulturens sammansättning av bakterier. Om ja, hur?**
  - **Regelbundet**
  - **Då och då**
  - **Aldrig**

- **Hur förvaras kulturen mellan olika produktionstillfällen?**
  - **Svar:**

  - **Ej relevant, använder alltid färsk kultur**

- **Tänker du i ditt arbete på att skydda din starterkultur från kontaminering av andra bakterier, jäst, mögel, fager etc?**
  - **Om ja, hur? Specificera**

- **Hur länge används samma kultur?**
  - **Svar**

- **Hur avgör du när det är dags att odla upp en ny kultur?**
  - **Svar**

- **Något du vill tillägga?**

Publishing and archiving

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