Flow of non-starter lactic acid bacteria

From to silage to raw milk to cheese

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Flow of non-starter lactic acid bacteria. From silage to raw milk to cheese

Flödet av medföljandefloran från ensilage, till mjölkråvara och till ost

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Abstract

Modern cheese manufacturing uses a known starter culture of lactic acid bacteria (SLAB) for the fermentation of lactose to lactic acid. There is, however, often a second fermentation in cheese manufacturing by non-starter lactic acid bacteria (NSLAB). These LAB are not added in the manufacturing, and it is not always clear where these LAB originate from. The main objective of this review was to study if there is a flow of NSLAB from silage to raw milk and to the resulting cheese. More specific objectives were to investigate how LAB in silage may affect the raw milk, if LAB stemming from silage can act as NSLAB in cheese, and if so, how they affect the cheese. *L. plantarum, L. paracasei* and *L. casei* were found to be the LAB most described as NSLAB in cheese, with the potential to persist in raw milk and occasionally survive pasteurisation. LAB in silage is crucial for the fermentation process, and NSLAB in cheese can enhance flavour of cheese but also cause defects during ripening. Most LAB from silage do not enter the milk, but some actually do. LAB in the milk could enter through other contamination sources but it possible that some LAB from silage survive and act as NSLAB in the cheese. More research is needed to confirm the flow of LAB in the value chain of milk and cheese.

Keywords: Non-starter lactic acid bacteria, herbage, silage, raw milk, cheese, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus casei*

Sammanfattning

I modern osttillverkning tillsätts i regel en kommersiell starterkultur av mjölksyrabakterier (SLAB) för fermentering av laktos till mjölksyra. Vissa lagrade ostar har dock en andra fermentering med hjälp av s.k. medföljarflora (non-starter lactic acid bacteria, NSLAB), för utveckling av sin karaktäristiska smak och textur. Dessa mjölksyrabakterier (LAB) tillsätts inte avsiktligen i processen, och det är inte alltid helt klart varifrån de kommer ifrån. Huvudsyftet med detta arbete var att undersöka om NSLAB i ost kan vandra från ensilage till mjölkråvara och till den resulterande osten. I studien ingick att undersöka effekter av LAB från ensilage i mjölkråvaran, om LAB från ensilage kan utgöra NSLAB i ost, och om så är fallet, deras effekt på osten. Resultatet visar att *L. plantarum, L. paracasei* och *L. casei* är de vanligaste NSLAB i ost. Dessa LAB har potential att existera i obehandlad mjölk och kan även emellanåt överleva pastörisering. LAB i ensilage är avgörande för fermenteringsprocessen, NSLAB i ost kan förbättra smaken och sänka pH men även orsaka defekter i osten under lagringen. De flesta LAB i ensilage kommer inte över via mjölken, men en del gör det. LAB i mjölkråvaran kan härröra från andra kontamineringskällor, men det är möjligt att en del LAB från ensilage överlever

och kan agera som NSLAB i osten. Mer forskning behövs för att beskriva flödet av LAB i mjölkens och ostens värdekedja.

Nyckelord: Medföljarflora, vallväxter, ensilage, obehandlad mjölk, ost, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus casei*

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Abbreviations

LAB	Lactic acid bacteria
NSLAB	Non-starter lactic acid bacteria
SLAB	Starter lactic acid bacteria
TBC	Total Bacterial Count
CFU	Colony forming units

1. Introduction

1.1 Introduction to lactic acid bacteria and their importance

Lactic acid bacteria (LAB) constitute a large and varied group of bacteria (Gopal 2022). Homofermentative LAB ferment glucose to lactic acid (Müller 1990) whereas heterofermentative LAB ferment glucose to lactic acid, ethanol/acetic acid and carbon dioxide. LAB have a diverse natural habitat (Gopal 2022), which includes raw milk, dairy products, herbage, and gastrointestinal tract of mammals. The LAB metabolism can be important for taste and texture in dairy products, and LAB are of importance in the silage fermentation process (Driehuis & Elferink 2000), where their activities secure a low pH. LAB could also have anti-clostridial activity which could supress and inhibit growth of spoilage bacteria such as *Clostridium tyrobutyricum* (Christiansen et al. 2005).

Because of their fermentation, it is of great interest to use LAB in dairy production, and to know which LAB that are active during fermentation in production. Moreover, because of variation in the metabolism of different LAB it is important to recognise the properties of the LAB used, to be able to generate reproducible products. Cheese manufacturers therefore often use commercial cultures of starter lactic acid bacteria (SLAB) for the production (Choi et al. 2020). However, these SLAB are usually not the only LAB that is found in the resulting cheese. Non-starter lactic acid bacteria (NSLAB) are LAB that also affects the products but are typically not added as starters by the manufacturer (Broadbent et al. 2016). To be able to guarantee a reproducible production of a cheese, it is therefore of interest to understand were these NSLAB come from.

1.1.1 Starter lactic acid bacteria

Traditional cheese production commonly used raw milk and an undefined starter culture (Martley & Crow 1993), or a form of back-slopping where some of the previous product was used as starter (Bintsis 2018). Drawbacks with this type of starters are fluctuations in quality and a short shelf life, since there is almost no

microbiological control (Martley & Crow 1993; Bintsis 2018). Commercial SLAB has therefore been of significant use, where the species and strains of SLAB are known and gives a recognised and consistent result. But this is not always the case.

SLAB is of immense importance in cheese production. The major role of the primary SLAB is to ferment lactose to produce large amounts of lactic acid (Lazzi et al. 2016). This helps make the milk into curd and lowers the pH which makes it hostile to pathogens, safe to eat and increases shelf life. This first fermentation is usually called primary fermentation (Martley & Crow 1993) and happens in the first hours of cheese manufacturing. During cheese ripening, a secondary fermentation takes place. This is especially the case for cheeses that mature for a longer period, e.g., several months or even years, with participation of an added secondary starter culture or by adventitious flora, e.g., NSLAB.

1.1.2 Non-starter lactic acid bacteria

As mentioned, NSLAB are adventitious bacteria that are usually not deliberately added in the cheese production (Broadbent et al. 2016) but in some way gain access during manufacturing. LAB is therefore a NSLAB when it is found in cheese without being added. NSLAB do not contribute to the primary fermentation in any significant degree. In contrast, they will increase to higher numbers during ripening and contribute to a secondary fermentation, if not a secondary starter culture is used and added. The primary starter culture will drop in numbers due to the presence of salt, lower temperatures, and no residual lactose, and this will make place for NSLAB to multiply (see figure 1.)

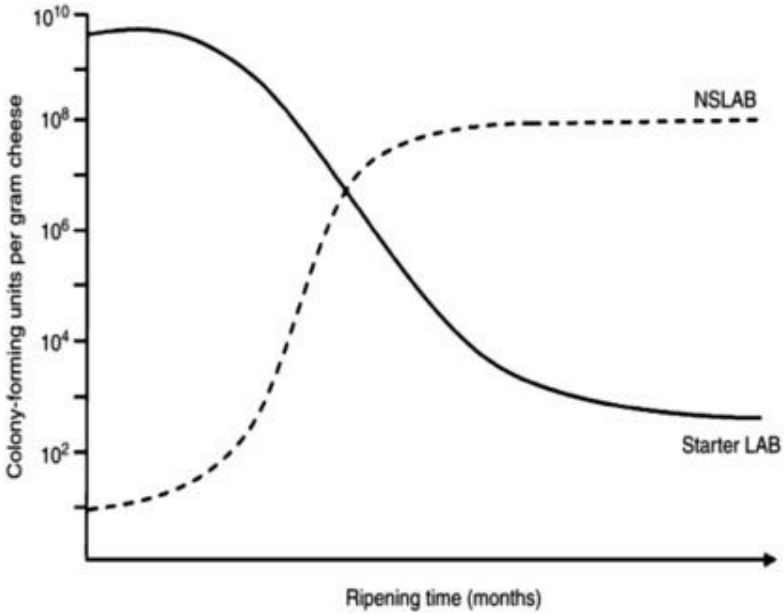

Figure 1: General illustration of the development of NSLAB and the decrease of SLAB during cheese ripening in months (Broadbent et al. 2016).

NSLAB usually tolerate the hostile environment during longer periods of ripening (De Angelis et al. 2001). It seems that some NSLAB survives higher temperatures even if most NSLAB are mesophilic (De Angelis et al. 2001; Broadbent et al. 2016). It has also been shown that enzymes of the mesophilic NSLAB have a greater adaptation to the conditions in cheese (De Angelis et al. 2001) than enzymes associated to SLAB. The metabolism of NSLAB is determined by intrinsic and extrinsic factors (Broadbent et al. 2016) and exactly how pH, moisture and salt content affect the development of NSLAB is still not fully understood.

In their fermentative metabolism, NSLAB must generate ATP to survive and grow, and SLAB have already consumed the residual lactose when NSLAB usually begin to grow in numbers, which means that NSLAB need to use other types of compounds for their energy. NSLAB could in theory use lactic acid, citric acid, fatty acids, amino acids, and other compounds in the fermentative metabolism. It has been shown that different types of NSLAB have different ways of tackling their metabolism to survive, and there is a large variety of developed NSLAB species and subspecies (Jonsson et al. 1990).

The fermentative metabolism of different NSLAB could affect the cheese in a variety of ways. It could increase the levels of amino acids and free fatty acids (De Angelis et al. 2001), leading to enhanced flavours in the cheese, but it could also lead to cheese defects. Since the origin of these adventitious NSLAB is often unclear, their presence and activities in cheese are exceedingly difficult to control and foresee.

1.2 Aim and research questions

The primary aim of the paper is to study the flow of NSLAB from silage to raw milk to cheese. This will include a study of the most common NSLAB, potential NSLAB in silage fermentation, and the microbiota in farm tank and dairy silo milk. It will also study treatment of the milk at the dairy, the role of NSLAB in cheese manufacturing and its impact on the resulting cheese. The impact of the shelf life of cheese and milk will be investigated, and potential negative impacts on milk and cheese of NSLAB and how NSLAB could improve sensory attributes in cheese. The main questionaries for this paper is therefore:

- Could NSLAB flow from Silage to raw milk to cheese?
- Which are the most common NSLAB?
- How do NSLAB help the development, shelf life/stability/preservation and sensory attributes of silage and cheese, and can NSLAB have a negative impact on cheese?
- Is NSLAB part of the microbiota in milk?
- How is the NSLAB population affected by pasteurisation?

1.2.1 Method

This is a literature review where primarily the databases; Web of science, Scopus and Google scholar were used. The articles that were used for the review come from these databases are published in scientifically reviewed journals, with a focus on experimental studies, but literature reviews and book sections were also used. Some sources suggested by the supervisor were also utilized. Keywords used when searching for relevant sources for this review were; LAB, SLAB, NSLAB, milk, raw milk, pasteurised milk, cheese, silage, herbage, milk microbiota, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus casei*. It should also be noted that the type of cheese that was mainly studied is cheeses that are manufactured using pasteurised, bovine milk.

2. Result

2.1 Types of potential NSLAB

There is a big variety of different LAB that could act as NSLAB (Jonsson et al. 1990), and there is also a great variety of different subspecies. Different LAB have different types of metabolism, which will affect cheese and silage in different ways. It is therefore desirable by manufacturers and farmers to know how different LAB affect their cheese or silage (Broadbent et al. 2016).

It is important to note, that there has been a reclassification of the *Lactobacillus* genus in recent time (Zheng et al. 2020). Using DNA analysis, it has been shown that many *Lactobacillus* species are only distantly related. The genus *Lactobacillus* has therefor been reclassified into 25 genera. This means that *Lactobacillus plantarum, Lactobacillus paracasei* and *Lactobacillus casei* are now called *Lactiplantibacillus plantarum, Lacticaseibacillus paracasei* and *Lacticaseibacillus casei.*

Several of the scientific journals that have been used for this review mention NSLAB species that are found in their research (table 1), and *Lactiplantibacillus plantarum, Lacticaseibacillus paracasei* and *Lacticaseibacillus casei* are the most usual NSLABS found. LAB can be found in silage and raw milk as potential NSLAB, but the highest definitive CFU counts of NSLAB are found in cheese, (figure 2). The explanation for the high numbers is that the cheese ripening process gives NSLAB time to grow in numbers (Choi et al. 2020), facilitating their detection and resulting in a larger impact on the resulting product, i.e. cheese. Most NSLABs are mesophilic and heterofermentative (Gagnon et al. 2020), but it should also be noted that some are thermophilic.

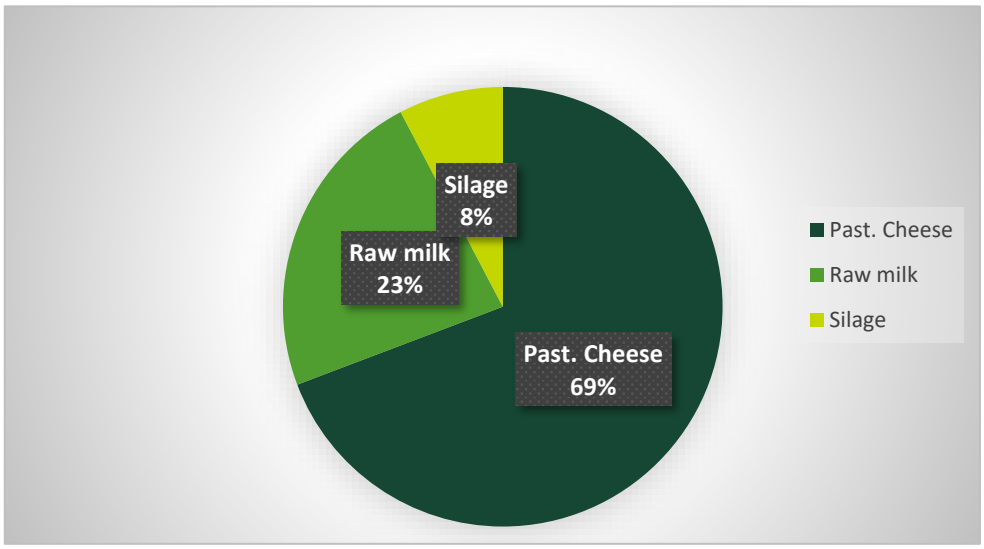

Figure 2: Type of matrix (silage, milk, cheese) in which the studies cited in this thesis have investigated the presence of non-starter lactic acid bacteria (NSLAB). Values represent % of total studies cited for silage, raw milk, and cheese, respectively. A total of 12 studies where used.

Table 1. Description of studies that have examined different species of potential NSLAB. References are marked with numbers and described below the table. The origin of NSLAB identified in the respective study is marked as P.C., R.M, and S. (pasteurised cheese, raw milk, and silage). The number of times the respective NSLAB was mentioned in the studies was counted and summarized as total number of times (Times found).

Reference:	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	Times found
NSLAB found in:	P. C.	P. C.	P. C.	P. C.	R. M	R. M & P.C.	P. C.	P. C. & S.	P. C.	R. M.	P. C.	S.	
L. plantarum	X	X		X		X	X	X	X	X	X		9
L. paracasei	X	X	X	X		X	X			X	X	X	9
L. rhamnosus	X	X		X	X					X			6
L. brevis	X		X	X					X		X		5
L. fermentum	X			X					X	X			4
L. casei	X	X		X	X	X	X		X	X		X	9
L. delbrueckii	X							X					2
L. curvatus		X	X	X			X				X		5
L. pentosus				X									1
L. parabuchneri				X								X	2
P. acidilactici					X					X			2
L. casei subsp.						X							1
L. lactis										X		X	2
L. cremoris										X			1
E. faecium										X			1
E. faecalis										X			1
E. durans										X			1
P. pediococus										X			1
P. pentosaceus										X		X	2
L. garlicum												X	1

References used were**:** 1. Aljewicz et al. 2016; 2. Crow et al. 2001; 3. Christiansen et al. 2005**;** 4. Choi et al. 2020; 5. Lazzi et al. 2016**;** 6. De Angelis et al. 2001**;** 7. **Broadbent et al. 2016; 8.** Gagnon et al. 2021; 9. Martley & Crow 1993; 10. Bluma & Ciprovica 2016; 11. Fitzsimons et al. 1999; 12. Gagnon et al. 2020

2.1.1 Lactiplantibacillus plantarum

L. plantarum is a heterofermentative mesophilic bacteria (Corsetti & Valmorri 2011) that grows between 15°C and 45°C and is commonly found in aged cheese (De Angelis & Gobbetti 2011). It is also one of the most common bacteria to add to silage and is also often found in herbage before ensiling. *L. plantarum* converts hexoses to lactic acid and pentoses to lactic acid and acetic acid (Corsetti & Valmorri 2011), using a variety of compounds as carbon energy sources. *L. plantarum* is well adapted to the environment in cheese and even uses metabolites from the activities of SLAB as energy source, which is why high amounts of *L. plantarum* can be found in cheese. *L. plantarum* is also a controlling bacterium in silage where it can quickly dominate the whole microbial population.

2.1.2 Lacticaseibacillus casei

L. casei is a heterofermentative mesophilic bacteria (Gobbetti & Minervini 2014) that grows between 2°C and 53°C. It is common in cheese and usually the dominant NSLAB in cheddar-like cheeses (Peterson & Marshall 1990). It uses hexoses and pentoses in a similar manner as *L. plantarum* (Gobbetti & Minervini 2014), mainly converting hexoses to lactic acid and pentoses to lactic acid and acetic acid. One of the reasons why *L. casei* is so common as NSLAB is its concurrent metabolism, e.g. *L. casei* first metabolises lactose then galactose and citrate and lastly just galactose (Broadbent et al. 2016). This ability makes *L. casei* a highly effective NSLAB in cheese, allowing numbers to rise fast.

2.1.3 Lacticaseibacillus paracasei

L. paracasei is closely related to *L. casei* (Gobbetti & Minervini 2014) as the name suggests. The taxonomy of *L. paracasei* is remarkably similar to *L. casei* which has led to some debate about the differences of the species, however, there is an accepted taxonomic division between the two species. *L. paracasei* is homofermentative and mesophilic and grows between 10°C and 37°C. The metabolism of *L. paracasei* is very similar to that of *L. casei* (Curry & Crow 2002), but the main distinction is that *L. paracasei* is not able to ferment as many different carbohydrates as *L. casei*. however, *L. paracasei* is still an effective NSLAB in cheese, and usually coexists with *L. casei.*

2.2 Silage microbiota and general fermentation process

Fermentation of herbage into silage can be performed in a variety of ways, either without additives, with chemical additives or with a starter culture. The principle of silage production is still the same, where a rapid pH drop and maintenance of anaerobic environment is crucial (Driehuis & Elferink 2000). The silage fermentation is divided into four phases. The first phase is the aerobic stage, where oxygen is trapped and then reduced by the herbage. This is because of the respiratory activity of plants, and of other aerobic organisms. The respiratory activity can be quite different in different types of herbage. The second phase is the fermentation phase, where anaerobic microorganisms compete for the nutrients in the herbage. LAB converts glucose, fructose, and sucrose to lactic acid, which will make the pH drop and clostridia and enterobacteria will diminish if LAB become dominant in this stage. The third phase is the storage phase were truly little occurs if the pH is maintained at a low level, and the silage is kept anaerobic. The fourth phase is the feeding phase where the anaerobic conditions are broken, and the pH rises. This means that aerobic microorganisms start to proliferate; as yeast, enterobacteria, moulds etc. The anaerobic condition is often broken before the fourth phase, because of the plastic not being fully airtight.

2.2.1 Fermentation without additives

Additives are not an absolute necessity for silage production (Driehuis & Elferink 2000). Quality herbage, good crop composition and good silage-making techniques will usually give a good result without additives. This is because LAB in the herbage will multiply fast if the right conditions exist. At the same time, it means that fermentation without any additives is associated to risks. Herbage needs to be processed into silage rapidly so that microorganisms like yeast and enterobacteria will not have the possibility to start multiplying in high numbers (Everitt et al. 2003). Herbage that has been fertilized with manure will therefore be at a higher risk of infection. The dry weight of silage also plays a significant role in silage, were dry weight under 40% can lead to infection of *Clostridium tyrobutyricum*, and other spoilage associated bacteria.

2.2.2 Fermentation with chemical additives

The use of additives is justified when the crop for some reason does not constitute a sufficiently good substrate for a safe ensiling process. Chemical additives can be split into two categories, salts, and acids. The most usual acids that are used are formic acid and propionic acid. Formic acid is efficient against bacteria, while propionic acid is more efficient against moulds and yeasts. Kofasil Ultra (Swedish

Agro 2015) is a mixture of different salts that are also often used as an additive, it contains sodium nitrite, hexamethylenetetramine, sodium benzoate and sodium propionate. Kofasil Ultra is efficient against *clostridium* and fungi (Everitt et al. 2003).

2.2.3 Fermentation with addition of starter culture

Addition of starter culture is a suitable alternative to control that the right microbial community develops in the silage and with a resulting drop in pH. LAB often has a harder time multiplying in wet environments, which means that the dry weight in the herbage needs to be sufficient for the use of starter culture. It is sufficient if dry weight is around 25-30% but not very sufficient at 15%. Usual inoculants in silage when using starter culture is *L. plantarum* and *L. buchneri* (Gagnon et al. 2020) because they are well adapted to thrive in the environment, and very effective producers of lactic acid (Driehuis & Elferink 2000).

2.2.4 NSLAB in Silage

The amount of LAB in silage varies a great deal between different herbages However, the amount of LAB that is transferred to milk from forage is debatable. Gagnon et al. (2020) could by use of RAPD typing show that some NSLAB were transferred to milk directly from silage, but only in small numbers. Their study suggested that the majority of NSLAB, originating from the silage and ending up in the milk, entered milk indirectly via faeces. However, since *L. plantarum, L. casei* and *L. paracasei* seem to be well adapted to both silage and milk, they could possibly also be transferred directly from silage to milk, e.g. in association to milking.

2.3 Microbiota in raw milk

Milk has a rather complex microbiota were LAB is one of the most common group of microorganisms (Quigley et al. 2013). Other bacteria, yeast and moulds are also usually present in milk, and there is a variety of contamination sources for these microorganisms. The cow teats, hides, and faeces can affect the microbiota of raw milk, and so can housing, feed and milk equipment. LAB that transfers to the milk is critical for the production of cheese and other milk products. Other bacteria as *Clostridium tyrobutyricum* and *Escherichia coli*, which can cause defects and serious disease in humans could also transfer to the milk. Milk in the udder of cows is not sterile as believed in the past (Skeie et al. 2019). This contamination is believed to enter the milk through the entero-mammary pathway, a gastrointestinal

track which involves the mammary gland through endogenous cellular route (Young et al. 2015).

It is important to note that the microbiota is vastly different in farm tanks/dairies and right before use in cheese making. This because the pasteurisation that happens after the raw milk has been sold eliminates a big number of microorganisms (Crow et al. 2001).

2.3.1 Microbiota in farm tank milk

The main microbial contamination of the milk in tanks take place on the dairy farms (Skeie et al. 2019). This could be through; milking equipment, teat surface and housing and it has been shown that the microbiota between farms milks differs significantly. A recent study reported higher TBC values in milk from herds with an automatic milking system because of inferior washing technique of the milking system. The brand of automatic milking robot also affected the milk microbiota, probably due to differences between the robots in the way teats were cleaned before milking (Sun et al. 2022). It has also been shown that the microbiota can be very different from day to day in milk tanks from the same farm (Skeie et al. 2019). There is a variety of factors that could affect the microbiota in farm tank milk, e.g., differences in the hygienic quality of animal feed, weather conditions, environment on farm and the animal's health. It is almost impossible to control the microbiota of raw milk, but teat preparation before milking and regular cleaning could at least help (Sun et al. 2022).

Specific LAB can be associated to a specific farm and be an essential part of the "house flora" (Martley & Crow 1993). Some sub-species of LAB have their origin in milk farms, and some studies have discovered that specific sub-species of potential NSLAB can be tracked to specific farms (Nikoloudaki et al. 2021). This suggests that the microbiota and quality attributes of milk products may be associated to the house flora from specific farms.

2.3.2 Microbiota in dairy silo milk

The milk that is collected on the farms is often delivered to a dairy processing plant where it may be stored and refrigerated for another period of time. Just as in the case of farm tank milk microbiota, the microbiota in the dairy silo milk can vary from day to day and from one dairy silo to another (McHugh et al. 2020). It has for example been shown that different dairies that have used milk from the same farm can have almost no species of LAB in common in milk after pasteurisation (Crow et al. 2001). Dairy silo milk typically has a higher amount of spoilage-associated bacteria than farm tank milk (McHugh et al. 2020). Plausible reason for this is that

the time elapsed after the transportation to the dairy gives *Pseudomonas* and *Acinetobacter* time to multiply.

Species of NSLAB could enter in the dairy as airborne flora (Fitzsimons et al. 1999) and also exist in the milk from the farm, meaning that NSLAB could enter the milk before cheese making.

2.4 NSLAB in heat treatment of milk

As mentioned, most NSLAB are mesophilic (Gagnon et al. 2020) which means that they grow at moderate temperatures around < 30 °C. This means that pasteurisation of milk could be a problem for the survival of NSLAB that are mesophilic.

2.4.1 Pasteurisation

Pasteurisation of milk can be performed at several combinations of time and temperature. The usual temperature of milk pasteurisation for cheese making is around 70-72 °C for 15-20 seconds (Desfossés-Foucault et al. 2013), this will lead to approximately 90% reduction of the bacterial population. As mentioned before, spoilage-associated bacteria are found in higher counts in dairy silo milk, but these bacteria are usually fully inactivated by the pasteurisation (McHugh et al. 2020). It is though, still possible that mesophilic NSLAB survives the pasteurising process (Desfossés-Foucault et al. 2013). Studies have shown that only a small number of *L. paracasei* needs to survive pasteurisation of milk for this bacterium to dominate the microflora in the resulting cheese. It has also been shown that some NSLAB may enter into a nonculturable state after pasteurising (Sun et al. 2022). It should also be noted that NSLAB could enter the milk after pasteurisation through equipment and as airborne flora (Fitzsimons et al. 1999).

2.4.2 Raw milk vs pasteurised milk

It should be noted that there is a difference in numbers of potential NSLAB in raw vs pasteurised milk (McHugh et al. 2020). Raw milk has a higher amount of NSLAB than pasteurised milk, for the simple reason that NSLAB have not been killed by heat treatment in the case of raw milk. Nevertheless, it has been reported that there is a larger diversity of NSLAB in cheese from pasteurised milk than in raw milk (Bluma & Ciprovica 2016). The reason for this is unknown, but it could be because smaller amounts of surviving NSLAB in pasteurised milk gives better chances for growth of all NSLAB, while in raw milk certain NSLAB are already dominant and give little room to other NSLAB.

2.5 The role of NSLAB in cheese

There is a large variety of NSLAB (Jonsson et al. 1990), affecting cheeses in different ways (Broadbent et al. 2016). It is still not fully understood how NSLAB enter cheese manufacturing, but a variety of possible sources have been suggested (Quigley et al. 2013; Broadbent et al. 2016).

2.5.1 Cheese manufacturing and ripening

Cheese can be manufactured in several ways and there is a wide variety of cheeses. According to Fox et al (1990) the standard manufacturing method of cheese can be divided in 8 steps. The milk is first pasteurised to kill pathogenic bacteria and then cooled down to the optimum temperature for the SLAB to grow. The SLAB is added to the milk, followed by rennet after a slight reduction in pH due to activation of the starter. Rennet enzymes make the milk coagulate and start forming a curd. The curd is cut and heated to help the whey separate from the curd. After collecting the cheese grains into molds, the cheese is salted, often set-in salt or in salt brine. The salt improves the flavour of the cheese and supresses spoilage bacteria.

The essential primary reaction in all cheese manufacturing is the fermentation of lactose to lactic acid which is done by SLAB during the first few hours of the production (Martley & Crow 1993). Fermentation of lactose to lactic acid is the primary role of a SLAB, and it has been estimated that SLAB ferment up to 98% of the lactose in the milk to lactic acid. Enzymes produced by LAB e.g., proteases and peptidases, will gradually break down citrate, lipid, and some proteins during the aging of the cheese (Broadbent et al. 2016). The breakdown of peptides and metabolism of amino acids is very important for some cheeses. The characteristic flavour of some cheeses may need months and up to some years to develop, and this step is done by the secondary fermentation were NSLAB metabolise free fatty acids, peptides, and amino acids.

2.5.2 Growth of NSLAB in cheese

Numbers of NSLAB are always low at the early stages of the cheese ripening (Broadbent et al. 2016), but will increase to high numbers, starting to dominate the LAB population in the cheese after some months (Figure 1). SLAB are usually not as well fitted to the environment in cheese and will therefore drop in numbers, while NSLAB are more fit for the harsh environment in maturing cheese. Numbers of NSLAB will be well below 10^2 cfu g^{-1} , rise to levels around 10^8 cfu g^{-1}, finally reaching a plateau (Crow et al. 2001; Broadbent et al. 2016) where the NSLAB no longer have the room for multiplication.

The population of NSLAB and the composition of its species in the cheese are almost uncontrollable and mostly random (Broadbent et al. 2016). It should be noted that a secondary flora can be added to cheese for a secondary fermentation, but the adventitious NSLAB flora will typically enter the fermentation even though it is not added.

NSLAB can affect the cheese in several ways, but there is often no superficial effect of NSLAB in cheese (Martley & Crow 1993), like the holes or developing moulds in Emmental cheese and mould-ripened cheese, respectively. It is rather the characteristic flavour of a cheese that is provided by the secondary fermentation, although some NSLAB could also give unwanted effects during ripening.

2.5.3 Improvement of cheese by NSLAB

NSLAB could improve the flavour and intensity of cheese which arguably is its most important role. This is generally possible through the ability of NSLAB to catabolize amino acids (Broadbent et al. 2016). The catabolism has a step where α-ketoglutaric acid is used as a receiver. The result is α-ketoacid being used by dehydrogenase and regenerating NAD+. This pathway leads to numerous productions of volatiles. One example is methionine, which is produced and associated with a strong cheese flavour. It is still unclear which NSLAB that may catabolize amino acids in a positive way, and there are several ways NSLAB can impact the cheese flavour negatively.

NSLAB can also extend the shelf life of cheese, through keeping the pH down in the cheese (Driehuis & Elferink 2000). This supresses spoilage associated bacteria and makes the environment in cheese more hostile. NSLAB can also have probiotic potential (Crow et al. 2001) which is good for human health. *L. paracasei* for example has probiotic potential and often acts as a NSLAB.

2.5.4 Defects in cheese by NSLAB

NSLAB can be a cause of defects in cheese (Gagnon et al. 2020). One example is the formation of calcium lactate crystals that are seen as small white spots on cheddar-like cheeses, a defect caused by racemisation of L-lactate produced by cows and converted to D-lactate by some specific NSLAB (Crow et al. 2001). NSLAB also have the potential to inhibit SLAB (Gagnon et al. 2020) in the early stages of cheese manufacturing, which could lead to failure in the lactic acid fermentation and product spoilage. The catabolism of amino acids cannot just lead to more flavour and intensity in the cheese, but could also lead to strong off-flavours

in cheese (Broadbent et al. 2016), and it is still unclear which NSLAB that produce these off flavours.

3. Discussion

It is a diverse group of LAB that can act as NSLAB in cheese, and how they end up in the final dairy product is under intensive discussion. It is desirable to know what effect different NSLAB have on the production, however, this is a challenge since a large group of LAB with many different supspecies and strains could act as NSLAB. NSLAB can affect the production differently in different steps in the value chain. As an example, *L. buchneri,* which is commonly used as an starter inoculant in silage (Gagnon et al. 2020), can be found in raw milk, but is usually not found in cheese. It is also known for producing histamine which could be dangerous if levels become too high in cheese. The large diversity of potential NSLAB also makes a review of their effects quite challenging. There are NSLAB that are more abundant and at higher numbers in silage, raw milk, and cheese (table 1). The most typical NSLAB, i.e., *L. plantarum L. casei* and *L. paracasei,* are well adapted to the environment in cheese and raw milk, and *L. plantarum* is particularly well fitted to the environment in silage as well.

One very notable aspect in this review is that the cited studies classify LAB very differently. Curell & Dammieras (1991) conclude that *L. brevis* is a very common SLAB, usually disappearing during secondary fermentation in cheese. Bluma & Ciprovica (2016) on the other hand says that *L. brevis* is one of the more common NSLAB found in raw milk and cheese. Gagnon et al. (2020) reported that *L. brevis* was the most dominating LAB in corn silage but did not find it at any significant numbers in raw milk. In this review, *L. plantarum, L. casei* and *L. paracasei* were the most common NSLAB (table 1), but 8 out of the 12 studies did not rank all 3 of these LAB as the most common NSLAB. This indicates that there is no clear consensus regarding which LAB that can act as NSLAB in cheese, and which LAB that are most common as NSLAB. Suggested further research could be to plot usual and important NSLAB in cheeses, for the understanding of their effects in different types of cheese.

Anaerobic fermentation of silage is dependent on the successful growth of LAB, lowering the pH (Driehuis & Elferink 2000), thereby supressing spoilage-associated bacteria. As already mentioned, ensiling can be done without additives, with additives or with starter culture. *L. plantarum,* which is an usual NSLAB, is

often added as starter culture to silage, but is also commonly found in corn and grass herbage (Gagnon et al. 2020). LAB could transfer from silage to cow's gastrointestinal tract, and exit via feces, and then enter the milk from contamination via feces. LAB is common in a variety of herbage, but this review does not discuss this to any high extent. Different herbage types have different types and amounts of LAB, and it is possible that the LAB in herbage will affect the silage and transfer to raw milk and cheese. Gagnon et al. (2020) for example found that corn herbage was associated to higher amounts of LAB that could possible transfer to the raw milk, while Bernardes et al. (2018) argued that there was a very similar flora of LAB in most silage types. One could speculate that LAB as *L. plantarum* has a greater potential of surviving the flow from silage to milk to cheese, and it is just LAB with similar properties that survive this.

The microbiota of milk is vast and complex (Quigley et al. 2013) LAB is one of the major types of bacteria in raw milk. LAB can transfer to farm tank milk through animal's teats, udder, milking equipment, housing, animal feed etc. The microbiota differs between different farms (Skeie et al. 2019) and even differs from day to day. The same goes for microbiota in dairy silo milk where the microbiota differs between different dairies (McHugh et al. 2020) and from day to day. LAB can transfer from almost everywhere into the raw milk and consequently also into the resulting pasteurised milk. The large variation in milk microbiota also makes it almost impossible to track the origin of bacteria in the raw and pasteurised milk. Studies report that the origin of specific NSLAB subspecies has been tracked to specific farms (Nikoloudaki et al. 2021), which indicates that NSLAB could originate from farms. Studies have also shown that only small numbers of *L. paracasei* need to survive pasteurisation to start multiplying and later become dominant in cheese (McHugh et al. 2020). Pasteurised milk also had a larger diversity of NSLAB than raw milk, even though numbers of NSLABs are greater in raw milk than in pasteurised milk. One could speculate that the potential NSLAB that stems from silage have longer time to multiply in the raw milk than NSLAB that stem from the dairy plant, which could enhance the chances of NSLAB from silage survives pasteurisation.

The cheese manufacturing process will vary between different cheese types, but there are steps in the process that are the same. One of these steps is the addition of SLAB which main purpose is to ferment lactose to lactic acid (Martley & Crow 1993). This takes place in the beginning of fermentation, after which NSLAB starts to multiply, eventually dominating with a plateau in their numbers after several months (Broadbent et al. 2016). It is not unusual that cheeses are left to ripen for several years, and the taste of these cheeses will be more complex. This could mean that NSLAB does not plateau fully after months, but instead keeps increasing. This

was, however, not explored in this review and further research is needed to find out if the amount of NSLAB decreases or increases after a period of months to years. It is possible that NSLAB enter during the cheese manufacturing although it has been shown that NSLAB stems from the milk (Gagnon et al. 2020). *L. plantarum L. casei* and *L. paracasei* are not well fitted for the primary fermentation and are not usually used as SLAB, and these NSLAB would probably not be optimal in ripening of all pasteurised cheese types. This is also suggested in table 1, since not all studies reported these 3 lactobacilli as NSLAB. NSLAB have some evident effects on cheese, e.g., keeping pH low (Driehuis & Elferink 2000), a probiotic potential (Crow et al. 2001) and improved flavour (Broadbent et al. 2016). NSLAB could also cause defects in cheese (Gagnon et al. 2020) and could also inhibit growth of SLAB. Studies classify potential NSLAB very differently. Whereas Broadbent et al. (2016) and Crow et al. (2001) reported the potential of NSLAB and their positive effects, Gagnon et al (2020) described NSLAB as spoilage flora. It is either not fully clear or not fully explored in this review which NSLAB that have these specific effects. Another question is if different NSLAB may collaborate with each other to create different effects; to answer this, more research is needed.

3.1 Conclusion

Species of LAB with the potential to act as NSLAB in cheese ripening are of great importance also in fermentation of silage, because of their ability to keep pH low, and enhance preservation. The most usual LAB that can act as NSLAB are *L. plantarum L. casei* and *L. paracasei.* This because of their good adaptation to the surrounding environment in silage, raw milk, and cheese. All LAB that are found in silage are not LAB that could function as NSLAB in cheese, but some are. SLAB can be added to silage, and herbage will also be a source of LAB in ensiling. Some NSLAB have probiotic potential, some can enhance flavour and intensity in cheese. But NSLAB could also cause defects in cheese, e.g., calcium lactate crystals and inhibition of SLAB. It seems that LAB from silage transfer in only small numbers to raw milk, and that pasteurisation eliminates a great deal of LAB in raw milk. However, LAB that may function as NSLAB in cheese have been found in silage. NSLAB in milk can possibly enter a nonculturable state after pasteurisation and could survive. NSLAB could therefore potentially flow from silage to raw milk to cheese. Further research to track NSLAB at the various stages in the dairy value chain is therefore needed.

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