Ensuring the microbial quality of forages and raw milk for profitable production of ripened cheese.
Säkrad mikrobiell kvalitet hos grovfoder och mjölkråvara för lönsam produktion av långlagrad ost.

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Del 1: Utförlig sammanfattning

Kunskap om faktorer som påverkar mjölkens mikroflora, särskilt förekomsten av vissa smakproducerande mjölksyrabakterier, är viktig för framgångsrik produktion av traditionell svensk långlagrad hårdost. Projektets övergripande syftet var att studera mikrofloran i hela värdekedjan; från grovfoder till mjölk, och den resulterande osten, samt att undersöka effekten av specifika bakterier på den slutliga kvaliteten hos en långlagrad hårdost. Mer specifika syften inkluderade att utforska mikrofloran i grovfoder i relation till metod för grovfoderproduktion, samt grovfodrets bidrag till mikrofloran i tankmjölken. Projektet syftade vidare att studera mikrofloran i den resulterande osten under lagring, undersöka om bakterier som härrörde från grovfodret och den obehandlade mjölken kunde återfinnas i osten samt utvärdera hur ostens mikroflora inverkade på ostens mognadsprocess.

Baserat på förväntad mjölkkvalitet utsågs 18 norrländska mjölkgårdar att medverka i studien, och dessa gårdar delades in i tre grupper; A) hög andel mjölksyrabakterier i mjölkens mikroflora, B) lågt antal klostridieliknande bakterier och hög proteinhalt i mjölken och C) mjölk med genomsnittlig bakterieflora och sammansättning. Syftet med detta upplägg var att generera största möjliga variation i den resulterande silomjölken på mejeriet. Mjölken från respektive grupp av gårdar hämtades separat och användes i fullskaliga ystningar på mejeriet vid tre tillfällen under 2017–2018. I anslutning till ystningarna analyserades mikrofloran i grovfoder och tankmjölk från de medverkande gårdarna, samt i den resulterande silomjölken. Information om besättningarna och deras mjölkproduktion samlades in, och rutiner och foderdata på gårdarna dokumenterades. Progressionen i ostarnas mikroflora, aromämnen och fria aminosyror analyserades därefter regelbundet fram till ca 20 månaders lagring.

Projekt har fått finansiering genom:
Stiftelsen Lantbruksforskning

Resultaten visade tydliga skillnader i mikrofloran mellan olika typer av grovfoder, varvid mikrofloran i balar uppvisade en högre diversitet, med en lägre andel *Lactobacillus* och en högre andel av exempelvis *Enterococcus*, *Hafnia-Obesumbacterium, Weisella*, *Leuconostoc*, och *Enterobacteriaceae* än mikrofloran i ensilagen. Mikrofloran i ensilagen från plan- och tornsilos hade en högre andel och dominerades i regel av *Lactobacillus*, samt innehöll mer mjölk-, ättiksyra, och ammoniumkväve än balarna. Även skördetillfälle påverkade grovfodrets mikroflora. Grovfodret som utfodrades i samband med det tredje ystningstillfället, hösten 2018, hade en avvikande mikroflora i jämförelse med grovfodren som användes vid de första ystningarna, som sannolikt härrörde från samma skördesäsong, dvs. sommaren 2017.

Tankmjölkens mikroflora påverkades delvis av grovfodret på gården, men även av besättningens inhysnings- och mjölkningssystem. Besättningar med lösdrift och robotmjölkning hade en annan mikroflora i tankmjölken i jämförelse med mjölken från gårdar med uppbundna kor. Mikrofloran i grovfodret och tankmjölken studerades även på ASV-nivå (amplikon-sekvens-variant) för att identifiera bakteriestammar förekommande i såväl grovfoder som tankmjölk. Gemensamma ASVs inkluderade flera mejeriteknologiskt intressanta mjölksyrabakterier, såsom *Lactococcus, Enterococcus, Pediococcus, Leuconostoc* och *Lactobacillus*, men även produktförstörare som *Enterobacteriaceae* och *Pseudomonas.* De gemensamma bakteriestammarna utgjorde i regel endast en liten andel av mikrofloran, och ASVs som dominerade i grovfodret var inte de samma som de som dominerade i tankmjölken. På mejeriet återspeglade mikrofloran i silomjölken mikrofloran i tankmjölken från de större gårdarna, dvs. de gårdar som bidrog med stora mjölkvolymer i mejerisilon. Resultaten visade en tydlig progression i ostarna under mognadsprocessen, varvid aromämnen och fria aminosyror frigjordes som ett resultat av den mikrobiella och enzymatiska aktiviteten. I motsats till resultaten i tidigare studier med samma typ av ost, utgjorde laktobacillerna endast en liten andel av ostens mikroflora. Den tillsatta starterkulturens mjölksyrabakterier dominerade även i de lagrade ostarna. Det saknades ett tydligt samband mellan å ena sidan ostarnas smakutveckling, utvärderad med en sensorisk panel, och å andra sidan progressionen i mikroflora, aromkomponenter och fria aminosyror i osten.

Sammantaget visade studien en tydlig effekt av metod för foderkonservering på grovfodrets mikroflora. Bakterier i grovfodret, inklusive flera mjölksyrabakterier av teknologiskt intresse i osttillverkning, återfanns även i mjölken och i den resulterande osten. Det kan inte helt säkerställas att detta förklaras av ett direkt flöde av bakterier; samma bakterier skulle t.ex. kunna finnas i miljön och kontaminera mjölken i olika steg. Den relativa förekomsten av mjölksyrabakterier i mjölken var som förväntat låg, men i motsats till tidigare erfarenheter var den relativa förekomsten av smakproducerade *Lactobacillus* mycket låg i den lagrade osten, och ostens mikroflora dominerades istället av mjölksyrabakterier från den tillsatta starterkulturen. Det kan finnas flera orsaker till detta och fortsatta studier är angelägna. Variationen i mikroflora och sammansättning hos mjölken som användes vid de olika ystningarna hade ingen tydlig effekt på ostmognadsprocessen. Resultaten visade inte heller några tydliga samband mellan de studerade variablerna i osten (mikroflora, aromkomponenter, fria aminosyror) och den sensoriska bedömningen av osten. Detta indikerar att mjölken som levereras till ysteriet är av hög kvalitet och att andra variabler än de som ingick i studien bidrar till variationen i ostarnas mognadsprocess.

En slutsats av studien är att bakterier i grovfodret även återfinns i den resulterande mjölken och osten. Denna kunskap skulle eventuellt kunna användas för att via grovfodret öka antalet av de smakproducerande laktobacillerna i långlagrad ost. I ett pågående industridoktorandprojekt med delfinansiering av SLF (R-18-26-1005) studeras effekten av utfodring med olika ensilage på

mikrofloran i den resulterande mjölken och osten. Tre olika typer av ensilage producerades sommaren 2020 (utan tillsats, med tillsats av syra, med tillsats av inokulat), och ensilagen användes i ett utfodringsförsök med 60 kor på Röbäcksdalens försöksbesättning våren 2021. Efter 3 veckors utfodring med respektive ensilage hämtades den resulterande mjölken till mejeriet och ostar tillverkades. I projektet analyseras mikrofloran i grovfoder, mjölk och ost, och förhoppningsvis kommer resultaten att bidra till att vi kan göra starkare slutsatser avseende betydelsen av grovfodrets mikroflora i ostmognadssammanhang. Våra resultat visar även på betydelsen av noggrannhet i produktionen av grovfoder och av god skötsel i besättningen, i synnerhet som besättningarna blir allt större och mjölken från en enda besättning får allt större genomslag i mejerisilon. Variationen i ostmognad förklarades inte enbart av de variabler som studerades i projektet, dvs mikroflora, aromämnen och fria aminosyror i osten. Den sensoriska bedömningen av ostens smak och textur är sannolikt mer komplex än så, och för att fungera i en forskningsstudie som denna skulle den sensoriska utvärderingen behöva vara konstruerad på ett annat sätt. Parallellt med de senaste årens gemensamma projekt utvärderar mejeriet f.n. nya tekniker för att utvärdera ostens mognadsgrad, såsom en hyperspektral kamera. I det längre perspektivet, är resultaten och kunskapen som genererats i våra gemensamma projekt viktiga att integrera i kvalitetssäkringen utmed mjölkens hela värdekedja, från gården till mejeriet och förädlingen av mjölken till olika produkter.

Del 2: Rapporten (max 10 sidor)

Introduction

This project has been unique in the way that it has covered the whole value chain for milk for cheese production with the microbiota in focus; from factors on the farm and the preserved forage, the raw milk in the farm bulk tank and in the dairy silo, to the resulting long-ripened cheese. It has been an integrated project, with participation from dairy industry and their farmers, the advisory organisation Växa Sverige, and different departments and faculties at SLU. Finally, different funding organizations (RJN, Family Kamprad Foundation, SLF) have supported our research between 2015 and 2021.

In the action plan *"Joint initiatives to develop Swedish milk production*", increased export of dairy premium products, such as long ripened cheese, was identified as an important step in developing the Swedish dairy sector (1). To procure a high price for the added values of such products, increasing volumes of suitable raw milk will be required and problems, e.g., the costly variation in ripening time of cheese must be managed. The traditional manufacture of long ripened cheese is a very complex process, in which both microbial and biochemical processes contribute to the characteristic flavour and texture of the cheese (2). The metabolic activity in cheese during ripening is derived from enzymes, e.g., native milk enzymes, added rennet, and enzymes from the added lactic acid bacteria (LAB) starter culture but also from non-starter lactic acid bacteria (NSLAB). NSLAB is believed to stem from the milk and either survived pasteurization or contaminated the pasteurized milk later in the process. It is a heterogeneous group of mesophilic bacteria consisting of *Lactobacillus, Pediococcus, Enterococcus* and *Leuconostoc*. Although NSLAB will not grow well in milk, they may increase about 4-5 orders of magnitude within a few months in the maturing cheese (3, 4). This means that populations present at very low levels in milk will develop and eventually become a significant part of the cheese microbiota with impact on the flavour and texture of the cheese (5). Raw milk contains a diverse and complex microbial population, and its specific composition will have a large impact on the subsequent process and quality of dairy products (6). Many of its bacteria are of technological importance, bringing distinctive flavours and aromas in traditionally manufactured long-ripened cheeses, whereas other bacteria may cause spoilage or disease. The total number of bacteria is the compulsory standard parameter indicating the microbial quality of the raw milk but providing no information about the microflora composition. This is a limitation, since the correlation between total number of bacteria in raw milk and the quality of the resulting product is poor. In contrast, knowledge about factors affecting the milk microbiota, especially the abundance of the flavour producing NSLAB, is important for successful ripening of the traditional Swedish cheese in this project.

Aim of this project

The overall objective of the project was to study the flow of microbiota, from field and farm to milk and the resulting cheese, and to investigate the impact of specific bacteria on the final quality and ripening time of a traditional Swedish long-ripened cheese. More specific objectives were to explore the composition of the forage microflora, its relationship with the production of the forage, and its contribution to the bulk milk microbiota. The project further aimed to explore the microbiota of the long-ripened cheese and investigate if bacteria stemming from the raw milk and the forage could be identified in the cheese. Finally, the project aimed to evaluate the impact of the cheese microbiota on the ripening time, taste, and texture of the cheese.

Material and methods

In the writing of this report, it is difficult to separate activities that were funded by SLF from those funded by RJN and the family Kamprad foundation. The three projects have run closely integrated, one project providing the basis for another. However, all work associated to the quality of forages and the microbiota in forages, milk, and cheese, as well as the aroma components and the free amino acids in cheese, was funded by SLF. Visits on the farms to characterize the production systems and management took place in previous project (RJN). Likewise, detailed data on milk composition and microbiota in bulk milk samples from farms delivering their milk to the cheese making facility were characterized in previous projects (RJN, Kamprad), and some of our results were recently published (7-9). The results from these studies have served as the basis for selection of farms to be included in this SLF study.

Study design

Farm clusters Individual farms were selected to participate in this study, based on recently generated data on detailed milk composition and farm characteristics in projects co-funded by RJN and Kamprad (7-9). To have sufficient volumes of milk for the full-scale cheese making trials, clusters of farms had to be created. Further, to create as large variation in the milk used for our cheese making trials as possible, we aimed for three different clusters of farms, producing milk different in various aspects. Cluster A consisted of farms which delivered milk which could be described as "average composition and microbiota". Cluster B consisted of farms, the majority tie-stall farms, delivering milk with higher fat and protein content, better coagulation properties and less clostridia. Finally, farms in cluster C were selected due to their milk having a higher total number of bacteria and a larger proportion of *Lactobacillus* (Table 1).

Table 1. *Milk selection criteria and basic characteristics associated to the farms constituting the different clusters, A-C. Farms belonging to cluster B, had significantly lower numbers of cows compared to the farms in clusters A and C, and fed significantly higher proportions of silage as bales compared to farms in cluster A.*

Farm cluster and selection criteria	**Number of farms**	**Average number of cows per herd (min-max)[1]**	**Dominating cow breed[2]** (number of farms)	**Milking system** (number of farms)	**Proportion of silage fed as bales[1]**
A - average milk quality, "control"	4	110 [a] (68-180)	SH (3) Mixed (1)	AMS (3) Milking parlour (1)	29 % [b]
B - less clostridia, higher protein content in milk	9	49 [b] (17-90)	SH (1) SRB (2) SKB (1) SJB (1) Mixed (4)	Tie-stall (6) AMS (2) Milking parlour (1)	73 % [a]
C - higher abundance of lactic acid bacteria in milk	5	84 [a] (41-127)	SH (4) SKB (1)	AMS (3) Milking parlour (2)	60 % [ab]

[1]Data with different superscripts differ at P<0.05
[2]SH=Swedish Holstein, SRB=Swedish Red, SKB=Swedish Mountain breed, SJB=Swedish Jersey

Cheese making trials Bulk milk was collected from participating farms on three occasions, November 2017 (trial 1), February-March 2018 (trial 2), and September 2018 (trial 3). On these occasions, bulk milk from the farms belonging to each of the individual clusters was collected separately every second day during the trial week, i.e., on three occasions, and transported to the cheese making facility. On each of these days, milk from the farms belonging to the same cluster was filled into a special dairy silo, processed into a traditional long-ripened cheese, and matured at a dedicated cheese ripening facility. For each of the three trial periods, milk from each farm cluster was used for full scale cheese production on three days, resulting in a total of 3 x 3 x 3 cheese batches.

Sampling of forages, milk, and cheese Representative forage samples were taken in connection to visits on each participating farm close in time to each of the three cheese making trials. Fresh forage samples were used for microbiological analysis (hygienic quality), and additional samples were stored frozen for later characterization of microbiota, fermentation quality and nutritional value. A representative bulk milk sample was taken by the milk truck driver on the participating farms each day the milk was collected for cheese making, i.e., 3 days per trial and farm. Likewise, the dairy silo milk used for each specific cheese batch was sampled. The resulting cheeses were sampled according to a special schedule, from fresh milk gel to cheese of different ages until 20 months. To avoid affecting the conditions for the cheese microbiota, the same cheese wheel was never sampled on more than 3 occasions, and thus, multiple cheeses from the same production batch (silo) had to be sampled during cheese maturation. From the age of 14 months and thereafter bi-monthly, cheeses were evaluated by a dairy sensory panel. For the other parameters/analyses samples were frozen for later characterization.

Characterization of forages, milk, and cheese samples

Microbiota analysis The microbiota, which was the focus of this project, was characterized by DNA sequencing of the bacterial 16S rRNA gene using the Illumina Miseq platform at SciLifeLab (Uppsala, Sweden) and at Macrogen (Seoul, Korea). A previously developed protocol describing bacterial DNA extraction in milk followed by PCR amplification (7) was optimized to allow characterization of the microbiota in forages and cheese in the project. This part of the project, including the bioinformatic process and data evaluation, was conducted at MolSci.

Quality attributes in addition to microbiota

Forage samples were analysed at the Dept of Animal Nutrition and Management, SLU Uppsala (HUV). The hygienic quality was evaluated in terms of yeast, mould, Enterobacteria, clostridia spores, and LAB. Fermentation quality was evaluated in terms of pH, volatile fatty acids, and ammonium-nitrogen, and nutritional value as dry matter, crude protein, neutral detergent fibre, energy, and water-soluble sugars.

Farm bulk and dairy silo milk samples were analysed for fat, protein, lactose, urea, free fatty acids, somatic cell count, total number and number of thermoresistant bacteria, at Eurofins. Milk coagulation properties, detailed milk protein profile (capillary electrophoresis), fatty acid composition (GC-MS), plasmin/ plasminogen activity, total proteolysis, and casein micelle size were analysed at MolSci. Fat globule size was determined at KTH and minerals at AgriLab.

Cheese samples were analysed for chemical composition (NIR), and a basic sensory evaluation was performed by a dairy sensory panel, both at Norrmejerier. Aroma component profile was analysed at University of Copenhagen (GC-MS-MS) and free amino acid profile (ion-exchange chromatography) at SGS Synlab.

Statistical methods

The large volumes of data generated in the project have required different multivariate statistical tools for evaluation, e.g., Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA, Bray Curtis and Generalised Unifrac distance matrixes) but univariate analysis has also been used to describe data and identify differences.

Results

Optimizing protocols for DNA sequencing of the bacterial 16S rRNA gene in forages and cheese

DNA extraction protocols for forages and cheese, respectively, were successfully adapted from our previous publication describing the extraction of bacteria DNA in milk (7). For DNA extraction of forages, three different extraction kits were compared for their DNA yield and PCR inhibitor removal efficiency, i.e., NucleoSpin® Soil (MACHEREY-NAGEL), FastDNA™ SPIN Kit for Soil (MP Biomedicals) and ZymoBIOMICS™ DNA Microprep Kit (Zymo Research). Based on results obtained in quantitative PCR, the NucleoSpin® Soil extraction kit performed better than the other two kits and was subsequently used in the study. For cheese, samples were processed by a stomacher homogenizer before DNA extraction, using PowerFood DNA isolation kit (Qiagen AB). Fat was included in the DNA extraction for better DNA yield and for inclusion of amplicon sequence variants (ASVs) associated to the fat, in agreement with the protocol for milk (10).

Exploring the forage microbiota and its associations with factors in the production and management of forage

The overall variation in forage microbiota indicated a clear effect of trial, i.e., the forage microbiota associated to trial 3 was different compared to the forage microbiota associated to trials 1 and 2. PCA also suggested an effect of farm, i.e., that microbiota in silages from the same farm was associated. This was, however, only true for trials 1 and 2, and explained by the fact the forages sampled during the first two trials were both produced in summer 2017. Silage samples often even originated from the same harvest occasion. In contrast, silages sampled in connection to trial 3 were in most cases produced in summer 2018, thus representing a new harvest year.

Type of forage had a clear effect on the microbiota, i.e., the microbiota associated to bales was very different from that in silages from tower or bunker silos (Figure 1).

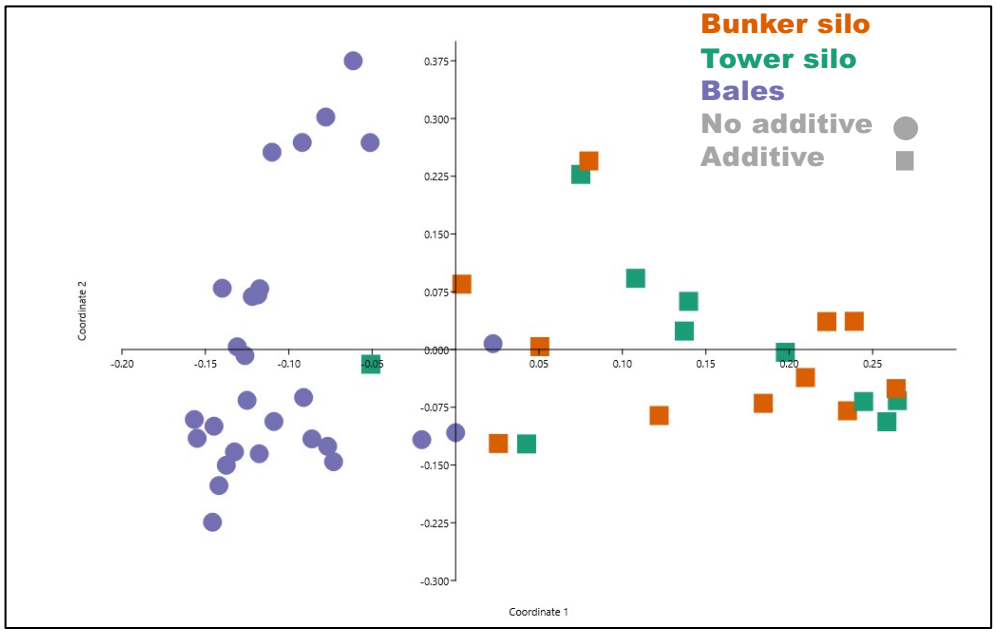

***Figure 1**. PCoA (Bray-Curtis) illustrating the total variation in forage microbiota, color representing type of forage, and shape illustrating use of additive used to facilitate ensiling. There was a clear effect of forage type on microbiota, bales having a microbiota different from that in forages produced in bunker and tower silos. However, this effect could not be distinguished from the effect of the use of additives in ensiling, since silages in bunker and tower silos were always produced by use of an additive, and bales always without an additive. The additives used in the study were acid or salt, never inoculant.*

Differences between the forages in terms of ensiling process, and thus pH, dry matter, volatile fatty acids (VFA) and sugar content, were as expected, and explained the effect of forage type on microbiota. This effect could, however, not be distinguished from the effect of additives, since the silages in bunker and tower silos in this study were always produced by use of chemical additives (acids and salts, no inoculants) in this study. PCA was used to evaluate associations between forage microbiota data (16S seq data) and the traditional forage quality parameters. In general, results agreed with expectations. Bunker and tower silages had a microbiota which was dominated by and associated to a higher relative abundance (RA) of *Lactobacillus*, more lactic and acetic acid, more ammonium-nitrogen, whereas bales had a more diverse microbiota, with lower RA of *Lactobacillus*, and higher RA of e.g., *Enterococcus*, *Hafnia-Obesumbacterium, Weisella*, *Leuconostoc*, and *Enterobacteriaceae*.

Exploring the microbiota composition in farm bulk milk. Is there a flow of bacteria from forage to milk?

PCoA (Bray-Curtis) showed an effect of farm cluster on bulk milk microbiota but no effect of trial. There was no clear effect of forage type on bulk milk microbiota, although there was a trend that milk from farms with forage in bunker silos had a different microbiota (Figure 2, to the left). The difference in bulk milk microbiota between farms with tie-stall vs. robotic milking (AMS) observed in our recent study (7) was confirmed also in this study (Figure 2, to the right). Since all forage produced in bunker silos originated from AMS farms, these two factors were confounded, and it was thus not possible to distinguish between the effect of milking system and forage type in this study.

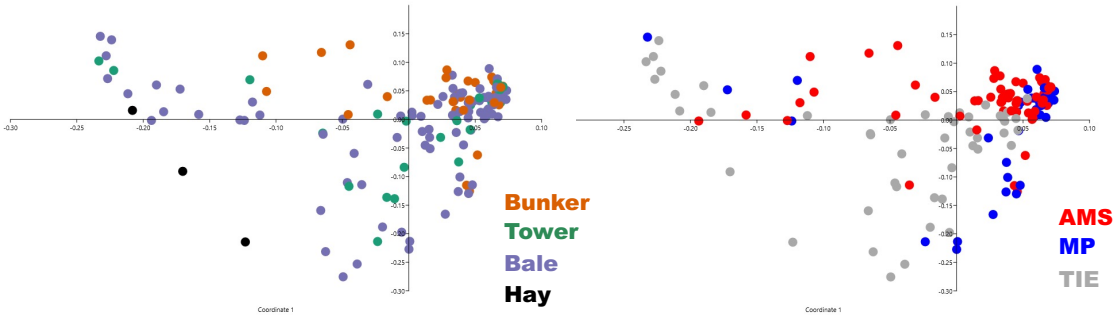

Figure 2. *Total variation in bulk milk microbiota, color representing type of forage (to the left) and milking system (to the right) on the farms, respectively. The PCoA plots suggest that forage type and milking system on farm were confounded factors, and it was not possible to distinguish their respective effect on the bulk milk microbiota.*

The association between forage and bulk milk microbiota was further investigated using a dataset on ASV level including both forage and bulk milk data. First, data was filtered to remove ASVs that were unique to either bulk milk or forage samples. Thereafter, a total data set of 50 pairs were created, i.e., forage and bulk milk samples collected from the same farm in the same trial period. ASVs found in both forages and milk included several LAB of potential technological importance, e.g. *Lactococcus, Enterococcus, Pediococcus, Leuconostoc* and *Lactobacillus*, but also spoilage bacteria e.g. *Enterobacteriaceae* and *Pseudomonas,* as well as different gut-associated bacteria. The RA of ASVs that were found in both forage and bulk milk samples was generally very low, i.e., the ASVs that were dominant in forage were not the same as the ASVs that were dominant in bulk milk.

Exploring the microbiota in dairy silo milk. Did we succeed in establishing great variation in the microbiota?

Evaluation by PCoA (Bray Curtis) suggested an effect of cluster but not of trial on dairy silo milk microbiota. Compared to the clear effect of cluster on bulk milk microbiota, a less clear effect of cluster on dairy silo milk microbiota could be explained by the dilution effect, i.e., mixing milk from different farms into one silo. The microbiota of the dairy silo milk is thus largely determined by the microbiota in bulk milk from farms contributing to a major part of a dairy silo. This can be illustrated by cluster A, where the dominance of *Pseudomonas* in the bulk milk from one of the smaller farms, was reflected in just a minor RA of *Pseudomonas* in the dairy silo. In contrast, *Staphylococcus,* which was dominant in bulk milk from another farm, was also dominant in the resulting dairy silo milk, due to a much larger volume of milk from that farm. In this context, it is important to also remember, that the results only take the RA of different bacteria into account, not bacteria numbers. Bulk milk from the farm with 76% RA of *Staphylococcus* had a very low total bacteria count and thus, the bulk milk was still of high quality.

Exploring the microbiota in cheese

The microbiota in cheese was very much dominated by lactic acid bacteria from the starter culture, i.e. *Lactococcus lactis* and *Leuconostoc*. Our results showed no effect of farm cluster on the microbiota of the resulting cheese, but a clear effect of trial (Figure 3). Whereas the microbiota in cheeses associated to trials 1 and 2 was more similar, the cheese microbiota associated to trial 3 was different, corresponding to the results for the forage microbiota. This difference was associated to a lower RA of different *Lactobacillus* and higher RA of some

Streptococcus in cheese from trial 3. Although it is possible that the change in forage could have contributed to the effect of trial, also other factors may have contributed, e.g., installation of new fermentation tanks for the starter culture at the dairy close in time to trial 3. However, it is important not to overinterpret these results, considering that the model in Figure 3 only explained 16% of the total variation along principal component 2, i.e., the y-axis.

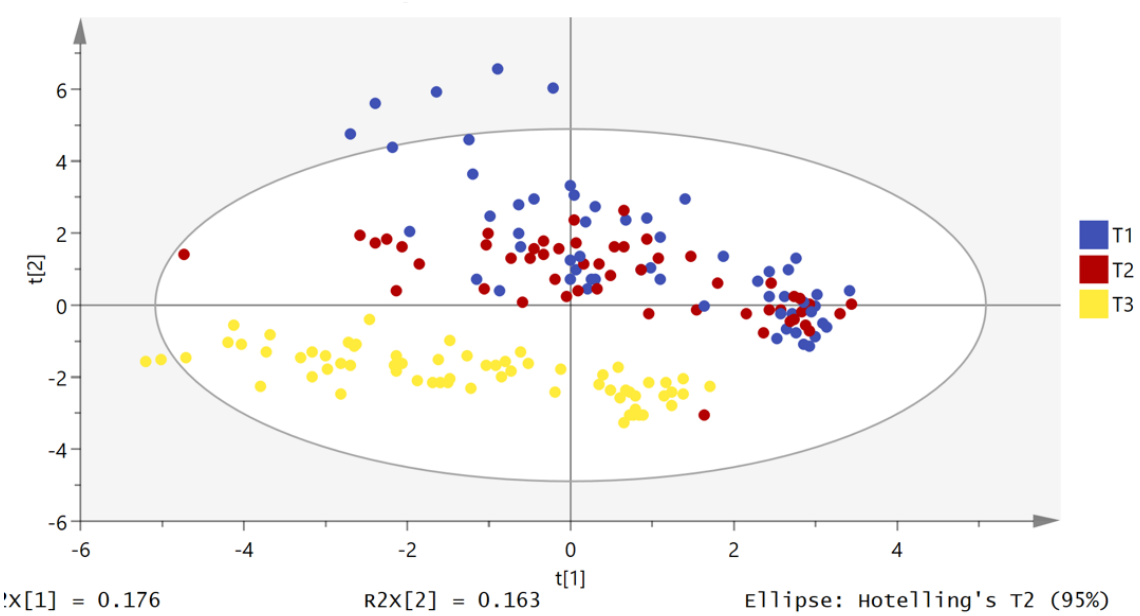

Figure 3. *PCA plot illustrating the total variation in cheese microbiota, color representing trial period. Cheese resulting from trial period 3 clearly had a different microbiota compared to cheese resulting from trial period 1 and 2.*

Comparing the cheese microbiota at different ages of the cheese, a progress in the microbiota with maturation of the cheese was quite clear for trials 1 and 2, but less so for trial 3. In general, the young cheeses (7 months) showed less variation in microbiota between batches, but with increasing age, the variation between the cheese batches increased. In addition to the effects of trial and ageing of the cheese, there was thus a strong batch effect on the cheese microbiota.
To identify bacteria existing both in silo milk and cheese, a dataset on ASV level including both dairy silo milk and the resulting cheese was used. In total, 64 ASVs were identified as present in at least one dairy silo and one corresponding cheese sample. In the case of ASVs with a RA <1%, many of them were batch specific, i.e., only associated to a certain batch of cheese. From the 64 ASVs, 22 had an average RA higher than 0.1%, and 8 ASVs higher than 0.5%. These 8 ASVs, identified in both dairy silo milk and the resulting cheese, consisted of *Leuconostoc* (2 ASVs), *Lactobacillus* (2 ASVs), *Lactococcus* (2 ASVs), *Staphylococcus* (1 ASV) and *Streptococcus* (1 ASV).

Exploring relationships between microbiota in cheese, development of aroma component and free amino acid profiles and age at approval of the cheese

Considering that there seemed to be a progress in the cheese microbiota with ripening (at least for trials 1 and 2), it was surprising that there was no association between cheese microbiota and age at approval of the cheese. The aroma component profile in cheese at different ages indicated a clear effect of ripening and a clear effect of trial, but no effect of cluster on aroma component

profile. Despite the effect of ripening on aroma component profile, there was no clear association between aroma component profile and age at approval of the cheese (Figure 4).

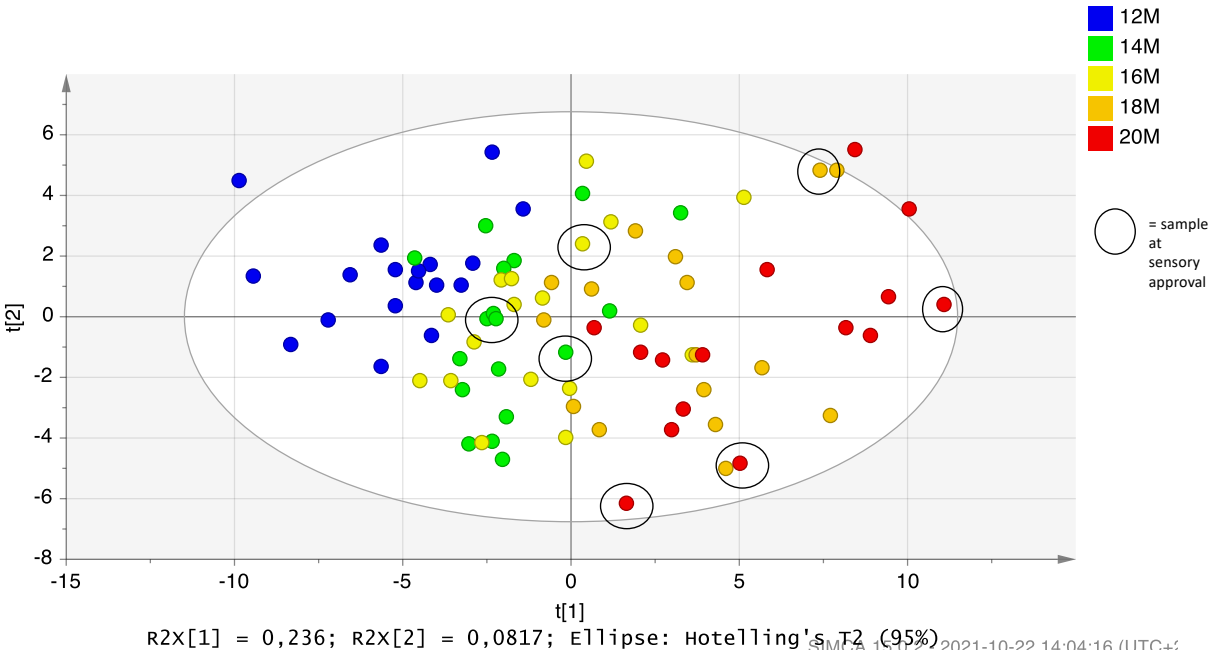

Figure 4. *PCA plot illustrating the total variation in aroma component profile of cheese of various age, each dot representing one cheese sample and color indicating the age of the cheese at analysis. The circle indicates when a cheese was approved.*

On recommendation of our reference group, free amino acid (FAA) profiles of the cheeses were determined, since free amino acids may also contribute to the flavor of the matured cheese. As for the aroma component profile, there was a clear progress in the development of FAA in the cheese during maturation. However, despite the effect of maturation on the FAA profile, there was no association between FAA profile and age at approval of the cheese. Combining data sets related to microbiota and FAA, partial least square analysis showed a positive association between *Lactobacillus* (1 ASV) and FAA levels, and a negative association between *Lactococcus* (2 ASVs) and *Acinetobacter* (1 ASV) and FAA levels. This may reflect the cheese ripening progress, in which *Lactococcus* is typically more active during the earlier stages of maturation, whereas *Lactobacillus* plays a major role in the later stages of maturation. When combining data sets for all three parameters (microbiota, aroma component and FAA profiles), a clear progress during maturation was observed but there was still no association with age at approval of the cheese.

Discussion

This study showed a clear impact of ensiling method on forage microbiota. The microbiota of bales was characterized by a higher microbial diversity, more non-lactic acid bacteria, and a lower relative abundance of *Lactobacillus,* clearly different from that of bunker and tower silages. Forage microbiota was affected by trial, most likely explained by differences in year and time of harvest. Several farms used the same cut in trials 1 and 2, explaining why forage

microbiota associated to trials 1 and 2 showed higher similarity compared to trial 3. The extreme weather conditions (drougth) during summer 2018 (trial 3) could also have contributed to a different forage microbiota associated to trial 3. There was no clear effect of forage type on bulk tank milk microbiota, but a trend that the microbiota in milk from farms with silage from bunker silos was different compared to the microbiota in milk from farms using bales or silage produced in tower silos. Since all forage stored in bunker silos was associated to AMS farms, we could not distinguish between the effect of milking system and forage type. A clear effect of milking system on bulk milk microbiota was reported in our recently published study (7), and it is likely that milking system influenced the results also in this study. Finally, we observed an effect of farm cluster on bulk milk microbiota but no effect of trial, despite the clear effect of trial on forage microbiota. ASVs found in both forages and bulk milk included several LAB of potential technological importance, e.g. *Lactococcus, Enterococcus, Pediococcus, Leuconos*toc and *Lactobacillus*, but also spoilage bacteria e.g. *Enterobacteriaceae* and *Pseudomonas.*

In general, there seemed to be an effect of both cluster and trial on the dairy silo milk microbiota. However, the microbiota of dairy silo milk was largely determined by the bulk milk microbiota associated to farms contributing to the major proportion of milk in the silo. This illustrates the importance of good hygiene on farms in general, and in particular on larger farms. We observed no effect of cluster on the microbiota of the resulting cheese, but a clear effect of trial. There was a progression in the cheese microbiota from month 7, with an increasing variation between batches during maturation, i.e., seemingly a strong batch effect on cheese microbiota. ASVs found in both dairy silo milk and cheese included several LAB with potential technological importance, e.g. *Lactobacillus, Leuconostoc* and *Lactococcus*, but also other bacteria, e.g. *Staphylococcus* and *Streptococcus.* There was a clear progression in both aroma component and the free amino acid profiles in cheese during ripening, but no clear association between these profiles and age at approval of the cheese. Combining data sets related to microbiota and free amino acid profile, a positive association was observed between *Lactobacillus* and free amino acid profile, and a negative association between *Lactococcus* and *Acinetobacter* and free amino acid profile. A few ASVs were observed in forage, bulk tank milk on farm, in the dairy silo and the resulting cheese. These included *Lactococcus*, *Enterococcus*, and *Leuconostoc*, but also also *Romboutsia* and *Turicibacter*, with unknown effects on the cheese. In contrast to previous studies on this traditional cheese, there was a very low abundance of *Lactobacillus* and a high abundance of lactic acid bacteria stemming from the starter culture in the maturing cheese. There are several possible reasons for these findings, including 1) method related aspects (is sequencing of DNA an inappropriate method, i.e., could DNA fragments of already dead starter culture bacteria in high numbers interfere with our results?) 2) starter culture related aspects (has work to develop the commercial starter cultures resulted in more robust and active LAB starters, with higher survival rates and later autolysis during cheese ripening, out-conquering NSLAB, e.g. the lactobacilli?) 3) raw milk microbiota related aspects (has work to improve the hygienic quality of the raw milk, reducing the total bacteria counts in bulk milk, also reduced the abundance of lactobacilli in raw milk, to numbers which are too low to “do their work”?) and 4) dairy process related aspects (have changes in early process parameters, and a stricter hygiene also at the dairy plant, affected the survival of lactobacilli, reducing their already low numbers in the cheese?). These questions are complex and interactions between the suggested factors likely exist.

Conclusions

Our results showed a clear impact of ensiling method on forage microbiota. Bacteria observed in the forage were also detected in milk and the resulting cheese, including LAB with a potential

role in cheese ripening. We can, however, not conclude whether the bacteria in the cheese have their origin in the forage, i.e., that there is a direct flow of the bacteria, since many of the bacteria are commonly occurring in both farm and dairy environments, and thus may have contaminated the milk and cheese by other ways than via the forage. The relative abundance of LAB in milk was low, in agreement with expectations. However, in contrast to earlier studies, the relative abundance of *Lactobacillus* in the cheese during ripening was very low and there was an unexpected dominance of starter LAB, e.g., *Lactococcus*, also at later stages of cheese ripening, where dominance of *Lactobacillus* was expected. Further studies are needed to find the reasons for this. In contrast to our expectations, we observed no associations between the measured variables in cheese and age at approval of the cheese. The results also showed that despite the variation in milk delivered from the participating farms, milk composition and milk microbiota showed very few associations with the sensory evaluation and ripening time of the resulting cheese. This suggests that the milk delivered for cheese production is of high quality, and that factors other than those covered by this study will contribute to the variation in the ripening time of the cheese.

Benefit for the industry and recommendations

One conclusion of this study is that bacteria observed in forages also exist in the resulting milk and cheese. The question which arises is, if knowledge generated in the project can be used to increase numbers of the desirable, flavor producing NSLAB in traditional Swedish long ripened cheese. Inspired by this SLF project, an industrial PhD project, co-funded by SLF (R-18-26-1005), and better designed to evaluate the effect of forage microbiota on the resulting cheese, was initiated in 2018. We are currently evaluating a feeding trial conducted at the dairy research farm in Röbäcksdalen (SLU). Three different types of silages were produced summer 2020, fed to 60 dairy cows spring 2021, and cheeses were produced by Norrmejerier from the milk resulting after feeding the different silages. The microbiota in forages, milk and the resulting cheese will be evaluated, and hopefully we will be able to draw stronger conclusions regarding the link between forage microbiota and cheese maturation.
The sensory evaluation for final approvement of the cheese is complex, clearly also taking aspects other than the variables investigated in this project into account. It appears, that the variables we have studied (microbiota, aroma component and free amino acid profiles in the cheese), are not the only decisive variables for the outcome of the sensory evaluation. To fit in our research study, a different approach for sensory evaluation would have been required. However, partly because of this project, additional work has been initiated by the cheese producer to find other measures and tools to control the stage of maturation of the cheese, e.g., spectral analyses (11, 12). Changes in the protocol of the sensory panel may also be considered in the future. Finally, work is needed to evaluate the effect of methods in future characterization of microbiota in cheese ripening, e.g., DNA vs RNA based techniques. There may be a risk, that even if the DNA of the starter culture bacteria will be degraded during cheese ripening, DNA fragments that exist may be sequenced and give a misleading picture of the composition of the microbial community in the cheese, i.e., underestimating the abundance of the flavour producing bacteria.

Although this study did not show a direct link between bacteria in forage and cheese quality, it draws attention to the importance of good management in silage production and management on the farm, especially as the dairy farms become larger. In the longer perspective, results and knowledge generated in this project, will be important to integrate in quality management along

the whole dairy value chain, from the dairy farms through the dairy facilities producing the valuable end products

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Del 3: Resultatförmedling

Projektet har genomförts med lönemedel från SLF för en doktorand (3 år) samt lönemedel från NJ-fakulteten vid SLU för en 2-årig post-doktor. En snabb och lyckosam rekrytering av en mikrobiolog till anställningen som post-doktor (Li Sun) medförde att SLF-projektet kunde komma i gång drygt ett halvt år innan doktoranden var på plats. Arbetet med att optimera metoderna för karaktärisering av mikrobiotan i grovfoder, mjölk och ost, samt utvärderingen av sekvenseringsdata med avancerade multivariata statistiska metoder, dvs. det arbete som ligger till grund för denna rapport, har därför även fortsättningsvis genomförts av Li Sun med lönemedel från SLU, SLF och Kamprad. Med SLFs godkännande har SLFs lönemedel till doktorandtjänsten bidragit till en doktorsavhandling som fokuserar på hur olika gårdsnära faktorer påverkar mjölkråvarans sammansättning och egenskaper samt den resulterande långlagrade osten (Hasitha Priyashantha, 2021). Sammanställningen nedan tar därför även upp publiceringar och kommunikation som inte enbart beskriver resultaten från denna rapport, men där projektet kommunicerats som en del i ett större sammanhang och där arbetet är helt eller delvis finansierat av SLF.

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