Slutrapport

Influence of forages on development of desirable and undesirable flavours in milk and dairy products

Grovfodrets betydelse för uppkomst av önskvärda och oönskade smaker i mjölk och mejeriprodukter

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Del 1: Utförlig sammanfattning

I projektet har vi studerat faktorer associerade med produktionen av grovfoder och grovfodrets roll för utvecklingen av önskvärda och oönskade smaker i mjölk och ost. Grovfodret utgör en viktig del av kornas diet, och dess hygieniska kvalitet har stor betydelse för mjölkens och mejeriprodukternas kvalitet. Vallensilage bestående av en blandning av gräs och klöver är idag det vanligaste grovfodret i mellersta Sverige och norröver. Vid ensilering fermenterar mjölksyrabakterier (LAB) vallväxternas kolhydrater till mjölksyra och andra organiska syror. Ensileringen sker spontant, men ibland används tillsatsmedel, främst organiska syror eller starterkulturer av utvalda LAB, för att gynna ensileringen. Dålig hygienisk grovfoderkvalitet har i tidigare studier kopplats till mjölk med "blåbärssmak", ett smakfel med allvarliga ekonomiska konsekvenser för drabbade mjölkproducenter, främst i Mellansverige och Västerbotten. Grovfodret har även föreslagits vara en bidragande faktor i den okontrollerade variationen i mognadstid för långlagrad ost. Den s.k. medföljarfloran, i första hand laktobaciller, är viktig för den karakteristiska smaken hos långlagrad ost och kontaminerar sannolikt mjölkråvaran på gården eller i mejeriet. Eftersom laktobaciller även förknippas med vallväxterna, var hypotesen att botanisk sammansättning och ensileringsmetod bidrar till variation i förekomsten av laktobaciller i mjölkråvaran och därmed också i ostmognadsprocessen.

Projekt har fått finansiering genom:

Stiftelsen Lantbruksforskning

Blåbärssmak i mjölkråvaran. Totalt 25 mjölkproducenter som fått anmärkning för smakfelet intervjuades i projektet avseende ett stort antal produktionsfaktorer. Av de drabbade gårdarna levererade 18 gårdar sin mjölk till Norrmejerier, 3 gårdar till Arla Foods och 4 gårdar till Falköpings mejeri. De flesta smakfelen rapporterades under stallperioden, och de flesta gårdarna drabbades vid minst 2 tillfällen, vissa gårdar vid 4 tillfällen eller fler. Storleken på drabbade gårdar varierade (13–300 kor), gårdar hade olika raser (vanligen fler än en på samma gård), det fanns hög- och lågavkastande gårdar, uppbundna kor eller i lösdsrift, och djurhälsan på gårdarna var generellt god. Variationen i insamlad data var stor och det var inte möjligt att påvisa samband med smakfelet. Med hjälp av avancerade metoder (GC-olfaktometri, GC-MS och GC-FID) analyserades oika lättflyktiga ämnen i mjölkprover med och utan blåbärssmak (RISE, Göteborg). Resultaten visade att i de flesta fall förekom estern etylbutanoat, i vissa fall även etylkaproat, i högre koncentrationer i mjölk med blåbärssmak jämfört med ett referensprov från samma gård. Resultaten för en andra uppsättning insamlade mjölkprover uppvisade inte en lika tydlig trend, möjligen för att smakfelet ofta rapporterades som svagt, och skillnaden i lukt mellan blåbärsmjölk och referensprov var otydlig. Foderprover från tre av de drabbade gårdarna skickades för hygienisk analys, och de högsta värdena för såväl jäst som mögel överskred rekommenderade gränser. Foderproverna skickades även till RISE för analys av de flyktiga ämnen som analyserats i mjölkproverna. De flesta av proverna innehöll inte bara höga koncentrationer av estrarna i mjölken utan även andra estrar. De högsta värdena av etylbutanoat kom från ett ensilage som utfodrats då gården i fråga hade problem med blåbärssmak. Estrar i ensilage bildas av alkoholer, främst etanol, och korta organiska syror, t.ex. smörsyra. Enzymer som katalyserar esterbildning återfinns hos jäst, mögel och mjölksyrabakterier i ensilaget. En hypotes, baserad på resultaten i projektet, är att blåbärssmaken i mjölk orsakas av höga halter av estrar i ensilaget. Estrarna absorberas, tas upp i blodet och övergår till mjölken. Svårigheten med att bevisa hypotesen beror på att estrarna sannolikt bildas lokalt i mindre partier i en fodersilo. Detta stöds av att problemet i regel försvinner när drabbade lantbrukare byter foderparti. Studierna fortsätter under 2024 genom utfodring av kor i Röbäcksdalen med ett mindre foderparti från en mjölkproducent som drabbats av smakfelet. Foder- och mjölkprover från utfodringsförsöket kommer att analyseras som tidigare, därutöver analyseras ensilaget för etanol, jäst, mögel och mikrobiota.

Flödet av mjölksyrabakterier från vallväxter och grovfoder till mjölkråvara och ost.
I en inledande studie användes SLU:s långliggande fältförsök för att studera mikrobiotan i gräs- och klövervallar. Växtmaterial skördades två gånger per säsong i Lönnstorp, Lanna, Säby och Röbäcksdalen åren 2018 och 2019. Den botaniska sammansättningen utvärderades, den kemiska sammansättningen analyserades, antalet mjölksyrabakterier bestämdes och DNA extraherades för analys av mikrobiota. Tre typer av experimentellt ensilage (utan tillsats, med tillsats av syra, med tillsats av starterkultur) framställdes i glasburkar och representativa prover togs ut för DNA-extraktion, bestämning av antal mjölksyrabakterier, kemisk sammansättning och fermenteringsprodukter. Extraherat DNA sekvenserades av SciLife Lab, Stockholm (Illumina Miseq Plattform). Den botaniska sammansättningen av växtmaterialet varierade signifikant mellan skördetillfälle och plats men tydliga samband mellan växternas mikrobiota, plats, år eller skördetid kunde inte påvisas. Förekomsten av LAB i växtmaterialet var mycket låg, <2% relativ förekomst (RA). En intressant observation var anrikningen över tid av *Xanthomonas* i Röbäcksdalen, möjligen förklarat av *Xanthomonas* förmåga att övervintra i fleråriga växter. Obehandlade ensilage uppvisade slumpmässiga ensileringsresultatet, medan de inokulerade ensilagen dominerades helt av LAB. Obehandlat ensilage visade en högre diversitet av LAB, medan LAB i det syrabehandlade ensilaget huvudsakligen bestod av släktet *Lactobacillus*. I vilken utsträckning den botaniska sammansättningen påverkade ensilagens mikrobiota är oklart.

Samma ensileringsmetoder som i studien med de experimentella ensilagen användes 2021 vid framställning av ensilage i plansilos inför en utfodringsstudie i Röbäcksdalen. Borrkärnprover från varje plansilo togs efter cirka tre månader och analyserades för kemisk sammansättning och hygienisk kvalitet. Ensilagen användes i ett 12 veckors utfodringsexperiment i foderblandningar tillsammans kraftfoder, rapsmjöl och mineraler. Varje ensilage användes i 3 veckor, utfodring med det inokulerade ensilaget upprepades även en andra 3-veckorsperiod. Korna (totalt 67) hölls i en lösdrift, båsen rengjordes manuellt med skrapa dagligen och täcktes med träspån. Mjölkningen skedde två gånger dagligen i mjölkgrop, och utrustningen rengjordes noggrant efter varje mjölkning. Provtagning av ensilage, foderblandning, använt strömaterial, och tankmjölk utfördes den sista veckan i varje 3-veckorsperiod. Fodrets kemiska sammansättning och hygieniska kvalitet analyserades, odling av bakterier utfördes, och bakteriellt DNA extraherades. På motsvarande sätt analyserades mjölkens sammansättning och DNA extraherades från mjölkens bakterier. Resultaten visade att växtmaterialets mikrobiota inte liknande mikrobiotan i något annat material, medan mikrobiotan i ensilage och foderblandning var överlappande. Mikrobiotan i mjölk uppvisade störst likheter med mikrobiotan i det använda strömaterialet. Högst totalantal bakterier återfanns i det använda strömaterialet (9,6 $\log_{10}$ cfu/g) och lägst i tankmjölenk (3,5 $\log_{10}$ cfu/g); det högsta antalet laktobaciller återfanns i ensilage och foderblandning (7,1 och 7,5 $\log_{10}$ cfu/g). Mikrobiotan i ensilaget återspeglades i motsvarande foderblandning, och de mest förekommande bakterierna i foderblandningen återfanns i det använda strömaterialet, men nästan inte alls tankmjölk. Ensilagens mikrobiota utgjordes huvudsakligen av de tre släktena *Lactobacillus, Prevotella* och *Pseudomonas*, men till vår förvåning påvisades inga signifikanta skillnader mellan de olika ensilagebehandlingarna. En närmare granskning av varianter av amplikonsekvenser, s.k. ASV, visade dock att de tre släktena bestod av många olika arter med varierande RA. *Lactobacillus fructivorans* var huvudsakligen associerad med syrabehandlat ensilage och en annan intressant observation i ensilage utgjordes av *Prevotella*, ett av de dominerande släktena i vommen. Mjölkens mikrobiota uppvisade högst diversitet, omfattande totalt 122 släkten, varvid *Lactobacillus* utgjorde det vanligaste släktet (RA 10,7 %) följt av *Pseudomonas* (RA 5,9 %). En filtrering av alla ASV som tillhör ordningen Lactobacillales resulterade i totalt 716 LAB i foderblandning, strömaterial och mjölk. Av de som förekom i mjölk (437) förekom endast 22 ASV vid en RA >0,1 %, och dessa ASV fanns i princip bara i mjölk. Sammanfattningsvis uteblev den förväntade effekten av de olika behandlingarna på ensilagets mikrobiota, och överföringen av bakterier från ensilage och foderblandning till den obehandlade mjölken var nära obefintlig. *Lactobacillus* var vanlig i både foder och mjölk, i de flesta fallen rörde det sig dock om olika genetiska varianter.

Mjölken från utfodringsförsöket hämtades i Röbäcksdalen sista veckan i varje 3-veckorsperiod och användes i tillverkningen av en långlagrad ost i Burträsk (Norrmejerier). Den inkommande mjölken standardiserades, pastöriserades och pumpades till ystningskaret. En mesofil startkultur tillsattes och ystmjölken fick förmogna i 90 min innan löpe, $CaCl_2$ och $NaNO_3$ tillsattes. Ostmassan fylldes i formar, pressades och ostarna saltades med saltlake, paraffinerades och lagrades. Mjölkprov från tankbilen togs i balanstanken vid ankomst till mejeriet och ett prov av den pastöriserade mjölken togs från ystningskaret. Prov togs efter förmognad och på färska ostkorn efter vassledränering. Den färska osten provtogs 24 timmar efter pressning och osten under lagring provtogs regelbundet fram till 22 månaders ålder. Mjölk- och ostprover analyserades för kemisk sammansättning och antal bakterier, DNA preparerades och sekvenserades. Sekvenseringsdata från de insamlade proverna grupperade sig i obehandlad mjölk, starterkultur, prover från ystningen (fermenterad mjölk, ostkorn, färskost) och lagrad ost (månad 4-22). Mjölkens mikrobiota skilde sig signifikant från mikrobiotan i övriga grupper, medan mikrobiotan i ystningsprover och lagrad ost delvis överlappade med starterkulturens

mikrobiota. Mjölkens mikrobiota bestod av ett stort antal släkten, varav de 20 mest förekommande förklarade ca. 75 % av RA. Överraskande observerades mer variation inom period mellan ystningsbatcherna än mellan perioderna då de olika ensilagen utfodrades. Mjölkråvarans diversa mikrobiota förändrades dramatiskt under osttillverkningen, då den kom att domineras av *Lactococcus*, följt av *Leuconostoc* och *Lactobacillus*. Sammanfattningsvis dominerade samma ASV som dominerande i proverna från tidig ystningsprocess, dvs. *Lactococcus* och *Leuconostoc*, även i den mogna osten och förekomsten av det aromproducerande släktet *Lactobacillus* var låg i ostens.

Hur sammanfattar man kort dessa studier? Arbetet med att finna orsaken till den s.k. blåbärsmjölken har i och med projektet tagit ett stort kliv framåt. Förhöjda nivåer av estrar har påvisats i mjölkprover med tydlig blåbärssmak och höga nivåer av samma estrar har återfunnits i foderpartier i samband med smakfelet. En hypotes som vuxit fram, är att jäst och mögel, men även mjölksyrabakterier, vid förhöjda nivåer av etanol i ensilaget producerar estrar som absorberas, tas upp i blodet och övergår i mjölken. I studierna av laktobacillernas flöde från vallväxter och ensilage till mjölkråvara och ost, stöds inte den ursprungliga hypotesen att de smakproducerade laktobacillerna härrör från grovfodret av projektets resultat. Förekomsten av laktobaciller i ensilagen var mycket hög, men sekvenseringsdata visar att dessa utgörs av andra genetiska varianter än de laktobaciller som är viktiga för ostmognaden. Sannolikt har laktobaciller anpassat sig till miljön på mejeriet och skapat en ekologisk nisch med andra egenskaper än laktobacillerna som finns i grovfodret. Frågan återstår nu varför antalet laktobaciller i osten är så lågt, och varför ostens mikrobiota domineras av de LAB som härrör från starterkulturen. En hypotes är att dagens kommersiella starterkulturer är mer robusta och motståndskraftiga än de tidigare, vilket gör det svårt för laktobacillerna att tillväxa under ostmognaden. Forskning pågår för att undersöka hur *Lacticaseibacillus paracasei's* transkriptionsprofil påverkas av värmestress, varvid värmebehandlingar som används i mejeriprocessen simuleras.

Del 2: Rapporten (max 10 sidor)

Background

The sensory attributes of the raw milk are of major importance for the quality of the dairy products. The “blueberry off-flavour” was initially reported across middle Sweden and Västerbotten in the 1990’ies. Since then, multiple studies were carried out to identify possible causes behind the blueberry taint; a phenomenon that cause severe economic consequences for the affected dairy farm (1). In previous investigations, poor hygienic quality of forages seems to be associated to most cases. Forage quality has also been suggested to contribute to the uncontrolled variation in ripening of a traditional Swedish cheese. Non-starter lactic acid bacteria, i.e., certain lactobacilli, play a vital role in the formation of the characteristic aroma components of the cheese. Since lactobacilli are also associated to herbage and forage, we hypothesized that factors e.g., composition of herbage and ensiling method, could influence on numbers of lactobacilli in the raw milk and thereby also affect cheese ripening.

This project has covered the whole value chain for milk, and successful collaboration between dairy industry, and different departments and faculties at SLU has been a prerequisite for the project. While the SLF grant did not cover costs for salaries, the project has been relying on additional funding. In addition to SLF, the Family Kamprad Foundation and RJN have supported our research associated to the blueberry off-flavour. To elucidate the origin of the lactobacilli responsible for the characteristic aroma of a traditional Swedish long-ripened cheese, the microbiota of leys and influence of geographical location and harvest occasion were explored. The effect of ensiling method on the microbiota was investigated in a laboratory study, and later, using the same methods, silages were produced at Röbäcksdalen and fed to dairy cows in a feeding experiment. The microbiota in leys, silage, feed mix, bedding material, and bulk tank milk were characterized. To investigate potential effects from feeding cows with the different silages on cheese production, the milk resulting from the feeding experiment was used in production of long-ripened cheese. This part of the SLF-project was performed as an industrial PhD project with grants from Livsmedelsstrategin (LivsID), SLU and Norrmejerier. Thomas Eliasson, industrial PhD student at Norrmejerier, will defend his thesis September 20.

Objective of the project

The overall objective of our research was to generate knowledge that can be used in integrated quality management, from forage quality to raw milk quality and the quality of the resulting dairy products. In this project, we have investigated factors associated with forage production and the role of forage in the development of desirable and undesirable flavours in milk and cheese.

Material and methods

Origin of the blueberry off-flavour

In total 25 farmers were interviewed about their milk production after being affected with the blueberry off-flavour (herd size, cow breed, milk yield, number of lactations, housing and milking system, conditions around calving, reproduction, use of advisory service in calculating diets, type and quality of forages, concentrate, hygiene in general, water to cows, routines associated to milking, cleaning of equipment etc, etc). The receiving dairies provided analytical data on milk from the affected herds, to investigate if milk with the taste defect differed from non-affected milk. Analytical data associated to forage quality on affected herds were provided

by Växa. One of the major challenges with the project was to find a laboratory that could identify the substance(s) associated to the off-flavour. RISE (Göteborg) was contacted, and using GC-olfactometry, GC-MS and GC-FID, volatile substances differing in concentration between affected milk and a reference sample collected close in time on the same farm were identified. The same method with a slightly modified protocol was also used to analyse a limited number of forage samples from affected farms.

The flow of lactic acid bacteria from herbage and forage to milk and cheese

Microbiota of Swedish grass and clover leys and the effect of silage additives on forage microbiota. A multi-site long-term field experiment managed by SLU was used to study the microbiota of Swedish grass and clover leys. Herbage samples were collected in Lönnstorp, Lanna, Säby, and Röbäcksdalen in 2018 and again in 2019, leys harvested two times each season (2). The botanical composition was evaluated, chemical composition and numbers of lactic acid bacteria were determined. To characterize the epiphytic microbiota, herbage samples were immediately frozen in liquid nitrogen. From the herbages, three types of silages were prepared in lab-scale silos; without additive, with addition of acid, and inoculated with a starter culture. Treated herbage was packed in autoclaved glass jars, sealed, and stored for 100 days at 20°C. Opening the jars, representative samples were taken for DNA extraction, viable counts of lactic acid bacteria, chemical composition and fermentation products analysed (Valio Oy). To prepare DNA, frozen herbage and silage samples were thawed. DNA in bacterial cells was extracted using NucleoSpin Soil Kit, and the DNA was used to construct a 16S rRNA library (3) which was sequenced at SciLife Lab, Stockholm, using Illumina Miseq Plattform. The bioinformatic process and statistical analyses is described in (2).

Effect of feeding different types of silages on microbiota of raw milk. Silages were produced and stored in bunker silos at Röbäcksdalen (SLU, Umeå) in June-July 2020, using the same treatments as in the lab-scale study. Fresh herbage samples were used for estimation of viable bacteria and epiphytic microbiota. Drill core samples from bunker silos were taken after approximately three months and chemical composition and hygienic quality were analysed. The silages were used in a 12 week's feeding experiment performed January-April 2021. Each silage was used in a partial mixed ration (PMR) with concentrate, rapeseed meal and minerals, and fed to the cows (n=67). Each silage was used for 3 weeks; the inoculated silage was fed during a second 3-week period to evaluate if a potential change in milk microbiota could be repeated. Cows were kept loose housed in an insulated barn, cubicles were cleaned with a scraper each day and covered with wood shavings. Milking took place twice daily in a milking parlor, and milking equipment and milking parlor were thoroughly cleaned after each milking. The milk was collected to the cheese producing facility every second day during the last week of each 3-week period. Sampling of silage, PMR, used bedding material, concentrate, rapeseed meal and wood shavings took place during the last week of each 3-week period. Chemical composition and hygienic quality were analysed (Eurofins). Culturing of bacteria in the samples was performed directly after sampling, lactobacilli were evaluated on MRS agar, total bacteria on modified milk plate count agar. DNA extraction was performed as described in (2).
Milk samples for microbiota analysis were taken from the bulk tank upon milk collection, samples were immediately frozen and stored at -80°C until DNA extraction. Milk composition was analyzed using mid-infrared spectroscopy and SCC with fluorescence-based cell counting (Eurofins Milk Testing). Number of viable bacteria in the milk was estimated using plate count agar (Norrmejerier). Microbial DNA in thawed milk samples was extracted using the PowerFood DNA isolation kit (Qiagen AB) following Sun et al. (4). All bacterial DNA was sent to Novogene (Cambridge) for library construction and sequencing using Illumina Nova Seq platform. Details related to the bioinformatic data processing can be found in (5). Alpha- and

Beta-diversity metrics were estimated, and Principal Coordinate Analysis (PCoA) performed. Faith's Phylogenetic Diversity Index was used to compare diversity, while the weighted UniFrac distance matrix and PCoA results were used to compare microbiota composition between and within materials (5).

The effect of feeding dairy cows different forages on ripening time and flavour development of the resulting cheese. Milk from the feeding experiment was used to produce a traditional long-ripened cheese (Burträsk, Norrmejerier). Cheese production took place during January-April 2021, while cheese ripening proceeded until February 2023. Raw milk was standardised, pasteurized, and pumped to the cheese vat. A mesophilic starter culture was added (Chr. Hansen) and the cheese milk was allowed to pre-ripen during 90 min. Liquid rennet (Caglificio Cerici) was added together with $CaCl_2$ and $NaNO_3$. Cheese making comprised long cooking periods at temperatures above 40°C. The resulting cheese curd was filled into moulds, pressed, and cheeses were brine-salted, paraffined and ripened in a dedicated cheese-ripening facility. A total of 12 batches of cheese were produced. Raw milk was sampled from the tanker truck upon arrival to the cheese making facility and from the balance tank, pasteurized milk was sampled from the cheese vat. Fermented milk was sampled after pre-ripening of the milk, and cheese grains were collected after whey drainage. Fresh cheese was sampled 24 h after pressing before transfer to the salt brine and cheese was sampled at intervals until the age of 22 months by use of a sterile cheese drill. Milk and cheese samples were analysed for chemical composition and bacterial numbers in the dairy laboratory. Chemical composition of the milk (MilkoScan) and cheese (FoodScan) was evaluated, milk and cheese samples for microbiota analysis were frozen at -80°C. Numbers of aerobic bacteria, thermoresistant bacteria, psychrotrophic bacteria, and Enterobacteriaceae in milk were estimated by culturing. In cheese, numbers of aerobic bacteria and lactobacilli were estimated. Milk and cheese samples were prepared for microbiota analysis using the PowerFood DNA isolation kit (Qiagen) according to (4). Extracted bacterial DNA was stored at -80°C until submitted for amplification and sequencing at Novogene (Cambridge). The bioinformatic process, data handling and statistical evaluation followed the same procedure as for data generated in the feeding experiment. Sensory evaluation of the cheese was performed by a panel at the dairy, free amino acid profile was analysed using NMR (SLU, Uppsala).

Results and discussions

Origin of the blueberry off-flavour of milk. The affected farms delivered milk to Norrmejerier (Norrbotten 4, Västerbotten 5, Jämtland 5 and Västernorrland 4), Arla foods (Gävleborg 2, Kronoberg 1), and Falköpings mejeri (Västra Götaland 4). Most of the taste defects occurred during the in-door period, and most farms were affected on at least 2 occasions, some farms on 4 occasions or more. The size of affected farms varied (13-300 cows), farms had different breeds (usually more than one on the same farm), there were high- and low-yielding farms, tied or loose cows, animal health on farms was in general good and when it comes to feeding, the variation between affected farms was large. It was not possible to find associations between collected data and the occurrence of the off-flavor. Results from the analysis of pairs of samples from the same farm, i.e., affected and reference milk, showed that in most cases, the ester ethyl butanoate, in some cases also ethyl caproate, were present at higher concentrations in samples with blueberry taint compared to the reference (Table 1). There was no correlation between off-flavour and hexanal and pentanal, both markers of fat oxidation. Another set of samples collected from affected farms during the indoor period 2022/23 was analysed. The trend was not as clear with these samples, however, the off-flavor was often reported as weak and the difference in odor between tainted and reference sample was often unclear. Forage samples were in some cases taken for the project, but in most cases, results from analysis of batches fed

in connection to the off-flavor already existed. Most parameters associated to chemical composition, fermentation products and minerals showed large variation. There were feedlots that in various ways may have contributed to a reduced nutritional supply for the cows, but there were also feed batches without any remark.

Table 1. *Quantification (ng/l) of substances based on GC-olfactometry. The upper value in each pair of milk samples refers to sample with blueberry flavor, the lower value refers to reference sample without flavor defects. Values are means of duplicate analyses*.

Farm	Sampling date	Ethyl-butanoate	Ethyl-2-methyl butanoate	Ethyl-caproate	Hexanal	Acetone	2-Butanone	Pentanal
O	Feb. 2021	140.3	0.04	20.3	7.1	486	3 445	11.5
	Maj 2021	6.7	0.01	0.5	12.0	2 839	402	21.5
H	April+June 2021	3.7	0.00	0.3	24.2	231	152	5.1
	May + July 2021	3.0	0.00	0.2	31.7	376	1 361	4.7
I	May 2021	1.7	0.02	0.0	76.3	436	92	7.7
	May 2021	1.5	0.00	0.0	94.1	330	74	7.3
J	July 2021	14.6	0.02	1.6	60.2	569	1 037	13.6
	Sept. 2021	2.3	0.00	0.0	42.2	281	496	7.1
G	Oct. 2021	22.1	0.14	1.0	16.7	755	4 043	6.7
	Nov. 2021	7.9	0.09	1.0	21.7	686	376	8.4
P	Nov. 2021	8.2	0.06	0.1	23.4	695	28 346	5.5
	Dec. 2021	3.5	0.01	0.0	20.0	360	2 282	5.7
F	Jan. 2022	171.4	0.15	29.3	50.4	424	702	10.2
	Feb. 2022	8.0	0.13	0.2	54.1	492	3 412	6.8

Eight forage samples from three affected farms were sent for hygienic analysis. The highest values for yeast and mold were above the recommended limits of 4.5 log cfu/g and 6.0 log cfu/g sample, respectively. Forage samples (n=14) were also sent to RISE for analysis of the volatile substances analysed in the milk samples. Most of the samples contained not only the relevant esters in high concentration, but also many other esters that may cause odors, while contents of acetone, 2-butanone and pentanal were below detection limits. The highest values of ethyl butanoate came from a silage fed during a period when the farm in question had problems with blueberry flavour.

Early studies (6) report that methyl sulphide, lower boiling aldehydes, ketones, alcohols, and esters comprised most of the volatiles from grass and corn silages. Krizsan et al. (7) investigated the effect of esters and other volatile compounds in grass silage on voluntary intake by growing cattle. The volatile compounds were mainly present in poorly fermented silages, however, none of compounds affected the voluntary intake. Bergamaschi and Bittante (8) studied the effect of dairy system and characteristics of individual cows on volatiles of model cheeses. The most important source of variation in volatiles of model cheeses was dairy system. Milk from farms using total mixed rations had higher contents of alcohols and esters compared with those using separate feeds. Esters in silage are formed from alcohols, mainly ethanol, and short organic acids, e.g., propionic and butyric acid. Esterases catalysing their formation are known to exist among yeast, mold and bacteria, e.g., lactic acid bacteria. Excess production of ethanol was

suggested to be responsible for the formation of large amounts of ethyl butyrate and ethyl hexanoate in Cheddar cheese with a fruity off-flavour (9).
This project is still on-going at SLU, Umeå, but our conclusion so far is that the blueberry off-flavour in milk is associated to formation of high levels of esters in the silage. The esters are absorbed in blood from which they pass to milk. The challenge is that ester production can take place locally in smaller lots in a feed silo making it difficult to detect, and this is supported by the fact that when affected farmers are asked to change batch of forage, the problem disappears.

The flow of lactic acid bacteria from herbage and forage to milk and cheese
Microbiota of Swedish grass and clover leys and the effect of silage additives on forage microbiota. The botanical composition of herbages collected on different harvesting occasions and locations varied significantly, but we found no clear association between site, year or harvesting time, and herbage microbiota (Figure 1).

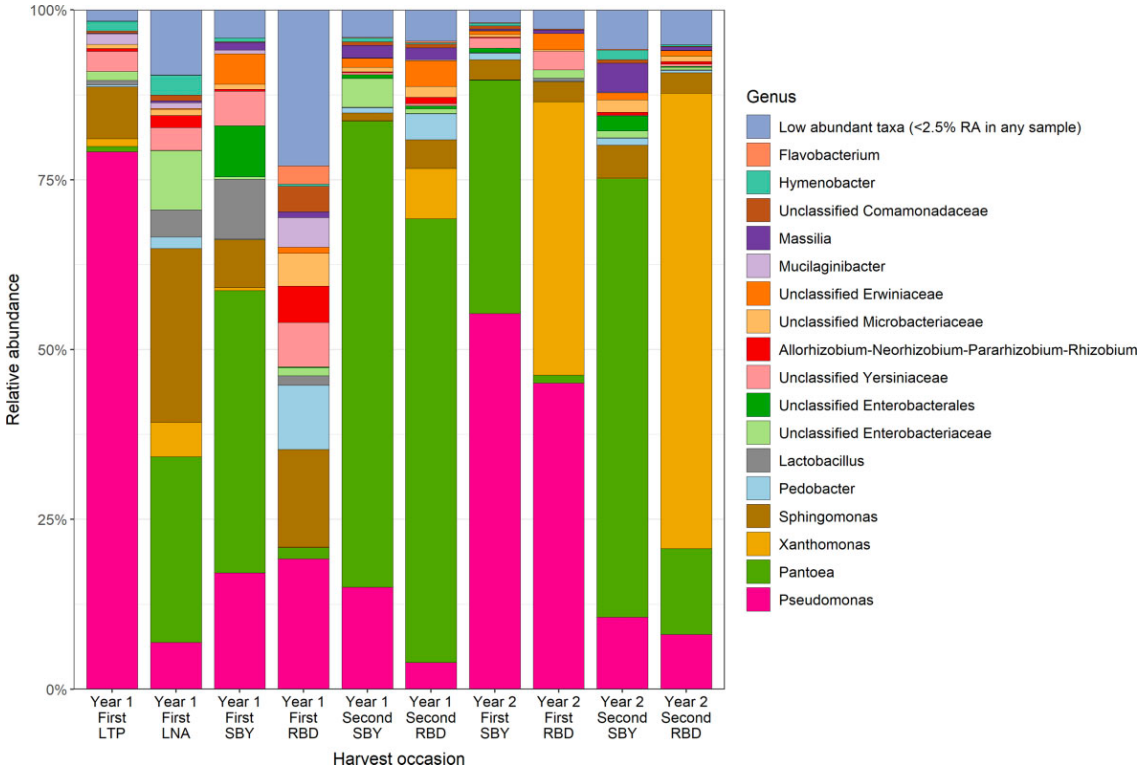

Figure 1. *Relative abundance of bacteria (genus level) in herbages prior to ensiling on each harvesting occasion. LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.*

Lack of effect of site on herbage microbiota agrees with Gaube et al. (10), who found the variation between parts of the plants larger than between different geographical regions. The first year of this study, i.e., 2018, the summer was unusually warm and dry, especially in the south. As an example, second harvest in Säby consisted of only 35% grass, the rest was legumes and weed. LAB were found at very low abundance in herbage, i.e., <2% relative abundance (RA). Low numbers of viable LAB in herbage were also reported in the past for timothy and meadow fescue (11). One interesting observation was the build-up of *Xanthomonas* in RBD over time, possibly explained by its ability to overwinter in perennial hosts, e.g., timothy. In agreement with recent studies on Nordic forage crops, our results confirmed that the most random fermentation outcome was associated to the untreated silages. Inoculated silages were completely dominated by LAB, whereas untreated and acid treated silages showed major

variation (Figure 2). Untreated silages showed a higher diversity of LAB, while LAB in the acid-treated silage mainly consisted of the genus *Lactobacillus*, possibly because its ability to resists a low pH.

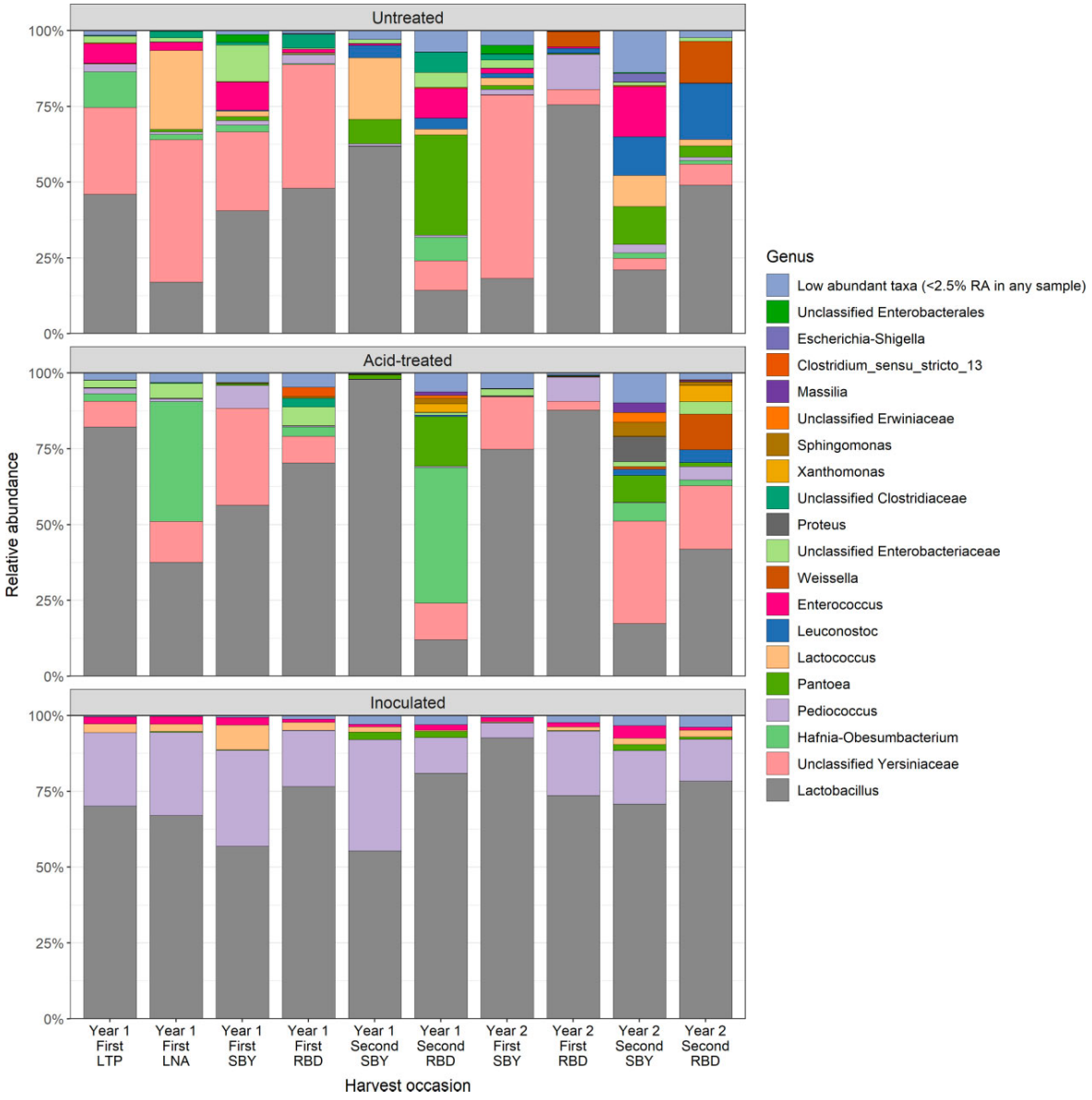

Figure 2. *Relative abundance of bacteria in the different silages; untreated, acid-treated and inoculated with starter culture. LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.*

The rather consistent proportions of bacteria in the inoculated silages, with *Enterococcus* almost disappearing after ensiling, deviated strongly from the proportions of the bacteria in the starter culture. Possible explanations include higher pH tolerance of *Pediococcus,* but also bacteriocin production inhibiting *Enterococcus*. For the inoculated silages, the fermentation parameters were mostly consistent with preferable values, while for the untreated and acid-treated silages, some bacterial genera drive the fermentation process in a more positive, other genera in a more negative direction (2). The extent to which herbage composition affected silage microbiota was

unclear, but a higher content of legumes and unwanted plants resulted in a more diverse LAB community in untreated and acid-treated silages.

Effect of feeding different types of silages on microbiota of raw milk. Surprisingly, there were only minor differences in the microbiota between the different silages produced. The silage microbiota was reflected in the microbiota of the corresponding PMR, and the major bacteria in PMR were also found in the used bedding material, while barely in milk. The alfa-diversity was significantly higher in milk than in the other materials, and milk microbiota was mostly related to that of the used bedding material. However, abundant bacteria in milk were often not found in the other materials. Highest numbers of total bacteria in the different sample types were found in the used bedding material (9.6 $\log_{10}$ cfu/g), the lowest in milk (3.5 $\log_{10}$ cfu/g), while the highest numbers of lactobacilli were found in silage and PMR (7.1 and 7.5 $\log_{10}$ cfu/g). Acid treated silage showed lower numbers of total bacteria than the inoculated silage, while numbers of lactobacilli in the untreated silage was higher compared to the other silages. The microbiota varied largely between the materials, the PCoA showing separation between three clusters of materials, 1; herbage, 2; silage and PMR, and 3; used bedding material and milk (Figure 3). The results showed that the microbiota of the used bedding material was closest to that of the milk.

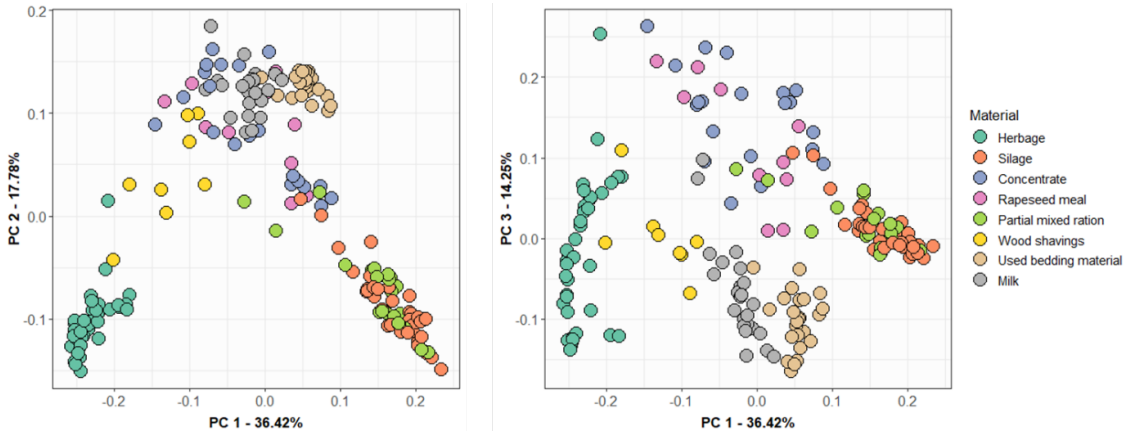

Figure 3. *Principal coordinate analysis of the weighted UniFrac distance matrix of the microbiota of the different materials. The figure includes sample replicates and shows the first three components*.

The top 30 bacterial genera in all sampled materials (herbage excluded) are presented in Figure 4 by silage treatment. The silage microbiota mainly comprised the three genera *Lactobacillus*, *Prevotella* and *Pseudomonas*, however, despite major variation in these genera, pairwise comparisons revealed no significant differences between the silage treatments. However, scrutiny at amplicon sequence variant (ASV) level showed that the major silage genera comprised many different species present at varying RA. In agreement with our first study, *Lactobacillus fructivorans* was mainly associated with ACID. Very few silage studies have reported *Lactobacillus fructivorans* in silages, possibly because it does not grow well on MRS (12). Another interesting observation was the finding of *Prevotella* in the silages, one of the dominant genera in the rumen. Contamination from the environment is a likely explanation, strengthened by the results in Krizsan et al., (13), reporting 34% RA of *Prevotella* in rumen samples collected 2 months before our study in the same dairy farm. Milk was the most diverse of all materials, comprising a total of 122 genera. While Ouamba et al. (14) estimated bacterial transfer at ASV level between feed and milk to be 18-31%, it was surprising that *Prevotella* and other core ASVs in PMR were barely detectable in the milk in our study.

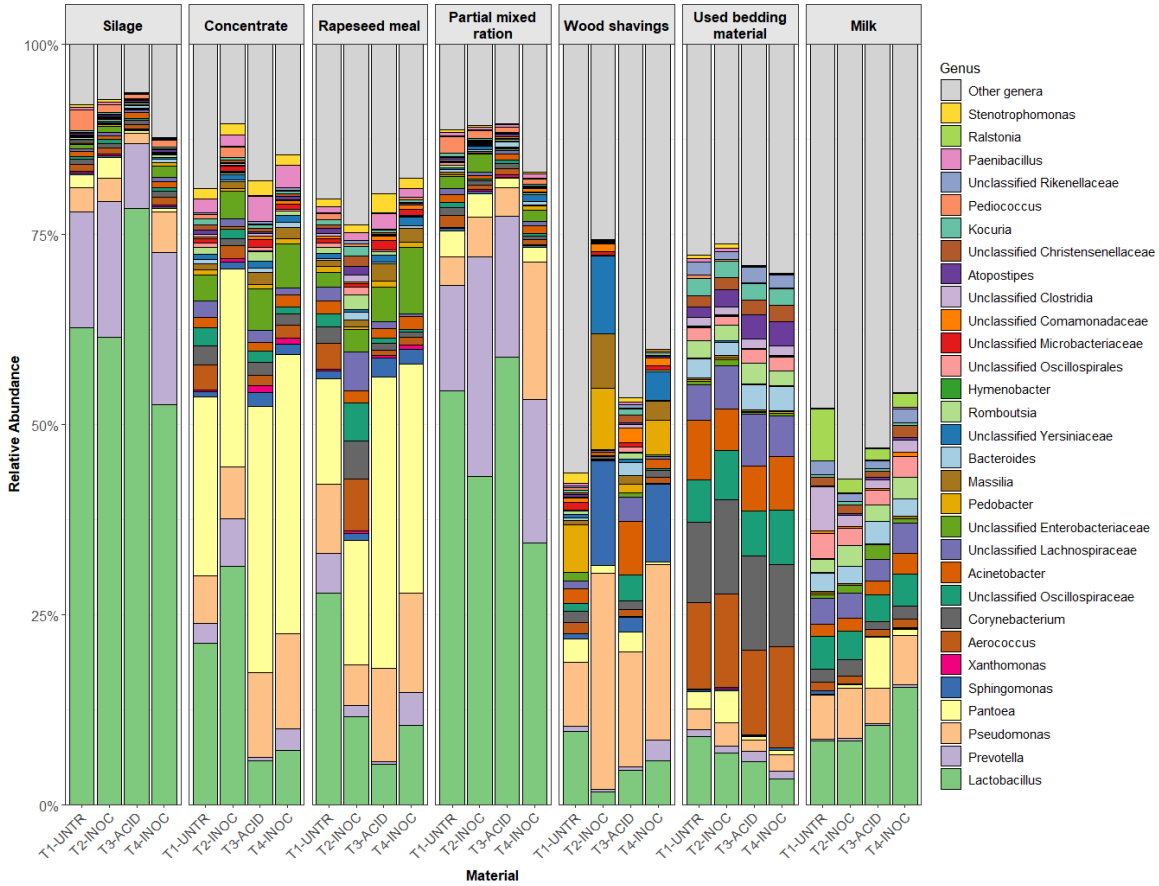

Figure 4. *Relative abundance of top 30 bacterial genera in all materials sampled the last week of each treatment during the feeding trial. Untreated silage (T1-UNTR), inoculated silage (T2-INOC), acid-treated silage (T3-ACID), and a repeat of inoculated silage (T4-INOC).*

In milk, *Lactobacillus* was the most abundant genera with RA of 10.7%, with no significant difference between silages, followed by *Pseudomonas* at RA of 5.9%. Filtering out ASVs belonging to the order *Lactobacillales*, a total of 716 LABs were found in PMR, bedding material and milk. Of those in milk (437), only 22 ASVs were present at RA >0.1%, and these ASVs were in principle only found in milk. In conclusion, we did not observe the expected of the different treatments effect on silage microbiota, and there was very limited transfer of bacteria from silage and PMR to the raw milk. *Lactobacillus* was a major genus in both feed and milk, however, investigations at ASV level showed that in most cases it was not the same ASV. The different materials harboured quite different microbiota, the milk microbiota being most diverse and showing highest resemblance with that of the used bedding material.

The effect of feeding dairy cows different forages on ripening time and flavour development of the resulting cheese. The objective of this study was to evaluate if milk from the previously described feeding trial would affect the quality and ripening of the resulting cheese. Sequencing data associated to the collected samples were categorized into groups, i.e., raw milk, starter culture, cheese making (fermented milk, cheese grains, fresh cheese), and cheese (month 4-22).

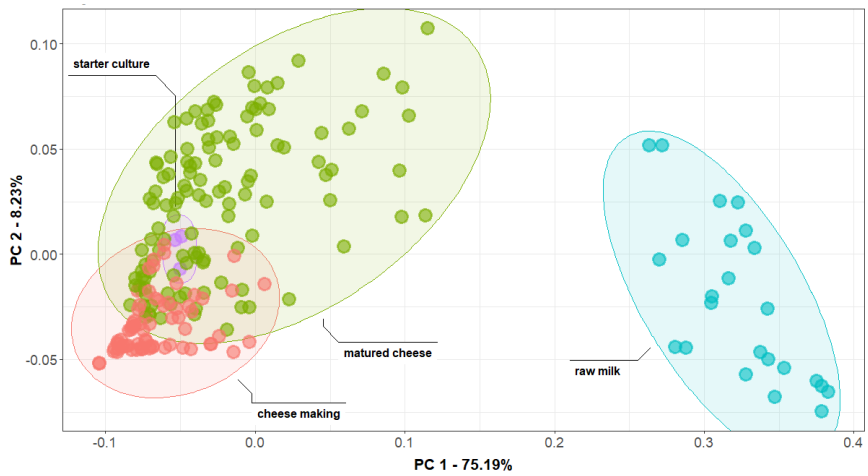

Figure 6. *Principal Component Analysis (PCoA) of the weighted UniFrac distance matrix for grouped sample types.*

PCoA of the weighted UniFrac distance matrix showed that the raw milk microbiota was different from that of the other three groups, while cheese making, and matured cheese partly overlapped with the starter culture (Figure 6). The microbiota is summarized with the average top 20 genera across all sample types in Figure 7. Raw milk microbiota was most diverse, and explained by many different genera, the top 20 explaining approx. 75 % of the relative abundance. Surprisingly, more variation was observed between the batches than between periods (data not shown). The microbiota of raw milk samples changed dramatically in cheese making samples, *Lactococcus* contributing to most of the RA, followed by *Leuconostoc* and *Lactobacillus*.

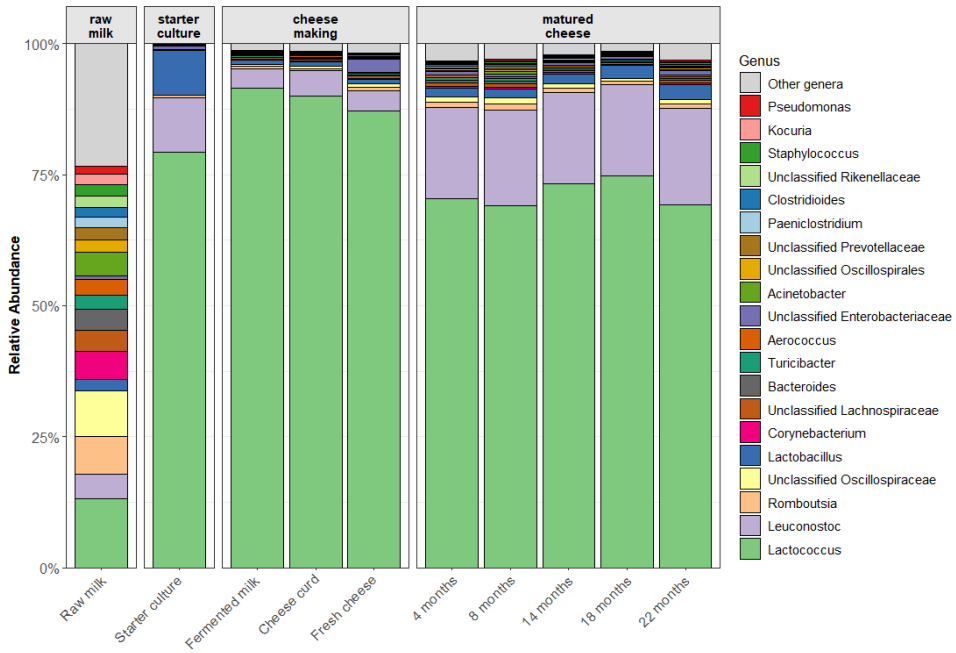

Figure 7. *Top 20 genera in grouped samples, with individual sample types plotted.*

In conclusion, ASVs dominant in early process samples, i.e., *Lactococcus* and *Leuconostoc,* were also dominant in the mature cheese. In agreement with our previous study (Sun et al.,

2023), the starter lactic acid bacteria were dominant also at the later stages of cheese ripening and the abundance of the aroma-producing genus *Lactobacillus* was low in the matured cheese. Evaluation of data is still on-going, and a manuscript is under preparation.

Benefit for the industry and recommendations

For many years, attempts were made to find reasons for the sporadically occurring blueberry off-flavor. One problem has been that there is little preparedness when the problem is reported. Forage and milk samples must be collected with short notice when a dairy farm is affected, and farmers interviewed. Voluntary participation from dairy farmers, dairy cooperatives Arla Foods, Falköpings mejeri, Norrmejerier, and the advisory organisation Växa Sverige is highly acknowledged in this project. A huge amount of data related to the affected farms has been collected in the project, showing that the problem seems to affect virtually any farmer. Most cases are, however, located to northern Sweden, where grass silages are most common. With the identification of the esters in milk samples with a clear off-flavour as well as in some of the silages, the project has generated a clear hypothesis that can be further evaluated. In a pilot trial which took place spring 2024, 10 cows were fed silage from one of the affected farms. Milk and samples are currently being analysed. Interestingly, some of the cows produced milk with the blueberry off-flavour during the trial (Hetta, personal communication). Studies associated to the off-flavour continue with funding from the Family Kamprad foundation and RJN until 2026.

Our results showed that the botanical composition of herbages vary with site, harvesting occasion, and year but without a clear association to microbiota. There was very low abundance of LAB in herbage, and it was not clear to which extent herbage composition affected silage microbiota. The effect of ensiling method on silage microbiota was clear in the laboratory trial but very limited in the bunker silages produced in Röbäcksdalen. In both cases, however, spontaneously fermented silages showed the most unpredictable results, whereas both acid treatment and inoculation with a starter culture resulted in more predictable silages, with a rather consistent microbiota. Interestingly, there was very limited transfer of bacteria from silage and PMR to the raw milk. Even if *Lactobacillus* was a major genus in both silage, PMR and milk, investigations at ASV level showed that in most cases it was not the same genetic variant. The different materials harboured quite different microbiota, the milk microbiota being most diverse and showing highest resemblance with that of the used bedding material. It is therefore not likely that the flavour-producing lactobacilli stem from the farm or the silage. The reason behind the dominance of starter lactic acid bacteria at the later stages of cheese ripening is most likely not be explained by the raw milk microbiota but seems to be associated to elements in the dairy process. Research is continuing to investigate the transcription profile of of *Lacticaseibacillus paracasei* under heat stress conditions simulating those in the dairy process.

Please note: while the limitations of the report (10 pages) made it impossible to include and discuss all findings, complete results from our different studies are available from the authors.

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Del 3: Resultatförmedling

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