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From farm to cheese

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From farm to cheese – exploring the bacteria in the dairy value chain

Abstract

This thesis investigated potential flows of bacteria from the field through silage and raw milk to the mature cheese, focusing on lactic acid bacteria (LAB) of importance for flavour development in a Swedish long-ripened hard cheese.

First, the herbage microbiota was compared to that of corresponding silages made with three different silage additives in laboratory-scale silos. Silages inoculated with starter culture were found to be dominated by the genera *Lactobacillus* and *Pediococcus*, irrespective of harvest location (four sites in Sweden), harvest occasion (first or second cut) or year. The other silage additives resulted in silages with fewer LAB and a more mixed microbiota.

Next, silages were made in concrete bunker silos, using the same silage additives as in the laboratory study. The resulting silage microbiota was found to be dominated by the genera *Lactobacillus* and *Prevotella*, irrespective of silage additive used. Finally, the silages were fed as partial mixed rations to 67 Swedish Red dairy cows for three weeks per silage, with one of the silages repeated. Analyses revealed that the raw milk microbiota had the highest bacterial diversity but the lowest bacterial load, with *Lactobacillus* and *Pseudomonas* being the most abundant genera.

An effect of silage additive treatment was evident to a minor extent in the used bedding material, but not in the raw milk. In cheese made from the raw milk, the microbiota mainly resembled that of the starter culture used in production. An effect of silage additives was not observed in the microbiota of the cheese produced, with the main variation arising between batches of cheeses.

Overall, the microbiota of the cheese was affected by factors not analysed in this thesis or factors at the dairy, with the starter culture used in the cheese making process acting as the main source of bacteria in the final cheese. However, a limitation of the study was the use of short-read sequencing, the resolution was not sufficient to distinguish bacteria fully.

Keywords: Microbiota, lactic acid bacteria, herbage, silage, milk, cheese.

Från gård till ost – en utvärdering av bakterierna i mjölkens värdekedja

Sammanfattning

Denna avhandling undersökte det potentiella flödet av bakterier från åkern via ensilaget och mjölken till den lagrade osten, med fokus på de mjölksyrabakterier som är viktiga för smakutvecklingen i en Svensk långtidslagrad hårdost.

Först jämfördes mikrofloran i växtmaterial och den motsvarande i ensilage, tillverkat med tre olika ensileringsmedel i laboratorieskala med glassilos. Ensilaget som inokulerades med starterkultur var dominerat av släktena *Lactobacillus* och *Pediococcus*, oberoende av skördeplats (fyra olika i Sverige), skördetillfälle (första eller andra) eller år. Användning av de andra ensileringsmedlen resulterade i ensilage med färre mjölksyrabakterier och en mer varierande mikroflora.

Därefter tillverkades ensilage i plansilos med samma ensileringsmedel som i den första studien. Mikrofloran i de resulterande ensilagen dominerades av släktena *Lactobacillus* och *Prevotella*, oberoende av ensileringsmedel. Ensilagen utfodrades tre veckor vardera tillsammans med kraftfoder och rapsmjöl till 67 SRB kor, med upprepning av ett ensilage. Mikrofloran i mjölken visade en hög mångfald i kombination med ett lågt antal bakterier, de mest förekommande släktena var *Lactobacillus* och *Pseudomonas*.

Ensileringsmedlen visade sig endast ha en liten effekt på mikrofloran i använt strömaterial, men inte på mjölkens mikroflora. Efter att ost producerats av mjölken visade sig mikrofloran vara mest lik den starterkultur som användes. Ingen effekt av ensileringsmedlen observerades i ostens mikroflora, däremot en effekt av batch.

Generellt påverkades mikrofloran i osten av faktorer på mejeriet eller faktorer som inte undersöktes i denna avhandling. Starterkulturen som användes under ystningen var huvudkällan till de bakterier som fanns i osten. En begränsande faktor i denna studie var valet av sekvenseringsmetod, tillräcklig information för att kunna särskilja olika arter av bakterier tillräckligt saknades.

Nyckelord: Mikroflora, mjölksyrabakterier, växtmaterial, ensilage, mjölk, ost.

Preface

"A bottle of wine contains more philosophy than all the books in the world."
- *Attributed to Louis Pasteur*

The term **microbiota** usually refers to:
"The assemblage of living microorganisms present in a defined environment."
- *Marchesi & Ravel (2015)*

Dedication

To my parents
Lasse Eriksson and *Anki Eliasson*

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Eliasson, T., Sun, L., Lundh, Å., Höjer, A., Hallin Saedén, K., Hetta, M. & Gonda, H. (2023). Epiphytic microbiota in Swedish grass-clover herbage and the effect of silage additives on fermentation profiles and bacterial community compositions of the resulting silages. *Journal of Applied Microbiology*, 134 (9), lxad196. https://doi.org/10.1093/jambio/lxad196

II. Eliasson, T., Sun, L., Lundh, Å., Gonda, H., Höjer, A., Hallin Saedén, K. & Hetta, M. (2024). Microbial communities in feed, bedding material, and bulk milk - experiences from a feeding trial. *Journal of Dairy Science*, X(X), X. https://doi.org/10.3168/jds.2024-25213

III. Eliasson, T., Lundh, Å., Höjer, A., Hallin Saedén, K., Gonda, H., Hetta, M. & Sun, L. Changes in microbiota during the cheese making process – from raw milk to the final cheese (manuscript).

All published papers are published open access.

The contribution of Thomas Eliasson to the papers included in this thesis was as follows:

I. Participated in planning the field study.
Conducted the main part of the work and sampling.
Analysed most of the samples.
Processed the data and summarised the results.
Drafted and submitted the manuscript.

II. Participated in planning the feeding trial.
Helped with the work and conducted most of the sampling.
Analysed most of the samples.
Processed the data and summarised the results.
Drafted and submitted the manuscript.

III. Participated in planning the cheese making trial.
Did not participate in work or sampling (Covid-19 restrictions).
Analysed some of the samples.
Processed the data and summarised the results.
Drafted parts of the manuscript.

List of tables

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Abbreviations

ASV	Amplicon sequence variant
LAB	Lactic acid bacteria
MPCA	Milk plate count agar
MRS	de Man, Rogosa and Sharpe
NSLAB	Non-starter lactic acid bacteria
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PMA	Propidium monoazide
PMR	Partial mixed ration
RA	Relative abundance
VBNC	Viable but not cultivable
vPCR	Viability PCR

1. Introduction

Cheese is a major food commodity world-wide, and a large proportion of global milk production is now processed into cheese. According to the latest report from the International Dairy Federation (2023), global milk production in 2022 amounted to 936 million tons, with milk from cows constituting 80% of the total. Cheese production from cow's milk during the same year was estimated to 23.2 million tons. Assuming an average of 10 L of milk per kg of cheese produced, around one-third of all cow's milk produced during 2022 was used in cheese production.

Cheese making, which dates back several thousand years, is today a highly evolved food biotechnology application. Traditional cheese making relies on a combination of only four ingredients, milk, rennet, microorganisms and salt, and yet more than 1,000 cheese varieties are produced worldwide. This immense diversity in cheese types is generated by variations in various factors, including raw milk species, microbiology, process parameters and time of ripening (McSweeney *et al.*, 2004).

Although cheese comes in many varieties, not all are dependent on microorganisms for their development. This thesis focused on cheese ripened by internal bacteria, specifically a traditional Swedish long-ripened hard cheese. During recent decades, the main producer of this cheese has experienced uncontrolled variation in ripening time and generally also a longer time for the cheese to mature. Improvements in hygiene on dairy farms and strict hygiene in the dairy facility have resulted in total numbers of bacteria in raw milk that are often below 10,000 per mL (Glantz *et al.*, 2020). However, to develop its characteristic aroma this cheese variety is dependent on lactic acid bacteria (LAB) that are not present in the starter culture used during production, referred to as non-starter lactic acid bacteria (NSLAB).

One hypothesis for the recent problems in production is that the numbers of NSLAB in the raw milk are nowadays insufficient to allow these bacteria to develop and become dominant in the ripening cheese in the same way as in the past (Rehn *et al.*, 2010). It has been shown that farm management has an impact on raw milk microbiota, so one way to increase the abundance of NSLAB important for the aroma of this particular cheese could be through altering the forage fed to dairy animals. The main aim of the work presented in this thesis was to improve understanding of the microbiota in the dairy value chain from farm to cheese, and in particular the role of NSLAB.

2. Background

The process of making long-ripened hard cheese starts at the dairy farm, with milk being produced and collected. When the raw milk arrives at the dairy facility, industrial processing usually starts with a standardisation step, in which the milk fat content is adjusted to a set value by partial removal of cream. The milk is then pasteurised (heat-treated) to eliminate harmful bacteria and cooled to a temperature that is optimal for the next step, fermentation.

The raw milk is fermented using a starter culture to lower the pH of the milk, both for product safety and to aid in the subsequent coagulation process. The starter culture is usually a mesophilic or thermophilic culture[1], depending on the temperatures used during the milk coagulation step. An adjunct culture can be also added, to provide bacteria that are useful in *e.g.* aroma development or formation of eyes (the characteristic holes inside some cheese varieties) during ripening of the cheese (Johnson, 2013).

At the end of the fermentation process, rennet[2] is added to coagulate the milk. When the milk reaches a certain level of coagulation (gel), cutting is performed to start the whey draining process. Additional stirring and heating steps are applied to increase whey drainage and shape the cheese curds to the desired size and density. The resulting cheese curds are transferred to moulds and pressed in a cheese press to form cheeses, commonly in the shape of

[1] These terms refer to the optimum growth temperature for the bacteria, where mesophilic means moderate (around 20-32 ºC) and thermophilic means high (around 37-45 ºC). However, the temperature range for bacterial survival in both cases is much wider (Johnson, 2013).

[2] Rennet is the coagulating agent used in the cheese making process, generally comprising the enzymes chymosine and pepsin, derived from calf stomachs. Chymosine cleaves off a small part of the milk protein kappa-casein, causing the molecules to attract to each other, while pepsin causes general cleavage of milk proteins, leading to improved coagulation (Uniacke-Lowe & Fox, 2017).

wheels or blocks. The cheese is then salted, usually by submersion in a brine solution for a set time, followed by drying off excess moisture.

Finally, the cheese reaches the most important step, ripening, which usually takes place in a facility with controlled temperature and humidity. During this process, the cheese further develops its specific characteristics. The combination of residual rennet, milk enzymes (*e.g.* plasmin) and bacteria from the starter culture and other microorganisms works to further break down proteins and fats to other compounds, leading to the development of aroma and texture (Fox & McSweeney, 2017).

2.1 Bacteria in the dairy value chain

The dairy value chain comprises many steps from farm to cheese, and bacteria are found in varying numbers in all these steps. The starting point is in the field, as the soil harbours a broad variety of bacteria depending on *e.g.* land use, soil composition and climate (Labouyrie *et al.*, 2023). These soil bacteria play a role in forming the plant microbiota, together with the surrounding environment and seed bacteria (Trivedi *et al.*, 2020). The dairy cow then consumes these plants, grown under differing soil and environmental conditions. A common way of preserving plant biomass for feeding animals throughout the year is in fermented form, as silage. During the silage fermentation process, the microbiota of the crops shifts from comprising many genera to just a few, mainly LAB (McAllister *et al.*, 2018).

Dairy cows are often housed in an environment where they consume silage and are also surrounded by it and by the excrement from digestion of their diet. It can thus be concluded that the silage plays a key role, directly or indirectly via the environment, in forming the microbiota in milk. However, at the time of initiating this thesis work (early 2018), there was a lack of consensus in the literature regarding the main source of milk microbiota. Some studies concluded that the teat surface is the main source of microbial contaminants in milk (Vacheyrou *et al.*, 2011; Doyle *et al.*, 2017), but the original source of bacteria on the teat surface is unclear. A study by Porcellato *et al.* (2021) investigating the raw milk microbiota on selected Norwegian farms found a persistent core microbiota that was mainly affected by udder health in the short-term. In the longer run the microbiota was affected by unidentified factors, possibly including feeding (Porcellato *et al.*, 2021). In more recent studies, Ouamba *et al.* (2023) reported notable

transfer of bacteria from silage to raw milk, while Sun *et al.* (2024) did not observe such transfer to the same extent.

In most commercial settings, raw milk is collected twice per day by milking equipment and pumped to a holding tank, where it is cooled to a temperature at which most harmful bacteria do not proliferate. After a few milking occasions, the bulk raw milk is transported to a dairy facility, where it is kept cold until processing. Processing generally includes a heat treatment step (pasteurisation at 70-72 ºC for 15-20 s) to eliminate harmful bacteria (Bylund, 2015). In principle, only thermoduric[3] bacteria survive pasteurisation, while pathogens in their vegetative form, all coliforms[4] and most, if not all, psychrotrophic[5] bacteria are destroyed (Grappin & Beuvier, 1997).

However, some bacteria possess the ability to survive in challenging environments, although they cannot grow on standard culture media, a phenomenon known as viable but non-cultivable (VBNC) state. Bacteria commonly enter VBNC state in nutrient-poor environments or under stressful conditions, *e.g.* heat treatment (Pazos-Rojas *et al.*, 2023).

2.2 Non-starter lactic acid bacteria (NSLAB)

There has been a suggestion that the specific taste and texture of some dairy products, specifically cheese, are related to the microbiota of the raw milk (Martley & Crow, 1993). Factors linked to animal management, generally in relation to the feed, may also have an influence on cheese flavour. A good example is provided by Serrapica *et al.* (2020), who evaluated the effect of forage preservation method on sensory properties of a traditional Italian Caciocavallo cheese. The resulting cheese composition was barely affected by the preservation method, but the appearance and taste were perceived as different, with a sensory panel preferring cheese produced from animals consuming forage preserved as hay over silage. These factors are of particular importance in the case of products with protected designation of origin (PDO) or protected geographical indication (PGI), since their specific

[3] Survives but does not necessarily grow at high temperatures, such as in pasteurisation.

[4] Group of bacteria commonly found in the environment, including soil, water and faeces, often used as an indicator of sanitary quality and potential contamination of food and water.

[5] Growth at low temperatures, usually below 7 ºC.

flavour is claimed to have close links with milk production conditions (Martin *et al.*, 2005). For example, the raw milk production guidelines for Parmigiano Reggiano are rather specific to guarantee the "correct" quality of the milk, *e.g.* limiting the geographical area in which it can be produced and the type of forage used. Cows may not be fed silage or fermented feeds, and no additives or preservatives may be used in the cheese production process (Popping *et al.*, 2017).

In the literature, NSLAB are often mentioned in this context. In most commercial cheese production practices, a starter culture comprising mainly LAB is used. Depending on production practices, the starter culture used is either defined, comprising a specified composition of bacteria, or undefined, comprising a mixture of unspecified bacteria (Powell *et al.*, 2016). In contrast, NSLAB are not added, but enter the system at some point on the way from farm to cheese, and are of high importance for certain cheese to develop its specific characteristics (Martley & Crow, 1993; Gobbetti *et al.*, 2018).

Research has been conducted to understand how and where in the dairy value chain these bacteria enter the system, but no consistent explanation has been found. Raw milk is acknowledged to be the main source of NSLAB, as it provides the vat milk with its microbiota and enriches the overall microbiota of the cheese-making environment (Montel *et al.*, 2014). According to Verdier-Metz *et al.* (2012), the NSLAB potentially come from the teat skin, but their original origin is unclear. For example, De Souza *et al.* (2024) found increased occurrence of NSLAB in milk passing from the farm bulk tank through the tanker truck to the dairy silo. In a study by Decadt *et al.* (2024), one source of NSLAB was found to be the brine used for salting the cheese, while another source was unknown but assumed to be the pasteurised milk.

2.3 Previous research

The cheese making process has been intensively reviewed in recent decades in order to further understanding of factors of importance for the final cheese. Our research group has recently been focusing on factors affecting the milk coagulation process and the bacteria found in the dairy value chain, and how these bacteria affect the cheese ripening process.

For example, Priyashantha *et al.* (2021a) investigated the effect of differences in raw milk composition, coagulation properties, enzymatic activity and total bacteria on the cheese ripening process. Among the factors investigated in that study, it was mainly the milk enzymes plasmin and plasminogen which affected the overall sensory score of the final cheese. The reasons for variations in different factors were evaluated further in relation to on-farm factors and seasonal variation, where the main finding was that milking system (*i.e.* robotic vs. tie-stall) contributed to changes in the raw milk (Priyashantha *et al.*, 2021b, 2021c).

The bacteria in the dairy value chain on commercial farms and their effect on the cheese making process were evaluated by Sun *et al.* (2023). They collected milk from three clusters of farms during three periods and processed the milk to long-ripened hard cheese at a dairy facility. They found that the raw milk microbiota was mainly affected by period, while the cheese microbiota was affected by both farm cluster and period. The observed effect of farm cluster on the final cheese indicated that factors at farm level are important for bacteria in the dairy value chain, although not observed in the raw milk in the study by Sun *et al.* (2023). Following the cheese microbiota study, two studies on raw milk were conducted by our research group to evaluate possible factors contributing to changes in the bacterial composition. The first, by Sun *et al.* (2022), investigated the effect of milking system and teat preparation before milking, and concluded that both had an effect on the raw milk microbiota, although only to a minor extent. In the later study, by Sun *et al.* (2024), the microbial relationship between silage and raw milk on commercial farms was investigated. The conclusion was that *Lactobacillus* in silage was not the major source of *Lactobacillus* in the milk, with generally very few bacteria present in common in the different samples.

2.4 Project design

Based on previous research, it can be concluded that factors at farm level affect the outcome of the cheese making process, both from a biochemical and bacterial point of view. However, the factors that actually affect the microbiota are not clear, as the cheese microbiota may differ between farm clusters but that of the raw milk does not. One possible reason could be that the many commercial farms included in previous studies, practising many

different variants of dairy farming, caused so much variation in the studied factors that they could not be evaluated properly in relation to the raw milk microbiota. Further, as the raw milk from all farms in each cluster was mixed at the dairy, it was impossible to evaluate fully which farm factors actually contributed to the observed changes in cheese microbiota.

Therefore, the major part of the work in this thesis was performed on one farm (a research farm where many factors could be controlled) and the cheese was produced in smaller batches in a test vat, to eliminate the need to include milk from multiple farms in order to obtain a sufficient volume for ordinary production.

2.4.1 Potential sources of the raw milk microbiota

In summary, the raw milk microbiota depends on many factors that involve direct contact (*e.g.* teat skin, milking equipment, farm bulk tank, transport truck and the dairy facility environment) and indirect contact (*e.g.* feed, water, bedding material and the farm environment in general). Many of these factors are discussed further in review papers by Quigley *et al.* (2013) and Parente *et al.* (2020). Forage has been suggested as an indirect source of microorganisms for raw milk, *e.g.* Hagi *et al.* (2010) observed changes in raw milk microbiota as cows were pasturing, while Vacheyrou *et al.* (2011) found that sources in the barn environment (*e.g.* the forage) could potentially contribute bacteria. Moreover, the milk microbiota is influenced by the overall management system of the farm, making it difficult to identify the influence of a single practice.

3. Aims

The overall aim of the work in this thesis was to improve understanding of the microbiota in the dairy value chain from farm to cheese, and in particular to identify the role of NSLAB. Specific objectives were to investigate the following interlinked research questions:

- The relationship between the microbiota of the forage crop and that of the resulting silage, including the effect of silage treatments (Paper **I**)

- Possible transfer of microbiota from feed and barn environment to the raw milk (Paper **II**)

- Whether variation in the feed microbiota affects the composition of the milk microbiota, and the properties of the resulting cheese (Paper **III**)

4. Methodology

The work in this thesis was divided into two separate parts. The first of these focused on the microbiota of herbage and the resulting silages after ensiling with different additives in laboratory scale (Paper **I**). The second focused on the microbiota of the dairy value chain, from the forage in field to the produced long-ripened hard cheese, and the effect of feeding silages with different microbiota (Papers **II** & **III**).

4.1 Experimental design – Paper **I**

The work in Paper **I** was performed at laboratory scale with glass silos, using plant biomass from experimental plots in an on-going long-term field experiment. The objective was to investigate whether geographical location and harvest time affect herbage and silage microbiota, and the effects of silage additives on the final silage microbiota. The study focused on the most common forage crops for Swedish dairy cows, *i.e.* a mixture of grass and clover. The study took place during early and late summer of two consecutive years (2018 and 2019). The initial plan was to use four locations in Sweden; Röbäcksdalen in the north, Säby in the mid-east, Lanna in the mid-west, and Lönnstorp in the south (Figure 1). However, after the first silage cut in year one, it became apparent that the trial would have to be limited for logistical reasons. This led to exclusion of two locations, Lanna and Lönnstorp, for the remaining cuts. The silage additives used were: i) no additives, ii) acid treatment, and iii) inoculation with starter culture.

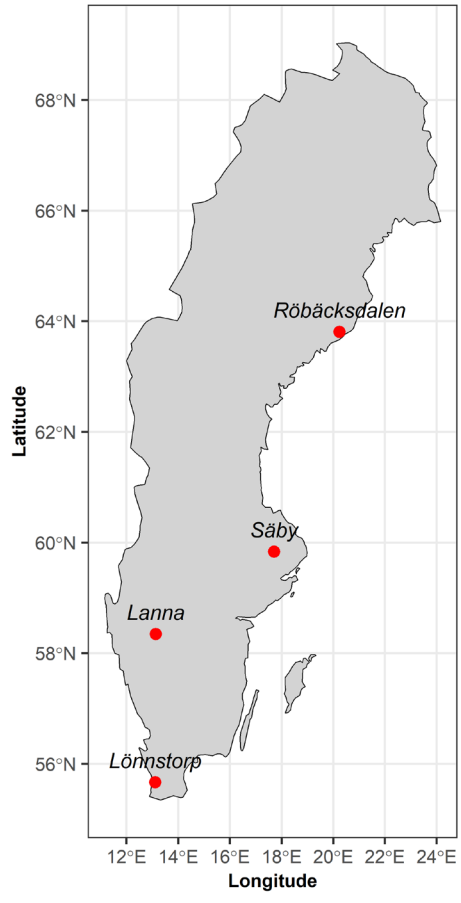

Figure 1. Map of Sweden showing the location of the four field stations and their coordinates.

4.1.1 Minimising the effect of unstudied factors

The main reason for choosing the four locations was that they were all able to provide herbage produced according to a standardised protocol. The herbage was collected from the on-going long-term field experiment R3-0021, run by the Swedish University of Agricultural Sciences, in which agronomic practices are kept as similar as possible across locations, as described further in Mattsson (2002). Selection of harvest time was set to the same stage of maturation at all locations, to avoid any effects of differences in plant maturation.

With herbage produced in a standardised way, independent of location, the next question was how to harvest the herbage in a representative manner. To avoid bias from using different cutting equipment at each location, a harvester was built in-house using a bicycle trailer and a battery-powered hack saw (Figure 2).

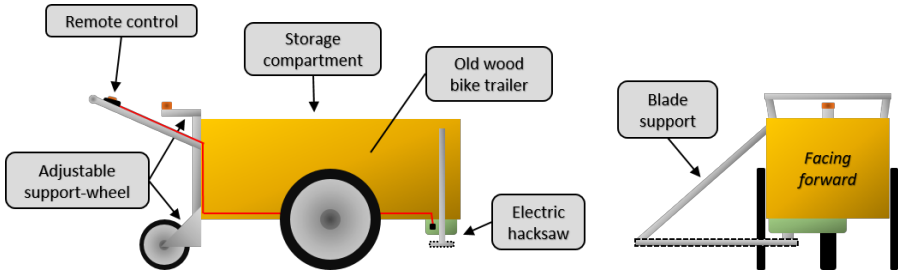

Figure 2. Schematic illustration of an in-house built harvester used to cut herbage at different locations.

Harvesting was performed by two individuals, one running the harvester and the second collecting the cut herbage into a plastic bag, which was stored in the storage compartment (Figure 2). The blade was mounted to the right of the harvester, so that cut herbage did not fall in the path of the harvester, and its operator. A compost grinder with a rotary knife was brought to each location, for standardised chopping of the herbage before ensiling. Finally, for standardised filling of the laboratory-scale silos, an in-house built press and a scale were used.

4.1.2 The ensiling process

In short, on each harvesting occasion, each field plot (n = 3) generated ~20 kg herbage (Figure 3). It was chopped in the compost grinder, followed by thorough mixing and extraction of three 3-kg samples, which were stored in plastic bags. Silage additive (same volume of liquid in all three cases) was sprayed into the bags and thorough mixing was performed. Each bag was then used to make two silos (glass jars), resulting in six silos from each field plot and 18 silos in total for each location. By using a scale, an even density was obtained during the filling process. The glass jars were stored in a climate controlled room, set at 20 ºC and 50% relative humidity. Silo opening was set to 100 days from the start of ensiling, ensuring that all jars fermented for the same time before sampling of the finished silage.

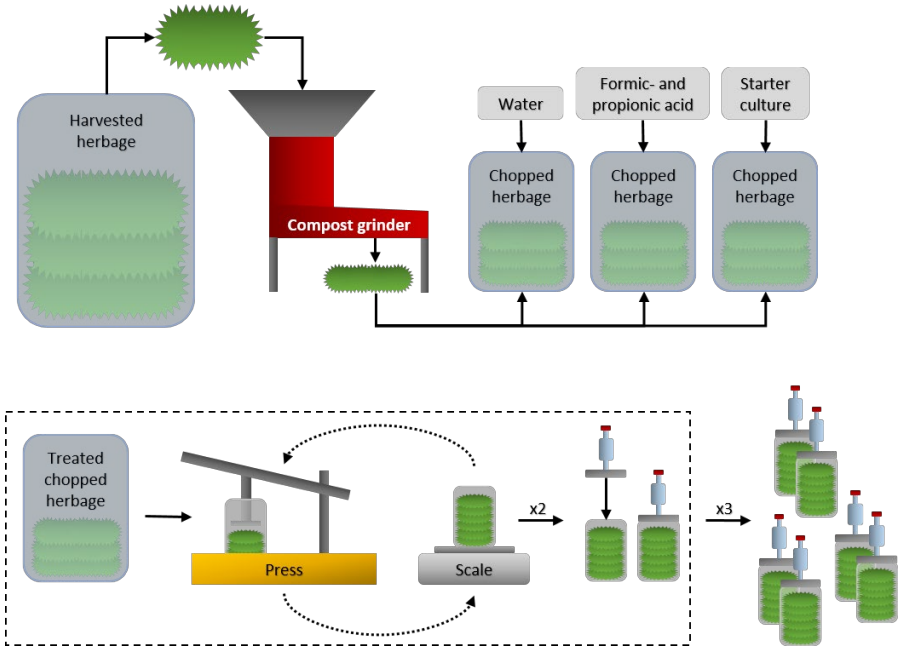

Figure 3. Ensiling process performed at each field station.

Sampling

Herbage was sampled just before cutting and directly frozen in liquid nitrogen to obtain an instant picture of the microbiota. Sampling of silages was performed directly after opening the jars, using the full volume.

4.2 Experimental design – Papers **II** & **III**

Following findings in Paper **I** that silage additive had a strong effect on the final silage microbiota, a combined feeding and cheese making trial was designed. As mentioned in section 2.3, previous work by our research group evaluated on-farm factors and their effect on milk and cheese microbiota in commercial farm settings. However, since many of the factors were confounded in those studies, it was not possible to draw conclusions regarding the effect of individual factors. Therefore, the objective of the work in Papers **II** & **III** was to evaluate whether silage with different microbiota, produced and fed to dairy cows on a research farm, affected the microbiota of the raw milk and the resulting cheese. The point at which bacteria important for the cheese ripening process enter the dairy value chain

and the extent of entry were also studied. The advantage of using a research farm, compared with previous studies on commercial farm, was that most factors could be controlled to a major extent. On the research farm, strict protocols associated with milk production during the feeding experiment were used to enable conclusions to be draw on bacterial transfer in the dairy value-chain from feed to raw milk, and the final cheese. Due to the large amount of samples obtained and data produced, the analysis was divided into two parts (Papers **II** & **III**).

4.2.1 From field to raw milk

Silages used in Paper **II** were produced at Röbäcksdalen research farm in summer 2020, using the same type of additives as in Paper **I** (no additive, acid-treatment and inoculation with starter culture). However, due to shortage of the Paper **I** starter culture at the manufacturer, another similar starter culture also had to be used. This silage was stored in a separate bunker silo and the two inoculated silages were always pooled 1:1 on a dry matter basis at feed-out. The experimental design and sampling points are summarised in Figure 4.

In short, grass was cut with a disc-mower conditioner and wilted, followed by windrowing and collection with a pick-up wagon. The treatments were applied through nozzles as herbage passed through the pick-up mechanism. Treated herbage was packed into concrete bunker silos and covered with plastic covers and sand bags. The silages from the additive treatments were fed for three weeks each as partial mixed rations (PMR) in feed bunks, to ~67 dairy cows housed in an insulated two-row barn. One of the silages was fed for a second three-week period, to evaluate whether any potential change in raw milk microbiota was repeated. The last week of each three-week feeding was a sampling week, with most sampling points covered every second day ($n = 3$). The raw milk produced during the sampling week of each silage treatment was transported to the dairy every second day for cheese making, with in total three milk deliveries per sampling week.

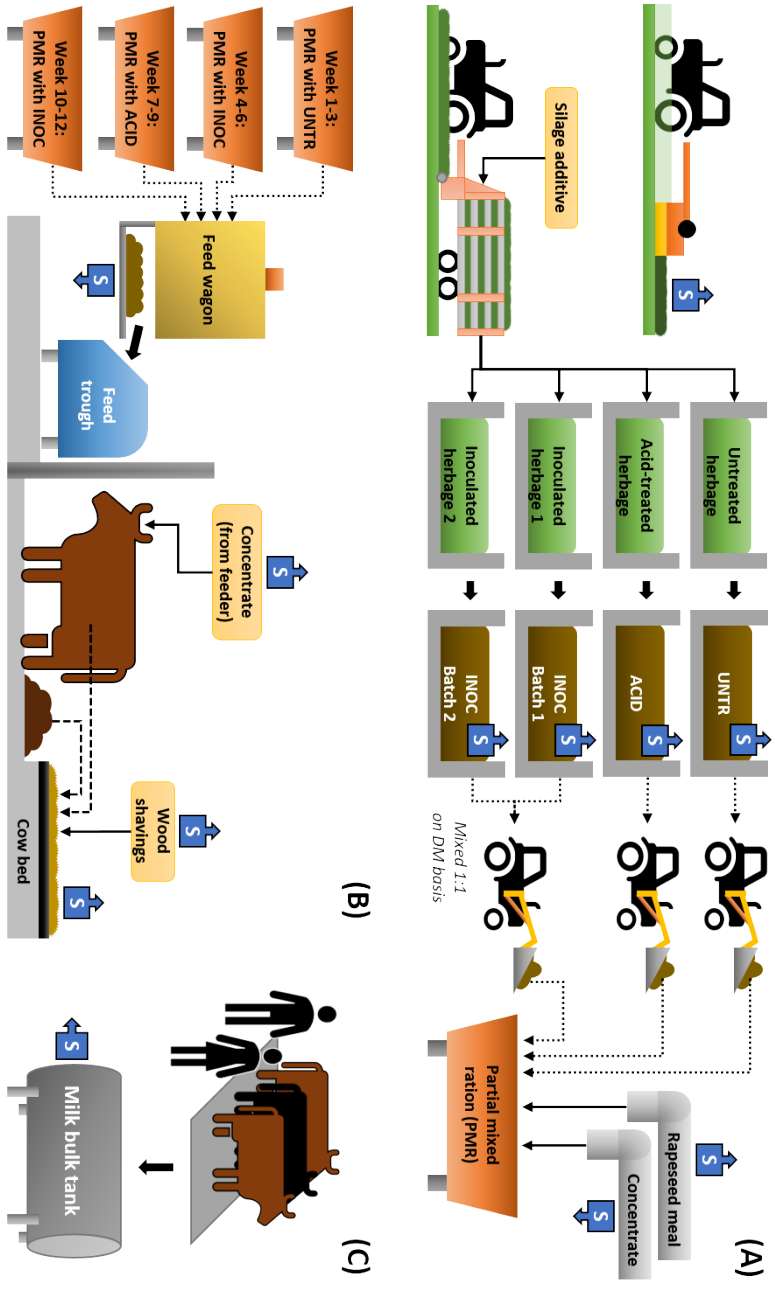

Figure 4. Schematic diagram of the feeding trial set-up. A) Production of silages (no additive; UNTR, acid treated; ACID, inoculated; INOC) and partial mixed rations (PMR), B) feeding and housing of cows, and C) milk collection. S: sampling points.

Additional sampling points not included in the analysis

The initial plan was to sample the water that the cows consumed, as water is a major component of dairy cow daily intake. Agenäs *et al.* (2003) estimated intake to be 58 ± 4 L of water per day in Swedish Red cows, the same breed as in Paper **II**. However, the Swedish Veterinary Agency (2023) stipulates that the hygienic quality of drinking water provided to dairy cows must be the same as that of water for human consumption, so this sampling point was excluded considering the low bacterial load in drinking water.

Another sampling point could have been some form of aerosol capture device, since Riccardi *et al.* (2021) found cultivable bacteria at concentrations of up to ~2,500 CFU/m^3 of sampled air in Italian cow sheds. These air-borne bacteria could potentially transfer to the milk, both directly by entering the raw milk in the bulk tank or indirectly by attaching to the udder of the cow and contaminating the milk during the milking process.

Finally, sampling points in the milking equipment (*e.g.* lines, pumps and sub-tank) were considered but excluded, as these were expected to add only minor numbers of bacteria and to be of limited value in bacterial transfer from feed and the farm environment to the milk.

4.2.2 From raw milk to the long-ripened hard cheese

In Paper **II**, the raw milk from the research farm was transported cooled in an insulated tanker truck to the dairy facility within 2-3 hours of collection. Processing began directly by pumping the milk from the tanker truck to a clean silo. From the silo, milk passed through a balance tank to keep an even flow to the process and was pre-heated in a plate heat exchanger and passed through a cream separator to obtain a standardised fat content. The standardised milk was pasteurised and cooled in the plate heat exchanger to a set temperature. It was then transferred to the cheese vat and starter culture was added. After fermentation for a set time, rennet was added and the milk coagulated, followed by a set of processing steps that included cutting, stirring, heating and whey drainage. Sampling points were set to capture possible changes in the milk microbiota during processing and in the event of contamination. The process and the sampling points are summarised in Figure 5.

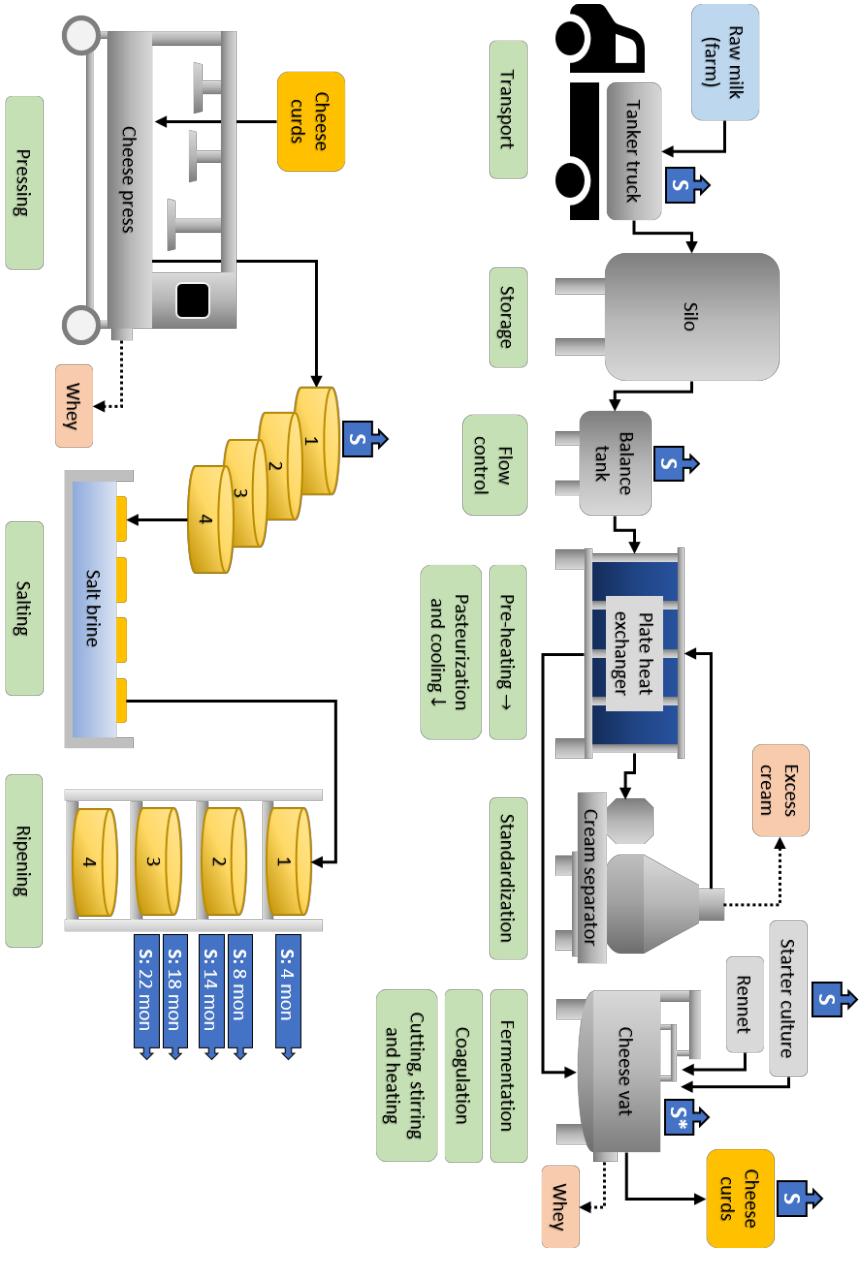

Figure 5. Illustration of the cheese making process from raw milk to cheese ripening. Processing steps are indicated by green boxes. S: sampling points. *Milk sampled after filling and after fermentation for a set time.

Cheese grains were transferred from the cheese vat to moulds, which were placed in a cheese press and the whey was drained off. The resulting cheese wheels were transferred to a brine bath with salt brine, after which the cheese was moved to storage shelves to allow excess moisture to dry off. Finally, the cheeses were ripened for 22 months in a dedicated storage facility, with samplings at set time-points. Each batch generated four 18-kg cheese wheels, and each was sampled a maximum of two times to avoid the interior of the cheese being affected by excessive sampling. One cheese from each batch was kept as a spare in case something happened to the other three.

Additional sampling points not included in the analysis

The water used in all processing steps, both for pushing product in the lines and rinsing of processing equipment and the tanker truck after cleaning, could potentially contaminate the milk or cheese grains. The dairy environment itself could contribute bacteria from the "house flora", as many processing steps take place exposed to the air in the facility, as discussed in a review by Kang & Frank (1989). The salt brine has been shown to be of interest in previous studies, *e.g.* Haastrup *et al.* (2018) found that bacteria in salt brine are an important source of contamination for surface-ripened cheeses and that they exert an plant-specific composition effect. The brine used in Paper **II** was sampled, but unfortunately the samples disappeared. The whey expelled from the cheese vat and the cheese press was not sampled, but analysis of this could improve understanding of the bacteria that affect the process before the final cheese. Da Silva Duarte *et al.* (2020) observed variation in both numbers of viable bacteria and their composition in whey samples from four dairy plants, sampled at different months.

4.3 Sampling and sample preparation

Sampling and preparation of samples for all analyses were tedious and time-consuming, as many steps were involved. Feedstuffs were generally obtained in large volumes that needed to be condensed from kilograms (Paper **I**) or tonnes (Paper **II**) to a few grams without affecting representation of the original material. The general approach was to take many samples from a large area or volume and mix these thoroughly and then take subsamples for use in the final analysis. A similar approach was applied for unused and used bedding material, but for milk and cheese only a few samples were taken, as

multiple sampling would increase the risk of contamination. Another reason for limiting the sample volume for milk was because a large volume would take a long time to freeze after sampling, which could introduce unwanted changes in the microbiota. For cheese, the volume was limited due to the risk of disturbing the environment inside the cheese.

Most samples needed some form of pre-processing before analysis. Feedstuffs generally needed to be dried and milled, except for the silage used in fermentation analysis. It was based on silage juice, which was obtained after pressing the samples in a hydraulic press. The majority of samples used for evaluation of microbiota were run in a stomacher, to obtain an emulsion which could be used for either bacterial enumeration or further processing before sequencing of bacterial DNA.

4.4 Evaluation of microbiota

The main focus of the work in this thesis was to identify bacteria present in different types of materials related to the dairy value chain. At the time of initiating the work, bacterial enumeration in combination with sequencing of the 16S rRNA gene in bacterial DNA was the most feasible option for identification of bacteria. It was also the most cost-efficient and was expected to yield the most information for the invested work and resources.

4.4.1 Bacterial enumeration

Estimation of total numbers of aerobic bacteria in all samples analysed in Papers **II** & **III** was performed on milk plate count agar (MPCA), a modified plate count agar with addition of skim milk powder to provide lactose as a carbohydrate source for the more dairy-specific bacteria. In Paper **I**, numbers of total aerobic bacteria were not estimated.

Estimation of lactobacilli[6] in both studies was performed on de Man, Rogosa and Sharpe (MRS) agar. In addition, Rogosa agar was used in Paper **I**, as it is more specific for *Lactobacillus* than MRS agar. A minimum of three dilution steps were plated for each unique sample and medium. All

[6] Genera in the family *Lactobacillaceae*; mainly *Lactobacillus* but also *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*.

media were incubated at 30 ºC for 48-72 hours. In addition, MRS and Rogosa agar were incubated inverted and in anaerobic conditions.

4.4.2 Sequencing of bacterial DNA

In total, more than 700 samples were analysed, from raw samples to samples taken at different points along the production chain. The analytical process comprised three major steps: i) extraction of bacterial DNA, ii) amplification and library preparation, and iii) sequencing. A simplified description of the most important parts of the process is provided below.

Extraction of bacterial DNA

For extraction of bacterial DNA, sample was homogenised with buffer solution in a stomacher. The emulsion obtained was mixed and a representative sub-sample was centrifuged to obtain a cell pellet, which was dissolved in buffer solution. The final cell slurry was lysed with a reagent together with bead beating, to release the DNA from the cells. The unclean DNA was then exposed to a set of washing steps to remove cell debris *etc.*, using different filter columns and reagents. The final clean DNA, bound to the last column, was eluted into a cryo-tube and frozen until further processing.

Amplification and library preparation

The length of bacterial DNA varies from a few hundred thousand to many millions of base pairs. For the purpose of identifying the bacteria in the samples analysed in this thesis, only a short fragment (the 16S rRNA gene[7] and specifically the V4 region[8]) was of interest. The amplification process uses primers to direct an enzyme to a specified section of the DNA (full details on the primers used can be found in Hugerth *et al.* (2014)). As the enzyme binds to the DNA, the section is copied, and for every time the amplification process runs, copies increase exponentially. After a set amount of runs, the amplified DNA is dominated by the specified section. To

[7] The 16S rRNA gene is ~1,500 base pairs long and is present in all bacteria. It contains conserved regions for universal detection and hypervariable regions for distinguishing between species, making it a key tool for bacterial identification and classification.

[8] The V4 region of the 16S rRNA gene is ~300 base pairs long and one of nine hypervariable regions. It is commonly used in microbial studies due to its balance of variability and manageable length, allowing for efficient sequencing and reliable bacterial identification.

facilitate sequencing of multiple samples at once, a unique barcode is bound to the amplified DNA together with adapters that the sequencing equipment can recognise (Caporaso *et al.*, 2012). The amplified DNA of all samples is then pooled into a DNA library.

Sequencing

The DNA library is loaded into a sequencer, in the present case, the Illumina Miseq platform. It is categorised as a high-throughput sequencer as it can read much DNA fast, although at the cost of only being able to read short fragments and thus amplification of a small part of the bacterial DNA. The length of the V4 region of the 16S rRNA gene is close to the maximum length that the platform can handle. After sequencing, the reads obtained in this thesis were de-multiplexed[9] and files with all reads separated by each unique sample were delivered to the laboratory.

Implications

The main issue with using the short-read sequencing approach is the lack of resolution. With less than 300 base pairs of information, it is often not possible to distinguish different species within the same genera from each other, and in some cases, not even different genera that are closely related. On the plus side, the platform gives major depth, as the amount of reads generated is up to 25 million per run, guaranteeing a good representation of the bacteria in the sample, even those present in low concentrations. A way of overcoming the lack of resolution is either to combine sequencing runs with different regions of the 16S rRNA gene amplified or to use another sequencing platform that gives longer reads. However, this generally involves a major increase in cost, which would lead to exclusion of samples.

4.4.3 Bioinformatics

Another time-consuming component of this thesis work was bioinformatics. In short, bioinformatics is the process that takes place after sequencing, when reads are turned into something useful. The process can be simplified into the following main parts: i) quality control, and cleaning, ii) feature table

[9] De-multiplexing refers to the process of sorting the mix of sequenced reads to its original samples by the unique bar codes used in the amplification of DNA.

construction, iii) taxonomic annotation and phylogenetic analysis, and iv) synthesis of results.

Quality control and trimming

The raw reads from sequencing contain left-over sequences that need to be removed (*e.g.* primers and adapters) and the sequencing produces some level of errors in the reads. By using different tools, these can be identified and removed, leaving just the DNA of interest to the investigation.

Feature table construction

After cleaning, the reads are much shorter than the initial sequencing length. However, the sequencer (in most cases) actually reads both sides of the DNA, meaning that each read has a corresponding mirror image. Additionally, the main part of the trimming takes place from opposite ends of these read pairs, meaning that they can be joined in the middle. This step is called read merging, and results in merged reads with the initial sequencing length. These are sorted into a table, where each unique sequence (called "feature") represents one row and each column a unique sample. The number in each cell represents the number of times a feature occurred in a sample, hence the commonly used term "abundance".

Taxonomic annotation and phylogenetic analysis

A table of features and their abundances does not give much information in itself. However, by using a taxonomic database, the sequences in the feature table can be matched against known taxonomic classifications, resulting in a new table with all features annotated at the highest possible taxonomic level. The SILVA ribosomal RNA database (version 138), described further in Quast *et al.* (2012), was used in this thesis. In addition to taxonomy, the phylogenetic relationship between the features, measured as distance, was of interest. The term "distance" refers to how similar each sequence is to another sequence, and by calculating the distance between all features, a phylogenetic tree can be constructed.

Synthesis of results

The feature table, taxonomic annotation and phylogenetic tree are used to perform various analyses:

- To measure and compare diversity within and between samples or groups of samples.
- To relate samples or groups of samples to each other by their phylogenetic distance and abundance.
- To investigate relationships with other studied variables (metadata).
- To visualise bacterial composition in *e.g.* bar charts or heat maps.

Considerations

The bioinformatic process is complex and time-consuming, and requires major computational power. Additionally, the tools used are undergoing constant development, *e.g.* during this thesis work the software pipeline used for the bioinformatic process, QIIME2 by Bolyen *et al.* (2019), underwent an average of three major updates per year. Therefore in many cases it is probably better to outsource this part of the work to a company or specialist department that works with bioinformatics on a daily basis, although with the risk of not understanding the results to the same extent.

4.4.4 Comparison of DNA amplification methods (Paper **III**)

The regular DNA amplification method, polymerase chain reaction (PCR), comes with the major limitation that there is no distinction between DNA from live and dead bacteria. To address this, viability PCR (vPCR) is used in conjunction with propidium monoazide (PMA), a dye that selectively penetrates dead cells and binds to their DNA, preventing it from being amplified (Nocker *et al.*, 2007). This approach allows the DNA amplification method to target and amplify DNA only from viable bacteria, providing a more accurate assessment of the microbiota in a sample. The vPCR with PMA approach was only used in Paper **III**, and mainly as a test to evaluate the application on dairy-related materials, such as cheese.

4.4.5 Thesis specific methodology

The feature tables, taxonomic annotations and metadata from Papers **I-III** were merged in R by an in-house written script and the *msa* package (Bodenhofer *et al.*, 2015; R Core Team, 2024). The method facilitates comparison of sequence data from different sequencing runs and bioinformatic pipelines. In summary:

- Feature table reads were converted to relative abundance
- Features present in <0.1 % relative abundance were removed
- Multiple sequence alignment was performed with ClustalOmega
- Sequences were trimmed to an even and comparable length
- Feature tables were merged by their aligned and equal length sequences, with taxonomic annotation and metadata included
- The merged table was used for *e.g.* plotting the most abundant features in the combined datasets

4.5 Other analytical methods

To further understand the microbial data obtained, other analytical methods were also used, as summarised in Table 1. Most of these analyses were performed at commercial laboratories or by the commercial dairy staff, with only the preparation done in the research laboratory.

Table 1. Summary of analytical methods, parameters measured and instruments/ techniques used in Papers **I-III**.

Analytical method	Parameters measured	Instruments/techniques
Weather data collection	Temperature, humidity, rainfall and global radiation	Monitoring at weather station
Estimation of herbage botanical composition	Proportions of plant species	Manual sorting (Paper **I**) Dry-weight-rank* (Paper **II**)
Analysis of chemical composition in herbage and silage	Dry matter	Oven drying
	Carbohydrate fractions, protein and ash	Near infrared reflectance spectroscopy and other commercial methods
Silage fermentation analysis	pH	pH-electrode
	Short-chain fatty acids and ammonia-nitrogen	Titration-method** (Paper **I**) Commercial methods (Paper **II**)
	Fermentation losses	Scale, before and after fermentation
Evaluation of dairy herd performance	Weight	Scale
	Feed intake	Individual feed troughs with scales
	Milk production	Milking equipment
	Fat, protein and somatic cells	Test-milking
Evaluation of raw milk quality for dairy processing	Total bacteria, Enterobacteriaceae, Psychrotrophic and thermoduric bacteria	Bacterial enumeration on plate count agar and other media
	Somatic cell count	Flow cytometry
	Fat, protein and lactose	MilkoScan
	pH	pH-electrode
Monitoring of the cheese ripening process	Total bacteria, Enterobacteriaceae, and lactic acid bacteria	Bacterial enumeration on plate count agar and other media
	Fat and protein	FoodScan
	pH	pH-electrode
	Sensory evaluation	Taste panel

*Mannetje & Haydock (1963). **Moisio & Heikonen (1989).

5. Main findings

This chapter presents the main findings in Papers **I-III** and discusses these findings in relation to past and present research. In section 5.4, the results from the three papers are combined to summarise the microbiota of the dairy value chain.

5.1 Microbiota of herbage and the resulting silage, and the effect of silage additives (Paper **I**)

The results obtained in Paper **I** played a major role in this thesis work, as they laid the foundations for Papers **II** & **III**. Unfortunately, the summer of 2018 (when the work in Paper **I** was performed) was unusually warm for Sweden and therefore a decision was taken to repeat that work during 2019, at the cost of delaying the second part of the work for a full year. This was deemed necessary in order to ensure that the results did not just reflect the effect of an extreme weather event.

5.1.1 Herbages contained mostly non-lactic acid bacteria, with no clear connection to growing location or harvest

The herbage microbiota was random and not significantly dependent on growing location or time of harvest (Figure 6). The main bacterial genera found were *Pantoea*, *Pseudomonas*, *Sphingomonas* and *Xanthomonas,* in varying proportions. Although the distribution appeared to be random, *Xanthomonas* was mainly found at Röbäcksdalen, while *Pantoea* was generally observed in higher relative abundance (RA)[10] in Säby. Herbage

[10] Relative abundance (RA) refers to the proportions of bacterial DNA in a specified sample or environment, and should not be confused with the actual numbers of bacteria.

samples comprised few viable LAB (0.8-4.1 $\log_{10}$ CFU/g), with a tendency for more viable bacteria in second-cut herbages. The *Lactobacillus*-specific Rogosa agar gave even lower numbers, indicating that the LAB comprised different genera and not just *Lactobacillus*. However, LAB were rarely observed in the herbage sequencing results.

The harvested field plots comprised a mix of timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*) and red clover (*Trifolium pratense*). In general, the first cut contained more of the grasses, while the second cut contained more of the red clover, with a tendency for more red clover at Säby than at the other locations. Similarly, a previous study by Mogodiniyai Kasmaei *et al.* (2017) on harvested herbage of the same plant species grown at a location close to Säby found low RA of LAB (<0.5%), while the microbiota was driven by bacteria belonging to the genera *Pantoea*, *Pseudomonas* and *Sphingomonas*. In a more recent study by Franco *et al.* (2022a) on harvested herbage of a red clover-dominated crop in Finland, the RA of LAB was higher (5-10%), while the majority of the bacteria belonged to *Pseudomonas* and unclassified genera of *Enterobacteriaceae*, *Yersiniaceae* and *Enterobacterales*. Unfortunately, neither of those studies estimated the number of viable LAB. However, an ensiling study with red clover by Wang *et al.* (2022) reported 4.25 $\log_{10}$ CFU/g of LAB in the harvested material, with the most abundant bacteria belonging to the genera *Methylobacterium*, *Pseudomonas* and *Sphingomonas*. An earlier study by Saarisalo *et al.* (2007) reported 2.83 $\log_{10}$ CFU/g of LAB in a harvested mixture of timothy and meadow fescue and, although the microbiota was not studied with sequencing, reported 7.24 $\log_{10}$ CFU of aerobic bacteria, which indicates a very low proportion of LAB.

It is clear that the numbers of viable LAB and their proportions are low in grass and clover herbages, but that they tend to be higher in legume-rich (red clover) herbages compared with grass-rich herbages. Finally, both *Pseudomonas* and *Sphingomonas* are commonly found in grass and clover herbages, and partly also *Pantoea*.

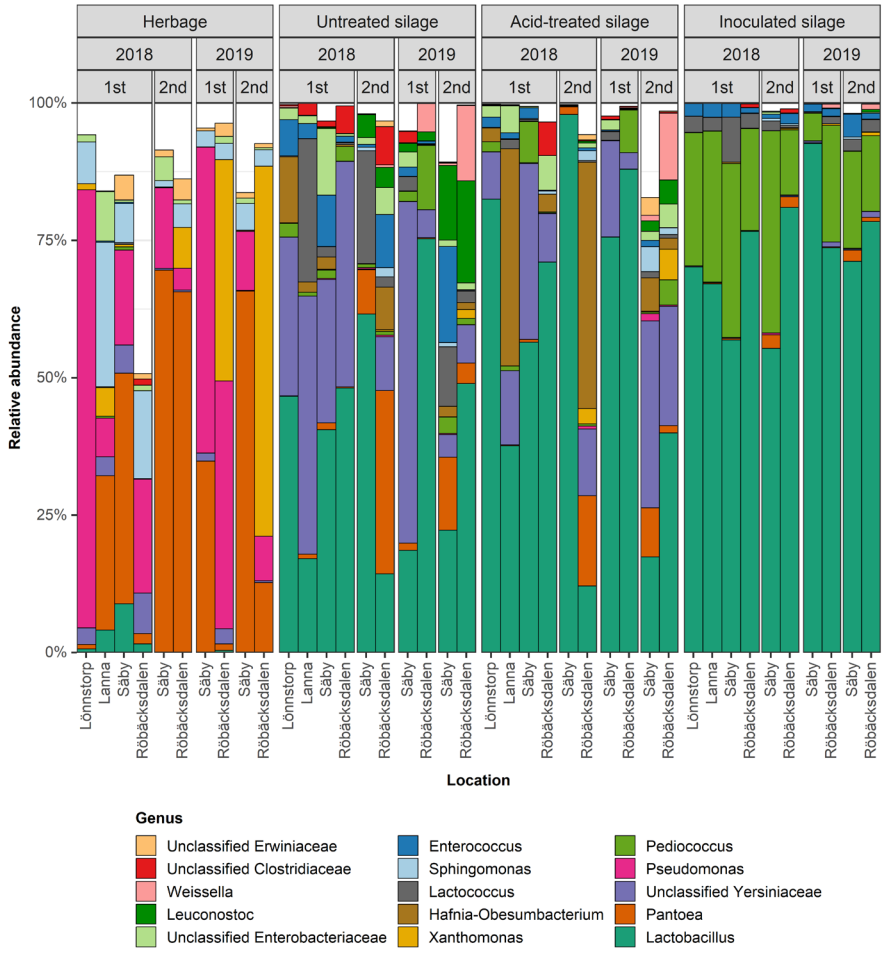

Figure 6. Summary of the 15 most abundant genera in herbage and the corresponding silages in Paper **I**, grouped by sample type, year and cut.

5.1.2 Contamination of chloroplast and mitochondria DNA

One major issue with the herbage samples analysed in Paper **I** was low read recovery after cleaning the sequencing data, with a major part of the reads consisting of non-bacterial DNA. It was concluded that this was probably an effect of plant cell contents (chloroplast and mitochondria) being present at high concentrations during bacterial DNA extraction, as described further in Beckers *et al.* (2016). In an ensiling study by Franco *et al.* (2022b), the extent of contamination from plant DNA was visible in the raw material before

ensiling. However, this was not an issue in the silage samples in this thesis, indicating that plant-related DNA was degraded during the ensiling process, as shown previously by Aufrère *et al.* (1994), while in herbage it was intact and released after the samples had been frozen. The main effect of low read recovery is less thorough representation of the bacteria in the samples, as DNA from bacteria at lower concentrations will potentially not be amplified and sequenced. This could explain why LAB were not observed in many of the herbage samples analysed in Paper **I**, and potentially also in other studies using the same techniques.

5.1.3 Silage additive had a major effect on the final silages

The strongest effect of silage additive was observed in the inoculated silages, which resulted in uniform LAB dominance with little variation between harvesting occasions (Figure 6). The main genera were *Lactobacillus* and *Pediococcus*, followed by minor RA of *Lactococcus* and *Enterococcus*. The proportion of *Enterococcus* decreased from 30% in the starter culture to an average of 1.9% in the silages. One possible reason for this was that the *Pediococcus acidilactici* included in the starter culture produced bacteriocins[11] with effects on *Enterococcus*, as described by *e.g.* Todorov *et al.* (2021). In general, the other silages comprised a more mixed microbiota with higher presence of non-LAB genera. The acid-treated silages comprised more LAB than the untreated silages but the diversity was higher in the untreated silages, with more frequent presence of the LAB genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus.*

Further analysis at amplicon sequence variant (ASV)[12] level revealed that much of the RA represented by *Lactobacillus* in all silages belonged to the same ASV. It was concluded that the *Lactobacillus plantarum* used in the starter culture and this ASV were probably not exactly the same bacteria, but very similar. The acid-treated silages often contained a different *Lactobacillus* ASV than the other silages, namely *Lactobacillus fructivorans*. This is a rather unique *Lactobacillus* as it can grow at high ethanol concentrations, as described by Suzuki *et al.* (2008) in relation to

[11] A bacteriocin is a protein produced by bacteria to kill or inhibit growth of other microorganisms.

[12] The term amplicon sequence variant (ASV) refers to a unique DNA sequence in the dataset. It should not be confused with operational taxonomic unit (OTU), where unique sequences are clustered together based on a defined level of similarity.

spoilage of alcoholic beverages. Ethanol concentration in silages was not evaluated in this thesis but Randby & Bakken (2021) showed that silages with the same acid treatment as in this thesis often contain more ethanol than silages subjected to other additive treatments. The literature on *Lactobacillus fructivorans* in relation to silage is scarce, but Wu & Nishino (2016) concluded that it does not grow well on MRS agar and grows better on liver-infused sake agar, which could explain the lack of literature on the species. However, a more recent study by Bayat *et al.* (2023) observed the genus *Fructilactobacillus* in higher proportions in acid-treated silages and feed mixes produced from these. This genus is new and is proposed in the re-classification of *Lactobacillus* by Zheng *et al.* (2020), in which the type species for this new genus is *Lactobacillus fructivorans*. With this new classification, future studies might be able to determine why this species is found in relation to acid types of silage additive.

5.2 Microbiota in feed, bedding material and bulk milk (Paper **II**)

Paper **II** sought to determine the point in the dairy value chain at which bacteria, and specifically NSLAB, potentially enter the system. As Paper **I** showed a strong effect of silage additive on the final silage microbiota, similar silage additives were used in Paper **II**. The main objective was to evaluate whether the observed differences in silage microbiota were maintained all the way to the raw milk.

5.2.1 Herbage analyses confirmed previous findings

The herbages used for ensiling came from 14 fields around the research farm in Röbäcksdalen, with in total more than 200 ha harvested to fill the four silos. The average microbiota in 75% of these fields is presented in Figure 7. Interestingly, the variation between the fields was in most cases rather minor (Paper **II**). The main genera were *Sphingomonas* and *Xanthomonas,* followed by *Pedobacter* and *Pseudomonas*. It was interesting to observe higher RA of *Xanthomonas* in these fields, as Röbäcksdalen was the only field station in Paper **I** to show major presence of this genus. It was hypothesised that the variant detected could be a pathogenic *Xanthomonas* species causing *e.g.* leaf streak in cereals that overwinters in straw or other grass species, as discussed by Sapkota *et al.* (2020). The findings in Paper **II**

supported this hypothesis. Further, the proportion of LAB in the herbages was low and enumeration of lactobacilli showed an average concentration of 4.3 $\log_{10}$ CFU/g, which is similar to the level in Paper **I**.

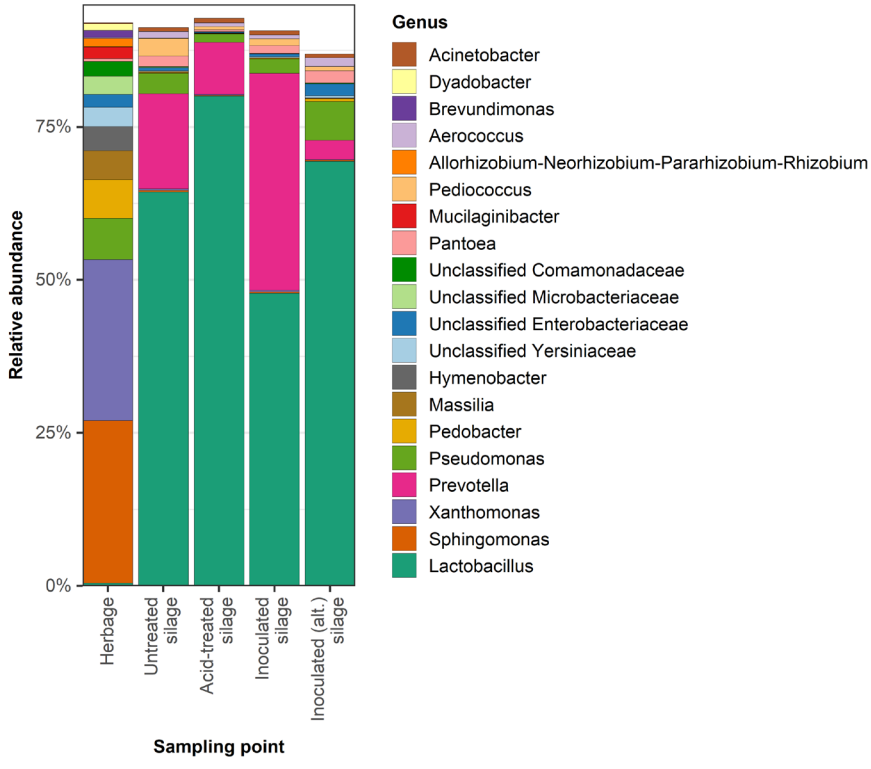

Figure 7. Summary of the 20 most abundant genera in herbage and silages in Paper **II**.

5.2.2 Silage additives behaved differently in bunker silos

The silages, on the other hand, did not turn out as expected, since the microbiota mainly comprised *Lactobacillus*, *Prevotella* and *Pseudomonas* (Figure 7). When the silages were further evaluated on ASV level, the effect of silage additive was more evident, but still not as clear as in Paper **I**. As in Paper **I**, *Lactobacillus fructivorans* was observed at high RA in acid-treated silage in Paper **II**, which further highlights the connection between this bacteria and silages made with acid additives. It is unclear why a strong effect of additives was not observed, but differences between the ensiling processes (see Table 2) probably played a role.

Table 2. Differences between the ensiling processes in Papers **I** & **II**.

	Paper I	**Paper II**
Cutting	Electric hack-saw	Disc mower conditioner
Wilting	None	Few hours
Collection	By hand	By pick-up wagon
Soil contamination	Minor	Potentially major
Type of silo	Sterilised glass jar	Concrete bunker silo
Treatment application	Spray bottles	Nozzles inside wagon
Filling	Manual with sterile gloves	Tractor with scoop
Packing	Sanitised piston-press	Tractor driving on herbage
Sealing	Sanitised water-lock lids	Plastic film and sand bags
Ensiling process*	Few hours	1-2 days
Ensiling conditions	Climate controlled room	Varying with climate
Fermentation time	100 days	180, 210 or 240 days
Usage after opening	Once	Multiple

*Time from start of harvesting to sealed silage silo.

The major proportion of *Prevotella* in the silages was a surprising finding, since the genus was not observed in Paper **I** and is rarely reported in the literature in relation to grass and clover silage. One theory is that the rumen microbiota somehow contaminated the environment in close proximity to the farm, and thereby also the silages. A study by Jami *et al.* (2013) of rumen microbiota in growing cattle found that already at two months of age, *Prevotella* is the dominant genus in the rumen. Similarly, Stevenson & Weimer (2007) observed high RA of *Prevotella* in rumen samples from lactating cows. By coincidence, Krizsan *et al.* (2023) studied rumen samples from the same cows as in this thesis two months earlier and also observed higher RA of *Prevotella*. However, as the most abundant *Prevotella* ASVs in this thesis were aligned with all sequences belonging to *Prevotellaceae* in their study, the closest match still differed in a few base pairs. This means that the *Prevotella* in the silage in this thesis did not originate from the rumen of the cows, or that the rumen microbiota shifted due to changes in feeding between the studies, favouring other species of *Prevotella*, as shown in the feeding trial by Chai *et al.* (2024).

5.2.3 Raw milk microbiota showed little resemblance to that in feedstuffs, but some to that in used bedding material

When the silages were fed to the dairy cows, the raw milk microbiota did not show any clear differences between the treatments. The conclusion was that the different silages had no effect on the microbiota of the raw milk (Paper **II**). However, as silage additives did not affect the silage microbiota as expected, an effect on raw milk microbiota could possibly be observed with silages showing more diverse microbiota.

To further understand the microbiota of the different materials in the dairy value chain, principal coordinate analysis (PCoA)[13] with weighted UniFrac distance matrix[14] was performed (Lozupone *et al.*, 2007). The PCoA was combined with measures of bacterial diversity (Faith's phylogenetic diversity index[15]), as presented in Figure 8. The results showed three clusters; i) herbage, ii) silage and PMR, and iii) used bedding material and raw milk. The values obtained for concentrate and rapeseed meal, which were mixed with the silage to make the PMR, were more spread out, as were those for the wood shavings used as bedding material. The tight clustering of silage and PMR indicated that the microbiota showed little difference between materials, despite the inclusion of concentrate and rapeseed meal. The long distance from the silage and PMR cluster to the herbage cluster (Figure 8) indicated that these materials shared very few bacteria. The raw milk and used bedding material clustered closely, but not as closely as silage and PMR, which means that these materials shared bacteria to some extent. In terms of bacterial diversity, raw milk was the most diverse, followed by used bedding material.

[13] Visualisation of similarities or differences between samples based on a distance matrix. It reduces complex data into a few dimensions, allowing patterns and relationships among samples to be easily interpreted.

[14] A distance matrix that takes into consideration the phylogenetic relationship, composition (presence/absence) and abundance of bacteria.

[15] Measure of the total evolutionary branch length spanned by the species in a community, capturing both species richness and their phylogenetic relationships (Faith, 1992).

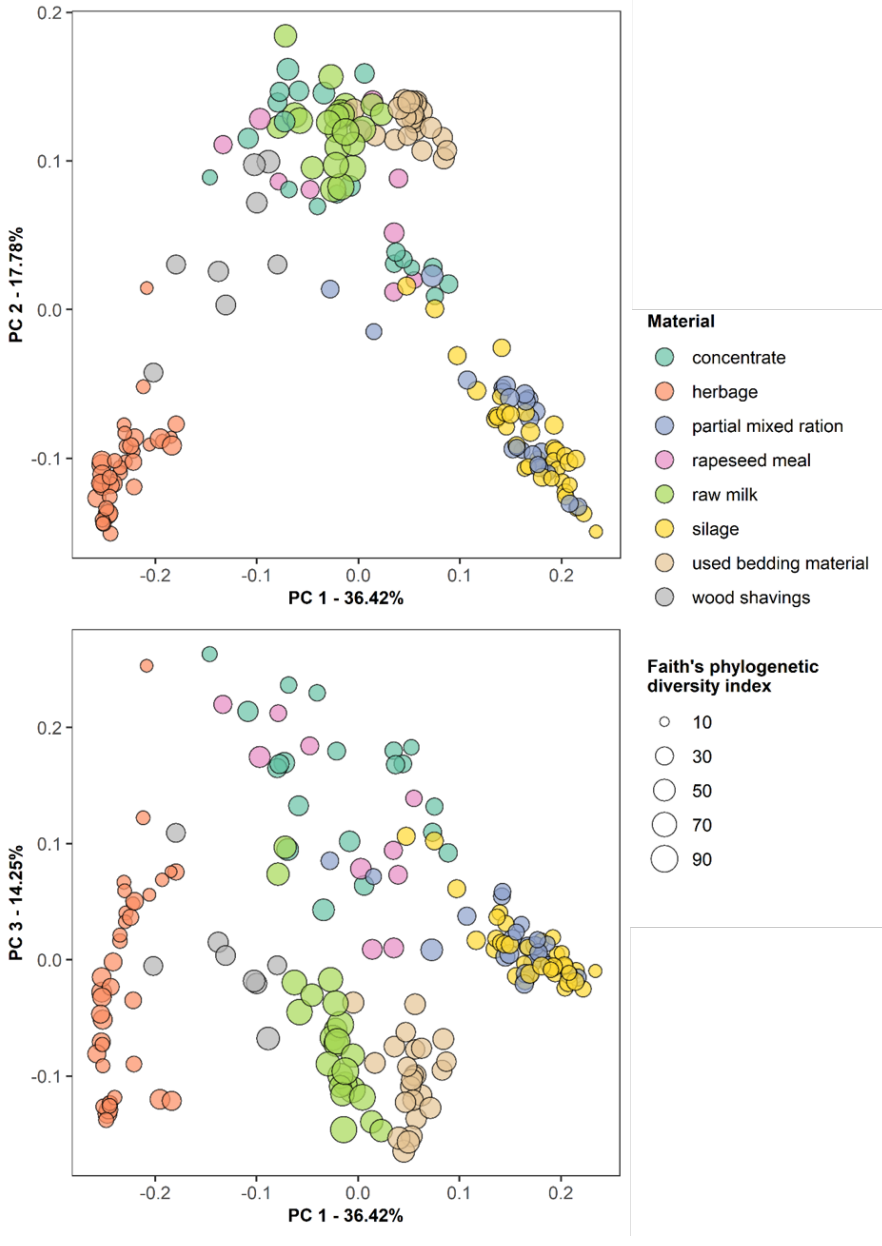

Figure 8. Principal coordinate analysis plots of the weighted UniFrac distance matrix for all samples in Paper **II**, coloured by material. The plots are combined with diversity metrics in the form of Faith's phylogenetic diversity index, shown by dot size.

The most abundant bacterial genera in all materials, except herbage, are summarised in Figure 9, together with the bacterial enumeration results. The proportion of LAB in relation to non-LAB correlated relatively well to the bacterial enumeration results. The silage and PMR were dominated by LAB and showed higher numbers of lactobacilli compared with total aerobic bacteria, while the opposite was observed for the other materials.

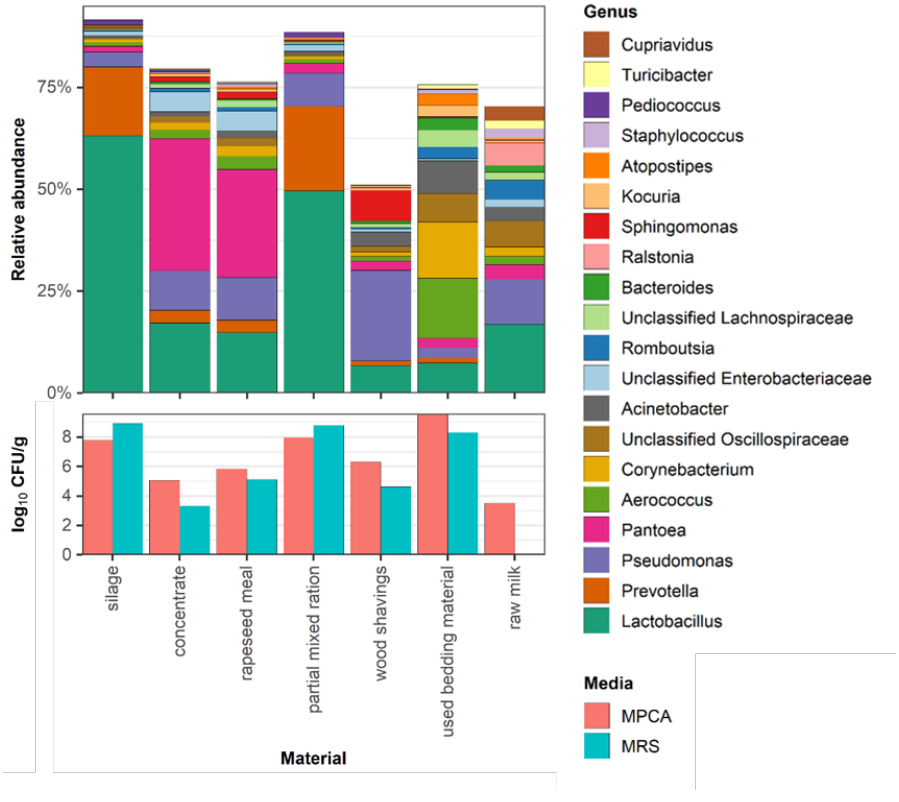

Figure 9. The 20 most abundant genera in all materials analysed in the feeding trial together with the results from the bacterial enumeration on different media.

The PMR showed close resemblance to the silage, with *Lactobacillus*, *Prevotella* and *Pseudomonas* being the most abundant genera. In comparison, Bayat *et al.* (2023) observed a more major shift in microbiota as silage was mixed with concentrate (dried barley). The concentrate and rapeseed meal were dominated by *Pantoea*, *Lactobacillus* and *Pseudomonas*. The finding of *Lactobacillus* was surprising, as the materials were dry (~87%

dry matter), but was confirmed by enumeration of lactobacilli (3.3 and 5.1 $\log_{10}$ CFU/g for concentrate and rapeseed meal, respectively). In the study by Bayat *et al.* (2023), *Lactobacillus* was not observed in the dried barley.

The main genus in wood shavings was *Pseudomonas* and the bacterial load was surprisingly high for such a dry material, especially total aerobic bacteria (6.3 $\log_{10}$ CFU/g). For comparison, Ferraz *et al.* (2020) reported 5.2 $\log_{10}$ CFU/g in wood shavings from Slovenia. The used bedding material and raw milk shared many genera, but in different proportions. *Aerococcus* and *Corynebacterium* were more frequent in the used bedding material, while *Lactobacillus* and *Pseudomonas* were more frequent in the raw milk. However, is important to consider the bacterial load when comparing the microbiota of these two materials, as total aerobic bacteria amounted to 9.6 $\log_{10}$ CFU/g in used bedding material, but only 3.5 $\log_{10}$ CFU/mL in the raw milk. Thus 1% of the microbiota in the used bedding material corresponded to ~10,000 times all bacteria in the raw milk. The low bacterial load in milk was surprising, but other studies have observed similar numbers, *e.g.* Glantz *et al.* (2020) reported 3.6-4.1 $\log_{10}$ CFU/mL and Sun *et al.* (2022) reported 3.5-5.0 $\log_{10}$ CFU/mL in milk samples collected on farms throughout Sweden. The *Prevotella* found in the silages was detected in all materials, except the raw milk. This was surprising, as Quigley *et al.* (2013) reported this genus in both raw and pasteurised milk.

5.2.4 The issue of low bacterial load and high diversity

The low bacterial load in raw milk in combination with very high bacterial diversity was troubling, as it raised questions regarding whether the few bacteria were represented correctly and whether the high diversity was an indication of bias from background contamination. Low bacterial load in raw milk and the implications for microbiota research is a known problem and filtering of the sequence data has been suggested as one mitigation approach (Dahlberg *et al.*, 2019). Other methods have also been proposed, although the need to evaluate the effectiveness and bias of the these methods has been highlighted (Marsh *et al.*, 2018). In addition to handling existing contaminants, another approach is to reduce their presence, *e.g.* Kurokawa et al. (2023) observed reduced contamination with addition of agar in the cell lysis step of the DNA extraction process. In this thesis, filtering was performed to remove *e.g.* sequences only occurring in few samples and at low abundance.

5.2.5 Bacterial transfer to raw milk mainly derived from used bedding material

The raw milk microbiota shared many bacterial genera with that in used bedding material and partly also other materials (Figure 9). However, one genus can contain many different bacteria, and therefore the most abundant ASVs in all materials, divided into non-LAB and LAB (order *Lactobacillales*), were evaluated further in a heatmap (Figure 10).

In comparison with the heatmaps in Paper **II**, this ASV comparison was performed on all materials. Thus, a better picture of the key bacteria in the materials and a better understanding of potential bacterial transfer were obtained. *Pantoea* (0951f), *Pseudomonas* (3973d) and unclassified *Enterobacteriaceae* (8622f) were clearly observed in all materials. Detection of *Pantoea* (0951f) is interesting, as it was one of the few ASVs showing a strong tendency to be more abundant during one of the silage treatments (acid additive, effect not significant; Paper **II**). Bacteria belonging to the genus *Pantoea* can potentially affect processes at the dairy, as it has been found in pasteurised milk (Masiello *et al.*, 2016). *Pseudomonas* (3973d) was the most abundant ASV in raw milk. This was unsurprising since, according to Yuan *et al.* (2019), studies on psychrotrophic bacteria in raw milk from around the world have found *Pseudomonas*. Interestingly, the drier materials, *i.e.* wood shavings (bedding), concentrate and rapeseed meal, showed the highest proportions of this ASV.

Many of the other ASVs found in the raw milk were also found in higher proportions in the used bedding material. Among these, *Aerococcus* (0cb4d) is closely related to the mastitis bacteria *Aerococcus viridans*, which has been found previously in cow bedding (Saishu *et al.*, 2015). The only potential NSLAB found in the raw milk were *Lactobacillus* (172f4), *Lactobacillus intermedius* (1646a) and *Lactococcus lactis* (dc6c4), but these were rarely found at all in the other materials analysed. In addition to these, *Lactobacillus* (6b62e) and (8427e) were found in the feedstuffs, but at such low RA in the raw milk that, when the bacterial load is considered, these could derive from contamination.

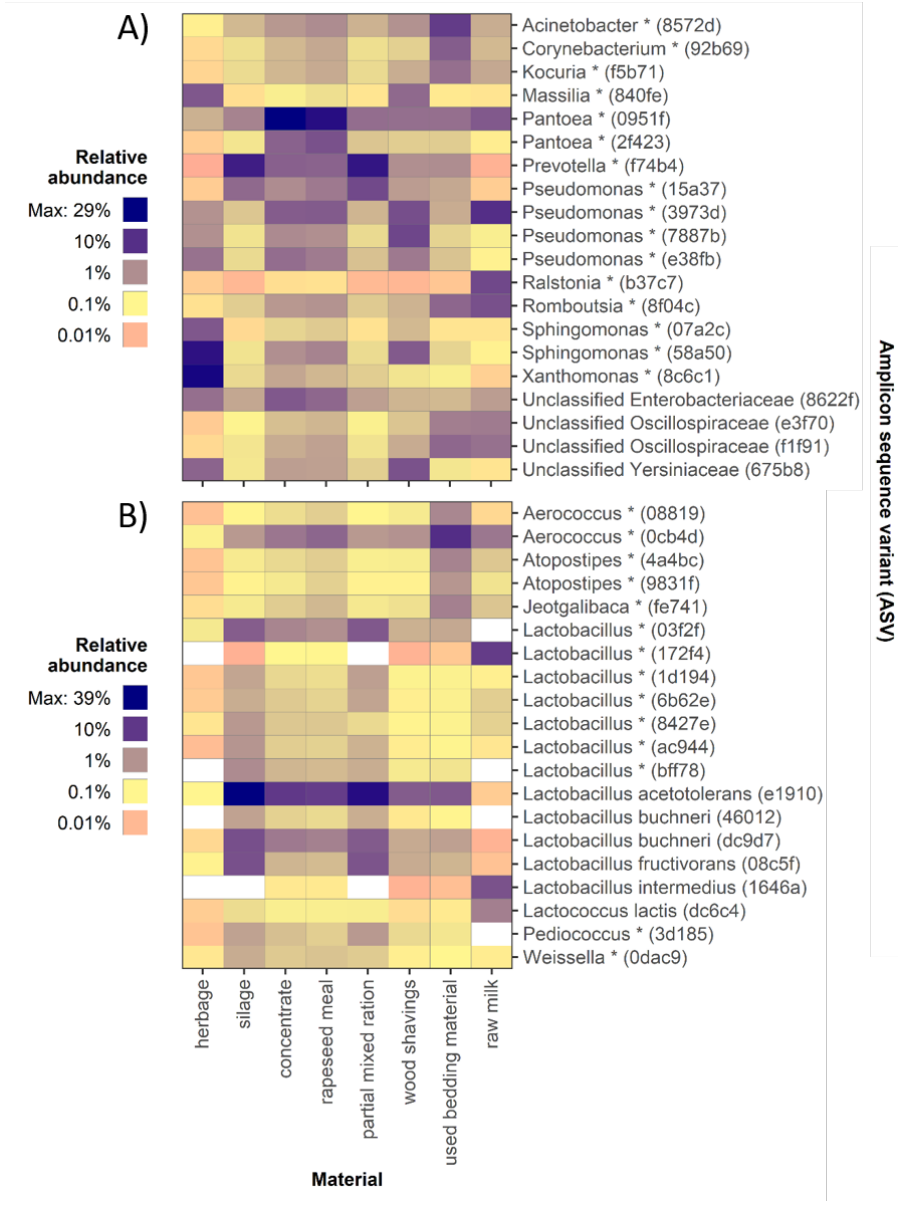

Figure 10. Heatmaps of the most abundant amplicon sequence variants in all materials analysed in Paper **II**. A) Top 20 non-lactic acid bacteria, and B) top 20 lactic acid bacteria (order *Lactobacillales*). The data were $\log_{10}$-transformed before plotting and converted back to relative abundance in the legends for easier interpretation.

5.3 Microbiota in raw milk and the resulting cheese (Paper **III**)

The work in Paper **III** was performed in parallel with that in Paper **II**, and started with the raw milk leaving the farm and entering the dairy facility. The main objective was to evaluate whether any variation in the milk microbiota from Paper **II** affected the properties of the resulting cheese. As this experiment was performed in direct proximity to the feeding trial in Paper **II**, the effect of feeding on raw milk microbiota was unknown.

5.3.1 Raw milk microbiota changed after leaving the farm

A comparison of raw milk ASVs from Papers **II** & **III** was performed by using the method described in section 4.4.5, to evaluate whether any changes occurred during transport. The comparison included milk from the bulk tank on the farm and in the tanker truck at arrival at the dairy facility (Figure 11).

Interestingly, an almost complete change was observed. Two of the potential NSLAB observed in Paper **II**, *Lactobacillus* (172f4) and *Lactobacillus intermedius* (1646a), were not found in the raw milk samples from the tanker truck in Paper **III**. *Lactococcus lactis* (dc6c4) was found, but at much higher RA, together with two new ASVs, *Lactobacillus nenjiangensis* (5f86a) and *Leuconostoc* (4b97e). A tendency for more LAB in raw milk from the repeated inoculated silage treatment was observed in Paper **II** and also in Paper **III**, but with different LAB in the latter. *Pantoea* (0951f), which showed a tendency for a treatment effect (acid-treated silage) in Paper **II**, was not observed after transport in Paper **III**.

A major change in the microbiota of raw milk during transport has been observed in previous studies. For example, Kable *et al.* (2016) observed changes in microbiota between dairy silos and tanker trucks delivering the raw milk to these, with the order *Lactobacillales* (including *Lactococcus*) showing a clear increase as milk was transferred to the dairy silos. Falardeau *et al.* (2019) observed major changes in the bacterial genera present in raw milk on the farm, compared with the dairy facility, with *Lactobacillus* and *Lactococcus* being present only after transport. Similarly, De Souza *et al.* (2024) evaluated the presence of NSLAB in raw milk sampled from farm tank, tanker truck and dairy silo and found low 2.3 $\log_{10}$ CFU/mL in the farm tank, but increasing levels in the tanker truck to reach 4.4 $\log_{10}$ CFU/mL in the dairy silo.

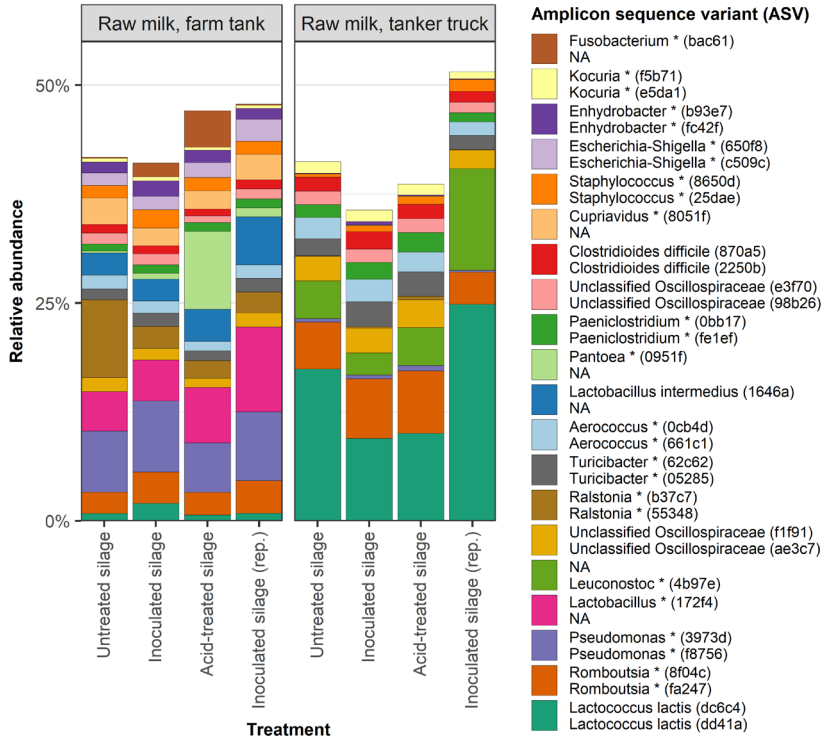

Figure 11. Comparison of most abundant amplicon sequence variants in raw milk from the farm bulk tank (Paper **II**) and raw milk from the tanker truck (Paper **III**). Values shown are means for each treatment. The legend shows the taxa for each ASV from Paper **II** (top) and Paper **III** (bottom), with "NA" indicating not found.

The average bacterial load was not affected during transport, with total aerobic bacteria count of 3.4 $\log_{10}$ CFU/mL. The similar bacterial load in Papers **II** & **III** indicates that transport of raw milk did not cause any major contamination. In comparison, Glantz *et al.* (2020) studied various raw milk collection occasions throughout Sweden and observed 3.6-4.1 $\log_{10}$ CFU/mL on-farm, but 4.2-5.3 $\log_{10}$ CFU/mL at dairy facilities.

Since there was no change in bacterial load, the difference in bacterial composition between Paper **II** & **III** is confusing. One possible reason could be the sampling procedure, as raw milk was sampled from the top of the bulk tank in Paper **II** and from the bottom of the tanker truck in Paper **III**. This could potentially lead to sampling of different fractions of the raw milk, as sedimentation occurs over time. Moreover, Sun *et al.* (2019) showed that the microbiota differs between the skim milk and cream fractions.

5.3.2 The cheese microbiota was dominated by the starter culture

To identify the most abundant bacteria in all steps, from raw milk in the tanker truck to the final cheese after ripening, a summary was performed on ASV level (Figure 12).

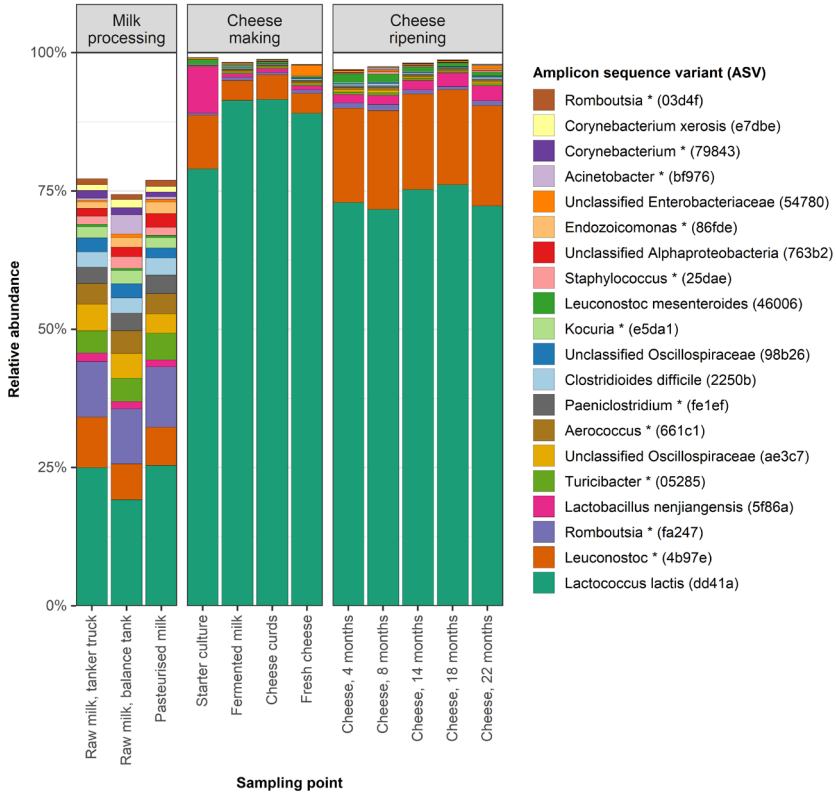

Figure 12. Summary of the 20 most abundant amplicon sequence variants in all samples obtained at the dairy facility in Paper **III**, grouped by processing step.

The microbiota showed a complete change after the starter culture was added to the pasteurised milk, with the two ASVs *Lactococcus lactis* (dd41a) and *Leuconostoc* (4b97e) dominating the cheese making process. The proportion of these two ASVs shifted during cheese ripening, while two other ASVs were also detected. *Lactobacillus nenjiangensis* (5f86a) at increasing RA during ripening and *Leuconostoc mesenteroides* (46006) at higher RA during the early stages of ripening. The first three ASVs were all found at high RA in the starter culture, while *Leuconostoc mesenteroides* (46006) was found,

but at lower RA. This is difficult to explain, as the starter culture should not have contained a *Lactobacillus*. The starter culture used was mesophilic and contained the following bacteria at undefined proportions: i) *Lactococcus lactis* ssp. *lactis*, ii) *Lactococcus lactis* ssp. *cremoris*, iii) *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*, and iv) unspecified *Leuconostoc*. The same batch of starter culture was used throughout the work in Paper **III** and three full envelopes were used in analysis of its composition, in which a consistent finding of the *Lactobacillus* was obtained.

Another finding that is difficult to explain is the presence of starter culture ASVs in the raw and pasteurised milk. One theory is that the bacteria in the starter culture, or similar strains of these bacteria, are part of the house flora of the dairy and their trucks. This is supported by Somers *et al.* (2001), who evaluated the survival of NSLAB in biofilms during cleaning and detected these bacteria on various surfaces at a dairy facility. They also found that NSLAB were persistent and contaminated future batches of cheese. In addition, the dairy environment housed various NSLAB, *e.g. Lactococcus lactis* and many *Lactobacillus* species (Somers *et al.*, 2001). Similarly, Kable *et al.* (2019) evaluated the effect of cleaning practices on the microbiota found at various processing steps in a dairy facility and found that a prolonged equipment standing time after cleaning affected the microbiota, which could be the case in *e.g.* the tanker truck used in Paper **III**.

The bacterial enumeration results and pH at all sampling points in the dairy are summarised in Table 3. Considering the low numbers of bacteria in raw and pasteurised milk, the theory of contamination as milk enters the dairy environment is not impossible, as only a few bacteria are needed to cause a shift in the microbiota. Further evaluation showed that both total aerobic bacteria and psychrotrophic bacteria counts were reduced by the pasteurisation step, but not completely eliminated. Estimation of lactobacilli was unfortunately not performed for milk samples. The numbers of bacteria in cheese decreased during cheese ripening, with total aerobic bacteria starting to decrease from four months of age, while lactobacilli started decreasing from eight months of age. These results slightly contradict findings by Rehn *et al.* (2010), who evaluated the same type of cheese and found that lactobacilli on MRS reached a peak of ~8 $\log_{10}$ CFU/g in the first month and then dropped to ~7 $\log_{10}$ CFU/g throughout the trial (13 months), with some minor exceptions. The average pH of cheeses in their study was higher at six months (5.51) and reached 5.63 at 13 months, whereas a similar

increase was not observed in Paper **III**. The biochemical processes behind an increase in pH during cheese ripening are complex, but it is generally accepted that NSLAB utilise lactic acid produced by the starter culture LAB during early fermentation and contribute to proteolysis (McSweeney, 2004). As NSLAB proliferate and become a major part of the microbiota, an increased level of proteolysis, together with reduced lactic acid concentration and production of ammonia, typically leads to a rise in pH. In Paper **III**, the pH readings and LAB enumeration in cheeses indicated that NSLAB did not proliferate significantly during the sampled timespan, as the pH increase was minor or absent in combination with declining numbers of LAB at an early stage of ripening.

Table 3. Bacterial enumeration on various media (log_{10} CFU per mL or gram) and pH in all samples obtained at the dairy facility in Paper **III**

Sampling point	MPCA	MRS	Entero.	Psychro.	pH
Raw milk, tanker truck	3.5		0.5	2.4	6.71
Raw milk, balance tank	3.6		0.9	2.8	6.72
Pasteurised milk	2.6		1.3	1.4	6.70
Fermented milk			1.5		6.62
Cheese curds	6.4				6.15
Fresh cheese	6.6	*6.7	3.5		5.38
Cheese, 4 months	5.6	7.1			5.42
Cheese, 8 months	4.3	5.7			5.48
Cheese, 14 months	3.3	4.7			5.43
Cheese, 18 months	4.3	4.4			5.44
Cheese, 22 months	2.9	2.8			5.44

Entero; *Enterobacteriaceae*, Psychro; Psychrotrophic bacteria.

*Plated on calcium citrate agar.

5.3.3 Cheese ripening differences were batch-dependent

Multiple attempts were performed to assess whether the different treatments in Paper **II** affected the outcome of the cheese ripening process. A multiple PCoA analysis of the weighted UniFrac distance matrix in Paper **III** showed that raw milk from the inoculated silage treatment separated from samples from the other treatments, but this effect dissipated later in the ripening process. The cheese microbiota was summarised by selecting the 15 most abundant ASVs in all ages of all cheeses (Figure 13).

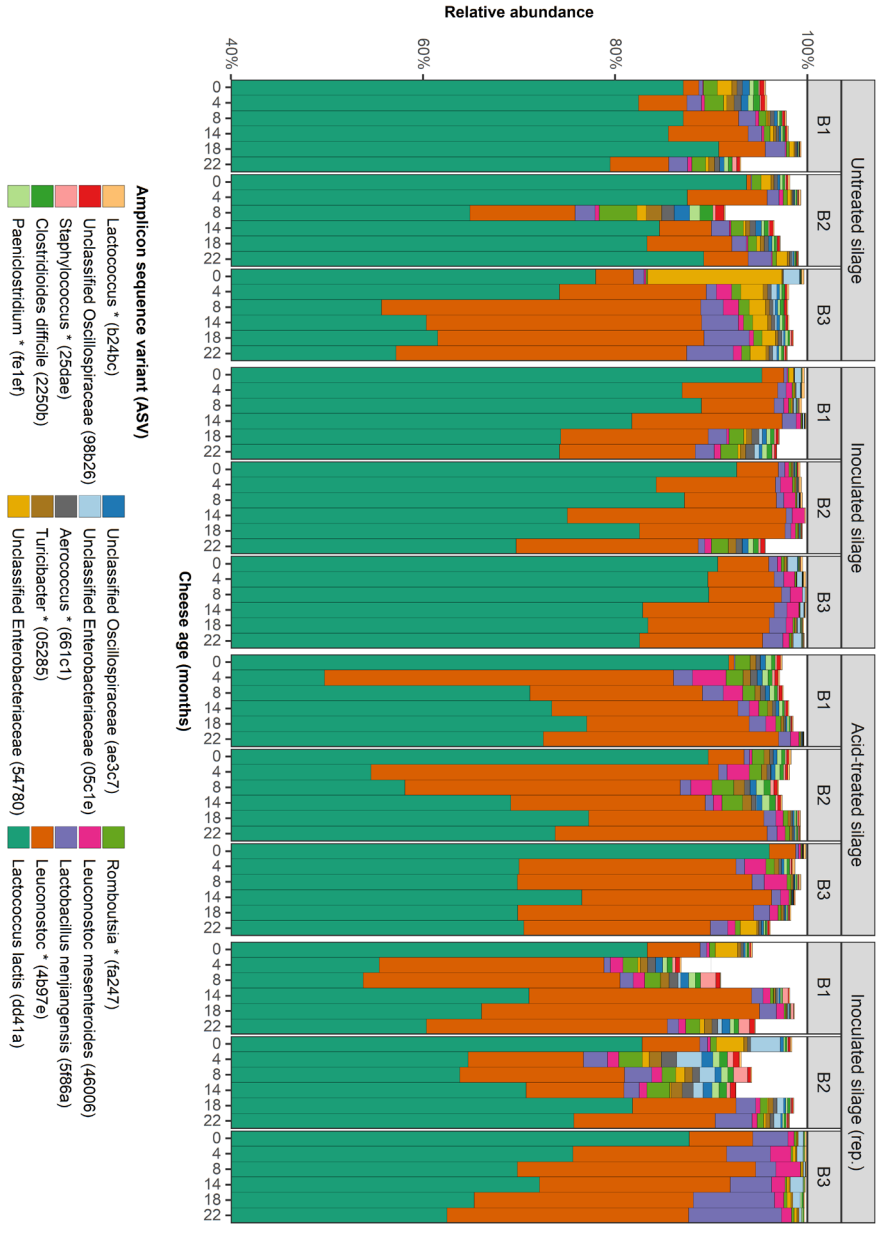

Figure 13. Summary of the 15 most abundant amplicon sequence variants in cheese during ripening, grouped by silage treatment and raw milk batch. Y-axis cut at 40% relative abundance for clarity.

Lactococcus lactis (dd41a) clearly dominated the cheese ripening process (>70% RA) in the beginning, while decreasing slightly in favour of *Leuconostoc* (4b97e) and *Lactobacillus nenjiangensis* (5f86a) as the ripening process proceeded. One reason for the slow start of *Leuconostoc* could be a competition for substrate (*e.g.* amino acids), in which the *Lactococcus* has an advantage due to its rapid growth, as suggested by Bellengier *et al.* (1997). Bacterial enumeration of fresh cheese samples was performed on calcium citrate agar, as the medium can be used to differentiate certain LAB, but *Leuconostoc* was not detected. However, an issue with the calcium citrate agar method is the high sample dilution, as the other LAB investigated are found at very high concentrations compared with *Leuconostoc*.

Only minor variation was observed as ripening proceeded, with the exception of batch B3 produced in the untreated silage treatment and batch B3 produced in the repeated inoculated silage treatment, where *Lactobacillus nenjiangensis* (5f86a) clearly increased throughout the ripening process. In addition, *Leuconostoc mesenteroides* (46006) was generally found at higher RA in 4-month-old cheeses and tended to decline towards the end of the ripening process. Two unclassified *Enterobacteriaceae* (54780 and 05c1e) were also found at rather high RA in the fresh cheese of some batches, especially B3 in the untreated silage treatment and B2 in the repeated inoculated silage treatment. The higher RA of these two ASVs in fresh cheese explains the increase in *Enterobacteriaceae* from pasteurised milk to fresh cheese (Table 3 and Paper **III**). The more abundant occurrence of *Enterobacteriaceae* in some batches could be explained by contamination from insufficiently cleaned cheese making equipment. For example, Cherif-Antar *et al.* (2016) investigated stainless steel surfaces after cleaning in a dairy facility and found various bacteria, including *Enterobacteriaceae*.

The relatively minor changes in microbiota during cheese ripening correlates well with the lack of pH increase normally caused by proliferation of NSLAB during cheese ripening, as discussed earlier in this chapter. A shift in microbiota was observed by Barzideh *et al.* (2022), who evaluated the microbiota of long-ripened Cheddar produced with a starter culture containing *Lactococcus lactis* and observed a major decrease in *Lactococcus* spp. and a major increase in other LAB genera, mainly *Lacticaseibacillus* spp. (up to 75% RA) as cheeses ripened. Those authors also compared non-treated and PMA-treated bacterial cells and found similar results when DNA

from dead bacteria was removed. However, it is not clear why NSLAB were not observed to a major extent in the cheeses analysed in Paper **III**.

The main variation observed in cheeses was between batches, which implies that the final cheese microbiota was mostly affected by factors acting after the milk entered the dairy facility, or factors not analysed in this thesis. The batches of cheese produced were evaluated by a taste panel, but no correlation to the microbiota was found. The amino acid composition of 22-months-old cheese was determined but not evaluated further in relation to the microbiota or other variables analysed, due to time limitations.

The main conclusion in Paper **III** was that the raw milk showed little variation and differed to a major extent from the samples taken a few hours previously at the farm. The cheese-making process seemed to be the major driver of the cheese microbiota, as most variation was found between batches without any clear connection to on-farm factors. The microbiota in the final cheeses mainly comprised the starter culture bacteria, with some exceptions.

5.3.4 Major differences in microbiota with different methods

Use of regular PCR for amplification of bacterial DNA has a major drawback in that dead bacteria or remnants of these are also amplified (Nocker *et al.*, 2007). In Paper **III**, a comparison was performed between amplification of DNA with and without PMA treatment, using cheese samples collected at 22 months of age. The results looked rather different when DNA from dead bacteria were filtered out, as shown in Figure 14.

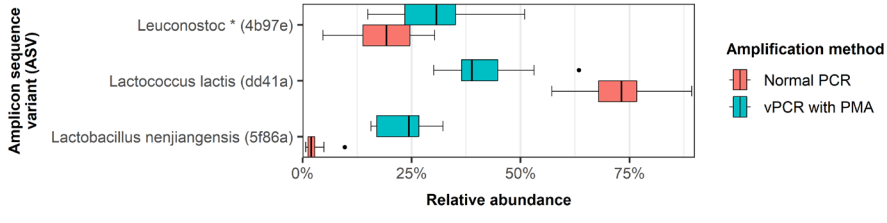

Figure 14. Boxplot comparison of DNA amplification methods, regular polymerase chain reaction (PCR) and viability PCR (vPCR) with propidium monoazide (PMA). The three most abundant amplicon sequence variants in cheese at 22 months were compared.

As can be seen in Figure 14, *Lactococcus lactis* (dd41a) was clearly over-represented when dead bacteria were not filtered out before amplification of DNA, while *Lactobacillus nenjiangensis* (5f86a) and *Leuconostoc* (4b97e)

were under-represented. This discrepancy is very important to consider, since only live bacteria contribute to further changes in the cheese ripening process. This is of course not of great value at a late stage of cheese ripening, as the numbers of bacteria are low, but if applied to samples taken earlier in the process it could provide valuable information on the direction in which ripening is heading. For greater accuracy, the PMA treatment should have been applied throughout this thesis, not only with the samples collected during cheese ripening, but also with milk collected at different processing stages, and especially the samples before and after pasteurisation.

5.4 The full dairy value chain (Papers **II** & **III**)

The individual papers analysed the microbiota of various materials and sampling points at the farm and the dairy separately. In this section, data from both Papers **II** & **III** are merged to evaluate the potential flow from field to cheese. The method used is described briefly in section 4.4.5.

In simple terms, all sequences obtained in Papers **II** & **III** were merged to a master table using multiple sequence alignment. The resulting table was used to identify the most abundant ASVs in the cheese at 22 months, and their corresponding RA at all other sampling points of the dairy value chain. The results are presented in Figure 15, together with the unique ASV IDs from Papers **II** & **III** to facilitate comparison with previous findings.

From the results, it is clear that most bacteria in the cheese derived from the dairy environment, from raw milk in the tanker truck onwards. Two of the main ASVs in the cheese, *Leuconostoc* (4b97e) and *Lactobacillus nenjiangensis* (5f86a), were not detected at all in the farm samples, *i.e.* in raw milk at the farm or in previous samplings. These findings further strengthen the conclusion that the forage microbiota is of little importance for the final cheese. *Lactococcus lactis* (dc6c4 | dd41a) was found in the raw milk at the farm, but at low RA. However, this ASV matched with many different *Lactococcus* sequences found in the 16S rRNA sequence database at NCBI (Zhang *et al.*, 2000) so it cannot be concluded that the exact same bacteria were present in both samples.

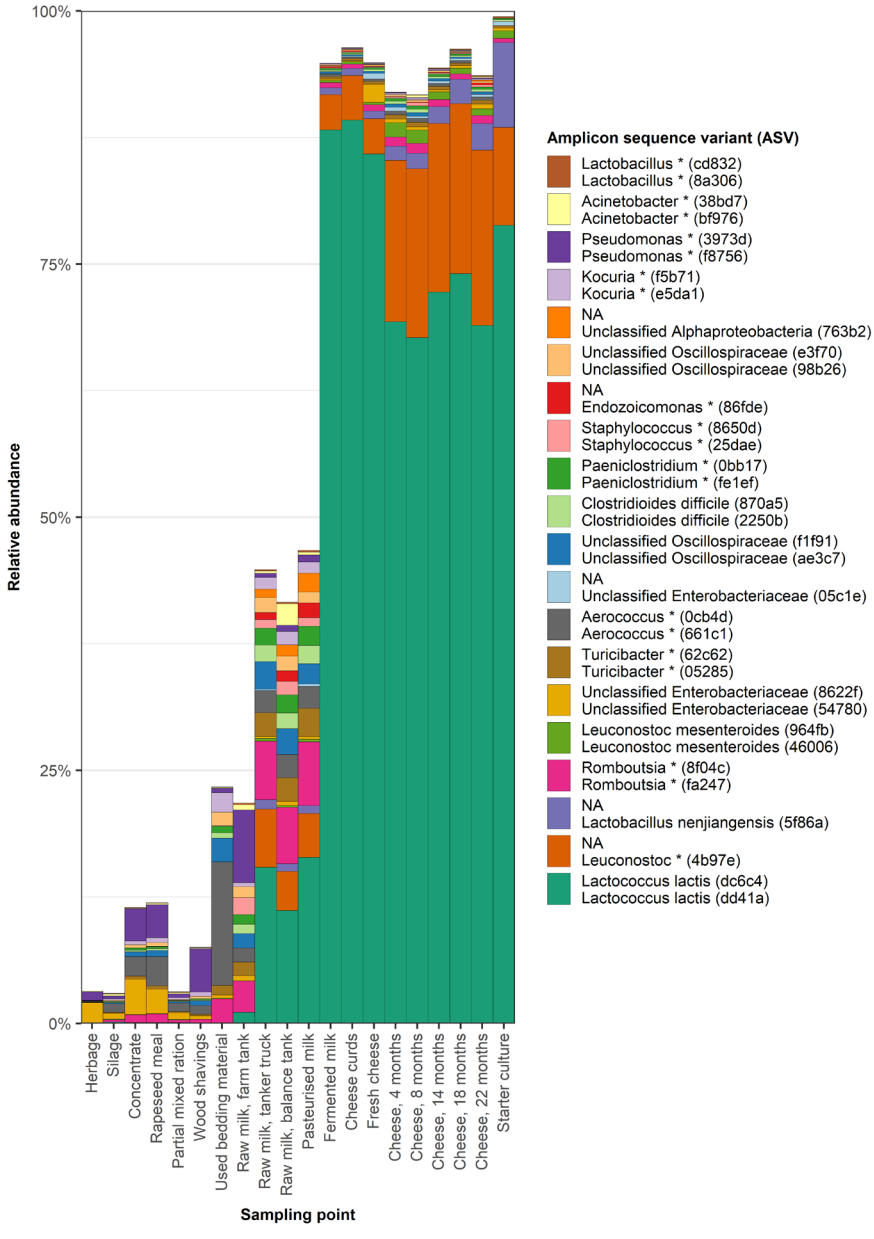

Figure 15. Comparison of the 20 most abundant amplicon sequence variants (ASVs) in cheese at 22 months and their occurrence in the other samples analysed in Papers **II** & **III**. Values shown are means for each sampling point. The legend show the taxa for each ASV from Paper **II** (top) and Paper **III** (bottom), with "NA" indicating not found.

6. Concluding remarks

The hypothesis that forage can alter the microbiota of cheese during ripening can be discarded based on the results presented in this thesis. Feeding dairy cows with silages produced using different additives did not affect the microbiota of either the raw milk or the cheese. In addition, transfer of bacteria from the farm to the dairy was rarely observed. The bacteria found in the final cheese were mostly present in the starter culture used in the cheese making process. However, differences between cheese batches were observed, which indicates that factors in the dairy environment most probably had an effect on the final cheese. These factors were not identified, but could be related to variations in *e.g.* equipment cleaning, presence of "house flora" or the cheese making process itself.

Another novel finding was that herbage and the resulting silage only had minor bacteria in common, with the microbiota of silage mainly comprising LAB and that in herbage mostly non-LAB. Use of silage additives allowed the ensiling process to be driven in a more favourable and predictable direction, especially when the additive comprised inoculation with a starter culture. However, the effect of silage additives was less clear in large-scale bunker silos compared with laboratory-scale glass silos.

The raw milk microbiota showed the strongest resemblance to the microbiota in used bedding material (based on wood shavings). Despite this similarity in composition, the bacterial load was extremely different, with used bedding material containing >1,000,000 times more bacteria per gram than raw milk.

6.1 Future research directions

The origin of NSLAB present in the dairy value chain is still unknown. In future research, other environmental sampling points should be included, especially in relation to the potential "house flora" at the dairy facility. This should include evaluation of airborne bacteria (*e.g.* dust sampling and surface swabs) and potential biofilm present in equipment in contact with the raw milk. This could probably also help to explain why the microbiota of the raw milk changed during transport between the farm and the dairy.

A short-coming of the work in this thesis was the use of short-read sequencing, as this method only gives limited information for differentiation of bacteria beyond genus level (in rare cases species level). More information could be obtained by using a different sequencing approach, *e.g.* sequencing additional regions of the 16S rRNA gene or other genes that could aid in further differentiation of bacteria. Another option is to change sequencing method to obtain longer reads, potentially the full 16S rRNA gene or even some full genomes. In addition, the PMA method should be used for as many samples as possible, as DNA from dead bacteria otherwise skew the analysis, as shown in both Porcellato *et al.*, (2015) and Barzideh *et al.*, (2022).

The bacteria in the starter culture did not seem to decrease much in proportion to other bacteria and there was no clear evidence of NSLAB developing during cheese ripening. *Lactococcus lactis* was still present and highly abundant in the older cheeses, which could be an indication of bacteriocin production. The composition of the starter culture was also different from the specification. It could therefore be informative to further investigate the starter cultures used in cheese making.

Another factor affecting the development of NSLAB could be the low bacterial load in raw milk, with so few of these important bacteria that they do not manage to proliferate later on in the cheese ripening process. One solution to mitigate the low occurrence of NSLAB could be to use adjunct cultures, containing bacteria known to contribute to the preferred cheese ripening, together with the starter culture. Di Cagno *et al.* (2006) used a Caciotta cheese model to evaluate various LAB and found that bacteria originating from sourdough could be suitable for improving cheese ripening. In a more recent study, Bettera *et al.* (2023) isolated strains of *Lacticaseibacillus* from raw milk designated for production of Grana Padano and found a few candidates that could benefit cheese ripening. In the case of the long-ripened Swedish hard cheese analysed in this thesis, a similar

approach could be adopted, where isolated NSLAB from "good" cheeses or raw milk used in their production are tested as adjunct cultures in ordinary production.

Finally, the work in this thesis mainly focused on the microbiota, points at which it enters where in the dairy value chain and the extent of transfer between selected sampling points. However, to fully understand the role of the microbiota in the steps from field to cheese, determining the numbers and composition of the bacteria is not enough. Instead, Afshari *et al.* (2020) suggest an approach they call "cheesomics", which involves the use of a combination of methods to deepen understanding of the microbiota present during cheese ripening. These methods includes analysis of gene expression (transcriptomics), protein synthesis (proteomics) and metabolic processes (metabolomics) in microorganisms of any given sample. The use of "multi-omics" approaches is also suggested by others, such as Jiang *et al.* (2023), and is perceived as a way forward in furthering understanding of what happens when milk becomes cheese.

6.2 Practical implications

There are some practical implications from the findings in this thesis. First, silage additives can be used to drive the microbiota of grass-clover silages in a certain direction, especially if a LAB starter culture is used (Paper **I**). Second, making silages intended for dairy cows without using an additive, with an acid additive or with inoculation with a starter culture does not affect the microbiota of the raw milk (Paper **II**). Third, cheese making is mainly affected by processes at the dairy facility and especially the starter culture used in production, while the raw milk used is not markedly affected by dairy cow feeding and does not affect the cheese ripening process (Paper **III**).

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Popular science summary

Swedish agriculture has a long tradition of producing high-quality cheeses with a good reputation and a high level of trust among chefs and consumers. Much of the milk produced in Sweden is converted into different types of cheese with unique properties and flavours, with long-ripened hard cheeses occupying a special position on the market. Increased knowledge of factors that contribute to variations in the milk value chain for hard cheese, from forage crops in the field, via the cow and the dairy to the consumer, is important for the Swedish dairy industry. One of the factors identified as important for development of the flavour and character of long-ripened hard cheese is the naturally associated microbiota of bacteria originating in fields, animal houses and the so-called house flora in commercial dairies.

The microbiota in cheese mainly originates from the starter culture added after pasteurisation of the milk. However, a small part of the natural microbiota in the raw milk survives pasteurisation, develops during cheese ripening and contributes to the taste and maturity of the cheese. A group of bacteria that are particularly important in this context are the lactic acid bacteria. Some of these are associated with the natural microbiota on leaves and stems of forage plants such as grass and clover, so the forage could contribute to variations in the presence of lactic acid bacteria in raw milk and in the resulting cheese. Microbiota composition can be analysed with different methods, such as traditional cultivation of bacteria on different types of agar or molecular techniques such as sequencing of DNA. Both types of methods were used in this thesis, for different purposes.

The work began with analysis of the microbiota on common forage plants and in the resulting silage. For this, forage samples from SLU's long-term field experiments, taken at four different locations in Sweden and during two growing seasons, were used. This plant material was later used in laboratory-

scale silos to produce three types of silages: without additive, with acid additive and with a lactic acid bacteria starter culture. The botanical composition of the plant material was found to vary between years, harvesting occasions and growing locations, but there were no clear structural relationships between the microbiota of the plant material and the silages resulting from the different treatments. In contrast, the different silages showed a clear pattern regarding the composition of their microbiota. Silages without an additive showed random bacterial composition with high diversity, while silages made with an additive were instead dominated by various lactic acid bacteria. Thus use of an additive in the ensiling process affected the composition of the microbiota in the forage more than the botanical composition of the forage.

In a next step, full-scale farm studies were performed in which the microbiota was examined from field to the finished cheese. The work began with silage production in bunker silos of the same types of silage as in the laboratory-scale silos, *i.e.* silage without additive, and with addition of acid or starter culture. The different silages were used in a feeding trial at Röbäcksdalen research farm, where they were included in feed mixtures for 67 dairy cows, with each type of silage fed to the animals for a 3-week period. The cows were milked twice daily in a milking parlour. Sampling for studies of the microbiota in silage, feed mixtures, used bedding material, and farm tank milk was carried out during the last week of each 3-week period. The results showed that the microbiota of the plant material in the field was different from the microbiota in all other materials on the farm or at the dairy. The microbiota in silage and feed mixture resembled each other in composition, while the microbiota of the milk differed significantly from the microbiota of the other samples collected on the farm. It consisted of a large number of different bacteria and was most similar to the microbiota found in used bedding material (based on wood shavings) in the cows' stalls.

The milk from the experimental cows was collected in a designated tank on the farm and transported every second day in the last week of each 3-week period to a commercial dairy for production of long-ripened hard cheese. Samples for analysis of the microbiota in milk, starter culture and cheese curds were taken during cheese making and the microbiota of the ripening cheeses was analysed regularly up to 22 months of age. In contrast to expectations, the variation in the milk microbiota between the repeated milking occasions within each feeding period was greater than between the

periods when the cows were fed the different silages. However, the composition of the microbiota changed drastically after fermentation in connection with cheese making. The microbiota in samples collected during cheese production and in cheese samples during ripening largely corresponded to the composition of the starter culture, which contained special lactic acid bacteria added to speed up and favour cheese production. Bacteria belonging to the genera *Lactococcus* and *Leuconostoc* dominated in samples taken early in the cheese making process, but also in cheese samples taken after 22 months of ripening, and the presence of bacteria belonging to the aroma-producing genus *Lactobacillus* was low. The results showed that although lactic acid bacteria were abundant in the forage, they did not include variants important for cheese ripening. This means that the forage is not a source of the bacteria that contribute to the characteristic flavour of the long-ripened hard cheese investigated.

Populärvetenskaplig sammanfattning

Svenskt lantbruk har en lång tradition av att producera högkvalitativa ostar med gott rykte och högt förtroende bland kockar och konsumenter. Mycket av den mjölk som produceras i Sverige förädlas till olika sorters ost med unika egenskaper och smaker, där långtidslagrade hårdostar har en särställning på marknaden. Ökad kunskap om faktorer som bidrar till variationer i mjölkens värdekedja för hårdost, från fodergrödor på fältet, via kon och mejeriet till konsumenten, är viktigt för den svenska mejeriindustrin. En av de faktorer som identifierats som viktiga för utvecklingen av smaken och karaktären hos långtidslagrad hårdost är den naturligt associerade mikrofloran av bakterier med ursprung från åkern, kostallet och den så kallade husfloran på mejeriet.

Mikrofloran i ost härrör huvudsakligen från startkulturen som tillsätts efter pastörisering av mjölken. En liten del av den naturliga mikrofloran i den obehandlade mjölken överlever pastörisering, utvecklas under ostmognad och bidrar till ostens smak och mognad. En grupp bakterier som är särskilt viktiga i detta sammanhang är mjölksyrabakterierna. Vissa av dem är förknippade med den naturliga mikrofloran på blad och stjälkar av vallväxter, exempelvis gräs och klöver. Vilket betyder att fodret kan bidra till variationer i förekomsten av mjölksyrabakterier i obehandlad mjölk och i den resulterande osten. Mikroflorans sammansättning kan analyseras med olika metoder, såsom traditionell odling av bakterier på olika typer av agar eller molekylära tekniker såsom sekvensering av DNA. Båda metoderna användes i denna avhandling för olika syften.

Arbetet började med analys av mikrofloran på vanliga vallväxter och i det resulterande ensilaget. För detta användes växtmaterial från SLU:s långvariga fältförsök, skördat på fyra olika platser i Sverige och under två växtsäsonger. Detta växtmaterial användes senare i laboratorieskala för att

producera tre typer av ensilage i glasburkar: utan tillsats, med tillsats av syra och med tillsats av en starterkultur med mjölksyrabakterier. Den botaniska sammansättningen av växtmaterialet visade sig variera mellan år, skördetillfällen och odlingsplatser, men det fanns inga tydliga strukturella samband mellan växtmaterialets mikroflora och ensilaget till följd av de olika behandlingarna. Däremot visade de olika ensilagen ett tydligt mönster vad gäller sammansättningen av deras mikroflora. Ensilage utan tillsats visade en mer slumpmässig mikroflora med hög mångfald av bakterier, medan ensilage tillverkat med tillsats dominerades av olika mjölksyrabakterier istället. Användningen av tillsats i ensileringen hade alltså en större effekt på mikrofloran i ensilaget, jämfört med effekten av skillnader i botanisk sammansättning.

I ett nästa steg genomfördes ett fullskaligt försök där mikrofloran undersöktes från åker till färdig ost. Arbetet började med ensilering i plansilo med samma typer av ensilage som i glasburkarna, det vill säga ensilage utan tillsats och med tillsats av syra eller starterkultur. De olika ensilagen användes i ett utfodringsförsök vid Röbäcksdalens forskningsladugård. Ensilagen ingick i foderblandningar till 67 mjölkkor, där varje typ av ensilage utfodrades under en treveckorsperiod. Korna mjölkades två gånger dagligen i en mjölkgrop. Provtagning för utvärdering av mikrofloran i ensilage, foderblandningar, använt strömaterial och gårdstanksmjölk utfördes under den sista veckan i varje treveckorsperiod. Resultaten visade att mikrofloran i växtmaterialet från åkern skilde sig från mikrofloran i alla andra prover från gården eller mejeriet. Mikrofloran i ensilaget och foderblandningen liknade varandra till sammansättning, medan den i mjölken skilde sig markant från mikrofloran i de andra proverna från gården. Den bestod av ett stort antal olika bakterier och liknade mest den mikroflora som fanns i använt strömaterial (baserat på kutterspån) i kornas liggbås.

Mjölken från försökskorna samlades in i en avsedd tank på gården och transporterades varannan dag under den sista veckan i varje treveckorsperiod till ett mejeri för produktion av långtidslagrads hårdost. Prover för analys av mikrofloran i mjölk, starterkultur och ostmassa togs under osttillverkningen. Provtagning och analys fortsatte med den färska osten och regelbundet under ostens mognad upp till 22 månaders ålder. I motsats till förväntningarna var variationen i mjölkens mikroflora mellan de upprepade mjölkningstillfällena inom varje utfodringsperiod större än mellan utfodringsperioderna. Sammansättningen av mikrofloran förändrades dock drastiskt efter syrning

av mjölken i samband med osttillverkningen. Mikrofloran i prover insamlade under osttillverkning och i ostprover under mognad motsvarade i stort sett densamma i starterkulturen. Starterkulturen som användes innehöll speciella mjölksyrabakterier, tillsatta för att påskynda och gynna osttillverkningen. Bakterier tillhörande släktena *Lactococcus* och *Leuconostoc* dominerade i prover tagna tidigt i osttillverkningen, men även i ostprover tagna efter 22 månaders mognad, och förekomsten av bakterier tillhörande det aromproducerande släktet *Lactobacillus* var låg. Resultaten visade att även om det förekom rikligt med mjölksyrabakterier i fodret, inkluderade de inte dem som är viktiga för ostmognaden. Det betyder att fodret inte är en källa till de bakterier som bidrar till den karakteristiska smaken hos den undersökta långtidslagrade hårdosten.

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As I approached my bachelor's thesis while working part-time at **Norrmejerier**, the idea emerged to make something sustainable out of the by-products of milk processing. But such an ambitious project didn't fit within the scope of a bachelor's thesis. That's when **Su-lin H** came into the picture. During one of your lectures on fungi, the idea of cultivating yeast on dairy residues to make fish feed was born. It became a large and well-executed project that further sparked my fascination with the world of microbes. It was also during this time I found my academic comrades, **Jonas O** and **Hasse J**. You showed me that academia can be fun and doesn't always

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Epiphytic microbiota in Swedish grass-clover herbage and the effect of silage additives on fermentation profiles and bacterial community compositions of the resulting silages

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Abstract

Aims: To investigate the epiphytic microbiota in grass-clover herbage harvested at different sites and occasions and to explore the effect of different silage additives on the resulting silage microbiota.

Methods and results: Herbage was harvested from grass-clover leys at geographically distributed sites in a long-term field experiment in Sweden, in early and late season of two consecutive years. Different silages were made from the herbage using: (1) no additive, (2) acid-treatment, and (3) inoculation by starter culture. Herbages were analysed for botanical and chemical composition, and the resulting silages for products of fermentation. Bacterial DNA was extracted from herbage and silage samples, followed by sequencing using Illumina 16S rRNA amplicon sequencing. Herbage microbiota showed no clear correlation to site or harvesting time. Silage additives had a major effect on the ensiling process; inoculation resulted in well fermented silages comprising a homogenous microbiota dominated by the genera *Lactobacillus* and *Pediococcus*. A minor effect of harvest time was also observed, with generally a more diverse microbiota in second-harvest silages. Untreated silages showed a higher relative abundance (RA) from non-lactic acid bacteria compared to acid-treated silages. In most silages, only a few bacterial amplicon sequence variants contributed to most of the RA.

Conclusions: The epiphytic microbiota in grass-clover herbage were found to be random and not dependent on site. From a microbial point of view, the most predictable and preferable silage outcome was obtained by inoculation with a starter culture. Acid-treatment with formic- and propionic acid surprisingly resulted in a less preferable silage. Silage making without additives cannot be recommended based on our results.

Impact Statement

This study contributes with new insights into the effect of different factors (e.g. herbage composition, site, harvest time, and year) on the microbiota in herbage and the resulting silages, including the effects of different silage-making methods on silage microbiota and quality.

Keywords: grass, clover, ensiling, silage additives, microbiota, lactic acid bacteria

Introduction

Nordic dairy cow rations contain a large proportion of ensiled forage, typically harvested from mixed leys of grasses and legumes (Rinne et al. 2002). Ley herbage is non-uniform in terms of botanical composition, which varies with year, site, and age of the ley (Hetta et al. 2004). Because of the short growing season in northern Europe, most ley forage is preserved by ensiling and fed as silage year-around. The anaerobic fermentation that occurs during ensiling, and the resulting combination of low pH and high concentration of short-chain fatty acids (mainly lactic acid), preserves the forage from microbial deterioration (McDonald et al. 1991). The fermentation process is carried out by the microbiota present in the herbage, which ferment available soluble carbohydrates to organic acids, i.e. lactic and acetic acid (Pahlow et al. 2003). The ensiling process usually proceeds spontaneously, but silage additives can be used to promote or inhibit fermentation. Typical fermentation promoters are inoculants based on lactic acid bacteria (LAB), either homofermentative (producing only lactic acid) or heterofermentative (producing both lactic and acetic acid) (Muck et al. 2018). Addition of inoculants strongly alters the microbiota and the fermentation products in the silage (Benjamim da Silva et al. 2022, Drouin et al. 2022). Typical fermentation inhibitors are mixtures of organic acids, e.g. formic and propionic acid, that reduce the pH in the forage directly, thereby preventing carbohydrates from being consumed by fermentation and leaving them as animal feed (Kung et al. 2003). The addition of organic acids to some extent also prevents the growth of undesired spoilage microorganisms in the silage (Muck et al. 2018).

During the past decade, there has been increasing interest in understanding and distinguishing the effects of botanical composition and epiphytic microbiota in the herbage on ensiling performance (Mogodiniyai Kasmaei et al. 2016, Ali et al. 2020). The microbial community in the forage may be of importance not only for feed quality, but also e.g. for

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Table 1. Date of harvest and weather data for each harvest occasion.

Year	1						2			
Harvest	First				Second		First		Second	
Site*	LTP	LNA	SBY	RBD	SBY	RBD	SBY	RBD	SBY	RBD
Date of harvest	May 22	May 31	Jun 02	Jun 09	Aug 21	Aug 15	Jun 05	Jun 24	Jul 31	Aug 09
Temperature**, avg. °C	16.4	20.0	21.8	13.2	13.3	12.5	20.2	14.9	13.6	14.2
Humidity**, avg. %	65.5	52.2	49.3	64.2	58.9	76.3	56.8	57.0	66.6	81.0
Rainfall***, acc. mm	122	297	14	67	73	156	119	154	179	210
Since first harvest					*59*	*89*			*60*	*56*
Global radiation**, MJ m^{-2}	23.3	29.7	37.0	25.0	25.0	18.9	30.8	24.0	12.9	13.3

*LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen
**Recorded at each site on the day of harvest
***Recorded as accumulated rainfall from start of each year until day of harvest

the microbial community in the raw milk and the resulting dairy products, due to transfer of bacteria from the field and farm environment to the udder (Hagi et al. 2010, Vacheyrou et al. 2011). Non-starter LAB (NSLAB), are known to play an important role during ripening of cheese (Beresford et al. 2001). Facultative heterofermentative lactobacilli are regularly found at low concentrations in milk but may reach concentrations of 8 $\log_{10}$ CFU g^{-1} at later stages of the cheese ripening process, and are vital for development of the characteristic flavours of many cheeses (Marilley and Casey 2004). NSLAB can be found in a variety of ecological niches, including forage crops. While Mordenti et al. (2017) reviewed the influence of forage on the milk microbiota in production of Parmesan/Parmigiano-Reggiano cheese, there are few studies that have dealt with LAB associated to forages and the impact of different ensiling processes on the occurrence and numbers of NSLAB in later stages of the dairy value chain.

The microbial community associated with the herbage will depend on many factors, such as plant species, geographical location, and time of harvest (Xu et al. 2022). For a better understanding of how factors associated with forage production influence later stages in the dairy value chain, it is important to study the diversity of bacteria associated with typical forage crops. In addition, since both bacterial inoculants and organic acids are additives for ensiling commonly used by Nordic dairy farmers, it is of interest to investigate the effects of these on the final silage microbiota. Herbage can harbour a wide variety of microorganisms, were bacteria are usually the most prevalent (Mir et al. 2022). The microbial composition of herbage and silage can be studied using conventional techniques such as plate cultivation, but modern molecular techniques enable more precise identification in more diverse and complex environments (McAllister et al. 2018). Long-term field experiments (LTE), where the same crop management practices have been applied at different sites, have been used in several studies to evaluate the long-term changes in parameters like soil organic matter content (Sandén et al. 2018) and soil microbiota (Nelkner et al. 2019). In contrast, only a few recent studies have used LTEs to evaluate the plant microbiome of a specific crop within different geographical regions, e.g. Gaube et al. (2021). Hence, the regional effect on the natural epiphytic microbiota of a forage crop and its corresponding silages have not been fully elucidated.

The aims of this study were to investigate the epiphytic microbiota in grass-clover herbage from a well established LTE at four different sites in Sweden on different harvesting occasions, and to assess changes in the microbial community after ensiling using three distinctively different preservation methods: (1) spontaneous fermentation, (2) addition of organic acids, and (3) inoculation with starter culture.

Materials and methods

Experimental sites and design of field experiments

A multi-site LTE focusing on soil fertility with different N levels, managed by the Swedish University of Agricultural Sciences, was used for this study and herbage was collected from experimental plots of grass-clover leys. The leys comprised a mix of timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*), and red clover (*Trifolium pratense*), and were cut twice per year. Herbage was sampled at four LTE sites throughout Sweden: Lönnstorp (LTP) in the south, 55°67′N, 13°11′E; Lanna (LNA) in the mid-west, 58°21′N, 13°08′E; Säby (SBY) in the mid-east, 59°49′N, 17°40′E; and Röbäcksdalen (RBD) in the north, 63°49′ N, 20°17′E. At each site, samples were taken from three field plots fertilized with 50 kg N^{-1} ha per year. More information on the experimental design and the growing conditions is described by Carlgren and Mattsson (2001) and Mattsson (2002).

Harvest of herbages and silage preparation

Herbage collection and preparation of the silages took place during 2018 (year 1) and 2019 (year 2), by harvesting two times per season (the normal procedure in the LTE). Detailed harvest information and basic weather data are provided in Table 1.The timing of each harvesting occasion followed the estimated harvesting pattern on a dairy farm at each site. To characterize the epiphytic microbiota, herbage samples (100 g) were randomly taken by hand with sanitized scissors (70% EtOH) before harvesting each field plot. The sampling was carried out by walking along the field plot and cutting four randomly selected 0.5 m^2 spots. Cutting was done as close to the ground as possible without touching the ground with the scissor. The collected sample was wrapped in a plastic bag and immediately frozen in liquid nitrogen. Herbage was then cut using an in-house built harvester comprising a bicycle trailer with a horizontally attached hedge trimmer (RHT6160RS, Ryobi®). Cutting continued until ∼20 kg fresh matter (FM) was collected in plastic bags. The plant material was first sampled for estimation of botanical composition (250 g) and then chopped with a compost grinder (TCS 2500, AL-KO). Samples of the chopped material were taken and stored at 4°C for estimation of dry matter (DM) content (250 g) and numbers of viable LAB (25 g). The remaining herbage was divided between three plastic bags, with 3 kg in each, and

Table 2. Ensiling treatments and composition of additives used when making silages.

Treatment	Added product	Details
Without additive (Untreated)	Water	De-ionized
Treated with organic acids (Acid-treated)	Promyr NT-570 (Perstorp, Sweden)	Propionic acid < 25% Formic acid 30–40% Sodium formate < 20%
Inoculation with a starter culture* (Inoculated)	Feedtech Silage F10 (DeLaval, Sweden)	*Enterococcus faecium* (M74 NCIMB 11181) at 3.0×10^9 CFU g^{-1} *Lactobacillus plantarum* (LSI NCIMB 30083) at 5.0×10^9 CFU g^{-1} *Lactobacillus plantarum* (L-256 NCIMB 30084) at 1.0×10^9 CFU g^{-1} *Pediococcus acidilactici* (33–11 NCIMB 30085) at 5.0×10^8 CFU g^{-1} *Pediococcus acidilactici* (33–06 NCIMB 30086) at 5.0×10^8 CFU g^{-1}

*Mixed with water to reach recommended target of 100 000 CFU g^{-1} herbage.

kept cold (4°C) until ensiling. Three types of silages were prepared in experimental laboratory-scale silos: (1) without additive (untreated); (2) treated with organic acids (acid-treated); and (3) inoculated with a starter culture (inoculated). Additives were applied in a dose of 6 mL kg^{-1} FM (Table 2), directly into the plastic bags using spray bottles, followed by thorough mixing. Treated herbage (two jars per treatment) was packed directly into autoclaved 1.7 L glass jars, using in-house customized equipment (jar holder with lever connected to a piston), to a target density of 650 g L^{-1}. The jars (silos) were sealed with disinfected lids with water-locks and stored for 100 ± 1 days at 20°C in a temperature-controlled room.

Immediately before opening, the jars were weighed to obtain a measure of fermentation losses. The jars were then opened and emptied out onto a table covered with sterile plastic film. The silage was mixed thoroughly and multiple samples were taken. First, three 30 g FM samples were pooled in a plastic bag and frozen at −80°C for microbial analysis. Second, 25 g FM were sampled into a stomacher bag and stored at 4°C for estimation of numbers of viable LAB. Third, 250 g FM were sampled for DM estimation and analysis of chemical composition. Finally, 100 g FM were sampled and frozen at −20°C for estimation of fermentation products. After thawing, samples for analyses of fermentation products were pressed in a hydraulic press to extract and collect silage juice, which was kept at −20°C until analysis.

Analyses of herbage and silage samples

The botanical composition of herbage from each of the harvested plots was calculated on a DM basis, after manually sorting each of the *ad hoc* collected herbage samples into grasses, legumes, and unsown species. For determination of chemical composition, samples were dried at 50°C to constant weight and sent for analysis with near-infrared spectroscopy (NIRS) at a commercial laboratory (Valio Oy, Seinäjoki, Finland). Fermentation products in silage juice were analysed by electrometric titration (Moisio and Heikonen 1989) at the same commercial laboratory. Numbers of viable LAB were estimated by running 25 g of sample with 225 g peptone water (1 g L^{-1} Oxoid™ Peptone Bacteriological, Thermo Scientific™) in a stomacher (Stomacher® 400, Seward) for 120 s at normal speed. Then 10 mL aliquots of the emulsion obtained were transferred to sterile glass vials and used for dilution series and spread-plating on De Man, Rogosa, and Sharpe (MRS) agar (54.6 g L^{-1} MRS agar, Merck) and Rogosa agar (59.6 g L^{-1} Rogosa agar and 1.3 ml L^{-1} 99.6% acetic acid, Merck). All agar plates were incubated in anaerobic jars at 30°C for 48 h, with Anaerocult™ A (Merck) as anaerobic medium, and colonies were counted.

Preparing DNA for bacterial community analysis

Frozen herbage and silage samples were thawed at room temperature for 4 h. Each sample was then thoroughly mixed and a 30 g subsample was transferred to a stomacher bag, together with 270 g of $\frac{1}{4}$ strength Ringer solution with 0.5 ml L^{-1} Tween® 80 (Merck), prepared according to O'Brien et al. (2007). The stomacher bag was run for 120 s on normal speed in a stomacher (Stomacher® 400, Seward) and then 100 mL of the emulsion were divided between two sterile 50 mL screw-cap tubes (Sarstedt) and centrifuged at 9000 g for 15 min. The supernatant was discarded and the pellets were dissolved in $\frac{1}{4}$ strength Ringer solution with 0.5 ml L^{-1} Tween® 80 (Merck), prepared as mentioned previously, giving a total slurry volume of 20 mL, pooled in one tube. Finally, 1500 µL of the slurry were aliquoted to sterile 2 mL screw-cap microtubes (Sarstedt) and frozen at −20°C until DNA extraction.

For extraction of DNA, one microtube of each sample was thawed at room temperature for 1 h and then centrifuged at 13 000 g for 5 min. The supernatant was discarded and 700 µL of SL 2 buffer (NucleoSpin™ Soil, Macherey-Nagel™) were added. The tube was gently vortexed and the resulting emulsion was transferred to a NucleoSpin™ Bead Tube Type A (Macherey-Nagel™). From this point, DNA extraction instructions provided with the NucleoSpin™ Soil Kit (March 2019/Rev. 08, Macherey-Nagel™) were followed, with two exceptions: Enhancer SX was not utilized for the silage samples and the drying step before elution of DNA was prolonged to 5 min for all samples.

Library construction, sequencing, and bioinformatic analysis

The extracted DNA was used to construct a 16S rRNA library with primers 515F and 805R (Hugerth et al. 2014). Illumina adapters and barcodes were used for amplification, following a two-step PCR approach as described previously by Sun et al. (2019). The 16S rRNA library was sequenced using the Illumina Miseq platform at SciLifeLab (Stockholm, Sweden). The raw sequencing data have been deposited at the National Center for Biotechnology Information (NCBI), with BioProject ID PRJNA989025.

Bioinformatic data processing was performed using QIIME 2 2021.8 (Bolyen et al. 2019). Raw demultiplexed reads were trimmed with Cutadapt to remove primer sequences (Martin

Table 3. Botanical and chemical composition, and numbers of viable LAB in herbage samples from each harvest occasion.

	Year	1						2					
	Harvest	First				Second		First		Second			
	Site*	LTP	LNA	SBY	RBD	SBY	RBD	SBY	RBD	SBY	RBD	*SEM*	*P-value*
Botanical composition	Grass, g kg^{-1} DM	980	900	850	920	350	890	930	980	590	770	*4.6*	*< 0.001*
	Legumes, g kg^{-1} DM	20	70	110	60	530	60	50	10	270	210	*5.7*	*< 0.001*
	Other, g kg^{-1} DM	0	40	40	20	110	50	10	0	140	20	*3.3*	*< 0.001*
Chemical composition	DM, g kg^{-1}	312	331	345	273	259	362	196	239	296	257	*14.1*	*< 0.001*
	CP, g kg^{-1} DM	145	122	140	157	175	109	143	89	120	117	*11.0*	*< 0.001*
	NDF, g kg^{-1} DM	512	555	521	461	461	508	566	616	488	468	*15.8*	*< 0.001*
	WSC, g kg^{-1} DM	147	119	118	198	80	145	98	136	152	194	*9.8*	*< 0.001*
	Indigestible NDF, g kg^{-1} DM	64	80	90	29	118	107	81	101	92	65	*13.5*	*< 0.001*
	Ash, g kg^{-1} DM	52	51	48	42	68	60	69	54	69	67	*3.8*	*< 0.001*
Viable lactic acid bacteria	MRS, $\log_{10}$ CFU g^{-1} FW	1.4	2.3	1.7	1.0	2.6	1.3	0.8	1.7	3.4	4.1	*0.76*	*< 0.001*
	Rogosa, $\log_{10}$ CFU g^{-1} FW	1.0	0.7	1.0	0.7	1.5	0.5	0.4	1.5	1.1	4.1	*0.95*	*< 0.01*

Values represent averages ($n = 3$) for three field plots on each harvesting occasion.
Analysis of variance was performed with harvest occasion as fixed factor, SEM and *P*-values are presented.
Abbreviations: DM, Dry matter; NDF, Neutral detergent fiber; WSC, Water-soluble carbohydrates; CFU, Colony-forming units; FW, Fresh weight.
*LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.

2011), and all reads containing non-identified bases or missing primer sequences were removed. Further trimming, denoising, de-replication, read merging, and removal of chimeras were performed with DADA2 (Callahan et al. 2016). Truncation length was set to 229 bp for forward reads and 174 bp for reverse reads. An additional trimming of the first 12 bp in reverse reads was performed before truncation, due to low sequencing quality. Taxonomy was assigned to amplicon sequence variants (ASVs) with q2-feature-classifier (Bokulich et al. 2018) using release 138 from the Silva database (Quast et al. 2012) as reference. A phylogenetic tree was built using FastTree and MAFFT (Price et al. 2010, Katoh and Standley 2013). A generalized UniFrac distance matrix and alpha diversity measures were generated using the QIIME2 diversity plugin (Bolyen et al. 2019). For ASVs with higher relative abundances (RAs) not passing species annotation by QIIME2, selected ASVs were elaborated further using Nucleotide BLAST and the 16S ribosomal RNA sequences database as reference (accessed 06-03-2023), where only hits with 100% query cover and identity were considered. Multiple sequence alignment with MUSCLE (Edgar 2004) was performed on selected ASVs for evaluation of similarity between ASVs of the same genus.

Statistical analysis

Data on botanical and chemical composition of herbage and silage, silage fermentation products, and viable LAB in herbage and silage were analysed using R version 4.2.3 with the packages *readxl*, *dplyr*, and *stats* (Wickham and Bryan 2019,R Core Team 2021, Wickham et al. 2023). Before statistical analysis was performed, the three factors site, year, and harvesting time were grouped into one factor called 'harvesting occasion' and silage silo replicates were pooled into averages. One-way analysis of variance (ANOVA) was performed for herbage samples, with harvesting occasion as a fixed factor. This was also done for silages within each treatment. Microbial data were evaluated by combining the annotated feature table from QIIME2 with all other data in Excel, followed by grouping of minor ASVs at a set threshold to avoid cluttering, and finally export of a complete dataset to R. A principal coordinate analysis was performed with QIIME2 on the generalized UniFrac distance matrix, and imported into R with the *qiime2R* package (Bisanz 2018). Spearman correlation analysis was performed between the top 25 genera and silage fermentation parameters for each treatment in R with the package *Hmisc* (Harrel Jr. 2023). Microbial data was preprocessed in two steps; (1) conversion of zero abundance data points to numbers lower than the detection limit by using the *unif* method as described by Lubbe et al. (2021), and (2) centered log-ratio transformation of abundances as microbiota data is compositional. Genera showing more than moderate skewness (<-1 or >1) were excluded from the analysis, only significant correlations ($P < 0.05$) were visualized in the correlation plot. Figures were produced with R and the packages *ggcorrplot* (Kassambara 2022) and *ggplot2* (Wickham 2016).

Results

Botanical and chemical composition of herbages

There was significant variation in the botanical composition of the herbage samples taken on different harvesting occasions (Table 3). The lowest grass proportion was found in second-harvest herbage from SBY in year 1, SBY in year 2, and RBD in year 2. Herbage samples from SBY always had the highest non-grass proportion of all herbages taken within the same year and harvesting time. The chemical composition of the herbage also showed significant variation, e.g. DM content ranged from 196 to 362 g kg^{-1}, crude protein (CP) content from 89 to 175 g kg^{-1} DM, neutral detergent fiber (NDF) content from 461 to 616 g kg^{-1} DM, water-soluble carbohydrate (WSC) content from 80 to 198 g kg^{-1} DM, indigestible NDF content from 29 to 118 g kg^{-1} DM, and ash content from 42 to 69 g kg^{-1} DM. Herbage samples from RBD always showed the highest WSC concentrations of all herbages taken within same year and harvesting time. Number of viable LAB ranged from 0.8 to 4.1 $\log_{10}$ CFU g^{-1} FW on MRS agar, and from 0.4 to 4.1 $\log_{10}$ CFU g^{-1} FW on Rogosa agar.

Epiphytic microbiota of herbages

Principal coordinate analysis of herbage microbiota in relation to site, year, harvesting time, and other herbage parameters did not reveal any clear associations (results not shown). RA of bacteria at genus level in herbage samples prior to ensiling on each harvesting occasion is presented in Fig. 1.

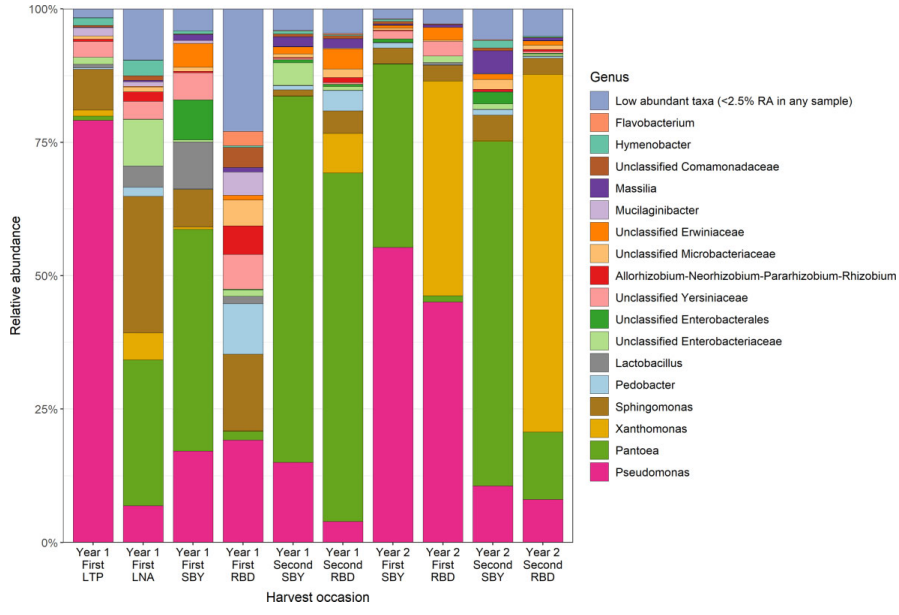

Figure 1. RA of bacteria at genus level for herbages prior to ensiling on each harvesting occasion. Values represent averages ($n = 3$) of herbage samples collected in three field plots per site. Bacteria without identified genus were allocated to the closest taxonomic level. Bacteria present in any of the samples at RA below 2.5% were pooled as 'Low abundant taxa'. Abbreviations: LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.

Herbage showed a diverse non-LAB flora mainly comprising the genera *Pantoea*, *Pseudomonas*, *Xanthomonas*, and *Sphingomonas*. Rarefaction curves for observed features in herbage on each harvesting occasion are presented in Fig. 2. The harvesting occasion with the highest microbial diversity (in numbers of observed features) was first harvest in year 1 at RBD, with >180 observed features. There was no clear dominant genus and a major proportion of 'low abundant taxa' (Fig. 1). In contrast, first-harvest herbage in year 2 at RBD had the lowest number of observed features, comprising mostly the genera *Pseudomonas* and *Xanthomonas*. Build-up of *Xanthomonas* was observed in herbage at the RBD site (Fig. 1), starting at second harvest (7.5%) in year 1, followed by first harvest (40.2%) and second harvest (67.0%) in year 2.

Performance of the different ensiling treatments

Fermentation parameters for the silages made on each harvesting occasion are presented in Table 4. The lowest final silage pH (on average pH 3.74) and highest concentration of formic and lactic acid (on average 63 g kg^{-1} DM) were observed in the inoculated silages. Irrespective of ensiling treatment, silages from the RBD site had very low pH values (3.52–4.07) for the first harvest in year 1 and for both the first and second harvests in year 2. Across harvesting occasions, mean fermentation weight losses and mean ammonia-nitrogen (ammonia-N) and volatile fatty acid (VFA) concentrations were higher in untreated than in acid-treated and inoculated silages. Fermentation weight losses in untreated silage (12.2 g kg^{-1} FW) were more than double those in inoculated (5.3 g kg^{-1} FW) and acid-treated (3.6 g kg^{-1} FW) silages. Ammonia-N concentration (index of protein break-down) was 62 g kg^{-1} DM in untreated silage, and 26 and 25 g kg^{-1} DM in acid-treated and inoculated silage, respectively. VFA concentration was 16 g kg^{-1} DM in untreated silage, 10 g kg^{-1} DM in inoculated silage, and 8 g kg^{-1} DM in acid-treated silage. The highest average WSC concentration was found in acid-treated silage (124 g kg^{-1} DM), double that in inoculated (63 g kg^{-1} DM) and untreated (57 g kg^{-1} DM) silage. Interestingly and irrespective of treatment, higher WSC concentrations were found in many of the lower-pH silages. Number of viable LAB was on average highest in untreated silage (5.9 $\log_{10}$ CFU g^{-1} FW), closely followed by acid-treated silage (5.3 $\log_{10}$ CFU g^{-1} FW), with a much lower number in inoculated silage (3.7 $\log_{10}$ CFU g^{-1} FW). The greatest variation among harvesting occasions within treatments was found in untreated silage, with respect to pH, fermentation weight losses, ammonia-N, formic and lactic acid, and VFA. For WSC, the variation was highest within acid-treated silages. For numbers of viable LAB, the variation was highest within acid-treated and inoculated silages.

Effect of ensiling treatment on microbial community in silages

The ensiling treatments had an effect on the resulting silage microbiota (Fig. 3). Inoculated silages clustered tight on one side of principal component (PC) 1, which explained 44.73% of the variance. Untreated and acid-treated silages did not cluster in the same way, indicating that factors other than treatment had a larger impact on the microbiota. Further investigation of the PCoA plots suggested that time of harvest also had an effect on the microbiota, as first- and second-harvest silages clustered, with a few exceptions, on

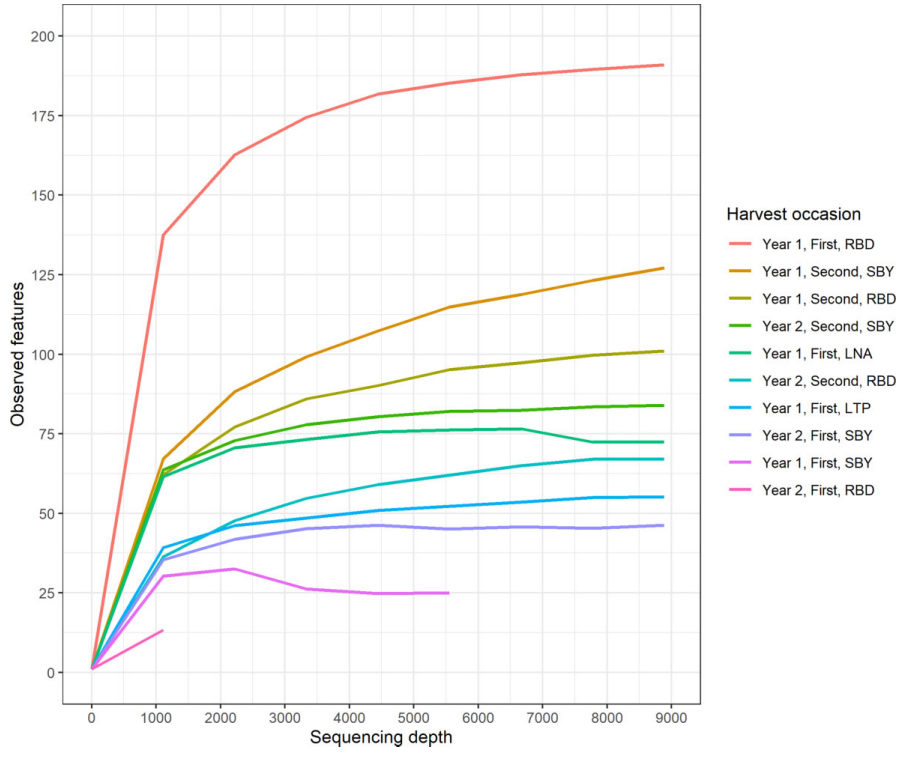

Figure 2. Rarefaction curves of observed features in herbage samples from each harvesting occasion. Values represents means of herbages collected from three field plots per harvesting occasion. Abbreviations: LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.

opposite sides from each other on PC 2, which explained 13.64% of the variance.

During fermentation, LAB in most cases proliferated to a high extent (Fig. 4). In untreated silages, *Lactobacillus* was the dominant genus among the LAB, followed by *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Weisella*, and *Pediococcus*. Non-LAB were also found in major proportions, comprising mainly unclassified *Yersiniaceae* and *Pantoea*. Samples from some sites showed rather major abundances of unclassified *Clostridiaceae*, unclassified *Enterobacteriaceae*, and *Hafnia–Obesumbacterium*. Compared with untreated silages, acid-treated silages on average contained more LAB (mostly *Lactobacillus*) than non-LAB. However, on some occasions, non-LAB (*Hafnia–Obesumbacterium*, unclassified *Yersiniaceae* and *Pantoea*) dominated. Inoculated silages showed complete dominance by LAB, mainly *Lactobacillus* and *Pediococcus*, followed by *Lactococcus* and *Enterococcus*. Non-LAB were found, although in very low RA. Untreated and acid-treated silages from the second harvest in year 1 at the SBY site and from the first harvest in year 2 at RBD showed particularly strong dominance by LAB. *Lactobacillus* contributed most to RA at both these sites, followed by *Lactococcus* and *Leuconostoc* at SBY, and *Pediococcus* and *Weisella* at RBD. Untreated silages from second-harvest herbage in year 2 at RBD and SBY also stood out by having a rich flora of LAB, including *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Weisella*, and *Lactococcus*. The genus *Weisella* was only found in major proportions in untreated and acid-treated silages from RBD during year 2. The lowest RA of LAB was found in acid-treated silages from second harvest in year 1 at RBD, where the non-LAB taxa *Hafnia–Obesumbacterium*, unclassified *Yersiniaceae*, and *Pantoea* dominated.

The starter culture used for making the inoculated silages comprised three different genera in the following proportions (calculated from Table 2): *E. faecium* (30%), *L. plantarum* (60%), and *P. acidilactici* (10%). After ensiling, the average RA of their corresponding genera showed a different pattern (Fig. 4), with *Enterococcus* contributing only 1.9%, *Lactobacillus* 72.4%, and *Pediococcus* 19.5%. These results indicated a major shift from the proportions in the starter culture, especially for *Enterococcus*. *Lactococcus* also showed rather consistent RA of around 2% in all inoculated silages, with the exception of that from first harvest in year 1 at SBY (8%). The abundance of *Lactococcus* was more random and variable in silages resulting from the other treatments.

Correlation analysis between silage microbiota and fermentation parameters

In the correlation analysis between the abundance of the top genera and silage fermentation parameters (Fig. 6) most correlations showed Spearman's Rho-values between −0.65 and

Table 4. Fermentation parameters and numbers of viable LAB in silages resulting from the different treatments.

Treatment	Year		1						2					
	Harvest		First				Second		First		Second			
	Site*	*Mean*	LTP	LNA	SBY	RBD	SBY	RBD	SBY	RBD	SBY	RBD	*SEM*	*P-value*
Untreated	pH	*4.32*	4.65	4.18	5.03	3.82	4.06	4.92	4.67	3.74	4.22	3.90	*0.366*	*< 0.01*
	Ferm. w. loss, g kg^{-1} FW	*12.2*	20.1	9.3	23.4	14.7	6.3	11.2	13.3	7.7	8.1	8.4	*3.92*	*< 0.001*
	Ammonia N, g kg^{-1} N	*62*	89	62	89	60	44	70	86	35	36	49	*24.5*	*0.067*
	Formic and lactic acid, g kg^{-1} DM	*43*	43	39	36	65	69	17	28	53	45	37	*12.3*	*< 0.01*
	VFAs, g kg^{-1} DM	*16*	30	7	17	13	16	6	30	16	10	11	*9.6*	*0.050*
	WSC, g kg^{-1} DM	*57*	17	46	10	62	22	150	3	46	82	131	*9.3*	*< 0.001*
	MRS, log$_{10}$ CFU g^{-1} FW	*5.9*	5.8	5.7	7.1	4.8	6.3	5.8	6.8	6.0	4.8	6.0	*0.51*	*< 0.001*
	Rogosa, log$_{10}$ CFU g^{-1} FW	*5.9*	5.8	5.6	7.0	4.7	6.3	5.8	6.8	5.9	4.8	6.0	*0.50*	*< 0.001*
Acid-treated	pH	*4.22*	4.35	4.23	4.56	4.06	4.09	4.42	4.17	3.93	4.33	4.07	*0.090*	*< 0.001*
	Ferm. w. loss, g kg^{-1} FW	*3.6*	7.9	2.3	2.7	3.5	4.4	3.3	3.7	2.8	2.3	3.1	*1.46*	*< 0.01*
	Ammonia N, g kg^{-1} N	*26*	36	37	37	34	13	34	25	12	12	25	*6.6*	*< 0.001*
	Formic and lactic acid, g kg^{-1} DM	*34*	40	26	29	40	55	20	41	34	28	26	*5.1*	*< 0.001*
	VFAs, g kg^{-1} DM	*8*	12	4	5	7	14	5	13	10	6	6	*2.5*	*< 0.001*
	WSC, g kg^{-1} DM	*124*	81	106	111	165	39	199	58	113	155	216	*26.6*	*< 0.001*
	MRS, log$_{10}$ CFU g^{-1} FW	*5.3*	5.3	5.5	5.1	3.1	8.0	4.6	5.8	6.1	4.4	4.8	*0.92*	*< 0.001*
	Rogosa, log$_{10}$ CFU g^{-1} FW	*5.3*	5.3	5.5	5.0	3.1	8.1	4.6	5.9	6.0	4.4	4.8	*0.91*	*< 0.001*
Inoculated	pH	*3.74*	3.82	3.73	3.78	3.65	3.96	3.67	4.08	3.56	3.71	3.52	*0.091*	*< 0.001*
	Ferm. w. loss, g kg^{-1} FW	*5.0*	6.0	4.4	6.1	4.6	4.6	4.7	5.4	4.3	5.0	4.6	*0.44*	*< 0.001*
	Ammonia N, g kg^{-1} N	*25*	24	27	25	33	29	26	52	5	9	23	*7.9*	*< 0.001*
	Formic and lactic acid, g kg^{-1} DM	*63*	73	59	67	80	67	50	50	62	62	61	*5.9*	*< 0.001*
	VFAs, g kg^{-1} DM	*10*	9	8	7	10	13	6	21	12	8	7	*1.9*	*< 0.001*
	WSC, g kg^{-1} DM	*63*	41	33	30	100	18	127	15	60	74	136	*9.0*	*< 0.001*
	MRS, log$_{10}$ CFU g^{-1} FW	*3.7*	2.4	4.0	3.7	2.7	5.6	2.8	6.5	3.7	2.7	2.9	*0.93*	*< 0.001*
	Rogosa, log$_{10}$ CFU g^{-1} FW	*3.7*	2.5	3.9	3.7	2.7	5.7	2.7	6.4	3.7	2.6	2.8	*0.95*	*< 0.001*

Values represent averages of two experimental silos from each of the three field plots at each harvest occasion ($n = 6$), treatment averages are shown in first column ($n = 60$).
Analysis of variance was performed within each treatment with harvest occasion as fixed factor, SEM and *P*-values are presented.
Abbreviations: DM, Dry matter; NDF, Neutral detergent fiber; WSC, Water-soluble carbohydrates; CFU, Colony-forming units; FW, Fresh weight.
*LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.

0.65. The strongest correlation (0.79) was found between the abundance of *Lactobacillus* and the content of formic and lactic acid in acid-treated silages. In general, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weisella* showed negative correlations with pH and ammonia N, and positive correlations with formic and lactic acid and VFA. The opposite was seen for *Enterococcus*, *Hafnia–Obesumbacterium*, Unclassified *Enterobacteriaceae* (not for VFA) and Unclassified *Yersiniaceae*, i.e. these genera in general showed positive correlations with pH and ammonia N, and negative correlations with formic and lactic acid and VFA. Fermentation weight losses were negatively correlated with most genera, except for *Enterococcus*, Unclassified *Enterobacterales*, Unclassified *Enterobacteriaceae,* and *Lactobacillus*. Water soluble carbohydrates was positively correlated with most genera, except for *Lactobacillus*, *Pediococcus*, Unclassified *Enterobacterales*, and Unclassified *Enterobacteriaceae*.

Dominant bacteria on ASV level in silage

Further investigation of the rarefied sequencing data indicated a total of 820 ASVs, of which 109 were LAB (order *Lactobacillales*). Figure 5 shows ASVs present at 2.5% RA or higher within treatments on each harvesting occasion (FASTA-sequences for all major LAB ASVs can be found in Table S1 in Supplementary Material). For the genus *Lactobacillus*, with few exceptions, ASV 9 was found to be the most abundant ASV and was observed in all silages. The exceptions were acid-treated silages from the second harvest in year 1 at SBY and the first harvest in year 2 at RBD, where ASV 43 (*Lact. fructivorans*) instead dominated. In inoculated silages from the first harvest in year 2 at SBY, ASV 99 (*Lact. buchneri*) showed the highest RA, and this was also the only silage showing higher RA of ASV 79 and 84 (*L. buchneri*). In untreated silages from the first harvest in year 2 at RBD, ASV 22 (*Lact. brevis*) was the dominant ASV. Interestingly, untreated and acid-treated silages from RBD at both first and second harvest in year 2 were the only silages showing higher RA of ASV 22 (*L. brevis*). Acid-treated silages from the second harvest in year 2 at RBD had six different major *Lactobacillus* ASVs.

Among the other LAB, *Pediococcus* ASV 93 was only found at higher RA in inoculated silages, while *Lactococcus* ASV 28 was found at rather high RA in untreated silages from the first harvest in year 1 at LNA. Untreated silages from the second harvest in year 1 at SBY showed high RA of both *Lactococcus* ASV 28 and 102 (*Lactococcus garvieae*). Two *Leuconostoc* ASVs were found at higher abundance in untreated silages from the second harvest in year 2, namely ASV 104 at SBY and ASV 65 (*leuconostoc mesenteroides*) at RBD. The untreated silages from the second harvest in year 2 at SBY also had the highest RA of *Enterococcus* ASV 58.

Non-LAB were mainly found in untreated and acid-treated silages (Fig. 4) and most of the RA associated with these bacteria were found to belong to just a few ASVs (data not shown). Unclassified *Yersiniaceae* comprised two main ASVs, one of which contributed to much of the RA in untreated silages from the first harvest in year 1 at LNA (44.1%) and at RBD (39.1%), and the first harvest in year 2 at SBY (47.8%). The same ASV also showed high RA in acid-treated silages from the first harvest in year 1 at SBY (24.8%) and the second harvest in year 2 at SBY (31.9%). Most of the *Pantoea*

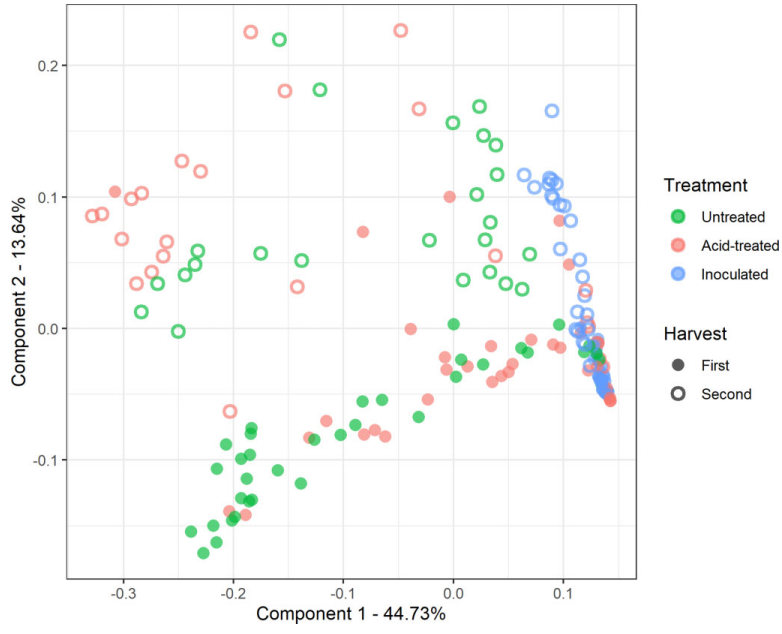

Figure 3. Principal coordinate analysis plot of the generalized UniFrac distance matrix associated to the silage microbiota. Each dot represents a unique silage sample from all harvesting occasions in the study. Dots colored according to ensiling treatment: green, untreated (no additive); red, acid-treated (addition of organic acids); blue, inoculated (with starter culture). Filled dots (●) indicate first, open dots (○) indicate second harvest.

originated from one ASV, showing major RA in untreated silages from the second harvest in year 1 at RBD (32.8%). *Hafnia–Obesumbacterium* comprised one main ASV, found at dominating RA in silage from the first harvest in year 1 at LNA (39.7%) and the second harvest in year 1 at RBD (46.1%). Unclassified *Enterobacteriaceae*, unclassified *Clostridiaceae*, *Xanthomonas*, and *Proteus*, found at higher RA in some silages, each also originated from one main ASV.

Discussion

Experimental design and harvest sites

Much effort has been made to establish LTEs in Sweden for studying long-term effects of crop rotation and management on soil fertility and agronomic performance (Bergkvist and Öborn 2011). In this experiment, we utilized an experiment with leys established from 1970 to 1981 at four sites in Sweden. These sites represent distinctly geographically separated agricultural regions in Sweden with large differences in length of the vegetation period and soil type. The experiment comprises many plot replicates, resulting in a relatively large biomass production per site that can be used for different scientific purposes, such as the ensiling study in this experiment. To our knowledge, this is the first approach to monitor the microbiota of forage crops in a well established LTE that covers different agricultural regions.

Though, the summer in year 1 was unusually warm and dry compared with the Swedish average, and offered very limited opportunities for the leys to grow on after the first harvest. This was obvious when comparing the number of days between the first and second harvest for each year (Table 1), i.e. 80 and 67 days in year 1, and only 56 and 46 days in year 2 for SBY and RBD, respectively. Botanical and chemical composition varied greatly within year and harvest (Table 3). The fact that leys were harvested only two times per season in the LTE in contrast to 3–4 times on commercial farms, resulted in a herbage with lower nutritional quality due to crop ageing.

Epiphytic microbiota of herbages in relation to studied factors

The original focus of this study was on LAB and their occurrence in herbage and resulting silages. However, LAB were found at a very low RA in the herbage samples, with on average <2% RA. There were at least two factors associated with the low RA of LAB, the first being that the fresh herbage contained very low numbers of viable LAB (Table 3). This has been reported previously by Müller and Seyfarth (1997) for timothy, Saarisalo et al. (2007) for timothy and meadow fescue, and more recently Wang et al. (2022a) for red clover, with red clover showing higher reported viable LAB counts than the two grass studies. Our study showed a similar trend, i.e. higher legume proportion in the herbage resulted in higher viable counts, especially on MRS agar (Table 3). The second factor associated with the low RA of LAB is that when working with fresh plant material and bacterial DNA, contamination with chloroplast and mitochondria DNA is common (Beckers et al. 2016). This includes cell organelles originating from ancestral bacteria, still containing preserved regions similar to bacterial DNA (Dyall et al. 2004). As a consequence, the already low-abundant LAB were not amplified at high

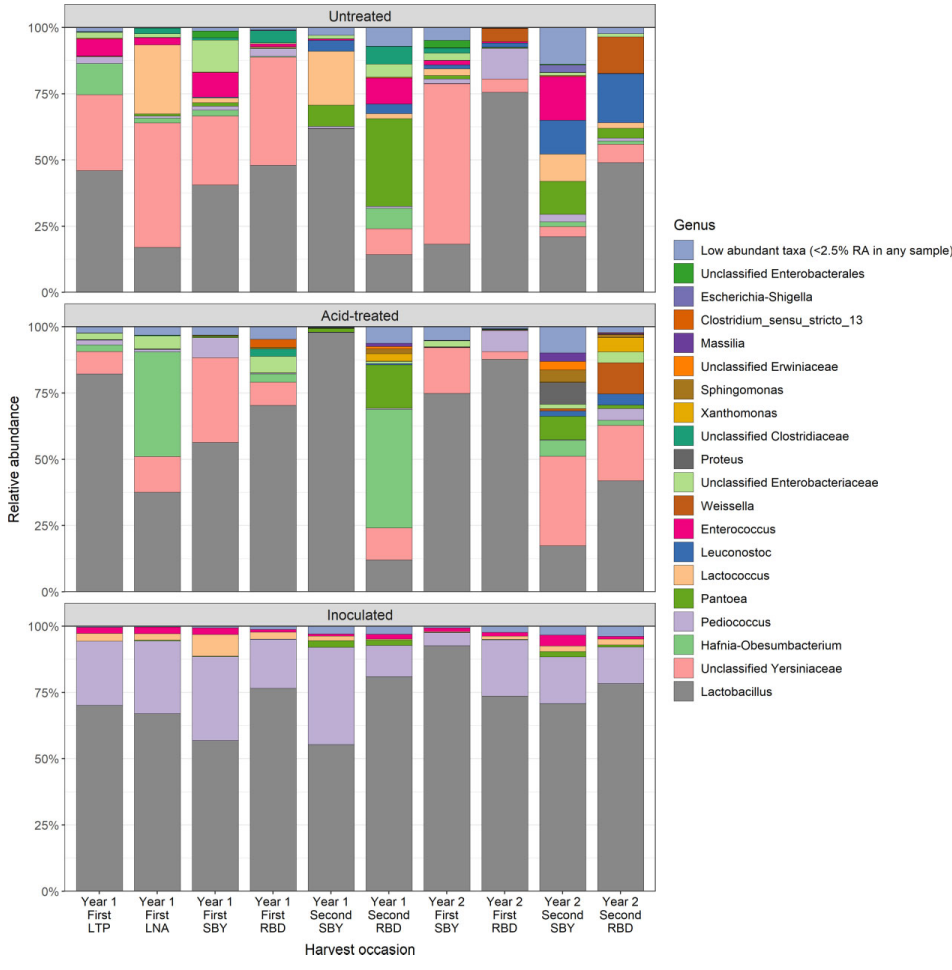

Figure 4. RA of bacteria at genus level for silages after ensiling with three different treatments: untreated (without additive), acid-treated (addition of organic acids), and inoculated (with starter culture). Bars represent averages ($n = 6$) based on duplicate samples from each of the three field plots per harvesting occasion. Bacteria without identified genus were allocated to the closest taxonomic level. Bacteria present in any sample at RA below 2.5% were pooled as 'Low abundant taxa'. Abbreviations: LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.

enough levels to get sequenced, since a major proportion of sequences belonged to chloroplast and mitochondria. Interestingly, contamination was not as problematic in the silages, possibly because chloroplast DNA is released and degraded during the ensiling process. This has been observed in a study by Aufrère et al. (1994), who found that proteins of chloroplast membranes were completely degraded after <48 h of ensiling. In contrast to the silages, the herbage samples in the present study were frozen until bacterial DNA extraction and homogenized directly after thawing, probably releasing their relatively intact chloroplast DNA during the process. Further, sequencing depth was rather low for some of the herbage samples after filtering of chloroplast and mitochondria, probably affecting the resolution of the results for those samples (Fig. 2).

Although some patterns were found, the diverse microbiota found in the harvested herbage samples showed no clear connection to site, year, or harvesting time alone (Fig. 1). SBY herbages showed high RA of *Pantoea* (around 37% in the first harvest and 65% in the second harvest). Interestingly, most of the RA originated from only one ASV. The most probable full match was *Pantoea agglomerans*, a bacterial species related to growth promotion and pathogen control in plants (Lorenzi et al. 2022). *Xanthomonas* was mainly found in RBD herbages during year 2, with RA mainly originating from one ASV. This ASV matched fully to a few species, among which the most probable matches were *X. translucens* and *X. albilineans*, both common plant pathogens causing leaf streak and leaf scald (Matsuoka and Maccheroni 2015, Sapkota et al. 2020). The observed build-up of *Xanthomonas* in RBD herbage over time

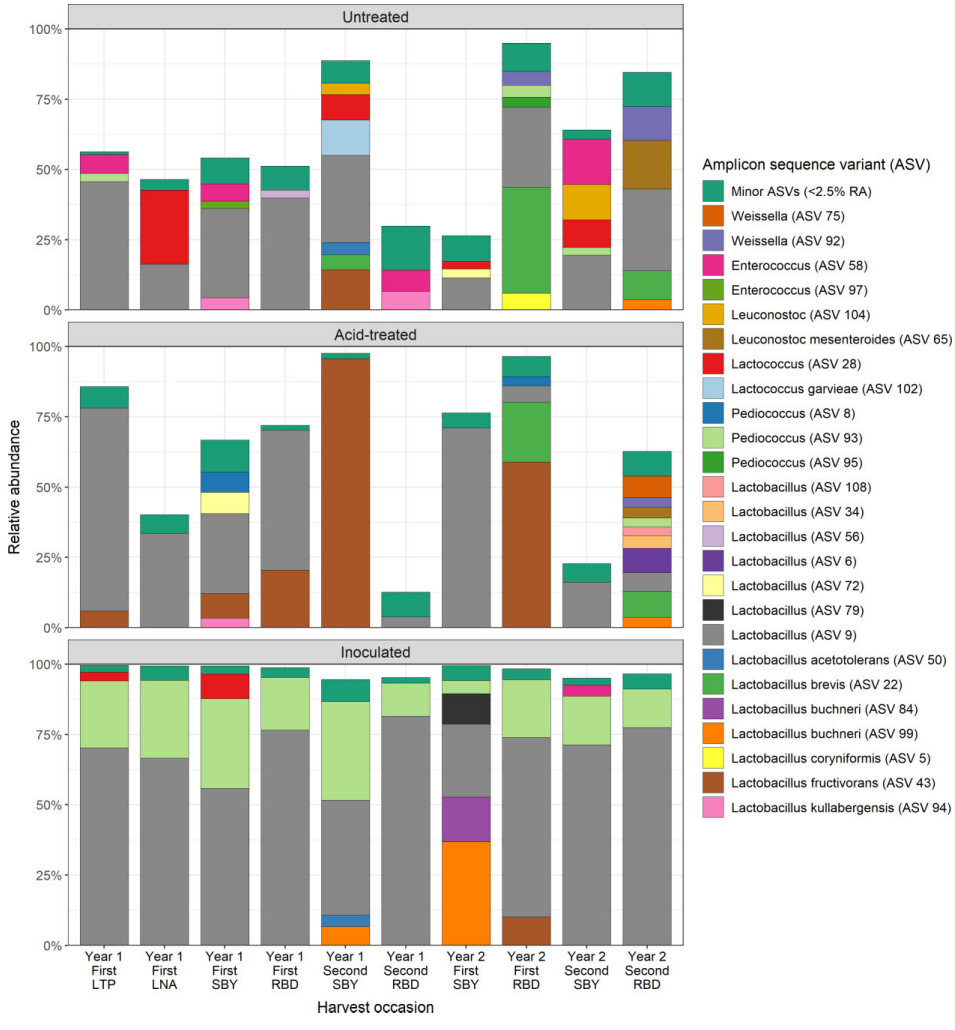

Figure 5. RA of LAB (order *Lactobacillales*) ASVs found in silages after ensiling with three different treatments: untreated (without additive), acid-treated (addition of organic acids), and inoculated (with starter culture). Values represent averages ($n = 6$) based on duplicate samples from each of three field plots per harvesting occasion. ASVs present in any sample at RA below 2.5% were pooled as 'Minor ASVs'. Abbreviations: LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.

might be explained by the ability of *X. translucens* to overwinter in perennial hosts (i.e. timothy), as reported by Duveiller (1997). Of the *Pseudomonas* found in all herbage samples there were three major ASVs. One of these ASVs dominated at the LTP site and the most probable full match was *Pseudomonas syringae*. The other two ASVs dominated in second-harvest herbage in year 2 at SBY, and first harvest herbage in year 2 at RBD, but it was not possible to draw conclusions on species due to the high number of full matches. Many of the matching *Pseudomonas* species have well-documented relationships to plants, mainly pathogenic but also beneficial (Schroth et al. 2006). For *Sphingomonas*, there was no finding at species level. One interesting observation was that there seemed to be 'competition' between *Pseudomonas*, *Pantoea*, *Xanthomonas*, and *Sphingomonas*. These four genera comprised a major proportion of RA on all harvesting occasions, with the exception of first-harvest herbage in year 1 at the RBD site.

Performance of the different ensiling treatments

Numerous studies have evaluated the effect of silage additives on Nordic forage crops (Hetta et al. 2003, Saarisalo et al. 2008, Franco et al. 2022a,b), and have shown that spontaneous fermentation is more unpredictable, while the addition of formic acid and various inoculants can contribute to a more

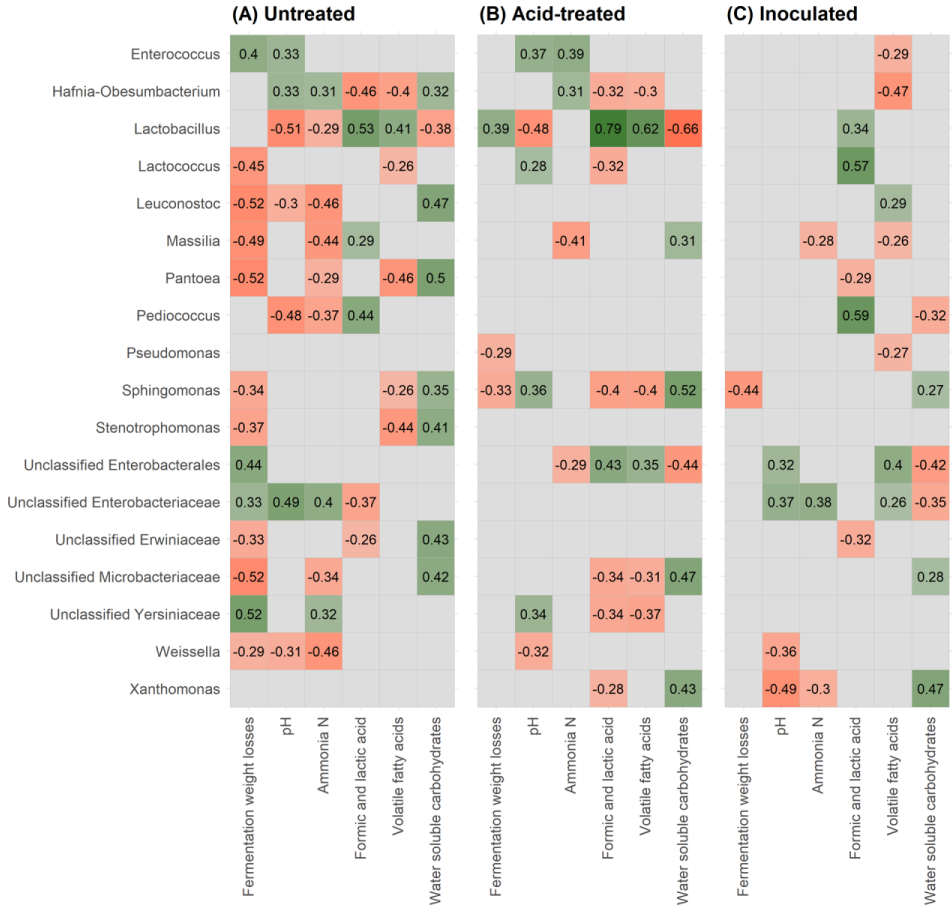

Figure 6. Plot resulting from Spearman correlation analysis between the top genera and the fermentation parameters associated to silages after ensiling with three different treatments: (A) untreated (without additive), (B) acid-treated (addition of organic acids), and (C) inoculated (with starter culture). Only significant correlations ($P < 0.05$) are presented, with Rho-values and colors indicating positive (green) or negative (red) correlations.

preferable ensiling process. Our results confirm these findings, as untreated silages showed the most random fermentation outcome and the largest variation between harvesting occasions for pH, formic and lactic acid, fermentation weight losses, ammonia-N, and VFA (Table 4). Observed variation between harvesting occasions was much lower for the acid-treated and inoculated silages. Weissbach (1996) and Kung et al. (2018) suggest that for high-quality silage, pH should be $<4.4 \pm 0.2$ (depending on herbage DM), ammonia-N content should preferably be below 80 g kg^{-1} N and not exceeding 120 g kg^{-1} N, and lactic acid content should be within the range 60–120 g kg^{-1} DM. Based on these recommendations, untreated silage from the first harvest in year 1 at LTP and SBY, the second harvest in year 1 at RBD, and the first harvest in year 1 in SBY cannot be considered as good silages due to their high pH and high ammonia-N concentration (Table 4). Most of the untreated and acid-treated silages did not meet the recommendation for lactic acid, although the measurement was performed as the sum of formic and lactic acid. Since the results of the VFA analysis were pooled by the commercial lab, the silages could not be evaluated on the basis of their VFA composition considering recommendations that refer to the individual acids (acetic, propionic, and butyric) in varying proportions.

Effect of ensiling treatment on silage microbiota

In general, there was an effect of ensiling treatment on the microbiota. Inoculated silages were completely dominated by LAB (mainly *Lactobacillus* and *Pediococcus*), showing rather small variation in the microbiota. In contrast, untreated and acid-treated silages showed major variation, with a tendency for more *Lactobacillus* in relation to total LAB in acid-treated silages. These findings partly agree with two recent ensiling studies with similar silage treatments and plant species. Franco et al. (2022b) found that inoculated silage showed major RA of *Lactobacillus*, but compared to our study, silages generally

showed a lower RA of LAB. Franco et al. (2022a) found higher RA of *Lactobacillus* in acid-treated than in untreated silage, but compared to our study, inoculated silage showed much lower RA from *Lactobacillus*, and other LAB in all silages mainly comprised *Weissella*.

Further investigation of possible associations between treatments and the resulting microbiota (Fig. 3) revealed a higher diversity of LAB in untreated silages, while acid-treated silages contained mainly the genus *Lactobacillus*. One possible explanation could be the ability of *Lactobacillus* to resist and function at lower extracellular pH compared with other LAB, as reported by McDonald et al. (1990) for a *Leuconostoc*, Cook and Russell (1994) for both a *Lactococcus* and a *Streptococcus*, and Yang et al. (2019) for both an *Enterococcus* and a *Pediococcus*. When acid treatment is applied, the pH drops dramatically before onset of fermentation. However, this pH drop did not seem to inhibit non-LAB to the same extent at the second harvest compared with the first harvest. Second-harvest acid-treated silages had higher RA from non-LAB, with the exception of those in year 1 at SBY. This may be due to an initial buffering effect of legumes, as previously reported by Hetta et al. (2003), explained by red clover containing high levels of glycerate and malate that may have a buffering effect during the ensiling process (Playne and McDonald 1966). Considering the higher average legume content in second-harvest herbage, this buffering effect could leave room for initial growth of non-LAB. However, on comparing herbages from year 1 at SBY and RBD the opposite was seen; non-LAB showed the lowest RA in the SBY silage, where legume content was extremely high compared with all other herbages.

The rather consistent proportions of bacteria found in the inoculated silages (Fig. 4) deviated strongly from the proportions in the commercial starter culture (Table 2), with *Enterococcus* almost disappearing. A similar finding was made by Bao et al. (2016) when ensiling alfalfa (*Medicago sativa*) with a similar starter culture, where *Enterococcus* was not among the major bacteria after ensiling, while a *Pediococcus* had become rather dominant. There could be many explanations for this and pH tolerance might be one. However, this contradicts observations by Yang et al. (2019) that an *Enterococcus* was more viable at lower pH than a *Pediococcus*. Another explanation could be that many *P. acidilactici* produce bacteriocins that inhibit *E. faecium*, as seen in studies by Albano et al. (2007); Aka-Gbezo et al. (2014); Todorov et al. (2021).

Interactions between silage microbiota and fermentation parameters

Both microbiota and fermentation parameters showed an even and rather predictable pattern for the inoculated silages. The microbiota in inoculated silages mainly consisted of *Lactobacillus* and *Pedioccocus* (Fig. 4), and fermentation parameters were mostly consistent with preferable values (Table 4). In contrast to expectations, the correlation analysis did not reveal many significant correlations between the two datasets (Fig. 6). The reason for this was probably the low variation in the data associated to both microbial community composition and fermentation parameters for the inoculated silages. When there is limited variation in the data, it becomes more challenging to detect meaningful correlations, especially with a small sample size. When the results were evaluated independently, however, it was rather clear that inoculation with the starter culture led to the most preferable silage (Table 4, Figs. 3 and 4).

For the untreated and acid-treated silages, the correlation analysis revealed that some bacterial genera will drive the fermentation process in a more positive, and other genera in a more negative direction. Higher abundance of most LAB led to a more preferable silage, and higher abundance of some of the non-LAB led to the opposite. Abundance of *Lactobacillus Pediococcus*, *Leuconostoc* and *Weisella* was negatively correlated with pH and ammonia N, which is in agreement with recent studies by Zheng et al. (2022) in the case of *Lactobacillus* and *Pediococcus*, Wang et al. (2022b) for *Pediococcus,* and Franco et al. (2022b) for *Lactobacillus*. Our results thus indicate that the decline in pH and prevention of growth of spoilage bacteria (ammonia N) in untreated and acid-treated silages in this study, is driven by many genera of LAB. This is in contrast to the previously mentioned studies, where fermentation seems to be more dependent on a few genera, while other LAB may even have a negative effect on the silage outcome. There may be many reasons behind the differences between studies, including factors associated to the fresh material itself that may conform the bacteria in a certain way. In this study, lactic acid production (formic and lactic acid), irrespective of silage type, was mainly positively correlated with *Lactobacillus*, in untreated silage also with *Pediococcus*, and in inoculated silage also with *Pediococcus* and *Lactococcus*. Abundance of *Lactobacillus* in untreated and acid-treated silages, and *Pediococcus* in inoculated silages, was negatively correlated with WSC, indicating that sugar is consumed as acid is produced. Surprisingly, abundance of *Lactobacillus* was negatively correlated with lactic acid content in Franco et al. (2022a), instead abundance of *Weisella* and *Lactococcus* showed the strongest positive correlation to lactic acid. In our study, only the inoculated silage showed a positive correlation between lactic acid (formic and lactic acid) and *Lactoccocus*. This is interesting, since this genus was not added with the starter culture (Table 2).

VFA showed a strong positive correlation with the abundance of *Lactobacillus* in untreated silage and especially in acid-treated silage, indicating production of other acids than lactic acid, e.g. acetic acid and propionic acid. Considering that propionic acid was a component of the additive used in production of the acid-treated silage, this may have contributed to the observed strong positive correlation between VFA and abundance of *Lactobacillus* in this silage. A similar formic and propionic acid-based additive was used for silage preservation in the study by Franco et al. (2022a). The authors observed a strong positive correlation between *Lactobacillus* and propionic acid, yet not with acetic acid, which further strengthens the assumption that propionic acid is linked to the silage treatment itself rather than to the abundance of *Lactobacillus*. Nevertheless, conversion of lactate to propionate by co-fermentation of different lactobacilli has been reported (Zhang et al. 2010). Since VFA were not analysed as independent acids, and the resolution of the taxonomic classification of bacteria in our study was too low, it is not possibe to draw any conclusions on the origin of the propionic acid; not in our study, nor in the study by Franco et al. (2022a).

It was interesting to see abundance of *Enterococcus* contributing to a higher pH in untreated and acid-treated silages, and also increased ammonia N in acid-treated silages, considering that this was one of the genera included in the commercial starter culture used for the inoculated silages. The

same correlation was observed in the studies by Franco et al. (2022b), Wang et al. (2022b), Zheng et al. (2022). The genera *Hafnia–Obesumbacterium* showed many non-preferable correlations in untreated and acid-treated silages. From the previously mentioned studies, only Wang et al. (2022b) reported *Hafnia–Obesumbacterium* among the top taxa. Similar to the results in our study, they found that abundance of *Hafnia–Obesumbacterium* was positively correlated with pH and ammonia N, and negatively with lactic acid. In a similar study by Zhao et al. (2021), *Hafnia–Obesumbacterium* was highly abundant in silages with poor performance, further confirming that the presence of this genus in silages is undesired. For the unclassified *Enterobacteriaceae* and unclassified *Yersiniaceae*, it is difficult to discuss any correlations, since there are many genera and species under each family of bacteria. In general, our results are in agreement with many recent studies, but there are also some discrepancies, highlighting the need for more research in the area of silage microbiota.

Evaluation of major silage ASVs

Lactobacillus ASV 9 was clearly a typical silage LAB in the study since, irrespective of silage treatment, it showed major RA in silage from most harvesting occasions. However, it was not possible to draw conclusions on species, as the ASV matched fully with many *L. plantarum* as well as a few other *Lactobacillus* species. *Lactobacillus plantarum* LSI and L-256, both included in the starter culture used for the inoculated silages, were not among these matches (Table 2). Though, since ASV 9 was dominant in the inoculated silages, to which the same species had been inoculated at a high level, it most probably represented a *L. plantarum*, although this could not be fully confirmed. The complete dominance by *L. fructivorans* (ASV 43) in acid-treated silages from the second harvest in year 1 at SBY was an interesting finding. At first, it seemed to be an effect of the extreme legume content, but acid-treated silages from the first harvest in year 2 at RBD, with botanical composition mainly comprising grass, showed similar dominance of ASV 43. According to Suzuki et al. (2008), *L. fructivorans* is commonly found as a spoilage bacteria in alcoholic beverages, with varying preferred growing conditions depending on strain. Many strains are reported to grow well and are even stimulated by rather high ethanol concentrations in the substrate, but unfortunately ethanol concentration in silage was not analysed in the present study. Henderson and McDonald (1971) showed that formic acid-treated silages of similar crops as used in our study, comprised higher ethanol concentrations than non-treated silages, which might explain the *L. fructivorans* dominance in some of the acid-treated silages. The observed major RA from *L. buchneri* (ASV 84 and 99) and ASV 79 in the inoculated silage from the first harvest in year 2 at SBY was surprising, as all other inoculated silages were dominated by ASV 9. No full matches were found for ASV 79, but multiple sequence alignment revealed that only one base pair (bp) differed from ASV 99 and two bp differed from ASV 84. The high RA of *L. buchneri* ASVs probably explained the higher pH value and higher ammonia-N and VFA concentrations observed in this silage compared with the other inoculated silages. In a comparison of silage inoculants by Nadeau and Auerbach (2013), a similar fermentation pattern was seen for grass-clover herbage inoculated with *L. buchneri*, but not with other LAB inoculants. The *L. brevis* (ASV 22) observed at higher RA in untreated and acid-treated silages from the first harvest in year 2 at RBD seemed to coincide with *L. fructivorans* (ASV 43), especially in the acid-treated silages. This is an interesting coincidence, considering that *L. brevis* also is a typical spoilage microbe in alcoholic beverages (Suzuki et al. 2008).

Based on the resulting microbiota in the inoculated silages (Figs. 4 and 5), it is tempting to conclude that *Pediococcus* (ASV 93) represented *P. acidilactici* 33–06 and 33–11 originating from the starter culture. However, the only full matches found for ASV 93 were one *Pediococcus stilesii* and one *P. pentosaceus*, although with full query cover and allowance of 1 bp mismatch, ASV 93 matched with a *P. acidilactici* (DSM 20284). Considering that ASV 93 was mainly found in the inoculated silages, and in rather uniform RA in all samples, it most probably represented a *P. acidilactici*. *Lactococcus* ASV 28 was observed in all untreated and inoculated silages and also in most acid-treated silages, although this is not visible in Fig. 5 for all silages due to filtering of minor ASVs (<2.5% RA) per sample. This ASV matched fully with many *Lactococcus* species, but it was not possible to draw conclusions on a specific species. *Lactococcus garvieae* (ASV 102) was the only other *Lactococcus* ASV found at a higher RA. A sequence alignment with ASV 28 revealed differences in six bp, indicating that they are rather distant from each other. *Leuconostoc* ASV 104 matched fully with *L. falkenbergense*, a species rather recently isolated in the Swedish city of Falkenberg by Wu and Gu (2021). Sequence alignment with *L. mesenteroides* (ASV 65) showed differences for two bp. From the LAB genera used in the starter culture (Table 2), *Enterococcus* was found to contribute least to RA in most inoculated silages (Fig. 4). On further evaluation, no major *Enterococcus* ASV was found in most inoculated silages (Fig. 5). However, a closer look at the raw data revealed that *Enterococcus* ASV 58, found at major RA in some untreated silages, was present in all inoculated silages at low and uniform RA, just under the filtering level for minor ASVs (<2.5% RA). This ASV matched fully with a major number of *Enterococcus* species, *E. faecium* being one. However, as this ASV was present at minor RA in inoculated silages and at major RA in many untreated silages, it probably represented more than one species of *Enterococcus*.

From the two major unclassified *Yersiniaceae* ASVs, one matched fully with many *Rahnella* and a few *Serratia*, *Rouxiella*, and *Yersinia* species. The other matched fully with many *Yersinia* and a few *Serratia* species. However, there were too many matches found to even consider any of them. The *Pantoea* was the same ASV as the dominating ASV associated with the herbage samples. The *Hafnia–Obesumbacterium* ASV only matched fully with two species, *Hafnia alvei* and *Obesumbacterium proteus*. According to Ridell (1999), *H. alvei* is a major species found in silages and *O. proteus*, a typical spoilage bacteria in breweries, belongs to biogroup 1 of *H. alvei*. It is not clear why the *Hafnia–Obesumbacterium* ASV was found at such high RA in two of the acid-treated silages. The presence of this bacteria in silages was recently discussed by Wang et al. (2022b), reporting higher pH and an increased protein degradation in silages with higher abundance of *Hafnia–Obesumbacterium*, similar to the results in our study. Interestingly, many of the matched species from unclassified *Yersinaceae* and *Hafnia–Obesumbacterium* were also identified by Heron et al. (1993), who used traditional methods to characterize silages with similar treatments as the ones in our study. The unclassified *Enterobacteriaceae* ASV matched fully with mostly *Klebsiella* and a few *Enterobacter*, but with

too many species to consider. The unclassified *Clostridiaceae* ASV did not match with any species. *Xanthomonas* was the same ASV as discussed for herbages. Finally, the *Proteus* ASV matched fully with *Proteus mirabilis* and *Proteus myxofaciens*, but no clear connection to plant material or silage was found in the literature.

Other factors affecting silage microbiota

The extent to which herbage composition affected the silage microbiota was unclear. In relation to botanical composition, it was found that herbages with a higher content of legumes and unwanted plants (Table 3) resulted in a more diverse LAB community in the untreated and acid-treated silages. However, this was not consistent, as the legume-rich herbage from the second harvest in year 1 at SBY resulted in silage with high RA of *Lactobacillus*. A more diverse LAB community in silages seemed to be associated with second harvest, with the exception of the previously mentioned extreme harvesting occasion in year 1. This could be an effect of growing climate, as the average temperature typically decreases towards second harvest. It could also be an effect of forages growing slower and closer to the ground (more affected by soil microbiota) after the first harvest. However, both hypotheses are difficult to evaluate from the data obtained in this study. The effect of herbage microbiota on the resulting silage microbiota was not clear, but seemed to be rather random. However, due to the low sequencing depth for some herbage samples and major contamination by non-bacterial DNA, accurate evaluation was a challenge.

In summary, this study did not reveal any clear effect of site (within Sweden), year or harvesting time on herbage microbiota, and there was no clear association between the botanical composition of the herbage and the microbiota of the corresponding silage. The lack of differentiated patterns in herbage microbiota between sites agrees with the findings of Gaube et al. (2021), who found that the variation between different plant parts was larger than between different geographical regions. Based on these findings, we agree with the suggestion by Schlechter et al. (2019), that future studies should have more emphasis on understanding how bacteria colonize plants. The benefit of using an ongoing LTE, which is continuously managed and documented, is that the findings of a particular study such as this can be reassessed. This is seldom possible when crops are established for a one-time study only. Use of silage additives had a clear effect on final silage microbiota, and inoculation with a starter culture resulted in a consistent and preferred outcome. In contrast to expectations, acid-treatment resulted in a more varying, and in some cases less preferable silage microbiota.

In a longer perspective, our on-going studies aim to investigate if feeding dairy cows with silages produced without additives, acid-treated silage, or silage produced by inoculation with a starter culture, affect the raw milk microbiota as well as the ripening of the resulting cheese. Although we could identify various LAB in the silages produced in this study, most of them will probably be of little importance for the cheese. Likewise, there may be ASVs of LAB with a role in cheese ripening, that due to their low abundance went undetected in this study.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest

Thomas Eliasson, Annika Höjer, and Karin Hallin Saedén are all employees of Norrmejerier. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

Thomas Eliasson (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing), Li Sun (Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing – review & editing), Åse Lundh (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing), Annika Höjer (Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing), Karin Hallin Saedén (Conceptualization, Funding acquisition, Supervision, Writing – review & editing), Mårten Hetta (Conceptualization, Funding acquisition, Supervision, Writing – review & editing) and Horacio Gonda (Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – review & editing).

Data availability

The data on herbage and silage compositions and plate counting will be shared on reasonable request to the corresponding author. The raw sequencing data is available in National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/, and can be accessed with BioProject ID PRJNA989025.

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II

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Microbial communities in feed, bedding material, and bulk milk - experiences from a feeding trial

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ABSTRACT

There is an increasing interest in the microbiota of the dairy value chain, from field to fork. Studies to understand the effects of environmental, feed and management factors on the raw milk microbiota have been performed to elucidate the origin of the bacteria and find ways to control the presence or absence of specific bacteria. In this study, we explored the microbiota in feedstuff, bedding material and milk on a Swedish dairy farm to investigate the effects of feeding different silages on the bacterial compositions throughout the dairy value chain. Three ensiling treatments were evaluated: without additive, with acid treatment, and with inoculation of starter culture. The silage treatments were fed as partial mixed rations to 67 dairy cows for 3 weeks each, with one treatment fed twice to evaluate if a potential change in milk microbiota could be repeated. The highest average total bacteria counts were found in the used bedding material (9.6 $\log_{10}$ cfu/g), while milk showed the lowest (3.5 $\log_{10}$ cfu/g). Principal coordinate analysis of the weighted UniFrac distance matrix showed clear separation between 3 clusters of materials: 1) herbage, 2) silage and partial mixed ration, and 3) used bedding material and milk. Surprisingly, the expected effect of the ensiling treatments on silage microbiota was not clear. Transfer of major bacteria from the silages and resulting partial mixed rations to the used bedding material was observed, but rarely to milk. The milk microbiota showed most resemblance to that of the used bedding material. *Lactobacillus* was a major genus in both feed and milk, but investigations at amplicon sequence variant level showed that in most cases the sequences differed between materials. However, low total bacteria count in the milk in combination with a high diversity suggests that results may be biased due to environmental contamination of the milk samples. Considering that the study was performed on a research farm, strict hygienic measures during the feeding experiment may have contributed to the low transfer of bacteria from feed to milk.
Keywords: Silage additive, silage microbiota, bulk tank milk microbiota

INTRODUCTION

There is an increasing interest in understanding the microbiota of the dairy value chain, from field to fork. Multiple studies have been conducted to explore microbial community composition in different environments and matrices, but also to determine the origin of milk microbiota (Ouamba et al., 2023). Specific attention has been devoted to non-starter lactic acid bacteria, which are responsible for formation of aroma components in many traditional cheeses (Bettera et al., 2023).

Lactic acid bacteria (**LAB**) are found in a variety of ecological niches associated with dairy production, including forage crops and the resulting silages. Dairy production in Sweden is distributed throughout the country and the botanical composition of forage leys varies between regions and farms. Ensiling is the most commonly used method to preserve forage crops in the Nordic countries, with LAB and water-soluble carbohydrates being crucial factors in making high-quality silage (Oliveira et al., 2017).

The microbiota in silage can be roughly divided into 2 groups, desirable and undesirable microorganisms. The desirable microorganisms are mainly LAB, e.g., *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Enterococcus*, epiphytic bacteria which occur naturally on forage crops and are important for the ensiling process. Undesirable microorganisms include *Clostridia*, *Enterobacteria*, and *Listeria*, as well as yeasts and molds (Driehuis and Elferink Oude, 2000). Factors of great importance for the hygienic quality of silage include pre-drying and dry

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

matter content of the herbage, and use of additives of the right type and dose (Kung et al., 2003). Other risk factors include contamination by soil and its associated microbiota under wet harvesting conditions, swathing, and extended pre-drying of herbage in the field (Pahlow et al., 2003). In a recent study, we investigated the epiphytic microbiota in grass clover herbages harvested at different sites and on different occasions in Sweden, to explore the effects of different silage additives on the microbiota of the resulting silages (Eliasson et al., 2023). The results showed that the epiphytic microbiota in grass-clover herbage was not dependent on site per se, although major variation was observed between sites and harvesting occasions. Silage additives had a clear effect, while the most predictable and preferable silage from a microbial perspective resulted from inoculation with a LAB starter culture. Surprisingly, acid treatment with formic and propionic acid resulted in less preferable silages (Eliasson et al., 2023).

Microorganisms can spread in the local environment on dairy farms to the cow udder and finally to the raw milk via various pathways, e.g., feed residues, manure, and bedding material (Ouamba et al., 2023). To our knowledge, few previous studies have examined the impact of silage additives on the numbers and relative abundance (**RA**) of natural LAB associated with forages, and flows of natural LAB through the dairy value chain. Ouamba et al. (2023) investigated the microbiota of different ration combinations and transfer rates of associated species to the raw milk, and found that silage-based forage rations shared more amplicon sequence variants (**ASV**) with the resulting raw milk than rations based on hay. They observed significant differences between milk samples associated with farms feeding different types of silage but, surprisingly, these differences were driven by *Enterobacteriaceae* and other *Proteobacteria*, rather than by LAB (Ouamba et al.., 2023).

Our starting hypothesis was that the microbiota of the feed affects the microbiota of the raw milk. To minimize variation in other factors than feed which could have a confounding effect on the milk microbiota, the study was performed in a dairy research farm. In this way, in contrast to performing the study in commercial dairy farms, such factors could be controlled and kept more or less constant. The specific objectives of the study were to (i) explore the microbiota in different samples on a Swedish dairy farm (herbage, silage, PMR and its ingredients, clean and used bedding material, and bulk milk) and (ii) investigate the effects of feeding silages produced with and without ensiling additives on microbial communities throughout the dairy value chain, but particularly LAB.

MATERIALS & METHODS

The experiment was conducted at Röbäcksdalen Research Centre in Umeå, Sweden (63°45′N, 20°17′E), which is part of Swedish Infrastructure for Ecosystem Science (SITES) within the Swedish University of Agricultural Sciences. Silages with different additives were made during June and July 2020 and the feeding experiment was carried out from January to April 2021. The full experiment is briefly described in the flowchart in Figure 1, with sampling points (S) marked.

Silage production

The herbage used in silage making was cultivated on the research farm, in a 5-year crop rotation with: 1) barley (*Hordeum vulgare*), 2) barley with an undersown forage mix comprising timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*), and red clover (*Trifolium pratense*), and 3–5) the forage mix as a ley cut 2 to 3 times per season. The soil type on the research farm is a silty loam with 2–5% clay, 3–6% organic matter, and mean pH of 6.1, with textural properties identical down to 100 cm. The agronomic setting is typical for the northern Swedish coastal region and river valleys. The arable land on the farm (approx. 200 ha) is divided into around 20 fields closely distributed around the dairy barn. More information on the agronomic conditions can be found in Zhou et al. (2019).

The silages were produced from the first (15–18 June) and second (24 July) cuts of the mixed grass leys. Actual cutting date was determined by phenological development of the crop, targeting forages with a high concentration of metabolizable energy (**ME** ≥ 11.0 MJ/kg DM). The leys were harvested with a disc mower conditioner, wilted in windrows aiming for a DM concentration of approximately 270 g/kg fresh matter (**FM**), and then precision-chopped to theoretical chop length of 16–32 mm. Three types of silages were produced: without additive (**UNTR**), with acid treatment (**ACID**), and with inoculation by starter culture (**INOC**). The ACID silage was produced with a formic and propionic acid-based additive (ProMyr NT-570, Perstorp, Sweden), added at a rate of 3 L per ton FM. The INOC silage was produced with commercial LAB-based starter culture. However, due to shortage of supply from the manufacturer, 2 different starter cultures were used. The first batch of INOC, produced during the first cut, was inoculated with Feedtech Silage F10 (DeLaval, Tumba, Sweden), comprising a mixture of *Lactobacillus plantarum*, *Enterococcus faecium*, and *Pediococcus acidilactici*. The second batch of INOC, produced during the second cut, was inoculated with SiloSolve MC (Svenska Foder, Lidköping, Sweden), comprising similar bacteria except

that *P. acidilactici* was replaced with *Lactococcus lactis*. Both INOC batches were prepared according to the instructions provided, with starter culture added at a rate of 2 L per ton FM, resulting in the inoculation of 100 000 cfu/g for Feedtech Silage F10 and 250 000 cfu/g for SiloSolve MC. All silages were stored in separate bunker silos as described by Hetta et al. (2007). Silage chemical composition and hygienic quality are presented in Table 1.

Table 1. Chemical composition and hygienic quality of the untreated (UNTR), acid-treated (ACID), and starter culture-inoculated (INOC) silages used in the feeding trial

Silage	UNTR	ACID	INOC	INOC
Batch	First	First	First	Second
pH	4.0	3.9	4.0	3.9
Lactic acid, g/kg DM	65.5	68.0	52.0	56.0
Acetic acid	18.0	13.5	15.5	17.0
Butyric acid	1.8	0.1	0.6	0.1
Nitrate	3.5	4.4	2.4	1.1
Yeast, log cfu/g	<2.0	<2.0	6.3	5.9
Mold	<2.0	<2.0	2.3	<2.0
Enterobacteriaceae	<2.0	<2.0	<2.0	<2.0
Escherichia coli	<1.0	<1.0	<1.0	<1.0
Aerobic spore-forming bacteria	<3.0	3.3	<3.0	3.7
Butyric acid spores[1]	<1.0	1.6	1.0	1.3
ME, MJ/kg DM	11.2	11.2	10.7	11.0
OM digestibility, %	77.0	77.2	73.4	76.3
DM, g/kg FM	277	306	320	280
NDF, g/kg DM	480	457	524	439
ADF	285	270	314	275
Crude fat	41	39	39	39
Water-soluble carbohydrates	15	18	16	29
Ash	77	77	69	85
CP	169	172	155	146
Soluble CP, g/kg CP	670	545	600	561
Ammonia-N, g/kg N	109	80	97	84

[1]Spore-forming bacteria that produce butyric acid.

Design of the feeding experiment

The feeding experiment was run for 12 weeks, with each of the 3 silages evaluated for 3 weeks (±1 d). The order of treatments was: T1) UNTR, T2) INOC, T3) ACID, and T4) INOC again. The INOC treatment was repeated to evaluate whether potential changes in milk microbiota were repeated. Each treatment was incorporated into a PMR that was fed to all animals included in the trial. The last week of each treatment was a sampling week in which data were recorded and samples were collected. On the day before the start of each treatment, the whole barn was thoroughly cleaned.

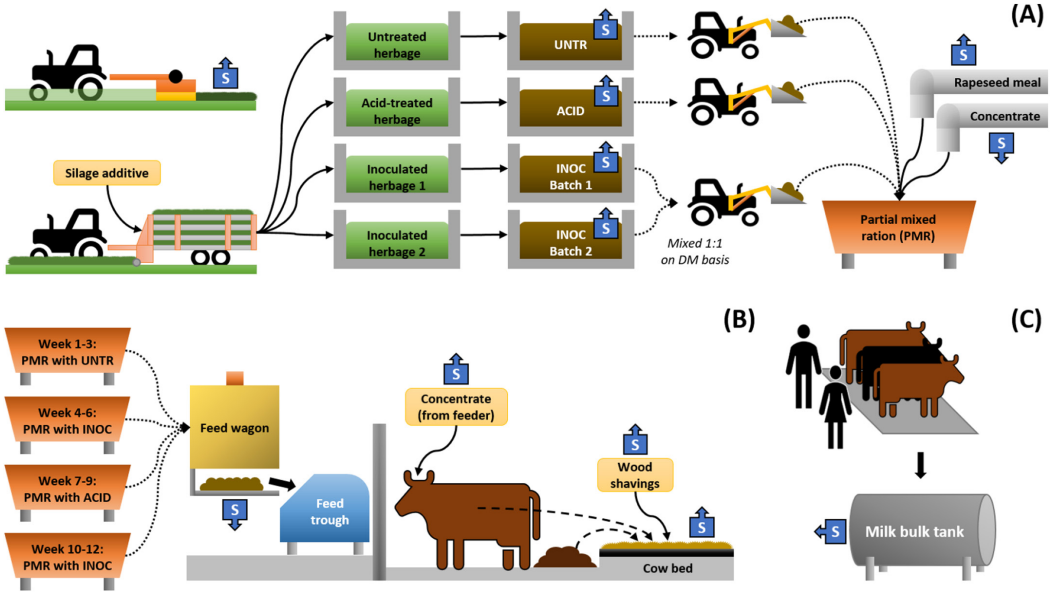

Figure 1. The experimental design, briefly summarized in 3 panels; (A) the ensiling process and preparation of partial mixed rations, (B) treatment schedule and animal housing, and (C) milk from last week of each treatment in bulk milk tank. Sampling points are marked with (S).

Table 2. Production averages for the cows during feeding of the untreated (UNTR), acid-treated (ACID), and starter culture-inoculated (INOC) silage treatments

Treatment	T1-UNTR	T2-INOC	T3-ACID	T4-INOC	SEM
Days, n/treatment	22	20	22	21	
Animals					
Cows, n/day	63.1	68.2	68.9	69.0	0.29
Weight[1], kg/cow	639	655	651	653	2.4
Feed intake[2]					
Total, kg DM/day	20.2	21.3	21.1	20.9	0.09
PMR	14.5	14.9	15.3	15.7	0.07
- *Silage*	*10.2*	*10.0*	*10.9*	*10.7*	*0.05*
- *Concentrate*	*3.4*	*3.3*	*3.5*	*3.6*	*0.02*
- *Rapeseed meal*	*0.8*	*1.4*	*0.8*	*1.3*	*0.01*
Concentrate	5.6	6.1	5.1	4.5	0.06
Test milking[3]					
Milk, kg	29.8	32.2	32.4	30.6	0.48
ECM, kg	32.8	34.6	35.7	33.1	0.47
Fat, %	4.60	4.37	4.58	4.45	0.065
Protein, %	3.77	3.79	3.81	3.75	0.049
Urea, mmol/L	5.0	4.9	4.6	5.0	0.09
SCC, 1000 cells/mL	125	115	147	125	12.4

[1]Weight was recorded approx. two times per cow during the last week of each treatment.
[2]Feed intake was recorded daily for each cow during the last week of each treatment.
[3]Test milking was performed during two consecutive milking occasions in the last week of each treatment.

Animals and diets

Approximately 67 (range 61–69) primi- and multiparous dairy cows (mainly Nordic Red) were included in the experiment. Average cow weight during the experiment was 653 kg (SEM 1.3 kg) and average milk production per cow was 32.8 L (SEM 0.12 L). The PMRs were produced using one of the treatments (UNTR, ACID, or INOC), concentrate, rapeseed meal, and a mineral premix. When the INOC was fed, the 2 different batches were mixed 1:1 on a DM basis. The PMR was designed to meet the basic nutritional needs of dairy cows producing 25 kg ECM per day, and all PMRs were set to be isocaloric (ME basis) and isonitrogenous. Additional concentrate was fed in proportion to milk yield. The PMR was fed through 30 feed bunks (Roughage Intake Control, Insentec B.V., Marknesse, the Netherlands) and additional concentrate through separate concentrate feeders. A stationary feed mixer (Nolan A/S, Viborg, Denmark) processed the PMR, which was delivered by automatic feeder wagons to the feed bunks 6 times per day. The amount of feed delivered was monitored daily, to avoid excessive leftovers. Detailed information on animals, feed intake, and milk production is provided in Table 2.

Housing and milk collection

The dairy barn where the experiment took place is insulated, with a controlled indoor temperature at 10–15°C. The cows were kept loose-housed in 2 aisles, one for eating and one for resting with cubicles and a rubber mattress for each cow bed. The cubicles were manually cleaned with a scraper each day and covered with wood shavings (pine and spruce) on a daily basis to keep the animals dry. The cows were milked twice daily in a milking parlor (2 × 8), at 06:00 h and 16:00 h. The milking procedure comprised: 1) udder wiping with clean wet cloth, 2) drying with clean dry paper, 3) pre-milking by hand, and 4) applying the milk liners. Individual milk production was recorded daily using gravimetric milk recorders (S.A. Christensen & CO, Kolding, Denmark). The milking equipment and the milking parlor were thoroughly cleaned and washed after each milking, and the milk was collected and transported to the dairy every second day.

Sampling and sample preparation

Herbage. The botanical composition of the ley from each individual field was evaluated just before harvest using the dry-weight ranking method developed by Mannetje and Haydock (1963). In short, the leys were assessed by walking across the field at 15-m intervals, with up to 30 observations per field using a 1 m^2 steel quadrat to assess the areal contribution of the major plant species.

Fresh herbage samples for estimation of total bacteria count and microbial community analysis were taken from every field directly after cutting. Grab samples (ca. 15 kg FM) were taken evenly with sterile nitrile gloves from the herbage swathes in each field and placed in plastic bags. The herbage sampled from each field was mechanically chopped into smaller pieces and mixed thoroughly, before further processing.

Feeds and bedding material. Samples of silage, concentrate, and rapeseed meal were collected for determination of DM at least once every week during the whole experiment, to maintain correct mixing proportions in the PMR. Drill core samples from the bunker silos designated for analysis of chemical composition and hygienic quality were taken by Eurofins Agro Testing (Kristianstad, Sweden) approximately 3 mo after the silos were closed (2 mo for the second INOC silo). The drill cores were taken from each silo by drilling from top to bottom in an evenly distributed pattern.

All sampling for estimation of total bacteria count and microbial community analysis was performed during the last week of each treatment. Silage, PMR, and used bedding material were sampled 3 times (every second day). Concentrate, rapeseed meal, and wood shavings were sampled once (mid-week). Silage was sampled from the opened bunker silos by grab sampling with sterile nitrile gloves at a minimum of 20 evenly distributed spots over the open surface just after silage was taken out. The PMR was sampled by grab sampling with sterile nitrile gloves from the outlet of the feeder wagon during one full filling round of the feed bunks. The silage and PMR samples were ground with a sanitized compost grinder before further processing. The used bedding material was sampled by taking grab samples with sterile nitrile gloves from the bottom half of every second cubicle, giving a sample comprising a mixture of wood shavings, manure, and various animal fluids. Concentrate and rapeseed meal were sampled from both the concentrate feeders and the individual lines going to the mixer. Sampling was performed by releasing a minimum of 5 kg from each source into a plastic bag. Clean wood shavings were sampled with sterile nitrile gloves from the most recently used bunker silo (2 in total) by grab sampling at a minimum of 20 evenly distributed spots on the open surface (top layer discarded).

Milk. Milk samples for microbial community analysis were sampled from the bulk tank in the morning of the same days as the silage, PMR, and used bedding material were sampled. On these occasions, the bulk tank contained milk from 4 milkings, 2 d of morning milk and 2 d of evening milk. Samples (40 mL) were drawn into duplicate sterile Falcon tubes (50 mL) and immediately stored frozen (−20°C). At the end of each sampling week, all milk samples were transferred to storage at −80°C. Additionally, a sample for estimation of total bacteria count was taken by Norrmejerier (Burträsk, Sweden) after transportation of the milk to the dairy (within 2 h from collection in the barn). Finally, test milking was performed during the last week of each treatment by measuring the yield and sampling the milk of each cow on 2 consecutive milking occasions (afternoon and morning).

Analytical methods.

Feed composition The chemical composition of silage was analyzed with near-infrared spectroscopy (NIR) by Eurofins Agro Testing (Wageningen, The Netherlands). An unspecified internal method was used for DM and ash, while no methods were specified for nitrate, butyric acid and ADF content. The ME content was calculated from the chemical composition by Eurofins Agro Testing (Kristianstad, Sweden). The hygienic quality of the silages was analyzed by Eurofins Food & Feed Testing (Jönköping, Sweden). The methods used were: unspecified for pH, NMKL 98 for yeast and mold, AFNOR 3M 01/06–09/97 for *Enterobacteriaceae*, AFNOR 3M 01/08–06/01 for *Escherichia coli*, and Internal Method 7 for spore-forming aerobic bacteria and butyric acid spores.

Weekly in-barn analysis of DM in silage, concentrate, and rapeseed meal was performed by oven-drying samples at 60°C to constant weight. Chemical composition of concentrate and rapeseed meal in each batch delivered was analyzed by the producer Lantmännen (Umeå, Sweden).

Milk composition Samples from test milking were analyzed by Eurofins Milk Testing (Jönköping, Sweden). Milk composition was analyzed with mid-infrared spectroscopy (Fourier Transform Infrared, FOSS, Hilleröd, Denmark) and SCC with fluorescence-based cell counting (Fossomatic, FOSS, Hilleröd, Denmark).

Estimation of total bacteria count Culturing of bacteria was performed directly after sampling and sample preparation for all materials except milk. Each sample was mixed thoroughly and 2 subsamples of 30 g each were placed in stomacher bags, mixed with 270 g peptone water (1 g/L Oxoid Peptone Bacteriological, Thermo Scientific), and run in a Stomacher (Stomacher 400, Seward) for 1 min. A 10-mL subsample from each bag was pipetted into a sterile glass vial and a dilution series was performed with peptone water, followed by spread-plating of selected dilutions (0.1 mL/plate). For lactobacilli, de Man, Rogosa, and Sharpe (MRS) agar (54.6 g/L MRS agar, Merck) was used. For total bacteria, a modified (0.08 g/L Delvocide, DSM) milk plate count (MPCA) agar (19.5 g/L MPCA agar, Liofilchem) was used. The MRS plates were placed inverted in sealed jars with anaerobic medium, while the MPCA plates were stacked in perforated plastic bags. All plates were incubated in a heating cabinet at 30°C for 48 h. For milk, estimation of total bacteria count was performed by Norrmejerier (Burträsk, Sweden) according to an internal protocol with plate count agar (PCA) and incubation at 30°C for 72 h.

Microbial community analysis For all materials except milk, preparation took place in connection with estima-

tion of total bacteria count. Two additional subsamples of 30 g each were placed in stomacher bags and mixed with 270 g of 1/4 strength Ringer solution with 0.5 mL/L Tween 80 (Merck), prepared according to O'Brien et al. (2007), and run in a Stomacher (Stomacher 400, Seward) for 1 min. A 50-mL subsample from each bag was pipetted into a sterile Falcon tube and run in a centrifuge at 7000 *g* and 10°C for 25 min. After centrifugation, the tubes were decanted without losing any pellet, and refilled to the 10 mL mark with Ringer solution. The tubes were vortexed until the pellet was dissolved and 1 mL from each was pipetted into a 2 mL cryo-tube. The cryotubes were frozen at −80°C until DNA extraction was performed, which was done as described in Eliasson et al. (2023).

The 50-mL Falcon tubes containing milk samples were thawed in a water bath at 25°C for 1 h. The thawed milk was then carefully mixed by inverting the tubes by hand a few times, and 1.8 mL subsamples were pipetted into 2-mL collection tubes provided with the PowerFood DNA isolation kit (Qiagen AB, Sollentuna, Sweden). This step was followed by the customized protocol described in Sun et al. (2023). Random samples were checked with a Nanodrop spectrophotometer to assure DNA extractions with sufficient yield and quality. Finally, the bacterial DNA was stored at −80°C until further analysis.

Library construction and sequencing

The bacterial DNA was sent to Novogene (Cambridge, UK) for library construction and sequencing. An initial quality control of the DNA was performed by agarose gel electrophoresis. The V4 region of the 16S rRNA gene was amplified using the primers 515f (GTGBCAGCMGCCGCGGTAA) and 805r (GACTACHVGGGTATCTAATCC), and a library was constructed. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Sequencing was performed on the Illumina NovaSeq PE250 platform (50k tags per sample). The raw reads were de-multiplexed before delivery. From the initial 184 samples sent to Novogene, all passed the quality control. The raw sequencing data were deposited in the Sequence Read Archive at the National Center for Biotechnology Information database, under accession number PRJNAXXXXXX.

Bioinformatics

Bioinformatic data processing was performed using QIIME 2 2022.11 (Bolyen et al., 2019). The raw demultiplexed reads were trimmed with Cutadapt to remove primer sequences (Martin, 2011), and all reads containing non-identified bases or missing primer sequences were removed. Further trimming, de-nosing, de-replication, read merging, and removal of chimeras were performed with DADA2 (Callahan et al., 2016). Truncation length was set to 160 bp for forward reads and 146 bp for reverse reads, as it gave the best read recovery after testing different levels of truncation. Phylogenetic trees were built using FastTree and MAFFT (Price et al., 2010; Katoh and Standley, 2013). Alpha and β diversity were estimated, and principal coordinate analysis (**PCoA**) was performed using the q2-diversity plugin. Faith's phylogenetic diversity index (**FPDI**; Faith, 1992) was used to compare diversity, while weighted UniFrac distance matrix (Lozupone et al., 2007) and PCoA results were used to compare microbiota composition between and within materials. Taxonomy was assigned to ASVs with q2-feature-classifier (Bokulich et al., 2018), using release 138 from the Silva database (Quast et al., 2012) as reference. For ASVs with higher RAs not passing species annotation by QIIME2, selected ASVs were elaborated further using Nucleotide BLAST and the 16S ribosomal RNA sequences database as reference (accessed 2024–02–28), where only hits with 100% query cover and identity were considered (Zhang et al., 2000).

Statistical analysis

The raw output files (.qza) from QIIME 2 were imported to R with the *qiime2R* package (Bisanz, 2018) together with all other data. Tables and diagrams were produced with R 4.3.1 (R Core Team, 2021), using the *tidyverse* package (Wickham et al., 2019). Statistical evaluation was performed with the additional packages *car* (Fox and Weisberg, 2019) and *emmeans* (Lenth, 2024).

Individual daily records of cow weight, feed intake, and milk production were first checked for outliers by using the z-score method for each variable and cow. Values deviating by ≥ 3 SD from the mean were discarded. Data from the last week of each treatment were filtered out and arithmetic means were calculated for each treatment together with SEM for all treatments, for each variable. The test milking results were filtered to only include complete records, i.e., cow data with missing values in any of the measured variables were discarded. Arithmetic means were calculated for each treatment and SEM for all treatments, for each variable. Compositional variables (fat, protein, urea, and SCC) were related to milk volume before calculation of treatment means and SEM.

For evaluation of total bacteria count, arithmetic means were calculated for each material and treatment. One-way ANOVA was performed for each material to evaluate the effect of treatment. For materials with a significant treatment effect, 2-tailed Welch *t*-tests were performed on all treatment combinations and *p*-values were adjusted to avoid falsely rejected hypotheses ac-

cording to Benjamini and Hochberg (1995), with $P < 0.05$ considered significant.

For α diversity (measured as FPDI), a boxplot was produced for evaluation of differences between materials and treatments. Arithmetic means and SEMs were calculated, and 2-tailed Welch *t*-tests were performed (as described above). Microbial composition was evaluated by pooling the reads by technical replicates (n = 2), followed by rarefication at the lowest sampling depth found in the data set (28806 reads/sample). Arithmetic means were calculated to the levels described in the diagrams, and data were evaluated descriptively. Taxa found below 0.1% RA (29 rarefied reads) were considered as detected, but not as clear findings. For evaluation of treatment effects, Quasi-Poisson regression and pairwise comparisons with Tukey adjustment were performed per genus or ASV.

RESULTS

Bacterial enumeration, composition and diversity

The bacterial enumeration on different media showed major variation between materials (Table 3). The highest average number of total bacteria was found in used bedding material (9.6 $\log_{10}$ cfu/g), while the lowest average was found in milk (3.5 $\log_{10}$ cfu/g), with both these differing significantly from all other materials. Mean number of total bacteria in silage and PMR (7.1 and 7.5 $\log_{10}$ cfu/g) was different from that in all other materials and from each other. The highest average number of lactobacilli was found in silage and PMR (8.8 and 8.7 $\log_{10}$ cfu/g, respectively), with both differing from the other materials. Among the silages, ACID had lower number of total bacteria than both batches of INOC, while UNTR had higher number of lactobacilli than the other silages. In PMR, differences in both total bacteria and lactobacilli were found between all silage treatments, except between the first and second INOC batches. Herbage was only randomly evaluated for lactobacilli during first cut (mean 4.3 $\log_{10}$ cfu/g, n = 26, SEM = 0.10).

The PCoA of the weighted UniFrac distance matrix explained 68.4% of the variation in bacterial composition by the first 3 principal coordinates (Figure 2). The PCoA plot revealed rather clear separation between 3 clusters of materials: 1) herbage, 2) silage and PMR, and 3) used bedding material and milk. Concentrate, rapeseed meal, and wood shavings were more spread in the PCoA plot, but were still rather separated from all other materials. Thus the microbiota of milk was closest to that of used bedding material.

Alpha diversity of the microbiota, measured as FPDI, varied widely between the materials, and to some extent also between the treatments (Figure 3, herbage excluded). Milk had the highest average FPDI (70.2), followed by used bedding material (44.1) and wood shavings (41.1). The FPDI of milk was different from that of all other materials, while used bedding material differed from all other materials except wood shavings. The lowest average FPDI was found in herbage (22.2, SEM 1.34), followed by silage (24.2), PMR (27.7), concentrate (32.9), and rapeseed meal (33.6). Herbage differed from all other materials except silage. Silage FPDI differed from that of PMR, concentrate, rapeseed meal, and wood shavings. The FPDI of PMR differed from that of wood shavings, but not from that of concentrate and rapeseed meal. In terms of FPDI, concentrate and rapeseed meal did not differ from each other or from wood shavings.

Table 3. Total bacteria and lactobacilli counts ($\log_{10}$ cfu/g) in the different materials during fedding of the untreated (UNTR), acid-treated (ACID), and starter culture-inoculated (INOC) silage treatments

Type	Material	T1-UNTR	T2-INOC	T3-ACID	T4-INOC	SEM	*p-value*
Total bacteria[1]	Silage	OG	7.3	6.4	7.4	*0.12*	*0.001*
	Concentrate	5.2	4.4	4.6	4.6	*0.09*	*0.005*
	Rapeseed meal	6.0	4.3	5.3	5.2	*0.16*	*0.001*
	Partial mixed ration	*8.4*[4]	7.7	6.8	8.0	*0.12*	*<0.001*
	Wood shavings	5.9	5.7	6.4	6.3	*0.13*	*0.153*
	Used bedding material	9.6	9.4	9.5	9.6	*0.03*	*0.023*
	Milk[2]	3.5	3.6	3.5	3.6	*0.09*	*0.952*
Lactobacilli[3]	Silage	9.2	8.8	8.6	8.7	*0.03*	*<0.001*
	Concentrate	3.5	3.3	3.9	3.3	*0.14*	*0.611*
	Rapeseed meal	5.4	3.9	3.8	NA	*0.21*	*<0.001*
	Partial mixed ration	9.0	8.8	8.4	8.6	*0.04*	*<0.001*
	Wood shavings	4.1	4.3	4.6	5.1	*0.12*	*0.003*
	Used bedding material	8.0	8.0	7.9	8.2	*0.06*	*0.413*

[1]Aerobic incubation on modified milk plate count agar (0.08 g/L Delvocide, DSM).

[2]Aerobic incubation on plate count agar.

[3]Anaerobic incubation on de Man, Rogosa, and Sharpe (MRS) agar.

[4]Counting only possible on one plate.

Abbreviations: OG = overgrown by unknown microorganism, NA = no colonies on plates.

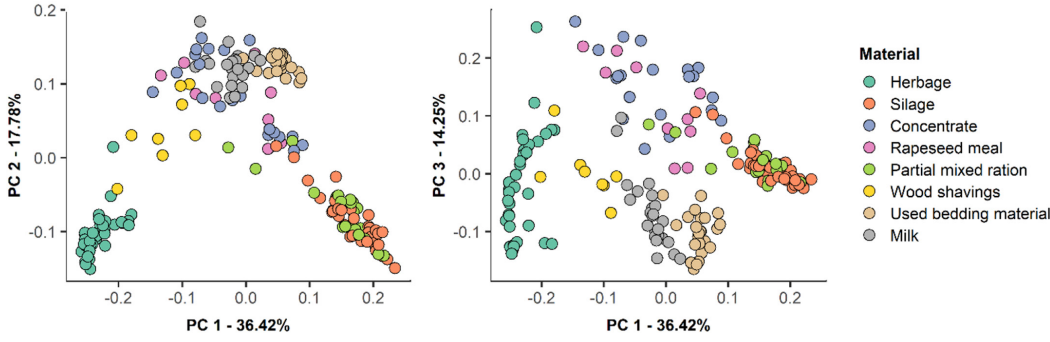

Figure 2. Principal coordinate analysis plots of the weighted UniFrac distance matrix of the microbiota of the different materials. The diagrams include sample replicates and show the first 3 principal coordinates (PC) and their contribution to the total variation in microbiota.

Herbage and silage composition

Evaluation of the botanical composition of herbage (Table S1in Supplementary Material) showed a major proportion of timothy (mean 70%, range 53–90%), while other plant species varied to a larger extent. Dandelions were mainly found in herbage J (21%), while annual bluegrass was mainly found in herbage F (27%). Herbage F was also the only herbage with tufted hairgrass (8%). Red clover was found in a high proportion in herbage L (21%), while white clover was only found in herbage C (13%). Herbage C also contained a high proportion of meadow fescue (16%). The proportions of other forage species varied from 3% to 15% in first-cut and from 21% to 41% in second-cut herbages.

The top 30 bacterial genera in herbages and the corresponding silages are presented in Figure 4. The second cut of herbages and the corresponding second INOC batch was not initially planned, but was necessary as the first INOC batch was not sufficient for the feeding experiment. Due to lack of communication, herbage was not sampled during the second cut. First-cut herbages showed a varying microbiota, with *Xanthomonas* and *Sphingomonas* contributing most to RA (mean 28.3% and 24.4%, respectively). Herbage J had high RA of unclassified *Yersiniaceae* (25.0%) and unclassified *Enterobacteriaceae* (19.6%). Other genera present in high average RA were *Pedobacter* (5.4%), *Pseudomonas* (5.4%), *Hymenobacter* (4.5%), and *Massilia* (3.7%), with mostly minor variation between herbages. The ge-

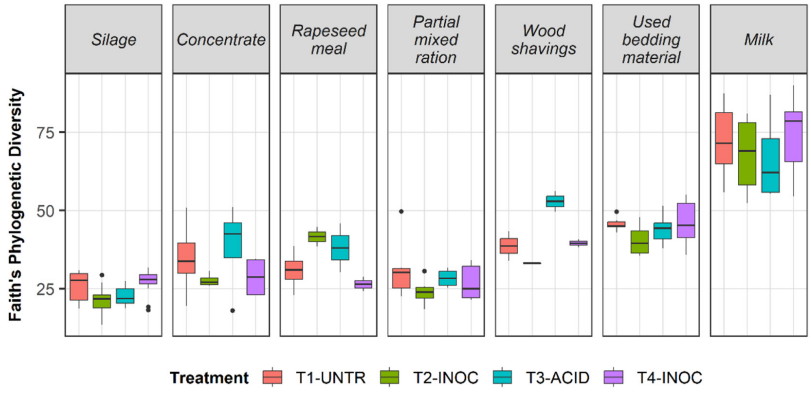

Figure 3. Boxplots of α diversity, estimated as Faith's phylogenetic diversity index, of the microbiota in feedstuffs, bedding material, and milk during the untreated (UNTR), acid-treated (ACID), and starter culture-inoculated (INOC) silage treatments in the feeding trial.

nus *Lactobacillus* was barely detected, except in herbage L, which was also the only herbage in which *Aerococcus* and *Corynebacterium* were found. The most abundant genera observed in the herbages were barely present in the corresponding silages.

The silage microbiota mainly comprised the 3 genera *Lactobacillus*, *Prevotella*, and *Pseudomonas*. *Lactobacillus* was found in average RA of 61.6% (range 43.8–78.4%), *Prevotella* in RA of 16.6% (range 0.7–34.9%), and *Pseudomonas* in RA of 3.6% (range 1.4–7.6%). Despite the major variation in these genera between silages, pairwise comparisons revealed no significant differences. However, differences were found for *Pediococcus*, which was present in higher RA in UNTR than in the other silages.

Microbiota of the different materials

The top 30 bacterial genera in all materials (except herbage) by treatment are presented in Figure 5. In general, there was little variation between treatments in microbiota within the different materials during the feeding trial. The RAs in the different silages in Figure 5 is the same as in Figure 4, with the exception of INOC which in Figure 5 is illustrated as the pooled value (1:1) of those 2 silage batches. For ACID, higher RA of *Lactobacillus* was observed in the silage, but the RA was not significantly different from that in the other silages. Concentrate and rapeseed meal showed similar microbiota and 3 main genera were observed at high average RA, namely *Pantoea* (30.4% and 24.6%, respectively), *Lactobacillus* (16.4% and 13.8%, respectively), and *Pseudomonas*

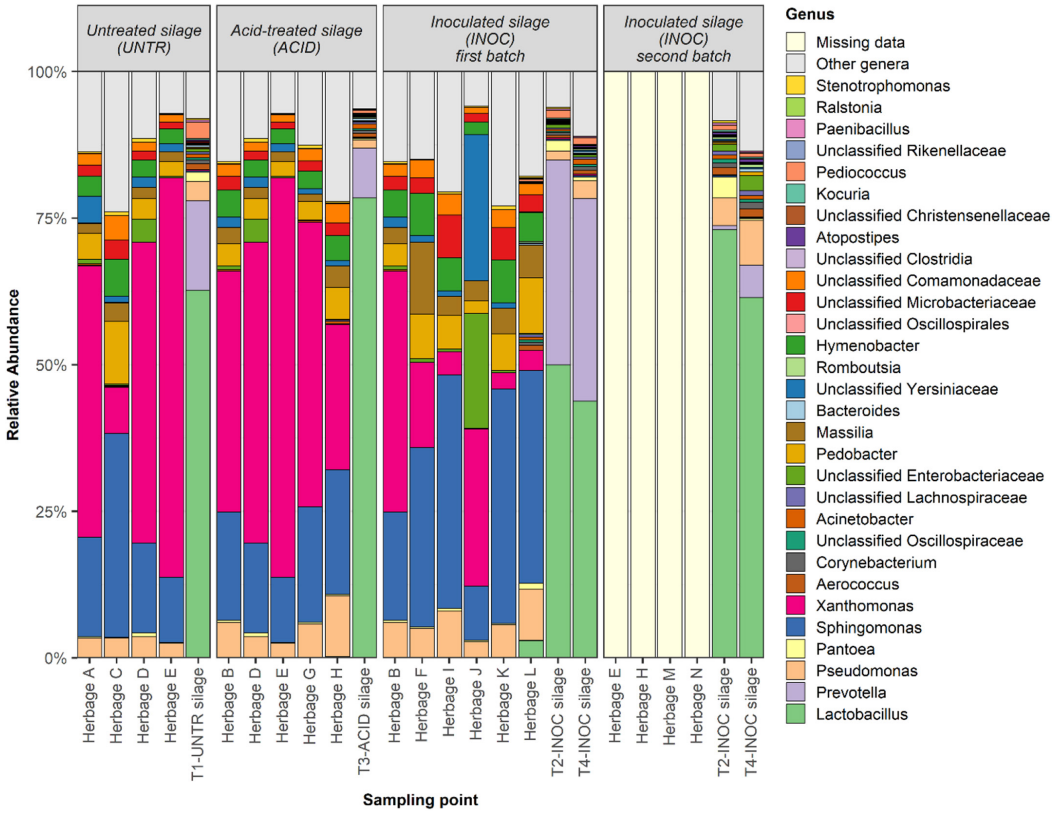

Figure 4. Relative abundance of the top 30 bacterial genera in the harvested herbages, and in the corresponding silages (sampled during each treatment). Figure represents sequence data pooled by technical replicate (n = 2). Silages were fed in the following order: T1) UNTR, T2) INOC, T3) ACID, and T4) INOC. The INOC silages were mixed 1:1 on dry matter basis during the T2-INOC and T4-INOC treatments.

(9.2% and 9.9%, respectively). The microbiota of the PMRs showed the strongest resemblance to that of the silages, despite the high inclusion of concentrate and rapeseed meal (Table 2). However, the number of genera with average RA > 0.1% increased from 43 in the silages to 58 in the PMRs. The 3 main genera in silage, i.e., *Lactobacillus*, *Prevotella*, and *Pseudomonas,* were observed at 47.8%, 20.0%, and 7.7% average RA, respectively, in PMR. As seen for the silages, the RA of *Pediococcus* was significantly higher in the PMR containing UNTR than in the other PMRs. Numerically high RA of *Pseudomonas* (18.1%) was observed in the PMR during the second INOC treatment, but RA was not significantly different from that in the other materials.

Wood shavings showed generally high average RA of *Pseudomonas* (18.8%). This genus and the genera *Sphingomonas*, unclassified *Yersiniaceae*, and *Massilia* showed a tendency to be present in higher RA in the INOC treatments. However, due to the low number of sampling occasions, this was not further evaluated. Additionally, the genera *Cellvibrio* and *Glutamicibacter* were found at higher RA in the wood shavings, but were not among the top 30 genera (shown in Figure 5). Used bedding material contained many genera, but none was clearly dominant, and it showed little resemblance to the wood shavings. The genera present in highest average RA were *Aerococcus* (12.0%) and *Corynebacterium* (11.5%), followed by *Acinetobacter* (6.5%), *Lactobacillus* (6.3%), unclassified *Oscillospiraceae* (6.3%), and unclassified *Lachnospiraceae* (5.6%).

Raw milk was the most diverse of all materials (Figure 3), comprising a total of 122 genera with average RA > 0.1%. Thus microbial diversity was much higher than in used bedding material (81 genera) or PMR (58 genera). The highest average RA was recorded for *Lactobacillus* (10.7%, range 8.4–15.5%), with a tendency for increas-

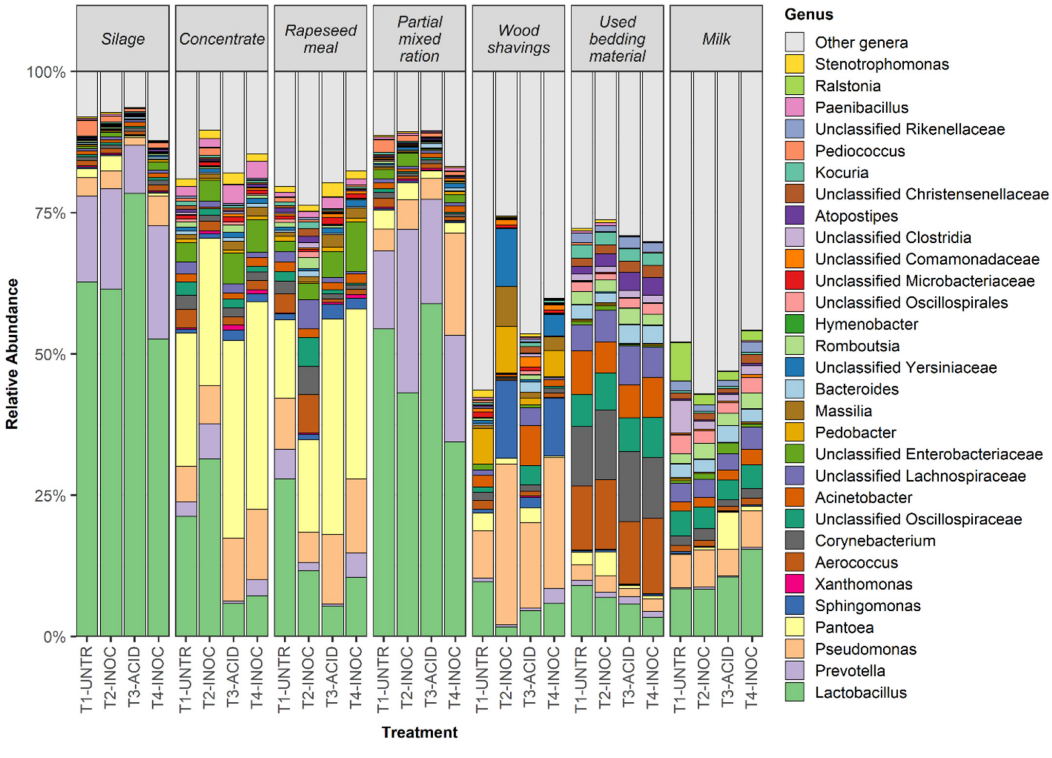

Figure 5. Relative abundance of the top 30 bacterial genera in feedstuffs, bedding material and milk during the last week of each treatment in the feeding trial. Figure represents sequence data pooled by technical replicate (n = 2). The treatments were: untreated silage (T1-UNTR), inoculated silage (T2-INOC), acid-treated silage (T3-ACID), and a repeat of inoculated silage (T4-INOC).

ing RA over the course of the experiment, but with no significant difference between treatments. This was followed by *Pseudomonas*, with average RA of 5.9%. The only significant difference in milk between treatments was found for unclassified *Clostridia*, which was present in higher RA when feeding the UNTR compared with the other treatments.

Most abundant ASVs found during the feeding trial

To further evaluate the flow of bacteria from feed to milk, an investigation on ASV level was performed. In total, 15766 ASVs were detected in PMR, used bedding material, and milk. Of these, only 151 were found at average RA > 0.1%, and only 15 at average RA > 1.0%. The top 50 ASVs were selected based on their average RA in all 3 materials, and a heatmap was produced (Figure 6). The overall finding was that the most abundant ASVs in PMR were to some extent also present in used bedding material, but rarely in the milk. However, several ASVs which were abundant in used bedding material were also abundant in the milk.

Effect of silage additive

At genus level, only a minor effect of additive was observed in the resulting silages. The microbiota of the silages was reflected in the corresponding PMR, but closer investigation on ASV level was performed to evaluate whether the silage additives separated the treatments. Among the 4 species of bacteria included in the starter cultures used for INOC, only *Lactococcus lactis* was found among the top 50 ASVs in the PMR (Figure 6). However, this ASV could represent another strain, and the other bacteria in the starter culture could be among the unidentified ASVs. The ASVs in PMR showing the highest RA were *Lactobacillus acetotolerans* (e1910) (RA range 20.4–36.0% and *Prevotella* (f74b4) (range 13.0–27.5%. Tendencies for differences between treatments were observed, but none of these was significant.

Among the remaining top 50 ASVs, only a few showed significant differences between treatments. *Lactobacillus* (03f2f) was less abundant in PMR in the ACID treatment than in the UNTR and the first INOC treatments. The RA of the ASV *Lactobacillus* (1d194) was significantly lower during the second INOC treatment compared with UNTR. *Lactobacillus* (6b62e) was mainly present during the UNTR treatment. The RA of *Lactobacillus buchneri* (dc9d7) was significantly lower during the ACID treatment compared with UNTR and the first INOC. *Lactobacillus fructivorans* (08c5f) was mainly present during the ACID treatment, and at a notably higher RA (16.7%). The RA of *Pediococcus* (3d185) was higher during UNTR than in the other treatments, while unclassified *Enterobacteriaceae* (8622f) was less abundant during the ACID treatment compared with UNTR. Only a few ASVs showed strong tendencies for higher abundance during one of the treatments, e.g., *Prevotella paludivivens* (5e89c) during the ACID treatment and *Pseudomonas* (15a37 and 3ae60) during the second INOC treatment. However, these differences were not significant, as one of the 3 sampling occasions typically contributed to the high average RA for a certain treatment.

Similarities between PMR and used bedding material

Lactobacillus acetotolerans (e1910), *Prevotella* (f74b4), and other highly abundant ASVs in the PMR were also found in used bedding material, but at lower RA. In addition, ASVs which were rarely found or only found at a low RA in PMR were found to be part of the microbiota in used bedding material. The most abundant ASVs in used bedding material were *Aerococcus* (0cb4d) and *Acinetobacter* (8572d), with average RA of 10.6% and 5.9%, respectively. Further, a group of *Corynebacterium* ASVs were observed, at total average RA of 8.8%.

A few significant differences between treatments were found for used bedding material. *Lactobacillus* (1d194 and 6b62e) and *Pediococcus* (3d185) showed higher RA during the UNTR treatment, while *Paeniclostridium* (0bb17) and *Turicibacter* (62c62) showed their highest RA during the ACID treatment. *Romboutsia* (8f04c) showed higher RA during the ACID treatment than during the second INOC treatment. Unclassified *Enterobacteriaceae* (8622f) showed higher RA during the first INOC treatment compared with both the ACID and second INOC treatments. Unclassified *Lachnospiraceae* (a411d) showed higher RA during the ACID treatment compared with the second INOC treatment.

ASVs in milk and their potential origin

The only ASVs present in high RA in all 3 materials were *Aerococcus* (0cb4d) and *Pantoea* (0951f), with the latter also showing a tendency for treatment differences (not significant). The most abundant ASV in milk was *Pseudomonas* (3973d), at average RA of 5.1%, and this ASV was also found in the other materials, although at lower RA. *Romboutsia* (8f04c) and *Turicibacter* (62c62) were also present at higher RA in used bedding material and milk, but with no significant treatment differences. Similar findings were made for *Clostridioides difficile* (870a5), *Paeniclostridium* (0bb17), and unclassified *Oscillospiraceae* (40610, e3f70, and f1f91). *Lactobacillus* (172f4) and *Lactobacillus intermedius* (1646a) were the most abundant LAB in milk, with average RA of 4.6% and 2.5%, respectively. However, they were not found in the other materials and showed no significant treatment

differences. Similar findings were made for *Lactococcus lactis* (dc6c4), although it was observed at low RA (>0.1%) in the PMR during all treatments except ACID. The only significant treatment difference in milk was observed for *Atopostipes* (4a4bc), which showed higher RA during the ACID and second INOC treatments compared with the first INOC. *Ralstonia* (b37c7) showed a strong tendency for higher RA in milk during UNTR, but the difference was not significant. A few more tendencies for differences between treatments were observed, but were not strong enough to overcome the variation between sampling occasions.

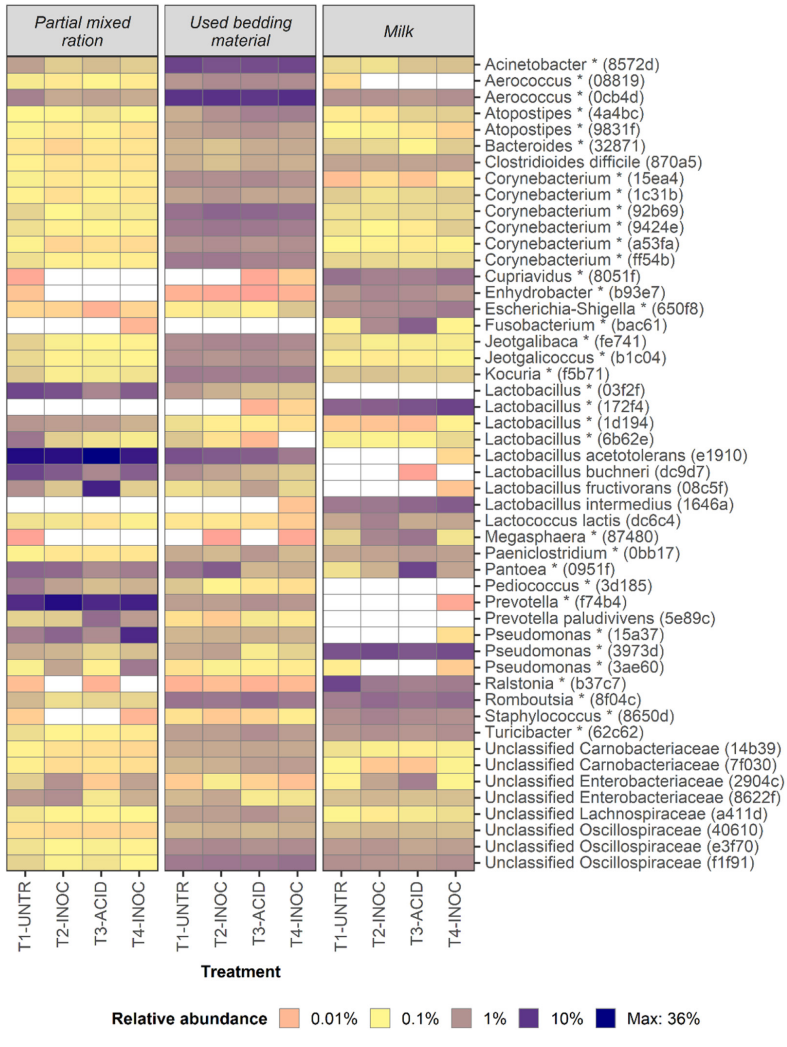

Figure 6. Heatmap showing $\log_{10}$-transformed relative abundance (RA) of the top 50 ASVs found in partial mixed ration, used bedding material, and milk during the feeding trial. The treatments were: untreated silage (T1-UNTR), inoculated silage (T2-INOC), acid-treated silage (T3-ACID), and a repeat of inoculated silage (T4-INOC). The legend scale was converted back to RA and non-present ASVs were colored white for easier interpretation. The (*) marks that the ASV was not classified further than genus-level.

Further investigation of the LAB ASVs found in milk

Further investigation was performed by filtering out ASVs belonging to the order *Lactobacillales*, resulting in a total of 716 detected LABs in the 3 materials, of which 437 were detected in milk. Of the LAB ASVs found in milk, only 22 were present in average RA > 0.1%, and these were summarized in a heatmap (Figure 7). Those present in highest RA were *Lactobacillus* (172f4), *Lactobacillus intermedius* (1646a), *Aerococcus* (0cb4d), and *Lactococcus lactis* (dc6c4). These 4 ASVs were already included in the heatmap in Figure 6, together with the less abundant *Atopostipes* (4a4bc), *Jeotgalibaca* (fe741), *Lactobacillus* (6b62e), and unclassified *Carnobacteriaceae* (14b39).

The remaining 14 of the 22 LAB ASVs in milk were not among the top 50 ASVs in Figure 6, most of them (10/14) being *Lactobacillus*. Among these, only *Lactobacillus* (8427e and cd832) was clearly found in both milk and the other materials, while the others were in principle only found in milk, which was also the case for *Streptococcus* (ac3e9 and f5123). *Enterococcus* (e0166) was found in the PMR, but barely detected in used bedding material. Unclassified *Aerococcaceae* (a59f9) in milk was found in both used bedding material and PMR. No significant treatment differences were observed for these ASVs in milk.

DISCUSSION

This study evaluated whether silages intended for dairy cows, and produced with different silage additives, affect the microbiota of the milk, and whether LAB are transferred from feed to milk. The silage treatments were each fed as PMRs to 67 dairy cows for 3 weeks, with one treatment fed twice to evaluate whether potential changes in milk microbiota were repeated. To our surprise, there were only minor differences in the microbiota of the different silages. The microbiota of the silages was reflected in that of the corresponding PMR, and the major bacteria in PMR were also found in used bedding material, but rarely in milk. The milk microbiota was mostly related to that of used bedding material. Abundant bacteria in milk, especially LAB, were often not found in the other materials.

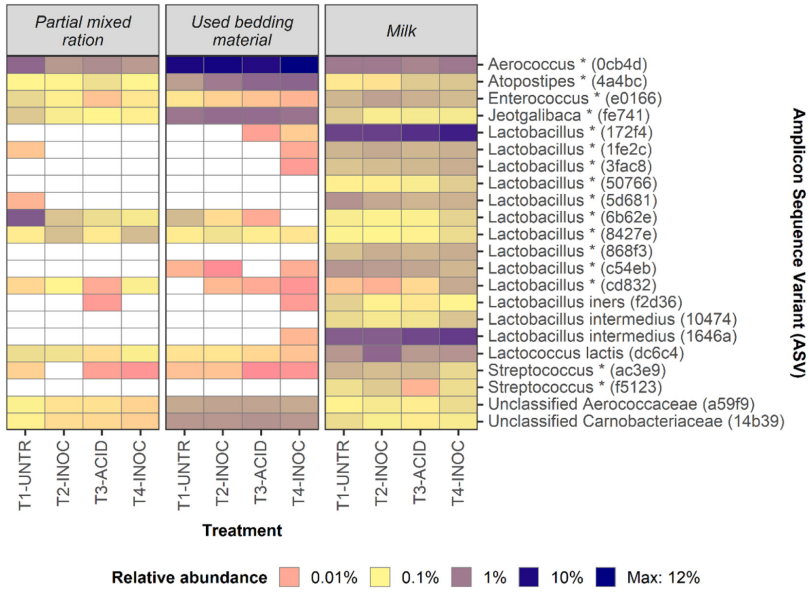

Figure 7. Heatmap showing $\log_{10}$-transformed relative abundance (RA) of Lactobacillales ASVs found at average RA > 0.1% in milk, and their concurrent RA in partial mixed ration and used bedding material during the feeding trial. The treatments were: untreated silage (T1-UNTR), inoculated silage (T2-INOC), acid-treated silage (T3-ACID), and a repeat of inoculated silage (T4-INOC). The legend scale was converted back to RA and non-present ASVs were colored white for easier interpretation. The (*) marks that the ASV was not classified further than genus-level.

Silage additives affected the silages less than expected

In a previous study evaluating the effect of silage additives typically used in the Nordic countries on the final microbiota in laboratory-scale silages, we observed a strong effect of ensiling additives on silage microbiota, while herbage microbiota showed little resemblance to that of the corresponding silage (Eliasson et al., 2023). In this study, we evaluated the microbiota of silages preserved in the same way as in our previous study, but in full-scale on a farm, and evaluated whether the different silages affected the microbiota of the milk when fed to dairy cows. The core microbiota of the herbage mainly comprised *Sphingomonas*, *Xanthomonas*, and a few other non-LAB genera, while the silage was dominated by *Lactobacillus*, *Prevotella*, and *Pseudomonas*. The effect of silage additives on the silage microbiota was not as clear as in our previous study, although some differences between the treatments were found. At first glance, ACID tended to differ from the other treatments, with higher RA of *Lactobacillus* in the silage. Scrutiny at ASV level showed that the major silage genera comprised many different species present at varying RA. However, only a few ASVs showed significant differences between the silage treatments. As in our previous study, *Lactobacillus fructivorans* was mainly associated with ACID, while various other LAB ASVs were found in UNTR and INOC in varying proportions. Although only a few observed differences were statistically significant, we believe that the microbiota of the silages differed. With the low number of replicates per sampling week (n = 6, 2 per sampling day) and sometimes large variation between these, differences had to be major to be statistically significant.

Prevotella and its potential origin

The finding of *Prevotella* in the silages was interesting, as this genus was not observed in our previous study (Eliasson et al., 2023) or in most other recent silage studies. In a laboratory-scale study by Franco et al. (2022) and the on-farm study by Kennang Ouamba et al. (2022), similar crops were ensiled but *Prevotella* was not detected in the final silages. However, closer scrutiny of results reported by Bayat et al. (2023) revealed a clear finding of *Prevotella* in some of their bunker silos and feed mixes. This is particularly interesting, as their study was similar to ours in many feed-related aspects. Based on the finding by Seshadri et al. (2018) that *Prevotella* is one of the dominant genera in the rumen, contamination of the barn environment by rumen bacteria is a likely explanation for the presence of *Prevotella* in feed in both our study and that by Bayat et al. (2023). Further support for this suggestion is provided by findings by Krizsan et al. (2023) of presence of *Prevotella* at average RA of 34.3% in rumen samples obtained from cows on the same dairy farm 2 mo before our study took place. Additionally, analysis of the raw data from the study by Ramin et al. (2023) of cow feces on the dairy farm during the period covered in the study by Krizsan et al. (2023) showed that sequences belonging to *Prevotellaceae* followed from the rumen to the feces. However, a multiple alignment with *blastn* (Zhang et al., 2000) of the most abundant *Prevotella* ASVs in our study with the ASVs of their studies, at its best, resulted in an alignment at 94% identity with full query cover, meaning that the sequences detected differed in at least 15 bp. This indicates that the bacteria in our study were rather distant from those found in the earlier rumen and feces samples from the same farm.

Lactobacillus fructivorans and acid-treated silage

Very few silage studies have reported *Lactobacillus fructivorans* in silages. This bacterium was detected in TMR-silage by Nishino et al. (2015), but was not discussed further until a study by Wu and Nishino (2016), who produced alfalfa silage using molasses. Interestingly, those authors found that *Lactobacillus fructivorans* did not grow well on MRS agar, but grew well on liver-infused sake agar. This could be one reason why it has not attracted much attention in previous silage studies. In both the present study and our previous ensiling study (Eliasson et al., 2023), *Lactobacillus fructivorans* was mainly found in silage made with formic and propionic acid as an additive. We found no clear connection between these acids and *Lactobacillus fructivorans* in the literature, although the bacterium is known to grow well at high ethanol concentrations (Suzuki et al., 2008). Unfortunately, ethanol in the silages was not analyzed in this or our previous study. However, Randby and Bakken (2021) found that silages made from crops similar to ours, with formic and propionic acid as additive, contain up to 30 g ethanol per kg DM. Following the reclassification of *Lactobacillus* into new genera (Zheng et al., 2020), *Lactobacillus fructivorans* now belongs to *Fructilactobacillus*. In the study by Bayat et al. (2023), using similar crops for ensiling, this new genus was found at the highest RA in silage made with formic and propionic acid. Thus there seems to be a rather clear connection between the bacterium and this type of silage additive, but it was not possible to evaluate the association further in this study.

Sampled materials in the feeding trial provided different bacterial niches

Analyses of total bacteria count, α diversity and microbial composition provided complementary information that was useful in characterization of the different materials. Silage and PMR both showed low diversity and higher numbers of lactobacilli than of total bacteria, with a major part of the RA explained by *Lactobacillus*. Used bedding material showed high diversity and higher numbers of total bacteria than of lactobacilli, with a major part of the RA explained by *Acinetobacter*, *Aerococcus*, *Corynebacterium,* unclassified *Lachnospiraceae*, and unclassified *Oscillospiraceae*. Concentrate, rapeseed meal, and wood shavings also showed high diversity and higher number of total bacteria than of lactobacilli, with a major part of the RA explained by *Pantoea*, *Pedobacter*, *Pseudomonas*, *Sphingomonas*, and unclassified *Yersiniaceae*.

The exceptionally high diversity found in milk, in combination with the low total bacteria count, highlighted an important consideration when evaluating milk microbiota. The high diversity indicated that DNA from many different bacteria was present, while the low total bacteria count indicated that the amount of DNA representing each unique bacterium was small. This could potentially lead to bias from background contamination, as discussed by Marsh et al. (2018). Alpha diversity showed greater variation within treatment for the milk samples than for the other materials, so bias due to background contamination could have arisen in our study.

The minor difference between the microbiota in silage and PMR, despite major inclusion of concentrate and rapeseed meal in the latter, was probably due to differences in the total bacteria count and DM content between silage and PMR. On an FM basis, silage contributed almost 3 times greater volume of material, together with bacterial concentrations that were many $\log_{10}$ cfu/g higher than in concentrate and rapeseed meal. The major differences between fresh wood shavings and used bedding material were probably explained by major inclusion in bedding of e.g., animal feces with much higher bacterial load than the wood shavings. Surprisingly, among all materials analyzed the microbiota in used bedding material showed the highest resemblance with that in milk, although the clustering of milk and used bedding material was not as tight as that for silage and PMR. The 2 clusters were also not close to each other, indicating that feed microbiota had little in common with milk microbiota.

According to Vacheyrou et al. (2011), bacteria which are useful in cheese-making, e.g., lactobacilli and propionic acid bacteria, are frequently present on the teat surface and in the milk, but rarely in other environments in the barn (air, dust, hay). A study by Doyle et al. (2017) confirmed the contribution of teats, but also identified feces as a major contamination source of the raw milk microbiota, while the contribution of grass or silage was minor. Gagnon et al. (2020) found that when a novel bedding material for dairy cows was used (recycled manure solids), the raw milk microbiota changed, while Sun et al. (2022) observed differences in bulk milk microbiota depending on milking system and hygiene routines applied on-farm. In agreement with these studies, we found that the microbiota of silage and the corresponding PMR had little in common with that of the milk, and that the microbiota of the milk was mainly associated with that of used bedding material.

Transfer of bacteria from feed to milk was rarely observed

Surprisingly, *Lactobacillus acetotolerans* (e1910), *Lactobacillus fructivorans* (08c5f), *Prevotella* (f74b4), and *Pseudomonas* (15a37) were barely detected in milk. They all showed exceptionally high RA (>10%) in PMR during at least one of the treatments, and all were clearly present in used bedding material. Ouamba et al. (2023) estimated bacterial transfer at ASV level between feed and milk to be 18–31%. The high RA of *Prevotella* in most materials indicated that these bacteria were well established in the barn and the surrounding environment. However, in comparison with the clear findings in both raw and pasteurized milk by Quigley et al. (2013), *Prevotella* and other core ASVs in PMR were barely detectable in the milk in our study.

Aerococcus (0cb4d) showed a clear tendency to transfer from feed to milk, and was also the most abundant ASV in used bedding material. This ASV matched fully with a few species, including *Aerococcus viridans* and *Aerococcus urinaeequi*, both described in relation to mastitis (Jahan et al., 2021; Alessandri et al., 2023). The high presence of this ASV in used bedding material could be due to its contamination by milk from cows with mastitis. Saishu et al. (2015) concluded that bedding material could be a source of *Aerococcus viridans*, based on findings from cow herds with clinical mastitis. *Acinetobacter* (8572d) also showed a clear tendency to transfer from feed to milk, and was the second most abundant ASV in used bedding material. It matched fully with *Acinetobacter lwoffii* and *Prolinoborus fasciculus*, with the latter being considered an erroneous classification (Glaeser et al., 2020). Previous studies have reported clear findings of this bacterium at both the teat apex and base of the udder (Dean et al., 2021), in manure and manure lagoon (Crippen et al., 2024), and in raw milk (Guo et al., 2021). These studies, together with our findings, suggest that *Acinetobacter lwoffii* in milk mainly originates from used bedding material, contaminating the teats of the cow,

but that the original source could be the feed. *Pantoea* (0951f) also showed a tendency to transfer from feed to milk, and the ASV matched fully with a few different species of *Pantoea*, mostly *Pantoea agglomerans*. This genus is mostly discussed in relation to plants (Lorenzi et al., 2022), with a few findings of *Pantoea* reported in raw milk and in e.g., pasteurized milk (Masiello et al., 2016).

CONCLUSIONS

To our surprise, we did not observe the expected effect of different ensiling treatments 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, but investigations at ASV level showed that in most cases the ASVs in these materials differed. The different materials harbored quite different microbiota, with the milk microbiota showing the highest resemblance to that of used bedding material. However, low total bacteria count in combination with high diversity indicated a risk of environmental contamination of the milk samples, and thus bias in the results. While the study was conducted on a research farm, rather than a commercial farm, strict hygienic measures during the feeding experiment could have contributed to the low transfer of bacteria from feed to milk.

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Supplementary Material: Botanical composition (DOI)

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Ethics statement: All use of animals in the study and the experimental protocol were approved by the Swedish Ethics Committee on Animal Research (Permit A 6-2021), represented by the Court of Appeal for Northern Norrland in Umeå, in line with Swedish laws and regulations implementing EU Directive 2010/63/EU on animal research.

Conflict of interest: Thomas Eliasson, Annika Höjer, and Karin Hallin Saedén are all employees of Norrmejerier. All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations: ACID = Silage with acid treatment, ASV = Amplicon sequence variant, FM = Fresh matter, FPDI = Faith's phylogenetic diversity index, INOC = Silage inoculated by starter culture, LAB = Lactic acid bacteria, PMR = Partial mixed ration, RA = Relative abundance, UNTR = Silage without additive

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The dairy value chain comprises many steps from the field to the final cheese. Bacteria are present in all of these steps at various concentrations and compositions. The work in this thesis aimed to understand where and how certain lactic acid bacteria, important for the development of the specific characteristics of a traditional Swedish long-ripened hard cheese, come into play. The results showed that silage additives can be used to control the microbiota of the ensiling process. However, factors at the farm, such as composition of herbage or use of silage additives, had little effect on the final cheese.

Thomas Eliasson received his postgraduate education at the Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), and obtained his Master of Science degree in Agriculture (Animal Science) at the same university.

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