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Research Article

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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, denosing, 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.

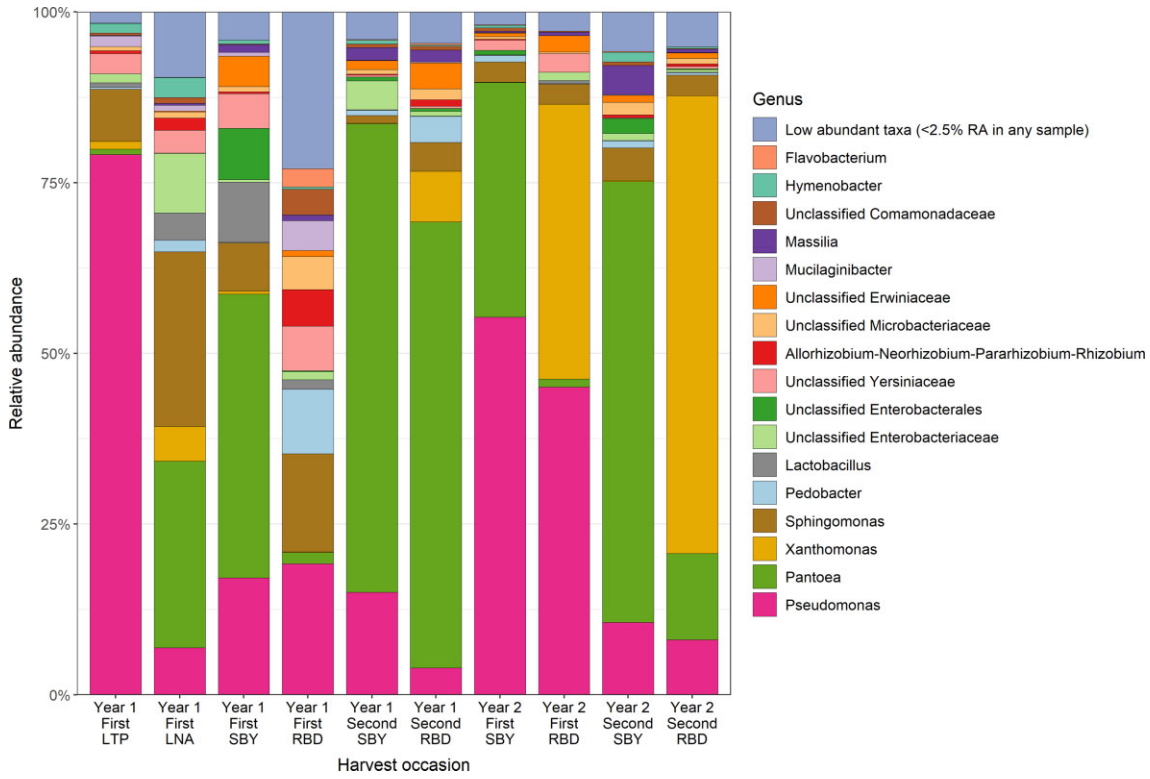

Figure 1. RA of bacteria at genus level for herbages prior to ensiling on each harvesting occasion. Values represent averages ($n = 3$) of herbage samples collected in three field plots per site. Bacteria without identified genus were allocated to the closest taxonomic level. Bacteria present in any 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

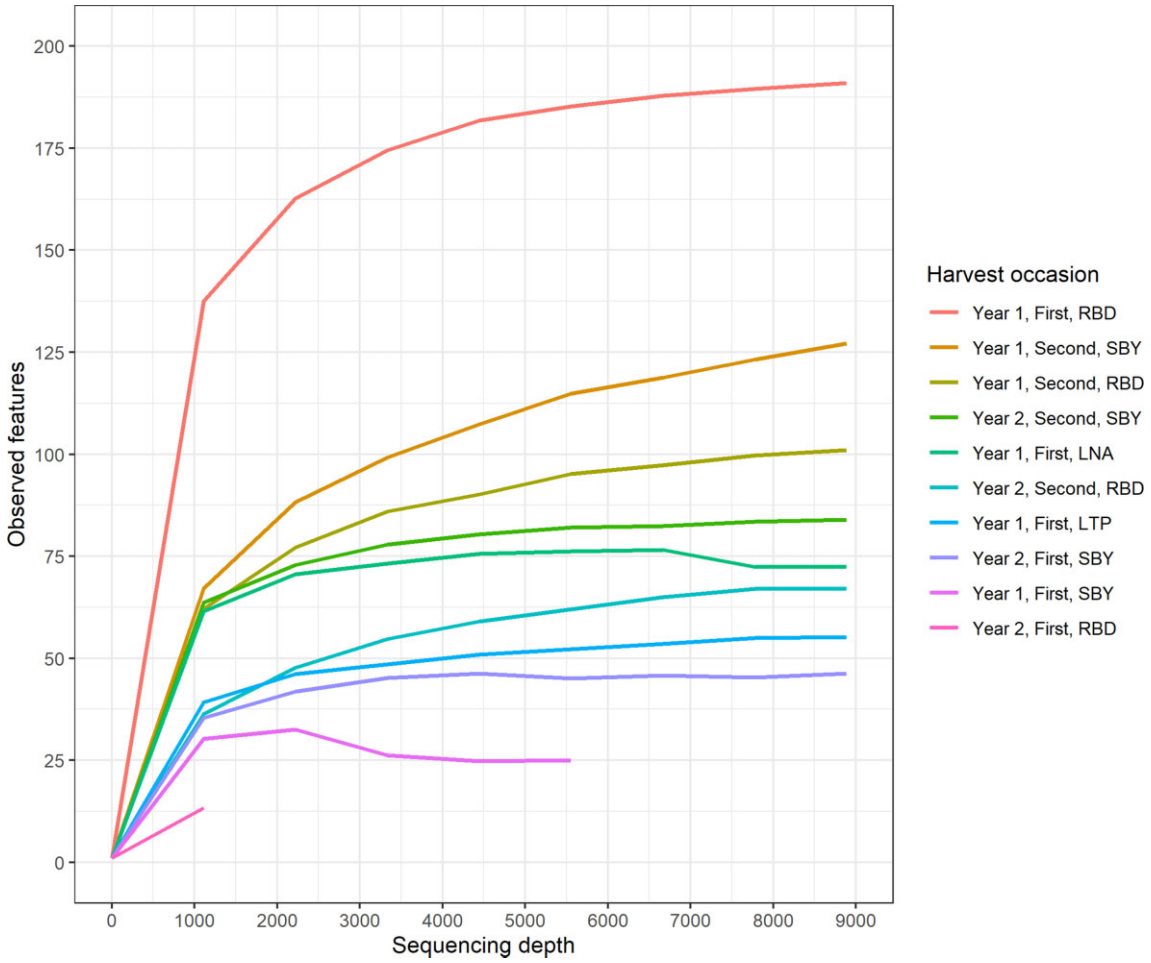

Figure 2. Rarefaction curves of observed features in herbage samples from each harvesting occasion. Values represents means of herbages collected from three field plots per harvesting occasion. Abbreviations: LTP, Lönnstorp; LNA, Lanna; SBY, Säby; RBD, Röbäcksdalen.

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*

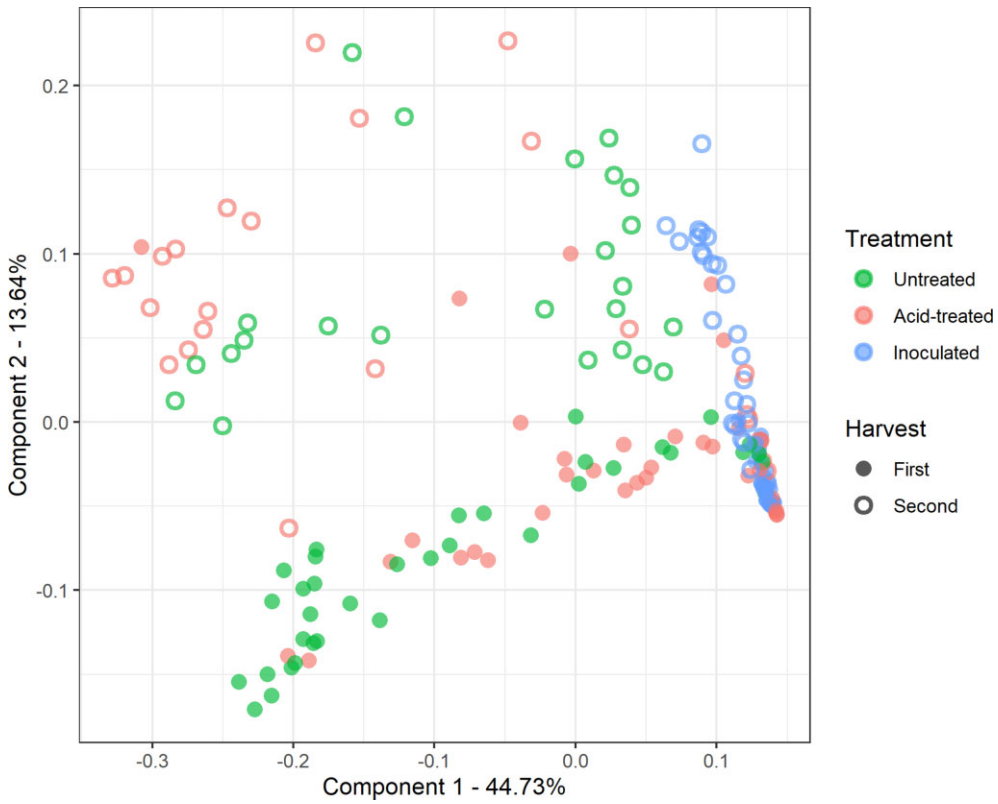

Figure 3. Principal coordinate analysis plot of the generalized UniFrac distance matrix associated to the silage microbiota. Each dot represents a unique silage sample from all harvesting occasions in the study. Dots colored according to ensiling treatment: green, untreated (no additive); red, acid-treated (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

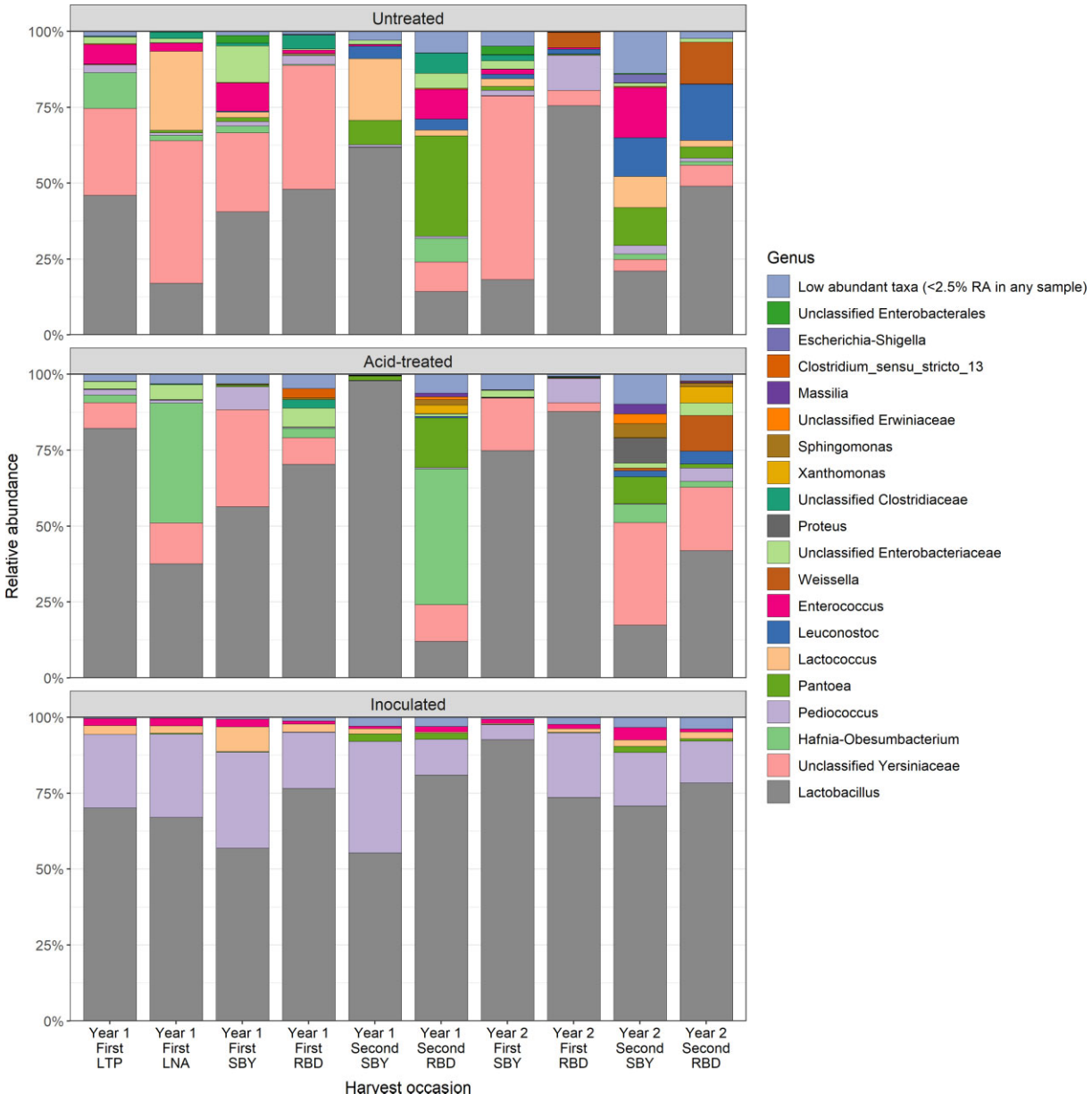

Figure 4. RA of bacteria at genus level for silages after ensiling with three different treatments: untreated (without additive), acid-treated (addition of organic acids), and inoculated (with starter culture). Bars represent averages ($n = 6$) based on duplicate samples from each of the three field 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

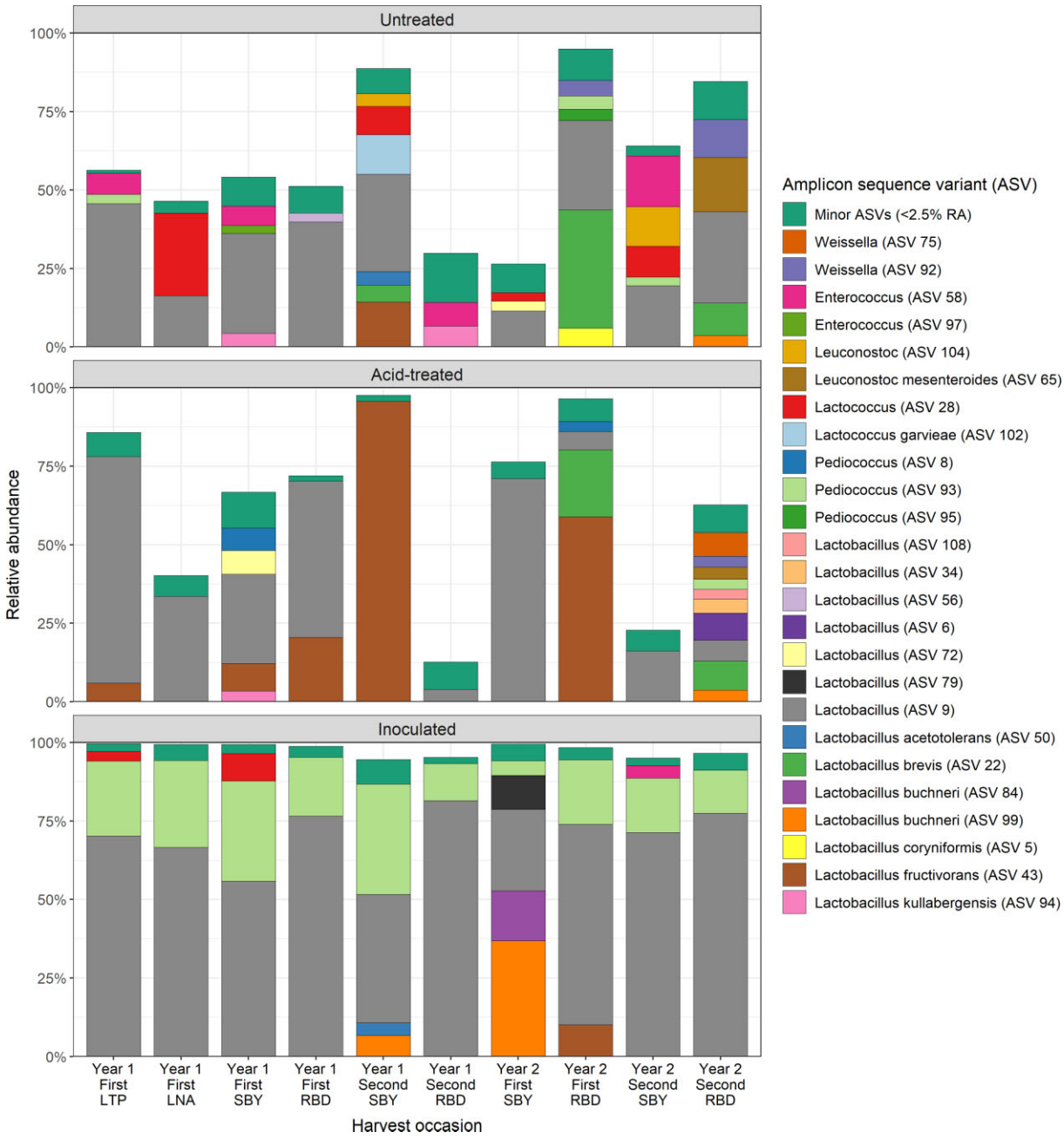

Figure 5. RA of LAB (order *Lactobacillales*) ASVs found in silages after ensiling with three different treatments: untreated (without additive), acid-treated (addition of organic acids), and inoculated (with starter culture). Values represent averages ($n = 6$) based on duplicate samples from each of 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

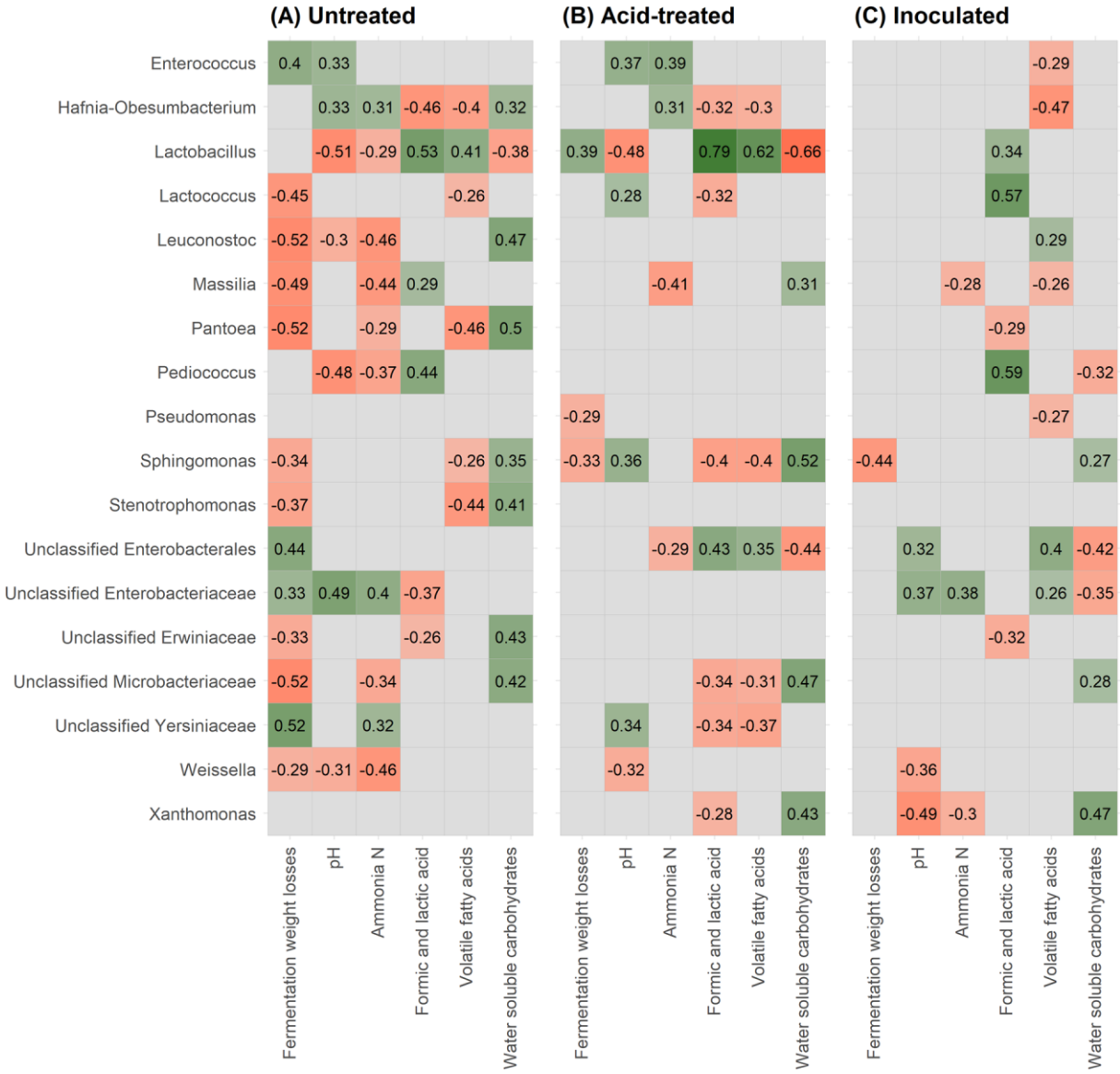

Figure 6. Plot resulting from Spearman correlation analysis between the top genera and the fermentation parameters associated to silages after ensiling with three different treatments: (A) untreated (without additive), (B) acid-treated (addition of organic acids), and (C) inoculated (with starter culture). 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 ± 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 addititives, 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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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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