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ORIGINAL ARTICLE

Changes to the microbiome of alfalfa during the growing season and after ensiling with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii* inoculant

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Abstract

Aims: This study evaluated changes in epiphytic microbial population of alfalfa (*Medicago sativa*) during the growing season. First cut forage was harvested to study the effects of an inoculant combining two obligate heterofermentative lactic acid bacteria strains on the bacterial and fungal communities and the fermentation of alfalfa silage.

Methods and Results: The epiphytic microbiome of alfalfa was evaluated 10-times during the growing season. Alfalfa wilted to 395.0 g/kg was treated with water (Control) or with a combination of *L. buchneri* NCIMB 40788 and *L. hilgardii* CNCM-I-4785 (LBLH). Mini-silos were opened after 1, 4, 8, 16, 32, and 64 days of ensiling. The relative abundance (RA) of the epiphytic bacterial and fungal families varied during the growing season. After 1 day, *Weissella* was the most abundant genus and present at similar RA in the two treatments (average 80.4%). Compared with Control, LBLH had a higher RA of *Lactobacillus* at day 1, 16, 32, and 64, and a lower RA of *Weissella* from day 8 to 64. Control contained more bacteria belonging to the *Enterobacteriales* than LBLH up to day 16. Inoculated silage had more acetate than Control at day 32 and 64. The fungal population were similar between treatments. The enhanced development and dominance of *Lactobacillus* in inoculated silage led to greater accumulation of acetate and propionate, which reduced the numbers of culturable yeasts but did not markedly affect the fungal community structure.

Conclusions: The bacterial community composition of alfalfa stands in the filed changed over time and was affected by cutting. For the ensiling trial, inoculation modified the composition of the bacterial community of alfalfa, increasing the RA of *Lactobacillus* while reducing the RA of *Weissella* and of *Enterobacteriaceae*.

Significance and Impact of Study: Inoculation increased the RA of *Lactobacillus*, hampering the dominance of *Weissella* in the early stages of ensiling, improving antifungal compounds production and reducing the numbers of culturable yeasts.

INTRODUCTION

Alfalfa (*Medicago sativa*) is one of the main crops used as animal feed and is often an important component of the diet of high-producing dairy cows (Albrecht & Beauchemin, 2003). However, as is usually the case for legumes, ensiling alfalfa can be challenging due to its high buffering capacity caused by the high concentrations of organic acids, proteins, and minerals (McDonald et al., 1991). For this reason, greater production of acids is needed to trigger a drop in the pH that is fast and low enough to prevent the development of undesirable microorganisms. However, the relatively low concentration of fermentable sugars in alfalfa (Lüscher et al., 2014) can limit the production of such acids, which may therefore not be enough to overcome its high buffering capacity (Hartinger et al., 2019).

Inoculants based on homofermentative or facultative heterofermentative lactic acid bacteria (LAB) are used in the alfalfa ensiling process to accelerate the drop in pH (Muck et al., 2018). In addition, obligate heterofermentative LAB, mainly *Lentilactobacillus buchneri* (previously *Lactobacillus buchneri*), are also applied to the alfalfa during ensiling mainly with the goal of producing antifungal organic acids that reduce aerobic spoilage (Schmidt et al., 2009; Zhang et al., 2009). However, the epiphytic microbial population of the plant can impact the effectiveness of inoculants (Muck et al., 2018). For example, low LAB numbers might cause poor fermentation, with high final pH, and high enterobacteria numbers might compromise silage nutritive value and sanitary quality.

Currently, inoculants containing different species of obligate heterofermentative LAB (e.g., *Lentilactobacillus hilgardii* and *Lentilactobacillus diolivorans*) combined with *L. buchneri* are being studied (da Silva, Smith, et al., 2021; Diepersloot et al., 2021; Ferrero et al., 2019). However, most published studies have used whole-plant corn silage, high moisture corn, and sorghum silage but not yet legume silage (da Silva, Costa, et al., 2021; da Silva, Smith, et al., 2021; Drouin et al., 2019; Ferrero et al., 2019).

Several authors have already analysed the effects of inoculants on the microbiota of alfalfa using high-throughput sequencing (Hu et al., 2020; Ogunade et al., 2018; Yang et al., 2020; Zhang et al., 2018). However, to the best of our knowledge, in the case of alfalfa silage, most studies focused only on the analysis of the bacterial community, not of the fungal community, and no studies have evaluated changes in microbiota dynamics caused by *L. buchneri* and *L. hilgardii* co-inoculation to date. The main objective of this study was thus to evaluate the effects of an inoculant combining *L. buchneri* NCIMB 40788 and *L. hilgardii* CNCM-I-4785 on the main fermentation parameters and the microbiota of alfalfa silage during the early stages of ensiling. We hypothesize that inoculating the silage with these bacteria would rapidly modify the microbiota of the silage and increase the production of antifungal organic acids, thereby more efficiently controlling the development of undesirable microorganisms that cause undesirable fermentation and aerobic spoilage. A second objective was to study the changes of the epiphytic microbial population at different sampling periods throughout the growing season.

MATERIALS AND METHODS

Standing crop epiphytic microbial population during the growing season

The epiphytic microbial community was studied by specifically sampling first cut alfalfa plants in in three different representative locations, in relation to forage composition, of a field containing alfalfa and tall fescue (86% alfalfa: 14% tall fescue). Each individual section measured 4 m^2 and was geo-referenced to be sure the same location was sampled at each period. The first sampling was done on May 23. In total, there were 10 sampling days: two in May, two in June, three in August, two in September, and one in October. In each section, 75–100 g of plants, with a minimum of 10 plants, was randomly collected within each section were cut manually 10 cm above ground level and chopped into fragments measuring 3 to 4 cm. The samples collected from the three locations of the field were pooled in a composite sample. The sub-samples weighing approximately 100 g were taken from the composite samples, and immediately stored at −80°C until DNA isolation.

Ensiling and experimental treatments

First cut alfalfa forage was cut at the early budding stage and a dry matter (DM) content of 239.0 g/kg DM. The maximum temperature on 2 days prior to harvesting was respectively, 21°C and 24°C, and the minimum temperature was 9°C. The forage was cut in the morning, without conditioning treatment, and wilted for approximately 6 h until mid-afternoon, when the DM content reached 395.0 g/kg DM. The forage was raked into a windrow, 60–90 min prior harvesting. The self-propelled forage harvester (John Deere 8700) was set to chop at a theoretical length of 30 mm.

The chopped forage was mixed and divided into ten 15-kg piles prior to treatment. The treatments consisted of water (Control) and of a combination of two *Lentilactobacillus* strains (*Lentilactobacillus buchneri* NCIMB 40788 and *Lentilactobacillus hilgardii* CNCM-I-4785) (LBLH) applied at 2×10^5 CFU/g of fresh forage each. The two strains were

received as a cell-only freeze-dried preparations directly from Lallemand Specialities Inc. (Milwaukee, USA). In the week prior to inoculation, the number of LAB was counted to adjust cell number at the targeted rate of application. Each treatment was applied into five forage piles, one for each replicate. Treatments were applied by hand by alternating spraying small volumes of the additive and mixing the forage. The volume of additive added to each pile was of 200 ml. After the entire volume was sprayed, the pile of forage was thoroughly mixed.

After treatment, a set of samples was immediately frozen (time 0). In addition, 300-g samples of forage were placed in plastic bags (20 × 30 cm, 2-ply 3 mil polyethylene, Western Brands LLC, Ohio), which were immediately vacuumed and sealed using a commercial vacuum sealer (Model 3000, Weston Brands LLC, Ohio). Six bag silos, one for each incubation period (1, 4, 8, 16, 32, and 64 days), were made from each of the five piles replicated in each treatment. The silos were stored in the dark at ambient temperature (~21°C) in a temperature controlled-storage room.

After a given incubation time, the corresponding silos were opened and the silage was thoroughly mixed. After mixing, 200-g sub-samples were collected from each individual silo for analysis of the fermentation profile and DNA isolation. The sub-samples were immediately frozen and stored at −80°C. Another set of 20 g sub-samples was collected, refrigerated and immediately transported to the laboratory for analysis and microbial counts.

Microbial counts

Sub-samples containing 20 g of forage (weighed at harvest) or of silage (weighed when the silo was opened) were mixed with 180 ml of NaCl buffer for two 60-s periods in a Stomacher 400 paddle blender mixer (Seaward, UK). Serial dilutions were then performed with NaCl buffer. Lactic acid bacteria from samples and inoculant were enumerated using De Man-Rogosa-Sharpe agar plates (Oxoid – Thermo Scientific, Hampshire, UK), containing 100 μg/L of cycloheximide (Drouin & Ferrero, 2020). Yeasts and moulds were enumerated on malt extract agar plates (Oxoid—Thermo Scientific, Hampshire, UK), containing 2 g/L of Rose Bengal (Fisher Scientific, USA), 100 μg/L of streptomycin and 50 μg/L of neomycin (Sigma-Aldrich, USA). Plates were incubated at 28°C and cell counts were performed after 72 h of incubation.

Fermentation profile

The concentrations of lactic, acetic, and propionic acids, 1,2-propanediol, and ethanol, and the pH were analysed at Cumberland Valley Analytical Services (Pennsylvania, USA). Representative 25-g silage samples were mixed with 200 ml deionized water and incubated in the refrigerator at 4°C for 2 h. Next, the mixture was blended for 2 min and filtered through coarse filter paper (20–25 μm particle retention). The concentration of L-lactic acid was determined in a mixture containing the extract and deionized water at a 1:1 ratio using a YSI 2700 Select Biochemistry Analyser (Champaign, IL, USA). For the quantification of acetic and propionic acids, 1,2-propanediol and ethanol 3 ml of the extract were filtered through a 0.2-μm filter membrane. Next, a 1.0-μl sub-sample was injected into a Perkin Elmer AutoSystem Gas Chromatograph (Perkin Elmer, USA) equipped with a Restek column packed with Stabilwax-DA (Restek, USA). The pH was measured prior to analysis of the titratable acidity using 30 ml of the previous extract by a Mettler DL12 Titrator (Mettler-Toledo, USA) using 0.1 N NaOH at pH 6.5.

DNA isolation

The DNA isolation and purification protocols were adapted from the methodology proposed by Romero et al. (2018) and Zhou et al. (2016). DNA was extracted from replicates 1, 2, and 3. Five grams of chopped forage or silage samples were weighed in a 50-ml conical centrifugation tube and mixed with 10 ml of sterile deionized water. The mixtures were then sonicated in a Branson model 8800 (Emerson Electric Co., St-Louis, MO, USA) ultrasonic water bath at 40 kHz for 5 min and vortexed for 1 min. A 3-ml aliquot of the homogenate was centrifuged. The resulting pellet was transferred to bead tubes in the PowerLyzer Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, NM, USA). Microbial lysis was optimized by 2-min mechanical lysis in a MixerMill 400 (Retsch, Inc., Haan, Germany) at a frequency of 15 cycles/second. DNA isolation then proceeded according to the manufacturer's protocol. The concentration of DNA was quantified on a spectrophotometer (Nanodrop Technology, Cambridge, UK) and its quality was checked by agarose gel electrophoresis (1% agarose). The concentration of DNA was standardized at 2 ng/μl for all samples.

High-throughput sequencing and bioinformatics analysis

The libraries were prepared for amplicon sequencing according to the Illumina 16S Metagenomic Sequencing Library Preparation guide (Part # 15044223 Rev. B), except that a Qiagen HotStar MasterMix (Toronto, Ontario, Canada) was used for the first PCR (amplicon PCR) and half the volume of reagents was used for the second PCR

(index PCR). The protocol included a PCR cleanup step that used AMPure XP beads to purify amplicons from free primers and primer dimers. The template-specific primers (without the overhang adapter sequence) for 16S amplification of the V4 hyper-variable region (Caporaso et al., 2011) specific to bacterial and archaeal organisms were as follows: 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). For the ITS region 1, the specific primers ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and 58A2R (5′-CTG CGT TCT TCA TCG AT-3′) (Yergeau et al., 2017) were used. The amplicon PCR reaction was carried out for 30 cycles with annealing temperatures of 55°C for 16S and 45°C for ITS. The diluted pooled samples were loaded on an Illumina MiSeq System (San Diego, California, USA), and sequenced using a 500-cycle MiSeq Reagent Kit v2 (San Diego, California, USA, adapted from Yergeau et al. (2017)). The average size of the amplicon sequences was 293 bp for the 16S regions and 276 bp for the ITS regions.

Sequencing data were analysed using an AmpliconTagger (Tremblay & Yergeau, 2019). Briefly, raw reads were scanned for sequencing adapters and PhiX spike-in sequences and the remaining reads were merged using their overlapping part with FLASH (Magoč & Salzberg, 2011). Primer sequences were removed from merged sequences, and the remaining sequences were filtered for quality. Sequences with an average quality (Phred) score of less than 30 or one or more undefined bases, or with more than 5 bases with a quality score of less than 15 were discarded. The remaining sequences were clustered at 100% identity and then clustered/denoised at 99% identity (DNACLUST v3, Ghodsi et al. (2011)). Clusters with abundances lower than three reads were discarded. The remaining clusters were scanned for chimeras with the UCHIME de novo and UCHIME reference (Edgar et al., 2011; Rognes et al., 2016) and clustered at 97% (DNACLUST) to form the final clusters/operational taxonomic units (OTU). A global read count summary is provided in Table S1 in supplementary data. Bacterial/Archaeal OTUs were assigned a taxonomic lineage with the RDP classifier (Wang et al., 2007) using AmpliconTagger 16S and ITS training sets (https://doi.org/10.528/zenodo.3560150). The RDP classifier attributes a score (0 to 1) to each taxonomic depth of each OTU. Each taxonomic depth with a score equal to or above than 0.5 was retained to reconstruct the final lineage. Taxonomic lineages were combined with the cluster abundance matrix to generate a raw OTU table. From the raw OTU table, an OTU table only containing bacterial organisms was generated. Five hundred 1000 read rarefactions were then performed on the latter OTU table and the average number of reads of each OTU for each sample was then computed to obtain a consensus rarefied OTU table. Alignments were filtered to keep only the hypervariable region of the alignment. Alpha (observed species) and Beta diversity metrics and taxonomic summaries were then computed using the QIIME v1.9.1 software suite (Caporaso et al., 2010; Kuczynski et al., 2011) and the consensus rarefied OTU table. Analysis of the ITS amplicons was performed similarly, but alignment was performed using the UNITE reference database (downloaded on May 17, 2017).

The 16S and ITS rDNA raw reads from the microbiota analyses have been deposited at the NCBI BioProject repository under accession number PRJNA693281.

Statistical analysis

Differences between the control and LBLH treatments were analysed using the non-parametric Kruskal–Wallis test under R version 3.3.3 (R Core Team, 2020). Data from each opening time were tested independently. The following fermentation parameters were tested individually: pH, lactic acid, acetic acid, the ratio of lactic acid to acetic acid (LA/AA), 1,2-propanediol, propionic acid, ethanol, LAB counts, and yeast counts. A logarithmic transformation was used for the microbial counts data. Differences were considered significant at $P < 0.05$ and biological trends at $P \geq 0.05$ and <0.10. The same non-parametric test was used to analyse differences in the microbiota data at the phylum, order, family or genus level and the alpha-diversity parameters between treatments. For statistical requirements, results below the detection level were replaced by a value equal to half the limit of detection.

According to our experimental design, differentially abundant OTUs were assessed with edgeR (v3.10.2) using its generalized linear models approach (Chen et al., 2016) and the OTU table raw count matrix as input. The OTUs with a logFC (log Fold-Change) ratio equal to or higher than 1.5 and a false discovery rate (FDR) lower than 0.05 were considered differentially abundant. Differential abundance analyses were performed between treatments at each opening period to look for statistical differences in OTU abundance. The logFC and CPM (counts per million—obtained with edgeR v3.10.2 (Robinson et al., 2010) values were used to generate histograms using in-house scripts, including ggplot functions. The output of the Bray–Curtis dissimilarity matrices was analysed statistically using permutational multivariate analysis of variance (PERMANOVA).

RESULTS

Dynamics of the epiphytic microbiota during the growing season

We monitored changes in the epiphytic microbial population of the stands in the field throughout the

growing season, both before and after harvesting the plants used for ensiling. The relative abundances (RA) of the bacterial and fungal families at each of the ten sampling dates are shown in Figure 1. The RA of the bacterial and fungal families varied over the growing season, the biggest changes being in the bacterial population. The RA of *Lactobacillaceae* varied over time and reached its highest level (54%) on May 29, the last sampling occasion before the first cut and harvest for ensiling. After the first cutting date, the RA *Lactobacillaceae* fluctuated from 1% to 30% over the sampling period. Like with *Lactobacillaceae*, the RA of two other families belonging to the *Proteobacteria* phylum (*Enterobacteriaceae* and *Xanthomonadaceae*) varied over time. The RA of the two families was relatively low in samples taken in May, whereas it was the most abundant families in samples collected in June and September, respectively, with RA of approximately 80%. Other bacterial families that presented great variability during the growing season were *Sphingomonadaceae*, *Methylobacteriaceae* and *Peptostreptococcaceae*. Families *Peptostreptococcaceae*, *Clostridiaceae* and *Erysipelotrichaceae*, known to be typical of animal gut microbiota, were the most abundant families following application of liquid manure on the field, on August 3rd. *Leuconostocaceae*, which includes the genus *Weissella*, was only observed early in the growing season and at a very low RA, which reached only 0.2% of the total population.

The composition of the epiphytic fungal microbiota changed over the growing season, but unlike the bacterial population, it did not fluctuate intensely, thus allowing a trend to be identified. The trend consisted of two different patterns: one observed in May and June and the other in the second half of the growing season, August, September and October. In the first stage of the growing season, there was a decrease in the RA of *Didymellaceae* and an increase in that of *Pleosporaceae* (mainly the genus *Epicoccum*) and *Davidiellaceae*. In the second half of the growing season, the most abundant family was *Pleosporaceae* (mainly the genus *Alternaria*), with an average RA of approximately 40%. The RA of *Montagnulaceae* and *Plectosphaerellaceae* increased markedly from the first to the second half of the growing season. Yeasts of the family *Saccharomycetaceae* were not detected on standing plants.

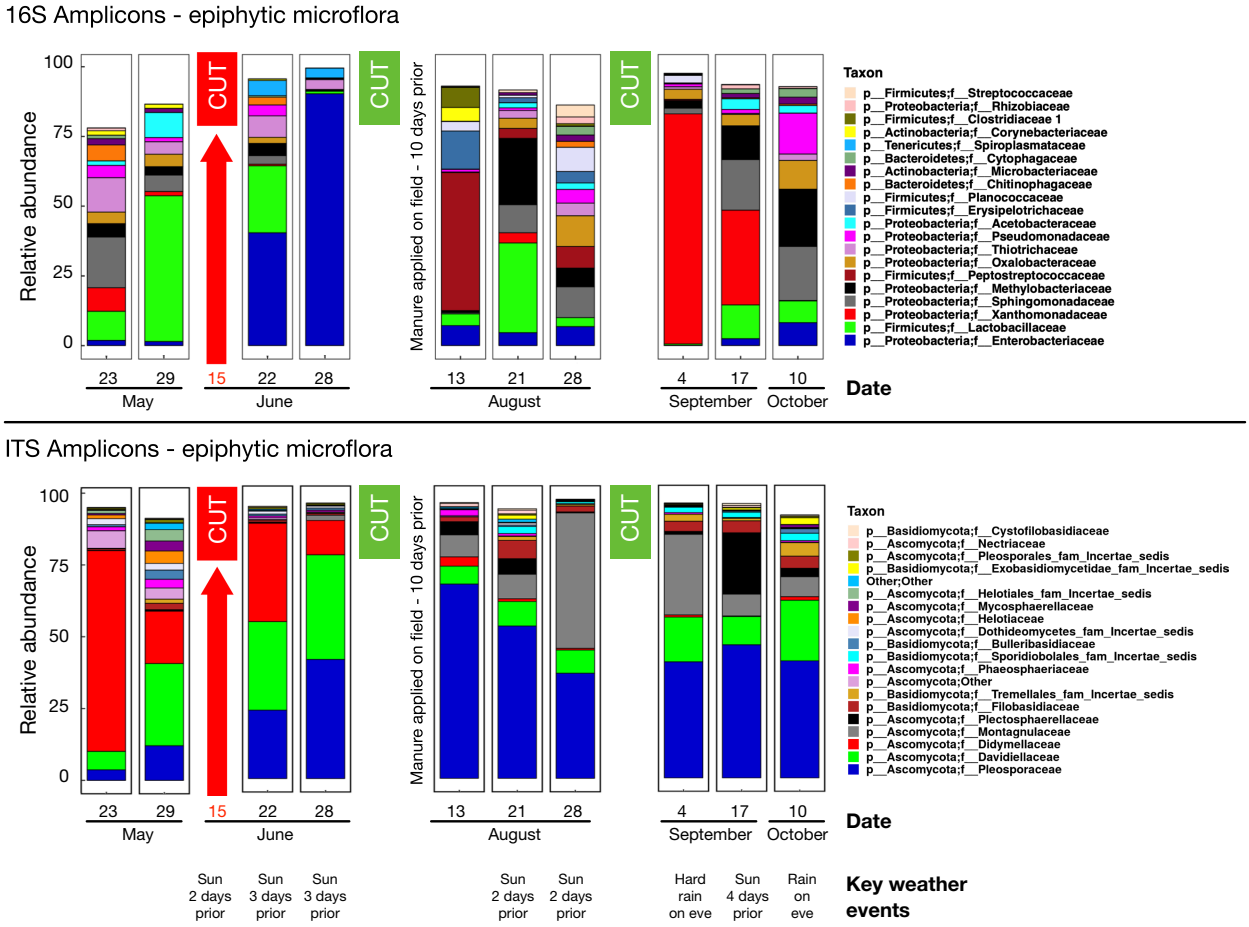

FIGURE 1 Taxonomic profiles of the 20 main epiphytic bacterial and the 20 main fungal families present on standing plants during the growing season. Sampling was performed on 10 different days during the growing season to observe changes in microbiota over time, due to weather conditions and field management, including harvesting and fertilization.

Dynamics of the bacterial population during ensiling

At harvest (Figure 2a), the *Proteobacteria* phylum was dominant in both Control and LBLH forage. In addition, most of the OTUs from the 16S amplicons were affiliated to the genus *Pseudomonas* (average RA of 47.6 ± 3.1%; $P = 0.154$), *Enterobacteriaceae* (comprising Erwinia-related OTU; average RA of 15.2 ± 4.1%; $P = 0.487$), and *Sphingomomas* (average RA of 11.9 ± 3.9%; $P = 0.326$). The RA of *Lactobacillus* in forage prior to ensiling was higher ($P < 0.001$) in LBLH forage (1.6%) than in Control forage (0.4%).

In the 24 h following ensiling, the composition of the microbiota changed considerably compared with that observed at harvest (Figure 2a). At harvest, the microbiota was dominated by bacteria belonging to the *Proteobacteria* phylum, but after 24 h of ensiling, it was dominated by *Firmicutes*. Indeed, the order *Lactobacillales* accounted for an average of 83.2% of the total bacterial population in the two treatments and did not differ ($P = 0.243$) between treatments. More precisely, after 24 h of fermentation, *Weissella* (order *Lactobacillales*) was the dominant genus, the RA being similar in both treated silages (average of 80.4%, $P = 0.436$) (Figure 3). In contrast, the RA of *Lactobacillus*-related OTUs was higher in LBLH than in Control silage (2.45 vs. 0.20%, $P = 0.002$, Figure 3).

From day 1 to day 64 of ensiling, the RA of *Lactobacillus* increased in LBLH silage at the expense

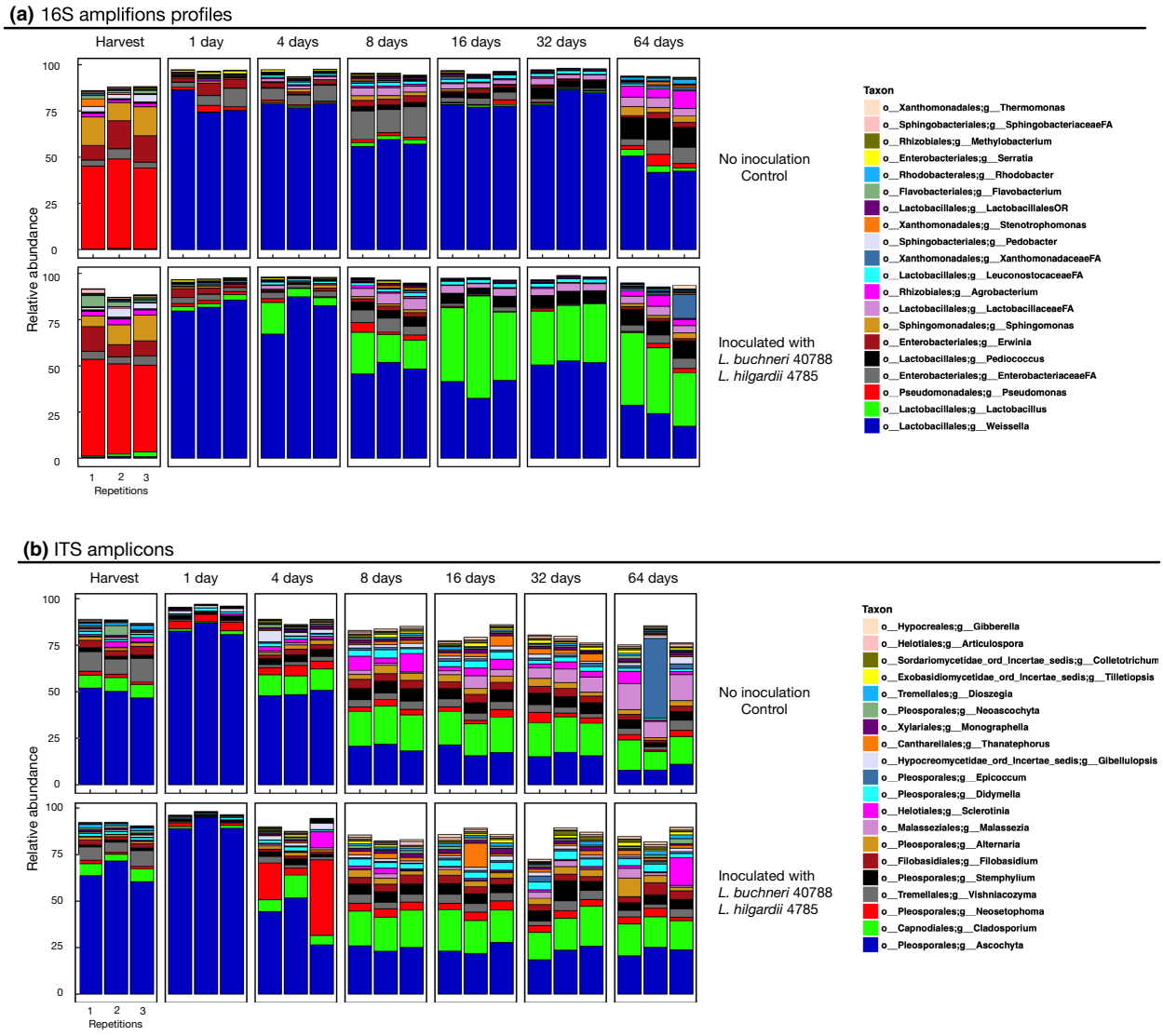

FIGURE 2 Taxonomic profiles with the main bacterial (a) and fungal (b) genera in uninoculated alfalfa (control) and alfalfa inoculated with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii*, at harvest and at days 1, 4, 8, 16, 32 and 64 of ensiling. ***Each panel shows the three independent repetitions of a specific fermentation condition in the inoculation treatment and time. The 20 most abundant taxa found in all 32 ensiling conditions, are shown.

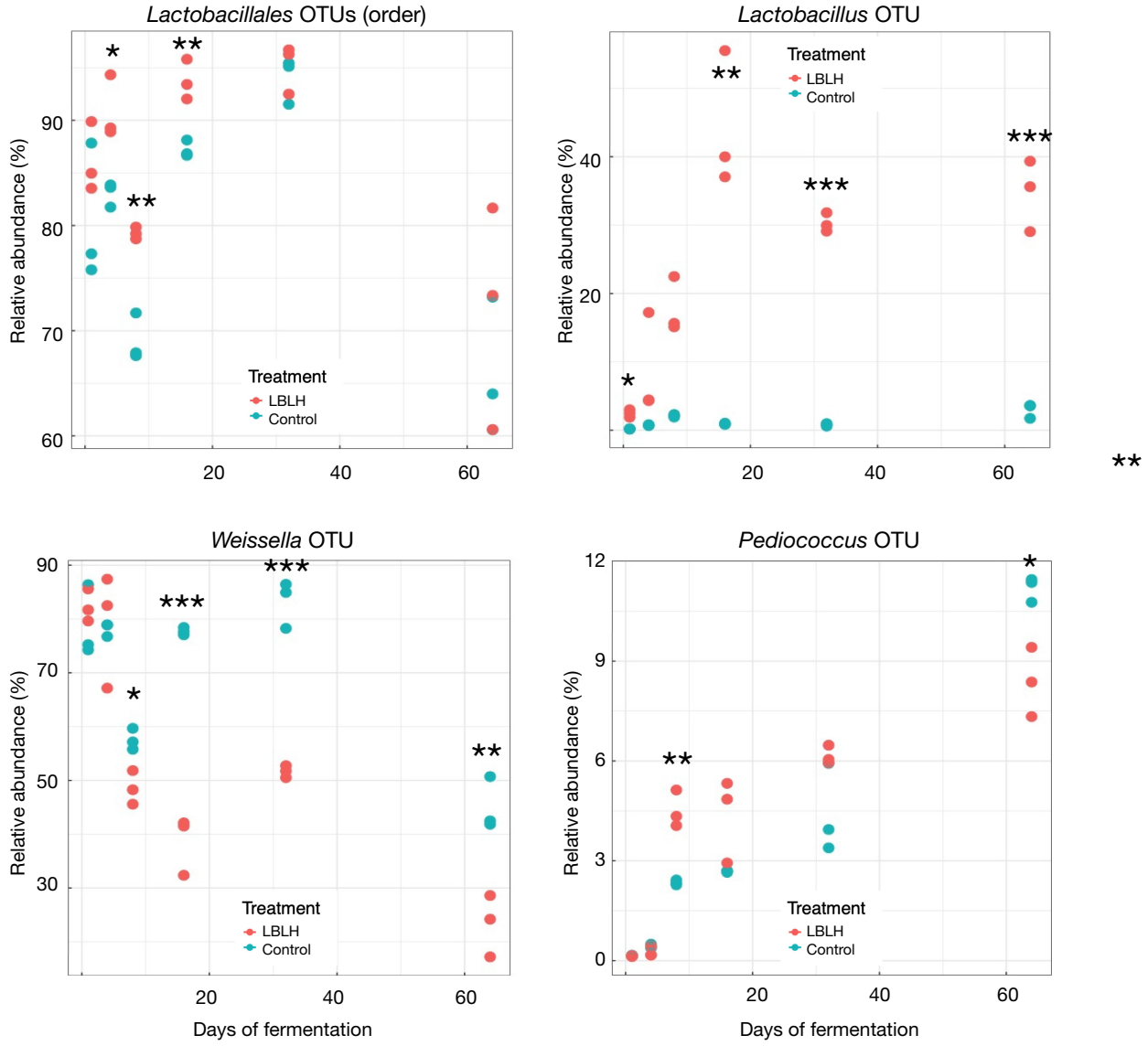

FIGURE 3 Changes in the relative abundance of the order *Lactobacillales* and the genera *Lactobacillus*, *Weissella*, and *Pediococcus* in uninoculated alfalfa (control) or alfalfa inoculated with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii* (LBLH) at days 1, 4, 8, 16, 32, and 64 of ensiling. Controls are in cyan and LBLH in red. *represents significant difference at 95%, ** at 99%, and *** at 99.9%.

of that in *Weissella* (Figure 3). Conversely, in Control silage, the RA of *Weissella* increased from 50% at day 8 to 77.7% at day 16, and 83.2% at day 32, whereas the RA of *Lactobacillus* remained low, reaching a maximum value of 3.0% at day 64 (Figure 3). Nevertheless, LBLH silage had a greater RA of *Lactobacillus* than Control silage at day 16 (44.2% vs. 0.9%, $P = 0.002$), day 32 (30.3 vs. 0.8%, $P < 0.001$), and day 64 (34.7% vs. 3.0%, $P < 0.001$), and a lower RA of *Weissella* than Control silage from day 8 to day 64 (average of 45.0 vs. 23.3%, $P = 0.008$). At day 8, the RA of *Pediococcus* was greater in LBLH than in Control silage ($P = 0.003$, Figure 3). The RA of *Pediococcus* increased over time in both treatments, and, at day 64, it was greater in Control than in LBLH silage (11.2% vs. 8.4%, $P = 0.012$). Finally, at day 64, the RA of the *Lactobacillales* was lower than at day 1 and was similar ($P < 0.001$) in LBLH (71.9%) and Control silage (65.9%) (Figure 3). However, the LAB community composition differed between treatments, as Control silage was dominated by *Weissella* (30%–35% and *Pediococcus* (5%), and LBLH was dominated by *Lactobacillus* (30%–40%) and *Weissella* (20%).

Concerning changes in Proteobacteria bacteria at day 64, the RA of specific OTUs increased in both Control and LBLH silage. More precisely, compared with day 32, there was an increase in the RA of *Agrobacterium* (from RA 0.5% to 5.4%; $P < 0.001$), *Sphingomonas* (from RA 0.3% to 2.9%; $P < 0.001$), *Erwinia* (from RA 0.5% to 1.9%; $P = 0.005$) genera, and of *Enterobacteriaceae* family (from RA 1.4% to 7.6%; $P < 0.001$) (Figure 2a).

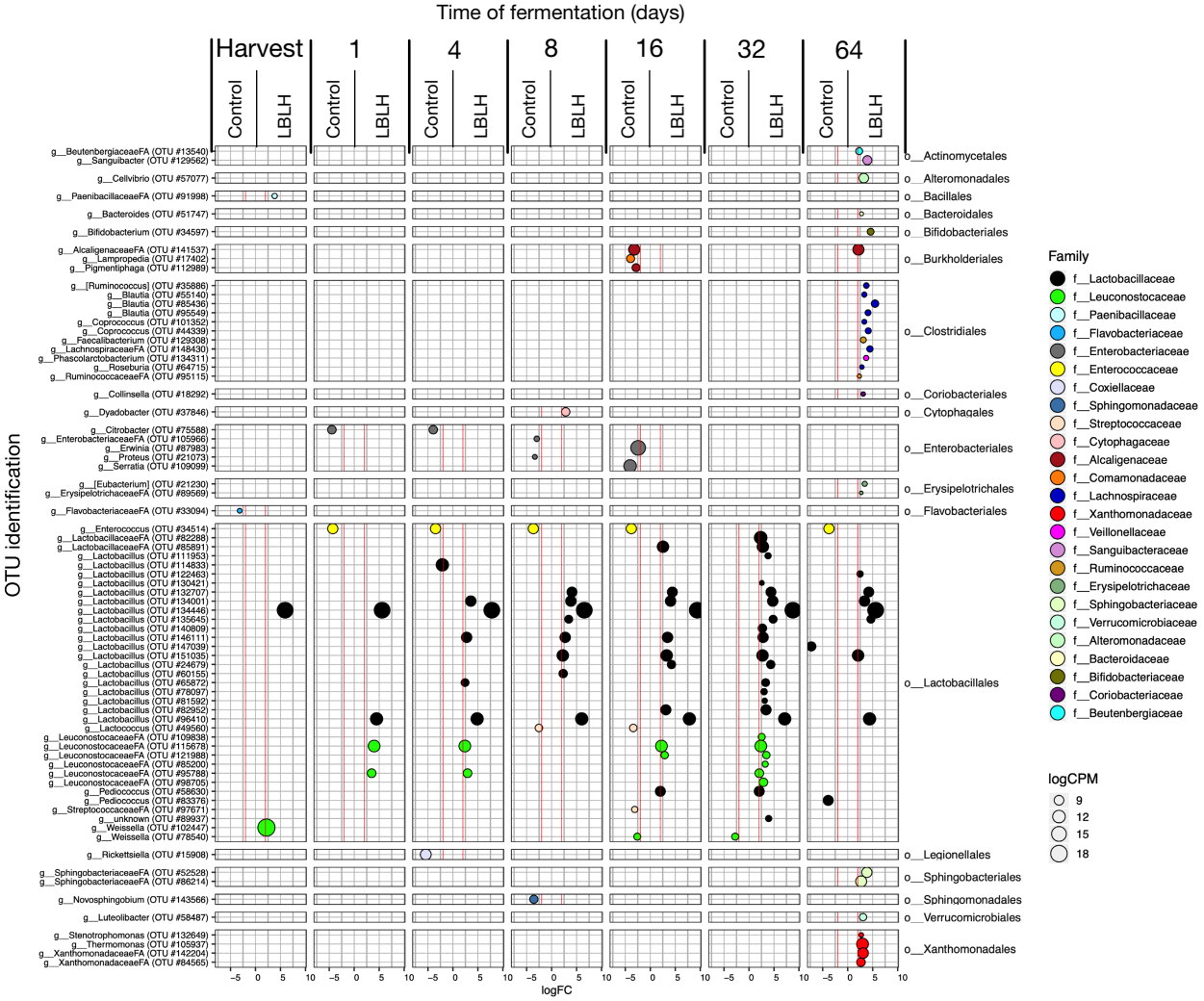

FIGURE 4 Differential expression analysis of the 16S operons using the edgeR package. The comparison was performed between uninoculated alfalfa (control) or alfalfa inoculated with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii* (LBLH), at harvest and at days 1, 4, 8, 16, 32, and 64 of ensiling. Only OTUs whose abundance differed significantly in the control and LBLH treatments are shown. LogFC (fold changes) and logCPM (counts per million) are shown. Only operational taxonomic units (OTUs) with a logFC ≤1.5 (red line in each panel) and false discovery rate (FDR) < 0.05 were considered as being differentially abundant. Colour-coding is based on the taxonomic classification of individual OTUs according to their taxonomic families.

The results of the edgeR test for over- or under-representation of OTUs are shown in Figure 4. These results confirmed that LBLH inoculation increased the frequency of *Lactobacillaceae*-related OTUs throughout the ensiling period. They also revealed that at least 19 different OTUs affiliated to the genus *Lactobacillus* were differentially represented in the treated silage, and that the samples collected at day 32 had the highest number of discriminant *Lactobacillus*-related OTUs. Two of these OTUs (#134446 and #96410) underwent an important logFC change and were consistently overrepresented in LBLH compared with Control silage at all ensiling periods. Although the length of the amplicons was short, OTU 134446 (278 bp) shared high homology with the *L. hilgardii—L. diolivorans—Lactobacillus farraginis* group according to the results of a BLASTn search on the NCBI database (100% homology), and OTU 96410 (271 bp) showed high homology with *Lactiplantibacillus plantarum* and *Lactobacillus mudanjiangensis* sequences (98.52% homology). One of the 19 *Lactobacillus* OTUs (#135645) was homologue to *L. buchneri* sequences (270 bp, homology 98.46%). The logFC values of this OTU were low during early ensiling, but increased after day 16. The OTUs related to *Lactobacillus coryneformis* were also frequent (#134001 and #82288). The Control silage had more bacteria belonging to the *Enterobacteriales* order. At the genus level, the Control silage had more *Citrobacter* at day 1 and day 4, more *Proteus* at day 8, and more *Erwinia* and *Serratia* at day 16. At day 64, OTUs related to the order *Clostridiales* were more abundant in LBLH silage than in

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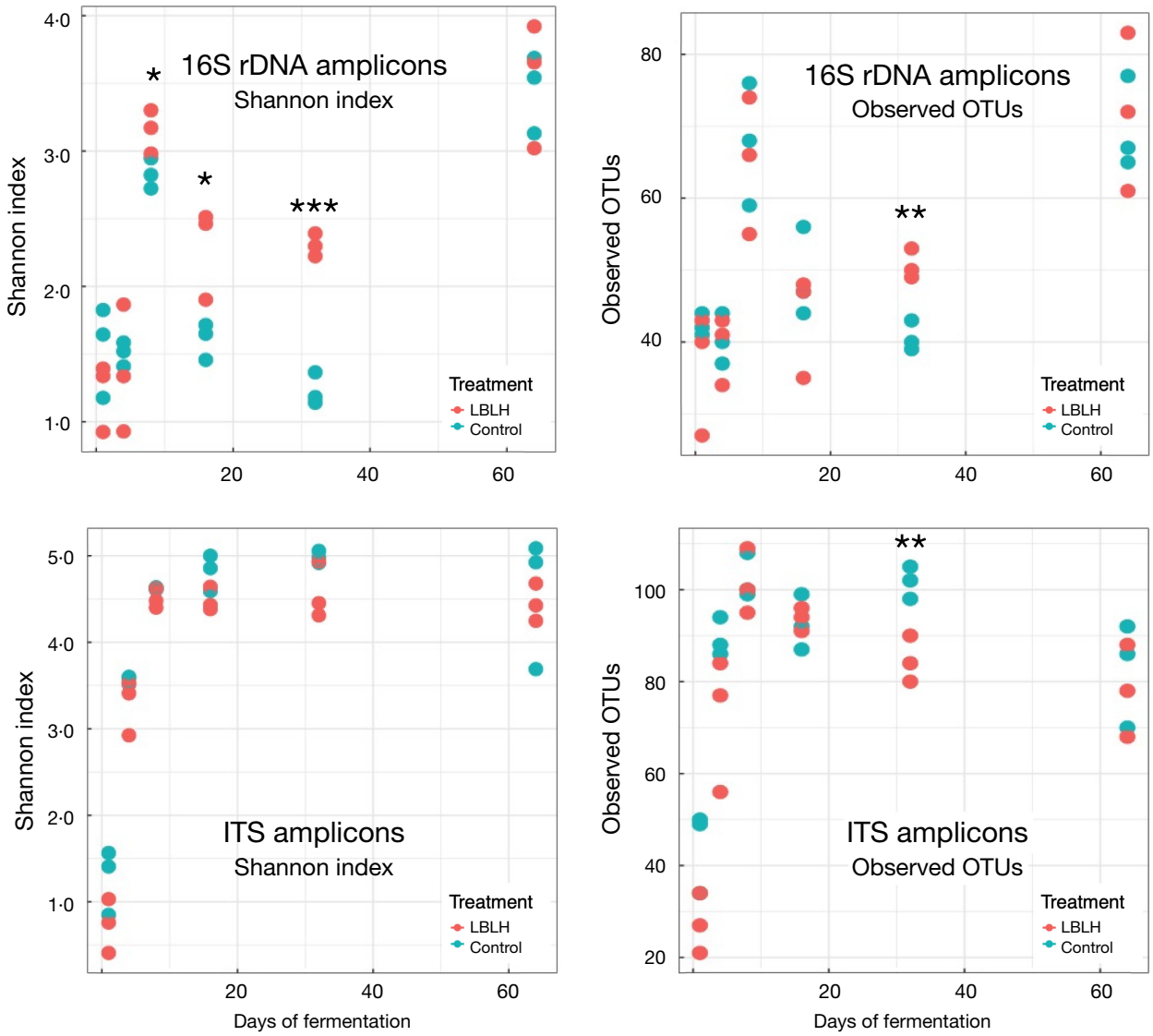

FIGURE 5 Alpha diversity parameters, the Shannon index, and the number of observed operational taxonomic units (OTUs) of the bacterial and fungal communities present in uninoculated alfalfa (control) or in alfalfa inoculated with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii* (LBLH) at harvest and at days 1, 4, 8, 16, 32, and 64 of ensiling. The alpha diversity results are based on the mean of three replicates. Controls are in cyan and LBLH in red. *represents significant difference at 95%, ** at 99%, and *** at 99.9%.

Control silage. These OTUs were assigned to the genera *Blautia*, *Coprococcus* and *Ruminococcus*.

Alpha and beta-diversities of the bacterial population

Two alpha-diversity measurements of the bacterial community (16S rDNA amplicons), Shannon index and observed OTUs, are shown in Figure 5. The Shannon index was similar in the LBLH and Control silage at days 1, 4 and 64, but higher at days 8, 16 and 32 in LBLH than in the Control silage ($P = 0.047$, 0.031, and <0.001, respectively). Similar numbers of OTUs were observed in the Control and LBLH silage at most of the ensiling times, but at day 32, LBLH silage had more OTUs than the Control.

The principal coordinate analysis (PCoA) plot based on the Bray–Curtis dissimilarity analysis is shown on the Supplementary Material (Figure S1). The PERMANOVA of the distance matrix was significant for treatment ($P < 0.001$) and for the length of fermentation ($P < 0.001$), but not for their interactions ($P = 0.186$), indicating that treatment and the length of fermentation influenced clustering separately. The PCoA plot revealed that the bacterial community had a similar structure in the Control and LBLH silage at harvest but that changed over the course of ensiling (first axis explained 90.55% of total variability). In addition, the effect of inoculation on the bacterial community structure became more

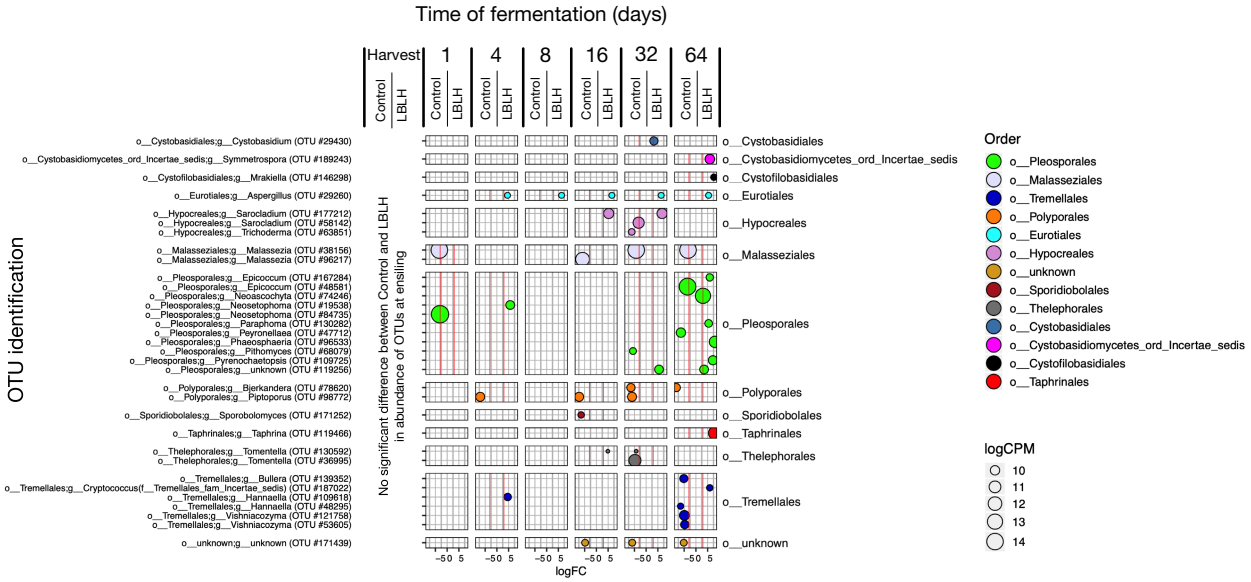

FIGURE 6 Differential expression analysis of the ITS operons using the edgeR package. The comparison was performed between uninoculated alfalfa (control) or alfalfa inoculated with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii* (LBLH), at harvest and at days 1, 4, 8, 16, 32, and 64 of ensiling. Only OTUs whose abundance differed significantly in the control and LBLH treatments are shown. LogFC (fold changes) and logCPM (counts per million) are shown. Only operational taxonomic units (OTUs) with a logFC ≤1.5 (red line in each panel) and false discovery rate (FDR) < 0.05 were considered as being differentially abundant. Colour-coding is based on the taxonomic classification of individual OTUs according to their taxonomic order.

obvious after 8 days of ensiling. No separate clusters were observed in control and LBLH silage at day 1 and day 4, but from day 8 on, Control samples clustered separately from LBLH samples.

Fungal population dynamics during ensiling

The fungal population of both Control and LBLH alfalfa prior to ensiling was dominated by the genus *Ascochyta* (59.3% RA), followed by *Vishniacozyma* (8.8% RA) and *Cladosporium* (6.4% RA) (Figure 2b).

The changes in the fungal population that occurred during ensiling were similar in Control and LBLH silage (Figure 2b). At day 1 of ensiling, the genus *Ascochyta* (order *Pleosporales*) was dominant in both silage groups (average RA of 87.2%, $P = 0.046$). At day 4, the RA of *Ascochyta* declined in both groups (average of 45.0%, $P = 0.344$), while the RA of the other fungus genera increased. Specifically, *Cladosporium* developed at higher rates in the Control silage and *Neosetophoma* developed at higher rates in LBLH silage. However, even though the RA of *Neosetophoma* was 24.1% in LBLH and only 4.5% in the Control silage, the difference was not statistically significant ($P = 0.188$). From day 8 of fermentation on, the fungal profile remained more stable but differed between Control and LBLH silage. In the Control group, the RA of *Ascochyta* declined numerically over time, and reached 8.9% RA at day 64, whereas the RA of *Malassezia* increased. Conversely, in the LBLH group, the RA of *Ascochyta* remained stable (between 20 and 25%) until day 64.

Figure 6 shows the results of the edgeR test for over- or under-representation of OTUs. The analysis brought to light subtle changes in the fungal community. More specifically, the genera *Hannaella* and *Vishniacozyma* (order *Tremellales*) were over-represented in Control silage at day 64. In addition, one of the OTUs belonging to the genus *Aspergillus* was over-represented in LBLH silage at all ensiling periods, but its RA was low in both treatments (0.032% in Control and 0.176% in LBLH).

Alpha and beta-diversities of the fungal population

Figure 5 shows two alpha-diversity parameters of the fungal community (ITS amplicons), the Shannon index and observed OTUs. Overall, there were no differences in the Shannon index or in the number of OTUs between the Control and LBLH silage, except at day 32, when more OTUs were observed in the Control silage than in LBLH silage. The Shannon index increased from 1.00 at day 1 to a mean of 4.38 between day 2 and day 64 ($P = 0.002$). The number of observed OTUs was lower at day 1, with a mean of 35.8 ($P = 0.056$) compared with the following ensiling periods. The mean number of observed OTUs was

80.8 ($P = 0.124$) at day 4, 101.8 ($P = 0.851$) at day 8, 93.2 ($P = 0.804$) at day 16, 93.2 at day 32 (101.7 in the Control silage and 84.7 in the LBLH silage, $P = 0.009$), and 80.3 ($P = 0.622$) after 64 days of ensiling (Figure 5).

The PCoA based on the Bray–Curtis dissimilarity analysis is shown in Supplementary Material (Figure S1). The PERMANOVA of the distance matrix was significant for treatment ($P = 0.039$) and length of fermentation ($P < 0.001$), but not for their interactions ($P = 0.086$), indicating that treatment and the length of fermentation influenced clustering separately. The beta diversity plot showed that the fungal community structure changed over the course of ensiling. Three clusters were detected: one cluster with silage fermented for 1 day, another cluster with samples collected at harvest plus samples ensiled for 4 days, and a third cluster with silage fermented for 8, 16, 32 and 64 days. No clear clustering due to inoculation was observed.

Fermentation parameters and counts of culturable microorganisms

The wilted alfalfa had a DM content of 395.0 g/kg ($P = 0.412$ between replicates) and a pH of 5.62 ($P = 0.698$ between replicates). The fermentation parameters for the Control and LBLH silage after 1, 4, 8, 16 and 32 days of ensiling are listed in Table 1. Inoculated silage had a higher ($P \leq 0.01$) pH than the Control silage at days 1, 16, 32 and 64 d of ensiling, but no differences were observed between treatments at day 4 or at day 8. At days 1, 4 and 16, the concentration of lactic acid was similar in the Control and LBLH silage, but at day 8, the LBLH silage had more ($P = 0.013$) lactic acid than the Control silage, and at days 32 and 64, the LBLH silage had less ($P < 0.001$) lactic acid than the Control silage. Up to 16 days of ensiling, the Control and LBLH silages had similar concentrations of acetic acid, but at day16, the LBLH silage tended ($P = 0.075$) to have a greater concentration of acetic acid than Control silage, and at day 32 ($P = 0.020$) and 64 d ($P = 0.011$), the LBLH silage had more acetic acid than the Control silage. Similarly, the lactic acid to acetic acid ratio (LA/AA) only differed between the two groups after 32 days of ensiling. At both day 32 ($P < 0.008$) and day 64 ($P < 0.001$), the ratio was lower in the LBLH silage than in the Control silage. Propylene glycol (1,2-propanediol) was detected only in LBLH samples and only after a minimum of 8 days of ensiling (average 1.0 g/kg DM). Similarly, propionic acid was only detected in Control samples after 64 days of ensiling (0.04 g/Kg DM), and was only detected in LBLH samples after at least 8 days of ensiling (average 1.2 g/Kg DM). At day 64, the concentration of propionic acid was higher in the LBLH silage than in the Control silage ($P = 0.015$).

Prior to ensiling, wilted alfalfa had an average of 3.3 $\log_{10}$ CFU/g fresh weight of LAB ($P = 0.724$ between replicates), 5.4 $\log_{10}$ CFU/g fresh weight ($P = 0.517$ between replicates) of yeast and 4.0 $\log_{10}$ CFU/g fresh weight ($P = 0.391$ between replicates) of moulds. The number of culturable LAB and yeasts in the Control and LBLH silage after 1, 4, 8, 16, 32 and 64 days of ensiling are listed in Table 1. Inoculated silage harboured more ($P < 0.014$) LAB than Control in all ensiling periods. The yeast counts were higher ($P = 0.022$) in LBLH than in Control silage at day 1. The number of yeasts dropped below the threshold of detection (2 $\log_{10}$ CFU/g fresh weight) after 16 days of ensiling in LBLH samples while in the Control samples, the number of yeasts remained detectable throughout the ensiling period (average of 3.77 $\log_{10}$ CFU/g fresh weight).

DISCUSSION

Epiphytic microbiota of the forage during the growing season and after harvesting and chopping

Epiphytic microbiota is known to impact the ensiling pattern of alfalfa, and can affect the effectiveness of inoculants (Muck et al., 2018; Wang et al., 2021). Therefore, because of its importance, we evaluated the epiphytic microbiota of alfalfa during the growing season and after harvesting and chopping. In a study using standard culture-based techniques, Lin et al. (1992) showed that the number of epiphytic microorganisms in alfalfa increased over the growing season but was not affected by cutting or maturity. In the present study, the structure of the bacterial community changed over time and was affected by cutting and field fertilization. Importantly, the *Lactobacillaceae* RA differed on the different sampling occasions over the growing season, the highest value being measured before the first cut.

After the first cut, the RA of *Lactobacillaceae* diminished, and *Enterobacteriaceae* became the most abundant bacterial family in the alfalfa stand. The increase in the RA of *Enterobacteriaceae* in June compared with May might have been due to the warmer and more humid summer conditions that facilitate the proliferation of enterobacteria, or to contamination of the soil from soil particles or through rainfall, for example. It is common for enterobacteria to dominate the epiphytic population of forage crops, but their development is undesirable, as they compete with LAB for substrate and in some cases, may be pathogenic (Queiroz et al., 2018). Clostridia are also undesirable in silage. These bacteria can be found in silage

TABLE 1 pH, concentrations of organic acids and alcohols, lactic acid/acetic acid ratio, and microbial counts in uninoculated alfalfa (control) and alfalfa inoculated with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii*, ensiled for 1, 4, 8, 16, 32, and 64 days

		Days					
Variables	**Treatments**	**1**	**4**	**8**	**16**	**32**	**64**
pH	Control	5.03^{b}	4.87	4.78	4.64^{b}	4.61^{b}	4.60^{b}
	LBLH	5.14^{a}	4.89	4.81	4.75^{a}	4.81^{a}	4.80^{a}
	P-value	0.005	0.234	0.616	0.010	<0.001	<0.001
Lactic acid	Control	16.7	27.7	34.3^{b}	50.7	65.0^{a}	65.3^{a}
(g/kg DM)	LBLH	18.3	34.0	38.3^{a}	45.0	51.0^{b}	52.0^{b}
	P-value	0.152	0.303	0.013	0.435	<0.001	<0.001
Acetic acid	Control	17.1	23.1	26.5	23.1	38.2^{b}	42.7^{b}
(g/kg DM)	LBLH	18.4	24.3	25.0	33.1	56.3^{a}	57.8^{a}
	P-value	0.441	0.213	0.669	0.075	0.020	0.011
Lactic acid/	Control	0.98	1.21	1.30	2.29	1.73^{a}	1.53^{a}
acetic acid	LBLH	0.99	1.40	1.59	1.39	0.91^{b}	0.90^{b}
ratio	*P*-value	0.836	0.504	0.281	0.141	0.008	0.001
1,2-propanediol	Control	ND	ND	ND	ND	ND	ND
(g/kg DM)	LBLH	ND	ND	1.0	1.1	0.7	1.2
	P-value	NA	NA	<0.001	<0.001	<0.001	<0.001
Propionic acid	Control	ND	ND	ND	ND	ND	0.4^{b}
(g/kg DM)	LBLH	ND	ND	0.2	0.7	1.6	1.6^{a}
	P-value	NA	NA	<0.001	<0.001	<0.001	0.015
LAB counts	Control	5.87^{b}	6.10^{b}	5.89^{b}	6.40^{b}	6.21^{b}	5.93^{b}
($\log_{10}$ CFU/g)	LBLH	6.54^{a}	7.21^{a}	7.18^{a}	7.63^{a}	7.43^{a}	7.24^{a}
	P-value	0.011	0.013	0.014	<0.001	<0.001	<0.001
Yeast counts	Control	4.29^{b}	3.31	3.27	4.10^{a}	4.33^{a}	3.33^{a}
($\log_{10}$ CFU/g)	LBLH	5.10^{a}	3.83	3.05	<2^{b}	<2^{b}	<2^{b}
	P-value	0.022	0.057	0.568	<0.001	<0.001	<0.001

Note: ^{a,b}Different letters between treatments within opening periods are significantly different according to a Dunn multiple comparison test at an alpha level of 0.05.

Abbreviations: LBLH, treatment with *Lentilactobacillus buchneri* NCIMB 40788 and *Lentilactobacillus hilgardii* CNCM-I-4785 at a ratio of 2×10^{5} CFU/g fresh forage each; NA, does not apply; ND, not detected—under the threshold of detection.

contaminated by soil or manure and can cause proteolysis and produce toxic compounds (Queiroz et al., 2018). In the present study, the highest *Clostridiaceae* RA was detected in the first samples collected after manure was applied. However, the *Clostridiaceae* RA decreased after about a week.

Unlike the bacterial population, the fungal population underwent no remarkable changes after the first cut, as the three families (*Davidiellaceae*, *Didymellaceae* and *Pleosporaceae*) that were the most abundant before the first cut were subsequently still the most abundant. However, some changes did take place after the second cut and application of manure. The RA of two main families (*Davidiellaceae* and *Didymellaceae*) decreased, *Pleosporaceae* RA increased, and *Montagnulaceae*, which was previously present at low abundance, became one of the three most abundant families. The families *Didymellaceae*, *Pleosporaceae* and *Montagnulaceae* are part of the *Pleosporales* order, which contains epiphytes, endophytes, parasites, and saprophytes species (Zhang et al., 2012). However, because of such a diversity of habitats, it is difficult to identify the role of those organisms in silage production and rumen activity.

From harvest to ensiling, there are several steps in which the epiphytic microbiota might be affected. In the present study, the steps that led to ensiling were the following: cutting, wilting, raking into a windrow, chopping, transport to the ensiling site, mixing for homogeneity and inoculation. These different processes and the several hours that separated harvesting from ensiling could explain the differences in microbiota observed in the plant stand (sampled 2 weeks prior to harvesting to

make the silage) and in the chopped material used for ensiling. More specifically, it could explain the greater RA of *Pseudomonas*, a strict aerobe, in the chopped material than in the standing plant. Even though the RA of *Lactobacillaceae* was high before the first cut, the number of culturable LAB in the chopped material was lower than expected based on prior studies (Schmidt et al., 2009). As expected, inoculation with *L. buchneri* and *L. hilgardii* increased the RA of *Lactobacillus* in inoculated forage over that in the Control forage prior to ensiling.

Microbial community dynamics during ensiling

The genus *Pseudomonas* dominated chopped forage prior to ensiling, but 1 day after ensiling, *Weissella* became the main genus in the silage. *Weissella* is a genus of obligate heterofermentative bacteria that mainly produce D-lactate, acetic acid and CO_2 during fermentation (Fusco et al., 2015), thereby contributing to spontaneous silage fermentation (Fessard & Remize, 2017). Although *Weissella* are not currently recognized as GRAS, a recent literature survey concluded that they are safe to use in animal feed (Sturino, 2018). In alfalfa at 270 g/kg DM, Wang et al. (2021) observed that after 3 days of ensiling, *Weissella* comprised less than 10% of the total bacterial population, whereas *Lactobacillus* was present at a high RA, i.e. approximately 40%. Such a decrease in the RA of *Weissella* and increase in the RA of *Lactobacillus* during ensiling was thus expected, but it did not occur in Control silages in the present study.

Among recent studies of the microbiota of alfalfa silage, several showed that *Weissella* and *Pediocccus* are often very abundant during the early stages of fermentation (Ogunade et al., 2018; Yuan et al., 2020). In the present study, the RA of *Weissella* was 45.0% in Control samples ensiled for 64 days.

Several factors may influence the RA of *Weissella* in silage. Zhang et al. (2018) ensiled alfalfa wilted to 450 g/kg in jars for 60 days at different temperatures. Their amplicon sequencing results indicated that the RA of *Weissella* was approximately 25% in untreated samples incubated at 30°C, *Weissella* RA decreased when the incubation temperature decreased from 30°C to 20°C and no *Weissella* OTUs were detected in samples incubated at 40 °C. Using cell culture techniques, Agarussi et al. (2019) characterized the LAB microflora of non-wilted (13.4 g/kg DM) and wilted (233 g/kg DM) alfalfa ensiled in vacuum bags for 56 days. These authors observed no difference in the number of *Weissella* isolates in non-wilted and wilted silages.

In the present study, the diversity of the fungal community increased from day 1 to day 4 of ensiling. While at day 1 of ensiling, one taxon (*Ascochyta*) dominated the silage, at day 4, the RA of this taxon decreased to less than 50%, while other taxa, such as *Neosetophoma* and *Cladosporium*, increased in abundance. We found only one other published study that evaluated the fungal community of alfalfa silage, however, the community was analysed at only one time point during ensiling (Bai et al., 2020). Consequently, we were unable to compare our findings regarding the changes in fungal community diversity over time with those reported in other studies of alfalfa silage. However, regarding the RA of different genera, a comparison was possible with the findings of Bai et al. (2020) in alfalfa ensiled for 60 days. Like in our study, Bai et al. (2020) observed the presence of *Ascochyta* and *Cladosporium*. *Cladosporium* has been identified in several types of silage (Dunière et al., 2017; Zhang et al., 2019), and is known to be able to produce mycotoxins (Alonso et al., 2013). *Ascochyta* is a recognized pathogen of grain legumes (Bretag et al., 2006), and *Neosetophoma* is part of the *Phoma* group, which contains several plant pathogenic species (de Gruyter et al., 2010). However, the role of *Ascochyta* and *Neosetophoma* in silage and in the rumen is not known.

Effects of inoculation on silage microbial community dynamics and fermentation profile

Like other legumes, alfalfa is considered to be hard to ferment (Améndola-Massiotti et al., 2019). Therefore, if the numbers or activity of LAB is not sufficient to drive fermentation, undesirable bacteria can develop and reduce silage quality. In addition, the process of wilting alfalfa can increase the number of yeasts (Lin et al., 1992) and cause aerobic instability. For these reasons, when producing alfalfa silage, using inoculants based on obligate heterofermentative LAB that produce antifungal compounds (e. g., acetic acid) thereby improving aerobic stability may be advantageous.

Inoculants might fail if they cannot outcompete certain members of the epiphytic community (Muck et al., 2018). However, in the present study, the inoculant quickly overcame the *Weissella* population. As a result, inoculated silages contained greater numbers of culturable LAB as soon as after 1 day of ensiling and higher RA of *Lactobacillus* than Control silage. Tohno et al. (2012) showed that strains identified as *Weissella* had lower tolerance to pH below 4.0 than *Lactobacillus* strains. Therefore, in inoculated silage (average pH of 4.86), the possibly greater tolerance of *Lactobacillus* to pH may have facilitated its dominance over the *Weissella* population. However, in the Control group, *Weissella* dominated the silage throughout

the ensiling period. Even though *Weissella* is not considered a hazard for the hygienic quality of silage, it can increase losses. Cai et al. (1998) reported that inoculating alfalfa with *Weissella paramesenteroides* did not improve the quality of the silage and was related to higher fermentation loss.

Inoculation reduced the representation of several genera belonging to the order *Enterobobacteriales*, including *Citrobacter*, *Erwinia*, *Serratia* and *Proteus*, during the early stages of ensiling. A faster decline in the enterobacteria population by inoculation with *L. buchneri* and *L. hilgardii* has already been observed in high-moisture corn and corn silages (da Silva, Costa, et al., 2021; da Silva, Smith, et al., 2021).

Inoculation may bring the fermentation process to a point at which the acidity level is sufficiently high to create physiological and biochemical constraints, opening specific metabolic niches for different species within the *Lactobacillaceae*, but also that limit the intrinsic metabolic function of other species, perhaps including reduced production of bacteriocins following accumulation of acetic acid (Ge et al., 2019). Subsequent changes to the microbial population can have unexpected results, for example, the increase in *Clostridiales* OTUs observed following differential expression analysis. Clostridia are mainly found in unwilted uninoculated legumes contaminated by soil or manure and can cause proteolysis, produce toxic biogenic amines, and cause enteric syndromes and botulism (Queiroz et al., 2018). The presence of these bacteria in silage is consequently a reason for concern. However, the genus *Clostridium*, which is of major concern, was not detected, and the *Clostridiales* OTUs found were assigned to the genera *Blautia*, *Coprococcus*, *Ruminococcus* and *Lachnospiraceae*. These OTUs are usually observed in microbial populations in the rumen, and like *Ruminococcus* (La Reau & Suen, 2018), and *Blautia*, may have probiotic potential (Liu et al., 2021).

Several studies showed that the facultative heterofermentative LAB *Lentilactobacillus plantarum* have a positive impact on the microbial community and on the quality of alfalfa silage (Yang et al., 2020, 2021; Zheng et al., 2017). Ogunade et al. (2018) and Zheng et al. (2017) observed that inoculation of alfalfa silage with *L. plantarum* reduced the RA of *Pediocccus* and *Weissella*. However, fewer studies on the impact of obligate heterofermentative LAB (that improve aerobic stability, e.g., *L. buchneri)* in alfalfa silage can be found in the literature than studies using facultative heterofermentative strains. Kung et al. (2003) reported that inoculation of alfalfa silage with *L. buchneri* 40,788 improved the aerobic stability of the total mixed ration made with that silage. Schmidt et al. (2009) reported that inoculation of alfalfa with *L. buchneri* 40,788 increased the number of *L. buchneri* amplicons detected by qPCR and that the maximum value was reached after 45 days of ensiling.

Like the study by Schmidt et al. (2009), other studies (Holzer et al., 2003; Wambacq et al., 2013) have also shown that the establishment and activity, including the conversion of lactic to acetic acid and 1,2-propanediol (Oude Elferink et al., 2001) of *L buchneri* require a longer period than facultative heterofermentative strains, such as *L. plantarum*. Additionally, recent studies have shown that combining *L. buchneri* with another obligate heterofermentative strain (*L. hilgardii*) improved the production of anti-fungal compounds in some trials, enabling earlier aerobic stability to be reached in whole-plant corn silage and high-moisture corn (da Silva, Costa, et al., 2021; da Silva, Smith, et al., 2021; Ferrero et al., 2019). Drouin et al. (2019) observed that the interactions between *L. buchneri* and *L. hilgardii* enhanced fermentation efficiency and positively influenced the microbiota succession in the first 2 months of whole corn plant ensilage. However, the effects of the combination of these two obligate heterofermentative strains have not yet been tested in alfalfa.

In the present study, compared with Control, inoculated silage had a higher concentration of acetic acid after 32 days of ensiling and more propionic acid after 8 days. Because of the higher production of these antifungal compounds, yeasts were inhibited in inoculated silage, reaching undetectable levels (<2 $\log_{10}$ cfu/g of fresh weight) after 16 days of ensiling. However, yeasts remained detectable in Control silage throughout the ensiling period. Aerobic stability was not tested in the present study; still, a reduction in the number of yeasts could be an indicator of improved stability. In addition, improvement in the aerobic stability of alfalfa by inoculation has already been reported (Blajman et al., 2020).

Even though inoculation reduced the number of culturable yeasts, overall, it had no marked impact on the structure of the fungal community. The sensitivity of certain fungi to low pH can modify the fungal community structure over the course of ensiling with the decline in pH. For example, in corn silage, Drouin et al. (2019) observed a marked drop in pH (from 5.01 to 4.24) from day 1 to day 2 of ensiling as well as significat changes in the composition of the fungal community. The absence of changes in the fungal community structure in the present study could be due to the fact that the pH did not vary much over the entire ensiling period: only from 5.09 at day 1 to 4.70 at day 64.

Some OTUs related to *Enterobacteriales*, which were epiphytic to alfalfa plants, persisted after ensiling. However, *Pseudomonas*, which was present at greater RA in the plant prior to ensiling, was quickly replaced by *Weissella* after ensiling in both Control and inoculated silages. This indicated that, in the present study, the epiphytic bacterial

population was not an impediment to natural LAB fermentation or to inoculant effectiveness.

We have shown that *Weissella* dominated the microbiota of uninoculated alfalfa silage. However, in silage inoculated with a combination of *L. buchneri* and *L. hilgardii*, *Weissella* dominance was hampered early in the ensiling process. Inoculation modified the composition of the bacterial community, increasing the RA of *Lactobacillus* while reducing the RA of *Weissella* and the presence of *Enterobacteriaceae*. In parallel, inoculation improved the accumulation of acetic and propionic acids, thereby preventing the development of yeasts, but had no marked effect on the structure of the fungal community.

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CONFLICT OF INTEREST

The authors Pascal Drouin and Emmanuelle Apper are employed by the Animal Nutrition business unit of Lallemand Inc. However, their affiliation did not impede their ability to follow journal guidelines or to remain impartial during the preparation of this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at the NCBI BioProject repository at https://www.ncbi.nlm.nih.gov/bioproject/, reference number PRJNA693281.

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