Interpretive Summary

Gagnon

Dairy farmers are commonly adding bacterial inoculants containing lactic acid bacteria (LAB) in silage. However, the possible transfer of these LAB in milk could have a negative influence on its quality. The aim of this study was to evaluate the impact of silage inoculation on LAB profiles of raw milk. This study demonstrated the plausible transfer of some LAB strains from silage to bulk tank milk. However, silage was a minor source of LAB contamination for raw milk. Inoculation has a positive effect on the quality of grass silage and seems to be an advantageous management practice for dairy farmers.

Prevalence and abundance of lactic acid bacteria in raw milk associated with forage types in dairy cow feeding

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ABSTRACT

Lactic acid bacteria (**LAB**) found in milk can be responsible for organoleptic defects in cheese. In order to identify the source of LAB that could potentially develop during cheese making, we evaluated their prevalence and abundance in milk according to the type of forage used in dairy cow feeding. Forages and bulk tank milk were sampled three times on 24 farms using either hay alone (control), or grass or legume silage supplemented or not with corn silage. Both types of silages were either noninoculated, or inoculated with commercial preparations containing at least a *Lactobacillus buchneri* strain along with *Lactobacillus casei, Lactobacillus plantarum, Enterococcus faecium,* or *Pediococcus pentosaceus*. Our results indicate that LAB viable counts in milk samples (2.56 log cfu/mL) did not differ according to the type of forage used. A total of 1239 LAB were isolated and identified by partial 16S rRNA gene sequencing. Although inoculation increased lactobacilli abundance in grass silage by 35%, we did not observe an effect on the LAB profile of milk. Indeed, there was no significant difference in milk LAB prevalence and abundance according to the type of forage ($P > 0.05$). Moreover, isolates belonging to the *L. buchneri* group were rarely found in bulk tank milk (3/481 isolates). Random amplified polymorphic DNA typing of 406 LAB isolates revealed the plausible transfer of some strains from silage to milk (~6%). Thus, forage is only a minor contributor to LAB contamination of milk.

Key words: silage, non-starter lactic acid bacteria, random amplification of polymorphic DNA, bacteriocin

INTRODUCTION

Raw milk is a complex matrix favorable to the development of microorganisms due to its high nutrient content and water activity. Milk microbiota is composed of diverse microorganisms belonging to fungi, bacteriophage, protists, archaea, and bacteria. Lactic acid bacteria (**LAB**) are one of the most common types of microorganisms in milk (Quigley et al., 2013). They are Gram-positive, oxidase and catalase-negative, asporogenous, and belong to the order of *Lactobacillales* (Mattarelli et al., 2014). Many genera of LAB such as *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus* are well-known because they are associated with the manufacturing of dairy products (Gobbetti et al., 2018). Non-starter LAB (**NSLAB**) found in cheese can come directly from the milk (Fox et al., 2017a; Desfossés-Foucault et al., 2013; Gobbetti et al., 2018). Indeed, they can resist heat treatment of milk before cheese making (Fox et al., 2017b). Heterofermentative NSLAB are occasionally responsible for cheese defects (Banks and Williams, 2004; Ortakci et al., 2015). Moreover, LAB are usually able to produce bacteriocins against closely related bacteria (Field et al., 2018). If NSLAB inhibit starters and adjunct cultures during cheese manufacturing, it could cause defects in end-products. Hence, it is essential to have a better understanding of the origin of NSLAB.

The origin of milk microbiota has been the subject of several recent scientific studies (Gleeson et al., 2013; Driehuis, 2013; Skeie et al., 2019). It seems that all components of the dairy farm environment, such as litter, milking systems, and cattle feeding influence this microbiota. In Eastern Canada, silage is a key element for feeding dairy cows (Fadul-Pacheco et al. 2017) and it is a potential contamination source of LAB for milk. It is obtained by fermentation of forage in bunkers or silos by epiphytic LAB. The composition

of these LAB is modulated by silage crops (McAllister et al., 2018). Silage in Eastern Canada endures a wide range of temperature and potentially could select for LAB able to resist heat treatment in milk. In recent years, it has become common practice to add bacterial inoculants in silage to improve its quality during storage. Facultative heterofermentative LAB such as *Lactobacillus plantarum* or obligate heterofermentative LAB such as *Lactobacillus buchneri* are often used (Muck et al., 2018). The *L. buchneri* inoculant is beneficial as this species decreases yeast number in silage through a high production of acetic acid (Dolci et al., 2011). While the impact of inoculants on silage quality is well described (Tabacco et al., 2011; Schmidt and Kung, 2010; Contreras-Govea et al., 2013), their direct impact on milk microbiota is not.

We hypothesized that there is a transfer of LAB from silage to milk, and that this transfer is exacerbated when silages are treated with commercial LAB inoculants. Therefore, this study investigated the impact of silage on raw-milk LAB prevalence and abundance. Lactic acid bacteria shown to be able to carry over from dairy cow feeding to milk were then isolated by a culture-dependent approach.

MATERIALS AND METHODS

Farm Sampling

Selection of herds. Commercial dairy farms (n = 24) in Quebec were recruited using the list provided by the Canadian DHIA (Lactanet, Sainte-Anne-de-Bellevue, QC, Canada), on which farmers consent for the sampling of forages and milk. Farms were divided into five feeding types according to the forages used, representing the common feeding practices in Eastern Canada (Figure 1). The first group was composed of farm using hay

(**H**; non fermented) as the sole source of forage, and was defined as control. The second group was farms feeding grass or legume silage (**GL**). The third group used grass or legume silage supplemented with corn silage (**GLC**). The fourth group used grass or legume silage supplemented with corn silage that was inoculated at the time of harvest (**GLCI**). Finally, the fifth group used grass or legume silage supplemented with corn silage, both inoculated at the time of harvest (**GLICI**). The inoculants added by the dairy producers of the GLCI and GLICI groups were those available on the market (Table 1), and included Biotal Buchneri 500 and Biotal Supersile (Lallemand Animal Nutrition, Milwaukee, WI), 11CFT, 11C33, and 11G22 (Pioneer, Johnston, IA). None of these herds had access to pasture during sampling periods.

Sampling, Isolation, and Quantification of Lactic Acid Bacteria. The dairy farms were sampled three times (Figure 1). Between the first and second samplings, three farms changed their feeding type. The farm 2H became 7GL, the farm 1GLCI became 7GLICI, and the farm 2GLCI became 4GLC. At each site, forages and raw milk were collected. Fermented forages were ensiled for at least 45 d before collections. All samples were placed in a cooler at 4°C until analysis at the laboratory and were processed within 24 h. Approximately 500 g of each forage were sampled directly from hay barns or silos. The samples were taken with sterilized shovel, or probe (Prov-Vac, St-Jean-Chrysostome, QC, Canada) for the baled material, and were transferred in a bag free from bacteria or other living microorganisms. A first subsample was sent to Lactanet laboratories for near-infrared reflectance spectroscopy analysis of DM and pH. A second subsample of 30 g was placed in a Whirl-Pak bag with a filter (Nasco, Fort Atkinson, WI) and homogenized in Stomacher 400 Circulator (Seward, West Sussex, UK) at 260 rpm for 5 min with 270 mL

of peptone-buffered saline containing 1 g/L of Bacto Peptone (BD Biosciences, San Jose, CA) and 9 g/L of NaCl (Thermo Fisher Scientific, Waltham, MA). The mixture was diluted in peptone-buffered saline for viable counts and plated on MRS agar with BromoPhenol Blue (MilliporeSigma, St. Louis, MO) (**MRS-BPB)** for total LAB (Lee and Lee, 2008) and a medium agar containing arginine (MilliporeSigma), bromocresol purple (MilliporeSigma), beef extract (BD Biosciences), and vancomycin (Thermo Fisher Scientific) (**ABEV** agar) for heterofermentative LAB (Sohier et al., 2012). However, viable counts on ABEV agar were revealed to correspond to total LAB because color-based distinction of homofermentative from heterofermentative colonies was difficult. The identification of isolated bacteria has shown that we recovered both types of fermentative LAB. Petri dishes for both media were incubated at 37°C for 48 h in an anaerobic glove box containing an atmosphere of 80% N_2, 10% H_2, and 10% CO_2 (Praxair Canada, Mississauga, ON, Canada).

The milk in the bulk tank was agitated for 5 min. Then, the tank tap was washed with water. Approximately 10-mL of milk was first discarded, and the milk sample (100 mL) was collected. From this sample, a 10-mL aliquot was homogenized in the Stomacher with 90 mL of 2% (w/v) sodium citrate (EMD Chemicals, Gibbstown, NJ) at 260 rpm for 5 min. Viable counts in milk were performed as described for forages.

For first and second sampling periods, LAB were isolated from Petri dishes used for viable counts with Harrison's disk to obtain representative isolates of the starting population from forage and milk samples (Ricciardi et al., 2015). The selected colonies were purified by plating on MRS-BPB agar and cultivated in MRS broth. The stock cultures were frozen at −80°C supplemented with 20% (v/v) glycerol (EMD Chemicals).

The isolates were labeled RKG (Roy, Kennang, and Gagnon) and were numbered 1 or 2 according to the sampling period followed by the order they were isolated. Samples of the commercial inoculants used on the farms were rehydrated in peptone-buffered saline and plated on MRS-BPB agar. Colonies were selected, purified and frozen as described for forage and milk samples, and were used to extract DNA for random amplification of polymorphic DNA (**RAPD**).

Bacterial Identification and Typing

All selected isolates were identified by partial 16S rRNA gene sequencing. First, genomic DNA was extracted with Geneaid Presto Mini gDNA Bacteria Kit (FroggaBio, Toronto, ON, Canada) with modifications. Mutanolysin (50 U/mL; MilliporeSigma) was added to the Gram+ buffer and the quantity of lysozyme was increased to 20 mg/mL. Finally, all incubation times in the sample preparation steps were doubled. Yield and quality of DNA were quantified by using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA was diluted to 25 ng/µL. The partial amplification of the 16S rRNA gene (~800 bp) with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 788R (5'-GGACTACCAGGGTATCTAA-3'), as well as the Sanger sequencing were performed according to Gagnon et al. (2020). Forward and reverse strand sequences were aligned with Geneious Pro R6 software (Biomatters, San Francisco, CA). Phylogenetic affiliation of the isolates was determined with the Nucleotide Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA gene sequences are available in GenBank under accession numbers MT044754 - MT045988.

Multiplex PCR assay targeting the housekeeping gene *recA* as described by Torriani et al. (2001) was carried out for better identification of isolates belonging to the *L. plantarum* group (*L. plantarum, L. paraplantarum,* and *L. pentosus*). The PCR amplification was performed with a Tgradient (Biometra, Montreal Biotech, Montreal, QC, Canada). Conditions and primers for PCR are presented in Supplemental Tables S1 and S2. The PCR products (*L. plantarum*: 318 bp, *Lactobacillus paraplantarum*: 107 bp, and *Lactobacillus pentosus*: 218 bp) were migrated through 2% agarose gel submerged in sodium boric acid buffer for 30 min at 135 mV. Positive controls were *L. plantarum* ATCC 14917 and *L. pentosus* ATCC 8041. Nuclease-free water was used as negative control.

A selection of LAB that could originate from silage was included for typing. For each farm, isolates of the same species collected both in forages and raw milk were selected (Figure 1). For example, if on a given farm, *L. plantarum* were isolated in milk, then all isolates from milk, forages, and the corresponding inoculants from this farm were typed. The RAPD with the M13 primer (5'-GAGGGTGGCGGTTCT-3'), as described by Ruiz et al. (2014), was used as a typing method. The PCR conditions are presented in Supplemental Tables S3 and S4. The band patterns obtained after migration at 135 mV during 60 min in a 2% agarose gel were normalized and compared with GelJ V.2.0 software (Heras et al., 2015). The similarity between isolates was determined by Pearson's Correlation Method (a coefficient working with the densitometric curve associated with the different lanes) and the clustering was performed by Unweighted pair group method with arithmetic mean. The genotype was defined at a minimum level of 80% similarity.

Antibacterial Agar Diffusion Assay

Antibacterial activity against indicator spoilage bacteria and cheese starters were screened for all isolates using the agar well diffusion method described by Fernandez et al. (2013). Briefly, after two subcultures, indicator strains were grown overnight in their optimal broth: Tryptic Soy Broth (BD Biosciences) with 0.6% yeast extract (Thermo Fisher Scientific) for *Listeria ivanovii* HPB28, Reinforced Clostridial Medium (HiMedia Laboratories LLC, West Chester, PA) for *Clostridium tyrobutyricum* ATCC 25755, Elliker (BD Biosciences) for *Lactococcus lactis* spp. *cremoris* SK11 and MRS for *Lactobacillus paracasei* ATCC 334. Then, inoculation at 0.6% on the same media with 0.75% agar was performed. Wells were dug in inoculated solidified agar plates, and 80 µL aliquot of isolate supernatant were added. The supernatant of overnight culture in MRS was obtained after centrifugation (10,000 × *g*, 10 min at 4°C). The plates were incubated 24 h at 37°C (under anaerobic conditions for *C. tyrobutyricum*). After incubation, inhibition zone diameters were measured. Bacteriocin-containing culture supernatants produced clear inhibition zones featuring well-defined margins with a diameter greater than 8 mm (Supplemental Figure S1). The positive controls used were supernatants of *Pediococcus acidilactici* UL5 producing pediocin PA-1, *L. lactis* ATCC 11454 producing nisin A, and *Escherichia coli* MC 4100 producing microcin J25.

Statistical analysis

Student's t-test, ANOVA, and Tukey's multiple comparisons or Honestly significant difference test on viable counts were used for data with normal distribution and equal variances. Otherwise, counts were analyzed with nonparametric Kruskal-Wallis-Wilcoxon test. Statistical analysis was performed with JMP version 14 Software (SAS Institute, Cary, NC). Data of taxonomic group relative abundances were transformed in the R environment

(R Core Team, 2013) using Phyloseq (McMurdie and Holmes, 2013) and Microbiome (Lahti and Shetty, 2017) packages. Relative abundances were then compared with STAMP version 2.1.3 (Parks et al., 2014). Significance was assessed using an ANOVA comparing multiple groups and Storey's FDR correction was applied to *P*-values.

RESULTS

Lactic Acid Bacteria Prevalence and Abundance in Forages

The characteristics of forages were different according to their type (Supplemental Table S5). In particular, the pH of C silage was lower than GL silage, but inoculation did not affect this parameter. The MRS-BPB agar was not specific for LAB (Supplemental Table S6); therefore, only viable counts obtained on the ABEV medium are presented. The LAB viable counts for H were lower (2.15 ± 1.83 log cfu/g) than both inoculated and non-inoculated silages (C + CI: 7.25 ± 1.30 log cfu/g; GL + GLI: 6.79 ± 1.05 log cfu/g; $P < 0.01$; Figure 2). Inoculated silage LAB viable counts were greater than non-inoculated silage only at the second sampling period (C vs. CI: $P < 0.02$; GL vs. GLI: $P < 0.01$).

A total of 758 LAB were isolated in forages from the first two sampling periods. They belonged to 42 taxa corresponding to six genera, e.g., *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella* (Figure 3A). Statistical analyses performed with STAMP indicated that LAB profiles differed between H and silages. At the genus level, the difference in mean proportions of *Enterococcus* spp. and *Leuconostoc* spp. were respectively 20 and 15% more abundant in H than silages (Figure 4A). The relative abundance of *Lactobacillus* spp. was greater in silages than in H. *Lactobacillus* was the dominant genus in both grass or legume silage (GL + GLI: 72%) and corn silage

(C + CI: 93%). At the species level, there were also significant features characterizing the forages (Figure 4B). *Enteroccocus mundtii*, *L. pentosus*, *Lactobacillus tucceti*, *L. lactis*, and *Leuconostoc mesenteroides/pseudomesenteroides* were specific to H. The *L. buchneri* group was a specific and a dominant taxon in silages whether inoculated or not (Figures 3B and 4B). Its relative abundance in grass or legume (GL + GLI) and corn (C + CI) silage samples was 48 and 38%, respectively. *Lactobacillus brevis/yonginensis/koreensis* was also found in silage, but it was more abundant in corn than grass or legume (C + CI: 11% vs. GL + GLI: 5%) silage. *Pediococcus pentosaceus* was more abundant in H (20%) and grass or legume (GL + GLI: 12%) than corn (C + CI: 1.5%). Finally, *Lactobacillus casei/paracasei* and *L. plantarum* were not exclusive to any forage type (Figure 3A). The prevalence and the abundance of LAB did not differ between C and CI silage samples. However, the relative abundance of specific genera was different between GL and GLI silage (Figure 5), particularly *Lactobacillus*, *Pediococcus*, and *Weissella* for the first two sampling periods. *Lactobacillus* spp. were 35% more abundant in GLI silage. On the other hand, *Pediococcus* spp. and *Weissella* spp. were more abundant in the GL silage. The LAB profile of GL silage samples was intermediate between H profiles and other silages. Indeed, as for hay, GL silage bacteria consisted of *Weissella paramesenteroides/thailandensis* and *P. pentosaceus* in high proportions, and enterococci were present. In addition, GL silage contained a high proportion of the *L. buchneri* group, similar to GLI, C and CI samples.

Lactic Acid Bacteria Prevalence and Abundance in Bulk Tank Raw Milk

There was no effect of sampling periods on LAB viable counts in bulk tank milk samples ($F = 0.32$; $P < 0.73$), the mean concentration being 2.56 ± 1.04 log cfu/mL. Viable counts of LAB differed among milk samples (~1 log cfu/mL) according to farm feeding

types (F = 5.16; $P < 0.005$; Figure 6). Milk from the GLICI group of farms contained significantly more LAB (3.22 ± 0.82 log cfu/mL) than GL (2.20 ± 0.58 log cfu/mL) and H (2.16 ± 1.38 log cfu/mL) groups.

For the first two samplings, 481 isolates of LAB were recovered from bulk tank raw milk samples. A total of 12 isolates of *Streptococcus* (*S. equinus/lutetiensis*, *S. macedonicus/gallolyticus*, *S. parauberis*, *S. sanguinis*, and *S. uberis*) were not compiled in LAB profiles as they are usually associated with mastitis and they are not found in forage (Cameron et al., 2016). The remaining 469 LAB isolates belong to 39 taxa (Figure 3B). Genera identified were the same as those found in forage i.e., *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. Even though there were significant differences in LAB profile among forage types, the difference was not so obvious for the milk types. *L. casei/paracasei* was the most abundant group, representing 25% of total. *Leuconostoc lactis/garlicum*, *P. pentosaceus*, *L. lactis*, *W. paramesenteroides/thailandensis*, *Lactobacillus parabuchneri*, and *L. plantarum* were also found in abundance in the different milk samples. Only three isolates belonging to the *L. buchneri* group were identified in all samples of milk (Figure 3B). One farm remained in GLCI category at the second sampling period where only *P. pentosaceus* isolates were identified. Lastly, the multiple comparison of relative abundance at genus- and species-levels identified only one significant difference in bulk tank milk samples according to the feeding types. The relative abundance of *Pediococcus parvulus* was greater (3.1%) in GLCI-milk samples than in milk from the other feeding types. However, only one milk sample contained *P. parvulus* (12%) in this group, and in other milk samples.

Screening for Bacteriocin Activity

Out of 1239 isolates collected during this study and from the commercial inoculants, 36 culture supernatants had a strong antibacterial activity against *L. ivanovii* HPB28 corresponding to a bacteriocin effect (Table 2). Most of these supernatants (64%) came from the *L. plantarum* group isolates. Half of those also inhibited the growth of *L. lactis* subsp. *cremoris* SK11. A total of 20 *Lactococcus* spp. also had a bacteriocin-like inhibitory effect on *L. lactis* subsp. *cremoris* SK11. Only *L. lactis* RKG 2-85 inhibited *C. tyrobutyricum* ATCC 25755 growth. Of all isolates exhibiting bacteriocin activity, 77% were isolated from milk. None of the culture supernatants exhibited a bacteriocin-like activity against *L. paracasei* ATCC 334. None of isolates from commercial inoculants had a bacteriocin-like inhibitory effect.

Typing of Bacteria Potentially Transferred from Forage to Milk

For each farm, isolates belonging to the same species, and shared in both milk and forages were typed. Out of 481 milk isolates, 172 were part of at least one forage or commercial inoculant and belonged to 18 taxa (Table 3). The 172 RAPD patterns were compared by species for each farm with Pearson's correlation analysis. Thus, 65 dendrograms were produced (Supplemental Figure S2). We identified 36 isolates (7%) more likely to be transferred from silage to milk as they shared more than 80% similarity (Table 4). Among these 36 isolates, few belonged to the same lineage; corresponding to 31 strains, which can be divided in nine taxonomic groups, i.e. *E. mundtii* (1), *L. brevis* group (1), *L. buchneri* group (1), *L. casei/paracasei* (5), *L. paraplantarum* (1), *L. plantarum* (5), *L. tucceti* (1), *P. pentosaceus* (9), and *W. paramesenteroides* group (7). They were associated with the five type of forages (H: 8 strains; GL: 8 strains; GLI: 4 strains; C: 4 strains; CI: 5 strains). Moreover, five strains were highly similar to

commercial inoculants. Screening for bacteriocin activity supports the possibility of a transfer from silage to raw milk of the strains RKG 2-212 and 2-227, as they both produced an antibacterial effect on *L. ivanovii* HPB28 and had 94% similarity. Bacteriocin screening also confirmed that four of these strains (RKG 1-375, 1-378, 1-380, and 1-500) did not come from forages or commercial inoculants because they had inhibitory activity on *L. ivanovii* HPB28, but not their corresponding isolates. Regarding inhibitory activity results, 27 strains were shared between silage and bulk tank raw milk.

DISCUSSION

In the present study, LAB presence in Eastern Canadian farm-scale silage samples were in agreement with a meta-analysis by Oliveira et al. (2017). The LAB count mean for non-inoculated silage were 7.04 log cfu/g, regardless of the forage type. Our results are also similar to data reported previously from Italian farms (Rossi and Dellaglio, 2007). In that study, LAB viable counts for corn and alfalfa non-inoculated farm-made mature silage were respectively 7.71 and 6.71 log cfu/g. Our LAB viable counts in C silage are also similar to Blajman et al. (2018) meta-analysis. According to their investigation, the mean LAB counts were 7.27 and 6.40 log cfu/g for inoculated and non-inoculated silage, respectively. In our work, unlike Reich and Kung (2010) and Guo et al. (2018), LAB viable counts were not much greater in inoculated than in non-inoculated silage samples. However, their studies were performed in lab-made instead of farm-made silage.

The LAB communities differed according to forage types (Figures 3, 4 and 5). Indeed, enterococci were associated with H and GL silage. They are known to be epiphytic in fresh alfalfa crops but are diminished during ensiling when *Lactobacillus* are growing (Yang et

al., 2019; Guo et al., 2018). *E. mundtii* was found specifically in association with H. This species was found to be epiphytic on fresh grass and legume crops (Guo et al., 2018; Muller et al., 2001). The greater relative abundance of *Enterococcus* spp. in H can be linked to their capacity to survive a long period under dry conditions (Wendt et al., 1998). Likewise, *Lactococcus lactis* abundance in H was probably due to their ability to survive high osmolarity and dehydration (Sanders et al., 1999). In the present study, *Leuconostoc mesenteroides/pseudomesenteroides* was also retrieved from H. Lin et al. (1992) found *L. mesenteroides* on fresh alfalfa and corn plants, but not after ensiling. This species can initiate the fermentation of forage, but it is not as acidotolerant as *Lactobacillus* spp. (Holzer et al., 2003). Past studies also showed the dominance of lactobacilli in corn and grass silage materials, between 50 and 98% (Yang et al., 2019; Hu et al., 2018; McAllister et al., 2018). *L. brevis*, *L. plantarum*, and *L. buchneri* are known to tolerate high acidity (Dunière et al., 2013). The *L. buchneri* group was the dominant species in silage, inoculated or not. Similar to the present study, Stevenson et al. (2006) showed that its relative abundance in inoculated alfalfa silage was comparable to the non-inoculated silage. *L. buchneri* became the dominant LAB in mature silage samples (Zhou et al., 2016; Muck et al., 2018). Genomic, transcriptomic and proteomic analysis of this species demonstrated its resistance against competing microorganisms by different mechanisms such as the production of lactic acid, acetic acid, and hydrogen peroxide (Heinl and Grabherr, 2017). Compared to the majority of LAB, this species can use lactic acid as an energy source (Muck et al., 2018), explaining its dominance in silage when the water-soluble carbohydrates are low. The *L. brevis* taxon was commonly found in grass and corn silage (Dunière et al., 2013). In our study, this obligate heterofermentative group was more

abundant in C silage than in GL silage. The pH of C silage was lower than GL silage and, as previously mentioned, *L. brevis* is an acidotolerant species. Furthermore, it was more abundant in the fall sampling. It could be linked to the warmer temperature, as Zhou et al. (2016) found a greater abundance of *L. brevis* when corn was ensiled between 15 and 25°C in comparison to 5 and 10°C.

The LAB profile did not differ between C- and CI-silage samples. This could be explained by the lower buffering capacity of corn as compared with alfalfa (Queiroz et al., 2018). Therefore, pH will decrease easily in both C and CI silage samples, quickly promoting acidotolerant lactobacilli. In their meta-analysis on inoculation with homofermentative and facultative heterofermentative LAB from silage materials, Oliveira et al. (2017) also demonstrated that the inoculation effect depends on the forage plants. Inoculation at harvest improved fermentation of grass and legumes silage, but not of corn silage. In our study, GL silage and H showed similarities, such as a high relative abundance of *P. pentosaceus* and *W. paramesenteroides/thailandensis. P. pentosaceus* is not exclusively epiphytic on grass and legume crops. Indeed, its proportion on alfalfa and corn were shown to be similar (Lin et al., 1992; Cai et al., 1999). When GL silages were inoculated, the relative abundance of *P. pentosaceus* decreased. Stevenson et al. (2006) and Yang et al. (2019) demonstrated similar results with a greater abundance of *Pediococcus* spp., *Enterococcus* spp., and *Weissella* spp. in non-inoculated alfalfa silage.

Even though LAB profile and concentration vary according to forage types, these differences were not reflected in raw milk. The concentration of LAB in raw milk is in agreement with data found in literature, which is to say between 10^1 CFU/mL and 10^4 CFU/mL (Quigley et al., 2013). *L. casei/paracasei* was the dominant LAB in raw milk,

as found by Vacheyrou et al. (2011) on French farms. This species and the other dominant LAB (*L. casei/paracasei*, *L. lactis/garlicum*, *P. pentosaceus*, *W. paramesenteroides/thailandensis*, *L. lactis*, *L. parabuchneri*, and *L. plantarum*) are often part of the NSLAB in cheese (Quigley et al., 2011; Fox et al., 2017a). We expected to find more *L. buchneri* in milk samples from silage fed herds as it was dominant in fermented forage and has already been found in cheese (Desfossés-Foucault et al., 2013; Blaya et al., 2018). However, this taxon was rarely isolated in raw milk. Its resistance mechanisms such as the use of lactic acid as an energy source and acid tolerance are not operational selective measures in raw milk compared to silage.

Silage has previously been identified as a critical contamination source of bacterial spores for milk (te Giffel et al., 2002; Driehuis et al., 2016). However, our RAPD typing results suggested that only a few LAB strains probably originating from silage were recovered in raw milk. Therefore, the use of silage did not seem to be the major contamination source of LAB in raw milk. Our study did not allow us to identify how this contamination occurred. It could be through transmission in the barn by direct contact of forages with cow's hair and skin, including udder and teats, or through fecal contamination. For example, spores from silage were previously found in cow feces suggesting their resistance to digestion (Driehuis et al., 2016). Spores are probably more resistant than bacteria to the conditions encountered on farms, particularly during ensiling, feeding and milking. Teat canals of dairy cows can contain LAB (Bouchard et al., 2015) although they are not in the principal bacteria of bovine mammary microbiota (Falentin et al., 2016).

Metagenomic analysis suggested that lactobacilli are niche specialists (Stefanovic et al., 2017). Their adaptation to specific niches such as milk result in the acquisition of new

genes by horizontal transfer and the loss of coding sequences that are not needed. Their survival capacity in environmental niches such as plant are reduced. This could explain that few forage isolates were collected in milk. Also, LAB specific to forage niche could lack genes important for intestinal tract survival. Therefore, their transfer opportunities from feces to milk should be reduced. The strains that seem adapted to silage and milk belonged to *L. casei/paracasei*, *L. plantarum*, *P. pentosaceus* and the *W. paramesenteroides* group. Stefanovic and McAuliffe (2018) demonstrated heterogeneity in the genome of three *L. paracasei* strains isolated in the same niche (Cheddar cheese). They were able to link the genome of one strain to a prior plant-based niche. In the case of *L. plantarum*, strains from different niches (insect gastrointestinal tracts, human feces, olives, fruits, sourdoughs, and cheese) were modulating their transcriptome to adapt to MRS medium (Filannino et al., 2018). Little information is available on the genomics of *P. pentosaceus* and *W. paramesenteroides/thailandensis* as well as their adaptation to ecological niches such as silage and milk.

In the present study, LAB prevalence and abundance in raw milk samples from farms using silage inoculated with *L. buchneri* was not different from other feeding types. Moreover, RAPD typing and the bacteriocin-screening eliminated all but two milk strains that can be linked to commercial inoculants. Therefore, this management practice should not negatively impact cheese making. In addition, this practice could be endorsed for the limitation of enterococci in GL silage types. *Enterococcus* spp. are known to be thermoresistant (Gagnon et al., 2020) and can cause defects during cheese making (Giraffa, 2003). More attention should be addressed, however, to the *L. casei/paracasei* and *L. plantarum* inoculants. These facultative heterofermentative species seem more adapted

to diverse ecological niches and are commonly found as NSLAB in cheese (Blaya et al., 2018; Stefanovic and McAuliffe, 2018). Moreover, some strains of *L. plantarum* isolated from silage were also able to inhibit the starter *L. lactis* subsp. *cremoris* SK11. The presence of those NSLAB could reduce starter activity and thus affect milk acidification during cheese manufacture.

CONCLUSIONS

Even though LAB prevalence and abundance differed according to forage type, this was not the case for bulk tank milk samples. The findings presented in this study confirm that silage is a minor source of contamination of LAB for raw milk. Out of 481 milk isolates, 27 strains could be associated with silage. They belong in majority to four taxonomic groups, i.e., *L. casei/paracasei*, *L. plantarum*, *P. pentosaceus*, and *W. paramesenteroides*. These strains should be investigated further for their heat resistance and their potential impact on cheese making. Finally, only two strains could originate from commercial inoculants and they were not identified as *L. buchneri*. Therefore, inoculation of silage with *L. buchneri* did not seem to increase milk contamination with obligate heterofermentative LAB.

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TABLES

Table 1. Description of commercial inoculants used on the dairy farms

Feeding type[1]	Farm number[2]	Inoculant[3]	Species in the inoculants				
			Lactobacillus buchneri	*Lactobacillus casei*	*Lactobacillus plantarum*	*Enterococcus faecium*	*Pediococcus pentosaceus*
GLCI	1	4	x		x	x	
	2	3	x	x	x		
	3	1	x				x
GLICI	1	1 and 2	x		x		x
	2	1	x				x
	3	1 and 3	x	x	x		x
	4	1	x				x
	5	1	x				x
	6	3 and 5	x	x	x	x	
	7	4 and 5	x		x	x	

[1]GLCI = Grass or legume silage, and corn silage inoculated; GLICI = Grass or legume silage inoculated, and corn silage inoculated.

[2]Farms were numbered sequentially within each feeding group.

[3]1 = Biotal Buchneri 500, and 2 = Biotal Supersile (Lallemand Animal Nutrition, Milwaukee, WI); 3 = 11CFT, 4 = 11C33, and 5 = 11G22 (Pioneer, Johnston, IA).

660 **Table 2.** Culture supernatants that have bacteriocin activity against *Listeria*
661 *ivanovii* HPB28, *Lactococcus lactis* subsp. c*remoris* SK11, or *Clostridium*
662 *tyrobutyricum* ATCC 25755

					Activity against		
RKG Isolate[1]	Taxonomic group	Feeding type[2]	Farm number[3]	Matrix[2]	HPB28	SK11	ATCC 25755
2-118	*Lactococcus lactis*	H	1	Milk		x	
2-124	*Lactococcus lactis*	H	1	Milk		x	
1-174B	*Lactobacillus plantarum*	H	2	H	x	x	
1-176	*Lactobacillus plantarum*	H	2	H	x		
1-175	*Pediococcus pentosaceus*	H	2	H	x		
1-174A	*Pediococcus pentosaceus*	H	2	H	x		
2-85	*Lactococcus lactis*	H	4	Milk	x	x	x
1-205	*Lactococcus lactis*	H	5	H		x	
1-135	*Lactococcus lactis*	H	6	Milk		x	
1-136	*Lactococcus lactis*	H	6	Milk		x	
1-138	*Lactococcus lactis*	H	6	Milk		x	
2-705	*Leuconostoc mesenteroides*	GL	7	H[4]		x	
2-707	*Leuconostoc mesenteroides*	GL	7	H[4]		x	
2-760	*Lactobacillus plantarum*	GL	7	Milk	x	x	
2-759	*Lactococcus lactis*	GL	7	Milk		x	
1-77	*Lactobacillus paraplantarum*	GL	1	Milk	x		
2-361	*Lactobacillus plantarum*	GL	1	Milk	x		
2-172	*Pediococcus pentosaceus*	GL	2	Milk		x	
2-181	*Lactobacillus casei/paracasei*	GL	2	Milk		x	
2-182	*Lactobacillus casei/paracasei*	GL	2	Milk		x	
1-243	*Pediococcus stilesii*	GL	3	Milk	x	x	
2-582	*Lactobacillus pentosus*	GL	3	Milk	x		
2-571	*Lactobacillus plantarum*	GL	3	Milk	x		
2-567	*Lactococcus raffinolactis*	GL	3	Milk		x	
2-572	*Lactococcus lactis*	GL	3	Milk		x	
2-574	*Lactococcus raffinolactis*	GL	3	Milk		x	
2-770	*Lactococcus lactis*	GL	4	Milk		x	
2-771	*Lactococcus lactis*	GL	4	Milk		x	
2-413	*Enterococcus mundtii*	GL	5	GL	x		
1-103	*Pediococcus pentosaceus*	GL	5	Milk	x		
1-541	*Pediococcus acidilactici*	GL	6	Milk	x		
2-433	*Lactococcus lactis*	GLC	2	Milk		x	
2-436	*Lactococcus lactis*	GLC	2	Milk		x	
2-212	*Lactobacillus plantarum*	GLC	4	Milk	x	x	
2-213	*Lactobacillus paraplantarum*	GLC	4	Milk	x		
2-229	*Lactobacillus plantarum*	GLC	4	C	x	x	

2-227	*Lactobacillus plantarum*	GLC	4	C	x	x
1-613	*Lactobacillus plantarum*	GLC	3	Milk	x	x
1-615	*Lactococcus lactis*	GLC	3	Milk		x
1-616	*Lactococcus lactis*	GLC	3	Milk		x
1-618	*Lactococcus lactis*	GLC	3	Milk		x
1-619	*Lactococcus lactis*	GLC	3	Milk		x
1-634	*Lactobacillus plantarum*	GLC	3	C	x	x
1-593	*Lactococcus lactis*	GLCI	1	Milk		x
1-592	*Lactococcus lactis*	GLCI	1	Milk		x
1-506	*Lactobacillus plantarum*	GLCI	2	Milk	x	x
1-500	*Lactobacillus plantarum*	GLCI	2	Milk	x	
1-478	*Lactobacillus plantarum*	GLCI	3	Milk	x	
2-664	*Lactobacillus plantarum*	GLICI	1	GLI	x	x
1-378	*Pediococcus pentosaceus*	GLICI	1	Milk	x	x
1-380	*Lactobacillus paraplantarum*	GLICI	1	Milk	x	x
1-371	*Lactobacillus plantarum*	GLICI	1	Milk	x	
1-375	*Lactobacillus plantarum*	GLICI	1	Milk	x	
2-726	*Enterococcus faecium*	GLICI	1	Milk	x	
2-336	*Lactobacillus zeae*	GLICI	2	Milk	x	
2-468	*Lactobacillus delbrueckii*	GLICI	3	Milk		x
2-471	*Lactobacillus delbrueckii*	GLICI	3	Milk		x
1-381	*Lactobacillus paraplantarum*	GLICI	4	CI	x	x
1-254	*Pediococcus acidilactici*	GLICI	4	Milk	x	
2-33	*Lactobacillus plantarum*	GLICI	5	GLI	x	
1-193	*Lactococcus lactis*	GLICI	6	Milk		x
1-196	*Lactobacillus casei/paracasei*	GLICI	6	Milk		x
2-297	*Lactobacillus casei/paracasei*	GLICI	6	Milk	x	
1-178	*Lactobacillus plantarum*	GLICI	6	CI	x	x

[1] The isolates were labeled RKG (Roy, Kennang, and Gagnon) and were numbered 1 or 2 according to the sampling period followed by the order they were isolated.

[2]H = Hay; GL = Grass or legume silage; C = Corn silage; GLI = Grass or legume silage, inoculated; CI = Corn silage, inoculated.

[3]Farms were numbered sequentially within each feeding group.

[4]Hay sample form farm 7GL.

669 **Table 3.** Number of milk isolates belonging to species also collected in forages or
670 commercial silage inoculants on a given farm

Feeding type[1]	Farm number[2]	*Enterococcus casseliflavus* group	*Enterococcus faecalis* group	*Enterococcus mundtii*	*Lactobacillus acidipiscis* group	*Lactobacillus brevis* group	*Lactobacillus buchneri* group	*Lactobacillus casei/paracasei*	*Lactobacillus coryniformis*	*Lactobacillus paraplantarum*	*Lactobacillus plantarum*	*Lactobacillus tucceti*	*Lactobacillus versmoldensis* group	*Lactococcus lactis*	*Leuconostoc mesenteroides* group	*Pediococcus parvulus*	*Pediococcus pentosaceus*	*Weissella hellenica/bombi*	*Weissella paramesentoides* group	
H	1			1													1			
	2							2			1				5		3		2	
	3	1		2				1									1			
	4							7												
	5							3				1		1			1		6	
	6																4		1	
GL	1					1			1		1						1		4	
	2																5		2	
	3										1						1		2	
	4													2			1		2	
	5																5	1		
	6							13												
GLC	1						1													
	2		4								1									
	3							5			1									
	4										2									
GLCI	1		1					5			1									
	2							2			2					1			4	
	3										1		1				10			
GLICI	1							1	1	1	2						2		2	
	2				1		1	3												
	3					1											2			
	4																2			
	5							9												
	6					1		14												
Total			1	5	3	1	3	2	65	2	1	13	1	1	3	5	1	39	1	25

671 [1]H = Hay; GL = Grass or legume silage; C = Corn silage; GLI = Grass or legume silage, inoculated;
672 CI = Corn silage, inoculated.

673 [2]Farms were numbered sequentially within each feeding group.

674

675 **Table 4.** Relationship of milk isolates with forage isolates or commercial silage inoculants

Farm number[1]	Feeding type[2]	Taxa	Milk isolate[3,4]	Forage/Inoculant isolate[5]	Sample type[2]	Similarity (%)[6]
1	H	*Pediococcus pentosaceus*	2-121	1-161	H	83
				1-162	H	84
2	H	*Lactobacillus casei/paracasei*	2-748*	1-658A	H	90
			2-749*	1-658A	H	90
		Weissella paramesentoides group	1-173*	2-704	H	91
			2-757*	2-704	H	91
3	H	*Enterococcus mundtii*	2-102	2-95B	H	92
5	H	*Lactobacillus tucceti*	1-198	1-206	H	94
		Weissella paramesentoides group	2-630	2-625	H	90
			2-635	1-208	H	96
6	H	*Pediococcus pentosaceus*	1-141*	2-811	H	91
			1-142*	2-811	H	84
			2-802*	2-811	H	89
1	GL	*Pediococcus pentosaceus*	1-74	2-379	GL	84
				2-380	GL	85
				2-383	GL	85
3	GL	*Weissella paramesentoides* group	1-240	2-552	GL	95
				2-555	GL	90
				2-547	GL	81
			2-581	2-552	GL	99
				2-555	GL	96
4	GL	*Pediococcus pentosaceus*	2-777	1-406	GL	82
				1-414	GL	87
				1-418	GL	96
5	GL	*Pediococcus pentosaceus*	2-399	1-83	GL	80
2	GLC	*Lactobacillus plantarum*	2-439	2-449	C	80
3	GLC	*Lactobacillus casei/paracasei*	1-623	1-640	C	89
			2-69	1-629	GL	86
				1-631	GL	85
4	GLC	*Lactobacillus plantarum*	2-211	**2-219**	C	86
				2-222	C	90
				2-227	C	93
			2-212	**2-219**	C	85
				2-222	C	91
				2-227	C	94
1	GLICI	*Lactobacillus casei/paracasei*	2-719	2-675	CI	95
		Lactobacillus paraplantarum	**1-380**	2-668	GLI	82
		Lactobacillus plantarum	**1-375**	2-671	GLI	84

				1-351	CI	82
				Ino2C	INO	88
				Ino2G	INO	84
		Pediococcus pentosaceus	1-374	Ino1E	INO	81
			1-378	Ino1E	INO	81
		Weissella paramesentoides group	2-708	2-674	CI	97
2	GLICI	*Lactobacillus buchneri* group	1-227	2-325	GLI	85
				2-329	GLI	92
				1-300	CI	81
				1-301	CI	83
				1-302	CI	85
3	GLICI	*Pediococcus pentosaceus*	2-474	Ino1C	INO	81
				Ino1E	INO	88
5	GLICI	*Lactobacillus casei/paracasei*	1-88*	1-89	GLI	88
			2-1*	1-89	GLI	80
6	GLICI	*Lactobacillus brevis* group	1-189B	1-180A	CI	92
2	GLCI	*Lactobacillus plantarum*	**1-500**	Ino3A	INO	85
				Ino3E	INO	89
				Ino3L	INO	88
		Weissella paramesentoides group	1-502	1-510B	GL	81
3	GLCI	*Pediococcus pentosaceus*	1-534	2-263	GL	89

[1]Farms were numbered sequentially within each feeding group.

[2]H = Hay; GL = Grass or legume silage; C = Corn silage; GLI = Grass or legume silage, inoculated; CI = Corn silage, inoculated; INO = Commercial inoculant.

[3]Isolates identified with a star (*) belong to the same strain on the same row.

[4]Codes of isolates producing antibacterial activity against *Listeria ivanovii* HPB28 are in bold.

[5]Ino1 = Biotal Buchneri 500, and Ino2 = Biotal Supersile (Lallemand Animal Nutrition, Milwaukee, WI); Ino3 = 11CFT (Pioneer, Johnston, IA).

[6]Milk isolates possessing more than 80% similarity to forage isolates or commercial silage inoculants as determined by the Pearson's Correlation Analysis applied to Random Amplified Polymorphic DNA results.

Captions for the figures

Figure 1. Flow chart of farm sampling along with quantification, isolation and characterization of lactic acid bacteria.

Figure 2. Box plot of lactic acid bacteria viable counts on ABEV agar in hay (H), grass or legume silage (GL), and corn silage (C) either non-inoculated or inoculated (I) for the three sampling periods (1: Spring 2015, 2: Fall 2015, 3: Spring 2016). See Materials and Methods for composition of ABEV agar. Means with distinct capital letters were significantly different ($P < 0.05$). The lines represent the median and the ends of the box represent the 1st and 3rd quartiles. The whiskers represent 1st quartile − 1.5 × (interquartile range) and 3rd quartile + 1.5 × (interquartile range), and the dots represent the outliers.

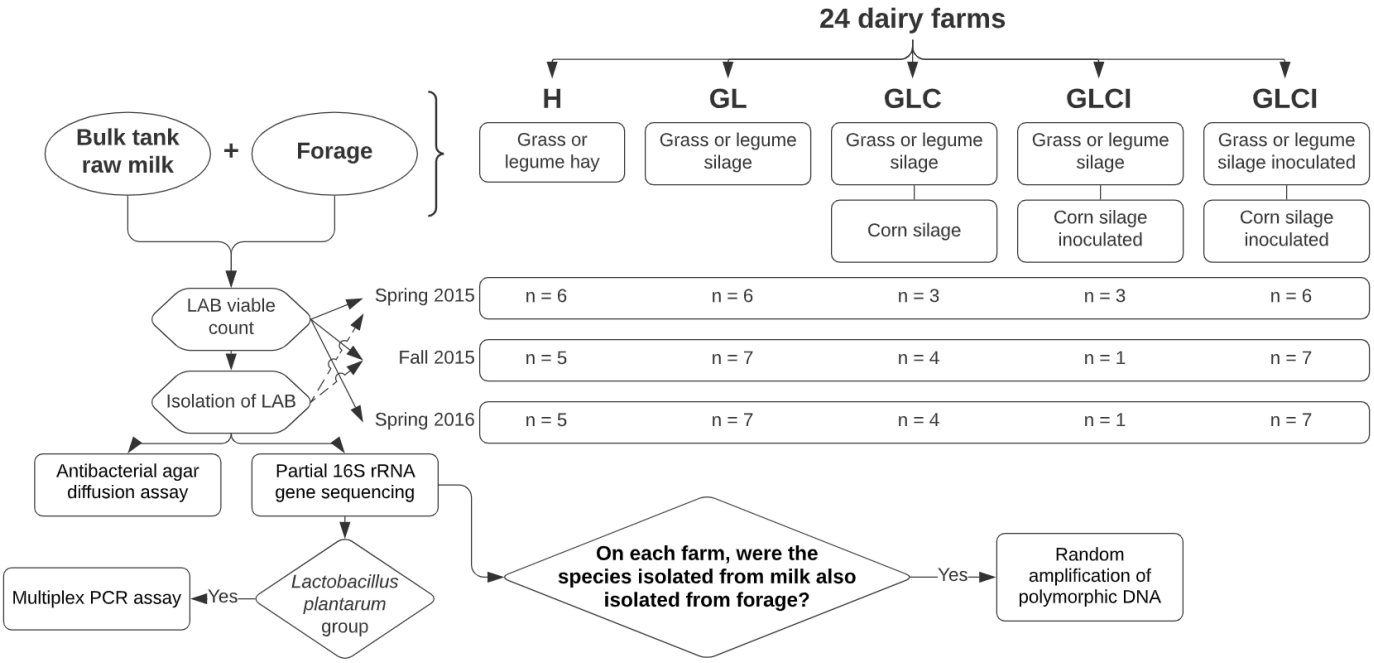
Figure 3. Relative abundance of lactic acid bacteria isolated from A) forages and B) bulk tank milk for the first (Spring 2015), and second (Fall 2015) sampling periods. H: hay; GL: grass or legume silage; C: corn silage; I: inoculated silage.

Figure 4. Comparison of lactic acid bacteria in the different forages: corn silage (white), grass or legume silage (grey) and hay (black). The extended error bar plot presented the lactic acid bacteria significantly different between forages at A) the genus level, and B) the species level. Statistical analysis was performed using STAMP software. Multiple test correction was Storey's FDR.

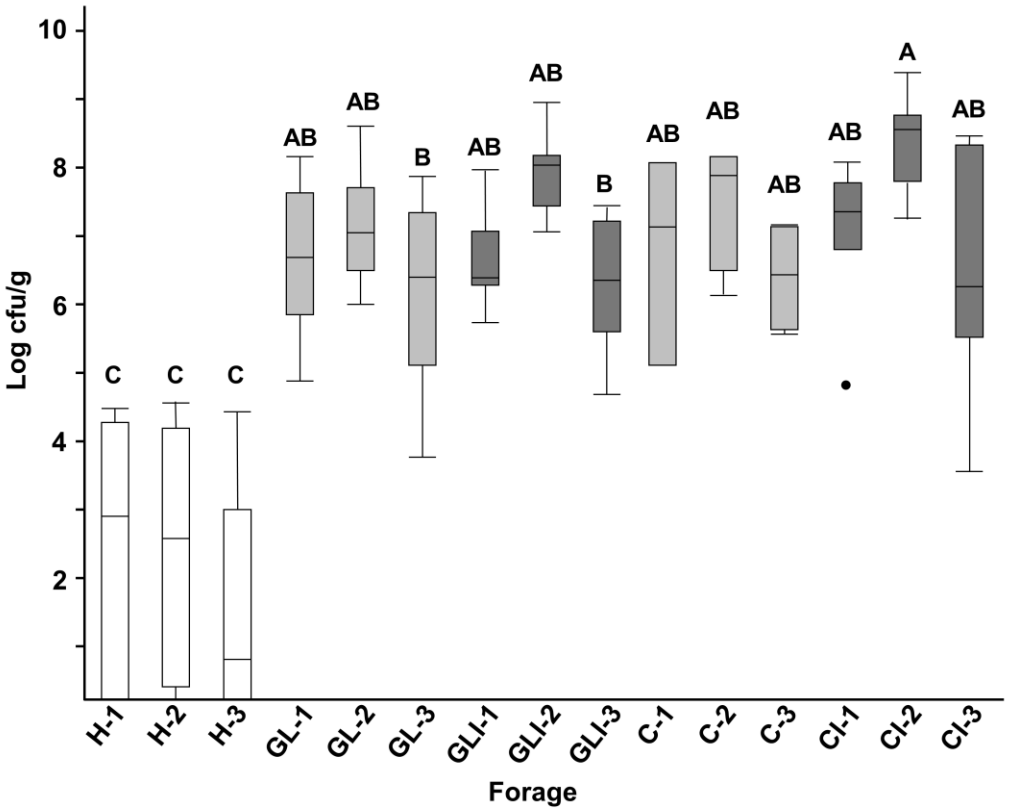
Figure 5. Effect of inoculation in grass or legume silage (GL). The extended error bar plot presented the lactic acid bacteria significantly different between inoculated (I) and non-inoculated silage at the genus level for first (S1; Spring 2015) and second (S2; Spring 2015)

709 sampling periods. Statistical analysis was performed using STAMP software. Multiple test
710 correction was Storey's FDR.

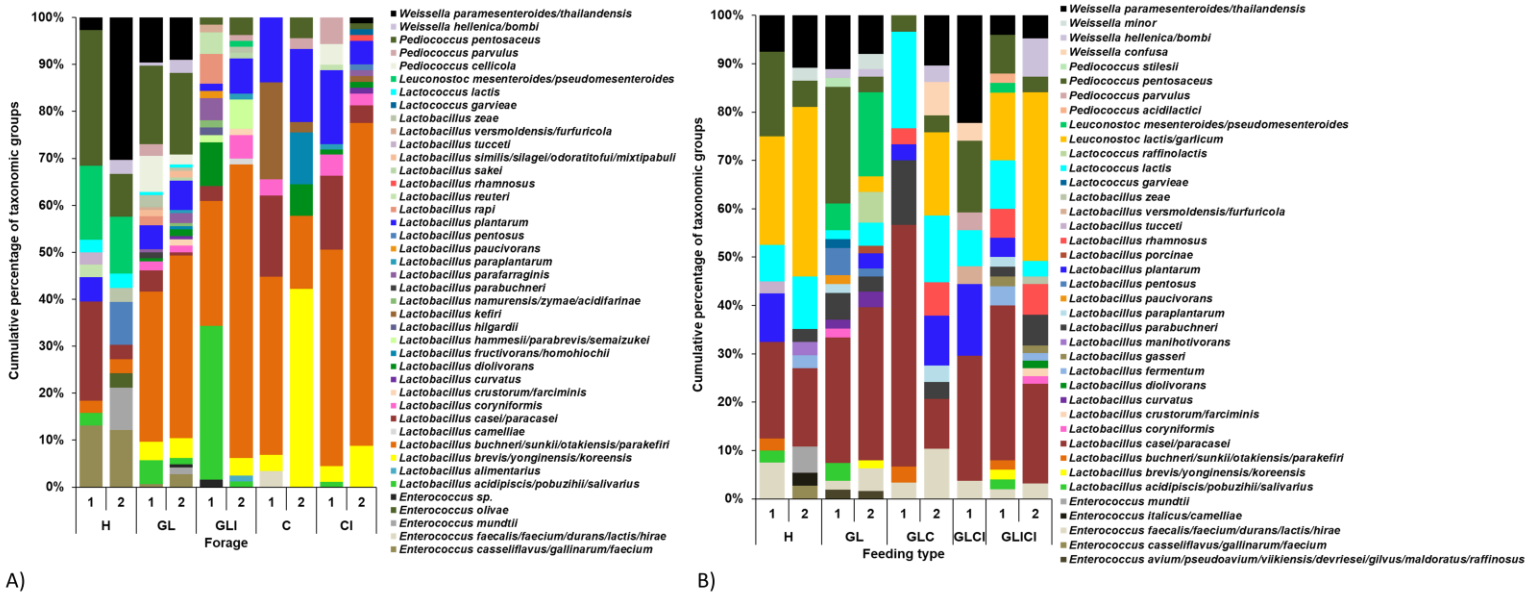
711 **Figure 6.** Box plot of lactic acid bacteria viable counts in bulk tank milk on ABEV agar
712 according to feeding types in hay (H), grass or legume silage (GL), and corn silage (C)
713 either non-inoculated or inoculated (I). See Materials and Methods for composition of
714 ABEV agar. Means with distinct capital letters were significantly different ($P < 0.05$).



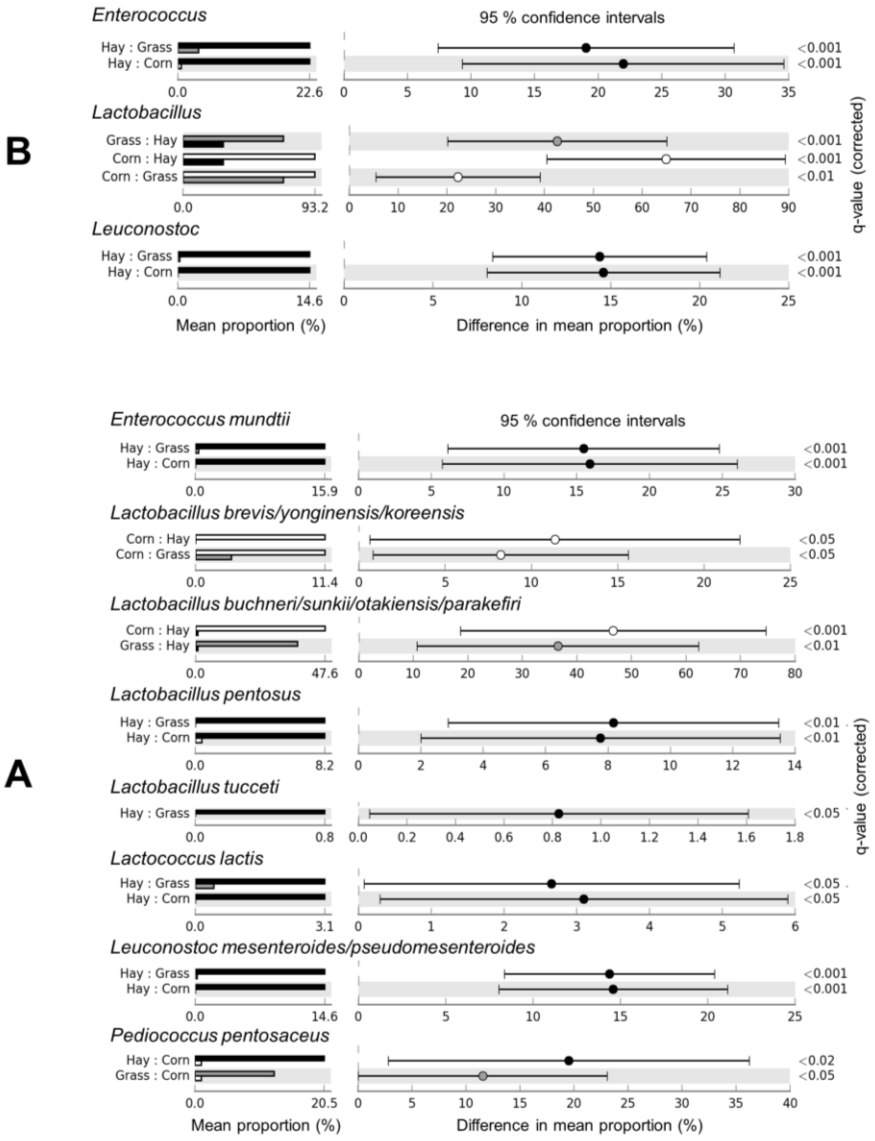
JDS.2019-17918.R1 Figure 1



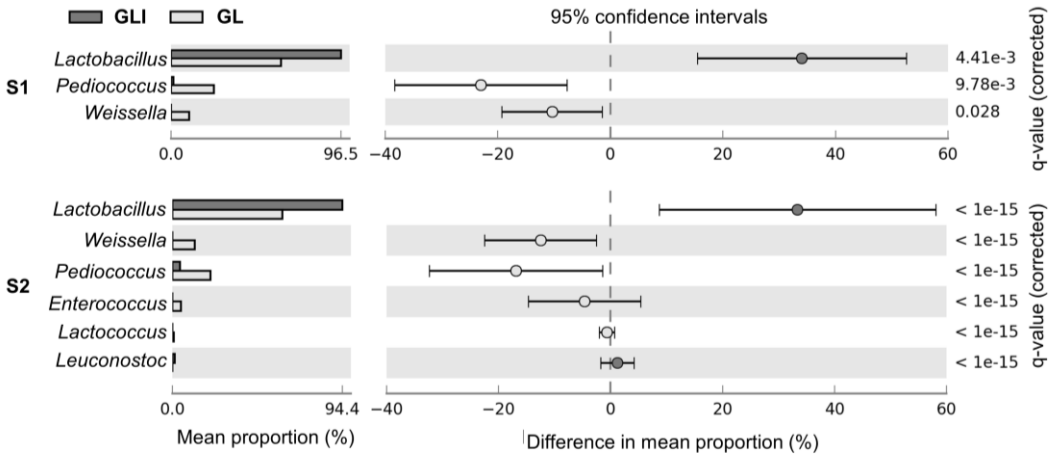
JDS.2019-17918.R1 Figure 2



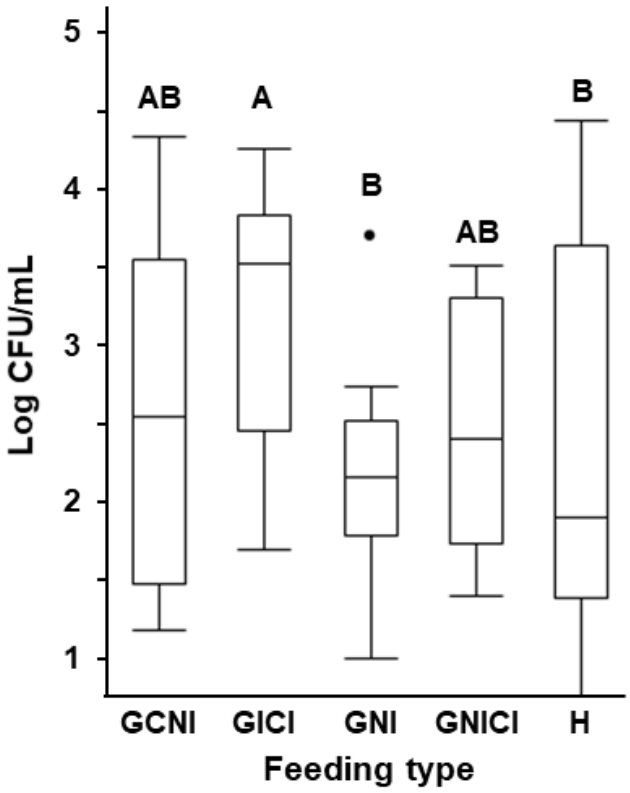
JDS.2019-17918.R1 Figure 3



JDS.2019-17918.R1 Figure 4



JDS.2019-17918.R1 Figure 5



JDS.2019-17918.R1 Figure 6