The development of lactic acid bacteria and *Lactobacillus buchneri* and their effects on the fermentation of alfalfa silage

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ABSTRACT

This study was conducted to document the development of populations of lactic acid bacteria (LAB) and *Lactobacillus buchneri* in alfalfa silage treated with various inoculants. Wilted and chopped alfalfa (45% dry matter) was treated with 1) distilled water (untreated, U), 2) *Lactobacillus buchneri* 40788 (4×10^5 cfu/g; LB), or 3) *L. buchneri* 40788 (4×10^5 cfu/g) and *Pediococcus pentosaceus* (1×10^5 cfu/g; LBPP). Forages were packed into triplicate vacuum-sealed, nylon-polyethylene bags per treatment, and ensiled for 2, 5, 45, 90, and 180 d. Viable (cfu) LAB in forage and silage were quantified by traditional plating on selective agar, and numbers of *L. buchneri* (cfu-equivalent, cfu-E) were quantified by real-time quantitative PCR. Fresh, untreated forage had 5.52 log cfu of LAB/g and 3.79 log cfu-E of *L. buchneri*/g. After 2 d of ensiling, numbers of LAB increased to >8 log cfu/g in all silages. In contrast, numbers of *L. buchneri* in U remained below 4 log cfu-E/g but reached approximately 7 log cfu-E/g in LB and LBPP. From d 5 onward, numbers of *L. buchneri* in U remained below 6 log cfu-E/g but approached 9 log cfu-E/g in LB and LBPP. The pH was lower in LBPP compared with U and LB after 2 and 5 d of ensiling, but pH was lower for U compared with LB and LBPP thereafter. Treatments LB and LBPP had more acetic acid than U at 45 d of ensiling, which coincided with detectable amounts of 1,2 propanediol. Inoculation with LBPP resulted in silage with the highest concentration of 1,2 propanediol after 180 d of ensiling. From d 45 onward, LB and LBPP silages had lower concentrations of residual water-soluble carbohydrates but had higher concentrations of ammonia-N than U. In conclusion, epiphytic *L. buchneri* can be detected in alfalfa but this population is unable to lead the silage fermentation. In contrast, when *L. buchneri* was added to silage as an inoculant, the numbers of *L. buchneri* (cfu-E) increased markedly but did not dictate fermentation until 45 d of ensiling. These findings help to explain why the re-

sponse (in increased acetic acid) from the addition of *L. buchneri* in silages is not immediate.

Key words: alfalfa silage, *Lactobacillus buchneri*, real-time quantitative PCR

INTRODUCTION

Alfalfa is a forage crop with high nutritive value and is often a major component of diets for high-producing dairy cows (Albrecht and Beauchemin, 2003). However, proper ensiling of this forage crop can be difficult because its high contents of organic acids, salts, proteins, and minerals result in a high buffering capacity (McDonald et al., 1991). Thus, the use of traditional homolactic bacterial inoculants as starters for alfalfa silage has been a recommended practice to ensure rapid fermentation during the early stages of ensiling and to minimize the loss of nutrients and DM (Muck and Kung, 1997; McAllister et al., 1998). However, the use of this type of inoculant may result in decreased aerobic stability because of insufficient production of short-chain fatty acids that are able to inhibit the growth of yeasts and molds (Weinberg et al., 1993). Thus, the heterolactic bacterium *Lactobacillus buchneri* has been used as an inoculant because it converts moderate amounts of lactic acid to acetic acid under anaerobic conditions (Driehuis et al., 1999). Acetic acid has good antifungal characteristics and has been shown to improve the aerobic stability of a variety of silages (Danner et al., 2003; Holzer et al., 2003). Combining traditional homolactic acid bacteria with *L. buchneri* has been proposed because inoculants with both types of organisms have the potential to improve the speed of fermentation and enhance the aerobic stability (Driehuis et al., 2001; Weinberg et al., 2002; Adesogan and Salawu, 2004).

Only a few reports document the effects of *L. buchneri* on the fermentation characteristics of alfalfa silage (Kung et al., 2003; Stevenson et al., 2006; Filya et al., 2007; Tyrolova and Vyborna, 2008). In addition, no studies have been conducted to explain why the expected effects of added *L. buchneri* occur only after 45 to 60 d of the fermentation process (Kleinschmit and Kung, 2006). Therefore, this experiment was conducted

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to investigate the effects of inoculating alfalfa with *L. buchneri* alone or combined with *Pediococcus pentosaceus* on the development of populations of lactic acid bacteria (**LAB**) and *L. buchneri* in alfalfa silage and their effects on fermentation end-products.

MATERIALS AND METHODS

Forage and Ensiling Conditions

Whole-plant alfalfa, cultivar Pioneer 54V46 (Pioneer Hi-Bred International, Des Moines, IA), was mowed at 1/10 bloom, wilted to 45% DM, and chopped to a theoretical length of 0.95 cm using a New Holland FP 240 pull-type forage harvester (New Holland, PA) at the University of Delaware Farm in Newark. Forage was divided into three 10-kg piles, which were assigned to one the following treatments: 1) deionized water (300 mL), untreated (**U**), 2) *Lactobacillus buchneri* NCIMB 40788 (Lallemand Animal Nutrition, Milwaukee, WI) to achieve 4×10^5 cfu/g of fresh forage (**LB**), and 3) *L. buchneri* NCIMB 40788 and *Pediococcus pentosaceus* NCIMB 12455 to achieve 4×10^5 and 1×10^5 cfu/g of fresh forage, respectively (**LBPP**; Lallemand Animal Nutrition). Microbial inoculants were plated on De Man, Rogosa, and Sharpe (**MRS**) agar (Oxoid CM0361, Unipath, Basingstoke, UK) beforehand to confirm their viability, and appropriate amounts of the inoculants were used to achieve the desired application rate. Inoculants were dissolved in 300 mL of deionized water and sprayed uniformly onto the forages under constant mixing. Approximately 400 g of forage was packed into a nylon-polyethylene bag (25.4 width $\times$ 35.6 cm height), vacuumed, and heat sealed (Doug Care Equipment Inc., Springville, CA). Triplicate experimental silos for each treatment were allowed to ensile for 2, 5, 45, 90, and 180 d at ambient temperature (20 to 25°C) in a closed barn. On each sampling date, silos were opened, their contents were thoroughly mixed, and samples were randomly obtained for the determination of microbial populations and fermentation end-products. Triplicate samples of fresh forage were collected from the initial pile, before its division into 10-kg piles for treatment, for chemical and microbiological analyses.

Chemical and Microbial Analyses

Dry matter content was determined on fresh forages and silages by drying duplicate samples in a 60°C forced-air oven for 48 h. Water extracts were prepared by combining 25 g of fresh forage or silage with 225 mL of 25% Ringer's solution (Oxoid BR0052G) and homogenizing for 1 min, as described by Kung and Ranjit (2001). The pH of the water extract was measured and

a portion of it (15 mL) was filtered through Whatman 54 filter paper (Clifton, NJ) and acidified with 100 μ L of 50% H₂SO₄ to reduce the pH of the extract to <2 before freezing (−20°C). Ammonia-N (NH₃-N) was analyzed by the phenol-hypochlorite procedure (Weatherburn, 1967) and water-soluble carbohydrates were determined as described by Nelson (1944). Water extracts from silage samples were also analyzed for VFA, 1,2-propanediol, ethanol, and lactic acid with HPLC (Shimadzu SCL-10 AVP) by Dairyland Laboratories (Arcadia, WI). The chromatograph was equipped with a refractive index detector (RID-10A), and a Bio-Rad Aminex ion exclusion HPX-87H (300 $\times$ 7.8 mm) column was used (Bio-Rad Laboratories, Hercules, CA) at 42°C. The flow rate of the mobile phase (0.015 *N* H₂SO₄ + 0.25 mM EDTA) was 0.6 mL/min.

Another portion of each water extract was filtered through a double layer of cheesecloth into 2 sets of sterile tubes for microbial analyses. One set was used for enumeration of viable LAB by pour plating on MRS agar and enumeration of yeasts and molds by pouring on malt extract agar (Oxoid CM0059) that had been acidified to pH 3.5 with 85% lactic acid (0.5% vol/vol) after autoclaving. Plates were incubated at 32°C for 48 h and numbers of colony-forming units were counted. The second set was frozen (−80°C) immediately for later extraction of bacterial DNA.

Bacterial DNA was extracted from the water extracts and served as template for real-time quantitative PCR (**qPCR**) using the primer pair specific for *L. buchneri* 186-LBF2 (5'-GAAACAGGTGCTAATACCGTATAACAACCA-3') and 316-LBR1 (5'-CGCCTTGGTAGGC-CGTTACCTTACCAACA-3'), according to Schmidt et al. (2008). Samples were run in a 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA), with the following conditions: 95°C for 30 s, 61°C for 30 s, and 56°C for 30 s for 40 cycles. The reaction mixture contained 12 μ L of SYBR Green PCR Master Mix (Applied Biosystems), 2 μ L of DNA of each silage sample, and 100 nM of each primer diluted in 5 μ L of H₂O. The amplification products were visualized using the software Sequence Detector 1.7 from the ABI Prism 7900 system (Applied Biosystems). The standard curve was constructed using Microsoft Excel 2000 (Microsoft, Redmond, WA) by plotting the number of input cells against the cycle threshold (*C_T*) value.

For quantification of *L. buchneri*, alfalfa silage (untreated) was sampled from a bag silo at the University of Delaware Dairy (Newark). *Lactobacillus buchneri* 40788 (Lallemand Animal Nutrition) was applied to this silage at rates of 1×10^3 to 1×10^8 cfu/g of silage. Isolation of DNA, PCR, and the visualization of the amplified product were performed on water extracts from the silage as described previously. Numbers of

Table 1. The DM content of alfalfa during ensiling

Time point	Treatment ¹			SE
	U	LB	LBPP	
0 d	45.35	45.35	45.35	ND ²
2 d	41.75 ^b	43.44 ^a	42.27 ^b	0.17
5 d	44.19	43.34	40.32	1.23
45 d	44.25 ^a	42.63 ^{ab}	41.34 ^b	0.75
90 d	43.77 ^a	42.82 ^a	41.72 ^b	0.30
180 d	45.62 ^a	42.22 ^b	41.57 ^b	0.37

^{a,b}Values with unlike superscript letters within a day differ ($P < 0.05$).

¹U = untreated silage; LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively).

²ND = not determined.

L. buchneri are presented as cfu-equivalents (**cfu-E**) because detected bacterial DNA can come from viable and dead bacteria.

Aerobic stability was not measured in this study because there were no detectable yeasts after 5 d of ensiling. The recovery of the DM was not measured because of the use of small-scale silos.

Statistical Analysis

All microbial data were transformed to log units and are presented on a wet weight basis. Chemical data are presented on a DM basis. Data from each day was analyzed separately as a completely randomized design by using the GLM procedure of SAS (SAS Institute, 1998). Tukey's test (Snedecor and Cochran, 1980) was used to test treatments, and significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

The DM content of silages is shown in Table 1 and although there were several significant differences among treatments, these differences were small and not biologically significant.

The population of viable LAB in untreated alfalfa increased rapidly from 5.52 log cfu/g in fresh forage to more than 9 log cfu/g of silage by d 2 of fermentation (Figure 1). Similar results in alfalfa silage have been reported by Kung et al. (2003). Subsequently, the population of LAB peaked after 5 d, declined slowly at 45 and 90 d, and was lowest (5.2 log cfu/g) after 180 d. The decrease in LAB over time was expected because low pH and lack of fermentable substrates result in death of bacteria (McDonald et al., 1991; Pahlow et al., 2003).

To our best knowledge, this study was the first to use a real-time qPCR technique to quantify the growth of

L. buchneri in alfalfa silage by creating a standard with pure cultures of *L. buchneri* grown on agar plates and applied to alfalfa silage. It is important to note that the cfu-E detected by real-time qPCR is not equal to a culturable, viable plate count on MRS agar because DNA from both live and dead *L. buchneri* contributes to the quantification. Stevenson et al. (2006) reported a decline in the presumptive population of *L. buchneri* within the first 48 h of fermentation in alfalfa silages that were inoculated with that bacterium but this was because they presented their data as a percentage of total bacterial DNA present in the silage. Because the primer pair utilized in this assay is not strain specific, naturally low levels of *L. buchneri* were found in fresh alfalfa (Figure 2). Using classic microbiological methods, Torriani et al. (1992) isolated LAB from alfalfa and over 20% of the isolates were classified as *L. buchneri*. In our study, the natural population of *L. buchneri* was never more than 5.52 log cfu-E/g of silage and it was not able to dominate the fermentation process. The population of *L. buchneri* cfu-E increased markedly after 2 d of ensiling in both inoculated silages and was almost 3 log higher compared with untreated silage and continued to increase in these silages after 5 d of ensiling. At 45, 90, and 180 d of ensiling *L. buchneri* cfu-E appeared to be the predominant bacterium in inoculated silages based on their fermentation profiles. The slow development of added *L. buchneri* most likely explains why the effects of this organism in silage are evident during the storage phase of ensiling (approximately 60 d), which is in agreement with the findings of Oude Elferink et al. (2001), in which the metabolism of *L. buchneri* in pure culture was studied.

Figure 1. The population of lactic acid bacteria (LAB) in alfalfa silages during ensiling. U = untreated silage (○); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; □); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; ◇). ^{a-c}Data points for specific days with unlike letters differ ($P < 0.05$).

Figure 2. The population of *Lactobacillus buchneri* in alfalfa silages during ensiling. U = untreated silage (○); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; □); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; ◇). ^{a-c}Data points for specific days with unlike letters differ ($P < 0.05$).

The effect of inoculation on silage pH is shown in Figure 3. Silage treated with LBPP had the fastest decrease in pH after 2 and 5 d, suggesting dominance from the addition of *P. pentosaceus*. In contrast, inoculation with LB resulted in the slowest decrease in pH compared with that of other silages, which agrees with previous findings with alfalfa (Kung et al., 2003). From d 45 on, silage pH was higher for both inoculated silages when compared with untreated silage. The concentration of lactic acid increased rapidly in all treatments (Figure 4) and was lowest for LB compared with LBPP and U at 2 d. Differences in concentrations of acetic acid (Figure 5) and 1,2 propanediol (Figure 6) between untreated and inoculated silages became more evident starting at 45 d of ensiling and continued through 180 d. These compounds are characteristic of silages inoculated with

Figure 3. The pH of alfalfa silages during ensiling. U = untreated silage (○); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; □); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; ◇). ^{a-c}Data points for specific days with unlike letters differ ($P < 0.05$).

Figure 4. The concentration of lactic acid in alfalfa silages during ensiling. U = untreated silage (○); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; □); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; ◇). ^{a,b}Data points for specific days with unlike letters differ ($P < 0.05$).

L. buchneri, although the increase in their numbers detected by qPCR was evident after just 2 d. During the initial phase of active fermentation, *L. buchneri* generates energy by fermenting simple sugars similar to other heterolactic acid bacteria, which results in an increase in their numbers. In the subsequent phase of ensiling, *L. buchneri* is induced by the low pH to ferment moderate amounts of lactic acid under anaerobic conditions, but this is a slow process and yields low amounts of energy (Oude Elferink et al., 2001); therefore, the enhancement in the concentration of the end-products from this pathway—acetic acid and 1,2-propanediol—has usually not been observed until approximately 45 to 60 d of storage (Driehuis et al., 1999; Kleinschmit and Kung, 2006). The present data on the concentrations of acetic acid and 1,2-propanediol in inoculated silages support

Figure 5. The concentration of acetic acid in alfalfa silages during ensiling. U = untreated silage (○); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; □); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; ◇). ^{a-c}Data points for specific days with unlike letters differ ($P < 0.05$).

Figure 6. The concentration of 1,2-propanediol in alfalfa silages during ensiling. U = untreated silage ($\circ$); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; $\square$); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; $\diamond$). ^{a-c}Data points for specific days with unlike letters differ ($P < 0.05$).

the existence of high populations of *L. buchneri* in those silages. For unknown reasons, the concentration of 1,2 propanediol, which is an end-product formed during the anaerobic conversion of lactic acid to acetic acid by *L. buchneri* (Oude Elferink et al., 2001), was greater from 45 d onward for LBPP compared with LB. Some naturally occurring organisms in silage can convert 1,2-propanediol to propanol and then to propionic acid (Krooneman et al., 2002). We did not detect propionic acid in our silages.

Concentrations of water-soluble carbohydrates (Figure 7) decreased after 2 d of ensiling, and differences among treatments generally reflected the production of acids. Inoculation resulted in some differences in the concentration of ethanol (data not shown), because ab-

Figure 7. The concentration of water-soluble carbohydrates (WSC) in alfalfa silages during ensiling. U = untreated silage ($\circ$); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; $\square$); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; $\diamond$). ^{a-c}Data points for specific days with unlike letters differ ($P < 0.05$).

Figure 8. The concentration of ammonia-N in alfalfa silages during ensiling. U = untreated silage ($\circ$); LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage; $\square$); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively; $\diamond$). ^{a-c}Data points for specific days with unlike letters differ ($P < 0.05$).

solute concentrations were low ($<0.30\%$) in all silages, especially during the later stages of storage. Inoculated silages had higher concentrations of $\text{NH}_3\text{-N}$ compared with control silage at 5, 45, 90, and 180 d of ensiling (Figure 8) and thus more deamination occurred in treated silages. Although adding classical homolactic acid bacteria to alfalfa has often resulted in a decrease in $\text{NH}_3\text{-N}$ (Muck, 1989), addition of the homolactic bacterium *P. pentosaceus* with *L. buchneri* in the current study could not prevent the increase apparently brought about by *L. buchneri*. Some previous studies have also reported that addition of *L. buchneri* increased $\text{NH}_3\text{-N}$ in alfalfa (Kung et al., 2003), grass (Driehuis et al., 2001), and corn and sorghum (Filya, 2003).

Yeasts and molds were only detected in silages through d 5 of ensiling (Table 2) and there were no differences among treatments. Low numbers of yeasts and molds in alfalfa silage is a common finding (Hoffman et al., 1998; Kung et al., 2003).

CONCLUSIONS

This study showed that the natural population of *L. buchneri* in alfalfa silage is unable to dominate the early fermentation process. However, inoculating silages with *L. buchneri* resulted in silages with higher levels of acetic acid and 1,2-propanediol. Accumulation of these end-products coincided with the slow but steady development of populations of *L. buchneri* in inoculated silages at 45 d of ensiling. The slow growth of added *L. buchneri* explains, in part, why its effects on fermentation are detected later in the ensiling process. Adding *P. pentosaceus* with *L. buchneri* resulted in a faster rate of fermentation during the very early stages of ensiling.

Table 2. The population of yeasts and molds (log cfu/g, wet basis) in alfalfa silages after 2 and 5 d of ensiling with 1 of 3 treatments¹

Item	Day 2				Day 5			
	U	LB	LBPP	SE	U	LB	LBPP	SE
Yeasts	4.30	2.71	5.09	0.82	3.71	3.54	3.67	0.25
Molds	3.34	3.58	4.20	0.28	3.14	3.04	3.37	0.26

¹U = untreated silage; LB = *Lactobacillus buchneri* (4×10^5 cfu/g of wet forage); LBPP = *L. buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of wet forage, respectively).

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