EPIPHYTIC LACTIC ACID BACTERIA SUCCESSION DURING THE PRE-ENSILING AND ENSILING PERIODS OF ALFALFA AND CORN[1]

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Summary

Twenty three species and 306 strains of epiphytic lactic acid bacteria (LAB) were found for two cuttings of alfalfa, each harvested at three stages of maturity, and three whole-plant corn hybrids. Epiphytic LAB counts were low and variable on the standing crops, particularly on alfalfa. Wilting increased LAB numbers slightly for alfalfa, but the chopping process increased counts dramatically for both crops. *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Enterococcus faecium*, and *E. faecalis* were predominant on both standing crops. The changes in LAB caused by wilting or chopping were mainly proportional changes in the four dominant species. Once the crops were ensiled, total LAB counts increased rapidly, reached a maximum within 1 day, and then declined after 7 days of fermentation. *Enterococcus* species decreased sharply or disappeared during the early fermentation. The species most prominent through day 7 were *L. plantarum* and *P. pentosaceus*. After 7 days, more species, i.e., *L. homohiochii*, *L. brevis*, and *L. gasseri*, joined the succession and became prevalent, depending on the crop.

Only two of the six alfalfa silages were adequately preserved, whereas all three corn hybrids fermented normally. No relationship was found between epiphytic LAB numbers or species and adequacy of fermentation. Neither were pH changes during the fermentation explained by the epiphytic LAB count or population succession. Rather, the well-fermented alfalfa silages were those ensiled at a high dry matter (DM) content (> 36%) and low buffering capacity (< 450 meq/kg of DM). Only a few of the LAB strains were consistently present, thus indicating that populations changed during fermentation to fit an ecological niche.

(Key Words: Epiphytic Lactic Acid Bacteria, Alfalfa, Corn, Silage.)

Introduction

Epiphytic LAB (i.e., lactobacilli, lactococci, enterococci, pediococci, streptococci, and leuconostocs) play a major role in silage fermentation. Their absolute and relative numbers might be important in predicting fermentation adequacy and in deciding whether or not to apply a silage bacterial inoculant. Epiphytic LAB counts are usually low and variable on silage crops, and LAB counts usually increase coincident to the chopping process.

Only limited information is available on epiphytic LAB succession during the ensiling of alfalfa and corn, the two major silage crops in the United States. Our objective was to investigate the epiphytic LAB succession during the pre-ensiling and ensiling periods for

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alfalfa and whole-plant corn.

Experimental Procedures

A second-year stand of Cody alfalfa was harvested at the 2nd and 4th cuttings and at the late-bud, 10% bloom, and 50% bloom stages of maturity within each cutting in the 1989 growing season. Following mowing, the alfalfa was wilted in the windrow for 5 to 6 hr prior to chopping. Three corn hybrids (Pioneer 3377, 3379, and 3389) were grown under irrigation in 1989 and directly chopped at the 2/3 milk line stage of kernel maturity. Samples were aseptically taken from standing crops, windrowed alfalfa prior to chopping, and chopped forages. The forages were ensiled in laboratory silos, stored, and sampled as described for the alfalfa and corn silages on pages 118 and 124, respectively, of this report.

Isolation of lactic acid bacteria. Each sample was homogenized with phosphate buffer (0.7 mM, pH 7.0) and serially diluted in the same buffer. Lactobacilli, pediococci, and leuconostocs were counted on Rogosa SL medium (Difco #480). Plates were overlaid with the same medium and incubated at 35 C for 2 days. Streptococci were counted on Slanetz & Bartley medium (Oxoid CM 377) following incubation at 35 C for 2 days.

Approximately 30 colonies from Rogosa SL medium and 20 from the Slanetz & Bartley medium were picked at random. Each colony was purified twice on Lactobacilli MRS medium (Difco #881) containing 1.5% agar (Difco #140). The pure cultures were grown in Lactobacilli MRS broth at 35 C for 20 hr, mixed with sterile glycerol in a ratio of 2:3, and stored as stock cultures at -22 C for further examination.

Morphology, soluble proteins, and biochemical tests. The cultures were examined for Gram reaction, morphology, and catalase production. Gram-positive, catalase-negative rods and cocci were studied further. For the isolates from Slanetz & Bartley medium, only Gram-positive, catalase-negative cocci were tested further.

Soluble cellular proteins from the pure cultures were analyzed by polyacrylamide gel electrophoresis, using a commercial *Pediococcus* strain as a control.

Cultures with different soluble protein patterns underwent final tests for lactic acid configuration, growth at 15 and 45 C in Lactobacilli MRS broth, gas production from glucose, fermentation of 32 carbohydrates, hydrolysis of esculin, and deamination of arginine.

Identification and counts of lactic acid bacteria. The identity of each isolate was determined by a dBASE III computer program that compared the tested characteristics of the LAB to respective phenotypic information in Bergey's Manual of Systematic Bacteriology. The percentage of each species or strain was calculated from the total LAB counts for each sample. Total LAB count was defined as count from Rogosa SL medium plus streptococci count from the Slanetz & Bartley medium.

Results and Discussion

LAB species succession during the preensiling and ensiling periods. Twenty three species and 306 strains of LAB were identified out of 3,400 colonies isolated from the crops and their silages (Table 1). Of the total LAB colonies isolated, more than 90% were homofermentative (produce only lactic acid). *Lactobacillus plantarum* was the predominant species, followed by *Pediococcus pentosaceus*. *Enterococcus faecium*, *E. faecalis*, *L. brevis*, and *L. homohiochii* contributed about 33% of the isolates. Remaining species were each 1% or less of the total. More LAB species were recovered from alfalfa than corn, although two of the species on corn (*L. casei* and *Streptococcus bovis*) were absent on alfalfa. Heterofermentive LAB were more numerous on alfalfa than corn, whereas the proportion of homofermentative *L. plantarum* was higher on corn. Only a few strains of *L. plantarum* and

P. pentosaceus were the same between the crops.

The LAB species or strains and their percentages of the total isolates at each period varied considerably among silage and showed profound changes as fermentation progressed (Tables 2 and 3). The second cutting, 10% bloom, standing alfalfa was inhabited only by heterofermentative *L. brevis* and *Leuconostoc mesenteroides* subsp. *mesenteroides*. The second cutting, 50% bloom and all the fourth cutting, standing alfalfas were dominated by homofermentative LAB species, *L. plantarum*, *P. pentosaceus*, and *E. faecalis*. Homofermentative LAB species predominated on all three standing corn hybrids. *P. pentosaceus* comprised 89 and 60% of the total isolates on 3389 and 3377, respectively, and *E. faecalis* comprising 96% of the isolates on 3379.

Table 1. Epiphytic LAB Species Isolated from the Six Alfalfa and Three Corn Hybrids during the Pre-ensiling and Ensiling Periods

	Total isolates		Alfalfa isolates		Corn isolates	
Species[1,2]	No. of strains	Percent-age	No. of strains	Percent-age	No. of strains	Percent-age
Lactobacillus plantarum	135	41.86	74	39.99	61	44.88
L. acidophilus	2	.02	2	.03	--	--
L. curvatus	2	.04	2	.07	--	--
L. gasseri	3	1.06	3	1.72	--	--
L. helveticus	3	.37	1	.26	2	.53
L. homohiochii	20	4.65	18	6.16	2	2.22
L. brevis	29	6.74	24	7.00	5	6.32
L. buchneri	3	.27	3	.44	--	--
L. viridescens	4	1.15	4	1.86	--	--
Leuconostoc mesenteroides subsp. *mesenteroides*	4	.45	3	.57	1	.26
Pediococcus pentosaceus	49	20.25	34	19.88	15	20.84
P. acidilactici	2	.10	2	.17	--	--
Enterococcus faecium	24	11.28	12	9.87	12	13.57
E. faecalis	12	9.63	10	10.18	2	8.74
Lactococcus lactis	5	.99	4	.57	1	.26

[1]Species having only one strain isolated from alfalfa were: *L. coryniformis* (subsp. *coryniformis*), *L. maltaromicus*, *L. confusus*, *L. collinoides*, *L. hilgardii*, and P. *inopinaius*.
[2]Species having only one strain isolated from corn were: *L. casei* (subsp. *casei*), *L. confusus*, and *Streptococcus bovis*.

Table 2. Epiphytic LAB Sucession during the Pre-ensiling and Ensiling Periods of the Second and Fourth Cutting Alfalfas

Species[1,2]	Cutting and stage of maturity	No. of strains	Pre-ensiling			Ensiling, days				
			ST[2]	WR[4]	CH[5]	1	3	7	42	90
			---------- percent of the total isolates ----------							
	Second									
L. plantarum	Late-bud	17	--	--	43	46	92	84	80	100
	10% bloom	12	--	--	5	48	66	72	89	100
	50% bloom	9	8	--	--	8	22	29	63	28
P. pentosaceus	Late-bud	5	--	--	14	23	--	16	--	--
	10% bloom	5	--	--	10	34	31	6	--	--
	50% bloom	3	23	--	11	76	75	61	15	--
Leu. mesenteroides subsp. *mesenteroides*	Late-bud	1	--	--	43	--	--	--	--	--
	10% bloom	2	33	--	5	--	--	--	--	--
L. brevis	10% bloom	3	67	--	10	--	3	--	5	--
	50% bloom	3	--	--	18	--	--	10	11	4
L. viridescens	10% bloom	2	--	--	65	18	--	--	--	--
L. homohiochii	Late-bud	4	--	--	--	--	--	--	20	--
	10% bloom	4	--	--	5	--	--	22	6	--
E. faecalis	50% bloom	3	69	--	71	--	--	--	--	--
L. gasseri	50% bloom	3	--	--	--	--	--	--	11	68
	Fourth									
L. plantarum	Late-bud	9	4	3	29	46	6	11	--	--
	10% bloom	14	1	--	53	29	76	91	20	47
	50% bloom	12	100	19	96	96	93	45	24	41
P. pentosaceus	Late-bud	9	62	--	37	54	76	63	4	--
	10% bloom	6	2	1	18	57	--	9	41	7
	50% bloom	7	--	--	4	4	--	50	32	19
E. faecium	Late-bud	7	21	86	26	--	--	18	2	3
	10% bloom	3	64	70	16	--	--	--	--	--
E. faecalis	10% bloom	3	32	29	--	--	--	--	--	--
	50% bloom	3	--	76	--	--	--	--	--	--
L. brevis	Late-bud	4	8	--	--	--	11	4	42	52
	50% bloom	7	--	1	--	--	--	5	16	29
L. homohiochii	Late-bud	5	--	--	8	--	5	4	50	41
	10% bloom	4	--	--	--	--	24	--	30	33

[1]Species contributing less than 14 percent of the total isolates at various stages of maturity and times during the pre-ensiling and ensiling periods of the second cutting alfalfa were: *L. confusus* (late-bud); *L. brevis* (late-bud); *L viridescens* (late-bud and 50% bloom); *E. faecium* (50% bloom); and *P. acidilactici* (50% bloom).

[2]Species contributing less than 14 percent of the total isolates at various stages of maturity and times during the pre-ensiling and ensiling periods of the fourth cutting alfalfa were: *L. lactis* (late-bud); *L. brevis* (10% bloom); *L. homohiochii* (50% bloom); *L. coryniformis*, subsp. *coryniformis* (late-bud); *P. inopinatus* (10% bloom); *L. acidophilus* (10% bloom); *L. collinoides* (10% bloom); *L. curvatus* (10% bloom); *L. hilgardii* (50% bloom); *L. maltaromicus* (50% bloom); *L. helveticus* (50% bloom); *L. buchneri* (50% bloom); and *P. acidilactici* (50% bloom).

[3]Standing alfalfa. [4]Windrow alfalfa. [5]Chopped alfalfa.

Wilting in the windrow caused a dramatic increase in *E. faecalis* and *E. faecium* counts on the fourth cutting alfalfa at all three stages of maturity. The chopping process changed the distribution of the main species (*L. plantarum*, *P. pentosaceus*, *E. faecalis*, *E. faecium*, and *L. brevis*) and caused recovery of a few more species (*L. homohiochii*, *L. viridescens*, and *P. inopinatus*). *E. faecalis* disappeared from the fourth cutting alfalfas and all three corn hybrids. *L. plantarum* numbers increased on all chopped alfalfa and corn. The proportions of *L. brevis* and *Leu. mesenteroides* subsp. *mesenteroides* on the second cutting, 10% bloom, standing alfalfa were greatly decreased by the chopping process, and *L. viridescens* became predominant. Also, some homofermentative species (i.e., *L. plantarum*, *P. pentosaceus* and *L. homohoichii*) were more numerous after chopping.

Dramatic changes in epiphytic LAB occurred during the ensiling period. *E. faecalis* disappeared within 1 day. *E. faecium* decreased and vanished within 3 days, except for the fourth cutting, late-bud alfalfa. *L. plantarum* predominated throughout the ensiling period of the second cutting, 10 and 50% bloom alfalfa silages and, with *P. pentosaceus*, dominated the other silages through day 7. After 7 days of fermentation, *L. brevis* and *L. homohiochii* increased and, along with *L. plantarum* and *P. pentosaceus*, became prevalent in several silages at 42 days. At the end of the ensiling period, two of the silages had *L. brevis* and one had *L. gasseri* as predominant LAB species. The other six were dominated by *L. plantarum*. *P. pentosaceus* disappeared from seven of the nine silages and was minor in the other two. None of the LAB strains remained predominant throughout the ensiling period in either alfalfa or corn.

Fermentation changes during the ensiling period (data not shown). For the second cutting alfalfa ensiled at 10 and 50% bloom, pH declined rapidly to 4.9 on the second day and dropped an additional .2 to .4 pH units by the end of the ensiling period. For the other four alfalfas, pH decreased to about 5.0 at 1 day but did not decline further. For the corn silages, the minimum pH was reached on day 3 of fermentation for hybrids 3377 and 3389; however, 3379 silage did not reach its lowest pH until day 7.

Lactic acid levels varied with both fermentation time and crop. For the second cutting, 10 and 50% bloom alfalfas, lactic acid increased rapidly during the first 3 days of fermentation and remained high (5.5 to 7.5% of the silage DM). For the other four alfalfa silages, lactic acid increased initially, then declined to only 2 to 3% of the silage DM at the end of the ensiling period. Lactic acid content in the second cutting, late-bud alfalfa silage remained almost constant during the final 87 days of fermentation, but in the fourth cutting, 50% bloom silage, lactic acid decreased from 8.5% of the silage DM on day 42 to only 2.5% on day 90. In all three corn silages, lactic acid concentration continued to increase throughout the ensiling period.

Ammonia-nitrogen increased throughout the ensiling period for all six alfalfa silages. However, only second cutting alfalfa ensiled at either 10 or 50% bloom had low enough ammonia-nitrogen values at the end of the fermentation to be acceptable (less than 12% of the total silage nitrogen). Before ensiling, these two alfalfas had the highest DM contents and lowest buffering capacities (Table 4). For all three corn hybrids, ammonia-nitrogen was produced slowly during fermentation and was less than 12% of the total nitrogen at 120 days.

Conclusions. These results indicate that the numbers and species of epiphytic LAB varied between alfalfa and corn and during the pre-ensiling and ensiling periods. Because knowing the numbers and species of LAB did not predict the outcome of the fermentations, further characterization of the epiphytic LAB strains and chemical composition of the ensiled crops, particularly their water soluble carbohydrate profiles and buffering capacity, is necessary.

Table 3. Epiphytic LAB Succession during the Pre-ensiling and Ensiling Periods of the Three Corn Hybrids

Species[1]	Hybrid	No. of strains	Pre-ensiling		Ensiling, days						
			ST[2]	CH[3]	.25	.5	1	3	7	42	120
			---------- percent of the total isolates ----------								
L. plantarum	3377	23	12	48	61	79	65	89	76	3	100
	3379	23	1	28	12	8	100	16	90	27	83
	3389	19	11	13	30	63	45	83	80	71	15
P. pentosaceus	3377	7	60	3	35	21	35	11	24	10	--
	3379	6	--	--	3	37	--	84	10	73	--
	3389	4	89	15	10	17	55	6	10	22	--
E. faecium	3377	2	24	49	--	--	--	--	--	--	--
	3379	7	3	72	49	43	--	--	--	--	--
	3389	2	--	31	--	--	--	7	--	--	--
E. faecalis	3379	1	96	--	36	12	--	--	--	--	--
L. homohiochii	3377	1	--	--	--	--	--	--	--	56	--
L. brevis	3377	2	--	--	--	--	--	--	--	17	--
	3389	3	--	--	50	20	--	4	7	7	85
L. confusus	3379	1	--	--	--	--	--	--	--	--	17
L. lactis	3389	1	--	36	--	--	--	--	--	--	--

[1]Species contributing less than 14 percent of the total isolates of the three hybrids at various times during the pre-ensiling and ensiling periods were: *E. faecalis* (3377); *L. casei*, subsp. *casei* (3377); *L. homohiochii* (3389); *L. helveticus* (3377); *lue. mesenteroides*, subsp. *mesenteroides* (3389); and *S. bovis* (3389).
[2]Standing corn. [3]Chopped corn.

Table 4. Dry Matter Content and Buffering Capacity of the Six Alfalfas

Cutting and item	Late-bud	10% bloom	50% bloom
Second			
Dry matter, %	31.2	36.7	44.4
Buffering capacity, meq/kg of DM	557	447	393
Fourth			
Dry matter, %	25.3	27.6	25.5
Buffering capacity, meq/kg of DM	559	492	468