Applied and Environmental Microbiology, Aug. 1998, p. 2982–2987 Vol. 64, No. 8
0099-2240/98/$04.00+0
Copyright © 1998, American Society for Microbiology. All Rights Reserved.

Influence of *Lactobacillus* spp. from an Inoculant and of *Weissella* and *Leuconostoc* spp. from Forage Crops on Silage Fermentation

YIMIN CAI,[1]* YOSHIMI BENNO,[1] MASUHIRO OGAWA,[2] SADAHIRO OHMOMO,[3]
SUMIO KUMAI,[4] AND TAKASHI NAKASE[1]

Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Wako, Saitama 351-0198,[1] Kyusyu National Agricultural Experiment Station, Nishigoshi, Kumamoto 861-1192,[2] National Institute of Animal Industry, Tsukuba, Ibaraki 305-0901,[3] and College of Agriculture, Ehime University, Matsuyama, Ehime 790-8566,[4] Japan

Received 17 February 1998/Accepted 26 May 1998

***Lactobacillus* spp. from an inoculant and *Weissella* and *Leuconostoc* spp. from forage crops were characterized, and their influence on silage fermentation was studied. Forty-two lactic acid-producing cocci were obtained from forage crops and grasses. All isolates were gram-positive, catalase-negative cocci that produced gas from glucose, and produced more than 90% of their lactate in the D-isomer form. These isolates were divided into groups A and B by sugar fermentation patterns. Two representative strains from the two groups, FG 5 and FG 13, were assigned to the species *Weissella paramesenteroides* and *Leuconostoc pseudomesenteroides*, respectively, on the basis of DNA-DNA relatedness. Strains FG 5, FG 13, and SL 1 (*Lactobacillus casei*), isolated from a commercial inoculant, were used as additives to alfalfa and Italian ryegrass silage preparations. Lactic acid bacterium counts were higher in all additive-treated silages than in the control silage at an early stage of ensiling. During silage fermentation, inoculation with SL 1 more effectively inhibited the growth of aerobic bacteria and clostridia than inoculation with strain FG 5 or FG 13. SL 1-treated silages stored well. However, the control and FG 5- and FG 13-treated silages had a significantly ($P < 0.05$) higher pH and butyric acid and ammonia nitrogen contents and significantly ($P < 0.05$) lower lactate content than SL 1-treated silage. Compared with the control silage, SL 1 treatments reduced the proportion of D-(−)-lactic acid, gas production, and dry matter loss in two kinds of silage, but the FG 5 and FG 13 treatments gave similar values in alfalfa silages and higher values ($P < 0.05$) in Italian ryegrass silage. The results confirmed that heterofermentative strains of *W. paramesenteroides* FG 5 and *L. pseudomesenteroides* FG 13 did not improve silage quality and may cause some fermentation loss.**

Silage is now the most common preserved cattle feed in many countries, including Japan. It is well established that lactic acid bacteria (LAB) play an important role in silage fermentation. Epiphytic microflora, the microorganisms naturally present on forage crops, are responsible for silage fermentation and also influence silage quality (3, 11, 15). Lactobacilli and lactic acid-producing cocci, e.g., leuconostocs, lactococci, streptococci, pediococci, and *Weissella* species, are major components of the microbial flora in various types of forage crops (3). Stirling and Whittenbury (21) reported that leuconostocs were the most numerous and widely distributed on forages and that lactobacilli occurred mostly on grasses. Cai et al. (3) examined a large number of forage crops and grasses and also found that the predominant LAB were lactic acid-producing cocci and that lactobacilli were the least numerous and mostly homofermentative. Ruser (17) found that although all LAB groups were present in chopped-maize samples, homofermentative lactobacilli and heterofermentative leuconostocs were present in the highest numbers.

In order to improve silage quality, many LAB-containing biological additives have been developed and are currently available (13, 20, 25). These inoculants may inhibit the growth of harmful bacteria and enhance lactic acid fermentation during ensiling periods. The epiphytic LAB influence the effectiveness of silage inoculants because the introduced bacteria must compete with these LAB (12). Therefore, the LAB species and their characteristics in the silage environment require further study. However, while an increasing number of studies have reported positive benefits from using some bacterial inoculants as silage additives, relatively few have reported the effect of epiphytic LAB, especially *Leuconostoc* and *Weissella* species, on silage fermentation. In the present study, the characterization of *Leuconostoc* and *Weissella* species isolated from forage crops and their influence on silage fermentation were examined.

MATERIALS AND METHODS

Materials. Corn (*Zea mays*) at the dough-ripe stage, sorghum (*Sorghum bicolor*) at the milk-dough stage, alfalfa (*Medicago sativa*) at the flowering stage, Italian ryegrass (*Lolium multiflorum*) at the heading stage, and Guinea grass (*Panicum maximum*) at the flowering stage were obtained from an experimental field at the National Grassland Research Institute (Tochigi, Japan) from July 1993 to December 1995. For the counts of viable microorganisms in the material, see Table 2.

Strains. A total of fifty-four strains (shown in Table 1) were isolated from forage crops and grasses, using GYP agar (10) and Lactobacilli MRS medium (Difco Laboratories, Detroit, Mich.) containing 1.5% agar incubated at 30°C for 2 days under anaerobic conditions. Each colony was purified twice by streaking on MRS agar. The pure cultures were grown on MRS agar at 30°C for 24 h, resuspended in a solution of nutrient broth (Difco Laboratories) and dimethyl sulfoxide at a ratio of 9:1, and stored as stock cultures at −80°C for further examination. As shown in Table 1, the *Leuconostoc* and *Weissella* type strains

* Corresponding author. Mailing address: Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-0198, Japan. Phone: (81) 48-467-9562. Fax: (81) 48-462-4619. E-mail: cai@ulmus.riken.go.jp.

TABLE 1. Strains used in this study

Strain(s)	Source
Isolates (n^a = 42)	Forage crop
Group A	
FG 5, FG 7, FG 22 to 24, FG 14, FG 15, FG 18, NG 8 to 10, NG 22 to 26, NG 33	
Group B	
FG 13, FG 16, FG 19, FG 20, FG 26 to 30, FG 42, FG 44, FG 55 to 57, NG 6, NG 7, NG 12 to 14, NG 16 to 19, NG 28, NG 29	
Type strains[b] (n = 11)	
Leuconostoc carnosum JCM 9698^T	Vacuum-packaged beef
Leuconostoc citreum JCM 9698^T	Food, blood
Leuconostoc fallax JCM 9694^T	Pickled cabbage
Leuconostoc gelidum JCM 9697^T	Vacuum-packaged beef
Leuconostoc lactis JCM 6123^T	Milk
Leuconostoc mesenteroides subsp. *cremoris* JCM 9898^T	Dried starter powder
Leuconostoc mesenteroides subsp. *dextranicum* JCM 9700^T	Kefir
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	Olives, silage, milk, blood
Leuconostoc pseudomesenteroides JCM 9696^T	Dairy food, blood
Weissella hellenica JCM 10103^T	Fermented sausage
Weissella paramesenteroides JCM 9890^T	Unknown

[a] n, number of strains tested.
[b] JCM, Japan Collection of Microorganisms.

were obtained from the Japan Collection of Microorganisms (JCM), The Physical and Chemical Research Institute, Wako, Saitama, Japan.

Microbiological analysis. The numbers of microorganisms were measured by the plate count method (26). Forage crops and grass samples (10 g) were shaken well with 90 ml of sterilized distilled water, and 10^{-1} and 10^{-8} serial dilutions were made in 0.85% sodium chloride solution. LAB were counted on a plate agar containing bromocresol purple (Nissui-seiyaku Ltd., Tokyo, Japan) and GYP-$CaCO_3$ agar (10) after incubation in an anaerobic box (TE-HER Hard Anaerobox, ANX-1; Hirosawa Ltd., Tokyo, Japan) at 30°C for 2 or 3 days. LAB were detected by a yellowish colony and a clear zone due to dissolving $CaCO_3$. Their physiological properties were then determined by the methods of Kozaki et al. (10). Clostridia were counted on EG agar (Eiken-kagaku Ltd., Tokyo, Japan) and BL agar (Nissui-seiyaku Ltd.) after incubation in an anaerobic box at 30°C for 3 to 5 days. They were detected by the methods of Ueno (24). Aerobic bacteria were counted on nutrient agar (Nissui-seiyaku Ltd.), and mold and yeasts were counted on potato dextrose agar (Nissui-seiyaku Ltd.). The agar plates were incubated at 30°C for 2 to 4 days. Colonies were counted as viable numbers of microorganisms (in CFU per gram of fresh matter [FM]).

Morphological, physiological, and biochemical tests. Morphological characteristics and Gram staining of LAB were examined after 24 h of incubation on MRS agar. Catalase activity, nitrite reduction, and gas production from glucose were determined by the methods of Kozaki et al. (10). Growth at different temperatures was observed in MRS broth after incubation at 10 and 15°C for 14 days and at 45 and 50°C for 7 days. To test for sugar fermentation, the strains were cultivated on liver broth (LB) at 30°C for 24 h, and the broth was then diluted 10-fold with sterile saline solution. Sugar fermentation patterns were examined by using a semiautomatic system for bacterial identification (1). Twenty-four sugars were tested in LB basal medium containing 0.5% (wt/vol) sugar. The LB basal medium was composed of 1,000 ml of 0.55% Bacto Liver (Difco) solution, 10 g of Proteose Peptone 3 (Difco), 5 g of Trypticase (BBL), 3 g of yeast extract (Difco), 1 g of Tween 80, 5 ml of salts solution, and 0.2 g of L-cysteine $HCl \cdot H_2O$; the pH was adjusted to 7.2. The salts solution contained 10 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.3 g of $MnSO_4$, and 250 ml of distilled water. The isomers of lactate formed from glucose were determined enzymatically by using reagents obtained from Boehringer GmbH, Mannheim, Germany.

DNA base composition and DNA-DNA hybridization. DNA was extracted from cells harvested from MRS broth incubated for 8 h at 30°C and purified by the procedure of Saito and Miura (18). DNA base composition was determined by the method of Tamaoka and Komagata (22) by high-performance liquid chromatography following the enzymatic digestion of DNA to deoxyribonucleosides. The equimolar mixture of four deoxyribonucleotides in a Yamasa GC Kit (Yamasa Shoyu Co., Ltd., Choshi, Japan) was used as the quantitative standard. DNA-DNA relatedness was determined by the method of Ezaki et al. (8) by using photobiotin and microplates.

Laboratory silage preparation and chemical analysis. Alfalfa and Italian ryegrass were harvested at the flowering stage in August 1996. Silage was prepared by using a small-scale system of silage fermentation (23). The strains FG 5, FG 13, and SL 1 isolated from a commercial inoculant, Snow Lact-L (*Lactobacillus casei*; Brand Seed Ltd., Sapporo, Japan), were used. MRS broth was inoculated with strains FG 5, FG 13, and SL 1 and incubated overnight. After incubation, the optical density at 700 nm of the suspension was adjusted with sterile 0.85% NaCl solution to 0.42. The inoculum size of LAB was 1 ml of suspension per kg of fresh matter (FM). Approximately 100-g portions of forage material, chopped into about 20-mm lengths, were packed into plastic film bags (Hiryu KN type, 180 by 260 cm; Asahikasei), and the bags were sealed with a vacuum sealer (BH 950; Matsushita). The silage treatments were designed as follows: (i) untreated control, (ii) FG 5, (iii) FG 13, and (iv) SL 1. The film bag silos were kept at 25°C, and three replicates per treatment were used for microbiological and chemical analysis.

The chemical composition of the forage crops and silages was determined by conventional methods (14). The dry matter (DM) content of the fresh forage was determined by oven drying at 70°C for 48 h, whereas that of the silages was determined by the removal of water, using toluene distillation with ethanol correction (5). The WSC and organic acid contents were measured by high-performance liquid chromatography (16). The ammonia nitrogen and lactic acid isomer contents were determined by enzymatic analysis, specifically, the F-Kit UV method (Boehringer GmbH). Gas production and DM loss were determined by the methods of Cai et al. (4).

Statistical analysis. Data on chemical composition of silage ensiled for 30 days were analyzed by analysis of variance, and the significance of differences among means was tested by the multiple-range test (6).

RESULTS

Counts of microorganisms. The counts of viable microorganisms in fresh forage crops are shown in Table 2. Overall, there were 10^5 to 10^6 aerobic bacteria, 10^2 to 10^5 mold and yeast, 10^3 to 10^5 enterococci and leuconostocs, $\leq 10^3$ lactobacilli, pediococci, and clostridia in each of the five forage crops (counts in CFU gram^{-1}).

TABLE 2. Microbiological analysis of the fresh forage crops

Forage	Mean (SD) count (log CFU g of FM^{-1}) of viable microorganisms						
	Lactobacilli	Enterococci	Leuconostocs	Pediococci	Clostridia	Aerobic bacteria	Mold and yeast
Corn	3.8 (0.4)	5.0 (1.1)	5.7 (0.8)	3.1 (0.6)	ND[a]	6.0 (0.5)	3.5 (0.2)
Sorghum	3.0 (0.6)	3.5 (0.8)	4.8 (1.2)	ND	3.5 (0.3)	6.8 (0.6)	4.3 (0.5)
Alfalfa	ND	3.2 (0.5)	5.2 (0.7)	3.6 (0.7)	3.3 (0.5)	5.3 (0.5)	2.8 (0.3)
Italian ryegrass	ND	4.0 (1.3)	3.6 (0.3)	ND	ND	5.9 (0.7)	4.2 (0.5)
Guinea grass	3.2 (1.2)	4.2 (0.9)	4.5 (0.4)	3.5 (1.5)	3.0 (0.2)	6.2 (0.7)	3.5 (0.3)

[a] ND, not detected.

TABLE 3. Characteristics of *Leuconostoc* and *Weissella* species[a]

Substrate	Acid produced[b] from substrate by:											
	Group A (n^c = 17)	Group B (n = 25)	*L. carnosum* JCM 9695[Td]	*L. citreum* JCM 9698[T]	*L. fallax* JCM 9694[T]	*L. gelidum* JCM 9697[T]	*L. lactis* JCM 6123[T]	*L. mesenteroides* subsp. *cremoris* JCM 9889[T]	*L. mesenteroides* subsp. *dextranicum* JCM 9700[T]	*L. mesenteroides* subsp. *mesenteroides* JCM 6124[T]	*L. pseudomesenteroides* JCM 9696[T]	*W. paramesenteroides* JCM 9890[T]
L-Arabinose	+	+	−	+	−	+	+	−	−	−	+	+
D-Ribose	w	+	+	−	−	+	−	−	−	w	+	w
D-Xylose	+	+	−	−	−	+	−	−	−	+	+	−
Gluconate	w	w	w	w	−	w	−	−	w	w	w	w
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	−	−	+	−	+	+
Galactose	+	+	−	w	−	+	+	−	−	−	+	−
Mannose	+	+	+	+	+	+	w	−	+	−	+	+
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−
Cellobiose	−	+	−	+	−	+	−	−	−	−	+	−
Lactose	−	+	−	−	−	+	+	−	−	−	+	−
Maltose	+	+	−	+	−	+	+	−	−	+	+	+
Melibiose	+	+	−	−	−	+	−	−	−	−	+	w
Sucrose	+	+	+	+	+	+	+	−	+	+	+	+
Raffinose	−	+	−	−	−	+	−	−	−	−	+	w
Trehalose	+	+	+	+	−	+	−	−	+	+	+	w
Mannitol	−	−	w	w	w	+	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	+
Esculin	−	+	−	+	+	+	−	−	−	+	+	−
Salicin	−	−	−	+	−	−	−	−	−	−	−	−
Amygdalin	−	−	−	+	−	−	−	−	−	−	−	−

[a] All strains were gram-positive, catalase-negative cocci that produced gas from glucose and formed more than 90% of their lactate in the D-isomer form.
[b] Tests were performed by using the Semi-Automatic Identification System. Readings were determined at 30°C for 7 days. +, positive; −, negative; w, weakly negative.
[c] n, number of strains tested.
[d] JCM, Japan Collection of Microorganisms. The superscript T indicates that the strain is the type strain.

Physiological and biochemical properties. Characteristics of *Leuconostoc* and *Weissella* species are shown in Table 3. All isolates were gram-positive, catalase-negative cocci that produced gas from glucose, produced more than 90% of their lactate in the D-isomer form, and did not grow below pH 4.5. These strains were divided into two groups on the basis of sugar fermentation patterns. Group A included 17 strains that did not produce acid from esculin, cellobiose, lactose, and D-raffinose, while group B included 25 strains that did produce acid from these sugars. The ability of group B to ferment carbohydrates was similar to that of *Leuconostoc pseudomesenteroides* JCM 9696[T], but group A isolates were easily distinguished from the type strains of all *Leuconostoc* and *Weissella* species. Characteristics of strains FG 5, FG 13, and SL 1 are shown in Table 4. Strain FG 5 from group A gave negative reactions for ammonia from arginine and dextran formation, whereas FG 13 from group B gave positive reactions. FG 5 and FG 13 did not grow below pH 4.5, but SL 1 could grow at pH 3.5. In addition, SL 1 was a homofermentative lactobacillus that formed lactic acid in the L-isomer form.

DNA base composition and DNA-DNA hybridization. DNA base composition and DNA-DNA hybridization data are shown in Table 5. Representative strains FG 5 and FG 13 from groups A and B had G+C contents in the range of 39.2 to 39.5 mol%. The data are within the range of 35 to 40 mol% G+C from the *Leuconostoc* and *Weissella* genera. Strains FG 5 and FG 13 showed high levels of DNA relatedness (84.5% and 85.8 to 88.4%) to the reference strain of *Weissella paramesenteroides* JCM 9890[T] and *L. pseudomesenteroides* JCM 9696[T], respectively, whereas homology values were low (8.8 to 36.8%) compared to other reference strains of the previously described species.

TABLE 4. Characteristics of strains FG 5, FG 13, and SL 1[a]

Character	FG 5	FG 13	SL 1
Shape	Coccus	Coccus	Rod
Ammonia from arginine	−	+	−
Dextran from sucrose	−	+	−
Gas from glucose	+	+	−
Fermentation type	Hetero	Hetero	Homo
Optical form of lactate	D	D	L
Growth at temp (°C):			
10	−	w	−
15	+	+	+
45	−	−	−
50	−	−	−
Growth in NaCl:			
3.0%	+	+	+
6.5%	+	−	+
Growth at pH:			
3.5	−	−	+
4.0	−	−	+
4.5	+	+	+
5.0	+	+	+
9.6	+	+	+

[a] −, negative; +, positive; w, weakly negative.

TABLE 5. DNA base compositions and levels of DNA-DNA homology for *Leuconostoc* species

Strain	G+C content (mol%)	% DNA-DNA reassociation with strain:			
		FG 5	FG 13	JCM 6124^T	JCM 9696^T
FG 5 (group A)	39.2	100	13.5	9.2	8.8
FG 13 (group B)	39.5	13.3	100	9.0	88.4
L. carnosum JCM 9695^T	38.6	16.9	16.5	36.8	22.3
L. citreum JCM 9698^T	37.9	20.8	33.3	28.1	26.9
L. fallax JCM 9694^T	39.1	20.4	15.5	44.2	23.8
L. gelidum JCM 9697^T	37.5	24.3	15.4	27.9	15.5
L. lactis JCM 6123^T	44.2	17.3	15.7	44.2	23.8
L. mesenteroides subsp. *dextranicum* JCM 9700^T	39.3	23.7	22.1	22.4	18.6
L. mesenteroides subsp. *mesenteroides* JCM 6124^T	39.0	15.9	22.9	100	21.0
L. pseudomesenteroides JCM 9696^T	39.0	24.8	85.8	30.6	100
W. hellenica JCM 10103^T	39.0	17.0	22.5	19.8	12.4
W. paramesenteroides JCM 9890^T	39.1	84.5	20.0	16.3	22.5

Changes in microbiological composition during silage fermentation. The changes in microbiological composition during silage fermentation are shown in Table 6. During silage fermentation of alfalfa and Italian ryegrass, the numbers of lactobacilli, leuconostocs, and weissellae in the treated silages were higher in the first few days of ensiling than those in the control silage. However, by day 10, the numbers of lactobacilli in FG 5- and FG 13-treated silages were lower than that in SL 1-treated silage. By day 30, the leuconostocs and weissellae had decreased to a low level (10^3 CFU g^{-1}) or were too few to count in all the silages. The number of aerobic bacteria decreased from day 2 after ensiling in all silages, and the most rapid decline in the aerobic bacteria was observed in the SL 1-treated silages. The number of clostridia decreased to a lower level ($<10^2$ CFU g^{-1}) in SL-treated silages during the first 10 days of incubation. However, in the FG 5- and FG 13-treated silages, the clostridia had increased to a high level (10^7 CFU g^{-1}) in the same time period.

Silage quality and dry matter loss. The silage quality and DM loss are shown in Table 7. In the alfalfa and Italian ryegrass silages, the SL 1 treatments gave the same values, the pH value and butyric acid and ammonia nitrogen contents were significantly ($P < 0.05$) lower, and the lactic acid contents were significantly ($P < 0.05$) higher than those of the control silage. However, in the FG 5- and FG 13-treated silages, these values were similar to those of the control. Compared with the control silage, SL 1 treatments reduced the proportion of D-isomer to total lactic acid, DM loss, and gas production significantly ($P < 0.05$) in two kinds of silage, but the FG 5 and FG 13 treatments resulted in similar values in alfalfa silages and increased them significantly ($P < 0.05$) in Italian ryegrass silages.

DISCUSSION

Generally, leuconostocs are found living in association with plant material and dairy products and several studies have reported leuconostocs as the dominant microbial population on forage crops and silage (3, 21). Some isolates from silage forages have been identified as *Leuconostoc mesenteroides*. In this study, the strains in group B were phenotypically similar to *L. pseudomesenteroides* JCM 9696^T. However, the strains in group A could not be identified to the species level on the basis of phenotypical characteristics. The DNA-DNA hybridization results demonstrated that the group A isolates and *Weissella paramesenteroides* and the group B isolates and *L. pseudomesenteroides* had DNA homology values of above 84 and 85%, respectively, showing that the strains in groups A and B could be assigned to the species *W. paramesenteroides* and *L. pseudomesenteroides*, respectively. This is the first report of the identification of *Weissella* strains from forage crops.

Lin et al. (12) reported that epiphytic LAB play a major role in silage fermentation, and the numbers of LAB have become a significant factor in predicting the adequacy of silage fermentation and determining whether to apply silage bacterial inoculants. Among epiphytic LAB, lactic acid-producing cocci, e.g., streptococci, leuconostocs, pediococci, lactococci, and enterococci, start lactate fermentation in silage, creating an aerobic environment suitable for the development of lactobacilli, although it was shown that they grew vigorously only in the early stage of ensiling processes (2). In contrast with these lactic acid-producing cocci, lactobacilli play an important role in promoting lactic acid fermentation for a longer time. Epiphytic lactobacilli counts on silage crops are usually low and variable when the lactobacilli reach a level of at least 10^5 CFU g of FM^{-1} silage stores well (9). However, as shown in Table 1, the low number of lactobacilli ($<10^3$ CFU g of FM^{-1}) and high

TABLE 6. Changes in microbiological composition during fermentation of silage

Forage crop and treatment	Change in microorganism count (log CFU g of FM^{-1}) at the indicated no. of days after ensiling															
	Lactobacilli				Leuconostocs and weissellae				Aerobic bacteria				Clostridia			
	2	5	10	30	2	5	10	30	2	5	10	30	2	5	10	30
Alfalfa (25°C)																
Control	7.3	8.9	8.2	7.2	7.8	4.5	ND[a]	ND	9.4	9.0	5.9	5.2	4.3	8.2	6.7	7.4
FG 5	7.6	8.2	7.4	6.9	9.8	6.5	3.6	3.2	8.3	6.3	4.23	4.3	5.5	8.2	6.7	5.4
FG 13	8.4	7.9	7.9	6.4	9.2	6.6	ND	ND	7.9	7.9	3.7	3.8	4.3	8.0	7.7	6.4
SL 1	9.9	9.3	8.6	7.5	8.3	ND	ND	ND	7.3	3.5	ND	3.4	3.2	ND	ND	ND
Italian ryegrass (25°C)																
Control	7.8	9.0	7.4	7.7	6.7	4.0	3.5	ND	9.7	9.8	6.7	6.8	3.8	7.5	5.4	5.3
FG 5	6.8	8.5	7.2	7.3	8.8	4.6	ND	3.2	9.0	8.1	5.9	5.7	4.7	7.4	7.4	6.5
FG 13	8.1	8.5	7.4	6.2	9.3	6.7	3.5	3.6	8.8	7.5	6.4	4.3	3.3	8.0	7.8	7.4
SL 1	9.8	9.5	8.5	7.8	6.5	3.4	ND	ND	7.4	6.4	4.8	3.6	ND	ND	ND	ND

[a] ND, not detected.

TABLE 7. Chemical composition of silage ensiled for 30 days[a]

Parameter	Alfalfa silage				Italian ryegrass silage			
	Control	FG 5	FG 13	SL 1	Control	FG 5	FG 13	SL 1
pH	5.0 A	5.2 A	5.1 A	4.2 B	4.6 A	4.6 A	4.4 A	3.9 B
DM (g kg of FM^{-1})	311.2	306.6	310.0	308.5	197.3	195.4	206.1	201.8
Lactic acid (g kg of DM^{-1}	8.3 B	8.2 B	10.0 B	24.3 A	21.7 B	15.3 B	24.2 B	59.4 A
Acetic acid (g kg of DM^{-1})	15.7 A	17.2 A	17.7 A	10.3 B	21.3	16.4	23.2	18.3
Butyric acid (g kg of DM^{-1}	17.2	13.5	15.3	ND	11.6 A	10.7 A	12.1 A	2.4 B
Ammonia N (g kg of total N^{-1})	108.5 A	115.5 A	98.2 A	44.2 B	73.5 A	73.4 A	87.4 A	39.6 B
D-Lactic acid/total lactic acid	70.4 A	74.0 A	71.2 A	30.3 B	60.3 B	67.7 A	71.8 A	37.8 C
Gas production (liter/kg of FM)	6.8 A	7.5 A	7.2 A	2.7 B	5.8 B	7.2 A	6.7 A	3.0 C
DM loss (%)	6.1 A	6.4 A	6.0 A	2.6 B	4.3 B	5.5 A	5.2 A	3.3 C

[a] Values are means of three silage samples. Means in the same row with different letters are statistically significantly different ($P < 0.05$).
[b] ND, not detected.

numbers of aerobic bacteria ($>10^5$ CFU g of FM^{-1}) present in the material suggested that the numbers of microbes during silage fermentation should be controlled.

In this study, higher numbers of LAB were observed at an early stage of ensiling in LAB-treated silages than in the control silage. The FG 5- and FG 13-treated silages were unable to inhibit the growth of aerobic bacteria and clostridia and to improve silage quality. The most plausible explanation lies in the physiological properties of LAB. The FG 5 and FG 13 strains were heterofermentative LAB which could not grow below pH 4.5. During silage fermentation, the leuconostocs grew vigorously only in the early stage of ensiling and they ferment WSC to produce D-lactate, CO_2, and acetate; in addition, the pH value of silage did not decline to less than 4.0, allowing the butyric fermentation by clostridia to occur. On the other hand, the SL 1-treated silages had significantly ($P <$ 0.05) lower pHs, ammonia nitrogen contents, DM losses, and gas production but significantly ($P < 0.05$) higher lactic acid content and higher proportions of L-isomer to total lactic acid compared with the control silage. These results may be evidence that the SL 1 strain used in this study was a homofermentative LAB which produced only L-lactic acid and may grow at low pH conditions. Therefore, inoculation with the SL 1 may result in beneficial effects by promoting the propagation of LAB and by inhibiting the growth of clostridia and aerobic bacteria, as well as by decreasing the amount of gas production and DM loss.

It is well-known that silage containing very large amounts of D-lactic acid may result in lactic acidosis in ruminants (7). Schadt and Johnson (19) found that the production of lactate in silage largely involves the D-isomer. Cai and Kumai (2) reported that on dairy farms, the proportion of D-isomer to total lactic acid in silage was 62 to 68%. In this study, the leuconostocs and weissellae were isolated from silage samples and when silage was reinoculated with these strains, the proportions of D-lactate increased. Therefore, the epiphytic *Leuconostoc* and *Weissella* species might change and influence the proportion of lactate isomer during silage fermentation.

These results confirmed that the inoculation with L-lactic acid-producing lactobacilli had beneficial effects on decreasing the proportion of D-isomer to total lactic acid and improving silage quality. However, the heterofermentative LAB strains *Weissella paramesenteroides* FG 5 and *Leuconostoc pseudomesenteroides* FG 13 did not improve silage quality and may cause some fermentation loss.

ACKNOWLEDGMENT

We thank J. A. Hudson (Environmental Science and Research Ltd., Christchurch Science Centre, New Zealand) for reading the manuscript.

REFERENCES

1. **Benno, Y.** 1996. A semi-automatic system for bacterial identification. Riken reviw. **12:**57–58.
2. **Cai, Y., and S. Kumai.** 1994. The proportion of lactate isomers in farm silage and the influence of inoculation with lactic acid bacteria on the proportion of L-lactate in silage. Jpn. J. Zootech. Sci. **65:**788–795.
3. **Cai, Y., S. Ohmomo, and S. Kumai.** 1994. Distribution and lactate fermentation characteristics of lactic acid bacteria on forage crops and grasses. J. Jpn. Soc. Grassl. Sci. **39:**420–428.
4. **Cai, Y., S. Ohmomo, M. Ogawa, and S. Kumai.** 1997. Effect of NaCl-tolerant lactic acid bacteria and NaCl on the fermentation characteristics and aerobic stability of silage. J. Appl. Microbiol. **83:**307–313.
5. **Dewar, W. A., and P. McDonald.** 1961. Determination of dry matter in silage by distillation with toluene. J. Sci. Food Agricul. **12:**790–795.
6. **Duncan, D. B.** 1955. Multiple range and multiple F test. Biometrics **11:**1–42.
7. **Dunlop, R. H.** 1972. Pathogenesis of ruminant lactic acidosis. Adv. Vet. Sci. Comp. Med. **16:**250–302.
8. **Ezaki, T., Y. Hashimoto, and E. Yabuuchi.** 1989. Fluorometric deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. **39:** 224–229.
9. **Hellings, P., G. Bertin, and M. Vanbelle.** 1985. Effect of lactic acid bacteria on silage fermentation, p. 932–933. *In* Proceedings of the 15th International Grassland Congress. Kyotosyppan, Kyoto, Japan.
10. **Kozaki, M., T. Uchimura, and S. Okada.** 1992. Experimental manual for lactic acid bacteria, p. 29–72. Asakurasyoten, Tokyo, Japan.
11. **Lin, C., K. K. Bolsen, B. E. Brent, R. A. Hart, J. T. Dickerson, A. M. Feyerherm, and W. R. Aimutis.** 1991. Epiphytic microflora on alfalfa and whole-plant corn. J. Dairy Sci. **75:**2484–2493.
12. **Lin, C., K. K. Bolsen, B. E. Brent, and D. Y. C. Fung.** 1992. Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize. J. Appl. Bacteriol. **73:**375–387.
13. **McDonald, P., N. Henderson, and S. Heron.** 1991. The biochemistry of silage, 2nd ed., p. 48–249. Chalcombe Publications, Marlow, Berkshire, Great Britain.
14. **Morimoto, H.** 1971. Experimental method of animal nutrition, p. 284–285. Youkendo, Tokyo, Japan.
15. **Muck, R. E.** 1989. Initial bacterial numbers on lucerne prior to ensiling. Grass Forage Sci. **44:**19–25.
16. **Ohmomo, S., O. Tanaka, and H. Kitamoto.** 1993. Analysis of organic acids in silage by high-performance liquid chromatography. Bull. Nat. Grassl. Res. Inst. **48:**51–56.
17. **Ruser, B.** 1989. Erfassung und Identifizierung des epiphytischen Milchsaurebakterienbesatzes auf Gras und Mais in abhangigkeit von Standort, Sorte, Entwicklungsstadium sowie Ernte- und Klimaeinflussen. Ph.D. Dissertation. Institute of Grassland and Forage Research, Braunschweig, Germany.
18. **Saitou, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
19. **Schaadt, H., and R. R. Johnson.** 1968. Effect of maturity treatment on D(−)

and L(+)lactic acid in corn silage. J. Dairy Sci. **51:**802–805.
20. **Sharp, R., P. G. Hooper, and D. G. Armstrong.** 1994. The digestion of grass silages produced using inoculants of lactic acid bacteria. Grass Forage Sci. **49:**42–53.
21. **Stirling, A. R., and R. Whittenbury.** 1963. Sources of lactic acid bacteria occurring in silage. J. Appl. Bacteriol. **26:**86–92.
22. **Tamaoka, J., and K. Komagata.** 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. **124:**11–16.
23. **Tanaka, O., and S. Ohmomo.** 1994. A repeatable model system for silage fermentation in culture tubes. Biosci. Biotechnol. Biochem. **58:**1407–1411.
24. **Ueno, K.** 1982. Procedures for isolation and identification of anaerobic bacteria, p. 79–93. Saikonsyupan, Tokyo, Japan.
25. **Weinberg, Z. G., G. Ashbell, Y. Hen, and A. Azrieli.** 1993. The effect of applying lactic acid bacteria at ensiling on the aerobic stabiling of silages. J. Appl. Bacteriol. **75:**512–518.
26. **Yamamoto, K. S., S. Utagawa, T. Kodama, and T. Morichi.** 1986. Isolation method of microorganisms, p. 435–444. R and D Planning, Tokyo, Japan.