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LACTIC ACID BACTERIA ISOLATED FROM FORAGE CROPS AND SILAGE FERMENTATION

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Abstract

Six strains, *Lactobacillus plantarum* CM 1, *Lactobacillus rhamnosus* CM 2, *Pediococcus acidilactici* CM 4 *Enterococcus faecalis* CM 5, *Leuconostoc pseudomesenteroides* CM 7 and *Weissella paramesenteroides* CM 8 isolated from forage crops were used as additives at $1.0x10^5$ cfu g^{-1} of fresh matter to Italian ryegrass and alfalfa, and their effect on silage fermentation was studied. The two silage's treated with strains CM 1, CM 2 and CM 4 were well preserved; had significantly lower pH values, butyric acid and ammonia N concentrations; and had significantly higher lactic acid content than did the respective control, strains CM 5, CM 7 and CM 8-treated silage's. Compared with the strain CM 5-treated silage's, strains CM 1, CM 2 and CM 4-treatments reduced the fermentation losses, but strains CM 7 and CM 8 increased the fermentation losses. The results confirmed that *P. acidilactici, L. plantarum* and *L. casei* were more effective to improve silage quality than *E. faecalis, L. pseudomesenteroides and W. paramesenteroides*.

Keywords: Lactic acid bacteria, forage crops, silage fermentation

Introduction

The preservation of forage crops as silage depends on the production of sufficient acid to inhibit activity of undesirable microorganisms under anaerobic conditions. The epiphytic lactic acid bacteria (LAB) that are present on forage crops convert sugar into lactic acid in the ensiling process. As a result, the pH is reduced, and the forage is preserved. Although a number of studies reported positive effects on silage quality from using some LAB inoculants as silage additives, relatively few have reported the effect of LAB isolated from forage crops on silage fermentation. In this study, six LAB strains isolated from forage crops were used as silage additives, and their effect on fermentation characteristics of silage was examined.

Material and Methods

Material and silage preparation

Alfalfa at flowering stage and Italian ryegrass at heading stage were obtained from an experimental field at the National Grassland Research Institute (Nishinasuno, Tochigi, Japan). Silages were prepared using a small-scale system of fermentation (Cai et al., 1999a). The silage treatments were designed as follows: (1) untreated control; (2) CM 1; (3) CM 2; (4) CM 4; (5) CM 5; (6) CM 7 and (7) CM 8. The film bag silos were kept at 25^{o}C for 30 days, and three replicates per treatment were used for microbiological and chemical analysis.

Microbiological analysis

The microorganism numbers were measured by the plate count method (Yamamoto et al., 1986). LAB was counted on plate count agar containing bromocresol purple (Nissui-seiyaku Ltd) and GYP-$CaCO_3$ agar (Kozaki et al., 1992) after incubating in an anaerobic box (TE-HER Hard Anaerobox, ANX-1; Hirosawa Ltd, Tokyo, Japan) at 35 ^{o}C for 2 to 3 days. Aerobic bacteria, and yeasts and mold were counted on nutrient agar (Nissui-seiyaku Ltd, Tokyo, Japan) and potato dextrose agar (Nissui-seiyaku Ltd, Tokyo, Japan), respectively. The

agar plates were held in an incubator at 30 ^{o}C for 2 to 3 days. Yeasts and molds were distinguished from bacteria by colony appearance and cell morphology. Colonies were counted as viable numbers of microorganisms {colony-forming units (cfu) g^{-1} FM}.

Chemical analysis

The chemical composition of the forage crops and silages were determined by conventional methods (Morimoto, 1971). The DM content of the fresh forage was determined by oven drying at 70^{o}C for 48 hours, whereas that of the silages was determined by the removal of water using toluene distillation with ethanol correction. The organic acid content was measured by high-performance liquid chromatography. The content of ammonia-nitrogen was determined by enzymatic analysis according to the UV method of F-Kit (Boehringer Mannheim Ltd, Germany). Gas production and dry matter loss were determined by a method of Cai et al. (1998).

Results and Discussion

The counts of microorganisms in fresh forages were 10^3 cfu g^{-1} FM and less than lactobacilli, 10^2 to 10^4 leuconostocs, enterococci and pediococci, 10^6 to 10^7 aerobic bacteria and 10^3 to 10^4 yeasts and molds in each of the two forage crops.

Generally, the lactobacilli play a more important role in fermentation processes and effectively promoted lactic acid fermentation for a longer time than lactic acid-producing cocci, eg. enterococci, streptococci, leuconostocs, Weissella and pediococci. When the lactobacilli reach a level of at least 10^5 cfu g^{-1} FM, silage can be well preserved. However, epiphytic LAB counts are usually low and variable on silage crops (Cai et al., 1999b). Our results also confirmed the fact that there were the low number of enterococci (10^4 cfu g^{-1} FM) and lactobacilli ($<10^3$ cfu g^{-1} FM), and high numbers of aerobic bacteria ($>10^6$ cfu g^{-1} FM) presented in these silage materials.

The physiological properties of the six LAB strains used in this study are shown in Table 1. All strains were Gram-positive and catalase-negative bacteria that produce lactic acid from glucose. Strains CM 2 and CM 5 exclusively formed lactic acid as L-isomer, strain CM 7 and CM 8 formed it as D-isomer, and strain CM 1 and CM 4 formed it as a racemic mixture of D- and L-lactic acid. Strains CM 1, CM 2, and CM 4 were able to grow at the lowest initial pH value of 3.5, but strains CM 5, CM 7 and CM 8 were unable to grow below pH 4.5. Compared with strains CM 5, CM 7 and CM 8, strains CM 1, CM 2 and CM 4 grow vigorously to produce more lactic acid and decrease the pH to a lower value in the MRS broth. Most strains fermented glucose, fructose and sucrose.

Chemical composition of 45-day silages is shown in Table 2. All the CM 1, CM 2 and CM 4-treated silage's of alfalfa and Italian ryegrass were well preserved, the pH value, acetic acid, butyric acid, propionic acid and ammonia-nitrogen contents were significantly ($P < 0.05$) lower and the lactic acid content was significantly ($P < 0.05$) higher than that of the control, CM 5, CM 7 and CM 8-treated silage. Compared with the each control silage, their CM 1, CM 2 and CM 4 treatments reduced DM loss and gas production significantly ($P < 0.05$), strain CM 5 increased these contents significantly ($P < 0.05$), the strains CM 7 and CM 8-treated silage's showed similar level of these contents in alfalfa and Italian ryegrass silages.

The addition of LAB inoculants at ensiling is intended to ensure rapid and vigorous fermentation that results in faster accumulation of lactic acid, lower pH values at earlier stages of ensiling and improved forage conservation (Cai et al., 1999b). The strains CM 1, CM 2 and CM 4 used in this study were homofermentative lactobacilli that able to grow under low pH conditions. Therefore, inoculation with these strains may result in beneficial effects by promoting the propagation of LAB and improving silage quality. In the present study, the enterococci, leuconostocs and Weissella-treated silage's were unable to improve silage quality. The most plausible explanation lies in their physiological properties. The

Enterococcus, *Leuconostoc* and *Weissella* strains could not grow at low pH (<4.5) environment. During silage fermentation, these strains grew vigorously only in the early stage of ensiling and may die below pH 4.5 of silage. Therefore, these strains unable to ferment WSC to produce sufficient lactic acid resulting in the pH value of silage not palling to less than 4.2, and so allowing the butyric acid fermentation by clostridia (Cai et al., 1999a). Furthermore, the heterofermentative leuconostocs and Weissella produce gas from glucose and may cause some fermentation losses.

The results obtained in the present study demonstrated that *P. acidilactici*, *L. plantarum* and *L. casei* were more effective to produce lactic acid and improve fermentation quality in the silage environment than *E. faecalis*, *E. faecium*, *L. pseudomesenteroides* and *W. paramesenteroides*.

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Table 1. Characteristics of lactic acid bacteria used in this study.

Characteristic	*L. plantarum* CM 1	*L. casei* CM 2	*P. acidilactici* CM 4	*E. faecalis* CM 5	*L. pseudomesenteroides* CM 7	*W. paramesenteroides* CM 8
Shape	Rod	Rod	Cocci	Cocci	Cocci	Cocci
Fermentation type	Homo	Homo	Homo	Homo	Hetero	Hetero
Optical form of lactate	DL	L	DL	L	D	D
Growth at						
10?	-	-	-	-	-	w
15?	+	+	+	+	+	+
45?	-	-	+	+	-	-
50?	-	-	+	-	-	-
Growth at pH						
3,0	-	-	-	-	-	-
3,5	+	+	+	-	-	-
4,0	+	+	+	-	-	-
4,5	+	+	+	+	+	+
5,0	+	+	+	+	+	+
6,0	+	+	+	+	+	+
Growth characteristics in MRS broth						
OD_{620}	2,02	1,95	1,98	0,79	0,74	0,83
Lactate production (g/L)	14,26	13,80	13,50	5,35	7,31	6,13
Final pH	3,60	3,85	3,7	4,75	4,65	4,60
Fermentation of sugar						
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Sucrose	+	+	-	+	+	+

+, Positive reaction; -, negative reaction; w, weakly positive reaction.

Table 2. Chemical composition and fermentation loss of 45-day silages stored at 25°C.

	Control	CM 1	CM 2	CM 4	CM 5	CM 7	CM 8
Alfalfa							
pH	5.0[a]	4.5[b]	4.4[b]	4.4[b]	5.0[a]	5.0[a]	5.0[a]
DM, g/kg of FM	210,4	211,2	210,6	210,9	210,5	210,5	210,5
Lactic acid, g/kg of DM	13.2[b]	20.5[a]	21.6[a]	19.8[a]	13.8[b]	13.0[b]	13.5[b]
Acetic acid, g/kg of DM	15.2[a]	10.9[b]	11.5[b]	12.0[b]	13.3[ab]	13.0[ab]	12.8[ab]
Butyric acid, g/kg of DM	15.0[a]	9.1[b]	10.6[b]	10.1[b]	13.9[a]	15.1[a]	14.5[a]
Propionic acid, g/kg of DM	7.5[a]	5.0[b]	5.4[b]	5.5[b]	6.9[a]	6.6[a]	7.6[a]
Ammonia N, g/kg of DM	4.9[a]	3.5[b]	3.3[b]	3.3[b]	4.7[a]	5.1[a]	4.7[a]
Gas production, L/kg of DM	6.3[b]	4.1[c]	3.7[c]	4.0[c]	6.4[b]	7.5[a]	8.0[a]
DM loss, g/kg of DM	95.3[b]	86.5[c]	84.3[c]	85.3[c]	93.2[b]	97.0[a]	97.8[a]
Italian ryegrass							
pH	4.7[a]	4.0[b]	4.2[b]	4.2[b]	4.7[a]	4.8[a]	4.7[a]
DM, g/kg of FM	220,3	221,2	220,8	221,8	221,0	220,5	220,8
Lactic acid, g/kg of DM	40.0[b]	45.7[a]	46.3[a]	46.0[a]	40.3[b]	37.8[b]	41.8[b]
Acetic acid, g/kg of DM	17.3[a]	12.0[b]	12.8[b]	12.2[b]	16.5[a]	17.5[a]	16.2[a]
Butyric acid, g/kg of DM	6.5[a]	3.4[b]	nd	nd	6.6[a]	6.8[a]	6.3[a]
Propionic acid, g/kg of DM	4,1	nd	nd	nd	3,8	4,3	4,0
Ammonia N, g/kg of DM	3.6[a]	2.5[b]	2.6[b]	2.8[b]	2.9[ab]	3.5[a]	3.7[a]
Gas production, L/kg of DM	5.0[b]	3.7[c]	3.5[c]	3.4[c]	5.1[b]	6.8[a]	6.5[a]
DM loss, g/kg of DM	54.6[b]	44.3[c]	40.7[c]	42.7[c]	50.8[b]	59.3[a]	58.8[a]

Values are means of three silage samples. Means in the same silage row with different superscripts are significantly different ($P < 0.05$).

FM, fresh matter; DM, dry matter; nd, not detected.