EFFECTS OF INOCULATION WITH *LACTOBACILLUS CASEI* SUBSP. RHAMNOSUS AT ENSILING ON FERMENTATION AND FLORA OF LACTIC ACID BACTERIA OF GRASS SILAGES

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ABSTRACT

The purpose of this study was to determine whether the inoculation with *Lactobacillus casei* subsp. *rhamnosus* (L.c.r) at ensiling would improve the fermentation and affect the flora of lactic acid bacteria of orchardgrass (*Dactylis glomerata* L) and timothy (*Phleum pratense* L) silages. The fermentation quality of orchardgrass and timothy silages were improved by the addition of L.c.r and L*actobacillus casei* subsp. *casei* (L.c.c) except orchardgrass silage stored at 15°C. In orchardgrass and timothy silages, the species of lactic acid bacteria in the control silages was different than the inoculated silages. At 15°C and 35°C, L.c.r was dominant in timothy silage with the addition of L.c.r-inoculant. The L.c.r-inoculant was deemed to have the same great potential with the L.c.c-inoculant.

KEYWORDS

Orchardgrass silage, timothy silage, fermentation quality, commercial inoculant, *Lactobacillus casei* subsp. *rhamnosus*

INTRODUCTION

In grass silage making on the farm, the use of silage additives based on freeze-dried culture of lactobacilli has been regarded as necessary for improving grass silage preservation. Either a single strain of *Lactobacillus plantarum* or mixture of *L. plantarum* and *Pediococcus* spp. or *Enterococcus* spp. is used in most commercial inoculants. Some workers have recommended addition of *Lactobacillus acidophilus* or *Lactobacillus casei* to enhance the fermentation of grass silage. Most species of lactobacilli grow at 15°C but not at 45°C. The optimum for these species is about 30°C. *Lactobacillus casei* subsp. *rhamnosus* is the only homofermentative lactobacilli which grow well at both 15°C and 45°C (Kandler and Wiss, 1986). This strain is more acid tolerant than the other strains (Ohmomo, 1995). The purpose of this experiment was to study the effects of inoculation with *L. casei* subsp. *rhamnosus* at ensiling on fermentation and flora of lactic acid bacteria of orchardgrass and timothy silages and to compare the resultant silages treated with *Lactobacillus casei* subsp. *casei* under two temperatures (15°C and 35°C) during ensilage.

MATERIALS AND METHODS

Preparation of silage: First cut orchardgrass (*Dactylis glomerata* L.; heading stage, DM 33.5% , CP 10.1%DM, WSC 7.3%DM) and timothy (*Phleum pratense* L.; heading stage, DM 36.7% , CP 8.5% DM, WSC 11.8% DM) were pre-wilted for 2 days in the room. Twenty-four laboratory silos (capacity 1l) for each control (no additive) and each inoculated silage were prepared in duplicate. The inoculants (Snow Brand Seed Co., Ltd) consisted of a single strain of the organism *Lactobacillus casei* subsp. *rhamnosus* (L.c.r) and *Lactobacillus casei* subsp. *casei* (L.c.c) was added at the rate of 0.3% of fresh grass respectively. Number of added organism L.c.r and L.c.c were $2.9x10^5$ and $6.0x10^5$ (cfu/g fresh grass) respectively. The silos were stored at 15°C and 35°C in the incubator and opened at 45 days after ensiling.

Chemical analysis: Water-soluble carbohydrate (WSC) content was estimated colorimetrically using anthrone (National Grassland Research Institute; NGRI, 1975). Lactic acid content was determined by the colorimetric method of Barker and Summerson (NGRI, 1975). Volatile basic nitrogen (VBN) was measured by a steam distillation (NGRI, 1975). Volatile fatty acids (VFA) was measured by gas chromatography (GC-12A, Shimadzu Co., Ltd.)(Kageyama et al., 1972).

Microbiological analysis: The plating media were glucose yeast extract peptone calcium carbonate agar for lactic acid bacteria, bouillon agar for aerobic bacteria and pepton yeast extract malt extract agar containing 0.5% lactic acid for moulds and yeasts. The viable colonies were counted by dilution plate method.

Identification of lactic acid bacteria isolated: One hundred twelve strains of lactic acid bacteria were isolated from silages. Cell form was determined by a microscopic observation. Gram staining, motility, catalase activity, nitrate reduction, CO_2 production from glucose, growth temperature, hydrolysis of arginine, growth at pH 4.8 and 9.6, fermentation type, optical form of lactic acid and fermentation of sugars were determined by procedures described by Experimental Manual of Lactic Acid Bacteria (Uchimura and Okada, 1992). The identification criteria used were in accordance with the systems used in Bergey's Manual of Systematic Bacteriology vol.2 (Garvie, 1986; Kandler and Wiss, 1986).

RESULTS

Chemical quality and viable counts of silages: The chemical quality and viable counts of orchardgrass and timothy silages is shown in Table 1. Both controls stored at 15°C and 35°C in orchardgrass silages were badly preserved. At 15°C the L.c.r and L.c.c-treated silages were badly preserved. At 35°C both inoculated silages had a lower pH and contained more lactic acid and less butyric acid than the control silage. Both controls stored at 15°C and 35°C in timothy silages were badly preserved. At 15°C and 35°C the L.r.c and L.c.c-treated silages had a lower pH and contained more lactic acid and less butyric acid than the control silages. The number of lactic acid bacteria was markedly higher in the silage stored at 15°C than that stored at 35°C regardless of the inoculants in both orchardgrass and timothy silages. The number of lactic acid bacteria in all the inoculated silages was not higher than that in the control silages. Aerobic bacterial and yeast counts in both orchardgrass and timothy silages were higher in the silages stored at 15°C than that stored at 35°C regardless of the inoculants. Mould counts were increased at 15°C in orchardgrass silages and at 35°C in timothy silages.

Identification of lactic acid bacteria isolated: The identification list of lactic acid bacteria isolated from orchardgrass and timothy silages are shown in Table 2. In orchardgrass silage stored at 15°C and 35°C, the control silages included different species. The L.c.r-treated silages included mainly *Lactobacillus casei* subsp. *casei* and *Lactobacillus coryniformis* subsp. *coryniformis* at 15°C and 35°C respectively. The number of isolated *Lactobacillus casei* subsp. *rhamnosus* was low. The L.c.c-treated silages included three and five strains of *L. casei* subsp. *casei* at 15°C and 35°C respectively. In timothy silage stored at 15°C and 35°C, the control silages included different species . The L.c.r-treated silages included mainly *L. casei* subsp. *rhamnosus* in both 15°C and 35°C. The L.c.c-treated silages included mainly *L. casei* subsp.*casei* and *Lactobacillus curvatus* at 15°C and 35°C respectively.

DISCUSSION

The fermentation quality of orchardgrass and timothy silages was improved by the additon of L.c.r and L.c.c with the excetion of orchardgrass silage stored at 15°C.The inoculated silages had a lower pH and contained more lactic acid and less butyric acid than the control silages. However, the number of lactic acid bacteria in the L.c.r and L.c.c-treated silages was lower than that in the control silages. This result indicates that the activity of lactic acid bacteria in inoculated silages is higher than that in the control silages. This trend agrees with the result reported by Masuko et al. (1992b). In orchardgrass and timothy silages, the species of lactic acid bacteria in the control silages was different than in the inoculated silages. The control silages contained mainly species of cocci. The inoculated silages included the same species with added lactic acid bacteria with the exception of the L.c.c-treated timothy silage stored at 35°C. The L.c.r-treated timothy silage stored at 15°C and 35°C included eight strains of *Lactobacillus casei* subsp. *rhamnosus*. *L. casei*

subsp. *rhamnosus* was dominant in these silages. However, the number of these species in the L.c.r-treated orchardgrass silage stored at 15°C and 35°C was two and one respectively. At 15°C the L.c.r-treated orchardgrass silage included five strains of *Lactobacillus casei* subsp. *casei*. At 35°C this silage included six strains of *Lactobacillus coryniformis* subsp. *coryniformis*. Masuko et al. (1992a, 1992b) reported that *L. coryniformis* was the dominant species in poor quality silage. In this experiment, though at 15°C and 35°C *L. casei* subsp. *rhamnosus* was not dominant in orchardgrass silage with the addition of L.c.r-inoculant, this strain was dominant in timothy silage. *L. casei* subsp. *rhamnosus* grew at 15°C and 45°C in the experiment of growth temperature. *L. casei* subsp. *casei* did not grow at 45°C. *L. casei* subsp. *casei* is used with commercially available inoculant. The inoculant with *L. casei* subsp. *rhamnosus* was deemed to have the same great potential with *L. casei* subsp. *casei*.

REFERENCES

Garvie, E. 1986. Bergey's manual of systematic bacteriology Vol.2. Pages 1071-1079 in P.H.A.Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt, eds. Williams and Wilkins Press, Baltimore.

Kageyama, K., H. Mori and K. Sato. 1972. Simultaneous determination of volatile fatty acids and lactic acid in silage by gas chromatography. Jpn. J. Zootech. Sci. **44**: 465-469.

Kandler, O. and N. Wiss. 1986. Bergey's manual of systematic bacteriology Vol. 2. Pages 1208-1234 in P.H.A. Sneath, N.S. Mair, M. E. Sharp and J.G. Holt, eds. Williams and Wilkins Press, Baltimore.

Masuko, T., T. Uchimura, S. Okada and K. Awaya. 1992a. Relationship between quality of grass silage and flora of lactic acid bacteria. Anim. Sci. Technol. (Jpn.) **63**: 629-637.

Masuko, T., S. Okada, T. Uchimura and K. Awaya. 1992b. Effects of inoculation with lactic acid bacterial culture at ensiling on the fermentative quality and flora of lactic acid bacteria of grass silage. Anim. Sci. Technol. (Jpn.) **63**: 1182-1187.

National Grassland Research Institute. 1975. Experimental method of silage. Mem. Nat. Grassl. Res. Inst. No.**50-3**, pp. 44-59.

Ohmomo, S. 1995. Biological additives in silage making. -Their development and improvement in future- . TechnoInnovation No.**17**, pp. 32-37.

Uchimura, T. and S. Okada. 1992. Experimental manual of lactic acid bacteria from isolation to identification. Pages15-16 in M.KOZAKI, ed. Asakurashoten, Tokyo

Table1
Chemical quality and viable counts of orchardgrass and timothy silages

Item	15°C			35°C		
	Control	L.c.r[1]	L.c.c[2]	Control	L.c.r	L.c.c
Orchardgrass silage						
pH	5.94	5.63	5.79	4.66	3.87	3.87
Organic acids (%DM)						
Lactic acid	0.68	1.15	0.87	0.87	2.46	3.96
Acetic acid	1.09	0.97	1.00	0.42	0.20	0.27
Propionic acid	0.02	0.01	0.01	0.57	0	0
Butyric acid	1.23	0.94	0.93	0.39	0.02	0.03
VBN[3]	0.22	0.19	0.17	0.14	0.06	0.08
Mark[4]	15.0	25.0	25.0	35.0	92.5	92.5
Viable counts (cells/gDM)						
Lactic acid bacteria	2.0×10^{10}	4.1×10^{9}	5.9×10^{9}	3.3×10^{8}	1.6×10^{5}	5.9×10^{5}
Aerobic bacteria	8.7×10^{9}	3.4×10^{10}	2.8×10^{10}	2.1×10^{5}	4.1×10^{5}	5.3×10^{5}
Yeasts	1.1×10^{5}	1.6×10^{3}	6.0×10^{2}	<10	<10	1.2×10^{2}
Moulds	2.9×10^{4}	1.6×10^{4}	2.4×10^{5}	1.6×10^{2}	<10	<10
Timothy silage						
pH	5.52	4.26	3.98	4.94	3.70	3.75
Organic acids (%DM)						
Lactic acid	1.51	3.04	3.78	1.12	6.32	5.86
Acetic acid	0.58	0.36	0.63	0.73	1.00	1.08
Propionic acid	0	0	0.01	0.33	0	0.01
Butyric acid	0.60	0.37	0.24	0.15	0.03	0.03
VBN[3]	0.13	0.09	0.07	0.10	0.04	0.04
Mark[4]	55.0	75.0	77.5	52.5	92.5	90.0
Viable counts (cells/gDM)						
Lactic acid bacteria	2.9×10^{10}	1.9×10^{10}	6.8×10^{9}	1.3×10^{8}	3.1×10^{5}	5.4×10^{7}
Aerobic bacteria	3.0×10^{10}	4.2×10^{8}	4.1×10^{7}	1.2×10^{8}	1.1x105	6.1×10^{3}
Yeasts	1.3×10^{3}	3.0×10^{3}	2.3×10^{3}	<10	<10	<10
Moulds	<10	<10	<10	1.9×10^{3}	8.0×10^{2}	<10

1 *Lactobacillus casei* subsp. *rhamnosus*.
2 *Lactobacillus casei* subsp. *casei*.
3 Volatile basic nitrogen.
4 According to Flieg's evaluation.

Table 2
Identification list of lactic acid bacteria isolated from orchardgrass and timothy silages

Treat-ments	Number of strains isolated	Identified species
Orchardgrass silages		
15°C		
Control	9	5 *Leuconostoc mesenteroides* subsp. *cremoris* 4 *Enterococcus faecalis*
L. c. r[1]	9	5 *Lactobacillus casei* subsp. *casei* 2 *Lactobacillus casei* subsp. *rhamnosus* 2 *Leuconostoc mesenteroides* subsp. *cremoris*
L. c. c[2]	9	3 *Lactobacillus casei* subsp. *casei* 3 *Lactobacillus viridescens* 2 *Lactobacillus curvatus* 1 *Lactobacillus amylophilus* 1 *Streptococcus agalactiae*
35°C		
Control	9	7 *Lactobacillus viridescens* 1 *Lactobacillus casei* subsp. *pseudoplantarum* 1 *Lactobacillus casei* subsp. *rhamnosus*
L. c. r	9	6 *Lactobacillus coryniformis* subsp. *coryniformis* 2 *Lactobacillus casei* subsp. *casei* 1 *Lactobacillus casei* subsp. *rhamnosus* 1 *Lactobacillus curvatus*
L. c. c	9	5 *Lactobacillus casei* subsp. *casei* 4 *Lactobacillus curvatus*
Timothy silages		
15°C		
Control	9	6 *Enterococcus faecalis* 3 *Lactobacillus viridescens*
L. c. r	10	8 *Lactobacillus casei* subsp. *rhamnosus* 2 *Lactobacillus coryniformis* subsp. *coryniformis*
L. c. c	10	8 *Lactobacillus casei* subsp. *casei* 2 *Lactobacillus animalis*
35°C		
Control	9	5 *Leuconostoc mesenteroides* subsp. *cremoris* 2 *Lactobacillus plantarum* 2 *Enterococcus faecalis*
L. c. r	10	8 *Lactobacillus casei* subsp. *rhamnosus* 1 *Lactobacillus curvatus* 1 *Lactobacillus* helveticus
L. c. c	10	7 *Lactobacillus curvatus* 2 *Lactobacillus* animalis 1 *Lactobacillus casei* subsp. *tolerans*

[1] and [2] see footnote of Table 1.