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

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Abstract In the first part of the study, forty samples of Italian ryegrass (*Lolium multiflorum* Lam.) and corn (*Zea mays* L.) silages were collected from dairy farms in Ehime Prefecture and these were used to analyze the quality and proportion of L- and D-lactate to total lactate in silage. Two other experiments were then conducted using Italian ryegrass and corn to investigate the effect of lactic acid bacteria (LAB) inoculation on the quality and proportion of L-lactate isomers in silage. In Experiment 1, the silage materials were treated with either inoculant SI containing *Lactobacillus casei* or inoculant Cy containing both *L. casei* and *Lactococcus lactis*. In Expt. 2, the silage materials were inoculated with one or two of the following : *Enterococcus faecium*, *L. lactis* and *L. casei*. The proportion of L-lactate to total lactate of the silage from dairy farms was 32–38%. In Expt.1 and Expt. 2, the silage samples inoculated with LAB had a lower pH value, more lactate and a higher FLIEG's score compared to their respective controls. In Expt. 1, the proportion of L-lactate in SI- and Cy-treatments was 5–10% ($P < 0.05$) higher than the controls. In Expt. 2, the proportion was 7–11% ($P < 0.05$) higher in lactate-producing cocci treatment and was 12–14% ($P < 0.05$) higher in lactobacilli or mixed treatment than their respective controls. The LAB inoculant mixture did not result in advantageous effect over the inoculation of lactobacilli alone. The results confirmed that the inoculation of LAB, especially lactobacilli, had the beneficial effect for increasing L-lactate content in silage, thus for improving silage quality.

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Two isomers of lactate, L(+)-lactate and D(–)-lactate, are produced by lactic acid bacteria (LAB) in silage. In animals, the L-lactate is more effectively utilized within the body than the D-lactate^{7,8,19,20,25}. In Japan, concentrates are widely used as the main component of the diet for cattle. Excessive intake of concentrates and silage containing high amounts of D-lactate leads to an unusual accumulation of D-lactate in the reticulorumen and blood, which may be associated with the development of lactic acidosis in ruminants^{7,8,25}. Recently,

a year-round silage feeding system has spread to many dairy farms in Japan. Under this feeding system, increasing L-lactate content in silage may introduce an important to ruminant health and nutrition problems. Research concerning the effects of inoculation of LAB on the quality of various silage has been reported^{2,4,9,11,13,22,23}, but there are very few reports on the L- and D-isomers of lactate in silage on dairy farms and the effects of LAB inoculation on lactate isomers. The objectives of this study were :

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(1) to investigate the proportion of lactate isomers in dairy farm silage, and (2) to investigate the effects of LAB inoculation on the proportion of L- and D-lactate isomers in silage made in laboratory silos.

Materials and Methods

Sample collection of dairy farm silages: A total of 40 samples, consisting of 6 high moisture Italian ryegrass silages (IRH), 10 medium moisture Italian ryegrass silages (IRM) and 24 corn silages were collected from dairy farms in Ehime Prefecture. In making these farm silages, the Italian ryegrass was cut at the heading or flowering stage and the corn was cut at the dough or yellow ripe stage. The IRH (78.3–82.9% moisture), IRM (61.9–68.9% moisture) and corn silage (66.7–74.7% moisture) were made on dairy farms between October 1989 and December 1990, without any additives including commercial inoculants, in large polyethylene vessels or tower silos. The silages were analyzed for the quality and proportion of L-lactate isomer.

Laboratory silage preparation: Expt. 1; Italian ryegrass and corn were grown in an experimental field of the Faculty of Agriculture, Ehime University. The second regrowth of Italian ryegrass at the heading stage was cut

on May 20, 1989. Corn at the silking stage (corn S) and ripe stage (corn R) were cut on August 12, 1989 August 8, 1990, respectively. The chemical composition and viable counts of microorganisms in silage materials are shown in Table 1. These materials were chopped into about 20 mm lengths and ensiled into 300 l capacity polyethylene vessels. Prior to ensiling, the materials were either untreated (Co) or treated with one of the following bacterial inoculants: SI, which contained a single strain of the organism *Lactobacillus casei* (Lc) or Cy, which contained a mixture of Lc and *Lactococcus lactis* (Ll). The SI was targeted to provide 1.0×10^5 colony forming units (cfu) of Lc. The Cy was added with 7.0×10^4 cfu of Lc plus 3.0×10^4 cfu of Ll g^{-1} on a fresh matter basis. There were four replications per treatment. The silos were opened 90 days after ensiling for chemical analyses of silage. Expt. 2; Italian ryegrass and corn were grown in a farmer's field in Kawauchi Town, Ehime Prefecture. The second regrowth of Italian ryegrass at the heading stage was cut on July 28, 1990 and wilted for 4 hours. The corn at early yellow ripe stage was cut on August 8, 1990. The chemical composition and viable count of microorganisms in the materials are shown in Table 1. These materials were chopped into

Table 1. Chemical composition (% DM) and viable count of microorganisms of materials (g^{-1} on a fresh matter basis)

Material	Mois- ture	Crude proten	Crude fat	Crude fiber	NFE ^{a)}	Crude ash	WSC ^{b)}	Aerobic bacteria	Mold & Yeast	Lacto- cocci ^{c)}	Lacto- bacilli
Exp. 1.											
Italian ryegrass	84.3	6.1	3.4	29.8	52.4	8.3	10.3	3.5×10^5	2.1×10^4	1.0×10^3	5.0×10^2
Corn S ^{d)}	85.6	11.2	2.2	32.6	44.8	9.2	6.0	6.7×10^6	3.3×10^5	5.6×10^3	1.2×10^3
Corn R ^{e)}	71.0	5.9	3.2	17.3	66.9	6.7	16.4	9.2×10^5	8.2×10^4	1.6×10^4	7.3×10^3
Expt. 2.											
Italian ryegrass	66.3	9.1	3.5	26.5	51.0	9.9	11.5	2.7×10^5	3.6×10^4	2.5×10^3	7.7×10^2
Corn	75.2	6.7	2.6	21.0	64.5	5.2	17.9	1.3×10^6	2.9×10^5	2.2×10^4	5.6×10^3

^{a)} : Nitrogen free extract.

^{b)} : Water soluble carbohydrates.

^{c)} : Including lactococci, enterococci, streptococci, leuconostocs and pediococci.

^{d)} : Corn at silking stage.

^{e)} : Corn at ripe stage.

about 20 mm length and ensiled into 1 l laboratory silos. Two species of lactate-producing cocci (*Enterococcus faecium* and L1) and one specie of lactobacilli (Lc) were used. Treatments were (1) Co, (2) *E. faecium* (Ef), (3) L1, (4) Lc, (5) Lc+Ef and (6) Lc+L1. The inoculants Ef, L1 and Lc were targeted to give 1.0×10^5 cfu of LAB, Lc+Ef and Lc+L1 with 1.0×10^5 cfu of Lc plus 5.0×10^4 cfu of Ef and L1 g^{-1} on a fresh matter basis, respectively. There were four replications per treatment. The silos were opened 90 days after ensiling.

Microorganism enumeration: Ten grams of the silage materials were shaken with 90 ml of sterilized 0.85% sodium chloride (NaCl) solution, and 10^{-1} – 10^{-8} serial dilutions were made in 0.85% NaCl solution. The plating media were nutrient agar²⁶⁾ for aerobic bacteria and potato dextrose agar²⁶⁾ for mold and yeast. Lactate-producing cocci and lactobacilli were counted using LEE's agar¹²⁾ and acetate agar¹⁶⁾, respectively. The microorganism counts were measured by the plate count method. The plated samples were incubated at 25–30°C for 3–4 days. The viable colonies were counted as viable counts of microorganisms g^{-1} on a fresh matter basis.

Chemical analyses: The chemical composition of materials was determined by the conventional method. Water soluble carbohydrate (WSC) in the material was estimated colorimetrically using anthrone reagent¹⁸⁾. The moisture content of the material and silage

were determined by the toluene distillation method¹⁷⁾. The measurement of pH was carried out with a glass electrode pH meter and organic acid content was measured by FLIEG's distillation method¹⁸⁾.

The L- and D-lactate isomers were determined according to the UV-method for determination of lactate as described below. Fifty grams of silage sample were mixed with 200 ml distilled water in a 500 ml volumetric flask and placed in a refrigerator for 12 h at 5°C with occasional shaking. After filtration, the extracted solution was placed in a water-bath at 80°C for 15 min to stop any enzymatic reaction. The solution was then centrifuged and the supernatant was used for the enzymatic assay²¹⁾.

Statistical analysis: Data were analyzed by analysis of variance and the significance of differences among means was tested by DUNCAN's Multiple range test⁶⁾.

Results

The quality and proportion of L-lactate in dairy farm silage is shown in Table 2. The IRM and corn silage had lower pH values, more lactate content and higher FLIEG's scores than IRH silage.

The proportions of L-lactate and D-lactate to total lactate content in IRH, IRM and corn silage were 32–38% and 62–68%, respectively. Thus, it was confirmed that D-lactate was the predominant isomer in these dairy farm silages.

Table 2. The quality and proportion of L-lactate in dairy farm silages

Silage	Number of samples	Moisture (%)	pH	Organic acid (% in fresh matter)						FLIEG's score	L(+) ^{c)} T–L
				Acetic	Butyric	Lactic	L(+) ^{a)}	D(–) ^{b)}	Total		
IRH ^{d)}	n= 6	80.6±2.3	4.8	1.08	0.01	1.62	0.52	1.10	2.71	70	32
IRM ^{e)}	n=10	65.4±3.5	4.0	1.09	0.00	2.82	1.07	1.75	3.91	88	38
Corn	n=24	70.7±4.0	4.1	0.58	0.00	2.58	0.92	1.66	3.16	95	36

^{a)} : L-lactate.

^{b)} : D-lactate.

^{c)} : L-lactate/total lactate×100%.

^{d)} : Italian ryegrass with high moisture.

^{e)} : Italian ryegrass with medium moisture.

The Proportion of Lactate in Silage

The effects of LAB inoculants on the quality and proportion of L-lactate in silage in Expt. 1 are shown in Table 3. Silages made with LAB inoculant had a lower pH value and a significantly ($P < 0.05$) higher lactate content than their respective controls. Compared with the control silage samples, the FLIEG's scores of Italian ryegrass, corn S and corn R silage samples treated with SI and Cy were 12, 25 and 5 points higher, respectively. The SI- and Cy-treatments significantly increased the proportion of L-lactate in silage by 5-6% ($P < 0.05$) for Italian ryegrass silage and 6-10% ($P < 0.05$) for corn silage over their respective controls.

The effects of LAB inoculation on the quality and proportion of L-lactate in silage in Expt. 2 are shown in Table 4. The FLIEG's score in all treatments was 100 points for Italian ryegrass silage and 95 points for corn silage. However, Lc, Cf and Cl treated silage had significantly ($P < 0.05$) lower pH values and significantly ($P <$

0.05) higher lactate content Co, Ef and Ll treatments. In Italian ryegrass silage, the proportion of L-lactate was 7-9% ($P < 0.05$) more for Ef and Ll treatments and 12-14% ($P < 0.05$) more for Lc, Lc+Ef and Lc+Ll treatments than those of the controls. In corn silage, the proportion of L-lactate was 9-11% ($P < 0.05$) more in Ef and Ll treatments, and 13-14% ($P < 0.05$) more in Lc, Lc+Ef and Lc+Ll treatments than those of the controls. Therefore, the inoculation of lactobacilli provided beneficial effects by increasing L-lactate content compared to lactate-producing cocci. The inoculation of lactobacilli plus enterococci or lactococci did not result in greater effect than lactobacilli inoculation alone.

Discussion

Proportion of L- and D-lactate in dairy farm silage: Lactobacilli and lactate-producing cocci such as lactococci, streptococci, enterococci, leuconostocs and pediococci mainly

Table 3. The quality and proportion of L-lactate of silages in Expt. 1^{a)}

Silage treatment	Moisture (%)	pH	Organic acid (% in fresh matter)						FLIEG's score	L (+) ^d
			Acetic	Butyric	Lactic	L (+) ^b	D (-) ^c	Total		T-L
Italian ryegrass										
Co ^e	82.4	4.1 ^A	1.09 ^A	0.00	2.82 ^B	1.07 ^B	1.75 ^B	3.91 ^B	88	38 ^B
SI ^f	81.8	3.8 ^A	0.66 ^B	0.00	3.59 ^A	1.54 ^A	2.05 ^{AB}	4.25 ^{AB}	100	43 ^A
Cy ^g	82.1	3.8 ^A	0.68 ^B	0.00	3.76 ^A	1.65 ^A	2.11 ^A	4.44 ^A	100	44 ^A
Corn S ^h										
Co	83.4	4.4 ^A	0.91 ^A	0.00	1.36 ^B	0.34 ^B	1.02 ^A	2.28 ^A	70	25 ^C
SI	83.4	3.8 ^B	0.54 ^B	0.00	1.92 ^A	0.67 ^A	1.25 ^A	2.46 ^A	95	35 ^A
Cy	83.3	3.8 ^B	0.51 ^B	0.00	1.94 ^A	0.67 ^A	1.27 ^A	2.45 ^A	95	34 ^A
Corn R ⁱ										
Co	69.2	3.8 ^A	0.70 ^A	0.00	2.54 ^B	0.84 ^B	1.70 ^A	3.24 ^A	95	33 ^C
SI	68.5	3.4 ^A	0.38 ^B	0.00	3.12 ^A	1.22 ^A	1.90 ^A	3.50 ^A	100	39 ^{AB}
Cy	68.7	3.5 ^A	0.40 ^B	0.00	3.20 ^A	1.34 ^A	1.86 ^A	3.60 ^A	100	42 ^A

^{a)} : Silo : 300 liter capacity polyethylene vessels ; silo opening : 90 days after ensiling.

^{b), c), d)} : Refer to Table 2.

^{e)} : Control.

^{f)} : *Lactobacillus casei* inoculant.

^{g)} : *L. casei* and *Lactococcus lactis* inoculant.

^{h)} : Corn at silking stage.

ⁱ⁾ : Corn at ripe stage.

^{ABC} : Means in the same column with different superscripts are significantly different ($P < 0.05$).

Table 4. The quality and proportion of L-lactate of silages in Expt. 2^{a)}

Silage treatment	Moisture (%)	pH	Organic acid (% in fresh matter)						FLIEG's score	L (+) ^{d)} T-L
			Acetic	Butyric	Lactic	L (+) ^{b)}	D (-) ^{c)}	Total		
Italian ryegrass (wilted)										
Co ^{e)}	64.4	4.2 ^A	0.69 ^A	0.00	4.27 ^B	1.41 ^C	2.86	4.96 ^B	100	33 ^C
Ef ^{f)}	64.8	4.0 ^A	0.58 ^A	0.00	4.58 ^B	1.92 ^B	2.66	5.16 ^B	100	42 ^{AB}
Ll ^{g)}	65.1	4.0 ^A	0.56 ^A	0.00	4.47 ^B	1.79 ^B	2.68	5.03 ^B	100	40 ^B
Lc ^{h)}	64.3	3.5 ^B	0.39 ^B	0.00	5.17 ^A	2.38 ^A	2.79	5.56 ^A	100	46 ^A
Lc + Ef	65.4	3.5 ^B	0.42 ^B	0.00	5.15 ^A	2.42 ^A	2.73	5.57 ^A	100	47 ^A
Lc + Ll	65.4	3.5 ^B	0.47 ^B	0.00	5.21 ^A	2.34 ^A	2.87	5.68 ^A	100	45 ^A
Corn										
Co	72.2	3.9 ^A	0.35 ^B	0.00	1.48 ^B	0.36 ^C	1.12	1.83 ^B	95	24 ^C
Ef	72.5	3.7 ^A	0.36 ^B	0.00	1.56 ^B	0.55 ^B	1.01	1.92 ^B	95	35 ^{AB}
Ll	71.8	3.7 ^A	0.38 ^B	0.00	1.59 ^B	0.52 ^B	1.07	1.97 ^B	95	33 ^B
Lc	72.0	3.5 ^B	0.45 ^A	0.00	1.77 ^A	0.67 ^A	1.10	2.22 ^A	95	38 ^A
Lc + Ef	71.7	3.6 ^B	0.43 ^A	0.00	1.73 ^A	0.65 ^A	1.08	2.16 ^{AB}	95	38 ^A
Lc + Ll	71.7	3.5 ^B	0.46 ^A	0.00	1.75 ^A	0.65 ^A	1.10	2.21 ^A	95	37 ^A

a) : Silo : 1 liter laboratory bottles ; silo opening : 90 days after ensiling.

b), c), d) : Refer to Table 2.

e) : Control.

f) : *Enterococcus faecium*.

g) : *Lactococcus lactis*.

h) : *Lactobacillus casei*.

ABC : Means in the same column with different superscripts are significantly different (P < 0.05).

participate in the lactate fermentation of silage^{5,14,15)}. The lactococci grew vigorously only in the early stage of ensiling, but the propagation of lactobacilli continued until silage ripening⁵⁾. Therefore lactobacilli has a large function in determining the period of silage fermentation. Among lactobacilli, *Lactobacillus casei* exclusively produces L-lactate. *L. lactis* exclusively produces D-lactate, and *L. plantarum* produces a racemic mixture of L- and D-lactate¹⁵⁾. These LAB are usually present in forage crops and grasses^{3,15)} and they ferment WSC to produce either L-, D-lactate or a mixture of both during silage fermentation^{4,5,14,15)}. In the present experiment, the proportion of L-lactate was below 40% in the three silage types. Therefore, LAB which exclusively produced D-isomer had numerical or functional superiority in fermentation over LAB which exclusively produced L-isomer. DUNLOP^{7,8)} and HELINGS *et al.*¹⁰⁾ stated that

silage containing very high amounts of lactate may result in the problem of lactic acidosis in ruminants, and that the D-isomer of lactate is very slowly metabolized by gluconeogenesis. SCHAADT and JOHNSON²⁴⁾ found out that, in general, the production of lactate in silage largely involves the D-isomer. ATAKU *et al.*¹⁾ reported that on Hokkaido dairy farms, the proportions of D-lactate in corn and grass silage were 55% and 56%, respectively. In the present experiment, the proportion of D-lactate in silage showed a higher range of 62-68%. The epiphytic LAB species might change and influence the proportion of lactate isomer in silage according to the location of silage preparation and the time of ensiling.

Influence of inoculation with LAB on proportion of L-lactate in silage : The authors^{3-5,11)} of the present study have reported that when LAB (especially lactobacilli) are present in very low numbers, the inoculation of lactobacilli

generally provides beneficial effects by promoting the propagation of lactobacilli and by inhibiting the growth of harmful bacteria such as aerobic bacteria, mold and yeast as well as improving the quality of silage. HELINGS *et al.*¹⁰ reported that at least a level of 10^5 g⁻¹ lactobacilli in fresh material is required for good quality of silage. In this experiment, high numbers of aerobic bacteria (10^5 – 10^6 g⁻¹), mold and yeast (10^4 – 10^5 g⁻¹), and low numbers of lactate-producing cocci (10^3 – 10^4 g⁻¹) and lactobacilli (10^2 – 10^3 g⁻¹) were found in the fresh materials, suggesting the necessity for inoculation with LAB. In Expt. 1, the silage samples inoculated with SI and Cy demonstrated a lower pH values and a significantly higher lactate content than those of the controls. Consistent with previous reports^{4,5}, inoculation with LAB increased the numbers of LAB, leading to production of more lactate which reduced pH values. Both SI- and Cy-inoculated silage samples showed a significantly higher proportion of L-lactate to total lactate content. The cause was attributed to Lc which is a homofermentative LAB, producing exclusively L-lactate¹⁵. These results indicated that the inoculation with LAB such as Lc increased the L-isomer of lactate and improved the silage quality.

In Expt. 2, the silage samples inoculated with LAB increased lactate content. This would tend to accelerate the decline in silage pH value and hence improve the silage quality. Compared with the control, the inoculation of the LAB examined in this trial had beneficial effects on the proportion of L-lactate. However, Lc, Lc+Ef and Lc+Ll resulted in a greater increase in the proportion of L-lactate than Ef and Ll. It is assumed that lactate-producing cocci acted as a starter for lactate fermentation in silage, which created an anaerobic environment suitable for the development of lactobacilli, although it was shown that they only played a short-time role at an early stage of the fermentation processes in silage⁵. On

the other hand, lactobacilli played an important role in all fermentation processes and promoted lactate fermentation for a longer time than lactate-producing cocci⁵. In the present experiment, lactobacilli plus lactate-producing cocci did not provide greater effects than lactobacilli alone. It was concluded that high levels of lactobacilli (10^6 g⁻¹) during ensiling enhance the lactate fermentation of silage. The bacteria species of *E. faecium*, *L. lactis* and *L. casei* are homofermentative LAB and can exclusively produce L-lactate. Therefore, silage with these LAB have a higher L-lactate content as well as an increased total lactate content.

In Expt. 1 and Expt. 2, the WSC content of material was relatively low but the lactate content of silage was much higher for Italian ryegrass than corn silage. The reason for this is unclear. However, McDONALD *et al.*¹⁵ found that because of the existence of hemicellulase in Italian ryegrass, a considerable amount of the original hemicellulose could be degraded during ensiling, and suggested that there are three possible sources of hemicellulose breakdown: 1) hemicellulases present in the original herbage, 2) bacterial hemicellulases, and 3) hydrolysis by organic acid produced during fermentation. It is possible that the enzyme degraded the cell wall polysaccharides to WSC, and that if the epiphytic or inoculated LAB can grow at low pH, a large amount of lactate will be produced from the cell wall component in silage. Further studies are needed to clarify the fermentation characteristics of epiphytic LAB and the activity of cell wall degrading enzymes during silage fermentation.

The present results confirmed that the inoculation of LAB, especially lactobacilli, had beneficial effects on decreasing D-lactate content, increasing L-lactate content and improving silage quality. Therefore, in silage preparation, inoculation of the L(+) optical type of homofermentative LAB such as *L. casei* is recommended from the view point that this

will improve the silage quality and will decrease the risk of lactic acidosis in ruminants.

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サイレージにおける乳酸異性体の産生比率ならびに 乳酸菌添加が L(+) 乳酸産生比率に及ぼす影響

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愛媛県下の酪農家からイタリアンライグラスとトウモロコシサイレージ 40 点を集めて、サイレージ中の乳酸異性体の産生比率を測定した。また、乳酸菌添加がサイレージ中の L(+) と D(-) 乳酸異性体の産生比率に及ぼす影響を明らかにするために、イタリアンライグラスとトウモロコシを材料草とし、2 回の実験を行なった。実験 1 で乳酸菌剤の SI と Cy の各添加区、実験 2 で乳酸球菌の *Enterococcus faecium* と *Lactococcus lactis* および乳酸桿菌の *Lactobacillus casei* の各単独添加区と球・桿菌混合添加区（混合区）をそれぞれ設け、サイレージを調製した。酪農家サイレージの全乳酸に占める L(+) 乳酸の産生比率は 32~38% と低かった。実験 2 は実験 1 とほぼ同様な傾向を示し、乳酸菌添加によってサイレージの pH が低下し、乳酸含量が高まった。またフリーク評点が向上し、サイレージの品質が明らかに改善された。サイレージ中の L(+) 乳酸産生比率は無添加区に比べ、実験 1 で乳酸菌剤添加区が 5~10% ($P<0.05$)、実験 2 で乳酸球菌添加区が 7~11% ($P<0.05$)、乳酸桿菌区と混合区が 12~14% ($P<0.05$) の範囲で有意に増加した。一方、乳酸桿菌区と混合区の添加効果については、両者間にほとんど差がなかったので、サイレージ発酵品質の改善効果は主として *L. casei* の添加によることが示唆された。以上の結果、酪農家サイレージは D(-) 乳酸を高い比率で含有することともに乳酸菌、とくに L(+) 乳酸のみを産生する乳酸桿菌の添加はサイレージの品質と L(+) 乳酸の産生比率を高めることが明らかになった。

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