Characteristics of lactic acid bacteria strains isolated from Tibetan Plateau and their effects on silage quality of Italian ryegrass (*Lolium multiflorum* Lam.) at low temperature

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Abstract. Temperature is an important factor affecting ensilage. In cold regions, low temperature could be an adverse environmental factor during ensiling. However, little research has focused on improving silage quality at low temperatures. This study aimed to examine two lactic acid bacteria (LAB) strains (LCG9 and TG1) isolated from the Tibetan Plateau, and evaluate their effects on the silage quality of Italian ryegrass (*Lolium multiflorum* Lam.) at three temperatures (10 ℃, 15 ℃ and 25 ℃). The isolated strains and one commercial inoculant (G, *Lactobacillus plantarum* MTD-1) were evaluated by morphological, physiological and biochemical tests. Strains G, LCG9, TG1 and their combination of LCG9+TG1 were added to Italian ryegrass for ensiling 90 days at various temperatures. All the isolates could grow normally at 5-20 ℃, pH 3.5-7.0 and NaCl (3.0%, 6.5%). Strains LCG9 and TG1 were identified as *Pediococcus pentosaceus* and *Lactobacillus plantarum* by sequencing 16S rDNA, respectively. Compared to the corresponding controls, all the inoculants improved the silage quality of Italian ryegrass at different temperatures, indicated by significantly (P<0.05) higher lactic acid (LA) contents and ratios of lactic acid/acetic acid (LA/AA) and lactic acid bacteria (LAB) counts, and lower pH and ammonia nitrogen (NH_3-N) contents and undesirable microorganism counts. At 10 ℃ and 15 ℃, strains TG1 and G performed better than other inoculants, indicated by significantly (P<0.05) higher LA contents and ratios of LA/AA and LAB counts, and lower pH and NH_3-N contents. Strains TG1 and G are recommended as starter culture for Italian ryegrass silage at low temperatures.

Introduction

Silage is a very important source of forage for ruminant animals, especially in areas where animals need to be wintered for long periods. However, ensiling is a fermentation process strongly influenced by temperature, and a moderate temperature from 20 to 30 ℃ is generally preferred for silage fermentation. In practice, low temperature (<20 ℃) could hinder silage production. Such silages are often found to have high pH value, low rate of pH decline and low acid production. Low temperature could also reduce fermentation efficiency by lowering the growth rate and enzymatic activity of microorganisms. Even, the commercial lactic acid bacteria (LAB) inoculants might be inhibited and are thus not effective at low ambient temperatures. Hence, it is necessary to isolate and examine the potential LAB capable of playing a positive role under low temperature conditions. The objective of this study was to examine the characteristics of isolated LAB strains, and evaluate their effects on silage quality of Italian ryegrass (*Lolium multiflorum* Lam.) at three storage temperatures (10 ℃, 15 ℃ and 25 ℃).

Methods and Study Site

A total of 36 LAB strains were isolated from naturally-fermented common vetch and tall fescue silages at Shigatse Grassland Station (Tibet, China: N 29°16', E 88°51', elevation 3836 meters, annual mean temperature 6.5 ℃ and average annual precipitation 400 mm). The 36 LAB strains isolated were examined for low temperature resistance. Strains LCG9 and TG1 were isolated from naturally-fermented common vetch and tall fescue silages after ensiling 30 days, respectively. *L. plantarum* MTD-1, a commercial LAB inoculant (defined as G; Ecosyl. Products. Inc. Madison, WI, USA) that is a high-performance silage inoculant and works well in both high- and low-dry matter ensilage materials over a wide pH (7.5 to 3.5) and temperature (7.8 ℃ to 45 ℃) range, was used as positive control for silage making. Gram stain, colony morphology, catalase activity and gas production from glucose were determined in five replicates. Growth at different pH, temperatures and NaCl concentrations, acid production and growth rates were determined.

Italian ryegrass was harvested at the boot stage. The ensilage materials were chopped into 2-3 cm lengths with a manual forage chopper, and were immediately transported to the laboratory. LAB inoculations (G, TG1, LCG9, and LCG9+TG1) and the control at different storage temperatures (10 ℃, 15 ℃ and 25 ℃) were designed as follows: all LAB strains were inoculated at 1×10^5 colony-forming units (cfu)/g of fresh material, and the same volume of distilled water was used for the control. After inoculation, the material was mixed thoroughly. Then, about 7.50 kg of fresh material was tightly packed into a plastic polyethylene bottle (10 L capacity) and sealed with a screw top and plastic tapes. Five replicates were made for each treatment, and seventy-five bottles in total (5 treatments ×3 temperatures ×5 replicates = 75) were prepared. Silos for each treatment were stored in the incubators according to the designed temperatures for 90 days.

Table 1. The characteristics of lactic acid bacteria strains.

Items	LCG9	TG1	G
Shape	Cocci	Rod	Rod
Fermentation type	Homo	Homo	Homo
Gram stain	+	+	+
Catalase activity	-	-	-
Gas from glucose	-	-	-
Growth at temperature :			
5 ℃	+	+	-
10 ℃	+	+	+
15 ℃	+	+	+
20 ℃	+	+	+
Growth at pH:			
3.0	W	+	-
3.5	+	+	+
4.0	+	+	+
4.5	+	+	+
5.0	+	+	+
6.0	+	+	+
7.0	+	+	+
Growth in NaCl:			
3% NaCl	+	+	+
6.5% NaCl	+	+	+

G, commercial inoculant *Lactobacillus plantarum* MTD-1. +, normal growth; w, weak growth; -, no growth. Homo, homofermentative.

Results and Discussion

Isolated LAB strains

All the isolated strains could grow normally even at 5 ℃ (Table 1), indicating their psychrotolerant nature. It might be related to their long-term evolution and natural selection on the Tibetan Plateau. Essentially, it might be associated with some cold stress genes. The role for the heat shock proteins (Hsps) in preventing damage of LAB from low temperature has been reported and *L. plantarum* strains can grow better at low temperature by overproducing Hsp 18.5, Hsp 18.55 and Hsp 19.3. The unique characteristics provide enormous potential for these isolated strains in practical production.

Effect of LAB inoculation on fermentation quality

The epiphytic LAB count on Italian ryegrass was lower (10^4 cfu/g FW) than the requirement (10^5 cfu/g

FW; Table 2). Among all silages, the inferior fermentation quality could be observed in three corresponding controls, especially at 10 ℃ and 15 ℃ conditions (Table 3). The inefficient silage fermentation at low temperatures might be attributed to the thermodynamic implication that low temperature inhibits bacterial metabolism. The membranes of the microorganism were usually equipped with low proton permeability in adverse growing conditions. For most bacteria, a temperature reducing causes a transient cell growth arrest, during which the general protein synthesis is severely inhibited, and low temperature affected the viability and acidification activity of bacteria. Therefore, to improve the silage quality at low temperatures, it is necessary to enhance the quantity and activity of LAB during fermentation.

Table 2. Chemical and microbial compositions of Italian ryegrass prior to ensiling.

Items	Italian ryegrass
Chemical compositions	
Dry matter (g/kg FW)	247$\pm$ 3.13
Crude protein (g/kg DM)	67.1 $\pm$ 1.34
Water soluble carbohydrate (g/kg DM)	106.5 $\pm$ 1.68
Buffering capacity (mEq/kg DM)	88.9 $\pm$ 1.05
Microbial compositions	
Lactic acid bacteria (Log cfu/g FW)	4.37 $\pm$ 0.09
Aerobic bacteria (Log cfu/g FW)	6.43 $\pm$ 0.58
Yeasts (Log cfu/g FW)	4.61 $\pm$ 0.13
Molds (Log cfu/g FW)	4.03 $\pm$ 0.12

FW, fresh weight; DM, dry matter; mEq, milligram equivalent; log, denary logarithm of the numbers; cfu, colony-forming units.

The inoculated silages had better fermentation quality than the corresponding controls at different temperatures. It demonstrated that the silage quality of Italian ryegrass could be improved by these LAB inoculants, even at low temperatures. This is in accordance with our initial goal of isolating these LAB strains. Nevertheless, they showed different performance in silages at various temperatures.

Strains G and TG1 performed better than strain LCG9 in improving silage quality of Italian ryegrass at 10 ℃ and 15 ℃. One possibility is that strains G and TG1 had obviously faster acid production and growth rates than strain LCG9. McDonald *et al*. (1991) suggested that the competitiveness of LAB could be enhanced if they had a faster growth rate and an extended pH range. It may result in their relatively stronger competitiveness than strain LCG9 in the silage. The second possibility may be ascribed to that strains G and TG1 had wider range of carbohydrate sources than strain LCG9. The ability of LAB to utilize different substrates present in the forage crop and to produce different metabolites could be an advantage in the competition with other microorganisms, especially when limited amounts of soluble carbohydrates are available (Arriola *et al.* 2011).

Albeit the combined inoculant LCG9+TG1 performed best at 25 ℃, it performed mediocre at 10 ℃ and 15 ℃, even not as well as strain TG1 alone. It was speculated that the low temperature condition probably caused a competition relationship between strains LCG9 and TG1, and this competition weakened their combined effect.

The inoculated silages had a more homofermentative pattern evidenced by greater LA concentration and lower concentrations of acetic acids, and decreased the NH_3-N contents compared with the corresponding controls. All the inoculants increased the residual WSC contents compared to the corresponding controls.

Conclusions

Italian ryegrass is difficult to ensile under low temperature condition. All the LAB inoculants could improve the silage quality of Italian ryegrass at different temperatures (10 ℃, 15 ℃ and 25 ℃), while strain TG1 and commercial inoculant G performed best at 10 ℃ and 15 ℃. Therefore, strains TG1 and G are recommended as starter culture for Italian ryegrass silage at low temperatures, and merit further research to investigate their effect on the silage quality of other forages at low temperatures.

Table 3. Effects of LAB inoculants on the silage quality (g/kg DM) of Italian ryegrass at different temperatures after 90 days.

Treatments	pH	Dry matter (g/kg FW)	Lactic acid	Acetic acid	LA/AA	NH_3-N (g/kg TN)	WSC
10 ℃							
Control	5.87a	230	42.0h	39.6a	1.06h	135.0a	8.2g
G	3.87f	232	103.5d	13.7e	7.58f	32.1f	17.1cd
TG1	3.84fg	232	105.5d	9.5fg	11.11e	31.9f	16.9d
LCG9	4.97b	226	68.4g	16.5d	4.16g	95.9c	11.9f
LCG9+TG1	4.45d	229	88.7e	8.2gh	10.88e	83.2d	14.2e
15 ℃							
Control	4.82c	230	63.9g	34.5b	1.85h	108.2b	10.5f
G	3.83fg	228	113.2c	10.4f	10.89e	35.9ef	18.1c
TG1	3.85f	226	115.1c	7.5hi	15.40d	38.8e	20.4b
LCG9	4.11e	229	91.6e	12.8e	7.17f	33.7f	17.7cd
LCG9+TG1	3.79fg	229	108.0d	7.0hi	15.52d	33.8f	19.2bc
25 ℃							
Control	4.12e	225	77.0f	27.0c	2.85gh	83.3d	14.6e
G	3.84fg	228	137.6b	6.5hi	21.10c	33.8f	20.3b
TG1	3.83fg	230	137.5b	6.0i	23.02b	32.3f	20.5b
LCG9	3.86f	232	137.3b	6.1i	22.44bc	33.5f	20.7b
LCG9+TG1	3.76g	231	142.8a	5.2j	27.42a	22.0g	24.6a
SEM	0.09	1.70	3.42	1.59	1.23	3.26	1.63
Temperature means							
10 ℃	4.60a	230	81.6c	17.5a	6.96c	75.6a	13.7c
15 ℃	4.08b	229	98.3b	14.4b	10.17b	50.1b	17.2b
25 ℃	3.88c	228	126.4a	10.2c	19.37a	41.0c	20.2a
LAB inoculant means							
Control	4.94a	228	61.0d	33.7a	1.92e	108.8a	11.10d
G	3.85d	229	118.1a	10.2c	13.19c	33.9d	18.50b
TG1	3.84d	229	119.4a	7.7d	16.51b	34.3d	19.27a
LCG9	4.31b	229	99.1c	11.8b	11.25d	54.4b	16.77c
LCG9+TG1	4.00c	230	113.2b	6.8e	17.94a	46.3c	19.33a
Significance							
Temperature	<0.001	0.704	<0.001	<0.001	<0.001	<0.001	<0.001
LAB	<0.001	0.981	<0.001	<0.001	<0.001	<0.001	<0.001
Temperature × LAB	<0.001	0.507	<0.001	<0.001	<0.001	<0.001	<0.001

DM, dry matter; FW, fresh weight; LA/AA, lactic acid/acetic acid; NH_3-N, ammonia nitrogen; TN, total nitrogen; WSC, water soluble carbohydrates.
LCG9, *Pediococcus pentosaceus*; TG1, *Lactobacillus plantarum*; G, commercial inoculant *L. plantarum* MTD-1.
Values in the same column with different following letters are significantly different (P<0.05). SEM, standard error of mean.

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