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ORIGINAL ARTICLE

Enhancing Nutritional Quality of Silage by Fermentation with *Lactobacillus plantarum*

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Abstract The present study was aimed to investigate the nutritive profiles, microbial counts and fermentation metabolites in rye, Italian rye-grass (IRG) and barley supplemented with *Lactobacillus plantarum* under the field condition, and its probiotic properties. After preparation of silage, the content of crude protein (CP), crude ash, acid detergent fiber (ADF), and neutral detergent fiber (NDF), microbes such as lactic acid bacteria (LAB), yeast and fungi counts, and fermentation metabolites lactic acid, acetic acid and butyric acid was assessed. Results indicated that the content of ADF and NDF were significantly varied between rye, IRG and barley mediated silages. The content of CP was increased in *L. plantarum* supplemented with IRG, but slightly decreased in rye and barley mediated silages. The maximum LAB count was recorded at 53.10×10^7 cfu/g in rye, 16.18×10^7 cfu/g in IRG and 2.63×10^7 cfu/g in barley silages respectively. A considerable number of the yeasts were observed in the IRG silages than the rye silages ($P < 0.05$). The amount of lactic acid production is higher in *L. plantarum* supplemented silages as compared with control samples ($P < 0.05$). It was confirmed that higher amount of lactic acid produced only due to more number of LAB found in the silages. *L. plantarum* was able to survive at low pH and bile salt and the duodenum passage with the highest percentage of hydrophobicity. Furthermore, the strain was sensitive towards the antibiotics commonly used to maintain the microbes in food industrial setups. In conclusion, supplementation of *L. plantarum* is most beneficial in rye, IRG and barley silage preparations and probiotic characteristics of *L. plantarum* was an intrinsic feature for the application in the preparation of animal feeds and functional foods.

Keywords *Lactobacillus plantarum* · Silage quality · Rye · Italian ryegrass · Lactic acid

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Introduction

In the developing countries, the livestock sector is one of the fastest growing segments of the agricultural economy. As demand for meat and dairy products continues to increase, the questions arise as to how this demand will be met and by whom. Access to a permanent forage base is a physiological priority for ruminants and an economic priority for farmers. Forage production is seasonal in many parts of the world, with surplus available during harvest

and deficient in winter or in the dry season. Whole-crop corn, Italian rye-grass (IRG), rye, alfalfa, crimson and barley are a major crop in many dairy and meat production industry and these crops were easily fermented into the silages, which is very useful for the preparation of valuable forage for dairy cattle and Hanwoo steers. In general, silages are very stable for a long period of time. Ensiling is a common preservation method for common forage crops. It is based on the conversion of water soluble carbohydrates (WSC) by lactic acid bacteria (LAB) into organic acids. Maintaining the nutritional value (energy, dry matter and crop quality) of silages during the storage period is essential to successful ensiling programs. The importance of silage to the dairy industry implies that any undesired microbial activity in silage can affect the individual herd as well as having fundamental implications for overall profitability of the industry.

Plants contain native LAB, and when silage is placed under the anaerobic conditions, this LAB produces lactic acid and acetic acid, reducing the pH to the level in which other bacteria are not able to survive. Microorganisms like Clostridia, Enterobacteria, yeast and molds, also are present in plants and compete with LAB for sugars. The food products produced from *Lactobacillus* strains have dominated applications in many industrial sectors. The start of new frontiers in agriculture and food biotechnology, the spectrum of *Lactobacillus* strains application has widened in many other fields, such as probiotics, bio-preservatives and medicinal applications. Especially the use of LAB supplementation during silage preparation is improving the silage fermentation and stability of silage for a long time. The number of LAB present in crops prior to ensiling may be too low. For that, first ensure the rapid and efficient preservation for silage making [1]. LAB typically associated with silage; belong to the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, and *Weissella* [2]. Some of this LAB is known to be the obligate homo-fermentative and can produce more than 90 % lactic acid but are unable to ferment pentoses. Facultative hetero-fermentative LAB can also produce the acetic acid, ethanol, hydrogen and carbon dioxide. Obligate hetero-fermentative species ferment the hexoses and the pentoses into the same end-product. LAB is characterized by their acid tolerance and final pH at the end of the silage fermentation stage [3]. As no survey has been conducted to determine the number of micro biota on rye, IRG and barley crops prior to ensiling in South Korea, prediction of potential effects of a bacterial inoculants on crops is not possible. Therefore, the objective of this work was to investigate the nutritive profile, microbial counts and their fermentative metabolites in silage prepared using rye, IRG and barley crops supplemented with *Lactobacillus plantarum* under field conditions of Korean livestock farms. This study also involved in evaluating the probiotic properties of *L. plantarum.*

Materials and Methods

Strain and Cultivation Condition

Lactobacillus plantarum strain was procured from Chung-Mi Bio Co., Korea. The strain was cultivated in Man Rogosa and Sharpe agar medium (MRSA) and incubated at 30 °C for 48–72 h, and maintained at 4 °C for routine use. Alternatively, the strain was re-suspended in 20 % glycerol and stored at −80 °C until further examination.

Collection of Crops and Process of Silage Preparation

Rye, IRG and barley were harvested at heading stages from different regions of Korea. The collected samples were chopped in the field condition and shade dried for 48 h. After drying, the samples were packed with *L. plantarum* in round bale wrapper using the silage wrapping machine. Previously, sufficient amount of *L. plantarum* was dissolved in sterile water and sprayed separately at the rate of 2.5 % (2.4×10^5 cfu/g sample) into the silage and tightly packed using wrapping machine. This machine automatically sprays the microbes to the silage during the packaging time. For each control (without addition of strains) and each sample with strains were prepared in ten replicates. After 3 months of incubation in the field conditions, the nutritive values, microbial counts and quality of silage were evaluated by directly collecting the samples.

Nutrient Composition Analysis of Silages

Samples were ground to pass through a 1 mm sieve prior to analysis of nutritive values. Physicochemical parameters like acid detergent fibre (ADF), neutral detergent fibre (NDF), relative feed value (RFV), ash content and crude protein (CP) content was evaluated.

Microbial Contents

Silage samples (10 g wet weight) were transferred to 250 mL sterile flasks containing 90 mL sterile water. The suspension was kept in an orbital incubator shaker at 150 rpm for 1 h. After incubation, tenfold dilution was prepared in sterile water, and samples (0.1 mL) were placed on selective media. LAB was enumerated on de Man, Rogosa, and Sharpe agar (Diffco) and Bromocresol purple blue agar medium and incubated at microaerobic condition at 28 ± 1 °C for 4 days. Yeasts were enumerated on 3 M petrifilm (3 M Microbiology Products, St.Paul,

USA), and following aerobic incubation at 28 ± 1 °C for 4 days. Fungi were grown on potato dextrose agar (PDA) [4 g/L of potato starch (Diffco), 20 g/L of starch (Diffco), and 20 g/L of agar (Diffco)] following aerobic incubation at 28 ± 1 °C for 4 days. Anaerobic bacteria were cultivated on reinforced clostridial medium (Diffco) at 30 ± 1 °C for 4 days under anaerobic condition.

Analyses of Fermentation Metabolites

Water extracts of silage samples were prepared by weighing 20 g of silage and 80 mL of deionized water into a blender and homogenizing for 2 × 30 s. The homogenate was kept in a refrigerator at 4 °C until centrifugation (8,000 rpm at 4 °C for 20 min). The pH of the supernatant was measured after centrifugation using a combination electrode. Water extracts were stored at −20 °C with and without stabilization with 5 % meta-phosphoric acid (final concentration). Fermentation products were analysed by HPLC (HP1100 Agilent Co. USA). The HPLC analysis was carried out with a flow rate of 0.5 mL/min at a column wavelength of 220 nm. The injection sling was 10 µL. Quantification of the different organic acids was based on peak areas and calculated as equivalents of standard compounds.

Probiotic Properties

Tolerance Level of L. plantarum to Low pH

Tolerance to low pH was determined using the plate count method. Briefly, active *L. plantarum* was grown in MRS broth and was inoculated (1 %) in 10 mL of fresh MRS broth adjusted to pH 2.5 with hydrochloric acid (1.0 N) and incubated at 30 °C for 3 h. Samples were determined for their initial bacterial population and residual cell population at 0, 30, 60, 120 and 180 min respectively, by plating suitable dilutions on MRS agar plates. The plates were incubated at 30 °C for 48 h, and the number of colonies that grew was counted. The experiment was performed in triplicate.

Bile Tolerance Level of L. plantarum

The ability of *L. plantarum* to grow in the presence of two different bile salts was studied according to the method of Vinderola and Reinheimer [4] with slight modification. MRS-thio broth (MRS supplemented with 0.2 % sodium thioglycollate) and MRS-thio broth supplemented with 0.3 % (w/v) Oxgall were freshly prepared and inoculated overnight with 1 % suspensions of *L. plantarum*. Samples without Oxgall were used as a control. After 12 and 24 h incubation at 30 °C, bacterial concentration was checked by a viable count determination on MRS agar by plating suitable dilutions. The experiment was performed in triplicate.

Haemolytic Activity of L. plantarum

Freshly prepared cells at 0.5 OD_{600} nm *L. plantarum* culture was streaked on agar plates containing 5 % (w/v) blood, and were incubated for 48 h at 30 °C [5].

Proteolytic Activity of *L. plantarum*

The proteolytic activity was measured by following the method of Arasu et al. 2013 [6]. Briefly, 1 % cells of the active *L. plantarum* was transferred into 10 % skim milk and incubated at 30 °C for 42 h. The absorbance was read at 650 nm with an ELISA reader (Bio-Radad). The results were expressed as milligrams/milliliter tyrosine by means of reference to a calibration curve.

Biogenic Amine Production Level of L. plantarum

The production of biogenic amines was assessed by taking, a 0.5 optical density at 600 nm (OD_{600}) aliquot of freshly prepared *L. plantarum* cells were inoculated on MRS agar medium supplemented with 1.0 % each of lysine, tyrosine, ornithine, and histidine. Tween 80 (0.1 %) was included in the medium to enhance bacterial growth. Bromocresol purple (0.006 %) was used as the pH indicator. The formation of a clear purple halo was considered a positive reaction, indicating the presence of the amino acid decarboxylase. Medium without supplementing amino acids was used as a negative control, and the experiment was performed in triplicate.

Evaluation of Cell Surface Hydrophobicity Level of L. plantarum

The cell surface hydrophobicity assay was conducted according to the method described by Lee et al. [7], with slight modifications. Briefly, freshly prepared cells were centrifuged at 8,000 rpm for 10 min. The cells were washed twice with PBS (pH 7.0). One mL of this suspension was used to determine the absorbance at OD_{580} nm. In duplicate assessments, a further 1 mL of this suspension was added to an equal volume of n-hexadecane (Sigma, USA) and was thoroughly mixed for 2 min using a vortex. The phases were allowed to separate at room temperature for 30 min, after which 1 mL of the upper phase was removed and the absorbance was determined at OD_{580}nm. Percentage hydrophobicity was calculated as follows: (OD580 reading 1 − OD580 reading 2/OD580 reading 1) × 100 = % hydrophobicity.

Table 1 Nutrient composition of silage prepared using rye, IRG and barley

Sample	Treatment	ADF (%)	NDF (%)	C. protein (%)	C. ash (%)
Rye	Control	40.70 ± 1.33	63.05 ± 2.05	11.70 ± 0.98	9.20 ± 0.10
	Inoculation	42.48 ± 1.16	63.43 ± 1.26	10.25 ± 0.69	11.75 ± 3.73
IRG	Control	39.40 ± 1.97	61.40 ± 1.27	7.70 ± 0.51b	6.97 ± 0.42b
	Inoculation	38.76 ± 0.57	60.28 ± 0.84	10.63 ± 0.75a	8.82 ± 0.39a
Barley	Control	35.13 ± 4.91	52.94 ± 6.19	8.88 ± 0.51	10.61 ± 1.85a
	Inoculation	34.62 ± 0.68	53.74 ± 0.75	8.31 ± 0.24	6.77 ± 0.32b

Values in each column followed by the same alphabets are significantly different by T-test at $P < 0.05$

ADF, acid detergent fiber, *NDF*, neutral detergent fiver

Enzymatic Activities of L. plantarum

Enzymatic activities were assayed using the kit method according to the manufacturer's instructions (BioMerieux). Freshly prepared cells at 0.5 OD_{600} were centrifuged at 8,000 rpm for 10 min, and the pellets were suspended in sterile distilled water for screening. Enzyme activities of each LAB strain were evaluated by transferring 50 µL of active cell suspension into each well, followed by a 4 h incubation at 30 °C. After the incubation, 20 µL of ZYM-A and ZYM-B reagent was added to each well and incubated at 30 °C for 5 min to measure enzyme activity.

Antibiotic Sensitivity Pattern of L. plantarum

Antibiotic sensitivity and resistance of *L. plantarum* was assayed by disc diffusion method [8]. Briefly, cells were prepared by growing in MRS medium for 24 h at 30 °C. Petri plates were prepared with 25 mL of sterile MRS medium (Sigma). The test cultures (100 µL) of suspension containing 10^8 cfu/mL bacteria were swabbed on the top of the solidified media and allowed to dry for 10 min. Different antibiotics loaded discs were placed on the surface of the medium and left for 30 min at room temperature for the diffusion of the antibiotics. The plates were incubated for 17 h at 30 °C. After incubation, the microbes were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone given in the standard antibiotic disc chart.

Statistical analysis

The results were statistically analyzed by *T* test at $P = 0.05$ with the help of SPSS 11.5 version software package.

Results

Nutrient Profiles of Different Silages

The changes in the nutritive values such as CP, ADF, and NDF were analyzed between the treated and untreated samples of the prepared silages. The ADF determined to be 40.70 % in control and 42.48 % in the bacteria inoculated rye sample, whereas; in IRG it was 39.40 and 38.76 % respectively Table 1. The average content of NDF did not reveal significant differences between control and *L. plantarum* supplemented silages. The content of CP increased in *L. plantarum* supplemented with IRG. But in rye silage, CP level was comparatively decreased.

Microbial Contents of Different Silages

The results of microbial counts in different silages are presented in Table 2. Total LAB counts were found as a dominant in silages. The LAB counts recorded in control and inoculated rye as $36.00 \pm 9.82 \times 10^7$ cfu/g and $53.10 \pm 7.86 \times 10^7$ cfu/g and in IRG it was recorded as $5.0 \pm 0.10 \times 10^7$ cfu/g and $16.18 \pm 3.38 \times 10^7$ cfu/g respectively. Total LAB counts were significantly increased in both bacteria treated samples ($P < 0.05$). The numbers of LAB increased after experiment periods as compared with initial counts. This increase of LAB may be due to the adaptive nature of the *Lactobacillus* strains during ensilaging duration. A significant number of yeast counts were observed in silages. It indicated that the environmental conditions were enhancing the growth of microbes. The manufactured silage contains less number of fungi. It is good for quality silage preparation because of fungi secrete the variety of toxic substances which can affect the quality of silage. Present study, we have observed the minimum number of fungi in the prepared silage.

Fermentation Metabolites of Different Silages

The pH level and the proportion of lactic acid, acetic acid and butyric acid in silages are presented in Table S1. Rye and IRG were considered as the best silage quality. Among the total fermentation acids, lactic acid detected as the dominant, and significantly higher in IRG (5.65 %). The average amount of acetic acid and butyric acid were comparatively similar in both silages. The amount of acetic

Table 2 Quantitative determination of microbial population in rye, IRG and barley silages

Sample	Treatment	LAB (10^7 cfu/g)	Yeast (10^2 cfu/g)	Fungi (10^2 cfu/g)	Anaerobic bacteria (10^2 cfu/g)
Rye	Control	36.00 ± 9.82b	0.42 ± 0.13a	0.04 ± 0.01	0.7 ± 0.01
	Inoculation	53.10 ± 7.86b	0.26 ± 0.05b	0.02 ± 0.01	0.27 ± 0.01
IRG	Control	5.93 ± 0.58b	0.43 ± 0.15a	0.03 ± 0.03	0.61 ± 0.03
	Inoculation	16.18 ± 3.38a	0.25 ± 0.08b	0.02 ± 0.01	0.19 ± 0.01
Barley	Control	0.59 ± 0.14b	0.03 ± 0.03b	0.04 ± 0.02	0.69 ± 0.02
	Inoculation	2.63 ± 0.81a	0.10 ± 0.02a	0.05 ± 0.01	0.11 ± 0.01

LAB, lactic acid bacteria, Mean ± standard error. Values in each column are significantly different by *T* test at $P < 0.05$

acid production was lesser than butyric acid. Moreover, pH, lactate, acetate and total fermentation acid ratio were affecting the quality of silages. The results concluded that the addition of LAB strain enhanced the production of lactic acid which plays an important role in the quality silage preparation.

Probiotic Properties

A good probiotic bacterium must survive the passage through the stomach gastric juice with respect to low pH, presence of bile salts, pepsin and pancreatin. Results revealed that the strain was able to grow between the pH ranges from 2.5 to 4.0, which indicated its survival ability in an acidic environment (Table S2). MRS broth at pH 6.0 served as control. Results indicated that a low pH level of 2.5 had consistent improvements in the viable growth of the *L. plantarum* cells. The effect of bile salts [Oxgall (0.3 %) and sodium taurocholate (0.3 %)] on the viability of *L. plantarum* is presented in Fig. 1. The results indicated that the strain had high levels of tolerance to sodium taurocholate bile salts even after 24 h of incubation in solid medium and was slightly sensitive to oxgall. The strain showed negative results for haemolysis of agar therefore it is considered that the strain is non virulent in nature. Proteolytic activity was determined as 0.061 mg/mL tyrosine liberation. A positive result for decarboxylase activity with tyrosine was observed, and exhibited a moderate level of hydrophobicity (62 %). It showed positive responses to β-glucosidase, β-glucuronidase, and α-galactosidase activity, but did not reveal activity to alkaline phosphatase, lipase, α-fucosidase, α-chymotrypsin, β-galactosidase, or α-mannosidase activities. Among the enzymes, β-glucuronidase, α-galactosidase and β-glucosidase documented the highest activity (20 nmol of substrate hydrolysed). This strain was sensitive to all the tested antibiotics suggested that the pathogenicity of this strain is less serious. The growth of the strain was inhibited by most of the tested antibiotics and exhibited highest sensitivity towards nitrofuron antibiotics, fluroquinolones and β-lactamase inhibitors, which interrupt either protein or cell wall biosynthesis in the bacteria. Therefore this strain is considered as safe in usage as a potential probiotic.

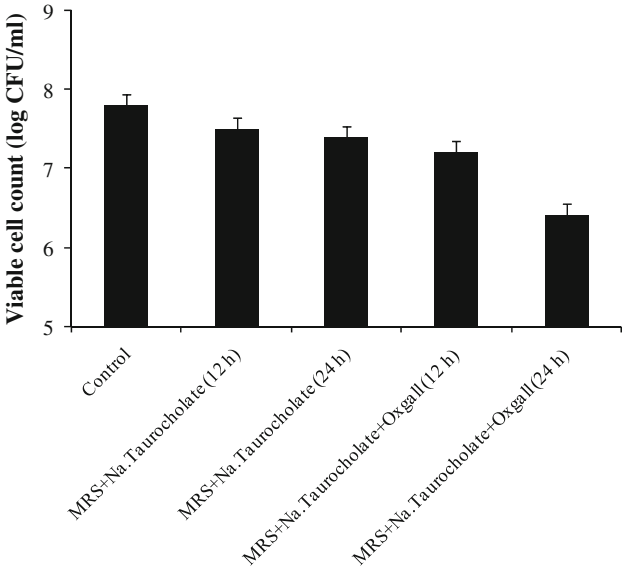

Fig. 1 Viable growth pattern of *Lactobacillus plantarum* in the presence of bile salts

Discussion

When compared to the concentrated animal feed, silage was cost effective and commonly available; therefore, farmers are very much interested in the preparation of silage using rye, IRG and barley. However, their nutritive values of these silages were altered depending upon the preparation methods and feed additives. Therefore, effect of *L. plantarum* on the preparation of silage using rey, IRG and barley were studied. The content of CA is more important than other nutrient values in the silage, because its content is associated with other materials such as soil and dust. Whereas, the results confirmed that the contents of the C. ash was higher in all the silages. Reports claimed that the addition of homo fermentative LAB enhances aerobic deterioration of silages and inhibit the growth of fungi. Indeed, acetic acid is known to play an important role in the aerobic stability of silages. The simultaneous production of lactic acid and acetic acid by the LAB maintain the aerobic stability in silages [9]. In this study, the prepared silages contained the comparable amount of

lactic acid, acetic acid and butyric acid with good stability. Results indicated that the addition of *L. plantarum* enhances the secretion of lactic acid thereby the pH decreases. Recent report claimed that, IRG extracts enhances the positive regulation of adipogenesis in 3T3-L1 cells, therefore the addition of *L. plantarum* in IRG further improves the nutritive value [10].

The nature of good probiotic microbes' was investigated under the conditions gastrointestinal tract such as stresses to microorganisms in the mouth, amylases in the oral cavity, low pH (1.5 and 3.0) in the stomach, bile secretions and pancreatic juice in the duodenal section of the small intestine. *L. plantaram* also showed a good adaptation to simulated gastric juice at pH 2.5, and bile salt tolerance, and the results are in accordance with the previous report [11]. The resistance to gastric acid is a rare probiotic property, and it is essential for the probiotic bacteria to have a protection system for low pH [12]. *L. plantaram* exhibited a moderate level of cell hydrophobicity. It is generally known that the mechanisms behind the adhesion properties of probiotic strains are the presence of complex matrix in the cell surfaces [13]. The epithelial cells present in the intestine contain binding attachment sites and the cell surface molecules such as S-layer proteins, lipoteichoic acid, and exopolysaccharides for the specific and nonspecific adhesion of gut bacteria. Extracellular mucus binding proteins such as the mannose specific adhesin (Msa) of *L. plantarum* are examples of important adhesion factors [14]. The antibiotic sensitivity pattern was found to be susceptible to most of the commonly used antibiotics which inhibit the cell wall synthesis and the protein synthesis, which is in coincides with the observations of many publications [15–17]. The antimicrobial sensitivity towards various antibiotics and negative results for haemolysis is rarely observed in food LAB.

Conclusion

LAB has several applications in the food, pharmaceutical industries. Among that *L. plantarum* has already been established as a potential source for the preparation of silages. Maintenance of nutritive values and fermentation metabolite concentration in silage has the added advantage of keeping the nutritive values and safety by inhibiting the growth of pathogenic microorganisms like fungi. Addition of *L. plantarum* maintains the nutritive values and enhances the silage fermentation, whereas the LAB count were comparatively increased from the initial inoculation, and by inhibiting the growth of other anaerobic and micro aerobic toxic metabolite producing bacteria. A result confirmed that during silage fermentation, *L. plantarum* improved silage quality and ferment to produce a good amount of lactic acid, resulting in the pH value of silage not falling to less than 3.9. Besides this, *L. plantarum* contained good functional probiotic properties like high tolerance to low pH, bile salts, sensitive towards various antibiotics and capable in producing extracellular enzymes. Therefore, *L. plantarum* holds a position for the preparation of animal foods and suitable to use various probiotic products.

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