JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 215-220

Enhancing Nutrient Quality by Combined Ensiling of Barley and Crimson Silage with *Lactobacillus plantarum* and Chlorella

Mariadhas Valan Arasu[1], Soundharrajan Ilavenil[2], Da Hye Kim[3], Hyung Su Park[2], Mariamichael Jane[2], Min-Woong Jung[2], Kyung Dong Lee[4], Naif Abdullah Al-Dhabi[1] and Ki Choon Choi[2]*

[1]Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia.
[2]Grassland and forage division, National Institute of Animal Science, RDA, Seonghwan-Eup, Cheonan-Si, Chungnam, 330-801, Republic of Korea.
[3]The United Graduate School of Agricultural Sciences, Tottori University, Tottori-Shi, 680-8553, Japan.
[4]Department of Oriental Medicine Materials, Dongsin University, Naju, 520-714, Republic of Korea.

(Received: 10 April 2014; accepted: 18 May 2014)

Effect of *Lactobacillus plantarum* together with different concentration of Chlorella on the varying proportions of barley and crimson silage quality were investigated under field condition. After silage preparation, the contents of crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), total digestible nutrient (TDN), *in vitro* dry matter digestibility (IVDMD) and microbes such as lactic acid bacteria (LAB), yeast and fungi counts, and fermentation metabolites such as lactic acid, acetic acid and butyric acids were analyzed. The nutrient profiles were significantly ($P < 0.05$) varied with respect to the concentration of Chlorella. Lactic acid bacteria counts were dominant in 0.25% Chlorella (6.99×10^7 cfu g^{-1}) in 100 % crimson and 4.74 and 2.88×10^7 cfu g^{-1} in 50% and 80% barley silages, respectively. However the counts of yeast and fungi were comparatively less. The pH ranged from 3.61-4.66 in all the prepared silages. Among the organic acids, lactic acid concentration was higher (14.59%) ranged from 2.33 to 14.59 % respectively. However, the amount of acetic acid and butyric acid was comparatively less in all the silages. It is confirmed that addition of *L. plantarum* together with Chlorella is most beneficial in barley and crimson silage preparation.

Key words: Silage quality, *Lactobacillus plantarum*, Chlorella, barley, crimson, Lactic acid.

Grasses and forages are the natural feeds for ruminants. The production of forage depends upon the season in many parts of the world, with surplus available during harvest and depleted in winter or in the summer season. Italian rey grass, barley rey, alfalfa, crimson, rice and corn are prepared for silages in many parts of the world, and are widely used as feed for dairy cow and Hanoo steers in Republic of Korea due to their high nutritional value and high ûber content. However, the preservation of forage crops as silage depends on the production of sufficient acids such as lactic acid, acetic acid and other volatile components to inhibit activity of undesirable microorganisms under anaerobic conditions. Lactic acid bacteria (LAB) play a major role in the preservation and maintaining the nutritive properties of the silages by protecting it from the undesirable microorganisms. Different species of LAB are involved in the silage fermentation are belonging to the genera *Lactobacillus*,

* To whom all correspondence should be addressed.
Tel: +82-41-580-6752; Fax: +82-41-580-6779;
E-mail: choiwh@korea.kr

Lactococcus, *Pediococcus*, *Leuconostoc*, *Enterococcus*, and *Weissella* (Tohno *et al*., 2012). Some of these LAB is known to be obligatory homo fermentative and can produce more than 90% lactic acid from water soluble components but are unable to ferment pentoses, whereas, facultatively hetero fermentative LAB can produce acetic acid, ethanol, hydrogen and carbon dioxide in addition to lactic acid. Obligately hetero fermentative species can ferment both hexoses and pentoses into the same end-product (Cai *et al*. 1999; Ennahar *et al*. 2003; Giraffa *et al*. 2010). Among the *Lactobacillus*, *Lactobacillus plantarum*, *L. brevis*, *L. casei*, *L. buchneri* and *L. pentosus* are mainly used in the preparation of silages. These *Lactobacillus* strains are characterized by their acid tolerance at pH values ranges from 3.5 – 4.0 at the end of the silage fermentation stage (Weinberg and Ashbell, 2003), whereas other unwanted bacteria such as Clostridia or Enterobacteria increase the pH value, with the acetic acid and butyric acid in silage and hence producing ammonia-N, amines, keto acids and fatty acids and decreasing the nutritional value (Arasu *et al*., 2013a; 2014a). The application of *Lactobacillus* strains in silage preparation has an advantage of its antimicrobial properties thereby inhibiting the growth of the undesirable microorganisms. These *Lactobacillus* strains also used to preserve the aerobic stability of open silos and speed the process of the secretion of organic acids such as lactic acid which preserve the silages. The growth of the LAB was achieved by the addition of growth promoting components which are the cost contributing in silage industry. Therefore, in this study, the effect of Chlorella together with *L. plantarum* on the nutritive values of barley and crimson silages were evaluated.

MATERIALS AND METHODS

Collection and preparation of silage

Lactobacillus plantarum strain was procured from Chung-Mi Bio Co., Korea. The strain was cultivated in Man Rogosa and Sharpe agar medium (MRSA) for 72 h at 30 °C. The observed pure colony was grown in liquid culture medium and stored in the lyophilized form for further experiment. For, silage preparation, fresh barley and crimson forage crops were harvested at the flowering stage and chopped into 1.0–1.5-cm pieces. The effect of *L. plantarum* on the forage crops were studied by mixing different proportions of the crops. Individually, one hundred grams of barley, one hundred grams of crimson, fifty grams of barley and fifty grams of crimson and eighty grams of barley and twenty grams of crimson were packed in an airtight bag. The lyophilized cells of *L. plantarum* (2.18×10^5) [colony forming units (CFU)/g sample], was dissolved in sterile water in sterile bottle and mixed with different concentration (0.25% and 0.5%) of Chlorella additive. The cells with Chlorella were sprayed at the rate of 2.5 % of fresh crops, and then sealed to prevent air flow. Each control (without addition of strains and Chlorella) and each of the samples with strains were prepared in triplicate. The samples were stored in underground and opened at 50 days post-ensiling for the analysis of nutrients, microbial counting and fermentation metabolites.

Analysis of nutrient composition

Physicochemical parameters like moisture content (%), dry matter (DM), acid detergent fiber (ADF), neutral detergent fiber (NDF), relative feed value (RFV), ash content and biochemical constituents like protein were evaluated by standard procedure. Samples were ground to pass through a 1mm sieve prior to analysis of nutritive values. Approximately 100g of the sample was dried at 70! for 3 days and weighed to determine the dry matter content. An NDF and ADF content on an ash-free basis was measured according to the standard method (AOAC, 1990 Arasu *et al*., 2014b).

Determination of microbial population

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 hr. After incubation, ten-fold dilutions were prepared in sterile water, and samples (0.1 mL) were plated on selective media. LAB was enumerated on de Man, Rogosa, and Sharpe agar (Diffco) and Bromocresol purple blue agar medium and incubated at micro aerobic condition at 30 ± 1°C for 3 d. Yeasts and molds were enumerated on 3M petrifilm (3M Microbiology Products, St.Paul, USA), and following aerobic incubation at 30 ± 1°C for 3 d. Coliforms (*Enterobacteriaceae*) were enumerated on McConkey agar (Merck) after aerobic incubation at 30 ± 1°C for 1 d. Fungi were

enumerated 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 30 ± 1°C for 4 d.

Fermentation metabolites analysis

Water extracts of silage samples were prepared immediately after arrival at the Institutes 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 (8000 rpm at 4°C for 20 min). The pH of the supernatant was measured after centrifugation using a pH meter. Water extracts were stored at "20°C with and without stabilization with 5% meta-phosphoric acid (final concentration) for the analysis of the metabolites. Fermentation by product lactic acid content was analysed by HPLC (HP1100 Agilent Co. USA). The contents of acetic acid and butyric acid were analyzed by Gas chromatography (GC-450, Varian Co., USA).

Statistical analysis

Numerical data obtained from the experiment was expressed as standard error of mean (mean ± SEM). Statistical difference between control and experiments were analyzed by SPSS/ 16 software hypothesis testing methods that included the analysis of variance (one ANOVA) followed by least significance difference test. P values of less than 0.05 were considered to show statistical significance.

RESULTS

Variation of nutrient profile in the silage

The changes in the nutritive values such as CP, ADF, NDF, TDN and IVDMD were analyzed between the control and different concentration of Chlorella added samples of the prepared silages (Table 1). The amount of CP was determined as 8.34, 9.61 and 8.83% for the control treatment and 10.1, 10.72 and 10.62% for 0.25% Chlorella treated silages and 10.63, 11.26 and 11.22% for 0.5% Chlorella treated silages respectively. The results clearly revealed that the nutrient profiles were significantly ($P < 0.05$) varied with respect to the concentration of Chlorella. The average content of ADF and NDF were comparatively higher in only *L. plantarum* treated barley and crimson silage and the contents of TDN and IVDMD were slightly higher in Chlorella added silages.

Microbial contents of different silages

The results of microbial counts in different combination of the silages are presented in Table 2. The results revealed that the counts of *L. plantarum* were dominant in all the combination of the silages. The highest number was noted in 100% crimson (6.99×10^7CFU/g) followed by barley (6.92×10^7CFU/g) and barley and crimson combinations (4.74×10^7CFU/g). The results were significantly ($P < 0.05$) higher than the control silages in all the treatments. Whereas, the counts

Table 1. Nutrient composition of silage prepared using different ratio of whole crop barley and forage crimson.

	Whole crop barley (100 %)					Only Crimson (100 %)				
Treatment	CP[3]	ADF[4]	NDF[5]	TDN[6]	IVDMD[7]	CP	ADF	NDF	TDN	IVDMD
	Percentage (%)									
LAB-IN[1] Control	8.34b	28.71	45.53	66.22	64.6	10.73b	28.92	37.02	66.05	79.09
CA[2] 0.25%	10.10a	27.06	44.78	67.52	64.34	12.81a	28.5	35.09	66.39	79.29
CA 0.50%	10.63a	27.07	44.81	67.51	66.39	12.76a	22.97	32.78	70.75	82.34

Whole crop barley (50%) & Crimson (50%)					Whole crop barley (80%) & Crimson (20%)				
CP	ADF	NDF	TDN	IVDMD	CP	ADF	NDF	TDN	IVDMD
Percentage (%)									
9.61b	30.72ab	49.81	64.63	64.54b	8.83c	32.42a	49.38a	63.29b	65.61b
10.72ab	31.24a	48.12	64.22	68.79ab	10.62ab	23.09b	39.49b	70.66a	71.26a
11.26a	28.06b	46.04	66.73	70.63a	11.22a	21.08b	34.85b	72.25a	73.27a

[1] addition of *Lactobacillus plantarum*, [2] Chlorella, [3] CP, Crude protein, [4] ADF; Acid detergent fiber, [5] NDF; Neutral detergent fiber, [6]TDN: Total digestible nutrient, [7]IVDMD: *in vitro* dry matter digestibility, Values in each column followed by the same alphabets are significantly different by T-test at $P < 0.05$.

Table 2. Quantitative determination of microbial count in different ratio of whole crop barley and forage crimson.

Treatment		Whole crop barley (100 %)					Crimson (100 %)				
		pH	Lactate (%/DM)	Acetate (%/DM)	Butyrate (%/DM)	Flieg2 s score	pH (%/DM)	Lactate (%/DM)	Acetate (%/DM)	Butyrate score	Flieg's
LAB-IN[1)]	Control	4.51	1.35b	0.47a	0	Excelent	3.61b	12.38b	0.66b	0.02	Excellent
	CA[2)] 0.25%	4.66	1.46ab	0.44a	0.03	Excelent	3.61b	14.59a	0.75b	0.01	Excellent
	CA 0.50%	4.33	1.85a	0.18b	0.02	Excelent	3.72b	14.46a	0.96ab	0.01	Excellent
		Whole crop barley 50% & Crimson 50 %					Whole crop barley 80% & Crimson 20%				
		4.02	2.33c	1.32a	0.02	Excelent	4.45	1.75b	0.8	0.02	Excellent
		4.19	4.81b	1.14ab	0.01	Excelent	4.26	3.92a	0.91	0.01	Excellent
		4.37	6.50a	0.92b	0	Excelent	4.11	3.78a	1.1	0.01	Excellent

[1)] addition of *Lactobacillus plantarum,* [2)] Chlorella, [3)] LAB, Lactic acid bacteria, [3)] CFU, Colony Forming Units. Values in each column followed by the same alphabets are significantly different by T-test at $P < 0.05$.

Table 3. Physiological condition and fermentative metabolite profile of different ratio of whole crop barley and crimson.

Treatment		Whole crop barley (100 %)			Forage Crimson (100 %)		
		LAB[3)] ($x10^7$CFU[4)]/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)	LAB ($x10^7$CFU/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)
LAB-IN[1)]	Control	4.10b	0.25b	0	3.28bc	0	0
	CA[2)] 0.25%	6.92a	0.20b	0.1	6.99a	0	0
	CA 0.50%	6.00a	1.16a	0	6.75a	0.2	0
		Whole crop barley 50% & Crimson 50 %			Whole crop barley 80% & Crimson 20%		
		LAB ($x10^7$CFU/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)	LAB[4)] ($x10^7$CFU/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)
		4.05b	0	0	1.05b	0	0
		4.74ab	0	0	2.88ab	0	0
		5.13a	0	0	3.16a	0.26	0

[1)] addition of *Lactobacillus plantarum,* [2)] Chlorella. Values in each column followed by the same alphabets are significantly different by T-test at $P < 0.05$

of yeast and fungi were comparatively lesser than the *L. plantarum* counts in the silages. Yeast cells were dominant in 100% barley. The addition of Chlorella significantly increases the counts of *L. plantarum* in all the treatments, therefore, it is confirmed that the Chlorella had influence in the growth of microbes. Collectively, *L. plantarum* might inhibit the growth of fungi, therefore, fungi counts were less in the silages.

Fermentation metabolites of the silages

The fermentation metabolites of different combination of the silages are shown in Table 3. Compared to the control silage the *L. plantarum* and Chlorella treated silages were well preserved and showed the pH range between 4.02 to 4.6. Control had the highest pH followed by the treated forages. In the case of 100% of barley, crimson and the mixed ratio the *L. plantarum* treatment had significantly ($P < 0.05$) higher lactic acid concentration, whereas the concentration of acetic acid was comparatively lower in the control and butyric acid concentration were significantly less. The results concluded that the addition of different concentration of Chlorella enhanced the production of lactic acid which plays an important role in the quality silage preparation.

DISCUSSION

The acidic nature of the silage is very important for the successful preservation, otherwise the spoilage microorganisms such as fungi and other anaerobic bacteria dominate when the crop moisture is relatively high, condition. The acidic condition of the silage environment prevents the survival of spoilage microorganisms because they are less tolerant to the acidic conditions than LAB (Cai *et al*., 1998; Gollop *et al*., 2005; Arasu *et al*., 2013b). Literature claimed that in the early fermentation stage, *Lactococcus* species, such as *Lactococcus lactis*, *Enterococcus faecalis*, *Pediococcus acidilactici*, *Leuconostoc mesenteroides*, and *Lactobacillus* species such as *Lactobacillus plantarum* and *L. cellobioses* grow together with aerobic microorganisms like yeasts, molds and aerobic bacteria. Under anaerobic condition, *Lactococcus* and *Lactobacillus* species population become dominant to and secrete organic acid such as lactic acid, acetic acid and succinic acid (Ohmomo *et al.* 2002). The presence of LAB is not always large enough, especially in some low WSC content and high buffering capacity (Nakui *et al.* 1988). The growth of LAB is mainly depends on the environmental condition and the presence of soluble carbohydrates together with the growth enhancing substances. In the present study, addition of *L. plantarum* strain together with different concentration Chlorella played an important role in the preservation of silage by its enhanced high cell density growth pattern. As shown in the results, after inoculation of LAB the counts were comparatively increased at day fifty. The increase in the total number of LAB confirmed that the strain was able to use the carbohydrates present in the grasses and ferment them to lactic acid and acetic acid. The metabolite profiles of the silage extracts were found to be highly dependent on whether the grass was inoculated with a *Lactobacillus* strain. Conversion of soluble carbohydrates in forages to lactic acid results in the lowest losses of dry matter and energy during silage fermentation. Previous report claimed that, good quality silage has little or no butyric acid. Similar to this statement in the present study the amount of acetic acid and butyric acid were comparatively less.

In conclusion, inoculation of homo fermentative bacteria *L. plantarum* together with varying concentration of Chlorella enhanced the nutritive values and silage fermentation, and its counts were comparatively higher from the initial inoculation, probably because this inoculant was relatively homologous and had the antagonistic property towards the epiphytic micro flora. In the present study, the *L. plantarum* treated silage's were able to improve silage quality and demonstrated that *L. plantarum* was more effective to produce lactic acid and improve fermentation quality in the silage environment.

ACKNOWLEDGEMENTS

We would like to thank the Minister for Food, Agriculture, Forestry and Fisheries, for their support to carry out this project under the grant No. PJ008445012013 & No. PJ008445032013. We also extend our gratitude to NIAS, RDA for their support.

REFERENCES

1. AOAC. Official methods of analysis (15th ed.). Association of analytical chemisits, Washington, DC, 1990.
2. Arasu MV, Jung M-W, Kim DH, Ilavenil S, Jane M, Park HS, Al-Dhabi NA, Jeon BT, Choi KC. Enhancing nutritional quality of silage by fermentation with *Lactobacillus plantarum*. *Indian J. Microbiol.*, 2014b; DOI 10.1007/s12088-014-0473-9.
3. Arasu MV, Jung MW, Kim DH, Park HS, Ilavenil S, Al-Dhabi NA, Choi K C. Identification and phylogenetic characterization of novel *Lactobacillus plantarum* species and their metabolite profile in grass silage. *Ann. Microbiol.*, 2014a; DOI 10.1007/s13213-014-0830-2.
4. Arasu MV, Kim DH, Kim PI, Jung MW, Ilavenil S, Jane M, Lee KD, Al-Dhabi NA, Choi KC. *In vitro* antifungal, probiotic and antioxidant properties of novel *Lactobacillus plantarum* K46 isolated from fermented sesame leaf. *Ann. Microbiol.*, 2013a; DOI 10.1007/s13213-013-0777-8.
5. Arasu VM, Jung MW, Ilavenil S, Jane M, Kim DH, Lee KD, Park HS, Hur TY Choi GJ, Lim YC, Al-Dhabi NA, Choi KC. Isolation and characterization of antifungal compound from *Lactobacillus plantarum* KCC-10 from forage silage with potential beneficial properties. *J. Appl. Microbiol.*, 2013b; **115** (5): 1172-1185.
6. Cai Y, Benno Y, Ogawa M, Kumai S. Effect of applying lactic acid bacteria isolated from forage crops on fermentation characteristics and aerobic deterioration of silage. *J. Dairy. Sci.*, 1999; **82**: 520-526.
7. Cai Y., Benno Y., Ogawa M., Ohmomo S., Kumai S. and Nakase T. Influence of *Lactobacillus* spp. from an inoculant and of Weissella and Leuconostoc spp. from forage crops on silage fermentation. *Appl. Environ. Microbiol.*, 1998; **64**: 2982-2987.
8. Ennahar S, Cai Y, Fujita Y. Phylogenetic diversity of lactic acid bacteria associated with paddy rice silage as determined by 16S ribosomal DNA analysis. *Appl. Environ. Microb.*, 2003; **69**:444–451.
9. Giraffa G, Chanishvili N, Widyastuti Y. Importance of lactobacilli in food and feed biotechnology. *Res. Microbiol.*, 2010; **161**(6): 480–487.
10. Gollop, N., Zakin, V., Weinberg, Z.G. Antibacterial activity of lactic acid bacteria included in inoculants for silage and in silages treated with these inoculants. *J. Appl. Microbiol.*, 2005; **98**: 662–666.
11. Nakui T, Masaki S, Aihara T, Yahara N, Takai S. The making of rice whole crop silage and an evaluation of its value as forage for ruminants. *Bull. Tohoku. Natl. Agric. Exp. Stn.*, 1988; **78**: 173–180.
12. Ohmomo S, Tanaka O, KItamoto HK, Cai Y. Silage and microbial performance, old history but new problem. *JARQ;* 2002; **40**(2): 59-71.
13. Tohno M, Kobayashi H, Nomura M, Uegaki R, Cai Y. Identification and characterization of lactic acid bacteria isolated from mixed pasture of timothy and orchardgrass, and its badly preserved silage. *J. Anim. Sci.* 2012; **83**: 318–330.
14. Weinberg ZG, Ashbell G. Engineering aspects of ensiling. *Biochem. Eng. J.* 2003; **13**: 181–188.