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Effect of Addition of Chlorella with *Lactobacillus plantarum* on Quality, Microbial Contents and Fermentation Metabolites of Barley and Pea Silages

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The present study aimed to investigate the effects of silage quality, microbial contents and fermentation metabolites of mixed ratio of barley and pea with the supplementation of chlorella with *Lactobacillus plantarum* under field conditions of livestock farmers and to monitor the responses of silage variables after forty five days. After silage preparation completed, the contents of dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), RFV (Relative feed value), crude ash (CA) 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. Results indicated that the nutritive profiles were significantly changed with respect to the concentration of chlorella. Addition of *L. plantarum* further improved the silage quality. Among the microbes, LAB showed dominant and counts were recorded in barley and pea silages (6.92 and 6.99 x 10^7 cfu g^{-1}) and 50 % barley with 50 % pea and 80 % barley with 20 % pea (6.869 and 4.12 x 10^7 cfu g^{-1}) respectively, whereas the average numbers of yeast and fungi significantly less. The pH of the samples was ranged from 3.6-5.45. Lactic acid detected as the dominant organic acids, detected higher amount (14.59%) in 0.25% chlorella supplemented pea silage. This result confirmed that silage preparation using different crops with the supplementation of chlorella and *L. plantarum* inoculation is most beneficial for farmers.

Key words: *L. plantarum*, Chlorella, Barley, Pea, Nutritive profile, Lactic acid, Acetic acid.

Microorganisms present in the silage play a key role in the successful outcome of the conservation process. They are classified into two groups based on the presence of the desirable and the undesirable microorganisms. The desirable microorganisms are the homo-fermentative lactic acid bacteria (LAB), is involved in the acidification and inhibition of spoilage microorganisms, whereas the undesirable microorganisms such as clostridia and enterobacteria, involved in anaerobic spoilage; yeasts, moulds and listeria species mainly responsible for aerobic spoilage[1]. These spoilage microorganisms, not only decrease the nutritional value of the silage, but also have a detrimental effect on animal health and/or its products. Microbial silage inoculants containing LAB have long been used to improve in silage fermentation. LAB inoculation affects not only plant fermentation but also animal performance as indicated by increased milk yield, weight gain and/

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or feed intake[2,3]. These LAB produces organic acids such as lactic acid, acetic acid and succinic acid, which reduces the surrounding environmental pH in which other bacteria cannot survive[4,5]. LAB commonly associated with silage belongs to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Enterococcus*[6]. Among the *Lactobacillus* genera, *Lactobacillus plantarum*, *L. acidophilus*, *L. casei*, *L. brevis* and *L. buchneri* classified into obligate homo-fermentative, facultative hetero-fermentative, and obligate hetero-fermentative species based on sugar fermentation[7]. An inoculation rate of 10^5–10^6 viable cells per gram crop is often sufficient for the inoculant LAB to overwhelm the epiphytic LAB and become the predominant population in the silage. LAB is characterized by their acid tolerance and final pH values reach 3.8 at the end of the silage fermentation stage[8]. As no survey has been conducted to determine the number of micro biota on barley, pea crops and different ratio 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 barley and pea crops supplemented with *L. plantarum* under field conditions of Korean livestock farms.

MATERIALS AND METHODS

Collection and preparation of silage

Lactobacillus plantarum strain was procured from Chung-Mi Bio Co., Korea. Fresh barley and pea were harvested at the flowering stage was chopped into 1.0–1.5-cm pieces. Individually, one hundred grams of barley, one hundred grams of pea, fifty grams of barley and fifty grams of pea and eighty grams of barley and twenty grams of pea were packed in an air-diffusible 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 grass, respectively, 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.

Nutrient composition analysis of silages

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[9]. 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 van Soest's procedures[10].

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 a orbital incubator shaker at 150 rpm for 1 hr. After incubation, ten-fold dilutions were prepared in sterile water by the technique of Miller and Wolin (1974), and samples (0.1 mL) were plated on selective media[11]. 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.

Analyses of metabolites

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 combination electrode. Water extracts were stored at "20°C with and without stabilization with 5% meta-phosphoric acid

(final concentration). Fermentation by product lactic acid content was analysed by HPLC (HP1100 Agilent Co. USA). The contents of acetic acid and butyric acid were analysed by Gas chromatography (GC-450, Varian Co., USA)[12].

RESULTS

Variation of nutritive profile

The change of nutritive values such as crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF) of different ratio of the silage was presented in Table 1. The CP level of only barley silage ranged 8.04, 8.66 and 9.04% for the non treated *L. plantarum*, 0.25 and 0.5% chlorella respectively, whereas, barley silage supplemented with *L. plantarum* and different concentration of chlorella significantly enhanced the CP level. Results indicated that the addition of different concentration of chlorella significantly enhanced the CP level ($p<0.05$). The average values of ADF, NDF, TDN and IVDMD did not exhibit significant variations in barley silage (100%). In the pea silage, without *L. plantarum* supplementation, the CP level was numerically higher than the CP values of the addition of *L. plantarum* together with 0.25 and 0.5% chlorella (10.73, 12.81, and 12.76% respectively) ($p<0.05$). The percentage of ADF, NDF, TDN and IVDMD did not exhibit considerable variations in the chlorella supplemented barley silage. The nutritive profile was comparatively better in an equal mixing ratio of barley and pea, whereas, higher ratio (80%) of barley and pea (20%) has less crude protein level confirmed its quality. Compared to the control treatment the addition of *L. plantarum* and proportional concentration of chlorella also had effect in the enhancement of nutritive profile in barley and pea mixed silage.

Microbial counts in different silage

The counts of lactic acid bacteria in control barley and pea were 1.59×10^5 cfu g^{-1} each whereas in *L. plantarum* added silage was 4.1×10^5 cfu g^{-1} and 3.28×10^5 cfu g^{-1} table 2. The count of yeast and fungi were comparatively less than lactobacilli. The number of *L. plantarum* in a mixed

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

Treatment		Whole crop barley (100 %)					Only forage pea (100 %)				
		CP[4]	ADF[5]	NDF[6]	TDN[7] MD[8]	IVD	CP	ADF	NDF	TDN	IVD MD
		Percentage (%)									
NON-IN[1]	Control	8.04b	28.38	46.37	66.48	64.52	11.19b	30.3	38.8	64.93	77.2
	CA[3] 0.25%	8.66b	27.34	45.02	67.3	64.7	13.02a	26.7	34.7	67.78	80.1
	CA 0.50%	9.04b	27.7	43.86	67.02	65.22	13.06a	27.1	35.1	67.52	80.05
LAB-IN[2]	Control	8.34b	28.71	45.53	66.22	64.6	10.73b	28.9	37	66.05	79.09
	CA 0.25%	10.1ab	27.06	44.78	67.52	64.34	12.81a	28.5	35.1	66.39	79.29
	CA 0.50%	10.63a	27.07	44.81	67.51	66.39	12.76a	23	32.8	70.75	82.34

	Whole crop barley (50%) & forage pea (50%)					Whole crop barley (80%) & forage pea (20%)				
	CP	ADF	NDF	TDN	IVDMD	CP	ADF	NDF	TDN	IVDMD
	10.94	27.85	39.54	70.72	66.9	9.26	#	46.71	66.7	60.9
	10.71	26.01	39.38	72.12	68.35	9.39	#	43.47	68.1	60.5
	10.96	25.21	39.3	76.28	68.98	9.53	#	44.61	67.6	62.1
	10.69	25.68	40.53	73.19	68.61	9.25	#	42.03	67	66.3
	11.32	25.01	36.14	73.94	69.14	9.68	#	43.01	68.5	69.2
	11.51	25.03	44.26	73.2	69.13	10.08	#	45.72	68.2	63.4

[1] Without addition of *Lactobacillus plantarum*, [2] addition of *Lactobacillus plantarum*, [3] chlorella, [4] CP, Crude protein, [5] ADF; Acid detergent fiber, [6] NDF; Neutral detergent fiber, [7]TDN: Total digestible nutrient, [8]IVDMD: *in vitro* dry matter digestibility, Values in each column followed by the same alphabets are significantly different by T-test at $P < 0.05$.

ratio of barley and pea were comparable. Interestingly, chlorella addition had extra influence in the growth of *L. plantarum* in all the treated condition with four fold higher in 100% barley and pea, six fold more in 50% barley and 50% pea respectively, however 80% barley and 20% pea has 2.5 fold higher in *L. plantarum* count.

Metabolite profile of different silage

The pH level and the proportion of lactic acid, acetic acid and butyric acid in total fermentation acids are presented in Table 3. Among the total fermentation acids, lactic acid detected as the dominant, ranged from 0.61-3.14.59%. The average amount of acetic acid and butyric acid were comparatively lesser than the amount of acetic acid. The amount of acetic acids was lesser compared to butyric acid. Moreover, pH, lactate, acetate and total fermentation acid ratio were affected the quality of silages.

DISCUSSION

The present study was conducted as part of a broader research objective, which is to find out how the addition of *L. plantarum* together with different concentrations of chlorella on barely and pea forage crops nutritive profile and microbial metabolites. In general, to improve the nutritive value and to reduce the risks during ensiling, silage microbial strains were used[13]. Among the strains, lactic acid bacteria have improved silage fermentation, but often reduce aerobic stability due to lower secretion of organic metabolites[14]. Reports claimed that, the lactic acid bacteria are essential for the silage fermentation[15]. During the silage fermentation process, prolong extension of the acidic nature reflects the viable count of LAB, and a lower rate of pH decline in silage allows more time for growth of anaerobic bacteria such as Clostridia species[6]. Depending on the crop, its pH in the field can range between 5 and 6, and decrease from 4.5 to 3.6 after ensiling[16]. The total counts and the relative abundance of LAB, yeast, and fungi in the present study were coincided with previous reports on the epiphytic micro flora of corn[17]. Silages had substantially greater counts of LAB, yeast, and fungi than plant sample[6,18]. The excretion of lactic acid and acetic acid together with marginal amounts of butyric acid content constitute the most important factors to reach

Table 2. Quantitative determination of microbial population in different ratio of whole crop barley and forage pea

Treatment		Whole crop barley (100 %)			Forage pea (100 %)		
		LAB[4] ($x10^7$CFU[5]/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)	LAB[4] ($x10^7$CFU[5]/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)
NON-IN[1]	Control	1.59c	0.1	0	1.59c	0.1	0
	CA[3] 0.25%	2.81c	0.07	0	3.01c	0.12	0
	CA 0.50%	3.65b	0.51	0	4.69ab	0	0
LAB-IN[2]	Control	4.10b	0.25	0	3.28bc	0	0
	CA 0.25%	6.92a	0.2	0.1	6.99a	0	0
	CA 0.50%	6.00a	1.16	0	6.75a	0.2	0

	Whole crop barley 50% & pea 50 %			Whole crop barley 80% & pea 20%		
	LAB[4] ($x10^7$CFU[5]/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)	LAB[4] ($x10^7$CFU[5]/g)	Yeast ($x10^4$CFU/g)	Fungi ($x10^4$CFU/g)
	1.59b	0.1	0	1.59b	0	0
	1.61b	0	0	2.59ab	0	0
	1.61b	0	0	3.94ab	0	0
	7.25a	0.28	0	2.67b	0	0
	6.89a	0	0	4.12a	0	0
	9.52a	0.48	0	4.21a	0	0

[1] Without addition of *Lactobacillus plantarum*, [2] addition of *Lactobacillus plantarum*, [3] chlorella, [4] LAB, Lactic acid bacteria, 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 forage pea

Treatment		Whole crop barley (100 %)					Forage pea (100 %)				
		pH	Lactate	Acetate (%/DM)	Butyrate score	Flieg2 s (%/DM)	pH score	Lactate	Acetate	Butyrate	Flieg2 s
NON-IN[1)]	Control	5.19a	0.61b	0.26	0.01	Excelent	4.25a	4.29c	1.13a	0.48	Excellent
	CA[3)] 0.25%	4.81ab	1.43a	0.42	0.03	Excelent	3.74b	10.45b	1.07a	0.03	Excellent
	CA 0.50%	4.99a	1.47a	0.3	0.06	Excelent	3.6b	13.39ab	0.71b	0.01	Excellent
LAB-IN[2)]	Control	4.51ab	1.35a	0.47	0	Excelent	3.61b	12.38b	0.66b	0.02	Excellent
	CA 0.25%	4.66ab	1.46a	0.44	0.03	Excelent	3.61b	14.59a	0.75b	0.01	Excellent
	CA 0.50%	4.33b	1.85a	0.18	0.02	Excelent	3.72b	14.46a	0.96ab	0.01	Excellent
		Whole crop barley 50% & Pea 50 %					Whole crop barley 80% & forage pea 20%				
		pH	Lactate	Acetate (%/DM)	Butyrate score	Flieg2 s (%/DM)	pH score	Lactate	Acetate	Butyrate	Flieg2 s
		5.45a	3.64c	0.57b	0.2	Excellent	5.12a	1.58c	0.49	0.04	Excellent
		4.30b	4.36bc	0.64b	0.19	Excellent	4.83ab	2.38b	0.48	0.02	Excellent
		3.99b	4.51bc	0.84ab	0.14	Excellent	4.52ab	2.31b	0.46	0.01	Excellent
		4.08b	4.13bc	0.49b	0	Excellent	4.58ab	2.17b	0.65	0	Excellent
		4.05b	5.4ab	0.94a	0.01	Excellent	4.28b	3.35a	0.51	0.01	Excellent
		3.88b	6.83a	1.07a	0	Excellent	4.3b	3.34a	0.65	0.02	Excellent

[1)] Without addition of *Lactobacillus plantarum*, [2)] addition of *Lactobacillus plantarum*, Values in each column followed by the same alphabets are significantly different by T-test at $P < 0.05$.

aerobic stability in silages[8,19]. Hetero fermentative lactic acid bacteria usually produce higher levels of acetic and lactic acid than untreated silages, which result in improved aerobic stability of the silage by inhibiting growth of yeasts at pH 4 [19]. In this study, silages contained the presence of lactic acid, acetic acid and butyric acid with good stability. However, the contents of lactic acids were comparatively higher than the other organic acid indicated the dominant of LAB. The presence of butyric acid indicates that the silage had undergone an anaerobic fermentation[6], but the concentration of butyric acid in the present study is normal as it is comparable to the well preserved silages[20].

In summary, inoculation of silage with LAB and chlorella should be encouraged in that it will result in maintenance of nutritive values, low pH and fermentation metabolite of the silage, with the added advantage by keeping the nutrients in safety by inhibiting the growth of pathogenic microorganisms like fungi. LAB treatments can be employed for improving the feeding value of low quality fibrous crop residues. In addition, focus should be given to develop a simple and economic technology for effective implementation especially at small and mixed farming systems in developing countries which may partially solve the ever increasing problems of feed crisis to livestock.

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