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Research Article
Effects of Bacterial Inoculants and Enzymes on the Fermentation, Aerobic Stability and *in vitro* Organic Matter Digestibility Characteristics of Sunflower Silages

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Abstract

Objective: This study was carried out to determine the effects of lactic acid bacteria inoculant, enzymes and lactic acid bacteria inoculant+enzymes mixture on the fermentation, cell wall content, aerobic stability and *in vitro* organic matter digestibility characteristics of sunflower silages. **Methodology:** Sunflower was harvested at the milk stage of maturity. The treatments were as follows: (1) Control (no additive), (2) Inoculation of lactic acid bacteria (LAB, 2 g t^{-1}, a mixture of *Lactobacillus plantarum* and *Enterococcus faecium* applied at a rate of 6.00 $\log_{10}$ CFU LAB g^{-1} of fresh material) (3) Enzyme (E, 150000 CMCU kg^{-1} for cellulase and 200000 SKB kg^{-1} for amylase) and (4) LAB+enzyme mixture (LAB+E, 2 g t^{-1} a mixture of *Lactobacillus plantarum* bacterium (6.00 $\log_{10}$ CFU g^{-1}) and 150000 CMCU kg^{-1} for cellulase and 200000 SKB kg^{-1} for amylase). After treatment, the chopped sunflower was ensiled in 1.0-l special anaerobic jars, equipped with a lid enabling gas release only. The jars were stored at 25±2°C under laboratory conditions. Three jars from each group were sampled for chemical and microbiological analysis for 2, 4, 8 and 60 days after ensiling. At the end of the ensiling period all silages were subjected to an aerobic stability test for 5 days. **Results:** In addition, *in vitro* organic matter digestibilities of these silages were determined. Both inoculants (LAB and LAB+E) increased characteristics of fermentation but impaired aerobic stability of sunflower silages. Lactic acid bacteria+enzymes mixture inoculants decreased neutral and acid detergent fiber content and than control silages. **Conclusion:** *In vitro* organic matter digestibility was numerically increased for treated than control silages.

Key words: Silage, sunflower, lactic acid bacterial inoculants, enzyme, fermentation, aerobic stability

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ensiling is a preservation technology for moist whole-plant forage crops which is based on lactic acid fermentation under anaerobic conditions, whereby Lactic Acid Bacteria (LAB) convert Water Soluble Carbohydrates (WSC) into organic acids, mainly lactic acid. As a result, pH decrease and thus forage is preserved for a long time[1]. The application of silage additives has become the conventional implement to control the ensiling process. Although the main objective in using silage additives is to ensure the fermentation process to produce well preserved silages, attention is also paid to methods of reducing ensiling losses and improving aerobic stability of silages during the feed-out period[2]. In order to improve the ensiling process various chemical and biological additives have been developed. Biological additives are advantageous because they are safe and easy to use, are non-corrosive to machinery, do not pollute the environment and are natural products[3]. Bacterial inoculants generally increase lactic acid and reduce pH, acetic acid, butyric acid and ammonia-nitrogen levels in silage[4,5]. Inoculation of forage crops with homofermentative LAB can improve silage fermentation if sufficient fermentable substrate (WSC) is available. Enzyme (E) mixture can partially degrade plant carbohydrates (cellulose, hemicellulose, pectin and starch) to release sugars for bacteria fermentation and should, therefore, act additively with inoculants LAB[6]. When LAB is combined with cell wall degrading enzymes a stronger effect should be expected by releasing fermentable sugars to produce more lactic acid in proportion to other products[7-9].

Sunflowers have been grown successfully as silage crop in many parts of the world. Sunflower, in comparison to corn, provides high dry matter yield and has better cold tolerant and more drought resistant. High fiber content of sunflower silage cause decreases in digestibility of nutrient matters[10].

The aim of this study was to determine the effects of LAB, enzymes and LAB+enzymes mixture on the fermentation, aerobic stability and nutritive value of sunflower silage.

MATERIALS AND METHODS

Sunflower forage (at the milk stage) was harvested by hand and cropped with laboratory type cropped to about 2.0 cm size and ensiled in 1.0 L special anaerobic jars (Weck, Wher-Oftlingen, Germany), equipped with a lid that enables gas release only. Each jar filled with about 450 g (wet weight) of cropped forage, without a head space. There were 48 jars per crop and they were stored at ambient temperature (25±2°C). Fresh and ensiled material (on days 2, 4, 8 and 60 after ensile, three jars per treatment for each time) were sampled for chemical and microbiological analysis. At the end of the ensiling period, the silages were subjected to an aerobic stability test for 5 days in a system developed by Ashbell *et al.*[11]. In this system, the numbers of yeasts and molds, change in pH and amount of CO_2 produced during the test are used as aerobic deterioration indicators.

The treatment groups were as follows: (1) Control (C), no additive, (2) Inoculation of lactic acid bacteria (LAB, 2 g t^{-1}, a mixture of *Lactobacillus plantarum* applied at a rate of 6.00 log_{10} CFU LAB g^{-1} of fresh material, Silaid WSTM, Global Nutritech Co., USA) (3) Enzyme (E, 150000 CMCU kg^{-1} for cellulase and 200000 SKB kg^{-1} for amylase, Silaid WSTM, Global Nutritech Co., USA) and (4) LAB+enzyme mixture (LAB+E, 2 g t^{-1} a mixture of *Lactobacillus plantarum* bacterium (6.00 log_{10} CFU g^{-1}) and 150000 CMCU kg^{-1} for cellulase and 200000 SKB kg^{-1} for amylase, Silaid WSTM, Global Nutritech Co., USA). The LAB, enzyme and LAB+enzyme were dissolved in 20 mL water and sprayed on the chopped sunflower fresh materials.

The pH values and ammonia nitrogen (NH_3-N) content of fresh and silage samples was determined, according to Anonymous[12]. The WSCs content of silages was determined by spectrophotometer (Shimadzu UV-1201, Kyoto, Japan) after reaction with an antron reagent[12]. Lactic and acetic acid were determined by the spectrophotometric method[13]. Laactobacilli, yeast and mold numbers were obtained according to the methods reported by Seale *et al.*[14]. The microbiological examination included enumeration of lactobacilli on pour plate Rogosa agar (Oxoid CM627 incubated at 30°C for 3 days), yeast and molds on spread plate malt extract agar (acidified with LA to pH 4.0 and incubated at 30°C for 3 days). The lactobacilli mold and yeast numbers of the silages were converted into logarithmic coli form unit (CFU g^{-1}). The DM content of the fresh and silage materials was determined by drying at 60°C for 72 h in a fan-assisted oven, followed by milling through a 1 mm screen and drying for another 3 h at 103°C. Ash content was obtained after 3 h at 550°C. Crude Protein (CP) content were determined following the procedure of Association of Official Analytical Chemists[15]. Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) was performed according to Goering and van Soest[16]. *In vitro* Organic Matter (OM) digestibility of the silages was determined with the procedure reported by Aufrere and Michalet-Doreau[17], with a three-stage technique: Pre-treatment with pepsin in hydrochloric acid (0.2% pepsin in 0.1 N HCl), starch hydrolysis, attack by cellulase (Onozuka R 10 from trichoderma viride, Merck).

The statistical analysis of the results included one-way analysis of variance and Duncan's multiple range tests, which were applied to the results using the Minitab statistical package program[18].

RESULTS AND DISCUSSION

The chemical composition of the fresh and ensiled sunflower is given in Table 1. The sunflower used for ensiling was characterized by DM content of 19.97%, concentration of CP of 10,40% and concentration of WSCs of 44.2 g kg^{-1} DM. The composition of structural carbohydrate in the cell wall was 42.93% NDF and 38.76% ADF in DM. All silages were well preserved. The addition of LAB, enzyme and LAB+enzyme mixture at ensiling improved the fermentation parameters of sunflower silages, with increasing lactic acid levels and decreasing acetic acid, NH_3-N and pH values ($p<0.05$) compared to control silage. The WSCs in all silages decreased with the decrease in pH. The addition of enzyme at ensiling had significantly higher WSCs compared with the LAB silage ($p<0.05$).

Microbial additives, such as bacterial inoculants have been added to silage in order to stimulate lactic acid fermentation, accelerating the decrease in pH and thus improving silage preservation[3]. The same trend was shown in this experiment. Both LAB inoculants (LAB and LAB+E) ensured rapid and vigorous fermentation that resulted in faster accumulation of lactic acid, lower pH values at an earlier stage of ensiling and improved forage preservation. Acetic acid and NH_3-N concentration in silage are also important criterions for evaluating silage fermentation quality. High concentration of acetic acid (>3-4% of DM) probably leads to poor energy and DM recovery[19]. In the present study, the concentrations of acetic acid of sunflower silages were significantly increased in control silage compared with other groups. Silage NH_3-N concentration, which reveals the extent of proteolysis in silage was significantly lower in silage treated with LAB, E and LAB+E compared with control. The low NH_3-N concentration may attribute to the pH sharp decline which made aerobic microorganism and plant enzymes inhibit rapidly, resulting in reduction in protein degradation during fermentation process[20]. Cell wall degrading enzymes, such as cellulases and hemicellulases, applied to herbage before ensiling decreased the cell wall content of ensiled crops[21]. Including cell wall degrading enzymes in silage additives has been used to increase WSCs available to LAB and as a method to degrade cell wall and subsequently improve the digestibility of OM and fiber[2,22,23]. In some studies, enzyme and LAB+enzyme mixture inoculants decreased cell wall contents of silages[24,25]. In contrast to these researcher's findings, some reports show that inoculants did not decrease significantly cell wall contents of silages[7,26]. At the end of the ensiling period, treatment with LAB+E mixture significantly decreased NDF and ADF concentration of sunflower silages compared with the control silages in present study.

The microbiological composition of the silages is given in Table 2. Lactobacilli numbers increased during the fermentation period. In the present study, the LAB and/or E treated silages increased lactobacilli of sunflower silages compared with the control silage ($p<0.05$). The addition of LAB and/or E had no influence on yeast numbers of the silages ($p>0.05$).

However, LAB, E and LAB+E mixture inoculants improved microbiological composition of sunflower silages compared with control silage. At the end of the ensiling period all the treatment (LAB, E and LAB+E) increased lactobacilli numbers of sunflower silages compared with the control silage. The addition of LAB or E had no influence on yeast numbers.

Table 3 gives the results of the aerobic exposure test. The pH change, CO_2 production and an increase in yeast and mold numbers are indicators of silage deterioration. In the present study, the LAB and/or E treated silages increased significantly CO_2 production, yeast and molds numbers in the sunflower silages compared to the control silage ($p<0.05$).

Aerobic deterioration of silage is a complex process which depends on many factors. Usually it is initiated by aerobic yeasts that can use either residual WSCs or lactic acid for their metabolism. Aerobic deterioration usually results in production of CO_2 and consequent DM losses[27]. Treatment with E and LAB+E mixture had high contents of both residual WSCs and lactic acid and therefore, tended to spoil more upon aerobic exposure, as indicated by more intensive CO_2 production.

Values for *in vitro* OM digestibility are given in Table 4. Inoculation with the LAB and or LAB+E did not affect *in vitro* OM digestibility ($p>0.05$).

There are various reports indicating that LAB or enzyme did not effect ruminal DM and OM degrabilities or digestibility of silages[24,28], however in some studies, LAB or enzymes treated silage improved, degradability or digestibility[9,22]. In the present study, the *in vitro* organic matter digestibility was numerically increased for treated than control silages.

Table 1: Results of the chemical analyses of the sunflower silages

Days of ensiling		pH	DM (%)	WSCs (g kg^{-1} DM)	NH_3-N (g kg^{-1} TN)	LA (%)	AA (%)	CP (%)	CA (%)	NDF (%)	ADF (%)
0	Treatments	5.52	19.97	44.2	-	0.70	0	10.40	11.91	42.93	38.76
2	Control	4.92±0.03^{a}	19.59±0.30	37.60±1.27	41.30±3.43	1.63±0.10	1.04±0.05^{a}	10.43±0.32	11.75±0.17	43.59±0.48	39.17±0.22
	LAB	4.38±0.03^{c}	19.60±0.14	39.06±2.18	30.86±2.99	2.06±0.08	0.50±0.09^{b}	10.31±0.20	12.23±0.26	42.49±0.43	37.76±0.71
	E	4.67±0.03^{b}	19.49±0.29	39.98±1.06	34.89±3.14	1.87±0.10	0.42±0.05^{b}	10.19±0.31	11.67±0.21	42.85±0.17	37.88±0.44
	LAB+E	4.39±0.03^{c}	19.46±0.17	38.92±1.35	30.87±1.12	1.97±0.13	0.76±0.05^{b}	10.31±0.38	12.24±0.33	43.31±0.30	37.84±0.49
4	Control	4.74±0.04^{a}	19.65±0.30	25.87±1.04^{b}	60.26±3.10^{a}	1.83±0.06^{b}	1.28±0.09^{a}	10.08±0.32	11.87±0.25	42.71±0.83	38.07±1.02
	LAB	4.20±0.05^{c}	19.51±0.14	36.63±0.97^{a}	24.50±1.42^{b}	2.96±0.12^{a}	0.69±0.06^{b}	10.40±0.20	11.75±0.36	42.67±0.77	37.76±0.68
	E	4.50±0.04^{b}	19.55±0.29	33.67±1.22^{a}	46.22±4.72^{a}	1.99±0.13^{b}	0.77±0.12^{b}	10.27±0.31	11.93±0.05	41.56±0.65	37.20±0.44
	LAB+E	4.19±0.03^{c}	19.54±0.17	24.73±1.42^{b}	25.94±1.89^{b}	2.81±0.06^{a}	0.85±0.03^{b}	10.33±0.38	11.90±0.20	41.16±0.16	38.04±0.60
8	Control	4.50±0.03^{a}	19.47±0.18	18.90±0.76^{b}	90.63±4.10^{a}	2.98±0.12^{b}	1.64±0.09^{a}	9.96±0.320	11.79±0.14	42.53±1.14	38.05±0.91
	LAB	4.22±0.05^{b}	19.39±0.15	24.60±0.89^{a}	41.80±3.88^{c}	4.34±0.10^{a}	1.20±0.05^{b}	10.33±0.20	11.76±0.18	42.56±2.14	37.17±1.67
	E	4.36±0.04^{ab}	19.76±0.23	26.73±0.71^{a}	63.39±3.06^{b}	4.15±0.15^{a}	1.18±0.04^{b}	10.22±0.31	12.03±0.21	40.68±1.28	36.17±0.63
	LAB+E	4.19±0.04^{b}	19.21±0.16	18.07±1.01^{b}	43.95±1.58^{c}	4.48±0.15^{a}	1.32±0.08^{b}	10.26±0.38	12.05±0.11	42.08±0.68	38.81±0.54
60	Control	4.41±0.05^{a}	18.98±0.25	15.70±0.87^{ab}	102.39±3.35^{a}	3.91±0.15^{c}	2.80±0.15^{a}	10.25±0.32	11.91±0.14	43.03±1.10^{a}	38.70±0.32^{a}
	LAB	4.09±0.05^{b}	19.34±0.11	13.80±0.85^{b}	53.66±4.62^{c}	5.60±0.14^{a}	1.44±0.07^{b}	10.01±0.20	11.38±0.27	40.36±1.18^{ab}	36.79±0.70^{ab}
	E	4.19±0.06^{b}	19.20±0.02	18.03±0.81^{a}	81.05±2.89^{b}	4.92±0.05^{b}	1.74±0.10^{b}	10.27±0.31	11.67±0.25	40.97±0.79^{ab}	38.17±0.42^{ab}
	LAB+E	4.10±0.07^{b}	19.32±0.20	16.20±0.79^{ab}	57.64±2.44^{c}	5.29±0.11^{ab}	1.48±0.05^{b}	10.34±0.38	11.76±0.18	38.52±0.41^{b}	35.89±0.68^{b}

LAB: Lactic acid bacteria, E: Enzyme, DM: Dry matter, WSCs: Water-soluble carbohydrates, NH_3-N: Ammonia-nitrogen, TN: Total nitrogen, LA: Lactic acid, AA: Acetic acid, CP: Crude protein, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, $^{a-c}$Within a column means followed by different letter differ significantly ($p<0.05$)

Table 2: Results of the microbiological analysis of the sunflower silages (log CFU g^{-1} DM)

Days of ensiling	Treatments	Lactobacilli	Yeast	Mold
0		4.2	2.8	>2.0
2	Control	4.9±0.07[b]	2.1±0.17	ND
	LAB	7.0±0.39[a]	2.0±0.03	ND
	E	5.1±0.04[b]	2.6±0.28	ND
	LAB+E	7.0±0.23[a]	2.2±0.16	ND
4	Control	5.3±0.28[b]	3.2±0.10	ND
	LAB	6.9±0.25[a]	3.1±0.11	ND
	E	5.5±0.05[b]	3.3±0.15	ND
	LAB+E	7.4±0.06[a]	3.3±0.16	ND
8	Control	5.4±0.05[b]	3.7±0.28	ND
	LAB	6.8±0.14[a]	4.4±0.20	ND
	E	6.4±0.20[a]	4.2±0.10	ND
	LAB+E	6.6±0.17[a]	4.0±0.15	ND
60	Control	5.7±0.02[b]	5.2±0.20	ND
	LAB	7.6±0.08[a]	4.9±0.06	ND
	E	7.5±0.06[a]	4.9±0.02	ND
	LAB+E	7.2±0.34[a]	4.8±0.25	ND

LAB: Lactic acid bacteria, E: Enzyme, NF: Not detection, [a-b]Within a column means followed by different letter differ significantly ($p<0.05$)

Table 3: Results of the aerobic stability test (5 days) of the sunflower silages

Treatments	pH	CO_2 (g kg^{-1} DM)	Yeast (log CFU g^{-1} DM)	Molds (log CFU g^{-1} DM)
Control	5.10±0.10	30.30±4.40[b]	5.9±0.51[b]	4.6±0.41[b]
LAB	5.20±0.12	38.73±3.71[ab]	7.5±0.33[a]	5.8±0.41[a]
E	5.38±0.21	45.77±2.90[a]	7.1±0.30[a]	6.2±0.42[a]
LAB+E	5.21±0.11	43.23±2.25[a]	7.8±0.31[a]	5.8±0.33[a]

LAB: Lactic acid bacteria, E: Enzyme, [a-b]Within a column means followed by different letter differ significantly ($p<0.05$)

Table 4: *In vitro* OM digestibility of the ensiled sunflower after 60 days of ensiling (%)

Treatment	*In vitro* OM digestibility
Control	53.32±0.80
LAB	53.81±2.00
E	55.55±1.46
LAB+E	54.82±1.12

LAB: Lactic acid bacteria, E: Enzyme

CONCLUSION

In conclusion, the result of this study show that both LAB inoculants and enzyme increased characteristics of fermentation, but impaired aerobic stability of sunflower silages. The LAB+enzyme mixture inoculants decreased NDF and ADF content of sunflower silages.

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