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Scientific Research

Effect of Cellulase and Lactic Acid Bacteria on Fermentation Quality and Chemical Composition of Wheat Straw Silage

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Abstract

The object of this study was to determine the effect of cellulase and lactic acid bacteria (LAB) on fermentation quality and chemical composition of wheat straw silage. Silages were prepared using a small-scale fermentation system and the moisture level was adjusted to 60% of fresh matter (FM) with deionized water. Treatments were designed as: control silage without additives, LAB inoculant *Lactobacillus casei* Z3-1 (1.0×10^6 cfu·g^{-1} of FM), commercial inoculant *L. plantarum* FG 1 (1.0×10^6 cfu·g^{-1} of FM), Z3-1 + cellulase and FG 1 + cellulase. The neutral detergent fiber (NDF), acid detergent fiber (ADF) and crude protein (CP) contents of the wheat straw prior to ensiling were 76.93%, 48.52% and 4.63% of dry matter (DM), respectively. After 30 days of fermentation, the silages treated with LAB and LAB + cellulase had a lower ($P < 0.05$) pH and higher ($P < 0.05$) lactic acid content than the control, and the coliform bacteria, yeast and mold were inhibited at the early stage of fermentation. Besides, silages treated with cellulase had lower ($P < 0.05$) values of ADF and NDF than the control. The results confirmed that the addition of cellulase and LAB contributed to improving the fermentation quality of wheat straw silage.

Keywords

Cellulase, Chemical Composition, Lactic Acid Bacteria, Silage Fermentation, Wheat Straw

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1. Introduction

In China, the annual yield of wheat (*Triticum aestivum* L.) straw is approximately 115.4 million tons [1]. However, about 50% of this abundant crop by-product is deserted directly in the field or burned for cooking, which cause seriously environmental problems [2]. With the rapid development of animal husbandry, feed shortage has become a limiting factor for livestock production [3]. Therefore, it is necessary to develop silage preparation technique to effectively use these local available feed resources for livestock production in China.

Ensilage has long been used to preserve forage crops for livestock. During fermentation process, the epiphytic lactic acid bacteria (LAB) on forage crops utilizes water-soluble carbohydrate (WSC) to produce lactic acid, the primary acid responsible for decreasing the pH in silage [4]-[6]. Silage additives have also been used to obtain high-quality silage, and inoculants and cellulase are the most popular additives on silage [4]. LAB inoculants applied to forage at ensiling promote homofermentation of major WSC transfer to lactic acid, which causes a rapid pH decline [7]. Wheat straw is mainly composed of cellulose, hemicellulose and lignin; hence, its low digestibility prevents the use in feedlots. The addition of cellulase to silage can partially degrade fiber to fermentable WSC used by LAB [8]. The mixtures of inoculants and cellulase had also been used to improve the fermentation quality of silage.

In the present study, we aimed to examine the effect of LAB and cellulose on the fermentation quality and chemical composition of wheat straw silage by using a small-scale fermentation system.

2. Materials and Methods

2.1. Materials and Silage Preparation

The wheat (cultivar: Yumai 19) straw was collected from a local farmland (Zhongmou County, Henan Province, China) on June 4, 2012. Deionized water was sprayed to the wheat straw and the final moisture level was approximately 60% of the fresh wheat straw. A commercial cellulase made from *Trichoderma reesei* (Heilongjiang Zhaodong Enzyme Product Co. Ltd, Zhaodong, China), and two selected strains: Z3-1 (*Lactobacillus* (*L.*) *casei*, Zhengzhou University, Zhengzhou, China), isolated from corn and FG 1 (*L. plantarum*) from a commercial inoculant (Chikuso-1, Snow Brand Seed Co. Ltd., Sapporo, Japan), were used as additives. Experimental treatments included control silage without additives, wheat straw + cellulase, wheat straw + Z3-1, wheat straw + FG 1, wheat straw + cellulase + Z3-1 and wheat straw + cellulase + FG 1 silages.

The lactobacilli de Man, Rogosa, Sharpe (MRS) broth inoculated with strains Z3-1 and FG1 was incubated overnight and the inoculum volume of LAB was 1 ml of suspension per kilogram of FM. The numbers of inoculated LAB Z3-1 and FG 1 were both 1.0×10^6 colony-forming units per (cfu) g of FM, respectively, while the added dosage of cellulase was 1.0 $g \cdot kg^{-1}$ of the fresh wheat straw. Silages were prepared using a small scale system of silage fermentation, approximately 100g portions of forge material chopped into about 20-mm length and packed into plastic bags. The bags were sealed with a vacuum sealer. The plastic bags were stored at room temperature and ten bags were used per treatment.

2.2. Morphological, Physiological and Biochemical Tests

Gram-staining response of LAB was examined after 24 h of incubation on MRS agar. Catalase activity and gas production from glucose were determined using the methods of Cai *et al.* [3]. Growth at different temperatures was observed in MRS broth after incubation at 5˚C and 10˚C for 10 days, and at 45˚C and 50˚C for 7 days. Growth at pH 3.0, 3.5, 4.0, 4.5, 5.0 and 8.0 was observed in MRS broth after incubation at 30˚C for 7 days. Salt tolerance of LAB was tested in MRS broth containing 3.0% and 6.5% NaCl.

2.3. Microbiological Analysis

One bag per treatment was opened on day 3, 5, and 10, and 3 bags per treatment were opened in the 30th day, respectively. The wheat straw sample (10 g) was blended with 90 ml of sterilized water, and serially diluted from 10^{-1} to 10^{-5} in sterilized water. The number of LAB was measured by plate count on MRS agar incubated at 30˚C for 48 h under anaerobic conditions (DG 250/min MACS, Don Whitley Science, England). Coliform bacteria were counted on blue light broth agar (Nissui Ltd., Tokyo, Japan), incubated at 30˚C for 48 h. Mold and

yeast were counted on potato dextrose agar (Nissui), incubated at 30˚C for 24 h, and yeast were distinguished from mold and other bacteria by colony appearance and the observation of cell morphology. Bacilli and aerobic bacteria were counted on nutrient agar (Nissui), incubated at 30˚C for 24 h under aerobic conditions. Colonies were counted as viable numbers of microorganisms in cfu·g^{-1} of fresh matter.

2.4. Chemical and Statistical Analysis

After 30 d of storage, three bags per treatment were opened for analyzing the chemical composition and fermentation quality. The wheat straw silage samples were dried in a forced-air oven at 65˚C for 48 h and ground to pass a 1-mm screen with a Wiley mill (ZM200, Retsch GmbH, Germany). Dry matter (DM), crude protein (CP), ether extract (EE) and organic matter (OM) were analyzed according to AOAC Methods 934.01, 976.05, 920.39 and 942.05, respectively [9]. The crude fiber was analyzed by the methods of Soest *et al*. [10]. Wet silage (10 g) was homogenized with 90 ml sterilized distilled water. The pH was measured with a glass electrode pH meter (pH 213, HANNA, Italy), and ammonia-N was determined by steam distillation of the filtrates. The organic acid contents were measured by HPLC (1200 series, Agilent, American) according to the methods described by Cai *et al*. [4].

The variance analysis and multiple comparisons of data were performed by the GLM procedures of SAS (SAS Institute Inc., Cary, NC, US).

3. Results

3.1. Characteristics of LAB Used in This Experiment

Morphological, physiological and biochemical properties of LAB used in this experiment are shown in **Table 1**, both of the isolated strains Z3-1 and FG 1 were Gram-positive, catalase-negative, homofermentative LAB and could not grow at temperatures of 5˚C, 10˚C and 50˚C. The strain Z3-1 could grow at the salinity of 6.5% and pH 3.0, while FG 1 could not.

3.2. Chemical Composition of the Wheat Straw

The chemical composition of the wheat straw prior to ensiling is shown in **Table 2**. The DM and OM contents of the wheat straw were 91.61% and 92.42% on DM basis, acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents were 48.5% and 76.9% on DM basis, respectively. However, contents of CP and EE were relatively low (4.63% and 0.49% of DM, respectively).

3.3. Changes in pH and the Microbiological Composition during Silage Fermentation

Changes in pH and the microbiological composition during silage fermentation are shown in **Table 3**. All the pH values of silages declined sharply after 3 days of fermentation, but at the day 30, the values of LAB (Z3-1 and of FG 1)- and cellulase + LAB-treated silages decreased to near 4.0, while the control and the cellulase-treated silages were 5.02 and 4.87, respectively. Before ensiling, 10^3 - 10^4 (cfu·g^{-1} of FM) LAB and mold and 10^5 coliform bacteria, aerobic bacteria and yeast were found in the fresh wheat straw. During the first 3 days of fermentation, numbers of LAB increased rapidly and reached to 10^8 - 10^9 cfu·g^{-1} of FM in all silages; at the day 5 fermentation, they remained at this high level, however, after 30 days fermentation, LAB in these silages decreased to 10^4 - 10^6 cfu·g^{-1} of FM. Mold and coliform bacteria were not detected in almost all silages after 3 days of fermentation. At the 30 days, the numbers of aerobic bacteria decreased to 10^3 - 10^4 cfu·g^{-1} of FM. Yeast were not detected in all the silages after 30 days of fermentation, except the control and cellulase-treated silages ranging from 10^4 to 10^5 cfu·g^{-1} of FM.

3.4. Chemical Composition and Fermentation Quality of Wheat Straw Silages Treated with LAB and Cellulase after 30 Days of Fermentation

The chemical composition and fermentation quality of wheat straw silages treated with LAB and cellulase after 30 days of fermentation are shown in **Table 4** and **Table 5**. The OM and EE contents did not differ among these six treatments. The CP contents of the LAB- and cellulase + LAB-treated silages were higher than control and

Table 1. Morphological, physiological and biochemical properties of LAB used in this experiment.

Characteristics	Z3-1	FG 1
Source	Rice silage	A commercial inoculation
Species	*Lactobacillus casei*	*Lactobacillus plantarum*
Shape	Rod	Rod
Gram stain	+	+
Gas from glucose	−	−
Catalase	−	−
Fermentation type	Homo	Homo
Growth at temperature		
5˚C	−	−
10˚C	−	−
15˚C	w	w
45˚C	+	+
50˚C	−	−
Growth at pH		
3.0	+	−
3.5	+	+
4.0	+	+
5.0	+	+
6.0	+	+
8.0	+	+
Growth in NaCl (%)		
3.0	+	+
6.5	+	−

+: positive; −: negative; w: weakly positive.

Table 2. Chemical composition of wheat straw prior to ensiling.

Item	Wheat straw
DM, %	91.61 ± 0.32
OM, % of DM	92.42 ± 0.54
CP, % of DM	4.63 ± 0.22
EE, % of DM	0.49 ± 0.03
ADF, % of DM	48.52 ± 1.07
NDF, % of DM	76.93 ± 1.22

DM: dry matter; OM: organic matter; EE: ether extract; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber.

cellulase-treated silages ($P < 0.05$). The ADF and NDF contents of the cellulase-treated silages were lower than control ($P < 0.05$).

The pH values of the silages treated with cellulase and LAB were lower than that of the control; the lowest pH value was found in the LAB- and LAB + cellulase-treated silages, nearly reaching to 4.0. The lactic acid content was highest ($P < 0.05$) in the silages treated with cellulase + Z3-1, increasing to 3.02% of FM, but the

Table 3. Changes in counts of viable microorganisms of wheat straw silages during fermentation process.

Item	Ensiling days	pH	Microorganism (cfu/g of fresh matter)				
			LAB	Coliform bacteria	Aerobic bacteria	Mold	Yeast
Control	0	6.18	1.0×10^3	1.0×10^5	2.8×10^5	2.3×10^4	1.5×10^5
	3	5.20	3.0×10^8	1.5×10^8	1.0×10^8	ND	2.5×10^8
	5	5.26	2.5×10^8	2.0×10^6	4.5×10^6	ND	1.5×10^6
	10	4.96	3.5×10^5	1.8×10^5	4.3×10^5	ND	2.5×10^4
	30	5.02	1.5×10^5	ND	3.5×10^4	ND	1.5×10^4
WS + cellulase	0	6.18	1.0×10^3	1.0×10^5	2.8×10^5	2.3×10^4	1.5×10^5
	3	5.23	2.5×10^8	4.5×10^8	6.0×10^8	ND	1.8×10^8
	5	5.13	2.6×10^8	2.7×10^7	5.5×10^6	ND	3.8×10^7
	10	5.03	2.2×10^7	1.2×10^4	2.1×10^6	ND	1.8×10^5
	30	4.87	1.2×10^6	ND	4.4×10^4	ND	5.0×10^3
WS + FG 1	0	6.18	1.0×10^3	1.0×10^4	2.8×10^5	2.8×10^5	2.3×10^4
	3	4.67	2.5×10^8	ND	2.5×10^3	ND	2.4×10^5
	5	4.25	2.0×10^8	ND	2.2×10^4	ND	1.3×10^4
	10	4.16	6.6×10^6	ND	1.5×10^3	ND	ND
	30	4.09	3.2×10^5	ND	5.0×10^3	ND	ND
WS + Z3-1	0	6.05	1.0×10^3	1.0×10^5	2.8×10^5	2.3×10^4	1.5×10^5
	3	4.55	2.6×10^9	ND	5.6×10^5	ND	4.3×10^4
	5	4.23	6.0×10^8	ND	1.2×10^4	ND	4.4×10^4
	10	4.15	2.3×10^7	ND	3.0×10^4	ND	2.4×10^5
	30	4.11	3.4×10^4	ND	5.0×10^3	ND	ND
WS + cellulase + FG 1	0	6.08	1.0×10^3	1.0×10^5	2.8×10^5	2.3×10^4	1.5×10^5
	3	4.25	3.5×10^8	ND	4.3×10^5	ND	3.5×10^5
	5	4.27	2.5×10^8	ND	5.3×10^4	ND	8.5×10^4
	10	4.16	8.0×10^7	ND	4.5×10^4	ND	ND
	30	3.98	2.1×10^4	ND	6.0×10^4	ND	ND
WS + cellulase + Z3-1	0	6.05	1.0×10^3	1.0×10^5	2.8×10^5	2.3×10^4	1.5×10^5
	3	4.31	1.0×10^9	ND	2.6×10^4	ND	1.0×10^4
	5	4.11	6.0×10^8	ND	3.0×10^4	ND	1.0×10^4
	10	4.07	5.0×10^6	ND	1.5×10^4	ND	5.0×10^3
	30	4.03	9.0×10^4	ND	1.4×10^4	ND	ND

WS: wheat straw; LAB: lactic acid bactria; ND: not detected.

acetic acid content of the silages treated with Z3-1 inoculant and cellulase did not show significant difference than the control. Propionic acid was not detected in all silages; the Butyric acid contents of all the treated silages, except the cellulase-treated silages, were lower than the control, and ammonia-N contents of all silages were no more than 0.2 $g \cdot kg^{-1}$ of FM.

Table 4. The chemical composition (% of DM) of wheat straw silage treated with LAB and cellulose after 30 days of fermentation.

Item	OM	CP	EE	ADF	NDF
Control	92.11 ± 0.55	4.52 ± 0.06^{a}	0.50 ± 0.09	43.11 ± 0.76^{a}	70.64 ± 0.76^{a}
WS + cellulase	92.09 ± 0.67	4.53 ± 0.04^{a}	0.53 ± 0.07	41.50 ± 0.89^{b}	69.17 ± 0.04^{b}
WS + FG 1	91.82 ± 0.76	4.69 ± 0.04^{b}	0.55 ± 0.03	41.55 ± 0.71^{b}	69.31 ± 0.34^{b}
WS + Z3-1	91.32 ± 0.75	4.63 ± 0.05^{b}	0.53 ± 0.07	42.81 ± 0.43^{a}	69.42 ± 0.21^{b}
WS + cellulase + FG 1	92.28 ± 0.68	4.64 ± 0.03^{b}	0.57 ± 0.04	40.33 ± 0.88^{b}	69.03 ± 0.65^{b}
WS + cellulase + Z3-1	92.21 ± 0.89	4.69 ± 0.05^{b}	0.54 ± 0.02	41.87 ± 0.61^{b}	68.88 ± 0.87^{b}

Means in the same column with different superscripts in lowercase letter are statistically significantly different ($P < 0.05$); LAB: lactic acid bacteria; OM: organic matter; CP: crude protein; EE: ether extract; ADF: acid detergent fiber; NDF: neutral detergent fiber.

Table 5. Fermentation quality of wheat straw silage prepared with LAB and cellulase after 30 days of storage.

Item	pH	Organic acid (% FM)				Ammonia-N (g/kg of FM)
		Lactic	Acetic	Propionic	n-Butyric	
Control	5.02 ± 0.12^{a}	0.93 ± 0.12^{c}	0.38 ± 0.08^{a}	ND	0.64 ± 0.04^{a}	0.18 ± 0.08
WS + cellulase	4.87 ± 0.08^{b}	0.91 ± 0.21^{c}	0.39 ± 0.06^{a}	ND	0.76 ± 0.21^{a}	0.11 ± 0.05
WS + FG 1	4.09 ± 0.11^{c}	2.59 ± 0.16^{b}	0.20 ± 0.12^{b}	ND	0.28 ± 0.12^{b}	0.18 ± 0.09
WS + Z3-1	4.08 ± 0.09^{c}	2.55 ± 0.27^{b}	0.44 ± 0.10^{a}	ND	0.20 ± 0.24^{b}	0.12 ± 0.06
WS + cellulase + FG 1	3.98 ± 0.13^{c}	2.77 ± 0.16^{b}	0.19 ± 0.13^{b}	ND	0.23 ± 0.13^{b}	0.16 ± 0.07
WS + cellulase + Z3-1	4.03 ± 0.04^{c}	3.02 ± 0.08^{a}	0.24 ± 0.08^{b}	ND	0.31 ± 0.13^{b}	0.07 ± 0.04

Means in the same column with different superscripts in lowercase letter are statistically significantly different ($P < 0.05$); FM: fresh matter; ND: not detected.

4. Discussion

The moisture content of silage material plays an important role influencing silage fermentation, because moisture is required by LAB for metabolic reactions and has a significant effect on the initial level and transport of oxygen during ensilage process [11]. Zheng *et al.* [12] reported that ensilage quality improved as the moisture content decreased from 80% to 60% for sugar beet pulp silage and from 60% to 45% for tomato pomace silage, and storage at below 30% of FM prevents almost all the microbial activity. However, moisture content of the material used in this study is only approximately 10% of FM which is much lower than the moisture requirement for high-quality silage (usually beyond 50% of FM). Therefore, the moisture of the wheat straw was moderated to 60% of FM by spraying distilled water.

Forages are fermented by a complex consortium of microorganisms, predominantly by LAB which convent WSC into organic acid. As a result, the pH is reduced and the forages are preserved. Generally, when LAB reaches to at least 10^5 (cfu·g^{-1} of FM), silages can be well preserved [13]. However, as shown in **Table 2**, LAB values 10^3 (cfu·g^{-1} of FM) present in the fresh wheat straw suggest that certain inoculants should be applied to this silage.

Lactobacilli are the dominant microbial population on forage crops and contribute to the lactic acid fermentation for a longer time than do lactic acid-producing cocci. Usually, *L. casei* and *L. plantarum* are found living in association with forage crops and silages [14] [15]. Many studies have shown the advantages of such inoculants [13], such as adding selected lactobacilli to ensilage forages can dominant or outnumber the epiphytic LAB. At the beginning of fermentation, production of lactic acid by homofermentative lactobacilli is preferred to reduce the pH value, which may inhibit the growth of harmful microorganisms such as mold, coliform bacteria and aerobic bacteria. In the present study, the LAB-treated silages have a relatively low pH at the earlier stage of fermentation, which inhibit the production of n-butyric acid and Ammonia-N. However, in the later days of fer-

mentation, the LAB- and LAB + cellulase-treated silages have lower LAB number than the control ($P < 0.05$). The plausible reason is that the lower pH prevents the growth of LAB its own. More surprise, the treatment with both two LAB inoculants reduces extents of ADF and NDF in a relative larger level compare to the cellulase treatment. Guan *et al*. [16] indicated that this may because the enzymes secreted by microbial degrading the crude fiber during fermentation process. Therefore, strains Z3-1 and FG 1 could be used as excellent inoculants for ensiling wheat straw.

Moreover, Sun *et al.* [17] reported the addition of cellulase increased substrate for LAB from NDF degradation, and propagation of LAB could be promoted in the early stage of ensiling, which resulted in a rapid increase in lactic acid and a drop of pH, so inhibiting activity of harmful bacteria and plant enzymes for proteolysis. However, the efficiency of such enzymatic hydrolysis has been shown to depend on various factors as substrate, environmental conditions and biomass physicochemical pretreatments [18]. That may mean in some cases enzymes cannot significantly reduce the NDF and ADF contents and improve the silage quality. For example, Mandebva *et al*. [19] and Kozelov *et al.* [20] found that fibrous enzyme had no effect on cell wall concentration of bermuda grass and lucerne silage, respectively. In the present study, the NDF and ADF values decreased significantly ($P < 0.05$) in the cellulase-treated silages compared with the control, indicating that the cellulase addition was effectively in degrading the cell wall carbohydrate. Silage treated by cellulase usually can reduce fiber composition and improve the digestibility, and the present experiment also proves the cellulase treatment silage, while raising the quality of fermented, also reduced the fiber composition and hardness of feed. The similar results were also found by Stokes *et al.* [21], Shepherd *et al*. [22] and Sun *et al.* [3]. However, the CP and lactic acid contents of cellulase-treated silages did not show a significant improvement than control, while the pH values were higher than cellulase + LAB (Z3-1 and FG 1)-treated silages. We consider the main reason of the high CP content of the sample is for the breed, because almost all fields in that area were treated with the same fertilizer cultivation. The results may indicate that the most epiphytic LAB present in the fresh wheat straw were heterofermentative, which could not effectively ferment the substrate provided by cellulase degrading the fiber, therefore, resulting in poor silage; in contrast, as described before, the homofermentative LAB Z3-1 and FG 1 inoculants could utilize the substrate sufficiently and improve the fermentation quality well.

The concentrations of butyric acid and NH_3-N in silages are also important criterions for evaluating silage fermentation quality, and they were usually associated with the activity of clostridia. In this study, addition of cellulase and inoculants can decrease the concentrations of butyric acid and ammonia-N at different extents compared with control. Usually, low digestive rate is one of the important factors of restriction wheat straw as livestock feed. This test using fresh straw as material, treated by lactobacillus and cellulase in the process of silage fermentation, not only improve the fermented quality, but promote the digestibility of wheat straw can be expected. The evaluating of the LAB and cellulase treated wheat straw silage through livestock digest test evaluation will be carried out in our laboratory.

5. Conclusion

LAB inoculation, especially the addition together with cellulase, could improve the fermentation quality of wheat straw silage better than only cellulase-treated silages. *L. casei* Z3-1 has a potential ability to be used as commercial inoculant, and its effectiveness on other crop silages is underway in our laboratory.

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