Enhancing the Co-ensiling Performance of Corn Stover and Cabbage Waste *via* the Addition of Cellulase

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Effects of cellulase addition were assessed relative to the co-ensiling performance of air-dried corn stover (DCS) and cabbage waste (CW). The DCS and CW mixtures were co-ensiled with 0 to 0.3% of cellulase addition, and changes in composition, intermediates, and biological activity were characterized. The results showed that the addition of cellulase enhanced the decomposition of cellulose and hemicellulose by 2.51 to 6.93% and 3.41%, based on different dosages and compared with the control. Thus, the content of water-soluble carbohydrates increased. The acid content also increased from 5.8% for the control to the range 5.16 to 8.51% for the samples containing cellulase. Moreover, there was a shift from homolactic to heterolactic fermentation with prolonged ensiling time, coupled with the dominant lactic acid bacteria shifting from *Paralactobacillus* and *Lactobacillus* to more of *Lactobacillus*. Thus, the addition of cellulase improved the relative abundance of *Lactobacillus*. An assessment of fermentation quality, therefore, suggested that cellulase addition can improve the silage quality of DCS/CW during co-ensiling.

Keywords: Cellulase; Ensiling quality; Microbial community; Lactic acid bacteria

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INTRODUCTION

Over the past few decades, human activity has led to an increasing amount of agricultural waste, such as crop stalk, fruit, and vegetable waste. In agricultural production, an urgent need has emerged to solve the problem of waste disposal (Hillion *et al*. 2018). Agricultural residues are regarded as an attractive alternative for bioenergy production due to their wide availability, environmental friendliness, low cost, and renewability, and because they do not compete with food crops (Hess *et al*. 2009; Vervaeren *et al*. 2010). However, the drawback of seasonal production and short harvest times negatively affects the large-scale utilization of agricultural waste. Consequently, controlling the long-term preservation and real-time disposition of agricultural waste is indispensable for ensuring year-round availability and sustainable food throughout the year for bioenergy production.

Ensiling is an anaerobic wet storage method and represents a feasible and cost-effective way to supply year-round biomass (Gallegos *et al*. 2018). During the ensiling process, lactic acid bacteria (LAB) convert water-soluble carbohydrates (WSCs) into organic acids, reducing the pH to a range of 3.5 to 4.2, which inhibits the growth of undesirable microorganisms and preserves the organic matter of biomass (Janke *et al*. 2019). Satisfactory silage preservation depends on intrinsic biochemical characteristics, such as the moisture, carbohydrate content, and the buffering capacity of biomass and

storage conditions, which includes temperature, time, and packing density. Among these, moisture content and WSCs are important for enhancing LAB activity and organic acid generation (Zhao *et al*. 2016). Studies have shown that ensiling with a moisture content ranging from 65% to 75%, and a rudimentary soluble carbohydrate content higher than 5% (dry matter, DM), is favorable for lactobacillus reproduction (Hillion *et al*. 2018). However, obtaining good silage quality on an industrial scale may is challenging, as the soluble sugar content in agro-industrial organic waste is insufficient for producing enough lactic acid. Thus, air-dried crop stalk is difficult to immediately ensile due to its low moisture, sugar, and high fiber content (Vervaeren *et al*. 2010). Although co-ensiling of dried crop stalk with other agro-industrial waste material, such as vegetable waste and sugar beet leaves, may achieve well-preserved silages, the natural cross-linked recalcitrant structure of dried crop stalk may hinder efficient lactic fermentation (Hillion *et al*. 2018; Ren *et al*. 2018; Chen *et al*. 2019).

Various additives, including lactic acid bacteria, enzyme, and molasses, have been used in ensiling to improve silage quality and break down the cell wall components (Li *et al*. 2014; Muck *et al*. 2018). Of these, fibrolytic enzymes can enhance LAB activity by promoting the release of sugar from lignocellulosic biomass, which improves ensiling performance at a lower pH and produces more lactic acid (Sun *et al*. 2011). Previous studies have confirmed that fibrolytic enzymes can increase the concentration of lactic acid and soluble carbohydrates and decrease the content of hemicellulose and cellulose in the silage (Lynch *et al*. 2015; Desta *et al*. 2016). Li *et al*. (2018) also reported that silage with added fibrolytic enzymes maintained lower levels of acid detergent lignin (ADL) throughout the ensiling process of *Pennisetum sinese*. In addition, fibrolytic enzymes can improve the digestibility of structural carbohydrates, especially neutral detergent fibers (NDF), during the first 24 h of incubation (Colombatto *et al*. 2004a; Romero *et al*. 2015). Therefore, before silage production, cellulolytic enzyme pretreatment is considered a beneficial biological pretreatment for lignocellulosic biomass for the production of bioenergy and biological products. This process can decompose the persistent structural matrix, change the composition of lignocellulose, and increase the conversion rate of cellulose (Sun *et al*. 2011; Desta *et al*. 2016; Wu *et al*. 2020). Thus, a better understanding of enzyme addition for altering the microbial communities in ensilage is needed in order to effectively improve silage quality.

Though reports have shown that both ensiling and the use of additives are effective strategies for the preservation and valorization of agricultural waste, and are a beneficial pretreatment method for downstream biorefining, to the best of our knowledge, there is a lack of research on the effects of additives applied during the ensiling of air-dried corn stover and cabbage waste. Therefore, the objectives of this study were to (1) determine the effect of cellulase addition for the co-ensiling performance of corn stover and cabbage waste in terms of fermentation characteristics and chemical composition, and (2) assess the evolution of microbial community diversity during co-ensiling with various cellulase dosages.

EXPERIMENTAL

Materials

Raw material and silage additives

Air-dried corn stover (DCS) was cultivated and harvested in October from local farms in Yuzhong County (latitude 35.34°N, longitude 103.49°E, and altitude 1874.4 m a.s.l., Gansu Province, China). The material was chopped mechanically into lengths of 10 to 20 mm. In this location, the average annual temperature is 7.9 °C, and the hottest and coldest monthly average temperatures are 35.8 °C (July) and −11 °C (January), respectively. In addition, the annual average precipitation is between 300 and 400 mm, the annual average solar radiation is 4800 MJ/m^2, and the region receives an annual average of 2562.5 h of sunshine. The cabbage waste (CW) was obtained from the Qilihe vegetable market in Lanzhou (latitude 35.55°N, longitude 103.50˚E, altitude 1800.4 m a.s.L., Gansu Province, China) and cut into 20 mm × 20 mm pieces. The moisture content values of DCS and CW before ensiling were 10.23% and 91.41% on a fresh mass basis, respectively. The commercial cellulase (Ningxia Imperial Jade Biotechnology, Ltd., Yinchuan City, China), which consisted of β-glucanase, α-arazyme, α-galactosidase, β-galactosidase, and β-xylanase (> 10,000 U/g), was supplied as a freeze-dried powder.

Methods

Co-ensiling of DCS and CW

The chopped DCS and CW were mixed thoroughly and divided into a total of 48 equivalent piles (3.23 kg of DCS and 9.87 kg of CW for each pile, wet basis) for experimental treatment according to our previously published methods (Ren *et al.* 2018). The three blended piles were immediately frozen at −20 °C for further analysis. The remaining 45 piles (5 ensiling time points × 3 dosage treatments × 3 replicates) were randomly assigned into the following treatment groups: (1) control without additives (CWA), (2) cellulase applied at a low dosage of 0.1% (w/w, fresh weight) (CLD), and (3) cellulase applied at a high dosage of 0.3% (w/w, fresh weight) (CHD). The cellulase for each treatment was dissolved in 10 mL of distilled water and uniformly sprayed on each pile of silage. For the control group, the same amount of distilled water was applied. All treatments were adjusted to an approximate DM content of 30%, compacted, and filled and sealed in 30 L cylindrical polyethylene plastic buckets (no headspace). All of the buckets were stored at 18 ± 1 °C and sampled after 30, 60, 90, 130, and 170 days.

Fermentation characteristics of silages

After opening the buckets on the designated ensiling day, a 50 g sample was homogenized with 450 mL of distilled water for 1 min (JJ-2; Guohua Instruments Co., Ltd., Suzhou, China). After filtration through four layers of medical gauze and centrifuged at 3,900 rpm for 10 min, the supernatant was obtained for pH measurements using a glass electrode pH meter (UB-7, DANFO, Beijing, China). Then, a portion of the supernatant was acidified by 50% H_2SO_4 (w/w) and filtered with a 0.22 μm dialyzer to analyze the organic acids (lactic, acetic, propionic, and butyric acid) with an Agilent 1260 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Inc., Waldbronn, Germany). The system was fitted with a refractive index detector (RSpak KC-811, Shodex, Tokyo, Japan; eluent: 3 mmol/L $HClO_4$, flow rate: 1 mL/min, injection volume: 5 μL, and temperature: 50˚C) (Li *et al.* 2019). After protein precipitation formed by trichloroacetic acid, ammonia nitrogen (AN) content was determined using the phenol-

hypochlorite colorimetric method and expressed per total nitrogen (g/kg TN) (Ren *et al*. 2021).

Chemical composition analysis

The chemical compositions of the dry matter (DM), total nitrogen (TN), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analyzed according to previously reported methods (Ni *et al*. 2017). Holocellulose (HoC) content was the sum of hemicellulose (HC) and cellulose (CL) content (Robinson *et al*. 2016). The biodegradation potential of the silage was estimated as the ratio of (hemicellulose + cellulose) to lignin (Vandecasteele *et al*. 2017). Thus, the carbohydrate content was extracted and used to measure the WSC content using the 3,5-dinitrosalicylic acid (DNS) method with glucose as the standard (Leyva *et al*. 2008). The TN was equivalent to the Kjeldahl nitrogen, which was determined by a Kjeldahl nitrogen analyzer (K9840, Hanon instruments Co., Ltd., Jinan, China) (Li *et al*. 2019). All analyses were performed in triplicate. A Flieg evaluation system was used to evaluate the quality of the silage by scoring the silage and by scoring the ratio of the lactic acid, acetic acid, and butyric acid to the total acid content (Agneessens *et al*. 2015).

Microbial Community Analysis

Metagenomic DNA extraction

Samples from the treatment of CWA and CHD were selected to analyze the dynamic changes in the microbial communities during the ensiling process, using high-throughput sequencing technology. A total of 20 g of samples was mixed with 180 mL of sterilized NaCl solution (0.85%) and then treated with a table concentrator at 120 rpm for 2 h. The suspension was then filtered to obtain the microbial cells for DNA extraction. Total DNA content was extracted using a water DNA extraction kit (Fuji Biotechnology Co., Ltd., Chengdu, China), and DNA quantity and quality were assessed by agarose gel electrophoresis. High-molecular-weight DNA samples (minimal concentration of 20 ng/μL) were used for sequencing.

PCR amplification

Amplification of the V3-V4 hypervariable region of the 16S rRNA was achieved using 338F (5-ACTCCTACGGGAGGCAGCA-3) and 806R (5-GGACTACHVGGGTW TCTAAT-3) primers. Amplification of the targeted region was achieved using the following reaction chemistry: 5 μL of Gotaq Green master mix (Promega, Madison, WI, USA), 11.9 μL of DNase free water, 0.5 μL of $MgCl_2$ (50 mM), 0.5 μL of deoxynucleotide triphosphates (10 mM), 1 μL of DNA forward and reverse primers (10 μM), and 5 μL of the DNA template were adjusted for all samples to a final average concentration of 1 ng/μL of total reaction volume. Reaction conditions for bacterial 16S amplification were as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s. A final extension was conducted at 72 °C for 10 min (He *et al.* 2015). After a second examination with gel electrophoresis, the polymerase chain reaction (PCR) products were denatured with NaOH to generate single-strand DNA fragments, which were sequenced using an Illumina MiSeq platform (Personalbio Biotechnology, Ltd., Shanghai, China).

High-throughput sequencing of metagenomics DNA

To obtain the high-quality clean reads, quality filtering of the raw reads was

performed under specific filtering conditions. Chimera sequences from the bacteria were removed using the UCHIME algorithm by comparing them with the Silva database. However, chimeral sequences of fungal materials were removed using the UCHIME algorithm by comparing them with the UNITE database. The effective sequences (reads) were counted and operational taxonomic units (OTUs) were clustered at 97%, at similar levels for performing the taxonomic analysis of different samples and obtaining microbial community composition at different levels. Alpha diversity analysis was conducted using QIIME software (http://qiime.org/) to analyze species richness (Chao1, ACE index) and diversity (Shannon and Simpson index). Beta diversities of both weighted and unweighted UniFrac were calculated using QIIME software. Subsequently, species with a relative abundance higher than 0.1% were selected for microbial community composition analysis.

Statistical Analysis

The experiment design consisted of a 3 × 5 factorial design with 3 dosages and 5 ensiling times. The statistical model follows,

$$Y = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \varepsilon_{ij}, \quad (1)$$

where Y represents the response variable, μ is the overall mean, α_i is the effect of the ensiling period, β_j is the effect of different additive dosages (treatments), $(\alpha \times \beta)_{ij}$ is the interaction effect between the ensiling period and additive dosages, and ε_{ij} is the residual error (Li *et al.* 2020). Data were analyzed using the general linear model procedure from the statistical package for social science (SPSS 18.0, SPSS Inc., Chicago, IL, USA), and one-way analysis of variance (ANOVA) was determined using Tukey's multiple comparison test at a significance level of 0.05.

RESULTS AND DISCUSSION

Comparison of Composition between the Mixtures and Raw Material

The chemical compositions of the DCS and CW are presented in Table 1. The dry matter of the DCS was 89.77 ± 0.01% (Table 1). DCS moisture content (10.23%) was lower than the proper value for ensiling (Adesogan and Newman 2014). Moreover, the WSC content of DCS was 8.05 ± 0.03%, which was lower than the essential WSC content for high-quality silage (8 to 10%). However, dry matter and WSC values in the CW were 8.59 ± 0.15% and 14.57 ± 0.02%, respectively. In general, the production of high-quality silage requires a raw material with 35 to 45% of DM content and 8 to 10% of WSC content (Agneessens *et al*. 2015). After mixing, the dry matter and WSC content of the mixtures were 29.56 ± 0.03% and 9.53 ± 0.20%, respectively. Compared with the chemical compositions of the raw materials, the mixture had more suitable moisture and WSC content, compared to DCS or CW alone, indicating that the mixtures had optimal compositions for lactic acid fermentation.

The contents of NDF, ADF, and ADL of DCS were 76.85%, 47.15%, and 8.12%, respectively (Table 1). After mixing, an increase of 22.54% in ADL content and decreases of 11.95% and 0.07% in NDF and ADF content were observed for the mixtures, respectively. Optimal cellulase content provides a sufficient source of lactic acid fermentation substrate, thereby promoting the reduction in pH and inhibiting harmful microorganisms (Li *et al.* 2020). Therefore, the co-ensiling of DCS and CW was proposed in this study as a simple and low-cost strategy for storing both waste products.

Table 1. Chemical Compositions of Air-dried Corn Stover and Cabbage Waste

Items	Cabbage waste	Air-dried corn stover	After mixing
Dry matter (% FW)	8.59 ± 0.15	89.77 ± 0.01	29.56 ± 0.03
Water soluble carbohydrate (% DM)	14.57 ± 0.02	8.05 ± 0.03	9.53 ± 0.20
Acid detergent fiber (% DM)	33.27 ± 0.97	47.15 ± 0.01	44.01 ± 1.09
Neutral detergent fiber (% DM)	36.25 ± 1.20	76.85 ± 0.02	67.67 ± 0.39
Acid detergent lignin (% DM)	16.21 ± 0.45	8.12 ± 0.03	9.95 ± 0.21
Cellulose (% DM)	17.06 ± 0.64	39.03 ± 0.02	34.06 ± 1.08
Hemicellulose (% DM)	2.98 ± 0.46	29.70 ± 0.02	23.66 ± 0.74
Holocellulose (% DM)	20.04 ± 0.53	68.73 ± 0.05	57.72 ± 0.34
ADF:NDF	0.91	0.61	0.65
ADL:NDF	0.45	0.11	0.15
ADL:ADF	0.49	0.17	0.23
BDP	3.89	2.62	2.93

DM: dry matter; FW: fresh weight

Effect of Cellulase on Co-ensiling Quality

Changes in lignocellulosic composition during the ensiling process

The effects of cellulase on the mixture composition during the ensiling process are listed in Table 2. With prolonged silage time, the NDF, ADF, and ADL content in the CWA groups ranged from 67.67 ± 0.39% to 74.52 ± 0.15%, 44.01 ± 1.09% to 48.04 ± 0.53%, and 4.65 ± 0.07% to 10.98 ± 0.85%, respectively, during ensiling periods of 30 to 170 days. Compared with the CWA group, CLD and CHD treatments had lower HC and CL content ($P < 0.05$), with decreases of 13.55%, 26.60%, and 44.33% in NDF, ADF, and ADL content, respectively. Similarly, compared with the CWA group, decreases of 17.31% and 9.1% in CL and HoC content were observed for the CLD and CHD treatment groups, respectively. Thus, the addition of cellulase could effectively promote the degradation of fiber content, and a higher dosage of cellulase could effectively reduce CL, ADF, and ADL contents. Cellulase can also promote fiber degradation, elevating the WSC production by enzymolysis and acid solubilization, which increases the availability of fermentation substrates for LAB to produce lactic acid (Li *et al*. 2019; Hu *et al*. 2021). The present results were in good agreement with a previous study by Kholif *et al*. (2017), where NDF, ADF, and CL contents were significantly decreased after ensiling. Based on the composition changes, the biodegradation potential of the various samples was assessed, and the results are presented in Table 2. The biodegradation potentials of the samples increased after 170 days of long-term co-ensiling. The CHD group met this requirement, but the CLD group did not obtain a similar result.

To further understand the effects of cellulase on DCS and CW co-ensiling, WSC content was quantified, and the results are presented in Fig. 1. WSC content in all three treatments decreased significantly ($P < 0.05$) during the first 30 days of co-ensiling, with descent percentages of 79.64%, 72.19%, and 60.13% in groups CWA, CLD, and CHD,

respectively. This was attributed to the quick growth and proliferation of aerobic microorganisms, with abundant fermentation substrates during the initial stage. With more ensiling time, the CLD and CHD groups exhibited increasing trends in WSC content after 30 and 90 days, respectively. Moreover, the CLD and CHD groups showed significantly higher amounts of WSC than CWA during the storage period of 30 to 170 days, which was consistent with the report that cellulase indirectly provided fermentable sugars from the degradation of NDF, ADF, and HC, and thus required more time (Hess *et al*. 2009). Weinberg and Chen (2013) suggested that cell-wall degradation enzymes can release fermentable sugars and cause a decrease in fiber content, which was responsible for the observed enhanced digestibility. Furthermore, the WCS content in the CLD group was significantly higher ($p < 0.05$) than in the CHD group between 60 and 170 days of co-ensiling. This indicated that a low dose of cellulase was beneficial for the preservation of WSC.

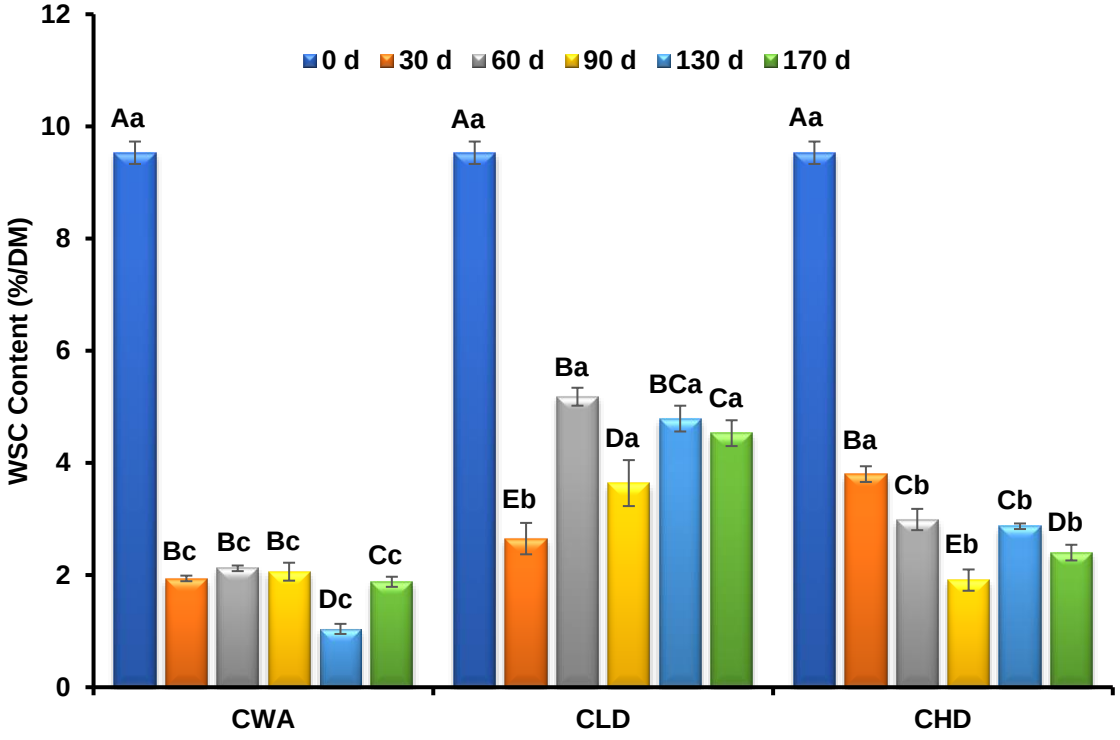

Fig. 1. Dynamic effects of cellulase dosage on WSC content: CWA contained no cellulase, CLD contained a low dose of cellulase (0.1%), and CHD contained a high dose of cellulase (0.3%). The different lowercase letters indicate significant differences at $p < 0.05$ between different groups at the same time, and different capital letters indicate significant differences at $p < 0.05$ for the same groups at different times.

Table 2. Compositions of Air-dried Corn Stover and Cabbage Waste Mixtures during Co-ensiling (%DM)

	Time (d)	NDF (%)	ADF (%)	ADL (%)	HC (%)	CL (%)	HoC (%)	Biodegradation potential
CWA	0	67.67 ± 0.39Da	44.01 ± 1.09Ca	9.95 ± 0.21Ba	23.66 ± 0.74Ca	34.06 ± 1.08Da	57.72 ± 0.34Da	5.80
	30	71.34 ± 0.14Ca	44.54 ± 0.43BCa	5.88 ± 0.26Eb	26.79 ± 0.56Aa	38.66 ± 0.69Ca	65.45 ± 0.14Ba	11.13
	60	71.13 ± 0.25Ca	45.49 ± 0.09Ba	10.98 ± 0.85Ab	25.64 ± 0.22Bc	34.51 ± 0.90Da	60.15 ± 0.77Ca	5.48
	90	74.52 ± 0.15Aa	47.40 ± 0.36Aa	4.65 ± 0.07Fc	27.12 ± 0.48Aa	42.75 ± 0.33Aa	69.88 ± 0.22Aa	15.03
	130	71.67 ± 0.01Ca	44.78 ± 0.04BCa	7.07 ± 0.35Db	26.89 ± 0.04Ab	37.71 ± 0.39Ca	64.60 ± 0.36Ba	9.14
	170	72.82 ± 0.53Ba	48.04 ± 0.53Aa	8.03 ± 0.07Cb	24.78 ± 1.06Bb	40.02 ± 0.46Ba	64.79 ± 0.59Ba	8.07
CLD	0	67.67 ± 0.39Aa	44.01 ± 1.09Ba	9.95 ± 0.21Ca	23.66 ± 0.74Da	34.06 ± 1.08Ba	57.72 ± 0.34Ca	5.80
	30	66.56 ± 0.13Bb	41.63 ± 0.09Cb	4.47 ± 0.01Fc	24.93 ± 0.09Cb	37.16 ± 0.08Ab	62.08 ± 0.12Ab	13.89
	60	64.84 ± 0.24Cb	35.26 ± 0.08Eb	6.86 ± 0.06Ec	29.58 ± 0.21Aa	28.39 ± 0.02Db	57.98 ± 0.22Cb	8.45
	90	67.68 ± 0.37Ab	42.15 ± 0.14Cb	12.40 ± 0.40Aa	25.53 ± 0.22Cb	29.75 ± 0.26Cc	55.27 ± 0.05Dc	4.46
	130	65.15 ± 0.15Cc	38.65 ± 0.10Dc	11.61 ± 0.25Ba	26.50 ± 0.22Bc	27.04 ± 0.14Ec	53.54 ± 0.34Ec	4.61
	170	67.43 ± 0.40Ab	46.06 ± 0.08Ab	8.55 ± 0.34Da	21.37 ± 0.48Ec	37.51 ± 0.32Ab	58.89 ± 0.61Bc	6.89
CHD	0	67.67 ± 0.39ABa	44.01 ± 1.09Aa	9.95 ± 0.21Ba	23.66 ± 0.74Da	34.06 ± 1.08Aa	57.72 ± 0.34Ca	5.80
	30	63.86 ± 0.04Dc	40.06 ± 0.09CDc	6.46 ± 0.21Da	23.81 ± 0.04Dc	33.60 ± 0.30ABc	57.40 ± 0.25Cc	8.89
	60	62.95 ± 0.23Ec	34.58 ± 0.01Ec	12.82 ± 0.10Aa	28.37 ± 0.23Ab	21.75 ± 0.09Cc	50.12 ± 0.32Dc	3.91
	90	67.28 ± 0.03BCb	41.31 ± 0.04Bc	7.30 ± 0.07Cb	25.97 ± 0.02Cb	34.01 ± 0.05Ab	59.98 ± 0.07Bb	8.22
	130	67.09 ± 0.19Cb	39.47 ± 0.04Db	6.65 ± 0.03Dc	27.62 ± 0.22Ba	32.82 ± 0.01Bb	60.44 ± 0.21ABb	9.09
	170	67.93 ± 0.41Ab	40.35 ± 0.04Cc	7.26 ± 0.21Cc	27.58 ± 0.36Ba	33.09 ± 0.25Bc	60.67 ± 0.61Ab	8.36

CWA: no cellulase addition; CLD: low dosage of cellulase (0.1%); CHD: high dosage of cellulase (0.3%); NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; HC: hemicellulose; CL: cellulose; and HoC: holocellulose.
Same column with different lowercase letters indicates significant differences at $p < 0.05$ between different groups at the same time, and the same column with different capital letters indicates significant differences at $p < 0.05$ for the same groups at different times.

Changes in pH and organic acid during the ensiling process

The pH values of the samples during co-ensiling were measured and are presented in Fig. 2. The results showed that the pH of all three groups decreased with prolonged ensiling time. The pH values of the CWA, CLD, and CHD groups after 30 days were 4.15, 4.08, and 3.91, respectively. In addition, the pH values of CWA, CLD, and CHD decreased to 3.92, 3.72, and 3.8 after 170 days of ensiling time, respectively. Furthermore, the pH values of the CLD and CHD groups were significantly ($p < 0.05$) lower than the CWA group, indicating that the addition of cellulase significantly reduced the pH of the silage. Thus, a low dose of cellulase (0.1%) showed better performance in maintaining a stable low pH compared to a higher (0.3%) dosage, which was consistent with the results reported by Colombatto *et al.* (2004b). The decrease in pH also promoted the degradation of lignocellulose and the production of soluble carbohydrate content, as indicated by the changes in WSC, NDF, ADF, and HC content. Sargsyan *et al*. (2016) reported that a long-term acidic environment with a low pH can loosen the lignocellulosic structure of fibrous polymers, which can help decompose the cell wall (cellulose and hemicellulose) due to the H^+ from the organic acid and the release of soluble carbohydrates.

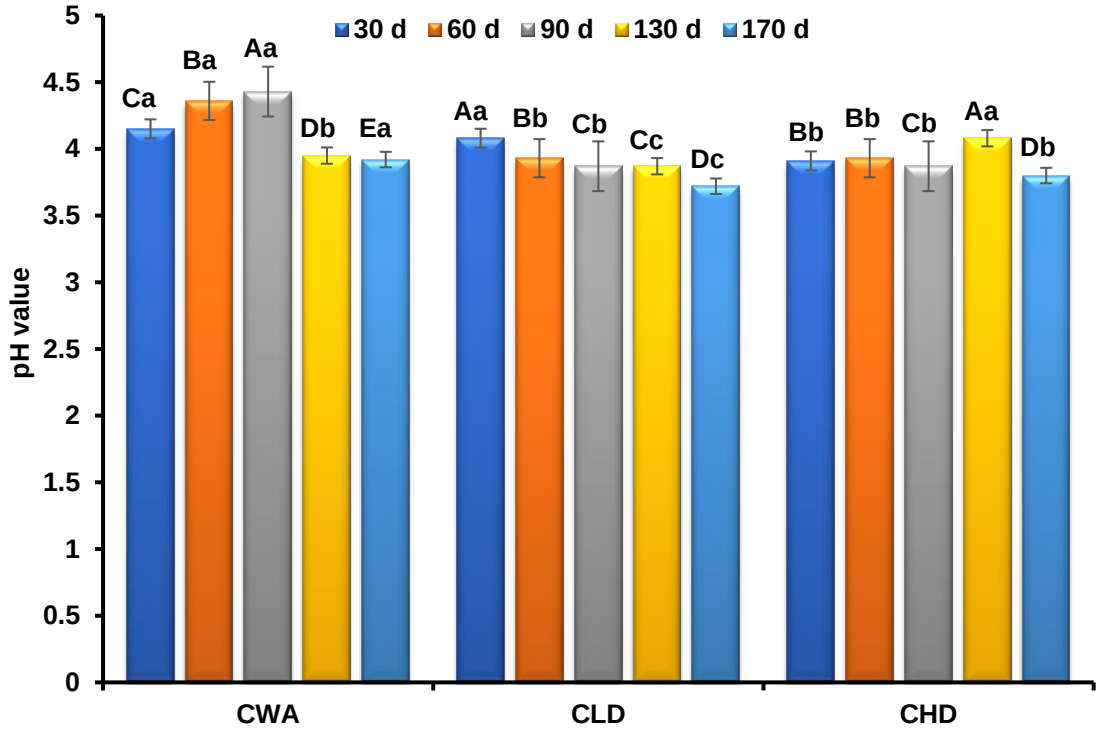

Fig. 2. Dynamic effects of cellulase dosage on fermentation pH values, where CWA contained no cellulase, CLD contained a low dosage of cellulase (0.1%), and CHD contained a high dosage of cellulase (0.3%). The different lowercase letters indicate significant differences at $p < 0.05$ between the different groups at the same time, while the different capital letters indicate significant differences at $p < 0.05$ for the same groups at different times.

Concentration changes in the organic acids during the ensiling process are listed in Table 3. The concentration of LA in all three treated groups initially increased, followed by some decreases, which was further followed by increases in their maximum values at

60 days. It should be noted that the concentrations of LA in the CLD and CHD groups were higher than in CWA, while the maximum value was obtained in the CHD group, except at 130 days. The results showed that the addition of cellulase enhanced the production of LA, which was consistent with the relative abundance of LA bacteria in Table 3. The concentration of acetic acid (AA) in CWA and CHD increased steadily, while in the CLD group, it showed a transient increase (30–90 days) followed by a decrease. On day 30, both CLD and CHD groups had higher AA content than CWA. However, between 60 and 130 days, the concentration of AA in the CHD group remained lower than in the CWA group.

Moreover, the concentrations of butyric acid (BA) in the CLD and CHD groups were higher than in the CWA group, while propionic acid (PPA) and BA content were less than 0.5% of the total concentration of organic acid during the entire process. These results were consistent with the requirements for high-quality ensiling (Agneessens *et al.* 2015). The ethanol (EA) content of CWA decreased continuously, while in the CLD and CHD groups, it increased. Based on the calculated contents of the organic acids, the ratios of lactic acid/acetic acid (LA/AA) and lactic acid/total organic acid (LA/TOA) decreased from 7.44 and 0.82 (30 days) to 4.06 and 0.75 (30 days), respectively, for all three groups during co-ensiling. These ratios were higher in CHD and CLD than in CWA.

Ammonia nitrogen (AN) content represents protein degradation, and a decline in AN content indicates lower CP consumption and better preservation of silage (Luo *et al*. 2021). Thus, as shown in Fig. 3, the AN value in the CWA group was stable from 30 to 90 days, followed by a significant decrease ($p < 0.05$) after 130 days, and then stabilized afterward. The AN values of the CLD group were stable from 1.20 to 1.25 between 30 and 60 days, which significantly ($p < 0.05$) increased on day 90 and stabilized afterward. In the CHD group, the AN values increased first to the maximum (2.83 ± 0.38) after 90 days, and then decreased. Furthermore, CLD and CHD had significantly ($p < 0.05$) lower AN values than the CWA group between 30 and 60 days, which increased and stabilized on day 90, and finally significantly ($p < 0.05$) increased on day 170. These results indicated that the addition of cellulase reduced protein decomposition during the co-ensiling stage between the 30- and 60-day period. Although the AN values increased after 90 days, the values were still within the recommended range (less than 10%) for high-quality ensiling (Ni *et al*. 2017). Generally, a successful ensiling process requires that the concentrations of LA, AA, and PPA be no more than 60%, less than 4%, and 1.5% of the total organic acid content, respectively. In addition, a ratio of LA/AA higher than two and a concentration of BA close to zero is required (Colombatto *et al*. 2004b). Filya (2003) reported that a high-quality ensiling process should have high LA (> 60%), low BA (< 2%), and low AN content (< 9%), and a low pH value (< 4.2). Thus, it was concluded that the co-ensiling of DCS and CW achieved good long-term storage performance (170 days), and the addition of cellulase promoted silage quality.

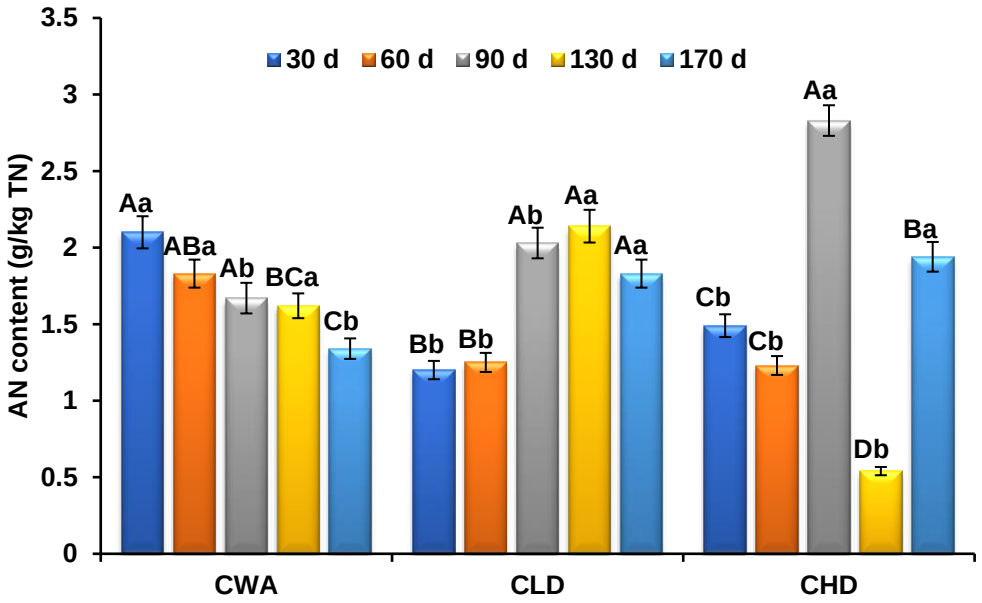

Fig. 3. Dynamic effects of cellulase dosage on the ammonia nitrogen (AN) content, where CWA had no cellulase addition, CLD contained a low dosage of cellulase (0.1%), and CHD contained a high dosage of cellulase (0.3%). The different lowercase letters indicate significant differences at $p < 0.05$ between the different groups at the same time, while the different capital letters indicate significant differences at $p < 0.05$ for the same groups at different times.

Table 3. Dynamic Effects of Cellulase Dosage on the Formation Patterns of Low-molecular Fermentation Products (mg/g DM)

	Time (d)	LA	AA	PPA	BA	EA	LA/AA	LA/TOA
CWA	30	3.78 ± 0.04Bb	0.77 ± 0.02Cb	0.09 ± 0.01ABb	0.25 ± 0.03Bb	0.71 ± 0.04Aa	4.91	0.77
	60	5.07 ± 0.03Ac	1.27 ± 0.05BCa	0.08 ± 0.01Bb	0.21 ± 0.01Cb	0.67 ± 0.05ABa	3.99	0.76
	90	3.05 ± 0.02Cc	1.45 ± 0.08Bb	0.11 ± 0.02Aa	0.32 ± 0.02Aa	0.50 ± 0.02Cc	2.10	0.62
	130	2.51 ± 0.02Db	1.11 ± 0.06BCa	0.10 ± 0.00ABa	0.15 ± 0.02Db	0.58 ± 0.05BCc	2.26	0.65
	170	3.77 ± 0.01Bc	2.06 ± 0.58Aa	ND	0.01 ± 0.00Eb	0.56 ± 0.09Cb	1.83	0.65
CLD	30	3.53 ± 0.07Dc	0.87 ± 0.02Da	0.09 ± 0.01Ab	0.23 ± 0.03Cb	0.33 ± 0.01Cc	4.06	0.75
	60	5.76 ± 0.01Ab	1.36 ± 0.10Ba	0.10 ± 0.01Aab	0.43 ± 0.11Aa	0.73 ± 0.06Ba	4.24	0.75
	90	4.21 ± 0.01Cb	1.76 ± 0.07Aa	0.11 ± 0.02Aa	0.13 ± 0.01Db	0.98 ± 0.02Aa	2.39	0.68
	130	4.23 ± 0.02Ca	1.16 ± 0.07Ca	0.12 ± 0.03Aa	0.35 ± 0.06Ba	1.07 ± 0.12Aa	3.65	0.72
	170	5.16 ± 0.01Bb	ND	ND	ND	ND	——	——
CHD	30	6.25 ± 0.02Ba	0.84 ± 0.03Ca	0.17 ± 0.02Aa	0.37 ± 0.06ABa	0.63 ± 0.03Cb	7.44	0.82
	60	7.13 ± 0.07Aa	1.06 ± 0.02Bb	0.11 ± 0.02Ba	0.40 ± 0.05Aa	0.66 ± 0.13Ca	6.73	0.82
	90	5.10 ± 0.10Da	1.15 ± 0.05Bc	0.09 ± 0.01Ba	0.11 ± 0.01Cb	0.82 ± 0.01Bb	4.43	0.79
	130	1.26 ± 0.01Ec	0.81 ± 0.08Cb	0.10 ± 0.02Ba	0.31 ± 0.04Ba	0.85 ± 0.07Bb	1.56	0.51
	170	5.96 ± 0.02Ca	2.49 ± 0.06Aa	ND	0.08 ± 0.03Ca	2.93 ± 0.04Aa	2.39	0.70

CWA: no cellulase addition; CLD: low dosage of cellulase (0.1%); and CHD: high dosage of cellulase (0.3%). LA: lactic acid; AA: acetic acid; PPA: propionic acid; BA: butyrate acid; EA: ethanol alcohol; and LA/TOA: lactic acid/total organic acid; and ND: not detected.
The same column with different lowercase letters indicates significant differences at $p < 0.05$ between different groups at the same time, and the same column with the same lowercase letters indicates no significant differences at $p > 0.05$ between different groups at the same time. The same column with different capital letters indicates significant differences at $p > 0.05$ for the same groups at different times, and the same column with the same capital letters indicates no significant differences at $p < 0.05$ for the same groups at different times.

Influence of Cellulase on Bacterial Community during Co-ensiling

Alpha diversity analysis

For the bacterial analysis, Chao and ACE indices were used to estimate the number of OTUs in the communities. The Simpson and Shannon indices were used to estimate one of the microbial diversity indices in the sample. As shown in Table 4, the Chao and ACE index values of the CWA group ranged from 223.00 to 716.51, and 246.64 to 730.51, respectively. However, the Chao and ACE index values of the CLD group ranged from 328.28 to 856.00, and 366.49 to 739.48, respectively. Compared to the CWA and CLD groups, a reduction in Chao and ACE index values was observed for the CHD group, except for the samples after 30 days. This indicated that the addition of cellulase enhanced the abundance of microbial communities (Du *et al.* 2020). The Simpson index ranged from 0.56 to 0.97 during the ensiling period, while for the Shannon index, a decrease was observed for the CHD group, indicating that the addition of cellulase improved LA fermentation, and thus reduced the diversity of the bacterial communities (Du *et al*. 2020; Wang *et al*. 2020).

Table 4. Alpha Index Diversities of the Samples

Time (d)	Group	Chao index	ACE index	Simpson index	Shannon index
30	CWA	434.27	453.28	0.82	3.53
	CLD	701.08	739.48	0.85	4.14
	CHD	576.13	596.83	0.82	3.70
60	CWA	506.77	505.16	0.82	3.63
	CLD	328.28	366.49	0.84	3.74
	CHD	445.73	458.19	0.83	3.49
90	CWA	223.00	246.64	0.92	4.58
	CLD	856.00	387.49	0.97	7.27
	CHD	214.00	253.63	0.86	3.37
130	CWA	564.80	613.14	0.88	4.57
	CLD	566.00	580.38	0.87	4.32
	CHD	513.06	510.56	0.69	2.93
170	CWA	716.51	730.51	0.70	3.27
	CLD	713.27	734.71	0.91	4.92
	CHD	617.52	648.88	0.56	2.58

CWA had no cellulase addition, CLD contained a low dosage of cellulase (0.1%), and CHD contained a high dosage of cellulase (0.3%).

Effect of cellulase on microbial communities during the co-ensiling process

The microbial communities at the phylum level of the co-ensiling process with and without cellulase are presented in Fig. 4. For the DCS, the dominant bacteria at the phylum level included Firmicutes and Proteobacteria, while for CW, Bacteroidetes and Proteobacteria were the primary bacteria. For both ensiling systems, phylum Firmicutes and Proteobacteria were the dominant bacteria, with a relative abundance of 87.9 to 99.4%

per total reads of bacteria. Meanwhile, the relative abundance of phylum *Bacteroidetes* ranged from 0.5% to 10.2% during the co-ensiling process. In addition, the relative abundance of phyla Actinobacteria, Cyanobacteria, and Verrucomicrobia was all less than 1.0%. Thus, compared to CWA, an increase of 5 to 174% in the relative abundance of Firmicutes was observed for the systems with cellulase addition (CLD and CHD), while a decrease of 8 to 68% in the relative abundance of Proteobacteria occurred. Phylum Firmicutes can degrade a variety of macromolecular compounds, such as cellulose, starch, and protein (Kung and Ranjit 2001). Therefore, an increase in the relative abundance contributed to the increase in the WSC concentration of the system with cellulase addition, coupled with a decrease in the amount of acid detergent and neutral detergent fibers. In contrast, phylum Proteobacteria, including *Escherichia coli* and *Salmonella*, negatively impacted ensiling, due to the competition for nutrients between the lactic acid bacteria and the production of biological amines (Kadivar and Stapleton 2003).

The microbial communities at the genus level for the raw material and co-ensiling process with and without cellulase are presented in Fig. 5. For the DCS, the dominant bacteria at the genus level included *Carnobacterium* and *Enterobacter*, while for the CW, genus Pseudomonas and Flavobacterium were the primary bacteria. For the co-ensiling system without cellulase (CWA), there were different microbial communities with a change in ensiling time. The dominant bacteria included *Paralactobacillus* (15.4 to 22.9%), *Lactobacillus* (20.7 to 35.4%), and *Enterobacter* (36.0 to 44.9%) between 30 and 60 days. Furthermore, the dominant bacteria shifted from genus *Enterobacter*, *Erwinia*, *Pseudomonas*, *Staphylococcus*, and the family, Moraxellaceae, to genus *Paralactobacillus*, *Lactobacillus*, *Klebsiella*, and *Rahnella* when the ensiling time increased from 90 to 130 days. At an ensiling time of 170 days, the relative abundance of *Lactobacillus* reached 62.1%. For the co-ensiling system with cellulase, genera *Paralactobacillus*, *Lactobacillus*, and *Enterobacter* were the primary bacteria. The dominant bacteria of the CLD and CHD groups were similar to those of the CWA group between 30 and 60 days, with a higher abundance of total LA bacteria and lower abundance of *Enterobacter* observed, compared to CWA. Notably, after 90 days, *Leuconostoc* and *Enterococcus* were newly identified in the CLD and CHD groups, and *Photobacterium* was identified in the CLD group. After 130 days, *Rahnella* was identified in the CLD and CHD groups, with relative abundance values of 15% and 11.8%, respectively. *Enterobacter* and *Lactococcus* disappeared from groups CLD and CHD between 130 and 170 days, while the relative abundance of *Leuconostoc* increased to 13.6% in the CLD group. The genus *Lactobacillus* is the preferred bacteria in silage, as it can metabolize WSCs to LA, and contributes to the decrease in pH and inhibits undesirable bacteria (Mu *et al*. 2020). In addition, *Paralactobacillus* is a homofermentative LA bacterium, producing L-(+)- as well as D-(−)-lactic acid from glucose, and a proposal for reclassification as genus *Lactobacillus* has been put forward (Zheng *et al*. 2020; Leisner *et al*. 2000; Haakensen *et al*. 2011). *Enterobacter*, *Pseudomonas*, and *Rahnella* are considered undesirable bacteria in silage, as they compete for available WSC with LAB, produce endotoxins, and reduce protein content (Zhao *et al*. 2021; Zi *et al*. 2021). Based on the proposed reclassification, a higher relative abundance of *Lactobacillus* was achieved for the system with cellulase addition. During the co-ensiling of DCS and CW, the addition of cellulase increased the abundance of LA bacteria, which eliminated *Enterobacter* after 130 days, and significantly ($P < 0.05$) improved the abundance of *Lactobacillus*. In the CHD group, the abundance of *Lactobacillus* reached 69.1% and 82.5% after 130 and 170 days, respectively, and the abundance of *Lactobacillus* was persistently higher in the CHD group than in the CLD and CWA groups. In addition,

a decrease of 11 to 22% and 20 to 57% in the relative abundance of *Enterococcus* and *Rahnella* was observed. The results indicated that the enhanced production of WSC caused by the addition of cellulase stimulated the growth of *Lactobacillus*. Similar results have been reported in previous studies. For example, Xu *et al*. (2020) reported that cellulase with *Lactobacillus plantarum* A1 performed well in fiber degradation during the co-ensiling of corn stalk and potato pulp. In addition, an improvement in the silage quality of mulberry leaves was observed with the application of cellulase and *Lactobacillus casei* LC (He *et al*. 2019).

Homo- and heterolactic fermentation are the common pathways for producing LA. Among them, LA is the primary production for homolactic fermentation, whereas heterolactic fermentation produces carbon dioxide, LA, ethanol, or AA (Muck *et al.* 2018). Kung and Ranjit (2001) reported that homolactic fermentation dominated with an LA/AA ratio higher than 3. Therefore, there was a shift from homolactic fermentation to heterolactic fermentation when the ensiling time increased from 30 to 60 days, and from 130 to 170 days, coupled with the dominant LAB changes from *Paralactobacillus* and *Lactobacillus* to *Lactobacillus* for both co-ensiling systems (Fig. 6). Furthermore, the addition of cellulase prolonged the homolactic fermentation time and improved the relative abundance of *Lactobacillus*.

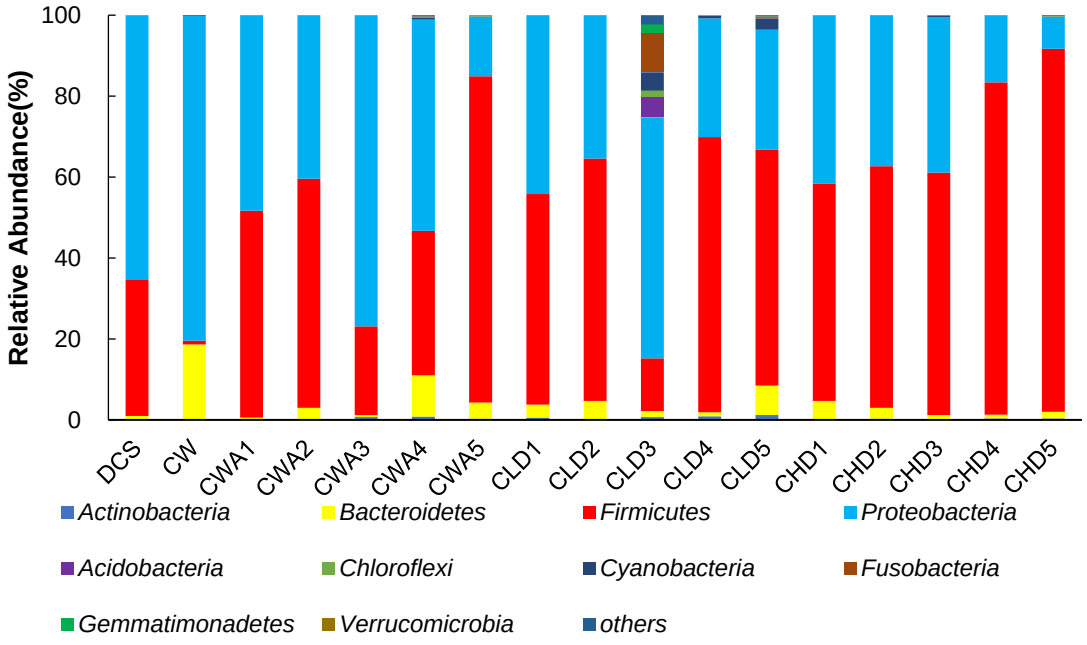

Fig. 4. Relative abundance of bacteria 16S rRNA genes at the phylum level, where DCS: dry corn stover; CW: cabbage waste; CLD: cellulase addition dosage of 0.1%; CHD: cellulase addition dosage of 0.3%); 1:30 d; 2:60 d; 3:90 d; 4:130 d; 5:170 d

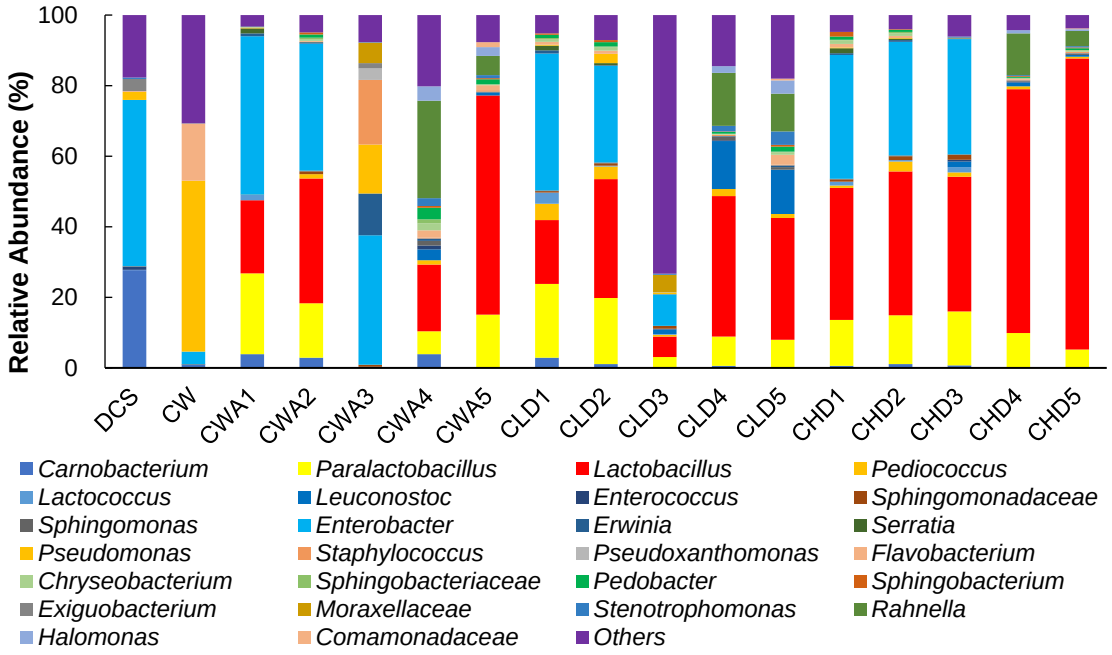

Fig. 5. Relative abundance of bacteria 16S rRNA genes at the genus level; DCS: dry corn stover; CW: cabbage waste; CLD: cellulase addition dosage of 0.1%; CHD: cellulase addition dosage of 0.3%; 1:30 d; 2:60 d; 3:90 d; 4:130 d; 5:170 d

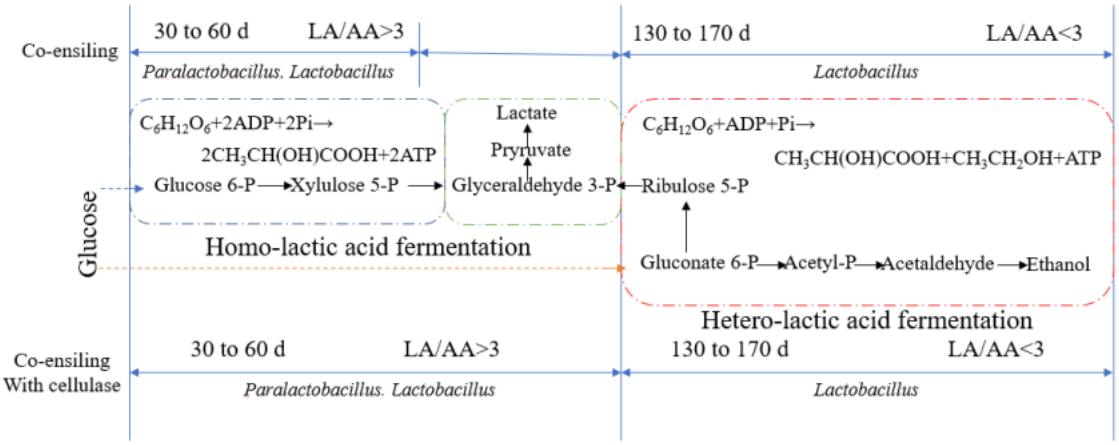

Fig. 6. The pathway of the co-ensiling system without and with cellulase

CONCLUSIONS

1. The experimental results showed that the co-ensiling performance of air-dried corn stover (DCS) and cabbage waste (CW) was improved by the addition of cellulase. Both high (0.3%) and low (0.1%) dosages of cellulase effectively preserved the water-soluble carbohydrates, promoted the production of lactic acid (LA), and significantly decreased the fermentation pH values and the neutral detergent and acid detergent fibers.

2. Furthermore, the addition of cellulase increased the relative abundance of desirable *Lactobacillus* and decreased the number of undesirable microorganisms such as *Enterobacter*, *Pseudomonas*, and *Rahnella.* This ultimately improved the quality of the DCS and CW silage.
3. Considering both the ensiling performance and enzyme cost, this study recommends a cellulase addition of 0.1% to fresh matter for the co-ensiling of DCS and CW, to enhance silage fermentation quality.

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