Effects of Cellulase on the Chemical Compositions Characteristics and Microbial Community of Saline-alkali Land Alfalfa Silage

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Abstract

Background We aim to assess the nutritional quality of alfalfa in saline-alkali and the main fermenting microorganisms acting on alfalfa in saline-alkali soils.

Results In this study, We tested the nutrient composition and microbial community of Zhongmu No. 3 (salt-tolerant) alfalfa, including the nutritional fermentation quality and microbial diversity analysis of alfalfa before and after ensiling (30 days and 60 days). Then we got closely genus that related to saline-alkali soils.The validity of the microbiological data was confirmed by alpha diversity analysis. From the Microbial network, we obtained the difference of the saline-type mantle type.

Conclusions we found that the silage quality of Saline-alkali alfalfa can be ensured by anaerobic fermentation with addition. The LABs that play a major role in the saline alkaline alfalfa silage are Lactobacillus, lactococcus and enterococcus.

Background

With the rapid development of the animal husbandry in the world, the availability and price of forages, particularly regarding adequate and high protein sources, have become serious problems for animal feed [1, 2]. Getting more protein feeds with limited resources has become increasingly important. Many plants, which alfalfa（*Medicago sativa* L.）has higher levels of crude protein, digestible nutrients, and minerals have been used to ameliorate this problem [3]. Alfalfa can be planted in a large amount on saline soil. As a high-quality pasture, it can fully adapt to the environment of saline-alkali land [4]. On the one hand, it can effectively get more forages from land, provide more high-protein glutinous feed, and can also be used for animal husbandry [5, 6].

According to the statistics of UNESCO and FAO, the saline-alkali soil resources are distributed in more than 100 countries around the world, and there is a global saline-alkali land area of 955 million hm^2(Wang,S J,2016) [7, 8], while China's total saline-alkali land area is about 99.14 million hm^2 [9], ranking third in the world. And as one of the important land resources, how to use saline-alkali resources and realize the sustainable uses of comprehensive agricultural resources has become an important research in today's society [10]. As a representative of leguminous grasses, alfalfa is distributed and planted in most parts of the world [11]. It is growing well in neutral saline-alkali or light saline-alkali soils. It is also a salt-tolerant forage.

The forage process has played a positive role in promoting [12]. Physiological characteristics of alfalfa can improve the saline-alkali land in the world, and greatly improve the utilization rate of saline-alkali land [13]. Therefore, Saline-alkali alfalfa may be a potential feed source. Ensiling is a method of preserving

fresh pastures, which is extended by anaerobic fermentation of lactic acid bacteria and effectively preserves nutrients [14, 15]. During anaerobic fermentation, water-soluble carbohydrates (WSC) are metabolically decomposed into lactic acid by LAB until the pH descend to around 4.5 (Herrmann et al., 2011) [16, 17]. Useing of silage additives is conducive to improving the quality of silage[18]. There are many types of additives on the market, which are divided into fermentation accelerators and fermentation inhibitors. Cellulase is an important additive for fermentation accelerators. It can effectively preserve the nutritional value of forage silage (Tian et al., 2017; Zheng et al.,2017) [19, 20].In current forage production, we need to maximize the benefits of existing resources. Therefore, it is of great significance to make full use of the resources of saline-alkali forage. We also hope to provide a method of using saline-alkali forage, which wish to be recognized by the society. The purpose of the current study was to investigate the influence of time and addition on the saline-alkali alfalfa fermentation characteristics and bacterial community of silage and to provide more-detailed information about alfalfa for ensiling.

Results

Nutritional and Microbial Quantity of fresh Alfalfa

The nutritional composition of fresh alfalfa and the composition of its microbes are visible in Table 1. The nutrients of raw alfalfa directly affect the quality of later silage. And its nutrient indicators are calculated based on the dry matter of the raw materials. The dry matter (DM) content of the raw material is 29.9 g/kg, And the crude protein (CP) content of alfalfa is 21.9, Protein content is relatively good, which may also be due to salt stress promoting protein accumulation in plants. The number of Lactic acid bacteria (LAB) is higher than yesat. If the operation is normal, this quantity of LAB is enough to ferment with additives.

Nutritional and Fermentation Quality of alfalfa Silage

After 30 and 60 days of anaerobic fermentation, the nutritional quality and microbial quantity of alfalfa silage have changed significantly. After 30 days of fermentation, most of the indicators, CP and WSC, changed significantly, which reduced fermentation by 12.9% and 7.6% under cellulase (Table 2), This is due to your metabolic fermentation of lactic acid bacteria, respectively. From the nutritional indicators of 30 days and 60, most indicators have no significant difference, except for WSC. And regardless of CK and cellulase treatment, the WSC content at the same time has significant differences.

However, the changes in the treatment groups at the same silage time were consistent. From the nutritional point of view of saline-alkali mantle, its overall nutritional quality treatment is better. It indicates that the saline-alkali mantle can be used as a good feed to feed the animals after good silage fermentation.

In addition to understanding the nutritional quality of silage, the quality of its fermentation is also crucial. Through the fermentation indicators, we can understand what the secreted products of the main fermenting microorganisms are. We would see the fermentation quality of the saline-alkali silage in Table 3. Among them, butyric acid, which has not detected, is bad for livestock, which indicates that the silage has good palatability. Lactic acid is the main product of lactic acid bacteria in the process of fermentation and metabolism. Under the same cellulase treatment, the lactic acid content of the 30-day and 60-day silage was 2.66 and 3.48, respectively, an increase of 23.5%. This also explains the reason for the decline in WSC in Table 2. WSC is decomposed into lactic acid water by glycolysis (EMP) or hexose phosphate (HMP) pathway. And it is obvious that after the silage fermentation of the additive, the NH_3-N and pH were significantly different from the control group ($P < 0.01$).

It can be distinguished from the silage fermentation index (Table 3). Cellulase has a significant effect on lactic acid content, pH and NH_3-N. As the silage time prolongs, the life activities of its microorganisms become more and more active. The key nutrients are slowly being consumed, but some changes such as propionic acid and Acetic acid do not differ. It is also apparent that there is no spoilage in the silage process, and the rapid propagation of lactic acid bacteria, low pH and anaerobic environment inhibit the growth of mold. It also shows that the saline-alkali mantle will have better quality after being filled with additives.

Effect of Cellulase on Bacterial Community After 30 and 60 Days of Ensiling

In this study, high-throughput determinations of variable regions 3 and 4 of 16s rDNA were performed to calculate and evaluate bacterial diversity after salinization of saline-alkali mantle. As can be seen from Table 4, the coverage of all samples was greater than 99%. It indicates that the sequencing width is relatively comprehensive, and the microbial high-throughput data is sufficient to represent the characteristics of the bacterial microbial community. And, it is obvious that it is known. After a long period of fermentation, the number of OTUs is significantly reduced, which may also be that LAB has become the dominant dominant flora, inhibiting the growth and reproduction of other harmful microorganisms. According to the number of OTU and Chao index, the bacterial community abundance after using the additive and different silage time is different.

Principle component analysis (PCA) analyzes the similarity and difference of bacterial communities after alfalfa silage through different treated sample points. As shown in Figure 1, PCA1's contribution to the interpretation of total variance reached 67.73%, while PCA2 explained 13.53% of the total variance.

Overall, there is a large difference between alfalfa raw materials and silage samples. And from the fermentation quality of the nutritional quality of Tables 2 and 3, there are differences in key indicators, such as WSC, CP and so on. The result is that they are most likely caused by these microbial flora.

After sequencing the DNA of the microbial community, we can know four phylums abundance contents have changed. It is also possible to understand the main role of the saline-alkali lining. The figure 2 shows the fresh alfalfa and silage microorganisms at the phylum level. The content of Firmicutes in the alfalfa raw material of saline-alkali is less than 0.1% of the total bacterial content. This is a very small amount. If no additives are added during anaerobic fermentation silage, the amount of silage beneficial bacteria is much smaller than that of the cellulase-treated treatment group.Before and after the silage, the microbes had similar species composition at the phylum level, and were composed of Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria. The only difference is the change in its content. This change in the species of different bacteria also represents the excitement of the microscopic world during the silage process.

Among them, the abundance of Proteobacteria was as high as 92.17%. However, after 30 days and 60 days of silage fermentation, the abundance of Proteobacteria decreased from 92.17% to 1.75-36.13%. Instead, the increase in firmicutes. It increased from 0.99% in raw material alfalfa to between 63.6% and 93.9%. And with the increase of silage time, whether it is the control group or the cellulase silage. The content of firmicutes has increased by 3%-13%. This also indicates that as the fermentation time is extended, the number of beneficial bacteria is also gradually increasing. And from the different experimental addition treatments, the firmicutes content of the silage added with cellulase is more rapid, so the 60-day firmicutes content is relatively low. This also indicates that the addition of cellulase can make silage fermentation more complete.

In pasture silage, some species are recognized as the main fermented lactic acid bacteria in the academic, such as Lactococcus, Enterococcus, and Lactobacteriaceae. As shown in Figure 3, only Pantoea's abundance is dominant in fresh alfalfa, and the advantage of LAB is that only insignificant Lactococcus and Enterococcus exist. It may also be that the lactic acid bacteria suitable for growth on the saline alfalfa are Lactococcus, Enterococcus. In the absence of additives, after 30 days and 60 days of silage, the dominant species became fermented feeds with Lactococcus and Enterococcus as the dominant lactic acid bacteria. And as time goes by, its dominant lactic acid bacteria does not change much. After adding the fermentation promoter cellulase, slightly different from the CK group, the dominant lactic acid bacteria increased Lactobacillus. It is undeniable that cellulase has a degrading effect, which degrades macromolecular carbohydrates such as cellulose, hemicellulose and lignin in the crude fiber of the stem into small molecules of monosaccharides or polysaccharides, thereby rapidly enhancing the fermentation activity of the lactic acid bacteria.

Through the inductive organization of silage sample microorganisms, 16S function prediction is carried out on the datas, through each OTU corresponding greengene id, from the eggNOG database to the description information of each COG, and its functional information, thereby obtaining functional abundance Spectrum (Figure 4). In the above, we know that alfalfa has undergone 30 days and 60 days of silage fermentation and its quality has changed. From 16S function prediction analysis, it is known that its microbial community mainly focuses on life activities between cells. It is the reproductive growth of these bacteria that leads to nutritional differences in the saline alfafa. The main difference is mainly in Energy production and conversion (C), Amino acid transport and metabolism (E), Carbohydrate transport and metabolism (G), Intracellular trafficking, secretion, and vesicular transport (U). These functions are related to microbial activity during silage. In particular, the differences in function G between these treatment groups, which is a clear distinction between bacteria and carbohydrates in plant nutrition. This also explains the difference between WSC in fresh alfalfa and post-silage WSC. LAB mainly ferments and metabolizes WSC, which will result in the production of carbohydrate transport and metabolism (G) and Amino acid transport and metabolism (E).

Discussion

Silage is a process in which saccharide is converted into organic acid-lactic acid-based process by LAB anaerobic fermentation in a closed environment to reduce the pH of the closed environment and inhibit the growth and reproduction of other harmful microorganisms. The loss of nutritional value of feed. Good silage preservation requires a LAB count of > 105 cfu / g FM [17,26]. In this study, The raw material has a low LAB content (<5.00 log cfu / g FM), which is less than the number of beneficial microorganisms to support the success of the experiment. The data shows that the content of E. coli is also high, which indicates that the alfalfa silage requires fermentation additives to ensure complete fermentation.

Adequate WSC is a key factor in forage silage, which provides sufficient nutrient substrate for LAB reproduction. When the content of WSC reaches 60-80 g / kg [27], the fermentation can be carried out normally. In this study, the WSC content of alfalfa was 32.7 g/kg DM, which was lower than the WSC content of silage corn(Zhang et al., 2018) [28]. Perhaps Salt stress in soil inhibits the accumulation of WSC in fresh alfalfa [29]. Therefore, the current WSC content is sufficient to ensure good preservation of the alfalfa under addition.

The time of alfalfa sliage has an important influence on silage fermentation quality and microorganism [30]. After 30 days of ensiling, CP and WSC have different declines, which is caused by bacterial lactic

acid fermentation in this study, This is similar to the results of M Maharlooei's research [31]. In this study, after 30 days of fermentation silage, the nutritional quality and fermentation quality did not change significantly. It should be noted that the change in quality on the surface is not too great. The main microorganisms in the silage of the saline-alkali mantle are still the advantage of Firmicutes, but the advantage is that it has changed from 30 days of Enterococcus to 60 days of Lactococcus. After the addition of cellulase, the dominant genus shifted from 30 days of Lactococcus to 60 days of Lactobacillus-preferred genus [32-35]. Jacxsens et al. revealed that Pantoea agglomerans would be metabolized to produce acetic acid, propionic acid and succinic acid [36]. And Enterobacteriaceae is also capable of producing sugar metabolism under anaerobic conditions. This is similar to the results of this study. The relative abundance of Enterobacteriaceae (especially Lactococcus and Pantoea) is higher in alfalfa silage under salt stress (Figure 3), which may explain the increase of organic acids.

Lactic acid is the main factor leading to the pH drop in silage environment, and pH is also an important indicator to determine whether anaerobic fermentation is complete. McDonald et al. also showed that pH is an important indicator of the degree of fermentation and the quality of silage [37].From the fermentation situation in this paper, the organic acid content of 60d was significantly better than 30d, and the quality of alfalfa silage after salt stress treatment was better. However, after up to 1 and 2 months of fermentation, the cellulase-added silage had a higher WSC content, but its CP content was lower than CK. This may be due to the fact that the addition of cellulase leads to a dramatic increase in the number of lactic acid bacteria and expands the effect of anaerobic fermentation [38]. On the other hand, alfalfa have been identified as having high antibacterial activity [39], which may inhibit the growth of cellulase. In the current experiment, as the silage time prolonged, the lactic acid content decreased and the pH value increased significantly. This may be because Lactobacilli can metabolize lactic acid conditions in the absence of sugar [40]. Therefore, the role of microbial bacteria in the silage fermentation needs further study.

High-throughput sequencing can provide a wealth of data for exploring taxonomic classifications and activities of silage microbial bacteria [41]. In this study, alpha diversity values indicate that the diversity of bacterial communities after silage is more abundant, consistent with previously reported results [42, 43]. St-Pierre found that Mencius, Bacteroides, Chloroflexi and Proteobacteria are dominant gates that play an important role in hydrolysis and acid production [44]. Among these, the Firmicutes phylum is the main gate in most grass silage [45]. Proteobacteria is the most abundant bacteria in fresh alfalfa, and its content is above 90%. Bao also found that Proteobacteria is the main phylum of fresh alfalfa in the study of alfalfa [46]. Proteobacteria is the most abundant bacteria in fresh alfalfa, and its content is above 90%. Bao also found that Proteobacteria is the main phylum of fresh alfalfa in the study of alfalfa [46]. And in this study, the main genus such as Lactobacillus, Enterococcus, Lactococcus and Weissella after

silage were also significantly inhibited during the fermentation process. The growth of spoilage bacteria such as Proteobacteria is similar to that of Yanbing et al. and Di M A. et al. on corn and ryegrass [47, 48].

LAB is an important member of the bacterial community and a key microorganism in the silage that dominates silage quality. Lactobacillus, lactococcus and enterococcus play a key role in the anaerobic fermentation of saline-alkali alfalfa. Even Enterobacteriaceae and pantoea play a role in it, and they also consume nutrients for their own growth and reproduction [49]. And their relative abundance is lower than the LAB content, which also shows that the feed is better fermented. And under the influence of cellulase in silage, Lactobacillus rapidly multiplies and becomes another dominant bacteria in addition to Lactococcus and Enterococcus. Our research results on saline-alkali silage microorganisms are different from previous studies. It is reported that lactococcus and Bacillus are the main genus of silage after fermentation [50, 51]. Therefore, the role of LAB silage fermentation requires further research. Whether it can also be said that Lactobacillus is more suitable for the growth and reproduction of alfalfa in saline soil as long as it has suitable living conditions. In these genus, there may be LAB which is halophilic or salt-tolerant lactic acid bacteria, which requires We conduct further research.

Conclusion

In conclusion, through our experiments, we are more eager to explore a way of using forages in saline-alkali land, and to maximize the utilization value of forage under salt stress and saline-alkali grasses with appropriate input.we found that the silage quality of Saline-alkali alfalfa can be ensured by anaerobic fermentation with addition. And all treatments will get a better fermentation quality. The LAB plays a major role in the saline alkaline alfalfa silage are Lactobacillus, lactococcus and enterococcus. In addition, the use of cellulase enhances the reproduction of Lactobacillus.

Methods

Silage Preparation

We selected varieties of the Zhongmu No. 3 (salt-tolerant), Alfalfa were harvested on July 15 2018, in an experimental fields of Inner Mongolia Agricultural University (40°17’N, 111°27’E). Fresh alfalfa treated with wilting for 4h to a moisture content of 60.8%. After treatment with cellulase or control(no cellulase), and ensiling for 30 days or 60 days. Use alfalfa to chop alfalfa to 2-3cm The material was then placed in a polyethylene plastic bag (20 x 30 cm), each bag containing 300 grams, 3 replicates per treatment. And use a vacuum packaging machine to seal it. Silage samples will be stored at 25 °C.

Analysis of Microbial Population, Organic Acid, and Chemical Composition

After the silage time of the sample is reached, the alfalfa silage bag is disassembled, 10 g of the sample is mixed with 90 ml of the sterile aqueous solution [22], and the fermentation broth is fully extracted using a homogenous slap apparatus. The bacterial solution was diluted from 10^{-1} to 10^{-5} and used for screening of the number of microorganisms. The amount of LAB was calculated using MRS medium under anaerobic conditions, and the amount of E. coli was calculated using a Rose Bengal Agar under aerobic conditions.

The fermentation broth obtained above was used to determine the fermentation quality of alfalfa, and the specific method was the same as Cai did [21], The content of the organic acid is determined by a liquid chromatograph. The value of the silage pH was determined using a glass electrode pH meter (STARTER 100/B, OHAUS, Shanghai, China). The dry matter (DM) content was calculated after drying the tantalum sample at 65 ° C for 48 h. The content of crude protein (CP) was determined by Latimer [23] method. The content of neutral detergent fiber (NDF) and acid detergent fiber (ADF) was determined as described by Van Soest et al. [24]. The content of Water Soluable Carbohydrate (WSC) was determined as described by Thomas [25].

DNA extraction and PCR Amplification

The silage microbial DNA was extracted according to the EZNA® kit (Omega Bio-tek, Norcross, GA, US) instructions. The concentration and purity of the DNA was determined using a NanoDrop 2000 (Thermo Scientific, Wilmington, USA). The DNA extraction quality was measured by 1% agarose gel electrophoresis. 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT

-3') Primers were used for PCR amplification of V3-V4 variable region. The amplification procedure was: pre-denaturation at 95 °C for 3 min, 27 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s), extended for 10min at the end of 72°C (PCR instrument: ABI GeneAmp® 9700). The amplification system is 20μL, 4μL 5*FastPfu buffer Solution, 2 μL 2.5 mM dNTPs, 0.8 ul primer (5 μM), 0.4 μL FastPfu polymerase; 10 ng DNA template.

The resulting PCR product was extracted from a 2% agarose gel and further purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and using QuantiFluorTM-ST (Promega, USA) according to the manufacturer. Quantification of the program.

Statistical Analysis

SAS 9.3 software was used to analyze the differences in the data in the article. The difference between the means was assessed by Tukey's multiple comparison test, a significant level of $p < 0.05$ [26].

Availability of data and materials

Analytical data during the study and are included in this article and its supplemental information files.

Abbreviations

LAB: Lactic acid bacteria

DM: Dry matter; CP: Crude protein

NDF: Neutral detergent fiber

ADF: Acid detergent fiber

WSC: Water-soluble carbohydrates

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Declarations

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Authors Contributions

YSJ designed the experiments and revised the manuscript. QL, QMC performed the experiments. QL wrote the manuscript. GTG and MLH carried out the data analysis. All authors reviewed and considered the manuscript.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Tables

TABLE 1 | Chemical and microbial compositions in the pre-ensiled samples.

items	*Medicago sativa* L.
DM (%)	29.9
Crude protein (g/kg DM)	21.9
Neutral detergent fiber (g/kg DM)	37.4
Acid detergent fiber (g/kg DM)	33.9
Fatty acid (g/kg DM)	7.4
Water-soluble carbohydrates (g/kg DM)	32.7
Lactic acid bacteria (log cfu/g FM)	4.57
Coliform bacteria (log cfu/g FM)	4.80
Yeast (log cfu/g FM)	2.24

DM, dry matter; cfu, colony forming units.

TABLE 2 | Chemical characteristics of silage prepared with and without Cellulase under ensiling for 30 and 60 days.

Items	30days		60days	
	CK	Cellulase	CK	Cellulase
DM (g/kg)	30.3a	28.8b	29.6b	28.3a
Crude protein (g/kg DM)	20.7a	19.1a	19.8a	18.4a
Neutral detergent fiber (g/kg DM)	42.3b	41.9a	41.9a	39.8a
Acid detergent fiber (g/kg DM)	38a	36.7a	39.3a	38.3a
Fatty acid (g/kg DM)	1.87a	2.18a	2.1a	1.95a
Water-soluble carbohydrates (g/kg DM)	27.06a	30.2a	25.3b	27.6b

Values within the same row under same ensiling days with different superscripts in lowercase letter differ significantly from each other at $P < 0.05$

TABLE 3 | Chemical fermentation characteristics of alfalfa silage prepared with and without Cellulase under ensiling for 30 and 60 days.

Items	30 days		60 days	
	CK	Cellulase	CK	Cellulase
pH	4.37a	4.27a	4.24b	4.1a
Lactic acid(% DM)	2.39b	2.66b	2.6a	3.48a
Acetic acid (% DM)	4.63a	3.48b	4.7a	4.85a
Propionic acid (% DM)	0.01b	0.01a	0.01b	0.01a
Butyric acid (% DM)	ND	ND	ND	ND
NH_3-N(g kg -1 DM)	2.38b	1.92a	2.45b	2.49a

“ND”, not detected; “—”, not analyzed; values within the same row under same ensiling days with different superscripts in lowercase letter differ significantly from each other at $P < 0.05$.

TABLE 4 | Alpha diversity of bacterial diversity at the day 60 and 120 days of ensiling.

		OTU	Shannon	Ace	Chao	Coverage
	M	132	2.16	112.7	111.35	0.99
30 days	CK	90	1.29	105.79	84.29	0.99
	Cellulase	131	1.71	102.95	98.11	0.99
60 days	CK	128	1.83	102.38	102.7	0.99
	Cellulase	110	1.48	103.57	93.32	0.99

M, pre-ensiled sample.

Figures

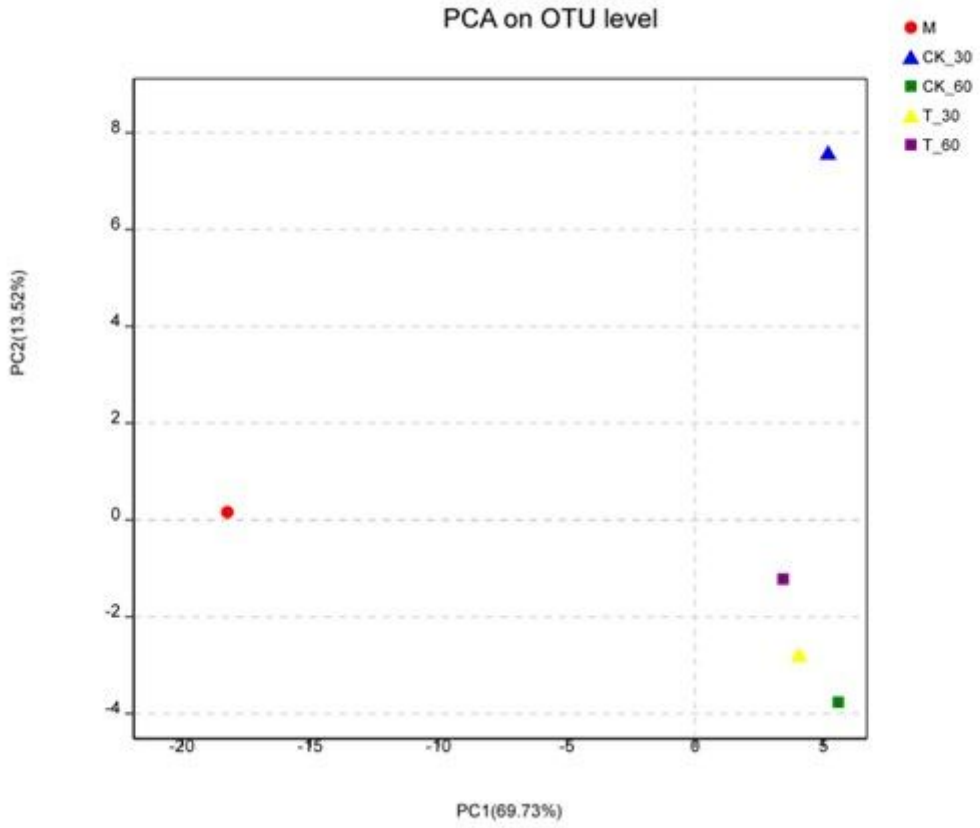

Figure 1

| Principle component analysis (PCA) of samples. PC1, principle component 1; PC2, principle component 2; CK, control (no addition); 30, ensiled for 30 days; 60, ensiled for 60 days; T_30, samples added for treatment,the same with other groups; CK_60, control ensiled for 60 days, the same with other groups.

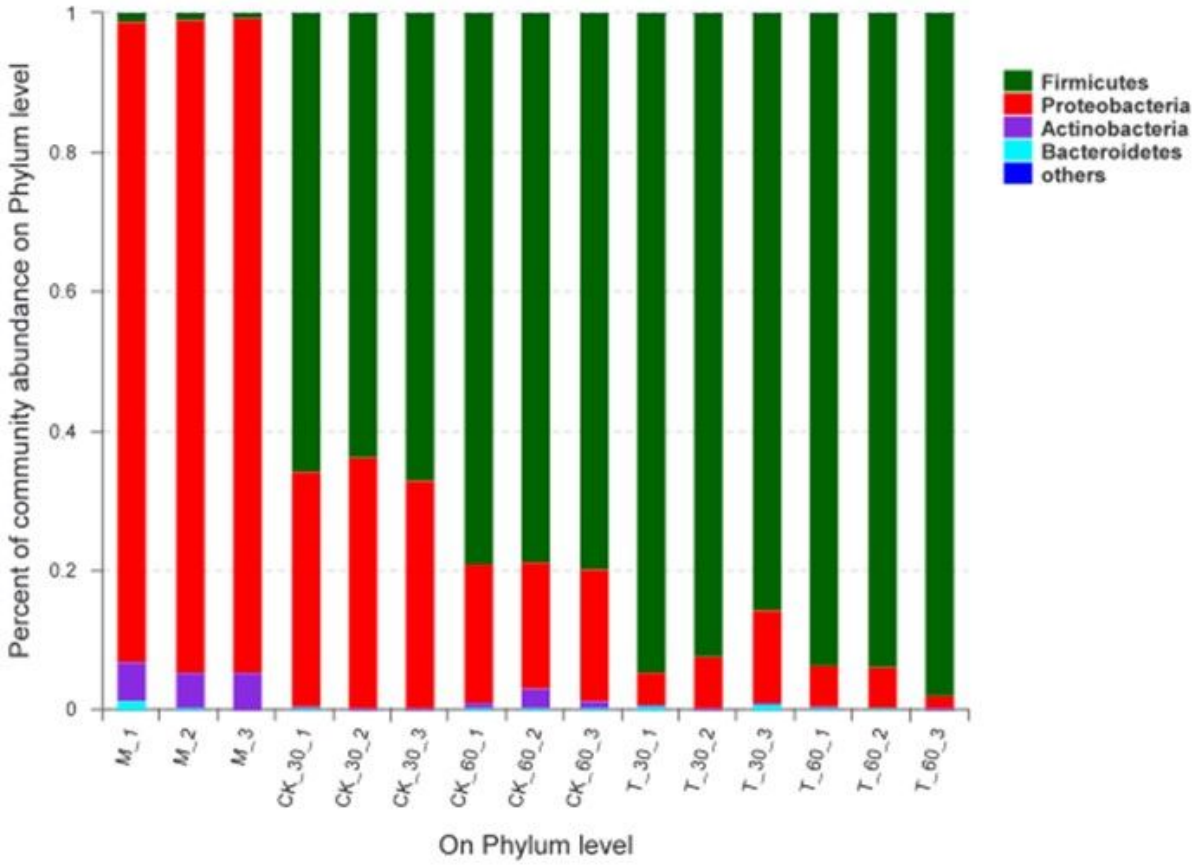

Figure 2

Relative abundance of bacterial at the phylum. M, Pre-ensiled sample; CK, control (no addition); T, with addition of cellulase. 30, ensiled for 30 days; 60, ensiled for 60 days; the same as other groups; 1, 2, 3, triplicate per treatment.

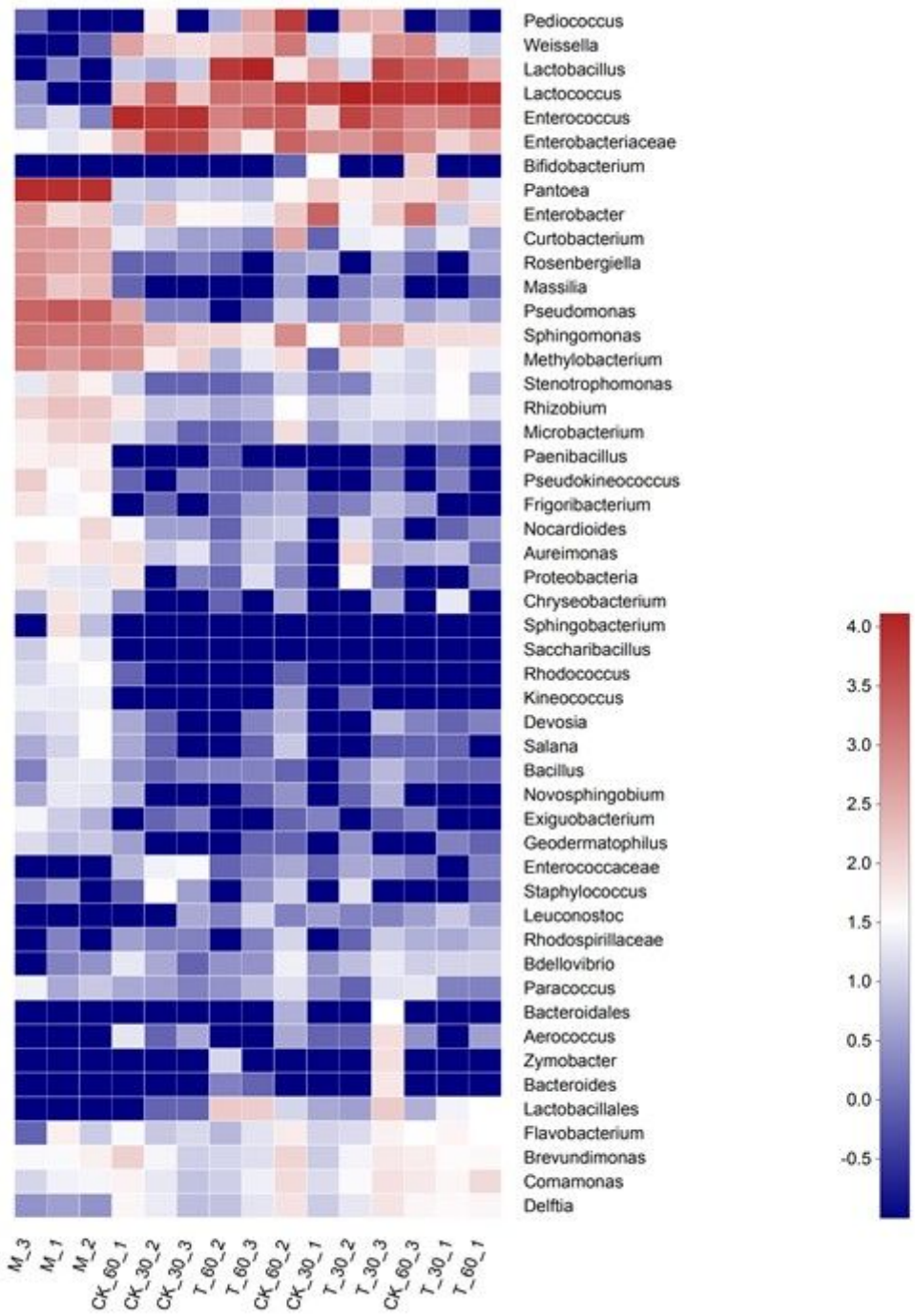

Figure 3

| Heatmap of bacterial at the Genus. M, Pre-ensiled sample; CK, control (no addition); T, with addition of cellulase. 30, ensiled for 30 days; 60, ensiled for 60 days; the same as other groups; 1, 2, 3, triplicate per treatment.

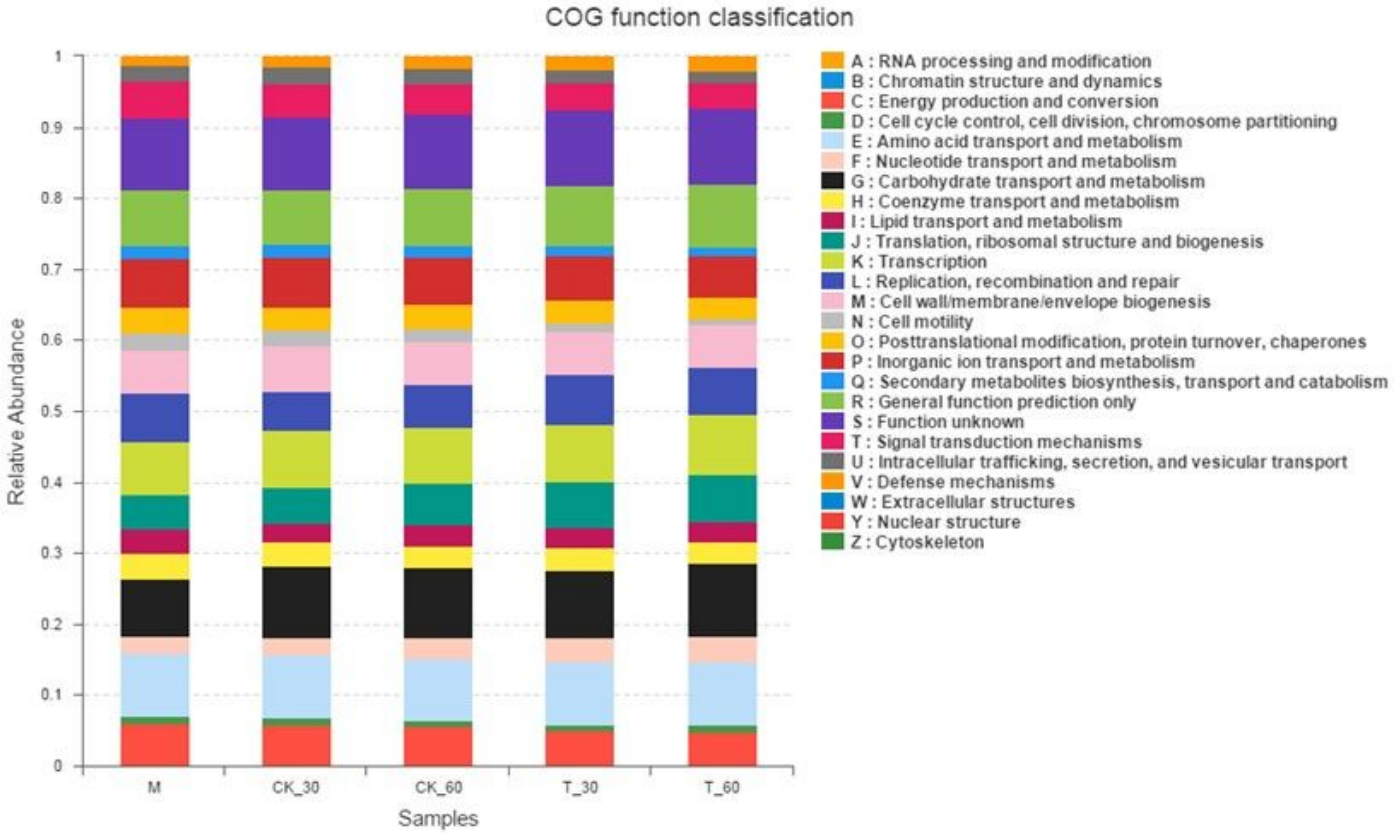

Figure 4

16S function prediction analysis.

Supplementary Files

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