

Article

Effect of Added Composite Bacteria on Fermentation Quality and Microbial Diversity of Mixed Microstorage of Reed Straw and Inferior Jujube

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Abstract: Reed straw is a potential roughage resource that limits its application in livestock production due to its higher fiber content. Inferior jujube is widely used as a non-conventional feed in livestock production due to its high nutritional value. In this study, a two-factor experimental design was used to investigate the effects of fermentation with Composite A (BA) and Composite B (BB) under the condition that the dry matter ratio of reed straw to residual red dates was 1:1, and mixed microstorage was fermented for different times (0 d, 30 d, and 60 d) with the effects of nutrient indexes, fermentation quality, and microbial community dynamics changes. A control group (CON) was set up, six replicates were set up for each treatment, and the amount of bacteria added was 1% of the dry matter basis. The results showed that compared with the control group, both composite bacterial liquids could effectively reduce the nutrient loss of micro-silage feeds, in which with the addition of composite bacterium B (BB) treatment group at the end of fermentation, the crude protein (CP) content increased by 1.91%, and the neutral detergent fiber (NDF) and the acid detergent fiber (ADF) were reduced by 11.32% and 10.61%, respectively. The volatile fatty acid content was significantly higher than that of the control group, which was 26.41 $\mu\text{g}\cdot\text{mL}^{-1}$. The BB group had the highest abundance of *Lactobacillus* during the fermentation process, which could produce a large amount of lactic acid and lower the pH to improve the quality of feed fermentation. In addition, the differences in the indexes were smaller in the BB group compared with the BA group, but the fermentation effect of the BB group was better than that of the BA group.

Keywords: composite bacteria; reed straw; inferior jujube; microbial community succession; vacuum fermentation



Academic Editor: Francesca Berini

Received: 14 January 2025

Revised: 14 February 2025

Accepted: 20 February 2025

Published: 28 February 2025

Citation: Sun, Y.; Zhang, N.; Shao, Y.; Wang, T.; Shen, W.; Wang, X.; Sun, X.; Hua, D. Effect of Added Composite Bacteria on Fermentation Quality and Microbial Diversity of Mixed Microstorage of Reed Straw and Inferior Jujube. *Fermentation* **2025**, *11*, 113. <https://doi.org/10.3390/fermentation11030113>

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1. Introduction

Reed (*Phragmites australis*), a perennial herbaceous plant from the genus *Phragmites* in the subfamily Arundinoideae of the family Poaceae [1], can spread and propagate rapidly in suitable areas. Reed straw refers to the above-ground part of the reed plant after flowering. Reed grass can not only be used as biofuel and soil fertilizer, but it also has a certain degree of ability to remediate soils with varying pH levels, including those contaminated with heavy metal ions [2]. Relevant research [3] shows that the crude protein content of fresh reed stems and leaves is 12.2%, while the crude fiber content is 26.8%, both of which are

higher than the average values for general forage grass. It indicates a high value of feed material development. In addition, fresh reed stems and leaves are rich in vitamins which are important for animal metabolism, promote the microbial ecological balance in the digestive tract, enhance intestinal peristalsis, and increase feed intake. However, reed straw contains a large amount of lignocellulose with a naturally resistant structure after flowering, leading to poor palatability and low digestibility, making it unsuitable for ruminant feeding. Moreover, excess reed that cannot be fully utilized not only causes a waste of resources and environmental pollution in wetland areas [4] but may also result in fire hazards in winter.

Jujube (*Zizyphus jujuba* Mill), commonly known as red dates, black dates, or jujubes, has high nutritional value, with sugar content ranging from 50.3% to 86.9%, protein content from 2.92% to 4.0%, and crude fiber content from 1.6% to 3.1% [5]. In addition, jujube is rich in cyclic adenosine monophosphate (cAMP), which possesses strong antioxidant properties, improves cardiac functioning, reduces fatigue, and inhibits the growth of cancer cells, among other functions [6]. Xinjiang is one of the main producing areas of Chinese jujube, but due to various factors such as insect pests, diseases, improper storage or processing, a significant quantity of residual jujubes including secondary, cracked, and diseased jujube is generated, which are collectively referred to as residual jujubes. Among harvested jujube, 30% are classified as residual, and their nutritional composition is similar to that of normal jujube, making them a valuable resource for livestock and poultry feed.

Microbial solid-state fermentation is a commonly used method for the biological treatment of feed. During the fermentation process, microbial activity secretes various enzymes and other active substances which can enhance the nutritional quality of feed, reduce cellulose content, and improve palatability. At the same time, it can synthesize microbial proteins using non-protein nitrogen and produce various metabolites, such as organic acids and alcohols [7,8]. Single microbial fermentation may encounter problems such as a limited range of enzyme-producing species and extended fermentation cycles. Additionally, there may be a synergistic effect among different bacterial strains that can shorten fermentation cycles and enhance fermentation outcomes [9]. The rich polysaccharide content of red dates can be used as an energy source for microbial fermentation, thereby reducing the cellulose content of reed straw and improving its nutritional quality and feeding value through microbial fermentation.

Therefore, by evaluating the effects of adding different types of complex bacteria on the mixed microstorage of reed straw and inferior jujube, we searched for better fermentation strains of reed straw. In addition, the second-generation sequencing technology was used to reveal the relationship between microbial life activities and feed nutritional indexes in order to improve the nutritional quality and feed utilization of reed straw and inferior jujube mixed microstorage feeds through microbial community succession and metabolic activities to a certain extent. The aim is to provide new ideas for the development and utilization of non-conventional feeds and to make full use of reed straw resources to obtain more benefits.

2. Materials and Methods

2.1. Experimental Materials

The inferior jujube and reed straw used in the experiment were sourced from the 53rd Corps, Third Agricultural Division of the Xinjiang Production and Construction Corps (Geographic location: 40°01'44" N, 79°24'50" E; temperate continental arid desert climate). The above-ground part of the reed straw was mowed, with a stubble height of 10–15 cm. The reed straw was subsequently crushed and kneaded wire to a size of 1–2 cm. Inferior jujubes were collected, crushed, and sieved through a 10-mesh sieve. The nutritional composition of the individual raw materials is shown in Table 1.

Table 1. Nutritional composition of reed straw and residual jujube (%DM).

Raw Material ¹	Index						
	DM	CP	NDF	ADF	Ash	EE	WSC
Reed straw	93.43	3.70	71.74	52.80	7.99	1.50	1.90
Inferior jujube	75.30	6.70	14.65	7.80	2.42	0.30	43.90

¹ DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; Ash, crude ash; EE, crude fat; WSC, soluble carbohydrate. All the parameters are on a dry matter basis.

This experiment was conducted using a completely randomized experimental design with two independent variables (inoculant and time), and the experimental site was located at Shihezi University, Shihezi City, Xinjiang, China (geographic location: 44°18' N 86°02' E; average elevation 450.8 m; temperate continental climate). The nutritional indexes of the reed straw and inferior jujube raw materials are shown in Table 1. The treated raw materials were fully mixed according to the dry matter mass of 1:1, and 1% composite bacterial solution A (BA) and composite bacterial solution BB (BB) were added on the basis of the dry matter of fermentation substrate, respectively, and the composition of bacterial strains is shown in Table 2. A control group (CON) was set up with three time gradients (0 d, 30 d, and 60 d), and six replicates of each treatment were set up, which resulted in a total of 54 test samples. In addition, 1% salt and 0.5% urea were added on a dry matter basis and the moisture content of the substrate was adjusted to 65%. The well-mixed feed samples were packed in 500 g packets into fermentation bags, vacuum sealed, and fermented at room temperature (20–25 °C). The samples were collected and analyzed at 0, 30, and 60 days of fermentation.

Table 2. Composition of two composite bacteria inoculants.

Items ¹	Viable Counts (cfu /mL)	
	Bacteria Complex A	Bacteria Complex B
<i>Lactobacillus acidophilus</i>	1.0×10^6	-
<i>Candida utilis</i>	1.5×10^6	-
<i>Saccharomyces cerevisiae</i>	2.5×10^8	2.5×10^8
<i>Bacillus subtilis</i>	3.5×10^8	3.5×10^8
<i>Geotrichum candidum</i>	1.3×10^6	1.3×10^6
<i>Monilia tropicalis</i>	-	1.5×10^6
<i>Lactiplantibacillus plantarum</i>	2.5×10^7	2.5×10^7
<i>Lactobacillus</i>	-	1.0×10^6

¹ both the composite bacteria inoculants were developed by the Forage Comprehensive Utilization Team of the College of Animal Science and Technology, Shihezi University, Xinjiang, China.

2.2. Nutrient Analysis

The feed samples were initially dried in an oven (LBAO-250; STIK Instrument Equipment Shanghai Co., Ltd., Shanghai, China) at 65 °C. After drying, the samples were pulverized using a grinder (FS200; Guangzhou Bomin Electrical and Mechanical Equipment Co. Ltd., Guangzhou, China) and passed through a 40-mesh sieve to ensure uniformity. The dry matter (DM), crude protein (CP), and ether extract (EE) were determined using the AOAC method [10]. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the method described by Van Soest [11].

2.3. Fermentation Quality Measurement

A total of 10 g well-mixed reed straw and inferior jujube samples were weighed and placed in 150 mL triangular flasks. Then, 100 mL of distilled water was added,

and the mixture was leached at 4 °C for 24 h. After leaching, the solution was filtered using 4 layers of gauze and qualitative filter paper. The pH value of the extract was immediately measured using a digital pH meter (PH8180-0-00; Smart sensor Co. Ltd., Dongguan, China). The remaining filtrate was transferred into a centrifuge tube and stored at −20 °C in a refrigerator. The concentration of ammoniacal nitrogen was determined by the phenol-sodium hypochlorite method [12]. The concentration of soluble sugar (WSC) was determined via the anthrone–sulfuric acid colorimetric method [13]. The lactic acid concentration was assessed utilizing a lactic acid kit (Nanjing Jianjian Bioengineering Institute, Nanjing, China). Volatile fatty acids were analyzed by high-performance liquid chromatography (1260 Infinity II; Agilent Technologies, Waldbronn, Germany) using the method described by Xie et al. [14].

2.4. Microbiological Data Analysis

Microbial DNA was extracted from the reed straw and inferior jujube microstorage samples using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The DNA samples were subjected to 16S rRNA sequencing at LinEn Biotechnology (Shanghai, China). The V4-V5 region of the bacterial 16S ribosomal RNA gene was amplified by PCR (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min) using primers 515F (5'-barcode- GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'), where the barcode represents an eight-base sequence unique to each sample. The PCR reactions were performed in triplicate in a 20 µL mixture containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions.

The purified PCR products were quantified by Qubit[®] 3.0 (Life Invitrogen, Carlsbad, CA, USA) and every twenty-four amplicons with different barcodes were mixed equally. The pooled DNA product was then used to construct an Illumina pair-end library following Illumina's genomic DNA library preparation protocol. The amplicon library was paired-end sequenced (2 × 250) on an Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd., Shanghai, China) according to standard protocols. The raw reads were deposited in the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA1160154).

2.5. Data Processing and Analysis

One-way analysis of variance (ANOVA) was performed using the SPSS 23.0 software to assess differences in chemical composition, fermentation quality, and alpha diversity. Multiple comparisons were performed using the Duncan method (SPSS 26.0, Chicago, IL, USA). Univariate analysis of interaction effects in general linear models was used. The results are expressed as mean ± standard error (SD) and the SEM values are presented. The interaction effects and significance are expressed as *p*-values, with *p* > 0.05 indicating no significant difference, *p* < 0.05 indicating a significant difference, and *p* < 0.01 indicating a highly significant difference. PCoA plots and LEfSe analysis were performed utilizing the online platform <https://cnsknowall.com/#/HomePage>. Clustering correlation heatmap with signs was obtained using the Omic Studio tools at <https://www.omicstudio.cn>.

3. Results

3.1. Chemical Composition

Under the same fermentation time treatment, the addition of different composite bacteria significantly increased the CP content of reed straw and residual red date microstorage feeds (*p* < 0.05) (Table 3), and significantly decreased the content of NDF, ADF, EE, and

WSC ($p < 0.05$). At 30 days of fermentation, there was no significant difference in the DM, CP, and ADF contents between the BA (Addition of composite bacteria A) and BB (Addition of composite bacteria B) groups ($p > 0.05$), NDF was significantly lower in the BB group than in the BA group ($p < 0.05$), while the WSC content was significantly higher than in the BA group ($p < 0.05$). At 60 days of fermentation, the DM content of the added composite bacteria treatment group was significantly higher than that of the CON and BA groups ($p < 0.05$), with no significant differences in the content of CP, NDF, ADF, and WSC between the BA and BB groups ($p > 0.05$).

Table 3. Effects of different composite bacteria inoculants on the nutritional composition of the reed straw and inferior jujube mixture over fermentation time.

Items	Groups ¹	Time			SEM	<i>p</i> -Value ²		
		0	30	60		T	B	T*B
Dry matter (%)	CON	40.42 ± 0.30 ^e	38.30 ± 0.99 ^{af}	31.13 ± 3.67 ^{cg}	0.529	<0.001	<0.001	<0.001
	BA	40.30 ± 0.58 ^e	36.53 ± 1.16 ^{bf}	34.08 ± 0.81 ^{bg}				
	BB	40.95 ± 0.58 ^e	37.50 ± 0.88 ^{abf}	36.28 ± 0.64 ^{ag}				
Crude protein (%DM)	CON	4.25 ± 0.13	4.47 ± 0.33 ^b	4.25 ± 0.25 ^b	0.126	<0.001	<0.001	<0.001
	BA	4.59 ± 0.12 ^f	4.68 ± 0.39 ^{abf}	5.93 ± 0.25 ^{ae}				
	BB	4.30 ± 0.32 ^g	5.23 ± 0.38 ^{af}	6.24 ± 0.23 ^{ae}				
Neutral detergent fiber (%DM)	CON	64.14 ± 1.21 ^e	61.95 ± 0.92 ^{af}	58.28 ± 1.28 ^{ag}	0.742	<0.001	<0.001	<0.001
	BA	64.00 ± 1.25 ^e	58.12 ± 0.30 ^{bf}	54.80 ± 1.28 ^{bg}				
	BB	64.40 ± 1.83 ^e	54.57 ± 1.77 ^{cf}	53.08 ± 1.00 ^{bf}				
Acid detergent fiber (%DM)	CON	43.61 ± 0.81 ^e	40.35 ± 1.61 ^{af}	37.76 ± 0.68 ^{ag}	0.641	<0.001	<0.001	0.01
	BA	43.57 ± 0.63 ^e	38.49 ± 1.40 ^{abf}	35.80 ± 1.47 ^{bg}				
	BB	44.40 ± 0.57 ^e	36.27 ± 1.19 ^{bf}	33.79 ± 1.35 ^{cb}				
Ether extract (%DM)	CON	1.47 ± 0.08	1.50 ± 0.18	1.67 ± 0.15	0.034	0.549	0.819	0.04
	BA	1.65 ± 0.26	1.70 ± 0.21	1.43 ± 0.25				
	BB	1.45 ± 0.058 ^f	1.47 ± 0.21 ^{ef}	1.73 ± 0.17 ^e				
Crude ash (%DM)	CON	5.35 ± 0.77	5.99 ± 0.45 ^b	6.95 ± 1.79 ^b	0.261	<0.001	<0.001	0.145
	BA	5.73 ± 0.15 ^g	7.93 ± 0.55 ^{af}	9.26 ± 0.76 ^{ae}				
	BB	5.72 ± 0.69 ^g	7.58 ± 0.49 ^{af}	8.97 ± 0.41 ^{ae}				
Water soluble carbohydrate (μg·mL ⁻¹)	CON	19.97 ± 0.65 ^e	15.70 ± 0.41 ^{af}	12.47 ± 0.38 ^{ag}	1.454	<0.001	<0.001	0.01
	BA	19.89 ± 0.66 ^e	13.10 ± 0.43 ^{bf}	11.70 ± 0.16 ^{bg}				
	BB	19.73 ± 0.47 ^e	12.20 ± 0.67 ^{cf}	11.53 ± 0.13 ^{bf}				

¹ CON, fermentation without the addition of composite bacteria; BA, fermentation treatment group with the addition of composite bacteria A; BB, fermentation treatment group with the addition of composite bacteria B. a–c indicates within the same column with different superscripts differ significantly ($p < 0.05$). e–g indicates within the same row with different superscripts differ significantly ($p < 0.05$). ² T denotes time treatment, B denotes composite bacteria inoculant treatment, and T*B denotes the interaction between time and inoculant.

Under the same strain treatment, as fermentation time increased, the chemical composition of each treatment group significantly differed from that of the 0-day treatment group. In particular, the NDF, ADF, and WSC contents were significantly lower than those of the control group at 60 days of fermentation for each treatment group ($p < 0.05$), CP was significantly higher than that of the 0-day treatment group ($p < 0.05$), and the crude ash was significantly higher than that of the 0-day treatment group at 60 days of fermentation for the BA and BB treatment groups ($p < 0.05$). And, there was an interaction effect between the DM, CP, NDF, ADF, and WSC fermentation time and the treatment of added composite bacteria ($p < 0.01$).

3.2. Fermentation Quality

With the same time treatment, as fermentation time increased (Table 4), the pH and ammonia nitrogen concentration of the added composite bacteria treatment group were significantly lower than those of the control group (CON), and the lactic acid and total volatile acid content were significantly higher than those of the control group ($p < 0.05$), with no significant differences between the BA and BB groups ($p > 0.05$). Butyric acid was not observed at 60 days of fermentation between the two groups.

Table 4. Effects of different composite bacteria inoculants on the fermentation parameters of the reed straw and inferior jujube mixture over fermentation time.

Items	Groups ¹	Time (d)			SEM	<i>p</i> -Value ²		
		0	30	60		T	B	T*B
pH	CON	6.25 ± 0.10 ^e	5.41 ± 0.38 ^{af}	4.78 ± 0.15 ^{ag}	0.139	<0.001	<0.001	0.002
	BA	6.25 ± 0.07 ^e	4.80 ± 0.08 ^{bf}	4.31 ± 0.15 ^{bg}				
	BB	6.23 ± 0.05 ^e	4.64 ± 0.18 ^{bf}	4.11 ± 0.24 ^{bg}				
Lactic acid (mmol·mL ^{−1})	CON	1.05 ± 0.22 ^g	4.38 ± 0.60 ^{bf}	7.15 ± 0.81 ^{be}	0.749	<0.001	<0.001	<0.001
	BA	1.05 ± 0.26 ^g	4.68 ± 0.70 ^{bf}	10.77 ± 0.88 ^{ae}				
	BB	1.02 ± 0.38 ^g	6.29 ± 0.15 ^{af}	12.85 ± 1.09 ^{ae}				
Acetic acid (μg·mL ^{−1})	CON	0.84 ± 0.05 ^g	12.56 ± 0.75 ^{bf}	16.44 ± 0.85 ^{be}	1.472	<0.001	<0.001	0.011
	BA	0.85 ± 0.10 ^g	17.2 ± 1.89 ^{af}	20.88 ± 1.05 ^{ae}				
	BB	0.84 ± 0.10 ^g	18.34 ± 2.15 ^{af}	22.99 ± 2.02 ^{ae}				
Propionic acid (μg·mL ^{−1})	CON	0.33 ± 0.02 ^f	3.07 ± 0.22 ^{be}	3.26 ± 0.14 ^{ae}	0.240	<0.001	<0.001	<0.001
	BA	0.33 ± 0.13 ^g	3.47 ± 0.15 ^{ae}	2.85 ± 0.23 ^{bf}				
	BB	0.33 ± 0.02 ^g	3.68 ± 0.08 ^{ae}	3.17 ± 0.15 ^{af}				
Butyric acid (μg·mL ^{−1})	CON	ND	ND	0.17 ± 0.03	-	-	-	-
	BA			ND				
	BB			ND				
TVFA (μg·mL ^{−1})	CON	1.17 ± 0.23 ^g	15.62 ± 0.99 ^{bf}	19.86 ± 1.40 ^{be}	1.697	<0.001	<0.001	0.008
	BA	1.18 ± 0.16 ^f	20.67 ± 2.26 ^{ae}	23.73 ± 0.70 ^{abe}				
	BB	1.17 ± 0.31 ^g	22.03 ± 2.64 ^{af}	26.41 ± 1.95 ^{ae}				
Ammonia nitrogen (μg·mL ^{−1})	CON	0.45 ± 0.17 ^f	0.79 ± 0.16 ^{ae}	0.76 ± 0.08 ^{ae}	0.024	<0.001	<0.001	0.002
	BA	0.45 ± 0.03	0.46 ± 0.11 ^b	0.49 ± 0.02 ^b				
	BB	0.44 ± 0.02 ^f	0.54 ± 0.10 ^{be}	0.53 ± 0.09 ^{bef}				

¹ TVFA, total volatile fatty acids; ND, indicates not detected; CON, fermentation without the addition of complex bacteria; BA, fermentation treatment group with the addition of complex bacteria A; BB, fermentation treatment group with the addition of complex bacteria B. a–c indicates within the same column with different superscripts differ significantly ($p < 0.05$). e–g indicates within the same row with different superscripts differ significantly ($p < 0.05$). ² T denotes time treatment, B denotes composite bacteria treatment, and T*B denotes the interaction effect between time and different composite bacteria.

Under the same composite bacteria treatment, there was a decreasing trend of pH among all the treatment groups with the change in fermentation time, and the pH of each treatment group was significantly lower ($p < 0.05$) and the content of lactic acid and volatile fatty acids was significantly higher ($p < 0.05$) compared to that before the non-fermentation (0 d), and butyric acid was detected at the 60th day of fermentation in the control group (CON), which was not treated with the addition of the composite bacteria. There was a highly significant interaction effect ($p < 0.001$) for lactic and propionic acids on time and complex bacteria treatment.

3.3. Microbial Communities

All the samples were annotated at different taxonomic levels (Figure 1A), with the proportion of the samples annotated at phylum and genus levels being 100% and 89.42%, respectively. The top 30 abundances at the genus level were selected to create a bar chart of the dominant species (Figure 1B), in which *Vibrionimonas* had the highest relative abundance, followed by *Lactiplantibacillus* and *Lacticaseibacillus*, which were the top 3 dominant species. At the phylum level, the thick-walled phylum was dominant (Figure 1C). At 30 days of fermentation, the abundance of thick-walled phylum in the group treated with the addition of Complex B was significantly higher than that in the CON and the BA groups ($p < 0.05$). At 60 days of fermentation, the relative abundance of the level of thick-walled phylum in the BB group was significantly lower, while the relative abundance of Bacteroidota was significantly higher. At the genus level (Figure 1D), at 30 days of fermentation, *Levilactobacillus* showed significantly higher abundance ($p < 0.05$) in the CON group than in the BA and BB groups. *Lactiplantibacillus* showed significantly higher abundance

($p < 0.05$) in the BA group compared to the CON and BB groups, and *Lacticaeibacillus* was significantly higher in the BB group than in the CON and BA groups. At 60 days of fermentation, *Lacticaeibacillus* remained the dominant genus in the BB group, while the relative abundance of *Lentilactobaillus* was significantly higher in the CON group.

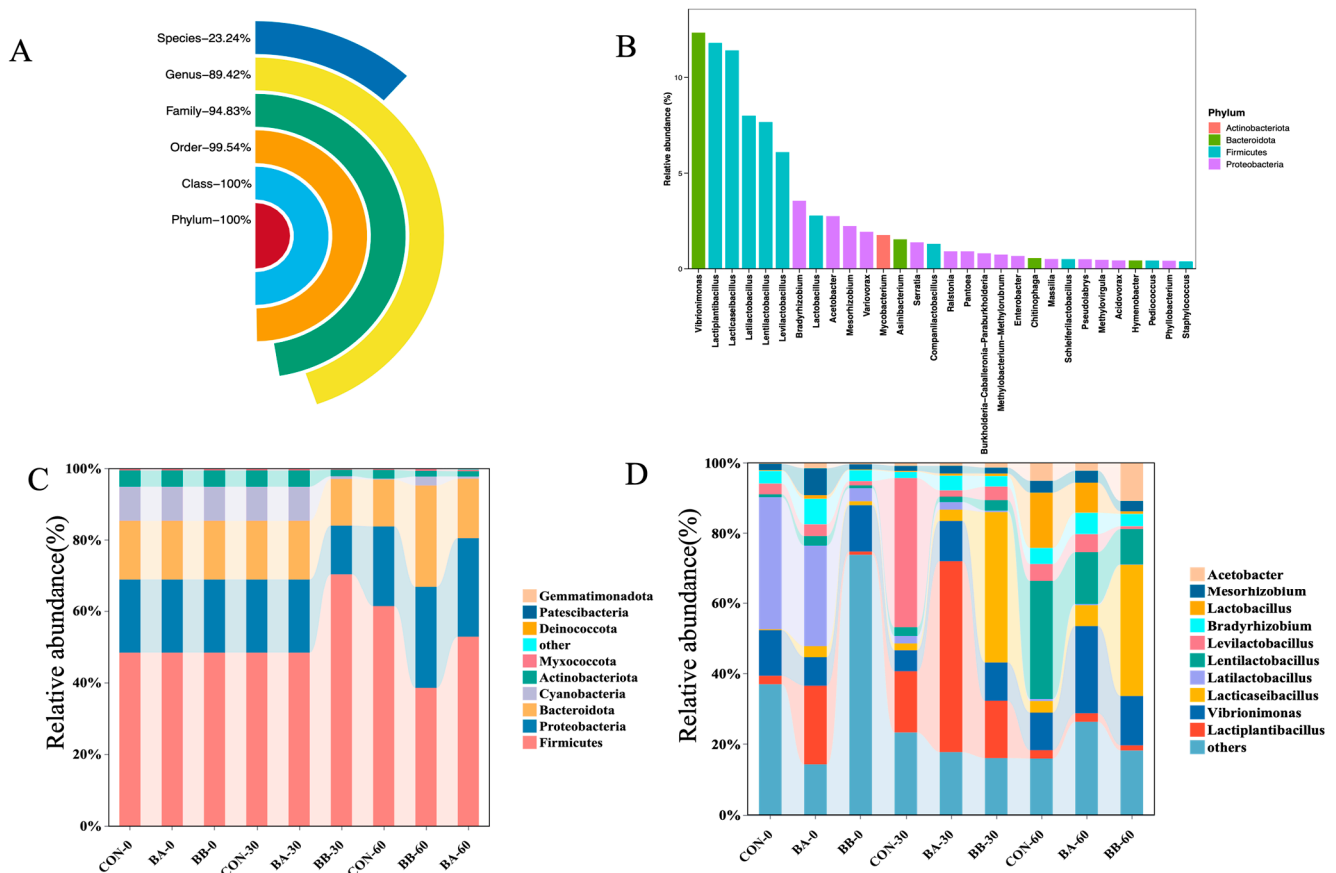


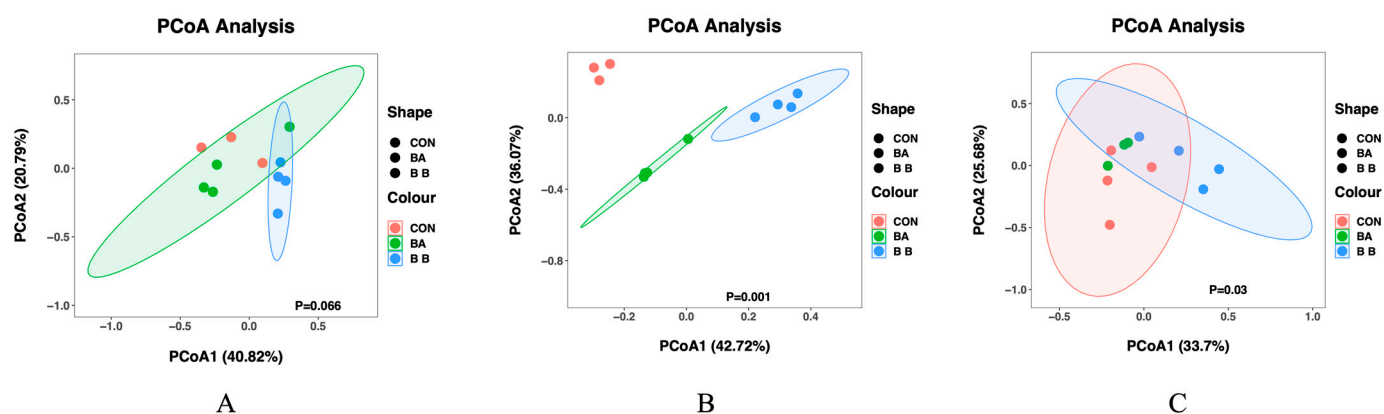
Figure 1. Bacterial community structure in the mixed microstorage of reed straw and inferior jujube by different composite bacteria inoculants. (A) Statistical graph of the annotated results; (B) the fractions of dominant bacterial genera; (C) the phylum structure; (D) the genus structure. CON: control group without the composite bacteria addition; BA: group with the addition of composite bacteria A; BB: group with the addition of composite bacteria B. The numbers 0, 30, and 60 refer to the 0 d, 30 d, and 60 d of fermentation, respectively.

In terms of the alpha diversity index (Table 5), under the same strain treatment, the Chao1 and ACE indexes of the CON and BB groups decreased significantly ($p < 0.05$) with the extension of fermentation time, with no significant differences observed between 30 and 60 days of fermentation ($p > 0.05$). There were no significant differences in the BA group across all the time periods during fermentation ($p > 0.05$). The Shannon and Simpson indexes varied over time, with significant differences observed between the BA and BB groups at all the time periods ($p < 0.05$). For β -diversity, a principal co-ordinates analysis (PCoA) (Figure 2) showed significant structural separation between the treatment groups at 30 days (Figure 2B) and 60 days (Figure 2C) of fermentation according to the Bray–Curtis index. Differential bacteria were identified from the kingdom to the genus level using an LFSE analysis (Figure 3A). By setting the Linear Discriminative Analysis (LDA) value to two and analyzing the differences in bacterial communities in the mixed microstorage of reed straw and residual red dates over different fermentation times, the effect of unfermented pre-fermentation (0 d) on the mixed microstorage bacterial communities was significant (Figure 3B).

Table 5. Effects of different composite bacteria inoculants on the microbial α -diversity of the reed straw and inferior jujube mixture over fermentation time.

Items ¹	Groups	Time (d)			SEM	p-Value		
		0	30	60		T	B	T*B
Chao1	CON	141.33 $\pm$ 36.36 ^a	89.00 $\pm$ 6.38 ^b	83.37 $\pm$ 24.94 ^b	10.470	<0.001	0.100	0.263
	BA	193.58 $\pm$ 79.76	84.33 $\pm$ 11.37	175.00 $\pm$ 114.53				
	BB	191.24 $\pm$ 11.39 ^a	84.50 $\pm$ 6.95 ^b	90.33 $\pm$ 28.45 ^b				
ACE	CON	141.39 $\pm$ 36.46 ^a	89.00 $\pm$ 6.38 ^b	83.51 $\pm$ 24.88 ^b	10.469	<0.001	0.100	0.262
	BA	193.69 $\pm$ 79.73	84.33 $\pm$ 11.377	175.06 $\pm$ 114.48				
	BB	191.42 $\pm$ 11.29 ^a	84.60 $\pm$ 6.94 ^b	90.48 $\pm$ 28.32 ^b				
Shannon	CON	2.99 $\pm$ 0.49	2.43 $\pm$ 0.17	2.65 $\pm$ 0.42	0.104	<0.001	0.403	0.191
	BA	3.40 $\pm$ 0.68 ^a	2.29 $\pm$ 0.61 ^b	3.16 $\pm$ 0.11 ^{ab}				
	BB	3.54 $\pm$ 0.41 ^a	2.30 $\pm$ 0.32 ^b	2.36 $\pm$ 0.57 ^b				
Simpson	CON	0.87 $\pm$ 0.06 ^a	0.77 $\pm$ 0.03 ^b	0.86 $\pm$ 0.06 ^a	0.167	<0.001	0.624	0.148
	BA	0.91 $\pm$ 0.03 ^a	0.72 $\pm$ 0.15 ^b	0.70 $\pm$ 0.11 ^b				
	BB	0.92 $\pm$ 0.04 ^a	0.75 $\pm$ 0.88 ^b	0.75 $\pm$ 0.12 ^b				

¹ CON, fermentation without the addition of composite bacteria; BA, fermentation treatment group with the addition of composite bacteria A; BB, fermentation treatment group with the addition of composite bacteria B. a–c indicates within the same row with different superscripts differ significantly ($p < 0.05$).

**Figure 2.** Beta diversity analysis of the mixed microstorage of reed straw and residual red dates for 0 d (A), 30 d (B), and 60 d (C) of fermentation. CON: control group without the composite bacteria addition; BA: group with the addition of composite bacteria A; BB: group with the addition of composite bacteria B.

According to the correlational analysis between the dominant genus species and chemical composition fermentation indicators (Figure 4), *Lacticaseibacillus*, *Acetobacter*, and *Lentilactobacillus* exhibited significant negative correlations with DM, pH, WSC, and ADF ($p < 0.05$). Furthermore, *Lacticaseibacillus* had a highly significant negative correlation with NDF ($p < 0.001$) and significantly positive correlations ($p < 0.05$) with PA, CP, TVFA, LA, and AA. Both *Acetobacter* and *Lentilactobacillus* were significantly positively correlated ($p < 0.05$) with AA and highly significantly positively correlated ($p < 0.01$) with LA. *Latilactobacillus* showed a significant negative correlation ($p < 0.05$) with $\text{NH}_3\text{-N}$ and AA and a significant positive correlation ($p < 0.05$) with DM, pH, and WSC.

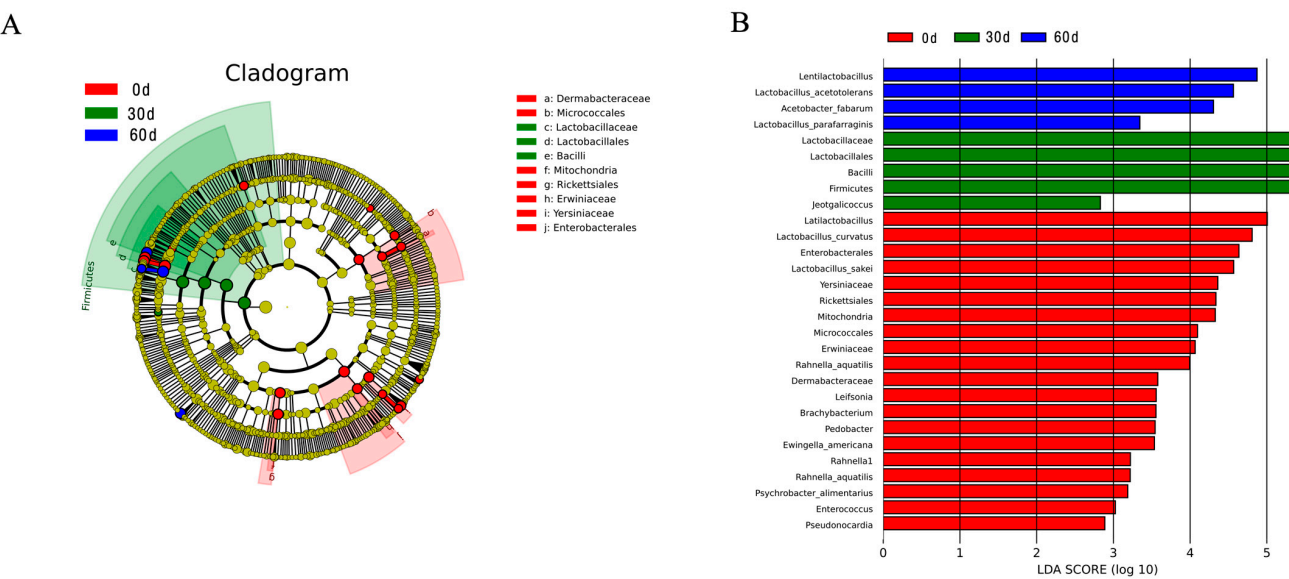


Figure 3. LEfSe analysis of the bacterial community in different fermentation times. Cladogram (A) shows the significantly enriched bacterial taxa from phylum to genus levels. In the histogram (B), the red color illustrates 0 days of fermentation, the green color illustrates 30 days of fermentation, and the blue color illustrates 60 days of fermentation.

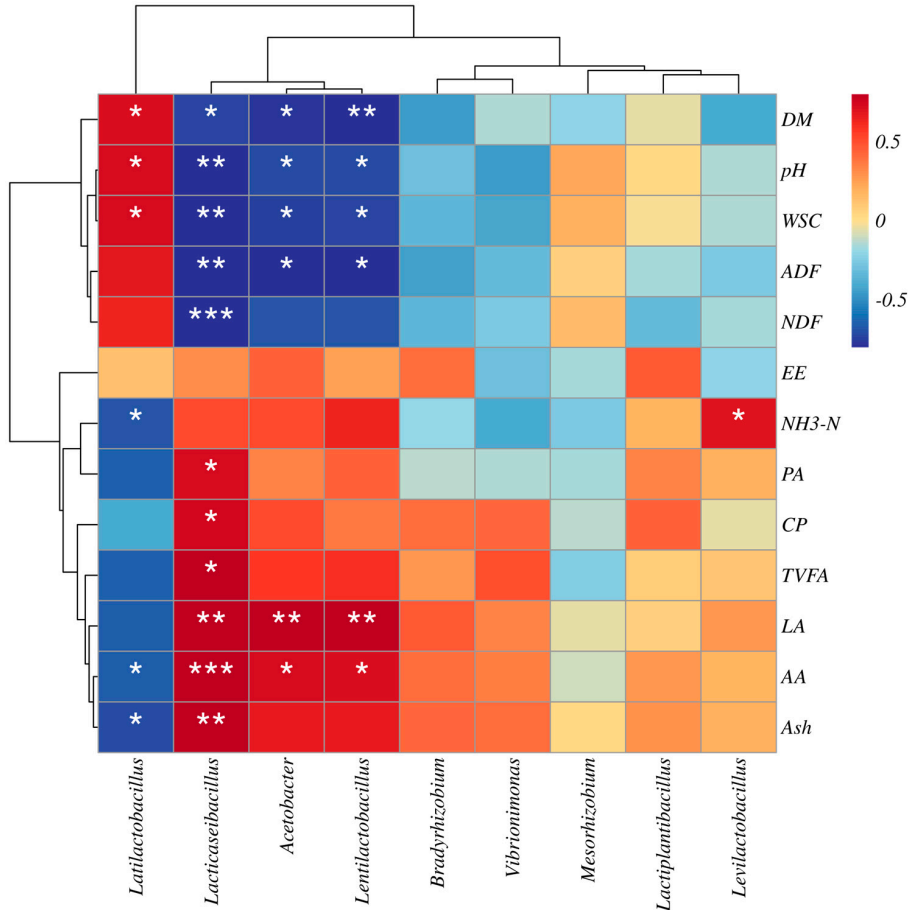


Figure 4. Correlation analysis between the bacterial genus and the feed nutritional composition or fermentation parameters. Note: each column represents a bacteria genus, and each row represents a feed nutritional composition or fermentation parameter. The color red means the correlation is positive, and the color blue means the correlation is negative. * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

4. Discussion

4.1. Chemical Composition

A good microstorage inoculant is crucial for the high quality of straw feed, with metrics such as crude protein (CP), dry matter (DM), neutral detergent fiber (NDF), and acid detergent fiber (ADF) being commonly assessed in microstorage feed research. The results indicate that the DM of reed straw and residual red dates fermented for 30 d and 60 d was lower than that of the control group on day 0. This drop may be attributed to the physiological and metabolic activities of microorganisms throughout the fermentation, which produced cellulase, xylanase, and protein hydrolase, leading to the breakdown of the cell wall and resulting in the transformation of soluble substances and cell wall components into gases and volatiles, thereby decreasing the DM content [15,16]. The addition of composite bacteria B (BB) resulted in significantly higher dry matter content than the CON and BA groups after 60 days of fermentation, possibly due to the composite bacteria B's higher efficiency in inhibiting harmful microorganisms in the raw materials, thus reducing DM loss [17]. The increase in crude protein content over the fermentation period is significant, indicating that microorganisms converted certain nutrients into microbial proteins during fermentation. NDF and ADF are indicators of feed digestibility, and lower contents are correlated with higher digestibility in animals. Dewar et al. [18] reported that microbial physiological activities, enzymes, and other biological or chemical treatments could contribute to the degradation of structural carbohydrates, consistent with our findings that the NDF and ADF contents significantly decreased in the CON, BA, and BB groups by the end of the fermentation. The crude fat content in the BB group increased significantly, possibly due to microbial decomposition and metabolic activities that produced a large number of alcohols, lipids, and organic acids, which in turn increased the feed palatability. The higher WSC content in residual red dates offers a valuable substrate for microbial fermentation. The WSC content decreased significantly in all the groups with extended fermentation time. Previous studies [19] have demonstrated that WSC serves as a crucial substrate for microbial life, supplying the necessary carbon source for their activities, which is consistent with our observations of significant reductions in the WSC levels across the treatment groups in this study.

4.2. Fermentation Quality

A number of indicators collectively determine the fermentation quality in plant feeds [20]. The pH and VFA are the most critical indicators for assessing the quality of microbial fermented feeds [21]. A lower pH indicates better feed preservation. Lv et al. [22] revealed that high-quality silage typically has a pH range of 3.5–4.2. In this study, after 60 days of fermentation, the BB group's pH decreased to 4.11, significantly lower than that of the other groups, which also demonstrated the most rapid decline during the initial 30 days of fermentation, likely due to reduced WSC content. The decreased pH within the first 30 days may also be linked to reduced WSC levels. Lactic acid bacteria in the microstorage system convert WSC to lactic acid leading to pH reduction, inhibiting harmful bacteria and minimizing the degradation of feed nutrients. The addition of composite bacteria significantly increased the lactic acid levels in the microstorage of reed straw and inferior jujube, with the BB group reaching content as high as 12.85 mmol/mL after 60 days of fermentation—much higher than that of the CON group—presumably due to the higher WSC presence in the raw material. Microbial metabolites produce organic acids which determine the quality of fermented feed; higher quality feeds are characterized by lactic acid dominance and lower acetic and butyric acid levels [23]. At both 30 and 60 days of fermentation, the content of acetic acid (AA) and propionic acid (PA) increased significantly, which may be related to the rapid decrease in pH, with a shift from homo-lactic fermenta-

tion to hetero-lactic fermentation, producing lactic acid (LA), AA, and a small amount of CO₂. After 60 days of micro-silage fermentation, the addition of composite bacteria significantly reduced the NH₃-N concentration, likely by minimizing the protein hydrolysis and enhancing nitrogen retention through amino acid deamidation and decarboxylation [19,24].

4.3. Microbial Communities

Microbial fermentation is a prevalent method of treating straw, and the nutritional quality changes in fermented feeds are intricately linked to microbial activity. The composition and abundance of dominant microbial species significantly affect fermented feed quality [25]. In assessing alpha diversity, a larger Chao 1 index indicates more species, a larger Shannon index suggests higher community diversity, a smaller Simpson index indicates higher community diversity, and a larger ACE index implies higher microbial abundance [26]. In this study, the Chao1 index and ACE index of the reed straw and inferior jujube feed with the composite bacteria B decreased significantly over fermentation time, which may be related to the inferior jujube's rich WSC content serving as ample carbon for microbial growth, promoting the rapid proliferation of lactic acid bacteria and resulting in significant lactic acid production, which in turn inhibited the growth of other microorganisms. At both 30 and 60 days of fermentation, the Shannon index of the BB group was lower than that of the CON group, indicating reduced microbial community diversity in the BB group. Related studies [27] have shown that increased dominance of microbial flora correlates with diminished community diversity, consistent with our observation that *Lacticaseibacillus* consistently remained dominant at both 30 and 60 days in the BB group.

Jazi et al. [28] found that fermentation favors the thick-walled bacterial phylum which plays an important role in anaerobic acid hydrolysis, including most of the bacteria involved in lactic acid fermentation responsible for metabolic influence and maintaining internal homeostasis through their unique structure, activity, and metabolites [29]. Meanwhile, the thick-walled bacterial phylum is an important decomposer of plant fibers. At 30 days of fermentation, the BB group exhibited significantly higher thick-walled phylum abundance compared to the CON and BA groups, corroborating the lower NDF and ADF content observed in the BB group's fermented feed. Proteobacteria, which contain a variety of harmful bacteria (like *Escherichia coli* and *Salmonella*), primarily decompose organic material, leading to silage quality deterioration [30]. In this study, the abundance of Proteobacteria significantly declined in the BB group at 30 days, indicating that the addition of composite bacteria B promotes the transition from *Aspergillus* phylum to thick-walled phylum during fermentation, effectively inhibiting harmful microorganisms and enhancing feed quality.

Studies indicate that *Lactobacillus* promotes lactic acid fermentation during silage, which implies favorable silage preservation [31,32]. Relevant studies reported that *Lactobacillus* is present throughout all the fermentation periods of fermented feeds, with dominance noted during storage from 30 and 45 days [33]. In this study, at 30 days of fermentation, *Levilactobacillus* was the dominant genus in the CON group, while *Lactiplantibacillus* was the dominant genus in group BA, and in group BB *Lacticaseibacillus* was the dominant genus. Notably, *Lacticaseibacillus*, *Lactobacillus*, and *Lactiplantibacillus* all belong to the *Lactobacillaceae* family. Furthermore, *Levilactobacillus* is associated with heterofermentation, producing CO₂, lactic acid, and acetic acid or ethanol during fermentation, often causing spoilage and degradation in beer production [34]. In this study, the addition of compound bacteria effectively reduced the relative abundance of *Levilactobacillus* over 60 days, indicating that the compound bacteria effectively inhibited feed heterofermentation. In addition, *Lacticaseibacillus* maintained dominance at both 30 and 60 days of fermentation with compound bacteria B, suggesting that this addition accelerates the

fermentation process of the reed straw and residual red dates mixture while minimizing nutrient loss and enhancing feed fermentation quality.

5. Conclusions

Under the condition of a 1:1 ratio of dry matter between reed straw and inferior jujube, both composite bacteria could reduce the nutrient loss, and at the end of fermentation, the CP of composite bacterium B treatment group was the highest, amounting to 6.24%, and NDF and ADF were reduced by 11.32% and 10.61%, respectively. Compared with compound bacterium A, compound bacterium B could significantly increase the abundance of *Lactobacillus* in microstorage feed, inhibit the proliferation of harmful bacteria, and make the microbial community in the fermented feed reach dynamic balance faster. The correlation analysis showed that microbial life activities could effectively improve the nutritional quality of reed micro-silage and increase its utilization rate, and that Composite B was more suitable for reed straw fermentation than Composite A. In addition, future studies should investigate the effects of reed straw fermented feed on rumen microorganisms and rumen function.

Author Contributions: Conceptualization, X.W. and X.S.; methodology, X.W. and X.S.; software, Y.S. (Yaoqiang Sun), N.Z. and Y.S. (Yongbin Shao); validation, Y.S. (Yaoqiang Sun), N.Z. and Y.S. (Yongbin Shao); formal analysis, Y.S. (Yaoqiang Sun) and N.Z.; investigation, D.H., X.W. and X.S.; resources, D.H., X.W. and X.S.; data curation, Y.S. (Yaoqiang Sun) and N.Z.; writing—original draft preparation, Y.S. (Yaoqiang Sun), N.Z., Y.S. (Yongbin Shao) and T.W.; writing—review and editing, Y.S. (Yaoqiang Sun), N.Z., D.H. and W.S.; visualization, N.Z.; supervision, X.W. and D.H.; project administration, Y.S. (Yaoqiang Sun), X.W. and X.S.; funding acquisition, Y.S. (Yaoqiang Sun), X.W. and X.S. All authors have read and agreed to the published version of the manuscript.

Funding: The Xinjiang Production and Construction Corps, “Three District” science and technology talent support program special (KJTP202315), the Xinjiang Production and Construction Corps, Key Areas of Technological Research and Development (2023AB008), the Third Division’s unveiling and leadership project (KY2024JBGS08), the 8th Group Key Areas of Technological Research and Development (2023NY03), and Demonstration and Promotion of Cotton Straw Micro-Storage Feed Utilization Technology for Common Prosperity in Yingjisha County.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequence data reported in this study were archived in the Sequence Read Archive (SRA) under the accession number PRJNA1160154.

Acknowledgments: We are grateful to Hongxing Jia, Kun Tai Mei Farmers’ Breeding Cooperative, Company 8, 224th Regiment, 14th Division, Xinjiang Production and Construction Corps, and others for providing experimental materials such as reed straws and defective red dates. We also thank every member of the Forage Comprehensive Utilization Team of the College of Animal Science and Technology of Shihezi University.

Conflicts of Interest: The authors declare no conflicts of interest.

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