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RESEARCH

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Bacterial inoculants and enzymes based silage cocktails boost the ensiling quality of biomasses from reed, corn and rice straw

Evan Y. Liu[1,2†], Shuiping Wang[2†], Shibo Wang[1,3], Nazir Ahmad Khan[1], Xiaoling Zhou[3], Shaoxun Tang[1], Chuanshe Zhou[1], Zhiliang Tan[1] and Yong Liu[1*]

Abstract

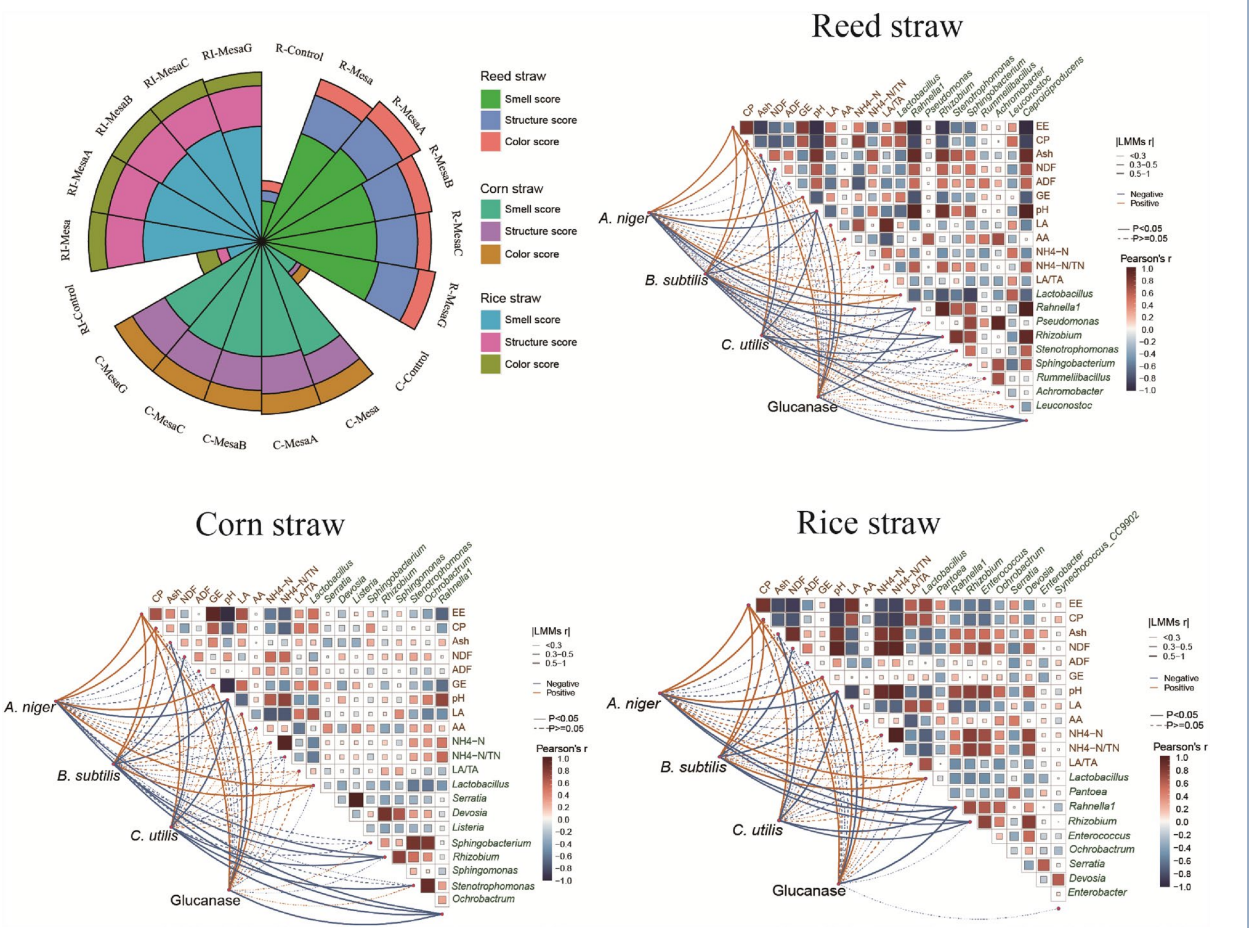
This study investigated the effects of bacterial inoculants and enzyme-based silage cocktails on the dynamics of fermentation, microbiome, and nutritional value of silages produced from low-quality biomasses of reed, rice, and corn straw. A 90-day ensiling trial was performed using five distinct combinations of six basal bacterial species (*Lactobacillus plantarum*, *Lactobacillus buchneri*, *Pediococcus pentosaceus*, *Aspergillus niger*, *Bacillus subtilis*, and *Candida utilis*) and three basal enzymes (xylanase, β-mannanase, and glucanase). Each type of biomass was ensiled with six different treatments, including the Control treatment without an ensiling agent, the basal silage cocktail treatment (Mesa), and Mesa with a double dose of *A. niger* (MesaA), *B. subtilis* (MesaB), *C. utilis* (MesaC) and glucanase (MesaG). The "Mesa" contained (per kg silage), 1.0×10^6 CFU of *L. plantarum*, 1.4×10^7 CFU *L. buchneri*, 3.0×10^5 CFU *P. pentosaceus*, 8.0×10^8 CFU *A. niger*, 1.6×10^6 CFU *B. subtilis* and 1.0×10^9 CFU *C. utilis*, three enzymes (5.0×10^4 U xylanase, 2.5×10^3 U β-mannanase, and 1.0×10^4 U glucanase), and 20 mL molasses. Addition of the silage cocktails significantly improved the fermentation and nutritional quality of the reed, corn, and rice straw silages. Notably, the silage cocktails increased ($P<0.01$) the contents of crude protein (CP), ether extract (EE), gross energy (GE), lactic acid (LA), ratio of LA to total acids and ensiling comprehensive evaluation scores, and decreased ($P<0.01$) the contents of neutral detergent fiber (NDF), acid detergent fiber (ADF) and pH of reed, corn, and rice straw silages. Regarding the silage microbiome, silage cocktails decreased the relative abundance of *Enterobacter* and *Rahnella1*, and increased the relative abundance of *Leuconostoc. A. niger*, and *B. subtilis* had a strong positive correlation with CP, EE, GE and *Lactobacillus,* and a negative correlation with pH, *Rhizobium*, and *Rahnella1* in reed, corn and rice straw silages. In comparison, *C. utilis* had a strong positive correlation with EE, and a negative correlation with pH, *Rhizobium, Stenotrophomonas,* and *Rahnella1*. Glucanase was positively correlated with LA, EE and GE, and negatively correlated with pH and *Rahnella1*. Silage quality characteristics and microbiome did not differ ($P>0.05$) due to the composition of silage cocktails. Based on the comprehensive membership function analysis, the silage comprehensive evaluation scores were highest for double doses of *B. subtilis* and glucanase for reed, corn, and rice straw. This study revealed that silage cocktails upgraded straw silage fermentation and nutritional quality, and provided a practical solution for the optimal utilization of low-quality straw biomass.

Keywords Straw silage, Fermentation quality, Nutritional quality, Microbial diversity, *Silage cocktails*

†Evan Y. Liu and Shuiping Wang have contributed equally to this work.

*Correspondence:
Yong Liu
y.liu@isa.ac.cn
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

China is one of the largest agricultural countries in the world, and the annual production of biomass residues is over 1.04 billion tons [1]. The annual production of corn straw (*Zea mays*), rice straw (*Oryza sativa*) and reed straw (*Phragmites australis*) in China exceeds 220 million tons [2], 270 million tons [3], and 3 million tons [4], respectively. The enormous amount of energy trapped in the highly lignified agricultural biomass residues is not optimally utilized as animal feed. Optimal utilization of agricultural lignocellulosic biomass in ruminant rations can ensure a more effecient utilization and recycling of nutrients in agricultural production systems and play a pivotal role in the long-term sustainability of global food production [5, 6]. Improving the utilization of low-quality biomasses such as reed, rice, and corn straw in ruminants has garnered considerable attention in recent decades due to their high yields, low costs and sustainable production [7]. However, a substantial proportion of the carbohydrates (cellulose and hemicellulose) in these biomass residues are protected from rumen fermentation by complexes with the recalcitrant lignin polymer [8], resulting in lower digestibility, dry matter (DM) intake and production performance of the animals. This represents a major challenge for researchers to produce high-quality silage from agricultural biomass residues.

Straw ensiling can optimize an eco-friendly utilization of agricultural biomass residues in ruminants [9]. Ensiling can loosen the complex structures of these biomasses and improve the availability of cellulose and hemicellulose for enzymatic saccharification and ruminal fermentation [10, 11]. Ensiling treatment of high-fiber lignin-rich biomass with fungi, bacteria, and their ligninolytic and/or fibrolytic enzymes has emerged as the most effective approach for increasing straw

utilization in ruminants, due to its eco-friendly, low-cost, simple and safer methodology [12, 13].

Lactic acid bacteria (LAB) are widely used as silage inoculum, due to their ability to accelerate the procution of lactic acid or acetic acid, decrease in pH, and prevent the decomposition of organic matter, inhibit harmful microbial activities, delay the spoilage of straw silage, and improve animal performance [14, 15]. LAB inoculants are categorized into homofermentative (HoLAB) and heterofermentative (HeLAB) bacteria based on their functions and fermentation patterns during ensiling. HoLAB, such as *Lactobacillus plantarum* and *Pediococcus pentosaceus*, rapidly produce a large amount of lactic acid (lactic fermentation) to lower silage pH, inhibit harmful microbial activities in the early ensiling process, and reduce the degradation of proteins and water-soluble carbohydrates [16–18]. HeLAB, for example, *Lactobacillus buchneri*, produces lactic acid and acetic acid during ensiling to delay the spoilage of straw silage and increase silage aerobic stability [16]. Different LAB genera show different functions and fermentation patterns during the ensiling process. Therefore, mixed inoculation cocktails of *L. plantarum*, *P. pentosaceus*, and *L. buchneri* were developed to improve silage quality and aerobic stability.

Aspergillus niger, a genus of fungi in the order Eurotiales, can utilize nutrients (nitrogen and carbon) from low-quality agricultural biomass residues (crop straw residues) to produce microbial by-products, citric acid and gluconic acid, and secrete many beneficial enzymes (glucoamylase, pectinases, and α-galactosidase) to break down recalcitrant lignin polymer [19–21]. *Bacillus subtilis* is a bacterial species with excellent fermentation properties [22], and produces various enzymes (e.g., amylase, xylanases, levansucrase, cellulases, β-glucanases and proteases), which can improve fermentation of straw silages [23, 24]. In addition, *Candida utilis* is a yeast widely used as a food additive to produce various metabolites and proteins [25]. Xylanase, β-mannanase, and glucanase are microbial extracellular enzymes that break down the recalcitrant lignin structure (xylan, mannan and large polysaccharides) in straws to enhance its digestibility [26–28].

This study was, therefore, designed to investigate the combinatorial effects of bacterial inoculants and enzymes-based silage cocktails on the fermentation characteristics, microbiome, and nutritional value of low-quality, recalcitrant lignin structure of straw silage. In addition, we investigated the relationship between silage fermentation characteristics and the inherent changes in bacterial communities at the genus level in response to the various additives to understand the mechanism. By comparing the impact of 5 distinct combinations of six basal bacterial species and 3 basal fibrolytic enzymes on bacterial community dynamics and silage fermentation, our research provides valuable insights into the sustainable utilization of these plentiful bioresources and addresses a significant research gap in this field.

Materials and methods

Straws, enzymes and microbial inoculants

The straws used in this study included (1) reed, harvested during the withered and yellow stage, (2) corn straw, harvested at the fully mature stage after kernel harvesting, and (3) rice straws, harvested at the mature stage after seed harvesting. All straws were harvested from research farms (113°00”23”E, 29°27”40”N) in Yueyang, China, in November 2021. After harvesting, the straws were chopped, with a theoretical length of cut ranging from 1 to 2 cm for reed straw, 2 to 2.5 cm for corn straw, and 4 to 5 cm for rice straw.

The bacterial inoculants used in this study were obtained from Weikai Hisilicon Biological Engineering Co., Ltd (Shandong, China). The specific bacterial strains and their respective colony-forming units per gram (CFU/g) were as follows: *Lactobacillus plantarum* (*L. plantarum*, 1.5×10^7 CFU/g), *L. buchneri* (1.2×10^8 CFU/g), *Pediococcus pentosaceus* (*P. pentosaceus*, 1.5×10^7 CFU/g), *Candida utilis* (*C. utilis*, 8.0×10^9 CFU/g), *Bacillus subtilis* (*B. subtilis,* 1.0×10^8 CFU/g), and *Aspergillus niger* (*A. niger*, 1.0×10^8 CFU/g).

The enzyme additives used in this study were purchased from Pan Asia Pacific Biotechnology Co., Ltd (Guangdong, China). The specific enzymes and their respective activity units per gram (U/g) were as follows: dextranase (1.0×10^4 U/g), xylanase (1.0×10^4 U/g), and β-mannanase (5.0×10^3 U/g). Molasses used in the experiment were procured from the market in Changsha, China. The experimental work was conducted at the Institute of Subtropical Agroecology Chinese Academy of Sciences in Changsha, China (113°04′59.48”E, 28°12′07.39”N).

Experimental design and ensiling procedure

Experimental design

A 90-day ensiling trial was performed using distinct combinations of six basal bacterial inoculants (*Lactobacillus plantarum*, *Lactobacillus buchneri*, *Pediococcus pentosaceus*, *Aspergillus niger*, *Bacillus subtilis*, and *Candida utilis*) and three basal enzymes (xylanase, β-mannanases, and glucanase) (Table 1). Each type of biomass was ensiled with six different treatments in triplicate silos. The six treatments include a Control treatment without an ensiling agent, a basal silage cocktail treatment (Mesa). The “Mesa” contained (per kg silage), 1.0×10^6 CFU of *L. plantarum*, 1.4×10^7 CFU *L. buchneri*, 3.0×10^5 CFU *P. pentosaceus*, 8.0×10^8 CFU *A. niger*, 1.6×10^6 CFU

Table 1 Experimental design with bacterial inoculants in combination with enzymes in straw silage

Biomass	Cocktail[a]	Straw (g)	Molasses (mL)	Water (mL)	Bacterial inoculants (CFU)						Enzymes (U)		
					L. plantarum	*L. buchneri*	*P. pentosaceus*	*A. niger*	*B. subtilis*	*C. utilis*	xylanase	β-Mannanases	glucanase
Reed straw	R-Mesa	500	20	500	1.0×10^6	1.4×10^7	3.0×10^5	8.0×10^8	1.6×10^6	1.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	R-MesaA	500	20	500	1.0×10^6	1.4×10^7	3.0×10^5	1.6×10^9	1.6×10^6	1.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	R-MesaB	500	20	500	1.0×10^6	1.4×10^7	3.0×10^5	8.0×10^8	3.2×10^6	1.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	R-MesaC	500	20	500	1.0×10^6	1.4×10^7	3.0×10^5	8.0×10^8	1.6×10^6	2.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	R-MesaG	500	20	500	1.0×10^6	1.4×10^7	3.0×10^5	8.0×10^8	1.6×10^6	1.0×10^9	5.0×10^4	2.5×10^3	2.0×10^4
	R-Control	500	/	500	/	/	/	/	/	/	/	/	–
Corn straw	C-Mesa	500	20	710	1.0×10^6	1.4×10^7	3.0×10^5	4.0×10^8	8.0×10^5	5.0×10^8	5.0×10^4	2.5×10^3	1.0×10^4
	C-MesaA	500	20	710	1.0×10^6	1.4×10^7	3.0×10^5	1.2×10^9	8.0×10^5	5.0×10^8	5.0×10^4	2.5×10^3	1.0×10^4
	C-MesaB	500	20	710	1.0×10^6	1.4×10^7	3.0×10^5	4.0×10^8	2.4×10^6	5.0×10^8	5.0×10^4	2.5×10^3	1.0×10^4
	C-MesaC	500	20	710	1.0×10^6	1.4×10^7	3.0×10^5	4.0×10^8	8.0×10^5	1.5×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	C-MesaG	500	20	710	1.0×10^6	1.4×10^7	3.0×10^5	4.0×10^8	8.0×10^5	5.0×10^8	5.0×10^4	2.5×10^3	2.0×10^4
	C-Control	500	/	710	/	/	/	/	/	/	/	/	–
Rice straw	RI-Mesa	500	20	570	1.0×10^6	1.4×10^7	3.0×10^5	6.0×10^8	1.2×10^6	1.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	RI-MesaA	500	20	570	1.0×10^6	1.4×10^7	3.0×10^5	1.2×10^9	1.2×10^6	1.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	RI-MesaB	500	20	570	1.0×10^6	1.4×10^7	3.0×10^5	6.0×10^8	2.4×10^6	1.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	RI-MesaC	500	20	570	1.0×10^6	1.4×10^7	3.0×10^5	6.0×10^8	1.2×10^6	2.0×10^9	5.0×10^4	2.5×10^3	1.0×10^4
	RI-MesaG	500	20	570	1.0×10^6	1.4×10^7	3.0×10^5	6.0×10^8	1.2×10^6	1.0×10^9	5.0×10^4	2.5×10^3	2.0×10^4
	RI-Control	500	/	570	/	/	/	/	/	/	/	/	–

[a] *R* reed straw, *C* corn straw, and *RI* rice straw; control group means the control without bacterial and enzyme supplementation; *Mesa* bacterial inoculant and enzyme silage additive (bacterial inoculants: 1.0×10^6 CFU *L. plantarum*, 1.4×10^7 CFU *L. buchneri*; 3.0×10^5 CFU *P. pentosaceus;* 8.0×10^8 CFU *A. niger;* 1.6×10^6 CFU *B. subtilis;* 1.0×10^9 CFU *C. utilis;* and enzymes: 5.0×10^4 U xylanase; 2.5×10^3 U β-Mannanases; 1.0×10^4 U glucanase), *MesaA* represents Mesa with a double or triple dosage of *A. niger* for reed, corn and rice straws, respectively, *MesaB* represents Mesa with a double dosage of *B. subtilis*, *MesaC* represents Mesa with a double dosage of *C. utilis*, *MesaG* represents Mesa with a double dosage of glucanase

B. subtilis and 1.0×10^9 CFU *C. utilis*, three enzymes (5.0×10^4 U xylanase, 2.5×10^3 U β-mannanase, and 1.0×10^4 U glucanase), and 20 mL molasses. The four fortified cocktail treatments contained "Mesa" with a double or triple dose of *A. niger* (MesaA), *B. subtilis* (MesaB), *C. utilis* (MesaC) and glucanase (MesaG) (Table 1). The reed, corn or rice straw was denoted with R-/, C-/, or RI prefix. The straw for each biomass was adjusted to a total weight of 500 g with a water content of 65%. The control group for reed silage was treated with 500 mL of distilled water, the control group for corn silage was treated with 710 mL of distilled water, and the control group for rice straw silage was treated with 570 mL of distilled water. The amounts of bacterial inoculants and enzymes added to the three types of straw are reported in Table 1, which was based on extensive literature research [17, 29–36].

Silage additive preparation, silage processing and sampling

Depending on the viable bacterial or enzyme activity, the bacterial inoculant and enzymes were weighed for each treatment, and transferred to 10 mL of distilled water to activate the bacterial inoculants or enzymes. Then, 20 mL of molasses and a suitable amount of distilled water were added to the ensiling additive mixture (bacterial inoculant and enzyme mixture) and mixed thoroughly to achieve a moisture content of 65% in the ensiling system for all treatments (corresponding to the moisture content of each straw, see Table 1). Then, 500 g of reed, corn, and rice straw were accurately weighed and mixed with the abovementioned silage additive mixtures (3 replicates for each cocktail), successively filled into individual silage bags, compacted, and vacuum sealed for ensiling. The silos were stored at room temperature (20 °C) for 90 days [36].

After 90 days of ensiling, three portions from each ensiling bag were quantitatively collected according to the quartering method. The first portion (approximately 200 g) was air-dried to constant weight at 65 °C for 48 h, ground through a 40-mm sieve, and stored for chemical composition analysis. The second portion (approximately 50 g) was used to determine silage fermentation quality parameters. The third portion (approximately 25 g) was frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

Measurements and methods

Nutritional analysis

The contents of DM (method 930.15), ash (method 942.05), ether extract (EE, method 920.39), and crude protein (CP, method 984.13) contents were analyzed according to the standard procedures of AOAC (2005). Briefly, DM content was measured after drying the sample at 105 °C to constant weight. The CP content was calculated as nitrogen × 6.25, and total nitrogen (TN) content were determined by the Kjeldahl method using the DK 42 digestion apparatus (VELP Scientifica, Italia). The EE was determined using the SOXTHERM SOX416 extraction instrument (Gerhardt, Germany), and ash content was calculated from the residues after complete burnout of the biomass at 550 °C. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined using the FIBRETHERM FT12 (Gerhardt, Germany) [37]. Gross energy (GE) was measured using the 5E-AC8018 calorimeter (Kaide Measurement and Control Instrument Co., Ltd, Changsha, China).

Data on the chemical composition of the un-ensilaged reed straw, corn straw and rice straw are presented in Table 2. All measured chemical components markedly varied among the three types of straw. Reed straw had the highest content of NDF (84.1%, DM), ADF (61.8%, DM), and GE (17.9 MJ/kg, DM). Corn straw had the highest content of CP (6.0%, DM). As for rice straw, it contained the highest contents of ash (15.3%, DM) and EE (2.7%, DM).

Sensory evaluation

After opening of the silage, the sensory quality of the experimental silages were evaluated using the traditional silage evaluation standards of the German Agricultural Society (Table 3). The three main aspects of evaluation were odor, texture and color. The specific evaluation standards are listed in Table 3. In total, 5 experts conducted an on-site sensory evaluation to avoid subject biases.

Table 2 Chemical compositions of un-ensilaged biomasses

Biomass	DM (%)	Chemical compositions (% DM)					GE, MJ/kg
		CP	EE	NDF	ADF	Ash	
Reed straw	39.31	1.50	1.38	84.12	61.81	3.76	17.91
Corn straw	37.10	6.02	0.38	73.76	45.71	9.70	17.00
Rice straw	38.77	5.92	2.65	62.66	40.18	15.25	13.85

CP crude protein, *EE* ether extract, *NDF* neutral detergent fibre, *ADF* acid detergent fibre, *GE* gross energy

Table 3 Field sensory evaluation standards

Item	Grading			Scores
Smell	No butyric acid odor, aromatic fruity			14
	Has a faint butyric acid odor			10
	The smell of butyric acid is quite strong			4
	Has a strong butyric acid or ammonia smell			2
Structure	Stem and leaf structure maintained in good condition			4
	Leaf structure is poorly maintained			2
	The stem and leaf structure are extremely poorly preserved			1
	Stems and leaves are rotten or seriously contaminated			0
Color	Similar to the raw materials			2
	Slightly discolored, light yellow or brownish			1
	Severe discoloration, dark green or fading to yellow			0
Total score	16–20	10–15	5–9	0–4
Grade	Excellent	Good	Medium	Corruption

Ensiling quality

A 50 g silage sample from each silo was mixed with 450 mL distilled water, sealed and shaken until complete homogenization to evaluate fermentation quality. The suspension was then refrigerated at 4 °C for 24 h. The supernatant liquid was filtered through four layers of medical gauze, and then filtered with quantitative filter paper to obtain the extraction solution. About 10 mL of extraction solution was collected and stored at −20 °C for subsequent analysis [38].

The pH of the extracted solution of silage samples was determined using a digital pH meter (Shanghai Ohaus Instrument Co., Ltd., China). Lactic acid (LA) of was analyzed by High-Performance Liquid Chromatography (HPLC; Agilent Technologies, USA), while acetic acid (AA), propionic acid (PA), and butyric acid (BA) were analyzed by 7890A gas chromatograph (Agilent Technologies, USA). The ammonia–nitrogen (NH_4–N) was determined using the 1290 Infinity II UHPLC (Agilent Technologies, USA). The values of silage quality parameters were converted to the concentrations based on the mass weight of silage samples.

Comprehensive evaluation analysis of membership function

To comprehensively evaluate overall effect of the different combinations of bacterial inoculants and fibrolytic enzymes on the fermentation quality and nutritional value of reed, corn, and rice straws silages, a comprehensive membership function analysis was conducted to determine the best treatment based on fermentation and nutritional indices [39].

For positively correlated indicators (CP, EE, GE, LA and AA), the membership function values were calculated using the following formula:

$$y_{(pos)} = \frac{x - x_{min}}{x_{max} - x_{min}}$$

For negatively correlated indicators (ADF, NDF, pH, ash and NH_4–N/TN), the membership function values were calculated using the formula:

$$y_{(neg)} = 1 - y_{(pos)}$$

In the formulas, the symbol (x) is the measured value of each index of the sample. The symbol ($y_{(pos)}$ or $y_{(neg)}$) represents the positive or negative correlation membership function value of each index. The symbol (x_{max}) represents the maximum measured value of that index in the same sample, and the symbol (x_{min}) represents the minimum measured value of that index in the same sample [40–43].

DNA extraction, PCR amplification, and library preparation and sequencing

Approximately 25 g sample from each silo was used for DNA extraction. A brief description of DNA extraction, PCR amplification, and high-throughput sequencing is given here. Bacterial DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, according to the CTAB protocol [44, 45]. Subsequently, bacterial 16S rRNA amplicon sequences were amplified using barcode (a 12-bp unique barcode)-tagged primer sets 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'), with the following program, initial denaturation at 98 °C for 1 min; denaturation at 98 °C for 10 s with 30 thermal cycles; annealing at 50 °C for 30 s; and elongation at 72 °C for 30 s and 72 °C for 5 min with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were purified with a Universal DNA Purification Kit (TianGen, China,

Catalog #: DP214) according to the manufacturer's protocols. The purity and concentration of DNA samples were assessed using 2% agarose gel electrophoresis. After qualification, the PCR products were sequenced on the Illumina NovaSeq 6000 platform with pair-end 250 bp mode (PE250) by Novogene Bioinformatics Technology (Beijing, China).

Bioinformatics analysis pipeline

The raw data of the established library of bacterial sequences were processed through data cleaning (cutting of unique barcode and truncated by cutting off the barcode and primer sequence using Cutadapt (https://github.com/marcelm/cutadapt/), data merging (FLASH, version 1.2.11, http://ccb.jhu.edu/software/FLASH/), data filtering (fastQ, version 0.23.1, https://github.com/LUMC/fastq-filter), and data denoising (100% similarity) with DADA2 from Bioconductor (version 3.16, https://github.com/benjjneb/dada2) [46] in QIIME2 (version 2017.6) with R program (version 4.2.0) to obtain clean amplicon sequence variants (ASVs) or feature sequences (with the abundance of each feature sequence, to get feature-table). Sequences with an abundance lower than 5 were filtered out to obtain the final ASVs [47]. For the obtained ASVs [48], species annotation was performed on the representative sequence of each ASV to obtain the corresponding species information and species-based abundance distribution. The alpha and beta diversity were calculated by "qiime diversity alpha" and "qiime diversity beta" commands from the rarefied feature-table. The NMDS results were calculated by "qiime diversity nmds" command and visualized by the "qiime emperor plot" command.

Statistical analysis

The original experimental data were organized using Microsoft Excel 2016 software. One-way analysis of variance (ANOVA) was performed using SPSS 25.0 software to determine the significance of differences among groups, and Duncan's post hoc test was used for multiple comparisons. Results shown in the tables are presented as means and standard errors of the means (SEM). Statistical significances are defined as follows: $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, respectively.

Silage processing score, composition and diversity of the bacterial community were visualized with R 4.2.0 using the ggplot2 package (https://CRAN.R-project.org/package=ggplot2). Spearman correlation coefficients between silage inoculants, dominant bacterial genera, and physicochemical properties were analyzed with R 4.2.0, and results were visualized with R 4.2.0 software using the ggcor package (https://github.com/hannet91/ggcor). Network plots of the dominant bacterial species were generated with R 4.2.0 software using the igraph package (https://CRAN.R-project.org/package=igraph). All graphics in this article were integrated and typeset using Adobe Illustrator 27.9 software.

Results

Silage processing score

As evident from Fig. 1, the smell score, structure score, color score, and total score were markedly ($P<0.01$) higher for all cocktails added reed, corn and rice straw silages, as compared to their respective control silage. Furthermore, all evaluated silage cocktails resulted in a notable improvement in silage quality, elevating it from a medium grade to an excellent one. For reed straw, supplementation of R-MesaA (*A. niger*, 1.2×10^{9} CFU) cocktail had the highest silage quality score, as compared to other additives. For rice straw, RI-MesaA, RI-MesaB and RI-MesaC supplemented silage had high silage quality scores; however, RI-MesaG had a lower silage score.

Effects of additives on chemical compositions of straw silages

The nutrient profiles of reed, corn and rice straw silage, as affected by different types of silage cocktails, are shown in Table 4. Overall, the addition of different cocktails caused similar changes in the nutrient composition of the silages. In particular, NDF, ADF, and ash decreased ($P<0.05$), while EE, CP, and GE increased ($P<0.05$) with supplementation of the different silage cocktails. It is worth noting that silages treated with higher levels of *B. subtilis* had significantly lower NDF content, highlighting its positive effects on fermentation quality and the nutritional value of straw silages.

Ensiling quality

Data on the effects of experimental cocktails on ensiling quality of reed, corn, and rice straw silages are summarized in Table 5. The addition of silage cocktails changed ($P<0.05$) all measured ensiling characteristics, except AA and NH_4–N in reed straw silage. In reed straw, application of silage cocktails decreased pH ($P<0.01$) and NH_4–N/ TN ratio ($P=0.04$), and increased LA ($P=0.02$) and LA/total acids (LA/TA) ($P=0.04$).In contrast, for corn straw and rice straw, supplementation of silage cocktails reduced ($P<0.05$) pH, NH_4–N, and NH_4–N/TN. Comparison of the silage cocktails revealed that cocktails with a higher proportion of *B. subtilis* had significantly lower NH_4–N/TN ratios than the other inoculants in corn straw silage. These findings demonstrated that the application of silage cocktails improved the quality of straw silage.

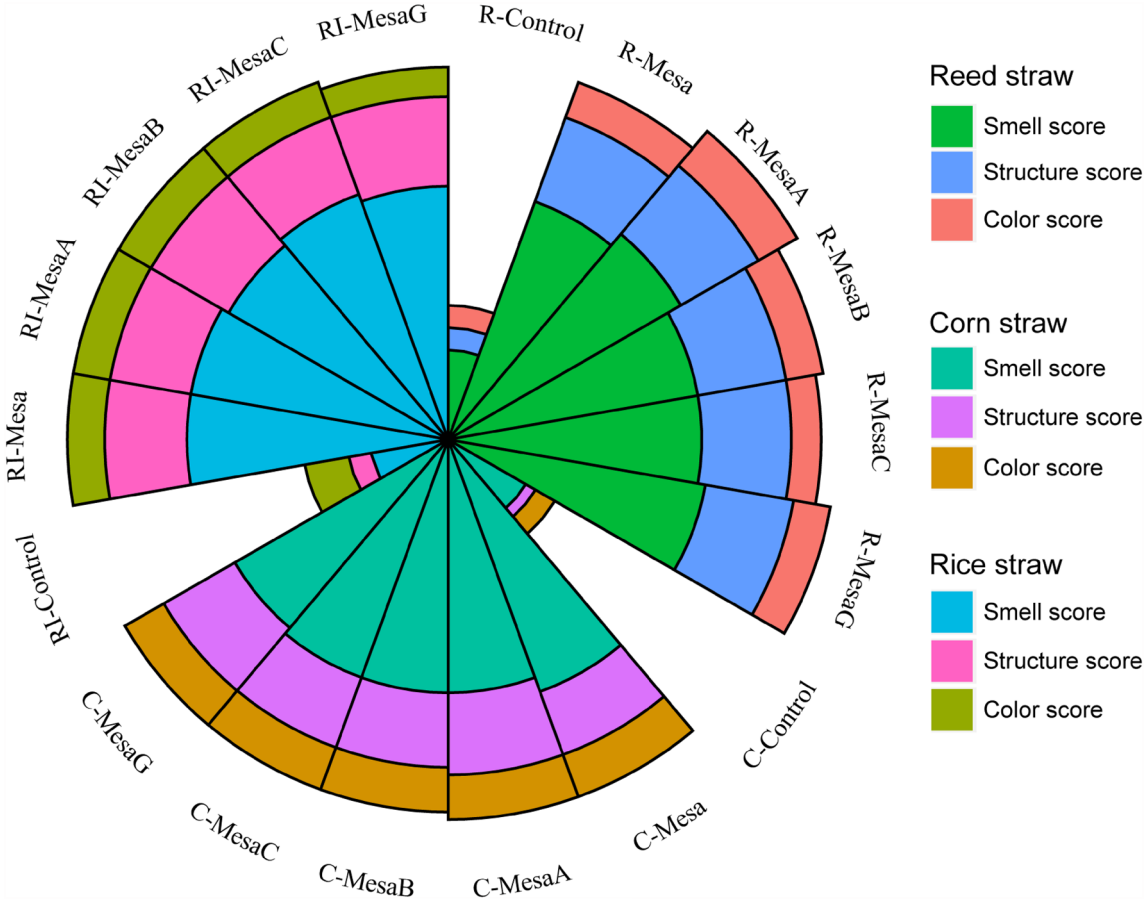


Fig. 1 Pie chart depicting the effect of experimental cocktails on silage sensory scores for reed straw, corn stover and rice straw. Control group means the control without bacterial and enzyme supplementation; Mesa, the basal silage cocktail treatment (containing 1.0×10^6 CFU *L. plantarum*, 1.4×10^7 CFU *L. buchneri*; 3.0×10^5 CFU *P. pentosaceus;* 8.0×10^8 CFU *A. niger;* 1.6×10^6 CFU *B. subtilis;* 1.0×10^9 CFU *C. utilis;* and enzymes: 5.0×10^4 U xylanase; 2.5×10^3 U β-Mannanases; 1.0×10^4 U glucanase); *R* reed straw, *C* corn straw, and *RI* rice straw, *MesaA* represents Mesa with a double or triple dosage of *A. niger* for reed, corn and rice straws, respectively, *MesaB* represents Mesa with a double dosage of *B. subtilis*, *MesaC* represents Mesa with a double dosage of *C. utilis*, *MesaG* represents Mesa with a double dosage of glucanase

Comprehensive membership function analysis

Data on the effects of the experimental cocktails on the comprehensive membership function analysis scores of ensiling quality and nutritional value of reed, corn, and rice straw silages are summarized in Table 6. For reed straw, the overall comprehensive evaluation score of R-MesaB inoculant was the highest (0.57). The most influential indices contributing to the quality of reed straw silage were NH_4–N/TN ratio and pH. The remaining inoculants were ranked in descending order as follows: R-MesaA, R-Mesa, R-MesaG, R-MesaC, and R-Control. The C-MesaB inoculant had the highest comprehensive evaluation score of 0.59 for corn straw silage. The key contributing factors for corn straw silage quality were the NH_4–N/TN ratio and LA content. The ranking of other inoculants, in descending order, was as follows: C-MesaA, C-Mesa, C-MesaG, and C-MesaC. The ranking of rice straw ensiling and nutritional quality revealed that the RI-MesaB silage had the highest comprehensive score of 0.52. The primary indicators contributing to the comprehensive evaluation score of the rice straw silage were pH and AA content. The remaining inoculants were ranked in descending order as follows: RI-Mesa, RI-MesaA, RI-MesaG, RI-MesaC, and RI-Control.

These comprehensive scores provide a quantitative assessment of the inoculants based on the ensiling quality and nutritional value of reed, corn, and rice straw silages, with certain indicators identified as major contributors to ensiling comprehensive evaluation scores. These findings highlight the varying impacts of different inoculants on the ensiling quality of straw biomasses, and can serve as a valuable reference for optimizing straw silage production processes.

Table 4 Effects of silage cocktails based on different combinations of bacterial inoculants and enzyme preparations on the nutritional value of straw silage

Biomass	Cocktails[d]	CP, %	EE, %	GE, MJ/kg	NDF, %	ADF, %	ash, %
Reed straw	R-Control	1.34[c]	4.90[b]	17.75[b]	87.73[a]	62.51[a]	6.11[a]
	R-Mesa	2.27[b]	5.77[a]	17.95[a]	82.19[b]	57.30[b]	5.38[b]
	R-MesaA	2.26[b]	5.76[a]	18.04[a]	82.89[b]	57.63[b]	5.41[b]
	R-MesaB	2.39[a]	5.82[a]	18.02[a]	79.94[c]	57.59[b]	5.39[b]
	R-MesaC	2.42[a]	5.82[a]	18.03[a]	83.63[b]	58.66[b]	5.26[b]
	R-MesaG	2.29[b]	5.77[a]	18.04[a]	83.18[b]	57.95[b]	5.43[b]
	SEM	0.05	0.04	0.02	0.37	0.39	0.04
	P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Corn straw	C-Control	6.23[c]	5.79[b]	17.37[b]	73.66[a]	43.71[a]	9.90[a]
	C-Mesa	6.86[b]	7.68[a]	17.68[a]	68.27[b]	39.94[b]	8.24[b]
	C-MesaA	6.89[b]	7.77[a]	17.69[a]	68.95[b]	40.42[b]	8.44[b]
	C-MesaB	7.65[a]	7.88[a]	17.64[a]	66.21[c]	40.01[b]	8.16[b]
	C-MesaC	7.51[a]	7.73[a]	17.69[a]	68.94[b]	40.23[b]	8.47[b]
	C-MesaG	7.07[b]	7.73[a]	17.66[a]	68.17[b]	40.51[b]	8.27[b]
	SEM	0.07	0.11	0.02	0.40	0.66	0.09
	P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Rice straw	RI-Control	6.31[c]	2.66[b]	13.66[b]	63.10[a]	41.16[a]	12.49[a]
	RI-Mesa	7.67[b]	3.74[a]	13.82[a]	55.77[a]	36.81[b]	11.42[b]
	RI-MesaA	7.66[b]	3.65[a]	13.87[a]	55.21[a]	36.85[b]	11.16[b]
	RI-MesaB	7.83[a]	3.65[a]	13.87[a]	53.55[c]	34.59[c]	11.30[b]
	RI-MesaC	7.86[a]	3.63[a]	13.88[a]	55.66[b]	36.33[b]	11.14[b]
	RI-MesaG	7.68[b]	3.61[a]	13.84[a]	55.70[b]	36.59[b]	11.21[b]
	SEM	0.09	0.06	0.02	0.47	0.50	0.09
	P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

CP crude protein, *EE* ether extract, *GE* gross energy, *NDF* neutral detergent fibre, *ADF* acid detergent fibre

[a–c] Mean the statistical differences in the same column within each straw type with a *P* value less than 0.05

[d] *R* reed straw, *C* corn straw, and *RI* rice straw, control group means the control without bacterial and enzyme supplementation; *Mesa* the basal silage cocktail treatment (containing 1.0×10^6 CFU *L. plantarum*, 1.4×10^7 CFU *L. buchneri*; 3.0×10^5 CFU *P. pentosaceus;* 8.0×10^8 CFU *A. niger;* 1.6×10^6 CFU *B. subtilis;* 1.0×10^9 CFU *C. utilis;* and enzymes: 5.0×10^4 U xylanase; 2.5×10^3 U β-Mannanases; 1.0×10^4 U glucanase), *MesaA* represents Mesa with a double or triple dosage of *A. niger* for reed, corn and rice straws, respectively, *MesaB* represents Mesa with a double dosage of *B. subtilis*, *MesaC* represents Mesa with a double dosage of *C. utilis*, *MesaG* represents Mesa with a double dosage of glucanase

The influence of inoculants on microbial community structure

Microbial diversity

Microbial diversity analysis revealed interesting findings for the effects of experimental cocktails on reed, corn, and rice straw silages (Fig. 2). The rarefaction curves reached saturation for all samples, indicating that the sequencing depth was sufficient to capture most of the amplicon sequences generated.

In the reed straw silage, the Shannon index of R-MesaA, R-MesaB, and R-MesaC inoculants was lower ($P < 0.05$) than that of R-Control, whereas R-Mesa and R-MesaG inoculants showed only a decreasing trend in microbial diversity (Fig. 3, panel A). In addition, non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distance algorithm demonstrated a significant separation of the groups (stress = 0.021), indicating significant differences in microbial communities among silage treatments for reed, corn, and rice straws (Fig. 2, panel B).

Overall, the addition of microbial agents and enzymes significantly influenced the microbial diversity of corn and rice straw silages during fermentation, resulting in a decrease in Chao1 and Shannon indices. NMDS analysis also indicated significant separations between the different treatment groups of corn straw (stress = 0.072) and rice straw (stress = 0.054) silages. These results indicate that the application of the experimental inoculants had a considerable impact on the microbial composition of corn and rice straw silages, and altered the overall microbial diversity during the fermentation process.

Profile of bacterial composition

Significant differences in community composition were observed across petal plots (Fig. 3). In reed straw silage, 134 common bacterial species were observed in the

Table 5 Effects of silage cocktails based on bacterial inoculants and enzyme mixtures on ensiling quality of reed, corn and rice straw

Biomass	Cocktail[d]	pH	LA (g/kg)	AA (g/kg)	NH_4–N (g/kg)	NH_4–N/TN (%)	LA/TA (%)
Reed straw	R-Control	5.76^{a}	1.48^{b}	7.24	0.55	2.54^{a}	0.21^{b}
	R-Mesa	3.64^{b}	23.57^{a}	7.7	0.74	1.68^{b}	3.47^{a}
	R-MesaA	3.64^{b}	16.9^{a}	5.38	0.72	1.98^{ab}	3.42^{a}
	R-MesaB	3.9^{b}	19.62^{a}	7.25	0.69	1.80^{b}	2.99^{ab}
	R-MesaC	3.66^{b}	13.73^{a}	8.69	0.61	1.57^{b}	2.05^{ab}
	R-MesaG	3.68^{b}	22.87^{a}	7.8	0.68	1.86^{b}	3.43^{a}
	SEM	0.10	2.08	0.46	0.02	0.06	0.41
	P value	< 0.01	0.02	0.42	0.13	0.04	0.04
Corn straw	C-Control	5.84^{a}	12.62^{b}	13.54	2.74^{a}	2.76a	0.84^{b}
	C-Mesa	3.69^{b}	44.11^{a}	14.33	2.18^{b}	1.99^{b}	3.98^{a}
	C-MesaA	3.68^{b}	55.12^{a}	15.94	2.23^{b}	2.02^{b}	4.38^{a}
	C-MesaB	3.67^{b}	59.29^{a}	10.21	2.01^{b}	1.64^{c}	4.66^{a}
	C-MesaC	3.64^{b}	33.47^{a}	11.32	2.57^{b}	2.14^{b}	5.67^{a}
	C-MesaG	3.70^{b}	52.33^{a}	15.83	2.15^{b}	1.90^{bc}	5.07^{a}
	SEM	0.12	4.6	1.01	0.06	0.06	0.68
	P value	< 0.01	0.04	0.49	< 0.01	< 0.01	0.04
Rice straw	RI-Control	5.59^{a}	2.11^{b}	10.83^{bc}	2.53^{a}	2.57^{a}	0.20^{d}
	RI-Mesa	3.68^{b}	48.37^{a}	6.81^{c}	1.43^{b}	1.16^{b}	7.20^{a}
	RI-MesaA	3.79^{b}	41.78^{a}	15.03^{a}	1.45^{b}	1.18^{b}	2.35^{cd}
	RI-MesaB	3.77^{b}	34.83^{a}	11.74^{ab}	1.09^{b}	0.87^{c}	3.12^{bcd}
	RI-MesaC	3.76^{b}	41.98^{a}	7.57^{c}	1.10^{b}	0.87^{c}	6.14^{ab}
	RI-MesaG	3.82^{b}	44.03^{a}	10.45^{bc}	1.35^{b}	1.10^{bc}	4.19^{abc}
	SEM	0.11	3.89	0.68	0.08	0.09	0.65
	P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

LA lactic acid, *AA* acetic acid, *NH_4–N* ammonia–nitrogen, *TN* total nitrogen, *TA* total acid, *SEM* standard error of the means

[a–c] Mean the statistical differences in the same column within each straw type with a *P* value less than 0.05

[d] *R* Reed straw, *C* Corn straw, and *RI* rice straw, control group means the control without bacterial and enzyme supplementation, *Mesa* the basal silage cocktail treatment (containing 1.0×10^{6} CFU *L. plantarum*, 1.4×10^{7} CFU *L. buchneri*; 3.0×10^{5} CFU *P. pentosaceus;* 8.0×10^{8} CFU *A. niger;* 1.6×10^{6} CFU *B. subtilis;* 1.0×10^{9} CFU *C. utilis;* and enzymes: 5.0×10^{4} U xylanase; 2.5×10^{3} U β-mannanases; 1.0×10^{4} U glucanase), *MesaA* represents Mesa with a double or triple dosage of *A. niger* for reed, corn and rice straws, respectively, *MesaB* represents Mesa with a double dosage of *B. subtilis*, *MesaC* represents Mesa with a double dosage of *C. utilis*, *MesaG* represents Mesa with a double dosage of glucanase

treatment groups, while the R-MesaG group exhibited 1206 unique species. Similarly, corn straw silages had 218 common bacterial species, and the C-MesaC group displayed the highest number (1347) of unique species. For rice straw, each group contained 197 similar bacterial species, with the RI-Mesa group exhibiting the most (1062) unique species.

The impact of silage cocktails on the dominant bacteria is crucial to understanding their influence on the ecological function of the dominant microbial community. Nine of the top 10 bacterial phyla in reed, corn, and rice straw silages were *Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Acidobacteria, Cyanobacteria, Myxococcota, Gemmatimonadota*, and *Chloroflexi*. The remaining bacterial phylum was *Desulfobacterota* in reed silage, *Methylomirabilota* in corn silage, and *Crenarchaeota* in rice straw silage.

Correlation analysis of bacterial composition with physicochemical properties

The correlation between physicochemical properties and phylum-level bacteria composition in reed, corn, and rice straw silages is shown in Fig. 4. A co-occurrence network of the dominant bacterial genera, with a relative abundance greater than 5%, was constructed to analyze the correlations between different bacterial genera. The findings revealed several significant correlations in reed straw. *Rahnella 1, Rhizobium* and *Caproiciproducens* were negatively correlated with EE, CP, and GE, and positively correlated with ash and pH.

In corn and rice straws, *Rahnella 1* demonstrated a negative correlation with EE, CP, and GE, and a positive correlation with ash and pH. In rice straw, *Rhizobium* demonstrated a negative correlation with EE, CP, and GE, and a positive correlation with ash and pH. In

Table 6 Effects of experimental cocktails on comprehensive membership function analysis scores of fermentation quality and nutritional value of silage reed, corn and rice straw

Biomass	Cocktail[a]	CP	EE	GE	NDF	ADF	Ash	pH	LA	AA	NH_4–N/TN	Comprehensive score	Order
Reed straw	R-Control	0.35	0.31	0.35	0.28	0.23	0.32	0.23	0.35	0.26	0.3	2.98	6
	R-Mesa	0.52	0.41	0.59	0.45	0.66	0.72	0.46	0.38	0.35	0.43	4.97	3
	R-MesaA	0.47	0.46	0.55	0.57	0.54	0.48	0.58	0.37	0.51	0.49	5.02	2
	R-MesaB	0.54	0.37	0.63	0.53	0.52	0.62	0.66	0.51	0.62	0.67	5.67	1
	R-MesaC	0.55	0.43	0.32	0.61	0.38	0.53	0.39	0.6	0.42	0.4	4.63	5
	R-MesaG	0.55	0.42	0.57	0.43	0.54	0.46	0.34	0.54	0.34	0.45	4.64	4
Corn straw	C-Control	0.32	0.35	0.32	0.31	0.51	0.42	0.31	0.33	0.24	0.28	3.39	6
	C-Mesa	0.56	0.47	0.52	0.62	0.54	0.46	0.43	0.6	0.45	0.64	5.29	3
	C-MesaA	0.59	0.52	0.54	0.52	0.58	0.62	0.48	0.66	0.55	0.63	5.69	2
	C-MesaB	0.64	0.61	0.53	0.55	0.53	0.5	0.53	0.67	0.63	0.7	5.89	1
	C-MesaC	0.6	0.43	0.55	0.51	0.57	0.44	0.4	0.52	0.33	0.71	5.06	5
	C-MesaG	0.6	0.42	0.52	0.41	0.63	0.53	0.39	0.64	0.41	0.66	5.21	4
Rice straw	RI-Control	0.37	0.24	0.45	0.44	0.36	0.33	0.22	0.21	0.47	0.13	3.22	6
	RI-Mesa	0.53	0.77	0.55	0.47	0.54	0.41	0.63	0.38	0.45	0.43	5.16	2
	RI-MesaA	0.49	0.4	0.62	0.58	0.34	0.52	0.69	0.35	0.55	0.37	4.91	3
	RI-MesaB	0.52	0.51	0.58	0.54	0.48	0.49	0.58	0.49	0.58	0.4	5.17	1
	RI-MesaC	0.5	0.42	0.5	0.57	0.36	0.47	0.35	0.36	0.38	0.28	4.19	5
	RI-MesaG	0.53	0.42	0.47	0.64	0.43	0.41	0.34	0.58	0.48	0.52	4.82	4

CP crude protein, *EE* ether extract, *GE* gross energy, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *LA* lactic acid, *AA* acetic acid, *NH_4–N* ammonia nitrogen, *TN* total nitrogen

[a] *R* reed straw, *C* corn straw, and *RI* rice straw, control group means the control without bacterial and enzyme supplementation, *Mesa* the basal silage cocktail treatment (containing 1.0×10^6 CFU *L. plantarum*, 1.4×10^7 CFU *L. buchneri*; 3.0×10^5 CFU *P. pentosaceus;* 8.0×10^8 CFU *A. niger;* 1.6×10^6 CFU *B. subtilis;* 1.0×10^9 CFU *C. utilis;* and enzymes: 5.0×10^4 U xylanase; 2.5×10^3 U β-mannanases; 1.0×10^4 U glucanase), *MesaA* represents Mesa with a double or triple dosage of *A. niger* for reed, corn and rice straws, respectively *MesaB* represents Mesa with a double dosage of *B. subtilis*, *MesaC* represents Mesa with a double dosage of *C. utilis*, *MesaG* represents Mesa with a double dosage of glucanase

all straw sialges, *Lactobacillus* was positively correlated with EE, CP, and GE, and negatively correlated with ash and pH.

Furthermore, significant negative correlations were observed between *Lactobacillus* and *Rahnella 1* in reed and corn straw silage. However, *Rhizobium*, and *Caproiciproducens* exhibited positive correlations in reed silage. These findings shed light on the inherent relationships between the predominant bacterial genera and physicochemical properties of reed, corn and rice straw silages, and provide valuable insights into their co-occurrence patterns and ecological interactions in the microbial population, as well as on their correlations with fermentation and nutritional quality parameters in straw silages.

From the holistic perspective presented in Fig. 4, it can be observed that the high doses of *A. niger*, *B. subtilis*, *C. utilis*, and glucanase in reed straw and rice straw, exhibited significant negative correlations with pH, NH_4–N/TN, ash, and ADF, and positive correlations with GE, EE, and CP ($P<0.05$). This correlation pattern was more pronounced in rice straw. In corn silage, *B. subtilis* had a significant negative correlation with pH and NH_4–N/TN, and a positive correlation with LA, GE, and EE ($P<0.05$).

Furthermore, we simulated the influence of the nutrient composition of un-ensiled straw (including corn straw, rice straw, and reed straw) on silage chemical composition, fermentation characteristics, microbial population (Fig. 5). The CP in the substrate was positively correlated with LA, NH_4–N, *Enterococcus*, *Sphingomonas* and *Ochrobacterum*. NDF, ADF and GE in the substrate were positively correlated with *Stenotrophomonas*, *Leuconostoc*, and negatively correlated with *Pantoea*, *Enterococcus*, and *Ochrobactrum*.

Discussion

Silage cocktails on the nutrient quality of reed, corn and rice straw silages

Improvement in the nutritional value of straws is a crucial aspect of silage research in recent years. Higher CP, and lower NDF and NH_4–N contents of the straw silages supplemented with the experimental cocktails, indicate better fermentation and nutritional quality. The NDF content of forages is negatively correlated with animal feed intake [49], while ADF in corn silage and forages is negatively correlated with digestibility [50]. Lower NDF promotes higher DM

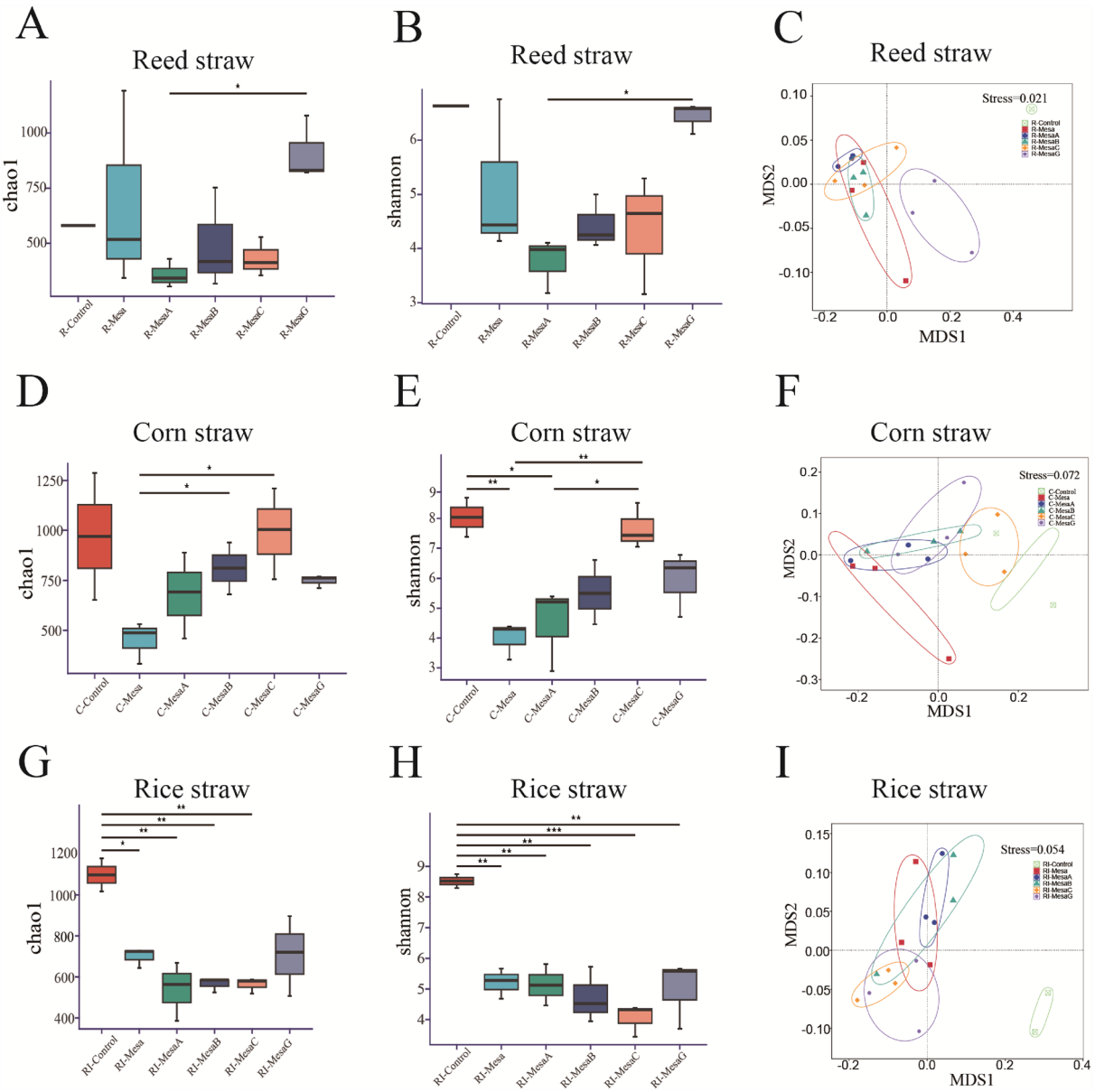


Fig. 2 Microbial diversity. Bacterial α-diversity (Chao1 and Shannon indices) of reed, corn, and rice straw silages (Chao1: **A**, **D**, and **G**; Shannon: **G**; **B**, **E**, and **H**, respectively). Bacterial beta diversity (NMDS) of reed, corn, and rice straw silages (**C**, **F**, and **I**, respectively). *R* reed straw, *C* corn straw, and *RI* rice straw, control group means, ensiled without bacterial and enzyme supplementation; Mesa, the basal silage cocktail treatment (the basal silage cocktail treatment bacterial inoculants: 1.0×10^6 CFU *L. plantarum*, 1.4×10^7 CFU *L. buchneri*; 3.0×10^5 CFU *P. pentosaceus;* 8.0×10^8 CFU *A. niger;* 1.6×10^6 CFU *B. subtilis;* 1.0×10^9 CFU *C. utilis;* and enzymes: 5.0×10^4 U xylanase; 2.5×10^3 U β-Mannanases; 1.0×10^4 U glucanase); MesaA, represents Mesa with a double or triple dosage of *A. niger* for reed, corn and rice straws, respectively; MesaB, represents Mesa with double dosage of *B. subtilis*; MesaC, represents Mesa with double dosage of *C. utilis;* MesaG represents Mesa with a double dosage of glucanase

intake, and lower ADF indicates higher digestible DM and a better ensiling effect [51, 52]. Research has demonstrated that *B. subtilis* can degrade cellulose in straw [53]. The *C. utilis* utilizes simple nitrogen sources for growth and reproduction, and contributes to the assimilation of bacterial protein in silages [54]. The addition of exogenous nitrogen sources and *C. utilis* (as a single-celled protein strain) contribute to CP production during ensiling. Cellulase and hemicellulase degrade cellulose and hemicellulose, and convert the resulting xylose and free monosaccharides into CP [55, 56]. Moreover, the CP in straw is expected to be more available to animals after ensiling due to the softening of straw and fermentation of fibrous carbohydrates.

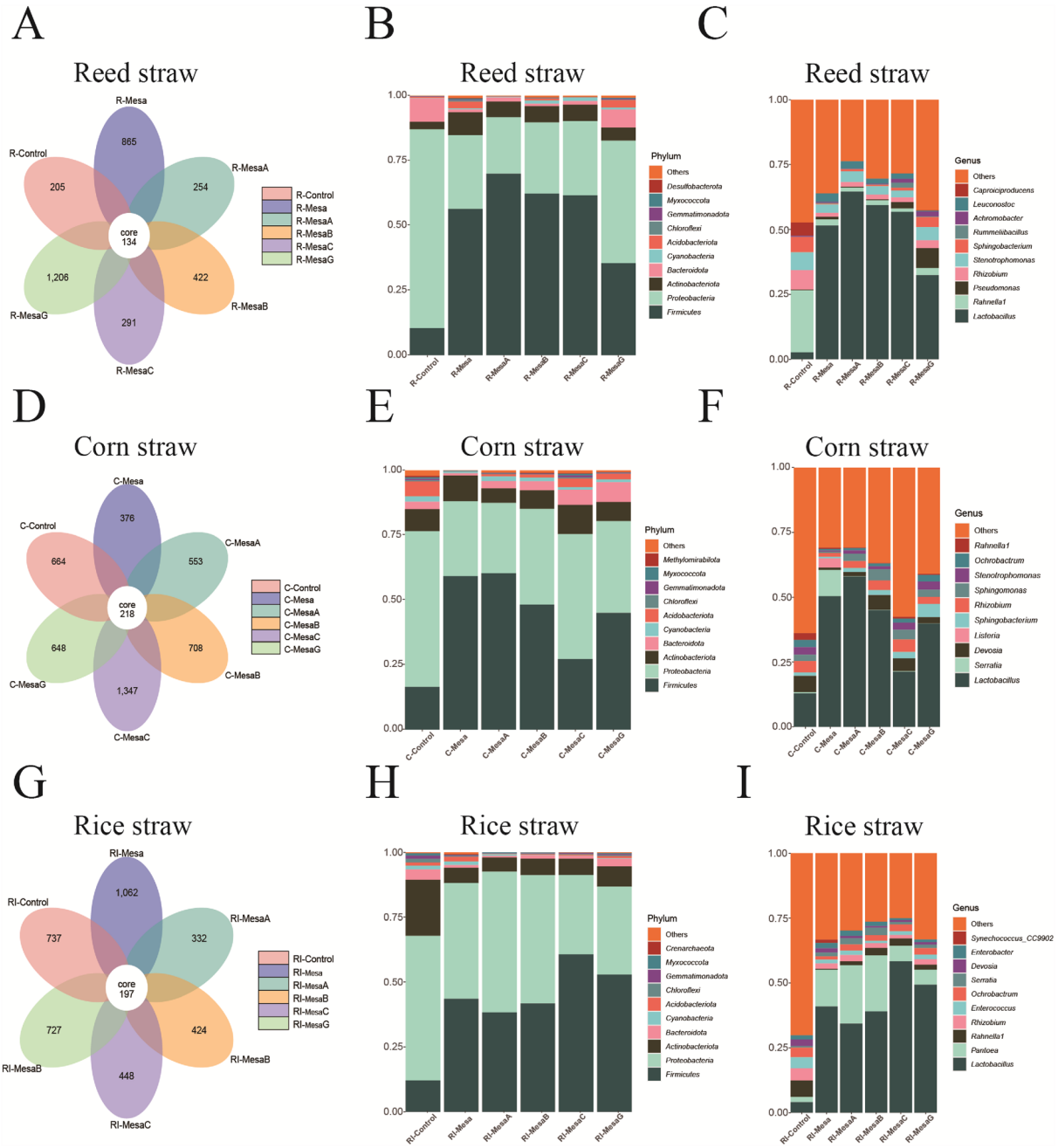


Fig. 3 Microbiome of straw silage. **A** Petal plot of bacterial species composition (**A**, **D**, **G**), phylum (**B**, **E**, **H**) and genus (**C**, **F**, **I**) levels in reed, corn, and rice straw silage, respectively. *R* reed straw, *C* corn straw, and *RI* rice straw; control group means the control without bacterial and enzyme supplementation; Mesa, the basal silage cocktail treatment (the basal silage cocktail treatment bacterial inoculants: 1.0×10^6 CFU *L. plantarum*, 1.4×10^7 CFU *L. buchneri*; 3.0×10^5 CFU *P. pentosaceus;* 8.0×10^8 CFU *A. niger;* 1.6×10^6 CFU *B. subtilis;* 1.0×10^9 CFU *C. utilis;* and enzymes: 5.0×10^4 U xylanase; 2.5×10^3 U β-mannanases; 1.0×10^4 U glucanase); MesaA, represents Mesa with a double or triple dosage of *A. niger* for reed, corn and rice straws, respectively; MesaB, represents Mesa with a double dosage of *B. subtilis*; MesaC, represents Mesa with a double dosage of *C. utilis;* MesaG represents Mesa with a double dosage of glucanase

Further comparison of the results revealed that, straw silages treated with the double dose of *B. subtilis* (R-MesaB, C-MesaB, RI-MesaB) and *C utilis* (R-MesaC, C-MesaC, RI-MesaC) had markedly higher CP content, while those treated with double dose of *B. subtilis* (R-MesaB, C-MesaB, RI-MesaB) exhibited the most significant reduction in NDF and ADF contents. An earlier study reported that silage cocktails with a higher

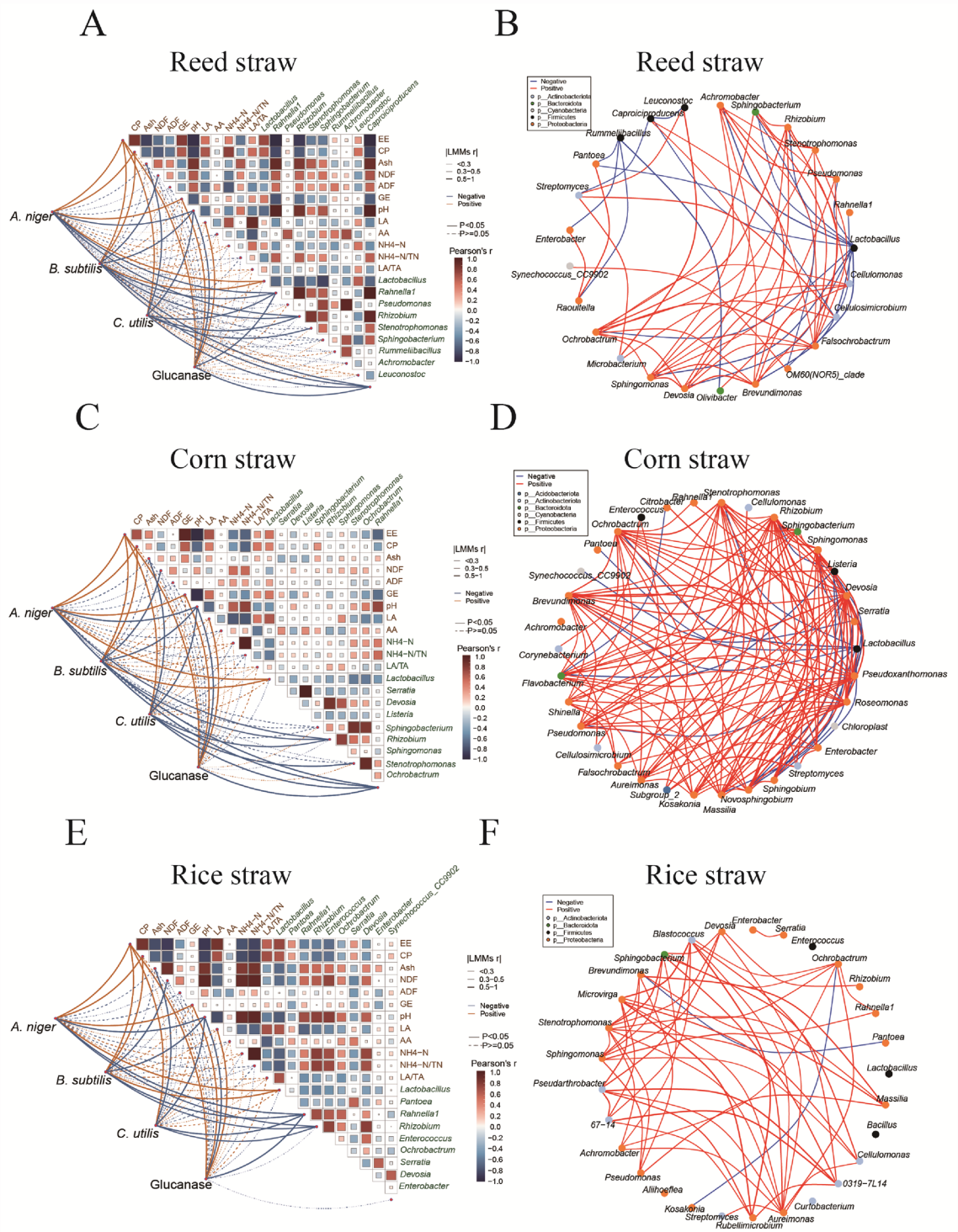


Fig. 4 Correlation analysis of silage cocktails on silage parameters and complex network of silage microbiome. Correlation analysis (mantel test and Pearson's correlation matrix: **A**, **C**, **E**) and association network analysis (**B**, **D**, **F**) of silage cocktails (silage agents with enhancing dosage of *A. niger*, *B. subtilis*, *C. utilis*, and glucanase) on dominant bacterial genera and physicochemical properties of reed, corn and rice straw silage

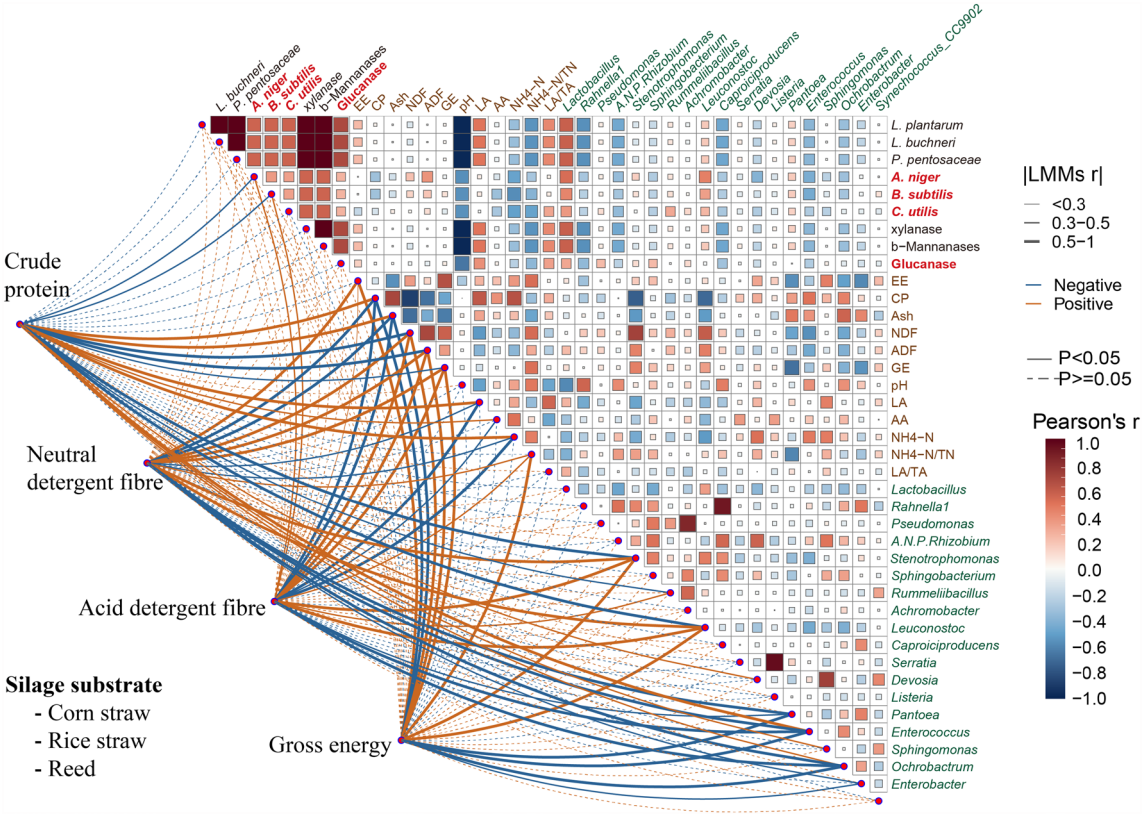


Fig. 5 Correlation analysis of nutrient components of un-ensiled straw with silage parameters. The nutrient components (crude protein, neutral detergent fibre, acid detergent fibre and gross energy) of un-esiled straw, including corn straw, rice straw and reed straw, are presented on the left side of the figure, while silage parameters are presented on the right side

dosage of *B. subtilis* or *C. utilis* increased CP content and lower NDF [53]. In this experiment, varying degrees of increases were observed in EE of reed, corn, and rice straws after ensiling. This increase is attributed to the conversion of carbohydrates, decomposed by lactic acid bacteria and yeast, into fat-soluble substances such as lactic acid and volatile fatty acids, which end up in EE [57]. In addition, the increase in EE and CP levels after ensiling contributed to higher GE levels in reed, corn, and rice straw silages.

Effects of silage cocktails on ensiling quality of reed, corn and rice straw

The pH is a fundamental and extremely effective parameter for assessing the quality of silage. A low pH (< 4.2) [58] in well-fermented silage indicates a harsh environment for the survival of harmful microorganisms, and prevents undesirable fermentation, which otherwise results in DM and nutrient losses [36]. During the anaerobic phase, lactic acid-producing bacteria are the primary microorganisms that convert forage carbohydrates into LA and AA, causing a decrease in silage pH and an improvement in ensiling quality [59, 60].

In this study, silage cocktails were evaluated for their potential to increase LA content and reduce pH below 4.2 during the ensiling of reed, corn, and rice straw. The combination of *L. plantarum* and *L. brucella* promoted the rapid proliferation of lactic acid bacteria in the initial phase of ensiling, and caused a rapid increase in LA content with a concomitant decrease in pH. In addition, *L. plantarum*, a homofermentative lactic acid bacterium, produces LA from fermentation of hexose sugars such as glucose [61]. Meanwhile, *L. brucella*, an obligate heterofermentative lactic acid bacterium, produces not only LA but also ethanol, AA, and carbon dioxide. The AA prevents the growth of aerobic microorganism, particularly yeast, and enhances the silage's aerobic stability during the feed-out period [62]. In this trial, the AA contents of all inoculated corn and rice straw silages were significantly higher than that of the control (non-inoculated) silages, possibly due to the addition of heterofermentative lactic acid bacteria [63]. Furthermore, when the pH is very low, the activity of most lactic acid bacteria with low acid resistance is inhibited in the later phase of ensiling, allowing acid-resistant strains to produce AA from LA [61]. However, there were no significant differences

in AA contents between the cocktails treated reed straw silage and the control group, which could be due to differences in chemical composition and epiphytic microorganisms of the reed straw [64].

The presence of BA and PA in ensiled straw can lead to a pungent odor and seriously impairs the palatability of silages. The content of BA and AA serves as a vital indicator for silage quality [52]. In this experiment, BA and PA were not detected in the corn and rice straw silages, while the control group of reed straw silages contained a small amount of BA and PA. The NH_4–N content is an indicator of protein degradation. A higher level of NH_4–N indicates greater degradation of nitrogenous nutrients, which in turn reflects poor fermentation quality [65, 66]. The NH_4–N/TN ratio represents the extent of protein degradation in the silage, and a high ratio indicates poor silage quality. This trial showed that the combination of different enzymes and bacteria preparations can reduce NH_4–N content during the ensiling of corn and rice straw. A plausible explanation is that, the silage cocktails accelerated the fermentation process and created a low-acid environment in a short time that inhibited the activity of the enzymes, thereby reducing the degradation of nitrogenous substances, such as protein [67].

Comprehensive evaluation of silage

In the evaluation of silage quality and nutritional value, several key indicators are usually taken into account, including CP, NDF, GE, pH, LA and AA. These indicators exert a significant impact on silage fermentation and nutritional quality. To comprehensively evaluate silage quality, membership functions are computed for each of these indicators using different formulas. These individual membership functions are then averaged to derive an overall evaluation score for the silage. This approach provides an effective and holistic means to assess silage quality and understand how it is impacted by the application of different additives.

The analysis of the membership functions and the subsequent comprehensive scores comprehensively evaluate the influence of silage cocktails on the overall quality of straw silages. This evaluation process helps to compare the effects of different silage cocktails, by highlighting the overall variations in the quality of straw silages [68]. The notably higher comprehensive evaluation scores for all inoculated reed, corn, and rice straw silages clearly indicate that the application of silage cocktails enhanced the fermentation quality and nutritional value of the low-quality straw biomasses.

Across the three types of straw silages, the R-MesaB, C-MesaB, and RI-MesaB achieved the highest comprehensive evaluations, demonstrating the positive impact of the double dose of *B. subtilis* on straw silage quality. The most important factors contributing to the comprehensive score of R-MesaB, C-MesaB, and RI-MesaB were NH_4–N/TN, LA, pH value, and AA. Demonstrating that a higher level of *B. subtilis* stimulate greater production of LA in reed straw, corn straw, and rice straw. Moreover, the double dose of *B. subtilis* also resulted in lower pH and NH_4–N/TN ratio, and inhibited AA production.

Effect of silage cocktails on microbial community

The silage process is primarily driven by microbial activity. High-throughput sequencing of 16S rRNA for microbial communities is a valuable tool to establish the relationship between microbial communities and the ensiling quality and nutritional value of silages. Changes in the α-diversity of bacterial communities were observed after the application of silage cocktails. The richness (Chao 1) and diversity (Shannon) of bacterial communities were reduced by the application of silage cocktails. In partial agreement with our findings, a decrease in the Chao1 index and Shannon index of corn straw silage with the addition of *B. subtilis* has been reported previously [69].

After 90 days ensiling of reed, corn and rice straw, the dominant bacterial phyla were *Firmicutes* and *Proteobacteria*. Microorganisms such as *Firmicutes* and *Proteobacteria* play a positive role in the degradation of fiber-like substances in an anaerobic environment [70]. This indicates that the changes in DM, NDF, and ADF content of the three straw silages in this experiment have an important relationship with the microorganisms of these two phyla [71].

In agreement with our findings, *Rahnella 1,* a facultatively anaerobic, nitrogen-fixing, Gram-negative rod from the *Enterobacteriaceae* family, was highly abundant in the mixed soybean and corn stover silage [72]. Other lactic acid bacteria associated with fermentation, such as *Lactobacillus, Pediococcus, Leuconostoc, and Enterococcus,* were also detected [72]. In this experiment, *Lactobacillus and Leuconostoc* were found in reed straw silage, while *Lactobacillus* and *Enterococcus* were detected in corn and rice straw silage. This indicates that the diversity of lactic acid bacteria during ensiling is also affected by the type of straw.

Furthermore, this study also analyzed the relationship between microbes. In particular, *Enterobacter,* a harmful microorganism, which negatively affects silage quality by degrading proteins and producing toxic compounds, and also competes with *Lactobacilli* for sugars. As the pH of silage decreased, *Enterobacteriaceae* were gradually replaced by *Lactobacilli*, resulting in a negative correlation between *Lactobacilli* and *Enterobacteriaceae*. Similar results were observed in mixed stalk silage of alfalfa and maize, where *Lactobacillus* was negatively correlated

with *Enterobacter,* and *Enterobacter* was gradually replaced by *Lactobacillus* during ensiling [73].

Conclusions

Based on nutrient composition, ensiling quality, membership function analysis, and microbiome analysis, it was observed that application of the experimental silage cocktails improved nutrient composition, ensiling quality, and beneficial bacteria, and decreased harmful bacteria in reed, corn and rice straw silages as compared to their respective control (untreated) silages. Silage cocktails containing a double dose of *B. subtilis* and glucanase resulted in greater improvement in straw silage quality, indicating that a double dosage of *B. subtilis* in combination with glucanase is suitable to achieve optimal silage outcomes for reed, corn, and rice straw.

Abbreviations

R	Reed straw
C	Corn straw
RI	Rice straw
Control group	The control without bacterial and enzyme supplementation
Mesa	Bacterial inoculant and enzyme silage additive
MesaA	Represents Mesa with a double dosage of *A. niger* for Reed straw, and a triple dosage of *A. niger* for corn and rice straws
MesaB	Represents Mesa with double dosage of *B. subtilis*
MesaC	Represents Mesa with a double dosage of *C. utilis*
MesaG	Represents Mesa with a double dosage of glucanase
L. plantarum	*Lactobacillus plantarum*
L. buchneri	*Lactobacillus buchneri*
P. pentosaceus	*Pediococcus pentosaceus*
A. niger	*Aspergillus niger*
B. subtilis	*Bacillus subtilis*
C. utilis	*Candida utilis*
CP	Crude protein
EE	Ether extract
GE	Gross energy
NDF	Neutral detergent fibre
ADF	Acid detergent fibre
LA	Lactic acid
AA	Acetic acid
NH_4–N	Ammonia–nitrogen
TN	Total nitrogen
TA	Total acid
LA/TA	The ratio of LA to TA

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Author contributions

SBW and EYL conducted animal experiments, analyzed data, and wrote the main manuscript. YL, SPW and XLZ designed the experiment, and reviewed and confirmed the manuscript. NAK, CSZ, and ZLT reviewed the manuscript. All authors approved the manuscript.

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Availability of data and materials

Data are provided in Supplementary materials. The sequencing data is publicly available at the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1036041).

Declarations

Ethics approval and consent to participate

All the protocols for the use of animal and experimental procedures in this study were approved by the Animal Care Committee according to the Animal Care and the Use Guidelines (CAS-ISA-2018-1250) of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (Changsha, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential competing interests.

Author details

[1]CAS Key Laboratory for Agro-Ecological Processes in Subtropical Region, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, Hunan, China. [2]Chongqing Key Laboratory of Herbivore Science, College of Animal Science and Technology, Southwest University, Chongqing 402460, China. [3]College of Animal Science, Tarim University, Alar 843300, China.

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