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Fermentation quality and bacterial community of delayed filling stylo silage in response to inoculating lactic acid bacteria strains and inoculating time

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Abstract

Background Filling silos generally takes much time in practice, which may negatively affect silage fermentation and feed intake. To clarify the effects of inoculating time, lactic acid bacteria (LAB) strains and filling time on the silage fermentation and microbes of stylo (*Stylosanthes guianensis*) and its silage, ensiling was quickly performed (quickly filling, QF) with or without LAB (*Lactobacillus plantarum* SXC48, *Lb. plantarum* CCZZ1 and *Enterococcus faecalis* XC124), and was performed after stylo inoculated with or without LAB was placed for 1 day (delayed filling, DF1) and 2 days (DF2).

Results Delayed filling significantly increased pH, buffering capacity, microbial counts and lactic acid, acetic acid and NH_3–N contents of stylo prior to ensiling. Inoculating *Lb. plantarum* SXC48 and CCZZ1 improved the fermentation quality of QF silage, indicated by more lactic acid, as well as lower pH and butyric acid content. Inoculating time significantly affected the pH and lactic acid content of silages. For the DF2 silages, inoculating SXC48 at filling was better than at chopping, while inoculating CCZZ1 had good fermentation quality, regardless of inoculating time. The results of 16S rRNA sequencing indicated that delayed filling enhanced the bacterial diversity of materials and silage, and inoculating significantly changed the composition of silage microbes. *Kosakonia*, *Pseudomonas* and *Pantoea* jointly dominated the fresh material, while *Pantoea* and *Lelliottia* jointly dominated the DF2 material. For the QF silages, inoculating SXC48 and CCZZ1 increased the relative abundance of *Lactobacillus* from 16.4% in the control silage to 76.5% and 82.0%, respectively. *Pantoea* and *Lelliottia* jointly dominated the DF silages. Inoculating SXC48 and CCZZ1 also increased the relative abundance of *Lactobacillus* in the DF stylo silages.

Conclusions Under the present research conditions, delayed filling increased the lactic acid content and reduced the acetic acid, propionic acid and NH_3–N contents of stylo silage, however, increased the bacterial diversity and relative abundance of undesirable bacteria, such as *Pantoea* and *Lelliottia*. The inoculating effectiveness varied with the LAB strains and inoculating time. Inoculating strian SXC48 at filling was better than at chopping, while inoculating strian CCZZ1 at both chopping and filling obtained the similar benefit.

Keywords Bacterial diversity, Delayed filling, Inoculating time, Quick filling, Stylo silage

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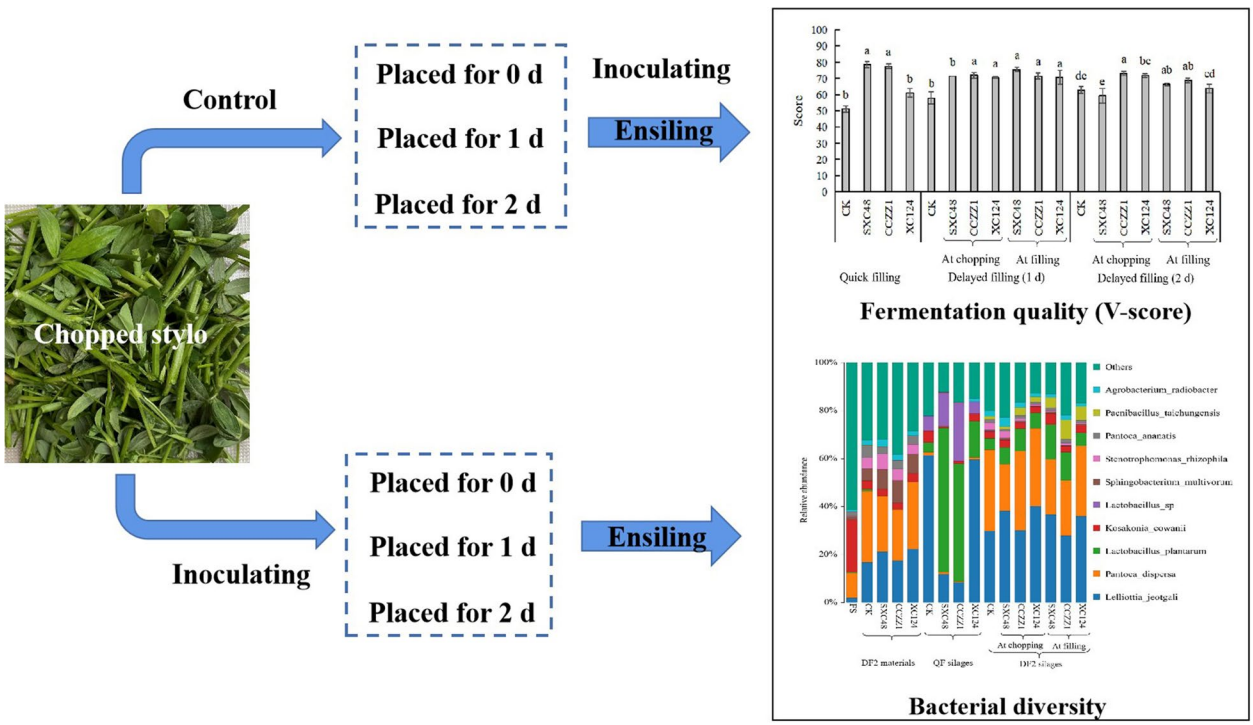
Graphical Abstract



Introduction

Stylo (*Stylosanthes guianensis*) is a very important tropical legume and feed source for local livestock because of its high crude protein content [1]. Feeding cattle with stylo-contained diet significantly improved the dry matter (DM) and N intake [2], and feeding pigs with stylo replacing soybean increased the weights of lung, large intestine and stomach [3]. Wilting is difficulty in the tropical and subtropical regions due to wet and rainy; therefore, ensiling may be a practical way to preserve forage [4]. Ensiling is an approach for long-term preservation of forage crops under the anaerobic conditions. Adding stylo silage to the diet could enhance goats production [5], and benefit the total tract apparent digestibility, N retention and energy digestibility in pigs [6]. In general, the silage well-preserved has lower pH and higher lactic acid content [4]. However, Liu et al. [7] and Pitiwittayakul et al. [8] reported that stylo silage had poor fermentation quality with high pH and NH_3–N content. The factors limiting the fermentation of legume were relatively low concentration of water-soluble carbohydrates (WSC), high buffering capacity and less epiphytic LAB, compared to grasses [9]. Therefore, additives have been widely used to enhance the preservation of stylo silage. Nevertheless, chemical additives have some shortage, such as high cost and poor safety [10]. LAB have been used to promote rapid and efficient fermentation during ensiling through producing more lactic acid [11].

Shortening the initial aerobic phase in silage making is necessary [12]. Several researches [4, 13, 14] reported that the chopped materials exposed to the air for several days might delay the onset of fermentation, encouraging growth of undesirable microbes, such as yeasts. Then, the built-up yeasts partly remained latent after sealing until the silo was opened for feed-out [15–17], which might lead to aerobic deterioration. However, for large-scale silage production, ensiling is difficult to be completed in a short time, at least several days even 1 week [18]. In addition, poor management, such as leaving chopped materials in wagons or piles, or rainfall at harvesting, will prolong the filling process and increase exposure period. The fermentation quality and nutrition losses of silage are easily influenced by delayed filling [13, 14, 18]. Although some losses are unavoidable, good management practices can reduce them, such as using additives [19]. Arbabi et al. [20] found that adding buffered propionic acid-based additive in whole-plant corn exposed to air for 1 d and 2 d before ensiling prevented

a decrease in DM digestibility of silage. Mills and Kung [13] also reported that adding buffered propionic acid-based additive affected the chemical compositions and yeast counts of whole-plant barley exposed to air for 1 d before ensiling, and prevented the reduction in in vitro digestion of silage, irrespective of adding either before or after exposure to air. Besides, the result of Cai et al. [16] showed that inoculating *Lactobacillus plantarum* did not improve the fermentation quality of silage sealed with 1 d delay. However, little is known about the inoculating time on the silage fermentation and microbial community of silage, and how bacterial community on forage crops change during aerobic exposure before ensiling.

Therefore, the objectives of this study were to investigate the effects of inoculating time, LAB strains and filling time on (1) the characteristics and microbial population of stylo before filling, and (2) the fermentation quality and bacterial diversity of stylo silage.

Materials and methods

Materials and silage preparation

Stylo was obtained from an experimental field at South China Agricultural University (23°260 N, 113°150 E, Guangzhou, China) in July 2021. The stylo harvested at the flowering stage was chopped into approximately 1 to 2 cm lengths using a mechanical chopper (9Z-0.4, Shentong Heavy Industry Co., Ltd., Zhengzhou, China). The chopped materials were thoroughly mixed and treated according to the experimental design. The experiment was designed as a 3×4×2 factorial study in a completely randomized design with three replicates per treatment. Ensiling time included quick filling (QF) within 6 h of harvesting, delayed filling after placed for 1 day (DF1) and 2 days (DF2). To test the differences in the fermentation quality among different strains, inoculating LAB strains (isolated by our laboratory) included *Lb. plantarum* SXC48 (SXC48), *Lb. plantarum* CCZZ1 (CCZZ1) and *Enterococcus faecalis* XC124 (XC124) at 10^5 cfu/g FM, and the equal amount of sterile water was added without LAB strain as the control (CK). Inoculating time was at chopping and at filling, the treated materials were packed into plastic film bags (30 cm×20 cm, Mingkang Packing Co. Ltd, Zhongshan, China), then degassed and sealed using a vacuum sealer (Mainfold Vacuum Sealer DZ-280/2SD, Yijian Packaging Machinery Co. Ltd, Dongguan, China). The bags were kept at an ambient temperature of 28–35 °C and were opened after 60 d of ensiling to analyze the fermentation quality and microbial community composition.

Chemical composition analyses

Pre-ensiling stylo samples were dried in a forced air oven at 70 °C for 48 h to determine dry matter (DM) content, and ground to pass a 1.0 mm mesh screen for chemical analyses. The crude ash and crude protein were analyzed by the methods 942.05 and 984.13 of AOAC [21], respectively. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were performed following the procedure of van Soest [22] with an ANKOM A200i fiber analyzer (ANKOM Technology, Macedon, NY, USA) and were expressed exclusive of residual ash. The WSC content was measured using anthrone colorimetry [23]. The buffering capacity was determined by the method of McDonald et al. [4].

Fermentation quality analyses

Twenty grams of samples were sampled and homogenized with 80 mL deionized water, and kept in a refrigerator at 5 °C overnight as described by Zhang et al. [24]. Then, the material was filtered, and the filtrate was used to measure the fermentation products of silage, including pH, ammonia nitrogen (NH_3–N) and organic acids. The pH value was measured using a glass electrode pH meter (FiveEasy Plus, Mettler Toledo Co., Ltd, Shanghai, China). The NH_3–N content was analyzed using a Kjeldahl apparatus [25], and the concentrations of organic acids including lactic acid, acetic acid, propionic acid and butyric acid were measured by high-performance liquid chromatography (HPLC) method as described by Zhang et al. [24]. using Eleven Organic Acids on Transgenomic COREGel 87H3 column (Shodex, Japan), RID-10A detector (210 nm, SPD-20A, Shimadzu Research Laboratory Co., Ltd, Kyoto, Japan), eluent (0.1 mmol/L HP_3O_4, 1.0 mL/min), temperature (40 °C). The fermentation quality of silage was evaluated by the V-score evaluation system [26]. The V-score was calculated based upon the

Table 1 Calculation of V-score

Index[a]	Value[b]	Individual index score[c]
Ammonia nitrogen (% TN)	$X_N \leq 5$	$Y_N = 50$
	$5 < X_N \leq 10$	$Y_N = 60–2X_N$
	$10 < X_N \leq 20$	$Y_N = 80–4X_N$
	$X_N > 20$	$Y_N = 0$
Acetic acid + propionic acid (% FM)	$X_A \leq 0.2$	$Y_A = 10$
	$0.2 < X_A \leq 1.5$	$Y_A = (150–100X_A)/13$
	$X_A > 1.5$	$Y_A = 0$
Butyric acid (% FM)	$0 \leq X_B \leq 0.5$	$Y_B = 40–80X_B$
	$X_B > 0.5$	$Y_B = 0$
V-score	$Y_N + Y_A + Y_B$	

[a] TN, total nitrogen; FM, fresh matter

[b] X_N, ammonia nitrogen content; X_A, acetic acid and propionic acid contents; X_B, butyric acid content

[c] Y_N, index score of ammonia nitrogen content; Y_A, index score of acetic acid and propionic acid contents; Y_B, index score of butyric acid content

contents of NH_3–N, acetic, propionic and butyric acid in silage using the formula in Table 1.

Microbial counts and bacterial diversity analyses

Ten grams of the sample was shaken well for 30 min with 90 mL of sterilized water, and serial dilutions (10^{-1}–10^{-5}) were made in sterile water. The LAB number was measured on de Man Rogosa Sharpe (MRS) agar incubated at 37 °C for 1–2 d under anaerobic conditions (Anaerobic Pack Rectangular Jar, 2.5 L, Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). Aerobic bacteria, yeasts and molds were counted on nutrient agar and Rose Bengal agar incubated for 2–3 d at 30 °C under aerobic conditions, respectively. These media were obtained from Guangdong Huankai Microbial Sci. and Tech. CO. Ltd. (Guangzhou, China). Yeasts were distinguished from molds by observation of colony appearance. Colonies were counted as viable numbers of microorganisms in log10 cfu/g of FM.

Microbial DNA extraction from grass and silage samples was extracted with the TGuide S96 Bacteria DNA isolation kit (DP812, Tiangen, Beijing, China) according to manufacturer instructions. The 27F: AGRGTT TGATYNTGGCTCAG and 1492R: TASGGHTACCTT GTTASGACTT universal primer set was used to amplify the full-length 16S rRNA genes from the genomic DNA extracted from each sample by single molecule real-time (SMRT) sequencing technology. Both the forward and reverse 16S primers were tailed with sample-specific PacBio barcode sequences to allow for multiplexed sequencing. The polymerase chain reaction (PCR) program and procedures were performed as described by Mu et al. [27]. After purification and quantification, amplicons were sequenced using PacBio Sequel (Pacific Biosciences, Menlo Park, CA, USA). The raw reads generated from sequencing were filtered and demultiplexed using the SMRT Link software (version 8.0) to obtain the circular consensus sequencing (CCS) reads. The quality was filtered using the Cutadapt quality control process (version 2.7) through the recognition of forward and reverse primers. The UCHIME algorithm (v8.1) was used in detecting and removing chimera sequences to obtain the clean reads. Sequences with similarity ≥ 97% were clustered into the same operational taxonomic unit (OTU) by USEARCH (v10.0). Taxonomy annotation of the OTUs was performed based on the Naive Bayes classifier in QIIME2 using the SILVA database with a confidence threshold of 70%. Alpha diversity was calculated based on Shannon–Wiener, Simpson's diversity, Chao1 and rarefaction estimators and displayed by R software. Data were analyzed using the free online BMK Cloud Platform (www. BMKbiocloud.com).

Statistical analyses

The effects of inoculating time, LAB strains and filling time on the chemical characteristics and microbes of stylo prior to ensiling, and on the fermentation parameters of stylo silage were analyzed with IBM SPSS 20.0 for Windows. The results were evaluated using analysis of variance (ANOVA). The means were compared for significance by Duncan's multiple range method. Statistical significance was considered at the $P < 0.05$ level. An online platform (http://www.biocloud.net/) was used to analyze the sequencing data of the bacterial community.

Results

Characteristics and microbial population of stylo prior to ensiling

The contents of DM, crude protein and crude ash, pH and buffering capacity were significantly influenced by filling time, whereas the NDF, ADF and WSC contents were not. The DM, crude protein and crude ash contents, pH and buffering capacity of the DF1 and DF2 materials tended to increase, compared to the QF material. The buffering capacity of DF2 material was over 940 mEq/kg DM, which was significantly higher ($P < 0.05$) than that of the DF1 and QF materials. In addition, lactic acid and acetic acid were detected in the QF material, and they were increased by delayed filling. The NH_3–N was not detected in the QF material, but was 46.5–55.8 g/kg TN in DF1 material and 70.4–88.5 g/kg TN in DF2 material, respectively. Inoculating significantly affected pH, buffering capacity, and the contents of crude protein, acetic acid and NH_3–N. The buffering capacity, acetic acid and NH_3–N contents of the DF1 and DF2 materials inoculated with SXC48 were higher than those of materials uninoculated and inoculated with CCZZ1 and XC124 (Table 2).

Delayed filling significantly increased the amounts of microbes in pre-ensiling materials, while inoculating did not affect the amounts of aerobic bacteria, yeasts and molds. Inoculating significantly increased ($P < 0.05$) the LAB number of the DF1 and DF2 materials, compared to the uninoculated one (Table 3).

Ensiling characteristics and microbial population of stylo silages

The fermentation quality of silage ensiled for 60 d is shown in Table 4. Inoculating *Lb. plantarum* SXC48 and CCZZ1 significantly decreased pH and NH_3–N content, increased the lactic acid content of QF silage. Uninoculated DF2 silage had more lactic acid (30.89 g/kg DM), lower pH (4.73) and less NH_3–N (134 g/kg TN) than the uninoculated DF1 and QF silages. Inoculating strains (except SXC48 before placing) decreased pH and increased lactic acid content of delayed filling silages

Table 2 Chemical compositions of stylo before ensiling[a]

Treatments[b]		DM	Crude protein	Crude ash	NDF	ADF	WSC	pH	Buffering capacity	Lactic acid	Acetic acid	NH_3–N
		(g/kg FM)	(g/kg DM)						(mEq/kg DM)	(g/kg DM)		(g/kg TN)
Fresh stylo		289.9d	10.11e	51.13e	706.8	491.8	15.82a	5.64b	397.8e	0.46b	0.93f	0.00d
Stylo inoculated at chopping and placed for 1 d	CK	319.5c	10.44cde	53.72cde	725.8	499.4	11.44ab	5.67b	666.5c	2.41a	1.98de	55.76c
	SXC48	318.1c	10.97ab	55.18bcd	686.8	482.0	10.66ab	6.03a	715.0c	2.38a	2.77bc	72.27b
	CCZZ1	325.3c	10.65abcd	53.21de	699.9	474.9	13.57ab	5.70b	573.2d	2.13a	1.76e	46.48c
	XC124	325.5c	10.50bcde	53.77cde	719.8	499.8	12.75ab	5.74b	692.0c	1.68a	2.23cde	49.46c
Stylo inoculated at chopping and placed for 2 d	CK	381.8ab	10.83abc	60.01a	700.5	488.2	10.67ab	6.12a	958.8b	1.69a	2.75bc	77.26b
	SXC48	365.4b	11.06a	57.01b	678.8	476.8	9.58b	6.32a	1033.6a	1.63a	3.32a	88.52a
	CCZZ1	376.6ab	11.03a	56.15bc	690.8	488.7	13.51ab	6.14a	966.0ab	1.81a	2.84ab	77.36b
	XC124	385.9a	10.31de	57.11b	681.4	466.1	13.92ab	6.10a	941.0b	1.73a	2.47bcd	70.43b
SEM[c]		1.837	0.076	0.283	3.918	4.430	0.562	0.029	7.587	0.119	0.058	1.175
Significance[d]	Delayed filling	**	*	**	NS	NS	NS	**	**	**	**	**
	Inoculation	NS	**	NS	NS	NS	NS	*	**	NS	**	**
	Interaction	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS

Different lowercase letters in the same column indicate significant difference ($P < 0.05$)

[a] DM, dry matter; FM, fresh matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water-soluble carbohydrates; NH_3–N, ammonia nitrogen; TN, total nitrogen

[b] CK, without inoculation; SXC48 and CCZZ1, inoculating *Lb. plantarum* SXC48 and CCZZ1; XC124, inoculating *En. faecalis* XC124

[c] SEM, standard error of the means

[d] $*P < 0.05$; $**P < 0.01$; NS, not significant

Table 3 Microbial counts of stylo before ensiling (lg cfu/g FM)[a]

Treatments[b]		LAB	Aerobic bacteria	Yeasts	Molds
Fresh stylo		4.06e	6.97c	4.68c	4.30c
Stylo inoculated at chopping and placed for 1 d	CK	6.03d	8.74b	6.65ab	5.31b
	SXC48	6.69c	8.76b	6.63ab	5.48b
	CCZZ1	6.64c	8.74b	6.29b	5.27b
	XC124	7.16b	8.74b	6.63ab	5.51b
Stylo inoculated at chopping and placed for 2 d	CK	5.97d	9.88a	6.41ab	6.04a
	SXC48	6.97bc	9.68a	6.99a	6.36a
	CCZZ1	7.01bc	9.71a	6.72ab	6.19a
	XC124	7.72a	9.68a	6.60ab	6.27a
SEM[c]		0.047	0.053	0.062	0.053
Significance[d]	Delayed filling	**	**	**	**
	Inoculation	**	NS	NS	NS
	Interaction	NS	NS	NS	NS

Different lowercase letters in the same column indicate significant difference ($P < 0.05$)

[a] FM, fresh matter; LAB, lactic acid bacteria

[b] CK, without inoculation; SXC48 and CCZZ1, inoculating *Lb. plantarum* SXC48 and CCZZ1; XC124, inoculating *En. faecalis* XC124

[c] SEM, standard error of the means

[d] $^{**}P < 0.01$; NS, not significant

compared to uninoculated one, and reduced the contents of propionic acid and butyric acid, especially inoculating at filling. However, the time of inoculating CCZZ1 had no significant effect on the fermentation quality of silage. The DF2 silage inoculated with SXC48 at filling had the lowest pH and the highest lactic acid content (Table 4).

For QF silages, the V-scores of silages inoculated with SXC48 and CCZZ1 were higher ($P < 0.05$) than those inoculated with XC124 and uninoculated. For DF1 silages, inoculating strains (except SXC48 at chopping) significantly increased the V-scores of silage compared to uninoculated silages. The DF2 silages inoculated with SXC48 at filling, XC124 at chopping and CCZZ1 at either time had higher V-score than uninoculated silage (Fig. 1).

Bacterial diversity of stylo and its silages

The alpha-diversity of the bacterial community in stylo before ensiling and its silages is summarized in Table 5. The coverage values of all samples were above 0.99. The bacterial community of DF2 materials before ensiling had higher ($P < 0.01$) Simpson and Shannon as well as lower ($P > 0.05$) OTUs and chao 1 than the QF materials, regardless of inoculating. For DF2 silages, Simpson and Shannon had an increasing trend compared to the QF silages.

PCA 1, PCA 2 and PCA 3 were 38.8%, 20.8% and 31.7% of the total variance in this study, respectively. PCA illustrated that the bacterial community of DF2 materials before ensiling or silages differentiated apparently from that of corresponding QF materials. Inoculating also led to the clear separation of bacterial community of the QF silages, while had no significant effect on the bacterial community of DF2 silages. Moreover, the bacterial community of QF silages inoculated with SXC48 and CCZZ1 was separated from those of silages inoculated with XC124 and uninoculated (Fig. 2).

As shown in Fig. 3A, Proteobacteria was the dominant phylum (74.1–80.3%) in the bacterial community of QF and DF2 materials, followed by Bacteroidetes (15.4–25.0%). However, Proteobacteria (67.1–85.3%) and Firmicutes (13.8–31.8%) were the top two phyla in all silages except the QF silages inoculated with SXC48 and CCZZ1 that had remarkably higher relative abundance of Firmicutes (79.5% and 84.2%). The bacterial community was altered by delayed filling and ensiling. In Fig. 3B, the genera with the relative abundance of above 1% in the materials before ensiling were *Kosakonia* (21.7%, mainly *Ko. cowanii*), *Pseudomonas* (13.3%), *Pantoea* (12.3%), *Chryseobacterium* (3.7%), *Lelliottia* (2.0%), *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* (1.8%), *Sphingobacterium* (1.5%) and *Paenibacillus* (1.1%). The relative abundance of *Kosakonia* was greatly decreased to 2.6–3.7% after the materials were placed for 2 d. The DF2 materials were jointly dominated by *Pantoea* and *Lelliottia*, mainly *Pa. ananatis* and *Le. jeotgali*, respectively (Fig. 4). The relative abundance of *Kosakonia* decreased from 21.7% to 0.8–4.7% in QF silages and 2.5–4.5% in DF2 silages. *Lelliottia* and *Lactobacillus* were the top two genera in QF silages. Differently, *Lelliottia* was the dominant genus in the QF silages uninoculated and inoculated with XC124 (59.5% and 61.4%), while *Lactobacillus* was the dominant genus in the QF silages inoculated with SXC48 and CCZZ1 (76.5% and 82.0%), and *Lb. plantarum* was main species. The undesirable bacteria *Lelliottia* (27.9–40.2%) and *Pantoea* (20.5–35.4%) were the top two genera in the DF2 silages, while the relative abundance of *Lactobacillus* was only 7.2–10.0% (Figs. 3B and 4).

Discussion

Characteristics of stylo prior to ensiling

The contents of DM and WSC, and buffering capacity of forage crops prior to ensiling play key roles in good silage fermentation [4]. The DM, crude protein and WSC contents of raw stylo in this study were lower than the values reported by Rufino et al. [28], but were comparable with those reported by Wu et al. [29]. In this study, delayed filling significantly increased the DM content and buffering capacity, decreased the WSC content of materials before ensiling. This might be because the amount of materials piled was less, and the moisture greatly lost

Table 4 Fermentation quality and microbial composition of stylo silages[a]

Treatments[b]		DM	pH	Organic acid (g/kg DM)				NH_3–N	Microorganisms (lg cfu/g FM)	
		(g/kg FM)		Lactic acid	Acetic acid	Propionic acid	Butyric acid	(g/kg TN)	LAB	Aerobic bacteria
Quick filling	CK	286.7i	4.98a	10.46 h	11.98a	1.40a	2.57ab	178.73a	6.20	5.07e
	SXC48	296.7hi	4.72def	21.65def	9.00b	0.08 fg	0.81ab	122.57e	6.43	5.19e
	CCZZ1	297.1hi	4.63 fg	19.81ef	8.02bc	0.03 g	0.99ab	124.59e	6.37	5.05e
	XC124	298.9hi	4.88b	12.12gh	9.14b	0.23defg	2.13ab	158.49b	6.31	5.13e
Delayed filling (1 d)	CK	312.7fgh	5.01a	14.36gh	9.46b	0.62bc	2.74ab	160.67b	6.27	6.35bcd
Inoculating at chopping	SXC48	307.6gh	5.00a	14.42gh	9.55b	0.74b	2.74ab	147.20bcd	6.11	6.13 cd
	CCZZ1	321.9efg	4.72def	19.60efg	7.65bc	0.17defg	0.54b	140.35bcde	6.10	5.93d
	XC124	327.0cdef	4.80bcd	22.07def	9.23b	0.43cde	0.74ab	141.92bcde	6.07	5.94d
Inoculating at filling	SXC48	322.5efg	4.71def	16.90fgh	6.32c	0.19defg	0.70ab	150.10bcd	5.90	6.17 cd
	CCZZ1	321.6efg	4.69ef	23.32def	8.35bc	0.20defg	0.60b	155.92bc	6.33	6.12 cd
	XC124	324.6def	4.78cde	21.65def	8.83bc	0.48bcd	0.67b	136.67cde	6.36	6.09 cd
Delayed filling (2 d)	CK	341.8bc	4.73def	30.89bc	7.58bc	0.58bc	2.41ab	133.96de	6.46	6.64abc
Inoculating at chopping	SXC48	328.3cdef	4.84bc	24.21de	7.02bc	0.38cdef	3.03a	131.18de	6.43	7.11a
	CCZZ1	356.5ab	4.47i	33.21bc	7.25bc	0.11efg	0.60b	141.02bcde	5.73	6.84ab
	XC124	363.2a	4.58gh	28.30 cd	7.27bc	0.26defg	1.34ab	142.24bcde	6.27	6.59abc
Inoculating at filling	SXC48	334.3cde	4.42i	40.81a	8.27bc	0.16efg	1.07ab	150.55bcd	5.92	6.45bcd
	CCZZ1	334.0cde	4.44i	32.70bc	7.01bc	0.14efg	1.00ab	145.62bcd	5.59	6.58abc
	XC124	340.9bcd	4.51hi	37.10ab	7.20bc	0.26defg	1.87ab	151.43bcd	5.53	6.52abcd
SEM[c]		1.234	0.008	0.502	0.178	0.023	0.162	1.481	0.072	0.044
Significance[d]	D	**	**	**	**	**	NS	NS	NS	**
	I	**	**	*	**	**	NS	**	NS	NS
	T	NS	**	**	NS	NS	NS	NS	NS	NS
	D×I	NS	NS	NS	NS	NS	NS	*	NS	NS
	D×T	**	NS	*	NS	NS	NS	NS	NS	*
	I×T	**	**	*	NS	**	*	NS	NS	NS
	D×I×T	NS	NS	*	*	NS	NS	NS	NS	NS

Different lowercase letters in the same column indicate significant difference ($P < 0.05$)

[a] DM, dry matter; FM, fresh matter; NH_3–N, ammonia nitrogen; TN, total nitrogen; LAB, lactic acid bacteria

[b] CK, without inoculation; SXC48 and CCZZ1, inoculating *Lb. plantarum* SXC48 and CCZZ1; XC124, inoculating *En. faecalis* XC124

[c] SEM, standard error of the means

[d] D, delayed filling; I, inoculation; T, inoculating time; *$P < 0.05$; **$P < 0.01$; NS, not significant

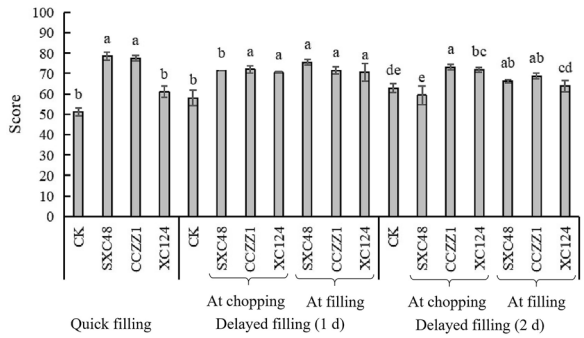


Fig. 1 V-scores of stylo silages. (Different letters indicate significant difference among the treatments of same material)

during placing. The microorganisms in the materials after placed significantly increased and produced more organic acids, resulting in an increase in buffering capacity and a decrease in WSC content. Mills and Kung [13] also reported that the WSC content of barley decreased by more than 50% as a result of exposure to air for 1 d.

Epiphytic LAB on the forage crops are also essential for its silage fermentation [30]. The LAB population (4.0 log cfu/g) in the present study was comparable to that reported by Wu et al. [29] (4.20 log cfu/g), but lower than 5.0 log cfu/g FM considered as adequate for the good fermentation of silage [31]. The LAB numbers of DF1 and DF2 materials increased to 5.0 log cfu/g, while the numbers of undesirable microorganisms such as aerobic bacteria, yeasts and molds all increased and were relatively high. This is consistent with the results reported by Cai et al. [16] and Pahlow et al. [15]. Moreover, aerobic bacteria dominated the microbial populations of delayed filling materials in this study, and reached 8–10 log cfu/g FM. Thus, lactic acid, acetic acid and NH_3–N were detected in delayed filling materials. Such results also occurred in the pre-ensiled soybean curd residue placed for 2 days [17]. NH_3–N indicated protein degradation by undesirable microorganisms, such as *Enterobacter* [32]. Inoculating SXC48 increased the NH_3–N content of delayed filling materials, which might be that inoculating strain SXC48 did not inhibit the growth of harmful bacteria during aerobic exposure, resulting in more protein hydrolysis.

Table 5 General information of sequence and bacterial diversity of stylo and its silage[a]

Samples	Treatments[b]	Effective Reads	OTUs	Chao 1	Simpson	Shannon	Coverage
Fresh stylo		10585	210	239	0.48b	2.45b	1.00
Stylo inoculated at chopping and placed for 2 d	CK	9974	197	231	0.87a	4.38a	0.99
	SXC48	9578	175	220	0.88a	4.32a	0.99
	CCZZ1	9769	179	203	0.90a	4.54a	1.00
	XC124	9482	174	218	0.85a	4.04a	0.99
SEM[c]		142.540	5.437	4.530	0.050	0.238	0.000
Significance[d]		NS	NS	NS	**	**	NS
Quick filling	CK	10530	120	160	0.59c	2.52c	1.00
	SXC48	9941	148	204	0.59c	2.37c	0.99
	CCZZ1	10279	148	184	0.68abc	2.63c	1.00
	XC124	10468	121	172	0.62bc	2.41c	1.00
Delayed filling (2 d)	CK	9994	128	167	0.78a	3.31abc	1.00
Inoculating at chopping	SXC48	10517	142	195	0.80a	3.65a	0.99
	CCZZ1	9779	120	222	0.78a	3.18abc	0.99
	XC124	9793	91	160	0.72abc	2.71bc	1.00
Inoculating at filling	SXC48	10120	100	161	0.76ab	2.97abc	1.00
	CCZZ1	10080	123	153	0.84a	3.57ab	1.00
	XC124	9652	105	148	0.77ab	3.07abc	1.00
SEM[c]		76.032	4.552	6.471	0.020	0.103	0.000
Significance[d]		NS	NS	NS	**	*	NS

Different lowercase letters in the same column and same material indicate significant difference ($P < 0.05$)

[a] OTUs, operational taxonomic units

[b] CK, without inoculation; SXC48 and CCZZ1, inoculating *Lb. plantarum* SXC48 and CCZZ1; XC124, inoculating *En. faecalis* XC124

[c] SEM, standard error of the means

[d] $*P < 0.05$; $**P < 0.01$; NS, not significant

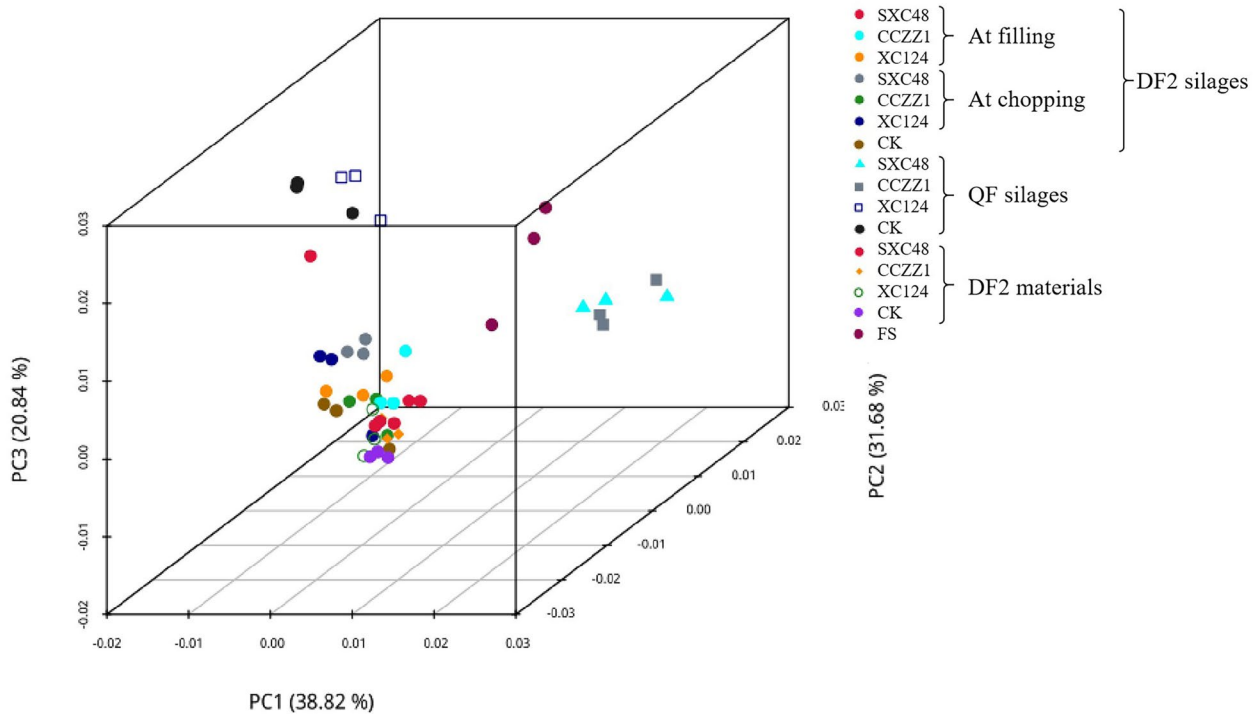


Fig. 2 Cluster analysis of bacterial communities in stylo before ensiling and its silage as assessed by a Principal Coordinate Analysis

Delayed filling would have a negative impact on the fermentation quality.

Fermentation quality and microbial population of stylo silage

Ensiling inhibits the activities of undesirable microorganisms mainly through producing lactic acid to reduce pH, so as to preserve nutrients [4]. In this study, relatively high pH (4.98) of the uninoculated QF silage indicated the low fermentation quality. Inoculating LAB promoted lactic acid production and reduced the NH_3–N content of QF silage, thus accelerating pH decline, even though their final pH were still higher than the ideal pH below 4.20 for high-quality silage [4]. This is consistent with the results of Wu et al. [29] and Liu et al. [7], who reported that stylo silage had the high pH of 4.84–5.39, and inoculating *Pediococcus pentosaceus* and *Lb. paraplantarum* significantly improved its fermentation quality. Inoculating and delayed filling reduced the pH value and increased the lactic acid content to different degree of stylo silage, which was different from the findings that delayed sealing resulted in poor silage fermentation [33, 34] or inoculating was not effective for delayed sealing silage [16]. The reason might be that the materials piled were not thick, and delayed filling might play a similar role to wilting, promoting lactic acid fermentation of silage [35].

Butyric acid is unfavorable to the silage quality because of the nutritional losses during butyric acid fermentation by clostridial activity [33]. All uninoculated silages had more butyric acid than the inoculated silages except the inoculation of SXC48 at chopping, which were probably related to the promotion of lactic acid fermentation and inhibition of butyric acid fermentation by LAB strains [4].

Bacterial community of stylo and its silage

Analyzing bacterial community would contribute to reveal the changes of bacteria in the materials during aerobic exposure and further understand the silage fermentation. The coverage for all samples in the present study were over 0.99, indicating that sequencing abundance was large enough to reflect the profile of the bacterial community. The alpha-diversity indices revealed stylo silages had lower bacterial community richness and higher diversity relative to fresh materials. Moreover, the bacterial community diversity (Shannon and Simpson) in stylo and its silages were altered by delayed filling and inoculating in this study. The significant increase in bacterial diversity of delayed filling materials might be attributed to the remarkable growth of harmful microorganisms, since their growth and survival generally require moderate pH level and aerobic condition. This result was consistent with the report by Du et al. [36]. As microbes

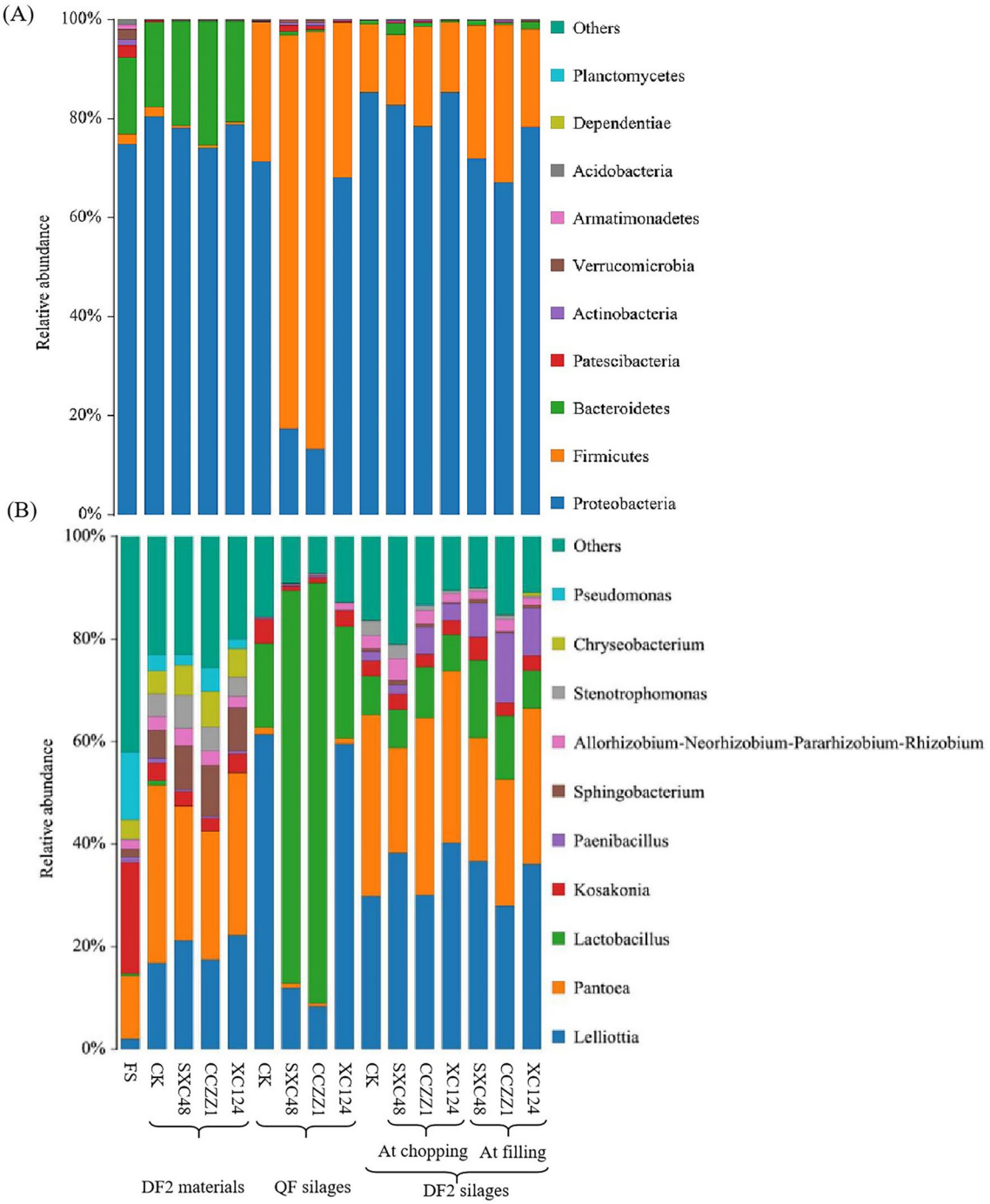


Fig. 3 Bacterial community at the phylum (**A**) and genus (**B**) level of stylo before ensiling and its silages

grow and compete during aerobic exposure or ensiling, inoculating LAB strains would shape the microbial community more desirable, resulting in the fermentation quality improvement [37]. Consistently, PCA analysis showed that the bacterial community of delayed filling materials and silages differentiated apparently from that of fresh material, and inoculating remarkably altered the bacterial community of silage.

In this study, Proteobacteria and Bacteroidetes were dominant in all DF2 materials before ensiling (>1% abundance), while Proteobacteria and Firmicutes were the main phyla in all the silages. Quickly ensiling and

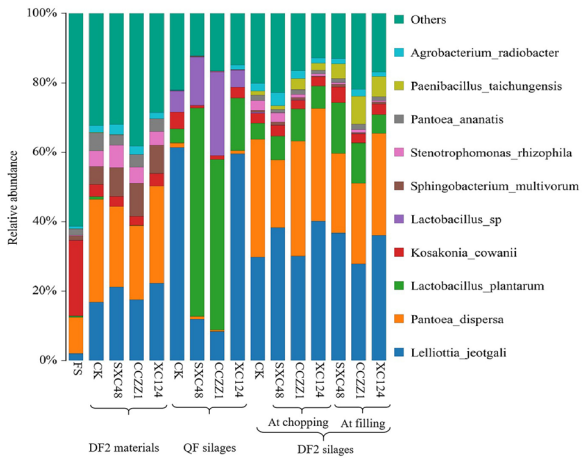


Fig. 4 Species-level microbes analyses of stylo before ensiling and its silages (FS, fresh stylo; CK, without inoculation; SXC48 and CCZZ1, inoculating *Lb. plantarum* SXC48 and CCZZ1; XC124, inoculating *En. faecalis* XC124)

inoculating SXC48 and CCZZ1 changed the dominant phylum from Proteobacteria to Firmicutes. The result of Zi et al. [9] also suggested that Firmicutes and Proteobacteria were dominant in all stylo silages, while the abundance of Proteobacteria was higher than that of Firmicutes in sucrose-treated silage. The presence of more Proteobacteria in silage indicates high pH value of silage as Proteobacteria prefer the neutral environment [38]. Moreover, gram-negative pathogenic Proteobacteria include a wide variety of pathogenic genera, such as *Escherichia*, *Salmonella*, *Vibrio*, and many others. The dominant phylum in the QF silages inoculated with strain SXC48 and CCZZ1, being the homofermentative LAB, was Firmicutes, which was generally considered desirable during ensiling, since they can inhibit the growth of *Clostridia* and decrease the content of NH_3–N. Thus, inoculating strain SXC48 and CCZZ1 decreased the NH_3–N content and improved the fermentation quality of QF silages.

The fresh stylo was jointly dominated by several genera, such as *Kosakonia*, *Pseudomonas* and *Pantoea*, while *Pantoea* and *Lelliottia* were main genera in the delayed filling materials. Bacteria such as *Kosakonia* and *Pseudomonas* in the DF1 and DF2 materials reduced, resulting in that the bacterial diversity changed during placing and then affected the silage fermentation. *Kosakonia* was also the main genera in stylo silage reported by He et al. [39] and Wang et al. [40]. *Kosakonia* is recently classified from the genus *Enterobacter* [41]. In this study, *Kosakonia* mainly included *Ko. cowanii*, which was rarely found in silage. Kumar et al. [42] reported *Ko. cowanii* promoted the plant growth. *Lelliottia* (mainly *Le. jeotgali*) dominated the QF silages uninoculated and inoculated with XC124, whereas *Lactobacillus* (mainly *Lb. plantarum*) was the overwhelming genus in the QF silages inoculated with SXC48 and CCZZ1. *Lelliottia* was also detected in stylo silage reported by Wu et al. [29], and it is separated from the genus *Enterobacter* and reclassified subsequently as a novel genus [43]. Thus, *Lelliottia* might play the similar function to *Enterobacter* in silage. The relatively higher pH and lower lactic acid content of the QF silages inoculated with XC124 and uninoculated might be attributed to more *Lelliottia.* Inoculating SXC48 and CCZZ1 made *Lb. plantarum* predominant to facilitate the fermentation of QF silages. *Lelliottia* and *Pantoea* jointly dominated the DF2 silages, and *Pantoea* was mainly *Pa. ananatis. Pantoea* is commonly found in stylo silage [9, 17, 44], and is undesirable microbes, because they compete the fermentation substrate with LAB [45]. However, some studies have claimed that *Pantoea* is beneficial to silage fermentation, owing to reduce the NH_3–N content and pH of silage [42]. *Pa. ananatis*, a gram-negative bacterium, provides various beneficial characteristics, such as the growth promotion of their host plants and increased crop yield [46]. Tao et al. [47] reported *Pa. ananatis* had the ability to degrade the lignin. It is seldom reported in silage and the role of *Pa. ananatis* in silage fermentation needs to be further researched.

Conclusions

Under the present research conditions, delayed filling increased the pH, buffering capacity, DM content, microbes number, lactic acid content, acetic acid content and NH_3–N content of stylo before ensiling. Delayed filling and inoculating LAB changed the bacterial community of stylo and its silages. Inoculating SXC48 and CCZZ1 largely increased the relative abundance of beneficial bacteria such as *Lactobacillus* and greatly reduced that of undesirable bacteria such as *Kosakonia*, *Pantoea* and *Lelliottia* in the QF silages. Delayed filling increased the lactic acid content and decreased NH_3–N content of silage, but increased the bacterial diversity and relative abundance of *Pantoea* and *Lelliottia.* The inoculating effectiveness of delayed filling silage varied with the inoculating time and LAB strains, inoculating strian SXC48 at filling was better than at chopping, while inoculating strian CCZZ1 at both chopping and filling obtained the similar benefit.

Abbreviations

LAB	Lactic acid bacteria
Lb. plantarum	*Lactobacillus plantarum*
En. faecalis	*Enterococcus faecalis*
QF	Quickly filling
DF1	Delayed filling for 1 d
DF2	Delayed filling for 2 d

DM	Dry matter
FM	Fresh matter
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
WSC	Water-soluble carbohydrates
NH_3–N	Ammoniacal nitrogen

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Author contributions
JT: methodology, visualization, data curation, and writing—original draft preparation; LH and RT: investigation, resources, and validation; JW and RT: software and formal analysis; JZ: conceptualization, reviewing and editing, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The data sets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors listed have read the complete manuscript and have approved submission of the paper.

Competing interests
No conflicts of interest declared by the authors.

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