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Insight into the key limiting factors affecting anaerobic fermentation quality and bacterial community of sweet sorghum by irradiation sterilization and microbiota transplant

Jie Zhao, Zhao-Di Jing, Xue-Jing Yin, Jun-Feng Li, Zhi-Hao Dong, Si-Ran Wang and Tao Shao*

Abstract

Biomass microbiota and chemical constituent are closely associated with final anaerobic fermentation performance. But the limiting factors affecting anaerobic fermentation quality and bacterial community have been rarely explored. This study aimed to elucidate the relative contribution of initial microbiota and chemical constituent of sweet sorghum on its final anaerobic fermentation quality. Sweet sorghum at two developmental stages (heading-stage, G_1; hard-dough-stage, G_2) was treated as follows: G_1 microbiota + sterilized G_1 (M_1C_1), G_2 microbiota + sterilized G_1 (M_2C_1), G_1 microbiota + sterilized G_2 (M_1C_2), and G_2 microbiota + sterilized G_2 (M_2C_2). The results showed that chemical constituent rather than microbiota changes remarkably influenced the production of lactic acid, propionic acid and ammonia-N, the relative abundance of *Lactobacillus*, *Weissella*, *Lactococcus*, *Pediococcus*, and *Pantoea* of sweet sorghum after anaerobic fermentation. The chemical constituent was the key limiting factor affecting the anaerobic fermentation quality of sweet sorghum. This study could provide a reference for clarifying the key limiting factors affecting anaerobic fermentation and making recommendations for production.

Keywords Sweet sorghum, Phyllosphere microbiota, Chemical constituent, Fermentation quality, Bacterial community

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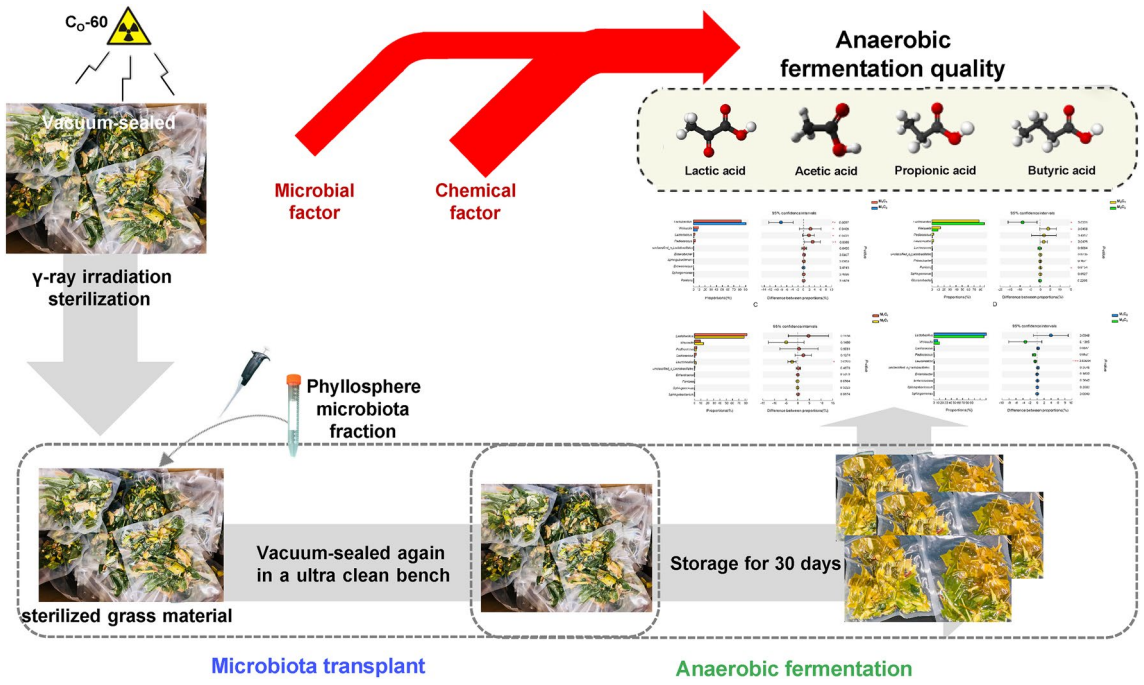
Graphical Abstract



Background

Sweet sorghum (*Sorghum bicolor* (L.) Moench) as a well-known forage or energy crop is widely planted in tropical, subtropical and temperate regions with humid, semi-humid and semi-arid climates due to its adaptable, fast-growing and high-yield properties [1]. It has been used in the production of sugar, food, fibrous products, biofuels and chemicals, especially feed. However, the toxic cyanogentic glycosides and antinutritional tannin in fresh sweet sorghum require anaerobic fermentation as the preferred processing method to degrade these components. Ensilage refers to the anaerobic fermentation of moist forages by epiphytic probiotics to produce mainly short-chain fatty acid (SCFA) from the sugars present [2]. However, it should be noted that when anaerobic fermentation is performed from forage harvested at different developmental stages, their fermentation patterns and final SCFA composition can be a far cry from. In the case of guinea grass, van Niekerk et al. [3] reported that the fermentation of guinea grass prepared at the early vegetative and boot stages was lactate-type, while the fermentation of guinea grass prepared at the full bloom stage was acetate type. These inconsistent results caused by developmental stages can ultimately be inferred from differences in the chemical and microbial compositions of forage at harvest. Namely, the developmental stage influences the phyllosphere microbiota and chemical constituent (including but not limited to dry matter (DM), water-soluble carbohydrates (WSC) and buffering capacity) of grasses, thereby affecting the final anaerobic fermentation quality. Therefore, it needs to clarify the relative contribution of initial phyllosphere microbiota and chemical constituent to final fermentation quality, which is of great significance for silage production.

Over the past 60 years, a series of research has tried to distinguish the role of chemical and microbial factors in the anaerobic fermentation quality. In order to suppress the disturbance of microbial factor, sterile material was gained through sterile cultivation [4], chemical sterilization [5], autoclaving [6], dry heat [7], etc. But the risk of chemical sterilization, performed by bactericides such as chloroform and toluene, limits its further application in related research. While the major challenge of sterile cultivation is the long-term maintenance of a totally sterile environment [4]. Although autoclaving and dry heat has desirable sterilization effects, they have been shown to cause significant changes in the physicochemical properties of forage [8]. Fortunately, γ-ray irradiation sterilization has recently been shown to disinfect forage material at appropriate doses without impacting plant enzyme activities and chemical constituent [9, 10], which is suitable for the preparation of sterile forage.

Microbiota transplantation, as the most effective method to reconstitute symbiotic microbiota, has been a prospective research tool in the field of medical and agricultural microecology [11]. Microbiota transplantation is not limited to the treatment of intestinal disease. Williams and Marco [12] assessed the feasibility of microbiota transplantation for phyllosphere microbial studies under laboratory conditions, and they found that it would be useful to elucidate the interactions of microbes on plants and important for agriculture and fermented food safety. These findings are also instructive for anaerobic fermentation studies. Mogodiniyai Kasmaei et al. [13] applied the microbiota transplant for the first time in anaerobic fermentation research and stated that the epiphytic microflora of forages can be transplanted effectively. In recent years, the next-generation sequencing (NGS) technique has been widely applied in sensitively and accurately monitoring microbial community succession, which makes it possible to evaluate the effects of phyllosphere microbiota and chemical constituent on microbial community structure and diversity.

Therefore, the current study was designed to elucidate the relative contribution of phyllosphere microbiota and chemical constituent to the anaerobic fermentation quality and bacterial community of sweet sorghum, which may be important in clarifying the key limiting factors affecting anaerobic fermentation and making recommendations for production.

Methods

Material preparation

Sweet sorghum was planted in the Baima National Agricultural-tech Zone (31° 61′ N, 119° 18′ E, a.s.l. 25.1 m, Jiangsu, China) on June 30, 2020. A 30 m^2 planting field was separated into three equal blocks (for replications) and each block was further divided into two equal plots (two developmental stages). Half of the sweet sorghum was mowed on August 28, 2020, and the left sweet sorghum was mowed on October 2, 2020 to obtain two batches of sweet sorghum (G_1, the heading stage; G_2, the hard dough stage). The harvest time was in the morning with clear weather, and the stubble height was about 15 cm. Each batch of fresh sweet sorghum was immediately cut into about 2 cm lengths by a feed cutter, mixed totally and split into three parts for phyllosphere microbiota collection, fresh sample analysis and experimental preparation, respectively.

Phyllosphere microbiota collection

Following the procedures of Mogodiniyai Kasmaei et al. [13], the phyllosphere microbiota were respectively eluted from fresh G_1 or G_2, namely M_1 or M_2. Concretely, 5 kg fresh G_1 or G_2 was mixed thoroughly and separated into 10 portions (500 g per portion). Subsequently, 3 L Ringer solution (formula: NaCl, 2.25 g; KCl, 0.105 g; $CaCl_2$, 0.06 g; $NaHCO_3$, 0.05 g, dissolved in 1 L distilled water, adjusted to pH 6.9, sterilized by autoclaving at 121 °C for 15 min) containing 0.05% v/v Tween-80 was mixed with 500 g fresh-cut G_1 or G_2. Then, microbial suspension of the above mixture was obtained by shaking at 150 rpm for 2 min. After centrifuging at 16,000×*g* for 90 min at 4 °C, microbial precipitation was obtained and resuspended with 3 mL Ringer solution. Before the transplant, all microbial precipitation was pooled together and homogenized.

Experimental preparation and microbiota transplantation

After thorough mixing, about 450 g of fresh-cut G_1 or G_2 was loaded into each UV-sterilized laboratory silo (polythene bag with a size of 300 × 400 mm) and vacuum-sealed. Based on the dose and duration of irradiation sterilization recommended by Junges et al. [14], totally 18 laboratory silos (fresh material: 2 developmental stages × 3 replicates; anaerobic fermentation: 4 treatments × 3 replicates) were exposed to a Co-60 source and sterilized by γ-ray irradiation at 32 kGy for 4 h (Nanjing Xiyue Technology Co., Ltd, Jiangsu, China). Designate γ-ray sterilized G_1 as C_1 and γ-ray sterilized G_2 as C_2.

In an ultra-clean bench, the γ-ray sterilized silos were opened and treated as follows (Fig. 1): (i) transplantation of M_1 to C_1 (M_1C_1); (ii) transplantation of M_2 to C_1 (M_2C_1); (iii) transplantation of M_1 to C_2 (M_1C_2); and (iv) transplantation of M_2 to C_2 (M_2C_2). The transplant volume of M_1 or M_2 was 3 mL per silo and the silos were resealed and kept at the surrounding temperature (24 ± 4 ℃). Triplicate silos per treatment were opened and sampled after 30 days of anaerobic fermentation for further analyses. The experimental flow chart is shown in Fig. 2.

Chemical, microbial and fermentative parameter analyses

The chemical composition and microbial population were analyzed according to our previous study [15]. For fermentation parameter analysis, 30 g fresh or ensiled material was extracted with 90 mL deionized water at 4 °C for 24 h. After filtering with 4 layers of medical cheesecloth and Whatman filter paper, the pH of fresh or ensiled material was recorded by a HI 2221 pH/mV/°C bench meter (Hanna Instruments Inc., Rhode Island, United States) immediately. The ammonia-N (NH_3-N) concentration of ensiled material was determined by colorimetry after a reaction with phenol and hypochloric acid [16]. The concentrations of ethanol and SCFA including lactic acid, acetic acid, propionic acid, n-butyric acid, and iso-butyric acid of ensiled material were determined by a 1260 Infinity II HPLC system (Agilent Technologies Inc.,

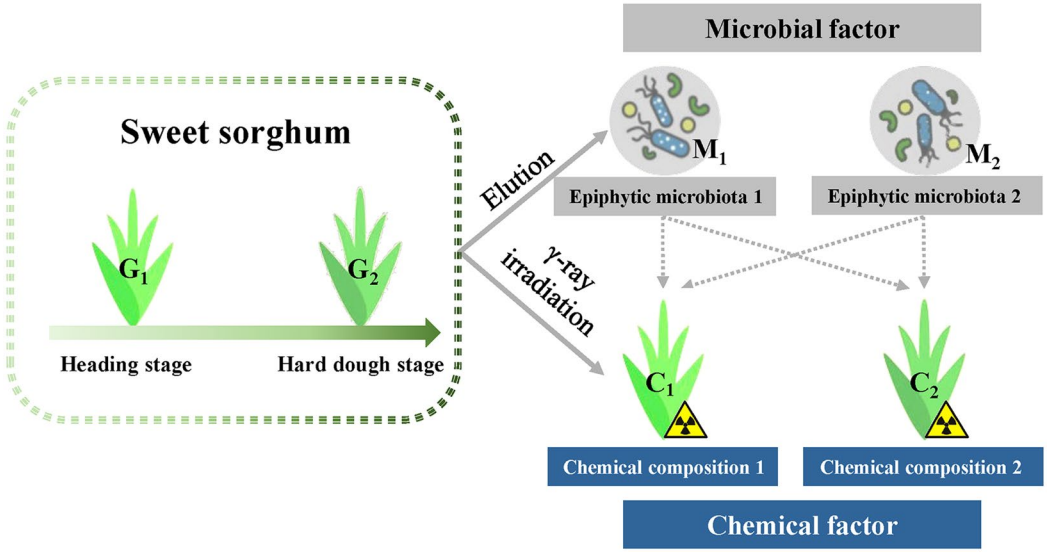


Fig. 1 Experimental design diagram of this study. G_1: fresh sweet sorghum harvested at the heading stage; G_2: fresh sweet sorghum harvested at the hard dough stage; M_1: phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage; M_2: phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage; C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage

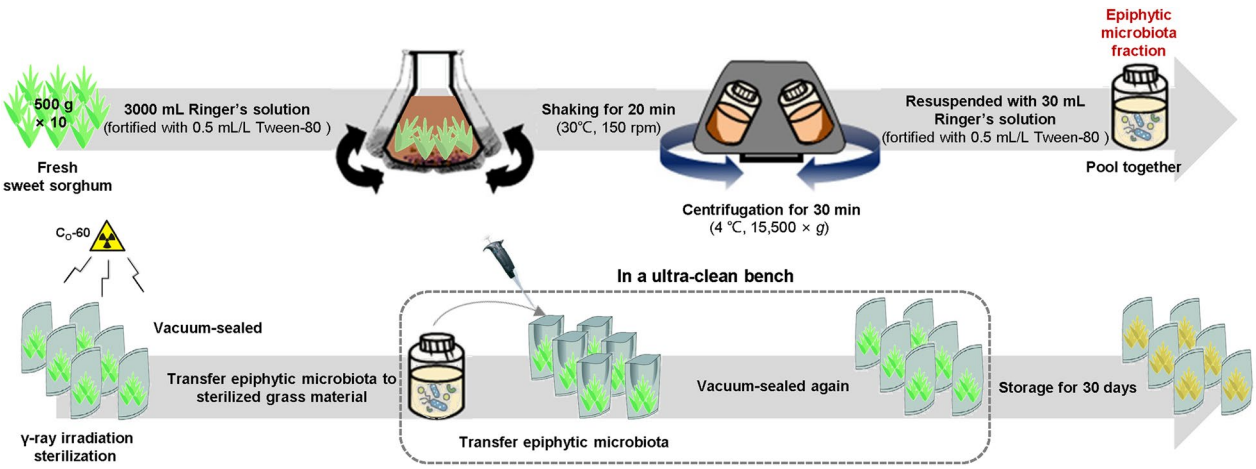


Fig. 2 Experimental flow chart of this study

California, USA). The mobile phase was 2.5 mM H_2SO_4, the flow rate of the mobile phase was 0.5 mL/min and the column temperature was 55 °C. The concentration of butyric acid was the sum concentration of n-butyric acid and iso-butyric acid.

The NGS analyses

Preparatory work before sequencing (bacterial DNA extraction and PCR amplification) was performed following the procedures reported by Zhao et al. [17]. Purified amplicons (equimolar concentrations) were paired-end sequenced on a MiSeq PE300 platform (Illumina Inc., California, USA) provided by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The generated two sequencing files per replicate were first merged using FLASH (v1.2.11). Then, the obtained raw sequence data were quality filtered by QIIME (v1.9.1) to retain sequences with quality scores > 20. Operational taxonomic units (OTUs) were clustered with 97% identities using UPARSE (v7.0.0) and chimeric sequences were identified and removed using UCHIME (v4.1). After matching with the 132 SILVA database, the taxonomy of OTUs was assigned using RDP classifier (v2.11). Bacterial α-diversities (Shannon, Chao and Coverage indices)

and Bray–Curtis metric β-diversity were calculated using QIIME (v1.9.1). Bray–Curtis distance metric nonmetric multidimensional scaling analysis (NMDS) plot was constructed using vegan R package (v4.1.2). Multispecies difference test histograms at the top 10 genera between treatments were constructed using STAMP (v2.1.3) to figure out the bacterial abundance differences.

Statistical analysis

The GLM of SAS (v9.2; SAS Institute Inc., North Carolina, USA) was applied to investigate the effects of irradiation sterilization, developmental stage and their interactions on the physio-biochemical characteristics, as well as the effects of phyllosphere microbiota, chemical constituent and their interactions on the fermentation parameters and bacterial α-diversities in a 2 × 2 factorial design.

The statistical model for Table 1 was as follows:

$$Y_{ij} = \mu + \gamma_i + D_j + (\gamma \times D)_{ij} + e_{ijk}$$

where Y_{ij} is the dependent variable; μ is the overall mean; γ_i is the effect of irradiation sterilization ($i=2$, with vs. without); D_j is the effect of developmental stage ($j=2$, G_1 vs. G_2); $(\gamma \times D)_{ij}$ are the interaction effects of irradiation sterilization and developmental stage; and e_{ijk} is the residual error.

The model for Tables 2 and 3 was as follows:

$$Y_{ij} = \mu + C_i + M_j + (C \times M)_{ij} + e_{ijk}$$

where Y_{ij} is the dependent variable; μ is the overall mean; C_i is the effect of chemical constituent ($i=2$, C_1 vs. C_2); M_j is the effect of phyllosphere microbiota ($j=2$, M_1 vs. M_2); $(C \times M)_{ij}$ are the interaction effects of chemical constituent and phyllosphere microbiota; and e_{ijk} is the residual error. The difference compared between treatments was performed using student *t*-test when the effects of phyllosphere microbiota or chemical constituent were significant. Means were statistically different at $P<0.05$.

Results

Phyllosphere characteristics of fresh sweet sorghum

Different from that the developmental stage had significant ($P<0.001$) effects on both phyllosphere chemical and microbial parameters, irradiation sterilization had no effects ($P>0.05$) on the chemical constituent of sweet sorghum (Table 1 and Additional file 1). As sweet sorghum grew, pH value, DM, WSC, neutral detergent fiber (NDF) and acid detergent fiber (ADF) content as well as lactic acid bacteria (LAB), aerobic bacteria, yeast, moulds and enterobacteria number significantly ($P<0.001$) increased, whereas CP content and buffering capacity significantly ($P<0.001$) decreased. There were no colonies found in the irradiated sweet sorghum through the culture-based method.

Chemical and fermentation parameters

As shown in Table 2, changes in the chemical constituent significantly ($P<0.05$) impacted the value of pH, the content of DM and WSC, the concentrations of lactic

Table 1 The phyllosphere characteristics of fresh sweet sorghum

Developmental stage	Heading		Hard dough		*P*-value		
Item and treatment	G_1	C_1	G_2	C_2	γ	*D*	γ × G
pH	5.64^A	5.60^A	5.23^B	5.21^B	0.403	< 0.001	0.822
Dry matter (g/kg FW)	192^B	189^B	319^A	315^A	0.629	< 0.001	0.914
Water soluble carbohydrates (g/kg DM)	156^B	159^B	284^A	288^A	0.463	< 0.001	0.917
Buffering capacity (mEq/kg DM)	73.4^A	71.2^A	50.9^B	49.1^B	0.575	< 0.001	0.964
Neutral detergent fiber (g/kg DM)	492^B	486^B	553^A	548^A	0.290	< 0.001	0.974
Acid detergent fiber (g/kg DM)	255^B	251^B	295^A	288^A	0.336	< 0.001	0.821
Crude protein (g/kg DM)	76.8^A	75.3^A	51.3^B	50.8^B	0.781	< 0.001	0.888
Lactic acid bacteria (Log_{10} cfu/g FW)	6.07^B	ND^C	8.59^A	ND^C	< 0.001	< 0.001	< 0.001
Aerobic bacteria (Log_{10} cfu/g FW)	7.64^B	ND^C	8.57^A	ND^C	< 0.001	< 0.001	< 0.001
Yeasts (Log_{10} cfu/g FW)	6.32^B	ND^C	7.86^A	ND^C	< 0.001	< 0.001	< 0.001
Moulds (Log_{10} cfu/g FW)	5.09^B	ND^C	6.00^A	ND^C	< 0.001	< 0.001	< 0.001
Enterobacteria (Log_{10} cfu/g FW)	6.46^B	ND^C	8.67^A	ND^C	< 0.001	< 0.001	< 0.001

$^{A-C}$Means with different uppercase in the same row differ at $P<0.05$

FW: fresh weight; DM: dry matter; cfu: colony-forming units. G_1: fresh sweet sorghum harvested at the heading stage; G_2: fresh sweet sorghum harvested at the hard dough stage; C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage. γ: the effect of irradiation, with vs. without; *D*: the effect of developmental stage, heading stage vs. hard dough stage; γ × *D*: the interaction between irradiation and developmental stage

Table 2 Effects of phyllosphere microbiota and chemical constituent on anaerobic fermentation quality and SCFA production

Item and treatment	C_1		C_2		SEM	*P*-value		
	M_1	M_2	M_1	M_2		C	M	C × M
pH	3.89^{A}	3.80^{A}	3.51^{B}	3.60^{B}	0.048	< 0.001	0.997	0.020
Dry matter (g/kg FW)	141^{B}	141^{B}	281^{A}	288^{A}	21.75	< 0.001	0.523	0.472
Lactic acid (g/kg DM)	52.3^{C}	64.4^{BC}	96.8^{A}	85.0^{A}	5.595	< 0.001	0.330	0.006
Acetic acid (g/kg DM)	20.6^{B}	31.6^{A}	12.0^{C}	22.1^{B}	2.453	< 0.001	< 0.001	0.656
Propionic acid (g/kg DM)	0.04^{C}	0.04^{C}	0.43^{B}	1.05^{A}	0.174	0.040	0.306	0.312
Butyric acid (g/kg DM)	ND	ND	ND	ND	–	–	–	–
SCFA (g/kg DM)	72.9^{C}	96.0^{B}	109^{A}	108^{A}	6.022	< 0.001	0.062	0.362
Ratio of lactic to acetic acid	2.57^{C}	2.04^{C}	8.31^{A}	3.87^{B}	0.402	0.001	0.004	0.013
Water soluble carbohydrates (g/kg DM)	69.1^{C}	69.8^{C}	210^{A}	178^{B}	19.18	< 0.001	0.055	0.007
NH_3-N (g/kg TN)	77.2^{B}	75.2^{B}	90.9^{A}	93.1^{A}	2.325	0.011	0.158	0.403
Lactic acid bacteria (Log_{10} cfu/g FW)	7.76	8.11	7.44	7.37	0.125	0.032	0.514	0.328
Aerobic bacteria (Log_{10} cfu/g FW)	< 2.00	< 2.00	< 2.00	< 2.00	0.403	–	–	–
Yeasts (Log_{10} cfu/g FW)	3.41^{A}	3.17^{A}	$< 2.00^{B}$	$< 2.00^{B}$	0.467	0.017	0.956	0.795
Moulds (Log_{10} cfu/g FW)	ND	< 2.00	ND	ND	0.300	–	–	–
Enterobacteria (Log_{10} cfu/g FW)	< 2.00	ND	ND	ND	0.217	–	–	–

$^{A-C}$Means with different uppercase in the same row differ at $P < 0.05$

FW: fresh weight; SCFA: short-chain fatty acid; NH_3-N: ammonia-N; TN: total nitrogen; cfu, colony-forming units. C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage; M_1: phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage; M_2: phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage. C: the effect of chemical constituent, C_1 vs. C_2; M: the effect of phyllosphere microbiota, M_1 vs. M_2; C × M: the interaction between chemical constituent and phyllosphere microbiota

Table 3 Effect of phyllosphere microbiota and chemical constituent on diversity and richness indices of the bacterial community

Item and treatment	M_1	M_2	C_1		C_2		*P*-value		
			M_1	M_2	M_1	M_2	C	M	C × M
Sequence number	56,553	47,956	66,141	70,021	69,325	69,717	0.353	0.182	0.267
OTUs	234	317	136^{A}	112^{AB}	93.7^{B}	101^{AB}	0.011	0.333	0.081
Shannon	3.48	2.89	1.27^{A}	1.31^{A}	0.87^{B}	1.11^{AB}	0.004	0.097	0.202
Chao1	277	398	195^{A}	179^{AB}	126^{B}	145^{AB}	0.004	0.887	0.218
Coverage	0.9987	0.9979	0.9991	0.9987	0.9986	0.9988	–	–	–

$^{A-C}$Means with different uppercase in the same row differ at $P < 0.05$

OTUs: operational taxonomic units. M_1: phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage; M_2: phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage; C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage. C: the effect of chemical constituent, C_1 vs. C_2; M: the effect of phyllosphere microbiota, M_1 vs. M_2; C × M: the interaction between chemical constituent and phyllosphere microbiota

acid, acetic acid, propionic acid and SCFA, the lactic acid–acetic acid ratio and the number of LAB and yeast, while changes of the phyllosphere microbiota significantly ($P < 0.05$) impacted the acetic acid concentration and the lactic acid–acetic acid ratio. Concretely, M_1C_2 and M_2C_2 contained higher ($P < 0.05$) levels of DM, WSC, lactic acid, SCFA, lactic acid–acetic acid ratio and NH_3–N, and lower ($P < 0.05$) levels of pH and yeast than M_1C_1 and M_2C_1. Compared with the M_1 transplant, the M_2 transplant increased the concentration of acetic acid and decreased the lactic acid–acetic acid ratio ($P < 0.05$). No or negligible (< 2.0 log_{10} colony-forming units (cfu)/g fresh weight (FW)) aerobic bacteria, yeast and enterobacteria were detected in all treatments.

Bacterial community structure and diversity

After NGS, 1,139,144 raw sequences were formed from 6 fresh materials and 12 ensiled materials (Table 3). Totally 645,156 quality sequences were got by quality filtering and subsequently clustered into 640 OTUs according to the 97% sequence identity threshold. The changes in

chemical constituent significantly ($P<0.05$) influenced OTUs number and Shannon and Chao1 indices; differently, the changes in phyllosphere microbiota did not influence ($P>0.05$) these α-diversity parameters. The indices of Shannon and Chao1 were peak in fresh material (phyllosphere microbiota) and followed by ensiled material. After 30-day anaerobic fermentation, M_1C_2 detected the lowest OTUs number and Shannon and Chao1 indices. The Coverage index of all sequenced samples was above 0.99.

The differences of bacterial β-diversity among treatments are shown in Fig. 3. A clear separation was observed between the sample points of the fresh and ensiled sample. Wherein the sample points of M_1 and M_2 were well separated, the sample points of M_1C_1 and M_2C_1 were slightly separated from those of M_1C_2 and M_2C_2.

Figure 4 shows that Proteobacteria (72.6%), Firmicutes (10.9%) and Actinobacteriota (10.2%) were the phyla with high relative abundance (RA) in M_1, while Proteobacteria (80.9%) and Bacteroidota (8.60%) were the phyla with high RA in M_2. With the growth of sweet sorghum, the RA of Firmicutes and Actinobacteriota decreased from 10.9% and 10.2% to 4.21% and 5.53%, respectively, but the RA of Proteobacteria and Bacteroidota increased from 72.6 and 5.86% to 80.9% and 8.60%, respectively. After 30-day anaerobic fermentation, Firmicutes (> 95%) were the overwhelmingly dominant phylum in all the microbial communities.

There were 21 and 14 genera with an RA greater than 1% in M_1 and M_2, respectively (Fig. 5). The most abundant genera in M_1 were *Acinetobacter* (20.4%), followed by *Sphingomonas* (10.9%), *Pseudomonas* (9.20%) and *Microbacterium* (8.77%), while *Pantoea* (28.8%) and *Enterobacter* (24.7%) were 2 genera with high abundance in M_2. As anaerobic fermentation proceeded, the original bacterial community of sweet sorghum was rapidly replaced by *Lactobacillus* and *Weissella*. The changes in chemical constituent significantly ($P<0.05$) impacted the RA of *Lactobacillus*, *Weissella*, *Lactococcus* and *Pediococcus* in M_1C_1 and M_1C_2, and *Lactobacillus*, *Weissella*, *Leuconostoc* and *Pantoea* in M_2C_1 and M_2C_2 (Fig. 6A and B). While the changes in phyllosphere microbiota only impacted ($P<0.05$) the RA

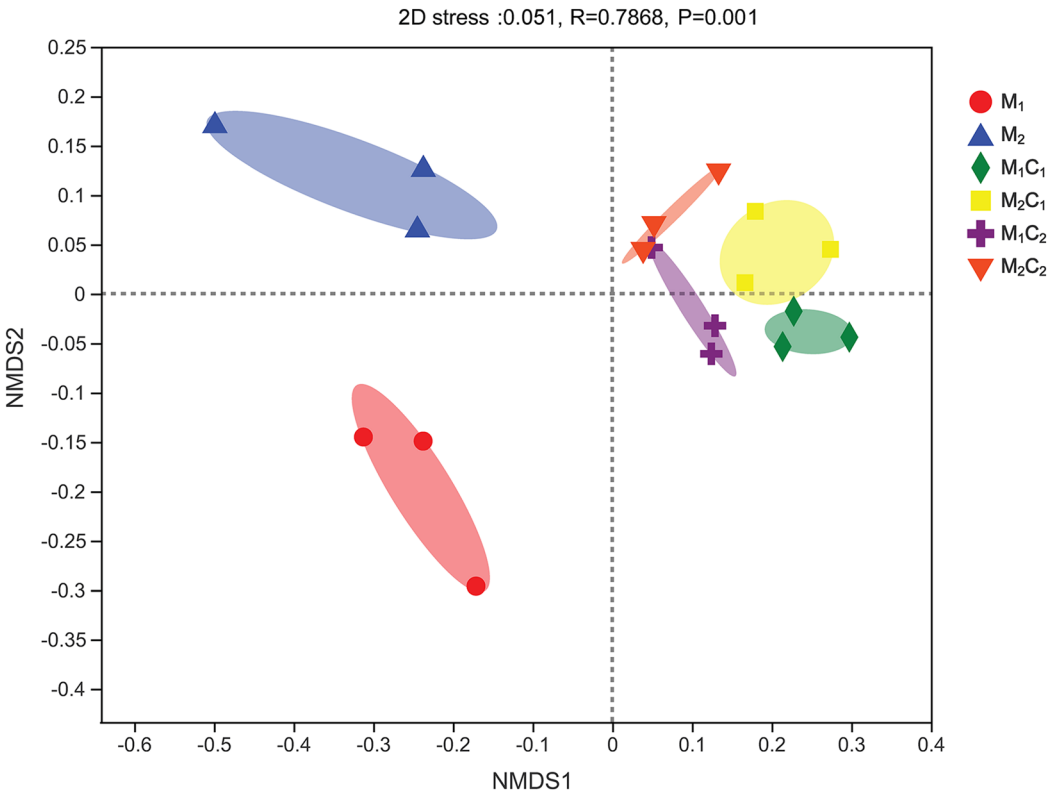


Fig. 3 Nonmetric multidimensional scaling (NMDS) plot based on the Bray–Curtis distance metric of bacterial community at genus level between samples (2D stress = 0.05). M_1: phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage; M_2: phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage; C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage; M_1C_1: transplantation of M_1 to C_1; M_2C_1: transplantation of M_2 to C_1; M_1C_2: transplantation of M_1 to C_2; M_2C_2: transplantation of M_2 to C_2

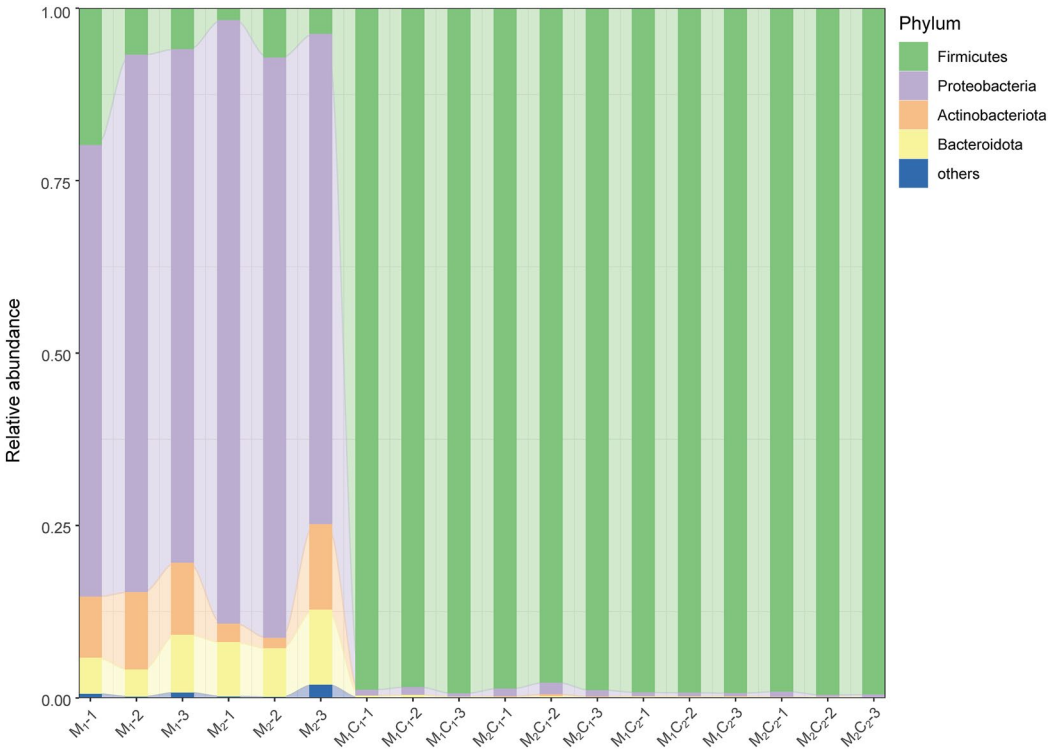


Fig. 4 Effect of phyllosphere microbiota and chemical constituent on the RA (%) of bacterial community at the phylum level in fresh and ensiled sweet sorghum. M_1: phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage; M_2: phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage; C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage; M_1C_1: transplantation of M_1 to C_1; M_2C_1: transplantation of M_2 to C_1; M_1C_2: transplantation of M_1 to C_2; M_2C_2: transplantation of M_2 to C_2

of *Weissella* in M_1C_1 and M_2C_1 as well as in M_1C_2 and M_2C_2 (Fig. 6C and D).

Discussion

Effects of irradiation sterilization and developmental stage on the chemical constituent and phyllosphere microbiota of fresh sweet sorghum

There were no distinct differences in chemical constituent between fresh and irradiated samples, and γ-ray irradiation at the dose of 32 kGy effectively sterilized forage grass because no microorganisms were cultured from the irradiated sweet sorghum, which was similar to previous studies [18, 19]. Comino et al. [20] found that forage maturity greatly influenced its chemical and microbial characteristics. In the current study, DM, WSC, NDF and ADF content increased while CP content decreased as sweet sorghum grew, which was possibly due to the increase of cell wall deposition and the decline of leaf-stem ratio [21]. The decrease in the CP content might in turn explain the decrease in BC as the CP content of materials is positively correlated with BC [22].

Throughout the growth cycle of forage, the external environment (e.g., solar radiation, temperature and rainfall) and internal environment (e.g., plant morphology, moisture content and leaf thickness) are changing, and they are reported to affect microbial colonization [23]. The number of LAB, aerobic bacteria, yeast and enterobacteria as well as the α-diversities of phyllosphere microbiota in this study increased with the maturity of sweet sorghum, which further confirmed the fact that the microbial number and diversity of phyllosphere can be sharply influenced by the developmental stages. This phenomenon can be partly explained by the increased WSC content and aging tissue proportion of sweet sorghum from the heading stage to the hard dough stage. Recent studies showed that sugar and volatile organic compounds secreted by forage play an important role in determining the microbial population of forage [24, 25]. Microbes are known to enrich on sugar-rich plants such as maize. Moreover, as Thompson et al. [26] reported, the nutrients such as sugar released from aging tissue and leaves were found to benefit the growth of phyllosphere microorganisms.

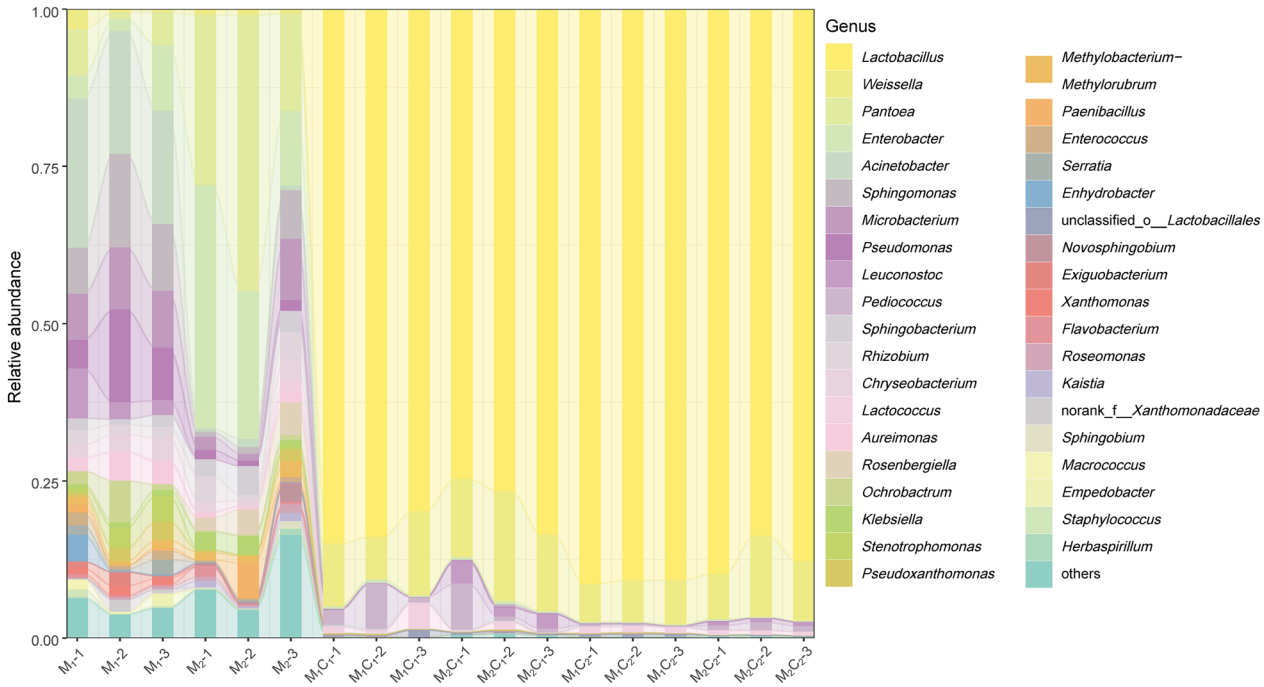


Fig. 5 Effect of phyllosphere microbiota and chemical constituent on the RA (%) of bacterial community at the genus level in fresh and ensiled sweet sorghum. M_1: phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage; M_2: phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage; C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage; M_1C_1: transplantation of M_1 to C_1; M_2C_1: transplantation of M_2 to C_1; M_1C_2: transplantation of M_1 to C_2; M_2C_2: transplantation of M_2 to C_2

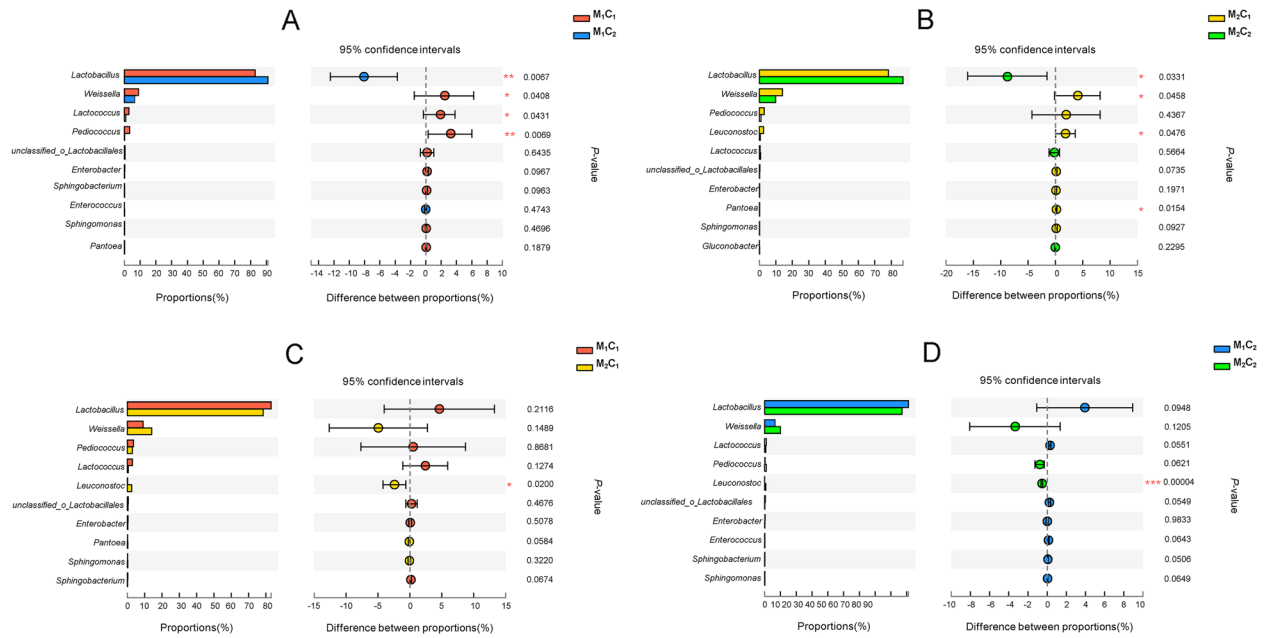


Fig. 6 Differential analyses bar plots at the genus level (top 10 genera) between treatments. * = $0.01 < P \leq 0.05$; ** = $0.001 < P \leq 0.01$. M_1: phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage; M_2: phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage; C_1: irradiated sweet sorghum harvested at the heading stage; C_2: irradiated sweet sorghum harvested at the hard dough stage; M_1C_1: transplantation of M_1 to C_1; M_2C_1: transplantation of M_2 to C_1; M_1C_2: transplantation of M_1 to C_2; M_2C_2: transplantation of M_2 to C_2

Effects of phyllosphere microbiota and chemical constituent on the anaerobic fermentation quality

Regardless of developmental stage, the anaerobic fermentation of sweet sorghum displayed desirable homolactic fermentation, reflected in low pH values (< 4.0) and high lactic acid-acetic acid ratio [27]. The higher DM content of M_1C_2 and M_2C_2 (C_2 anaerobic fermentation) than that of M_1C_1 and M_2C_1 (C_1 anaerobic fermentation) was associated with the higher DM content in C_2 than that in C_1. Likewise, the higher WSC content, lactic acid concentration and SCFA production in M_1C_2 and M_2C_2 than those in M_1C_1 and M_2C_1 could ascribe to the higher WSC content in C_2 than that in C_1. The acetic acid concentration of M_1C_1 and M_2C_1 was higher than that of M_1C_2 and M_2C_2. Meanwhile, a higher acetic acid concentration was also observed in M_2C_1 and M_2C_2 (M_2 transplant). These results suggested that the acetic acid generation of sweet sorghum after anaerobic fermentation in this study was associated with the chemical constituent and phyllosphere microbiota of sweet sorghum at harvest. This is not difficult to understand because the acetic acid in desirable anaerobic fermentation is mainly driven by the fermentation of pentose and heterofermentative LAB. Pentose fermentation converts pentose to the intermediate of D-xylose-5-phosphate and then to lactic acid and acetic acid [22]. Although the data of pentose were not shown in this study, the higher abundance of *Leuconostoc* in M_2C_1 and M_2C_2 (M_2 transplant) still partly explained the higher acetic acid generation because *Leuconostoc* are heterofermentative LAB [28].

Benefiting from homolactic fermentation, the NH_3-N level in all treatments was below the maximum acceptable limit (100 g/kg TN) [22]. The protein degradation and NH_3-N formation during anaerobic fermentation are a complex biochemical process, involving the activities of plant protease, clostridia, enterobacteria, etc. Regardless of phyllosphere microbiota, the NH_3-N concentration of M_1C_2 and M_2C_2 was always higher than that of M_1C_1 and M_2C_1, which was in line with the finding of Jia et al. [29] that the silage produced by more mature whole-crop oat had higher NH_3–N concentration. The higher proteolysis degree at the mature stage reported by Faria et al. [30] probably explained the above higher NH_3–N concentration. As O_2 is depleted, the number of aerobic bacteria and moulds that cannot tolerate anaerobic conditions rapidly decreased to a negligible level [31]. While the undetected or negligible number of enterobacteria in all treatments was associated with the low pH value. Although yeasts are resistant to low pH, SCFA such as lactic acid and acetic acid can pass through the yeast cell membrane in the form of non-dissociation and release H+ to reduce intracellular acidity, finally suppress or kill the yeast [22]. Correspondingly, the higher concentrations of SCFA in M_1C_2 and M_2C_2 were accompanied by the lower number of yeasts.

Different from the acetic acid concentration, which was impacted by both chemical constituent and phyllosphere microbiota changes, other fermentation parameters such as pH, lactic acid, propionic acid, SCFA and NH_3-N were only impacted by the chemical constituent changes.

Effects of phyllosphere microbiota and chemical constituent on the bacterial community structure and diversity

All coverage indices were above 99%, implying that most of the bacterial community had been fully captured. And the number of OTUs in M_2 was higher than that in M_1, suggesting that the bacterial composition in G_2 was more complex and abundant. Anaerobic fermentation process decreased the species richness and diversity of the bacterial community, reflected by the reduced Shannon and Chao1 indices, and this could be associated with the disappearance of acid-intolerant aerobes [32]. Low pH conditions are mainly responsible for the reduced microbial diversity in acidic habitats [33], which could in turn explain the lowest Shannon and Chao1 indices in M_1C_2 with the lowest pH value.

According to Shannon and Chao1 indices, changes in chemical constituent rather than phyllosphere microbiota remarkably affected both the bacterial richness and diversity of sweet sorghum after anaerobic fermentation. This indicated that the difference in bacterial α-diversities in this study mainly resulted from the chemical constituent changes of fresh sweet sorghum at harvest. The Shannon and Chao1 indices of M_1C_2 and M_2C_2 were lower than those of M_1C_1 and M_2C_1, which could be explained by the superiority of C_2. The lower buffering capacity and higher WSC content of C_2 stimulated the proliferation of LAB, accelerated the initial LA production and acidification, thereby further decreasing the α-diversities of the bacterial community.

The clear separation of sample points M_1 and M_2 in the NMDS plot showed great differences in the composition of bacterial community for fresh sweet sorghum at two developmental stages, and this could be associated with climate, the physio-biochemical characteristics of forage grass, or other factors [19]. Meanwhile, the separated clustering between the fresh and ensiled sample was as abovementioned attributed to the disappearance of acid- and anaerobic-intolerant phyllosphere bacteria during anaerobic fermentation. The sample points of M_1C_1 and M_2C_1 or M_1C_2 and M_2C_2 were clumped together, suggesting that the composition of the bacterial community was similar in C_1 or C_2 anaerobic fermentation.

Proteobacteria dominated the phyllosphere microbiota of fresh sweet sorghum (M_1 and M_2), while Firmicutes

were the overwhelmingly dominant phylum after anaerobic fermentation. The succession from *Proteobacteria* to *Firmicutes* under anaerobic environments has been extensively documented [8, 17, 18, 34–36]. The anaerobic fermentation process benefited the growth of Firmicutes because this phylum preferred the anaerobic and acid environments [37]. The apparent succession of bacterial community from Proteobacteria to Firmicutes after anaerobic fermentation could ascribe to the inhibition of aerobic genera (*Acinetobacter*, *Sphingomonas*, *Pseudomonas*, etc.) and the bloom of LAB (*Lactobacillus*, *Weissella*, *Pediococcus*, etc.). *Lactobacillus*, *Weissella* and *Pediococcus* are the 3 most common genera in silages [38, 39]. *Weissella* and *Pediococcus* are generally considered early colonizers during ensiling [34, 40] due to their weaker tolerance to acid compared with *Lactobacillus* [41–43]. The initial acid environment established by *Pediococcus* and *Weissella* is suitable for the growth of *Lactobacillus* [44]. Thus, *Lactobacillus* dominated the bacterial community of sweet sorghum after anaerobic fermentation, followed by *Weissella* and *Pediococcus*.

To clearly elucidate the relative contribution of chemical constituent and phyllosphere microbiota on the bacterial community of sweet sorghum after anaerobic fermentation, the multispecies difference test was used to show the differences of bacterial community composition between treatments. Chemical constituent changes have a significant impact on the RA of *Lactobacillus*, *Weissella*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and *Pantoea*, while phyllosphere microbiota changes only impacted the RA of *Leuconostoc* in sweet sorghum after anaerobic fermentation. Lin et al. [45] analyzed the bacterial population of alfalfa and maize before and after ensiling and found that the epiphytic microbiota could not predict the final fermentation outcome due to the chemical constituent (WSC, BC, etc.) differences of fresh material. A similar finding was also obtained in the current study, that is, the differences in the anaerobic fermentation quality, SCFA production and bacterial community of sweet sorghum harvested at various developmental stages were primarily attributed to different chemical constituents at harvest. This study may provide the first record for further understanding the relative contribution of chemical and microbial factors to the bacterial community and anaerobic fermentation quality of sweet sorghum, which can guide subsequent production practices to regulate anaerobic fermentation and improve silage production. The main limitation of the present study is the failure to trace down the microbial community to the species level using third-generation sequencing, which needs to be addressed in future studies (Additional file 1).

Conclusions

In the current study, the chemical constituent changes had remarkable effects on the concentration of lactic acid, acetic acid, propionic acid and NH_3-N as well as the RA of *Lactobacillus*, *Weissella*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and *Pantoea*. While the phyllosphere microbiota changes only affected the concentration of acetic acid and the RA of *Leuconostoc*. Thus, the differences in the bacterial community and anaerobic fermentation quality of sweet sorghum at the two developmental stages were primarily driven by the chemical factors at harvest. The selection and breeding of new varieties of sweet sorghum for anaerobic fermentation may pay more attention to the improvement of chemical and nutritional parameters. Certainly, future work is required to further verify the findings of this study.

Abbreviations

ADF	Acid detergent fiber
C	The effect of chemical constituent, C_1 vs. C_2
C_1	Irradiated sweet sorghum harvested at the heading stage
C_2	Irradiated sweet sorghum harvested at the hard dough stage
C × M	The interaction between chemical constituent and phyllosphere microbiota
cfu	Colony-forming units
CP	Crude protein
DM	Dry matter
FW	Fresh weight
G_1	Fresh sweet sorghum harvested at the heading stage
G_2	Fresh sweet sorghum harvested at the hard dough stage
LAB	Lactic acid bacteria
M	The effect of phyllosphere microbiota, M_1 vs. M_2
M_1	Phyllosphere microbiota eluted from sweet sorghum harvested at the heading stage
M_2	Phyllosphere microbiota eluted from sweet sorghum harvested at the hard dough stage
NDF	Neutral detergent fiber
NGS	Next-generation sequencing
NH_3-N	Ammonia nitrogen
NMDS	Nonmetric multidimensional scaling analysis
OTUs	Operational taxonomic units
RA	Relative abundance
RDP	Ribosomal database project
SCFA	Short-chain fatty acid
TN	Total nitrogen
WSC	Water-soluble carbohydrates

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-023-00392-w.

Additional file 1: Table S1. The chemical, microbial and fermentative parameters of sweet sorghum (untreated) after 30 days of anaerobic fermentation. **Table S2.** The chemical, microbial and fermentative parameters of sweet sorghum (irradiated) after 30 days of anaerobic fermentation.

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Author contributions
JZ: methodology, formal analysis, visualization, writing-original draft preparation. ZJ and XY: validation, methodology, software. JL: investigation, formal analysis. SW and ZD: methodology, visualization. TS: conceptualization, project administration, resources, data curation, supervision, funding acquisition, writing—review and editing. All authors read and approved the final manuscript.

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Declarations

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Not applicable.

Consent for publication
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Competing interests
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