Effects of ambient temperature and available sugar on bacterial community of *Pennisetum sinese* leaf: An *in vitro* study

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Abstract

The present *in vitro* study investigated the effects of temperature and available sugar on the bacterial community of *Pennisetum sinese* leaf during fermentation. *P. sinese* leaves were cultured in MRS broth containing 0.4 and 1.6 g sugar and incubated at 25°C and 45°C for 9, 18, and 36 h. The results showed that the dominant phyla during sugar fermentation were Firmicutes, followed by Proteobacteria and Bacteroidetes. Compared to a low incubation temperature (25°C), a high incubation temperature (45°C) decreased the relative abundances of *Exiguobacterium* and *Acinetobacter* and increased those of *Bacillus* and *Paenibacillus*. Leaf samples incubated at 25°C showed higher bacterial alpha diversity indices than those incubated at 45°C. Principal coordinate analysis revealed that the bacterial community structure was altered by the high incubation temperature. Sugar concentration of 1.6g/50ml increased the relative abundances of *Bacillus* and *Klebsiella* but decreased those of *Paenibacillus* and *Serratia* as compared to sugar concentration of 0.4g/50ml. pH was the primary factor that influenced the succession of bacterial communities during sugar fermentation in *P. sinese* leaves. In conclusion, ambient temperatures（25℃ and 45℃）and high sugar concentration restructured the bacterial communities on *P. sinese* leaves by facilitating the dominance of *Bacillus* and *Paenibacillus*. This study provided insight into the mechanisms by which bacterial communities on *P. sinese* leaves are enriched.

Introduction

The degree of silage fermentation highly depends on the epiphytic microflora of the ensiled forage material (Eikmeyer et al., 2013; Muck et al., 2013). The natural microflora of forage crops participates in silage fermentation. Ohshima et al. (1997) found that adding the fermented juice of epiphytic LAB (FJLB) to silage effectively improved its fermentative quality. This definition implies that silage quality may depend on the types of microorganisms present on the leaf surface. Therefore, it is crucial to study the microorganisms that adhere to the leaf surface to enhance silage quality. In the fermentation process, maintaining the appropriate temperature is essential to facilitate efficient bacterial growth and metabolite production. Seale et al. (1986) showed that soluble sugar is a limiting factor for obtaining high-quality fermentation products; this implies that soluble sugar is another key factor that influences fermentation quality. Understanding the epiphytic and inherent microorganisms of forage leaves during *in vitro* fermentation is an interesting and critical issue for producing high-quality silage. Therefore, the present study investigated the bacterial communities present on the surface of *Pennisetum sinese* (king grass) leaves under different incubation temperatures and soluble sugar concentrations. We hypothesized that sugar concentration and temperature might affect the abundance of bacterial communities on the leaf surface of *P. sinese*.

Materials and methods

The experimental material *P. sinese* was cut from Guanling County, Anshun City, Guizhou Province (25.94° N, 105.61° E) on July 28, 2021. A bacterial culture medium was used for culturing the bacterial species from *P. sinese* leaves, and the temperature gradient and incubation temperature were controlled by a constant temperature incubator. The harvested *P. sinese* leaves were brought to the laboratory under aseptic conditions at a low temperature. The

samples were prepared on an ultra-clean bench and the relevant materials and instruments required for the experiments were sterilized. The collected *P. sinese* leaves were cut into 135 square pieces of size 1 × 1 cm. The bacterial culture medium was prepared as follows: nutrient broth CM 124 powder (18.0 g) was added to 1 L distilled water, and the mixture was heated and boiled until the powder completely dissolved. The prepared broth was then distributed in separate flasks and autoclaved at 121°C for 15 min. Five square leaflets were taken and cultured in the prepared liquid medium with the following soluble sugar concentration: S1: 0.4 g/50 mL and S2: 1.6 g/50 mL. Three biological replicates were used for each concentration gradient. The cultures were subjected to shaking conditions at 25°C and 45°C for 9, 18, and 36 h. Sample aliquots were taken from the culture medium at the three time points to determine pH, concentrations of ammonia-N and soluble sugar, abundance of microbial communities, and other indicators. Three replicates were used for each treatment.

Results and discussion

Alpha diversity of bacterial community

Table 1 shows the alpha diversity of bacterial communities detected on *P. sinese* leaves. At the same soluble sugar concentration, the Chao1 index, abundance-based coverage estimator (ACE) index, Shannon index, and Simpson index of the samples incubated at 25°C were higher than those of the samples incubated at 45°C. This implies that the bacterial community richness and diversity were generally higher at 25°C than at 45°C. This finding can be attributed to bacterial growth inhibition at high temperatures. Previous studies have also reported that high temperatures inhibit bacterial growth. Weinberg et al. (2001) demonstrated that moderate temperatures of 20°C to 30°C are generally preferred for silage fermentation. In the present study, at the same temperature, the alpha diversity of the bacterial community increased with the increasing sugar concentration (Chao1, ACE, Shannon, and Simpson indices were higher at 1.6-g sugar concentration than at 0.4-g sugar concentration). In conclusion, temperature and sugar content altered the alpha diversity of bacterial communities on *P. sinese* leaves.

Table 1 Alpha diversity of bacterial communities around leaves

Groups	OTU number	Coverage	Chao1 index	Ace index	Shannon index	Simpson index
T1S1D9	951	0.999	412.68	383.35	3.24	0.78
T1S1D18	938	0.999	398.02	400.48	2.47	0.69
T1S1D36	640	0.999	282.37	373.98	2.42	0.68
T1S2D9	737	0.998	304.49	311.17	2.51	0.72
T1S2D18	708	0.998	320.44	323.19	2.47	0.69
T1S2D36	671	0.999	330.03	360.57	2.72	0.77
T2S1D9	717	0.999	299.52	298.52	0.91	0.24
T2S1D18	785	0.998	359.05	345.59	1.71	0.54
T2S1D36	809	0.999	363.5	366.13	1.96	0.55
T2S2D9	724	0.998	320.32	323.91	1.27	0.41
T2S2D18	637	0.999	294.18	298.72	1.64	0.54
T2S2D36	816	0.998	369.75	367.77	1.87	0.5

Incubation temperature T1：25℃；T2 ： 45 ℃ respectively; Soluble sugar concentration S1：0.4g: S2 :1.6g; Incubation time D9:9h; D18:18h: D36 :36h

Bacterial community composition

Figure 1a shows the relative abundances of bacterial communities in *P. sinese* silage at the phylum level. In this study, Firmicutes and Proteobacteria were the dominant phyla in the fermentation broth of *P. sinese*. At the culture temperature T1 (25°C) and soluble sugar content S1 (0.4 g) and S2 (1.6 g), each incubation time period (9h, 18h, 36h) the main bacterial phyla in the fermentation broth were Firmicutes and Proteobacteria. At culture temperature T2 (45°C) and soluble sugar content S1 (0.4 g) and S2 (1.6 g), the dominant phylum in the fermentation broth was Firmicutes. The high temperature increased the relative abundance of Firmicutes and reduced the proportion of Proteobacteria in the fermentation broth.

As shown in Figure 1b, at the incubation temperature T1 (25°C) and soluble sugar content S1 (0.4 g), the dominant bacteria at the genus level were *Serratia*, *Bacillus*, *Acinetobacter*, and *Exiguobacterium*. At the soluble sugar content S2 (1.6 g), the dominant bacterial genus was *Exiguobacterium* with the relative abundance of 41%. Notably, after the temperature was

controlled, the abundance of LAB increased when the sugar concentration was increased.

At the soluble sugar content of 0.4 g, the relative abundances of *Bacillus* and *Paenibacillus* in the fermentation broth increased by 38.77% and 35.50%, respectively, with the increase of culture temperature. At the soluble sugar content of 1.6 g, the relative abundances of *Bacillus* and *Paenibacillus* in the fermentation broth increased with the increase of culture temperature (63.16% and 41.96%, respectively).

The present study also found that increasing the culture temperature and sugar concentration in the green juice fermentation broth did not increase the relative abundance of LAB and did not promote the growth of LAB, but significantly increased the relative abundance of *Bacillus* and *Paenibacillus*.

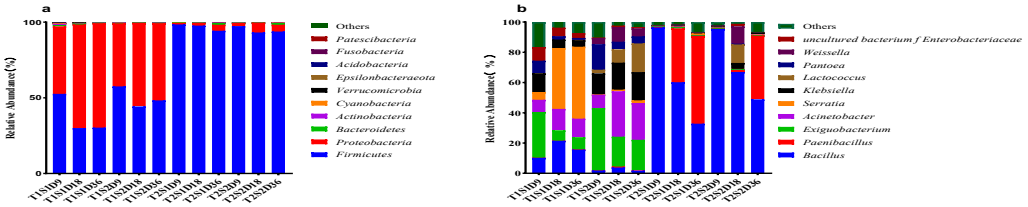

Fig. 1 Relative abundances of species *Pennisetum sinese* at the phylum level (a) and at the genus level (b) after fermentation of at different temperatures and sugar concentrations. T1, 25°C; T2, 45°C; S1, 0.4g; S2, 1.6g; D9, 9h; D18, 18h; D36, 36h.

Bacterial community structure

The PCoA map shows the bacterial community structure of the fermentation broth samples incubated at 25°C and 45°C (Fig. 2a). The x-axis and y-axis explained 72.39% and 9.40% of the variance in the bacterial community structure, respectively. Both temperature and sugar concentration promoted changes in bacterial communities, with temperature contributing to the effect of sugar concentration on bacterial growth. According to the functional prediction (Fig. 2b), the main functions of bacterial communities in the fermentation broths treated with temperatures T1 and T2 were metabolism, followed by environmental information processing and genetic information processing. This result showed that different temperatures did not influence the metabolic functions of bacteria, thus indicating that bacteria were metabolically active at both temperatures.

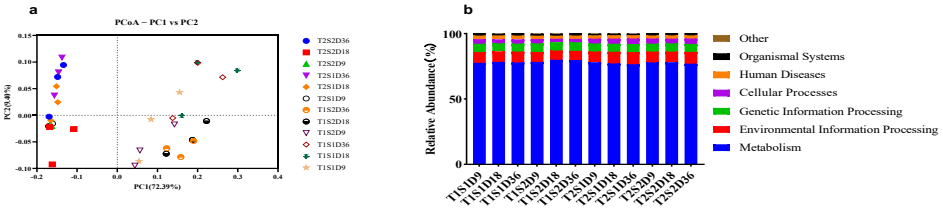

Fig. 2 PCoA analysis (a) and Functional gene prediction (b).

Key environmental factors and functional prediction of bacterial community

As shown in Fig. 3a, *Bacillus, Paenibacillus, Exiguobacterium, Pantoea*, and *Klebsiella* were positively correlated with pH. Among these species, *Pantobacillus* showed the strongest correlation value of 0.389. *Serratia*, *Lactococcus*, *Acinetobacter*, *Weissella*, and *Unculture* were negatively correlated with pH, and *Serratia* showed the weakest correlation value of -0.424. The ammonia-N content was positively correlated with *Bacillus*, *Lactococcus*, *Serratia*, *Weissella*, and *Unculture*. *Weissella* showed the strongest correlation value of 0.525, while *Exiguobacterium*, *Paenibacillus*, *Klebsiella*, *Pantoea*, and *Acinetobacter* showed a negative correlation, and the correlation was the weakest (-0.263). The soluble sugar concentration showed a positive correlation with *Paenibacillus, Bacillus, Exiguobacterium, Klebsiella*, and *Pantoea*, and *Pantoea* showed the strongest correlation value of 0.389. The relative abundances of *Weissella* and *Exiguobacterium* were positively and negatively correlated with ammonia-N in the fermentation broth, respectively. As shown in Fig. 3b, environmental information processing, cellular processes, human diseases, and organic systems showed a positive correlation with pH, and among these, cell transformation showed the strongest correlation value of 0.288. pH was negatively correlated with metabolism and genetic information

processing, and the correlation with metabolism was the weakest at -0.251. Ammonia -N showed a positive correlation with environmental information processing, cellular processes, and human diseases, and the correlation of ammonia-N with human diseases was the strongest (0.050). The main function of the bacterial community in the fermentation broth was metabolism, with a relative abundance of 79.98%, followed by environmental information processing and genetic information processing, with a relative abundance of 9.3% and 6.75%, respectively. Metabolism was negatively correlated with WSC, indicating that the higher the bacterial activity, the lower the WSC content.

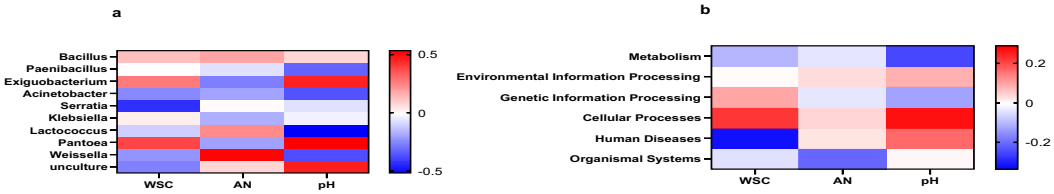

Fig. 3 Spearman correlation between WSC, pH and AN in the fermentation broth and the relative abundance of the top 10 bacterial genera (b) and functional predictions of bacteria (b).

Silage pH is an important indicator of fermentation quality, and silage with a pH of 4.2 or lower is considered to be well fermented. In the present study, pH gradually decreased with the increase of incubation time and temperature (Fig. 3c). In general, the pH of the fermentation broth at 45°C was lower than that at 25°C, while the pH at the soluble sugar concentration of 1.6 g was lower than that at 0.4 g, probably due to the promotion of LA production by high sugar concentration, which lowered the pH of the broth. The addition of soluble sugars promotes the reproduction and growth of LAB, resulting in an increased LA content, which decreases pH (Li et al., 2014; Ni et al., 2017; Wang et al., 2019).

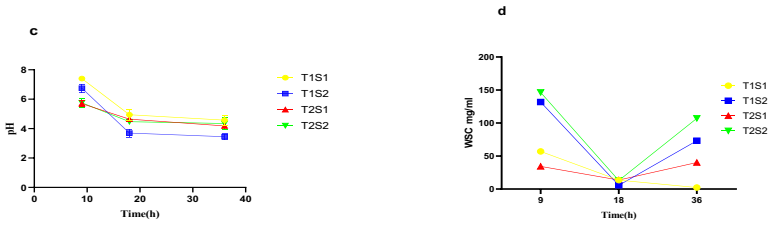

Fig. 3 Trends in pH (c) and WSC content (d) of the fermentation broth with increasing incubation time.

As the main effective nutrient for LAB growth, WSC concentration showed an increasing trend with both incubation temperature and sugar concentration; it first decreased and then increased with the increase of incubation time (Fig. 3d). Madigan et al. (2017) reported that Proteobacteria are gram-negative bacteria that include pathogenic bacterial species such as *E. coli* and *Salmonella*, which compete with LAB to utilize WSC and thus decrease CP content and increase ammonia-N content. In conclusion, both temperature and sugar content affect pH and WSC. As temperature and sugar content increase, pH decreases and WSC first decreases and then increases, which affects the composition and succession of microorganisms.

4. Conclusion

Ambient temperature and available sugar concentration affected the bacterial composition of *P. sinese* leaves. The best fermentation quality was achieved by adding 1.6 g of soluble sugar at 25°C, which increased the relative abundance of LAB and *Weissella*. However, the proportion of *Bacillus* and *Paenibacillus* in the fermentation broth was also found to be relatively large. The use of PFJ may be unsuitable, because it contains a low percentage of LAB associated with silage and a high percentage of other pathogenic anaerobic bacteria. These discoveries suggest that green juice fermentation broth is not suitable as a silage additive.

Reference

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