Assessing the quality of *Astragalus* fermented using *Lactobacillus plantarum* and *Enterococcus faecium* by its physicochemical properties and monitoring the bacterial composition with single molecule, real-time sequencing technology

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Background. *Astragalus* was a well-known traditional herbal medicine, widely used in human s , livestock and poultry in China and E ast Asia. Fermentation could improve health-promoting biological substance by probiotics. **Methods.** We investigated *Astragalus* that was fermented using probiotics including *Enterococcus faecium* , *Lactobacillus plantarum* and *E nterococcus faecium* + *L actobacillus plantarum* and applied the PacBio single molecule, real-time sequencing technology (SMRT) to evaluat e the quality of *Astragalus* fermentation production. **Results.** We found the production rates of acetic acid, methylacetic acid , ethylacetic acid and lactic acid using *E. faecium* + *L. plantarum* fermentation were 1866.24 mg/kg on day 15 , 203.80 mg/kg on day 30 , 996.04 mg/kg on day 15 and 3081.99 mg/kg on day 20 , respectively. Other production rates were: polysaccharide s, 9.43%, 8.51% and 7 . 59% on day 10; saponins , 19.6912 mg/g, 21.6630 mg/g and 20.2084 mg/g on day 15; and flavonoid s, 1.9032 mg/g, 2.0835 mg/g and 1.7086 mg/g on day 20 using *E . faecium* , *L . plantarum* and *E. faecium* + *L. plantarum* , respectively. According to SMRT analysis of the microbial composition s of nine *Astragalus* samples, we found after fermentation on day 3 , *E. faecium* and *L. plantarum* became the most prevalent species. Moreover, *E. faecium* + *L. plantarum* gave more positive effects than single strains in the *Astragalus* solid state fermentation process. **Inclusion.** Our data have demonstrate d that the SMRT sequencing platform is applicable to assessing the quality of *Astragalus* fermentation.

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Short Title: Assess quality of Astragalus fermented

Abstract

Background. *Astragalus* was a well-known traditional herbal medicine, widely used in humans, livestock and poultry in China and East Asia. Fermentation could improve health-promoting biological substance by probiotics. **Methods.** We investigated *Astragalus* that was fermented using probiotics including *Enterococcus faecium*, *Lactobacillus plantarum* and *Enterococcus faecium* + *Lactobacillus plantarum* and applied the PacBio single molecule, real-time sequencing technology (SMRT) to evaluate the quality of *Astragalus* fermentation production. **Results.** We found the production rates of acetic acid, methylacetic acid, ethylacetic acid and lactic acid using *E. faecium* + *L. plantarum* fermentation were 1866.24 mg/kg on day 15, 203.80 mg/kg on day 30, 996.04 mg/kg on day 15 and 3081.99 mg/kg on day 20, respectively. Other production rates were: polysaccharides, 9.43%, 8.51% and 7.59% on day 10; saponins, 19.6912 mg/g, 21.6630 mg/g and 20.2084 mg/g on day 15; and flavonoids, 1.9032 mg/g, 2.0835 mg/g and 1.7086 mg/g on day 20 using *E. faecium*, *L. plantarum* and *E. faecium* + *L. plantarum*, respectively. According to SMRT analysis of the microbial compositions of nine *Astragalus* samples, we found after fermentation on day 3, *E. faecium* and *L. plantarum* became the most prevalent species. Moreover, *E. faecium* + *L. plantarum* gave more positive effects than single strains in the *Astragalus* solid state fermentation process. **Inclusion.** Our data have demonstrated that the SMRT sequencing platform is applicable to assessing the quality of *Astragalus* fermentation.

Keywords: *Astragalus*; fermentation; *Lactobacillus plantarum*; *Enterococcus faecium*; single-molecule, real-time technology

INTRODUCTION

Astragalus, a well-known traditional herbal medicine, has long been widely used in humans, livestock and poultry in China and East Asia. It contains polysaccharides, saponins, flavonoids, anthraquinones, alkaloids, amino acids, β-sitosterol and metallic elements(Ibrahim et al. 2013; Li et al. 2014; Li et al. 2009). Moreover, *Astragalus* has anti-inflammatory (Kim et al. 2014), immunostimulant (Qin et al. 2012), antioxidative (Kim et al. 2005) and antiviral activities (Sanpha et al. 2013). Fermentation is often used in the various fruits. However, accumulating evidence has shown that some herbs can also be fermented, for example *Flos Lonicera* and *Rhizoma Atractylodis Macrocephalae* (Wang et al. 2015; Wang et al. 2014) .

Solid state fermentation (SSF) is unique process with cultivation of microorganisms on a moist solid base. It is considered to be superior to submerged fermentation technology (HÖlker et al. 2014). SSF possesses several advantages, including the cultivation of microorganisms specialized for water-insoluble substrates, higher product concentrations, higher fermentation productivity, higher product stability, lower catabolic repression and lower demands on sterility (HÖlker et al. 2014). Furthermore, this process can also increase the activities of biological substrates by modifying naturally occurring molecules such as isoflavones, polysaccharides and saponins.

In the literature, assessment of the quality of fermented *Astragalus* was typically based on determining the changes in microbial compositions and various physiological parameters including pH and water content (Chen et al. 2014). However, little is known about the content of organic acids, the yields of active substances and the microbiota composition after lactic acid bacteria (LAB) fermentation. Although culture-dependent methods and quantitative real-time polymerase chain reaction (PCR) methods have been used to study the microbial compositions, these methods are time-consuming and the results are sometimes inaccurate,

especially target bacterial counts (Bose et al. 2014).

Recently, there has been a revolution in next-generation sequencing platforms such as the Sanger sequencing method (Heather et al. 2016), a high-throughput platform based on the Roche GS20 454 sequencer (Loman et al. 2012) , Illumina GA and MiSeq and HiSeq platforms (White et al. 2016). However, these methods were restricted with respect to genus precision due to the low taxonomical resolution of the traditional DNA sequencing technique, which could only determine the partial sequence of the 16S rRNA genes (Bao et al. 2016). Moreover, the third NextGen was emerging, the Pacific Biosciences (PacBio) single molecule, real-time sequencing (SMRT) technology, which is faster and more informative sequencing technology. PacBio currently offers long DNA sequence reads that are able to depict the bacterial profiles of target samples to the species level (Hou et al. 2015).

This method has been fully tested by applying the PacBio SMRT in evaluating the quality of silage production (Bao et al. 2016). In the present study, apart from analyzing the quality of fermented *Astragalus* using conventional indicators such as pH, organic acid contents and active substance yields, we specifically focused on detecting and comparing the bacterial microbiota composition of *Astragalus* produced by adding *Enterococcus faecium* and *Lactobacillus plantarum* using the PacBio SMRT method.

MATERIALS AND METHODS

Preparation of fermented *Astragalus*

Astragalus was obtained from a northwest Chinese medicine market (Minxian, Gansu, China), and it was identified by Dr. Zhang Jing Yu (Henan University of Traditional Chinese Medicine, Zhengzhou, Henan, China). The fermentation of the *Astragalus* was performed following our laboratory-optimized procedure as described previously. Briefly, *Astragalus* was ground into powder using a 100-mesh screen. Then, the dried

powder (7500 g) was divided into three groups: A, B and C. The A group was inoculated with 10^6 colony-forming units (CFU)/g of *E. faecium* (CGMCC 1.130), the B group was inoculated with 10^6 colony-forming units (CFU)/g of *L.s plantarum* (CGMCC 1.557), and the C group was inoculated with 10^6 CFU/g *E. faecium* + *L. plantarum*, which were isolated and deposited in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China). Fermentation was conducted in 35×45-mm plastic film bags (Jinhu Co., Zhejiang, China), and the bags were evacuated and sealed using a vacuum packing machine. Subsequently, the mixtures were incubated for 30 days at 37 °C under anaerobic conditions to produce the fermented *Astragalus* in which the starter bacterial population was kept alive to achieve the *Lactobacillus* effects. The three groups were sampled at days 0, 3 and 30 and labeled A1, A2, A3, B1, B2, B2, C1, C2 and C3, respectively.

Fermentation changes in organic acid contents and pH value

In order to perform organic acid analysis, 5 g of fermented day 3 and day 30 samples from A2, A3, B2, B3, C2 and C3 were mixed with 60 mL of deionized water, followed by heating in a water bath for 20 min. Then, the filtrate was centrifuged at 80 000×*g* for 20 min. 10 mL of supernatant were filtered through a 0.45-μm membrane before chromatographic analysis. Separations by high performance liquid chromatography (HPLC) were performed on an Agilent 1260 Series LC system with a preparative XB-C18 column (4.6mm×150mm, i.d. 5 μm, Waters, USA). Solvent A was phosphate buffer solution (pH 2.70), and solvent B was methanol solution. Elution was performed with a gradient of 97:3, while the analytical column temperature was 20 ℃, and the flow rate was 0.80 mL/min. Absorbance was detected at 210 nm. Acetic acid, methylacetic acid, ethylacetic acid and lactic acid were determined. Fermented *Astragalus* samples (25 g) from the three groups were dissolved in 225 mL of deionized water. After vortex mixing for 30 min, a pH meter was used for the measurements.

Active substance analysis of fermented Astragalus

Astragalus polysaccharide yield analysis

The dried and fermented *Astragalus* was kept in distilled water (with the ratio of 1:8) for 24 h and extracted

3 times with distilled water in a boiling water bath. The extract was collected by centrifugation at 5000×*g* for 15 min and the supernatant was concentrated through rotary evaporation. Then 95% ethanol (3-fold volume) was added to the concentrated solution and the mixture was stored at 4 °C for 24 h and then centrifuged at 5000×*g* for 20 min. The precipitate was dried at 60 °C and ground into a powder. The amounts of polysaccharides in the extracts were determined using the phenol-sulfuric acid method.

Total saponins yield analysis

Astragalus membranaceous methyl glucoside reference substance (5 mg) was added to a 25-mL volumetric flask, and methanol was added diluted to scale, and the mixture was shaken well and used as a reference substance solution. 0.1-, 0.2-, 0.4-, 0.6-, 0.8-, 1.0- and 1.2-mL aliquots of the reference substance solution were placed in 10-mL calibrated test tubes, dried in a water bath and cooled. 0.2 mL of freshly prepared 5% vanillin glacial acetic acid solution was added to 0.8 mL of perchloric acid and shaken well. Then the mixture was heated at 70 °C in a water bath for 20 min, and cooled with ice water for 5 min, then 5 mL of glacial acetic acid were added and the mixture was shaken well as the blank control. A standard curve was constructed with absorbance at 580 nm as the ordinate (Y) and the concentration of reference substance as the abscissa (X). According to the standard curve the production of glycosides from *Astragalus* could be calculated.

Flavonoid yield analysis

A 10-mg sample of the rutin reference substance was kept in a 50-mL volumetric flask, with a 60% ethanol solution metered volume, and shaken well as the reference solution. 0-, 2-, 4-, 6-, 8-, 10- and 12-mL aliquots of the reference solution were placed in 25-mL volumetric flasks, and then 1.0 mL of 5% sodium nitrite solution was added and shaken well for 6 min, 1.0 mL of 10% aluminum nitrate solution was added and shaken well for 6 min, and 10 mL of 4% sodium hydroxide solution in 60% ethanol were added and shaken for 15 min. A standard curve was constructed with absorbance at 510 nm as the ordinate (Y) and concentration of the reference substance as the abscissa (X), and according to the standard curve the production of astragaloside IV was calculated.

Flavonoids yield = [flavonoid concentration (mg/mL) × flavonoid solution volume (mL)] / sample quality (g) × 100%

SMRT analysis of microbial composition

A total of nine samples, A1, A2, A3, B1, B2, B3, C1, C2 and C3, were collected. The samples were immediately frozen at −196℃ until DNA extraction. A total of 200 mg of fermented *Astragalus* from each group was utilized for DNA isolation. DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The quality of extracted DNA was assessed by 0.8% agarose gel electrophoresis and spectrophotometry (optical density at 260 nm/280 nm). All extracted DNA samples were stored at −20 ℃ prior to further analysis.

The bacterial 16S rRNA was amplified by PCR for barcoded SMRT sequencing with the forward 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse 1492R (5'-ACCTTGTTACGACTT-3') primers. The PCR program was as follows: 95 ℃ for 2 min; 30 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 30 s with a final extension of 72 ℃ for 5 min.

The entire 16S rRNA lengths of the community were sequenced by Pacbio Sequel platform at Personalbio, Inc. (Shanghai, China). The raw data were taken for Circular Consensus Sequencing and corrected so that the correctness of the forecast was not less than 90%. The extraction of high-quality sequences was performed with the QIIME package (Quantitative Insights into Microbial Ecology, v1.8, http://qiime.org/acripts/pick_oyus.html), and they were clustered into operational taxonomic units (OTUs). All sequences were compared against the Greengenes reference database (release 13.8, http://greengenes.secondgenome.com/) . Taxa summarization, alpha diversity, beta diversity and a taxon differential distribution analysis were performed using all of the available sequences for each sample. The raw sequence reads have been deposited in the National Center for Biotechnology Information Short Read Archive under the accession number SAMN07411593-SAMN07411601.

158 **Statistical analysis**

159 Experimental data were analyzed using SAS software (SAS version 9.0, SAS Institute Inc., Cary, NC,
160 USA), and the statistical significances were tested using analysis of variance. The chemical composition of
161 each sample was tested three times, and the results were expressed as the mean ± standard deviation.

162 Sequences were rarefied prior to calculation of alpha and beta diversity statistics. Alpha diversity indexes
163 were calculated in QIIME from rarefied samples using for diversity the Shannon index and for richness the
164 Chao 1 index. Beta diversity was calculated using weighted and unweighted UniFrac and principal coordinate
165 analysis. An unweighted pair group method with arithmetic mean tree was constructed from the beta diversity
166 distance matrix.

167 **RESULTS**

168 **Fermentation changes in Astragalus pH**

169 The changes in pH of fermented *Astragalus* are shown in Figure 1. Generally, the addition of one or two
170 LAB additives (*L. plantarum, E. faecium, L. plantarum + E. faecium*) resulted in varying degrees of
171 fermentative changes in pH. After fermentation the pH decreases (to below 5.0) were significant from days 6
172 to 30 in the *L. plantarum, E. faecium* and *L. plantarum + E. faecium* groups.

173 **Fermentation changes in organic acid contents**

174 **Acetic acid analysis**

175 There were drastic rises in acetic acid contents in the *L. plantarum* group, *E. faecium* group and *L.*
176 *plantarum + E. faecium* group, reaching peaks of 1723.01 mg/kg, 1329.61 mg/kg and 1866.24 mg/kg on day
177 15, respectively, as shown in Figure 2 (a). However, there was no significant change in the control group.
178 These results demonstrated that *L. plantarum* and *E. faecium* promoted the production of acetic acid.

Methylacetic acid analysis

There was a gradual rise in methylacetic acid in the *L. Plantarum* group, *E. faecium* group and *L. plantarum* + *E. faecium* group, reaching peaks of 173.29 mg/kg, 123.88 mg/kg and 203.80 mg/kg on day 30, respectively, as shown in Figure 2 (b). The production of methylacetic acid may continue to increase with extension of the fermentation. These results suggested that *L. plantarum* and *E. faecium* promoted the production of methylacetic acid.

Ethylacetic acid analysis

There were drastic rises in ethylacetic acid in the *L. plantarum* group, *E. faecium* group and *L. plantarum* + *E. faecium* group, reaching peaks of 616.07 mg/kg, 445.74 mg/kg and 996.04 mg/kg on day 25, respectively, as shown in Figure 2 (c). These results indicated that *L. plantarum* and *E. faecium* promoted the production of ethylacetic acid.

Lactic acid analysis

There were gradual rises in lactic acid in the *L. plantarum* + *E. faecium* group and *L. plantarum* group, reaching peaks of 3081.99 mg/kg on day 20 and 1946.17 mg/kg on day 15, respectively, as shown in Figure 2 (d), but the *E. faecium* group exhibited no significant change compared with the control group. These results indicated that *L. plantarum* + *E. faecium* promoted the production of lactic acid.

Active substance yields of fermented Astragalus

Polysaccharide yield analysis

The polysaccharide yield was higher in the *L. plantarum* fermentation than in the control group as shown in Figure 3 (a). Moreover, the polysaccharide yield was 9.43% on day 10, which was 2.3-fold higher than that of the control group, and it remained steady to reach a maximum on day 30. These results illustrated that the

polysaccharide yield changed significantly through *L. plantarum* fermentation ($p<0.05$).

Total saponins yield analysis

The total saponins yield in the *L. plantarum* + *E. faecium* group sharply increased on day 15, and reached 21.6630 mg/g as shown in Figure 3 (b). Moreover, this was a 125.68% increase compared to the control group. These results meant that the combination of the *L. plantarum* + *E. faecium* was superior to either *L. plantarum* or *E. faecium* alone ($p<0.05$).

Flavonoid yield analysis

The flavonoid yield in the *E. faecium* group sharply increased and reached 2.0835 mg/g as shown in Figure 3 (c). Moreover, this was 1.44-fold higher than the yield for the control group. There seems to be a tendency for the flavonoid yield to reach two peaks, during the initial and later stages of the fermentation. These results revealed that the flavonoid yield changed during the *E. faecium* fermentation ($p<0.05$).

Changes in microbial composition after Astragalus fermentation

SMRT sequencing of the full length 16S rRNA genes was performed to obtain accurate bacterial profiles of the *Astragalus* samples at species level. A total of 2,945,166 sequence reads were obtained from nine *Astragalus* samples, with an average of 8888 reads for each sample. The ACE, Chao 1, Shannon and Simpson indexes were calculated, and a different richness for each of the nine groups was observed (Table 1). These results indicated that the samples showed a high bacterial biodiversity.

The total OTUs obtained were as follows: 1505 in the A group (*E. faecium* fermentation), 1866 in the B group (*L. plantarum* fermentation), 1853 in the C group (*E. faecium* + *L. plantarum* fermentation). As shown in Figure 4, a total of 203 OTUs were common among the three groups, whereas the number of OTUs present only in one group varied from 1162 to 1470.

Microbial Beta diversity analysis

Using R software, the partial least squares discriminant analysis (PLS-DA) discriminant model was constructed based on the species abundance matrix and the sample packet data as shown Figure 5. And the variable importance in projection (VIP) coefficient was calculated for each species. The results showed that the VIP coefficients of A1, A2, A3, C1, C2 and C3 were greater than 1; however, the VIP coefficients for B1, B2 and B3 were less than 1. These results showed that the shorter the distances between the same groups, the further the distances between the points of the different groups, indicating that the classification model works well.

Bacterial community compositions

As shown in Figure 6 (a), at the genus level an analysis of the most abundant taxa revealed the highest abundances were of *E. faecium* (94.0%) in the A2 sample, *L. plantarum* (71.0%) in the B2 sample and *L. faecium* + *E. plantarum* (98.7%) in the C2 sample. However, the compositions of the microbes in the A3, B3 and C3 samples tended to be more consistent at day 30. These results indicated that *Stanieria* was the most abundance genus in the A1, B1 and C1 samples before fermentation, whereas after fermentation on day 3, *Enterococcus* and *Lactobacillus* were the most abundance genera in the A2, B2 and C2 samples.

At the species level, the relative abundances of microbes in the three groups are shown in Figure 6 (b). *E. faecium* exhibited dynamic changes in the A group, and its proportion was 44.8%. *L. plantarum* displayed slight changes in the B group, and its proportion was 35.3%. *E. faecium* + *L. plantarum* underwent great changes in the C group, and its proportion was 47.35%. Clearly, the prevalent species that existed in fermented *Astragalus* were highly dependent on the original bacterial composition.

Community compositional Heat Map combined with cluster analysis

As shown in Figure 7, the top 50 species according to abundance were clustered and plotted using R software. Red represents the species with higher abundances in the corresponding samples, and green represents the species with lower abundances. From the heat map, it can be seen that *Enterococcus* and *Lactobacillus* were more abundant in the A2, B2 and C2 samples than in the other samples. However, with time the fermentation bacteria were reduced in numbers and other natural bacteria began to grow.

On day 30 of the fermentation, we concluded that the fermentation bacteria were dominant on day 3 of the fermentation, and this indicated *Astragalus* could be fermented for 3 days under the conditions of the fermentation process.

DISCUSSION

The Chinese herb *Astragalus* has been widely used as a dietary supplement in Asia (I-Chuan et al. 2011). In this study, to assess the nutritional value and organic content of fermented *Astragalus*, *E. faecium* and *L. plantarum* were added to aid the fermentation process. The full 16S rRNA gene-SMRT sequencing method was applied to monitor the quality of *Astragalus* production, as traditional methods including culture-dependent methods are inaccurate and the results may sometimes be difficult to interpret and ambiguous.

In this study, we found the fermentation of *Astragalus* with *E. faecium* and *L. plantarum* additives caused a decrease in pH due to the production of organic acids during the process. In general, the decrease in pH value was mainly due to the production of lactic acid during fermentation, and the low pH is advantageous as *Astragalus* are better preserved and more stable. Thus, the organic acid production of fermented *Astragalus* is dependent upon the type of bacteria used. *L. plantarum* is a well-known homo-fermentative LAB (Park et al. 2012), which efficiently produces lactic acid from fermented *Astragalus*. Moreover, the fermentation conducted with *E. faecium* + *L. plantarum* was better than the use of a single strain. It has been reported that

there is a natural synergy between different probiotics when they are present at certain proportions (Bielecka et al. 1999; Rajagopal et al. 1990). It is common practice to apply mixed bacterial strains to a fermentation system that is used to biodegrade natural cellulose, as using only a single bacterial strain imposes certain limitations on the fermentation process due to the effects of some enzymes (Alakomi et al. 2000). It is quite important to determine the appropriate ratios of bacteria because unsuitable proportions would reduce the rates of production of organic acids and perhaps even inhibit the fermentation (Kibeom 2005). Use of the combination of *E. faecium* + *L. plantarum* in the fermentation could improve the odor and provide an acidic taste and flavor.

Moreover, the current study highlighted that *Astragalus* produces polysaccharides, flavonoids and total saponins, which increased gradually during fermentation and were higher than the control group during the entire fermentation process when *E. faecium* and *L. plantarum* were applied to SSF. The reason may be that free polysaccharides and extracellular polysaccharides were produced due the degradation of cell wall cellulose by digestive enzymes during the fermentation process of *E. faecium* + *L. plantarum* (Xue et al. 2015). At a later stage of fermentation, the yield of active substances decreased, which may be due to their utilization by *E. faecium* and *L. plantarum* or their transformation into other compounds or generation of secondary glycosides or more active substances.

The microbiota profile is another indicator that reflects the quality of fermented *Astragalus*. The current study focused on the composition of microbiota in the fermentation of solid *Astragalus* powder using *E. faecium* and *L. plantarum*. The results showed that *Astragalus* may greatly promote the growth of *E. faecium* and *L. plantarum*, as the sources of organics, carbon, inorganic salts and polysaccharides from *Astragalus* are indispensable for the growth of *E. faecium* and *L. plantarum* (Timmerman et al. 2014; Choi et al. 2007).

Preparation of traditional Chinese medicines mostly incorporates extraction and prefractionation (Xu et al. 2017) , processes associated with an irritating odor (Qiu 2007) that is not conducive to animal feeding. Thus, fermentation of herbal medicines by probiotics may also be an effective processing method (Kim et al. 2012). Moreover, *Astragalus* has a sour taste after fermentation that depends upon acid production and is more conductive to animal feeding.

SMRT analysis of the microbial composition of nine fermented *Astragalus* samples showed that the major bacterial species depended on the original bacterial composition. After day 3 of fermentation, the original bacterial such as *E. faecium* and *L. plantarum* were dominant. Moreover, the microbiota diversity was greater when *E. faecium* + *L. plantarum* was used for fermentation compared to a single bacterium. These results confirmed that multiple strains have positive effects on the *Astragalus* fermentation process. So, *Astragalus* promoted the growth of bacteria to exert prebiotic-like effects that selectively stimulate the growth of symbiotic beneficial bacteria (Cockburn et al. 2016).

However, the microbiota composition tended to remain constant after fermentation for 30 days. The reason is that lactic acid bacteria are relatively fragile, with a short growth period (Zhang et al. 2014; Hu et al. 2015). By 30 days, the inherent bacteria of *Astragalus* had begun to grow and multiply. Compared with the traditional method of colony counting (Fu et al. 2014) , SMRT analysis can reflect the microbiota composition during the *Astragalus* fermentation process.

CONCLUSION

In summary, *E. faecium* and *L. plantarum* have positive effects on the fermentation of *Astragalus*. Although only nine of the samples were analyzed using the SMRT sequencing technology, our data have shown that this is a prospective method for assessment of the quality of fermented *Astragalus*.

ADDTIONAL AFORMANTION AND DECLARATIONS

Funding

This work was supported by the Henan Province Natural Science Planning Fund Projects [grant NO. 162300410128] and by the veterinary discipline key construction project of Henan University of Animal Husbandry and Economy [grant NO.MXK2016102].

Grant Disclosures

The following grant disclosures information was disclosed by the authors:

Henan Province Natural Science Planning Fund Projects: 162300410128.

The veterinary discipline key construction project of Henan University of Animal Husbandry and Economy: MXK2016102.

Competing interests

The authors declare no conflicts of interest.

Authors' contributions

Conceived and designed the experiment: Hongxing QIAO, Chuanzhou BIAN. Performed the experiments: Xiaojing ZHANG, Yuzhen SONG,. Data analysis: Hongtao SHI. Wrote the paper: Hongxing QIAO.

Date Availability

The data generated or analysed during this study are included in this article.

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Figure legends

Figure 1. Determination of pH using *E. faecium*, *L. plantarum* or *L. plantarum* + *E. faecium*. Samples were taken for analysis after 0, 3, 6, 9, 12, 15, 18, 21 24, 27, 30 days. Data are expressed as the mean ± SD from three independent experiments.

Figure 2. Determination of organic acids in *Astragalus* fermented using *L. plantarum*, *E. faecium* or *L. plantarum* + *E. faecium* compared to the control group. Samples were taken for analysis after 5, 10, 15, 20, 25, 30 days. Data are expressed as the mean ± SD from three independent experiments. (a): Acetic acid analysis; (b): methylacetic analysis; (c): ethylacetic acid analysis; (d): lactic acid analysis.

Figure 3. Analysis of active substance yields of *Astragalus* fermented using *L. plantarum*, *E. Faecium* or *L. plantarum* + *E. faecium* compared to the control group. Samples were taken for analysis after 0, 5, 10, 15, 20, 25, 30 days. Data are expressed as the mean ± SD from three independent experiments.

Figure 4. Shared OTU analysis of the different groups. The number of species in the A group is 1505; the number of species in the B group is 1866; the number of species in the C group is 1853; the number of species common to the A and B groups is 295; the number of species common to the A and C groups is 251; the number of species common to the B and C groups is 335; a total of 203 OTUs were common to the three groups.

Figure 5. PLS-DA discriminant model from A1, A2, A3, B1, B2, B3, C1, C2 and C3 samples. VIP coefficients of A1, A2, A3, C1, C2, C3 were greater than 1; however, those of B1, B2, B3 were less than 1.

Figure 6. Genus-level and Species-level analysis of the samples.

(a) **Genus-level analysis of the nine samples.** Overall microbiota composition of fermentation samples at the genus level for: A1, on day 0 using *E. faecium*; A2, on day 3 using *E. faecium*; A3, on day 30 using *E. faecium*; B1, on day 0 using *L. plantarum*; B2, on day 3 using *L. plantarum*; B3, on day 30 using *L. plantarum*; C1, on day 0 using *L. plantarum* + *E. faecium*; C2, on day 3 using *L. plantarum* + *E. faecium*; C3, on day 30 using *L. plantarum* + *E. faecium*. The relative abundances of *E. faecium*, *L. plantarum* are shown on the *y*-axis.

(b)**Species-level analysis of the three groups.** A: Overall microbiota composition of fermentation samples at the species level using *E. faecium*. B: Overall microbiota composition of fermentation samples at the species level using *L. plantarum*. C: Overall microbiota composition of fermentation samples at the species level using *L. plantarum* + *E. faecium*. The relative abundances of *E. faecium* and *L. plantarum* are shown on the *y*-axis.

Figure 7. Heat map analysis of the nine samples. Heat map showing that the abundances of the top 50 species are clustered and plotted using R software. Red represents species with higher abundances in the corresponding sample, and green represents species with lower abundances. *Enterococcus* and *Lactobacillus* were present at higher abundances in groups A2, B2, C2 than in the other groups.

Figure 1

Determination of pH using *E. faecium* , *L. plantarum* or *L. plantarum* + *E. faecium* .

Samples were taken for analysis after 0, 3, 6, 9, 12, 15, 18, 21 24, 27, 30 days. Data are expressed as the mean ± SD from three independent experiments.

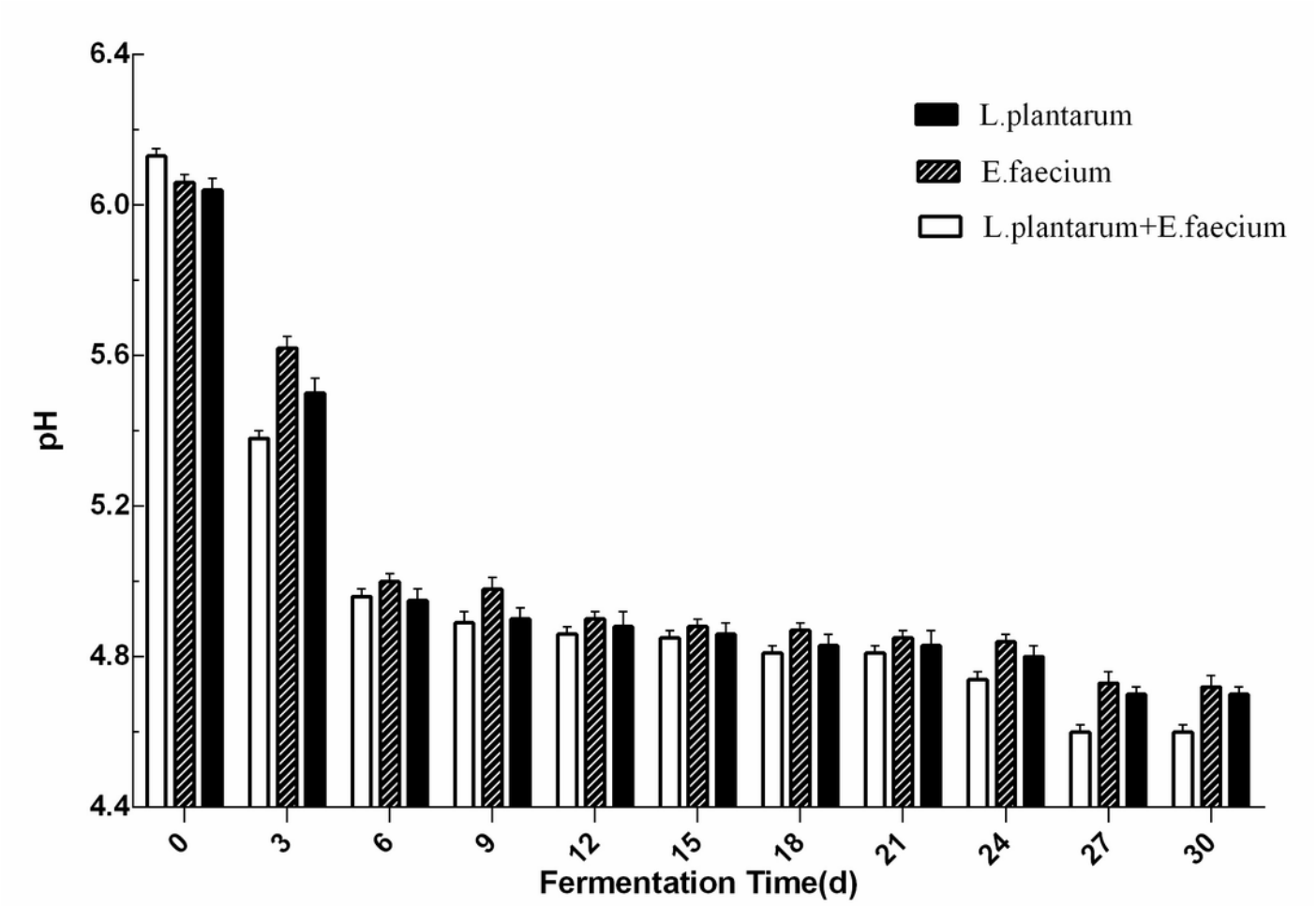
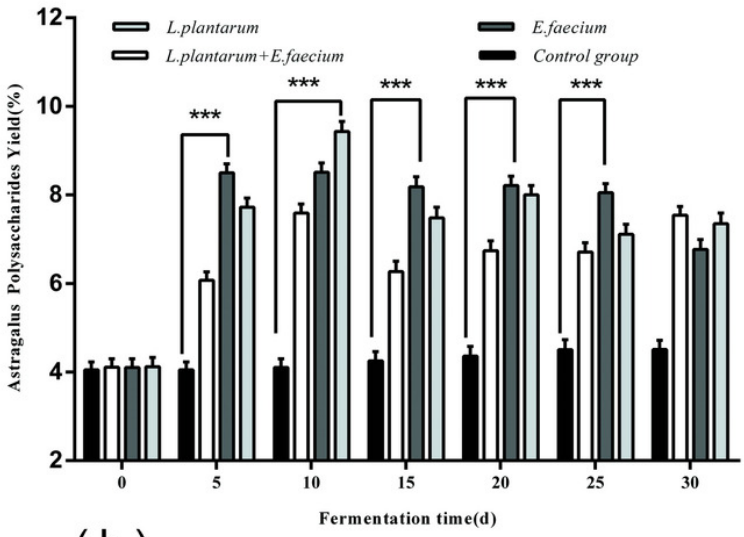


Figure 2

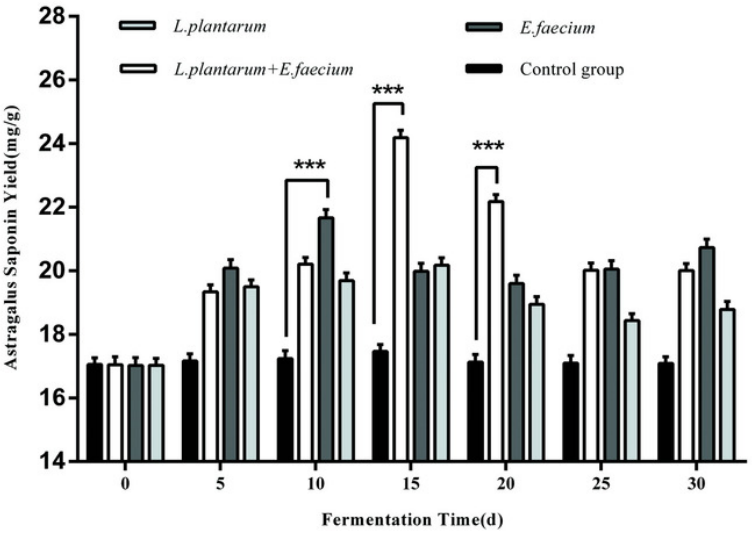
Determination of organic acids in *Astragalus* fermented

Samples were taken for analysis after 5, 10, 15, 20, 25, 30 days. Data are expressed as the mean ± SD from three independent experiments. (a): Acetic acid analysis; (b): methylacetic analysis; (c): ethylacetic acid analysis; (d): lactic acid analysis.

(a)



(b)



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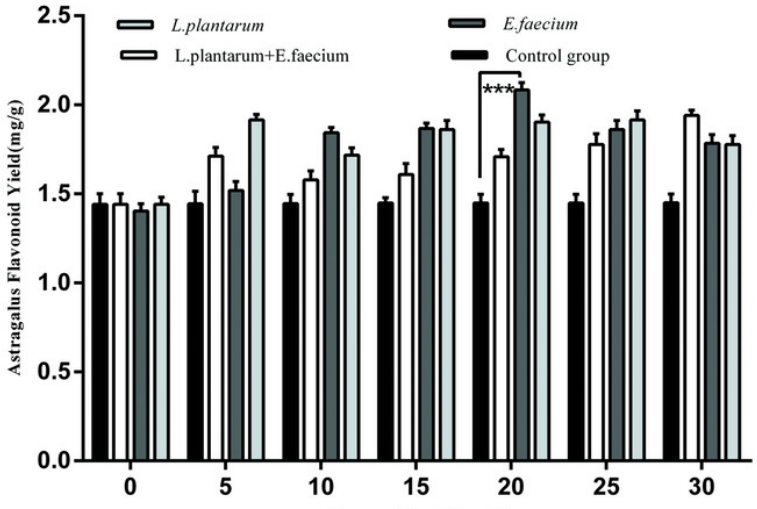
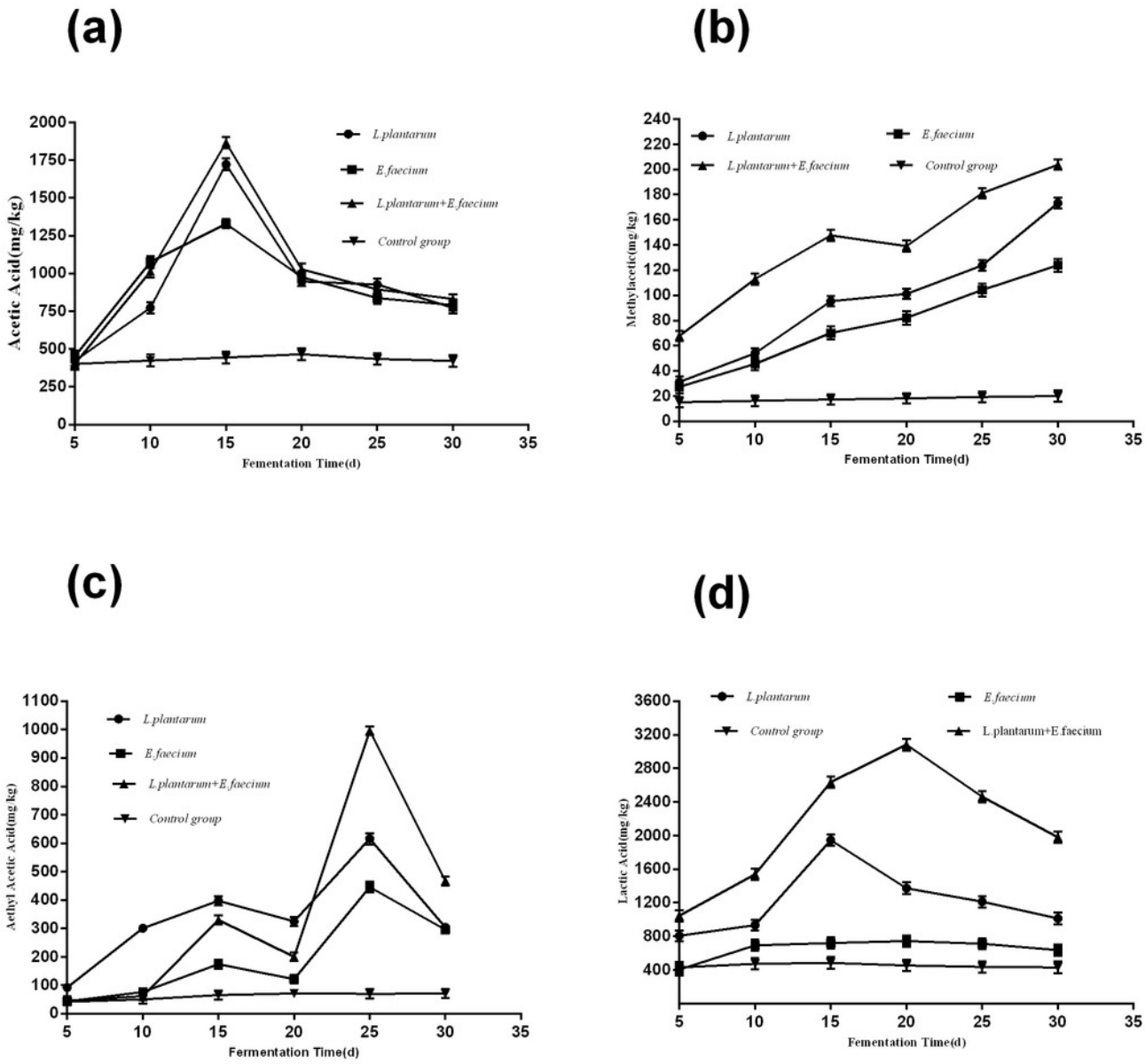


Figure 3

Analysis of active substance yields of *A stragalus* fermented

Samples were taken for analysis after 0, 5, 10, 15, 20, 25, 30 days. Data are expressed as the mean ± SD from three independent experiments.



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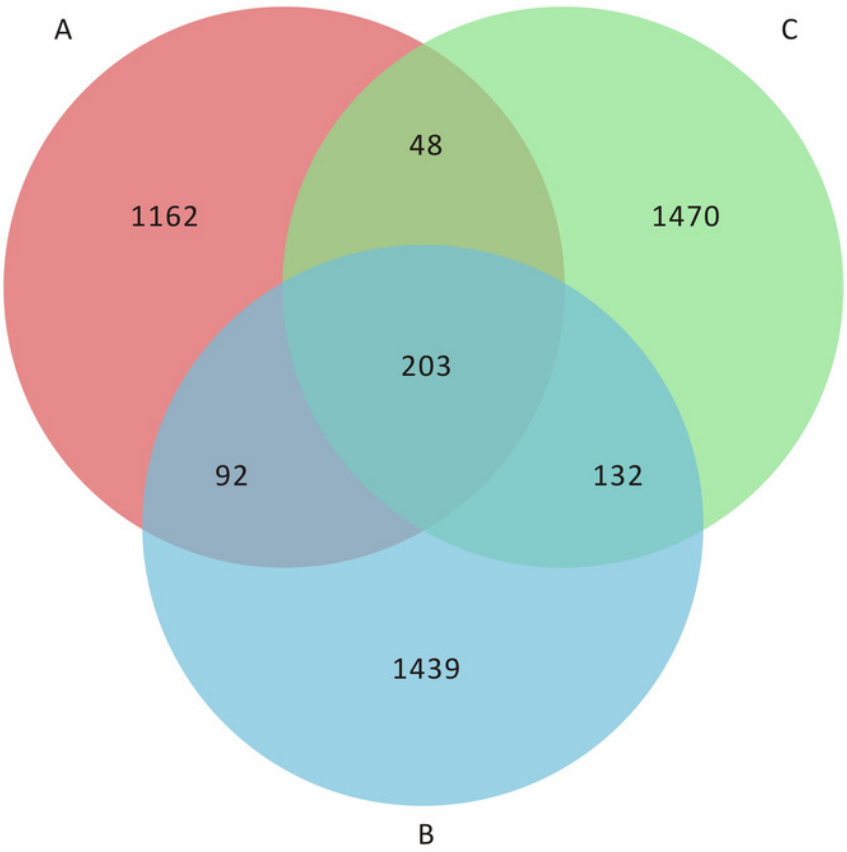
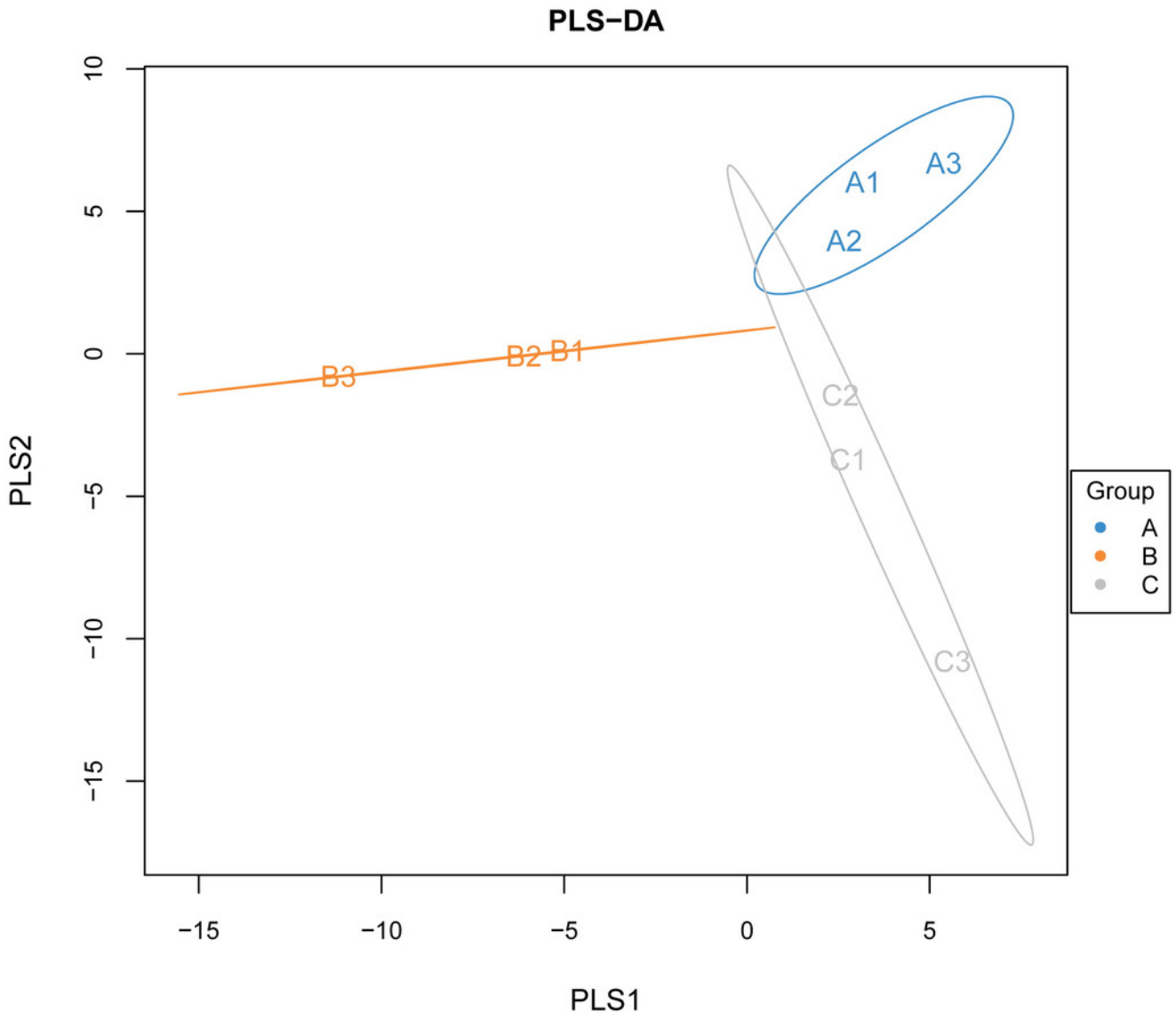


Figure 5

PLS-DA discriminant model from A1, A2, A3, B1, B2, B3, C1, C2 and C3 samples.

VIP coefficients of A1, A2, A3, C1, C2, C3 were greater than 1 ; however, those of B1, B2, B3 were less than 1.



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(b) **Species -level analysis of the three groups .** A : Overall microbiota composition of fermentation samples at the species level using *E. faecium* . B : Overall microbiota composition of fermentation samples at the species level using *L. plantarum* . C : Overall microbiota composition of fermentation samples at the species level using *L. plantarum* + *E. faecium* . The relative abundances of *E . faecium* and *L . plantarum* are shown on the *y* -axis.

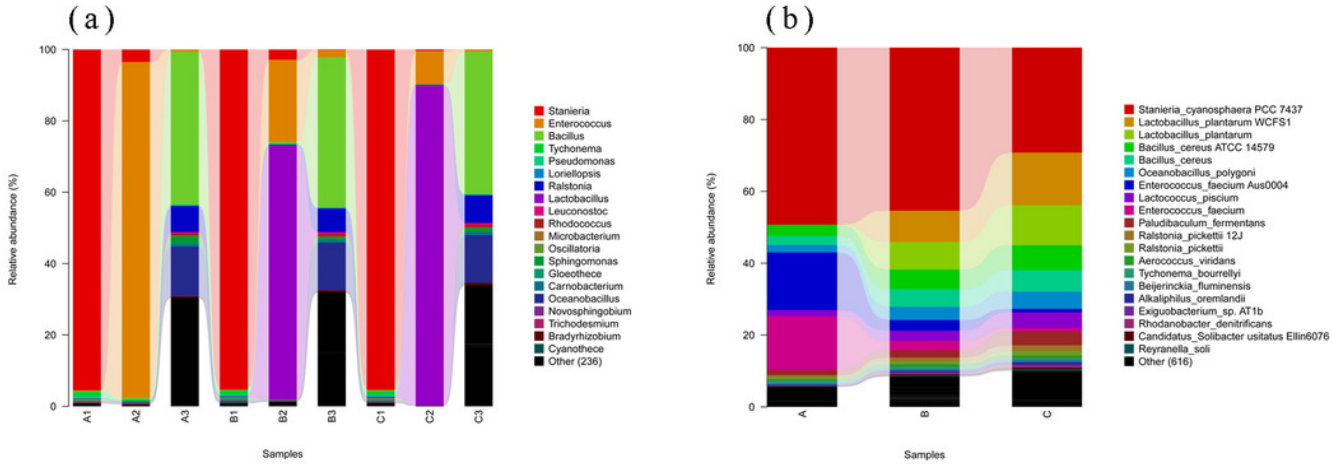

Figure 7

Heat map analysis of the nine samples.

Heat map showing that the abundances of the top 50 species are clustered and plotted using R software. Red represents species with higher abundances in the corresponding sample, and green represents species with lower abundances. *Enterococcus* and *Lactobacillus* were present at higher abundance s in group s A2, B2, C2 than in the other groups .

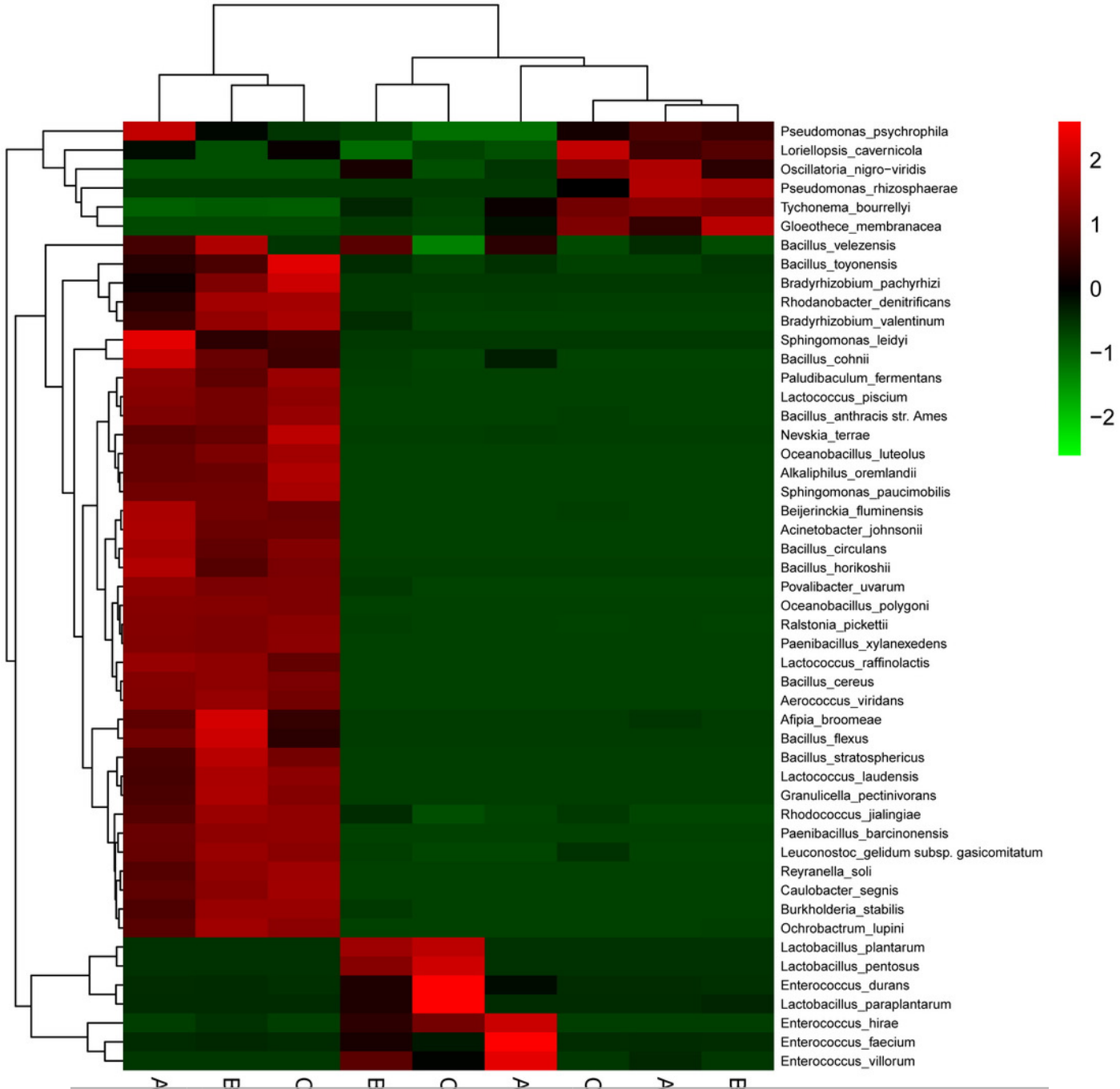

Table 1(on next page)

Diversity estimation of the 16S rRNA gene libraries of the nine samples from the sequencing .

Note: A1: *Astragalus* samples fermented using *E. faecium* on day 0; A2: *Astragalus* samples fermented using *E. faecium* on day 3; A3: *Astragalus* samples fermented using *E. faecium* on day 30; B1: *Astragalus* samples fermented using *L. plantarum* on day 0; A2: *Astragalus* samples fermented using *L. plantarum* on day 3; A3: *Astragalus* samples fermented using *L. plantarum* on day 30; C1: *Astragalus* samples fermented using *E. faecium* + *L. plantarum* on day 0; C2: *Astragalus* samples fermented using *E. faecium* + *L. plantarum* on day 3; C3: *Astragalus* samples fermented using *E. faecium* + *L. plantarum* on day 30 .

Table 1. Diversity estimation of the 16S rRNA gene libraries of the nine samples from the sequencing.

Sample	ACE	Chao 1	Shannon	Simpson
A1	60.07	29	1.18	0.51
A2	53.19	31	1.44	0.55
A3	249.41	178	4.9	0.92
B1	87.7	27	1.19	0.52
B2	224.59	79	2.35	0.72
B3	321.63	190	4.95	0.92
C1	54.53	31	1.21	0.52
C2	167.45	110	2.51	0.67
C3	294.69	178	4.89	0.93

Note: A1: *Astragalus* samples fermented using *E. faecium* on day 0; A2: *Astragalus* samples fermented using *E. faecium* on day 3; A3: *Astragalus* samples fermented using *E. faecium* on day 30; B1: *Astragalus* samples fermented using *L. plantarum* on day 0; A2: *Astragalus* samples fermented using *L. plantarum* on day 3; A3: *Astragalus* samples fermented using *L. plantarum* on day 30; C1: *Astragalus* samples fermented using *E. faecium* + *L. plantarum* on day 0; C2: *Astragalus* samples fermented using *E. faecium* + *L. plantarum* on day 3; C3: *Astragalus* samples fermented using *E. faecium* + *L. plantarum* on day 30.