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Effect of *Broussonetia papyrifera* Silage on the Serum Indicators, Hindgut Parameters and Fecal Bacterial Community of Holstein Heifers

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Abstract

This study investigated the effects of substitution of whole corn silage (CS) with *Broussonetia papyrifera* silage (BPS) in different ratios on the serum indicators, hindgut fermentation parameters (pH, ammoniacal nitrogen, and volatile fatty acids), and fecal bacterial community of Holstein heifers. Sixteen heifers (8-month-old, 220±30 kg) were randomly divided into four treatments according to different BPS substitution ratios of feed basis (0%, 25%, 50%, and 75%). The experiment consisted of a 7-day preliminary feeding period and a 30-day experimental period. On the last day of the trial, the blood samples were collected from caudal vein, and the feces samples were collected from rectum. With the increasing of BPS content, the concentration of malondialdehyde (MDA) and interleukin-1β (IL-1β) in serum decreased ($P<0.05$), and the immunoglobulin A (IgA) and IL-4 content of serum increased ($P<0.05$); and the hindgut pH value increased ($P<0.05$). 16S rDNA sequencing found that the dominant phyla were *Firmicutes*, *Bacteroidetes*, and *Verrucomicrobia*; and the dominant genera were *Ruminococcaceae_UCG-005*, *Ruminococcaceae_UCG-010*, and *Rikenellaceae_RC9_gut_group*. Linear Discriminant Analysis Effect Size (LEfSe) analysis found 12 differential operational taxonomic units (OTUs) which have strong correlation with some serum and hindgut indicators. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) found that BPS have impacts on the pathways, such as carbohydrate transport and metabolism, and promotes amino acid transport and metabolism. To sum up, inclusion of BPS in heifer diets can affect the fecal bacterial

community, and further improve serum anti-oxidant and immune indicators in Holstein heifers.

Key words *Broussonetia papyrifera* silage, Holstein heifers, Serum indicators, Hindgut fermentantion, Fecal bacterial community.

1. Introduction

With the increase of population and the decrease of arable land per capita, conventional feed such as forage and grain can no longer meet the need of animal husbandry (Dong et al. 2019; Zhai et al. 2020). In order to meet the growing demand for animal products, it will be necessary to make novel and unconventional feed resources available (Araújo et al. 2019). Unconventional feed materials are those that are not commonly used in formulations or have little research on their nutritional properties and feeding value. However, unconventional feeds have characteristics of high yield, wide distribution, and various varieties. Currently, various new feed materials, including by-products like Chinese jujube meal, pomegranate residue, orange leaves, olive leaves have been reported (Xie et al. 2018; Fernández et al. 2019; Hukerdi et al. 2019; Khorsandi et al. 2019). The above studies showed that those unconventional feed can promote the production performance of livestocks to some extent with no adverse effect.

Broussonetia papyrifera (Paper mulberry), a deciduous tree of *Moraceae* family, is widely distributed in China, Japan and other Asian countries (Yao et al. 2017). Many biologically active compounds contained in *B. papyrifera*, such as flavonoids, lignans, polysaccharides, and terpenoids have antimicrobial, anti-inflammatory, and antioxidant

53 properties and can reduce the growth of tumors (Mei et al. 2009; Sohn et al. 2010; Wang
54 et al. 2010; Xu et al. 2010; Sun et al. 2012; Guo et al. 2013; Han et al. 2016). For this
55 reason, *B. papyrifera* is already widely used in the pharmaceutical industry. Due to its
56 high-quality fiber, *B. papyrifera* is also used as important raw material for the
57 production of paper (Peng et al. 2019). *B. papyrifera* has advantages of rapid growth,
58 strong adaptability and disease resistance, and high protein content (approximate 18–
59 22%, including both leaves and stems) (Peng et al. 2019), which makes it a potential
60 candidate for new feed resourse.

61 Intestinal bacteria, which are diverse and abundant, play vital parts in maintaining
62 the homeostasis of the digestive system and the function of the immune system (Liu et
63 al. 2019). Intestinal bacteria have two main functions: converting nutrients into energy
64 and resisting the invasion of pathogenic microorganisms (Guarner 2007). The stability
65 of intestinal flora is directly related to animal physical condition, thus affecting
66 production performance. In ruminant, intestinal flora is influenced by feed ingredients.
67 Xie et al. (2020) fed Holstein heifers by substituting different proportions (0%, 5%, and
68 10%) of corn silage (CS) with herbal tea residue, finding that the microbial composition
69 of the three treatments was significantly different. Moreover, Sun et al. (2017) fed
70 Holstein cows different percentages of ensiled *Moringa oleifera* and found a strong
71 correlation between the presence of *Akkermansia* and *Prevotella* in total milk yield and
72 milk protein, which indicates that some bacterial groups could be associated with
73 enhanced milk production performance.

74 Whole crop CS is the most common forage used in ruminant production in China.

However, with the development of intensive and large-scale animal husbandry, CS is unable to meet the requirement gradually. As a feed material with high protein, *B. papyrifera* has been used as fodder for thousands of years (Peng et al. 2019). Nevertheless, studies on the effects of feeding *B. papyrifera* silage to Holstein cows are still limited. In the present study, effects of substitution of CS with different proportions of *B. papyrifera* silage (BPS) on the serum anti-oxidant and immune indicators; hindgut fermentation parameters including pH value, ammoniacal nitrogen (NH_3-N), and volatile fatty acid VFA); and fecal bacterial community of Holstein heifers were studied, providing a reference for the application of *B. papyrifera* in production. In depth, we analyzed the correlation between bacterial community and various serum and hindgut indicators, and predicted the function of bacterial community, in order to reveal the cause of differences caused by feed on the microbial level.

2. Materials and Methods

All experimental procedures used in this study were approved by the Committee of Animal Experiments of South China Agricultural University (No. 201004152).

2.1. Experimental materials

B. papyrifera silage was purchased from a feed company (Heyuan, GD, China). Hybrid *B. papyrifera* was cut off when it reached a height of 1–1.5 m, and the trunk and branches were removed by a straw chopper, leaving the front 20–30 cm thin branches and leaves, and cutting them into 1 cm per segment. After air drying moderately, the mince, including leaves and twigs, was made into silage via stretch-film-wrapped silage technology (30.00±5.00 kg per bale). Then the packaged silages were preserved in a

dry and room-temperature indoor environment for 60 days.

2.2. Experimental animals

This experiment was carried out in a commercial dairy farm (Yangjiang, GD, China) and adopted a completely randomized block, including a 7-day preliminary feeding period and a 30-day experimental period. Sixteen healthy 8-month-old Holstein heifers (220±30 kg) were randomly assigned to four treatments. In the four groups, the substitution ratios (feed basis) of BPS for CS in dietary was 0% (T0), 25% (T25), 50% (T50), and 75% (T75), respectively. The total mixed ration (TMR) were formulated based on Chinese feeding standards (China Standard NY/T34, 2004). The nutrient composition of CS, BPS, and TMR were analyzed. Thereinto, dry matter (DM), crude protein (CP), ether extract (EE) were measured according to Association of Official Analytical Chemists (AOAC 2002); neutral detergent fiber (NDF), acid detergent fiber (ADF), and hemicellulose (HC) were tested based on Van Soest et al. (1991); calcium (Ca) was measured via EDTA complexometric titration method; phosphorus (P) was determined via vanadium molybdate yellow colorimetric method. The nutrient content of CS and BPS were shown in Table 1. The ingredient and nutrient content of TMRs were shown in Table 2. Heifers were fed twice per day (10:00 and 16:00), and had *ad libitum* access to feed and water throughout the experimental period. The heifers were kept in a free-stall barn with natural ventilation and the excrement was cleaned artificially every day. The cowshed took natural light during the day and artificial light at night, in order to ensure all-day illumination. The serum and feces samples were collected at 4 h after morning feeding on the last day of experimental period. The blood

(approximately 20 mL) was collected from caudal vein; after centrifugation (4000 r/min, 15 min), the upper serum was separated and stored at -20℃. The feces samples were collected from rectum, approximately 2 g of each samples were stored at -80℃ for the determination of microbial flora, another 10 g was taken and added into 20 mL of double steamed water for the determination of fecal parameters, including pH, NH_3-N, and VFAs.

2.3. Serum indicators determination

Serum antioxidant indicators, including malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and total antioxidant capacity (T-AOC), were tested via a commercial kit (Nanjing Jiancheng Bio-Engineering Co. Ltd., Nanjing, China) (Peng et al. 2020). Serum immune indicators, including immunoglobulin A (IgA), interleukin 1β (IL-1β), IL-2, IL-4, IL-6, IL-10, and IL-17, were tested via enzyme-linked immunosorbent assay (ELISA) kit (Jiangsu Jingmei Biotechnology Co. Ltd., Yancheng, China) (Dong et al., 2019). The detailed operating steps of kits were showed in manufacture's protocols in detail.

2.4. Hindgut fermentation parameters determination

Approximately 10 g of each fresh feces sample was mixed with 20 mL distilled water, and then shaked up and centrifuged (5,400 rpm×10 min). The supernatant was collected to test pH-value and the content of NH_3-N and VFAs, including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid.

The determination of NH_3-N content were according to the method of Broderick and Kang (1980). Briefly, a spectrophotometer (UV-2600, Unico, Shanghai) was used

for colorimetry, and the standard curve was obtained according to OD value of standard ammonia solution. The prepared phenol reagent and sodium hypochlorite reagent were successively added to the supernatant mentioned above, after water bath, OD value of the solution was calculated at the wavelength of 630 nm.

The determination of volatile fatty acids (VFA) was based on the method described by Erwin et al. (1961), and the column selection was adjusted and some chromatographic operating conditions were optimized. The chromatographic column was HP-INNOWax capillary column and set to constant flow mode, flow: 2.0 mL/min, mean linear velocity: 38 cm/s. The parameters of gas chromatograph were set as follows: carrier gas, N_2; injection volume, 0.6 μL; injection temperature, 220°C; split ratio, 40:1. In this research, 2-ethyl butyric acid (2-EB) was selected as the internal standard. Based on the established integral parameter and correction curve, the content of each component of the unknown sample was obtained by internal standard calculation method.

2.5. Bacterial community analysis

The total DNA of bacterial community was extracted via E.Z.N.A.® DNA Kit (Omega Biotek, Norcross, GA, USA) according to the specification. The ABI GeneAmp®9700 PCR Amplifier was adopted to amplify the 16S rDNA V3-V4 regions. The primers were designed as follows: forward primer, 338F (ACTCCTACGGGAGGCAGCAG); and reverse primer, 806R (GGACTACHVGGGTWTCTAAT). PCR system was as follows: 5×FastPfu buffer, 4 μL; 2.5 mM dNTPs, 2 μL; forward primer (5 μM), 0.8 μL; reverse primer (5 μM), 0.8

163 μL; FastPfu polymerase, 0.4 μL; bovine serum albumin (BSA), 0.2 μL; template DNA,
164 10 ng; ddH_2O, fill to 20 μL. The reaction was performed under the following condition:
165 95℃ for 3 min; 95℃ for 30 s, 55℃ for 30 s, 72℃ for 45 s for 27 cycles; 72℃ for 10
166 min. After purification, detection, and quantification, the PCR products were sequenced
167 via Illumina Miseq platform. After sequencing, the raw data was analyzed according to
168 Sun et al. (2017).

169 *2.6. Statistical analysis*

170 Serum and fecal parameters were analyzed using SAS 9.4 software (SAS Institute
171 Inc., Cary, NC, USA). The model used for data processing is: $Y_{ij} = \mu + T_i + \varepsilon_{ij}$, thereinto,
172 Y_{ij} is the dependent variable value of the test heifers in different treatments; μ is the
173 overall mean; T_i is the dietary treatment effects; ε_{ij} is the random error. Orthogonal
174 polynomial contrasts (linear, quadratic, and cubic) were used to analyze the effects of
175 the different BPS inclusion levels on the serum and fecal parameters. The experimental
176 data were presented in tables with mean value and mixed standard error (MSE), $P<0.05$
177 indicated significant differences.

178 Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis was performed
179 via an online tool (http://huttenhower.sph.harvard.edu/galaxy/) in order to obtain
180 differential operational taxonomic units (OTU). The correlation analyses between
181 differential OTU and various indicators were carried out via WGCNA package in R
182 3.6.1 software; and the results showed P-Value and correlation coefficient (C_{XY}).
183 Clusters of Orthologous Groups (COG) functional annotation and Kyoto Encyclopedia
184 of Genes and Genomes (KEGG) sample abundance statistics were computed via

185 Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
186 (PICRUSt) on Majorbio (Shanghai, China) online platform (http://www.i-sanger.com).

187 **3. Results**

188 *3.1. Serum indicators and hindgut fermentation parameters*

189 The MDA content of T50 declined linearly ($P<0.05$) compared to T0 (Table 3), no
190 significant differences were found between T25 and T75. Other antioxidant indicators
191 had no significant differences in the four treatments. For serum immune indicators
192 (Table 2), compared with T0, the IgA content of T25, T50, and T75, the IL-4 content of
193 T75 increased (linear, $P<0.05$); the IL-1β content of T50 and T75 decreased (linear,
194 $P<0.05$). The IL-10 content of T75 was significantly lower than that in T25 ($P<0.05$).
195 For hindgut fermentation parameters (Table 4), the pH value of T25 was higher than
196 that in T0 ($P<0.05$); the NH_3-N content of T50 and T75 increased compared with T25
197 ($P<0.05$), there was no difference between T0 and the other three group treated with
198 BPS. Other indicators did not differ significantly between the four treatments.

199 *3.2. 16S rDNA Gene Sequencing and Analysis*

200 Miseq high-throughput sequencing was performed on the V3-V4 region of 16S
201 rDNA, and a total of 424,032 effective sequences were obtained, 26,502 for each
202 sample. According to high-throughput sequencing results, cluster analysis of OTUs was
203 conducted, and a total of 15 phyla, 26 classes, 39 orders, 67 families, 185 genera, 341
204 species, and 1356 OTUs were obtained. The Venn diagram of this study was shown in
205 Figure 1. As shown in the figure, the number of OTU that all the four treatments possess
206 was 1186, and it accounted for 87.46% of the total amount.

207 The rarefaction curve is commonly used to reflect the sequencing depth and
208 coverage of test samples. Figure 2 shows that the rarefaction curve of 16 samples
209 sequenced in this test did not enter the plateau phase when the number of sequencing
210 reads reached 25,000, indicating that the sequencing data volume cannot absolutely
211 represent all OTUs in the bacterial community of feces, and it is still possible to find
212 new OTUs by increasing the sequencing data volume. However, the growth rate of
213 rarefaction curve has slowed, suggesting a sufficient level of species richness.
214 According to the coverage curve in Figure 3, when the number of sequencing reads
215 reached 10,000, the coverage reached 97%, and when the number of sequencing reads
216 continued to grow to 25,000, the coverage was close to 100%.It shows that the depth
217 and coverage of sequencing data are reasonable and the measured data can be used for
218 subsequent analysis. The rank-abundance curve of fecal samples was shown in Figure
219 4. It can be seen from the figure that the microbial diversity of the 4 treatments is similar.
220 Shannon index, Ace index, and Chao index were calculated and the results were
221 shown in Table 5. As we can see from the table, there were no significant differences in
222 the above three indexes, indicating BPS had no remarkably effect on the diversity and
223 abundance of fecal bacteria community.
224 The composition of bacterial community on the phylum and genus levels were
225 shown in Figure 5. *Firmicutes* and *Bacteroidetes* were the two most dominant phyla,
226 accounting for more than 95% of the bacterial community. On the genus level, in
227 different treatments, *Ruminococcaceae_UCG-005*, *Ruminococcaceae_UCG-010*, and
228 *Rikenellaceae_RC9_gut_group* were always the first three dominant genera. With the

increasing of BPS content, some phyla, including *Firmicutes*, *Bacteroidetes*, *Tenericutes*, *Proteobacteria*, and *Verrucomicrobia*, and some genera, including *Paeniclostridium*, *Phocaeicola*, and *Norank_f_bacteroidales_BS11_gut_group*, became more abundant; while other genera, such as *Ruminococcaceae_UCG-013*, *Ruminococcaceae_UCG-010*, and *Ruminococcaceae_UCG-009* decrease.

3.3. Correlation between differential OTUs and various indicators

LDA score represented the influence of significantly different OTUs. The OTU, which its LDA score was greater than the set point, can be regarded as statistically significant biomarker. In the present study, the set point was 2, and 12 differential OTUs (Figure 6), including *Peptostreptococcaceae* (family), *Paeniclostridium* (genus), *Thermoanaerobacteraceae* (family), *Unclassified_f_peptoccaceae* (genus), *Tyzzerella* (genus), *Gelria* (genus), *Thermoanaerobacterales* (order), *Roseburia* (genus), *Alphaproteobacteria* (class), *Ruminococcaceeae* (family), *Saccharofermentants* (genus), and *Coprococcus_3* (genus) were obtained. Figure 6a was the LDA score distribution histogram, the different colors represented their respective groups; and the length represented the LDA score, which is the degree of influence of the differential OTUs between the four treatments. Figure 6b was the cladogram, the circle radiating from the inside to the outside represented the classification level from phylum to genus, and the diameter of the circle represented the relative abundance. The OTUs with no significant difference were uniformly colored yellow, and the differential OTUs followed the treatment.

In this study, differential OTUs were performed correlation analysis with serum

antioxidant and immune indicators, and hindgut fermentation parameters (Figure 7). For serum antioxidant indicators, *Thermoanaerobacteraceae*, *Thermoanaerobacterales*, and *Gelria* were positively correlated with MDA (C_{XY}=0.63, P=0.009; C_{XY}=0.63, P=0.009; C_{XY}=0.63, P=0.009, respectively); *Unclassified_f_peptoccaceae* was negatively correlated with CAT (C_{XY}=-0.53, P=0.04). For serum immune indicators, *Peptostreptococcaceae*, *Paeniclostridium*, *Thermoanaerobacteraceae*, *Gelria*, and *Thermoanaerobacterales* had positive correlation with IL-4 (C_{XY}=0.58, P=0.02; C_{XY}=0.56, P=0.03; C_{XY}=0.62, P=0.01; C_{XY}=0.62, P=0.01; C_{XY}=0.62, P=0.01, respectively); *Roseburia* had positive correlation with IL-6 (C_{XY}=0.58, P=0.02); *Saccharofermentants* had positive correlation with IL-1β and IL-6 (C_{XY}=0.56, P=0.03; C_{XY}=0.57, P=0.03, respectively); *Coprococcus_3* had positive correlation with IL-1β (C_{XY}=0.69, P=0.004); *Unclassified_f_peptoccaceae* had negative correlation with IL-10 (C_{XY}=-0.61, P=0.02); *Tyzzerella* had negative correlation with IL-2 (C_{XY}=-0.56, P=0.03); *Coprococcus_3* had negative correlation with IL-4 (C_{XY}=-0.57, P=0.03). For hindgut fermentation parameters, *Coprococcus_3* was positively correlated with propionic acid (C_{XY}=0.68, P=0.005); *Peptostreptococcaceae* was negatively correlated with acetic acid and propionic acid (C_{XY}=-0.55, P=0.03; C_{XY}=-0.61, P=0.02); *Paeniclostridium* was negatively correlated with propionic acid (C_{XY}=-0.57, P=0.03).

3.4. 16S function prediction

16S function prediction was performed via PICRUSt software, which stores a series of databases. By comparing information with different databases, COG information, KEGG Ortholog (KO) information, and pathway information of

differential OTUs can be obtained and matched. The abundance of functional category can be calculated according to the abundance of differential OTUs. The results of 16S function prediction were shown in Figures 8. As shown in Figure 8a, with the increaing of BPS content, the abundance of some COG functions, such as Inorganic ion transport and metabolism, Chromatin structure and dynamics, and Coenzyme transport and metabolism, increased; others, like Cell motility, Cytoskeleton, and Replication, recombination and repair, decreased. In this study, a total of 222 pathways were obtained, and they involved in metabolism, genetic information processing, environmental information processing, and cellular processes; and the first 20 abundant pathways were shown in Figure 8b.

4. Discussion

In the process of cell metabolism, the body will produce a large number of free radicals like reactive oxygen species (ROS), which have a strong oxidation ability. These free radicals have strong toxicity and cause damage to biological macromolecules such as carbohydrates, proteins, lipids, and DNA, and finally leading to oxidative stress and the recession of physiological function, immunity, and production performance, thus causing diseases (Thannickal and Fanburg 2000; Gill and Tuteja 2020). In order to respond to the adverse effects of oxidative stress, the anti-oxidative defence system (AOS) release various enzymes such as CAT, GSH-Px, and SOD (Prokić et al. 2018). Superoxide dismutase can convert superoxide anion radical ($\cdot O_2^-$) into hydrogen peroxide (H_2O_2), subsequently, H_2O_2 is broken into H_2O by CAT or GSH-Px (Olsvik et al. 2005). Malonaldehyde is the final product of lipid

peroxidation caused by ROS, the content of MDA can reflect the degree of lipid peroxidation and cell damage in the body, and the increase of MDA content marks the aggravation of cell damage (Esterbauer et al. 1991; Rio et al. 2005; Castillo et al. 2006). Total antioxidant capacity is a comprehensive indicator to measure the antioxidant capacity in vivo, reflecting the dynamic balance between pro-oxidants and anti-oxidants, as well as the free radical metabolism state (Ghiselli et al. 2000). In this study, the MDA content of T25, T50, and T75 are lower than T0, indicating that BPS can enhance the resistance of lipid peroxidation in experimental heifers. However, another study reported that replacing WSC with 5%, 10%, and 15% BPS can improve the anti-oxidant indicators (T-AOC, CAT, GSH-Px, SOD) remarkably (Si et al. 2018). The anti-oxidant capacity in the present study was not improved as the increasing of BPS content, which might due to the substitution ratio of BPS is not in a suitable range.

Interleukins (IL) are a group of cytokines which are mainly secreted by leukocytes, playing important roles in immune response and tissue repair. IL-1β is a pro-inflammatory cytokine which features in inflammatory and infectious diseases, leading immune cell recruitment and bacterial clearance eventually (Zhang et al. 2019). IL-4 is an anti-inflammatory cytokine, which is related to regulating immune cells, cancer and trophic responses (Granja et al. 2009; Shamoun et al. 2018). Oviedo-Boyso et al. (2007) found that when cows develop bacteria-induced inflammation, the levels of pro-inflammatory cytokines like IL-1β, IL-2 and IL-6 in the body increased, and the overexpression of pro-inflammatory factor caused tissue damage. A large number of studies showed that IL-4 and IL-10 can inhibit the secretion of pro-inflammatory

317 cytokines like IL-1β and IL-6 (Cawston et al. 1996; Mak and Saunders 2006; Borghaei
318 et al. 2010). The above findings suggest that an increase in IL-1β or a decrease in IL-4
319 may indicate inflammation. In the present study, the IL-1β content of T25, T50, and
320 T75 decreased significantly compared with T0; the IL-4 and IgA content of T25, T50,
321 and T75 increased, demonstrating that BPS has the potential to improve immunity of
322 heifers. The immunomodulatory function of *Broussonetia papyrifera* is derived from
323 its bioactive substances, similar reports had been reported in other woody forages like
324 *Moringa oleifera* and *Neolamarckia cadamba* (Pandey and Negi 2016; Valdivié-
325 Navarro et al. 2020).

326 Corn is a starch-rich feed material. Starch that has not been digested by rumen
327 microorganisms and small intestinal enzymes will enter the hindgut and continue to
328 ferment, leading to the decreasing of fecal pH value (Petri et al. 2019). Superfluous
329 fermentable carbohydrates can cause hindgut acidosis (Sulzberger et al. 2016). In the
330 present study, the fecal pH value of T0 is lower than the other three treatments,
331 presumably because the fermentable carbohydrates content of T0 is the highest among
332 the four treatments. VFAs mainly come from the decomposition of carbohydrates.
333 Rumen microorganisms can convert carbohydrates such as starch, cellulose, and
334 soluble sugar into pyruvate, which can be converted into different VFAs due to different
335 metabolic pathways. Moreover, VFAs play important roles in maintaining the integrity
336 of intestinal epithelial morphology and function (Sato et al. 2009; Missotten et al. 2010).
337 Fecal NH_3-N is derived from the hydrolysis of amino acids and proteins by proteolytic
338 enzyme and deaminase; and is correlated with N intake strongly (Weiss et al. 2009). In

addition, according to another research, when animals ingest diets containing high NDF, a large number of microorganisms related to fiber degradation in the rumen take advantage of NH_3-N as the main nitrogen source for metabolism, resulting in the decrease of NH_3-N in the rumen and intestinal track (Hristov and Ropp 2003). In this study, compared with T0, NH_3-N content in the other three treatments occurred no significant changes. In conclusion, the substitution of CS for BPS had no significant effect on the intestinal fermentation parameters of heifers.

Gastrointestinal tract is an important habitat for bacteria, intestinal bacteria play important roles in the health and growth of host. Learning the microbial community structure of feces is important for reducing foodborne pathogens through dietary changes. In the present study, no significant differences were presented in Shannon index, Ace index, and Chao index, indicating the BPS had no obvious effect on α-diversity, and the result is similar to another two studies. Sun et al. (2017) and Li et al. (2017) fed cows and steers with ensiled *M. oleifera* and ensiled mulberry leaves respectively, finding that there were no conspicuous differences on α-diversity. According to Figure 1, 87.46% of OTU in the four treatments is uniform. The results above demonstrate that the component of coarse fodder cannot affect the fecal bacterial community significantly.

On the phylum level, the dominant phyla is *Firmicutes* and *Bacteroidetes*, according to a report, the two phyla are predominant in the large intestine of numerous mammals such as human being, ruminant, pig, and mouse (Ban-Tokuda et al. 2017). *Firmicutes* and *Bacteroidetes* contain various carbohydrate utilizing enzymes, play a

vital part in fiber degradation (Xu et al. 2019). The addition of BPS has no effect on the species of dominant phyla in feces, but the relative abundance of dominant phyla can be improved. *Verrucomicrobia* is the third dominant phylum, with the increasing of BPS content, the relative abundance of this phylum improves accordingly. A previous study found that *Verrucomicrobia* has the potential to induce and regulate immunity, it may be a target of intestinal microbial intervention to improve the regulation of immunity (Lindenberg et al. 2019). The increasing of *Verrucomicrobia* may be associated with improved intestinal immunity. On the genus level, the dominant phyla is *Ruminococcaceae_UCG-005*, *Ruminococcaceae_UCG-010*, and *Rikenellaceae_RC9_gut_group*. The result is different from another two studies (Li et al. 2012; Zhao et al. 2017). In addition, the dominants of different treatments in this study present significant difference, indicating dietary has a great influence on the genus level of fecal bacterial community of heifers (Kim et al. 2014). According to Li et al. (2018), with the severity of diarrhea, the relative abundance of *Ruminococcaceae_UCG-005*, and *Rikenellaceae_RC9_gut_group* in the musk deer feces decrease, in this study, the relative abundance of above two genera in T25 is higher than that in T0, T50, and T75, indicating that moderate BPS may be able to relieve diarrhea, however, this deserves further study.

LEfSe analysis is an analytical tool for the discovery and interpretation of high-dimensional data biomarker. It emphasizes statistical significance and biological correlation, and was able to look for biomarkers that differed statistically from group to group. In this study, 12 differential OTUs including one class, one order, three

families, and seven genera are obtained. *Paeniclostridium* is considered a potential pathogen, it may be related to soft tissue infection and toxic shock (Kim et al. 2017). *Gelria* (*Thermoanaerobacterales* Order, *Thermoanaerobacteraceae* Family) is known to be related to the metabolism of VFAs (FitzGerald et al. 2019). *Roseburia* is a common butyrate-producing bacteria in the intestinal tract (Hatziioanou et al. 2013; Sheridan et al. 2019). *Ruminococcaceae* are common in both rumen and hindgut of ruminants, playing important parts in degrading starch and cellulose (Zhang et al. 2020). In another study, Han et al. (2018) found that *Ruminococcaceae* is related to the balance of Treg/Th17, suggesting that *Ruminococcaceae* may be related to the immune system. Reports and researches about function of the other differential OTUs is limited, however, these differential OTUs can be investigated as biomarkers in depth. As we can see in Figure 5a, *Gelria* is positively correlated with MDA content, and shows a weak negative correlation with SOD, GSH-Px, and T-AOC, thus, it can be researches as a potential antioxidant biological target. Another thing worth noting is shown in Figure 5b, *Coprococcus_3* is positively correlated with IL-1β while negatively correlated with IL-4. We have described in the preceding text that IL-1β is a proinflammatory cytokine, and IL-4 can inhibit its secretion. Therefore, it can be speculated that the increasing of *Coprococcus_3* indicates the occurrence of inflammation.

PICRUSt is an analysis method in order to predict the gene function profile of archaea and bacteria based on the measured bacterial genome of 16S rDNA sequence. As shown in Figure 6a, BPS inhibits carbohydrate transport and metabolism, and promotes amino acid transport and metabolism; the metabolites of carbohydrate and

amino acid in the hindgut is VFA and NH_3-N, respectively. Presumably, the change of bacterial community causes the change of gene function abundance, thereby causing the change of metabolites. As shown in Figure 6b, the abundance of Methane metabolism decreases with the increasing of BPS content, may indicating BPS can reduce methane production; and this confirm the previous point of view.

In conclusion, this research reveals that replace CS with a certain proportion of BPS is beneficial to the health of Holstein heifers; and the BPS content has no impact on the dominant phyla of fecal bacterial community. In-depth research, 12 differential OTUs are obtained in the four treatments, and they have correlation with some indicators of serum and hindgut to some extent. These differential OTUs can be researched as potential biomarkers in order to observe the changes in healthy status. Finally, we find that BPS changes the abundance of genes and pathways related to various life activities. Presumably, BPS can affect serum indicators and hindgut fermentation because of the changing of fecal bacterial community.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The sequences in this study were submitted to the Sequence Read Archive (SRA) and a BioProject number PRJNA594421 was obtained.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HT, YC, BS, NZ and DL conceived and designed the study; HT, YC, NZ, YG, MD and GL performed the experiments; YL and MD organized the database and performed the statistical analysis; and HT, YC wrote the manuscript.

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675 **Figure Legends**

676 **Figure 1** Venn map of operational taxonomic units (OTU) in the four treatment. T0:
677 BPS replaces 0% of CS; T25: BPS replaces 25% of CS; T50: BPS replaces 50% of CS;
678 T75: BPS replaces 75% of CS. BPS, Broussonetia papyrifera silage; CS, whole corn
679 silage.

680 **Figure 2** Rarefaction curves of feces samples.

681 **Figure 3** Coverage curves of feces samples.

682 **Figure 4** Rank-abundance curves of feces samples.

683 **Figure 5** Relative abundance of fecal bacterial community on the phylum and genus
684 level. Figure 5a is the relative abundance on the phylum level; Figure 5b is the relative
685 abundance on the genus level. T0: BPS replaces 0% of CS; T25: BPS replaces 25% of
686 CS; T50: BPS replaces 50% of CS; T75: BPS replaces 75% of CS. Numbers 1, 2, 3 and
687 4 in names of samples refer to individual heifers per set of treatment. BPS, *Broussonetia*
688 *papyrifera* silage; CS, whole corn silage.

689 **Figure 6** Comparison of microbial variations using the LEfSe online tool. T0: BPS
690 replaces 0% of CS; T25: BPS replaces 25% of CS; T50: BPS replaces 50% of CS; T75:

691 BPS replaces 75% of CS. Numbers 1, 2, 3 and 4 in names of samples refer to individual
692 heifers per set of treatment. BPS, *Broussonetia papyrifera* silage; CS, whole corn silage.
693 **Figure 7** Correlation analyses of differential OTUs with serum anti-oxidant indicators
694 (7a), serum immune indicators (7b), and hindgut fermentation parameters (7c). Each
695 cell contains Pearson correlation coefficient and P-value (within brackets). Figure 7a:
696 MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase;
697 CAT, catalase; T-AOC, total antioxidant capacity. Figure 7b: Ig, immunoglobulin; IL,
698 interleukin. Figure 7c: AA, acetic acid; PA, propionic acid; IBA, isobutyric acid; BA,
699 butyric acid; IVA, isovaleric acid; VA, valeric acid; NH3-N, ammoniacal nitrogen.
700 **Figure 8** Heatmap of 16S rDNA gene-predicted functional (8a) and pathway-predicted
701 (8b) profiles obtained via Phylogenetic Investigation of Communities by
702 Reconstruction of Unobserved States (PICRUSt). T0: BPS replaces 0% of CS; T25:
703 BPS replaces 25% of CS; T50: BPS replaces 50% of CS; T75: BPS replaces 75% of
704 CS. Numbers 1, 2, 3 and 4 in names of samples refer to individual heifers per set of
705 treatment. BPS, *Broussonetia papyrifera* silage; CS, whole corn silage.

706 Table 1 The nutrient content of CS and BPS

Item	CS	BPS
DM	30.50	30.86
CP (%/DM)	10.70	14.34
EE (%/DM)	3.30	2.33
NDF (%/DM)	60.10	49.23
ADF (%/DM)	38.90	31.21
Ash (%/DM)	11.37	11.52

707 CS, whole corn silage; BPS, *Broussonetia papyrifera* silage; DM, dry matter; CP, crude

708 protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber.

709 Table 2 The ingredient and nutrient content of TMRs

Item[1]	Dietary treatment[2]			
	T0	T25	T50	T75
Ingredient (as fed-basis %)				
Hay	13.07	13.14	13.22	13.29
Soybean meal	3.40	3.07	2.91	2.66
Salt	0.17	0.18	0.18	0.18
Mineral premix[3]	0.09	0.09	0.09	0.09
Indian meal	2.61	2.41	1.98	1.77
DDGS	2.26	2.28	2.29	2.30
Brewer's grains	8.71	8.76	8.81	8.86
BPS	0.00	17.52	35.26	53.14
CS	69.69	52.55	35.26	17.71
Nutrient content				
CP (%DM)	13.44	14.92	13.30	15.44
EE (%DM)	2.10	1.89	1.91	1.94
NDF (%DM)	51.48	50.46	50.14	50.34
ADF (%DM)	30.02	31.32	30.20	30.78
HC (%DM)	21.46	19.14	19.94	19.56
Ca (%DM)	0.78	0.79	0.80	0.78
P (%DM)	0.45	0.43	0.43	0.44
RUP (%CP)	42.27	42.65	43.22	43.59

ME (mcal/kg DM)	1.86	1.87	1.88	1.89

[1] DDGS, distillers dried grains with solubles; BPS, *Broussonetia papyrifera* silage; CS, whole corn silage; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; HC, hemicellulose; RUP, rumen undegradable protein; ME, metabolizable energy.

[2] T0, BPS replaces 0% of CS; T25, BPS replaces 25% of CS; T50, BPS replaces 50% of CS; T75, BPS replaces 75% of CS. BPS, *Broussonetia papyrifera* silage; CS, whole corn silage.

[3] Mineral premix provided the following per kg of concentrate, vitamin A, 120-180 KIU; vitamin D3, 40-60 KIU; vitamin E, ≥1,102 mg; Cu, 459-613 mg; Mn, 918-1,225 mg; Zn, 1,840-2,455 mg; Se, 12-18 mg; I, 22.5-30 mg; Co, 5.4-7.34 mg.

720 Table 3 Effects of BPS on the serum indicators of Holstein heifers (n=4)

Item[1]	Dietary treatment[2]				MSE[3]	P-value		
	T0	T25	T50	T75		linear	quadratic	cubic
Serum anti-oxidant indicators								
MDA (mmol/L)	2.61[a]	2.24[ab]	1.79[b]	2.34[ab]	0.20	0.018	0.675	0.242
SOD (U/mL)	88.90	88.93	87.48	85.46	2.68	0.332	0.708	0.942
GSH-Px (U/mL)	58.45	50.31	44.50	43.63	7.40	0.134	0.631	0.941
CAT (U/mL)	0.47	0.50	0.37	0.42	0.05	0.286	0.827	0.161
T-AOC (mmol/L)	0.46	0.41	0.43	0.41	0.02	0.128	0.513	0.221
Serum immune indicators								
IgA (g/L)	0.11[b]	0.13[a]	0.14[a]	0.14[a]	0.01	0.019	0.224	0.774
IL-1β (ng/L)	46.86[a]	45.61[a]	38.77[b]	40.08[b]	2.20	0.011	0.572	0.210

IL-2 (ng/L)	292.84	290.91	281.16	252.76	15.47	0.054	0.974	0.718
IL-4 (ng/L)	77.99^{b}	83.62^{b}	93.19^{ab}	106.34^{a}	5.21	0.001	0.482	0.988
IL-6 (ng/L)	11.39	10.36	10.46	9.88	0.87	0.263	0.796	0.657
IL-10 (ng/L)	43.93^{ab}	48.03^{a}	43.90^{ab}	41.44^{b}	1.90	0.122	0.483	0.558
IL-17 (ng/L)	56.05	51.56	45.33	52.55	3.29	0.254	0.097	0.341

721 [1] MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; T-AOC, total antioxidant capacity; Ig,
722 immunoglobulin A; IL, interleukin.

723 [2] T0, BPS replaces 0% of CS; T25, BPS replaces 25% of CS; T50, BPS replaces 50% of CS; T75, BPS replaces 75% of CS. BPS, *Broussonetia*
724 *papyrifera* silage; CS, whole corn silage. Different letters mean significant difference.

725 [3] MSE, mixed standard error.

726

727 Table 4 Effects of BPS on the hindgut fermentation parameters of Holstein heifers (n=4)

Item[1]	Dietary treatment[2]				MSE[3]	P-value		
	T0	T25	T50	T75		linear	quadratic	cubic
pH	6.79[a]	7.18[b]	7.02[ab]	7.05[bc]	0.10	0.174	0.091	0.146
Acetic acid (mmol/L)	12.26	11.48	11.34	11.00	1.24	0.470	0.858	0.887
Propionic acid (mmol/L)	2.51	2.17	2.28	2.17	0.26	0.432	0.655	0.584
Isovaleric acid (mmol/L)	0.34	0.35	0.39	0.37	0.03	0.304	0.566	0.481
Butyric acid (mmol/L)	1.14	0.87	0.79	0.98	0.16	0.428	0.173	0.906
Isovaleric acid (mmol/L)	0.28	0.29	0.32	0.31	0.02	0.212	0.711	0.731
Valeric acid (mmol/L)	0.20	0.18	0.18	0.21	0.03	0.830	0.286	0.948
NH_3-N (mg/dL)	5.52[abc]	4.47[a]	5.40[b]	5.98[bc]	0.45	0.250	0.093	0.291

728 [1] NH_3-N, ammoniacal nitrogen.

729 [2] T0, BPS replaces 0% of CS; T25, BPS replaces 25% of CS; T50, BPS replaces 50% of CS; T75, BPS replaces 75% of CS. BPS, *Broussonetia*

730 *papyrifera* silage; CS, whole corn silage. Different letters mean significant difference.

731 [3] MSE, mixed standard error.

732

733

734 Table 5 Effects of BPS on the α-diversity of hindgut bacterial community of Holstein heifers (n=4)

Item	Dietary treatment[1]				MSE[2]	P-value		
	T0	T25	T50	T75		linear	quadratic	cubic
Shannon	5.64	5.58	5.48	5.56	0.06	0.603	0.227	0.155
Ace	1144.55	1145.67	1143.72	1153.51	24.18	0.794	0.860	0.978
Chao	1150.60	1154.06	1147.21	1165.92	25.20	0.647	0.767	0.964

735 [1] T0, BPS replaces 0% of CS; T25, BPS replaces 25% of CS; T50, BPS replaces 50% of CS; T75, BPS replaces 75% of CS. BPS, *Broussonetia*

736 *papyrifera* silage; CS, whole corn silage.

737 [2] MSE, mixed standard error.

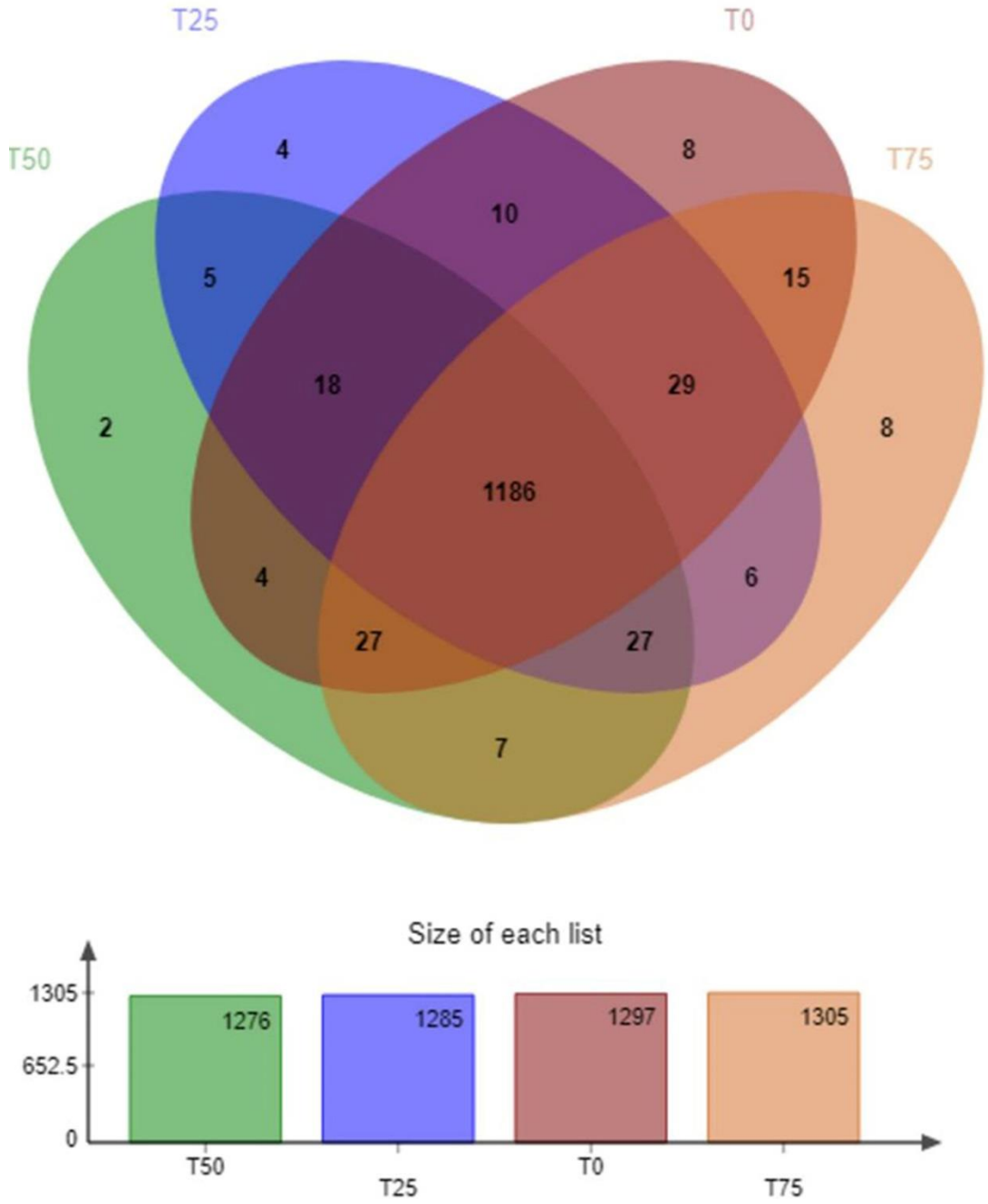

T25
T0
T50
T75
4
8
10
5
15
18
29
2
8
1186
4
6
27
27
7
Size of each list
1305
652.5
0
1276
1285
1297
1305
T50
T25
T0
T75

Figure 1

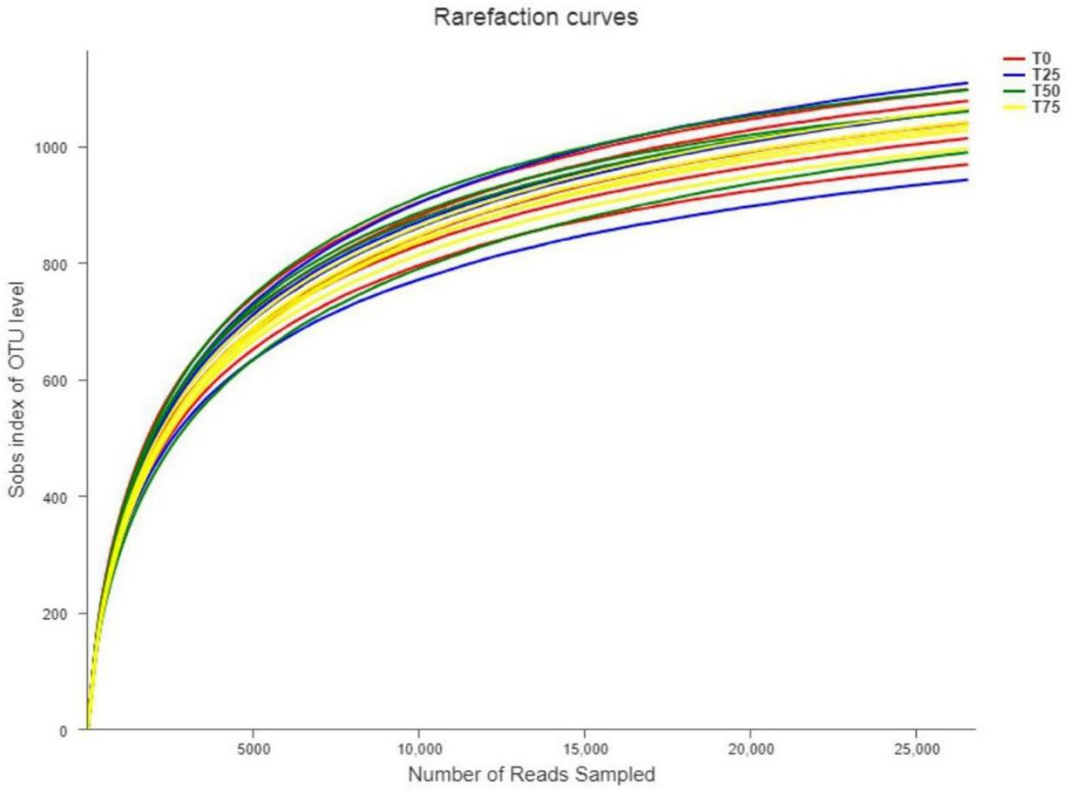

Rarefaction curves
T0
T25
T50
T75
Sobs index of OTU level
0
200
400
600
800
1000
5000
10,000
15,000
20,000
25,000
Number of Reads Sampled

Figure 2

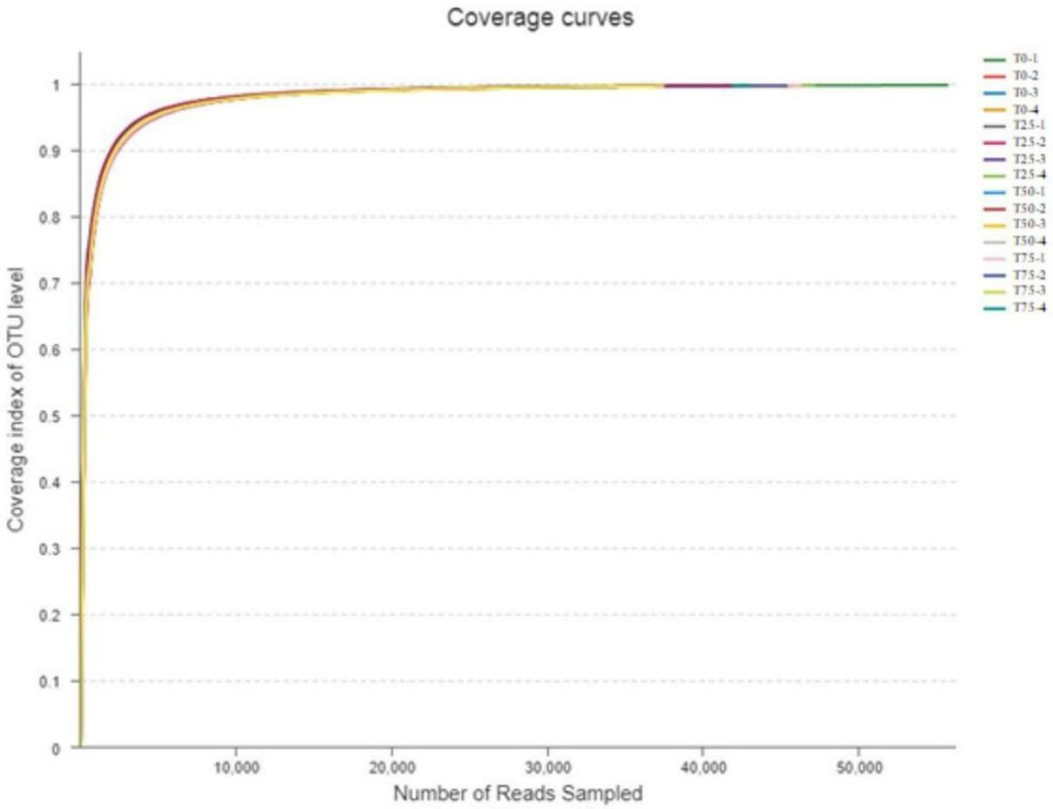

Coverage curves
Coverage index of OTU level
Number of Reads Sampled
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
10,000
20,000
30,000
40,000
50,000
T0-1
T0-2
T0-3
T0-4
T25-1
T25-2
T25-3
T25-4
T50-1
T50-2
T50-3
T50-4
T75-1
T75-2
T75-3
T75-4

Figure 3

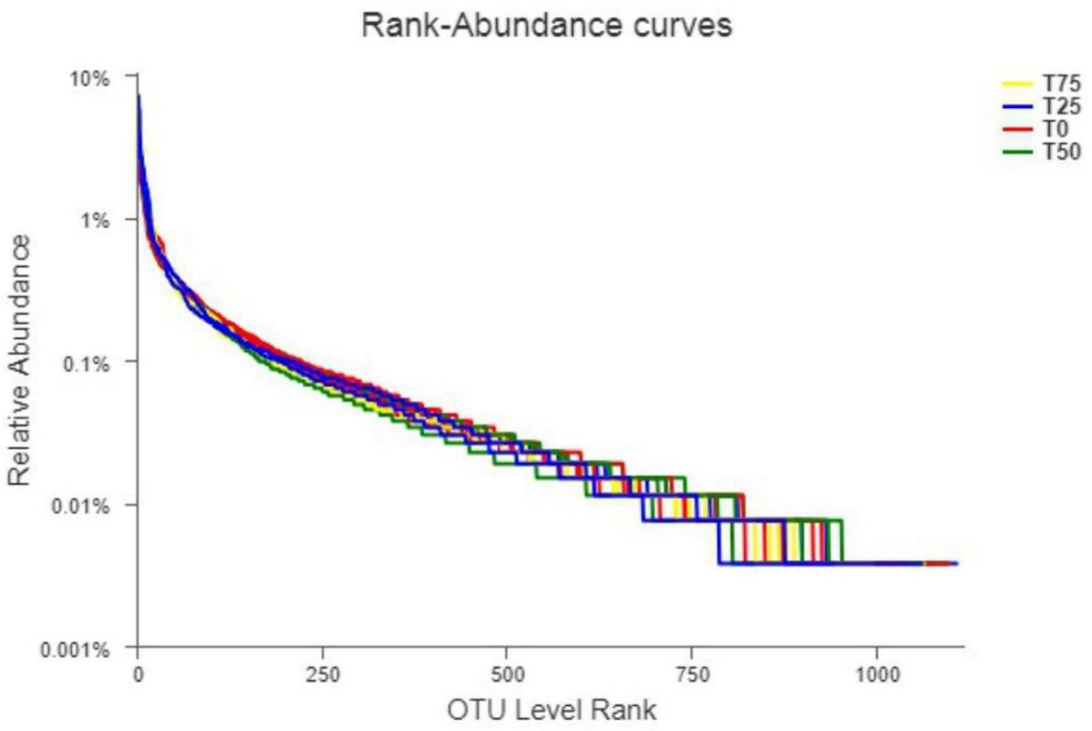

Rank-Abundance curves
10%
1%
0.1%
0.01%
0.001%
Relative Abundance
0
250
500
750
1000
OTU Level Rank
T75
T25
T0
T50

Figure 4

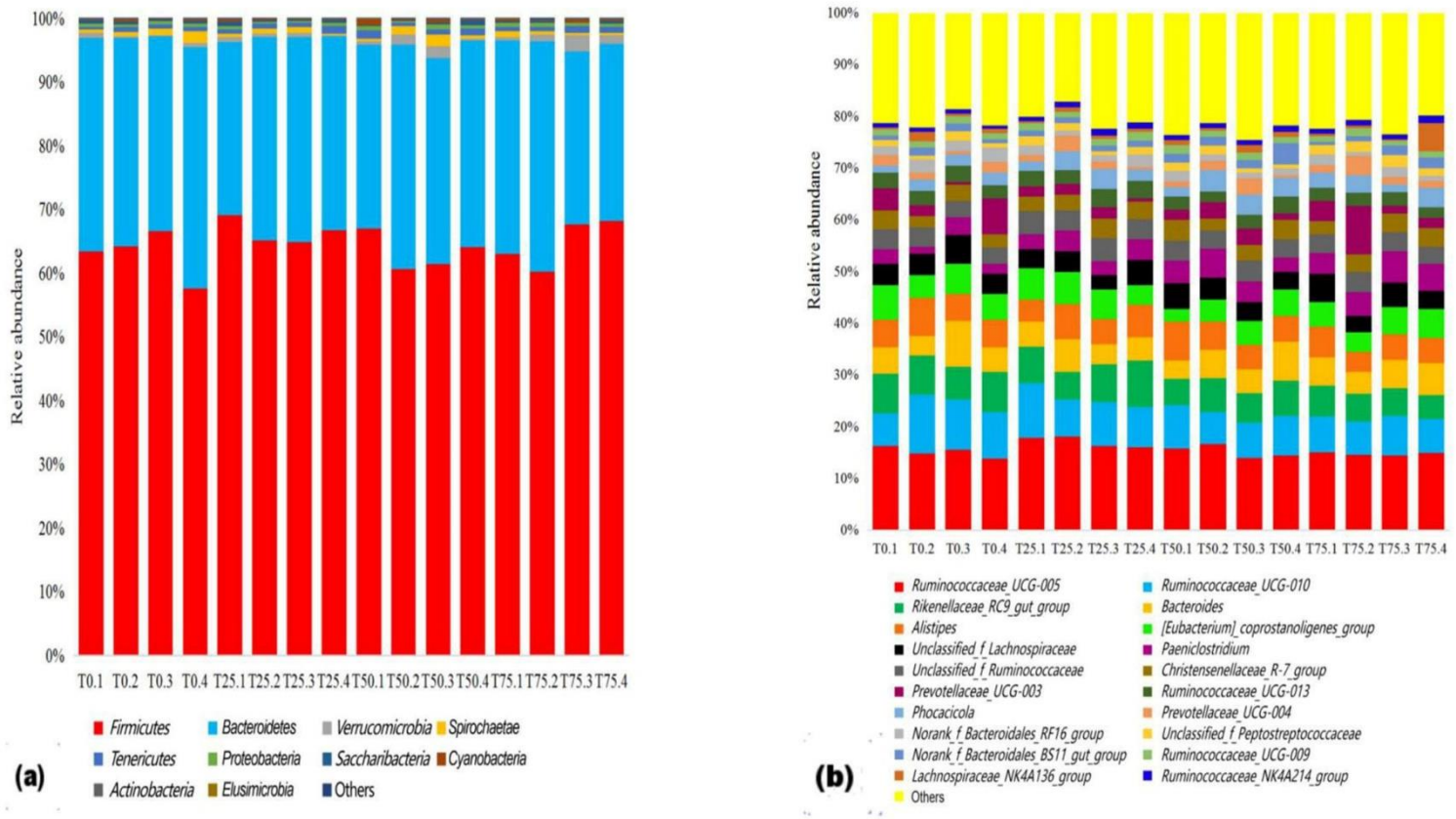

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
T0.1 T0.2 T0.3 T0.4 T25.1 T25.2 T25.3 T25.4 T50.1 T50.2 T50.3 T50.4 T75.1 T75.2 T75.3 T75.4
Firmicutes
Bacteroidetes
Verrucomicrobia
Spirochaetae
Tenericutes
Proteobacteria
Saccharibacteria
Cyanobacteria
Actinobacteria
Elusimicrobia
Others
(a)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
T0.1 T0.2 T0.3 T0.4 T25.1 T25.2 T25.3 T25.4 T50.1 T50.2 T50.3 T50.4 T75.1 T75.2 T75.3 T75.4
Ruminococcaceae_UCG-005
Ruminococcaceae_UCG-010
Rikenellaceae_RC9_gut_group
Bacteroides
Alistipes
[Eubacterium]_coprostanoligenes_group
Unclassified_f_Lachnospiraceae
Paeniclostridium
Unclassified_f_Ruminococcaceae
Christensenellaceae_R-7_group
Prevotellaceae_UCG-003
Ruminococcaceae_UCG-013
Phocaicola
Prevotellaceae_UCG-004
Norank_f_Bacteroidales_RF16_group
Unclassified_f_Peptostreptococcaceae
Norank_f_Bacteroidales_BS11_gut_group
Ruminococcaceae_UCG-009
Lachnospiraceae_NK4A136_group
Ruminococcaceae_NK4A214_group
Others
(b)

Figure 5

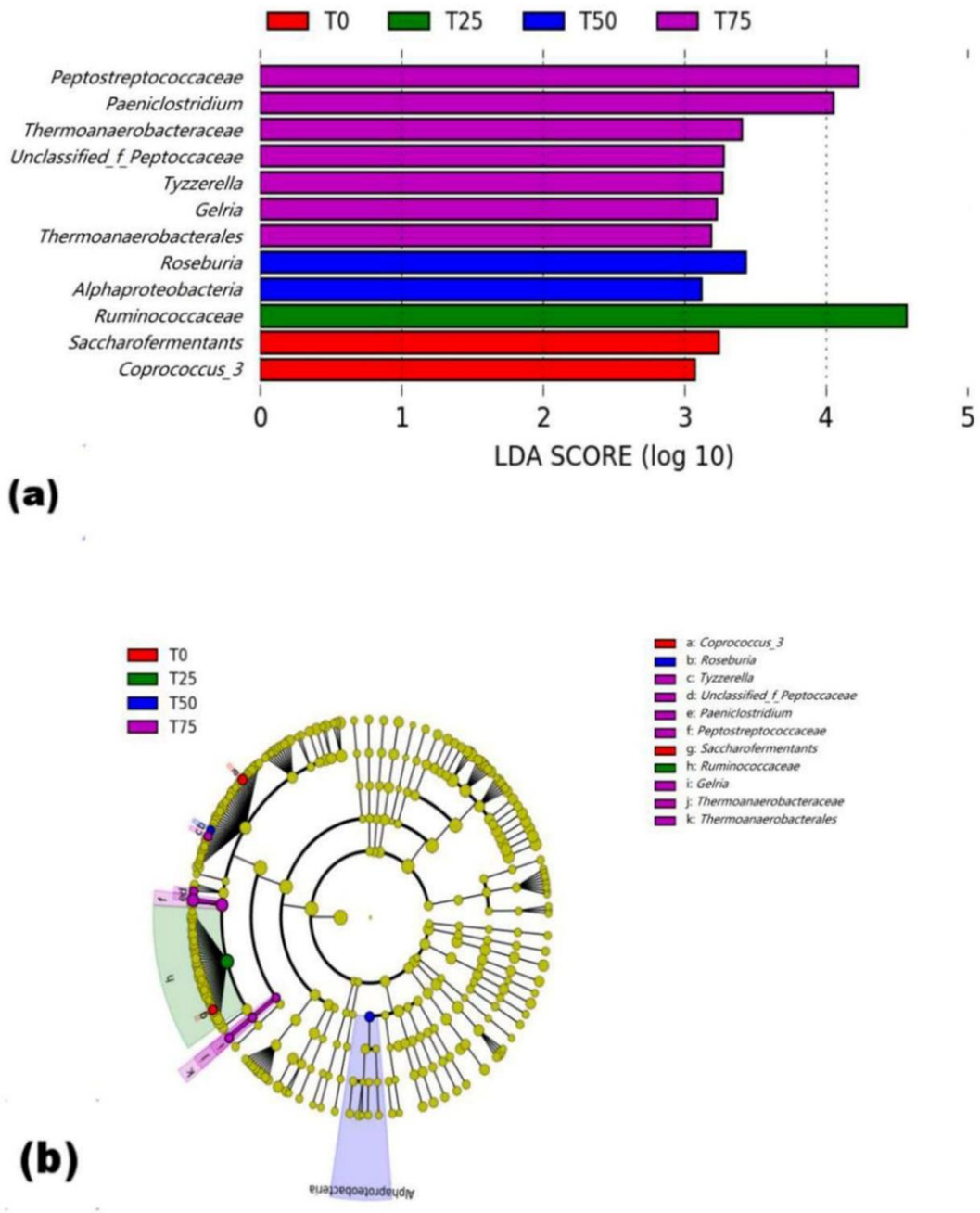

T0
T25
T50
T75
Peptostreptococcaceae
Paeniclostridium
Thermoanaerobacteraceae
Unclassified_f_Peptoccaceae
Tyzzerella
Gelria
Thermoanaerobacterales
Roseburia
Alphaproteobacteria
Ruminococcaceae
Saccharofermentants
Coprococcus_3
0
1
2
3
4
5
LDA SCORE (log 10)
(a)
T0
T25
T50
T75
a: Coprococcus_3
b: Roseburia
c: Tyzzerella
d: Unclassified_f_Peptoccaceae
e: Paeniclostridium
f: Peptostreptococcaceae
g: Saccharofermentants
h: Ruminococcaceae
i: Gelria
j: Thermoanaerobacteraceae
k: Thermoanaerobacterales
(b)

Figure 6

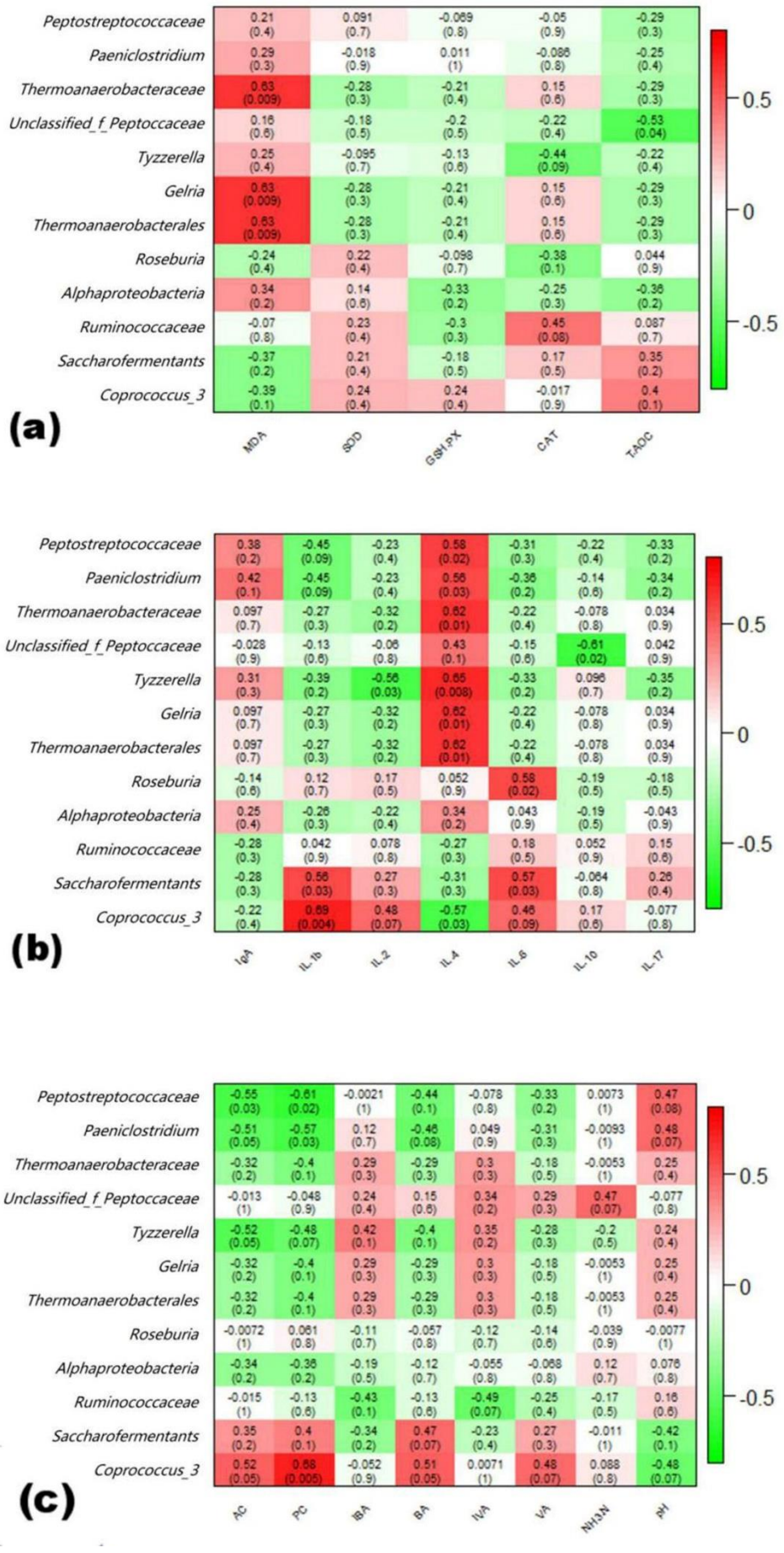
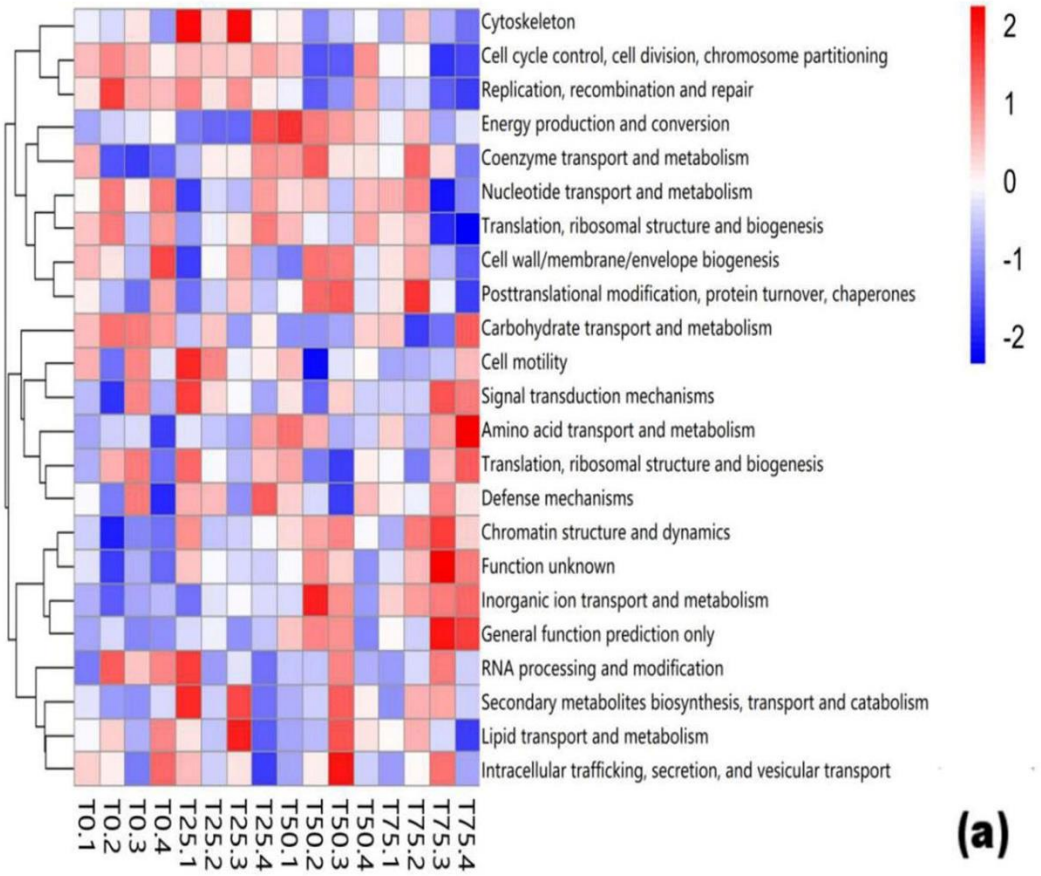

Peptostreptococcaceae
Paeniclostridium
Thermoanaerobacteraceae
Unclassified_f_Peptoccaceae
Tyzzerella
Gelria
Thermoanaerobacterales
Roseburia
Alphaproteobacteria
Ruminococcaceae
Saccharofermentants
Coprococcus_3
MDA
SOD
GSH-PX
CAT
TAOC
(a)
IgA
IL-1b
IL-2
IL-4
IL-6
IL-10
IL-17
(b)
AC
PC
IBA
BA
IVA
VA
NH3-N
pH
(c)
0.5
0
-0.5

Figure 7


Cytoskeleton
Cell cycle control, cell division, chromosome partitioning
Replication, recombination and repair
Energy production and conversion
Coenzyme transport and metabolism
Nucleotide transport and metabolism
Translation, ribosomal structure and biogenesis
Cell wall/membrane/envelope biogenesis
Posttranslational modification, protein turnover, chaperones
Carbohydrate transport and metabolism
Cell motility
Signal transduction mechanisms
Amino acid transport and metabolism
Translation, ribosomal structure and biogenesis
Defense mechanisms
Chromatin structure and dynamics
Function unknown
Inorganic ion transport and metabolism
General function prediction only
RNA processing and modification
Secondary metabolites biosynthesis, transport and catabolism
Lipid transport and metabolism
Intracellular trafficking, secretion, and vesicular transport
T0.1
T0.2
T0.3
T0.4
T25.1
T25.2
T25.3
T25.4
T50.1
T50.2
T50.3
T50.4
T75.1
T75.2
T75.3
T75.4
2
1
0
-1
-2
(a)

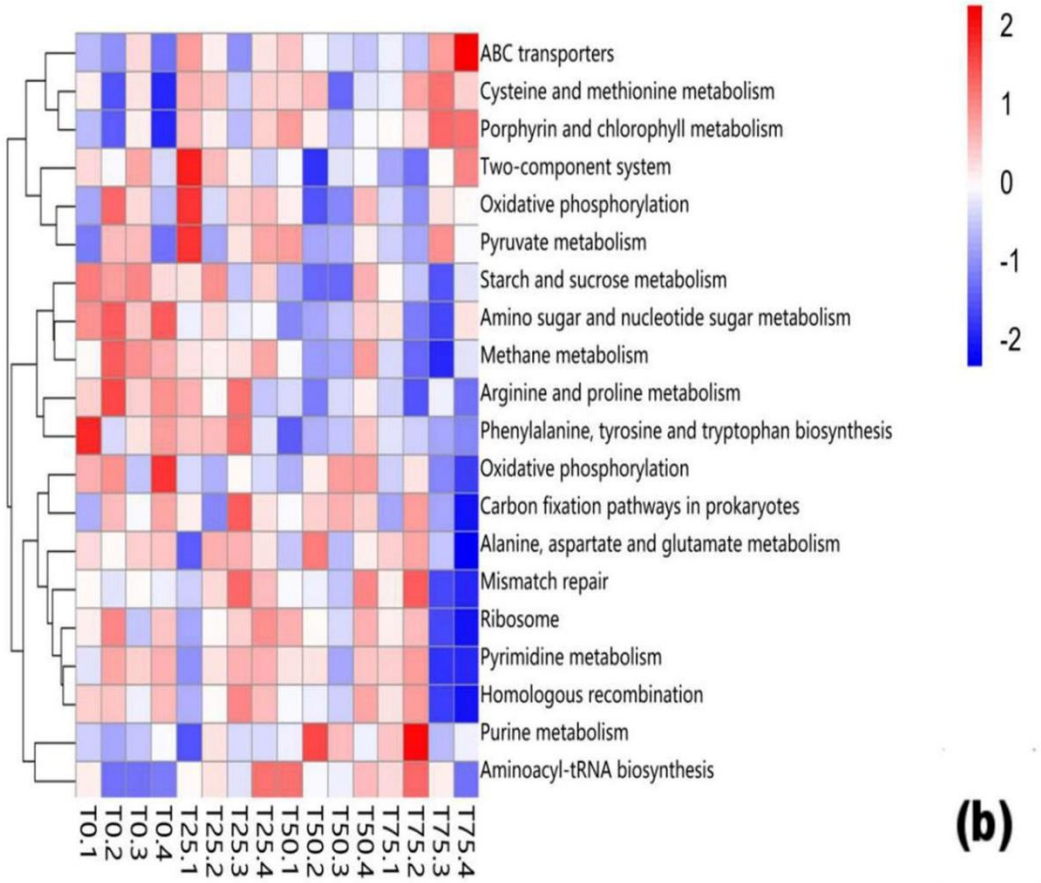

ABC transporters
Cysteine and methionine metabolism
Porphyrin and chlorophyll metabolism
Two-component system
Oxidative phosphorylation
Pyruvate metabolism
Starch and sucrose metabolism
Amino sugar and nucleotide sugar metabolism
Methane metabolism
Arginine and proline metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
Oxidative phosphorylation
Carbon fixation pathways in prokaryotes
Alanine, aspartate and glutamate metabolism
Mismatch repair
Ribosome
Pyrimidine metabolism
Homologous recombination
Purine metabolism
Aminoacyl-tRNA biosynthesis
T0.1
T0.2
T0.3
T0.4
T25.1
T25.2
T25.3
T25.4
T50.1
T50.2
T50.3
T50.4
T75.1
T75.2
T75.3
T75.4
2
1
0
-1
-2
(b)

Figure 8

Figures

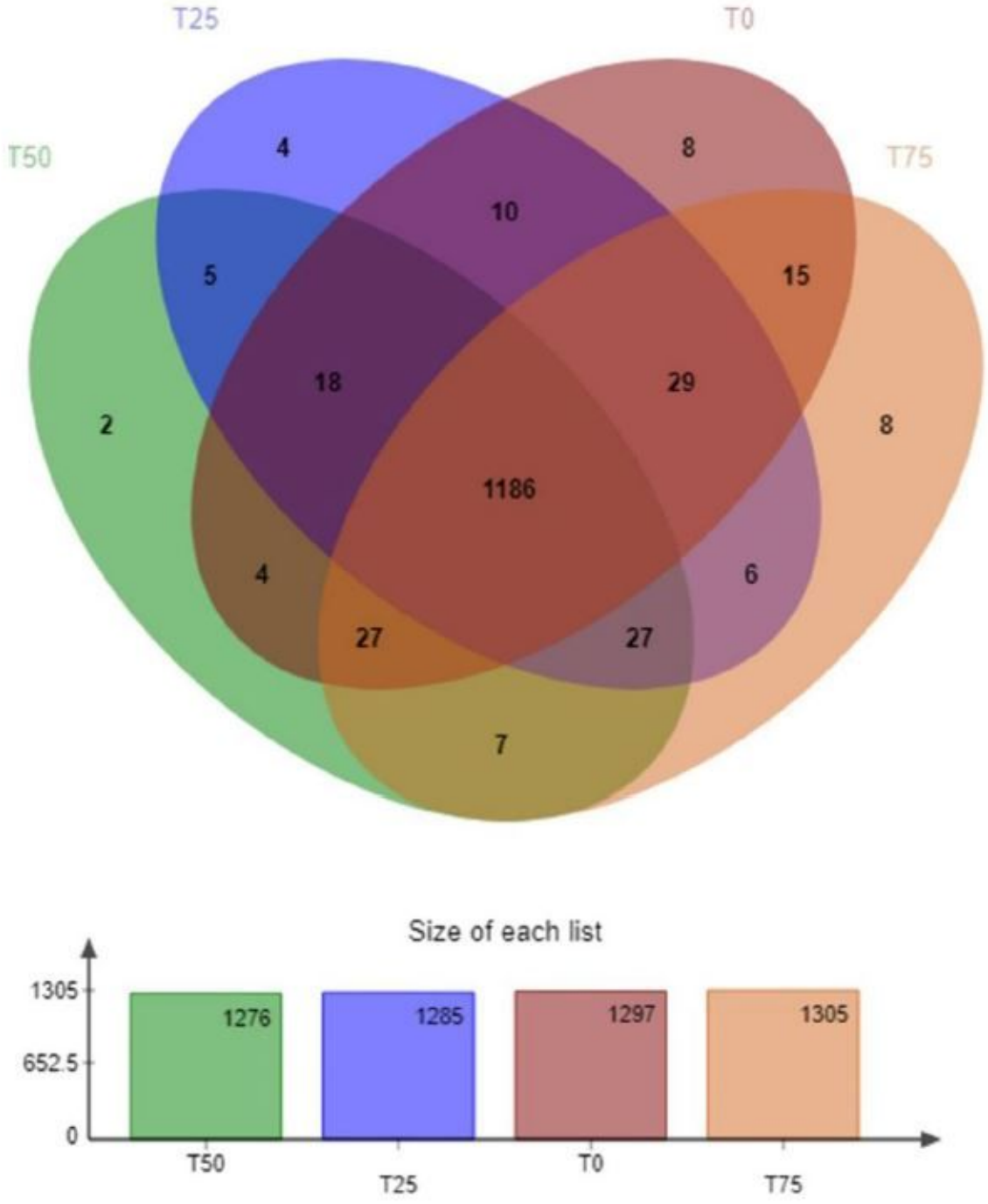


Figure 1

Figure 1

Venn map of operational taxonomic units (OTU) in the four treatment. T0: BPS replaces 0% of CS; T25: BPS replaces 25% of CS; T50: BPS replaces 50% of CS; T75: BPS replaces 75% of CS. BPS, Broussonetia papyrifera silage; CS, whole corn silage.

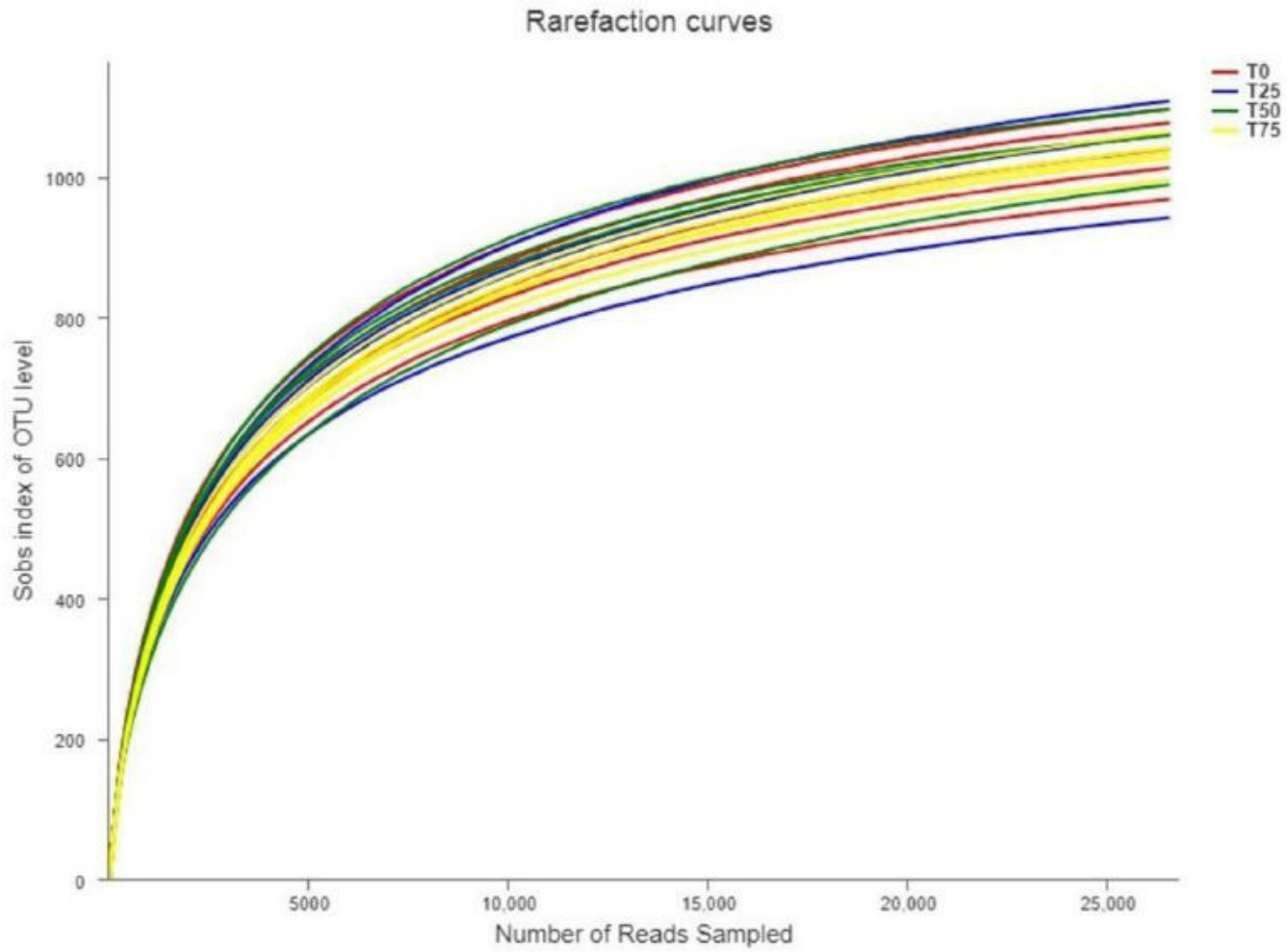


Figure 2

Figure 2

Rarefaction curves of feces samples.

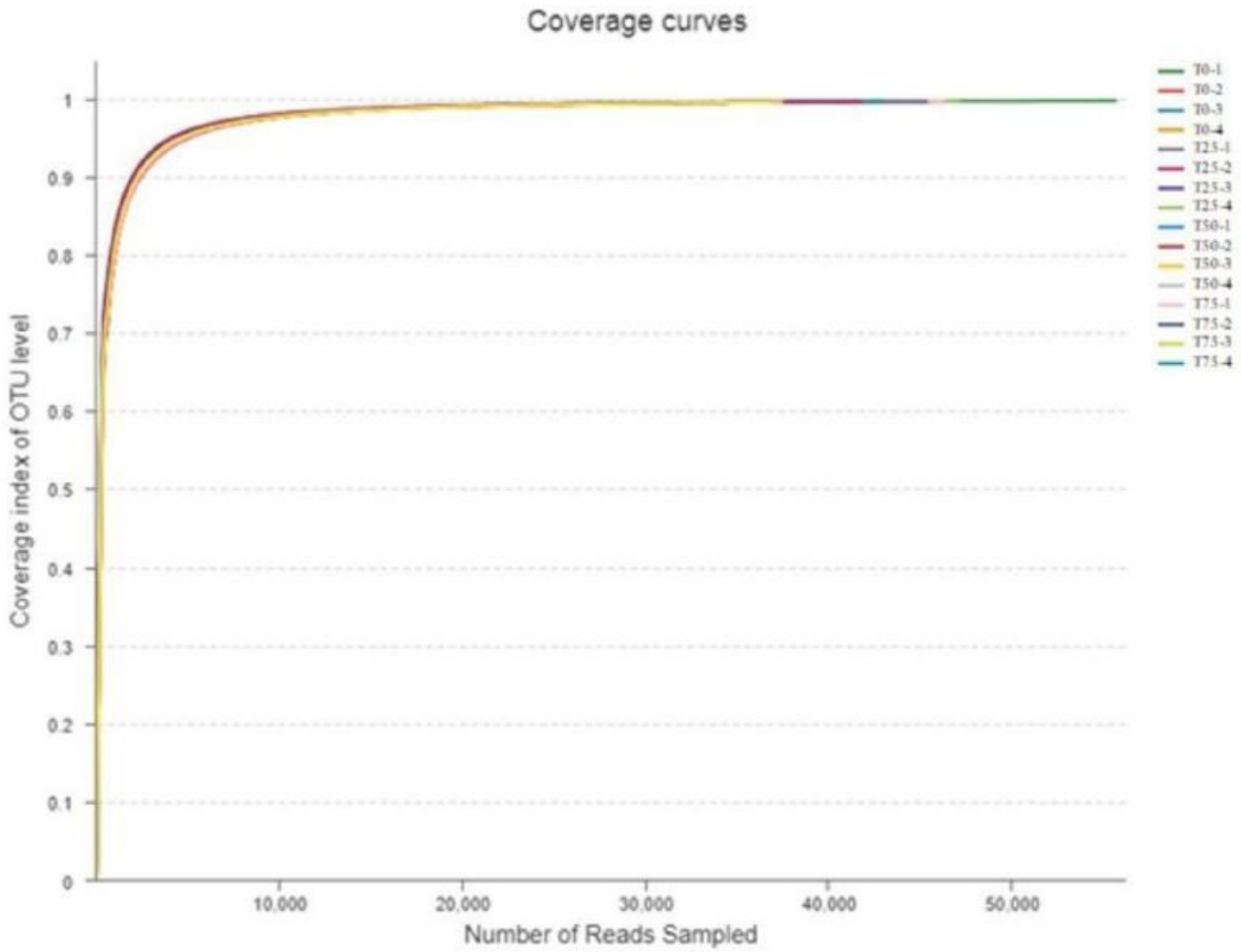


Figure 3

Figure 3

Coverage curves of feces samples.

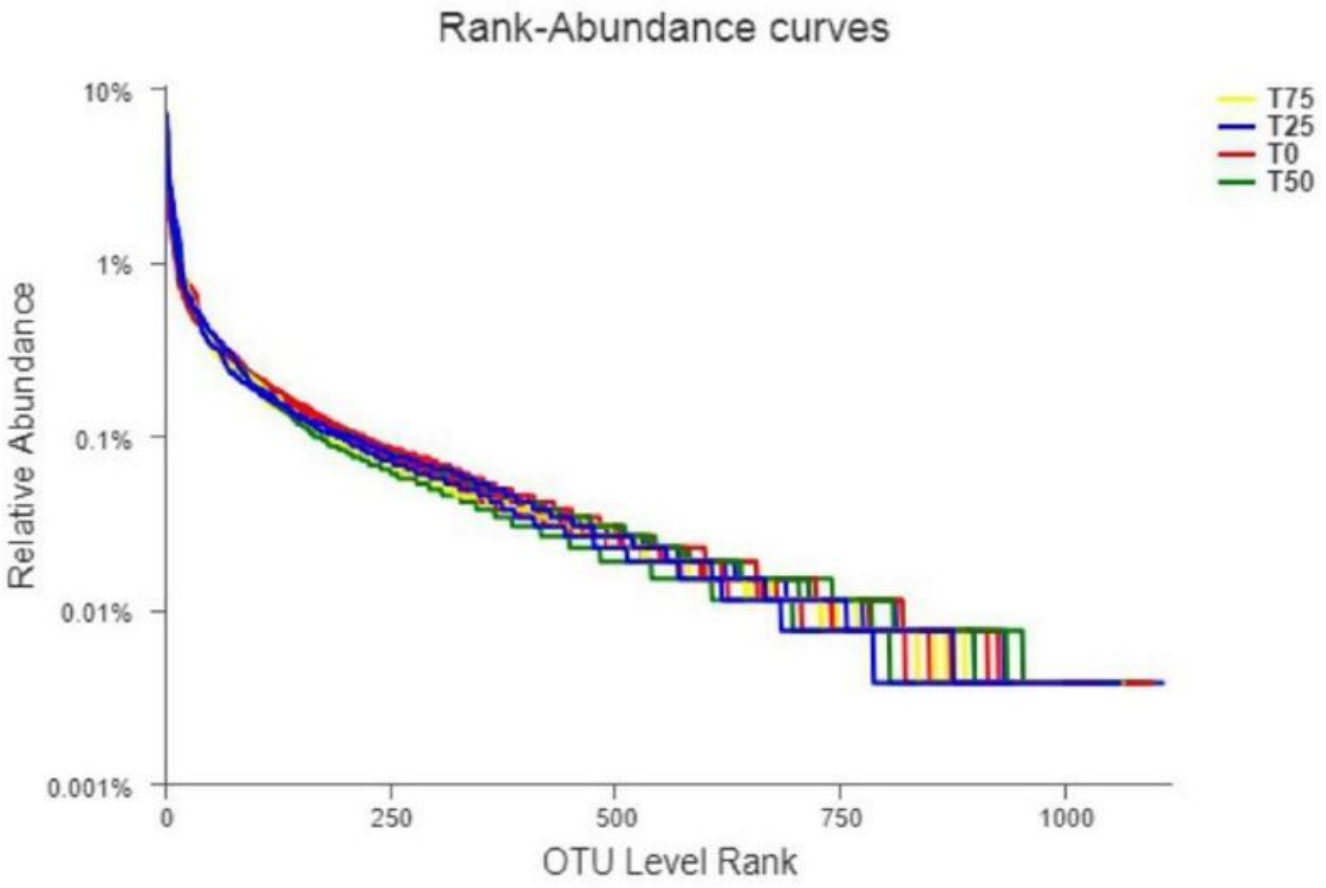


Figure 4

Figure 4

Rank-abundance curves of feces samples.

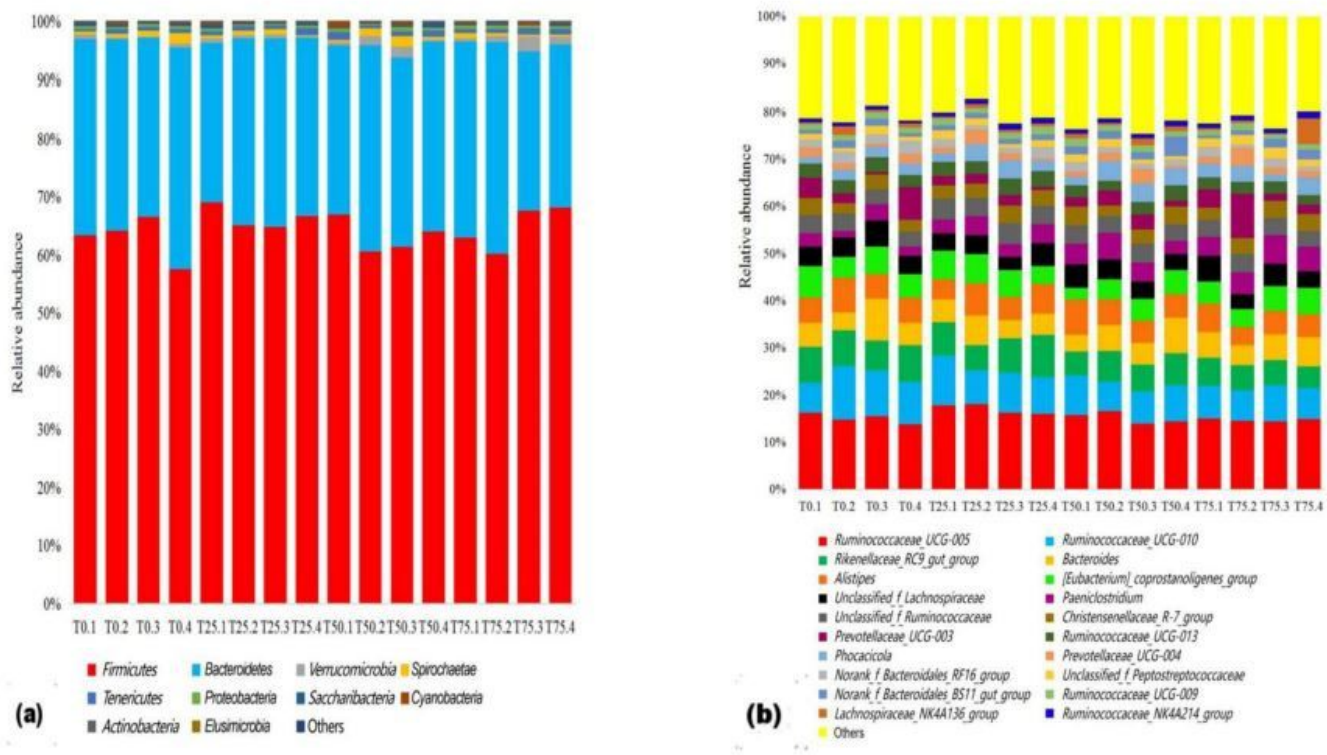


Figure 5

Figure 5

Relative abundance of fecal bacterial community on the phylum and genus level. Figure 5a is the relative abundance on the phylum level; Figure 5b is the relative abundance on the genus level. T0: BPS replaces 0% of CS; T25: BPS replaces 25% of CS; T50: BPS replaces 50% of CS; T75: BPS replaces 75% of CS. Numbers 1, 2, 3 and 4 in names of samples refer to individual heifers per set of treatment. BPS, Broussonetia papyrifera silage; CS, whole corn silage.

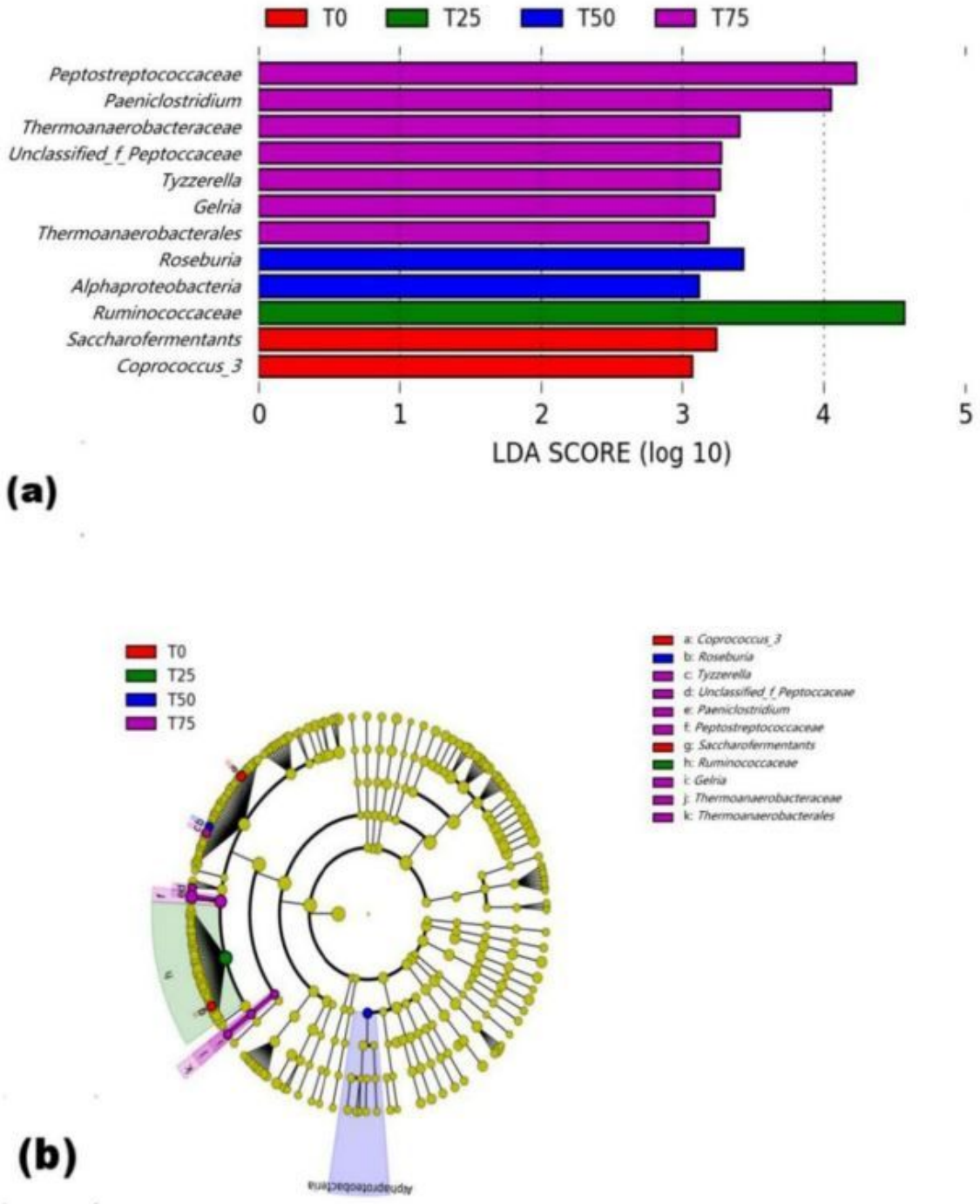


Figure 6

Figure 6

Comparison of microbial variations using the LEfSe online tool. T0: BPS replaces 0% of CS; T25: BPS replaces 25% of CS; T50: BPS replaces 50% of CS; T75: BPS replaces 75% of CS. Numbers 1, 2, 3 and 4 in names of samples refer to individual heifers per set of treatment. BPS, Broussonetia papyrifera silage; CS, whole corn silage.

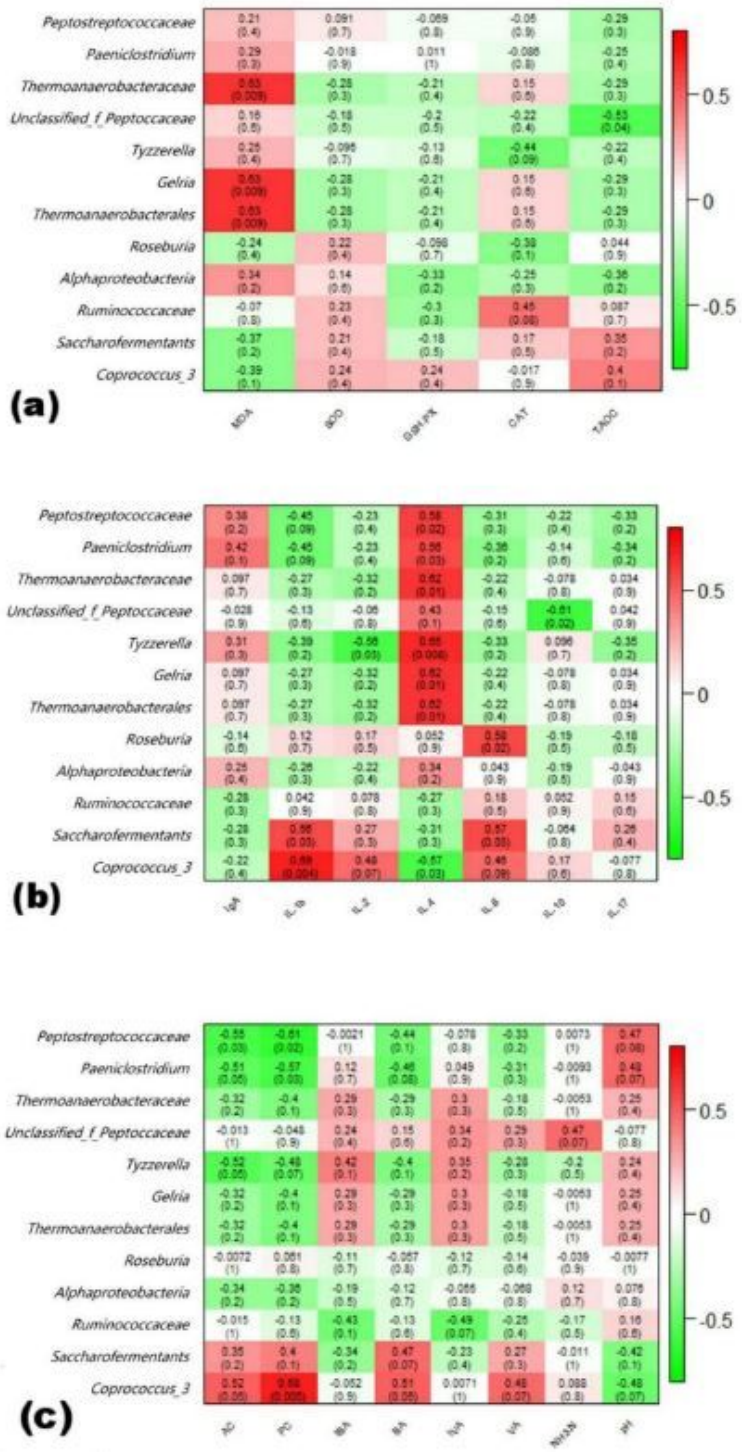


Figure 7

Figure 7

Correlation analyses of differential OTUs with serum anti-oxidant indicators (7a), serum immune indicators (7b), and hindgut fermentation parameters (7c). Each cell contains Pearson correlation coefficient and P-value (within brackets). Figure 7a: MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; T-AOC, total antioxidant capacity. Figure 7b: Ig,

immunoglobulin; IL, interleukin. Figure 7c: AA, acetic acid; PA, propionic acid; IBA, isobutyric acid; BA, butyric acid; IVA, isovaleric acid; VA, valeric acid; NH3-N, ammoniacal nitrogen.

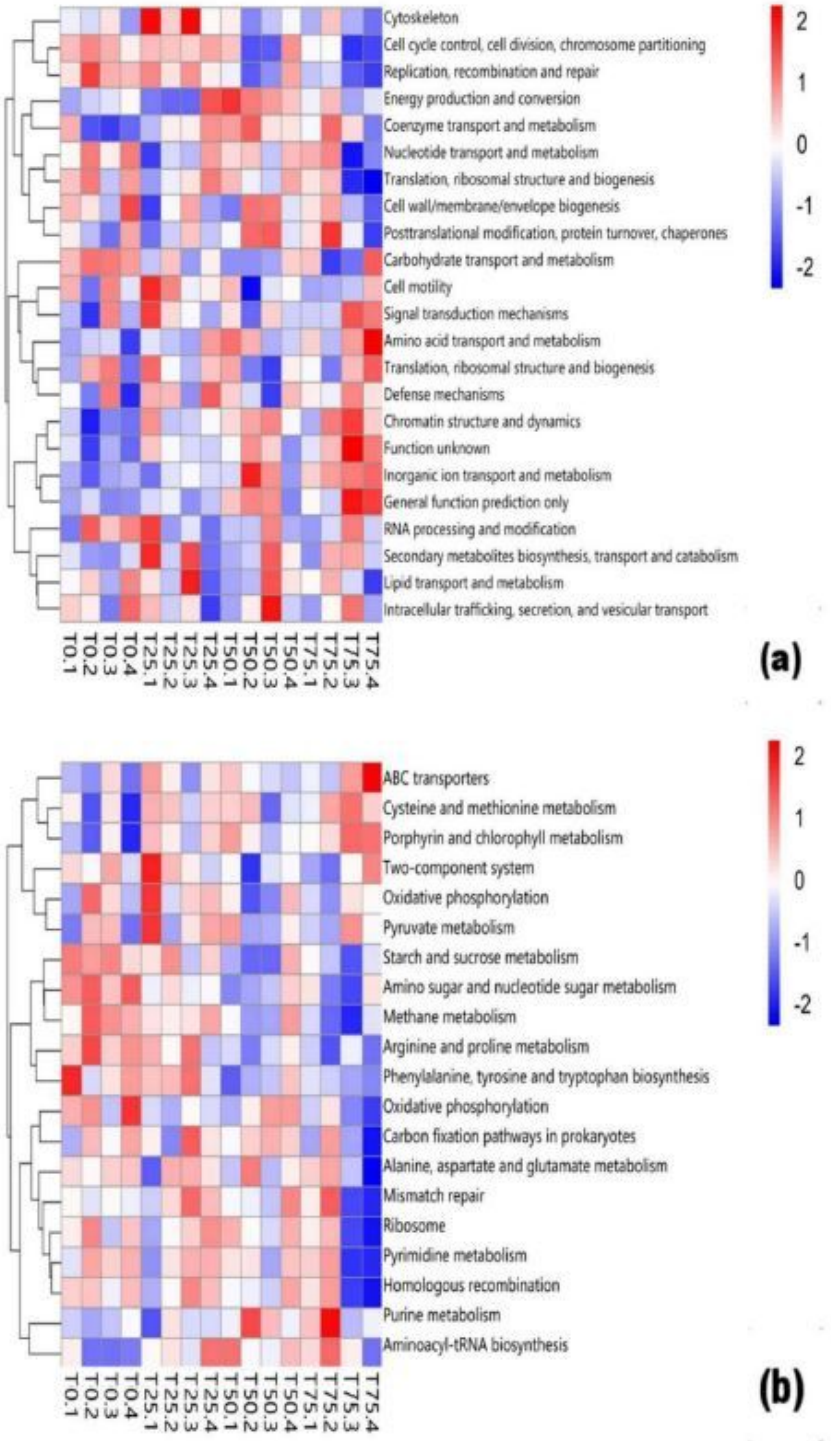


Figure 8

Figure 8

Heatmap of 16S rDNA gene-predicted functional (8a) and pathway-predicted (8b) profiles obtained via Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). T0: BPS replaces 0% of CS; T25: BPS replaces 25% of CS; T50: BPS replaces 50% of CS; T75: BPS replaces 75% of

CS. Numbers 1, 2, 3 and 4 in names of samples refer to individual heifers per set of treatment. BPS, Broussonetia papyrifera silage; CS, whole corn silage.