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Effects of anthocyanins from the fruit of *Lycium ruthenicum* Murray on intestinal microbiota

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ABSTRACT

Simulated digestion and fermentation *in vitro* by human intestinal microbiota of anthocyanins from *Lycium ruthenicum* Murray were investigated in this study. It was shown that the anthocyanins from *L. ruthenicum* were not digested in simulated saliva, gastric and small intestine fluids. Under fermentation *in vitro*, the anthocyanins from *L. ruthenicum* interacted with intestinal microbiota and promoted the production of short-chain fatty acids (SCFAs) due to microbial fermentation. The anthocyanins from *L. ruthenicum* significantly increased the relative abundances of *Bifidibacterium* and *Allisonella*, and reduced the relative abundances of *Prevotella*, *Dialister*, *Megamonas* and *Clostridium*. Moreover, the anthocyanins from *L. ruthenicum* not only exhibited similar effect as inulin on the production of SCFAs (promoting the formation of acetic, propionic and butyric acids), but also showed a dynamic and multiple regulatory effects on intestinal microbiota. Therefore, the anthocyanins from *L. ruthenicum* might have a positive role in maintaining intestinal health.

1. Introduction

The human intestines especially the large intestine are inhabited by a large number of bacteria. It is estimated that the human microbiota contains about 10^{14} bacterial cells, approximately 10 times that of the total cells in human body. Therefore, the gut microbiota is an extremely complex ecosystem of microbes and plays a major role in human health and disease (Clemente, Ursell, Parfrey, & Knight, 2012). It has been reported that the human microbiota is mainly composed of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia. Firmicutes and Bacteroidetes are the 2 most abundant phyla in the gut (accounting 90% of intestinal microbiota) (Gill et al., 2006). Diet is considered to be one of the most important factors that affect the establishment and composition of gut microbiota throughout the lifespan (Kałużna-Czaplińska, Gątarek, Chartrand, Dadar, & Bjørklund, 2017; Lankelma, Nieuwdorp, de Vos, & Wiersinga, 2015; Oriach, Robertson, Stanton, Cryan, & Dinan, 2016). For example, it has been reported that diet polyphenols can alter the composition of microbiota through simultaneous promotion of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* (Zhang et al., 2013), and inhibition of pathogenic bacteria, such as *Staphylococcus*, *Salmonella*, *Helicobacter pylori* and *Bacillus cereus* (Nohynek et al., 2006; Puupponen-Pimiä et al., 2005), in human intestine. Due to such reason, there is broad agreement that dietary polyphenols, in particular anthocyanins, have the ability to modulate gut microbiota (Jamar, Estadella, & Pisani, 2017).

Anthocyanins, a particular class of flavonoids, have been widely found in fruits and vegetables that make them vivid red to blue in color. The anthocyanin derivatives may have different aglycones (anthocyanidins such as cyanidin, delphinidin, petunidin, pelargonidin, peonidin and malvidin), glycone moieties (attached sugar residues such as glucose, galactose and arabinose), type and number of aliphatic or aromatic acids (such as *p*-coumaric, caffeic and ferulic acids) (Hu, Zheng, Li, & Suo, 2014; Koponen, Happonen, Mattila, & Törrönen, 2007; Wu, Lv, Wang, & Wang, 2016). Accordingly, over 700 anthocyanins are identified in nature. It has been reported that anthocyanins possess a wide range of biological functions, including anti-oxidant, anti-inflammatory, anti-tumor, reduction in the risk of several chronic diseases such as obesity, diabetes and cardiovascular diseases (Jung, et al., 2015; Zheng et al., 2011). To achieve these health-promoting characteristics, the interaction between anthocyanins and gut microbiota must be considered to understand their biological functions (Kahle, et al., 2006; Parkar, Trower, & Stevenson, 2013). In fact, it has been

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reported that only a small number of dietary anthocyanins is absorbed at the upper gastrointestinal tract (Kahle et al., 2006), while most of the anthocyanins reach the large intestine intact and interact with microbiota to be biotransformed and metabolized before being absorbed across the intestinal mucosa (Faria, Fernandes, Norberto, Mateus, & Calhau, 2014). *Lycium ruthenicum* Murray, a member of *Lycium* genus in the family of Solanaceae and mainly cultivated in northwestern of China, is the newly discovered resource of *Lycium* and medicine food homology with high economic value (Wang et al., 2018). It has been reported that the fruits of *L. ruthenicum* are rich in anthocyanins with high scavenging activities against 1,1-diphenyl-2-picrylhydra-zyl, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and superoxide radicals (Hu et al., 2014; Wu et al., 2016; Zheng et al., 2011). However, there is little information on their bioavailability and interactions with gut microbiota. In the present study, therefore, the *in vitro* digestion model was used to investigate the possible changes of anthocyanins from *L. ruthenicum* under simulated environments of mouth, stomach and small intestine. Based on the results of digestion, the effects of anthocyanins from *L. ruthenicum* on intestinal microbiota were evaluated by anaerobic fermentation and high-throughput sequencing. Furthermore, the effects of anthocyanins on production of short-chain fatty acids (SCFAs) were investigated.

2. Materials and methods

2.1. Materials and reagents

α-Amylase, pepsin, pancreatin and standards of acetic, propionic, *n*-butyric, *i*-butyric, *n*-valeric, *i*-valeric and 2-ethylbutyric acids were purchased from Sigma Co. (St. Louis, MO, USA). The fruits of *L. ruthenicum* were kindly provided by National Wolfberry Engineering Research Center (Yinchuan, China). Inulin was obtained from Nanjing Oddfoni Biological Technology Co., Ltd. (Nanjing, China). The anaerobic cultivation system including anaerobic sealed-box, anaerobic gas-producing package and oxygen indicator was purchased from Mitsubishi Gas Chemical Co., Inc. (Tokyo, Japan). The lactic acid assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The TIANamp Stool DNA Kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China).

2.2. Preparation of anthocyanins

The procedures for preparation of anthocyanins were performed according to the reported method (Tang et al., 2017) with some modifications. Briefly, 20 g dried fruits of *L. ruthenicum* were mixed with 80% aqueous ethanol (v/v, with 0.1% formic acid) at a ratio of 1:40 (m/v). The extraction was carried out at 50 °C in water bath for 3 h, and the extract was filtered through muslin cloth and concentrated with vacuum rotary evaporator. After concentration, the concentrate was diluted to 10 mL and loaded onto an AB-8 macroporous resin column (5 × 30 cm). The column was first washed with 2 times bed volume of deionized water to remove strong polar constituents. Then, the target constituents (anthocyanins) were eluted with 85% aqueous ethanol solution (v/v, pH 2.0) at a flow rate of 2.0 mL/min, collected, concentrated and freeze-dried, affording the anthocyanins for following experiments.

2.3. Determination of total anthocyanins content

The content of total anthocyanins was determined by the reported spectrophotometric pH differential method (Ravanfar, Tamaddon, Niakousari, & Moein, 2016). Briefly, 0.4 mL sample solution was mixed with 3.6 mL 0.025 M KCl solution (pH 1.0) or 0.4 M sodium acetate solution (pH 4.5), and the absorbance (Abs) of resulting solution was measured at 519 nm against distilled water as the blank by using a spectrophotometer. The total anthocyanins content was calculated according to the following formula:

$$\text{Total anthocyanins content (mg/L)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times \iota}$$

where $A = Abs_{519}$ (pH 1.0) – Abs_{519} (pH 4.5), MW represents the relative molecular weight of cyanidin-3-glucoside (C3G, 433.2 g/mol), DF is the dilution multiple (60), ε is the extinction coefficient of C3G (26,900 L cm^{-1} mol^{-1}), and ι indicates the length of the optical path (cm).

2.4. Simulated saliva digestion

The procedures of simulated saliva digestion were performed according to our reported method (Xie et al., 2016) with some modifications. Firstly, the artificial saliva was prepared by dissolving NaCl (0.12 g/L), KCl (0.15 g/L), mucin (1.0 g/L) and α-amylase (2.0 g/L) in deionized water. Then, 3.0 mL artificial saliva was mixed with 3.0 mL anthocyanins solution (2.0 mg/mL). The mixture was incubated in a water bath oscillator of 37 °C (vibrating at speed of 60 rpm). During the digestion, 1.0 mL mixture each time was taken out at 0, 0.5, 1, 2 and 4 h, respectively, and mixed with 2.0 mL methanol (acidified with 0.1% HCl) in order to inactivate α-amylase activity. The content of total anthocyanins was determined by spectrophotometric pH differential method.

2.5. Simulated gastric digestion

The procedures for simulated gastric digestion were carried out according to the reported method (Hu, Nie, Min, & Xie, 2013) with some modifications. Firstly, 0.31 g NaCl, 0.11 g KCl, 0.015 g $CaCl_2$, 0.06 g $NaHCO_3$, 1.0 mL CH_3COONa (1.0 M, pH 5.0), 25.0 g stomach lipase and 23.6 g pepsin were dissolved in 100.0 mL distilled water, and the pH of the resulting solution was adjusted to 3.0 by 0.1 M HCl solution, affording the simulated gastric medium. Then, the anthocyanins from *L. ruthenicum* were dissolved with the simulated gastric medium to afford a solution of 1.0 mg/mL anthocyanins, and the resulting mixture was incubated in a water bath oscillator of 37 °C (shaking at speed of 60 rpm). During the digestion, the pH of the solution was remained at 3.0. Moreover, 1.0 mL mixture each time was taken out at 0, 0.5, 1, 2, 4 and 6 h, respectively, and mixed with 2.0 mL methanol (acidified with 0.1% HCl). The content of total anthocyanins was determined as mentioned above.

2.6. Simulated small intestine digestion

The procedures for simulated intestinal digestion were carried out according to the method (Chen et al., 2018) with some modifications. Firstly, 0.54 g NaCl, 0.065 g KCl and 0.033 g $CaCl_2$ were dissolved in 100.0 mL distilled water, and 0.1 M NaOH solution was used to adjust the pH to 7.0, affording the small intestine electrolyte solution. Then, 20.0 g small intestine electrolyte solution was mixed with 20.0 g pancreatin solution (7%, w/w), 2.6 mg trypsin and 80.0 g bile salt solution (4%, w/w). The pH of solution was adjusted with 0.1 M NaOH solution to 7.5, affording simulated small intestine medium. Finally, 1.5 mL simulated small intestine medium was mixed with 5.0 mL gastric digested solution at 6 h (the pH was adjusted to 7.0). Then, the simulated small intestinal digestion was carried out as described above for simulated gastric digestion, and the content of total anthocyanins was analyzed by spectrophotometric pH differential method.

2.7. Fermentation in vitro

The procedures for simulated large intestinal fermentation were done according to the reported method (Xie et al., 2017) with some modifications. Firstly, the fresh feces for experiments were collected from 5 healthy volunteers (3 men and 2 women, 22–28 years old) not

taken antibiotics at least three months. They were diluted 10 times with sterilized modified physiological saline solution (containing NaCl 8.5 g/L and cysteine hydrochloride 0.5 g/L), stirred evenly and centrifuged at 4 °C (500 rpm) for 5 min. The resulting supernatant was used as the fecal slurry in the following fermentations, and the microbial community composition of the fecal slurry was recorded as OR. Secondly, solutions of anthocyanins (1.0 g/L) and inulin (10.0 g/L, positive control) were prepared by dissolving in autoclaved basal nutrient growth medium (peptone 2.0 g/L, yeast extract 2.0 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, $MgSO_4$ 0.01 g/L, $CaCl_2$ 0.01 g/L, $NaHCO_3$ 2.0 g/L, hemim 0.02 g/L, cysteamine hydrochloride 0.5 g/L, bile salts 0.5 g/L, resazurin 1.0 mg/L, Tween 80 2.0 mL/L and vitamin K_1 10 μL/L), respectively. Thirdly, 1.0 mL fecal slurry suspension was added to 9.0 mL of nutrient growth medium containing anthocyanins (recorded as ACN) or inulin (recorded as INL) in triangular bottle for fermentation *in vitro*. The basal nutrient medium without any other carbon source was used as blank control (recorded as BLK). The triangular bottles were placed in an Anaero Pack system and incubated at 37 °C for 24 h. The oxygen indicator was added to ensure that the oxygen pressure in the sealed box was below 0.1%, and the box was shaken gently every 6 h. The samples at fermentation of 0, 6, 12 and 24 h were taken out for further study (analysis of SCFAs or bacterial DNA extraction).

2.8. Determination of contents of SCFAs and lactic acid

The contents of SCFAs including acetic, propionic, *n*-butyric, *i*-butyric, *n*-valeric and *i*-valeric acids in fermentation fluids were determined by gas chromatography (GC) with calculating curves of standards. Before GC analysis, the sample was acidified with HCl solution. The conditions of GC analysis were as follows: HP-INNOWAX capillary column (30 m × 0.25 mm × 0.25 μm, Agilent), flame ionization detector (FID), nitrogen as the carrier gas with flow velocity of 19.0 mL/min; in the initial 1 min, column temperature remained at 100 °C; 1–16 min, column temperature rose to 180 °C at 5 °C /min; 16–20 min, column temperature remained at 180 °C. The content of lactic acid was determined with lactic acid assay kit according to the manual instruction.

2.9. Analysis of intestinal microbiota

After 24 h fermentation, the DNA of total bacteria in fermentation medium was extracted with TIANamp Stool DNA Kit. For 16S rDNA sequencing, the DNA samples were sent to Center for Genetic & Genomic Analysis, Genesky Biotechnologies Inc. (Shanghai, China) under -20 °C preservation and dry ice conditions. High fidelity polymerase chain reaction (PCR) was utilized to amplify bacterial 16S rDNA hypervariable region 4 (V4) with the primers. High-throughput sequencing was performed on Illumina Miseq platform with 2 × 250 bp paired-end method after the library was quantified, mixed and quality checked. All the results were based on sequenced reads and operational taxonomic units (OTUs).

2.10. Statistical analysis

All the tests were performed in triplicates, and the data are expressed as mean ± standard deviation (SD). Statistical comparisons of data were estimated by single factor variance analysis of one way (ANOVA) or Duncan's multiple-range tests by using SPSS 20. It was considered to be statistically significant with $p < 0.05$.

3. Results and discussion

3.1. Digestion of anthocyanins in simulated saliva, gastric and small intestine fluids and fermentation in vitro

After digestion in simulated saliva, gastric and small intestine fluids, there was no significant difference for total contents of anthocyanins (Table 1). Thus, it could be regarded that the anthocyanins from *L. ruthenicum* were not digested in the mouth, stomach and small intestine. It has been reported that only a small part of anthocyanins was absorbed in the mouth, stomach and small intestine, while a large part of anthocyanins entered into the large intestine and metabolized there by intestinal microbiota, possible resulting in the low bioavailability of anthocyanins (Faria et al., 2014; Kahle et al., 2006). For examples, anthocyanins extracted from blueberries and apples are not digested in the upper parts of the gastrointestinal tract (Bouayed, Hoffmann, & Bohn, 2011; Kahle et al., 2006; Liu et al., 2014). Furthermore, acylation with *p*-coumaric acid or a second sugar moiety in anthocyanin molecule could make the corresponding anthocyanin more stable in the gut (Selma, Espin, & Tomas-Barberan, 2009). Petunidin3-*O*-[rhamnopyranosyl-(*trans*-*p*-coumaroyl)]-5-*O*-[*β*-*D*-glucopyranoside], the main anthocyanin in *L. ruthenicum*, is an anthocyanin acylated with *p*-coumaric acid and with three sugar moieties (Tang et al., 2017). Accordingly, it might be the reason that the anthocyanins from *L. ruthenicum* exhibited stability in upper digestive tract. Moreover, most anthocyanins are not easily absorbed under high glycemic index level, and they can reach the large intestine intact (Faria et al., 2014). However, the total content of anthocyanins decreased significantly from 85.39 ± 0.98 (0 h) to 20.40 ± 0.09 mg/L ($p < 0.05$) after 24 h fermentation *in vitro*. The results indicated that the anthocyanins from *L. ruthenicum* could be degraded in the large intestine by gut microbiota, resulting in the metabolites absorbed thereby for use (Kay, 2006; Mosele, Macià, Romero, & Motilva, 2016). Thus, the effects of the anthocyanins from *L. ruthenicum* on intestinal microbiota were investigated in the present study.

Table 1
Contents of anthocyanins in samples under different digestion and fermentation times (mg/L).

Time	Simulated saliva digestion	Simulated gastric digestion	Simulated intestinal digestion	Fermentation *in vitro*
0	85.65 ± 0.97a	84.55 ± 0.34a	55.15 ± 1.37a	85.39 ± 0.98a
0.5	84.98 ± 0.56a	83.10 ± 0.68a	55.08 ± 1.05a	/
1	85.55 ± 1.48a	83.82 ± 0.34a	54.94 ± 1.73a	/
2	85.05 ± 1.12a	83.34 ± 0.68a	54.74 ± 0.68a	/
4	85.17 ± 1.01a	82.37 ± 1.02a	54.67 ± 1.36a	/
6	/	82.13 ± 1.68a	54.34 ± 0.68a	25.44 ± 0.48b
12	/	/	/	23.62 ± 1.63b
24	/	/	/	20.40 ± 0.09c
Control	85.24 ± 1.09a	83.94 ± 0.54a	54.99 ± 1.26a	85.26 ± 0.76a

The same letter in each column indicates that there is not significant difference in anthocyanins content between different times ($p < 0.05$).

3.2. Overall difference in microbial community due to fermentation in vitro

The overall differences of microbial communities were compared at OTU level, and the results are displayed as shown in Fig. 1. After fermentation, the composition of intestinal microbiota was significantly changed for ACN or INL group compared with that of BLK group. Moreover, the ACN group and INL group were close to OR group based on the principal component analysis (PCA, Fig. 1A), which indicated that the anthocyanins from *L. ruthenicum* and inulin could regulate the composition of intestinal microbiota in keeping the intestinal healthy. The results also suggested that the anthocyanins from *L. ruthenicum* were beneficial to the intestinal microbiota, like inulin as prebiotics. As shown in Fig. 1B, INL group was the most similar to OR group in the intestinal microbiota, ACN group was the second similar to OR group, while BLK group was the most different with OR group. The results are consistent with the results obtained by PCA.

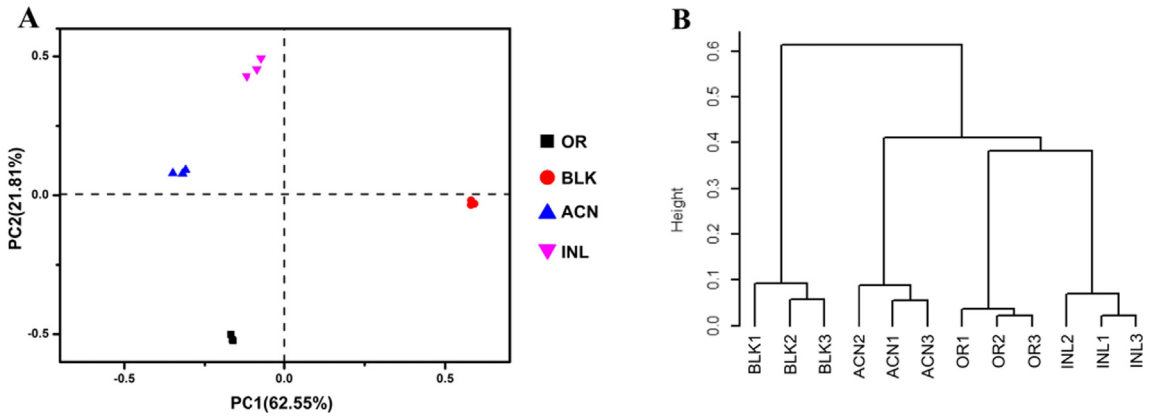

Fig. 1. Difference of microbial community structure based on principal component analysis (A) and cluster analysis (B).

3.3. Effects of anthocyanins from L. Ruthenicum on gut microbiota

At the level of phylum, the intestinal microbiota was found to be mainly composed of Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Fusobacteria (Fig. 2A). It can be seen from Fig. 2B that the anthocyanins from *L. ruthenicum* significantly reduced ($p < 0.05$) the ratio of Firmicutes/Bacteroidetes (from 0.57 to 0.28), suggesting that the anthocyanins from *L. ruthenicum* might have the effects of reducing fat and losing weight. It has been reported that the ratio of Firmicutes/Bacteroidetes, considered as a useful biomarker for obesity (De Filippo et al., 2010), was positively correlated with obesity caused by high-calorie diet (Ley, Turnbaugh, Klein, & Gordon, 2006). In addition, dietary fibers and probiotics have been reported to promote the growth of Bacteroidetes and Actinobacteria and inhibit the growth of Firmicutes, thereby achieving the role of prevention of obesity (Barczynska, et al., 2015; Renata, Janusz, Mieczyslaw, Katarzyna, & Mieczyslaw, 2016). In the present study, it was found that the relative abundance of Actinobacteria in the ACN group was significantly higher than that of the other group ($p < 0.05$, Fig. 2C). The anthocyanins from *L. ruthenicum* showed similar effects as reported in the literatures, suggesting that the anthocyanins from *L. ruthenicum* should have potential anti-obesity effect.

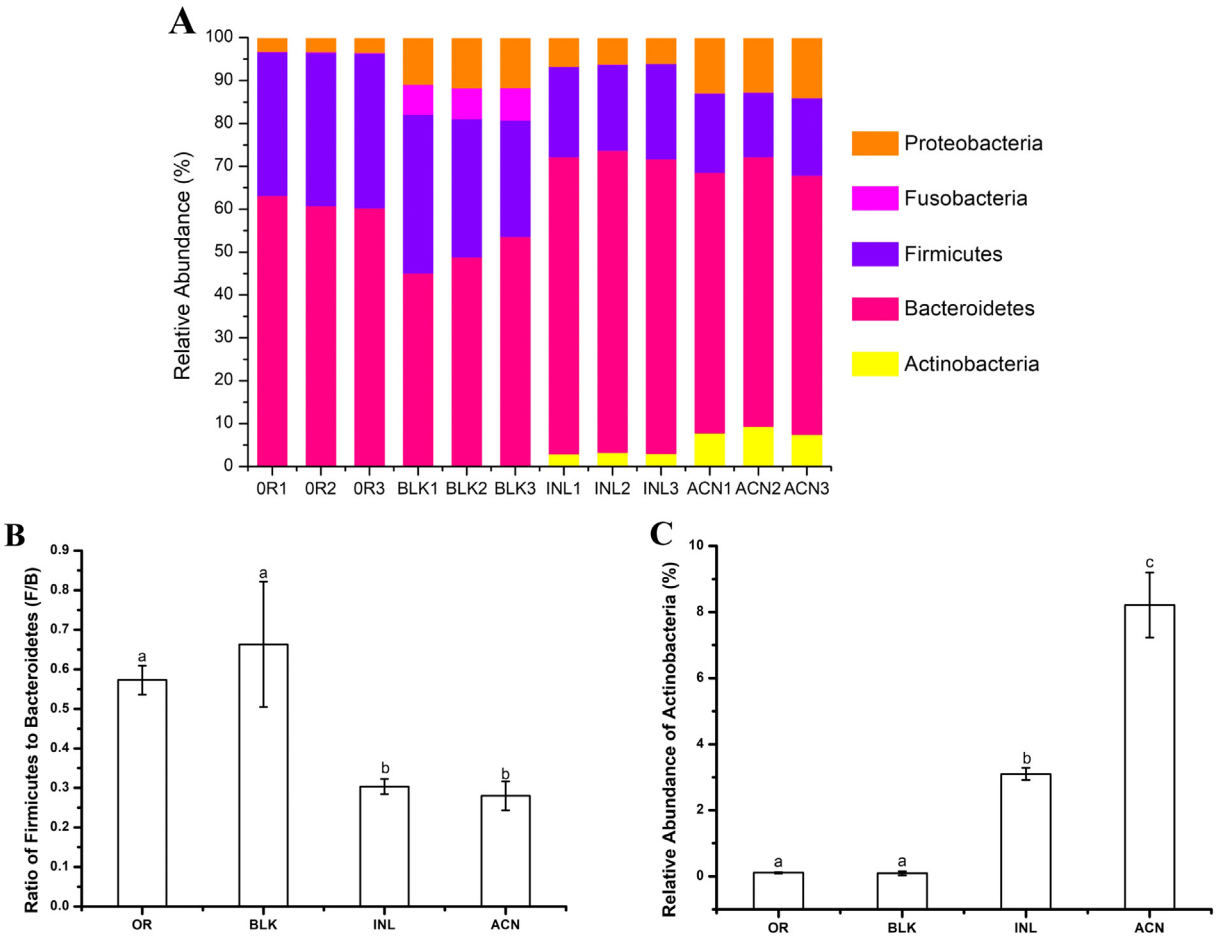

Fig. 2. Microbial compositions of ACN group, INU group, BLK group and OR at phylum level. A, Column diagram of microbial composition; B, Ratio of Firmicutes/Bacteroidetes; C, Relative abundance of Actinobacteria.

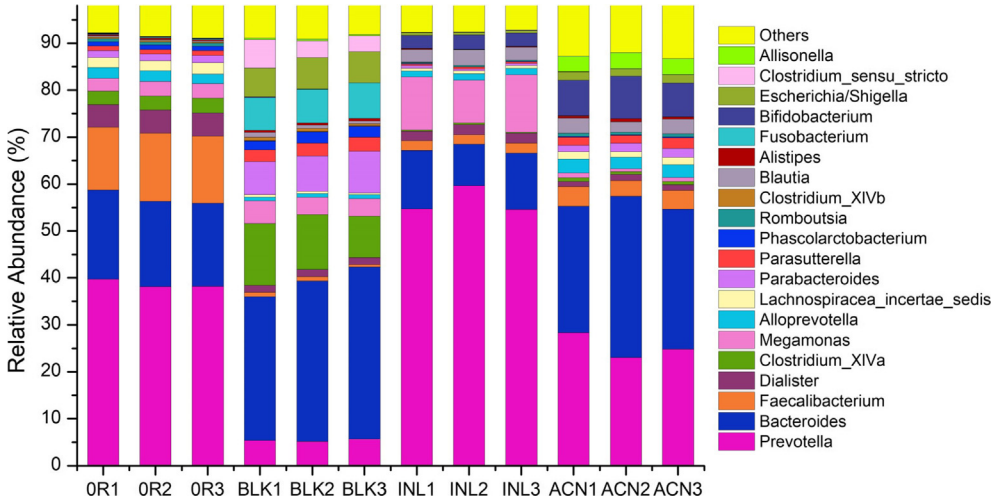

Fig. 3. Column diagram of microbial composition at genus level.

On the level of genus, the intestinal microbiota were mainly composed of *Bacterides*, *Prevotella*, *Bifidobacterium*, *Faecalibacterium*, *Allisonella*, *Dialister*, *Megamonas*, *Escherichia/Shigella*, *Parabacteroides*, *Clostridium_sensu_stricto*, *Fusobacterium* and *Clostridium_XlVa* (Fig. 3). Compared with OR, the anthocyanins from *L. ruthenicum* significantly increased the relative abundances of *Bifidibacterium* and *Allisonella* (Fig. 4A and B), especially *Bifidobacterium* that is considered to be one of the most important bacterial groups associated with human health (Hidalgo et al., 2012). The increase in *Bifidobacterium* induced by anthocyanins has been reported previously in literatures (Botoordóñez, et al., 2014; Morais, Rosso, Estadella, & Pisani, 2016). It has been demonstrated that *Bifidobacterium* could enhance intestinal barrier function, stimulate the host immune system, prevent diarrhea or allergies, participate in the activation of vitamins and regulate lipid metabolism (Gibson, 2008). Moreover, the anthocyanins from *L. ruthenicum* reduced the relative abundances of *Prevotella*, *Dialister*, *Megamonas* and *Clostridium* ($p < 0.05$, Fig. 4C–F). From the heatmap of cluster stacking at genus level, it was obvious that the anthocyanins from *L. ruthenicum* increased the relative abundances of *Allisonella*, *Sutterellaceae* and *Blautia*, and decreased the relative abundances of *Phascolarctobacterium*, *Lachnospiraceae* and *Faecalibacterium* (Fig. 5). It is newly discovered that *Blautia* is associated with body mass index related plasma metabolites that can be considered as the mediators between intestinal microbiota and obesity (Ottosson et al., 2018). The changes of the relative abundances of microbiota induced by the anthocyanins from *L. ruthenicum* might be caused by the metabolites of anthocyanins produced by the intestinal microbiota. The bacteria possibly associated with the metabolism of anthocyanins were *Bacteroides*, *Clostridium*, *Eubacterium*, *Ruminococcus* and *Eggertheilla* (Blaut & Clavel, 2007). A lot of studies have pointed out that *Clostridium* and *Eubacterium* are two common bacteria in the metabolism of several phenolic compounds (Selma et al., 2009). Based on pilot results of linear discriminant analysis (LDA) effect size (LEfSe), *Bifidobacterium*, *Collinsella*, *Ruminococcus2*, *Alloprevotella*, *Lachnospiracea_incertae_sedis*, *Roseburia*, *Romboustsia* and *Feacalibacterium* were the dominant genus in ACN group (Fig. 6). What's more, the anthocyanins from *L. ruthenicum* increased the relative abundance of *Bacteroides* and reduced the relative abundance of *Clostridium*, but the relative abundances of *Eubacterium*, *Ruminococcus* and *Eggertheilla* were less affected. Therefore, the anthocyanins from *L. ruthenicum* could ameliorate the structure of the intestinal microbiota not only through by adjusting beneficial microbiota but also proliferating the microbiota related with the metabolism of anthocyanins. It has been reported that anthocyanins might promote the growth of certain specific bacteria, such as *Akkermansia Muciniphila*, but similar phenomenon was not found in the present study, perhaps due to the different structures of anthocyanins or culture conditions. Certainly, it is worth to study further.

3.4. Effects of anthocyanins from L. Ruthenicum on production of SCFAs

SCFAs, including acetic, propionic, *n*-butyric, *i*-butyric, *n*-valeric, *i*-valeric and lactic acids, are the final products of dietary fiber due to anaerobic enteric microbial fermentation. These SCFAs are believed to promote fiber intake and contribute to colon health and metabolic health. At the same time, SCFAs can affect some metabolic pathways such as energy regulation, lipid metabolism and inflammation (Kasubuchi, Hasegawa, Hiramatsu, Ichimura, & Kimura, 2015). Thus, SCFAs play a key role in human intestinal health. As shown in Table 2, the contents of acetic, propionic and *n*-butyric acids in each group were increased with the increase of fermentation time, and the content of lactic acid was increased firstly and then decreased with the increase of the fermentation time. What's more, *i*-butyric, *n*-valeric and *i*-valeric acids were all in small amounts. At the end of fermentation, there was no significant difference in total SCFAs contents between INL group and ACN group ($p > 0.05$). But the total SCFAs content in ACN group was significantly higher than that in BLK group ($p < 0.05$). For ACN group, SCFAs accumulated with fast rate during 0–6 h fermentation and then increased slowly. This might be related to the activity of β-glucosidase in the intestinal microbiota, so that the rapid metabolism of anthocyanins occurred in the first 4–5 h fermentation (Hidalgo et al., 2012). The result is also consistent with the change of total anthocyanins content during fermentation (Table 1).

Obviously, acetic, propionic and *n*-butyric acids were the main fermentation products. At the end of fermentation, there was no significant difference between INL group and ACN group in content of acetic acid ($p > 0.05$). Contents of propionic and butyric acids for ACN group were slightly lower than those for INL group ($p < 0.05$). However, the total content of acetic, propionic and *n*-butyric acids in ACN group was almost the same with that in INL group. The results indicated that the anthocyanins from *L. ruthenicum* could promote the generation of SCFAs like inulin. Acetic acid is the final product of *Bifidobacterium* fermentation, and the increase of acetic acid content might be related to the increase of relative abundance of *Bifidobacterium* (Sanz et al., 2005). High concentration of acetic acid can promote the expression of AMP kinase and inhibit fat synthesis, so that acetic acid may be closely related to the regulation of weight (Yamashita et al., 2007). Similarly, propionic acid has been shown to increase satiety, thus it can reduce energy intake and body weight (Hosseini, Grootaert, Verstraete, & Van, 2011; Lin et al., 2012). The efficacy of these SCFAs is achieved by activating FFAR2 and FFAR3 (Lin et al., 2012). In addition,

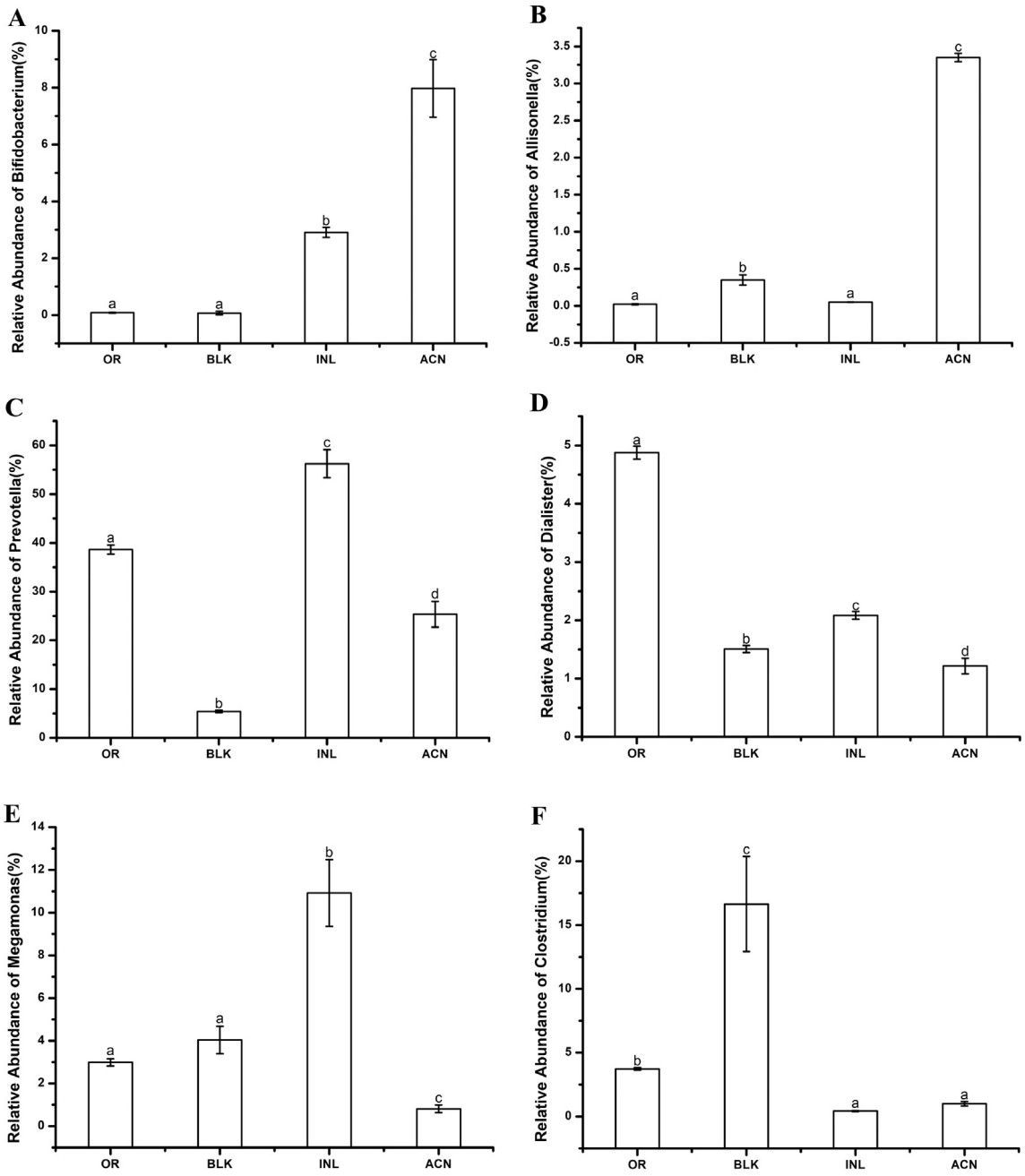

Fig. 4. Microbial composition at genus level. A, Relative abundance of *Bifidobacterium*; B, Relative abundance of *Allisonella*; C, Relative abundance of *Prevotella*; D, Relative abundance of *Dialister*; E, Relative abundance of *Megamonas*; F, Relative abundance of *Clostridium*.

SCFAs can regulate gene expression by binding to FFAR2 and FFAR3 which have been identified as endogenous receptors for SCFAs (Tremaroli & Bäckhed, 2012). Thus, it provides a pathway for intestinal microbes to manage obesity. Butyric acid can increase energy consumption by improving insulin sensitivity (Gao et al., 2009). Moreover, butyric acid is the main energy source of colon and participating in the maintenance of healthy colon mucosa. It is an important regulatory factor for gene expression in host cells (Vital, Howe, & Tiedje, 2014). Furthermore, several kinds of bacteria under phylum level of Actinobacteria are identified as the producers of butyric acid (Lee et al., 2015). Thus, the increased relative abundance of Actinobacteria in ACN group might be the reason for the increase of butyric acid. It has been reported that phenols were highly toxic to the *Clostridium* that could promote the production of butyric acid (Bourriaud et al., 2005). In the present study, the relative abundance of *Clostridium* was decreased in ACN group as mentioned above, which might be related to the slightly lower content of butyric acid. Lactic acid is also an important acid, but it is easily converted into other SCFAs by bacteria. It is the key intermediate of acetic, propionic and butyric acids in fermentation, and the increase of acetic, propionic and butyric acids are usually accompanied with its decrease (Brestenský et al., 2017; Kasubuchi et al., 2015). The trends of the changes of these acids and the relationship with the microbiota in this study are basically consistent with the results reported in literatures. In general, the anthocyanins from *L. ruthenicum* showed similar effects as the prebiotics of inulin and promoted the generation of SCFAs by regulating the intestinal microbial flora. The SCFAs produced by intestinal microbiota were not only served as energy sources, but also played a significant role in reducing fat and losing weight. What's more, the anthocyanins from *L. ruthenicum* were not just induced a single inhibition or promotion on the intestinal microbiota that

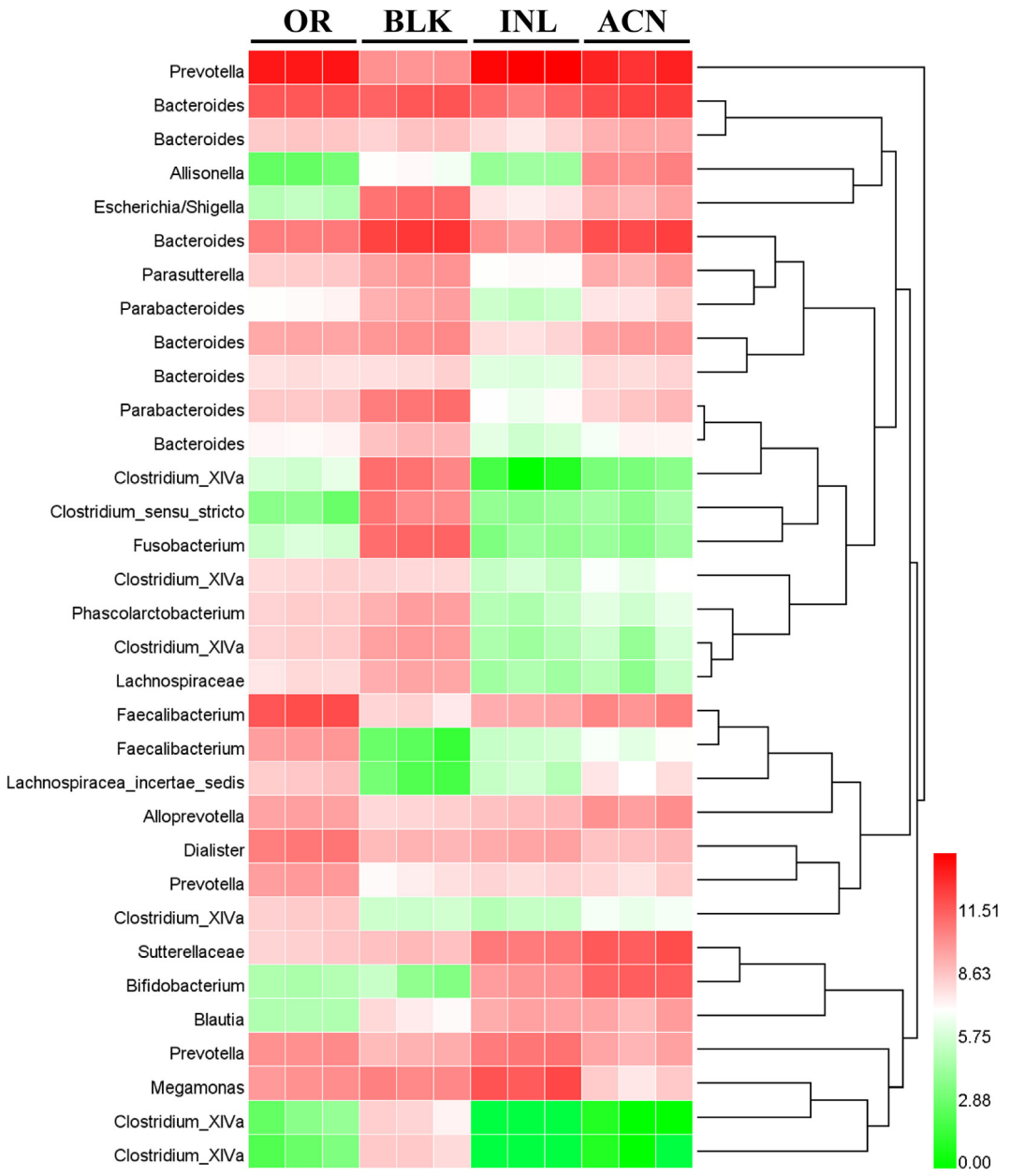

Fig. 5. Heatmap of cluster stacking at genus level.

produce SCFAs, but inhibited some microbiota while promoted others. In the present study, *Bifidobacterium* associated with acetic acid production was promoted, while the relative abundances of *Dialister*, *Megamonas* and *Clostridium* were decreased. Among them, *Dialister* is one of the main strains of producing propionic acid (Vital et al., 2014), *Megamonas* is a bacterium associated with the generation of SCFAs, possibly correlated positively with content of lactic acid (Lee et al., 2015). *Clostridium* is a bacterium that is reported to produce butyric acid (Bourriaud et al., 2005). The present study proved that the regulation of intestinal microbiota by the anthocyanins from *L. ruthenicum* was a dynamic and multivariate result.

4. Conclusion

The anthocyanins from *L. ruthenicum* were not digested in the simulated saliva, gastric and small intestine fluids, but they could be degraded by intestinal microbiota in the large intestine. The anthocyanins from *L. ruthenicum* promoted the growth of beneficial bacteria in the intestines such as *Bifidobacterium* and the production of SCFAs through intestinal microbiota, exhibiting similar effects as probiotics that regulate the structure of gut microbiota. In short, the anthocyanins from *L. ruthenicum* might have beneficial effects on intestinal microbiota and achieve the goal of maintaining intestinal health and reducing fat. Therefore, it is necessary to study the metabolism and potential effects *in vivo* of the anthocyanins from *L. ruthenicum* in the future.

Conflict of Interest

The authors declared that there is no conflict of interest.

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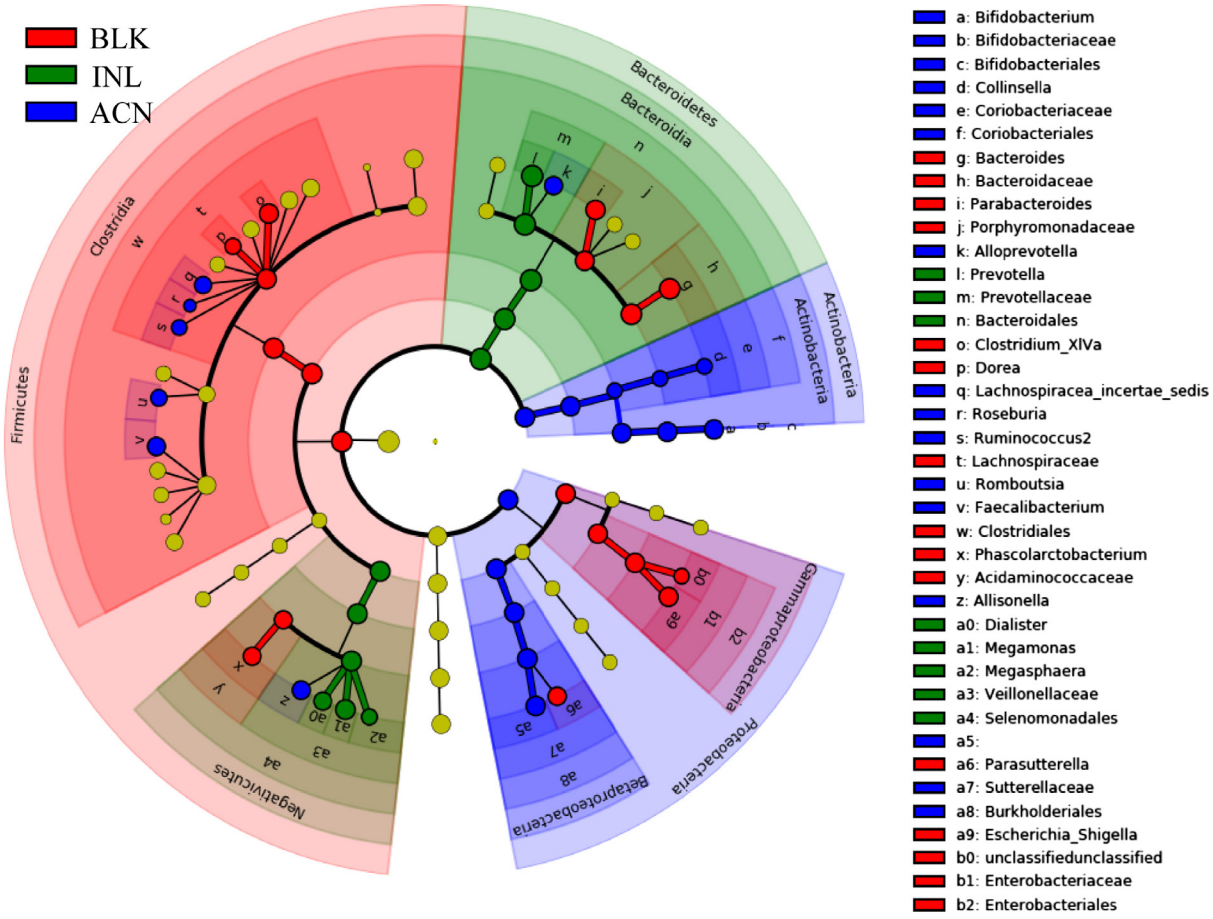

Fig. 6. Pilot results of LEfSe analysis at genus level.

Table 2
Contents of short-chain fatty acids, lactic acid and total acids in fermentation *in vitro* (mM).

Treatment	Time (h)	SCFAs (mM)							
		Acetic acid	Propionic acid	*i*-Butyric acid	*n*-Butyric acid	*i*-Valeric acid	*n*-Valeric acid	Lactic acid	Total
INL group	0	3.15 ± 0.24a	0.59 ± 0.34a	0.00 ± 0.00a	0.11 ± 0.03a	0.00 ± 0.00a	0.00 ± 0.00a	0.56 ± 0.19a	4.41 ± 0.31a
	6	10.89 ± 1.25c	10.04 ± 0.87e	0.00 ± 0.00a	1.22 ± 0.18b	0.00 ± 0.00a	0.00 ± 0.00a	4.05 ± 0.20b	26.20 ± 2.07d
	12	18.83 ± 1.68d	10.32 ± 1.22f	0.00 ± 0.00a	6.38 ± 0.16d	0.43 ± 0.07b	4.43 ± 0.14d	0.73 ± 0.04a	44.45 ± 1.89f
	24	19.08 ± 1.07d	13.42 ± 0.63e	0.47 ± 0.02bc	9.49 ± 1.3e	0.86 ± 0.08d	7.95 ± 0.60e	0.47 ± 0.15a	48.38 ± 1.57f
BLK group	0	3.15 ± 0.24a	0.59 ± 0.34a	0.00 ± 0.00a	0.11 ± 0.03a	0.00 ± 0.00a	0.00 ± 0.00a	0.56 ± 0.19a	4.41 ± 0.31a
	6	5.49 ± 0.83ab	2.90 ± 0.24b	0.00 ± 0.00a	1.38 ± 1.07b	0.00 ± 0.00a	0.00 ± 0.00a	1.04 ± 0.22a	10.80 ± 1.11b
	12	8.5 ± 1.21bc	4.45 ± 0.42c	0.05 ± 0.00c	2.20 ± 0.12b	1.10 ± 0.04e	0.00 ± 0.00a	0.42 ± 0.06a	17.21 ± 1.61c
	24	9.70 ± 1.50c	4.63 ± 0.44c	0.08 ± 0.02d	3.29 ± 0.08c	1.61 ± 0.05f	1.20 ± 0.11b	0.33 ± 0.05a	21.76 ± 2.1 cd
ACN group	0	3.15 ± 0.24a	0.59 ± 0.34a	0.00 ± 0.00a	0.11 ± 0.03a	0.00 ± 0.00a	0.00 ± 0.00a	0.56 ± 0.19a	4.41 ± 0.31a
	6	9.30 ± 0.83c	7.82 ± 0.84d	0.00 ± 0.00a	1.86 ± 0.90b	0.00 ± 0.00a	0.00 ± 0.00a	4.22 ± 0.81b	23.20 ± 2.07d
	12	16.40 ± 1.21d	11.99 ± 1.70f	0.00 ± 0.00a	1.76 ± 0.12b	0.00 ± 0.00a	0.24 ± 0.06a	3.80 ± 0.92b	34.18 ± 2.50e
	24	19.09 ± 1.12d	12.53 ± 0.96f	0.43 ± 0.02b	7.35 ± 0.19d	0.78 ± 0.07c	3.48 ± 0.26c	1.04 ± 0.38a	44.57 ± 2.18f

Different letter indicates a significant difference in the amount of short-chain fatty acids or total acids among different groups ($p < 0.05$).

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