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In vitro fermentation of the polysaccharides from *Cyclocarya paliurus* leaves by human fecal inoculums

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ABSTRACT

In vitro fermentation of polysaccharide from *Cyclocarya paliurus* leaves by human fecal inoculums was investigated by determining the changes in contents of neutral and reducing sugar and pH value, consumption of monosaccharide and production of short-chain fatty acids (SCFAs). During fermentation, the content of neutral sugar and reducing sugar decreased as fermentation time increased except that the content of reducing sugar increased within the fermentation time 0.5 h. The pH value significantly dropped from 7.2 to 6.04. Remarkably, the greatest yields and the fastest consumption of galacturonic acid were found and the yield of glucose and arabinose were relatively high. The dominant SCFAs, which were acetic acid, propionic acid and *n*-butyric acid, significantly increased. These results showed that polysaccharide was partly fermented, glycosidic bonds with galacturonic acid being more susceptible to be attacked by gut bacteria and galacturonic acid might be deemed as the main producer of acetic acid.

1. Introduction

Recently, there has been increasing interest in the microbial fermentation of carbohydrate due to its potential health benefit to human large intestine. It was reported that the end products of carbohydrate fermentation, such as various gases and short-chain fatty acids (SCFAs) in the colon exerted a far-reaching impact on the physiological and metabolic functions of animals and humans (Cherbut, Salvador, Barry, Doulay, & Delort-Laval, 1991; Cummings & Macfarlane, 1991; Nishina & Freedland, 1990; Sakata, 1987). Moreover, it was also found that polysaccharide regarded as prebiotics are capable to balance colonic bacteria, lower pH value and stimulate colonic immune system in the large intestine, which are important in reducing the risk of allochtonous microorganisms potentially causing diseases in the gut (Gibson & Roberfroid, 1995; Wong & Jenkins, 2007). Furthermore, the degradative behavior of fiber depended on its physical form, three-dimensional arrangement and physicochemical properties (Guillon, Barry, & Thibault, 1992; Jonathan et al., 2012). Therefore, it was essential to determine the fermentability of carbohydrate and its fermentation behavior with the purpose of investigating its action in the colon.

Cyclocarya paliurus (Batal.) *Iljinskaja* is unique plant species in China. Polysaccharides extracted from *C. paliurus* leaves have been found to have anticancer, immunomodulatory, antioxidant and lowering blood sugar activities, with little side effect (Ge et al., 2011; Han et al., 2009; Huang, Nie, Xie, Han, & Xie, 2009; Liu et al., 2007; Shi et al., 2009; Tian et al., 1998; Zhang, Duan, Liu, & Xiao, 2010). Our previous works had prepared a water-soluble polysaccharide from *C. paliurus* leaves that had been found it mainly contained two homogenous polysaccharides, namely CPP-1 and CPP-2. The main fraction CPP-1 with the molecular weight of 1167 kDa, was found to be composed of consisted of D-xylose, L-arabinose, D-glucose, D-galactose, L-rhamnose and D-mannose in a molar ratio of 3.23, 31.2, 31.3, 16.0, 10.6, and 8.72%, respectively (Xie, Xie, Nie, Shen, & Wang, 2010). To our knowledge, there are no reports regarding fermentation of the polysaccharide from *C. paliurus* and relationship between monosaccharide consumption and SCFAs production. The aim of this study was to delineate the fermentation behavior of polysaccharide from *C. paliurus* leaves by examining pH change, degradation of polysaccharide and relationship between monosaccharide consumption and SCFA production.

2. Materials and methods

2.1. Materials

The leaves of *C. paliurus* were provided by Jiangxi Xiushui Miraculous Tea Industry Co, Jiang xi Province, China. Monosaccharide standards including rhamnose (Rha) (99.9% purity), arabinose (Ara) (99.8% purity), xylose (Xyl) (99.8% purity), mannose (Man) (99.8%

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purity), glucose (Glc) (99.8% purity), galactose (Gal) (99.9% purity) and galacturonic acid (GalA) were obtained from Pharmacia (Uppsala, Sweden). SCFA standards including acetic acid (100% purity), propionic acid (100% purity), *i*-butyric acid (99.9% purity), *n*-butyric acid (100% purity), *i*-valeric acid (100% purity) and *n*-valeric acid (99.9% purity) were purchased from Sigma. All other reagents used were of analytical grade and purchased from Shanghai Chemicals and Reagents Co. (Shanghai, China).

2.2. Extraction of polysaccharides

Polysaccharide from *C. paliurus* leaves was prepared according to Xie et al.s' (2010) method with some modifications. The dried leaves of *C. paliurus* were defatted and decolorized by immersing into 80% ethanol for 72 h, and then dried naturally. The pretreated dry leaves were extracted three times with deionized water (with a matter to water ratio (g/ml) of 1:10) at a 90 °C water bath for 2.5 h. The extraction solution was concentrated to a quarter of volume, and polysaccharide was precipitated by adding 95% ethanol to a final concentration of 80% (v/v) at 4 °C for 12 h. The polysaccharide was repeatedly precipitated four times. Finally, polysaccharide was lyophilized in a freeze dryer. The obtained polysaccharide was used for fermentation.

2.3. Human fecal inoculum

The fresh fecal samples were collected from four healthy donors who had no history of bowel disorders. The donors, who consumed normal diets, did not receive antibiotics for at least 3 months. Fecal samples were pooled with an equal amount of feces from each donor to obtain human inoculum. Hereafter fecal slurry was prepared by diluting fecal sample with 10% (v/v) Dulbecco's phosphate buffered saline to obtain a 20% (w/w) solution, and subsequently was homogenized with a blender for 1 min. The inoculum was then filtered with two layers of cheesecloth, and filtrate was added into a fermentation medium without carbohydrate source with a ratio of 1:1 (v/v). D-PBS and fermentation medium, which were prepared according to Bianchi's method with some modifications (Bianchi, Dall Asta, Del Rio, Mangia, & Scazzina, 2011), were sterilized at 121 °C for 30 min before use. The human fecal inoculum was immediately stored in an anaerobic jar until use.

2.4. In vitro fermentation

One milliliter of 20 mg/ml polysaccharide dissolved with autoclaved ultra-pure water and 9 ml human fecal inoculums were introduced to tubes and mixed thoroughly through a vortex mixer. All processes were carried out under conditions of 10% H_2, 10% CO_2 and 80% N_2 in Forma Anaerobic System (Thermo Electron Corp., Marietta, OH, USA) (Hughes et al., 2007). The control group was treated with 1 ml autoclaved ultra-pure water instead of polysaccharide. Subsequently, the different anaerobic sealed tubes were incubated at 37 °C, 250 rpm using TC-2112B Thermostat shaker (Shanghai Nuoji Corp., Shanghai, China) for 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h.

2.5. Analytical methods

2.5.1. Determination of physicochemical property

The contents of total neutral sugar, uronic acid, reducing sugar and protein in the polysaccharide were determined by the phenol–sulfuric acid assay with glucose standard, carbazole–sulfuric acid assay with galacturonic acid standard, dinitrosalicylic acid method with glucose standard and Coomasie brilliant blue-based colourimetric assay with Bovine serum albumin standard, respectively (Miller, 1959; Xie et al., 2010). Additionally, the contents of moisture and ash were measured using AOAC methods (AOAC 934.01; AOAC 94 2.05), and the pH value was determined by a pHS-3Bmeter (Shanghai Leici Apparatus Corporation, Shanghai, China).

Table 1
The condition of the gradient elution solution.

Retention time (min)	Elution solution		
	250 mM NaOH (%)	Water (%)	1 M CH_3COONa (%)
0–20	0.8	99.2	0
20–20.1	0.8	94.2	5
20.1–30	0.8	74.2	20
30–30.1	80	20	0
30.1–50	80	20	0

2.5.2. Determination of pH value

The fermentation products of the polysaccharide and the control group were collected at 0, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h fermentation. These fermentation products were centrifuged at 9000 × *g* for 10 min. Then, the supernatants were transferred into 10 ml screw cap tubes, and the pH values were measured by a pHS-3Bmeter (Shanghai Leici Apparatus Corp., Shanghai, china).

2.5.3. Determination of neutral and reducing sugar contents

The contents of neutral and reducing sugar of fermentation products at 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h were analyzed using the phenol–sulfuric acid assay and dinitrosalicylic acid method (DNS), respectively, with some modifications (Miller, 1959; Xie et al., 2010). The procedures of determining reducing sugar level were carried out as follows: 1 ml supernatant was added into 0.75 ml DNS, and then placed into a 100 °C water bath for 5 min. After cooled down, the mixture was diluted to 10 ml with ultra-pure water. Ultimately, the absorbance of the mixture was measured at a wavelength of 540 nm. The net concentration of neutral or reducing sugar in the fermentation product was calculated using the equation as below:

$$C = C_2 - C_1$$

where C is the net concentration of neutral or reducing sugar, and C_2 and C_1 is the concentration of neutral or reducing sugar in the fermentation products of polysaccharide and the control (water), respectively.

2.5.4. Determination of monosaccharide content

The composition and content of free monosaccharide, which was produced during polysaccharide fermentation, were determined by a high-performance anion exchange chromatography (HPAEC) equipped with a Dionex CarboPac PA20 column (3 mm × 150 mm) and a Dionex gold electrode detector. The volume of injected sample was 25 μl, and it would run for 50 min at a flow rate of 0.5 ml/min (Dai, Liang, Liu, Chen, & Wang, 2006). The condition of the gradient elution solution was presented in Table 1.

2.5.5. Determination of SCFA contents

Contents of SCFAs in the fermentation products were determined according to Baere et al.'s method with some modifications (Bianchi et al., 2011). Samples were treated with 100 μl concentrated HCl, and then mixed for 15 s by a vortex mixer. Thereafter, the samples were extracted for 20 min by adding into 5 ml diethylether. After the samples were centrifuged at 3500 rpm for 5 min, the supernatant was shifted to another Pyrex tube and added into 500 μl of 1 M NaOH. Subsequently, the samples were extracted again for 20 min, followed by a centrifugation step. The aqueous phase was transferred to an autosampler vial and added into 100 μl

Table 2
Physicochemical property of the polysaccharide.

Main components (%)	Polysaccharide
Moisture	10.38 ± 0.02[a]
Ash	11.74 ± 0.01
Protein	10.11 ± 0.05
Uronic acid	38.44 ± 0.03
Reducing sugar	20.48 ± 0.04
Neutral sugar	31.17 ± 0.03

[a] Values were mean ± 459 standard deviations ($n = 3$).

concentrated HCl, followed by a vortex mixing step. Contents of SCFAs in the pretreated samples were analyzed by Agilent 6890 N gas chromatography (GC) system consisting of a flame ionization detector (FID), an N10149 automatic liquid sampler (Agilent Technologies, USA) and a HP-INNOWAX column (30 m × 0.32 mm I.D) (Agilent Technologies, USA) coated with 0.50 μm film thickness. Nitrogen, with a split ratio of 1:10, was supplied as the carrier gas at a flux rate of 19.0 ml/min. The initial oven temperature was 100 °C, and kept for 0.5 min, then rose to 180 °C at a rate of 4 °C/min. Both of the temperatures of the FID and the injection port were 240 °C. The flow rates of hydrogen and air were 30 and 300 ml/min, respectively. The injected sample volume was 0.1 μl, and the running time for each analysis was 20.5 min (Hu, Nie, Min, & Xie, 2012).

2.6. Statistical analysis

All the experiments were performed in triplicate. The results were expressed as mean ± standard deviations (SD). Data were evaluated by univariate analysis of variance (ANOVA), and differences were deemed significant when $p < 0.05$ using SPSS (version 17.0, Chicago, USA).

3. Results and discussion

3.1. Composition of polysaccharide from C. paliurus

As shown in Table 2, moisture, ash and protein accounted for 10.38 ± 0.02, 11.74 ± 0.01 and 10.11 ± 0.05%, respectively. Polysaccharide was testified as the pectic polysaccharide due to the content of uronic acid being 38.44 ± 0.03% (Table 2). The total carbohydrate content, which composed of neutral sugar and uronic acid (Wu, Cui, Tang, Wang, & Gu, 2007), was 69.61%. It was reported that the polysaccharide consisted of seven kinds of monosaccharide including Aha, Ara, Glc, Xyl, Man, Gal and GalA and the dominant monosaccharide was Ara, Gal, Glc and GalA (Xie et al., 2010), which was identical with the result of polysaccharide composition and dominant monosaccharide in this study (data not shown).

3.2. pH change during fermentation

As a whole, the pH value of polysaccharide fermentation (the experiment group) slightly reduced (from 7.2 to 6.04) and was significantly different from the control group (water) at the initial fermentation (4 h, $p < 0.05$). There were not different between the two groups at other time points evaluated ($p > 0.05$). Another finding was that pH value of polysaccharide fermentation group was significantly lower compared with water (the control group) (Fig. 1, $p < 0.05$). Similar results were found in two previous studies (Hu, Nie, Min, & Xie, 2013; Hu, Nie, Li, & Xie, 2013; Karppinen, Liukkonen, Aura, Forssell, & Poutanen, 2000).

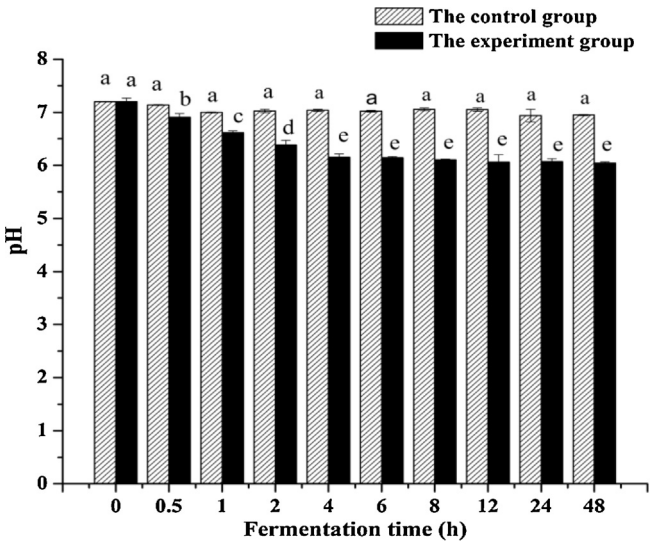

Fig. 1. pH change in vitro fermentation at different time points compared with control group (water). Data were mean ± SD ($n = 3$). Univariate ANOVA and Bonferroni tests were used to determine significant differences. Data with different letters indicated significant differences ($p < 0.05$).

3.3. The contents of neutral and reducing sugar of fermentation products

The content of reducing sugar significantly increased at 0.5 h as compared with polysaccharide (0 h) ($p < 0.05$) (Table 3). And Aha, Ara, Gal, Glc, Man and GalA were found in fermentation products at 0.5 h while Xyl was detected at 1 h (Table 4). Some studies have shown that the breakdown of glycosidic bonds must result in the increasing number of reducing ends (Chen et al., 2012a,b; Hu et al., 2013a,b). Consequently, it could be inferred that fermentation of the polysaccharide may involve in the breakdown of glycosidic bond, which was consistent with previous studies (Cummings & Macfarlane, 1991; Macfarlane, Macfarlane, & Gibson, 1998; Olano-Martin, Mountzouris, Gibson, & Rastall, 2000). Interestingly, it was found that the content of reducing sugar declined from 0.424 ± 0.020 mg/ml (0.5 h) to 0.263 ± 0.009 mg/ml (48 h), and the disappearance of reducing sugar was faster within 4 h (excluding 0 h) compared to the rest time points (6–48 h). Overall, the content of neutral sugar tended to decrease (from 0.427 ± 0.001 mg/ml at 0 h to 0.192 ± 0.021 mg/ml at 48 h). And its consumption was faster within fermentation time of 1 h compared to the rest time points (Table 3). It has been reported that the content change of carbohydrate could be demonstrated by determining

Table 3
The contents of reducing and neutral sugar during in vitro fermentation.

Time (h)	Reducing sugar (mg/ml)[a]	Neutral sugar (mg/ml)[a]
0	0.294 ± 0.002 ab[b]	0.427 ± 0.001 a
0.5	0.424 ± 0.020 c	0.321 ± 0.010 b
1	0.358 ± 0.005 d	0.263 ± 0.002 bc
2	0.343 ± 0.021 ad	0.268 ± 0.006 bd
4	0.305 ± 0.002 bd	0.260 ± 0.019 bc
6	0.290 ± 0.004 ab	0.220 ± 0.002 cd
8	0.290 ± 0.011 ab	0.229 ± 0.023 cd
12	0.285 ± 0.003 b	0.192 ± 0.028 c
24	0.264 ± 0.004 b	0.206 ± 0.019 cd
48	0.263 ± 0.009 b	0.192 ± 0.021 cd

Data with different letters indicated significant differences ($p < 0.05$) in the same column.

[a] Values were mean ± standard deviations ($n = 3$).

[b] Univariate ANOVA and Bonferroni test were used to determine significant differences.

Table 4
Composition and content of free monosaccharide in fermentation culture during fermentation.

Monosaccharide[a] (μg/ml)	Fermentation time (h)									
	0	0.5	1	2	4	6	8	12	24	48
Rha	ND[b]	4.540 ± 0.100 a[c]	3.900 ± 0.020 b	2.310 ± 0.400 c	ND	ND	ND	ND	ND	ND
Ara	ND	5.650 ± 0.090 a	1.560 ± 0.070 b	1.070 ± 0.010 c	0.310 ± 0.060 d	0.240 ± 0.070 d	0.250 ± 0.030 d	0.060 ± 0.010 e	ND	ND
Gala	ND	3.320 ± 0.150 a	2.660 ± 0.240 b	1.310 ± 0.170 c	1.500 ± 0.020 c	1.330 ± 0.130 c	1.160 ± 0.210 c	1.120 ± 0.120 c	ND	ND
Glc	ND	5.465 ± 0.114 a	4.269 ± 0.588 b	2.818 ± 0.045 c	2.325 ± 0.637 c	1.756 ± 0.034 c	ND	ND	ND	ND
Xyl	ND	ND	0.142 ± 0.119 a	0.894 ± 0.034 b	0.408 ± 0.089 c	0.148 ± 0.048 a	ND	ND	ND	ND
Man	ND	3.550 ± 0.130 a	1.570 ± 0.110 b	0.300 ± 0.040 c	ND	ND	ND	ND	ND	ND
Galac	ND	36.640 ± 0.120 a	23.480 ± 0.610 b	1.650 ± 0.090 c	0.360 ± 0.040 d	0.410 ± 0.020 d	0.300 ± 0.020 d	0.070 ± 0.010 d	ND	ND

Data with different letters indicate significant differences ($p < 0.05$) within a row.
[a] Values were mean ± standard deviations ($n = 3$).
[b] ND, not detectable.
[c] Significances are determined using univariate ANOVA and Bonferroni test.

the content of neutral sugar using the phenol–sulfuric acid assay (Hughes et al., 2007). Accordingly, it might be deduced from the content change of reducing and neutral sugar that polysaccharide could be partly fermented by human inoculum. The result was similar to some carbohydrate fermentation by human fecal microbiota (Hu et al., 2013a,b; Olano-Martin et al., 2000).

3.4. Monosaccharide consumption

The extent of fiber utilization could be indicated by the amount of monosaccharide consumption in the fermentation solution during fermentation. Generally, the levels of monosaccharide (Aha, Ara, Gal, Glc, Xyl, Man and GalA) decreased as fermentation time increased, and the difference of consumption of seven kinds of monosaccharide within initial fermentation (4 h) was significant ($p < 0.05$). As shown in Table 4, Aha, Ara, Gal, Glc, Xyl, Man and GalA in fermentation products were not detected at fermentation of 4, 24, 24, 8, 8, 4 and 24 h, respectively, and none of monosaccharide was detected at 24 h by HPAEC. Notably, the levels of reducing and neutral sugar remained unchanged ($p > 0.05$) (Table 4), but total SCFAs increased significantly ($p < 0.05$) (Fig. 2) after 24 h (from 24 to 48 h). It was found that the breakdown of glycosidic bonds to produce monosaccharide did not occur, but monosaccharide was utilized to synthesize SCFAs, especially *n*-butyric acid ($p < 0.05$), *i*-valeric acid ($p < 0.05$) and *n*-valeric acid ($p < 0.05$) after the fermentation 24 h. And the yield of GalA increased rapidly (36.64 ± 0.12 μg/ml at 0.5 h), and consumption rate of GalA was much higher (decreased from 36.64 ± 0.12 to 0.36 ± 0.04 μg/ml within 4 h) compared with the other monosaccharides (Table 4). On the contrary, the yield of Xly increased relatively slow, and its consumption rate was low (declined from 0.894 ± 0.034 at 2 h to 0.408 ± 0.089 μg/ml at 4 h). These results were similar to previous reports (Cherbut et al., 1991; Salvador et al., 1993). It was found that the rate and extent of carbohydrate degradation may depend on its chemical and physical arrangement in the fiber matrix (Salvador et al., 1993). It was also reported that incomplete hydrolysis of polysaccharide occurred in gum karaya, soy fiber, gum arabic and citrus pectin may be associated with glycosidic linkage of uronic acids. The hydrolysis of polysaccharide was also observed in this study, and the monosaccharide linked to side chain was more susceptible to be degraded to monomers or dimmers compared with linked to backbone (Bourquin, Titgemeyer, & Fahey, 1996; Gulfi, Arrigoni, & Amadò, 2007; Jonathan et al., 2012). Thus, it could be hypothesized that some of GalA may link to side chain in polysaccharide from *C. paliurus*, glycosidic linkage to GalA was prone to be broken down, and GalA was facile to be utilized or degraded by human fecal bacteria.

3.5. SCFA production

As shown in Fig. 2, the amount of total SCFAs and individual SCFAs in the experiment group (polysaccharide) continuously increased as the fermentation time increased. The levels of total SCFAs and individual SCFAs in the polysaccharide group were significantly different from the control group at each time point ($p < 0.05$). Markedly, the predominant SCFAs were found to be acetate (from 17.71 ± 0.86 mM at 0.5 h to 29.02 ± 1.33 mM at 48 h), followed by propionate and *n*-butyrate. The yields of propionate and *n*-butyrate were increased from 1.77 ± 0.19 mM at 0.5 h to 4.02 ± 0.23 mM at 48 h and from 1.52 ± 0.14 mM at 0.5 h to 3.17 ± 0.15 mM at 48 h, respectively (Fig. 2). The yield of *i*-butyrate was similar to *n*-valerate, but lower than *i*-valerate (from 0.29 ± 0.02 to 0.54 ± 0.03 mM, Fig. 2A). These results were in agreement with previous studies (Bourquin, Titgemeyer, & Fahey, 1993; Weimer, Stevenson, Mertens, & Hall, 2011). It was reported that the production rates of individual SCFAs were closely correlated

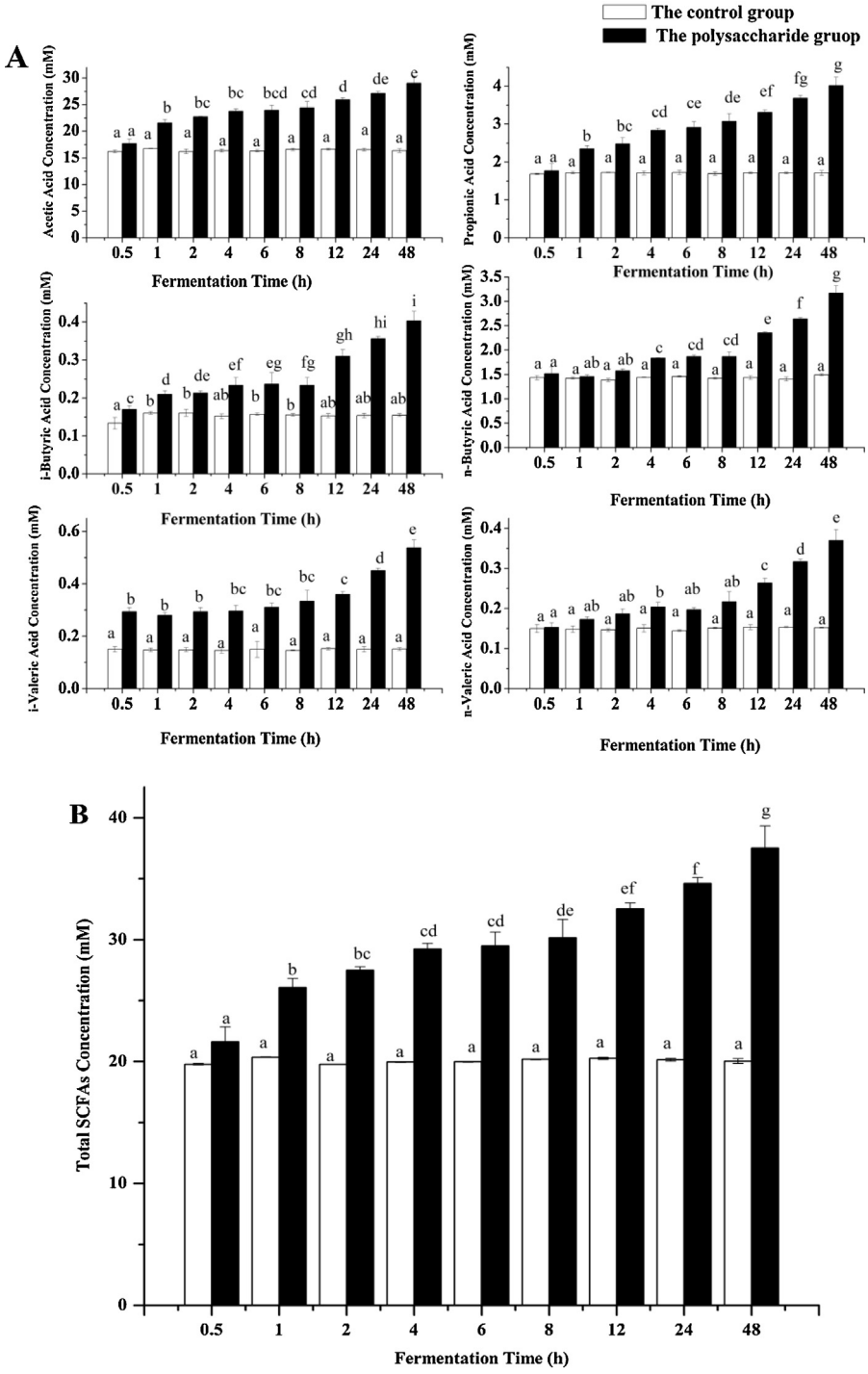

Fig. 2. Individual SCFAs (A) and total SCFAs (B) at 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h in vitro fermentation of polysaccharide from *Cyclocarya paliurus* leaves as compared with the control (water). Values were mean ± standard deviations of three independent experiments. Significances are determined using univariate ANOVA and Bonferroni test. Data with different letters indicate significant differences ($p < 0.05$) within a column.

with the fermentability of polysaccharide (Salvador et al., 1993). Fermentation of carbohydrate in vitro has also shown that uronic acids and pectins induced the production of acetic acid, and production of propionic acid could be promoted by the fermentation of glucose and arabinose (Chen et al., 2012a,b; Gulfi, Arrigoni, & Amad, 2005; Salvador et al., 1993). As a consequence, GalA may be principally involved in the production of acetic acid owing to its high content of uronic acid (38.44%). Furthermore, Ara and Glc might be closely correlated to propionic acid and *n*-butyric acid formation.

4. Conclusion

This study has for the first time investigated the fermentation behavior of polysaccharide from *C. paliurus* leaves by evaluating the production of SCFAs, pH change and relationship between SCFAs formation and monosaccharide by human fecal cultures. The results showed that pH value declined as fermentation time increased, and polysaccharide was partly fermented. In addition, acetic acid, propionic acid and *n*-butyric acid, which were the dominant SCFAs, significantly got a rise and galacturonic acid might be deemed as the

main producer of acetic acid. Future work should include determination of the structure and configuration of polysaccharide in detail, and further explain the relationship between monosaccharide and production of SCFAs.

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