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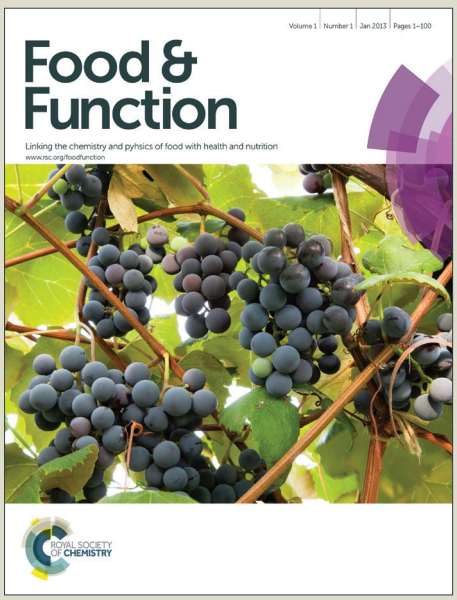

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Plant Polyphenols Altering a Pathway of Energy Metabolism by Inhibiting Fecal Bacteriodetes and Firmicutes *In Vitro*

Bin Xue [a*], Jinli Xie [a*], Jiachen Huang, Long Chen[a], Lijuan Gao[a], Shiyi Ou[a], Yong Wang[a, b], Xichun Peng[a**]

a. Department of Food Science and Engineering, Jinan University, Guangzhou 510630, China

b. Guangdong Engineering Research Center for Oil and Fat Biorefinery, Jinan University, Guangzhou 510630, China

[*]The two authors equally contributed to this paper.

[**] Corresponding author

Tel. +86 15899975229

Email: tpxchun@jnu.edu.cn

Abstract

The function of plant polyphenols in controlling body weight has been focused for a long time. The aim of this study was to investigate the effect of plant polyphenols on fecal microbiota utilizing oligosaccharides. Three plant polyphenols, quercetin, catechin and puerarin, were added into liquid media for fermenting 24hrs respectively. The pH values, OD_{600} of cultures and the content of carbohydrates at 0, 6, 10, 14, 18 and 24 hrs were determined. The abundances of Bacteroidetes and Firmicutes in each culture were quantified with qPCR after 10 hrs' fermentation, and the bacterial composition was analyzed using software Quantitative Insights Into Microbial Ecology. The results revealed that all the three plant polyphenols could significantly inhibit the growth of Bacteroidetes (P<0.01) and Firmicutes (P<0.01), at the same time down-regulate the rate of Bacteroidetes to Firmicutes (P<0.01). But the fecal bacteria could maintain the ability of hydrolyzing fructo-oligosaccharide (FOS) *in vitro*. Among the tested polyphenols, catechin presented the most intensive inhibitory activity towards the growth of Bacteroidetes and Firmicutes, and quercetin is the second. Only the samples with catechin had a significantly lower ability of energy metabolism (P<0.05). In conclusion, plant polyphenols can change the pathway of degrading FOS or even energy metabolism *in vivo* by altering gut microbiota composition. It may be one of the mechanisms that plant polyphenols can result body weight loss. It's the first report to study gastrointesintalmicrobiota *in vitro* fermenting dietary fibers under the intervention of plant polyphenols.

Keywords: plant polyphenols, FOS, feacal bacteria, energy metabolism, *in vitro* fermentation.

Introduction

Obesity is considered as a multifactorial disease caused by imbalance between calories consumed through eating and calories burned through physical activity Some studies suggest the health human GI tract microbiota could have impact on the development of excessive body weight [1-3]. The majority of mammals' gut bacteria belong to two phyla: the Bacteroidetes phylum and the Firmicutes phylum[4]. The microbiota acts as a metabolic organ which extracts energy from dietary material that we cannot digest [5]. *In vitro* and clinic studies have confirmed that changes in the microbiota ratio might affect normal food digestion process [6-15]. There is numerous evidence that the gut microbiota in obese patients involves higher of Firmicutes/Bacteroidetes ratio [2, 16, 17].

Bacteroidetes can degrade complex polysaccharides, which are resistant to the cleavage action of human digestive enzymes and can reach the colon relatively intact [18]. The microbiota, especially Bacteroidetes are believed to complement eukaryotic genomes with degradation enzymes targeting resistant dietary polymers, many of which are plant cell wall compounds (e.g., cellulose, pectin, and xylan) [18]. The bacteria-mediated fermentation of these food-derived polysaccharides in the colon leads to the generation of volatile, short-chain fatty acids (mainly acetate, propionate, and butyrate) that are reabsorbed by the host as energy resources. Therefore，these bacteria species help the host to gain energy from refractory carbohydrate sources [1-3, 5]. Hence, it is reasonable to assume that the host's body weight can be controlled by inhibiting carbohydrate- degrading bacteria (especially Bacteroidetes) in microbiota.

Many studies show that body weight reduces when ingesting puerarin and tea polyphenols such as catechins [19, 20]. No reported studies revealed that the other plant polyphenols can also induce body weight loss. But various plant phenolic compounds, including quercetin, ferulic acid, anthocyanins, (−)-epicatechin, caffeic acid and (+)-catechin, have been shown to modify the composition of gut microbiota [21-23].

Our previous studies discovered that a high fat diet induced host to shape gut

microbiota composition with a low ratio of Bacteroidetes to Firmicutes; higher content of Bacteroidetes in healthy animals attributes to body weight when the hosts ingest a high- fructo-oligosaccharide (FOS)/Soybean fiber diet [24-26]. The aim of this study was to investigate the effect of several plant polyphenols on gut microbiota fermenting FOS. The mechanism that plant polyphenols induce body weight loss will be studied and revealed

Materials and Methods

Fecal Batch-Culture Fermentation

Fermentation experiments were carried out using feces from a healthy volunteer, who had not ingested antibiotics for at least 6 months before the study and had no history of gastrointestinal disorder. Four parts of fresh feces were individually collected once and were immediately diluted 1:10 (w/v) with anaerobic phosphate buffer (1 M; pH 7.2) and homogenized in a stomacher for 2 min. Resulting fecal slurry was used to inoculate batch-culture vessels. The protocol described by Tzounis et al [27] was used for fecal fermentations with minor modification. Briefly, sixteen 50-mL plastic tubes were equally divided into 4 groups (Control, Q, CAT and P). Each vessel was filled with 49.5 mL of a prereduced sterile medium (peptone water (2 g/L), yeast extract (2 g/ L), NaCl (0.1 g/L), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), $NaHCO_3$ (2 g/L), $MgSO_4 \cdot 7H_2O$ (0.01 g/L), $CaCl_2 \cdot 6H_2O$ (0.01 g/L), Tween 80 (2 mL/L), hemin (50 mg/L), vitamin K (10 μL/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), resazurin (1 mg/L), fructo-oligosaccharide [FOS, Quantum Hi-Tech (China) Biological Co., Ltd] (10g/L), and distilled water). Quercetin, catechin, or puerarin (Sigma-Aldrich Chemical Co.) was individually added into the above liquid media of Group Q, CAT and P, 0.15g/L in each vessel[28]. The medium was adjusted to pH 7.0. The vessels of Group Control were prepared without any polyphenols (negative control). All media were deoxidized by ultrasonic and each vessel was filled up to avoid oxygen. Batch cultures were run in an anaerobic incubator for a period of 24 h during which samples were collected at six time points

(0, 6, 10, 14, 18, and 24 h) for determination of pH value, OD_{600} and carbohydrates. For this later analysis, samples were stored at −70 °C until required.

Carbohydrate analysis

The carbohydrate analysis was analyzed by the method described by Le et al [29] with minor modification. The standard curve was prepared as follows: FOS anhydrous (25 mg) is accurately weighed and then dissolved in 25 ml of double distilled water as stock standard solution (1 mg/mL). Accurately draw FOS solution 1.0, 0.5, 0.25, 0.125, 0.0625, 0 mL to six 10 mL tubes, individually add water to the volume of 1.0 ml, precisely add 4 mL of anthrone–sulfuric acid [0.25mg anthrone per 1mL of sulfuric acid (98%) in a 100 ml flask], heated for 15 min, then remove and put in ice-water to cool for 15 min, with the corresponding reagent as control. Determine the absorbance in the 625 nm wavelength and make it as the ordinate, concentration as abscissa to establish a standard curve.

Samples from the above six time-points were centrifuged to remove precipitates. Their supernatants were individually collected to remove protein with Sevage reagents for carbohydrate analysis. Precisely measure 1 mL of the sample solution, put it into a 10 mL test tube. Follow the method of establishing the standard curve, as the "precisely add 4 mL of anthrone–sulfuric acid" begin to determine absorbance. Then calculate the content of the polysaccharide according to the standard curve.

Extraction of DNA from cultures

Genomic DNA were extracted from the aforementioned fecal bacteria in the 10^{th} hours' cultures with a TaKaRa minibest bacterial genomic DNA extraction kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. The final elution volume was 100 μL, and the concentration was determined by spectrophotometer (Beckman Coulter DU 800, Fullerton, CA).

PCR conditions

The primers and probe sets specific for all groups used are described by Guo et al [30]. Oligonucleotide primers and probe were ordered from Sangong (Shanghai,

China). Amplification and detection of DNA by real-time PCR were performed with the ABI-Prism 7900 Sequence Detection System (Applied Biosystems) using optical grade 384-well plates. Triplicate samples were routinely used for the determination of DNA by real-time PCR, and the mean values were calculated. The PCR reaction was performed in a total volume of 10 μL. Bacteroidetes and Firmicutes were detected by using the Power SYBR PCR Master Mix (Applied Biosystems), with 100 nmol/L of each of the forward and reverse primers and 1 ng DNA for each reaction. The PCR reaction conditions for amplification of DNA were 50 ℃ for 2 min, 95℃ for 10 min and 40 cycles of 95℃ for 15 s and 60 ℃ for 1 min. A melting curve analysis was done after amplification. The threshold cycle (C_T) values and baseline settings were determined by automatic analysis settings. Data analysis was performed using Sequence Detection Software (version 2.3) supplied by Applied Biosystems.

16S rDNA gene PCR amplification and sequencing

The primers F515 (59-CACGGTCGKCGGCGCCATT-39) and R806 (59-GGACTACHVGGGTWTCTAAT-39) [31] were used to amplify the V_4 domain of bacterial 16S rDNA. PCR reactions contained 5-100 ng DNA template, 1×GoTaq Green Master Mix (Promega, Madison, WI), 1 mM $MgCl_2$, and 2 pmol of each primer. Reaction conditions consisted of an initial 94 ℃ for 3 min followed by 35 cycles of 94 ℃ for 45 sec, 50 ℃ for 60 sec, and 72 ℃ for 90 sec, and a final extension of 72 ℃ for 10 min. All samples were amplified in triplicate and combined prior to purification. Amplicons were purified using the Qiaquick 96 kit (Qiagen), quantified using PicoGreen dsDNA reagent (Invitrogen, Grand Island, NY), all according to the manufacturers' instructions. Purified libraries were sequenced on the Illumina GAIIx platform.

16S rDNA gene analysis

Raw Illumina fastq files were demultiplexed, quality-filtered, and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) [32]. Sequences that were shorter than 55 bp, contained primer mismatches, ambiguous bases or uncorrectable

barcodes, were removed. 16S rDNA gene sequences were assigned to operational taxonomic units (OTUs) using UCLUST with a threshold of 97% pair-wise identity [33], and then classified taxonomically using the Ribosomal Database Project (RDP) classifier 2.0.1 [34].

Alpha diversity estimates were calculated with Shannon value. Principal Coordinates Analysis (PCoA) and heat map was performed to present differences between the gut microbial communities of the two groups. These analyses were conducted by Gene Denovo Co. (Guangzhou, China).

Statistical analysis

Results are expressed as mean values and standard deviations. The statistical analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, IL). T-tests were conducted to compare the bacterial phenotypes in different vessels and all statistical tests were two-tailed. Statistical significance was set at a P value of < 0.05. All data are presented in the text as the means ± s.e.m.

Results

The effects of three tested plant polyphenols, including quercetin, catechin, and puerarin, on the growth of human gut bacteria were shown on Figure 1-6. Comparing to the control group, three groups of samples didn't significantly reduce the total bacteria amount based on their OD_{600} (Fig. 1A). All four groups presented the similar tendency of pH change (Fig. 1B). The content of carbohydrates in Group Q, CAT and P presented significant difference from the control group at the 6th and 14 th (P<0.01). After 24 hours' fermentation, all carbohydrates can't be detected; but at the first 10 hours' fermentation, the contents of carbohydrates in cultures were different. The control group showed the higher rate of depleting carbon source than the others (Fig. 1C).

Real-time PCR analysis was performed to quantify Firmicutes and Bacteroidetes after 10 hrs' fermentation. The 16S rDNA gene copies of both Firmicutes and Bacteroidetes in all three groups decreased after introducing the polyphenols(P<0.01)

(Fig. 2).

In order to furtherly study which bacteria were repressed by polyphenols, the cultured microbiota were identified by 16S rDNA gene analysis. Among 6 phyla detected in each group, the relative abundance of Bacteroidetes was significantly lowered down from 41.7% in the control samples to 23.1%, 11.6% and 23.5% in Q, CAT and P in order; the relative abundance of Firmicutes didn't present regular changes, 21.3% in the control samples, 25.1%, 19.6% and 23.6% in Group Q, CAT and P (Fig. 3A). Furthermore, at the level of genus it was discovered that the relative abundance of *Bacteroides spp*. significantly decreased in Group Q, CAT and P than in the control group, from 30.5% down to 18.4%, 8.8% and 18.1% in order. The relative abundances of other genera of Bacteroidetes, including *Parabacteroides spp*., *Prevotella spp*., *Rikenellaceae spp*, *Butyricimonas spp*. and *Odoribacter spp*., were also reduced at different extents (Fig. 3B). But the abundances of *Bifidobacterium spp*. belong to Actinobacteria phyla were individually up-regulated from 6.6% in the control group to 24.0%, 21.7% and 8.3% in Group Q, CAT and P in order. The abundances of some other bacteria, such as *Enterococcus spp*. and *Streptococcus spp*. belong to Firmicutes phyla and *Collinsella spp*. also belong to Actinobacteria phyla, also increased in the tested groups (Fig. 4).

Based on the PCoA analysis, each group presented a distinct but similar constitution after *in vitro* fermentation (Fig. 5). Especially, the major difference was from Group CAT, and followed by Group P in contrast with the control group. The evolution and abundance of bacteria species in different groups were showed in Fig 4.

The KEGG analysis presented Group CAT had a lower ability ($P<0.05$) of carbohydrates metabolism, energy metabolism, glycan biosynthesis and metabolism, and lipid metabolism than the others; Goup Q and P didn't show the similar tendency (Fig. 6).

4. Discussion

The function of plant polyphenols such as tea polyphenols in controlling body weight have been focused for years. But the involved mechanisms are still unclear. In this study, when three plant polyphenols were respectively added into liquid media, the growth of Bacteroidetes and Firmicutes was significantly repressed and the rate of Bacteroidetes to Firmicutes was also down-regulated. However，the overall fecal bacteria microbiota grow normally and was able to hydrolyze FOS *in vitro* with a similar rate compared to the control sample. Among the three plant polyphenols, catechin presented potent activity in inhibiting Bacteroidetes and Firmicutes, followed quercetin. Only the samples with catechin had a significantly weaker ability of energy mechanism than the others including the control sample.

In this study, FOS was the only carbon source in all media. FOS is a notable non-digestive carbohydrate by human, but can be hydrolyzed by healthy microbiota [24, 26, 35]. The results showed that the percentage of Bacteroidetes *spp*. in total bacteria was the highest in the control group, which was consistent with our previous studies on its ability of digesting complex carbohydrates[18, 24, 26] . Bacteroidetes (41.7%), Proteobacteria (29.1%) and Firmicutes (21.3%) dominated in the control group, which is similar with the reported bacterial composition in mammals' gut [36].

Polyphenolic compounds are plant nutraceuticals processing a huge structural diversity, including chlorogenic acids, hydrolysable tannins, and flavonoids (flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, and flavones). Marín et al [22] and Parkar et al [37] reported the antibacterial ability of many plant polyphenols, including quercetin and catechin. Quercetin can reshape faecal gut microbiota composition reportedly [38]; tea polyphenols can significantly repressed growth of certain pathogenic bacteria like *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides spp.*, while less severely affected commensal anaerobes like *Clostridium spp*., *Bifidobacterium spp*. and probiotics such as *Lactobacillus* species [21]. Tang et al [39] also reported the anti-bacterial activity of puerarin. Our results showed agreement with these studies. The three plant polyphenols in this study, quercetin, catechin and puerarin, also altered the composition of fecal bacteria. Our experiments furtherly discovered that since the introduction of three plant

polyphenols, each of them independently reshaped the fecal bacteria composition (Fig. 3 and 4). The three polyphenols all significantly inhibited the growth of Bacteroidetes and Firmicutes (Fig 4), but the degradation of FOS was less affected in *in vitro* fermentation (Fig. 1C and 6). Therefore, other bacteria that can secrete carbohydrate hydrolytic enzymes to break FOS were certainly activated. Fig. 3B and 5 presented that the abundance of several genera of bacteria increased, including *Bifidobacterium spp*., *Enterococcus spp*., *Streptococcus spp*. and *Collinsella spp*., since the intervention of three polyphenols. For example, Turroni et al. proposed that *Bifidobacteria spp*. have quite a versatile carbohydrate breakdown machinery [40]. It is reasonable to speculate that the addition of three plant polyphenols in this study altered the pathway of degrading FOS. Besides, quercetin and catechin especially cooperate with FOS to faciliate the growth of *Bifidobacterium spp*.

The activity of plant polyphenols, especially tea polyphenols, on controlling body weight has been proved by mounting data, and the relative mechanism was discussed from various aspects [20, 21, 41, 42]. One hypothesis involved anti-bacterial activity of plant polyphenols. For instance, Rastmanesh et al argued that polyphenols may modulate microbiota balance through the biased promoting effects on Bacteroides, while phenolic compounds may exert their effect through biased suppressing effects on Firmicutes [41], which is not able to explain the observation in our previous studies [24-26]. In our studies, lean rats with a higher Bacteroidetes/Firmicutes rate can gain more weight, indicating that Bacteroidetes could help harvesting extral energy [24, 26]. This study showed that the three polyphenols all significantly inhibited the growth of Firmicutes and Bacteroidetes with a down-regulated ratio of Firmicutes to Bacteroidetes. It might be a part of mechanisms that polyphenols can result in body weight loss. However, it is still not sure whether the FOS was exhausted after 10 hrs fermentation. Since most of Firmicutes and Bacteroidetes were inhibited, how the other bacteria maintain the ability of carbohydrate metabolism and energy metabolism in each group is still in question. The pathway of other bacteria utilizeing FOS and their metabolites is still unknown. Obviously, organic acids were still produced in each experimental group

based on the similar pH change with the control group. When both plant polyphenols and dietary fibers (for example, FOS) were ingested, the pathway of fecal bacteria *in vivo* metabolizing carbohydrates deserves to be furtherly researched.

Conclusion

This is the first reported study of gut microbiota *in vitro* fermenting dietary fibers under the stress of plant polyphenols. Three plant polyphenols, quercetin, catechin and puerarin, can inhibit the growth of Bacteroidetes and Firmicutes and down-regulate the rate of Bacteroidetes to Firmicutes. But the fecal bacteria could still maintain the ability of hydrolyzing FOS *in vitro*. Among the three plant polyphenols, catechin presented the strongest activity of reshaping fecal microbiota, and quercetin was the second. Only the samples with catechin had a significantly lower ability of energy mechanism. Hence, plant polyphenols can change the pathway of degrading FOS or even energy metabolism *in vivo* by altering gut microbiota composition. It may be one of the mechanisms that plant polyphenols can lead to body weight loss.

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Fig. 1. Changes of OD_{600} (A), pH values (B) and carbohydrate content (C) during 24 hrs' *in vitro* fermentation.

Fig. 2. Copies of Bacteroidetes and Firmicutes. **, means $P<0.01$ when compared to the control group.

Fig. 3. Composition of cultural gut microbiota at the level of phyla (A) and genus (B). Legends are labeled only when the relative abundance of genus is higher than 1% in the control group.

Fig. 4. Evolution and abundance of bacteria species in different groups.

Fig. 5. Fermented fecal bacteria distribution by PCoA analysis. Control: the control group including A_{1-4}; Q: the group of samples with quercetin, including Q_{1-4}; CAT: the group of samples with catechin, including Sample C_{1-4}; P: the group of samples with puerarin, including Sample P_{1-4}.

Fig. 6. KEGG analysis of functional genes.

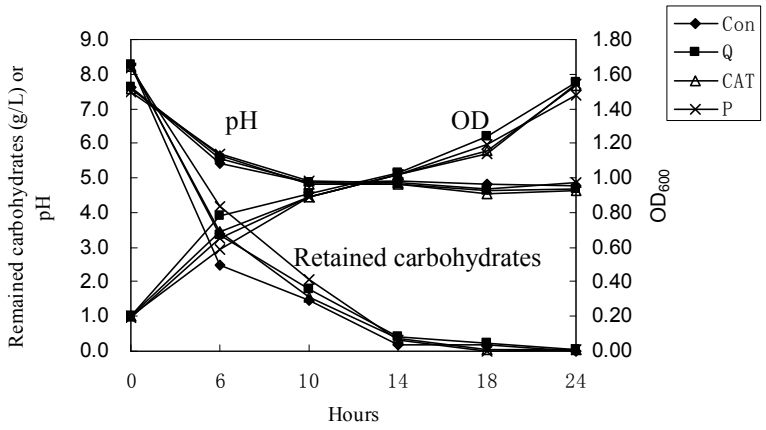


Fig. 1

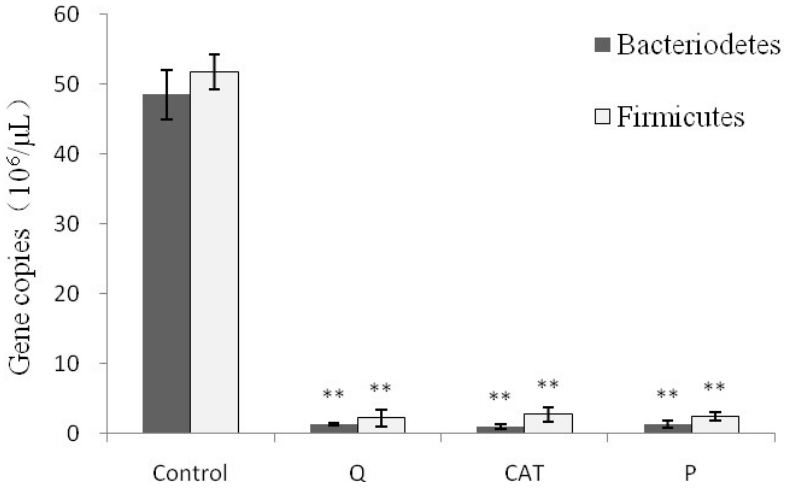
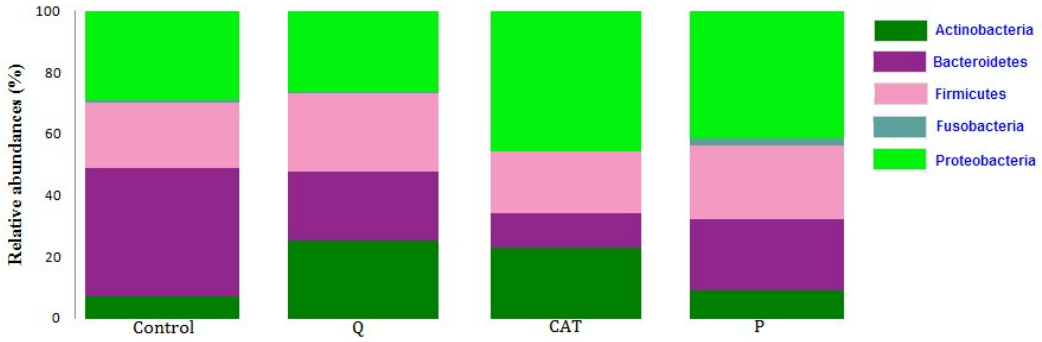
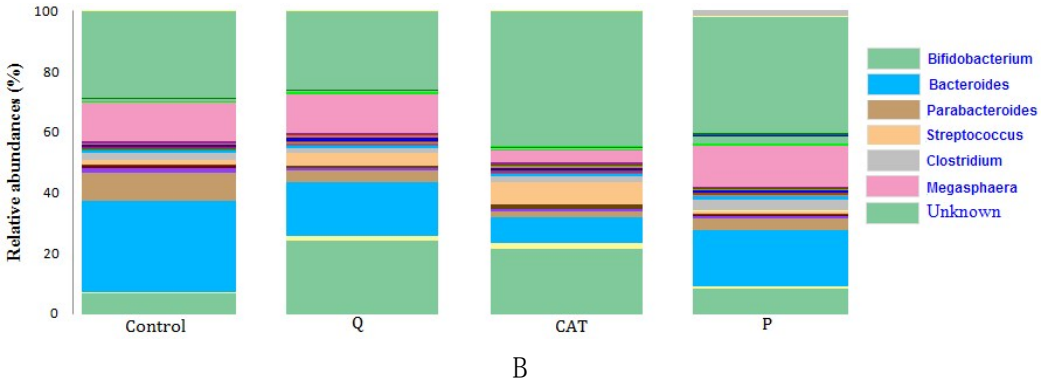


Fig. 2



A



B

Fig. 3

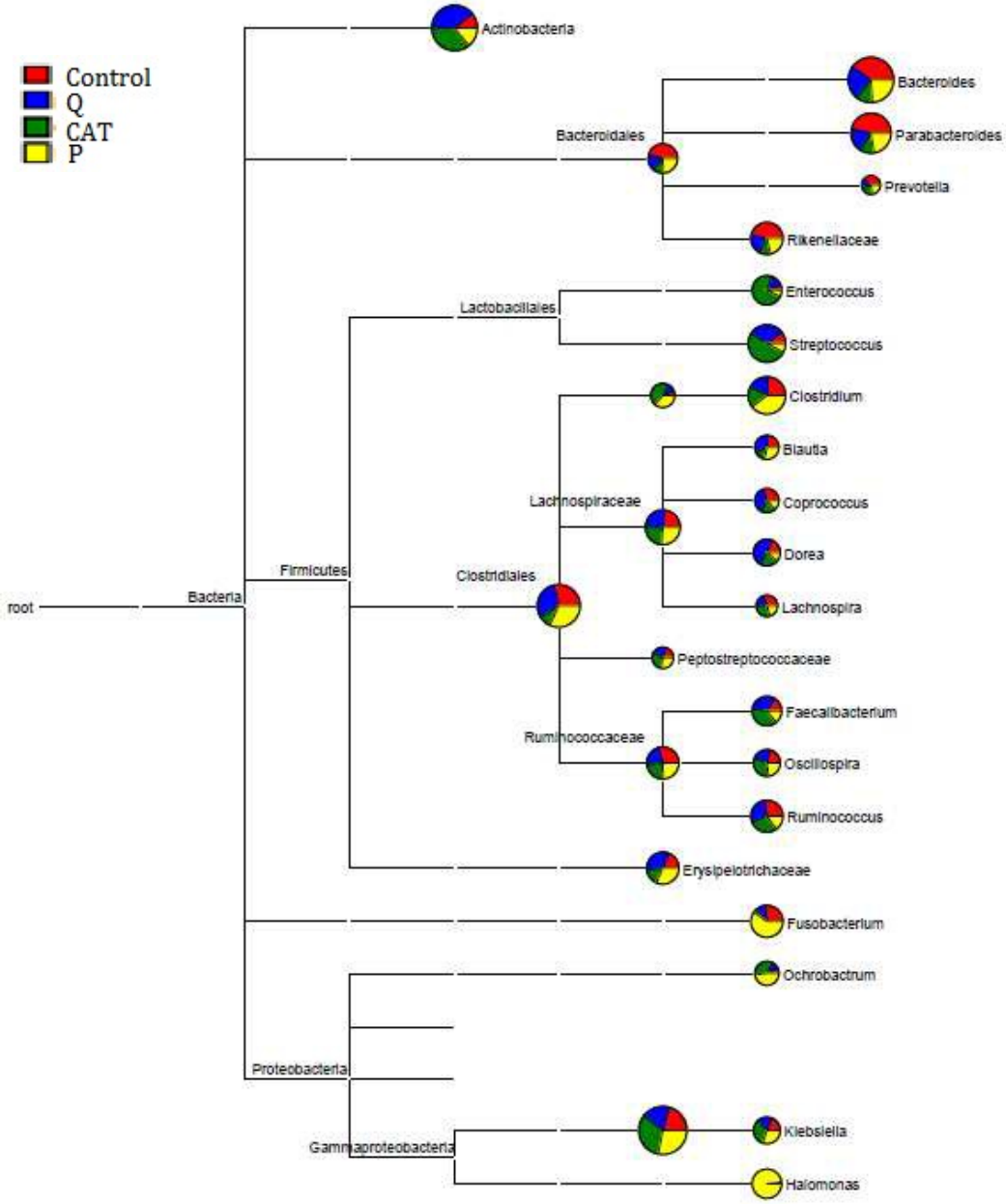


Fig. 4

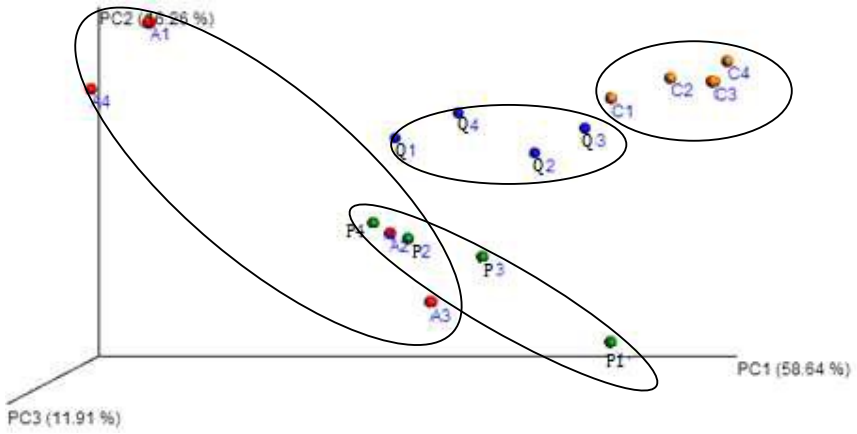


Fig.5

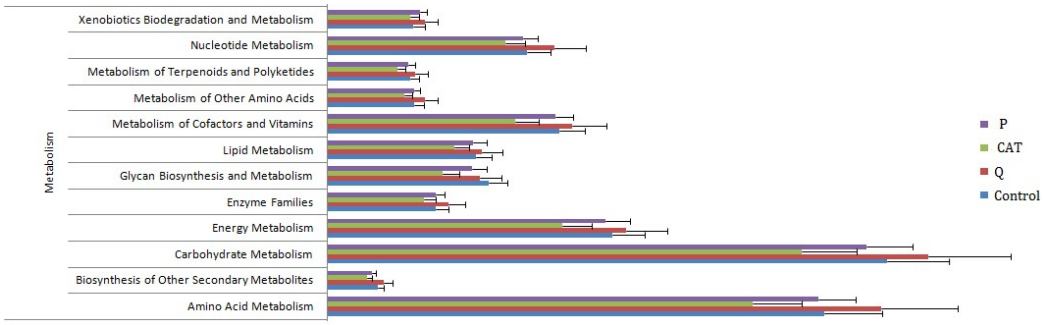
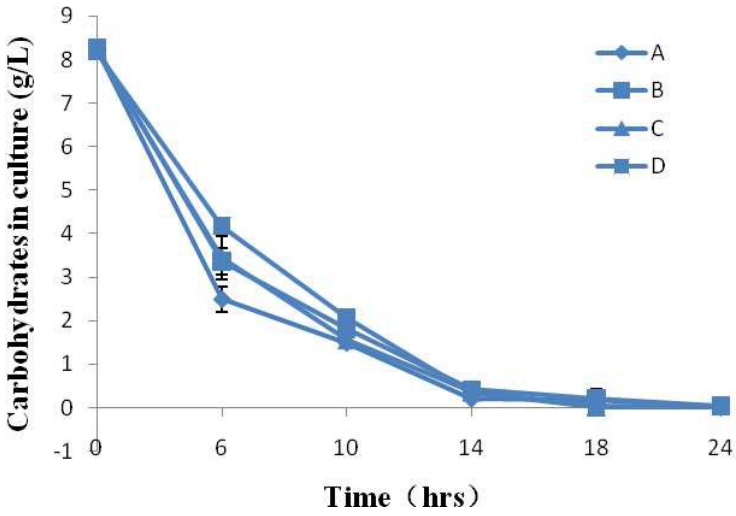


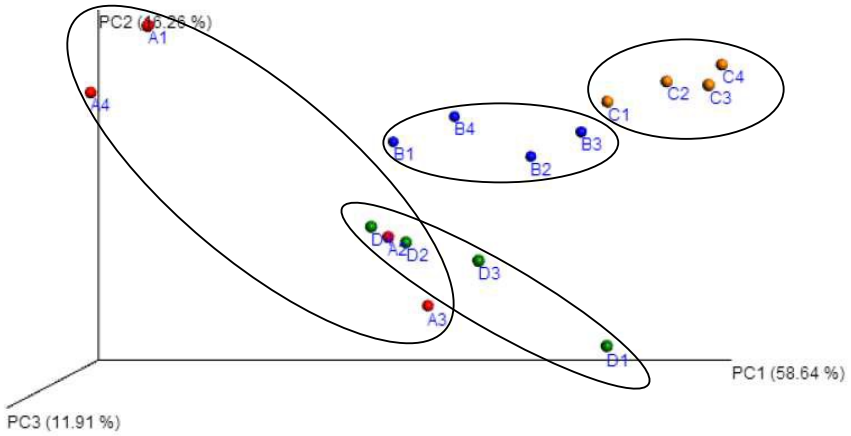
Fig.6

Graphical Abstract

This study was to investigate the effect of plant polyphenols on faecal microbiota metabolizing oligosaccharide. Three plant polyphenols, quercetin, catechin and puerarin, were individually added into liquid media for fermenting 24hrs. The pH values, OD_{600} of cultures and the content of carbohydrates in 0, 6, 10, 14, 18 and 24 hrs were respectively determined. The abundances of Bacteriodetes and Firmicutes in each culture were quantified and the bacterial composition in each culture was analyzed. The results showed plant polyphenols can change the pathway of degrading FOS or even energy metabolism *in vivo* by altering gut microbiota composition. It may be one of the mechanisms that plant polyphenols can lead to body weight loss.



Changes of carbohydrates concentration during in 24 hrs' vitro fermentation. A: the control group; B: the group of samples with quercetin; C: the group of samples with catechin; D: the group of samples with puerarin.



Fermented fecal bacteria distribution by PCoA analysis. A: the control group including Sample $A_{1\text{-}4}$; B: the group of samples with quercetin, including Sample $B_{1\text{-}4}$; C: the group of samples with catechin, including Sample $C_{1\text{-}4}$; D: the group of samples with puerarin, including Sample $D_{1\text{-}4}$.