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Full Length Research Paper

Effects of six flavonoid compounds addition on short-chain fatty acids production and human fecal microbial community change during *in vitro* fermentation

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Flavonoid has been proposed to have beneficial effects on human health, including antimicrobial activity. To clarify whether the flavonoid addition has an impact on the volatile fatty acids (VFA) production and the composition of the human fecal microbiota, six flavonoid compounds including baicalin, quercetin, icraiin, luteolin, amygdalin, naringin were investigated using a batch-culture fermentation system. 16S ribosomal RNA (rRNA) gene based polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE) and real-time PCR were used to monitor the changes in the composition of microbiota after the flavonoid addition. The results showed that incubation of six flavonoids compounds (40 or 160 mg/l) with faecal bacteria, led to a general increase in TVFA production (except baicalin), and improved the acetate, propionate (except baicalin and aringin) and butyrate (except baicalin) production. The analysis of DGGE profiles revealed that fingerprints of the fecal bacteria communities had a similarity of >89% between the control and the flavonoid treatment. As compared with the control, icraiin addition improved the Shannon index of diversity, while no significant differences were observed with baicalin, quercetin, luteolin, amygdalin or naringin addition, the Q-PCR results showed that the numbers of the total bacteria was increased by the naringin addition, and there is a tendency for increased in the bacteria number with the quercetin addition, while no any significant changes were observed on the bacteria number with the baicalin, icraiin, luteolin or amygdalin addition. These observations suggest that the consumption of flava-onid-rich foods may support gut health through their ability to exert prebiotic actions, but this flavonoid induced effect may depend on the type of the flavonid. Future human intervention studies will provide further insight into the potential of flavonoid monomers to act as prebiotics in the human large intestine *in vivo.*

Key words: Flavavonid, prebiotics, faecal microflora, Microbial diversity, polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

Flavonoids are a large group of polyphenolic compounds that are widely distributed in all plants. Fruits, vegetables and beverages (fruit juices, wine, tea, and coffee) are major sources of flavonoids in the human diet, and over 500 of these compounds have been reported to date. Adequate intakes of fruits and vegetables are reported to be associated with reduced risks of cardiovascular disease and cancer. These observations may be attributed, in part, to the effects of the flavonoid metabolites caused by gut microbial community.

It is well known that the colon is populated by a range of commensal and probiotic bacteria −10^{10} to 10^{11} cfu/g of colonic content (Isolauri et al., 2004). The balance among these bacterial species has been linked to both beneficial and detrimental effects. For example, certain members of

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Abbreviations: PCR, polymerase chain reaction; **DGGE,** denaturing gradient gel electrophoresis; **VFA,** volatile fatty acids; **TVFA,** total volatile fatty acids; **SCFA,** short chain fatty acid.

the *Clostridium* group, whilst others, such as *Bifidobacterium* and *Lactobacillus* spp., are known to exert beneficial effects in the colon (Saggioro, 2004) and have been utilized in the development of probiotic functional foods (Marteau, 2002). In addition, there has been much recent interest in the development of 'prebiotics', defined as non-digestible food ingredients that have a beneficial effect on the host by selectively stimulating the growth of a limited number of specific bacterial strains in the large intestine (Gibson et al., 2004). Presently, the most widely used prebiotics are fructo-oligosaccharides (FOS) and Insulin, and studies have indicated the anti-cancer potential of these carbohydrates (Kleessen et al., 2001). However, whilst soyabean isoflavones have been shown to possess prebiotic potential *in vivo* (Clavel et al., 2005), there is limited information regarding the potential prebiotic potential of other classes of flavonoids.

Previous studies have indicated that the flavonoid may influence gut microflora. De Boever et al. (2000) used a colonic model to investigate the prebiotic effect of soyagerm powder, and they observed an overall increase in all bacterial groups compared with controls, especially in *Lactobacillus* spp. Wells et al. (1999) showed that the isoflavone genistein can inhibit the internalization of enteric bacteria by human Caco-2 and HT-29 enterocytes. Clavel et al. (2005) reported that simultaneous consumption of isoflavones and functional foods led to marked changes in the dominant intestinal ecosystem of postmenopausal women. Tzounis et al. (2008) suggest that the consumption of flavanol-rich foods may support gut health through their ability to exert prebiotic actions. The purpose of the present study was to investigate the potential of the six falvonoid compounds including baicalin, quercetin, icraiin, luteolin, amygdalin and naringin, to influence the fermentation characteristics and microbial composition in batch-culture fermentation system. We detail the influence of flavonoid compounds addition on the short fatty acid production and diversity of the fecal microbiota and show that they are capable of inducing positive changes in VFA production and the balance of bacterial groups.

MATERIALS AND METHODS

The plant extracts (98% HPLC pure) were purchased from Qinche Co, Ltd (Nanjing, China). Sterilisation of media and instruments was achieved by autoclaving at 121°C for 15 min.

Faecal sample preparation

Faecal samples were collected from two separate individuals. The volunteers were in good health and had not ingested antibiotics for at least 3 months before the study. Samples were collected on the day of the experiment and were used immediately. The samples were mixed and diluted 1:10 (w/v) with anaerobic phosphate buffer (0·1 M; pH 6.9). Resulting faecal slurries were used to inoculate the batch-culture bottle.

Batch-culture fermentation

Batch-culture fermentation bottle consisted of 50 ml basal nutrient 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), $MgSO_4.7H_2O$ (0.01 g/L), $CaCl_2.6H_2O$ (0.01 g/L), Tween 80 (2 ml/L), haemin (50 mg/L), vitamin K1(10 ml/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), resazurin (1 mg/L) and distilled water) together with 0.2 g glucose The pH of the basal medium was adjusted to 6.9. Medium was then gassed overnight with O_2-free N_2, and autoclaved before inoculation. Each plant extracts (40 and 160 ug/mL) was added to reflect lower and upper levels of flavonoid monomer intake. Control bottles had the same ingredients except no flavonoid. Both flavonoid treatment and the control have three replicate bottles. Before the addition of faecal slurry samples, the temperature of the basal nutrient medium was set to 37°C. Each bottle were inoculated with 10 ml faecal slurry (1:10, w/v) and incubated at 37°C for 24 h. After 24 h of incubation, the fermentation was stopped by putting the bottles on ice. The sample was mixed and collected to analyze concentration of VFA and to extract the DNA. Total DNA was extracted from samples and used as templates to amplify fragments of the 16S rDNA gene. PCR products of V6–V8 regions were analyzed by DGGE. The DGGE profiles were analyzed for similarities between the samples by a computer program (BioNumerics 4.0).

DGGE (denaturing gradient gel electrophoresis)

Primers 968-GC (5'CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and 1401 (5'CGG TGT GTA CAA GAC CC) were used to amplify the special regions of the lactobacillus 16S rRNA. PCR was performed with *Taq* DNA polymerase kit (Promega, USA). The samples were amplified in a T1 Whatman Biometra (Gőttingen, Germany) using the following program: 94°C for 5 min, and 35 cycles of 94°C for 30 s, 56°C for 20 s, 60°C for 40 s, and 68°C for 7 min last extension. Aliquots of 5 µl were analyzed by electrophoresis on 1.2% agarose gel (w/v) containing ethidium bromide to check the sizes and amounts of the amplicons.

Amplicons of special regions of 16S rRNA gene were used for sequence-specific separation by DGGE according to the specifications of Muyzer et al. (1993), using a Dcode DGGE system (Bio-Rad, USA). DGGE was performed in 8% polyacrylamide gels containing 37.5:1 acrylamide-biacrylamide and a denaturing gradient of 38 to 53% of urea. The electrophoresis was initiated by pre-running for 10 min at a voltage of 200 V, and subsequently ran at a fixed voltage of 85 V for 16 h at 60°C. The gel was stained with $AgNO_3$ after completion of electrophoresis.

Banding pattern analysis and statistics

DGGE banding patterns were analyzed with Molecular Analyst software (BioNumerics 4.0). In order to compensate for internal distortions during electrophoresis, we aligned the gels by using an external reference pattern. The pattern was composed of pooled PCR products from five lactobacillus species loaded in at least five different lanes distributed along the gel. Subsequently, subtraction of the nonlinear background was achieved by using the rolling disk mechanism with an intensity of 8. For the completion of the gel analysis, identification and quantification of the bands present in each lane were performed by setting the tolerance and optimization at 0.75%.

Similarity indices were calculated for pairs of DGGE profiles. The similarity between the DGGE profiles was determined by calculating a band similarity coeffcient (SD) (Dice: SD =2nAB/(nA+nB), where nA is the number of DGGE bands in lane 1, nB represents the

number of DGGE bands in lane 2, and nAB is the number of common DGGE bands (Simpson et al., 1999; Gillan et al., 1998). As a parameter for the structural diversity of the microbial community, the Shannon index of general diversity, HP (Shannon and Weaver, 1963; Eichner et al., 1999; McCracken et al., 2001), was calculated using the following function: HP =-∑Pi log Pi, where Pi is the importance probability of the bands in a lane. HP was calculated on the basis of the bands on the gel tracks that were applied for the generation of the dendrograms by using the intensities of the bands as judged by peak height in the densitometric curves. The importance probability, Pi, was calculated as: Pi = ni/HP, where ni is the height of a peak and HP is the sum of all peak heights in the densitometric curve.

Real-time PCR assay for quantification of total bacteria

Real-time PCR was performed on an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenen-daal, the Netherlands) as described by Suzuki et al. (2000). A reaction mixture (25 ml) consisted of 12.5 ml of IQ SYBR Green Supermix (Bio-Rad), 0.2 mM of each primer set and 5 ml of the template DNA. The amount of DNA in each sample was determined in triplicate, and the mean values were calculated. A standard curve was generated by using the serially diluted 16S rRNA gene amplicons obtained from *Lactobacillus sobrius* strain. Universal primers, Bact1369 and Prok1492 (Suzuki et al., 2000) were used to estimate the total number of copies of the bacterial 16S rRNA gene in each sample. PCR was performed with an initial denaturation step of 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s.

Analysis of fermentation end products

VFA were measured by using a capillary column high performance liquid chromatography (HPLC) chromatograph.

Statistical analysis

The data were subjective to the analysis of variance using the general linear models (GLM) of SPSS 15.0. Significant differences were declared if $P<0.05$ and tendencies if $P<0.10$.

RESULTS

The effect of the flavonoid addition on VFA production

The results showed that the quercetin, icraiin, luteolin, amygdalin or naringin addition significantly improved the acetate, propionate (except naringin), butyrate and TVFA production ($P<0.05$) (Table 1). As compared with the control, baicalin addition improved the acetate production, while no significant difference was observed in propionate, butyrate and TVFA production with baicalin addition.

The effect of the flavonoid addition on the fecal microbial community structure

Following fermentation for 24 h, the analysis of DGGE profiles revealed that fingerprints of the fecal bacteria communities had a similarity of ≥89% among control, naringin and quercetin treatment, and higher than 91% between the control and baicalin,luteolin, amygdalin and icraiin trematment (Figures 1, 2 and 3). It can be clearly seen that bands from the control, naringein, quercetin, baicalin and luteolin group tended to cluster separately, except in the case of baicalin 160 C and quercetin 160 B.

The effect of the flavonoid addition on the Shannon index of diversity and quantity of the total bacteria during the fermentation of the flavonoid

As compared with the control, icraiin addition improved the Shannon index of diversity(H'), while no significant differences were observed with baicalin, quercetin, luteolin, amygdalin or naringin addition (Table 2), The Q-PCR results showed that naringin addition improved the the numbers of the total bacteria, and there is a tendency for increased the bacteria number with the quercetin addition, while no any significant changes were observed on the bacteria number with the baicalin, icraiin, luteolin or amygdalin addition (Table 3).

DISCUSSION

In recent years there has been much interest in the development of nutritional products that are based on a prebiotic principle. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of, beneficial bacteria in the colon. Although the effects of many carbohydrates have been investigated as potential prebiotics, for example inulin and FOS, few studies have considered the prebiotic potential of other dietary components. Flavonoids are potential prebiotic candidates as they are known to undergo limited absorption and metabolism in the upper gastrointestinal tract (stomach, duodenum and jejunum), with the majority reaching the large intestine intact where approximately 200 g of material (water, bacteria, food particles and mucus) are present at any given time (Frayn, 2003). Many studies estimated that gut bacteria can hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones, and they carry out ring-cleavage, reduction, decarboxylation, demethylation, and dehydroxylation reactions (Aura, 2008; Lampe et al., 2007; Rechner et al., 2004).The hydrolysis of glycosides results in metabolites that are potentially more biologically active than the apparent compounds. Further bacterial transformation of aglycones can lead to production of more or less active compounds, depending on the substrate being metabolized and the products formed (Lampe et al., 2007; Rechner et al., 2004). Phytochemicals and their derived products can also affect the intestinal ecology as a significant part of them are not fully absorbed and are

Table 1. Effect of flavonoid addition on the acetate, propionate, butyrate and TVFA production in *in vitro* fermentation.

Flavonoid	Acids	Dosage(μg/ml)				P value
		0	40	160	S.E.M	
Baicalin	Acetate	13.87	15.05	15.22	0.262	0.042
	Propionate	3.33	3.50	3.37	0.063	0.595
	Butyrate	3.27	3.62	3.37	0.073	0.108
	TVFA	20.47	22.17	21.96	0.359	0.089
Quercetin	acetate	13.87	15.33	15.80	0.329	0.011
	propionate	3.33	3.68	3.57	0.064	0.046
	butyrate	3.27	4.01	3.94	0.125	0.001
	TVFA	20.47	23.03	23.31	0.500	0.006
Icraiin	acetate	13.87	14.22	15.16	0.214	0.007
	propionate	3.33	3.33	3.74	0.072	0.001
	butyrate	3.27	3.59	3.88	0.104	0.022
	TVFA	20.47	21.14	22.79	0.374	0.004
Luteolin	acetate	13.87	13.90	16.28	0.410	<0.001
	propionate	3.33	3.31	3.84	0.090	<0.001
	butyrate	3.27	3.62	4.06	0.123	0.003
	TVFA	20.47	20.83	24.18	0.606	<0.001
Amygdalin	acetate	13.87	15.20	15.54	0.289	0.012
	propionate	3.33	3.74	4.24	0.147	0.010
	butyrate	3.27	3.84	3.72	0.093	0.003
	TVFA	20.47	22.77	23.50	0.503	0.006
Naringin	acetate	13.87	14.39	16.11	0.355	0.001
	propionate	3.33	3.08	3.37	0.070	0.167
	butyrate	3.27	3.49	4.00	0.120	0.006
	TVFA	20.47	20.95	23.48	0.493	0.001

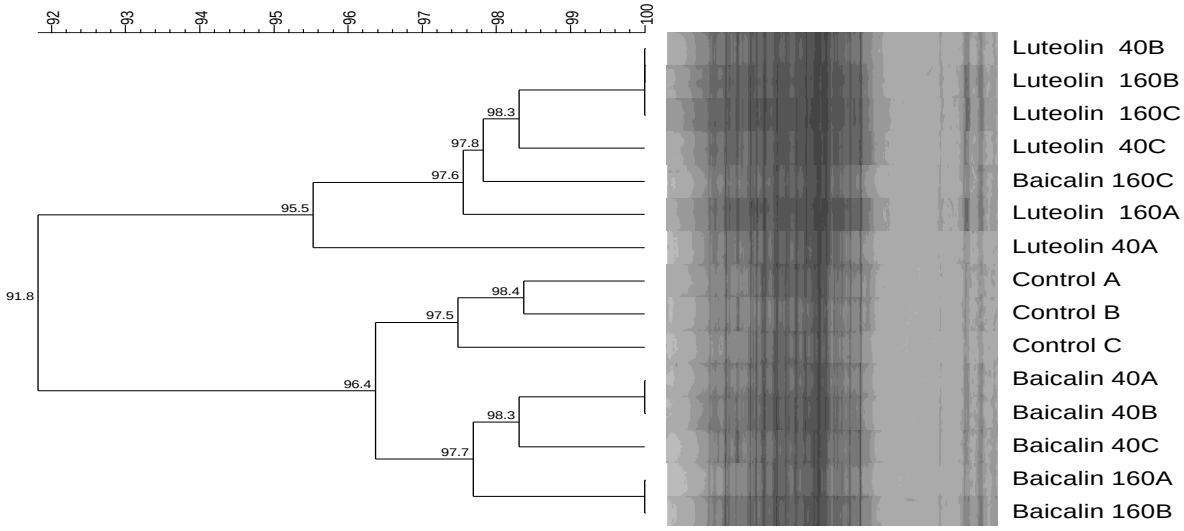

Figure 1. Similarity index of DGGE profiles obtained from the sample from *in vitro* fermentation. A, B, C means the replicates in control or flavonoid treatment, and 40,160 means the dosages of the flavonoid used in this study.

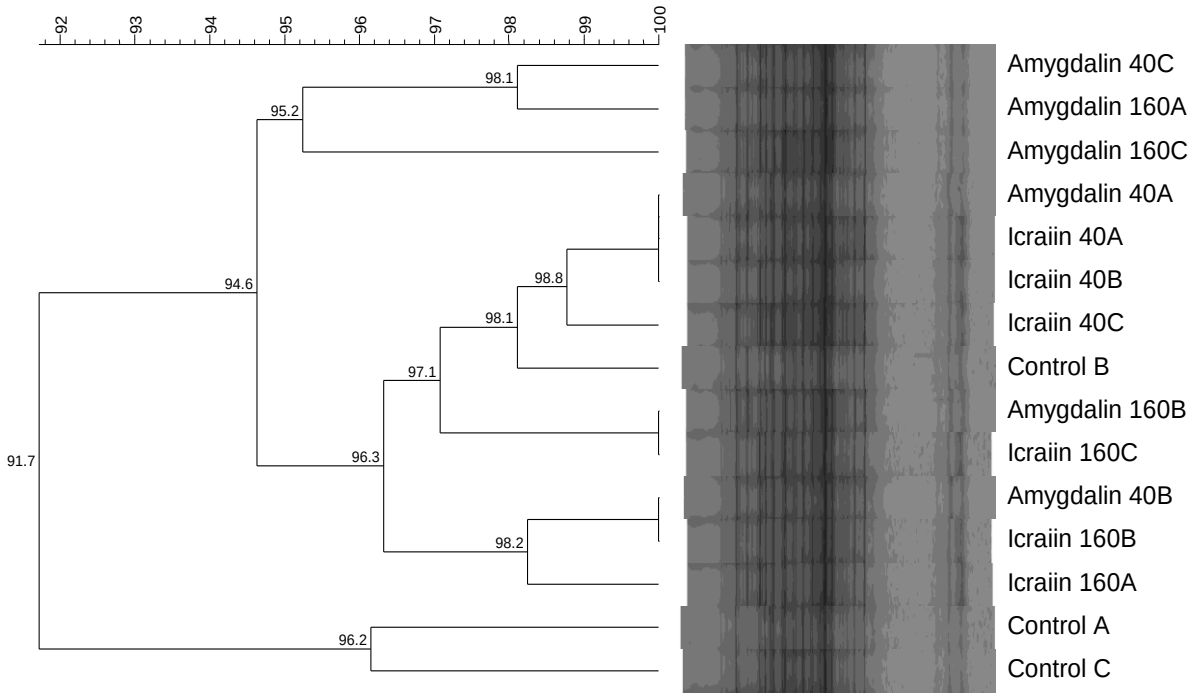

Figure2. Similarity index of DGGE profiles obtained from the sample from *in vitro* fermentation. A, B, C means the replicates in control or flavonoid treatment, and 40,160 means the dosages of the flavonoid used in this study.

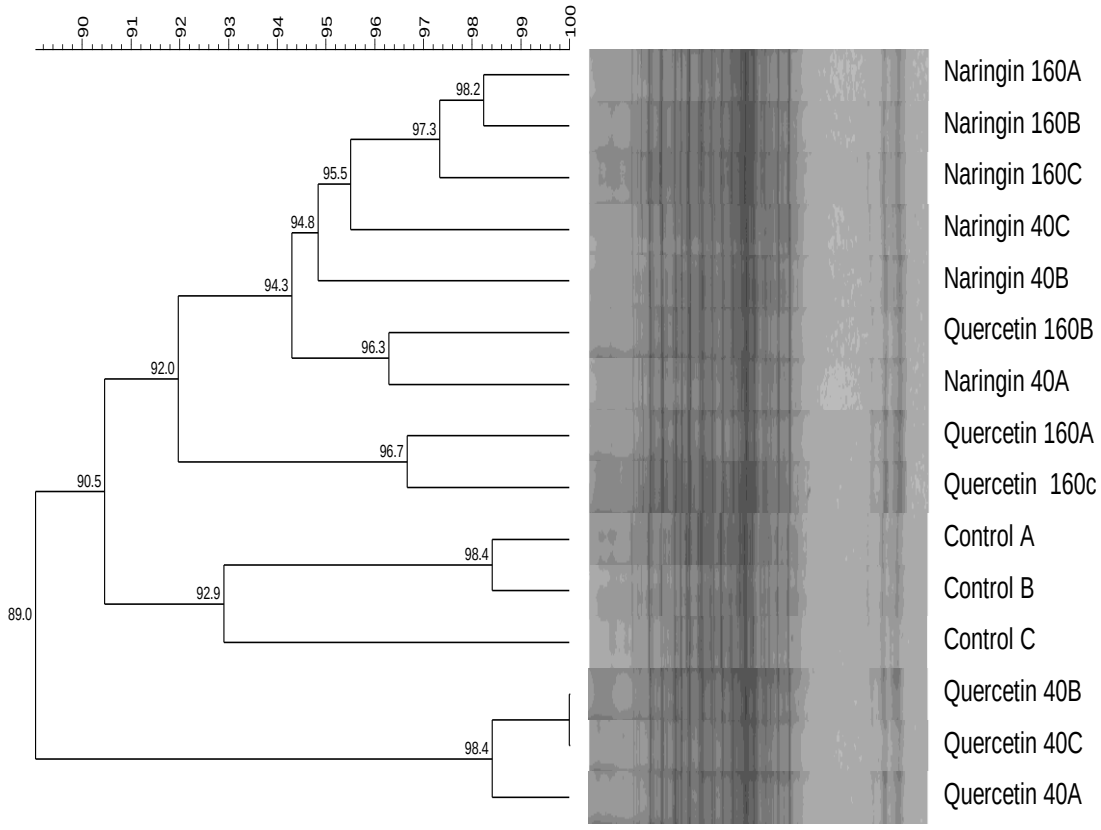

Figure3. Similarity index of DGGE profiles obtained from the sample from *in vitro* fermentation. A, B, C means the replicates in control or flavonoid treatment, and 40,160 means the dosages of the flavonoid used in this study.

Table 2. The effect of flavonoid addition on the changes of Shannon index of diversity.

H'	Dosage(μg/ml)			S.E.M	P value
	0	40	160		
Baicalin	3.12	3.13	3.12	0.016	0.983
Luteolin	3.12	3.14	3.13	0.014	0.856
Amygdalin	3.12	3.15	3.18	0.016	0.298
Icraiin	3.12	3.15	3.23	0.021	0.037
Quercetin	3.12	3.20	3.21	0.020	0.134
Naringin	3.12	3.15	3.09	0.014	0.369

Table 3. Quantitative real-time PCR analysis of total bacteria in fermentation samples [Lg (copies/ml)].

Q-PCR	Dosage(μg/ml)			S.E.M	P value
	0	40	160		
Baicalin	10.56	10.50	10.62	0.036	0.491
Luteolin	10.56	10.52	10.47	0.032	0.587
Amygdalin	10.56	10.54	10.60	0.033	0.754
Icraiin	10.56	10.51	10.65	0.039	0.412
Quercetin	10.56	10.75	10.73	0.040	0.061
Naringin	10.56	10.28	10.32	0.052	0.032

metabolised in the liver, excreted through the bile as glucuronides and accumulated in the ileal and colorectal lumen (Bazzocco et al., 2008). Tzonuis et al. (2008) investigated that the intake of flavonol-rich foods has been shown to improve the TVFA production, exerting prebiotic-like effects. Mao et al. (2007) showed that daidzein addition can improve the propionate proportion during *in vitro* fermentation by rumen bacteria, and increase cumulative gas production and the specific growth rate to half of the gas pool with the rumen anaerobic fungus *Neocallimastix* sp. In the present study, we showed that the SCFA production was improved by quercetin, icraiin, luteolin, amygdalin, naringin addition, and this indicated that these flavonoid addition may affect the metabolism of the bacteria in the large intestine, this flavonoid induced event believed to be beneficial primarily due to a rise of the SCFA concentration because these acids, especially butyric acid, are the main energy source for colonocytes and influence colonic function by stimulating water and sodium absorption and modulating motility (Cherbut et al., 1997). Furthermore, butyric acid induces differentiation, stimulates apoptosis of cancerous cells *in vitro* and thus arrests the development of cancer (Scheppach et al., 1995).

Unabsorbed dietary flavonoid and their metabolites, in addition to their direct beneficial effect on the human tissues, exert significant effects on the intestinal environment by modulation of the microbiota (Lee et al., 2006; Clavel, 2005). This is the case of tea flavonoid, including epicatechin and catechin, which have been identified as responsible for the repression of *Clostridium perfringens*, *Clostridium difficile,* and *Bacteroides* spp. growth, whereas commensal anaerobes such as *Clostridium* spp. and *Bifidobacterium* spp., were less severely affected (Lee et al., 2006). Similar results were obtained in another study in which (þ)-catechin incubation affected the growth of selected microbiota, resulting in a significant increase in the growth of the *Clostridium coccoides-Eubacterium* rectale group, *Bifidobacterium* spp., and *Escherichia coli*, as well as a significant inhibitory effect on the growth of the *Clostridium histolyticum* group (Tzounis et al., 2008). The increase of the *C. coccoides-Eubacterium* rectale group could be related to its capacity for metabolizing these flavonoid compounds. The stilbenoid resveratrol, the ellagitanins of pomegranate, and their main microbiota derived metabolite urolithin A have also been identified as responsible for changes in intestinal microbiota in rats with an increase of *Bifidobacterium* and *Lactobacillus* levels (Larrosa et al., 2009). However, in the present study, the DGGE analysis results revealed that fingerprints of the fecal bacteria communities had a similarity of ≥89% between Control and the flavonoid treatment, and as compared with the control without any flavonoid addition, except for the icraiin, no any significant effect was observed on the Shannon index of diversity(H') with other five types of plant extracts addition, The Q-PCR results showed that six types of flavonoid using in the present study(except for the Quercetin) addition did not affect the numbers of the total bacteria. The mechanism whereby quercetin, luteolin, amygdalin and naringin addition significantly influenced

the TVFA production, whereas produced no significant changes on diversity of the fecal microbial community is not yet fully understood. Indeed, all the six flavonoid compounds used in the present study can be metabolized by human intestinal bacteria *in vitro* (Liu et al., 2000; Aura et al., 2008), however, the response of the microbial by flavonoid addition on the TVFA production and the diversity of fecal microbial community is inconsistent. This reflects that the effect of the flavonoid on the gut health may depend on the itself such as the chemical structure or its metabolites by gut microbial, and the quercetinm and icaritin may influence the growth of specific large-intestinal bacteria and that this ability may be linked to specific metabolic transformations.

In spite of our results it cannot be excluded with certainty that the fecal bacteria composition was yet affected after the baicalin, luteolin, amygdalin, quercetin or naringin addition, but that possible changes were not detected because of the limitations of PCR-DGGE. It has to be kept in mind that only dominant groups in the ecosystem will result in a noticeable band in the DGGE gel. Moreover, bacterial diversity may be underestimated by PCR-DGGE, because heterologous sequences may comigrate and denature at the same position in the gel. It is also not really possible to quantitate the bacteria in a given sample by PCR-DGGE. Shannon similarity index, which was chosen to analyze the presented data, only considers the presence or absence of bands, but not their intensity. It can therefore not be excluded that flavonoid caused changes in the size of the microbial populations underlying the DGGE bands. Although hundreds of different bacterial species exist in the gastrointestinal tract, only a few species predominate, while most of the bacterial species occur in small numbers. It has been estimated that 90–99% of the bacterial community are detectable with PCR-DGGE (Zoetendal et al., 1998). However, only dominant fractions of the population can be visualized (Zoetendal et al., 1998). In other hand, in this present study, we only determined the changes of the diversity and quantified the numbers of the total bacteria composition, indeed, some previous study showed that several phenolics have been recognized as potential antibacterial compounds able to repress pathogenic bacteria in the human gut (Lee et al., 2006), therefore, more different bacteria species including some pathogenic bacteria such as *Staphylococcus* spp., *Salmonella spp*., *Helicobacter pylori,* and *Bacillus cereus* or probiotics such as lactobacillus should be investigated in the future study.

In general, the current study showed the higher TVFA production following incubation with the bacteria suggests that quercetin, icraiin, luteolin, amygdalin, naringin may be potential nutrient sources for the bacteria. However, the diversity of fecal total bacteria was not significant affected by the addition of quercetin, icraiin, luteolin, amygdalin and naringin. These observations suggest that these activity effect of the flavonoid on the diversity and the number of total bacteria were dependent on the type of the flavonoid.

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