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Identification of rutin deglycosylated metabolites produced by human intestinal bacteria using UPLC–Q-TOF/MS

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ABSTRACT

In this paper, rutin was metabolized by human intestinal bacteria and five isolated strains including *Bacillus* sp. 52, *Bacteroides* sp. 45, 42, 22 and *Veillonella* sp. 32, the metabolites were identified using ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC–Q-TOF/MS). As a result, *Bacillus* sp. 52 and *Bacteroides* sp. 45 could metabolize rutin to quercetin 3-*O*-glucoside and leucocyanidin. *Bacteroides* sp. 42 and *Veillonella* sp. 32 could convert rutin to leucocyanidin. *Bacteroides* sp. 22 could hydrolyze rutin to quercetin-3-*O*-glucoside. In order to further explain the metabolism pathway of rutin, the β-D-glucosidase and α-L-rhamnosidase activities of five strains were determined. *Bacteroides* sp. 22 could produce α-L-rhamnosidase but did not produce β-D-glucosidase or β-D-glucosidase activity was too low to be detected. The other four strains all demonstrated α-L-rhamnosidase and β-D-glucosidase activities. Furthermore, α-L-rhamnosidase and β-D-glucosidase activities of *Veillonella* sp. 32 and *Bacteroides* sp. 42 were higher than those of *Bacteroides* sp. 45 and *Bacillus* sp. 52. Based on these results, we can propose the deglycosylated rout of rutin: rutin was metabolized to be quercetin-3-*O*-glucoside by α-L-rhamnosidase produced from these bacteria, thereafter, quercetin-3-*O*-glucoside was further metabolized by β-D-glucosidase to form leucocyanidin. Because of the higher enzyme activity in *Veillonella* sp. 32 and *Bacteroides* sp. 42, quercetin-3-*O*-glucoside was completely metabolized to leucocyanidin by these two bacteria. Due to the lack of β-D-glucosidase activity, *Bacteroides* sp. 22 could not further metabolize quercetin-3-*O*-glucoside to leucocyanidin. This study will be helpful for understanding the deglycosylated rout of rutin and the role of different intestinal bacteria on the metabolism of natural compounds.

1. Introduction

Flavonoids are polyphenolic compounds of natural origin that are present in most Chinese herbal medicine. Flavonoids possess anti-allergic, anti-inflammatory, cardiovascular and anti-cancer activities [1,2]. Rutin is a quercetin rutinoside that exhibits multiple activities including antioxidative, anti-hyperglycemic, anti-inflammatory, hepatoprotective, and neuroprotective effects [3–8].

In order to clarify the biological activity of rutin, it is necessary to study the absorption and metabolism profile of rutin. Rutin is poorly absorbed by intestinal tract but largely degraded by the intestinal bacteria after oral intake [9,10]. It is hydrolyzed to aglycone quercetin or further degradation products such as phloroglucinol, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, and 3,4-dihydroxybenzaldehyde which could be absorbed easily by the intestinal tract [11–14]. The metabolites of rutin degraded by human intestinal bacteria exert more potent anti-platelet activity and cytotoxicity than the parental compound [15]. Therefore, the intestinal bacteria play an important role in the metabolism and action of rutin.

The intestinal tract contains more than 400 bacterial species, in which *Bacteroides*, *Streptococcus*, *Lactobacillus*, *Bifidobacteria* are major species, most of them to be strict anaerobes [16,17]. These bacteria have excellent enzymatic system, which contributes to their enormous catalytic and hydrolytic potential. *Escherichia coli* and *Enterococcus casseliflavus* were isolated from human feces and could cleave the ring system of rutin to phloroglucinol and 3,4-dihydroxyphenylacetic acid [18]. *Bacteroides* JY-6 and *Fusobacterium* K-60 produced α-rhamnosidase and β-glucosidase, which hydrolyze rutin to lose sugars [19–21]. According to a report [22], β-glucosidase could rapidly hydrolyze glucose conjugates such as quercetin 3-*O*-glucoside, but could not cleave other sugar conjugates such as rutin which was rhamnoside conjugate. Therefore, the lack of α-rhamnosidase activity may lead to the inability of

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Table 1
UPLC/ESI-MS, retention time (RT) and fragment ions of rutin and its metabolites.

No.	RT	*m/z* calculate	*m/z* found	Identification	Formula
M1	2.9	610.1534	609.1508	Parent	$C_{27}H_{30}O_{16}$
M2	5.91	758.1906	757.1753	Rutin monoglucuronide	$C_{32}H_{38}O_{21}$
M3	9.97	706.1051	705.0914	Rutin sulfate	$C_{27}H_{30}O_{20}S$
M4	8.5	690.1102	689.1041	Rutin sulfate	$C_{27}H_{30}O_{19}S$
M5	9.93	624.1326	623.1219	Methylated rutin	$C_{28}H_{32}O_{16}$
M6	2.99	612.169	611.1563	Hydrogenated rutin	$C_{27}H_{32}O_{16}$
M7	9.48	464.0955	463.1079	Quercetin-3-glucoside	$C_{21}H_{20}O_{12}$
M8	2.06	306.074	305.0728	Leucocyanidin	$C_{15}H_{14}O_7$
M9	6.14	288.0634	287.0493	5,7,3′,4′-Tetrahydroxy flavonone	$C_{15}H_{12}O_6$
M10	1.98	168.0423	167.0484	3,4-Dihydroxyphenylacetic acid	$C_8H_8O_4$

bacteria to utilize rutin as the sole carbon and energy source [23]. Based on this fact, the deglycosylation of rutin is the first and vital step in the whole process of its metabolism.

In this paper, we attempted to isolate different pure bacteria from human feces and study their ability and characteristic in the deglycosylation of rutin. In order to fast analyze the metabolites of rutin by the isolated intestinal bacteria, ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF/MS) with automated data analysis (MetabolynxTM) was used [24].

2. Experimental

2.1. Materials

The HPLC-grade acetonitrile was purchased from TEDIA Company Inc. (Fairfield, USA); formic acid was obtained from Merck KGaA (Darmstadt, Germany); ultra-pure water was purified by an EPED super purification system (Nanjing, China). Other reagents were of analytical grade. AnaeroPack Rectangular Jars were purchased from Mitsubishi Gas Chemical Company Inc. (Japan). Rutin was isolated in our laboratory. *p*-Nitrophenyl β-D-glucoside (PNP-β-D-glu) was provided by Baomanbio Bioscience & Technology Co. (Shanghai, China). *p*-Nitrophenyl α-L-rhamnoside (PNP-α-L-rham) was provided by Solarbio Bioscience & Technology Co. (Shanghai, China) and *p*-nitrophenol (PNP) was provided by Jianglai Bioscience & Technology Co. (Shanghai, China). The general anaerobic medium (GAM) used for all fermentation experiments consists of 10.0 g tryptone, 3.0 g soya peptone, 10.0 g proteose peptone, 13.5 g digestibility serum powder, 5.0 g yeast extract, 2.2 g beef extract, 1.2 g beef liver extract powder, 3.0 g glucose, 2.5 g KH_2PO_4, 3.0 g NaCl, 5.0 g soluble starch, 0.3 g L-cysteine hydrochloride, 0.3 g sodium thioglycolate, and 1000 mL distilled water, then the pH was adjusted to 7.3 before autoclaving at 121 °C for 20 min.

2.2. Preparation of human intestinal bacterial mixture

Fresh human fecal sample was obtained from a healthy female volunteer who had not taken any medicine in three months and avoided all alcohol and food rich in polyphenols for 48 h before fecal collection. 4 g fecal sample was weighed and suspended in a centrifuge tube covered with 20 mL sterile physiological saline, then was homogenized adequately by a vortex-mixer. The mixture was centrifuged at 2000 × *g* for 10 min and the suspension was used as human intestinal bacterial mixture. An aliquot of the bacterial mixture sample was directly used to the experiment, the remaining sample was preserved by adding glycerol as a cryoprotectant with a final concentration of 20% (v/v).

2.3. Isolation of human intestinal bacteria

The bacterial mixture was serially diluted in sterile water and each of the dilutions was spread on GAM agar plates. The plates were incubated in anaerobic jars under anaerobic condition at 37 °C for 48 h. About one hundred different types of bacterial colonies which developed on plates were picked up [25].

2.4. Incubation experiments

The standard solution of rutin was prepared by dissolving accurately weighed rutin in DMSO to give a final concentration of 10 mg/mL. The bacterial mixture and one hundred different bacterial colonies were inoculated into 0.5 mL of GAM broth containing 0.1 mM rutin, respectively, and the media were anaerobically incubated at 37 °C for 72 h [26].

2.5. Assay for glucosidase and rhamnosidase activities

The strains were cultured for 24 h in 1 mL of GAM at 37 °C, then the suspensions were centrifuged at 5000 × *g* for 10 min. The supernatant (0.3 mL) was diluted five times in phosphate buffer (0.2 M, pH 7.3). The suspensions were incubated with 0.25 mM PNP-β-D-glu and 0.25 mM PNP-α-L-rham at 37 °C, respectively. Aliquots of the reactive solutions were collected at different time and were diluted with water (1:10) in a 96-well microplate. The absorbance of each well was measured with a microplate reader at 405 nm. The absorbance of a series of different concentrations of PNP was used to calculate the enzymatic activity.

2.6. Preparation of supernatants for UPLC–MS

After 72 h, the cultures were mixed with 1 mL acetonitrile, and centrifuged at 12,000 × *g* for 10 min, the supernatant was removed and dried at 50 °C. The residues were dissolved in 0.2 mL of 70% MeOH, centrifuged at 12,000 × *g* for 10 min, and the supernatant was analyzed by UPLC–MS.

2.7. Ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) analysis

The liquid chromatography was carried out on a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA). An ACQUITY UPLC HSS T3 column (100 nm × 2.1 mm i.d., 1.8 mm; Waters Corp., Milford, MA, USA) was used for separation. The column temperature was maintained at 35 °C. The autosampler was set at 4 °C. Acetonitrile (solvent system A) and water containing 0.1% formic acid (solvent system B) served as the mobile phase with a 12-min gradient elution of 10% A at 0–7.5 min, 40% A at 7.5–9 min, 10% A at 9–12 min. The flow rate was 0.4 mL/min and the injection volume was 10 μL.

2.8. Mass spectrometric conditions

A Waters ACQUITYTM Synapt mass spectrometer (Waters Corp., Manchester, UK) was connected to the UPLC system via an

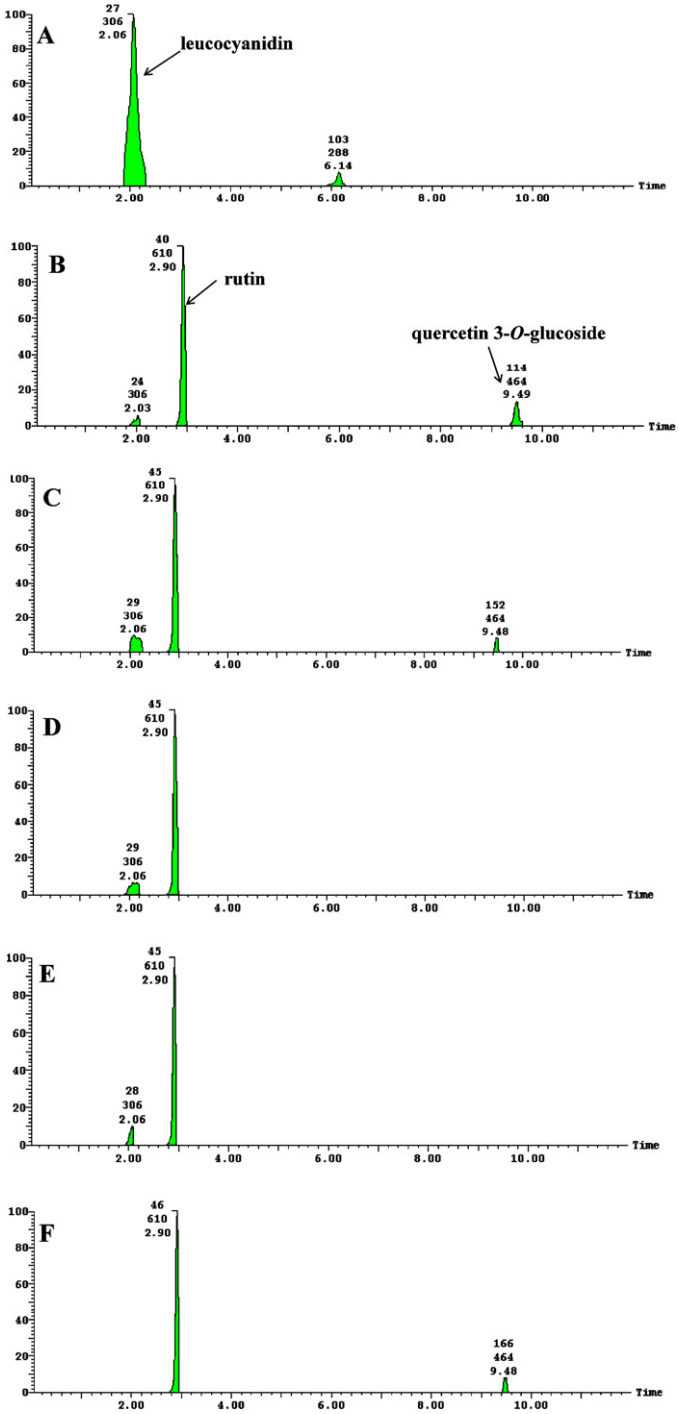

Fig. 1. UPLC/MS chromatograms of rutin and its metabolites: (A) intestinal bacteria mixture, (B) strain 52 sample, (C) strain 45 sample, (D) strain 42 sample, (E) strain 32 sample, and (F) strain 22 sample.

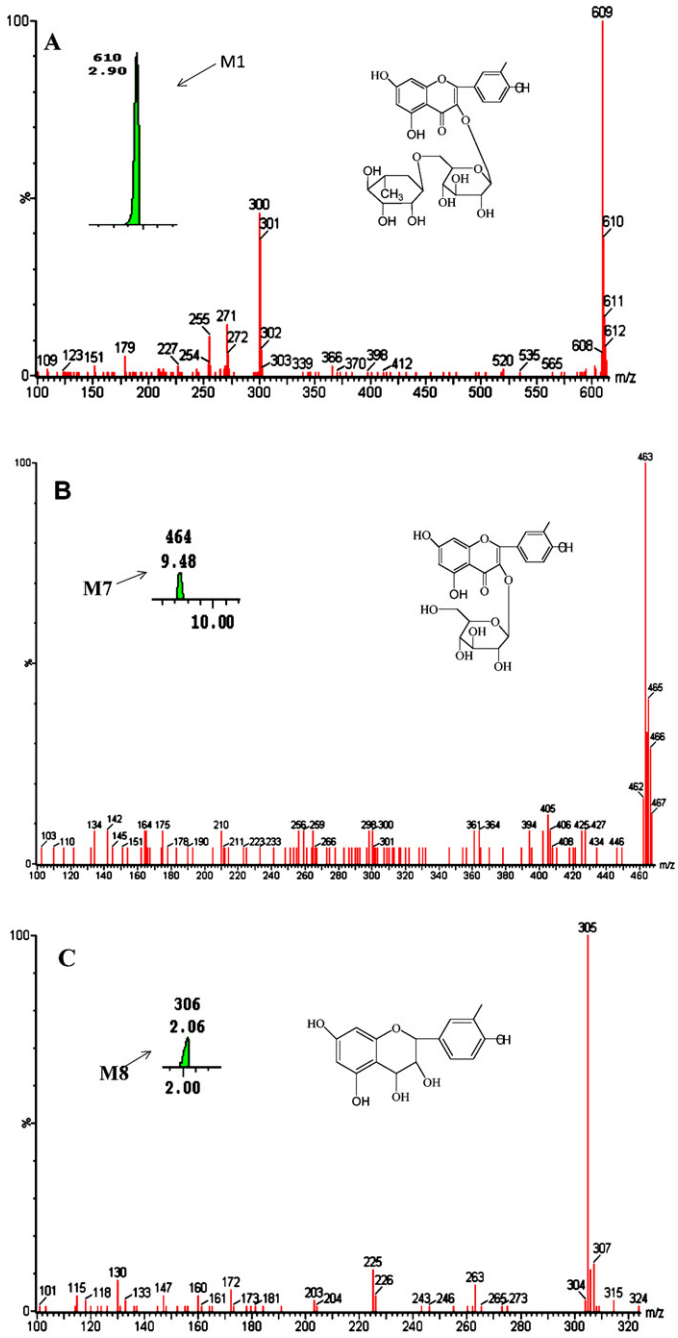

Fig. 2. UPLC–MS/MS spectra: (A) M1 (m/z 609), (B) M7 (m/z 463), and (C) M8 (m/z 305).

electrospray ionization (ESI) interface. The ion source can be operated in negative mode and the mass selective detector MS can be operated in SIM. The MS parameters were as follows: source temperature 120 °C, desolvation temperature 350 °C, cone voltage 40 V, capillary voltage 3 kV, the gas flows of cone and desolvation were 50 and 600 L h^{-1}, respectively. Leucine-enkephalin was used as the lock mass to generate an $[M-H]^-$ ion (m/z 554.2615). In addition, the MS^E experiment was necessary to be carried out for obtaining the parent and fragment mass information of the compounds. Function 1: m/z 100–1000, 0.5 s scan time, 0.02 s inter-scan delay, 6 V collision energy; function 2: m/z 100–1000, 0.5 s scan time, 0.02 s inter-scan delay, collision energy ramp of 15–40 V. All data collected in centroid mode were acquired using Masslynx™ NT 4.1 software (Waters Corp., Milford, MA, USA).

2.9. Data analysis

Post-acquisition analyses were performed using a Metabolynx™ (v4.1) program which employs an extensive list of potential biotransformation reaction consisting of deglycosylation, ring cleavage, dehydroxylation, decarbonylation, methylation, sulfation, hydrogenation and hydroxylation. In combination with the elemental composition of the substrate molecules generate a series of extracted ion chromatograms (XICs). These XICs are

Table 2
The metabolites of rutin by the isolated bacteria.

Strain	Metabolites		
Bacillus sp. 52	610	464	306
Bacteroides sp. 42	610	306	
Bacteroides sp. 22	610	464	
Bacteroides sp. 45	610	464	306
Veillonella sp. 32	610	306	

compared between the control and sample to eliminate those chromatographic peaks in the sample that also appear in the control.

Data evaluation with Metabolynx™ in this study can be divided into a three-stage process. Firstly, the acquired data are processed using a user-defined parameter file, to generate a preliminary report file. This involves an automated comparison of analyte LC/MS chromatograms with appropriate control samples. Secondly, this report is displayed in the browser, and the output is refined by a variety of data filters. Finally, a large number of peaks were generated which must be determined manually whether they are likely to be compound-related metabolites.

3. Results and discussion

After the comprehensive analyses of the peaks from one hundred different bacteria by the Metabolynx™ program, we found the parent compound (M1) and nine metabolites (M2–M10) (Table 1). These metabolites were likely to be rutin conjugates with methyl (M5), sulfate (M3, M4), hydrogen (M6), glucuronyl (M2) groups and the rutin catabolites: quercetin-3-glucoside (M7), leucocyanidin (M8), 5,7,3′,4′-tetrahydroxy flavonone (M9), 3,4-dihydroxyphenylacetic acid (M10) (Table 1). Because we only attempted to investigate the deglycosylation of rutin, five bacteria capable of inducing rutin to lose sugars were screened from one hundred bacteria, and two metabolites quercetin-3-glucoside (M7) and leucocyanidin (M8) were further identified.

3.1. UPLC chromatograms of metabolites of rutin in the bacterial mixture and five different bacterial samples

Here we investigated the deglycosylation of rutin by the intestinal bacterial mixture and five different bacterial strains: *Bacillus* sp. 52, *Veillonella* sp. 32 and *Bacteroides* sp. 45, 22, 42. UPLC chromatograms of metabolites of rutin in these bacterial samples are illustrated in Fig. 1. M7 and M8 were obtained in the samples of the bacterial mixture, *Bacteroides* sp. 45 and *Bacillus* sp. 52. M7 as the only metabolite of rutin existed in the sample of *Bacteroides* sp. 22. In addition, rutin was degraded to the uniqueness product M8 present in the samples of *Bacteroides* sp. 42 and *Veillonella* sp. 32 (Table 2). As shown in Fig. 1A, rutin was completely metabolized to leucocyanidin by the mixed intestinal bacteria. By comparing the results obtained from the mixed intestinal bacteria with that from the five pure bacteria, we can conclude that the metabolic activity of mixed bacteria was higher than that of single bacterium in our study.

3.2. UPLC–Q-TOF/MS identification of the metabolites of deglycosylation

3.2.1. Parent compound (M1)

Rutin (M1) was detected based on the same retention time (2.9 min) with authentic standard. As illustrated in Fig. 2, the $[M-H]^-$ ion of M1 was at *m/z* 609, and the mass spectrum yielded fragment ions at *m/z* 301, 151. The fragment ion at *m/z* 301 was

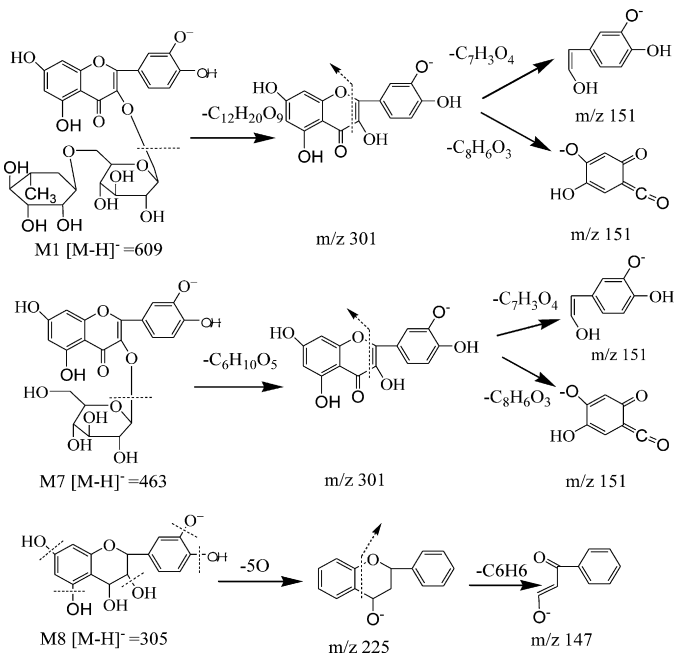

Fig. 3. Proposed mechanism for the decomposition of the *m/z* 609, 463, 305 $[M-H]^-$ ions of rutin.

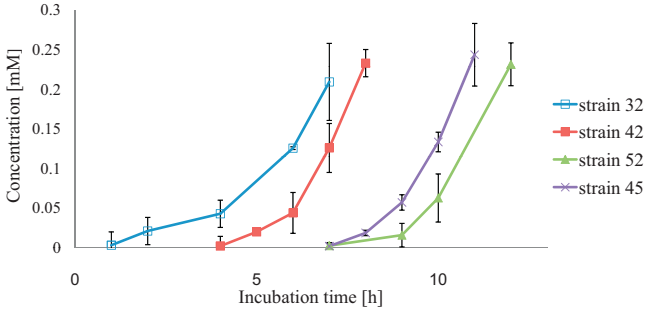

Fig. 4. Time-dependent release of PNP after incubation of PNP β-D-glu (0.25 mM) with five strains ($n = 3$, mean ± SD).

generated by deglycosylation from the loss of *m/z* 308. The *m/z* 151 was generated after the Retro-Diels Alder (RDA) cleavage (Fig. 3).

3.2.2. Metabolite (M7)

According to Fig. 2, the $[M-H]^-$ ion of M7 was at *m/z* 463 (retention time 9.48 min) which was suspected to be quercetin-3-glucoside. The high collision energy scan fragment ions were *m/z*

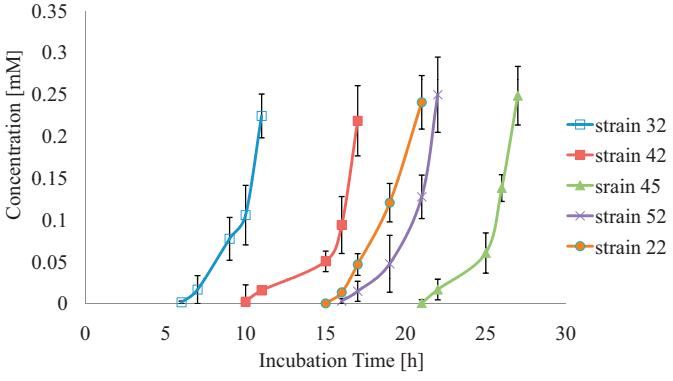

Fig. 5. Time-dependent release of PNP after incubation of PNP α-L-rham (0.25 mM) with five strains ($n = 3$, mean ± SD).

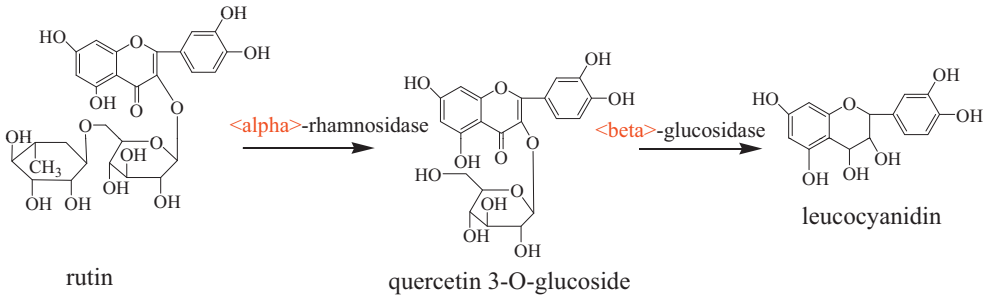

Fig. 6. Rutin deglycosylated pathway by human intestinal bacteria.

301, 151. The fragment ion at *m*/*z* 301 was generated by deglycosylation from the loss of *m*/*z* 162. The *m*/*z* 151 was generated after the Retro-Diels Alder (RDA) cleavage (Fig. 3). M7 as the metabolite of rutin degraded by the intestinal bacteria was not formerly reported, which was generated by the loss of rhamnose.

3.2.3. Metabolite (M8)

Besides M7, there was another peak (M8, retention time 2.06) that has not been reported as rutin metabolite previously. The mass spectra of M8 are listed in Fig. 2 and its $[M-H]^-$ ion was at *m*/*z* 305. The daughter ion *m*/*z* 225 was observed in the mass spectrum, which was generated from the loss of pentakis-hydroxy. The *m*/*z* 225 was further fragmented to *m*/*z* 147 after the Retro-Diels Alder (RDA) cleavage (Fig. 3). Based on the fragmentation pathway, the structure of M8 could be leucocyanidin, which was hydrogenated product of quercetin. So M8 was the metabolite of rutin losing disaccharide which has not been detected previously.

3.3. β-D-Glucosidase and α-L-rhamnosidase activities

The analysis was performed using PNP-α-L-rham and PNP-β-D-glu as substrates for corresponding enzymes. Figs. 4 and 5 show the release of PNP after incubation of PNP-β-D-glu and PNP-α-L-rham with the five stains. As shown in Figs. 4 and 5, *Bacteroides* sp. 22 did not produce β-D-glucosidase or its β-D-glucosidase activity was so low that the amounts of PNP were not traced in 12 h. The other four strains all produced α-L-rhamnosidase and β-D-glucosidase. Furthermore, the amounts of PNP released from PNP-glu and PNP-rham reached the highest level faster (at 7–8 h and 11–17 h) in *Veillonella* sp. 32 and *Bacteroides* sp. 42 test in comparison with the other two bacterial strains, respectively, which showed that enzymic activities were higher in *Veillonella* sp. 32 and *Bacteroides* sp. 42.

3.4. Analysis of the pathway of rutin deglycosylation

After incubation with *Bacteroides* sp. 45 and *Bacillus* sp. 52, both quercetin-3-*O*-glucoside and leucocyanidin were identified as rutin deglycosylated metabolites. And in β-D-glucosidase and α-L-rhamnosidase activities assay, *Bacteroides* sp. 45 and *Bacillus* sp. 52 showed moderate activities. From these results we can deduce that rutin was metabolized to be quercetin-3-*O*-glucoside by α-L-rhamnosidase produced from these bacteria, thereafter quercetin-3-*O*-glucoside was metabolized by β-D-glucosidase to form leucocyanidin.

Leucocyanidin was identified as the only metabolite of rutin after incubation with *Veillonella* sp. 32 and *Bacteroides* sp. 42. According to the β-D-glucosidase and α-L-rhamnosidase activities assay, the enzymic activities of *Veillonella* sp. 32 and *Bacteroides* sp. 42 was higher. From these results we can conclude that rutin was metabolized to be quercetin-3-*O*-glucoside by α-L-rhamnosidase, then quercetin-3-*O*-glucoside was completely metabolized by β-D-glucosidase to form leucocyanidin.

Only quercetin-3-*O*-glucoside was detected as rutin metabolite after incubation with *Bacteroides* sp. 22. The lack of β-D-glucosidase activity may lead to the inability of bacterium to further metabolize quercetin-3-*O*-glucoside.

4. Conclusion

In the study, we used ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF/MS) with automated data analysis (MetabolynxTM) for fast analysis of the metabolism of rutin by incubating with the intestinal bacterial mixture from the human feces and one hundred different bacteria which were isolated from the bacterial mixture. After the automated analysis, quercetin 3-*O*-glucoside and leucocyanidin were first identified as the products of rutin metabolized by intestinal bacteria.

According to the deglycosylated products of five strains and their enzymic activities, we know that the different bacteria can metabolize rutin to different metabolites, which owns to their enzyme system. Moreover, we can conclude that when incubating with human intestinal bacteria, rutin can be degraded to quercetin 3-*O*-glucoside by losing rhamnose at first, then quercetin 3-*O*-glucoside was further metabolized to leucocyanidin by losing glucose (Fig. 6).

In our study, the deglycosylation pathway and the metabolites of rutin produced by bacteria isolated from the human feces were investigated. Further study is needed to identify other metabolism pathway and enzyme system which contribute to the metabolism.

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