**Note**

Isolation of a Quercetin-metabolizing Bacterium 19 – 20 from Human Feces

Motoi Tamura*, Yosuke Matsuo, Hiroyuki Nakagawa, Chigusa Hoshi and Sachiko Hori

Food Research Institute, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki 305-8642, Japan

Received August 10, 2016 ; Accepted September 26, 2016

Much attention has been focused on the potential health-promoting effects of quercetin, a flavonoid that is metabolized by the intestinal microbiota. However, there are few reports on the intestinal bacteria that degrade quercetin. We isolated the quercetin-degrading bacterium strain 19 – 20 (Accession no: LC175305) from human feces. Based on comparative 16S rRNA gene sequence analysis, the bacterium exhibits 100% similarity to *Clostridium orbiscindens* (Accession no: Y18187). Quercetin degradation was observed to increase in a time-dependent manner when co-incubated with strain 19 – 20 *in vitro*; the majority of quercetin was degraded within 24 hr. Strain 19 – 20 quercetin degradation was inhibited by resveratrol. Co-incubation of quercetin and resveratrol significantly reduced strain 19 – 20 quercetin degradation. This is the first report of the inhibitory effect of resveratrol on quercetin degradation by intestinal bacteria.

Keywords: Quercetin, intestinal bacterium, resveratrol

**Introduction**

Much attention has been focused on the potential health-promoting effects of quercetin. Further, the antioxidant activity of quercetin has been reported. The plasma total antioxidant status of rats fed a diet containing 0.2% quercetin was higher than that of control rats (Morand et al., 1998). Quercetin has also been reported for its anti-allergic immune response (Mlcek et al., 2016). Meanwhile, another study reported that visceral and liver fat accumulation was reduced by 0.05% quercetin supplementation to a Western high-fat diet (Kobori et al., 2011). Thus, the recent research indicates that dietary quercetin may have positive biological effects.

Inter-individual variability of quercetin pharmacokinetics has been reported in a human study (Kaushik et al., 2012). Meanwhile, rats orally administered [14C]-quercetin showed more than 30% decomposition of [14C]-quercetin, yielding 14CO2 (Ueno et al., 1983). Moreover, the bioavailability of quercetin seems to show inter-individual variation.

The intestinal microbiota also affects the metabolism of quercetin (Parkar et al., 2013) and its derivative (rutin) (Aura et al., 2002). Rutin is metabolized by the intestinal microbiota, and deglycosylation, ring fission, and dihydroxylation occur during rutin fermentation (Aura et al., 2002). The intestinal microbiota metabolizes various polyphenols, including quercetin. Quercetin was metabolized to known phenolic acid breakdown products in the *in vitro* fermentation of human intestinal microbiota (Parkar et al., 2013). *Clostridium orbiscindens* has been isolated from human feces, and biotransforms quercetin and taxifolin to 3,4-dihydroxyphenylacetic acid (Schoefer et al., 2003). When testing for the presence of *C. orbiscindens* in human fecal samples, eight of 10 samples contained *C. orbiscindens*, and the mean number of *C. orbiscindens* was more than 10^8 cells g/dry feces (Schoefer et al., 2003). This report indicates that *C. orbiscindens* is part of the normal bacterial flora of humans and affects the metabolism of quercetin in the lower gut. However, there are few other reports of quercetin-metabolizing bacteria belonging to the normal gut microbiota.

Quercetin biotransformation appears to be important for its...
bioavailability and other biological effects. Moreover, for increased bioavailability, it may be important to reduce quercetin degradation by intestinal microbiota. While there is an incredible diversity of bacteria in the large intestine, few reports have focused on quercetin-degrading human intestinal bacteria.

Grapes contain resveratrol, and its many biological effects have been reported (Rayalam et al., 2008; Bujanda et al., 2008; Gambini et al., 2015). Further, resveratrol seems to be a potent functional food component.

In this report, we isolated and newly identified a quercetin-degrading bacterium from human feces. Further, using this quercetin-degrading bacterium, we determined that resveratrol inhibits quercetin degradation.

Materials and Methods

Chemicals Quercetin and resveratrol were purchased from Funakoshi (Tokyo, Japan). Genistein was purchased from LC Laboratories (Woburn, MA, USA).

Isolation of fecal bacteria We used a fecal tube (54 mm × 28 mm) with a small hole for stool examination (Sarstedt K.K., Tokyo, Japan). The fecal tubes were autoclave-sterilized. Fecal samples from a healthy female were sheet-collected and immediately transferred into sterilized fecal tubes. Fecal tubes were placed in an AnaeroPouch® with a CO₂ generator (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan), and transported to the Food Research Institute, NARO within 24 hr at < 10°C. An approximately 0.1-g fecal sample was transferred to a sterilized glass homogenizer containing 3 mL of a pre-reduced anaerobic medium. The anaerobic medium was prepared as follows: brain heart infusion (37 g), agar (1 g), L-cysteine HCl·H₂O (0.5 g), and Na₂CO₃ (4 g) dissolved in 1,000 mL distilled water. Aliquots of the medium (5 mL) were then distributed to test tubes, gassed with O₂-free CO₂, sealed with butyl rubber stoppers, and autoclave-sterilized. Feces were homogenized under gassing with O₂-free CO₂.

Isoflavone and arctigenin are polyphenols. It has been reported that an isoflavone-metabolizing bacterium (Yokoyama and Suzuki, 2002) was isolated from human feces by adding isoflavone to the fecal homogenate, while an arctigenin-metabolizing bacterium (Jin et al., 2002) was isolated from human feces by adding arctigenin to the anaerobic culture medium. Quercetin is a type of polyphenol; thus, to isolate quercetin-metabolizing bacteria, we added quercetin to the anaerobic medium.

To isolate quercetin-metabolizing bacteria, we first identified candidate bacteria by selecting for the de-colorization of quercetin's native yellow color in the incubation solution after anaerobic incubation of bacteria with quercetin. As the second step, quercetin degradation was confirmed by high-performance liquid chromatography (HPLC). Quercetin (20 mg) was dissolved in 1 mL dimethyl sulfoxide (DMSO). The quercetin solution (2 μL of each) was then transferred into 0.2 mL diluted fecal suspension in an anaerobic medium (as described above). The fecal suspensions with quercetin were anaerobically incubated under a CO₂ atmosphere (AnaeroPack® system, Mitsubishi Gas Chemical Company Inc.) for 24 hr at 37°C. The fecal solutions were then serially diluted 10-fold using an anaerobic dilution medium. The diluted samples were spread onto the surface of modified Gifu Anaerobic Medium (GAM) agar non-selective medium plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The plates were anaerobically incubated under a CO₂ atmosphere (AnaeroPack® system, Mitsubishi Gas Chemical Company Inc.) for 3 days at 37°C. Colonies were picked by an inoculating loop and transferred into 0.2 mL anaerobic medium and 2 μL quercetin solution (as described above). Bacterial solutions were anaerobically incubated under a CO₂ atmosphere (AnaeroPack® system, Mitsubishi Gas Chemical Company Inc.) for 2 to 3 days at 37°C. We obtained candidate quercetin-metabolizing bacteria by selecting for the de-colorization of quercetin’s native yellow color in the incubation solution after anaerobic incubation of bacteria with quercetin. Next, quercetin degradation was confirmed by high-performance liquid chromatography (HPLC). The HPLC analysis was the same as used for the in vitro qualitative incubation of intestinal bacteria with quercetin (as described in the next paragraph). The study was performed under the guidelines of the Declaration of Helsinki. The Human Investigations Review Board of the Food Research Institute, NARO approved the study protocol (HU2014-07), and informed consent was obtained from the subject.

In vitro qualitative incubation of intestinal bacteria with quercetin We isolated a quercetin-metabolizing bacterium (designated as strain 19–20), and measured its quercetin-degrading activity. Strain 19–20, previously incubated on modified GAM agar plates for 24 hr, was picked by inoculating loop and suspended in the anaerobic medium. Quercetin (20 mg) was dissolved in 1 mL DMSO. The quercetin solution (2 μL of each) was then transferred into 0.2 mL of the strain 19–20 anaerobic medium suspension, and incubated anaerobically at 37°C for 0, 4 or 24 hr. We confirmed quercetin degradation using anaerobic medium alone (as control). The quercetin solution (2 μL) was then transferred into 0.2 mL anaerobic medium and incubated anaerobically at 37°C for 0 or 24 hr. After incubation, methanol-acetic acid (100:5, v/v) was added into the reaction mixture to a total volume of 1.0 mL. The mixture was vortexed for 120 s and centrifuged at 11,000 x g at 4°C for 10 min. The supernatant was subjected to high-performance liquid chromatography (HPLC) analysis as follows: 20 μL of each preparation was injected into a 250 × 4.6 mm Capcell Pak UG C18 5 μm column (Shiseido, Tokyo, Japan). A Jasco MD-2015 photodiode array detector (Jasco Co., Ltd., Tokyo, Japan) was used to detect quercetin by monitoring spectral data from 200 to 400 nm for each peak. Spectral data at 254 nm were used to quantify quercetin, and pure quercetin was used as a standard. The mobile phase consisted of methanol/acetic acid/water (35:5:60, v/v/v). The HPLC system was operated at a
Newly Isolated Quercetin-metabolizing Bacterium

In vitro qualitative incubation of intestinal bacteria with quercetin, genistein or resveratrol Strain 19 – 20, previously incubated on modified GAM agar plates for 24 hr, was picked by inoculating loop and suspended in the anaerobic medium. Quercetin (20 mg) was dissolved in 1 mL DMSO. Resveratrol (20 mg) and genistein (20 mg) were also dissolved in 1 mL DMSO. In the quercetin and co-incubation experiment, 1 μL quercetin solution and 2 μL genistein, 2 μL resveratrol, or 2 μL DMSO (as control) were then transferred into 0.2 mL of strain 19 – 20 anaerobic medium suspension and incubated anaerobically at 37°C for 24 h.

After incubation, methanol-acetic acid (100:5, v/v) was added to the reaction mixture to a total volume of 1.0 mL. The mixture was vortexed for 120 s and centrifuged at 11,000 x g at 4°C for 10 min. The supernatant was subjected to HPLC analysis (as described above).

DNA extraction from bacteria Strain 19 – 20 was selected from the modified GAM agar plates and suspended in 1 mL sterilized distilled water. Bacterial template DNA for the polymerase chain reaction (PCR) was extracted using InstaGene matrix (Bio-Rad Laboratories, CA, USA) in accordance with the manufacturer’s instructions.

PCR amplification of 16S rRNA PCR was used to amplify the 16S rRNA. The PCR mixture (50 μL) was composed of each dNTP at a concentration of 200 μM, the primers 27f (5′-AAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGCTACCTTGTGTTACGACTT-3′) at a concentration of 0.30 μM, template DNA and 1.25 U of Takara EX Taq™ DNA polymerase and EX Taq™ buffer (Takara Bio Inc., Otsu, Japan). PCR was carried out using the Dice PCR System (Takara Bio Inc.). The amplification program consisted of one cycle at 94°C for 1 min, followed by 30 cycles at 94°C for 1 min, 65°C for 1 min, 72°C for 1.5 min, and a final cycle at 72°C for 2 min. The amplification products were subjected to gel electrophoresis in 0.7% agarose followed by ethidium bromide staining.

16S rRNA sequence analysis The PCR products were purified using QIAquick spin columns (Qiagen KK, Tokyo, Japan) according to the manufacturer’s instructions. Purified DNA was used for 16S rRNA sequence analysis, performed using an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA), and the following sequencing primers: r1L (5′-GTATTACCGCGCTGCTGG-3′), r2L (5′-CATCAGTTCAGTGAAGC-3′), r3L (5′-TCCTAGCTCCTTGAGGTG-3′), r4L (5′-ACGGCGGCGGTGTTATAAGC-3′), and f3L (5′-GTCCCCAACCAGCAGCACA-3′). The sequences were determined using an ABI Prism 310 DNA sequencer (Applied Biosystems). The assembled partial 16S rRNA sequences were compared with sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The ClustalW (Thompson et al., 1994) program was used to construct a distance dendrogram, which was visualized using the MEGA6 program (Tamura et al., 2013).

Statistics Data are expressed as the mean ± standard error (SE). All data were analyzed using Sigma Plot 13 (Systat Software, Inc., San Jose, CA, USA). The data were analyzed by the one-way analysis of variance and all pairwise multiple comparison procedures (Bonferroni t-test). Statistical significance was set at a p-value < 0.05.

Results

Identification of bacterium Strain 19 – 20 colonies on the modified GAM agar plates were grey in color. Colonies were also observed to be circular, convex, grey or white, and smooth. The cells were rod-shaped. Sequence data was aligned, and the assembled partial 16S rRNA sequence of strain 19 – 20, isolated from human feces, showed 100% similarity to that of Clostridium orbiscindens (Accession no: Y18187) Thus, the strain was identified as belonging to the genus Clostridium. A similarity-based tree was constructed using the neighbor-joining method within ClustalW (Thompson et al., 1994) based on the partial 16S rRNA sequences of strain 19 – 20 (1463 bp) (Accession no: LC175305) and several related bacteria. A phylogenetic tree was constructed using the MEGA6 program (Tamura et al., 2013) (Fig. 1).

In vitro incubation of strain 19 – 20 with quercetin In vitro incubation of strain 19 – 20 with quercetin showed that quercetin was metabolized within 4 hr. More than 90% of quercetin was metabolized within 24 hr. In contrast, most quercetin was not metabolized within 24 hr in the in vitro incubation of control medium (medium plus quercetin). Quercetin degradation by strain 19 – 20 increased in a time-dependent manner (Fig. 2). These results indicate that strain 19 – 20 has strong metabolic activity against quercetin in vitro.

In vitro incubation of strain 19 – 20 with quercetin and genistein or resveratrol During in vitro incubation of 19 – 20 with quercetin and DMSO (control), most quercetin was metabolized within 24 hr. However, in vitro incubation of 19 – 20 with quercetin and resveratrol showed suppressed quercetin degradation after 24 hr of anaerobic incubation (Fig. 3). Quercetin degradation by strain 19 – 20 was significantly reduced in the presence of resveratrol to a greater degree than with DMSO. However, supplementation with genistein did not strongly inhibit strain 19 – 20 quercetin degradation, indicating that genistein is not a strong inhibitor of strain 19 – 20 quercetin degradation. In contrast, supplementation with resveratrol inhibited quercetin degradation by strain 19 – 20.

Discussion

There are few reports focusing on inhibitory polyphenols of...
quercetin degradation by intestinal bacteria. We isolated strain 19–20 as a quercetin-metabolizing bacterium from a healthy human. Quercetin is metabolized by the intestinal microbiota (Parkar et al., 2013). For example, quercetin aglycones are degraded by 70% in the cecal contents of rat (Matsukawa et al., 2009). Quercetin was metabolized to known phenolic acid breakdown products during in vitro fermentation of the human intestinal microbiota (Parkar et al., 2013). C. orbiscindens biotransforms quercetin and taxifolin to 3,4-dihydroxyphenylacetic acid (Schoefer et al., 2003). There are inter-individual variations in the degradation of quercetin-3-rutinoside (Rechner et al., 2004). The bioavailability of quercetin seems to be affected by the metabolic activity of intestinal microbiota. Therefore, the inhibition of quercetin degradation by intestinal bacteria might increase quercetin bioavailability. Quercetin degradation of strain 19–20 was significantly inhibited by the addition of resveratrol to the incubation solution. An inhibitory effect of resveratrol against quercetin-degrading bacteria in the lower gut may be expected with the simultaneous intake of quercetin and resveratrol. We expect quercetin bioavailability to increase in the lower gut as a consequence. However, strain 19–20 quercetin degradation was not inhibited by the addition of genistein to the incubation solution. Thus, quercetin bioavailability is not expected to increase in the lower gut with the simultaneous intake of genistein.

It has been reported that dietary supplementation of resveratrol suppressed 1,2-dimethylhydrazine-induced colon carcinogenesis in rats. It has also been suggested that quercetin was protective against colon carcinogenesis in colon cancer rat models (Turner et al., 2009). Resveratrol reduced strain 19–20 quercetin degradation in the present study. This raises the possibility that simultaneous intake of dietary quercetin and resveratrol may provide synergic preventive effects against colon carcinogenesis.

Dietary polyphenols affect the intestinal microbiota. Polyphenols have been reported to affect bacterial short chain fatty acids (SCFAs) production (Parkar et al., 2013). In turn, SCFAs are thought to affect the intestinal microbiota. Bacteroides/Prevotella...
counts are reported to be negatively correlated with fecal total SCFAs (Fernandes et al., 2014). Dietary polyphenols might affect the composition of the intestinal microbiota through modification of bacterial SCFA production. Quercetin has been reported to inhibit the growth of Ruminooccus gauvreaui, Bacteroides galacturonicus, and Lactobacillus sp. (Duda-Chodak, 2012). Dietary resveratrol also seems to affect the intestinal microbiota. Lactobacilli and Bifidobacteria counts have been reported to increase after the intake of dietary resveratrol (Larrosa et al., 2009). To summarize, both resveratrol and quercetin affect the intestinal microbiota. Therefore, co-administration of resveratrol and quercetin may also affect the composition of the intestinal flora. Further studies are needed to clarify the effects of resveratrol and quercetin co-administration on the intestinal microbiota.

It has also been reported that quercetin is metabolized by Eubacterium ramulus. E. ramulus produced 3,4-dihydroxyphenylacetic acid from quercetin by anaerobic reaction (Braune et al., 2001). C. orbiscindens isolated from human feces was reported to biotransform quercetin and taxifolin to 3,4-dihydroxyphenylacetic acid (Schoefer et al., 2003). Thus, both E. ramulus and C. orbiscindens degrade quercetin. Meanwhile, E. ramulus was reported to metabolize hesperetin dihydrochalcone 4′β-D-glucoside to hesperetin dihydrochalcone, whereas C. orbiscindens did not metabolize hesperetin dihydrochalcone 4′β-D-glucoside within a period of 43 h (Braune et al., 2005). Different levels of enzyme/polyphenol substrate specificity may exist among quercetin-metabolizing E. ramulus and C. orbiscindens.

Inter-individual variation of the intestinal microbiota has been reported (Lozupone et al., 2012). The lower gut houses a complex bacterial community. By extension, quercetin metabolism in the lower gut is also complex. Nonetheless, diet affects the intestinal microbiota of humans (David et al., 2014). Diets may contain many kinds of polyphenols, and dietary polyphenols could influence both the metabolism and composition of the intestinal microbiota. Further investigation of the interactions between food components and quercetin metabolism by the intestinal microbiota is warranted to improve quercetin bioavailability in humans.

Acknowledgements This study was financially supported by a Research Project on Development of Agricultural Products and Foods with Health-promoting Benefits (NARO)

References


