Effect of probiotics on pharmacokinetics of orally administered acetaminophen in mice

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Abbreviations: LC/MS/MS, liquid chromatography-tandem mass spectroscopy. LC/QTOF/MS, liquid chromatography quadrupole time-of-flight mass spectrometer

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Abstract

Orally administered probiotics change gut microbiota composition and enzyme activities. Thus, coadministration of probiotics with drugs may lead to changes in the pharmacokinetic parameters of the drugs. In this study, we investigated the pharmacokinetics of acetaminophen in mice treated with probiotics. Oral administration of probiotics changed the gut microbiota composition in the mice. Of these probiotics, *Lactobacillus reuteri* K8 increased the numbers of clostridia, bifidobacteria, and enterococci, and *Lactobacillus rhamnosus* K9 decreased the number of bifidobacteria, determined by culturing in selective media. Next, we performed a pharmacokinetic study of acetaminophen in mice orally treated with K8 and K9 for three days. Treatment with K8 reduced the area under the curve (AUC) of orally administered acetaminophen to 68.4% compared to normal control mice, whereas K9 did not affect the AUC of acetaminophen. Oral administration to mice of K8, which degraded acetaminophen, increased the degradation of acetaminophen by gut microbiota, whereas K9 treatment did not affect it. Treatment with K8 increased the number of *Lactoacillus reuteri* adhered in the upper small intestine, whereas the number of *Lactobacillus rhamnosus* was not affected by treatment with K8 or K9. K8 increased the number of cyanobacteria, whereas K9 increased the number of deferribacteres. These results suggest that the intake of probiotics may make the absorption of orally administered drugs fluctuate through the disturbance of gut microbiota-mediated drug metabolism and that the subsequent impact on microbiota metabolism could result in altered systemic concentrations of the intact drug.

Introduction

Orally administered drugs are absorbed through the gastrointestinal tract (GI) into the blood. Absorption depends on the solubility, stability, and permeability of the drug as well as on its metabolism by enzymes secreted by the body and gut microbiota (Al-Hilal et al., 2013; Davis, 2005; Kim, 2015; Lennernäs and Abrahamsson, 2005). Nevertheless, the effects of gut microbiota on the absorption of drugs from the GI into the blood have not been studied sufficiently. The ability of gut bacteria to metabolize xenobiotics, particularly drugs, is comparable to that of any organs in the body, including the liver (Kim, 2015; Saad et al., 2012; Sousa et al., 2008).

Drug metabolism has been thought mainly to occur in the liver. However, recent studies have reported that orally administered xenobiotics are metabolized by gut microbiota before their absorption into the blood (Joh and Kim, 2010; Tralau et al., 2015). The metabolic reactions performed by the gut microbiota are quite different to those of the liver; the liver primarily catalyzes the formation of hydrophilic metabolites through oxidative and conjugative reactions, including glucuronidation and sulfation, whereas the gastrointestinal microbiota mainly produce hydrophobic metabolites through reductive and hydrolytic reactions (Joh and Kim, 2010). Therefore, the former is suggested to be a detoxification reaction, while the latter is a toxification or activation reaction.

Acetaminophen is the most common analgesic and antipyretic. Orally administered acetaminophen undergoes sulfation and glucuronidation mediated by Phase II enzymes, including arylsulfotransferase and UDP-glucuronyl transferase (Mitchell et al., 1973; Watari et al., 1983; Klaassen and Cui, 2015). These enzymes detoxify orally administered acetaminophen in the intestinal mucosae and enhance its excretion into the bile duct and urine.

A little acetaminophen is metabolized into N-acetyl-p-benzoquinoneimine, which is detoxified by conjugation with glutathione (Hjelle and Klaassen, 1984; Hjelle et al., 1985; Goon and Klaassen, 1990; Boles and Klaassen, 2000; Klaassen and Cui, 2015). However excretion of acetaminophen and its metabolites into the urine was significantly decreased in rodents treated with antibiotics (Kim and Kobashi, 1986; Gauhar et al., 2014). Intraperitoneal injection of p-nitrophenyl sulfate increases the excretion of acetaminophen-sulfate into the urine, and when acetaminophen is orally administered in humans, the excretion of acetaminophen-sulfate and cresol-sulfate into the urine is competitive (Clayton et al., 2009). The sulfation of phenolic compounds such as cresol, acetaminophen, and tyrosine is also catabolized by the arylsulfate sulfotransferase of gut bacteria such as Eubacterium rectale A-44 or Enterobacter amnigenus AR (Kim et al., 1992; Kwon et al., 1999). The pharmacokinetic parameters of orally administered acetaminophen may be affected by endogenous and exogenous factors of fluctuating gut microbiota.

Probiotics, frequently found in yogurt, kimchi, and human gut microbiota, are considered beneficial microorganisms because they exhibit beneficial biological effects for constipation, diarrhea, colitis, and diabetes and suppress the harmful enzyme production of gut microbiota. Therefore, the use of probiotics is increasing in medical practice (Goldin and Gorbach 2008; Bron et al., 2017; Wasilewski et al., 2015). Probiotics can in fact simultaneously be used in treatment with therapeutic drugs. But, they interact with many drugs in the gut and may cause the pharmacokinetic parameters of orally administered drugs to fluctuate (Lee et al., 2012; Stojančević et al., 2014); some probiotics affect drug metabolism involving certain drug-metabolizing enzymes, for example, the metabolism of nifedipine by CYP3A in the intestinal mucosa or the metabolism of sulfasalazine by gut microbiota (Kato et al., 2007). Therefore, to understand the effects of probiotics on the pharmacokinetics of frequently used drugs, we

evaluated the effect of probiotics on the pharmacokinetics of orally administered

acetaminophen in mice.

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Materials and Methods

Materials

Acetaminophen, formic acid, and dextrose were purchased from Sigma (St. Louis, MO, USA). QIAamp Fast DNA stool Mini Kit was purchased from QIAGEN (Hilden, Germany). BL agar (BA) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). m Enterococcus Agar (EA) was purchased from BD (Franklin Lakes, NJ). Clostridia agar (CA) was purchased from MB cell (Los Angeles, CA). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Distilled water was prepared using a Milli-Q purification system (Millipore, Billerica, MA, USA). Lactobacilli (K1, *L. acidophilus* KCTC3140; K2, *L. casei* KCTC3109; K3, *L. gasseri* KCTC3148; K4; *L. delbueckii* subsp. *bulgaricus* KCTC3635; K5, *L. helveticus* KCTC3545; K6, *L. fermentum* KCTC3112; K7, *L. paracasei* KCTC13169; K8, *L. reuteri* KCTC3679; K9, *L. rhamnosus* KCTC3237; and K10, *L. salivarius* KCTC5922) were purchased from Korea Collection for Type Cultures (Daejeon, Korea).

Animals

Male C57BL/6 mice (22-25g, 8-week old) were purchased from RaonBio Inc. (Seoul, Korea) housed in a wire cage under controlled condition (light/dark, every 12 h; temperature, 20 - 22°C; and humidity, 50 ± 10%), and acclimated for 1 week before the experiments. Mice fed standard laboratory chow and water *ad libitum*. The experiment was performed according to the protocol of the Kyung Hee University Guideline for Laboratory Animals Care and Usage. The experiment protocol was ethically approved by the Committee for the Care and Use of Laboratory Animals (KHPASP(SE)-16-027). Pharmacokinetic experiments were performed

in accordance with the Hanyang University Guidelines for Laboratory Animal Care and Use, and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Hanyang University (2016-0151).

Analysis of gut microbiota composition

Mice were orally gavaged probiotics (1×10^9 CFU/mouse) once a day for 5 days. The fresh feces (approximately 0.1 g) were collected 6 h before probiotics treatment, 1 or 3 days after the final gavage of probiotics. For the analysis of gut microbiota composition by using selective media was performed according to the method of Lim et al. The fresh feces were gently suspended in 0.9 mL of cold GAM broth (BD, Sparks, MD, U.S.A.). The suspended solution was diluted with the broth and inoculated into Clostridium agar (CA) for clostridia, BL for bifidobacteria, and m Enterococcus agar (ME) for enterococci. BL and CL agars were anaerobically cultured for 2 and 3 days, respectively. EA was aerobically cultured for 24 h.

For the analysis of gut microbiota composition by qPCR, fecal DNA was extracted from the fresh feces using QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) and analyzed Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes by qPCR. qPCR was performed according to the method of Yang et al. (Yang et al., 2015), utilizing Qiagen thermal cycler, which used SYBR premix agents, as per the instructions from Takara biology incorporation: activation of DNA polymerase at 95°C for 30 s and 40 cycles of amplification at 95°C for 5 s and at 63°C for 30 s. The normalized expression of the assayed genes, with respect to bacterial rRNA, was computed for all samples using the Microsoft Excel data spreadsheet. Primers were used as follows: Firmicutes forward, 5'-GGAGYATGTGGTTTAATTCGAAGCA-3', reverse, 5'-AGCTGACGACAACCATGCAC-3'; Bacteroidetes forward, 5'- GTTTAATTCGATGATACGCGAG-3' reverse, 5'-

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TTAASCCGACACCTCACGG-3'; Actinobacteria forward, 5'-TGTAGCGGTGGAATGCGC-3', reverse, 5'-AATTAAGCCACATGCTCCGCT-3'; γ/δ-Proteobacteria forward, 5'-GCTAACGCATTAAGTRYCCCG-3', reverse 5'-GCCATGCRGCACCTGTCT-3'; and bacterial rRNA forward, 5'-AGAGTTTGATCCTGGCTCAG-3', reverse 5'- AAGGAGGTGWTCCARCC-3'.

Preparation of mouse fecalase and intestinal enzyme fraction

The feces of each mouse dosed with or vehicle following the treatment with probiotics (approximately 0.3 g) were collected in plastic tubes, suspended in 2.7 mL of cold saline and centrifuged at $500{\times}g$ and 4℃ for 5 min. The supernatant was sonicated for 2 min five times and then centrifuged at $10,000{\times}g$ 4℃ for 20 min. The supernatant was used for the enzyme activity assay. Small intestines and colons were removed from the mice, washed with PBS trice, homogenized in 50 mM Tris-HCl buffer (pH 7.4), and centrifuged at $10,000 \times g$ and 4°C for 30 min. The resulting supernatants were used as a crude enzyme fraction of intestinal tissues.

Acetaminophen-metabolizing activity assay

The reaction mixture (total volume of 2.5 mL), which is consisted of 0.25 mL of 0.25 mM acetaminophen, 2 mL of 0.1 M potassium phosphate buffer (pH 7.0) and 0.25 mL of fecal suspension or a probiotic K8 or K9 cultured in MRS broth ($1{\times}10^{11}$ CFU/mL), was incubated at 37°C for 12 h, stopped by the addition of 1.5 mL of MeOH, and centrifuged ($3000{\times}g$ for 15 min). The amount of acetaminophen in the resulting supernatant was assayed by high-performance liquid chromatography (HPLC) or thin-layer chromatography.

Enzyme activity assay in the feces and intestinal tissues

For the activity assay of fecal β-glucuronidase, and sulfatase, the reaction mixture (total volume of 0.5 mL), which contained 0.1 mL of 1 mmol/L p-nitrophenyl-β-D-glucuronide for β-glucuronidase or p-nitrophenyl sulfate for sulfatase, 0.3 mL of 0.05 mol/L phosphate buffer, pH 7.0, and 0.1 mL of the fecalase or intestinal enzyme fraction was incubated at 37℃ for 20 min. For the activity assay of fecal arylsulfate sulfotransferase, the reaction mixture (total volume of 0.5 mL), which contained 0.1 mL of 1 mmol/L p-nitrophenyl sulfate, 0.1 mL of 1 mmol/L acetaminophen, 0.2 mL of 0.05 mol/L phosphate buffer, pH 7.0, and 0.1 mL of the fecal suspension or enzyme solution was incubated at 37℃ for 30 min. The reaction was stopped by the addition of 0.5 mL of 10 mmol/L NaOH and centrifuged at 3000×g for 2 min; the absorbance was measured at 405 nm (UV–vis spectrophotometer, Shimadzu UV-1201). Enzyme activity was indicated as the amount required to catalyze the formation of 1.0 nmole of p-nitrophenol per hour under the standard assay conditions. Specific activity was defined in a term of mmol/h/g wet feces.

Fecal sample analysis by LC/QTOF/MS

The fecal incubation samples were analyzed using LC/QTOF/MS. The instrument consisted of an Agilent 1260 HPLC system and an Agilent 6530 QTOF mass spectrometer with an electrospray interface (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a Poroshell 120 EC-C18 column (2.7 μm, 2.1 × 100 mm; Agilent Technologies, Santa Clara, California, USA). Column oven temperature was maintained at 40℃. The HPLC mobile phases consisted of 0.1% formic acid in distilled water (A) and 90% acetonitrile in 0.1% formic acid (B). A gradient program was used at a flow rate of 0.2 ml/min, as follows: 5% B to 85% B for 15 min, followed by a 7-min re-

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equilibration. Mass detection was performed in the positive ion mode. The drying gas (nitrogen) temperature was set at 350℃. The drying gas flow was set at 10 L/min. The nebulizer pressure was set at 20 psi. The capillary and fragmenter voltage were 3500 V and 110 V, respectively. High-purity nitrogen was introduced into the collision cell as fragmentation gas.

Pharmacokinetic experiments

Mice pretreated with probiotics or vehicle (n=6) were administered an intravenous (0.5 mg/kg) or an oral (10 mg/kg) dose of acetaminophen dissolved in saline (5 mg/mL) 24 h after completing the 5 consecutive days treatment with 1% dextrose (control) or each probiotic (K8 or K9) dissolved in 1% dextrose (1×10^9 CFU/mouse). The whole blood samples were taken from the orbital vein into heparinized tubes at 0.03, 0.16, 0.5, 1, 2, 4, 6, and 8 after intravenous injection or at 0.16, 0.5, 1, 2, 4, 6, and 8 hours after oral administration. The plasma was harvested by centrifugation at 13,200 rpm for 5 min and stored at -70℃ until analyzed for acetaminophen.

Blood sample preparation and calibration curves

A 20-μL aliquot of plasma samples were deproteinized with 20 μL acetonitrile containing 10 ng/mL bupropion (internal standard, IS) and added with 20 μL distilled water. The sample was vigorously vortex-mixed, and then centrifuged at 13,200 rpm for 5 min. The resulting supernatant was transferred to LC vials and a 5-μL aliquot was injected into the LC/MS/MS analysis system. The calibration curve of acetaminophen in mouse plasma was constructed using seven calibration standards at concentrations of 5, 10, 20, 100, 500, 1000, and 6000 ng/mL. Calibration curves were generated by plotting the peak area ratios of the analyte to

the IS versus the analyte concentrations, and the calibration curve equation was generated by linear least squares regression. The regression coefficient (r^2) was greater than 0.999. The accuracy and the precision of the calibration standard curves were reliable (less than $\pm$ 15% RSD) for all the concentration points tested.

LC/MS/MS analysis for pharmacokinetic samples

The LC/MS/MS system consisted of an Agilent 1260 Infinity HPLC system with an Agilent 6460 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies). Chromatographic separation was achieved with a Halo C8 (2.7 μm, 2.1 × 100 mm); oven temperature was maintained at 40℃. The mobile phase consisted of (A) 0.1% formic acid in distilled water, and (B) 0.1% formic acid in acetonitrile. A gradient program was used as follows: 10% B to 90% B at 2 min, to 10% B at 0.1 min, held at 10% B for 4.9 min, with a flow rate of 0.3 mL/min. Drying gas (nitrogen) temperature was set at 300℃, drying gas flow at 100 L/min, nebulizer pressure at 20 psi, and capillary voltage at 3500 V. Fragment voltages were set at 90 for acetaminophen and 130 for bupropion. Multiple-reaction monitoring detection was used, with nitrogen as the collision gas; the precursor-product ion pairs monitored were 152>110 for acetaminophen and 240>184 for bupropion.

Pharmacokinetic analysis

The maximum plasma concentration (C_{max}), the time taken to reach max (T_{max}) and area under plasma concentration-time curve from time 0-last (AUC_{last}) for acetaminophen were estimated directly from the plasma concentration-time profiles. The Phoenix WinNonlin Enterprise Program v5.3 (Pharsight Inc., St. Louis, MO, USA) was used with a

noncompartmental statistical model to determine other kinetic parameters of the mouse plasma samples.

Acetaminophen metabolite profiling in plasma, urine, and feces

Following an overnight fast, the mice were orally dosed with 10 mg/kg of acetaminophen and housed individually in metabolic cages equipped with a urine and feces separator. Heparinized samples of blood were collected at 30 min after dose administration. Urine and feces were collected and weighed at the following intervals: before dose administration, and 0-24 h after dose administration. All samples were stored separately at -20℃ until analysis. In the plasma samples, 40 μL of acetonitrile was added to 40 μl of plasma. Then it was vortex-mixed for 30 s and centrifuged of 13,200 rpm for 5 min and a 50 μL aliquot of supernatant was diluted to 50 μL distilled water. After urine sample collections, the urine were purified with solid-phase extraction (SPE) using Oasis HLB 96-well μElution extraction plate (Water, Milford, MA). Urine was loaded to SPE plate which was pretreated with 1 mL methanol. SPE columns were washed twice with water and eluted 1 mL methanol. The elution was collected and evaporated to dryness under nitrogen stream at 45℃. These residues were dissolved in 100 μL of 20% methanol in water. For feces samples, three volumes of methanol were added to the feces, followed by homogenized and then centrifugation at 1000 × g for 5 min. The supernatant was filtered using 0.2 μm PTFE syringe filter and diluted to water. The samples were analyzed using liquid chromatography-tandem mass spectrometry analyses according to a previously reported method (An et al., 2012).

Statistics

All the data were expressed as the mean ± standard derivation, and statistical significance

was analyzed by Student's t-test or ANOVA coupled with Tukey's post-hoc test with

statistical significance set at P<0.05.

Results

Effects of probiotics on the pharmacokinetics of acetaminophen

To understand the effects of probiotics on the pharmacokinetic of acetaminophen in mice, we first selected probiotics which influenced the gut microbiota composition. Ten kinds of probiotics were gavaged to mice, the fresh feces were cultured using selective media, and the consequent changes in the number of the representative gut bacteria were periodically analyzed (Fig. 1). Most of the probiotics increased the number of bifidobacteria grown in BL whereas K5 and K9 gradually reduced the number of bifidobacteria grown in BL. K8 significantly increased the number of clostridia grown in CA. Several probiotics including K4 and K10 significantly increased the number of enterococci grown in EA. Taken collectively, K8, and K9 were supposed to be the most characteristic probiotics, and thus, we selected these two probiotics for subsequent studies, where we investigated the effects of K8 and K9 on the pharmacokinetics of acetaminophen (Fig. 2).

The plasma concentration levels of acetaminophen were determined after oral administration to the vehicle or probiotic-treated mice. The mean plasma concentration-time profiles of acetaminophen in the control and probiotic-treated mice groups are shown in Fig. 2A, and the resultant pharmacokinetic parameters are described in Table 1. The C_{max} and AUC values of acetaminophen in the control group were 4.72 ± 0.83 μg/mL and 3.51 ± 0.47 μg·h/mL, respectively. As for K8-treated mice, the Cmax and AUC values were 3.30 ± 0.57 μg/mL and 2.40 ± 0.26 μg·h/mL, respectively; both parameters significantly decreased compared to those in the control group. Meanwhile, the Cmax and AUC values in the K9-treated mice did not show any significant difference from the control values, although some differences were observed between their mean values. As for Cl/F and $t_{1/2}$, K8-treated group

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showed the increased values of Cl/F compared to other groups. However, the $t_{1/2}$ values were comparable among three groups.

Meanwhile, when acetaminophen was intravenously administered after probiotics treatment, the plasma concentration profile of acetaminophen was not changed (Fig. 2B). These data indicate that the pharmacokinetic changes of acetaminophen in probiotics-treated mice resulted mainly from the alterations in the GI tract. The resulting pharmacokinetic parameters are summarized in Table 2.

Effects of probiotics on the acetaminophen metabolism in the gut

To understand how probiotic K8 could inhibit the absorption of acetaminophen from the GI tract into the blood, we first incubated acetaminophen with mouse fecal suspension for 24 h and the amount of acetaminophen remaining was measured (Fig. 3A). The remaining amount of acetaminophen decreased after the 24 h incubation; mouse fecal suspension significantly degraded acetaminophen. Based on these data, the acetaminophen-metabolizing activities of mouse fecal suspension were measured as 0.029 ± 0.028 nmol/h/mg.

Next, to investigate the in vivo effects of probiotics on the metabolic activity of acetaminophen by gut microbiota, we orally administered probiotics to mice and measured fecal acetaminophen-metabolizing activity 24 h after the final oral administration of the probiotics (Fig. 3A). The oral administration of probiotics caused some of the activity to fluctuate; treatment with K8 significantly increased the acetaminophen-metabolizing activity compared with normal control mice, whereas K9 treatment did not significantly influence the activity. We also measured the acetaminophen-metabolizing activity of probiotics themselves. Although the acetaminophen-degrading activity of the probiotics was weaker than that of the fecal suspension, they also degraded acetaminophen (Fig. 3B). Of the probiotics tested, K8

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significantly increased the metabolic activity of acetaminophen. These findings on the

changes in gut microbial acetaminophen-metabolizing activities by probiotics are consistent

with the pharmacokinetic results following probiotic treatment.

In addition, to confirm the effects of K8 on the gut microbial metabolism of

acetaminophen, we measured the acetaminophen concentration in the contents of different

parts of the gastrointestinal tract after oral administration of acetaminophen to control or

probiotics-treated mice (Table 3). The mean acetaminophen concentration in the stomach of

K8-treated mice was lower than control and K9-treated mice but statistical significance was

not shown. There was no difference of the concentrations in the small intestine between K8

and control groups. Meanwhile, the mean acetaminophen concentration in cecum was

somewhat higher than other two groups, which suggest that K8-treatment might stimulate the

peristaltic movements of the gastrointestinal tract and the acetaminophen moved more

quickly to the lower part of the intestine.

Metabolite profiles of acetaminophen in plasma, urine, and feces

The concentrations of acetaminophen metabolites were measured in plasma, urine and feces

of control and K8-treated mice. Acetaminophen and its six metabolites were determined and

their levels were plotted as a relative percentage based on peak area (Fig. 4). In plasma and

urine, the percentage of acetaminophen in K8-treated mice was lower than that in control

mice, which is consistent with the plasma pharmacokinetic results. The portion of urinary

concentration of the cysteine conjugate was elevated about twofold in K8-treated mice. In

addition, there were significant differences in the ratios of urinary concentration of

acetaminophen glucuronide and plasma concentration of the *O*-methylated metabolite. In

feces, the conjugate metabolite ratios were generally decreased by K8-treatment, which

reveals that K8 or K8-inducing alteration of gut microbiota might promote the degradation of the conjugate metabolites by gut microbial enzymes.

Effects of probiotics on the enzymes involved in the deconjugation of acetaminophen

As for the enzymes that may be involved in the conjugation of acetaminophen, both K8 and K9 significantly increased the fecal sulfatase and arylsulfate sulfotransferase activities. However, β-glucuronidase activities were reduced by treatment with K8 or K9 (Fig. 5). Subsequently, effects of probiotics on the intestinal β-glucuronidase, sulfatase and arylsulfate sulfotransferase activities were investigated. Fig. 6 showed that the treatment with K8 or K9 did not affect the intestinal metabolic enzyme activities. This finding suggests that the alteration of acetaminophen metabolism observed in the K8-treated mice may be due to the modulation of gut microbial enzyme activities rather than the modulation of the intestinal enzyme activities.

Effects of probiotics on the population of gut microbiota

Next, by qPCR, we analyzed the composition of fecal microbiota in control and probiotic-treated mice (Fig. 7). At the phylum level, probiotics treatment with K8 significantly increased the number of Cyanobacteria, whereas K9 treatment increased the number of Deferribacteres.

To investigate whether orally administered probiotics adhered into the gastrointestinal tract, we orally administered probiotics to mice and analyzed the amount of K8 or K9 in the epithelia of the stomach and upper small intestine using PCR. Treatment with K8 increased the number of *Lactoacillus reuteri* adhered in the upper small intestine, whereas the number of *Lactobacillus rhamnosus* was not affected by treatment with K8 or K9.

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Discussion

Acetaminophen is catalyzed to hydrophilic metabolites in the intestinal mucosae and liver by Phase II enzymes including sulfotransferase and UDP-glucuronyl transferase (Goon and Klaassen, 1990). These enzymes, respectively, require 3'-phosphoadenosine-5'-phosphosulfate and UDP-D-glucuronic acid as donor substrates. These reactions are limited, apparently because of the depletion of donor substrates such as glutathione (Chen et al., 2013). Non-conjugated acetaminophen ($< 10\%$) is transformed to N-acetyl-p-benzoquinoneimine (ABQ) by the cytochrome P-450 enzymes (CYP2E1, 1A2, and 3A4). ABQ, a reactive electrophile, is detoxified by conjugation with glutathione. However, when glutathione levels are depleted, ABQ binds to macromolecules such as protein and DNA in hepatocytes, resulting in liver necrosis (Hjelle and Klaassen, 1984; Hjelle et al., 1985). Moreover, the excretion of acetaminophen conjugates such as acetaminophen-O-sulfate in the urine is affected by antibiotics treatment (Kim and Kobashi, 1986; Gauhar et al., 2014), and its excretion into the urine is competitive with cresol-O-sulfate (Clayton et al., 2009). Cresol and acetaminophen are sulfated by liver arylsulfotransferase or bacterial arylsulfate sulfotransferase and are glucuronated by liver UDP-glucuronidase (Goon and Klaassen, 1990; Kim et al., 1992). Orally administered acetaminophen is mainly conjugated by sulfation, and acetaminophen-sulfoconjugates are mainly excreted into the intestine via the bile duct and urine (Kim and Kobashi, 1986; Kim et al., 1992). The conjugated acetaminophens, including acetaminophen-sulfoconjugates, are retoxified by β-glucuronidase and sulfatase of the liver and bacteria (Lee et al., 2003).

In the intestine, acetaminophen conjugates excreted via the bile duct are hydrolyzed to acetaminophen by bacterial β-glucuronidase and sulfatase (Kim and Kobashi, 1986; Lee et al.,

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2012). However, for acetaminophen conjugates, sulfoconjugates may be a donor substrate for bacterial arylsulfate sulfotransferase, which catalyzes the transfer of sulfoconjugates to phenolic compounds and produces free acetaminophen (Kim et al., 1992; Kwon et al., 1999). Meanwhile, the free acetaminophen can be sulfated by bacterial arylsulfate sulfotransferase (Kim and Kobashi, 1986). In the present study, we found that the fecal arylsulfate sulfotransferase activity in K8-treated mice is greater than that in control mice. This finding reveals the possibility that acetaminophen might be sulfoconjugated by bacterial arylsultransferase prior to being absorbed, which might limit the absorption of acetaminophen. In this context, the sulfation of free acetaminophen in the GI tract may be dependent on the concentration of phenyl sulfate esters such as cresol-O-sulfate and bacterial arylsulfate sulfotransferase (Kim and Kobashi, 1986; Kim et al., 1992). Accordingly, the absorption of deconjugated acetaminophen from the intestine into the blood may be dependent on the concentration of phenolic compounds and arylsulfate sulfotransferase-producing bacteria. This can be supported by reports that treatment with antibiotics decreased the number of arylsulfate sulfotransferase-producing bacteria in the GI tract and inhibited the excretion of acetaminophen into the urine (Kim and Kobashi, 1986; Gauhar et al., 2014). These results suggest that the factors that change gut microbiota composition could affect the pharmacokinetics of acetaminophen. Our results on pharmacokinetic parameters also showed that K8 treatment increased the oral clearance of acetaminophen and decreased the systemic exposure of acetaminophen.

Interestingly, the acetaminophen metabolite profile data showed that the portion of the urinary acetaminophen-cysteine conjugated metabolite was increased in K8-treated mice. This result suggests the possibility that K8-treatment might promote the clearance of acetaminophen through glutathione conjugation to decrease the plasma levels of

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acetaminophen. Lutgendorff et al. (2008) reported that probiotics enhance pancreatic glutathione biosynthesis and increase plasma gluthathione levels. This report may partly explain the mechanisms for the increase of the cysteine conjugated metabolite in K8-treated mice. Further study should be followed to clarify its underlying mechanisms.

In the present study, we found that orally administered probiotics K8 and K9 attached in the GI tract. Of these, K8 resided in the upper small intestine, not the stomach, in more significant amounts than K9. These probiotics also caused the composition of gut microbiota to fluctuate; the probiotics increased Firmicutes and Bacteroides phyla in the colon but inhibited the Proteobacteria phylum. These probiotics also changed the activities of their drug-metabolizing enzymes such as β-D-glucuronidase and arylsulfate sulfotransferase. Of these probiotics, K8 increased the degradation of acetaminophen by itself and significantly increased gut bacterial acetaminophen-degrading and arylsulfate sulfotransferase activities without changing the intestinal metabolic activities. These results suggest that probiotics could affect the absorption of acetaminophen as well as the sulfoconjugate level of acetaminophen in the intestine by modulating the gut microbial metabolic activities. Oral administration of acetaminophen in mice treated with K8 significantly reduced the AUC of the acetaminophen compared to that in mice treated in the absence of K8. These results suggest that K8 may inhibit the absorption to the blood of acetaminophen from the GI tract including the stomach, 1) by promoting the metabolism of acetaminophen directly or via modulation gut microbial enzyme activities or 2) by increasing its sulfoconjugation in the GI tract. In addition, the data from Table 3 suggests a possibility that the decrease of the bowel transit time due to stimulation of the peristaltic movements by K8 might disturb the absorption of acetaminophen. If sustained-release acetaminophen is used for treatment, the sulfoconjugation of orally administered acetaminophen may increase in the GI tract, which

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may lead to a decrease in the absorption of the acetaminophen.

Authorship contributions

Participated in research design: Kim DH and Yoo HH

Conducted experiments: Kim JK, Choi MS, Jeong JJ and Lim SM

Contributed new reagents or analytic tools: Kim IS

Performed data analysis: Kim JK, Choi MS, Lim SM

Wrote or contributed to the writing of the manuscript: Kim JK, Choi MS, Kim DH and Yoo

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Footnotes

Figure Legends

Fig. 1. Effects of commercially available probiotics on the composition of gut microbiota in mice. (A) Effects on the number of bifidobacteria grown in BL agar plates; (B) Effects on the number of clostridia grown in CA agar plates; (C) Effects on the number of enterococci grown in EA agar plates. Probiotics (1×10^9 CFU/mouse) was orally administered to mice once a day for 3 days. The fresh stools were collected on before and 1 and 3 days after the final treatment with probiotics. The plates were anaerobically incubated at 37^oC for 2 to days. □ , before; ■, 1 day; ▨, 3 days.

Fig. 2. Plasma concentration profiles of acetaminophen in mice treated with or without probiotics. Acetaminophen was (A) orally (10 mg/kg) or (B) intravenously (0.5 mg/kg) administered to mice after the final administration of probiotics. Probiotics K8 (*L. reuteri*) or K9 (*L. rhamnosus*) (1×10^9 CFU/mouse) was orally administered to mice once a day for 3 days before treatment with acetaminophen. Data represent the mean ± SD (n=6).

Fig. 3. Effects of probiotics on the acetaminophen-metabolizing activity of gut microbiota. (A) Fecal acetaminophen-metabolizing activity in mice after treatment of probiotics. (B) The acetaminophen-metabolizing activity of K8 and K9. Data are shown as mean ± SD (n = 6). $^{*}p < 0.05$ vs. control group.

Fig. 4. Metabolite profiles of acetaminophen in (A) plasma, (B) urine, and (C) feces of control and K8-treated mice. Data are shown as mean ± SD (n = 4). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group. APAP, acetaminophen; APAP-Sul, acetaminophen sulfate; APAP-Glc,

acetaminophen glucuronide; APAP-OME, 3-methoxy acetaminophen; APAP-NAC, 3-(N-acetyl-L-cystein-S-yl) acetaminophen; APAP-Glth, acetaminophen glutathione; APAP-Cys, 3-cysteinylacetaminophen.

Fig. 5. Effects of probiotics on the fecal (A) sulfatase, (B) arylsulfate sulfotransferase, and (C) β-glucuronidase activities in mice treated with or without probiotics. Probiotics (1×10^9 CFU/mouse) was orally administered to mice once a day for 3 days. The fresh stools were collected on before, 1 and 3 days after the final treatment with probiotics. Data are shown as mean ± SD (n = 6). [*]$p < 0.05$ vs. control group.

Fig. 6. Effects of probiotics on the intestinal (A) sulfatase, (B) arylsulfate sulfotransferase, and (C) β-glucuronidase activities in mice. Con, Control group; A, Acetaminophen was orally administered; A+K8, Acetaminophen was orally administered to mice treated with K8; A+K9, Acetaminophen was orally administered to mice treated with K9. Data are shown as mean ± SD (n = 5).

Fig. 7. Effects of probiotics on the gut microbiota composition in mice. (A) Effects on fecal gut microbiota composition, which was analyzed by qPCR. Effects of K8 and K9 treatment on the number of *L. rhamnosus* and *L. reuteri* adhered in (B) stomach and (C) upper small intestine. The numbers of gut bacteria were analyzed by qPCR. Data are shown as mean ± SD (n = 6). [*]$p < 0.05$ vs. control group.

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Table 1. Pharmacokinetic parameters of acetaminophen after oral administration in mice pretreated with or without probiotics (n=6).

Parameters	Acetaminophen (10 mg/kg)		
	Control	K8	K9
Tmax (h)	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00
Cmax (µg/mL)	4.72 ± 0.83	3.30 ± 0.57*	3.96 ± 0.96
AUC (µg·h/mL)	3.51 ± 0.47	2.40 ± 0.26**	3.65 ± 0.98
Cl/F (L/h/kg)	2.89 ± 0.39	4.14 ± 0.49**	2.89 ± 0.71
$T_{1/2}$ (h)	1.09 ± 0.35	1.65 ± 0.70	1.22 ± 0.24

* $p<0.05$ vs control

** $p<0.01$ vs control

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Table 2. Pharmacokinetic parameters of acetaminophen after intravenous injection in mice pretreated with or without probiotics (n=6).

Parameters	Acetaminophen (0.5 mg/kg)		
	Control	K8	K9
AUC (μg·h/mL)	0.76 ± 0.13	0.85 ± 0.16	0.80 ± 0.11
Cl (L/h/kg)	0.67 ± 0.14	0.60 ± 0.10	0.63 ± 0.08
$T_{1/2}$ (h)	0.94 ± 0.15	1.00 ± 0.16	0.94 ± 0.08
Vz (L/kg)	0.92 ± 0.26	0.86 ± 0.17	0.86 ± 0.15
MRT (h)	0.58 ± 0.05	0.53 ± 0.04	0.53 ± 0.05

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Table 3. The concentration of acetaminophen in the contents from different parts of the gastrointestinal tract of control and probiotics-treated mice after oral administration of acetaminophen (n=5)

	Acetaminophen (ng/mL)			
	Stomach	Small intestine	Cecum	Large intestine
Control	3902.6 ± 3648.2	159.5 ± 96.7	2.1 ± 2.2	0.0 ± 0.0
K8	574.3 ± 979.6	156.0 ± 166.6	447.1 ± 485.8	2.2 ± 2.6
K9	8147.0 ± 12794.2	304.7 ± 278.5	173.1 ± 133.9	14.2 ± 20.4

The gastrointestinal contents were collected at 1 h after the administration of acetaminophen.

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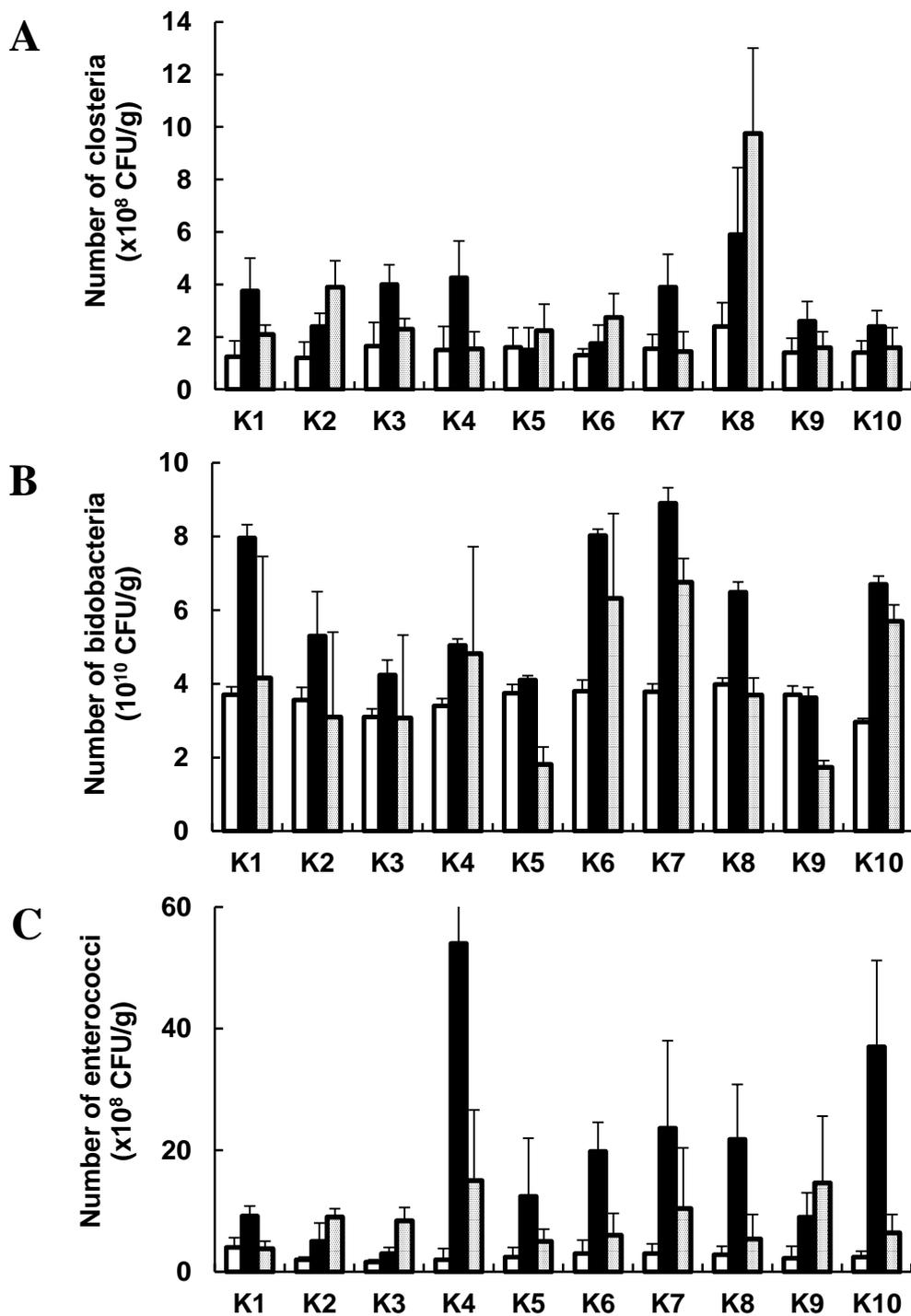
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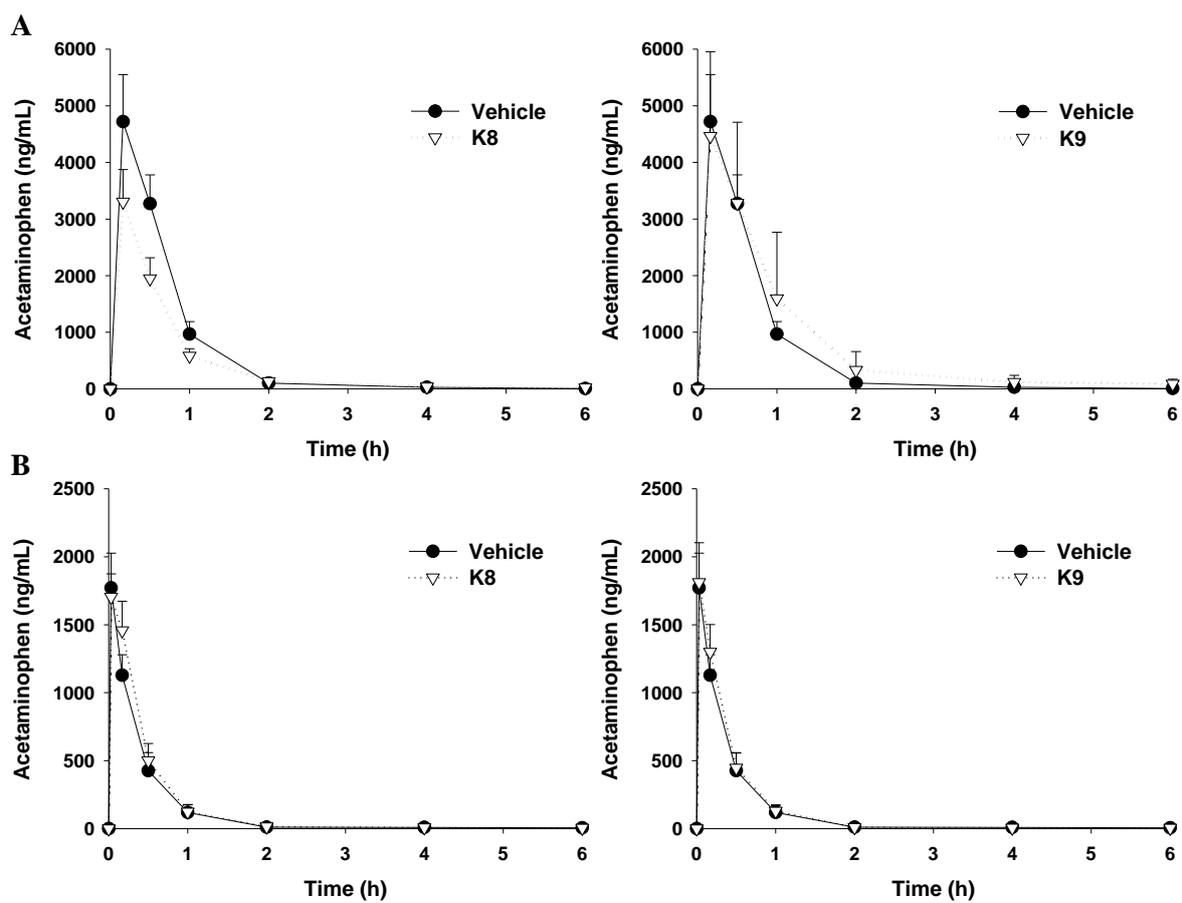
Figure 1

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Figure 2

(A) Plasma
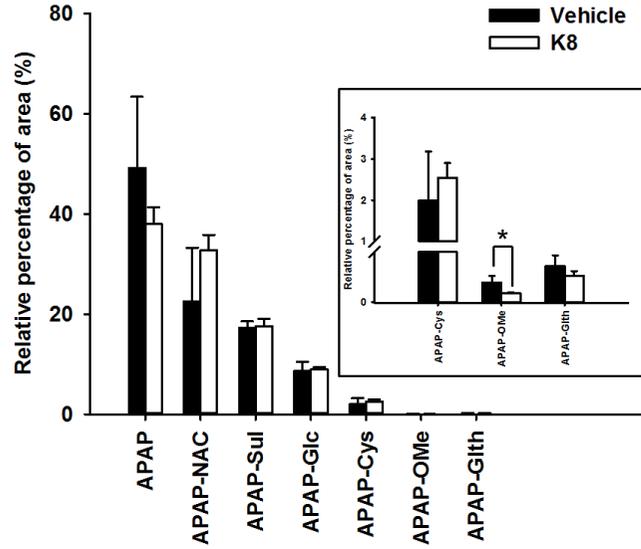
(B) Urine
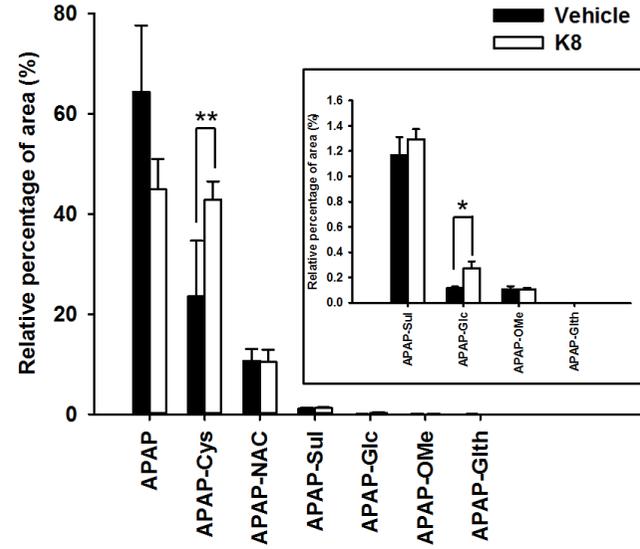
(C) Feces
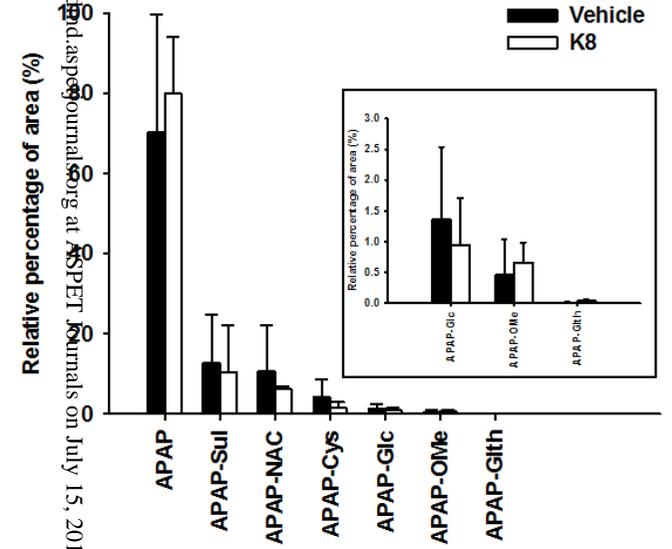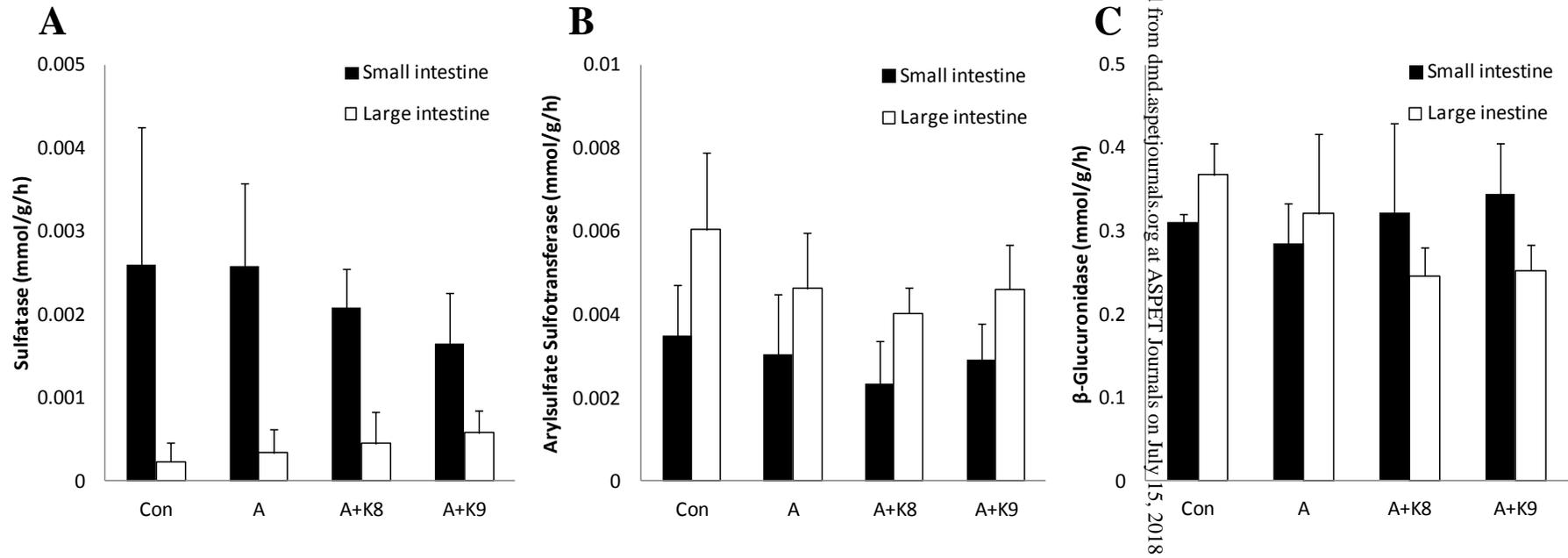

Figure 3

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Figure 4

A

B

C

Figure 5

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A

B

C
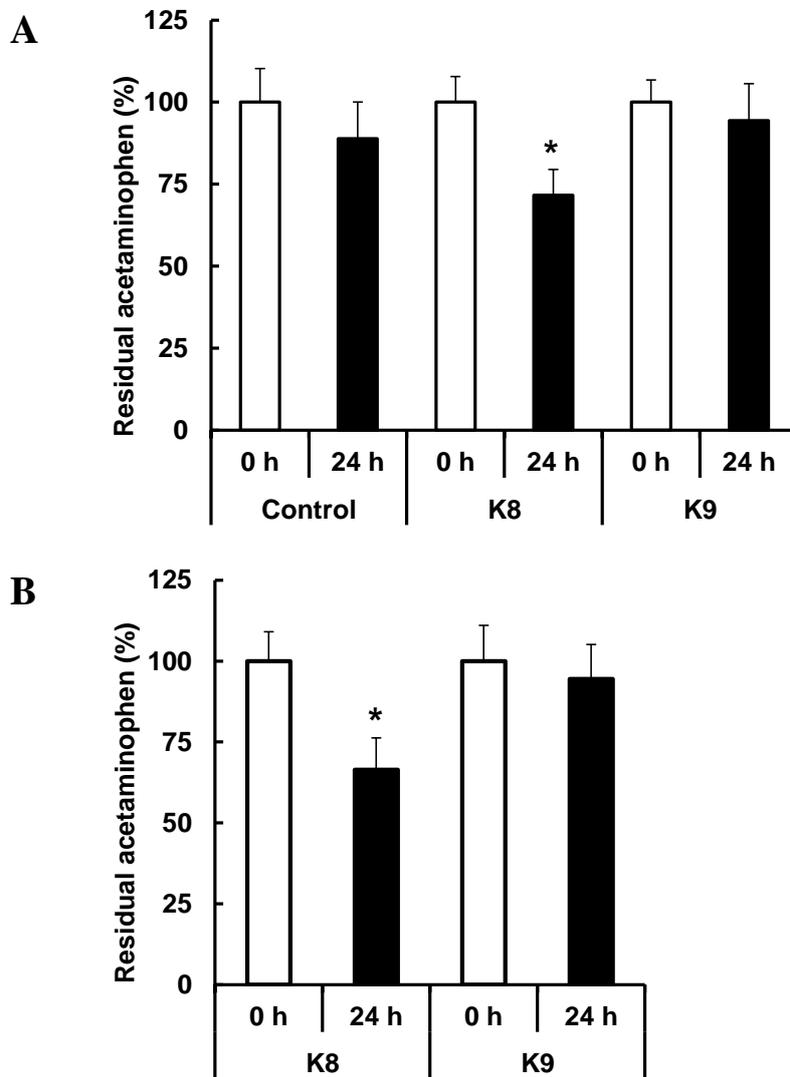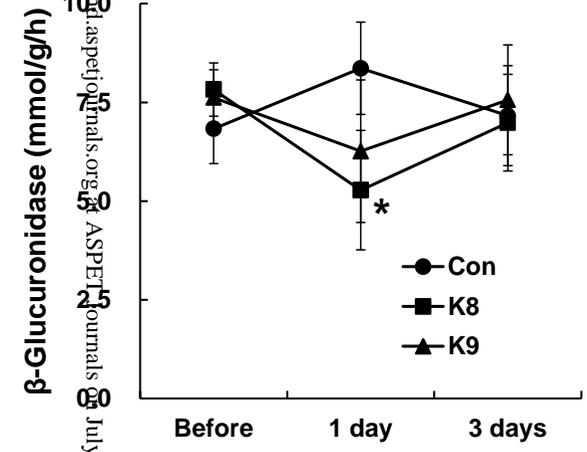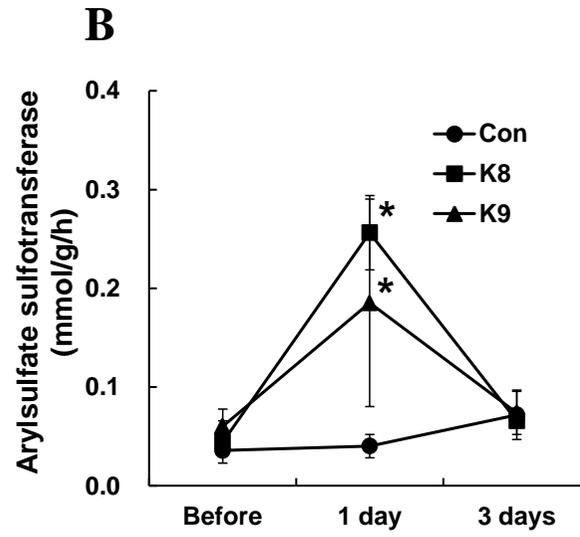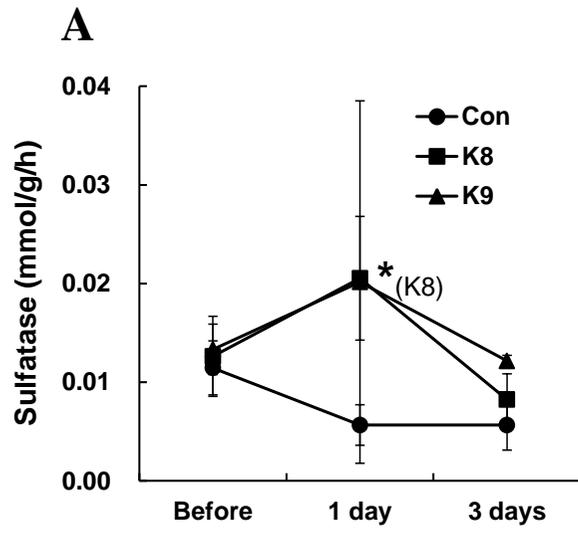
Figure 6

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Figure 7