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# Biotransformation of Rutin in In Vitro Porcine Ileal and Colonic Fermentation Models

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**ABSTRACT:** Quercetin, a polyphenol antioxidant, is widely distributed in food in the form of glycoside rutin, which is not readily absorbed in the gastrointestinal tract. The microbiota of the colon is known to biotransform rutin, generating quercetin aglycones that can be absorbed. We investigated the role of the ileal and colonic microbiota in rutin biotransformation using established in vitro fermentation models. Overall, a higher rate of rutin biotransformation was observed during colonic fermentation compared with ileal fermentation. The colonic microbiome showed higher potential for rutin conversion to quercetin through an increased abundance of $\alpha$-rhamnosidase- and $\beta$-glucosidase-encoding genes compared to the ileal microbiome. Nonetheless, rutin metabolism occurred rapidly during ileal fermentation (~20% rutin disappearance after 1 h). The appearance of quercetin varied depending on the ileal inoculum and correlated with an increased abundance of Firmicutes, suggesting that quercetin absorption could be improved via modulation of the ileal microbiota.

**KEYWORDS:** *ileal fermentation, colon fermentation, ileal microbiome, fecal microbiome, polyphenol biotransformation*



## 1. INTRODUCTION

Polyphenols have gained much scientific attention due to their potential health benefits, including antioxidative and anti-inflammatory properties.[1] One of the most abundant polyphenols in the diet is the flavonoid quercetin. Quercetin is present in most plant products, including fruits, vegetables, herbs, cereals, legumes, tea, and coffee, and accounts for an estimated 75% of flavonoid intake in humans.[2] Moreover, quercetin is considered highly beneficial due to its suggested protective effects against cancer, diabetes, obesity, asthma, and cardiovascular and neurodegenerative diseases.[3] However, the benefits of polyphenols are determined by their bioavailability, which is partially dependent on their absorption in the gut.[4]

Quercetin can occur either in glycosylated form (i.e., linked with a sugar moiety) or as an aglycone without bound sugars.[2] In foods, quercetin primarily occurs as glycosides. The type of sugar moiety in quercetin glycosides is a major determinant in their small intestinal absorption.[5] Quercetin in its aglycone form and quercetin glucosides (glycosides derived from glucose) are partially absorbed in the small intestine. However, the absorption of rutin, a common quercetin glycoside formed with the disaccharide rutinose (a dimer of rhamnose and glucose), is thought to occur mainly in the colon.[6] Upon entering the colon, rutin is hydrolyzed by the colonic microbiota, following which the quercetin aglycone can be absorbed.[7] However, as the colon has a smaller surface area and a reduced number of transporters compared to the small intestine, quercetin absorption in the colon is considered less efficient than in the small intestine.[4] In line with this, the bioavailability of rutin is only 20% of that of quercetin glucosides that are absorbed in the small intestine.[5]

In addition to polyphenols, plant-based foods contain dietary fiber that confers beneficial health effects via interactions with the gut microbiota. These dietary fibers, while serving as a substrate for fermentation by gut microbes, also play an important role in the modulation of the microbiota composition.[8] Although polyphenols and dietary fiber are typically consumed together, there is limited research investigating the interactions between these two substrate groups and the microbiota. A few in vivo and ex vivo studies have established that dietary fiber can affect the biotransformation of polyphenols by the colonic microbiota.[9] In line with this, specific fibers are thought to enhance the bioavailability of quercetin from rutin by altering the gut microbiome. In a study in which mice were fed a diet supplemented with either pectin and rutin or cellulose and rutin, plasma quercetin levels were found to be higher in the pectin-rutin diet group. The fecal microbiota of the mice differed between the two diet groups, leading the authors to speculate that the microbiota in the pectin-rutin diet group was more efficient at metabolizing rutin, thus allowing for greater absorption of quercetin.[10]

It should be noted that while the aforementioned studies utilized fecal samples, which are considered to be representative of the colonic microbiota,[11] emerging evidence suggests that the ileal microbiota may also have an important role in the fermentation of dietary substrates.[12] Thus, we hypothesized

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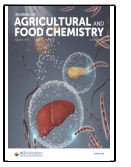





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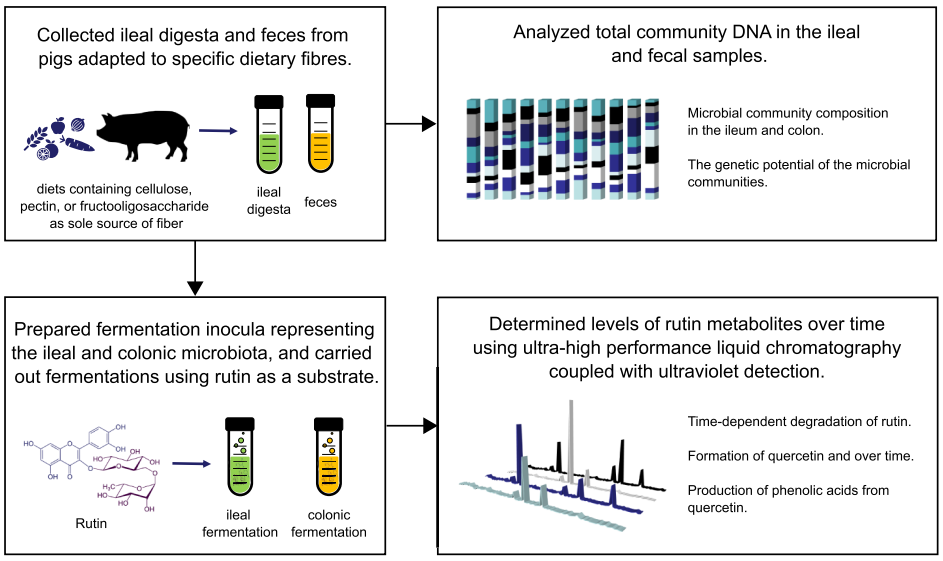


**Figure 1.** Overview of the study.

that both the ileal and colonic microbiota play roles in the biotransformation of rutin. As the small intestine is better adapted than the colon for the absorption of quercetin aglycones, a better understanding of the role of the ileal microbiota in the biotransformation of rutin has implications for the modulation of quercetin bioavailability. Here, we aimed to investigate the effects of both ileal and colonic microbiota on rutin biotransformation by using mixed-culture fermentation models. The models utilized ileal digesta and fecal samples obtained from ileal cannulated pigs, which were adapted to specific dietary fiber diets. We also undertook a shotgun metagenomic analysis of the ileal digesta and fecal samples to gain insights into specific microbial communities or functional groups that may play an important role in modulating quercetin bioavailability.

## 2. MATERIALS AND METHODS

An overview of the study is presented in Figure 1. Porcine ileal digesta and fecal samples were used to prepare inocula for in vitro ileal and colonic fermentations, respectively, using rutin as a substrate to assess its biotransformation. In addition, the microbial composition and function of the ileal and fecal samples were assessed.

**2.1. Chemicals.** All chemicals were purchased from Sigma-Aldrich (Auckland, New Zealand). Rutin (quercetin-3-rhamnoglucoside monohydrate) was used for in vitro fermentation experiments. Standards used for ultrahigh-performance liquid chromatography coupled with ultraviolet detection (UHPLC-UV) determinations were rutin trihydrate (quercetin-3-rhamnoglucoside trihydrate), quercetin dihydrate, 3′,4′-dihydroxyphenylacetic acid (3,4DHPA), 3′-hydroxyphenylacetic acid (3HPA), and 3-(3′-hydroxyphenyl)propanoic acid (3HPPA).

**2.2. Collection of Ileal Digesta and Fecal Samples.** A feeding study in ileal cannulated pigs was undertaken, as described in the Supporting Information Methods. Ileal digesta and feces were collected to prepare anaerobic inocula representing the microbiota of the ileum and colon, respectively. Samples were collected on day 10 during an 11 day diet cycle where pigs were fed one of five experimental diets containing cellulose (6%), pectin (3 and 6%), or fructooligosaccharide (FOS; 3 and 6%) as sole sources of fiber ($n = 6$ per diet group). Feces were collected directly from the anus into plastic bags flushed with $CO_2$ following anal stimulation. In the pectin (6%) diet group, feces could only be collected from five of the six animals. The samples were immediately placed on dry ice to minimize degradation and stored at −80 °C until use. Ileal digesta samples (~30 mL) were collected in $CO_2$-filled bags attached to the cannula of each pig over 1 h, starting 4−5 h after the final meal of the day. The bags were subsequently frozen at −80 °C until use.

**2.3. Preparation of Substrate for Fermentation.** The substrates used for the in vitro fermentations were (a) rutin solubilized in methanol and (b) methanol only (i.e., blank). The rutin was solubilized in methanol at a concentration of 26.5 mg/mL. Based on the average amount of ileal digesta collected per pig, it was calculated that adding 100 μL of the solubilized rutin to 5 mL of the ileal inoculum was equivalent to consuming 500 mg of rutin, the recommended daily dosage of rutin for humans.[13] Aliquots of prepared substrate were placed in sealed autoclaved serum bottles, and the headspace was gassed with $N_2$ for ~10 min to make the solutions anaerobic.

**2.4. In Vitro Fermentation Assays.** The amounts of ileal digesta and feces obtained from some animals were insufficient for in vitro fermentation. Thus, to obtain a representative ileal or colonic microbiota population for a given dietary treatment, the ileal digesta or feces from all animals in each diet group were pooled to provide a composite sample. We then used established methods of in vitro fermentation representing the ileum[14] and colon.[15] Pooled ileal digesta were added to 0.1 M anaerobic phosphate buffer (0.5 g/L L-cysteine, pH 7) at a concentration of 220 g/L to prepare an ileal inoculum. Due to the lack of sufficient feces, colonic inocula were prepared at a reduced concentration of 160 g/L. The samples were homogenized using a hand-held blender prior to being filtered through four layers of sterile surgical gauze to remove particulate matter under a constant flow of $CO_2$ to maintain anaerobic conditions. Once prepared, the ileal and colonic inocula were used immediately for in vitro fermentation assays.

The fermentation assays were carried out in Hungate culture tubes (16 mm diameter, 100 mm long; BellCo glass, USA), sealed with butyl rubber stoppers and perforated plastic caps. Aliquots (5 mL) of the inoculum were dispensed into $CO_2$-filled Hungate tubes containing 100 μL of the substrate (rutin solubilized in methanol or methanol only as a blank). After the tube was sealed, the inoculum and substrate were mixed by inverting, and 500 μL of the sample was collected (0 h time point) using a needle and syringe. The Hungate tubes were kept upright in the shaking incubator at 38 °C with 50 strokes/min for 2 h (ileal fermentation) or 38 h (colonic fermentation). Aliquots of fermentation samples (500 μL) were collected after 1, 2, 14, 24, and 38 h. All samples were placed on ice immediately after collection to reduce microbial activity and stored at −80 °C until analysis.

**2.5. Quantification of Polyphenols and Phenolic Acids in Fermentation Samples.** The in vitro fermentation samples were


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defrosted on ice, mixed to ensure homogenization, and quantitatively transferred to 2 mL microcentrifuge tubes. Samples were centrifuged at 4 °C for 15 min at 12,700 rpm. Two hundred μL of supernatant was then transferred to a new 2 mL microcentrifuge tube containing 200 μL of acidified methanol (containing ~6.5% $H_3PO_4$). Tubes were then vortexed for 30 s and stored at 4 °C for 30 min to encourage protein precipitation. For the pectin (3 and 6%) diet groups, gel formation was observed after the addition of acidified methanol in the ileal digesta but not in the fecal samples. Samples were then centrifuged at 4 °C for 15 min at 12,700 rpm to pellet any protein, pectin, or fines, and an aliquot of the supernatant was transferred to a 2 mL autosampler vial containing an insert for analysis. However, for the samples with pectin gel, less supernatant was available due to the binding of water in the gel. This led to a smaller volume of extract for analysis. In addition, from each extract, a 25 μL aliquot was reserved for the preparation of two pooled quality control samples: one for ileal digesta extracts and one for fecal extracts.

Single analyte stock standards of rutin and the three phenolic acids (3,4DHPA, 3HPA, and 3HPPA) were prepared in methanol. Mixed analyte working standards containing these four analytes were then prepared in methanol/water (50:50, v/v). Due to the lower solubility of quercetin in methanol and methanol/water (50:50, v/v), single analyte stocks and working standards of quercetin were prepared using 100% ethanol.

Rutin, quercetin, 3,4DHPA, 3HPA, and 3HPPA were determined by UHPLC-UV. The UHPLC system consisted of a Thermo Scientific Dionex HPG-3400SD Pump and a VWD-3400 UV detector equipped with a WPS-3000TSL autosampler (Thermo Fisher Scientific, New Zealand). The column used was a HypersilGOLD C18 (100 mm × 2.1 mm, 3 μm, Thermo Fisher Scientific). Five μL was injected into the HPLC column. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was 400 μL/min, and the elution gradient was as follows: initial 5% B for 2 min; 5−43% B over 9 min; 43−90% B over 1 min; 90% B for 2 min; 90−5% B over 1 min; followed by a column re-equilibration step at 5% B for 5 min. The UV absorbance data were measured at 360 nm for rutin and quercetin and at 285 nm for 3,4DHPA, 3HPA, and 3HPPA.

Where the sample concentration was below the limit of detection (LOD), the concentration was considered to be 0 μg/mL. Where the concentration was below the limit of quantification (LOQ), the concentration was considered to be half of the LOQ. Following adjustment of LOD and LOQ values, rutin disappearance in each fermentation tube at each time point was calculated using the following formula

$$\text{rutin disappearance}_{Ti}\,(\%) = \frac{((\text{rutin}_{T0} - \text{rutin}_{\text{blank }T0}) - (\text{rutin}_{Ti} - \text{rutin}_{\text{blank }Ti}))}{(\text{rutin}_{T0} - \text{rutin}_{\text{blank }T0})} \times 100 \quad (1)$$

where $\text{rutin}_{T0}$ and $\text{rutin}_{Ti}$ are the rutin concentrations at the 0 h and $i$ h time points, and $\text{rutin}_{\text{blank}}$ is the average concentration of the rutin in the blank (methanol only) fermentations at the defined time point. For the other phenolic compounds, the average concentration of the compound in the blank fermentations at a given time point was subtracted from the concentration of the compound in each fermentation tube at that time point.

**2.6. Microbiome Analysis.** Metagenomic DNA was extracted from ileal digesta (~250 μL) and fecal (~100 mg) samples that were thawed on ice using the NucleoSpin Soil DNA extraction kit (Macherey-Nagel, Germany) as per the manufacturer's instructions. DNA was assessed for shearing by gel electrophoresis, and a NanoDrop ND-1000 spectrophotometer (software version 3.8.1; Thermo Fisher Scientific, USA) was used to determine the DNA purity. A 260/280 ratio of 1.8−2.0 was accepted as pure for library construction. Following quantification of DNA using a Qubit dsDNA Assay Kit (Thermo Fisher Scientific) in a Qubit 2.0 Fluorometer (Life Technologies, USA), 1 μg of DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. In brief, following fragmentation of DNA samples by sonication to a size of 300 bp, DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. PCR products were purified (AMPure XP, Beckman Coulter), and libraries were analyzed for size distribution using an Agilent2100 Bioanalyzer and quantified using real-time PCR. The clustering of the index-coded library samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. Following cluster generation, library preparations were sequenced on an Illumina HiSeq X platform to generate 150 nt paired-end reads (Annoroad Gene Technology Co. Ltd., Beijing, China).

Quality control of sequencing data was performed by KneadData[16] using paired-end mode. First, contaminants and human reads were filtered out by aligning to the *Sus scrofa* reference genome and contaminant and mitochondria databases available from KneadData. Only reads with both pairs passing quality control were retained for further analysis. The forward and reverse reads were then concatenated into a single file using Microbiome Helper's concat_paired_end.pl with flags (-n).[17] Reads following quality control and concatenation were classified using DIAMOND[18] blasting against the NCBI nr database (November 23, 2018). Finally, output DAA files were analyzed in MEGAN Ultimate Edition[19] using absolute counts against the taxonomic and KEGG databases.

**2.7. Statistical Analysis.** Linear mixed models were used to analyze rutin disappearance and levels of quercetin and phenolic acids over time using SAS (SAS/STAT version 9.4; SAS Institute Inc.). The full statistical model included the effect of inoculum [cellulose (6%), pectin (3 and 6%), and FOS (3 and 6%)], time (up to quadratic order for ileal fermentation and up to quintic order for colonic fermentation), and their interactions as a fixed effect, and the fermentation tube as a random effect. For each response variable, the most appropriate covariance structure was selected after fitting the models by the restricted maximum likelihood method and comparing them using the log-likelihood ratio test. The final selected polynomial model was selected after comparing higher-order models with reduced-order models using the log-likelihood ratio test. The model diagnostics (e.g., normal distribution and homogeneity of variance) for each selected polynomial model were tested using the ODS graphics procedure of SAS. When the model assumptions were not fulfilled, transformation of the raw data was carried out. A predictor effect was considered significant at $P < 0.05$.

For microbiome analysis, the assignments of reads to KEGG pathways were exported from MEGAN in the CSV format and imported to R (version 3.6.3),[20] where read counts were converted to proportions of reads in level 1. Taxonomic classification and abundance data were exported from MEGAN in BIOM format and converted using the BIOM conversion tool[21] to adjust classifications into the correct format. The phyloseq R package[22] was used to merge OTUs at the genus level and convert counts to relative abundance. KEGG pathways are present at less than 0.001%. Taxa present at less than 1% were removed. Bray−Curtis distances and Adonis significance testing were computed using the vegan package.[23] Community and functional differences were represented on a two-dimensional ordination plot using Principal Coordinates Analysis (PCoA) with the Bray−Curtis distance between samples in the phyloseq and ggplot2 R packages. Boxplots depicting differences in KEGG orthologs between sample types were drawn using the microbiome R package,[24] and means were compared using the Wilcoxon rank-sum test in the ggpubr package.[25]

Correlations between the ileal or colonic microbial communities and rutin biotransformation were analyzed. This analysis was based on the assumption that the microbial taxa in the fermentation inoculum were equivalent to the average of the microbial taxa in the ileal digesta or fecal samples used to prepare the fermentation inoculum. The average relative abundance of taxa per diet was obtained in phyloseq by merging OTUs at their phylum and genus levels, summing the


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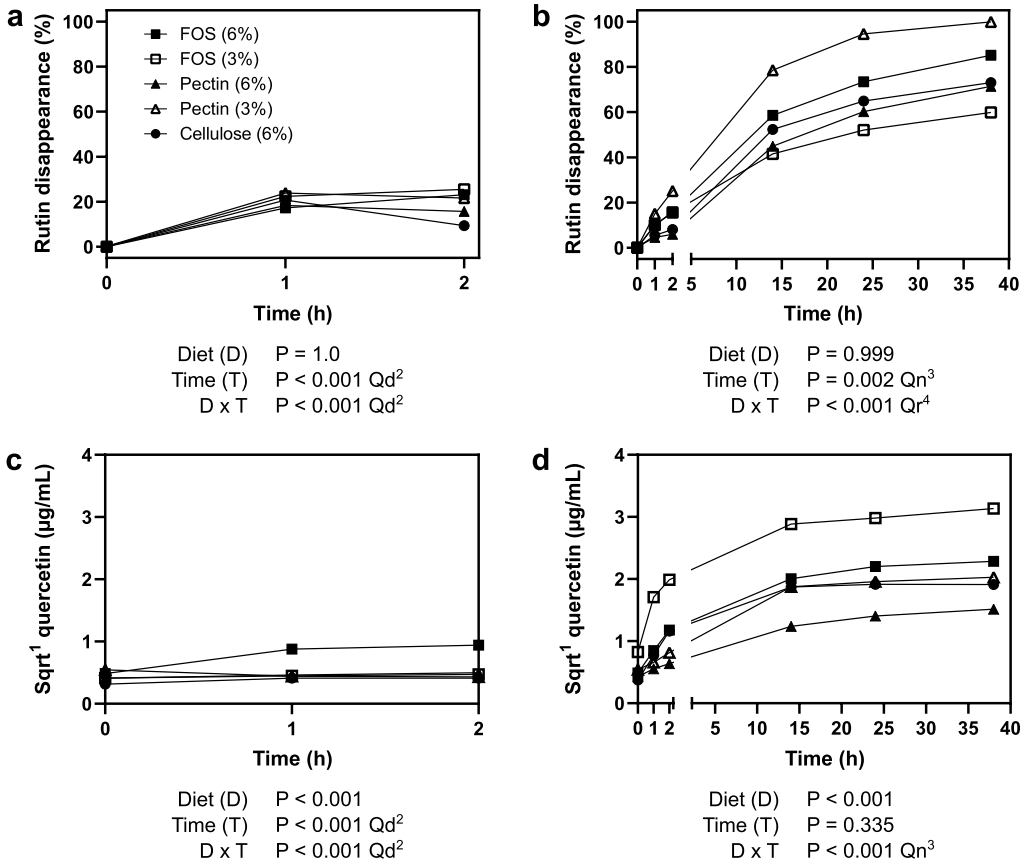


**Figure 2.** Rutin biotransformation in ileal and colonic fermentation in vitro. Graphs show the mean ± standard error (error bars are smaller than the symbol size) of rutin disappearance over time during in vitro ileal (a) and colonic (b) fermentations. The graphs also show the mean ± standard error (error bars are smaller than the symbol size) concentration of quercetin present at each time point during in vitro ileal (c) and colonic (d) fermentations. The fermentation inocula were prepared using ileal digesta (for ileal fermentations) and feces (for colon fermentations) from pigs fed diets containing cellulose (6%), pectin (3 or 6%), or fructooligosaccharides (FOS; 3 or 6%) as the sole fiber source ($n$ = 5 fermentations per diet, except for pectin (3%) colon fermentations, $n$ = 3).[1] The raw data were square-root transformed (Sqrt) to fulfill the model assumptions.[2] quadratic.[3] quintic.[4] quartic.

abundances for a given diet group, and transforming counts to relative abundance. Following filtering of low-abundance taxa (<1% in a given diet group), microbiota and phenolic associations based on Spearman's correlation were performed using the corrplot R package.[26]

## 3. RESULTS AND DISCUSSION

We investigated the conversion of rutin to quercetin and how this differed between the ileum and colon using established in vitro fermentation models. To enable the concurrent collection of ileal digesta (representative of the microbiota of the distal small intestine) and feces (considered to be representative of the microbiota of the colon), we used ileal cannulated pigs fed diets containing cellulose (6%), pectin (3 or 6%), or FOS (3 or 6%) as the sole fiber source (Table S1 in the Supporting Information). Numerous studies have used pigs as a model for the human intestinal tract, as pigs have similar digestive physiology, organ structure, and associated metabolic functions.[27] Moreover, the gut microbiome of pigs shows similarities to that of humans.[28] As the pigs had been fed controlled diets, this enabled us to investigate whether diet-induced changes in the gut microbiota influence rutin biotransformation in the ileum or colon.

**3.1. Phenolic Compound Determination Using UHPLC-UV.** A UHPLC-UV method was used to quantify the levels of rutin, quercetin, 3,4DHPA, 3HPA, and 3HPPA in in vitro fermentation samples. The LOD for both rutin and quercetin was 0.04 μg/mL, while the LOQ was 0.1 μg/mL. For 3HPA, 3HPPA, and 3,4DHPA, the LODs were 0.3, 0.5, and 0.13 μg/mL, respectively, and the LOQs were 0.9, 1.4, and 0.4 μg/mL, respectively.

Prior to analyzing the fermentation samples, we carried out spiking experiments to determine whether the phenolic compounds could be detected in the various fermentation inocula (Figure S1). The average recoveries of rutin, 3,4DHPA, 3HPA, and 3HPPA were 99−104% (RSD values ranged from 2.7 to 10%); however, quercetin detection was low (40%). This was likely due to quercetin being bound to proteins precipitated in the pellet and thus not being injected into the HPLC column. The protein binding of polyphenols has been studied extensively and is reduced by acidic conditions of ∼pH 3.[29] Thus, we increased the acidity of the system by means of acidified methanol (containing ∼6.5% $H_3PO_4$), as described in Section 2.5. This approach was successful in releasing the quercetin, as evidenced by the increased spike recovery (83%). However, for the pectin (3 and 6%) diet groups, gel formation was observed after the addition of acidified methanol to the ileal digesta. This phenomenon was not observed in the fecal samples. The difference was likely due to the dietary pectin not being digested in the small intestine compared to the colon.[30] Acidic


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conditions would promote the gelling of pectin in the ileal digesta sample due to the suppression of ionization of carboxylic acid functionalities present in the sugar chains of the pectin, reducing repulsion forces and promoting interlinking of the chains.[31] As gel formation caused the water in the extract to be bound, we observed a reduction in the available extract for analysis following sample centrifugation. Despite this, we were able to extract a sufficient sample to conduct the analyses, as described in Section 2.5.

**3.2. Biotransformation of Rutin to Quercetin in Ileal and Colonic Fermentation Models.** Rutin disappearance in the fermentation models indicated the amount of rutin metabolized by the microbiota. Rutin disappearance during in vitro ileal fermentation was initially rapid, reaching ∼20% disappearance after 1 h of in vitro ileal fermentation before plateauing (Figure 2a). On the contrary, in the in vitro colonic fermentation, rutin disappearance, though slightly slower compared to the ileum in the first hour (∼5−15%), continued to increase ($P < 0.01$) over the 38 h fermentation period (interaction effect significant, $P < 0.001$) (Figure 2b). In the colonic fermentations, differences were observed between diet groups over time ($P < 0.001$), with rutin disappearance reaching close to 100% for the pectin (3%) group, while only reaching 60% for the FOS (3%) group. The large differences between the diet groups in the colonic fermentation suggest that microbial variation in the colonic inocula has a bigger impact on rutin metabolism than the comparable variation in the ileal inocula. It is important to note here that the concentration of feces utilized for the colonic fermentations (160 g/L) was lower than the recommended concentration of 320 g/L[15] and thus may influence the rate of rutin biotransformation. However, as the amount of feces used was the same for all colonic fermentations, we expect that the differences between the fermentation reactions would be similar to those observed if the recommended amount of feces was used. Overall, the results indicate that biotransformation of rutin occurs to a greater extent in the ileum compared to the colon during the first hour of fermentation. However, when considering the retention time of the contents in vivo, the biotransformation of rutin that occurs in the colon is greater than that of the ileum.

To determine how much of the metabolized rutin was converted to quercetin, we also measured the levels of quercetin over time in the fermentation models. The quercetin levels detected in the ileal fermentation model were negligible, except for the FOS (6%) diet group, where quercetin levels continuously increased over the fermentation period, reaching 0.9 μg/mL (3.0 nmol/mL) (back-transformed value) after 2 h (Figure 2c). Quercetin levels in the colonic fermentation model were considerably higher, reaching 9.8 μg/mL (32.4 nmol/mL) (back-transformed value) after 38 h of fermentation in the FOS (3%) diet group (Figure 2d). Quercetin levels increased, on average, between 0.2 μg/mL (0.7 nmol/mL; pectin (6%) group) and 2.0 μg/mL (6.6 nmol/mL; FOS (3%) group) per hour in the first 2 h. Between 2 and 14 h, the average rate of quercetin increase was between 0.1 μg/mL (0.3 nmol/mL; pectin (6%) group) and 0.4 μg/mL (1.3 nmol/mL; FOS (3%) group), following which the level of quercetin tended to plateau. The level of quercetin at 38 h varied between diet groups, with the pectin (6%) group attaining a maximum level of 2.3 μg/mL (7.6 nmol/mL) (back-transformed value).

The diet groups with the fastest rate or highest level of rutin disappearance did not have the fastest rate of quercetin increase or achieve the highest maximum quercetin levels. As such, rapid rutin disappearance does not necessarily equate with the increased availability of quercetin. The disappeared rutin may have been converted to the intermediary metabolite isoquercitrin[32] and/or subsequently metabolized into phenolic acids. For example, human studies have indicated that hydroxyphenylacetic acids originate in the colon, where quercetin undergoes microbiota-mediated ring fission (between C2-ring oxygen and C3−C4), producing 3,4DHPA, which is dehydroxylated by the microbiota to produce 3HPA.[6] An alternative mode of ring fission by the colonic microbiota (between C2-ring oxygen and C4-ring A) results in 3HPPA, another major end product of rutin.[33]

**3.3. Microbiota-Derived Quercetin Metabolites.** Quercetin metabolites may have bioactive properties that differ from those of quercetin. For example, 3,4DHPA possesses significant reducing power and free radical scavenging activity and is thought to be responsible for the antioxidant capacity of quercetin-rich foods,[34] and 3HPPA possesses vasodilatory activity (associated with lower cardiovascular mortality) that is more potent than quercetin.[35] Hence, we quantified the levels of selected metabolites (3,4DHPA, 3HPA, and 3HPPA) in the in vitro ileal and colonic fermentation samples.

The compound 3HPPA was detected in the ileal fermentations but only in the pectin (6%) diet group. Here, the mean (±standard error) level of 3HPPA increased by 1.8 ± 0.02 μg/mL (6.0 nmol/mL) per hour. The presence of 3HPPA in only one diet group suggests that the metabolism of quercetin is dependent on the specific microbiota composition. Low levels of 3,4DHPA were detected only in the pectin (6%) group after 2 h of fermentation [0.17 ± 0.10 μg/mL (0.56 nmol/mL)] as well as the cellulose (6%) group after 1 h of fermentation [0.25 ± 0.08 μg/mL (0.83 nmol/mL)]. These results contrast with a human study where the appearance of 3,4DHPA following consumption of rutin was observed in the urine of individuals with an intact colon but not individuals with ileostomies.[6] This finding implied that 3,4DHPA cannot be formed in the small intestine. On the other hand, our data indicate that the production of beneficial phenolic acids from rutin can occur in the small intestine, but this process is likely dependent on the specific microbial composition of the ileum.

We did not detect 3HPA or 3HPPA in any of the colonic fermentation samples. The compound 3,4DHPA was detected (average values per diet group ranged between 0.2 and 1.3 μg/mL at 0 h to 9.3−26.9 μg/mL at 38 h) in the colonic fermentations containing the rutin substrate; however, it was also detected in the blank fermentations (average values per diet group ranged from 0.2 to 1.3 μg/mL at 0 h to 10.8 and 30.7 μg/mL at 38 h). The detection of 3,4DHPA at increasing levels over time in the blank fermentations indicates that 3,4DHPA was likely not produced as a direct result of rutin fermentation; rather, it was produced by other compounds natively present in the fecal inoculum. The absence of 3HPA, 3HPPA, or 3,4DHPA produced as a result of rutin fermentation was unexpected, as these phenolic acids have been detected in previous studies where in vitro fermentation models of the colonic microbiota (human fecal inocula) were utilized to investigate the metabolism of rutin.[33,34,36] It is possible that the rutin substrate in our colonic fermentation was rapidly converted to other metabolites that were not targeted in our analysis. However, it should also be noted that


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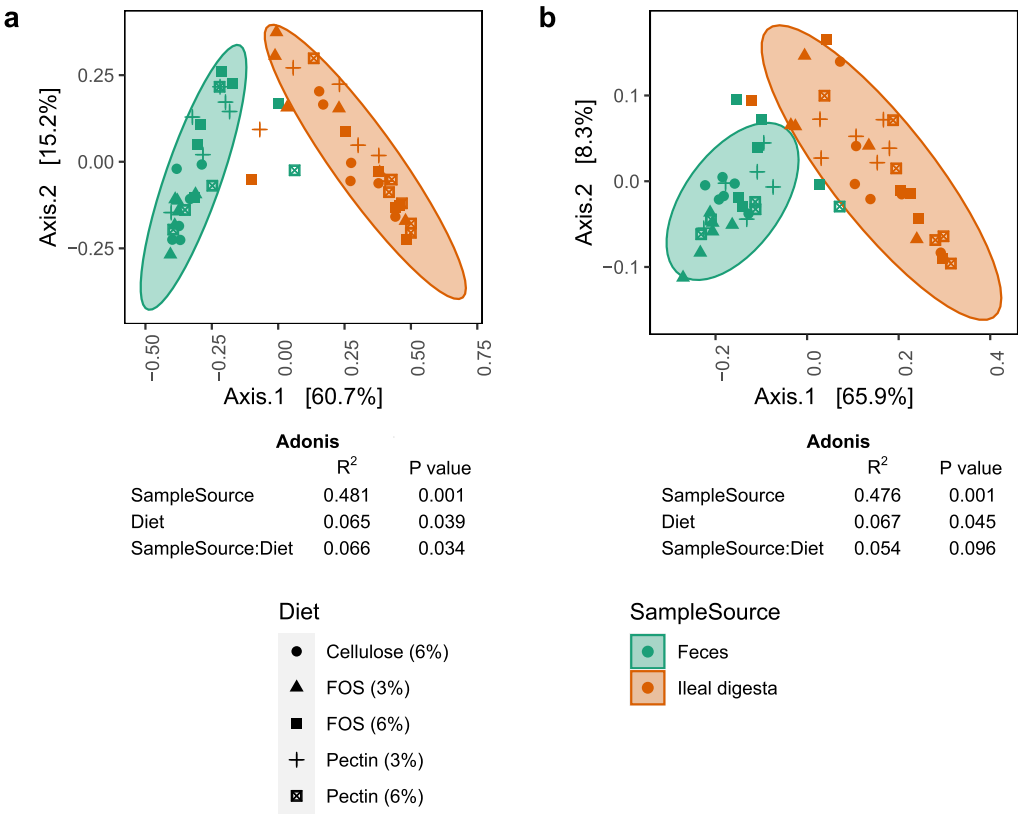


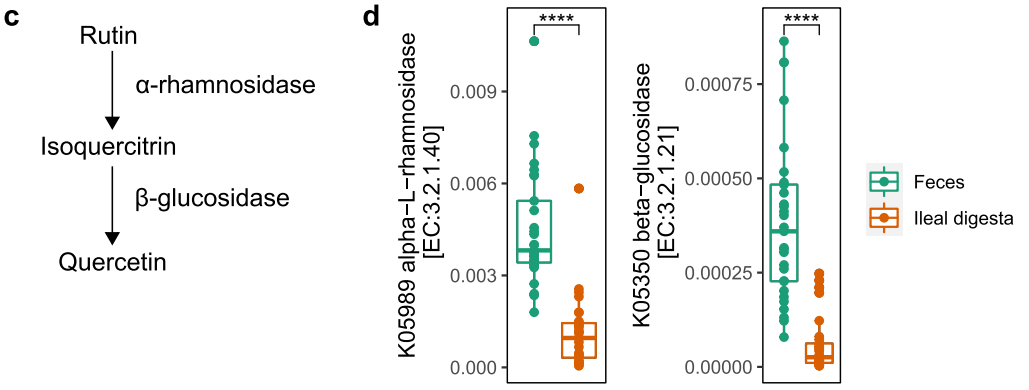


**Figure 3.** Differences between fecal and ileal microbiomes with regards to rutin biotransformation. Principal coordinates analysis (PCoA) plot and Adonis variance analysis (999 permutations) of (a) microbial genera and (b) KEGG (L4) pathway profiles of porcine fecal and ileal digesta samples based on Bray−Curtis distance. Pigs were fed diets containing cellulose (6%), pectin (3 or 6%), or fructooligosaccharides (FOS, 3 or 6%) as the sole fiber source. (c) Hydrolytic pathway from rutin to quercetin via isoquercitrin by two enzymes. (d) Relative abundance of KEGG orthology associated with the conversion of rutin to quercetin. ****$P < 0.0001$.

the detection method can impact the level of phenolic acids detected, contributing to the observed disparity from those of previous studies. For example, in a study by Aura et al.,[36] HPLC−MS analysis showed different ratios for 3,4DHPA and 3HPA compared with the ratios obtained by HPLC analysis from the same sample.

**3.4. Effect of Diet and the Microbiome on Rutin Biotransformation.** We examined the composition and functional potential of the microbiomes within each of the individual ileal digesta and fecal samples by extracting metagenomic DNA and analyzing these DNA samples by shotgun sequencing. PCoA and Adonis variance analysis of the microbial genera (Figure 3a) and KEGG pathways (Figure 3b) showed that the samples clustered by sample source and that the sample source explained ∼48% of the variation (Adonis; $P < 0.01$). This indicates, as expected, that the composition and functional potential of the fecal microbiome are distinct from those of the ileal microbiome. The sample source × diet interaction, however, explained only 6.6% of the variation in microbial genera (Adonis; $P < 0.05$) and 5.4% of the variation in KEGG pathways (Adonis; $P < 0.1$), indicating that diet was not likely associated with specific microbial genera or functions in the ileal or fecal microbiome.

To understand how differences between the microbiomes of the small intestine and colon translated to differences in rutin biotransformation, we focused on the KEGG orthology


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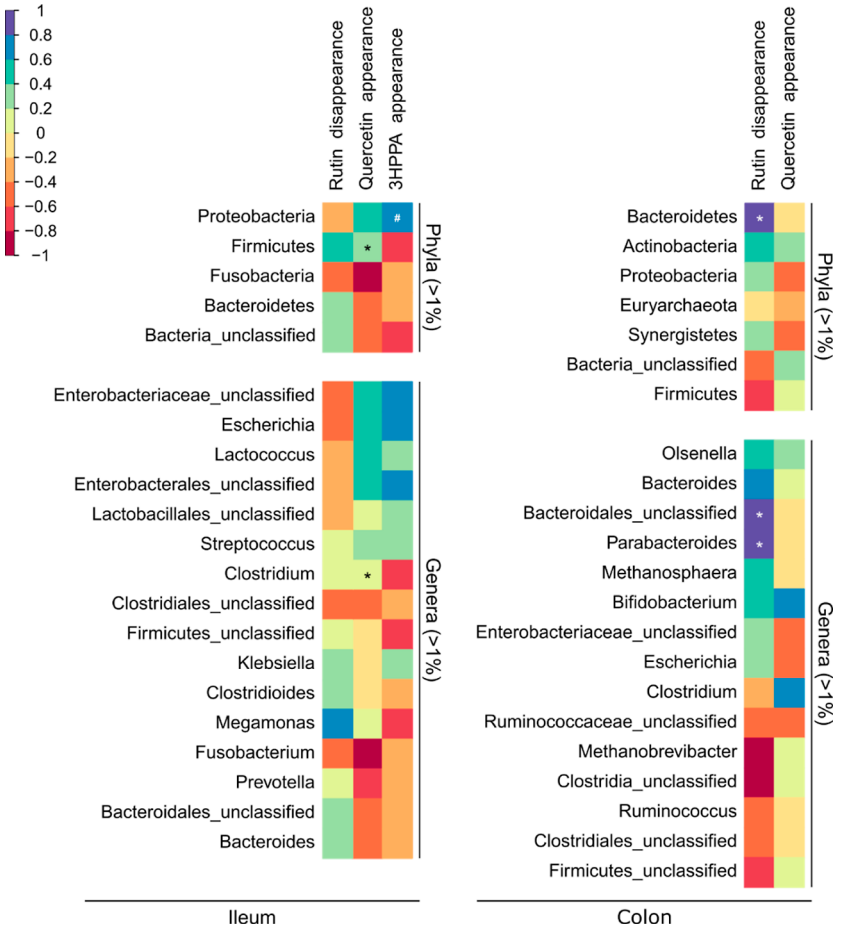


**Figure 4.** Correlation matrix heatmaps of microbiota and rutin biotransformation. The color represents Spearman's correlation coefficient between the mean relative abundance of microbial taxa in fecal/ileal samples of pigs, and the average level of phenolic compounds in the in vitro fermentations for each diet. Rutin disappearance refers to the total rutin disappearance over the in vitro fermentation duration. Quercetin and 3HPPA appearances refer to the concentrations of quercetin and 3HPPA at the final in vitro fermentation time point. The statistical significance of the correlation is denoted ($^{\#}P < 0.1$ and $^{*}P < 0.05$).

associated with the conversion of rutin to quercetin. As depicted in Figure 3c, rutin can be derhamnosylated by α-rhamnosidase to produce isoquercitrin, which can then be deglucosylated by β-glucosidase to produce quercetin.[32] The proportion of reads mapping to α-rhamnosidase and β-glucosidase (KEGG orthology K05989 and K05350, respectively) was significantly higher in the fecal samples compared to the ileal digesta samples (Figure 3d). Thus, the increased rutin disappearance and quercetin appearance in the in vitro colonic fermentation compared to those in the ileal fermentation may have been due to the increased abundance of bacteria possessing these enzymes in the colon compared to the ileum.

We then examined correlations between the microbiota (phyla and genera) of the ileal or fecal samples and the disappearance of rutin and the appearance of quercetin and 3HPPA during in vitro fermentation (Figure 4). In the ileum, the Firmicutes phylum (specifically, the *Clostridium* genus) positively correlated with the appearance of quercetin. Interestingly, a recent study by Riva et al.[37] showed that the related Lachnospiraceae family, belonging to the clostridial cluster XIVa of the Firmicutes phylum,[38] was associated with quercetin production. Ileal Proteobacteria tended to be positively correlated to 3HPPA appearance. In the colon, rutin disappearance showed a strong positive correlation with Bacteroidetes, specifically *Parabacteroides*, and other unclassified genera from the Bacteroidales order. This is in keeping with the data of Bokkenheuser et al.,[39] which showed that *Parabacteroides distasonis* produce both isoquercitrin and quercetin via α-rhamnosidase and β-glucosidase activity.

Overall, our data indicate that the microbial species involved in the biotransformation of rutin in the ileum are likely different from those responsible for the rutin biotransformation in the colon. Studies that have previously identified bacterial species involved in the biotransformation of rutin have almost exclusively focused on fecal isolates.[39,40] However, as the microbiome of the ileum is distinct from that of the colon, it is plausible that the rutin biotransformation in the ileum, albeit limited, is carried out by species that have not previously been recognized as important in rutin biotransformation. Whether species within the Firmicutes phylum are involved in the conversion of rutin to quercetin, as suggested by our correlation analysis, is yet to be determined.

**3.5. Limitations of the Study.** In this study, we sought to investigate the conversion of rutin to quercetin and how this differed between the ileum and colon using in vitro fermentation models. Although these models have been validated and optimized,[14,15] they have limitations. While pigs are widely used as a model of the human intestinal tract,[27,28] there may be sufficient differences across species that


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influence the biotransformation of polyphenols. However, this is an inherent limitation in the use of any model. The detection of phenolic acids was also limited by the method of detection used, and thus, the sensitivity of the UV detector used was the main limitation of the study. As discussed above, the amount of metabolite detected can depend on the method of detection, which may explain the lack of 3HPA or 3HPPA detected in the colonic fermentation samples.[33] This limitation can be overcome in future studies using a mass spectrometry detector for improved sensitivity and specificity.

As is the approach taken with most studies of the gut microbiome, we utilized fecal samples as representatives of the colonic microbiota. The limitation here is that the fecal microbiota is thought to only partially replicate the community of the colon.[11] However, fecal samples can be collected noninvasively, which, in the case of this study, allowed us to concurrently investigate both the ileal and colonic microbiota. Combining samples from donors of the same diet group to obtain a representative inoculum for each diet was another major limitation of this study as it masked any interindividual variation in the rutin disappearance and quercetin appearance. Unfortunately, preparing an ileal and colonic inoculum from each donor was not possible in this instance due to the lack of sufficient ileal digests and feces.

It should also be noted that the models utilized a fixed duration for in vitro fermentation, regardless of the diet group. This is a potential limitation, as in vivo, the duration of fermentation could vary between diet groups depending on intestinal transit time.[41] The addition of pure rutin substrate to the colonic fermentations also assumes that the rutin is not biotransformed in the proximal intestine. However, our results showed that ∼20% of the rutin was metabolized by the microbiota in the ileum and thus would not enter the colon.

As we did not have the microbial community composition data for the fermentation inocula, we based our correlation analysis on the assumption that the microbial taxa in the fermentation inoculum were equivalent to the average of the microbial taxa in the ileal digesta or fecal samples used to prepare the fermentation inoculum. The accuracy of this assumption is debatable, as the microbiota composition of the fermentation inocula can shift during the in vitro fermentation, likely determined by the specific substrates present in the inocula.[42] Nonetheless, metagenomic analysis of the individual fermentation inocula would have revealed the relative abundance of bacterial taxa (including that of any nonviable bacterial cells) present prior to the in vitro fermentation reaction and would not have indicated how the microbiota composition evolves during fermentation. Similarly, our approach of calculating the average microbial taxa provides an estimate of the microbial community in the initial fermentation inocula.

In summary, the physiological effects of polyphenols are, to some extent, dependent on their bioavailability, which in turn is partially dependent on their absorption in the gut. Quercetin glycosides, such as rutin, need to be biotransformed to the aglycone form before being absorbed in the intestine. Here, we investigated the biotransformation of rutin to quercetin and phenolic acids in vitro using fermentation models of the ileum and colon using ileal digesta and feces, respectively. The capacity of rutin to be metabolized was substantially greater during colonic fermentation compared to ileal fermentation, likely due to the increased abundance of $\alpha$-rhamnosidase and $\beta$-glucosidase enzymes encoded by the colonic microbiome. However, contrary to previous reports that rutin metabolism is carried out exclusively by the microbiota of the colon, in our experiments, ∼20% of the rutin present at the start of the fermentation was metabolized following 1 h of ileal fermentation, indicating that the microbiota of the ileum also plays an important role. Moreover, the level of rutin metabolites (e.g., quercetin and 3HPPA) detected during ileal fermentation was dependent on the microbial composition of the ileal inoculum used. This finding suggests that rutin bioavailability in the small intestine could be improved by modifying the small intestinal microbiome, for example, via diet. The rate of rutin biotransformation in the colonic fermentation model was also influenced by the microbial inocula, with total rutin disappearance ranging from 60 to 100% depending on the inoculum used. Correlation analysis suggested that microbial species important in rutin biotransformation in the colon (e.g., *Parabacteroides*) were different from those species involved in rutin biotransformation in the small intestine (e.g., Firmicutes). Thus, although rutin biotransformation occurs primarily in the colon, the role of microbiota in the ileum should not be overlooked, and further investigation of the small intestinal microbial taxa involved in polyphenol metabolism is warranted.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c00980.

Description of the porcine feeding study method, composition of experimental diets, and representative chromatograms (PDF)

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### Notes

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