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COMPOSITION OF RABBIT CAECAL MICROBIOTA AND THE EFFECTS OF DIETARY QUERCETIN SUPPLEMENTATION AND SEX THEREUPON

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Abstract: The purpose of this study was to add to the current understanding of rabbit caecal microbiota. This involved describing its microbial composition and linking this to live performance parameters, as well as determining the effects of dietary quercetin (Qrc) supplementation (2 g/kg feed) and sex on the microbial population. The weight gain and feed conversion ratio of twelve New Zealand White rabbits was measured from 5 to 12 wk old, blood was sampled at 11 wk old for the determination of serum hormone levels, and the rabbits were slaughtered and caecal samples collected at 13 wk old. Ion 16S™ metagenome sequencing was used to determine the microbiota profile. The dominance of *Firmicutes* (72.01±1.14% of mapped reads), *Lachnospiraceae* (23.94±1.01%) and *Ruminococcaceae* (19.71±1.07%) concurred with previous reports, but variation both between studies and individual rabbits was apparent beyond this. Significant correlations between microbial families and live performance parameters were found, suggesting that further research into the mechanisms of these associations could be useful. Negative correlations with the caecal flavonoid content were found, but the latter was not affected by diet, and the effects of quercetin supplementation on the microbiota were very limited, possibly due to the absorption of the quercetin-aglycone from the gastrointestinal tract prior to the caecum. Nonetheless, *Clostridiales Family XIII. Incertae Sedis* was more abundant in the quercetin-supplemented rabbits (Control: 0.003±0.003%; Qrc: 0.020±0.000; *P*=0.005), as was the genus *Anaerofustis* (Control: 0.000±0.002; Qrc: 0.010±0.002; *P*=0.003). Serum cortisol levels were higher in females, and several microbial families differed between the sexes. Most were more abundant in female rabbits, including the most abundant, the family *Eubacteriaceae* (Male: 2.93±0.40; Female: 4.73±0.40; *P*=0.01).

Key Words: flavonoid, growth, gut, microbiota, *Oryctolagus cuniculus*, rabbit.

INTRODUCTION

Rabbits rely on their commensal gut microbiota for assistance in the digestion of fibrous plant material and thus the supply of important nutrients, with the majority of microbial fermentation taking place in the caecum (Combes *et al.,* 2013). Caecal short-chain fatty acid production is suggested to account for 30-50% of maintenance energy requirements, while bacterial proteins, which are consumed during caecotrophy (the consumption of specifically produced soft faeces), can contribute 15% or more of the total ingested nitrogen in adults (Combes *et al.,* 2013). The caecal bacterial population is also an essential source of vitamins, particularly biotin, thiamine, cobalamin, riboflavin, folic acid and niacin (McBee, 1971).

The complexity and stability of the gut microbial ecosystem is also important for the prevention of disease, as it serves as a protective barrier and plays a role in development of the immune system and intestinal mucosa (Combes *et al.,*

2013). This aspect of host-bacteria interaction is of particular interest in rabbits, for which post-weaning mortalities due to digestive disturbances are very high (Michelland *et al.,* 2010).

It is consequently important that the composition of the caecal microbiota is understood, and that the effects of any dietary manipulation are elucidated, particularly in the case of substances with antimicrobial properties, such as flavonoids (Cushnie and Lamb, 2005; Lee *et al.,* 2006). Flavonoids have been reported to both alter the gut microbial profile (Duda-Chodak, 2012; Espley *et al.,* 2014; Oteiza *et al.,* 2018), and be affected by it, as the gut microbiota can metabolise flavonoids and thereby alter their absorption and biological effects (Lin *et al.,* 2003; Oteiza *et al.,* 2018).

The purpose of this study was to describe the composition of the rabbit caecal microbiota and investigate the link between this and live performance, as well as to investigate the possible effects of dietary quercetin supplementation and sex on this microbial population.

MATERIALS AND METHODS

Ethical clearance for this study was obtained from the Stellenbosch University Animal Care and Use Committee (protocol number SU-ACUD16-00094).

Rearing and sampling

Twelve (6 male, 6 female) New Zealand White rabbits reared at the Mariendahl Experimental Farm outside Stellenbosch in the Western Cape of South Africa were used for the study. They were weaned at 5 wk old, and assigned to treatment groups according to sex and weaning weight, so that there were 3 male and 3 female rabbits per dietary treatment group, and the average weights of the rabbits in each treatment group were comparable. The treatment diets were provided *ad libitum* from weaning, and consisted of two complete, pelleted feeds, one control (Ctrl) and one supplemented with 2 g quercetin dihydrate/kg feed (Qrc). The control diet contained predominantly alfalfa meal (36.2%), wheat bran (35.6%) and sunflower meal (12.6%), with 88.3% dry matter (DM), 19.0% crude fibre, 18.5% crude protein, 8.3% ash and 3.7% ether extract (see North *et al.*, 2018 for full composition). The quercetin dihydrate was extracted from *Sophorae japonica* flowers (Chengdu Okay Plant and Chemical Co., Ltd, Chengdu, China), and was added to the feed during mixing and prior to pelleting.

The rabbits were housed in individual cages in two rooms with 12L:12D light regimes during the growth period, with the males and females and dietary treatments being distributed between the two rooms. Each rabbit was assigned an identifying code based on the dietary treatment (Q or C), sex (M or F) and cage number, resulting in the following 12 codes: CM9, CM35 and CM 83 (control males), CF14, CF58 and CF88 (control females), QM11, QM37 and QM63 (quercetin males), and QF16, QF60 and QF86 (quercetin females). During the growth period, from 5 to 12 wk of age, the live weight (LW) of the individual rabbits was determined weekly, as was the daily feed intake, so that the feed conversion ratio (FCR) could be calculated. At 11 wk old, blood samples were collected from the rabbits via the central ear artery, and the free thyroxine (fT4), free triiodothyronine (fT3), somatotropin (GH) and cortisol contents of the serum were determined, as described by North *et al.* (2018).

The temperature and humidity of the grower rooms was measured during the growth period using automatic loggers (LogTag humidity and temperature recorder, Model HAXO-8). The average temperature and humidity in room A (rabbits CM9, CM35 CF14, QM11, QM37 and QF16) was 14.9±0.21°C and 72.3±0.96%, and in room B (rabbits CM 83, CF58, CF88, QM63, QF60 and QF86) was 16.4±0.14°C and 63.4±0.73%. All the rabbits used in this study were healthy throughout the growth period.

At 13 wk of age the rabbits were slaughtered, without prior fasting, at an abattoir on the experimental farm. They were electrically stunned and exsanguinated via the carotid arteries and jugular veins, and the gastrointestinal tracts were removed and collected. Samples of the caecal content for microbial DNA extraction were collected in a sterile manner from approximately 3 cm from the ileocaecal junction. Additional samples were collected for determination of the total flavonoid content, and the pH of the caecal content was measured using a calibrated Crison PH25 portable pH meter with a 50 54 electrode (Crison Instruments S.A., Barcelona, Spain).

Total flavonoid content determination

Caecal content samples were lyophilised (CHRIST, model ALPHA 1-4/LDC-1M) until a constant pressure of 0.037-0.040 mbar was attained (*ca.* 72 h), indicating complete drying. They were ground using a mortar and pestle

prior to flavonoid extraction. Flavonoid extraction was performed according to the method of Vasantha Rupasinghe *et al.* (2008), with modifications as described in full by North *et al.* (2018). The colorimetric assay was performed as described by Herald *et al.* (2012), as also described by North *et al.* (2018), with the exception that 25 μL, rather than 10 μL, of flavonoid extract was used for the assay. The total flavonoid content was quantified using a quercetin (Sigma-Aldrich, Steinheim, Germany) standard (25-500 μg/mL, $R^2 = 0.997$), and is expressed as mg quercetin equivalents/g DM.

16S™ metagenome sequencing

Microbial DNA was extracted from fresh caecal content samples using the PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific) according to the manufacturer's protocol (MAN0014266 Rev. A.0). Briefly, *ca.* 100 mg of each sample was homogenised with 700 μL S1 Lysis buffer in a bead tube. The DNA was bound to the column and washed, and subsequently eluted in 100 μL buffer using two sequential 50 μL elution steps. The total DNA contents of the extracts were quantified with the Qubit 2.0 Fluorometer, using the Qubit dsDNA HS assay kit according to the protocol (MAN0002326 REVA.0). To confirm the presence of bacterial gDNA in the sample material, the Femto™ Bacterial DNA Quantification Kit was used according to the manufacturer's protocol (Ver. 1.0.0). The bacterial DNA quantity and quality was considered sufficient for library construction. The Ion 16S™ Metagenomics Kit was used to amplify hypervariable regions from the polybacterial samples according to the protocol (MAN0010799 REV C.0). Two primer sets were used, one which amplified hypervariable regions V2, V4 and V8, and the other V3, V6, V7 and V9. Target regions were amplified from 2 μL gDNA across 25 cycles on the SimplyAmp Thermal Cycler (ThermoFisher Scientific). The presence of amplified products was verified on the PerkinElmer LabChip GXII Touch using the DNA NGS 3K LabChip and Reagent Kit according to the protocol (CLS145099 Rev. D). Following verification, PCR products were pooled and purified with Agencourt™ AMPure™ XP reagent and eluted in 15 μL nuclease-free water. Purified amplicons were quantified with the Qubit 2.0 Fluorometer using the Qubit dsDNA HS assay kit.

Library preparation was performed from 50 ng pooled amplicons for each sample using the Ion Plus Fragment Library Kit according to the protocol (MAN0006846, REV B.0). Briefly, amplified fragments were end-repaired in preparation for blunt-end ligation to IonCode™ Barcode Adapters. The adapter-ligated, barcoded library was purified with Agencourt™ AMPure™ XP reagent and subsequently quantified using the Ion Universal Library Quantitation Kit. The StepOnePlus™ Real-time PCR system was used to perform the quantitative polymerase chain reaction (qPCR) amplification. Libraries were diluted to a target concentration of 10 pM, and the 16S libraries were combined in equimolar amounts for template preparation, which was performed using the Ion 520™ and Ion 530™ Chef Kit. In brief, 25 μL of the pooled library was loaded onto the Ion Chef liquid handler using reagents, solutions and supplies according to the protocol (MAN0010846, REVD.0). Enriched, template positive ion sphere particles were loaded onto an Ion 530™ Chip. Massively parallel sequencing was performed on the Ion S5™ System using the Ion S5™ sequencing solutions and sequencing reagent kits according to the protocol (MAN0010846, REVD.0). Flow space calibration and base caller analyses were performed using default analysis parameters in the Torrent Suite Version 5.6.0 Software. Run data was uploaded to an IonReporter™ cloud account for mapping and annotation of the identified sequences, with the Basic Local Alignment Search Tool (BLAST) being used to align sequences to the curated MicroSEQ® 16S v2013.1 and Greengenes v13.5 reference libraries. Reads with fewer than ten copies were ignored. Alpha and beta diversity analysis was performed using the QIIME 1.9.1 open-source bioinformatics pipeline, through IonReporter™. The bacterial composition (per operational taxonomic unit, OTU) is reported as the percentage of the total mapped reads (for all primers) per sample.

Statistical analysis

Each rabbit constituted an experimental unit for statistical analysis. Data was analysed using Statistica version 13 software, with normality being tested using normal probability plots, and homoscedasticity using Levene's test. Univariate tests of significance for the main effects (diet and sex) and their interaction, were performed using the general linear model procedure, with Fisher's LSD test being performed to compare the individual diet-sex treatment groups. Pearson's correlation coefficients were calculated to measure the strength and direction of any relationships between the bacterial families and the total LW gain from 5 to 12 wk old, the average FCR from 5 to 12 wk, the serum hormone levels and the caecal flavonoid content and pH. Main effects and interactions with $P<0.05$ were considered significant, and values are reported as least square means (LSM), with the standard error of the mean (SEM) provided.

Table 1: Live performance, caecal content conditions and serum hormone levels of male and female New Zealand White grower rabbits fed control or quercetin-supplemented (2 g/kg) diets.

	Diet					Sex			
	Overall	Ctrl	Qrc	SEM	*P*-value	Male	Female	SEM	*P*-value
Total live weight gain (g)	2039	2032	2046	166	0.95	1970	2108	166	0.57
Average FCR	4.04	3.86	4.21	0.21	0.27	4.19	3.88	0.21	0.31
Caecal flavonoid content (mg Q/g DM)	3.90	4.11	3.69	0.23	0.22	4.01	3.79	0.23	0.52
Caecal pH	5.59	5.61	5.57	0.08	0.73	5.49	5.69	0.08	0.10
Serum fT3 (ng/mL)	3.38	2.94	3.83	0.80	0.45	3.92	2.85	0.80	0.37
Serum fT4 (ng/mL)	4.70	5.61	3.78	0.61	0.07	5.16	4.23	0.61	0.31
Serum GH (ng/mL)	1.25	1.13	1.36	0.30	0.60	1.10	1.40	0.30	0.49
Serum cortisol (ng/mL)	8.50	6.84	10.16	2.00	0.27	4.28	12.72	2.00	0.02

Ctrl: Control; Qrc: Quercetin-supplemented; SEM: Standard error of the mean; FCR: Feed conversion ratio
mg Q/g DM: mg quercetin equivalent/gram dry matter; fT3: Free triiodothyronine; fT4: Free thyroxine; GH: Somatotropin.

RESULTS

The LW gain, FCR and serum fT3, fT4 and GH contents did not differ between the sexes or dietary treatment groups (Table 1), while the cortisol content was higher in females than males (*P*=0.02). Neither the caecal pH nor caecal flavonoid content differed between the diets or sexes, with an average pH of 5.59 and an average flavonoid content of 3.90 mg quercetin equivalent/g DM being found.

For the metagenome sequencing, there was an average of 625 783±119 957 total reads for the caecal microbial DNA samples, with 399 460±76 621 valid reads and 162 266±36 149 mapped reads per sample. The alpha diversity plots (Figure 1A and B) indicated that the sequencing depth used was sufficient, as indicated by the plateau in the rarefaction measures. The treatment groups did not appear to differ in diversity at the family level (Figure 1); however,
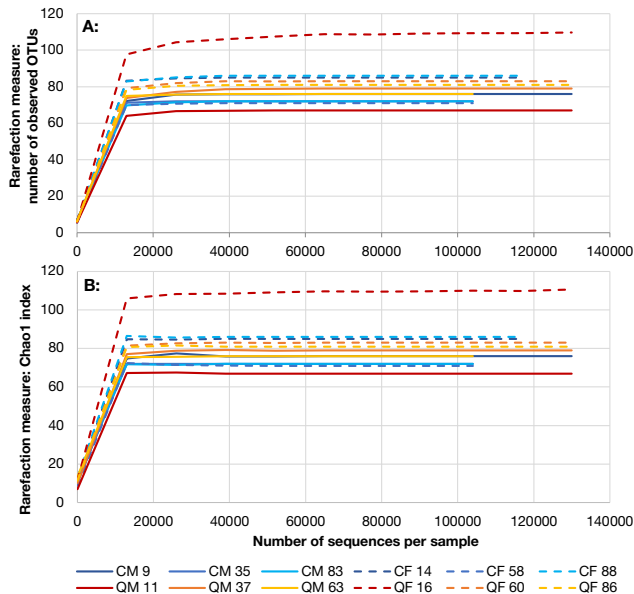

Figure 1: Alpha diversity at family level of the caecal microbiota of male (M) and female (F) New Zealand White grower rabbits, fed either a control (C) or quercetin-supplemented (Q, 2 g/kg feed) diet: Rarefaction curves of average (A) observed operational taxonomic units (OTUs) at 97% similarity, and (B) Chao1 index values for each treatment group.

one sample, QF 16, had considerably higher levels of microbial diversity than the other samples, with a larger number of OTUs being identified.

In accordance with the alpha diversity results, no clear separation of the treatment groups was seen at the species level in the Bray-Curtis beta diversity principle coordinate plots (Figure 2), suggesting that the overall diversity was more due to individual than treatment differences. However, two samples from Qrc rabbits, QM37 and QF16, had very different microbiota compositions at the family level to the other samples, with the latter concurring with the higher diversity seen in Figure 1.

At the phylum level (Figure 3), the dominant group in all the samples was *Firmicutes*, making up an average of 72.01±1.14% of the total mapped reads, followed by *Proteobacteria* (11.14±0.96%), *Bacteroidetes* (9.82±1.54%), *Actinobacteria* (3.67±0.26%), *Tenericutes* (1.42±0.22%) and *Verrucomicrobia* (1.28±0.16%). While some of the phyla were only present at very low levels, but were detected in all the samples, such as *Spirochaetes* (0.08±0.01%) and *Synergistetes* (0.13±0.03%), *Deinococcus-Thermus* (0.22±0.12%) was only present in a few of the samples, and *Nitrospinae* was only detected in one sample (0.01% in QF60), and was consequently not included in Figure 3.

As expected, families falling within the *Firmicutes* phylum (indicated in shades of green) dominated the microbiota (Figure 4), particularly *Lachnospiraceae* (23.94±1.01%), *Ruminococcaceae* (19.71±1.07%) and *Clostridiaceae* (10.17±0.74%), all of which also fall within the order *Clostridiales* (indicated by dotted shades of green), which was the most prevalent order (62.27±1.39%). The remaining prominent families were *Desulfovibrionaceae* (7.65±0.77%), *Eubacteriaceae* (3.83±0.38%), *Bacteroidaceae* (4.19±0.91%) and *Porphyromonadaceae* (4.05±0.63%), with both of the latter belonging to the phylum *Bacteroidetes,* (indicated by shades of blue). The latter two also both belong to the order *Bacteroidales* (9.05±1.47%, indicated in striped shades of blue), which was the second most prevalent order. The majority of the families detected (67 of 83) had abundances of less than 1% of the total mapped reads, and many were only present in a few of the samples, and were consequently not statistically analysed.

Only *ca*. 3.8% of the total mapped reads were identified to the species level, with 85.13±1.02% having family level identification and 10.92±0.78% having genus level identification. The proportion of the total mapped reads identified as species also varied widely between individuals, from 2.49% to 6.76%.
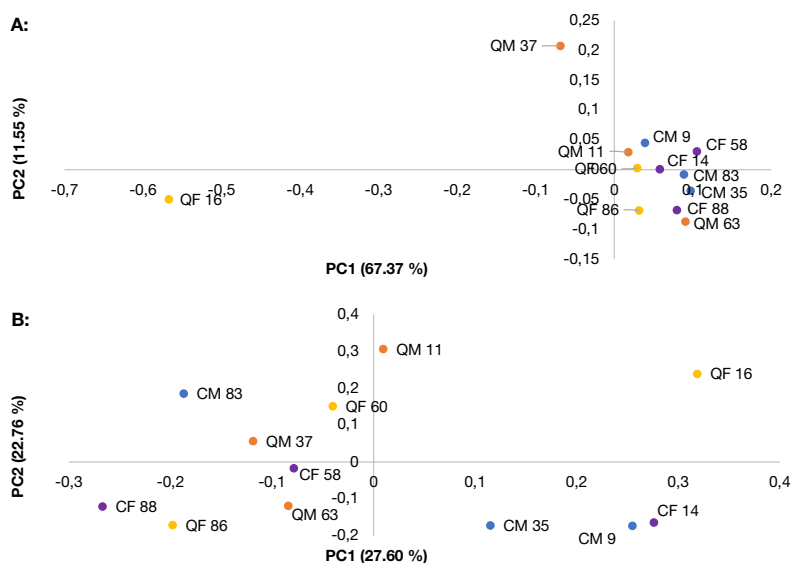

Figure 2: Bray-Curtis plots indicating the beta diversity at (A) family and (B) species level of the caecal microbiota of male and female New Zealand White grower rabbits fed either a control (Ctrl) or quercetin-supplemented (Qrc, 2 g/kg) diet. Points indicate individual samples, with blue circles representing Ctrl males (CM), purple circles Ctrl females (CF), orange circles Qrc males (QM) and yellow circles Qrc females (QF).
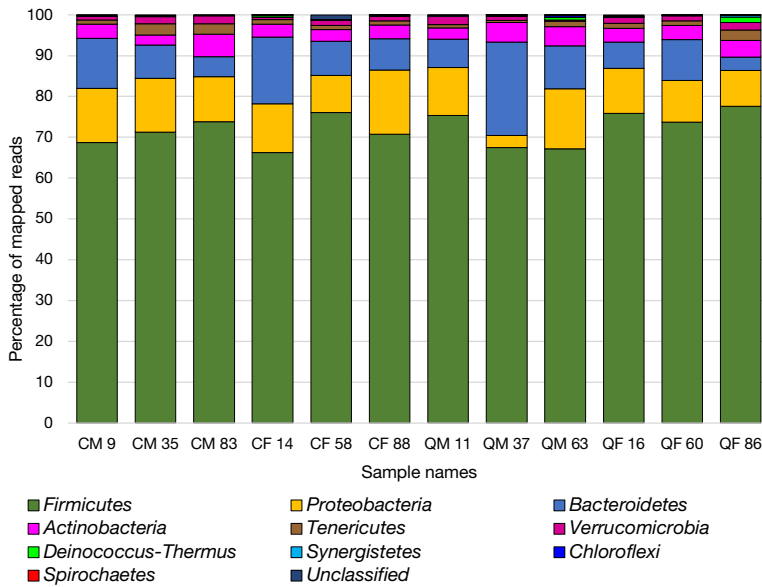
Figure 3: Relative abundance of the major bacterial groups in the rabbit caecum at the phylum level. Phyla are organised vertically from most to least prevalent (average across treatment groups) and samples are grouped horizontally by treatment (C: control; Q: dietary quercetin supplemented at 2 g/kg feed; M: male; F: female).

Figure 4: Relative abundance of the major bacterial groups (those contributing more than 1% of total mapped reads) in the rabbit caecum at family level. Families are organised vertically from most to least prevalent (average across treatment groups), and samples are grouped horizontally by treatment (C: control; Q: dietary quercetin supplemented at 2 g/kg feed; M: male; F: female).
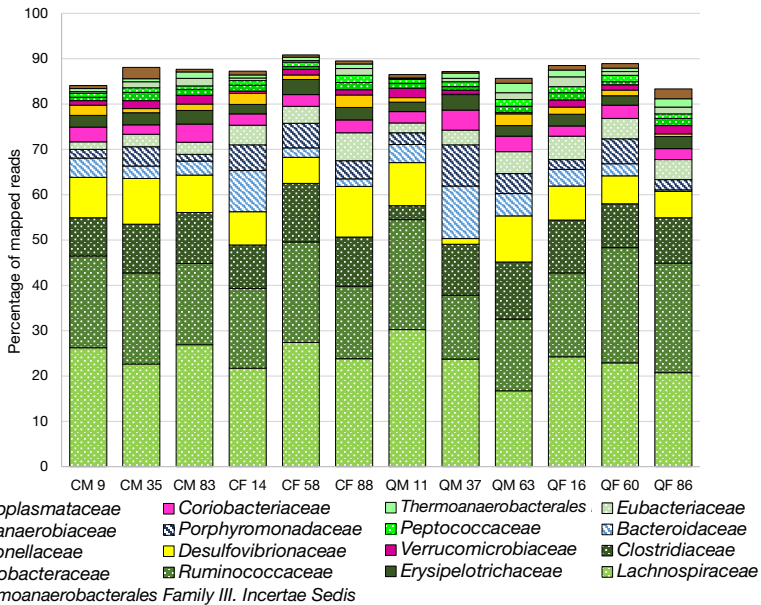
Figure 5: Relative abundance of the major bacterial species identified in the rabbit caecum. Species are organised vertically from most to least prevalent (average across treatment groups) and samples are grouped horizontally by treatment (C: control; Q: dietary quercetin supplemented at 2 g/kg feed; M: male; F: female).
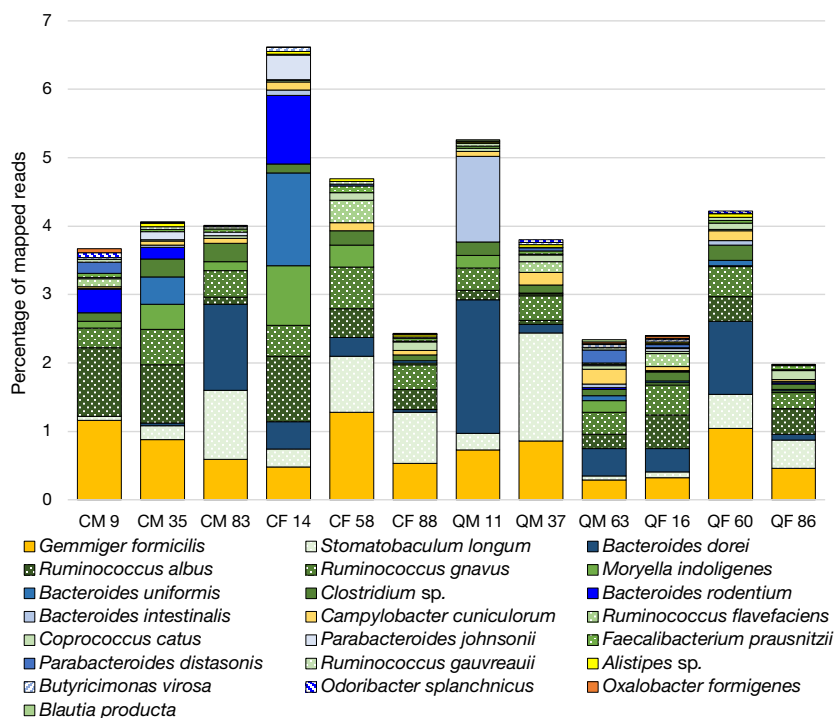
The composition at the species level (Figure 5) was far more diverse than at the phylum or family levels. Some species, such as *Gemmiger formicilis* (0.72±0.09%), *Stomatobaculum longum* (0.50±0.13%) and *Ruminococcus gnavus* (0.40±0.03%) were relatively consistently present in all the samples, whereas others, such as *Bacteroides dorei* (0.50±0.18%), *Bacteroides uniformis* (0.17±0.11%) and *Bacteroides rodentium* (0.13±0.08%) were present at high levels in some samples, but barely detected in others. *Ruminococcus* (family *Ruminococcaceae*) was the most prevalent genus (2.17±0.28%), with four identified species, of which *Ruminococcus albus* (0.44±0.10%) was the 4th most prevalent overall, whereas *Ruminococcus* (proposed taxonomy, family *Lachnospiraceae*, 2.03±0.17%), was the 2nd most abundant genus, but only had only a single identified species, *R. gnavus*. In contrast, eight of the identified species belonged to the genus *Bacteroides*, despite it being only the 5th most prevalent genus identified (1.32±0.43%). *Moryella indoligenes* was the only identified species in the genus *Moryella*, and the 6th most abundant species overall (0.19±0.07%). A number of species (18 of 38) were only identified in a few of the samples, generally at very low levels, although some, such as *Bacteroides fragilis* and *Akkermansia muciniphila,* were abundant in the few samples in which they were detected.

Despite the small sample number, several of the bacterial families correlated significantly with the live performance data (Table 2). *Eubacteriaceae, Natranaerobiaceae, Peptococcaceae* and *Syntrophomonadaceae* all had strong positive Pearson's correlations with the total weight gain, suggesting that rabbits for which these families were more abundant tended to gain more weight from weaning to 12 wk old. In contrast, rabbits with higher proportions of *Hyphomicrobiaceae* tended to grow less. Only three families correlated with the FCR, *Clostridiaceae, Erysipelotrichaceae* and *Haloplasmataceae*, suggesting that a higher abundance of these families coincided with a lower FCR, and thus an improvement in the efficiency of feed utilisation.

Table 2: Significant Pearson's correlation coefficients between the abundance of bacterial families and the live performance data and caecal content conditions of New Zealand White grower rabbits (*P*-values provided in parentheses).

	Overall abundance	Total weight gain	Average FCR	Caecal flavonoids (mg Q/g DM)	Caecal pH
Clostridiaceae	10.17±0.74	-	-0.67 (0.02)	-	-
Eubacteriaceae	3.83±0.38	0.62 (0.03)	-	-	-
Coriobacteriaceae (class Actinobacteria)	2.92±0.20	-	-	0.64 (0.02)	-
Erysipelotrichaceae	2.63±0.13	-	-0.61 (0.04)	-	-
Veillonellaceae (order Selenomonadales)	1.21±0.12	-	-	-0.66 (0.02)	-
Peptococcaceae	1.11±0.08	0.60 (0.04)	-	-	-
Natranaerobiaceae	1.08±0.19	0.70 (0.01)	-	-	-
Mycoplasmataceae	1.01±0.19	-	-	-0.69 (0.01)	-
Hyphomicrobiaceae	0.74±0.09	−0.62 (0.03)	-	-	-
Clostridiales Family XI. Incertae Sedis	0.25±0.06	-	-	-	0.64 (0.03)
Acetobacteraceae	0.18±0.04	-	-	-0.74 (<0.01)	-
Microbacteriaceae	0.17±0.04	-	-	-0.59 (0.04)	-
Haloplasmataceae	0.14±0.10	-	−0.68 (0.02)	-	-
Synergistaceae	0.13±0.03	-	-	-0.62 (0.03)	-
Erythrobacteraceae	0.10±0.02	-	-	-0.60 (0.04)	-
Desulfuromonadaceae	0.07±0.02	-	-	-	0.69 (0.01)
Syntrophomonadaceae	0.06±0.02	0.63 (0.03)	-	-	-
Spiroplasmataceae	0.06±0.01	-	-	-	0.72 (<0.01)
Veillonellaceae (order Clostridiales)	0.05±0.01	-	-	0.61 (0.03)	-

No.=12 for all correlations; Families listed from most to least abundant (abundance provided as least square mean±standard error of the mean). Total weight gain: weight gain from 5 to 12 wk of age; Average FCR: Average feed conversion ratio from 5 to 12 wk of age; mg Q/g DM: mg quercetin equivalent/gram dry matter.

There were also several correlations between bacterial families and serum hormone levels (Table 3). *Carnobacteriaceae, Coriobacteriaceae* (class *Coriobacteriia*), *Streptosporangiaceae*, *Thermoanaerobacterales Family III. Incertae Sedis*, *Thermoanaerobacterales Family IV. Incertae Sedis* and *Thermosporotrichaceae* all correlated positively with serum fT3 levels, whereas *Entomoplasmataceae* and *Hyphomicrobiaceae* both had strong negative correlations. *Carnobacteriaceae*, although correlating positively with fT3 levels, had a negative correlation with fT4 levels, as did *Campylobacteraceae* and *Clostridiales Family XVI. Incertae Sedis*. In contrast, *Acidaminococcaceae, Peptostreptococcaceae* and *Sutterellaceae* tended to be more abundant when serum fT4 levels were higher. Only *Clostridiales Family XII. Incertae Sedis* (*r*=0.65) correlated with serum GH levels. *Alicyclobacillaceae* correlated negatively with the serum cortisol level, while *Flavobacteriaceae, Rhizobiaceae* and *Rhodospirillaceae* correlated positively with this hormone.

Several bacterial families correlated with the caecal pH and flavonoid content. *Acetobacteraceae, Erythrobacteraceae, Microbacteriaceae, Mycoplasmataceae, Synergistaceae* and *Veillonellaceae* (order *Selenomonadales*) all tended to be less abundant in samples with higher flavonoid contents, while proportions of *Coriobacteriaceae* (class *Actinobacteria*) and *Veillonellaceae* (order *Clostridiales*) were higher. Three families correlated with the pH of the caecal content, *Clostridiales Family XI. Incertae Sedis*, *Desulfuromonadaceae* and *Spiroplasmataceae*, with all three being more abundant at higher pH levels. There was no apparent correlation between the pH and the caecal flavonoid content.

Diet had a very limited effect on the microbiota (Table 4), with only *Clostridiales Family XIII. Incertae Sedis* (*P*=0.005) and *Anaerofustis* (*P*=0.003) being more abundant in the Qrc group. Somewhat more extensive differences were found between the sexes, with this in most cases involving higher levels of the particular OTU being found in caecal samples from female rabbits. Females had higher levels of *Flavobacteriia* (*P* = 0.032), due to a higher abundance of *Flavobacteriales* and *Flavobacteriaceae*, as well as higher levels of *Desulfuromonadales* (*P*=0.038), *Eubacteriaceae* (*P*=0.013) and *Geobacteraceae* (*P*=0.023).

Table 3: Significant Pearson's correlation coefficients between the abundance of bacterial families and the serum hormone levels of New Zealand White grower rabbits. *P*-values provided in parentheses.

	Overall abundance	Serum fT3	Serum fT4	Serum GH	Serum cortisol
Thermoanaerobacterales Family III. Incertae Sedis	1.06±0.16	0.58 (0.05)	-	-	-
Hyphomicrobiaceae	0.74±0.09	−0.61 (0.03)	-	-	-
Flavobacteriaceae	0.54±0.14	-	-	-	0.62 (0.03)
Acidaminococcaceae	0.41±0.07	-	0.58 (0.05)	-	-
Campylobacteraceae	0.11±0.02	-	−0.58 (0.05)	-	-
Thermosporotrichaceae	0.09±0.03	0.67 (0.02)	-	-	-
Carnobacteriaceae	0.09 ±0.04	0.66 (0.02)	−0.64 (0.02)	-	-
Sutterellaceae	0.09±0.01	-	0.62 (0.03)	-	-
Peptostreptococcaceae	0.09±0.04	-	0.65 (0.02)	-	-
Clostridiales Family XII. Incertae Sedis	0.06±0.01	-	-	0.65 (0.02)	-
Alicyclobacillaceae	0.05±0.01	-	-	-	−0.65 (0.02)
Coriobacteriaceae (class Coriobacteriia)	0.05±0.02	0.69 (0.01)	-	-	-
Rhodospirillaceae	0.05±0.01	-	-	-	0.70 (0.01)
Entomoplasmataceae	0.04±0.01	−0.68 (0.01)	-	-	-
Rhizobiaceae	0.03±0.01	-	-	-	0.60 (0.04)
Thermoanaerobacterales Family IV. Incertae Sedis	0.03±0.01	0.62 (0.03)	-	-	-
Streptosporangiaceae	0.02±0.01	0.61 (0.04)	-	-	-
Clostridiales Family XVI. Incertae Sedis	0.02±0.01	-	-0.58 (0.05)	-	-

No. = 12 for all correlations; Families listed from most to least abundant (abundance provided as least square mean±standard error of the mean). fT3: free triiodothyronine; fT4: free thyroxine; GH: somatotropin.

DISCUSSION

The microbiota profile

At phylum level, the dominance of *Firmicutes* found in this study (Figure 3) was in agreement with previous studies of the rabbit caecal microbiota, and its relative abundance fitted within the previously reported range (Monteils *et al.,* 2008; Bäuerl *et al.,* 2014; Zhu *et al.,* 2015). The abundance of *Proteobacteria* and *Bacteroidetes* also concurred with some existing literature (Monteils *et al.,* 2008; Zhu *et al.,* 2015), although other studies reported considerably lower proportions of *Proteobacteria* (Massip *et al.,* 2012; Bäuerl *et al.,* 2014). It is notable that *Proteobacteria* tended to be more abundant in rabbits exhibiting the symptoms of epizootic rabbit enteropathy (ERE) (Bäuerl *et al.,* 2014); however, ERE did not occur in this study.

The abundance of *Actinobacteria* and *Tenericutes* was high relative to the results of Massip *et al.* (2012) and Zhu *et al.* (2015); however, Bäuerl *et al.* (2014) and Badiola *et al.* (2016) found a higher proportion of *Tenericutes* but minor amounts of *Actinobacteria*. Reports of *Verrucomicrobia* abundance also vary widely, possibly due to differences in rabbit age at sampling and the starch to fibre ratios of the diets used, as both have been reported to affect the abundance of this phylum (Monteils *et al.,* 2008; Massip *et al.,* 2012; Bäuerl *et al.,* 2014; Zhu *et al.,* 2015).

At the family level (Figure 4), the predominance of *Lachnospiraceae* and *Ruminococcaceae* concurred with the findings of Zhu *et al.* (2015), Massip *et al.* (2012) and Bäuerl *et al.* (2014), although the proportions were much lower than reported by the latter two. These families are thought to play an important role in fibre digestion, particularly of peptose and cellulose (Gosalbes *et al.,* 2011), and are significant producers of short-chain fatty acids (Zhu *et al.,* 2015). In addition, *Ruminococcaceae* has been associated with antibiotic biosynthesis in a metatranscriptomic study on the human gut microbiota, suggesting a role in gut health (Gosalbes *et al.,* 2011).

Clostridiaceae, the third most abundant family, was not reported as a main bacterial family in rabbits by Massip *et al.* (2012); however, it was present in rabbit caeca (Bäuerl *et al.,* 2014), and was a dominant family in the capybara (García-Amado *et al.,* 2012) and healthy horses (Steelman *et al.,* 2012). Members of the *Clostridiaceae* family have

Table 4: Abundance (percentage of total mapped reads) of bacterial classes, orders, families or genera, in the caecal microbiota of New Zealand White grower rabbits, which differed significantly between males and females, or rabbits fed control or quercetin-supplemented (2 g/kg) diets.

	Overall	Diet				Sex			
		Ctrl	Qrc	SEM	*P*-value	Male	Female	SEM	*P*-value
No.	12	6	6			6	6		
Class									
Flavobacteriia	0.54	0.56	0.53	0.16	0.90	0.25	0.84	0.16	0.03
Order									
Flavobacteriales	0.54	0.56	0.53	0.16	0.90	0.25	0.84	0.16	0.03
Desulfuromonadales	0.25	0.19	0.32	0.06	0.22	0.14	0.37	0.06	0.04
Family									
Eubacteriaceae	3.83	3.58	4.07	0.40	0.41	2.93	4.73	0.40	0.01
Flavobacteriaceae	0.54	0.56	0.53	0.16	0.90	0.25	0.84	0.16	0.03
Geobacteraceae	0.18	0.13	0.23	0.04	0.14	0.09	0.26	0.04	0.02
Clostridiales Family XIII. Incertae Sedis	0.012	0.003	0.020	0.003	0.005	0.008	0.015	0.003	0.17
Genus									
Anaerofustis	0.005	0.000	0.010	0.002	0.003	0.003	0.007	0.002	0.20

Ctrl: Control; Qrc: Quercetin-supplemented; SEM: Standard error of the mean.

been found to prefer simpler carbohydrates, rather than the fibrous components utilised by the *Lachnospiraceae* and *Ruminococcaceaea*, and its prevalence in the caecum was thus somewhat surprising (Biddle *et al.,* 2013).

Desulfovibrionaceae was also detected by Bäuerl *et al.* (2014) and Massip *et al.* (2012), and was linked to feed intake levels by the latter, and *Bacteroidaceae* was found at similar levels by Badiola *et al.* (2016). Both *Bacteroidaceae* and *Porphyromonadaceae* have been found to be among the most active of the human gut microbiota (Gosalbes *et al.,* 2011). However, Badiola *et al.* (2016) did not detect any *Porphyromonadaceae* in the rabbit caecum. *Eubacteraceae* have also been suggested to be functionally versatile (De Vos *et al.,* 2009; Gosalbes *et al.,* 2011), and were detected in the rabbit caecum by Bäuerl *et al.* (2014), but were not by Badiola *et al.* (2016) or Zhu *et al.* (2015).

Of the remaining families contributing more than 1% of the total mapped reads, *Coriobacteraceae, Erysipelotrichaceae, Oxalobacteraceae*, *Verrucomicrobiaceae*, *Veillonellaceae* and *Peptococcaceae* have been previously detected in rabbit caecum, although at widely varying abundances (Massip *et al.,* 2012; Bäuerl *et al.,* 2014; Badiola *et al.,* 2016). In contrast, *Natranaerobiaceae*, *Thermoanaerobacterales Family III. Incertae Sedis* and *Mycoplasmataceae* do not appear to have been previously found to form part of the rabbit caecal microbiota.

The greatest extent of individual variation was seen at the species level (Figure 5), with not only the abundance of the different species varying, but also the proportion of the sequences that were identified to this level. Only between 2.0% and 6.8% of the sequences were identified to the species level, which supports the speculation that rabbit caeca contain a large proportion of undescribed bacterial species (Michelland *et al.,* 2010), and suggests that the composition of these novel species may vary between individuals.

Overall, five species contributed 67% of the total identified at the species level, with the two most prevalent being *Gemmiger formicilis* and *Stomatobaculum longum*, although the abundance of the latter varied widely. Neither species appear to have been identified in rabbit caeca before, although *G. formicilis* has been found in chicken ceca (Clench and Mathias, 1995) and human faeces (Salanitro *et al.,* 1976).

The variable abundance of *Bacteroides dorei* was notable, as the genus *Bacteroides* has been suggested to be largely responsible for pectinolytic activity in the rabbit caecum (Sirotek *et al.,* 2001). Furthermore, Badiola *et al.* (2016) observed that the inoculation of rabbit kits with *B. dorei* reduced the expression of pro-inflammatory cytokines and increased the expression of MHC II, a surface antigen, as well as increasing immunoglobulin diversity, thereby possibly helping reduce the risk of ERE.

The prevalence of the genus *Ruminococcus* concurred with Bäuerl *et al.* (2014), and *R. albus*, the most abundant species identified in this genus and 4th most abundant overall, is generally recognised as being one of the main cellulolytic bacteria in the rumen environment and has been previously reported in the rabbit caecum (Abecia *et al.,* 2005; Monteils *et al.,* 2008; Rodríguez-Romero *et al.,* 2013). In contrast, *R. gnavus*, the fifth most abundant species, does not appear to have been previously detected in rabbits.

The remaining species detected in rabbit caecum were only present at much lower levels (<0.2% of mapped reads), and varied greatly in abundance between individuals.

Correlations with live performance and caecal conditions

Four families had significant, positive correlations with the total weight gain (Table 2), with *Eubacteraceae* being the most abundant (3.83±0.38% total mapped reads). Similar results have been previously reported for *Eubacterium*, a genus in the family *Eubacteraceae*, which correlated positively with the average milk fat yield and residual feed intake (RFI) in dairy cows (Jami *et al.,* 2014). Only *Hyphomicrobiaceae* was more abundant in rabbits showing lower growth.

Three families correlated negatively with the FCR (Table 2), indicating a beneficial association with feed efficiency. The genus *Clostridium*, which was the only member of the *Clostridiaceae* family detected in this study, was not found to significantly correlate with the FCR of dairy cattle (Jami *et al.,* 2014). However, it did have a moderately positive correlation with the RFI, which contrasts with the findings of this study.

A number of families correlated significantly with serum levels of fT3, fT4, GH and cortisol (Table 3). While these specific correlations do not seem to have been previously investigated, and no highly abundant families were involved, such possible interactions have been suggested in previous studies. Specifically, links between the gut microbiota and serum leptin and cortisol levels have been reported (Sanz *et al.,* 2010), and intestinal bacteria are known to deconjugate excreted thyroid hormones, thus allowing their reabsorption and possibly increasing circulating levels (Yen, 2001). However, the existence of these correlations alone does not necessarily indicate a physiological link or causation, and considerable further research is required before any assumptions of a direct link between serum hormones and the gut microbiota can be made.

Considering the known antimicrobial properties of the flavonoids (Cushnie and Lamb, 2005), the predominantly negative correlations with the total flavonoid content of the caecum were unsurprising (Table 2). The negative correlations with *Veillonellaceae* and *Mycoplasmataceae* concurred with previous findings that these families decreased in abundance in response to dihydromyricetin (Fan *et al.,* 2018), and an essential oil high in flavonoids (De Nardi *et al.,* 2016), respectively. In contrast, *Coriobacteriaceae* (class *Actinobacteria*) was more prevalent in samples with a higher total flavonoid content, concurring with the results of Huang *et al.* (2016), who found that quercetin, catechin and puerarin had no inhibitory effect on this family.

Few families correlated with the caecal pH (Table 2), with *Clostridiales Family XI. Incertae Sedis*, *Desulfuromonadaceae* and *Spiroplasmataceae* all being more abundant for higher pH values (Table 2). This general preference for a higher pH is in agreement with the known detrimental effects of acidic conditions on rumen microbial function (Russell and Wilson, 1996).

Effects of diet

Only two OTUs differed significantly between the dietary treatment groups, and both made only minimal contributions to the total mapped reads (Table 4). *Clostridiales Family XIII. Incertae Sedis* was more abundant in the Qrc rabbits, which loosely concurred with previous findings that quercetin increased the abundance of *Firmicutes* (the phylum to which it belongs), as well as the abundance of another member of the order *Clostridiales*, *Ruminococcaceae* (Huang *et al.,* 2016). The genus *Anaerofustis*, which belongs to the family *Eubacteraceae*, was also more abundant in the Qrc rabbits (Table 4). This concurred with previous findings for another species within this family, *Eubacterium ramulus*, which has been found to increase in relative abundance in response to the ingestion of flavonoids, including quercetin (Simmering *et al.,* 2002). This stimulatory effect of flavonoids on the growth of *E. ramulus* may be linked to its proposed role as a key species responsible for the degradation of flavonoids in the gut (Braune *et al.,* 2001), as

it can use the ingested flavonoids as a substrate for growth (Simmering *et al.,* 2002). A similar interaction may have been responsible for the higher levels of *Anaerofustis* in the supplemented rabbits found in this study.

The negligible effects of dietary quercetin on the caecal microbiota found in this study, in conjunction with the lack of effect on the caecal total flavonoid content, may have been due to the quercetin aglycone being absorbed from the gut prior to the caecum. As the absorption of flavonoid glycosides in the small intestine has been suggested to be limited by the presence of the sugar moiety, these glycosides may accumulate in the caecum, whereas the aglycone may not (Oteiza *et al.,* 2018).

Effects of sex

The only sex effect on a non-microbiota parameter was on the serum cortisol level, which was much higher in females than males. The same was found for the larger population from which the 12 rabbits used in this study were sampled, as discussed by North *et al.* (2018). Similar sex differences in serum cortisol levels have been reported in humans (Gunn *et al.,* 2016; Kirschbaum *et al.,* 1992) and a variety of other mammals (Tilbrook *et al.,* 2000; Green & McCormick, 2016).

The most abundant bacterial family to differ between the sexes was *Eubacteraceae*, which was more prevalent in females than males (Table 4). Considering the versatility of function of this group it is difficult to identify how or whether this would impact the overall health and efficiency of the microbiota (De Vos *et al.,* 2009; Gosalbes *et al.,* 2011). However, as *Eubacteraceae* did correlate with improvements in weight gain, this sex difference may be linked to the tendency for a higher growth rate in female rabbits towards the end of the growth period, as found by North *et al.* (2018).

Flavobacteriaceae and *Geobacteraceae* were also both more abundant in females, with this effect carrying over to the order (*Flavobacteriales, Desulfuromonadales*) and class (*Flavobacteriia*) level. *Flavobacteraceae* has not previously been found to be a major family in the rabbit caecal microbiota, with Bäuerl *et al.* (2014) only detecting it at very low levels. Badiola *et al.* (2016) also only detected *Flavobacteraceae* in younger rabbits (25 or 39 d old), and by 70 d of age it was no longer present. Unfortunately, sex was not reported by Badiola *et al.* (2016), as it would have been interesting to see whether the evolution in the abundance of *Flavobacteraceae* with age differed for males and females. *Geobacteraceae* similarly does not appear to be common species in the gut environment (Bäuerl *et al.,* 2014; Zhu *et al.,* 2015; Badiola *et al.,* 2016).

CONCLUSION

The comparison of the composition of the microbiota found in this study to those in previous research suggested that certain bacterial groups, such as *Firmicutes* at phylum level and *Lachnospiraceae* and *Ruminococcaceae* at family level, are relatively consistently dominant in the rabbit caecum. However, consensus beyond this was extremely limited, even between individuals within this study. This absence of a clear, consistent microbiota profile will present a challenge for any future research attempting to understand or manipulate this population. Nonetheless, the correlations between live performance traits and bacterial families, particularly *Eubacteraceae* and *Clostridiaceae,* suggest that further research may be of value. The correlations with serum hormone levels found, while not necessarily suggesting causation, should nonetheless also be kept in mind during future research.

The largely negative correlations between the caecal flavonoid content and the various bacterial families concurred with the antimicrobial properties of these compounds. However, there was a lack of any clear pattern in this relationship between studies, and this correlation did not translate into a difference between the dietary treatment groups, for either the caecal flavonoid content or the caecal microbiota. This minimal effect of diet may have been due to the use of the quercetin-aglycone, which may have been absorbed from the gastrointestinal tract prior to the caecum. Nonetheless, *Clostridiales Family XIII. Incertae Sedis* and *Anaerofustis* were both more abundant in the Qrc rabbits, with the latter possibly using the flavonoid as a substrate for growth.

The sex differences largely involved higher levels of OTUs in samples from female rabbits. *Eubacteraceae* was one of the most abundant families affected by sex, along with *Flavobacteriaceae*, but in the absence of genus or species-level identification, relating these differences to functional changes in the caecum was not possible.

Overall, the practical implications of this study are limited, as is typical of exploratory studies of this kind. However, the information presented provides avenues for further research, and adds to the limited picture of the rabbit caecal microbiota currently available.

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