Previous studies in different species have emphasized the importance of the intestinal microbiota on animal health. While there are publications characterizing the bacterial microbiota of the rabbit gastrointestinal (GI) tract and broader anatomy, the eukaryotic microbiota of rabbits remains largely unexplored. Furthermore, studies analyzing the impact of diet on the rabbit GI tract bacterial microbiota focused mainly on the primary site of fermentation, the cecum, and hard feces. Rabbits, like guinea pigs and chinchillas, are herbivores and classified as hindgut cecal fermenters. Like all herbivores, rabbits have a symbiotic relationship with the gut microbiota that facilitates the breakdown of the cellulose components of their plant-based diets, which provide their main energy source. GI disease is one of the most common issues faced by domestic rabbits and other exotic companion mammals; thus, a better understanding of the microbiota

OBJECTIVE
This study aimed to characterize the bacterial and eukaryotic microbiota of the gastrointestinal (GI) tract in domestic rabbits and to evaluate the effect of different diet characteristics, such as pelleting, extrusion, and hay supplementation.

ANIMALS
30 New Zealand White rabbits (15 male and 15 female; 6 to 7 months old) were fed 1 of 6 diets (5 rabbits per diet) for 30 days after an initial acclimation period. At the end of the trial, samples were collected from the stomach, small intestine, cecum, large intestine, and hard feces.

METHODS
The samples were analyzed using 16S rRNA and internal transcribed spacer 1 region-targeted amplicon sequencing.

RESULTS
The bacterial microbiota was distinct between the foregut and hindgut. The most abundant bacterial genera included an unclassified genus in the Bacteroidales order and Alistipes. Candida was the most abundant genus in the eukaryotic dataset. In the bacterial dataset, diet No Hay/Pellet E was shown to have lower diversity (Shannon diversity, P < .05) compared to all diet groups except for No Hay/Pellet M. Few significant differences in alpha-diversity indexes between diet groups were detected in the eukaryotic dataset.

CLINICAL RELEVANCE
Our findings demonstrated that feeding hay had a significant effect on the beta diversity of the bacterial microbiota. Given the prevalence of gastrointestinal disease in the domestic rabbit population, furthering our understanding of what constitutes a healthy rabbit microbiota and the effects of different diets on the microbial community can help veterinarians implement better intervention strategies and allow pet owners to provide the best level of care.

Keywords: rabbit, microbiota, diet, hay supplementation, pellet processing

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in healthy rabbits and how different diets can affect the composition of the microbial populations can help veterinarians provide the best level of care and medical treatment.16

Certain GI disorders affecting rabbits are thought to be associated with abnormal populations of the microbiota (dysbiosis) throughout the GI tract, most commonly affecting the hindgut (cecum and colon).17 Examples of such disorders include mucoid enteritis and epizootic rabbit enteropathy.18,19 Dysbiosis may be associated with and due to various conditions, such as a low-fiber diet, excessive dietary protein or simple carbohydrates, environmental stress, inappropriate antibiotic usage, and dehydration. These conditions can lead to or be associated with a shift in the pH of the hindgut, further leading to an imbalance of the microbiota, thereby negatively affecting the bacterial fermentation processes, which is essential for the production of volatile fatty acids and other important fermentation products.16 An imbalance can be defined either as a change in the composition of the microbiota or a change (usually a lack of) in the microbial diversity. Restoring the GI environment and microbial populations to normalcy is a key goal of treating many GI diseases in rabbits. Therefore, the aim of this study was 2-fold: the first goal was to characterize the bacterial and eukaryotic microbiota of various segments of the GI tract in healthy domesticated rabbits, and the second goal was to evaluate the impact of diet on the microbiota. For both aims, we studied the composition of the bacterial microbiota using 16S rRNA gene-targeted amplicon sequencing and the eukaryotic microbiota using internal transcribed spacer 1 (ITS1) region-targeted amplicon sequencing.

Methods

Animals

This research was approved by the St. Matthew’s University School of Veterinary Medicine IACUC. Thirty (15 male and 15 female) clinically normal 6- to 7-month-old New Zealand White rabbits (Oryctolagus cuniculus) were used for this study. The rabbits were originally reared for meat production by a third party and weighed 1.47 to 2.14 kg at the onset of the study. The rabbits were obtained and housed at a single USDA-approved facility. Rabbits were housed individually in suspended wire cages with solid floor substrate and nest boxes in a ventilated metal building. Cecotrophy was not prevented in animals during the entire length of this study. Before the study began, all rabbits were fed a diet that consisted solely of alfalfa-based pellets. Spot cleaning was completed daily, and full cage and floor cleaning was completed twice weekly.

Rabbits were randomly selected to participate in the study. When assigning the 30 (15 male and 15 female) rabbits to the 6 diet groups, the randomization was stratified by sex resulting in 5 rabbits per diet. The assigned number of males and females to each diet group is shown (Table 1). Immediately before the start of the study, each rabbit was weighed and assessed as healthy based on a complete physical examination. Rabbits were transitioned from the alfalfa-based pelleted diet to each of their specific dietary treatment groups from days 0 to 42. During week 1 (day 0) of the transition period, the rabbits were fed 3 parts alfalfa-based pellets with 1 part new pellets (E, M, or P). By the end of week 1 (day 7), the rabbits were transitioned to 2 parts alfalfa-based pellets with 2 parts new pellets. By the end of week 2 (day 14), the rabbits were transitioned to 1 part alfalfa-based pellets with 3 parts new pellets. By the end of week 3 (day 21), the rabbits were fully transitioned to their new diets until the end of the trial (day 72). For diets that received hay, hay was offered ad libitum throughout the trial (days 0 to 72). Water was offered ad libitum. The feeding trial began on day 43 and was completed on day 72.

On day 43 of the study, 1 rabbit from the Hay/Pellet E group was found dead during morning feeding. The rabbit had exhibited no clinical signs of disease and had no history of previous illness. Necropsy and histopathology were completed, and lesions associated with pneumonia were isolated and considered the presumptive cause of death. All samples from this animal were excluded from the study. No other rabbits exhibited any clinical signs, all rabbits transitioned successfully to the study diets, and all other rabbits completed the study. Three different

Table 1—Animal groups and diets fed.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Diet</th>
<th>Number of males/number of females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hay-Only</td>
<td>Free-choice timothy hay daily, no pellets</td>
<td>3 male/2 female</td>
</tr>
<tr>
<td>Hay/Pellet P</td>
<td>Free-choice timothy hay daily</td>
<td>2 male/3 female</td>
</tr>
<tr>
<td></td>
<td>High-fiber uniform pelleted diet&lt;sup&gt;a&lt;/sup&gt; (113 g daily)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Hay/Pellet E</td>
<td>Free-choice timothy hay daily</td>
<td>3 male&lt;sup&gt;c&lt;/sup&gt;/2 female</td>
</tr>
<tr>
<td></td>
<td>High-fiber uniform extruded diet&lt;sup&gt;a&lt;/sup&gt; (100 g daily)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>No Hay/Pellet P</td>
<td>No hay</td>
<td>2 male/3 female</td>
</tr>
<tr>
<td>No Hay/Pellet E</td>
<td>No hay</td>
<td>2 male/3 female</td>
</tr>
<tr>
<td>No Hay/Pellet M</td>
<td>No hay</td>
<td>3 male/2 female</td>
</tr>
<tr>
<td></td>
<td>Low-fiber muesli mix diet&lt;sup&gt;a&lt;/sup&gt; (100 g daily)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Base formula: Oxbow Essentials Adult Rabbit Food. <sup>b</sup>Calculated based on the caloric density of the product and energy needs of the animals. <sup>c</sup>One male found deceased.
pellet groups were tested during this study (Pellet P, Pellet E, and Pellet M). Pellet P (Pelleted diet) and Pellet E (Extruded diet), both nutritionally complete timothy hay-based pellets, consisted of the same formula (Oxbow Essentials Adult Rabbit Food; www.oxbowanimalhealth.com) but underwent different processing. Pellet P was produced using a standard pelleting process, which utilizes steam, pressure, and temperature to shape pellets by compression through dies. Pellet E was produced using extrusion, which modifies starch in the feed by applying higher temperatures coupled with pressure and steam. This process solubilizes the starch and is known as gelatinization. In contrast, Pellet M was a muesli-type mixed pellet. The Hay-Only treatment group was fed only free-choice timothy hay and no pellets. Experimental groups Hay/Pellet P (Pelleted diet) and Hay/Pellet E (Extruded diet) were fed free-choice timothy hay in addition to their allotted amount of pellets. The amount of timothy hay ingested was not recorded in any groups that were offered hay. Experimental groups No Hay/Pellet P, No Hay/Pellet E, and No-Hay/Pellet M were fed their controlled amount of test diet without timothy hay (Table 1). The nutrient composition of each dietary component is listed (Table 2). Each rabbit was fed to meet energy requirements and maintain body weight. Rabbits were weighed weekly throughout the duration of the study.

**Sample collection**

After 10 weeks of the study (day 72), all rabbits were humanely euthanized by carbon dioxide inhalation. Within 1 to 2 minutes following euthanasia, the GI tract of each rabbit was isolated for sample collection before the carcass was processed for meat. The same standardized collection process was used to limit variability between rabbits. The GI tract of each rabbit was isolated for sample collection. Within 1 to 2 minutes following euthanasia, the GI tract of each rabbit was isolated for sample collection before the carcass was processed for meat. The same standardized collection process was used to limit variability between rabbits. The GI tract of each rabbit was isolated for sample collection before the carcass was processed for meat. The same standardized collection process was used to limit variability between rabbits.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pellet P</th>
<th>Pellet E</th>
<th>Pellet M</th>
<th>Timothy hay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>9.5</td>
<td>5.9</td>
<td>11.5</td>
<td>11.3</td>
</tr>
<tr>
<td>Dry matter (%)</td>
<td>90.6</td>
<td>94.1</td>
<td>88.5</td>
<td>88.7</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>14.2</td>
<td>14.8</td>
<td>18.6</td>
<td>13.7</td>
</tr>
<tr>
<td>Acid hydrolysis fat (%)</td>
<td>2.0</td>
<td>3.0</td>
<td>7.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Crude fiber (%)</td>
<td>29.1</td>
<td>29.9</td>
<td>13.6</td>
<td>29.9</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>8.2</td>
<td>8.2</td>
<td>6.2</td>
<td>10.9</td>
</tr>
<tr>
<td>Sulfur (%)</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Phosphorous (%)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Potassium (%)</td>
<td>1.7</td>
<td>1.6</td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Magnesium (%)</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium (%)</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Iron (ppm)</td>
<td>770.4</td>
<td>571.7</td>
<td>309.6</td>
<td>135.3</td>
</tr>
<tr>
<td>Manganese (ppm)</td>
<td>101.0</td>
<td>106.3</td>
<td>98.4</td>
<td>103.3</td>
</tr>
<tr>
<td>Copper (ppm)</td>
<td>34.2</td>
<td>31.7</td>
<td>82.6</td>
<td>7.8</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>235.1</td>
<td>235.9</td>
<td>96.7</td>
<td>55.4</td>
</tr>
<tr>
<td>Total starch (%)</td>
<td>1.1</td>
<td>1.4</td>
<td>21.2</td>
<td>-</td>
</tr>
</tbody>
</table>

Nutrient analysis was conducted at Midwest Laboratories (Omaha, NE).

**Data availability**

The 16S rRNA gene and ITS1 region amplicon sequences were deposited in the Sequence Read Archive (SRA) under BioProject accession Nos. PRJNA274503 and PRJNA964774, respectively.

**Sequence analysis**

16S rRNA gene amplicon sequencing—Raw sequences were demultiplexed using the QIIME2 (v2021.4.0) command “cutadapt.” The demultiplexed sequences were then processed following the mothur (v1.43.0) MiSeq Standard Operating Procedure. A cutoff of zero ambiguities, a minimum length of 350 bp, and maximum homopolymer run of 8 bp was used for the “screen.seqs” command. The “chimera.vsearch” command was used in combination with the SILVA-gold content was removed for microbiota analysis. Each sample was placed in a labeled sterile plastic tube that was then immediately frozen at −20°C. The 5 collection sites were the stomach (midbody along greater curvature), small intestine (jejunum), cecum (midbody), ascending large intestine, and distal descending colon. In the distal descending colon, hard feces, rather than liquid ingesta, were collected for analysis.

**DNA extraction and amplicon sequencing**

DNA was extracted from the samples with a MoBio Power Soil DNA isolation kit (MoBio Laboratories) following the manufacturer’s instructions. The 16S rRNA gene PCR reactions were performed using the primers 27F (5’-CTGAGCCAMGAGCTACTACG-3’) and 319R (5’-CAGCKCGCAGCCTTACG-3’) in a 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. The ITS1 region was amplified using the primer ITS1F (5’-CTTGGTCATTTAGAGGAAGTAA-3’) in a PCR reaction using the AccuPrime high-fidelity Taq polymerase (Invitrogen). The reaction conditions were as follows: 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, 56°C for 60 seconds, and 72°C for 90 seconds, and then a final elongation step at 72°C for 10 minutes was performed. After amplification, PCR products were checked in a 2% agarose gel to determine the success of amplification and the relative intensity of bands. The samples were then pooled in equal molar ratios. Pooled samples were purified using calibrated Ampure XP (Agencourt) beads. The pooled and purified PCR products were used to prepare libraries following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed on an Illumina MiSeq sequencer using 300-bp read length paired-end sequencing for the 16S rRNA gene and 300-bp read length single read sequencing for the ITS1 region at MR DNA (Molecular Research LP).
reference database to remove chimeric sequences. After initial filtering, 3.6 million high-quality sequences were aligned and classified using the SILVA SSU NR database (v138). The sequences then underwent de novo clustering into operational taxonomic units (OTUs) with a cutoff of 99% 16S rRNA gene similarity. Samples with less than 3,000 sequences after quality control were removed, resulting in a loss of 17 samples. The shared and taxonomy files generated were imported into R for microbial community visualization using the Phylloseq (v1.38.0),24 vegan (v2.5.7),25 and ggplot2 (v3.4.0)26 packages.

**ITS1 region amplicon sequencing**—The raw sequences were uploaded to the Galaxy webserver and demultiplexed using “Barcode Splitter” in the Galaxy Toolbox. The mothur software (v1.43.0)22 was used for all subsequent sequence analysis. The demultiplexed sequences were randomly subsampled to 10,000 reads per sample. A cutoff of zero ambiguities, minimum length of 150 bp, and a maximum homopolymer run of 8 bp were used for the “screen.seqs” command. The “unique.seqs” command was used to minimize redundant sequences and the UNITEV8_sh_97 dataset provided by UNITE was used to classify sequences with the “classify.seqs” command.28

**Statistical analysis**

For the 16S rRNA dataset, all OTUs with less than 10 sequences were removed before statistical analysis. For both the 16S rRNA and ITS1 datasets, the microbial abundance data were normalized to minimize biases due to differences in sampling sequencing depth. The raw abundances were normalized by dividing by the corresponding sample library size, resulting in “relative abundance” values used for the analysis’s duration. Differences in beta diversity along the GI tract and across the various test diets were analyzed using Bray-Curtis distances and visualized through principal coordinate analysis (PCoA) plots. The assigned Bray-Curtis distances were also used to complete a permutational multivariate ANOVA (PERMANOVA) with the command “adonis2.” The “Pairwise.adonis” was used for pairwise comparisons between levels of the variable groups. The resulting P values were adjusted using Bonferroni’s multiple-comparison correction. To ensure that a significant finding in the PERMANOVA is not simply due to differences in variance, a permutational multivariate analysis of dispersion (PERMDISP2) was completed with the command “beta.disperser.”

Phylloseq was used to generate alpha-diversity measurements for the number of observed species (Observed), Chao species richness (Chao1), Simpson evenness, and Shannon diversity.24 The least-square means of all alpha-diversity measurements for each individual experimental group were compared using the PROC MIXED procedure in SAS. The resulting P values from the pairwise comparisons were corrected for multiple comparisons using Tukey honest significant difference test. Adjusted P values were considered significant if P < .05.

**Results**

**Bacterial microbiota**

**Microbiota in different sections of the GI tract**—After quality control, the average sequencing depth per sample was 17,287 with a standard deviation of 44,246 sequences. After removing OTUs with less than 10 sequences, 16,381 OTUs remained from the 128 samples of the 5 GI tract regions (n = 20 stomach, 24 small intestine, 28 cecum, 28 large intestine, and 28 fecal pellet; Supplementary Table S1). The OTUs were classified into 10 phyla with 21% of the sequences remaining unclassified. Interestingly, 50% of the stomach and small intestine sequences, but only 12% of the large intestine, cecum, and fecal sequences were unclassified. Firmicutes (Bacillota) (39%) and Bacteroidota (34%) were the most abundant phyla, followed by Actinobacteriota (2%; Supplementary Table S2). The PCoA plot (Figure 1) from all samples, regardless of diet, revealed a clear and separated clustering between the upper GI tract (stomach and small intestine) and lower GI tract (cecum, large intestine, and hard feces) microbial communities. Due to this distinct clustering, we decided to split the dataset into upper (foregut) and lower (hindgut) GI tracts for subsequent analysis. A significant PERMANOVA P value (P = < .0001) confirmed the rationality of grouping the samples this way. It is important to note that the PERMDISP2 P value of < 0.0001 suggests that the sampling locations have unequal dispersions.

The microbial communities of the foregut and hindgut, regardless of diet, showed distinct compositions when compared at a phylum level (Supplementary Figure S1). Firmicutes and Bacteroidota were found to be lower in abundance in the foregut (24% and 19%, respectively) than in the hindgut (44% and 39%, respectively). Actinobacteriota made up a larger percentage of the foregut microbiota (5%) than the hindgut (1%). When further characterized into genera, the most abundant genus for the foregut (10%) and the hindgut (21%) was an unclassified genus in the Bacteroidales order (Figure 2). *Alistipes* was the second most abundant genus for both the foregut and the hindgut accounting for 4% and 8% of total reads, respectively. The third most abundant genus differed between the foregut and the hindgut, with *Clostridia_UCG-014* accounting for 3% of total reads in the foregut and an unclassified genus in the Lachnospiraceae family accounting for 6% of total reads in the hindgut. The classifications for the 15 most abundant genera are shown (Supplementary Table S2).

Significant differences (P < .05) for alpha-diversity indexes were detected between the foregut and hindgut (Supplementary Figure S2). For example, the number of observed species, predicted species richness, and estimated diversity were higher in the foregut when compared to the hindgut (Supplementary Table S3).

**Effect of diet on the bacterial microbiota in the foregut and hindgut**—In addition to different
regions of the rabbit GI tract, an analysis of dietary impact on the microbial community was conducted. The number of samples per diet after quality control is shown (Supplementary Table S1). PCoA plots revealed the effects of the various diets on the composition of the foregut and hindgut microbiota (Figure 3). Generally, there were overlaps between most diets with little to no clustering evident visually. PERMANOVA results \((P < .05)\) point toward diet having a significant effect on the beta diversity of the GI tract microbiota. Pairwise contrasts between the diets are shown (Supplementary Table S4). In the hindgut, significant differences in community composition were observed for all 6 diet groups. When considering the same pellet type, Hay versus No Hay induced a significant change in community composition across all sample locations. Likewise, when the effect of feeding hay was kept constant, the inclusion or exclusion of pellets induced a significant difference in the microbiota. The differences in pellet type (E, M, or P), when the effect of feeding hay is kept constant, imposed a significant effect on the beta diversity in the hindgut but not in the foregut.

Significant differences in alpha-diversity indexes between diet groups were detected (Supplementary Figure S3). In the foregut, No Hay/Pellet E had lower diversity (Shannon, \(P < .05\)) and evenness (Simpson, \(P < .05\)) when compared to all diet groups except for No Hay/Pellet M. In the hindgut, No Hay/Pellet E had significantly lower numbers of observed species (Observed, \(P < .05\)) when compared against all other diets. No Hay/Pellet E also had significantly lower richness (Chao1, \(P < .05\)) when compared against all other diets except for No Hay/Pellet M. Additionally, No Hay/Pellet E had lower diversity (Shannon, \(P < .05\)) when compared to Hay/No Pellets, Hay/Pellet E, and No Hay/Pellet P (Supplementary Table S5).

**Eukaryotic microbiota**

**Microbiota in different sections of the GI tract**— After subsampling and quality control, 1,286,911 ITS1 sequences remained from 141 samples of the 5 GI tract regions (n = 28 stomach, 29 small intestine, 27 cecum, 29 large intestine, and 28 fecal pellet; Supplementary Table S6). The sequences were classified into 2 phyla with 54% of the sequences remaining unclassified. Across the entire GI tract, 41% of the sequences were classified into the Ascomycota phylum and 5% were classified into Basidiomycota. In general, the composition of the eukaryotic microbial communities of the stomach was significantly different from the other regions.
of the rabbit GI tract. In the stomach, the most abundant genus was *Candida* (68%), followed by *Malassezia* (8%) and *Humicola* (8%) (Figure 4). The abundance of *Candida* in the other regions of the GI tract was much lower. Excluding unclassified sequences, the 3 most abundant genera in the small intestine, large intestine, and cecum were *Candida* (17%, 11%, and 12%, respectively), an unclassified genus in the Ascomycota phylum (10%, 12%, and 13%, respectively), and *Malassezia* (1.4%, 5%, and 2%, respectively). A summary of the eukaryotic taxa on the genus level is shown (Supplementary Table S7). A comparison of the beta diversity of the eukaryotic microbial communities using PCoA plots (Supplementary Figure S4) did not reveal the same distinct clustering between the foregut and hindgut microbial communities as seen in the 16S rRNA sequencing data. PERMANOVA results point toward sampling location having a significant (*P* < .001) effect on beta diversity.

Overall, the eukaryotic microbiota of the rabbit GI tract had lower levels of alpha diversity compared to the bacterial community. The stomach had the lowest number of observed species when compared
to all other sampling locations (Observed, $P < .05$) and the lowest predicted species richness (Chao1, $P < .05$) when compared to the small intestine, large intestine, and fecal pellet samples (Supplementary Figure S5). The large intestine, cecum, and fecal pellets were all found to have lower estimated diversity (Shannon, $P < .05$) and evenness (Simpson, $P < .05$) than the stomach (Supplementary Table S8).

Figure 3—Beta diversity of foregut (A) and hindgut (B) rabbit bacterial microbial communities in response to different dietary treatments visualized as principal coordinate analysis plot. Distances between samples denote Bray-Curtis dissimilarity measures based on 16S rRNA gene amplicon sequencing. Only minor effects of the diets on the bacterial microbiota were revealed. A summary of the pairwise contrasts is provided (Supplementary Table S4).

Effect of diet on the eukaryotic microbiota—Diet had only a minor impact on the eukaryotic microbial communities in the different sections of the rabbit GI tract (Supplementary Figure S4). For the eukaryotic dataset, the number of samples per diet after quality control is shown (Supplementary Table S6). Similar to the 16S rRNA dataset, there was little to no clustering evident with most of the diets.
overlapping to some degree. When comparing the eukaryotic microbial communities of each diet on a whole-community level, PERMANOVA results showed a significant difference between the diets ($P < .001$) as well as the interaction of sampling location and diet ($P = .009$). Pairwise contrasts between the diets are shown (Supplementary Table S9). When comparing No Hay/Pellet M against Hay/No pellets ($P = .030$) and Hay/Pellet E ($P = .045$), there was a significant change in community composition across all sample locations. In the eukaryotic dataset, few significant differences in alpha-diversity indexes between diet groups were detected (Supplementary Figure S6). No Hay/Pellet M was found to have a lower number of observed species (Observed, $P = .011$) and estimated richness (Chao1, $P = .014$) when compared to Hay/No pellets. Alternatively, Hay/Pellet P had a higher number of observed species than No Hay/Pellet E (Observed, $P = .046$) and No Hay/Pellet M (Observed, $P = < .001$; Supplementary Table S10).

**Discussion**

In this study, the composition of the bacterial and eukaryotic intestinal microbiota of New Zealand rabbits was studied in different sections of the GI tract, and the response to different dietary treatments was evaluated. Despite their popularity as pets and meat animals, the GI tract microbiota of rabbits is still comparatively understudied.

**Bacterial microbiota of the rabbit foregut**

Interestingly, the majority (up to 50%) of obtained sequences from the foregut were unclassified bacteria. In contrast, the percentage of unclassified bacteria was much lower for the hindgut (less than 12% of sequences). Similar results have been reported recently; for example, Crowley et al determined that across 7 wild rabbit GI sampling sites, the mean number of unclassifiable reads was 36.2%. When considering 12 GI sampling sites from Belgian gray rabbits, Hu et al found that 52.4% of sequences were unclassifiable at a genus level. One explanation for the high percentage of unclassified bacterial reads in our study, specifically in the foregut, may be that the diversity of the rabbit microbiota for the foregut is not yet adequately represented in the current 16S rRNA gene reference sequence databases (SILVA v138 reference database). In contrast, 2 other studies that also surveyed the New Zealand rabbit GI tract microbiota did not find substantial amounts of unclassifiable sequences in their datasets. However, in these 2 studies, this can be linked to the use of closed-reference OTU clustering, resulting in a loss of all sequences that do not have homologs in the reference database.

Our study revealed distinct bacterial microbiota in the different sections of the rabbit GI tract. Above all, the microbiota in the foregut was clearly distinct from the lower GI tract sections. Our findings are consistent with several recent studies that showed that the bacterial rabbit microbiota is different between different sections of the GI tract.

It is well known that rabbit's consumption of cecotropes results in microorganisms from the hindgut appearing in the stomach. Given the low pH of the adult rabbit stomach (pH 1 to 2), many of the microorganisms present in the cecotropes are likely to survive and populate the stomach. This process, known as cecotrophy, is a unique feature of rabbits and is essential for the digestion of fibrous plant materials. The stomach is an important site for the digestion and fermentation of plant materials, and the presence of these microorganisms is crucial for the breakdown of hemicellulose and cellulose, which are major components of plant cell walls.

**Figure 4** — Relative abundance of rabbit eukaryotic microbial communities on genus level across different locations of the rabbit gastrointestinal tract based on internal transcribed spacer 1 region amplicon sequencing. Only the 10 most abundant genera are shown. The composition of the eukaryotic microbiota of the stomach was dominated by *Candida*, which showed much lower abundance in the other sections of the gastrointestinal tract.
killed. A limitation of DNA-based 16S rRNA ampli-con sequencing analysis is that it cannot distinguish between live and dead bacteria. Indeed, a recent study found that a lower percentage of bacteria in the rabbit foregut were alive (1% to 3%) compared to the hindgut (19% to 25%).

**Bacterial microbiota of the rabbit hindgut**

In the hindgut, our results are similar to those reported by Hu et al with Firmicutes and Bacteroidota being the 2 most abundant phyla. Interestingly, Hu et al found Firmicutes to account for 78% of hindgut sequences with our results only accounting for only 44%. This substantial change in relative abundance likely stems from differences in the locations of where the samples were taken within the hindgut (in Hu et al, the cecum, cecal appendix, rectum, and colon; in the current study, cecum (midbody), ascending large intestine, and hard feces). Compared to our findings, Cotozzolo et al described nearly identical distributions of Firmicutes (approx 44%) and Bacteroidota (approx 39%) in the hindgut despite using different methods of OTU clustering (de novo vs closed reference). Many of the same genera appear in both hindgut datasets such as Ruminococcaceae NK4A214 group, Akkermansia, and Christensenellaceae R-7 group. Markedly, the genus Alistipes (9%) does not appear in Cotozzolo et al. It is important to note that Lactobacillales (lactic acid bacteria) was minimally present in the rabbit GI tract in our study. This finding is also reflected in other studies surveying the rabbit GI tract microbiota. Thus, the efficacy of probiotics that contain lactic acid bacteria should be questioned.

**Influence of fasting and cecotrophy on rabbit microbiota studies**

One seemingly surprising finding of the current study was that the alpha diversity of the bacterial communities in the foregut was higher than in the hindgut. So far, most of the rabbit microbiota datasets have shown that the alpha diversity is higher in the hindgut. The most likely explanation for the higher alpha diversity we observed lies in the fact that our study did not fast rabbits or inhibit cecotrophy before sample collection as most previous studies did. It is plausible that the living and dead microorganisms present in the cecotropes are responsible for the increased alpha diversity in the foregut. One can assume that the input of the microorganisms originating from the rabbit’s cecotrope on the alpha diversity will be most prominent in the foregut, which has naturally lower species richness and diversity than the hindgut. As the digesta passes through the different sections of the GI tract, the contribution of the microorganisms from the cecotrope to general alpha diversity will likely decrease.

**Effect of diet on the bacterial microbiota along the GI tract**

The second objective of this study was to characterize how different diets influence the microbiota in various segments of the GI tract. As mentioned, Pellet P and Pellet E were both nutritionally complete timothy hay-based pellets sharing the same ingredient profile that underwent different processing (pelleting vs extrusion) whereas Pellet M was a muesli-type mixed pellet. The extrusion process can result in improved fiber quality and increased digestibility. However, in our study, given that the baseline amount of starch in the nutritional formula was relatively low (1% to 1.28%), the potential for extrusion to significantly impact digestibility is decreased. Notably, when comparing No Hay/Pellet E to No Hay/Pellet P, No Hay/Pellet E was linked to lower bacterial diversity (P < .05), and the 2 diets were found to have diverging bacterial compositions in the hindgut (P < .05).

Muesli diets (Pellet M) have been associated with negative health outcomes such as obesity, dental disease, and reduced GI motility. A study evaluating the impact of diet found that rabbits fed a muesli diet had smaller fecal pellets and reduced cecotrope ingestion. Thus, the muesli diet may predispose rabbits to digestive disorders. It is worth noting that while Pellet P and Pellet E did not significantly differ in nutrient composition, Pellet M differed significantly in several nutrients (Table 2). Protein was 28.5% higher in Pellet M compared to Pellet P and E, while fat and starch were 3 and 17 times higher in Pellet M, respectively. Furthermore, Pellet M contained less than half the amount of fiber as Pellet P and E. Our study also compared the effect of feeding or withholding hay alongside these diets. Field-dried timothy hay should be offered free choice and comprise a majority of the rabbit diet. It was not offered to the rabbits fed the muesli diet to simulate the “worst-case scenario” diet that pet rabbits may receive. Many muesli diets are nutritionally inappropriate and imbalanced, especially considering the selective feeding patterns of rabbits. Overall, diet had a smaller influence on the composition of the bacterial microbiota when compared to the effect size of the sampling location. This can be concluded from the lack of tight clustering by diet groups and the relatively small amount of variation explained by the PCoA plots (Figure 3). Furthermore, there was overlap between the various diets and no consistent pattern across all segments of the GI tract. Based on the pairwise contrasts on whole community level (Supplementary Table S4), feeding hay had a significant effect on beta diversity across the GI tract. The differences in pellet type (E, M, or P) also induced a significant change in the overall bacterial composition but only in the hindgut. Cremonesi et al also found that the beta diversity in the hindgut of New Zealand White rabbits (n = 28) was affected by dietary changes (Goji berry supplementation). These results are consistent with the hindgut microbiota’s pivotal role in enteric fermentation. A study comparing the cecal microbiota between healthy rabbits and those with diarrhea...
reported that *Alistipes* was more abundant in healthy rabbits. The authors also found that acetic acid production in the cecum was significantly lower in the rabbits with diarrhea. This reduction in *Alistipes* abundance coupled with a decrease in acetic acid production has been shown in human disease as well.42 Another study43 evaluating the effects of epizootic rabbit enteropathy on the rabbit cecal microbiota found a significant decrease in *Alistipes* in rabbits with epizootic rabbit enteropathy when compared to healthy rabbits. These findings indicate that the genus *Alistipes* might be positively correlated with rabbit health and cecal function. Considering the positive association *Alistipes* has with rabbit health, it should be noted that diet Hay/Pellet P resulted in the highest abundance of *Alistipes* across the GI tract.

A possible limitation of this study lies in the lack of histology and its length. The rabbits were given their respective diets exclusively for only 29 days. One study46 revealed the deleterious effects of feeding muesli kept their rabbits on the diet for 17 months. This suggests that the negative effects of an incomplete or unbalanced diet may develop after longer exposure than applied in the current study.

**Eukaryotic microbiota**

In addition to studying the bacterial microbiota, we were also interested in the eukaryotic microbiota in this study. For this, we applied ITS1 region amplicon sequencing. To the best of our knowledge, this study is the first to perform amplicon sequencing targeting the eukaryotic microbiota of the rabbit GI tract. In general, the eukaryotic microbiota of the rabbit GI tract is characterized by low diversity and dominated by a few highly abundant taxa. *Candida* was the most abundant genus across the GI tract. The *Candida* genus is a core member of the human GI microbiota and other animals like the horse.43,44 As this study is the first to perform amplicon sequencing on the eukaryotic rabbit GI microbiota, no sequencing data for comparison are currently available for rabbits. Although normally commensal, microbiota perturbation or a compromised immune system may trigger *Candida* overgrowth and pathogenesis.43 Similar to the 16S rRNA sequencing data, a high number of unclassified sequences was also detected for the eukaryotic microbiota. In contrast to the bacterial microbiota, the abundance of unclassified sequences was lowest in the stomach. Nevertheless, the stomach showed a distinct eukaryotic community compared to the other sections of the GI tract and was clearly dominated by *Candida*. Overall, when evaluating the effect of diet on the composition and the alpha diversity of the eukaryotic microbiota, there were very few significant comparisons between diet groups.

In summary, the different diets used in our study had a minimal effect overall on the intestinal microbiota of rabbits, but further studies, potentially longer in length, are needed to better understand the impact of these changes. Furthermore, the microbiota associated with the differing diets needs to be challenged by the comparison of healthy to diseased rabbits. Importantly, other essential members of the microbiota may need to be evaluated, such as viruses, as they also may have a significant impact on GI health.45–46 It may be possible that the diets induced more important shifts on a functional level, rather than a microbiota compositional level, and future studies will be useful to measure metabolomic changes in the GI tract.

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**Disclosures**

MRK is an employee of Oxbow Animal Health, CI was an employee of Oxbow Animal Health at the time of the study, and JG is a consultant to Oxbow Animal Health. No AI-assisted technologies were used in the generation of this manuscript.

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