Effect of Feeding Insoluble Fibre On The Microbiome And Metabolites of The Caecum And Faeces of Rabbits Recovering From Epizootic Rabbit Enteropathy Relative To Non-Infected Animals

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Abstract

Background:

This study aimed to investigate the effect of feeding insoluble fibre on the microbiome and metabolites of the caecum and faeces of rabbits recovering from epizootic rabbit enteropathy relative to non-infected animals.

Methods:

Animals which had either recovered from epizootic rabbit enteropathy, or ones which had never had epizootic rabbit enteropathy were fed on a diet of 32% or 36% neutral detergent fibre until they were 70 days of age. At this point short chain fatty acid and ammonia levels were measured in caecotroph and faecal samples and compared using 2x2 ANOVA. The microbial composition of the samples was also analysed using next generation sequencing and compared by PERMANOVA.

Results:

Caecotrophic samples from previously affected animals on lower fibre diets had higher short chain fatty acid content and higher species diversity index values for some indices ($P < 0.05$), although faecal samples showed lower species diversity levels ($P < 0.05$). In addition, PERMANOVA analyses demonstrated that differences were detected in the microbial composition of both faecal and caecotrophic samples depending on the disease status at the outset of the experiment ($P < 0.05$).

Conclusions:

The results of this work show that although there is some potential in the use of high fibre diets for treatment of animals which have had epizootic rabbit enteropathy, they are not able to produce the same digestive tract properties as those seen in animals which have never had the condition. This is true even after animals have recovered from epizootic rabbit enteropathy.

Introduction

Epizootic Rabbit Enteropathy (ERE) is a digestive disease that has negatively affected international rabbit production since the 1990s [1], with morbidities of up to 90% and mortalities of 80% [2]. ERE generally affects rabbits aged 3 to 7 weeks, reducing their daily food intake [3]. The earliest reports of ERE in Mexico were from late 2001 and early 2002 [4], and currently ERE represents 32% of the digestive disease cases involved in rabbit production in Mexico, with loss numbers of weaned rabbits being around 12–20% [5]. Although the aetiology of ERE has not been fully described and identified, the most common treatment of ERE involves using oral antibiotics [6], indicating a probable bacterial origin. In keeping with the assumption that ERE has a bacterial basis, we have shown that several bacterial phyla are associated with this disease [7], although no single organism was identified as being the cause. However, since there is a trend towards avoiding antibiotics in animals' diets, because there is a fear that overuse could help to promote resistant bacterial strains [8], this means that in large outbreaks the extensive use of the treatment could be problematic in the longer term. In addition, inclusion of antibiotics in the food can lead to changes in the diversity of the normal gastrointestinal microbiota causing a microbial imbalance, which can lead to dysbiosis [9]. The effects of antibiotics have already been investigated in rabbits, with different levels of effects being reported for different antibiotics [10, 11], with some showing a clear impact on the microbial populations. ERE is also characterized by a marked imbalance in the microbial community of the caecum [2, 7], which in turn changes the profile of the metabolites produced and absorbed in the caecum [12]. Combining these pieces of information has led to the hypothesis that an increase in the neutral detergent fibre (NDF) within the diet modulates the microbial communities, and the metabolites they produce. This then has the potential to reduce the effects of ERE in rabbits during the fattening stage of their production for meat. In most nutritional studies with herbivores, the change in dietary fibre content is assumed to impact mainly on the gut microbial community [13], although this in turn can influence the metabolites available for use by the host animal, and can have additional benefits to the host, such as influencing the host's anti-inflammatory signalling pathways [14].

While this increase in dietary fibre shows some promise as a means of impacting on ERE levels, the underpinning microbiology behind it is less clearly understood. Therefore, the objective of this study was to compare the effect of low (32% NDF) and high (36% NDF) fibre diets, in conjunction with rabbits either having previously shown, but having now recovered from ERE, versus those which have never shown any clinical signs of ERE, to determine the impact on the microbial community and the metabolites they produce.

This was performed by a combination of assessing short chain fatty acid (SCFA) concentrations produced as a means of examining metabolites arising from digestion of food, and the microbiome to evaluate the organisms involved in performing these roles.

Materials And Methods

The animal experimental work was carried out at the Amazcala Campus of the College of Natural Sciences, Autonomous University of Queretaro. Ethical conditions were approved using criteria established by the Ethics Advisory Committee for Animal Experimentation, University of Zaragoza [15]. The experimental protocol was approved by the Bioethics Committee of the College of Natural Sciences, Autonomous University of Queretaro (number 93FCN2016). Sixteen New Zealand rabbits aged 32-days-old were housed as groups of 4 rabbits in American type cages (90cm x 60cm x 40cm) equipped with feeders and drinking fountains. Rabbits were allocated in a 2x2 design experiment with, 4 animals per group. At the outset of the experimental work (i.e., when rabbits were 32 days old) animals were allocated to a group based on either having ERE or being clear of it (deemed healthy), and on a diet of 32% NDF which is recommended for the intestinal health of the rabbit [16], or 36% NDF which has been suggested for treatment of ERE [17]. Although it was not possible to balance animals for weight between ERE and healthy groups, due to ERE animals being significantly lighter at the outset, animals were balanced as closely as possible for dietary regimes. The diets were formulated with the same ingredients; alfalfa meal, canola meal, wheat bran, sunflower meal, sodium chloride, calcium carbonate, vitamins and minerals (Table 1). No antibiotics or anticoccidial drugs were included in the diet. All animals were given *ad libitum* access to food and water.

Table 1
Proximal chemical composition of diets ¹.

Proximal chemical analysis	Diet 1 (32%)	Diet 2 (36%)
Dry weight	91.2	91.3
Neutral Detergent Fibre (NDF)	31.7	35.8
Acid Detergent Fibre (ADF)	23.5	25.6
Hemi cellulose	8.2	10.2
Crude Protein	15.8	16.0
Ash	8.8	8.6
Lignin	7.8	7.8
Fat Content	1.7	1.8
Phosphorus	0.85	0.86
Calcium	1.42	1.43
Selenium	0.35	0.35
Vitamin A UI/kg	5167	5147
Vitamin D3 UI/kg	974	974
Vitamin E mg/kg	53.3	55.2
Vitamin B12 mg/kg	4.3	4.5

¹ Data were obtained in the Animal Nutrition Laboratory, Autonomous University of Querétaro following the AOAC manual [18]

Table 2

Volatile fatty acid, ammonia and pH values for cecotroph samples collected. Mean and standard deviation values are shown.

Metabolite / Parameter	T1	T2	T3	T4	P value - Diet	P value - Disease	P value - interaction
Total VFA $\mu\text{mol/g}$	9.75 $\pm$ 0.14	13.35 $\pm$ 0.16	14.36 $\pm$ 0.19	10.08 $\pm$ 0.16	0.034	0.001	< 0.001
Acetic acid $\mu\text{mol/g}$	3.65 $\pm$ 0.10	4.35 $\pm$ 0.01	4.95 $\pm$ 0.09	3.82 $\pm$ 0.10	0.028	0.001	< 0.001
Propionic acid $\mu\text{mol/g}$	1.71 $\pm$ 0.09	2.68 $\pm$ 0.03	2.00 $\pm$ 0.10	1.85 $\pm$ 0.08	0.007	< 0.001	< 0.001
Butyric acid $\mu\text{mol/g}$	1.24 $\pm$ 0.03	2.76 $\pm$ 0.02	1.25 $\pm$ 0.02	1.24 $\pm$ 0.02	< 0.001	< 0.001	< 0.001
Isobutyrate $\mu\text{mol/g}$	0.43 $\pm$ 0.02	0.41 $\pm$ 0.01	0.97 $\pm$ 0.02	0.43 $\pm$ 0.02	< 0.001	< 0.001	< 0.001
Valeric acid $\mu\text{mol/g}$	1.02 $\pm$ 0.01	1.32 $\pm$ 0.01	2.00 $\pm$ 0.04	1.03 $\pm$ 0.02	< 0.001	< 0.001	< 0.001
Isovalerate $\mu\text{mol/g}$	0.90 $\pm$ 0.01	0.82 $\pm$ 0.01	1.27 $\pm$ 0.02	0.97 $\pm$ 0.01	< 0.001	< 0.001	< 0.001
Ammonia %	0.06 $\pm$ 0.01	0.04 $\pm$ 0.01	0.16 $\pm$ 0.07	0.07 $\pm$ 0.01	NS	NS	NS
pH	6.58 $\pm$ 0.17	6.46 $\pm$ 0.07	6.43 $\pm$ 0.06	6.43 $\pm$ 0.13	NS	NS	NS

T1 = healthy animals on a diet with 32% NDF; T2 = healthy animals on a diet with 36% NDF; T3 = animals which had ERE at the outset on a diet with 32% NDF; and T4 = animals which had ERE at the outset on a diet with 36% NDF. NS denotes values were not significantly different: i.e. $P > 0.05$.

The experimental groups were designated as follows: T1 = healthy with 32% NDF; T2 = healthy with 36% NDF; T3 = ERE clinical signs with 32% NDF; and T4 = ERE clinical signs with 36% NDF. The presence of diarrhoea (an ERE clinical sign) was checked by measuring the severity (0 = no diarrhoea, 1 = slight, 2 = moderate, 3 = severe). At the start of the experimental work, all animals categorized as having ERE were around the 1 to 2 boundaries of severity. Information regarding productivity values which were recorded or measured were: initial weight (IW); weekly weight gain; final weight (FW); food consumption (FC) and daily weight gain (DWG).

At 70 days of age, samples of caecotrophs were collected from all rabbits. Collections were achieved by temporarily (07:00 a.m. to 11:00 a.m.) moving rabbits to individual cages and placing polyurethane conical collars (6cm narrow diameter and 27cm wide diameter) on the necks of the rabbits to prevent them ingesting their caecotrophs. Caecotrophs were identified on the basis of their consistency and appearance; they were expelled in the form of clusters with a shiny appearance. During this collection period a sample of faecal waste was also collected, with these being identified as dry and rough, and expelled as individual pellets. The pH of the caecotrophs was measured following collection of 2g samples and mixing with 5 ml of distilled water.

For the determination of SCFA content, 2 g of sample were collected in a 15 ml conical centrifuge tube with 5 ml of HPLC-grade acetone and 1 ml of HPLC-grade water. This was mixed by vortexing for 15 seconds, followed by addition of 100 μl of 85% [w/v] phosphoric acid and the solution was mixed again by vortexing for 10 seconds and centrifuged for 23 min at 21,000 g at 4°C; the supernatant was separated by passing through a 0.20 μm filter. The filtrate was subsequently placed in amber vials with a lid to carry out the liquid chromatography analysis and stored at -80°C until used. Liquid chromatography was performed using an Agilent 6890 gas chromatograph with a flame ionization detector and a DB-FFAP 30 m $\times$ 0.25 mm $\times$ 0.25 μm column (Agilent Technologies, Wilmington, North Carolina, USA) for the analysis of the filtered supernatant with a 0.2 μm syringe filter [19]. Conditions followed a split mode (20:1) with an inlet temperature of 220°C and pressure of 168 kPa. A volume of 1 μl was injected, with a constant flow of 1.4 ml/min, using helium as a carrier. The detector temperature was 250°C. The column initially operated at 35°C, with a hold period of 30 sec, before increasing at 10°C/min until 90°C was reached. This temperature was held for 2 min, and was followed by an increase of 12°C/min until 230°C was reached and this was held for 6 min.

For the analysis of ammonium content, a Kjeldahl approach was used [18, 20]. Initially 2 g of caecotrophs were placed in a Kjeldahl flask to which 200 ml of distilled water at 20°C was added, followed by addition of 4 glass boiling beads of 3 mm diameter (PROLAB) and 2 g of MgO. This was then distilled using a recovery rate of 99.5%. The solution was neutralized by addition of 20 ml 0.05 M sulfuric acid for 4 min. The products of the distillation process were collected in 500 ml Erlenmeyer flasks. To each flask 100ml of 2% [w/v] H₃BO₃ and 3 drops of indicator solution (composed of 20ml of 0.05% [w/v] methyl red and 4ml of 0.2% [w/v] methylene blue) were added to ensure the solution was neutral. Titrations were performed using 0.1M H₂SO₄ which was dripped with a graduated pipette until the colour of the indicator solution changed. These values were compared against a blank (i.e., without caecotrophs), and the N% of ammonia value was calculated using the equation below:

$$\text{Equation 1: } N\% = [(A-B) * 0.014 * N * 100] / M$$

Where: A and B are the volumes of 0.1M H₂SO₄ (ml) used in the titration of the caecotroph and control samples respectively, N is the normality of H₂SO₄ and M is the weight of the caecotroph sample measured in grams.

The data were analysed using a 2x2 ANOVA factorial design, using IBM SPSS Statistics for Windows (Version 19.0. Armonk, NY: IBM Corp). In the case of values which showed significant differences, *post hoc* analyses were performed.

The rest of the caecotrophs were placed in 1.5 ml tubes which were free of both RNases and DNases and immediately frozen in liquid nitrogen. They were then transported to the Veterinary Microbiology Laboratory of the College of Natural Sciences, Autonomous University of Queretaro, where they were stored at -80°C. DNA was extracted from caecotrophs using Qiagen stool kits with silica and micro pearl columns, according to the manufacturer's protocol. All caecotrophic and faecal samples (i.e., 16 caecotrophic and 16 faecal samples) were used for sequence analysis.

Next generation DNA sequencing of the V3-V4 region of the 16S *rRNA* gene was performed on an Illumina MiSeq® System by the Sequencing and Identification Unit, National Institute of Genomic Medicine, Mexico City. The sequencing process consisted of amplicon processing, Ampure beads purification, nucleic acid quantification by fluorescence, automated chip electrophoresis and loading onto the Illumina MiSeq system. The library preparation was performed by the bridge method, with the Nextera™ Flexible DNA Library Prep Kit (Illumina Inc. USA). Sequences were filtered with Trimmomatic [21] to remove low quality reads, followed by removal of the sequencing adapters, leaving reads of ~ 480bp. A further quality inspection was performed with FastQC [22]: involving trimming; filtering; identifying unique sequences; constructing tables of operational taxonomic units; removing chimeric sequences; assigning taxonomic identification; determining abundance and diversity using the R software Project and the specialized library DADA2 [23]. In addition, PERMANOVA was carried out following the methodology described in the manual of the Vegan package [24]. In the case of the analysis of bacterial diversity this was assessed by calculating the Shannon Index, the Simpson Index, the Chao1 Index and the Abundance-based Coverage Estimators (ACE), following the methodology of Oksanen et al. [24]. The data obtained were compared using a 2x2 factorial design ANOVA using IBM SPSS Statistics for Windows (Version 19.0. Armonk, NY: IBM Corp). A rarefaction curve was produced with the rarefy script: Rarefaction Species Richness in Vegan: Community Ecology Package [24].

Results

The initial weight of the rabbits which had had ERE was significantly lower ($P < 0.001$) than the weight of those which were healthy (Fig. 1). This observation continued throughout the experiment, even though animals had recovered, with rabbits which had originally had ERE being significantly lighter than those which were healthy ($P < 0.001$). The daily weight gain was significantly higher ($P = 0.023$) in healthy animals, but only those fed on the 36% NDF diet.

Rabbits in groups T3 and T4 presented with light (severity = 1) or moderate (severity = 2) diarrhoea on the first day of the trial and during the subsequent 3 days but showed a reduction in symptoms over the next few days. By day 9 none of the animals had diarrhoea or stool mucus and no further symptoms were detected for the rest of the experiment. Groups T1 and T2 did not present diarrhoea or signs associated with ERE during the duration of the experiment.

3.1. Caecotrophic fermentation

The results of the fermentation parameters are shown in Fig. 2 and Table 3. No statistically significant differences ($P > 0.05$) were observed between the groups for caecotroph pH values. In the case of the percentage of ammonia present, values were not significantly different ($P > 0.05$). However, there was a significant difference ($P < 0.001$) in the total production of SCFAs, with groups T2 and T3 having higher concentrations. Moreover, there were disease dependent differences ($P < 0.001$) as well as a differences in the interaction of the variables ($P < 0.001$).

Table 3

Summary of sequences identified and comparison of microbial diversity indices in cecal samples. Mean values are shown, with standard deviations shown in parenthesis.

	T1	T2	T3	T4	P value - Diet	P value - Disease	P value - interaction
Number of Sequences	164,382	259,136	272,098	150,978			
Number of Operational Taxonomic Units (OTU)	467	462	767	471			
Number of Identifiable genera	33	35	72	35			
Shannon Index	4.66 (0.53)	4.89 (0.54)	10.02 (3.05)	5.38 (1.14)	0.022	0.004	0.013
Simpson Index	4.01 (0.24)	4.02 (0.28)	4.33 (0.28)	4.07 (0.21)	0.349	0.165	0.312
Abundance-based Coverage Estimators (ACE)	0.40 (0.40)	0.56 (0.32)	1.42 (0.76)	1.23 (0.67)	0.963	0.011	0.544
Chao1 Index	165 (34.4)	159 (29.5)	464 (205.2)	178 (55.0)	0.020	0.013	0.024
T1 = healthy animals on a diet with 32% NDF; T2 = healthy animals on a diet with 36% NDF; T3 = animals which had ERE at the outset on a diet with 32% NDF; and T4 = animals which had ERE at the outset on a diet with 36% NDF.							

3.2. Caecotrophic samples

A total of 846,594 DNA sequences were obtained from the 16 caecotrophic samples, comprising 80 bacterial genera. This information is summarised in Table 4, with more detailed information contained in Table S1. Following rarefaction curve analysis, an almost horizontal asymptotic curve was obtained for almost all individuals (Fig. 3), thereby indicating that Operational Taxonomic Unit (OTU) detection had reached its peak.

Table 4

Summary of sequences identified and comparison of microbial diversity indices in fecal samples. Mean values are shown, with standard deviations shown in parenthesis.

	T1	T2	T3	T4	P value - Diet	P value - Disease	P value - interaction
Number of Sequences	256,412	268,214	384,230	243,146			
Number of Operational Taxonomic Units (OTU)	818	1018	842	858			
Number of Identifiable genera	63	64	66	58			
Shannon Index	9.11 (1.53)	11.50 (1.21)	9.61 (0.38)	9.74 (1.28)	0.055	0.306	0.081
Simpson Index	4.02 (0.25)	4.58 (0.19)	2.82 (0.87)	3.92 (0.16)	0.004	0.002	0.279
Abundance-based Coverage Estimators (ACE)	0.66 (0.30)	1.87 (0.87)	0.84 (0.51)	0.64 (0.36)	0.092	0.082	0.025
Chao1 Index	375 (105.6)	575 (103.3)	399 (38.4)	415 (93.7)	0.033	0.155	0.062
T1 (healthy animals on a diet with 32% NDF) = healthy animals on a diet with 32% NDF; T2 (healthy animals on a diet with 32% NDF) = healthy animals on a diet with 36% NDF; T3 (animals which had ERE at the outset on a diet with 32% NDF) = animals showing symptoms of ERE on a diet with 32% NDF; and T4 (animals which had ERE at the outset on a diet with 36% NDF) = ERE animals showing symptoms of ERE on a diet with 36% NDF.							

Depending on the index studied, significantly higher levels of diversity were seen in the animals originally with ERE on the low fibre diet relative to the other caecotrophic samples. Both the Shannon and Chao1 indices showed significantly higher values ($P = 0.022$ and $P = 0.020$ respectively), but no such difference was seen for ACE and Simpson values. This was true for the differences between diets (low versus high fibre), health status (healthy versus initially ERE infected) and for the diet*health interactions in all cases. All indices for caecotrophic samples were highest in those collected from animals which had initially had ERE on low fibre diets, although in the case of the Simpson and ACE indices these were not significantly higher.

3.3. Faecal samples

A total of 1,152,002 DNA sequences were obtained, comprising 90 bacterial genera. This information is summarised in Table 5, with more detailed information contained in Table S1. Following rarefaction curve analysis, an almost horizontal asymptotic curve was obtained for almost all individuals (Fig. 4) thereby indicating that OTU detection was reaching its peak.

Table 5

Percentage abundance of the major phyla based on next generation sequence analysis; numbers in parenthesis are SEM values.

Cecal Samples					Fecal Samples			
Phylum	T1	T2	T3	T4	T1	T2	T3	T4
Actinobacteria	-	-	0.007 (0.007) ^a	-	0.04 (0.02) ^b	0.04 (0.01) ^b	0.08 (0.03) ^b	0.04 (0.02) ^b
Bacteroidetes	10.2 (0.22) ^{ab}	8.8 (0.28) ^a	12.0 (0.59) ^b	10.7 (0.60) ^{ab}	5.6 (0.59) ^c	6.7 (0.16) ^d	6.0 (1.28) ^{cd}	5.2 (1.23) ^{cd}
Cyanobacteria	8.0 (0.54) ^{ab}	7.3 (0.25) ^a	10.2 (0.93) ^b	7.7 (1.08) ^{ab}	5.3 (0.61) ^a	6.3 (0.13) ^b	5.8 (1.32) ^{ab}	4.9 (1.09) ^{ab}
Epsilonbacteraeota	-	-	0.004 (0.002)	-	0.007 (0.007)	0.013 (0.008)	-	0.008 (0.008)
Euryarchaeota	-	-	-	-	-	0.011 (0.008)	0.002 (0.002)	0.004 (0.002)
Firmicutes	49.2 (1.6) ^a	55.9 (2.1) ^a	43.3 (0.8) ^a	47.0 (2.9) ^a	67.6 (3.7) ^b	61.0 (0.89) ^c	64.8 (7.8) ^{bc}	69.4 (7.0) ^{bc}
Lentisphaerae	0.004 (0.004)	-	0.002 (0.002)	-	-	-	-	-
Not Identified	10.2 (0.2) ^{ab}	8.7 (0.3) ^a	12.0 (0.6) ^b	10.7 (0.6) ^{ab}	5.3 (0.6) ^c	6.3 (0.1) ^d	5.7 (1.3) ^{cd}	4.9 (1.1) ^{cd}
Patescibacteria	3.6 (1.3) ^a	3.2 (1.0) ^a	7.0 (1.7) ^a	3.4 (1.9) ^a	0.003 (0.003) ^b	-	0.008 (0.008) ^b	0.010 (0.010) ^b
Proteobacteria	7.9 (0.9) ^{ab}	6.4 (0.8) ^a	7.7 (1.5) ^b	8.8 (1.1) ^{ab}	5.3 (0.6) ^c	6.5 (0.2) ^d	5.8 (1.3) ^{cd}	5.1 (1.1) ^{cd}
Synergistetes	-	-	0.013 (0.013) ^a	0.199 (0.118) ^a	0.003 (0.003) ^b	-	0.011 (0.006) ^b	0.005 (0.005) ^b
Tenericutes	6.8 (1.2)	5.8 (1.0)	4.9 (1.4)	7.3 (1.5)	5.5 (0.6)	6.6 (0.2)	5.9 (1.3)	5.2 (1.2)
Verrucomicrobia	4.2 (0.9) ^{abc}	4.0 (0.8) ^{abc}	3.1 (0.8) ^{abc}	4.2 (1.1) ^{abd}	5.4 (0.61) ^{de}	6.6 (0.15) ^{abcd}	5.9 (1.29) ^{abcd}	5.2 (1.20) ^{acbcd}
T1 = healthy animals on a diet with 32% NDF; T2 = healthy animals on a diet with 36% NDF; T3 = animals which had ERE at the outset on a diet with 32% NDF; and T4 = animals which had ERE at the outset on a diet with 36% NDF.								

Depending on the index studied, significantly different diversities were observed in the faecal samples. Shannon indices showed no significant differences for comparisons between diets, health status or diet*health interactions. Simpson diversity values were higher in healthy animals relative to initially ERE infected animals, as well as animals on higher fibre diets than on low fibre diets, but no significant differences in the diet*health interactions were observed. No significant differences were detected for ACE values for dietary differences, or health status differences, although differences were detected for diet*health interactions, with healthy animals on a high fibre diet showing a significantly higher index value than the other 3 groups. Finally the Chao1 index was significantly higher for animals on high fibre diets, but there was no significant difference based on health status or diet*health interactions. In the case of the faecal samples all indices were highest for those from the healthy animals on high fibre diets, although this was only significantly so for the ACE values.

3.4. Sequence comparisons in caecotrophic and faecal samples

The most abundant phyla in all caecotrophic groups were Firmicutes and Bacteroidetes (Fig. 5 and Table 6). Typically, the most abundant families were Ruminococcaceae, Bacteroidaceae, Clostridiales_vadinBB60 group and Barnesiellaceae (Table S1).

However there was variation within groups regarding the abundance of OTUs and following PERMANOVA testing there was a difference when comparing the healthy with the originally ERE affected ($P < 0.05$). The individual dietary comparisons ($P = 0.080$) and health*diet interactions ($P = 0.600$) did not present significant differences.

The most abundant phylum in all faecal groups was the Firmicutes (Fig. 6 and Table 6). Typically, the most abundant families were Ruminococcaceae, Verrucomicrobiaceae, Clostridiaceae and Lachnospiraceae (Table S1). However there was variation within groups regarding the abundance of OTUs and following PERMANOVA testing there were differences when comparing the healthy with the originally ERE affected ($P = 0.03$), by diet ($P = 0.01$) and healthy versus ERE infected ($P = 0.03$).

Discussion

4.1. General observations

A recent review of factors associated with ERE, and how to treat it [25] included the use of elevated fibre levels as one mechanism of treatment. This approach constituted the underpinning experimental design of this work. In addition to examining fibre levels, the presence or absence of ERE at the outset of the experiment impacted on the productive parameters evaluated in this experiment. This effect of ERE is in agreement with a previous study [26], where it was reported that rabbits that survive ERE show lower weights compared to healthy rabbits in the same system. In the case of food consumption, groups with initial signs of ERE had a lower intake than healthy animals (data not shown). Those groups which were healthy at the outset, T1 and T2, had values in keeping with those reported previously for animals on diets with lower ($\leq 30\%$) NDF [27], which are associated with a shorter retention time in the caecum, and in turn an increase in food intake [28]. By way of contrast the current work looks at the differences in the gut microbiome, and associated metabolome, in rabbits which had recovered from ERE versus those which have never suffered from it. As such it is not an investigation of the causal agents of ERE, but rather how closely animals respond and return to normality after an infection, together with the role that different dietary fibre levels can promote. For this reason, we had an interest in examining both the hard faeces which are defaecated and lost, and also the caecotrophs which are re-ingested, as the post-ERE recovery is poorly understood in both.

Animals which had ERE and were used on this trial had no more than level 2 symptoms. The reason for this was to maximise the likelihood that animals would recover from ERE and then survive to the end point of the experiment (70 day old), as those showing severe symptoms (level 3) are much less likely to survive, and our interests lay in examining the similarities between recovered animals and those which had never had symptoms. The effect that the diets had on diarrhoea and mucus in faeces is consistent with that reported previously [29], although the previous work involved a reduction of NDF from 30–25%. This produced an increase in the biodiversity of the microbiota in the ileum, but reduced biodiversity in the caecum. In addition to this, a decrease in the presence of diarrhoea (from 31–0.8%) has been reported at day 20 in rabbits with ERE supplemented with 33.6% of NDF [30]. This was associated with a change in the caecotrophic ecosystem, leading to a higher production of volatile fatty acids [30], because of a change in the microbial profile of the caecum.

4.2. Metabolites

The concentrations of ammonia observed in groups T1 and T2 are similar to those obtained previously [31], with clinically healthy rabbits. These values are associated with a high absorption of ammonia from the intestinal mucosa of the rabbit [12]. Here the ERE groups (T3 and T4) showed higher values than those reported in clinically healthy rabbits, although these differences were not significantly different. Differences might have been expected under these circumstances, as this could be associated with two different factors triggered by the ERE. Firstly, there could have been damage to the intestinal mucosa, where apoptosis of epithelial cells has been observed, together with atrophy, fusion and destruction of intestinal villi, and a reduced infiltration of inflammatory cells [32]. This resulted in poorer absorption of digestive products into the bloodstream. Secondly, changes in the intestinal microbiota can also have an impact [33, 2] with an increase in the abundance of bacteria of the genus *Clostridium* causing lesions in the intestinal mucosa due to the toxins generated [34] as well as an increase in ureolytic activity [32, 34].

The indices for diversity values observed are in keeping with previous experimental work, where changes in the concentration of NDF led to changes in clinically healthy rabbits [4]. Regarding the changes in the richness of the microbiota, these agree with that reported in previous studies [2, 17, 33] for the ERE group that received the diet with 36% NDF. However, the genera affected in our

study differ from those reported in previous studies [2, 33]. Use of 36% NDF makes it possible to manipulate the caecotrophic microbiota of rabbits with clinical ERE. This in turn may be a reflection of a decrease in proteolytic activity and an increase in the hemicellulolytic communities of rabbits which are positive for ERE.

4.3. Microbial populations

It has already been documented that the rabbit gut microbial community is different from that of other domesticated herbivores such as ruminants or horses [36, 37], with differences seen between different areas of the digestive tract of the rabbit [38]. In addition, previous next generation sequencing approaches have shown differences between caecotrophic samples from 4 virulent (i.e., affected by ERE) versus 2 non-virulent animals [39]. In the current work, in terms of population diversity of caecotrophic samples, the highest value of the Shannon Index (Table 4) was seen in the T3 group (animals which had originally had ERE and were fed on the low fibre diet). This is particularly interesting as the Shannon index places a high emphasis on species richness [36], but without the same emphasis on evenness that is seen in the Simpson index. This would suggest that there is still a very diverse population present, but that the spread of species has been affected. In keeping with this hypothesis, it would be anticipated that T3 would probably also have a higher Chao1 value, which measures numbers in the population. This is indeed the case (Table 4). These differences manifested at the level of the dietary consideration ($P = 0.022$ and $P = 0.020$ for Shannon and Chao1 respectively); ERE status ($P = 0.004$ and $P = 0.013$ for Shannon and Chao1 respectively); and the dietary*disease interaction ($P = 0.013$ and $P = 0.024$ for Shannon and Chao1 respectively).

It is also interesting to note that when the Abundance-based Coverage Estimators (ACE) index is used, there is a difference between the samples from the animals which had previously had ERE relative to those which had never had it. The ACE index is used to measure the number of rare OTUs detected [40], and the data in Table 4 suggest that there are differences in rare OTUs on the basis of whether an animal has had ERE or not. Moreover, there were no differences detected with the Simpson index which looks at abundance of dominant species [41], therefore suggesting that there has been no significant change in the number of abundant species. Thus, in some respects the post-ERE microbial population is still different from that seen in animals which have never had the condition. Interestingly the faecal samples did not show a similar pattern (Table 5), with higher Simpson Indices in T1 and T2 samples (both groups have animals which have never suffered from ERE) indicating certain species are more dominant than others in this group relative to those which had been seen in the animals which had originally suffered from ERE ($P = 0.004$). This pattern was also seen for animals with the higher fibre content in their diet ($P = 0.002$) with an increase in species dominance. However, this was not observed for a dietary*disease interaction ($P = 0.279$). Interestingly the Chao1 value is also higher for the animals which never suffered from ERE ($P = 0.033$), and this is a measure of the number of species within the population [42]. This would suggest that although there is a general level of dominance of species there are also a larger number of species present, possibly with some at approaching trace levels. The largest Chao1 value is in T2 (animals which had never had ERE and were fed a 36% NDF diet), although this does not allow a significant difference to be seen in terms of dietary*disease interaction ($P = 0.062$). However, a significant difference in terms of this interaction was seen using the Abundance-based Coverage Estimator ($P = 0.025$), which is a measure of the detection of species above a certain minimum baseline [42]. This is despite not showing significant differences for either the diet ($P = 0.092$) or disease status ($P = 0.082$). However, no significant differences were observed for Shannon Indices which consider species richness [36].

Taken together, these results suggest differences between caecotrophic and faecal samples in terms of how they continue to be affected by the host animal having had ERE. In the caecotroph samples from animals which had previously had ERE and were fed on a lower fibre diet showed an increased species richness (Shannon Index) and diversity (Chao1) values without an increase in dominance of certain species. Conversely the faecal samples showed an ERE effect when using the Simpson Index and a similar pattern for Chao1 values. In addition, the higher fibre diet from animals which had never had ERE showed a higher level of low abundance OTUs (Abundance-based Coverage Estimator) in faecal samples. In terms of identifying an individual species associated with the condition, as with other work, instead we identify more than one organism which has been affected significantly in terms of its abundance even after the animal has recovered from ERE symptoms. What is clear though is that differences detected in sequence data from the caecotrophic samples do not translate to the equivalent information in faecal samples (Table 6). In this context it is evident that there was no large-scale change to the organisms involved in causing ERE persisting at the level of the phylum. Clearly any differences must be at a lower taxonomic level. It remains impossible to pinpoint a single organism as the causal agent, although one of the most interesting is probably *Akkermansia muciniphila* which has high abundance in the ERE positive groups in previous work. This is a microorganism that has been also associated with animals fed on lower fibre and

which developed ERE [43] and we have previously reported as an important microorganism related with ERE in Mexico [7]. It is a member of the phylum Verrucomicrobia, a phylum which showed no obvious patterns in terms of abundance between the various samples collected in terms of caecotroph versus faecal, post-recovery from ERE versus never having been infected, and the two levels of fibre in the diet (Table 6). Moreover, *Akkermansia* was the only genus observed within the sequences identified as belonging to the phylum Verrucomicrobia (Table S1).

Another organism of interest from our previous work [7] *Cloacibacillus porcorum* (which is a member of the phylum Synergistes) was not detected in the current work as the only genus detected here from the phylum Synergistes was *Synergistes* (Table S1). Members of this phylum were present at very low levels in all faecal samples (irrespective of ERE status) and caecotroph samples which had recovered from ERE, but not in those which had never had ERE. However, specifically for *Cloacibacillus porcorum*, its absence in the current work may be an indication that it has been lost following recovery from ERE in those animals which had previously had it.

The final group of organisms which we highlighted as a potential causal agent for ERE were members of the genus *Clostridium*. Although the Firmicutes were present as one of the most abundant phyla (in some cases the most abundant phylum) in all samples, this was due primarily to the high abundance of members of the Class Ruminococcaceae. In terms of members of the genus *Clostridium*, these were only present at very low levels in faecal samples and in the caecotrophs collected from previously ERE rabbits on the low fibre diet. Furthermore, due to the wide range of species of bacteria within the genus *Clostridium*, these cannot be assumed to be the same species as had been identified in the previous work.

Therefore, none of the organisms we had highlighted in our previous work [7] as being potential causal agents for ERE could be detected in the samples here, even for animals which had recovered from ERE. This means that, within the limits of detection here, those organisms were not present in animals which had recovered from ERE.

In the wider context, our data identify similar levels of Bacteroides but lower levels of Firmicutes than were seen in previous work for both faecal and caecal samples [e.g., 44], although these authors used a much lower fibre content in the diet (18.7%) than either of our sets of conditions. However, it is worth noting that in our previous work [7] we observed a lower abundance of Firmicutes than has been published previously, which we speculated may be, at least in part, be due to the removal of a PCR step prior to sequencing; as PCR stages can introduce a bias in terms of amplification. The values we see here for Firmicutes in faecal samples are in keeping with those we reported previously. However, the caecotroph values are lower than that, although similar to those seen in wild rabbits [38]. Due to the differences detected in terms of Firmicutes relative to other work this makes any inferences to the Firmicutes : Bacteroides ratio, or the impact SCFA production relative to Firmicute numbers, difficult to achieve.

Conclusions

In conclusion, even though the rabbits which had previously suffered from ERE had made a full recovery in terms of no longer having diarrhoea, they are still smaller than animals which never had it and also have a different gut microbiome and metabolome produced by these organisms.

Abbreviations Used Within The Manuscript

ACE (Abundance-based Coverage Estimators); ANOVA (Analysis of Variance); DWG (Daily Weight Gain); ERE (Epizootic Rabbit Enteropathy); FC (Food Consumption); FW (Final Weight); HPLC (High Performance Liquid Chromatography); IW (Initial Weight); NDF (Neutral Detergent Fibre); OTU (Operational Taxonomic Unit); SCFA (Short Chain Fatty Acid).

Declarations

Ethical Approval and Consent to participate:

The experimental protocol was approved by the Bioethics Committee of the College of Natural Sciences, Autonomous University of Queretaro (number 93FCN2016).

Consent for publication:

All authors have read and agreed to the published version of the manuscript”.

Availability of supporting data:

Abundance of sequence information is contained within supplementary data (Table S1).

Competing interests:

The authors have no conflicting or competing interests to declare.

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Author Contributions:

Conceptualization, A.M.O.-R. and N.R.McE.; Methodology, A.M.O.-R., G.M.-L.; G.M.N.-M., J. J.M.; Bioinformatic and statistical analysis X-H.D.P.-P., R.C.A.-M.; Formal analysis, X.-H.D.P.-P., G.M.-L.; Resources, A.M.O.-R., G.M.-L.; Data curation, X-H.D.P.-P.; Writing—original draft preparation, XPPP, N.R.McE.; Writing—review and editing, N.R.McE. and A.M.O.-R.; Funding acquisition, A.M.O.-R., X-H.D.P.-P.

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Not applicable.

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Tables

Table 6 is not available with this version.

Figures

Figure 1

The mean weight of rabbits per group. T1 = healthy animals with a 31% NDF diet; T2 = healthy animals with a 36% NDF diet; T3 = animals which had ERE at the outset on a diet with 31% NDF; and T4 = animals which had ERE at the outset with a 36% NDF diet

Figure 2

Volatile fatty acid (VFA) profile per group in $\mu\text{mol} / \text{g}$. T1 = healthy animals with a 31% NDF diet; T2 = healthy animals with a 36% NDF diet; T3 = animals which had ERE at the outset on a diet with 31% NDF; and T4 = animals which had ERE at the outset with a 36% NDF diet.

Figure 3

Rarefaction curve for sequence analysis of cecotrophs. The vertical axis shows the number of different species (Operational taxonomic units, OTUs) that would be expected to be found after sampling the number of sequences shown on the horizontal axis. The curvature towards the horizontal indicates the greater sequencing effort required to observe new species. The curve was obtained with the Rarefaction Species Richness script from the Vegan package on the Rstudio platform [19]. Group identification: T1 (healthy animals on a diet with 32% NDF): 3, 5, 6, 7. T2 (healthy animals on a diet with 32% NDF): 8, 9, 10, 14. T3 (animals which had ERE at the outset on a diet with 32% NDF): 15, 18, 19, 20. T4 (animals which had ERE at the outset on a diet with 36% NDF): 21, 23, 24, 26.

Figure 4

Rarefaction curve for sequence analysis of feces. The vertical axis shows the number of different species (Operational taxonomic units, OTUs) that would be expected to be found after sampling the number of sequences shown on the horizontal axis. The curvature towards the horizontal indicates the greater sequencing effort required to observe new species. The curve was obtained with the Rarefaction Species Richness script from the Vegan package on the Rstudio platform [19]. : T1 (healthy animals on a diet with 32% NDF): 10, 20, 30, 40. T2 (healthy animals on a diet with 32% NDF): 50, 60, 70, 80. T3 (animals which had ERE at the outset on a diet with 32% NDF): 90, 100, 110, 120. T4 (animals which had ERE at the outset on a diet with 36% NDF): 130, 140, 150, 160.

Figure 5

Microbial profile of the abundances of the cecal samples by groups. The most abundant phyla are shown in each experimental group. T1 = healthy animals with a 32% NDF diet; T2 = healthy animals with a 36% NDF diet; T3 = animals which had ERE at the outset on a diet with 32% NDF; and T4 = animals which had ERE at the outset on a diet with 36% NDF.

Figure 6

Microbial profiles of the abundances of faecal samples by groups. The most abundant phyla are shown in each experimental group. T1 = healthy animals with a 32% NDF diet; T2 = healthy animals with a 36% NDF diet; T3 = animals which had ERE at the outset on a diet with 31% NDF; and T4 = animals which had ERE at the outset on a diet with 36% NDF.

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