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PYROSEQUENCING STUDY OF CAECAL BACTERIAL COMMUNITY OF RABBIT DOES AND KITS FROM A FARM AFFECTED BY EPIZOOTIC RABBIT ENTEROPATHY

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Abstract: Epizootic Rabbit Enteropathy (ERE) is a disease of unknown aetiology that mainly affects post-weaning animals. Caecotrophs from animals in a farm affected by ERE were analysed to identify changes in the microbiological profile of growing rabbits. Does and kits at weaning (28 d) and the same rabbits ten days later (38 d) were used for a comparison using Roche 454 pyrosequencing of hypervariable V3-V5 regions of the *16S rRNA* genes. The caecal bacterial community was dominated by the Firmicutes phylum (about 80%), followed by Bacteroidetes (15%), although relative abundances changed according to animal age (among does and kits at 28 and 38 d) and health status (affected or not by ERE). Two dominant families were classified within the Firmicutes phylum: Ruminococcaceae and Lachnospiraceae (50 and 20% of the sequences, respectively). In kits affected by ERE, relative abundance of *Ruminococcus* and *Bacteroides* genera decreased and increased, respectively, compared to healthy kits at the same age (28 and 38 d). The principal coordinate analysis plot revealed that kits at 28 d of age cluster together and apart from the does and the healthy 38-d rabbit groups. When only growing rabbits are considered, kits that showed symptoms of ERE clustered separately. Results suggest a different caecal bacterial community of rabbits affected by ERE. These findings highlight the need to identify different stages of the disease.

Key Words: epizootic rabbit enteropathy (ERE), microbiota, pyrosequencing, caecum, rabbit.

INTRODUCTION

Despite some digestive pathological processes in growing rabbits having been attributed to specific pathogens such as enteropathogenic *Escherichia coli, Clostridium spiroforme* or *C. perfringens* (Marlier *et al.,* 2006; Gallois *et al.,* 2008; Romero *et al.,* 2009), the aetiological agent of a non-specific, multifactorial enteropathy (Epizootic Rabbit Enteropathy, ERE) is still unknown. A bacterial infection has been hypothesised based on the effectiveness of some antibiotics in preventing ERE, on the fractionation of the reference inoculum in discontinuous sucrose gradient, and that the disease has been reproduced by experimental inoculation (Licois *et al.,* 2005; Szalo *et al.,* 2007; Huybens *et al.,* 2009), but it was not possible to reproduce the disease by inoculating rabbits with specific isolated microorganisms (Marlier *et al.,* 2006; Bovera *et al.,* 2010). Using amplification of the V5 and V6 regions of *16S rDNA* from virulent and non-virulent caecal samples, the resolution of the pyrosequencing technique seems too low to identify this agent, indicating that it could be a particular strain of a known species (Huybens *et al.,* 2013). However, Bäuerl *et al.* (2014) recently published an exhaustive pyrosequencing study relating bacterial caecal diversity with inflammatory response at the mucosa.

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The establishment of a healthy, stable and diverse microbiota in the digestive tract helps prevent the development of enteric diseases, especially at stressful periods such as weaning (Gaskins, 1997; Sekirov and Finlay, 2009). In rabbits, the microbial population of the caecum plays a key role in the host digestive physiology, by both providing with nutrients and acting as a barrier against pathogens and promoting the development of immunity (Forthun-Lamothe and Boullier, 2007). The establishment of caecal microbiota in milking kits is markedly determined by that of the doe (Abecia *et al.,* 2007). This link is highlighted by the fact that 50% of postweaning on-farm mortality occurred in only 14% of the litters (De Blas *et al.,* 2012). The transition from milking to a solid diet modulates the diversity and stability of the microbiota (Combes *et al.,* 2011), and thus further knowledge of the bacterial shifts after the weaning period would help to establish a strategy to preserve the gastrointestinal health.

The aim of the present study was to compare the bacterial microbiota from animals at different ages from a farm affected by ERE using pyrosequencing of the *16S rDNA* gene amplicons. This objective was approached both by using soft faeces (caecotrophs) as samples, thus avoiding slaughter of animals and allowing for a second sampling of the same animal, and by using animals from the same litter to reduce variability and homogenise the disease conditions.

MATERIALS AND METHODS

Experimental protocols followed the 2010/63/EU Directive on the protection of animals used for scientific purposes and the recommendations for applied nutrition research in rabbits described by the European Group on Rabbit Nutrition (Fernández-Carmona, *et al.,* 2005) and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València.

Animals and diets

Animals were housed in an experimental farm having a history of ERE. Environment was controlled within a range of 12 to 25°C and daily cycles of 16 h of light. Does were inseminated at the 11th day postpartum. Litters were standardised to 9-10 kits at birth. Until weaning, does and litters were kept together in 50×70×32 cm cages provided with nesting boxes. Does received a commercial reproduction diet (Cunilactal-UPV) and young rabbits had free access to it. Litters were weaned at the 28th d of lactation and the weaned rabbits were then moved to collective cages (50×80×32 cm, one for each litter) and received a commercial growing diet (Cunimed-UPV) until 38 d of age. Diets included zinc bacitracin (100 ppm) and robenidine (66 ppm).

Sampling and processing

Assuming that soft faeces are representative of caecal microbial environment (Rodríguez-Romero *et al.,* 2009), on the 28th d of lactation, caecotrophs from 6 does were sampled immediately after excretion with the help of neck collars fitted at 7:00 h. In addition, caecotrophs from growing rabbits of litters from these does were sampled both at 28 d of lactation and 10 d after weaning (38 d), while animals received solid feed only. Rabbits were classified in 3 different groups according to their health status: H=healthy, animals with no apparent ERE symptoms in the litter through the experimental period; NH=unhealthy, animals whose caecotrophs presented a clear massive layer of mucus and the bunch shape was missing; D=doubtful, animals apparently healthy at 28 d but that did not survive at 38 d, or those which did not present any ERE symptom but with a lot of dead siblings in their litter at 38 d. Sample selection is described in Table 1.

Samples for DNA extraction were freeze-dried and thoroughly homogenised by physical disruption using a bead beater (Mini-bead beater 8, BioSpec Products). Extraction was performed using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer´s instructions. The lysis temperature was increased to 95°C.

Pyrosequencing and sequence analysis

The V3-V5 regions of the bacterial *16S rRNA* gene were amplified using the primer pair 357F and 926R as described (Sim *et al.,* 2012). Analysis of the sequences was performance as described by Martínez-Fernandez *et al.* (2015) using the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso *et al.,* 2010). Briefly, sequences were filtered to exclude those with mismatches in the primer sequence, exceeding 6 homopolymer base runs or

Table 1: Selection of animals for sampling caecotrophs and initial classification of growing rabbits (kits), grouped as healthy (H), non-healthy (NH) or doubtful (D) and corrected pup grouping (H, healthy; NH, unhealthy), together with the number of sequences and diversity indices of bacterial communities in samples from does (M) at 28 d and growing rabbits (K) at 28 or 38 d.

	Seqs/Sample	Chao1	Observed sp.	Shannon	Doubtful kits	Corrected grouping
Does (28 d):						
M157	6378	2620	1316	7.88		
M45	5262	2394	1201	7.92		
M49	4167	2228	1024	7.68		
M87	14587	3551	2052	7.89		
M111	13410	2254	1387	7.02		
M59	15989	2040	1189	6.25		
Kits						
M157: K1.28 d	18116	3015	1534	7.87		H [1]
K1.38 d	10186	3153	1475	7.81		H
K2.38 d	14374	3257	1734	8.42		H
M45: K1.28 d	5277	1880	910	7.15		H [2]
K4.28 d	4280	1808	949	7.08	D	H
K1.38 d	5423	1858	907	7.30	D	H
K4.38 d	4556	1822	936	7.61	D	H
M49: K1.28 d	4792	1372	727	6.61	D	H [2]
K4.28 d	4884	2180	1101	7.69	D	H
K1.38 d	4327	1520	805	6.84	D	H
K4.38 d	5192	1904	1000	7.50		NH [3]
M87: K1.28 d	15682	3144	1910	7.86		H [1]
K2.28 d	16749	3356	2036	7.75		H
K1.38 d	16532	3480	2049	7.81		H
K2.38 d	3902	1517	715	7.18		H
M111: K1.28 d	12922	3051	1791	7.21	D	H [4]
K3.28 d	16415	2475	1385	6.25	D	H
K3.38 d	15824	1818	1086	6.36		NH [3]
M59: K1.28 d	14392	2200	1418	6.89		NH [4]
K3.28 d	13729	1954	1213	6.66		NH [5]
K4.28 d	15370	1844	1144	5.56		NH [5]
K7.28 d	17879	1664	1055	5.60		NH [4]
M123: K2.28 d	7869	1585	772	7.30		NH
K2.38 d	9371	1508	1006	6.21		NH [3]

[1] Healthy cage.

[2] Two kits in the cage died before 38 d.

[3] Sample with mucus at 38 d.

[4] One kit in the cage died before 38 d.

[5] Sample with mucus at 28 d

sequences containing ambiguous bases. The libraries were split according to the 10nt barcode incorporated into the forward primer. The error-corrections of amplicon pyrosequences were made using Acacia (Bragg *et al.,* 2012). The OTUs (Operational Taxonomic Unit) were generated by clustering at 97% sequence identity using UCLUST (Edgar *et al.,* 2011). The number of reads per sample was normalised to the sample with the lowest number of sequences. Representative sequences were aligned to the reads of the GreenGenes database (DeSantis *et al.,* 2006) using PyNAST (Caporaso *et al.,* 2010). Taxonomic classification was according to Basic Local Alignment Search Tool (BLAST). Rarefraction and alpha diversity (i.e., diversity within a sample) indices (Chao1, Shannon and observed species) were generated with the QIIME pipeline.

Statistical analysis

Diversity indices of kits according to their health status in the initial (H, NH and D groups, n=8) and corrected (H, n=16; NH, n=8) grouping calculated using normalised data were compared by ANOVA using Statistix 10 (Analytical Software, 2010). Distribution of the taxonomic groups of caecal bacteria of healthy and non-healthy kits at 28 or 38 d were compared statistically by t-test. Significant differences are declared when $P<0.05$. Beta diversity was used to create principal coordinate analysis (PCoA) plots among health status of rabbits, using unweighted UniFrac distances. The Unifrac phylogenetic method (Lozupone and Knight, 2005), which considers phylogenetic lineages and not just shared OTU, was used for community-level comparisons with the trees constructed during the OTU picking script. MANOVA was performed on the PCA generated to assess the significance of the grouping between health status.

RESULTS

Community structure in does at weaning

On average, 9966±5256 sequences of 450 base pair (bp) were observed per sample of soft faeces from does at weaning (28 d after parturition, n=6; Table 1). The sequences obtained in this work have been deposited at MG-RAST server (*http://metagenomics.anl.gov*) (project ID 19166). Average diversity values for Chao1 (species richness), observed species (unique OTUs) and Shannon index were 2514±410, 1362±90 and 7.44±1.15, respectively.

Taxonomic analysis of the bacterial community using BLAST (Table 2) revealed that the most representative phylum in the caecum of does was Firmicutes (about 86% of total bacteria), followed by Bacteroidetes (13%) and minor proportions of Tenericutes (0.68%), Proteobacteria (0.18%) and Verrucomicrobia (0.10%). Within the Firmicutes phylum, Clostridia was the predominant class, with 2 dominant families, Ruminococcaceae and Lachnospiraceae (50 and 22%, respectively), whereas families Rikenellaceae (3.9%) and Barnesiellaceae (2.8%) were the most abundant among Bacteroidetes. Up to 54.9 and 6.9% of the total sequences were unclassified at family level among the Firmicutes and Bacteroidetes phyla, respectively. A large individual variability was observed in the relative abundance of selected taxa identified by 454 pyrosequencing in caecotrophs from does at weaning.

Community structure in growing rabbits

Between 3902 and 18116 sequences per sample (on av., 10752±5341) were obtained from caecal contents of *growing rabbits* at weaning (28 d; n=14) and at 38 d of age (n=10). The average values (±standard deviation) for Chao1, observed species and Shannon index at weaning (28 d) were 2252±647, 1282±415 and 6.96±0.76, respectively, whereas at 38 d these diversity values were, on average, 2184±786, 1171±436 and 7.30±0.68. If these indices are averaged according to the corrected health status of growing rabbits from both ages (H, n=16 *vs.* NH, n=8), a significant reduction was observed by disease for Chao and Shannon diversity indices (2450±758 *vs.* 1810±222, $P=0.035$ and 7.40±0.55 *vs.* 6.51±0.71, $P=0.003$).

Firmicutes phylum represented roughly 81% of the total bacteria in caecotrophs of healthy rabbits at 28 d, followed by Bacteroidetes phyla (18%), whereas Tenericutes, Proteobacteria and Verrucomicrobia represented less than 1% of the total sequences (Table 2). The 2 dominant families of Firmicutes were Ruminococcaceae and Lachnospiraceae (48 and 16% of total sequences), and the 2 families most observed among Bacteroidetes were Bacteroidaceae (10.3%) and Rikenellaceae (3.5%). The microbial profile was similar in healthy animals after weaning (38 d), but with a higher proportion of Firmicutes phylum (93%) at the expense of Bacteroidetes (5%). Ruminococcaceae and Lachnospiraceae (52 and 23% of total sequences) were also the dominant families. Healthy animals were compared at 28 and 38 d and only genus *Bacteroides, Parabacteroides* and *Clostridium* varied significantly ($P=0.036$, 0.007 and 0.05, respectively). Also, between both ages, the Ruminococcaceae family increased ($P=0.02$) from 26 to 42%.

However, proportions changed according to the health status of growing rabbits. In NH also including D kits at 28 d, Firmicutes and Bacteroidetes phylum represented around 60 and 40% of total bacteria, respectively. Besides, the relative abundance of Ruminococcaceae and Lachnospiraceae was modified in these animals, both being around 24%. The genus *Bacteroides* was the microbial group that significantly increased ($P=0.028$) to a higher extent at 28 d NH compared to H kits (from 7 to 32%), although this response was very variable among individuals (from 1 to

Table 2: Distribution of the taxonomic groups (%) of caecal microbiota of does (28 d postpartum) and growing rabbits (kits) at 28 and 38 d of age, according to their health status.

Class/Order/Family/Genus[1]	Does	SEM	H[2] Kits 28 d	SEM	NH Kits 28 d	SEM	H Kits 38 d	SEM	NH Kits 38 d	SEM	28 d H *vs.* NH *P*-value	38 d H *vs.* NH *P*-value
Number of samples	6		9		5		7		3			
Phylum Bacteroidetes:	11.9	3.31	24.2	2.95	43	8.45	5.8	1.0406	37.2	14.4	*	**
O. Bacteroidales, unclass.	2.7	0.0134	2.5	0.0112	---	---	1	0.0003	0.3	0.0078	*	0.243
F. Bacteroidaceae	0.4	0.0019	5.4	0.0287	1.6	0.0140	0.3	0.0013	8.1	0.0254	0.493	0.246
Bacteroides	0.8	0.0024	7.3	0.0262	34	0.0913	0.4	0.0016	11.2	0.0340	*	0.233
F. Porphiromonadaceae												
Parabacteroides	0.1	0.0006	0.6	0.0022	0.2	0.0015	---		0.4	0.0015	0.096	0.492
F. Rikenellaceae, unclass.	3.7	0.0193	1.2	0.0038	0.5	0.0023	1	0.0087	6.4	0.0261	0.189	0.470
Rikenella	0.1	0.0005	0.5	0.0021	1.4	0.0031	0.5	0.0010	4.3	0.0168	0.414	0.375
S24-7	3.1	0.0117	1.5	0.0067	5.4	0.0250	0.5	0.0023	4.4	0.0146	0.137	0.340
F. Barnesiellaceae	2.8	0.0068	5.1	0.0122	0.2	0.0121	2	0.0016	1.2	0.0045	0.075	0.346
F. Odoribacteriaceae												
Butyricimonas	---	0.0002	---		0.3	0.0008	---		0.5	0.0021	0.095	0.408
Odoribacter	---	0.0003	0.1	0.0007	---		---		0.1	0.0005	0.231	0.447
C. Flavobacteria, unclass.	---		---		---		0.1	0.0010	0.2	0.0008	0.841	0.704
C. 4COd-2, Order YS2	---	0.0002	0.1	0.0006	---		0.2	0.0012	0.3	0.0015	0.263	0.615
Phylum Firmicutes:	86.9	3.35	74.8	2.9	56.5	8.39	93.4	1.1959	61.4	14.05	*	**
C. Bacilli												
O. Turicibacterales												
Turicibacter	---		---		---		---		0.3	0.0013		0.447
C. Clostridia, unclass.	2.1		1.8	0.0028	5.9	0.0115	2.8	0.0021	7.3	0.0234	**	0.653
O. Clostridiales, unclass.	2.8	0.0026	2.9	0.0047	1.1	0.0023	3.5	0.0026	2.6	0.0053	*	0.087
F. Christenellaceae	0.3	0.0006	0.4	0.0009	---		0.4	0.0008	---		**	0.786

Table 2 continued on next page.

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Class/Order/Family/Genus[1]	Does	SEM	H[2] Kits 28 d	SEM	NH Kits 28 d	SEM	H Kits 38 d	SEM	NH Kits 38 d	SEM	28 d H *vs.* NH *P*-value	38 d H *vs.* NH *P*-value
F. Clostridiaceae, unclass.	0.3	0.0008	0.2	0.0005	0.2	0.0004	0	0.0005	0.7	0.0012	0.367	0.260
Clostridium	---	0.0003	---		---		0.1	0.0003	0.3	0.0016	0.732	0.661
F. Dehalobacteriaceae, unclass.	---	0.0002	---		0.3	0.0014	---		0.1	0.0002	0.127	0.242
Dehalobacterium	0.1	0.0003	0.1	0.0003	---		0.1	0.0003	---		0.556	0.077
F. Eubacteriaceae, unclass.	1.6	0.0105	---		---		0.4	0.0006	---			0.488
Anaerofustis	---	0.0002	0.1	0.0003	0.1	0.0002	0.1		---		0.735	0.060
F. Lachnospiraceae, unclass.	16.4	0.0428	13.6	0.0269	11.4	0.0137	10.7	0.0234	6.9	0.0214	0.531	0.143
Anaerostipes	0.1	0.0004	0.1	0.0003	0.1	0.0008	0.1	0.0004	0.2	0.0008	0.916	0.649
Blautia	0.9	0.0012	1.3	0.0038	7.7	0.0166	2.3	0.0069	6.5	0.0190	**	0.643
Coprococcus	0.2	0.0005	0.4	0.0010	0.8	0.0014	0.5	0.0012	1	0.0023	0.111	0.791
Dorea	0.1	0.0003	0.3	0.0008	4.8	0.0130	0.4	0.0019	1.4	0.0051	**	0.550
[Ruminococcus]	3.8	0.0088	2.9	0.0077	3.9	0.0084	6.1	0.0145	5.4	0.0129	0.652	0.147
rc4-4	---	0.0002	0.1	0.0004	---		0.1		---			*
F. Peptostreptococcaceae	---		---		---		---		0.8	0.0038		0.447
F. Ruminococcaceae, unclass.	35.9	0.03	27.8	0.0375	10.3	0.0717	38.4	0.0563	10.9	0.0776	0.198	0.538
Anaerotruncus	---		---		---		---		0.2	0.0008		0.295
Oscillospira	3	0.0095	1.7	0.0050	2.4	0.0049	3.4	0.0131	3.1	0.0068	0.561	0.133
Ruminococcus	10.6	0.0045	11.5	0.0183	3	0.0088	11.5	0.0136	5.2	0.0131	**	*
O. Coribacteriales, unclass.	5.9	0.0058	9	0.0191	3.2	0.0075	9.5	0.0239	2.3	0.0185	*	0.369
F. Coriobacteriaceae, unclass.	0.3	0.0025	0.1	0.0004	0.4	0.0009	0.8	0.0009	0.2	0.0037	0.068	0.152
Adlercreutzia	0.2	0.0007	0.3	0.0006	---		0.5	0.0010	0.9	0.0020	**	0.182
C. Erysipelotrichi												
O. Erysipelotrichales												

Table 2 continued on next page.

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Class/Order/Family/Genus[1]	Does	SEM	H[2] Kits 28 d	SEM	NH Kits 28 d	SEM	H Kits 38 d	SEM	NH Kits 38 d	SEM	28 d H *vs.* NH *P*-value	38 d H *vs.* NH *P*-value
F. Erysipelotrichaceae, unclass.	0.1		---		---		0.1	0.0005	---		0.135	**
[Eubacterium]	---		---		0.1	0.0006	---		0.6	0.0019	0.089	0.220
F. [Coprobacillaceae], unclass.	---		---		0.3	0.0011	0.1	0.0008	4.4	0.0139	*	0.260
Coprobacillus	---		0.2	0.0012	0.3	0.0025	---		0.2	0.0005	0.761	0.299
C. RF3, O. ML615J-28	0.1	0.0004		0.0002							0.652	
Phylum Proteobacteria:	0.3	0.0877	0.2	0.0317	0.1	0.0321	0.3	0.0425	0.6	0.0861	0.093	**
C. Alphaproteobacteria, unclass.	---		---		---		---		0.2	0.0008		0.772
O. RF32, unclass.	0.1										0.408	0.312
C. Betaproteobacteria												
F. Alcaligenaceae												
Sutterella	0.1	0.0004	0.1	0.0002	---		0.1	0.0003	---		0.228	*
C. Deltaproteobacteria												
F. Desulfovibrionaceae				0.0001		0.0003						
Bilophila	---		---		---		---		---	0.0010		0.312
Phylum Tenericutes:	0.6	0.1734	0.5	0.107	0.2	0.0756	1.4	0.3299	0.2	0.20	*	0.061
C. Mollicutes												
F. Anaeroplasmateaceae	0.1	0.0002	---		---		---		---			
Anaeroplasma		0.0005	---	0.0002	---		0.3	0.0014	---	0.0023	0.215	0.815
O. RF39, unclass.	0.4	0.0017	0.5	0.0013	0.1	0.0009	1.1	0.0013	0.3	0.0036	0.064	0.419
Phylum Verrucomicrobia:	0.1	0.0111	0.1	0.0219	0		0		0.1	0.0756	*	0.176
C. Verrucomicrobia												
F. Verrucomicrobiaceae												
Akkermansia	0.1	0.0002	0.1	0.0003	---		0.1	0.0003	0.2	0.0007	*	0.128

[1]C=Class; O=Order; F=Family; G=Genus.
[2]Healthy=H; unhealthy=NH.

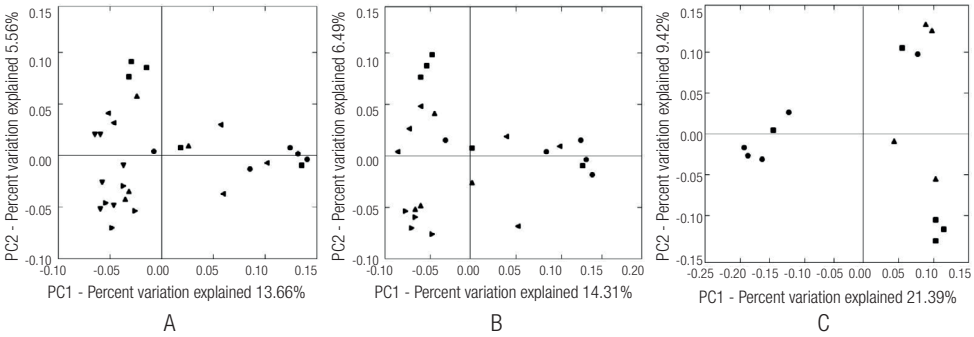

Figure 1: Principal coordinate analysis plots of kits classified initally as healthy (H), unhealthy (NH) or doubtful (D), using unweighted Unifrac distances. A) All samples: Does at 28 d (▼) and kits at 28 d (●=NH, ▲= H, ■=D) and 38 d (◄= NH, ►= H), Clusters according to health status of the animals. Does and healthy rabbits at 38 d grouped together suggesting that caecal microbiota at 38 d is completely developed. B) Only kits at 28 and 38 d. Animals were grouped according to their health status (H, NH and D) showing differences between 28 and 38 d of age. C) Only kits at 28 d. Groups of kits mainly according to their health status (H, NH and D) and secondly kits were grouped by litter.

27% in H and from 4 to 57% in NH group). The genera *Blautia* and *Dorea* (from Lachnospiraceae family) as well as unclassified Clostridia also increased (*P*=0.004, 0.005 and 0.014, respectively) in NH at weaning. In contrast, genus *Ruminococcus* decreased from 15% to 4% (*P*=0.003).

In NH rabbits at 38 d, around 67 and 30% of total bacteria were classified under Firmicutes and Bacteroidetes phyla, respectively, whereas the relative abundance of the families Ruminococcaceae and Lachnospiraceae was reduced to 30 and 17%, respectively. The higher increase in NH at this age was observed in the genus *Bacteroides*, which increased ten-fold from 0.4 to 8% (*P*=0.05) whereas the genus *Ruminococcus* decreased from 13 to 7% (*P*=0.05). It is worth noting that, despite the low proportion of sequences belonging to these groups, sequences of genera *Butyricimonas* (from the phylum Bacteroidetes) and *Anaerotruncus* and unclassified *Coprobacillaeceae* (from phylum Firmicutes) were only present in NH (or D) at both 28 and 38 d of age. Relative abundance of genus *Clostridium* did not vary among rabbit groups, representing less than 0.2% in all H, NH and D. Proportions did not change in NH rabbits when the same animals were compared at 28 and 38 d, and only the relative abundance of the family Clostridiaceae significantly increased (*P*=0.0002) from 0.13 to 0.73%.

The PCoA plot (Figure 1A) illustrates clusters according to health status of the animals, showing that does and healthy rabbits at 38 d grouped together. PCoA plot including kits at 28 and 38 d (Figure 1B) illustrates groups according to their health status (H, NH and D), and later by age. Figure 1C included only animals at weaning (28 d) and showed that 4 out of 5 NH rabbits grouped together. In this group, a rabbit from a different litter (M123) was included, which presented mucus in the caecotrophs at 38 d. In the other NH rabbit cluster next to the littermate (M111), 10 d later one of them was dead and the other one presented mucus.

DISCUSSION

Enteric diseases frequently occur in rabbits around weaning, leading to extensive use of antibiotics in rabbit breeding. However, the maintenance of a healthy gut is complex and relies on a delicate balance between the mucosa (including the absorptive epithelium and the digestive immune system), gut microbiota and environmental factors including diet. Guarner and Malagelada (2003) reported that the initial colonisation is very relevant to the final composition of the permanent microbiota in adult humans. Indeed, pioneer bacteria can modulate the expression of genes in host epithelial cells, thus creating a favourable habitat for themselves, and can prevent growth of other bacteria that are

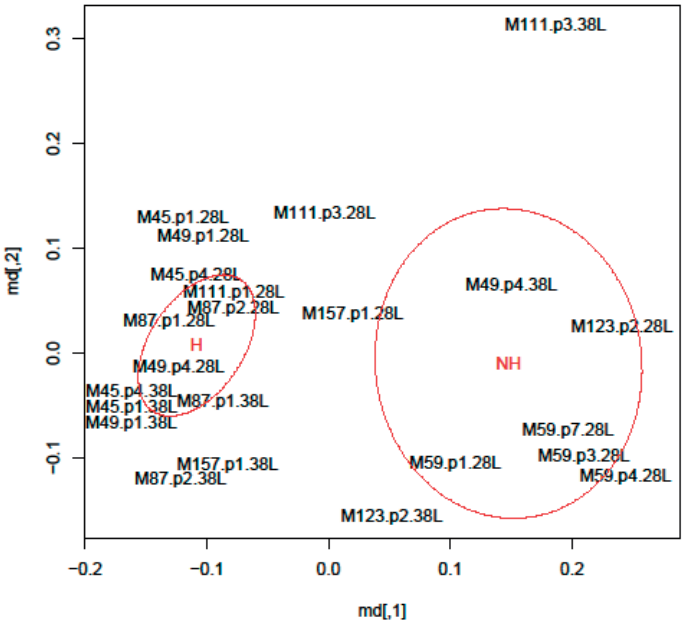

Figure 2: Principal coordinate analysis plot obtained of the mean distances (md) of the relative abundance of the genera for kits classified as healthy (H) and unhealthy (NH) at 28 and 38 d old.

later introduced into the ecosystem (Hooper and Gordon, 2001). The maternal intestinal microbiota and surroundings are the main source of bacteria colonising the newborn's intestine (Abecia *et al.,* 2007).

In healthy animals, individual variability in the relative abundance of the major groups was observed. This fact and the low number of experimental animals makes it difficult to observe a clear picture of the microbial evolution in the caecum of growing rabbits during the critical period from 28 to 38 d. Indeed, a progressive age-related change in the composition and relative abundance of species of the bacterial community was reported by Combes *et al.* (2011), who demonstrated that the rabbit caecal bacterial community evolved towards a more complex and regular community from the neonatal stage to 10 wk of age. Tannock (2005) also observed a high within-group distance between the bacterial communities in the neonatal period, indicating an unchecked proliferation of bacteria that proceeds initially in the neonatal gut, resulting in a heterogeneous collection of bacterial species. Curtis and Sloan (2004) suggested that communities in physically identical environments, such as the gut of newly born mammals, will have different compositions if they are formed randomly from large metacommunities. This model would help to explain the high level of individual variability detected in the neonatal rabbits and a progressive development of stability with increasing age.

Combes *et al.* (2011) described the age-related changes on Bacteroidetes and Firmicutes groups that could be detected from the second day of rabbit's life. The Bacteroides – Prevotella group's maximal density was reached 3 wk after birth (10-11 $\log_{10}$), whereas Firmicutes groups seemed to be established from the second week after birth and remained stable thereafter. The balance between Bacteroidetes and Firmicutes groups in young and adult rabbits was already described by Monteils *et al.* (2008), who showed that Bacteroidetes and Firmicutes represented 4% and 94% of the sequenced clone, respectively.

The ERE symptoms usually appear 2 wk after weaning and include bloat, relatively low body weight, distension of stomach and small intestine, epithelial integrity disruption and either liquid or compacted caecal contents (Licois *et al.,* 2006; Dewrée *et al.,* 2007; Chamorro *et al.,* 2010). Although the disease has not been obtained by inoculating specific pathogen free rabbits with isolated microorganisms (Licois *et al.,* 2005; Marlier *et al.,* 2006; Bovera *et al.,*

2010), ERE symptoms have been reproduced experimentally with inocula originating from intestinal contents of affected animals or from dust collected in contaminated farms, in which bacterium *Clostridium perfringens* was detected (Marlier *et al.,* 2006; Szalo *et al.,* 2007), also showing that proliferation of *C. perfringens* could be a consequence of ERE and may be associated to the mortality caused by this disease. Furthermore, Licois *et al.* (2006) did not detect the presence of other frequent digestive pathogens. In contrast, our work detected a low (<0.2%) relative abundance of *Clostridium* genus, even in NH rabbits. We must bear in mind that, in this preliminary study, sampling of caecotrophs from H and NH animals allowed us to compare the microbial caecal profile and to follow the evolution of the symptoms of intestinal disorders of the experimental animals, instead of getting a single sample from slaughtered animals.

Our results, however, should be taken into account with some considerations. Some samples that were initially classified as D rabbits might actually belong to H group, due to the criteria we used: being a survivor in a cage with most siblings dead at collection time (see Table 1 for initial and corrected classification details). From this new grouping, the comparison of Chao1, number of observed species and Shannon index for H *vs.* NH 38 d rabbits would be 2373 *vs.* 1743, 1232 *vs.* 1031 and 7.57 *vs.* 6.69, respectively, thus maintaining similar differences to the initial classification. If this consideration is applied in Figure 1A, the separation between H and NH animals would be clear, as 3 D samples present in H section should then belong to rabbits that did not show symptoms in the samples. A similar picture would be observed in Figure 1B and Figure 1C. However, due to the experimental design, animals were not monitored from 28 d to 38 d, and therefore the information in this period was missed.

With this evidence, data from kits at weaning were reanalysed grouping animals in only 2 categories, H and NH, as described in Table 2. In this case, *Bacteroides* genus increased from 7.3 to 36.1% and Ruminococcaceae family decreased from 31.7 to 10.1% (particularly, *Ruminococcus* genus decreased from 11.4 to 2.7%) in NH rabbits. On the other hand, family Lachnospiraceae, particularly genera *Dorea* and *Blautia*, increased in unhealthy rabbits. To illustrate the cluster according to health status of the animals, including kits at 28 and 38 d, a second analysis was presented in Figure 2. These data confirm our results and hypothesis of the occurrence of the disease as explained below.

Based on these results, it appears that rabbits affected by ERE presented a different microbiota compared to H. A clear dysbiosis was observed in this study, with an increase in the relative abundance of *Bacteroides* in those NH rabbits presenting mucus in their caecotrophs. Dysbiosis of intestinal microbiota is a possible disease factor (Round and Mazmanian, 2009) and has been related with alteration in gut barrier integrity and metabolic disturbances (Everard *et al.,* 2013). Our results are in agreement with a recent study by Bäuerl *et al.* (2014) who reported a remarkable dysbiosis in rabbits affected by ERE. *Bacteroides* spp. are involved in many important metabolic activities in the gut and has been associated with abdominal infections (Wang *et al.,* 2000). Diagnosis and treatment are complicated due to the slow growth of *Bacteroides*, the increasing resistance to antibiotics (e.g. resistance to penicillin, mostly due to the production of beta-lactamase), and the polymicrobial nature of an infection with *Bacteroides* (Chaudhry and Sharma, 2011). However, in this work we were unable to show a direct association of a single bacterial species or a specific group of microorganisms to the onset of ERE symptoms.

CONCLUSIONS

Comparison of the microbial communities in healthy rabbits and in those affected by ERE around weaning reveals marked differences in microbial diversity and relative abundance of the microbial groups. These data suggest that ERE-affected rabbits presented a dysbiosis, mostly with an increase in relative abundance of *Bacteroides* and a reduction in taxonomic diversity. However, at this level it is not possible to determine if one or several bacteria were directly implicated as aetiological agents in the onset of ERE. To our knowledge, this work is a first intensive non-culture based analysis of gastrointestinal microbiota for rabbit affected by ERE at different ages (28 and 38 d), and provides a framework for understanding the role of digestive microbiota in rabbit health.

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