Housing conditions, level of feeding and presence of antibiotics in the feed shape rabbit cecal microbiota

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Abstract

Background: the effect of the production environment and different management practices in rabbit cecal microbiota remains poorly understood. While previous studies have proved the impact of the age or the feed composition, research in the housing conditions and other animal management aspects, such as the presence of antibiotics in the feed or the level of feeding, is still needed. Characterization of microbial diversity and composition of growing rabbits raised under different conditions could help better understand the role these practices play in cecal microbial communities and how it may result in different animal performance.

Results: four hundred twenty-five meat rabbits raised in two different facilities, fed under two feeding regimes (*ad libitum* or restricted) with feed supplemented or free of antibiotics, were selected for this study. A 16S rRNA gene-based assessment through the MiSeq Illumina sequencing platform was performed on cecal samples collected from these individuals at slaughter. Different univariate and multivariate approaches were conducted to unravel the influence of the different factors on microbial alpha diversity and composition at phylum, genus and OTU taxonomic levels. The animals raised in the facility harboring the most stable environmental conditions had greater, and less variable, microbial richness and diversity. Bootstrap univariate analyses of variance and sparse partial least squares-discriminant analyses endorsed that the farm exerted the largest influence on rabbit microbiota since the relative abundances of many taxa were found differentially represented between both facilities at all taxonomic levels characterized. Furthermore, only five OTUs were needed to achieve a perfect classification of samples according to the facility where animals were raised. The level of feeding and the presence of antibiotics did not modify the global alpha diversity but had an impact on some bacteria relative abundances, albeit in a small number

of taxa compared with the farm, which is consistent with the lower sample classification power according to these factors achieved using microbial information.

Conclusions: this study reveals different degrees of influence attributable to environment and animal management. It highlights the importance of offering a controlled breeding environment that reduces differences in microbial cecal composition that could be causative of different animal performance.

Keywords

cecal microbiota, meat rabbit, housing conditions, feed restriction, antibiotics, 16S MiSeq Illumina sequencing, analysis of variance, multivariate approach

Background

Microbial communities that inhabit the gastrointestinal tract (GIT) of animals constitute a complex ecosystem whose members constantly interact between them and with their host [1-Gaskins, 1997]. These interactions ensure homeostatic balance maintenance since GIT ecosystem components are involved in many physiological and immunological processes [2-Belkaid and Hand, 2014]. In the case of the domestic meat rabbit (*Oryctolagus cuniculus*), a small herbivorous mammalian belonging to the family *Leporidae*, cecum is the main organ for microbial fermentation. Thus, it is not surprising that the rabbit cecum hosts the richest and the most diverse microbial community of its GIT [3-Gouet and Fonty, 1979]. For this reason, the cecum has been the organ preferably chosen in previous rabbit gut microbiota

assessments [4-Abecia et al., 2007; 5-Zou et al., 2016; 6-Zhu et al., 2017; 7-Chen et al., 2019].

Thanks to the development of the next generation sequencing (NGS) technologies, and their rapidly decreasing costs, it is currently possible to characterize the gut microbiota of a large number of animals. This characterization allows a deeper comprehension of the differences between animals concerning their microbial composition and diversity. It is hypothesized that the production environment could partially mediate these differences. Our general aim is to provide further evidence of the effect of different management and environmental factors on the cecal microbial composition and diversity. In relation to this topic, there is a certain amount of information already published. A growing number of studies have revealed changes in rabbit cecal microbial communities exerted by the age [8-Combes et al., 2011] or the type of feed provided to the kits after weaning [6-Zhu et al., 2017; 7-Chen et al., 2019]. Another factor that causes variation is the administration of antibiotics in the feed. Different molecules have been widely administrated in rabbit meat production, especially after weaning to curb mortality peaks (sometimes over 20%) as a result of the onset of gastrointestinal symptoms [9-Gidenne et al., 2010]. Multiple studies have shown alterations caused on gut microbiota by the administration of antibiotics in the feed [5-Zou et al., 2016; 10-Eshar & Weese, 2014]. Despite the European Union banned their use as growth promoters in animal feeds since 2006 (EC 1831/2003), the administration of one type of antibiotic molecule is still allowed to prevent or treat the emergence of potential infectious diseases on farms. However, substantial efforts are being made towards searching for efficient alternatives which allow a complete withdrawal of antibiotic in animal feeds. In this context,

68 the application of feed restriction during the growing period was proposed as an interesting
69 alternative to the use of antibiotics. Quantitative feed restriction is a widely applied
70 commercial practice that consists of reducing by a certain percentage the amount of feed the
71 animal would consume when the food is provided *ad libitum*. Gidenne et al. (2009) [11-
72 Gidenne et al., 2009] demonstrated that feed restriction, despite penalizing animal growth,
73 improves feed efficiency and reduces mortality due to enteric disorders. It is
74 hypothesized that these positive effects could be partially explained by changes in gut
75 microbial composition or activity originated by the application of feed restriction. However,
76 techniques used so far to study this possible association have not found evidence of it [11-
77 Gidenne et al., 2009].

78 This study, which comprises a large number of animals in an experimental design involving
79 different management and environmental factors, is intended to unravel changes in diversity
80 and composition of rabbit cecal microbial communities associated with these factors. It will
81 allow a better understanding of how the housing conditions associated with the farm where
82 the animal was raised, the presence of antibiotics in the feed, and feed restriction shape the
83 cecal microbiota of growing rabbits.

84

85 ## Results

86 ### Sequence processing

87 After the removal of doubletons and samples with low sequence counts, 425 rabbit cecal
88 samples (Additional file 1) were represented on 14,928,203 sequence counts clustered into
89 963 different OTUs. Each sample had on average 35,125 final sequences (range: 10,157-

678,798) and 677 OTUs (range: 197-841) (Additional files 2 and 3). Figure 1 shows two histograms representing the sample richness and the proportion of OTUs present across samples. Most of the samples had more than 700 different OTUs (mode = 748) and nearly 140 OTUs were present in all the samples.

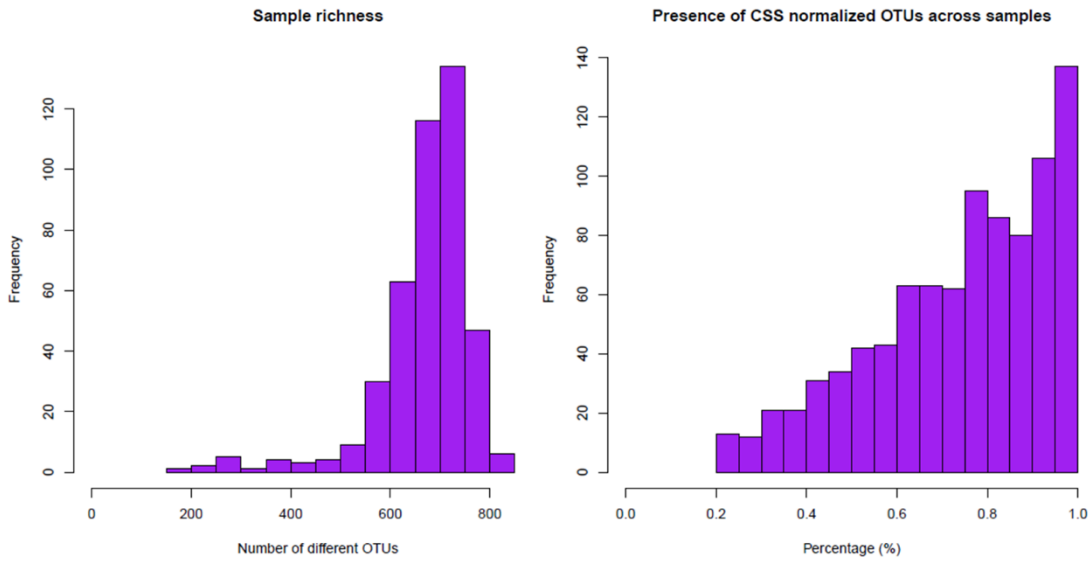
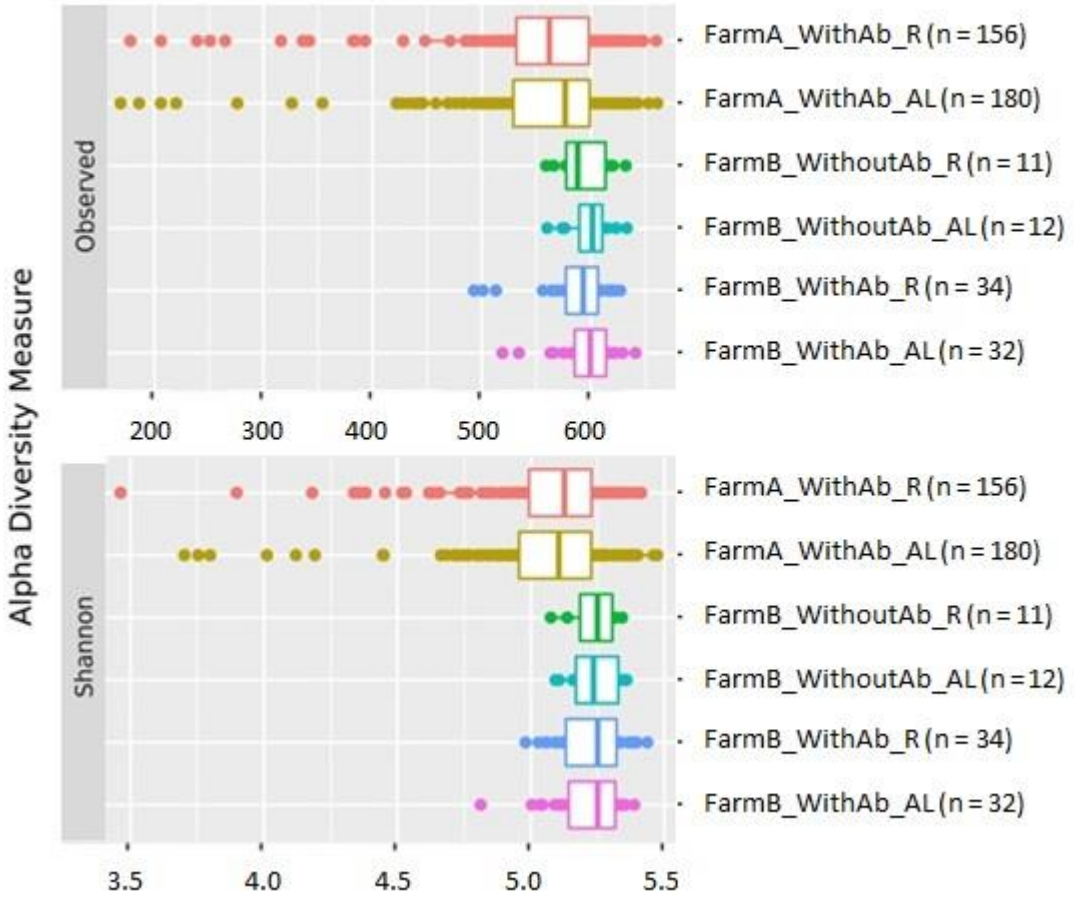


Figure 1 Sample richness and presence of CSS-normalized OTUs across samples.

Taxonomic assignment of representative OTUs against the Greengenes reference database gg_13_5_otus (Additional file 4) revealed the presence of 8 different known phyla with an average of 8 phyla per sample (range: 7-8) (Additional file 5), and 28 different known genera with an average of 24 genera per sample (range: 17-28) (Additional file 6).

Animal management and farm environment shaping cecal microbial alpha diversity

104 The study of alpha diversity was performed after rarefying the prefiltered and unnormalized
105 OTU table to 10,000 sequences per sample. Rarefaction generated a table which contained
106 the sequence counts of 963 different OTUs for 425 samples. The average (standard deviation)
107 number of observed OTUs within animal was 560.52 (75.03) and the average Shannon index
108 within animal was 5.09 (0.26). The comparison of alpha diversities revealed that the group
109 of animals raised in farm B had greater alpha diversity than the group of animals raised in
110 farm A (estimated differences of 40.20 (9.83) observed OTUs and 0.17 (0.03) Shannon index;
111 $P_{FDR} < 0.001$). Furthermore, larger variability in both indexes was observed in farm A than
112 in farm B. No significant differences for the two alpha diversity indexes were found between
113 feeding regimes within both farms (Figure 2, $P_{FDR} > 0.05$), nor between the presence and the
114 absence of antibiotics in the feed within the farm B (Figure 2, $P_{FDR} > 0.05$).
115



116

117 **Figure 2 Microbial richness and diversity between samples grouped according to**
118 **management that animals received.** The cecal microbial richness and diversity were
119 estimated by the observed number of different OTUs and the Shannon indexes, respectively.

120

121 **Animal management and farm environment shaping cecal microbial composition**

122 According to the taxonomic assignment of representative sequences (Additional file 4)
123 performed with the UCLUST consensus taxonomy assigner on the Greengenes reference
124 database gg_13_5_otus, *Firmicutes* (76.74%), *Tenericutes* (7.22%) and *Bacteroidetes*

(6.26%) were the predominant phyla, accounting for more than 90% of the microbial diversity, in the rabbit cecal samples studied (Figure 3).

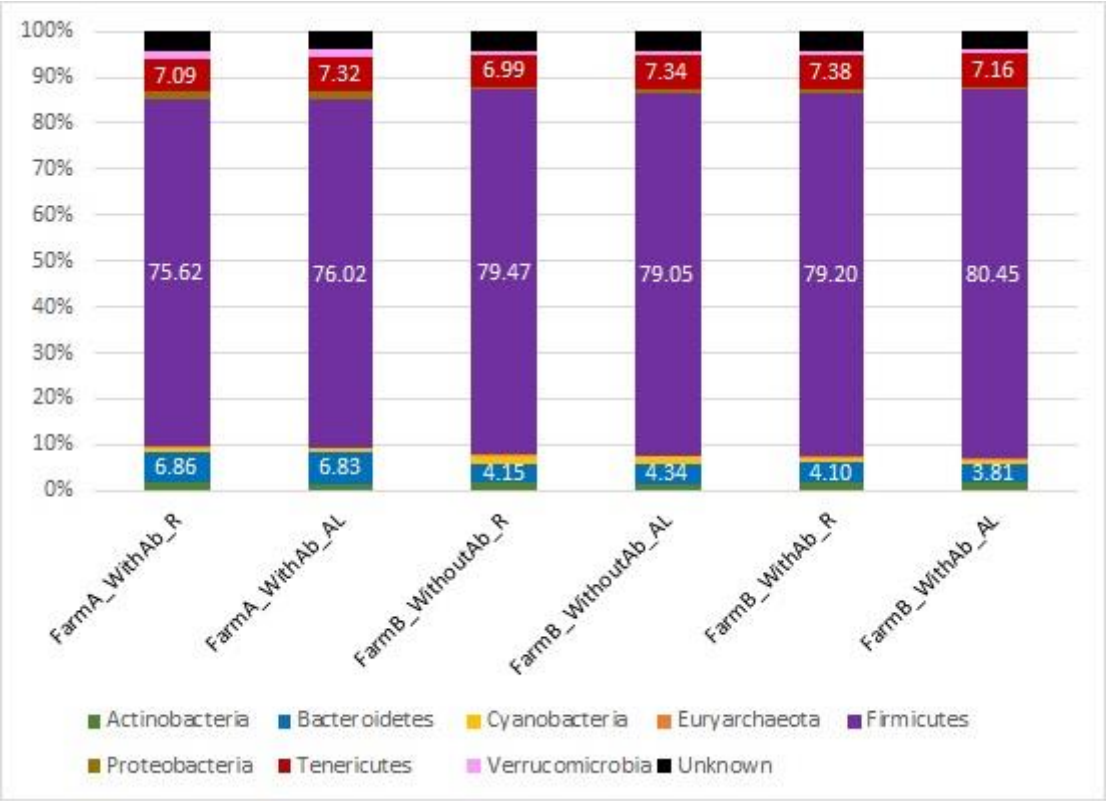


Figure 3 Phyla relative abundances of samples grouped according to farm, level of feeding and presence of antibiotics in the feed.

Differential growth and cecal microbial composition across farms

The facility where the animals were raised affected their growth performance. Animals raised in farm B exhibited a faster growth (47.11 grams/day) than those raised in farm A (44.19 grams/day). The estimated average daily gain difference between farm B and farm A was

2.92 ± 0.94 grams per day ($P < 0.005$). Cecal samples of rabbits raised in farm A showed an overrepresentation of phyla *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* while phyla *Euryarchaeota*, *Cyanobacteria* and *Firmicutes* were found to be overrepresented in cecal samples of rabbits raised in farm B (Table 1).

Table 1 Microbial composition at phylum level in cecal samples of rabbits grouped by farm.

Phylum	Mean relative abundance in farm A (%) (SD)	Mean relative abundance in farm B (%) (SD)	Estimated difference farm A - farm B ± SE	P_{FDR}
Actinobacteria	1.62 (0.67)	1.84 (0.33)	-0.14 ± 0.08	0.09
Bacteroidetes	6.84 (1.81)	4.03 (0.70)	2.74 ± 0.22	0.00
Cyanobacteria	0.77 (0.40)	1.05 (0.36)	-0.39 ± 0.05	0.00
Euryarchaeota	0.13 (0.19)	0.44 (0.17)	-0.28 ± 0.02	0.00
Firmicutes	75.83 (3.34)	79.66 (1.53)	-3.78 ± 0.41	0.00
Proteobacteria	1.83 (0.62)	0.66 (0.12)	1.14 ± 0.07	0.00
Tenericutes	7.21 (1.47)	7.25 (0.93)	0.00 ± 0.18	0.99
Verrucomicrobia	1.62 (0.45)	0.91 (0.24)	0.68 ± 0.05	0.00

Genera *Ruminococcus* (4.32%), *Blautia* (2.96%) and *Oscillospira* (2.37%) dominated the meat rabbit cecal microbiota. Most of the relative abundance differences at genus level were found differentially represented between animals raised in the different farms: genera *Bacteroides*, *Parabacteroides*, *Rikenella*, *Anaerofustis*, *Anaerostipes*, *Clostridium*, *Coprobacillus*, *Anaeroplasma* and *Akkermansia* were overrepresented in cecal samples of rabbits raised in farm A while genera *Adlercreutzia*, *Butyricimonas*, *Odoribacter*, *Methanobrevibacter*, *Blautia*, *Butyrivibrio*, *Coprococcus*, *Dehalobacterium*, *Dorea*, *Oscillospira*, *rc4-4* and *Oxalabacter* were overrepresented in cecal samples of rabbits raised

in farm B. Interestingly, genera *Epulopiscium*, *p-75-a5*, *Phascolarctobacterium*, *Campylobacter* and *Desulfovibrio* were only found in samples collected from farm A (Table 2).

Table 2 Relative abundances of genera, grouped by phylum, differentially represented between farms (P_{FDR} < 0.05).

Genus	Mean relative abundance in farm A (%) (SD)	Mean relative abundance in farm B (%) (SD)	Estimated difference farm A - farm B ± SE
Actinobacteria			
Adlercreutzia	0.89 (0.47)	1.14 (0.23)	-0.19 ± 0.06
Bacteroidetes			
Bacteroides	1.88 (0.67)	0.80 (0.35)	1.10 ± 0.08
Butyricimonas	0.16 (0.19)	0.35 (0.17)	-0.19 ± 0.02
Odoribacter	0.23 (0.21)	0.44 (0.20)	-0.21 ± 0.03
Parabacteroides	0.25 (0.18)	0.07 (0.07)	0.18 ± 0.02
Rikenella	0.39 (0.24)	0.18 (0.13)	0.25 ± 0.03
Euryarchaeota			
Methanobrevibacter	0.13 (0.19)	0.44 (0.17)	-0.28 ± 0.02
Firmicutes			
Anaerofustis	0.12 (0.08)	0.08 (0.04)	0.03 ± 0.01
Anaerostipes	0.17 (0.08)	0.12 (0.04)	0.06 ± 0.01
Blautia	2.86 (0.67)	3.22 (0.46)	-0.36 ± 0.08
Butyrivibrio	0.10 (0.07)	0.13 (0.06)	-0.03 ± 0.01
Clostridium	1.09 (0.26)	0.87 (0.13)	0.21 ± 0.03
Coprobacillus	0.20 (0.27)	0.14 (0.08)	0.08 ± 0.03
Coprococcus	1.96 (0.42)	2.26 (0.29)	-0.28 ± 0.05
Dehalobacterium	0.05 (0.08)	0.18 (0.03)	-0.13 ± 0.01
Dorea	0.46 (0.12)	0.51 (0.09)	-0.05 ± 0.02
Epulopiscium	0.14 (0.11)	0.00 (0.00)	0.15 ± 0.01
Oscillospira	2.11 (0.53)	2.85 (0.31)	-0.79 ± 0.07
p-75-a5	0.13 (0.06)	0.00 (0.00)	0.13 ± 0.01
Phascolarctobacterium	0.27 (0.24)	0.00 (0.00)	0.26 ± 0.03
rc4-4	0.13 (0.06)	0.23 (0.03)	-0.10 ± 0.01
Proteobacteria			
Campylobacter	0.08 (0.08)	0.00 (0.00)	0.08 ± 0.01

Desulfovibrio	0.58 (0.22)	0.00 (0.00)	0.57 ± 0.03
Oxalabacter	0.10 (0.06)	0.13 (0.03)	-0.03 ± 0.01
Tenericutes			
Anaeroplasma	0.23 (0.18)	0.10 (0.09)	0.12 ± 0.02
Verrucomicrobia			
Akkermansia	1.62 (0.45)	0.91 (0.23)	0.68 ± 0.05

158

159 The analyses on the CSS-normalized OTUs revealed that 648 out of the 946 OTUs showed
160 signatures significantly different between farms. Out of these, 276 were overrepresented in
161 farm A, while 372 were overrepresented in farm B. Table S1 shows the estimated difference
162 between farms for these OTUs, their sequences and their assignment at the lowest taxonomic
163 level. Only 9 of them could be assigned at species level and 129 were assigned to known
164 genera. These results showed remarkable coincidences with those obtained from the analyses
165 directly performed on the relative abundance of taxa at phylum and genera levels. An
166 example that illustrates this match is the overrepresentation of genus *Akkermansia* in farm
167 A. This genus is encompassed by phylum *Verrucomicrobia* that was also overrepresented in
168 rabbits raised in farm A, as well as 6 out of the 7 OTUs assigned to this phylum.

169

170 Differential growth and cecal microbial composition across feeding regimes

171 The feeding regime affected the rabbits' growth performance in both facilities. Animals fed
172 AL had a higher growth (48.74 and 55.77 grams/day in farms A and B, respectively) than
173 those fed R (38.95 and 38.65 grams/day in farms A and B, respectively). The estimated
174 average daily gain difference between AL and R groups was 9.79 ± 0.58 and 17.12 ± 1.08
175 grams per day in farms A and B, respectively ($P < 0.001$). An overrepresentation of phyla
176 *Cyanobacteria* (estimated difference R - AL = 0.11 ± 0.04; $P_{FDR} = 0.04$) and

177 *Verrucomicrobia* (estimated difference R - AL = 0.11 ± 0.05; P_{FDR} = 0.04) was found in cecal
178 samples of rabbits fed R and raised in farm A. On the other hand, phylum *Euryarchaeota*
179 was overrepresented in animals fed R and raised in farm B (estimated difference R - AL =
180 0.14 ± 0.04; P_{FDR} < 0.001). At genus level, the only significant contrast was observed for
181 *rc4-4* which resulted overrepresented in samples from animals fed AL in farm A (estimated
182 difference R - AL = -0.03 ± 0.01; P_{FDR} < 0.001) while in farm B none of the genera resulted
183 differentially represented (P_{FDR} > 0.05) between feeding regimes. The contrasts based on the
184 CSS-normalized OTUs revealed 51 and 9 OTUs differentially represented between feeding
185 regimes within farms A and B, respectively. Within the farm A, 32 OTUs were
186 overrepresented in cecal samples of rabbits that were fed AL and 19 OTUs in the samples
187 from rabbits fed R. Within the farm B, 7 OTUs were overrepresented in cecal samples of
188 rabbits that were fed AL and 2 OTUs were overrepresented in rabbits that were fed R. Table
189 S2 shows the estimated difference between feeding regime within farm of these OTUs, their
190 sequences and their assignment at the lowest taxonomic level. The analyses based on the
191 CSS-normalized OTUs within the farm A were in full accordance with the analyses
192 performed at genus level given that all OTUs assigned to genus *rc4-4* (phylum *Firmicutes*)
193 were overrepresented in cecal samples of rabbits fed AL.

194

195 Effect of the presence of antibiotics in the feed

196 The effect of the presence of antibiotics in the feed could only be assessed within the farm B
197 given that all rabbits raised in farm A received feed supplemented with antibiotics. Animals
198 that received antibiotics had a slightly higher growth (47.29 grams/day) than those that did
199 not received (46.59 grams/day). The estimated average daily gain difference between groups

was not significant (0.69 ± 2.43 grams per day; $P = 0.78$). Cecal samples of rabbits that received feed free of antibiotics showed an overrepresentation of phyla *Cyanobacteria* compared to those that received feed supplemented with antibiotics (estimated difference without antibiotics - with antibiotics = 0.49 ± 0.09; $P_{FDR} < 0.001$). In addition, the analyses on the CSS-normalized OTUs revealed an overrepresentation of 15 and 29 OTUs in cecal samples of rabbits that received a feed supplemented or free of antibiotics; respectively. Table S3 shows the estimated difference between the presence and the absence of antibiotics in the feed for the OTUs in which the differences reached the significance threshold. The OTU sequences as well as their assignment at the lowest taxonomic level are also shown in Table S3. Only 1 of these OTUs could bet assigned at species level (*Bacteroides fragilis*) and 2 OTUs at genus level (*Oscillospira* and *Coprococcus*).

Microbial information as a classifier of cecal samples according to farm environment and animal management

Sparse partial least squares-discriminant analyses (sPLS-DA) on the CSS-normalized OTUs were conducted to discriminate samples according to the factors considered in this study (i.e., the farm where the animal was raised, the presence or the absence of antibiotics in the feed and the feeding regime). The tuning process of the sPLS-DA conducted to discriminate samples according to the farm where the rabbits were raised selected 5 OTUs for component 1 and 1 OTU for component 2 (Figure 4). Component 1 explained 7.00% of the total variance while component 2 explained 0.67%. The classification performance of this sPLS-DA could be said to be perfect since its overall and balanced error rate (BER) per class across 1000

222 replicates of 5-folds cross-validation runs was 0.00 (0.00). Furthermore, two OTUs of
223 component 1 had a stability higher than 0.9.

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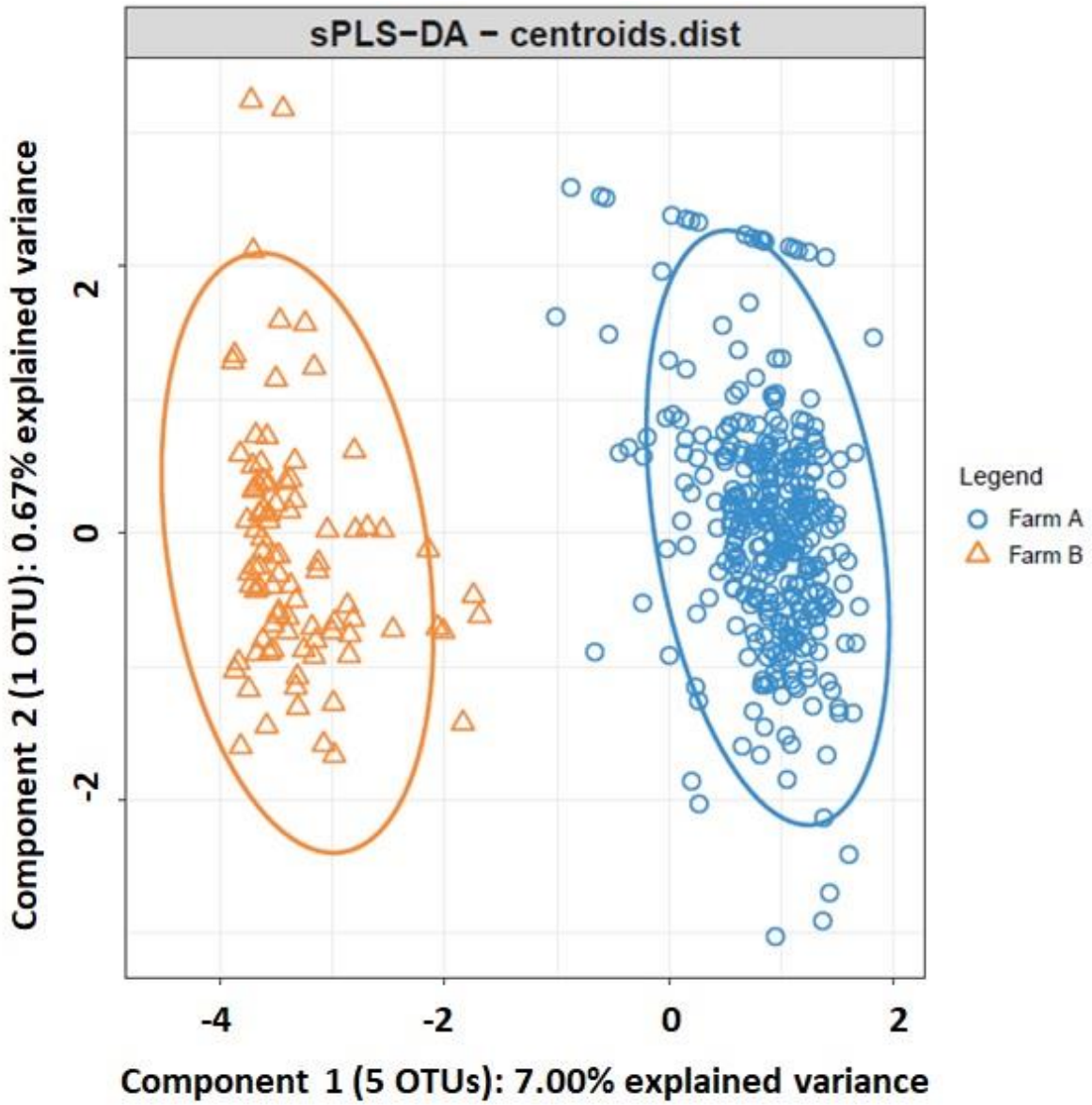

225

226 **Figure 4 Sparse partial least squares discriminant analysis representing cecal samples**
227 **of rabbits raised in farm A (blue) and in farm B (orange).**

228

229 The sPLS-DA performed to discriminate samples across feeding regimes within the farm A
230 selected 70 OTUs for component 1 and 65 OTUs for component 2 (Figure 5). Component 1
231 explained 2.34% of the total variance while component 2 explained 5.58%. The cross-
232 validation assessment of the classification performance of this sPLS-DA showed an overall
233 and BER per class of 0.27 (0.02). The stability of 18 and 5 OTUs selected in components 1
234 and 2, respectively, across the different cross-validation folds was higher than 0.9.

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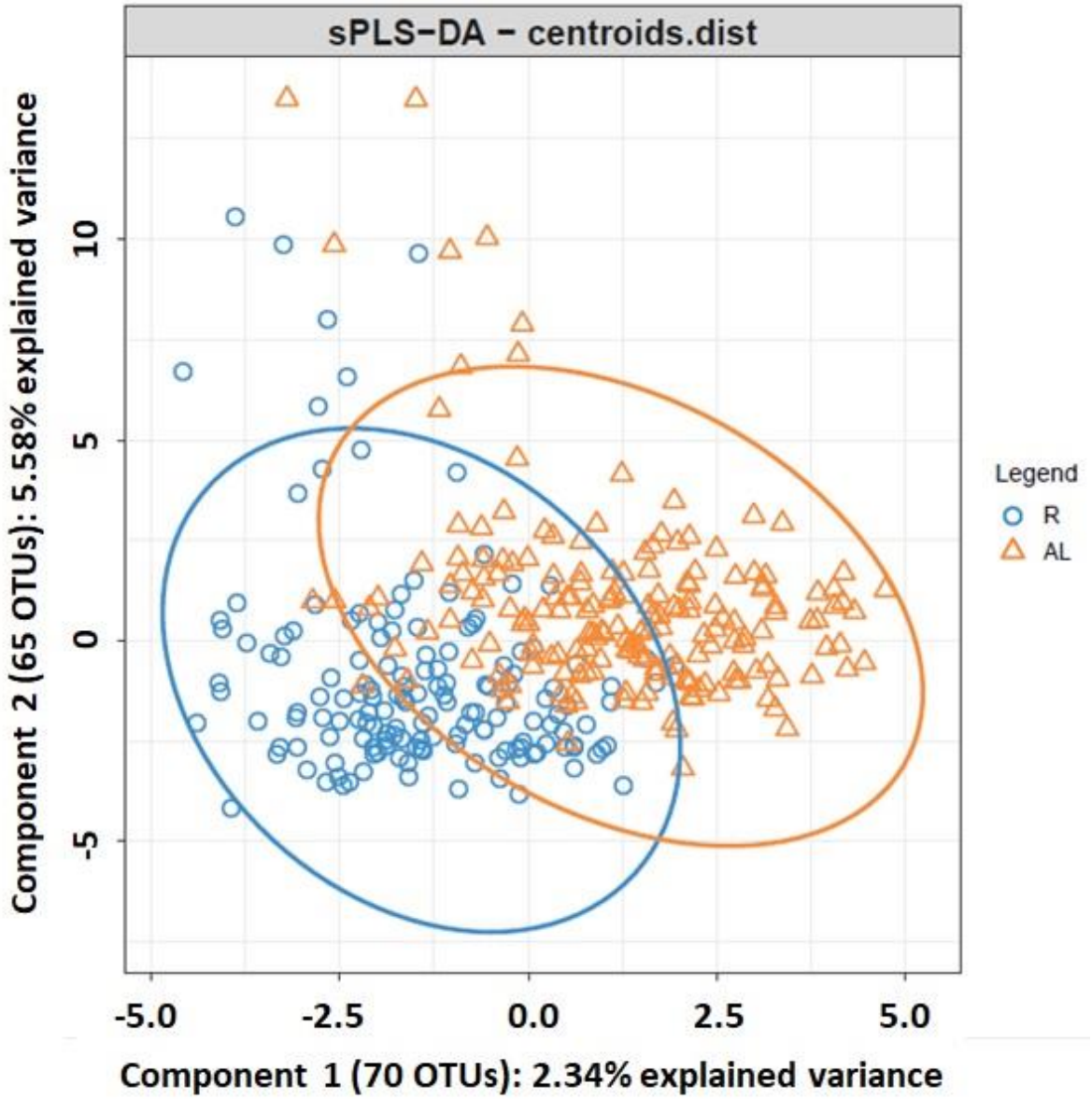

Figure 5 Sparse partial least squares discriminant analysis representing cecal samples of rabbits raised in farm A and fed R (blue) or AL (orange).

Finally, the sPLS-DA conducted to discriminate samples of animals raised within the farm B according to the combination of the presence or not of antibiotics in the feed and the feeding regime selected 9 OTUs for component 1 and 70 OTUs for component 2 (Figure 6).

243 Component 1 explained 3.05% of total variance and defined the discrimination between
244 samples from animals fed with antibiotics and those fed without antibiotics. On the other
245 hand, component 2 explained 3.05% of total variance and defined the discrimination between
246 samples from animals fed R and those belonging to animals fed AL. The cross-validation
247 assessment of the classification performance of this sPLS-DA showed an overall BER of
248 0.32 (0.15). The BER per class was 0.34 (0.12) for samples fed R without antibiotics, 0.46
249 (0.14) for samples fed AL without antibiotics, 0.29 (0.11) for samples fed R with antibiotics,
250 and 0.20 (0.07) for samples fed AL with antibiotics. The stability of 3 and 11 OTUs selected
251 in components 1 and 2, respectively, across the different cross-validation folds was higher
252 than 0.9.

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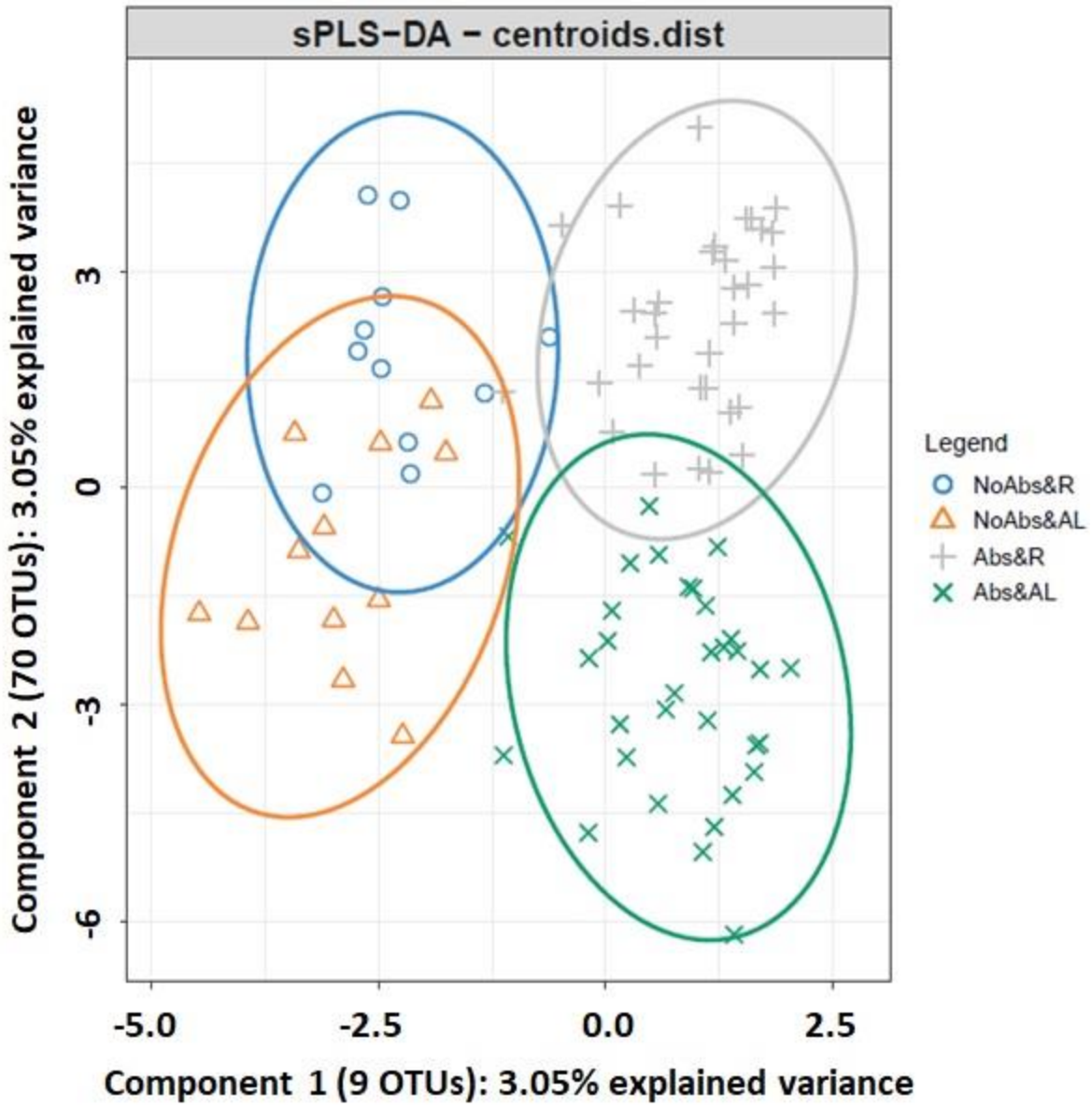

254

255 **Figure 6 Sparse partial least squares discriminant analysis representing cecal samples**
256 **of rabbits raised in farm B and fed R without antibiotics (blue), fed AL without**
257 **antibiotics (orange), fed R with antibiotics (gray) and fed AL with antibiotics (green).**

258

259

Discussion

The influences of farm environment and common commercial practices of animal management on their gut microbiota are not yet well known in many livestock species. In this study, we have aimed to disentangle potential changes in microbial diversity and composition of meat rabbit cecal communities as a result of being raised in different farms and subjected to different handling during their growing period. To shed light on this matter, we conducted a microbiota comparison of a large number of rabbits raised under different housing conditions, feeding regimes, and fed with feed supplemented or free of antibiotics.

16S rRNA gene-based characterization of meat rabbit cecal microbiota

The Illumina MiSeq sequence processing of samples collected from these animals revealed that phyla *Firmicutes*, *Tenericutes* and *Bacteroidetes* dominate the growing meat rabbit cecal ecosystem representing more than 90% of its entire microbial composition. This fact is in accordance with previous studies that have characterized the rabbit cecal microbiota [5-Zou et al., 2016; 7-Chen et al., 2019; 12-Velasco-Galilea et al., 2018] and reported *Firmicutes* as the predominant phylum. However, there are discrepancies between studies in establishing which other phyla are also prevalent in this ecosystem. Whereas we found phyla *Tenericutes* and *Bacteroidetes* representing 7.22% and 5.93% of the cecal microbial composition, respectively, Chen et al. 2019 [7] and Zou et al. 2016 [5] reported *Bacteroidetes* as the second predominant phylum representing 18% and 20% of New Zealand White and Rex rabbit cecal microbial composition, respectively. Conversely, other studies that have previously characterized meat rabbit fecal microbiota identified phyla *Proteobacteria* and

Verrucomicrobia in higher relative abundances [13-Kylie et al., 2018; 10-Eshar and Weese, 2014]. Velasco-Galilea et al. 2018 [12] reported *Firmicutes* (76.42%), *Tenericutes* (7.83%) and *Bacteroidetes* (7.42%) as the predominant phyla of meat rabbit fecal and cecal microbial communities. These discrepancies found across studies could be attributed to technical issues (e.g., pair of primers, sequencing platform, bioinformatic pipeline employed to process raw sequences or reference database used for the taxonomic assignment of the representative sequences) or to purely biological reasons (e.g., breed, age or section of the GIT sampled). Nonetheless, Kylie et al. (2018) [13] depicted that the relative increase in less beneficial phyla, such as *Proteobacteria*, could be related to seasonal climate changes that directly impact rabbits' health. This impact affects the susceptibility to enteritis and possibly feed conversion efficiency. In any case, this phylum was more prevalent in farm A where the animals were more exposed to changes in climate conditions.

Farm environment modify alpha diversity

Regarding the alpha diversity assessment, Shannon and the observed number of OTUs indexes revealed the existence of significant differences between housing conditions (i.e., the experimental farm where the rabbits were raised). Cecal samples collected from rabbits raised in farm B had greater richness and diversity than those belonging to animals raised in farm A. This could be explained by more stable environmental conditions in farm B (i.e., facility better insulated) than in farm A. This combined with the fact that samples of animals raised in farm A were collected from rabbits produced in 4 different batches, could also explain the larger variability in both indexes observed in this farm [13-Kylie et al., 2018]. Despite not having observed significant differences between the presence or not of antibiotic in the feed,

nor between feeding regimes, it is noteworthy to mention that samples collected from animals fed AL in both farms had a greater, although not significant, richness than those fed R. This fact is consistent with previous studies in mice that observed a lower alpha diversity in animals with a restricted level of feeding [14-O'Neil et al., 2017; 15-Chen et al., 2016; 16-Zarrinpar et al., 2014]. Surprisingly, but in agreement with our results, studies performed in pigs [17-Soler et al., 2017], chicken [18-Kumar et al., 2018] and Rex rabbits [5] also did not show clear significant differences on alpha diversity indexes between animals fed on diets with antibiotics with respect to those on diets free of antibiotics. Nevertheless, these studies were able to detect differences in the relative abundances of some specific species between diets. For example, Kumar et al. 2018 [18] found that the inclusion of bacitracin in the feed did not affect the chicken bacterial phyla. However, they observed differences between the control and the bacitracin-fed group in the ileal and cecal bacterial populations at lower taxonomic levels. It is worth noting that the antibiotic withdrawal at the beginning of the last week of the rabbits' lives equalized the diets of both groups and possibly their microbial populations, which may explain some lack of differences between them.

Farm environment has a large impact on rabbit cecal microbiota

Despite the lack of differences in microbial diversity and richness across management factors (except for the farm); univariate studies revealed differential microbial composition across the studied factors. In addition, the performed multivariate analysis evidenced a certain classification power of the samples on the different levels of management and environment factors based on the microbial composition of the samples.

As it might be expected, analyses of variance confirmed that the largest modification of meat rabbit cecal microbial composition is generated by the housing conditions (in this case represented by the farm factor). Our results revealed that the relative abundances of 6 out of 8 phyla are differentially represented between both farms. At genus level, we detected significant differences in the relative abundances of almost all of them. Genera *Bacteroides*, *Parabacteroides*, *Rikenella*, *Anaerofustis*, *Anaerostipes*, *Clostridium*, *Coprobacillus*, *Anaeroplasma* and *Akkermansia* were enriched in cecal samples of rabbits housed in farm A. The first three belong to phylum *Bacteroidetes* and genus *Bacteroides* is the most abundant of them in meat rabbit cecum. Species of this genus are anaerobic Gram-negative members of the family *Bacteroidaceae* that play an important role in the degradation of vegetal polysaccharides and amino acid fermentation in the mammal GIT [19-Fang et al.,2017; 20-Dai et al., 2011]. Moreover, this genus is involved in propionic acid and lactate formation depending on nitrogen organic availability. Nonetheless, some authors showed that great amounts of *Bacteroides* could predict obesity tendency. *Parabacteroides* is also an anaerobic Gram-negative bacterium (family *Porphyromonadaceae*) involved in amino acid transport and metabolism, energy production and conversion, lipid transport and metabolism, recombination and repair, cell cycle control, cell division, and cell motility in the intestinal microbiota of the growing rabbit [21-Sun et al., 2020]. This genus was specifically found in the cecal microbiota of mice raised in conventional conditions and absent in those raised in pathogen-free facilities in a study performed under different housing conditions [22- Müller et al., 2016].

Within the phylum *Firmicutes*, genus *Clostridium* (family *Clostridiaceae*) is an anaerobic Gram-positive bacterium that inhabits the GIT of many mammals where it acts by degrading

cellulose. However, some *Clostridium* species (e.g., *C. perfringens* and *C. difficile*) are pathogenic, and an enrichment of this genus has previously been described in rabbits affected by epizootic rabbit enteropathy [23- Bäuerl et al., 2014]. This genus, together with genus *Bacteroides*, was found enriched in the cecal microbiota of mice housed in open cages compared with those kept in individual ventilated cages [24-Thoene-Reineke et al., 2014]. Both genera have been associated with an exacerbation of the intestinal inflammatory response in mammals [25-Terán-Ventura et al., 2010]. Genus *Anaerofustis* (family *Eubacteriaceae*) has been found enriched in cecal samples of rabbits affected by paratuberculosis infection (*Mycobacterium avium*) [26-Arrazuria et al., 2016].

Within the phylum *Verrucomicrobia*, genus *Akkermansia* is an anaerobic Gram-negative bacterium that encompasses mucin degrader species [27-Belzer et al., 2012]. In the cecum, a proper enrichment of this genus could maintain a suitable mucosal turn-over, thus exerting a protective effect that could help the animal to deal with inflammatory processes.

It is worth mentioning that we have detected genera *Epulopiscium*, *p-75-a5*, *Phascolarctobacterium*, *Campylobacter* and *Desulfovibrio* only in the cecal samples of rabbits housed in farm A. The first three are encompassed within the phylum *Firmicutes.* Genus *Epulopiscium* is a large size Gram-positive bacterium that has a nutritional symbiotic relationship with surgeonfish that eats algae and detritus. This bacterium is physically similar to the phylogenetically related *Metabacterium polyspora* which is an endospore-producing bacterium isolated from the cecum of guinea pigs [28-Angert et al., 1996]. On the other hand, genera *Campylobacter* and *Desulfovibrio* are Gram-negative bacteria that belong to phylum *Proteobacteria*. Some species of these genera are pathogens responsible for infections and diarrheas in mammals. The exclusive presence of these genera in farm A could indicate the

existence of a potential dysbiosis of the animals raised in that facility that could affect their sanitary status and growth. While farm A was a semi-open-air facility, farm B was artificially ventilated and offered more controlled environmental conditions that favor animal growth. Moreover, the presence of sulfate-reducing bacteria (SRB) such as *Desulfovibrio* could be enhanced by sulfate-secreting bacteria (SSB) such as *Rikenella* in farm A where this genus is significantly more predominant. It is noteworthy to mention that SRB could also obtain sulfate via *cross-feeding* mediated by *Bacteroides*-encoded sulfatases [29-Rey et al., 2013], and interestingly, this phylum is more prevalent in farm A.

Regarding sample classification based on the sPLS-DA study, given the important differences in gut microbial composition found between farms, a perfect classification of the samples can be achieved with only 5 OTUs. One of these 5 OTUs was overrepresented in farm B and belonged to family *S24-7* (phylum *Bacteroidetes*). The remaining 4 were overrepresented in farm A and belonged to family *Barnesiellaceae* (phylum *Bacteroidetes*), order *Bacteroidales* (phylum *Bacteroidetes*), and genera *Desulfovibrio* (phylum *Proteobacteria*) and *Bacteroides* (phylum *Bacteroidetes*). It is worth mentioning that these 5 OTUs were also declared as differentially represented between farms by the univariate analyses.

Administration of antibiotics impact on some taxa relative abundances

Within the farm B, the effect of the presence of antibiotics in the feed was assessed by comparing the microbial cecal composition of rabbits fed with antibiotics with that of some animals that received feed without antibiotics. As stated above, we did not detect significant

differences in alpha diversity, nor in genera relative abundances, between both groups. However, some significant differences were observed at phylum and OTU levels. An overrepresentation of phylum *Cyanobacteria* was found in rabbits fed without antibiotics. The detection of this bacterial phylotype, commonly assigned to photosynthetic activity, in the rabbit cecum could suggest contamination during the GIT sampling. However, Zeng et al. 2015 [30] previously reported its presence in rabbit feces. In the present study, all OTUs taxonomically assigned to phylum *Cyanobacteria* are as well encompassed in the order *YS2*. Interestingly, it was demonstrated that this order does not really have photosynthetic capacity and it is currently classified within the candidate phylum *Melainabacteria* [31-Di Rienzi et al., 2013]. The non-photosynthetic cyanobacteria *YS2*, now named *Gastranaerophilales*, is a fermenter gut-associated order present in humans and other animals such as squirrels, where its exact role is unknown but it has the capacity to produce hydrogen, fix nitrogen and synthesize vitamins B and K [31-Di Rienzi et al., 2013; 32-Monchamp et al., 2019; 33-Liu et al., 2020]. Our results, in accordance with Kylie et al. 2018 [13], revealed that rabbits fed without antibiotics exhibited higher abundances of OTUs assigned to phylum *Bacteroidetes* than those fed with antibiotics. In addition, samples of rabbits that received antibiotics had a significant increase of an OTU taxonomically assigned to genus *Coprococcus.* Interestingly, a study that evaluated the differences in bacterial communities of Rex rabbits fed with different antibiotics also found an overrepresentation of this bacterium in animals treated with zinc bacitracin [5-Zou et al., 2016]. *Coprococcus* is an anaerobic bacterium that may protect against colon cancer in humans by producing butyric acid [34-Ai et al., 2019]. We hypothesized that the administration of antibiotics could modulate the abundance of some *Coprococcus* species to provide intestinal protection on meat rabbits. However, it is important to recognize that the reduced sample size of the group of rabbits fed without

antibiotics may have limited the statistical power to detect microbial composition differences associated with this factor.

Feed restriction modify *Euryarchaeota* and some bacteria relative abundances

Within the farm B, the effect of the feeding regime in microbial composition was also assessed by comparing samples of animals fed R with those fed AL. The main difference found was for phylum *Euryarchaeota* which was overrepresented in animals fed R in farm B. All *Euryarchaeota* species found in the rabbit cecum belong to genus *Methanobrevibacter* that encompasses different hydrogenotrophic methane-producing species. Previous studies in humans [35-Shen and Maitin, 2015] and cattle [36-McCabe et al., 2015; 37-McGovern et al., 2017] found an overrepresentation of *Methanobrevibacter* species in individuals submitted to feed restriction and a negative correlation between the abundance of this bacterium and body mass index. A prevalence of *Methanobrevibacter* species could be a positive indicator of a healthy microbiota since restricted animals showed an overrepresentation of this genus. The main purpose of applying feed restriction is to improve intestinal health, reducing weaning mortality. The growth of *Methanobrevibacter* is supported by fermenters such as *Gastranaerophilales* and butyrate-producing bacteria such as *Anaereostipes* via interspecies formate/hydrogen transfer [37-Bui et al., 2019]. A study in mice determined that *Methanobrevibacter smithii* facilitates *Bacteroides thetaiotaomicron* capacity to digest glycans resulting in increased production of short-chain fatty acids [38-Samuel and Gordon, 2006]. The same study defined *M. smithii* as a "*power broker*" that regulates polysaccharide fermentation efficiency that influences the fat stores. The lower prevalence of methanogenic archaea in farm A could be explained by the high presence of

442 SRB that outcompetes with methanogens for hydrogen consumption. This fact could favor
443 hydrogen sulfide production and compromise the rabbits' health.

444

445 Regarding the sample classification based on the sPLS-DA study conducted within the farm
446 B, component 1 and component 2 discriminated between animals that did or did not received
447 antibiotics in the feed and between feeding regimes, respectively. It is worth mentioning that
448 8 out of 9 OTUs selected in component 1 were also declared as differentially represented
449 between the presence or the absence of antibiotics in the feed by the univariate analyses.
450 Within the farm A, an sPLS-DA was also performed to classify samples according to the
451 feeding regime using microbial information. Although a large number of OTUs were selected
452 as classifier variables in the tuning process of this sPLS-DA, the classification error rate was
453 high. It implied a poor discrimination capacity of samples according to the feeding regime
454 the animal received. Nevertheless, bootstrap univariate analyses of variance detected some
455 significant differences at all taxonomic levels analyzed between feeding regimes within the
456 farm A. At genus level, *rc4-4* was overrepresented in animals fed AL. This genus belongs to
457 phylum *Firmicutes* and it is known as an obesity-associated bacterium [39- Ziętak et al.,
458 2016] and as a pathogenic candidate identified in mice with multiple sclerosis [40-Gandy et
459 al., 2019]. A potential pro-inflammatory role has been proposed for this genus [40] what
460 could be related to a reduced incidence of enteric disorders when feed restriction is applied.
461 It is worth mentioning that family *Peptococcaceae*, which encompasses genus *rc4-4*, is
462 strongly related to total rabbit weight gain from weaning to 12-week old [41-North et al.,
463 2019]. Although in our study this genus was prevalent in animals fed AL, its association with

weight gain is not clear since the greater growth exhibited by these animals was consequence of higher feed intake.

Rabbit cecal microbiota is shaped by farm environment and animal management

Different approaches have been applied in this study to evaluate the effect of different environments and management practices, commonly used in rabbit production, in their cecal microbial composition and diversity. Our results confirmed that the most important effect is exerted by the environment provided by the farm where the animals were raised. Those raised in the best insulated facility (farm B) appear to have a microbiota characteristic of healthier animals than those raised in the open-air facility (farm A). It is worth mentioning that the rabbits were housed in cages interspersed with feeding regime. This fact could make possible the exchange of microorganisms between animals of different feeding regimes and therefore have reduced the differences observed between regimes. However, the joint consideration of 70 OTUs in the sPLS-DA made possible a certain discrimination power of samples according to the level of feeding received by each animal raised in farm A. It implies the existence of cecal microbiota content patterns characteristic of each regime which could be revealed thanks to the univariate analyses conducted at different taxonomic levels. Similarly, the sPLS-DA performed within the farm B also involved the consideration of 70 OTUs to discriminate samples according to the amount of feed consumed. Within this farm, the classification of samples regarding the presence or the absence of antibiotics in the feed needed a smaller number of OTUs than the feeding regime but greater than the farm. This suggests that the effect of the presence of antibiotic in feed is stronger than the feeding level. The implication of the discussed microbial composition and diversity differences originated

by the studied management and environmental factors on the animals' performance still needs to be investigated. In future studies the role of specific groups of bacteria in rabbit growth and feed efficiency will be analyzed.

Conclusions

The analysis of a large number of animals from a paternal rabbit line has allowed a deeper comprehension of the role played by different management and environmental factors shaping the composition and diversity of cecal microbial communities. It reveals that the housing conditions offered to the rabbits during their growing play a key role that can result in different microbial alpha diversity and composition of almost all species that inhabit the rabbit GIT. This highlights the importance that a stable and controlled environment could have in the intestinal health and, consequently, in animal performance. It seems clear that the better insulated conditions of farm B favored the presence of a gut microbiota characteristic of healthier animals. Although the level of feeding and the presence of antibiotics in the feed did not modify the global diversity of cecal microbial communities, these factors can increase or decrease the prevalence of specific bacteria which could lead to a microbial composition potentially beneficial for the animal or, at the other extreme, to an origin of future intestinal dysbiosis.

Methods

Animals and experimental design

All biological samples used in the study were collected from animals of an experiment conducted at the Institute of Agrifood Research and Technology (IRTA) in different periods and involving two different farms. The objective of that experiment was to estimate the effect of the interaction between the genotype and the feeding regime (i.e., the amount of feed provided during fattening) on growth, feed efficiency, carcass characteristics, and health status of the animals [42-Piles and Sánchez, 2019]. For this particular study, 425 meat rabbits from Caldes line [43-Gómez et al., 2002] of that experiment were randomly selected. Most of them (336) were raised in 4 different batches in a semi-open-air facility (farm A). The remaining animals (89) were produced in a single batch in another facility under better controlled environmental conditions (farm B). Rabbits raised in farm A were housed in collective cages containing 8 kits each one while those raised in farm B were housed in cages with 6 kits each one. All animals were raised under the same management conditions and received the same standard pelleted diet. Twenty-three rabbits raised in farm B received a diet free of antibiotics and the remaining sixty-six received the same diet but supplemented with antibiotics. Those raised in farm A received oxytetracycline, valnemulin, and colistin while those in farm B received oxytetracycline, valnemulin and neomycin. At the time this experiment was conducted, it was possible to use up to four types of molecules to prevent or treat the emergence of potential infectious diseases on farms. However, nowadays, only one antibiotic molecule is allowed. During the last fattening week all the animals received an antibiotic free diet. Feed was supplied once per day in a feeder with three places for the 4-5 weeks that the fattening lasted. Water was provided *ad libitum* during the whole fattening period. The animals were under two different feeding regimes: (1) *ad libitum* (AL) or (2) restricted (R) to 75% of the AL feed intake. The amount of feed supplied to the animals under R feeding regime in a given week for each batch was computed as 0.75 times the average

feed intake of kits on AL from the same batch during the previous week, plus 10% to account for a feed intake increase as the animal grows. Kits were randomly assigned to one of these two feeding regimes after weaning (32 days of age). They were categorized into two groups according to their size at weaning (big if their body weight was greater than 700 g or small otherwise) aiming to obtain homogenous groups regarding animal size within feeding regime. A maximum of two kits of the same litter were assigned to the same cage in order to remove the possible association between cage and maternal effects on animal growth during the fattening period. The distribution of these animals across the different levels of management factors is shown in Table 3. The body weight of each animal was weekly recorded. The individual average daily gain was computed as the slope of the within animal regression of all body weight measurements recorded during the growing period.

Table 3 Distribution of rabbits in groups according to different management factors.

Farm	Batch	Feed	Feeding regime	Number of rabbits
A	1	With antibiotics	Ad libitum	27
A	1	With antibiotics	Restricted	30
A	2	With antibiotics	*Ad libitum*	35
A	2	With antibiotics	Restricted	41
A	3	With antibiotics	*Ad libitum*	61
A	3	With antibiotics	Restricted	53
A	4	With antibiotics	*Ad libitum*	57
A	4	With antibiotics	Restricted	32
B	5	With antibiotics	*Ad libitum*	32
B	5	With antibiotics	Restricted	34
B	5	Without antibiotics	*Ad libitum*	12
B	5	Without antibiotics	Restricted	11

545 Sample processing, DNA extraction and sequencing

546 Animals were slaughtered (at 66 and 60 days of age in farm A and farm B, respectively) and
547 cecal samples of each rabbit were collected in a sterile tube, kept cold in the laboratory (4ºC)
548 and stored at -80ºC. DNA extraction, amplification, Illumina library preparation and
549 sequencing followed methods described previously [12-Velasco-Galilea et al., 2018]. Whole
550 genomic DNA was extracted from 250 mg of each cecal samples using ZR Soil Microbe
551 DNA MiniPrep™ kit (ZymoResearch, Freiburg, Germany) according to manufacturer's
552 instructions with the following modification: cecal samples were mechanically lysed in a
553 FastPrep-24™ Homogenizer (MP Biomedicals, LLC, Santa Ana, CA, United States) at a
554 speed of 1 x 6 m/s for 60 s allowing an efficient lysis of archaea and bacteria species. Integrity
555 and purity of DNA extracts were measured with a Nanodrop ND-1000 spectrophotometer
556 equipment (NanoDrop products; Wilmington, DE, United States) according to Desjardins
557 and Conklin's protocol [44- Desjardins and Conklin, 2010]. All DNA extracts had adequate
558 integrity and purity (absorbance ratio 260 nm/280 nm > 1.6) to avoid PCR inhibition issues.

559

560 A fragment of the 16S rRNA gene including the V4-V5 hypervariable regions was amplified
561 with F515Y/R926 primer combination (5'-GTGYCAGCMGCCGCGGTAA-3', 5'-
562 CCGYCAATTYMTTTRAGTTT-3') [45-Parada et al., 2016] and then re-amplified in a
563 limited-cycle PCR reaction to add sequencing adaptors and 8 nucleotide dual-indexed
564 barcodes of multiplex Nextera® XT kit (Illumina, Inc., San Diego CA, United States)
565 following manufacturer's instructions. The initial PCR reactions were performed for each
566 sample using 12.5 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl forward primer, 5 µl reverse
567 primer and 2.5 µl template DNA (5 ng/ µl). The initial PCR conditions were as follows: initial

denaturation for 3 minutes at 95 ºC, 25 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 2 minutes at 72 ºC. The addition of indexes and sequencing adaptors to both ends of the amplified regions took place in a second PCR by using 25 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl index i7, 5 µl index i5, 10 µl PCR Grade water and 5 µl concentrated amplicons of initial PCR. The second PCR conditions were as follows: initial denaturation for 3 minutes at 95 ºC, 8 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 5 minutes at 72 ºC. Final libraries were cleaned up with AMPure XP beads, validated by running 1 µl of a 1:50 dilution on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa Clara, CA, United States) to verify their size, quantified by fluorometry with PicoGreen dsDNA quantification kit (Invitrogen, Life Technologies, Carlsbad, CA, United States), pooled at equimolar concentrations and paired-end sequenced in 5 parallel plates in a Illumina MiSeq 2 x 250 platform at the Genomics and Bioinformatics Service (SGB) of the Autonomous University of Barcelona (UAB).

Bioinformatic pipeline for OTU calling

Sequence processing was performed using QIIME software (version 1.9.0) [46- Caporaso et al., 2010]. In a first step, the resulting paired-ended V4-V5 16S rRNA gene reads were assembled into contigs with the python script *multiple_join_paired_ends.py*. Then the contigs were curated using the script *split_libraries.py* with default parameters in order to assign them to samples and to discard those with a low-quality (Q19 was the minimum acceptable quality score). Chimeric sequences generated during the process of DNA amplification were detected with UCHIME algorithm [47- Edgar et al., 2011] and removed.

591 The totality of filtered contigs were clustered into operational taxonomic units (OTUs) with
592 a 97% similarity threshold using the script *pick_open_reference_otus.py* with default
593 parameters [48- Rideout et al., 2014] that grouped, through UCLUST algorithm [49- Edgar,
594 2010], the sequences against Greengenes reference database (version gg_13_5_otus) and also
595 made a *de novo* clustering of those that did not match the database. The generated OTU table
596 was filtered at: (1) sample level: by discarding samples with less than 5,000 final sequence
597 counts and at (2) OTU level: by removing the doubleton ones. The filtered OTU table
598 contained the sequence counts of 963 OTUs for 425 samples. Taxonomic assignment of
599 representative sequences of each OTU defined (963) was conducted by mapping them to the
600 Greengenes reference database gg_13_5_otus with the UCLUST consensus taxonomy
601 assigner (QIIME default parameters). The raw sequence data were deposited in the sequence
602 read archive of NCBI under the BioProject accession number PRJNA524130. Metadata, the
603 prefiltered and normalized OTU tables, and corresponding taxonomic classifications are also
604 included as Additional files 1, 2, 3 and 4, respectively.

605

606 Models and statistical methods

607 In order to study differences in diversity and richness between rabbits grouped according to
608 the farm environment and the management that they received, two alpha diversity indexes
609 (Shannon and the observed number of OTUs) were computed from the OTU table rarified to
610 10,000 sequences per sample with "phyloseq" R package [50-phyloseq]. The statistical
611 method chosen to assess alpha diversity differences between these groups of animals was an
612 analysis of variance that included a factor resulting from the combination of four factors (the

613 farm where the animal was raised, the batch, the presence or the absence of antibiotics in the
614 feed and the feeding regime). The significance threshold was set at 0.05 for type I error.

615

616 Different approaches were considered to assess the influence of the environments and
617 management factors on microbial composition. A bootstrap analysis of variance was
618 individually implemented for each OTU to test whether it was differentially represented
619 between the different categories of the factors studied. This univariate analysis was
620 conducted by normalizing the OTU table with the cumulative sum scaling (CSS) method [51-
621 Paulson et al., 2013] and only for those OTUs which were detected in at least 5% of the
622 samples and had a sum of its counts resulting in a frequency greater than 0.01% of the total
623 sum of all OTUs counts across all samples. It was implemented by fitting a model defined
624 by the combination of the four aforementioned factors by using *lm()* function in R [52- R].
625 Then, the differences between the CSS-normalized OTUs counts in the different levels of the
626 studied factors were tested. The significance between the levels of the main factors: farm,
627 presence of antibiotics in the feed and feeding regime was assessed using an F statistic. When
628 the involved interaction terms were significant, the contrasts of interest were studied nested
629 within the levels of other interacting factors, i.e. feeding regime were studied within farm
630 levels. When the interaction terms were not significant, the effects of the different levels were
631 averaged, i.e. the effects of the levels of the batches within the farm A were averaged to
632 present the effect associated with this farm. In the performed F tests instead of relying on the
633 theoretical distribution of the statistic under the null hypothesis to define the p-values, they
634 were empirically computed using bootstrap after 1,000 permutations of the dependent
635 variable with respect to the design matrix of factors in the model. The use of bootstrapping

enabled the hypothesis test to be done without the need of assuming that data are normally distributed, which is an assumption that fails for OTUs counts. *P*-value was defined as the proportion of bootstrap rounds having an F statistic value equal or greater than that obtained with the original dataset. *P*-values were corrected defining a false discovery rate (FDR) of 0.05 [53- Benjamini and Hochberg, 1995]. This bootstrap analysis of variance approach was also implemented to study the effect of the management factors on the relative abundance of bacteria at phylum and genus levels.

The value of the microbial information to classify samples into the three factors considered in our study was explored using multivariate techniques. In particular, sparse partial least squares-discriminant analysis (sPLS-DA) [54-Le Cao et al., 2008] was used to find the combination of OTUs that allowed the best classification of cecal samples according to: (1) the farm where the animals were raised, (2) the feeding regime within the farm A and (3) the combination of feeding regime and the presence or absence of antibiotics in the feed for the animals raised in farm B. This approach was implemented through the R package "mixOmics" [55-mixomics]. In a first step, the function *tune.splsda()* was used to select the optimal sparsity parameters of the sPLS-DA model: the number of components and the number of variables (OTUs) per component. For the tuning process, a 5-fold cross-validation repeated 10 times was performed one component at a time, with a maximum of 4 components, on an input grid of values that indicate the number of variables to select on each component. The sparsity parameters were defined, based on the BER and centroids distance, and then included in the final sPLS-DA model. Samples were represented on the first two components and colored according to their class (e.g., R or AL in the case of the feeding

regime) in a sample plot with the function *plotIndiv()*. The performance of the sPLS-DA model was assessed with a 5-fold cross-validation repeated 1,000 times that randomly split the data in training and validation sets. In this data partition, it was ensured that 20% of the samples within each level of the discriminant factor were assigned to the validation set. Five different partitions were performed for each replicate to guarantee a different sample distribution in each validation set. The sPLS-DA model with the sparsity parameters previously defined was adjusted in the training set and its classification performance was assessed in the validation set using the overall and BER per class as criteria. The stability of the OTUs selected on each component was also assessed in the cross-validation by computing the selection frequency of each variable across the replicates.

Additional files

Additional file 1: metadata.txt. Metadata associated with the 425 rabbit cecal samples analyzed in this study.

Additional file 2: otu_table_prefiltered_unnormalized.txt. Prefiltered and unnormalized OTU table used for statistical analyses in this study.

Additional file 3: otu_table_filtered_CSSnormalized.txt. Filtered and CSS-normalized OTU table used for statistical analyses in this study.

Additional file 4: rep_OTUs_tax_assignments.txt. Taxonomic assignments for all OTUs in Additional file 2.

Additional file 5: phyla_table.txt. Relative abundances phyla table built from the collapse of the filtered and CSS-normalized OTU table at phylum level.

Additional file 6: genera_table.txt. Relative abundances genera table built from the collapse of the filtered and CSS-normalized OTU table at genus level.

Additional file 7: Table S1. OTUs differentially represented between farms.

Additional file 8: Table S2. OTUs differentially represented between feeding regimes within farms.

Additional file 9: Table S3. OTUs differentially represented between the presence and the absence of antibiotics in the feed within the farm B.

List of abbreviations

AL: *ad libitum*

BER: balanced error rate

CSS: cumulative sum scaling

FDR: false discovery rate

GIT: gastrointestinal tract

NGS: next generation sequencing

OTU: operational taxonomic unit

PCR: polymerase chain reaction

R: restricted

sPLS-DA: sparse partial least squares-discriminant analysis

SRB: sulfate-reducing bacteria (SRB)

SSB: sulfate-secreting bacteria (SRB)

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of the animal care and use committee of the Institute for Food and Agriculture Research and Technology (IRTA). The protocol was approved by the committee of the Institute for Food and Agriculture Research and Technology (IRTA).

Consent for publication

Not applicable.

Availability of data and materials

The raw sequence data were deposited in the sequence read archive of NCBI under the accession number SRP186982 (BioProject PRJNA524130). Metadata, the prefiltered and unnormalized OTU table, the filtered and CSS-normalized OTU table and corresponding

717 taxonomic assignments have all been included as Additional files 1, 2, 3 and 4, respectively.
718 Relative abundances phyla and genera table have also been included as Additional files 5 and
719 6, respectively. OTUs differentially represented between the studied factors, their sequences
720 and their assignment at the lowest taxonomic level have been included as Additional files 7,
721 8 and 9.

722

723 ***Competing interests***

724 The authors declare that they have no competing interests.

725

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733

734 ***Authors' contributions***

735 JS, MP and OR conceived the experimental design. JS, OR, MP and MVG collected
736 biological samples. MVG, OGR, MP, MG and AS processed the samples in the laboratory.
737 MVG processed and analyzed the sequencing data, interpreted data, prepared figures and

tables, and wrote the manuscript. JS and YRC helped analyzing the sequencing data. JS, MG, MP, MV and YRC helped interpreting the data, and wrote and revised the manuscript. All authors read and approved the final manuscript.

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References

[1] Gaskins, H. R. Immunological aspects of host/microbiota interactions at the intestinal epithelium. Gastrointestinal microbiology;1997;*2*:537-587.

[2] Belkaid, Y., Hand, T. W. Role of the microbiota in immunity and inflammation. Cell;2014;*157*(1):121-141.

[3] Gouet, P. H., Fonty, G. Changes in the digestive microflora of holoxenic* rabbits from birth until adulthood. Annales de biologie animale biochimie biophysique;1979;*19*:553–566.

[4] Abecia, L., Fondevila, M., Balcells, J., Lobley, G. E., McEwan, N. R. The effect of medicated diets and level of feeding on caecal microbiota of lactating rabbit does. Journal of applied microbiology;2007;*103*(4):787-793.

[5] Zou, F., Zeng, D., Wen, B., Sun, H., Zhou, Y., Yang, M., et al. Illumina Miseq platform analysis caecum bacterial communities of rex rabbits fed with different antibiotics. AMB express;2016:*6*(1):100.

[6] Zhu, Y., Sun, Y., Wang, C., Li, F. Impact of dietary fibre: starch ratio in shaping caecal archaea revealed in rabbits. Journal of animal physiology and animal nutrition;2017;*101*(4);635-640.

[7] Chen, S. Y., Deng, F., Jia, X., Liu, H., Zhang, G. W., Lai, S. J. Gut microbiota profiling with differential tolerance against the reduced dietary fibre level in rabbit. Scientific reports;2019;*9*(1):1-9.

[8] Combes, S., Michelland, R. J., Monteils, V., Cauquil, L., Soulié, V., Tran, N. U., et al. Postnatal development of the rabbit caecal microbiota composition and activity. FEMS microbiology ecology;2011;77(3):680-689.

778 [9] Gidenne, T., García, J., Lebas, F., Licois, D. Nutrition and feeding strategy: interactions
779 with pathology. Nutrition of the rabbit;2010;*20*:179.

780

781 [10] Eshar, D., Weese, J. S. Molecular analysis of the microbiota in hard feces from healthy
782 rabbits (Oryctolagus cuniculus) medicated with long term oral meloxicam. BMC veterinary
783 research;2014;*10*(1):62.

784

785 [11] Gidenne, T., Combes, S., Feugier, A., Jehl, N., Arveux, P., Boisot, P., et al. Feed
786 restriction strategy in the growing rabbit. 2. Impact on digestive health, growth and carcass
787 characteristics. Animal;2009;*3*(4):509-515.

788

789 [12] Velasco-Galilea, M., Piles, M., Viñas, M., Rafel, O., González-Rodríguez, O.,
790 Guivernau, M., et al. Rabbit microbiota changes throughout the intestinal tract. Frontiers in
791 microbiology;2018;*9*:2144.

792

793 [13] Kylie, J., Weese, J. S., Turner, P. V. Comparison of the fecal microbiota of domestic
794 commercial meat, laboratory, companion, and shelter rabbits (Oryctolagus cuniculi). BMC
795 veterinary research;2018;*14*(1):143.

796

797 [14] O'Neil, D. S., Stewart, C. J., Chu, D. M., Goodspeed, D. M., Gonzalez-Rodriguez, P. J.,
798 Shope, C. D., et al. Conditional postnatal deletion of the neonatal murine hepatic circadian

gene, Npas2, alters the gut microbiome following restricted feeding. American journal of obstetrics and gynecology;2017;*217*(2):218-e1.

[15] Chen, J., Toyomasu, Y., Hayashi, Y., Linden, D. R., Szurszewski, J. H., Nelson, H., et al. Altered gut microbiota in female mice with persistent low body weights following removal of post-weaning chronic dietary restriction. Genome medicine;*2016*;8(1):103.

[16] Zarrinpar, A., Chaix, A., Yooseph, S., Panda, S. Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. Cell metabolism;2014;*20*(6):1006-1017.

[17] Soler, C., Goossens, T., Bermejo, A., Migura-García, L., Cusco, A., Francino, O., et al. Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. PloS one;2018;*13*(5).

[18] Kumar, S., Chen, C., Indugu, N., Werlang, G. O., Singh, M., Kim, et al. Effect of antibiotic withdrawal in feed on chicken gut microbial dynamics, immunity, growth performance and prevalence of foodborne pathogens. PLoS One;2018;*13*(2).

[19] Fang, S., Xiong, X., Su, Y., Huang, L., Chen, C. 16S rRNA gene-based association study identified microbial taxa associated with pork intramuscular fat content in feces and cecum lumen. BMC microbiology;2017;*17*(1):162.

[20] Dai, Z. L., Wu, G., Zhu, W. Y. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. Frontiers bioscience;2011;*16*(1):1768-86.

[21] Sun, X., Shen, J., Liu, C., Li, S., Peng, Y., Chen, C., et al. L-Arginine and N-carbamoylglutamic acid supplementation enhance young rabbit growth and immunity by regulating intestinal microbial community. Asian-Australasian journal of animal sciences;2020;*33*(1):166.

[22] Müller, V. M., Zietek, T., Rohm, F., Fiamoncini, J., Lagkouvardos, I., Haller, D., et al. Gut barrier impairment by high-fat diet in mice depends on housing conditions. Molecular nutrition & food research;2016;*60*(4):897-908.

[23] Bäuerl, C., Collado, M. C., Zuniga, M., Blas, E., Martínez, G. P. Changes in cecal microbiota and mucosal gene expression revealed new aspects of epizootic rabbit enteropathy. PLoS One;2014;*9*(8).

[24] Thoene-Reineke, C., Fischer, A., Friese, C., Briesemeister, D., Göbel, U. B., Kammertoens, T., et al. Composition of intestinal microbiota in immune-deficient mice kept in three different housing conditions. PloS one;2014;*9*(11).

[25] Terán-Ventura, E., Roca, M., Martin, M. T., Abarca, M. L., Martinez, V., Vergara, P. Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats. Microbial ecology;2010;*60*(3):691-702.

[26] Arrazuria, R., Elguezabal, N., Juste, R. A., Derakhshani, H., Khafipour, E. Mycobacterium avium subspecies paratuberculosis infection modifies gut microbiota under different dietary conditions in a rabbit model. Frontiers in microbiology;2016;*7*:446.

[27] Belzer, C., De Vos, W. M. Microbes inside—from diversity to function: the case of Akkermansia. The ISME journal;2012;*6*(8):1449-1458.

[28] Angert, E. R., Brooks, A. E., Pace, N. R. Phylogenetic analysis of Metabacterium polyspora: clues to the evolutionary origin of daughter cell production in Epulopiscium species, the largest bacteria. Journal of bacteriology;1996;*178*(5:1451-1456.

[29] Rey, F. E., Gonzalez, M. D., Cheng, J., Wu, M., Ahern, P. P., Gordon, J. I. Metabolic niche of a prominent sulfate-reducing human gut bacterium. Proceedings of the national academy of sciences;2013;*110*(33):13582-13587.

[30] Zeng, B., Han, S., Wang, P., Wen, B., Jian, W., Guo, W., et al. The bacterial communities associated with fecal types and body weight of rex rabbits. Scientific reports;2015;*5*:9342-9350.

[31] Di Rienzi, S. C., Sharon, I., Wrighton, K. C., Koren, O., Hug, L. A., Thomas, B. C., et al. The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria. Elife;2013;*2*:e01102.

[32] Monchamp, M. E., Spaak, P., Pomati, F. Long term diversity and distribution of non-photosynthetic cyanobacteria in peri-alpine lakes. Frontiers in microbiology;2019;*9*:3344.

[33] Liu, P. Y., Cheng, A. C., Huang, S. W., Lu, H. P., Oshida, T., Liu, W., et al. Body-size Scaling is Related to Gut Microbial Diversity, Metabolism and Dietary niche of Arboreal folivorous flying Squirrels. Scientific reports;2020;*10*(1):1-12.

[34] Ai, D., Pan, H., Li, X., Gao, Y., Liu, G., Xia, L. C. Identifying gut microbiota associated with colorectal cancer using a zero-inflated lognormal model. Frontiers in microbiology;2019;*10*:826.

[35] Shen, Q., Maitin, V. Obesity-associated gut microbiota: characterization and dietary modulation. In Diet-Microbe Interactions in the Gut. Academic press;2015:149-171.

881

882 [36] McCabe, M. S., Cormican, P., Keogh, K., O'Connor, A., O'Hara, E., Palladino, R. A.,
883 et al. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an
884 uncharacterised Succinivibrionaceae and an increase of the Methanobrevibacter gottschalkii
885 clade in feed restricted cattle. PloS one;2015;*10*(7).

886

887 [36] McGovern, E., McCabe, M. S., Cormican, P., Popova, M., Keogh, K., Kelly, A. K., et
888 al. Plane of nutrition affects the phylogenetic diversity and relative abundance of
889 transcriptionally active methanogens in the bovine rumen. Scientific reports;2017;*7*(1):1-10.

890

891 [37] Bui, T. P. N., Schols, H. A., Jonathan, M., Stams, A. J., de Vos, W. M., Plugge, C. M.
892 Mutual Metabolic Interactions in Co-cultures of the Intestinal Anaerostipes rhamnosivorans
893 With an Acetogen, Methanogen, or Pectin-Degrader Affecting Butyrate Production.
894 Frontiers in microbiology;2019;*10*:2449.

895

896 [38] Samuel, B. S., Gordon, J. I. A humanized gnotobiotic mouse model of host–archaeal–
897 bacterial mutualism. Proceedings of the national academy of sciences;2006;*103*(26):10011-
898 10016.

899

900 [39] Ziętak, M., Kovatcheva-Datchary, P., Markiewicz, L. H., Ståhlman, M., Kozak, L. P.,
901 Bäckhed, F. Altered microbiota contributes to reduced diet-induced obesity upon cold
902 exposure. Cell metabolism;2016;*23*(6):1216-1223.

903

904 [40] Gandy, K. A. O., Zhang, J., Nagarkatti, P., Nagarkatti, M. The role of gut microbiota in
905 shaping the relapse-remitting and chronic-progressive forms of multiple sclerosis in mouse
906 models. Scientific reports;2019;*9*(1):1-17.

907

908 [41] North, M. K., Dalle Zotte, A., Hoffman, L. C. Composition of rabbit caecal microbiota
909 and the effects of dietary quercetin supplementation and sex thereupon. World rabbit
910 science;2019;*27*(4):185-198.

911

912 [42] Piles, M., Sánchez, J. P. Use of group records of feed intake to select for feed efficiency
913 in rabbit. Journal of animal breeding and genetics;2019;*136*(6):474-483.

914

915 [43] Gómez, E. A., Rafel, O., Ramon, J. The Caldes strain (Spain). Options
916 Méditerranéennes. Série B: Etudes et recherches (CIHEAM);2002.

917

918 [44] Desjardins, P., Conklin, D. NanoDrop microvolume quantitation of nucleic
919 acids. Journal of visualized experiments: JoVE;2010;*45*.

[45] Parada, A. E., Needham, D. M., Fuhrman, J. A. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environmental microbiology;2016;*18*(5):1403-1414.

[46] Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. QIIME allows analysis of high-throughput community sequencing data. Nature methods;2010;*7*:335-336.

[47] Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., Knight, R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics;2011;*27*:2194-2200

[48] Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., et al. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. PeerJ;2014;*2*:e545.

[49] Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. Bioinformatics;2010;*26*:2460–2461.

939 [50] McMurdie, P. J., Holmes, S. phyloseq: an R package for reproducible interactive
940 analysis and graphics of microbiome census data. PloS one;2013;*8*(4):e61217.

941

942 [51] Paulson, J. N., Stine, O. C., Bravo, H. C., Pop, M. Differential abundance analysis for
943 microbial marker-gene surveys. Nature methods;2013;10(12),1200.

944

945 [52] R Development Core Team. R: A Language and Environment for Statistical Computing.
946 2010[http://cran.r-project.org].

947

948 [53] Benjamini, Y., Hochberg, Y. Controlling the false discovery rate: a practical and
949 powerful approach to multiple testing. Journal of the Royal statistical society: series B
950 (Methodological);1995;57(1):289-300.

951

952 [54] Lê Cao, K. A., Rossouw, D., Robert-Granié, C., Besse, P. A sparse PLS for variable
953 selection when integrating omics data. Statistical applications in genetics and molecular
954 biology;2018;*7*(1).

955

956 [55] Rohart, F., Gautier, B., Singh, A., Le Cao K. A. mixOmics: An R package for 'omics
957 feature selection and multiple data integration. PLoS computational
958 biology;2017;*13*(11):e1005752.