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

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1 # Abstract

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

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

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

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

30

31 **Keywords**

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

34

35 **Background**

36 Microbial communities that inhabit the gastrointestinal tract (GIT) of animals constitute a
37 complex ecosystem whose members constantly interact between them and with their host [1-
38 Gaskins, 1997]. These interactions ensure homeostatic balance maintenance since GIT
39 ecosystem components are involved in many physiological and immunological processes [2-
40 Belkaid and Hand, 2014]. In the case of the domestic meat rabbit (*Oryctolagus cuniculus*), a
41 small herbivorous mammalian belonging to the family *Leporidae*, cecum is the main organ
42 for microbial fermentation. Thus, it is no surprising that rabbit cecum hosts the richest and
43 the most diverse microbial community of its GIT [3-Gouet and Fonty, 1979]. For this reason,

44 cecum has been the organ preferably chosen in previous rabbit gut microbiota assessments
45 [4-Abecia et al., 2007; 5-Zou et al., 2016; 6-Zhu et al., 2017; 7-Chen et al., 2019].
46
47 Thanks to the development of the next generation sequencing (NGS) technologies, and their
48 rapidly decreasing costs, it is currently possible to characterize the gut microbiota of a large
49 number of animals. It allows a deeper comprehension of the differences between animals
50 concerning their microbial composition and diversity, which could partially be mediated by
51 the production environment where the animals are raised. Our general aim is to provide
52 further evidence of the effect of different management and environmental factors in the cecal
53 microbial composition and diversity. In relation to this topic, there is a certain amount of
54 information already published. A growing number of studies have revealed changes in rabbit
55 cecal microbial communities exerted by the age [8-Combes et al., 2011] or the type of feed
56 provided to the kits after weaning [6-Zhu et al., 2017; 7-Chen et al., 2019]. One of the
57 important factors of variation is the administration of antibiotics in the feed. In rabbit meat
58 production, antibiotics have been widely administrated to curb mortality, especially after
59 weaning when it often reaches peaks of over 20% as a result of the onset of gastrointestinal
60 symptoms [9-Gidenne et al., 2010]. Multiple studies have shown alterations caused on gut
61 microbiota by the administration of antibiotics in the feed [5-Zou et al., 2016; 10-Eshar &
62 Weese, 2014]. Despite the European Union banned their use as growth promoters in animal
63 feeds since 2006 (EC 1831/2003), the administration of one type of antibiotic molecule is
64 still allowed to prevent or treat the emergence of potential infectious diseases on farms.
65 However, substantial efforts are being made towards searching for efficient alternatives
66 which allow a complete withdrawal of antibiotic in animal feeds. In this context, application

67 of feed restriction during the growing period was proposed as an interesting alternative to the
68 use of antibiotics. Gidenne et al. (2009) [11-Gidenne et al., 2009] demonstrated that feed
69 restriction, despite penalizing animal growth, improves feed efficiency and reduces mortality
70 due to enteric disorders. It is hypothesized that these positive effects could be partially
71 explained by changes in gut microbial composition or activity originated by the application
72 of feed restriction although techniques used so far to study this possible association have not
73 found evidence of it [11-Gidenne et al., 2009].

74

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

81

82 Results

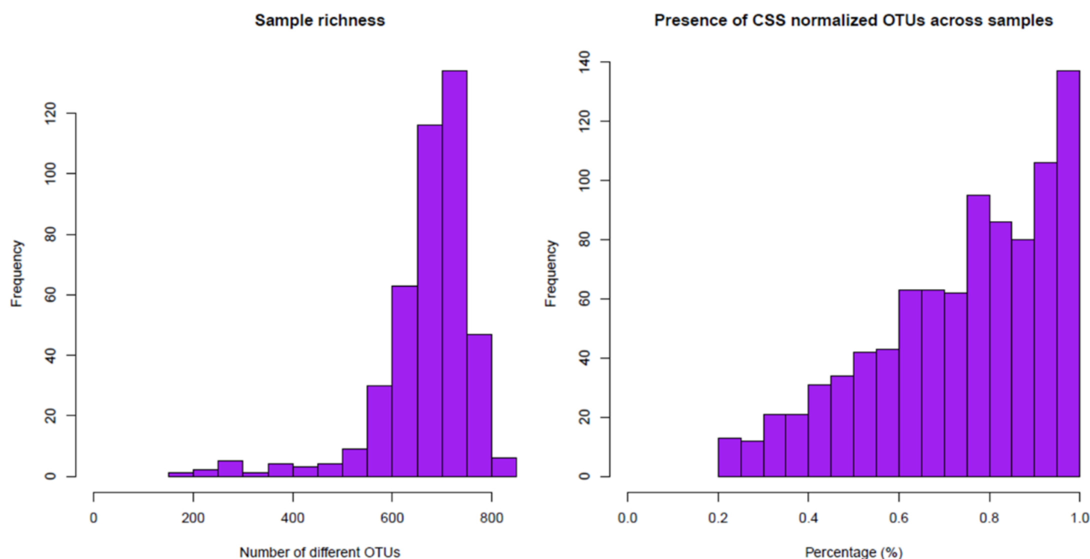
83 Sequence processing

84 After the removal of doubletons and samples with low sequence counts, 425 rabbit cecal
85 samples (Additional file 1) were represented on 14,928,203 sequence counts clustered into
86 963 different OTUs. Each sample had on average 35,125 final sequences (range: 10,157-
87 678,798) and 677 OTUs (range: 197-841) (Additional files 2 and 3). Figure 1 shows two
88 histograms representing the sample richness and the proportion of OTUs presence across

89 samples. Most of the samples had more than 700 different OTUs (mode = 748) and nearly

90 140 OTUs were present in all the samples.

91



92

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

94

95 Taxonomic assignment of representative OTUs against the Greengenes reference database

96 gg_13_5_otus (Additional file 4) revealed the presence of 8 different known phyla with an

97 average of 8 phyla per sample (range: 7-8) (Additional file 5) and 28 different known genera

98 with an average of 24 genera per sample (range: 17-28) (Additional file 6).

99

100 **Animal management and farm environment shaping cecal microbial alpha diversity**

101 The study of alpha diversity was performed after rarefying the prefiltered and unnormalized

102 OTU table to 10,000 sequences per sample. Rarefaction generated a table which contained

103 the sequence counts of 963 different OTUs for 425 samples. The average (standard deviation)
104 number of observed OTUs within animal was 560.52 (75.03) and the average Shannon index
105 within animal was 5.09 (0.26). The comparison of alpha diversities revealed that the group
106 of animals raised in farm B had greater alpha diversity than the group of animals raised in
107 farm A (estimated differences of 40.20 (9.83) observed OTUs and 0.17 (0.03) Shannon index;
108 $P_{FDR} < 0.001$). Furthermore, larger variability in both indexes was observed in farm A than
109 in farm B. No significant differences for the two alpha diversity indexes were found between
110 feeding regimes within both farms (Figure 1, $P_{FDR} > 0.05$), nor between the presence and the
111 absence of antibiotics in the feed within farm B (Figure 1, $P_{FDR} > 0.05$).

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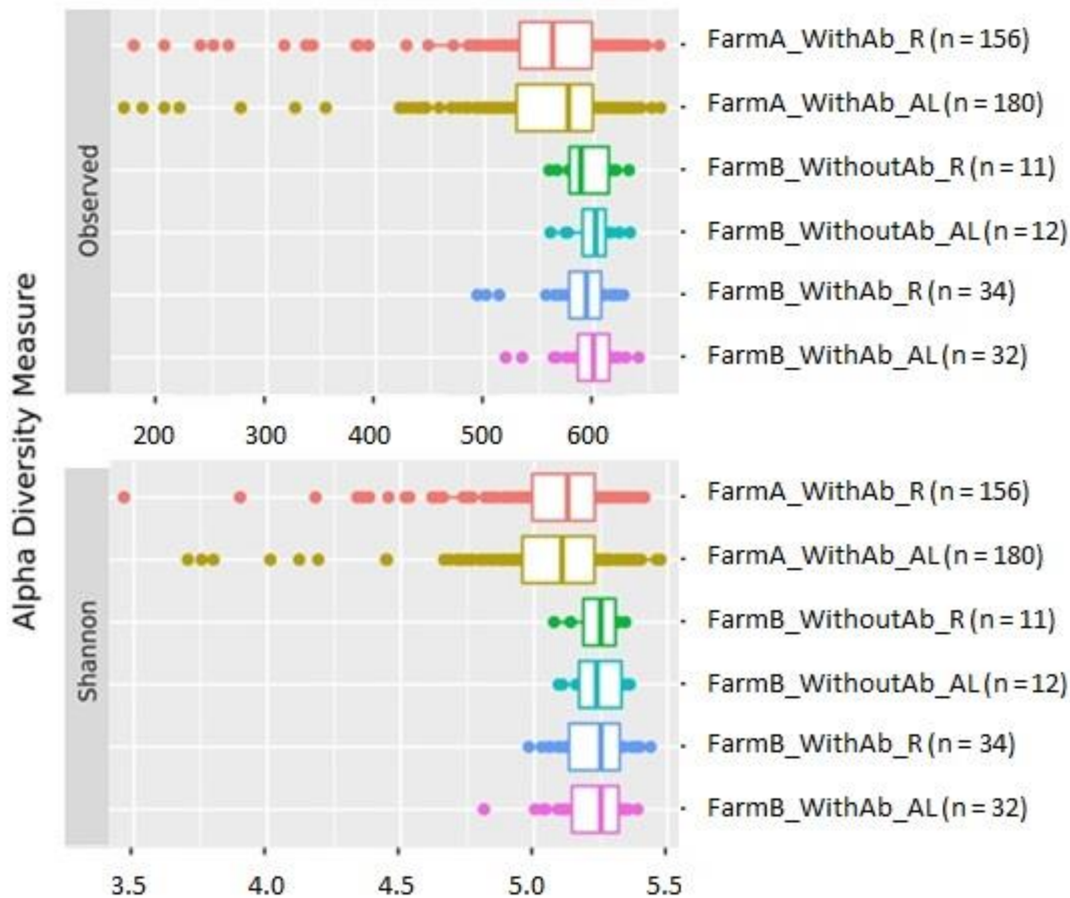

113

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

117

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

119 According to the taxonomic assignment of representative sequences (Additional file 4)
120 performed with the UCLUST consensus taxonomy assigner on the Greengenes reference
121 database gg_13_8_99_otus, *Firmicutes* (76.74%), *Tenericutes* (7.22%) and *Bacteroidetes*

122 (6.26%) were the predominant phyla, accounting for more than 90% of the microbial
123 diversity, in the rabbit cecal samples studied (Figure 3).

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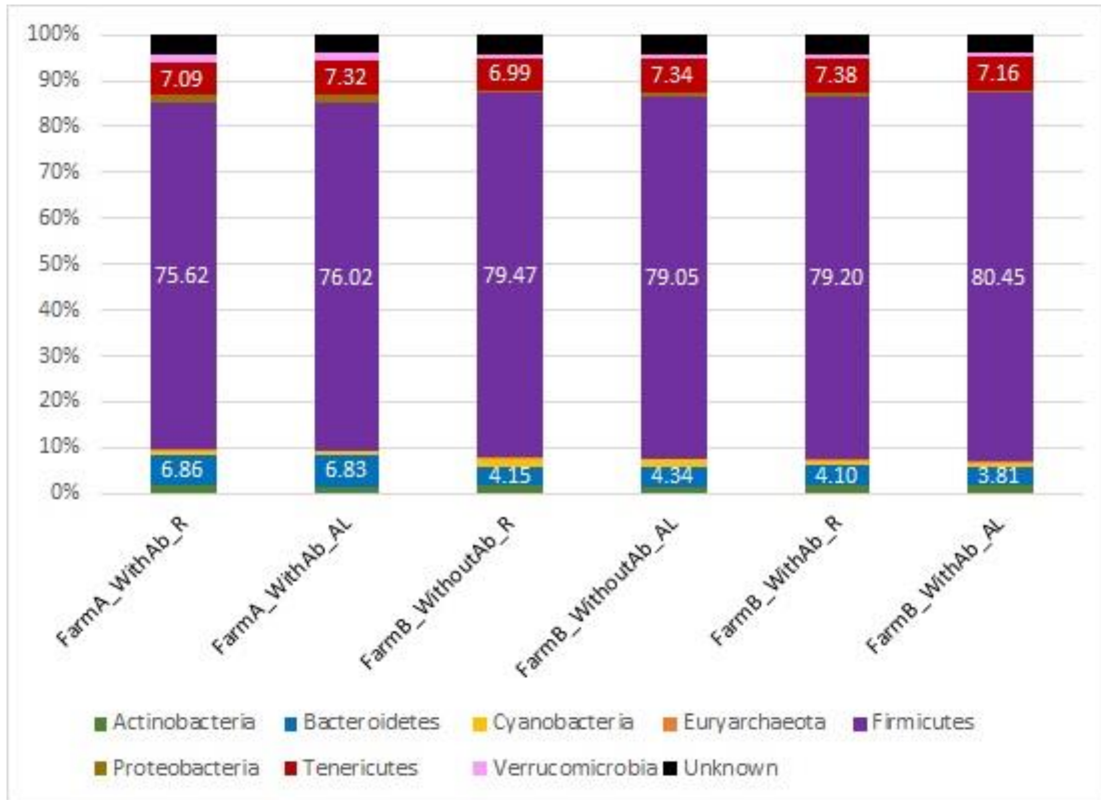

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126 **Figure 3 Phyla relative abundances of samples grouped according to farm, level of**
127 **feeding and presence of antibiotics in the feed.**

128

129 Differential cecal microbial composition across farms

130 Cecal samples of rabbits raised in farm A showed an overrepresentation of phyla
131 *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* while phyla *Euryarchaeota*,

132 *Cyanobacteria* and *Firmicutes* were found to be overrepresented in cecal samples of rabbits

133 raised in farm B (Table 1).

134

135 **Table 1 Microbial composition at phylum level in cecal samples of rabbits grouped by**

136 **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

137

138 Genera *Ruminococcus* (4.32%), *Blautia* (2.96%) and *Oscillospira* (2.37%) dominate meat

139 rabbit cecal microbiota. Most of the relative abundance differences at genus level were found

140 differentially represented between animals raised in the different farms: genera *Bacteroides*,

141 *Parabacteroides*, *Rikenella*, *Anaerofustis*, *Anaerostipes*, *Clostridium*, *Coprobacillus*,

142 *Anaeroplasma* and *Akkermansia* were overrepresented in cecal samples of rabbits raised in

143 farm A while genera *Adlercreutzia*, *Butyricimonas*, *Odoribacter*, *Methanobrevibacter*,

144 *Blautia*, *Butyrivibrio*, *Coprococcus*, *Dehalobacterium*, *Dorea*, *Oscillospira*, *rc4-4* and

145 *Oxalabacter* were overrepresented in cecal samples of rabbits raised in farm B. Interestingly,

146 genera *Epulopiscium*, *p-75-a5*, *Phascolarctobacterium*, *Campylobacter* and *Desulfovibrio*

147 were only found in samples collected from farm A (Table 2).

148

149 **Table 2 Relative abundances of genera, grouped by phylum, differentially represented**

150 **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

151

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

162

163 <u>Differential cecal microbial composition across feeding regime</u>

164 An overrepresentation of phyla *Cyanobacteria* (estimated difference R - AL = 0.11 ± 0.04;
165 $P_{FDR} = 0.04$) and *Verrucomicrobia* (estimated difference R - AL = 0.11 ± 0.05; $P_{FDR} = 0.04$)
166 was found in cecal samples of rabbits fed R and raised in farm A. On the other hand, phylum
167 *Euryarchaeota* was overrepresented in animals fed R and raised in farm B (estimated
168 difference R - AL = 0.14 ± 0.04; $P_{FDR} < 0.001$). At genus level, the only significant contrast
169 was observed for *rc4-4* which resulted overrepresented in samples from animals fed AL in
170 farm A (estimated difference R - AL = -0.03 ± 0.01; $P_{FDR} < 0.001$) while in farm B none of
171 the genera resulted differentially represented ($P_{FDR} > 0.05$) between feeding regimes. The
172 contrasts based on the CSS-normalized OTUs revealed 51 and 9 OTUs differentially

173 represented between feeding regimes within farms A and B, respectively. Within farm A, 32
174 OTUs were overrepresented in cecal samples of rabbits that were fed AL and 19 OTUs in the
175 samples from rabbits fed R. Within farm B, 7 OTUs were overrepresented in cecal samples
176 of rabbits that were fed AL and 2 OTUs were overrepresented in rabbits that were fed R.
177 Table S2 shows the estimated difference between feeding regime within farm of these OTUs,
178 their sequences and their assignment at the lowest taxonomic level. The analyses based on
179 the CSS-normalized OTUs within farm A are in full accordance with the analyses performed
180 at genus level given that all OTUs assigned to genus *rc4-4* (phylum *Firmicutes*) were
181 overrepresented in cecal samples of rabbits fed AL.

182

183 Effect of the presence of antibiotics in the feed

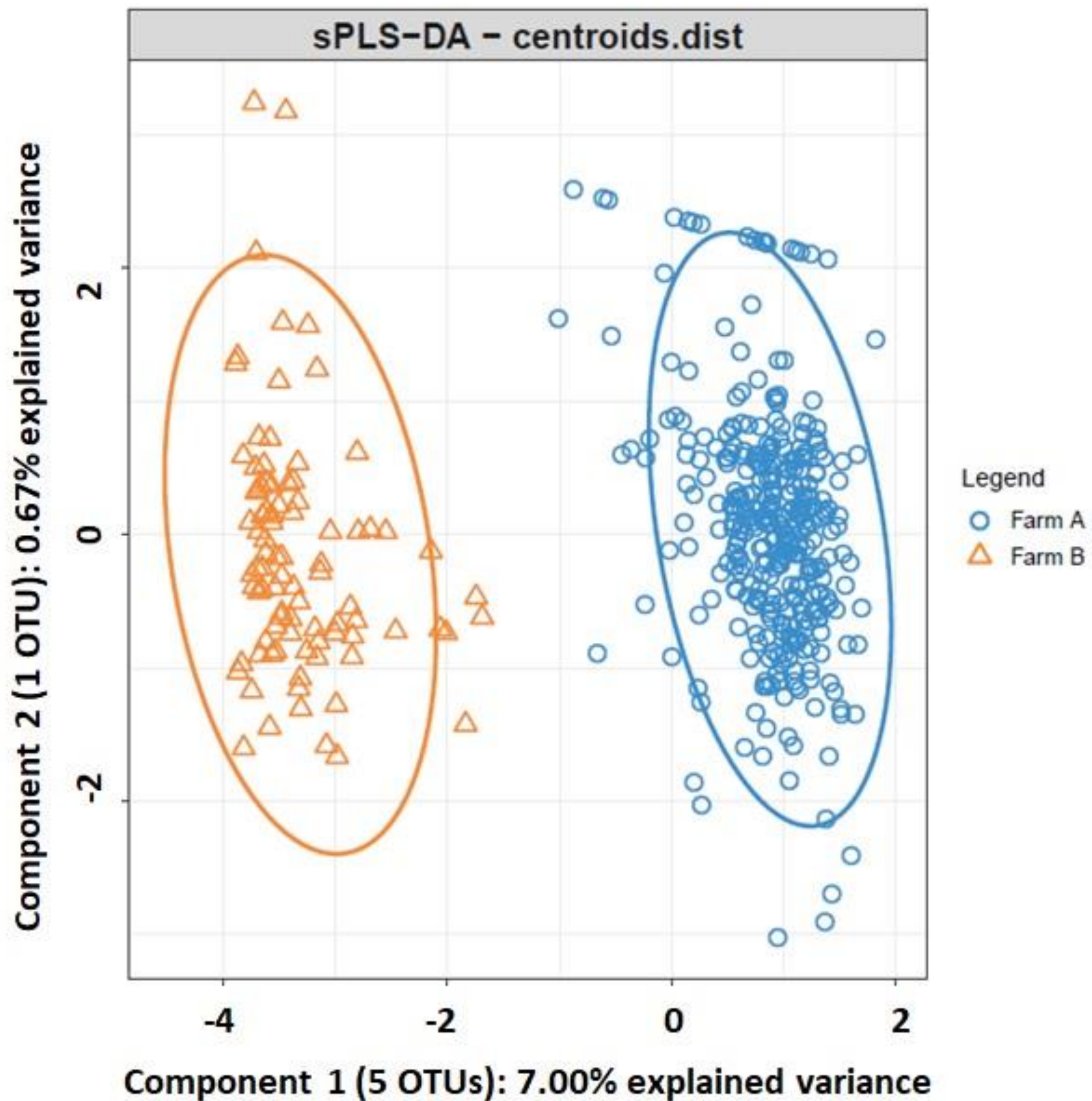
184 The effect of the presence of antibiotics in the feed could only be assessed within farm B
185 given that all rabbits raised in farm A received feed supplemented with antibiotics. Cecal
186 samples of rabbits that received feed free of antibiotics showed an overrepresentation of
187 phyla *Cyanobacteria* compared to those that received feed supplemented with antibiotics
188 (estimated difference withoutAb - withAb = 0.49 ± 0.09; $P_{FDR} < 0.001$). In addition, the
189 analyses on the CSS-normalized OTUs revealed an overrepresentation of 15 and 29 OTUs in
190 cecal samples of rabbits that received a feed supplemented or free of antibiotics; respectively.
191 Table S3 shows the estimated difference between the presence and the absence of antibiotics
192 in the feed for the OTUs in which the differences reached the significance threshold. The
193 OTU sequences as well as their assignment at the lowest taxonomic level are also shown in
194 Table S3. Only 1 of these OTUs could bet assigned at species level (*Bacteroides fragilis*) and
195 2 OTUs at genus level (*Oscillospira* and *Coprococcus*).

196

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

199 Sparse partial least squares-discriminant analyses (sPLS-DA) on the CSS-normalized OTUs
200 were conducted to discriminate samples according to the factors considered in this study (i.e.,
201 the farm where the animal was raised, the presence or the absence of antibiotics in the feed
202 and the feeding regime). The tuning process of the sPLS-DA conducted to discriminate
203 samples according to the farm where the rabbits were raised selected 5 OTUs for component
204 1 and 1 OTU for component 2 (Figure 4). Component 1 explained 7.00% of the total variance
205 while component 2 explained 0.67%. The classification performance of this sPLS-DA can
206 be said to be perfect since its overall and balanced error rate (BER) per class across 1000
207 replicates of 5-folds cross-validation runs was 0.00 (0.00). Furthermore, two OTUs of
208 component 1 had a stability higher than 0.9.

209



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

213

214 The sPLS-DA performed to discriminate samples across feeding regimes within farm A
215 selected 70 OTUs for component 1 and 65 OTUs for component 2 (Figure 5). Component 1
216 explained 2.34% of the total variance while component 2 explained 5.58%. The cross-

217 validation assessment of the classification performance of this sPLS-DA showed an overall
218 and BER per class of 0.27 (0.02). The stability of 18 and 5 OTUs selected in components 1
219 and 2, respectively, across the different cross-validation folds was higher than 0.9.

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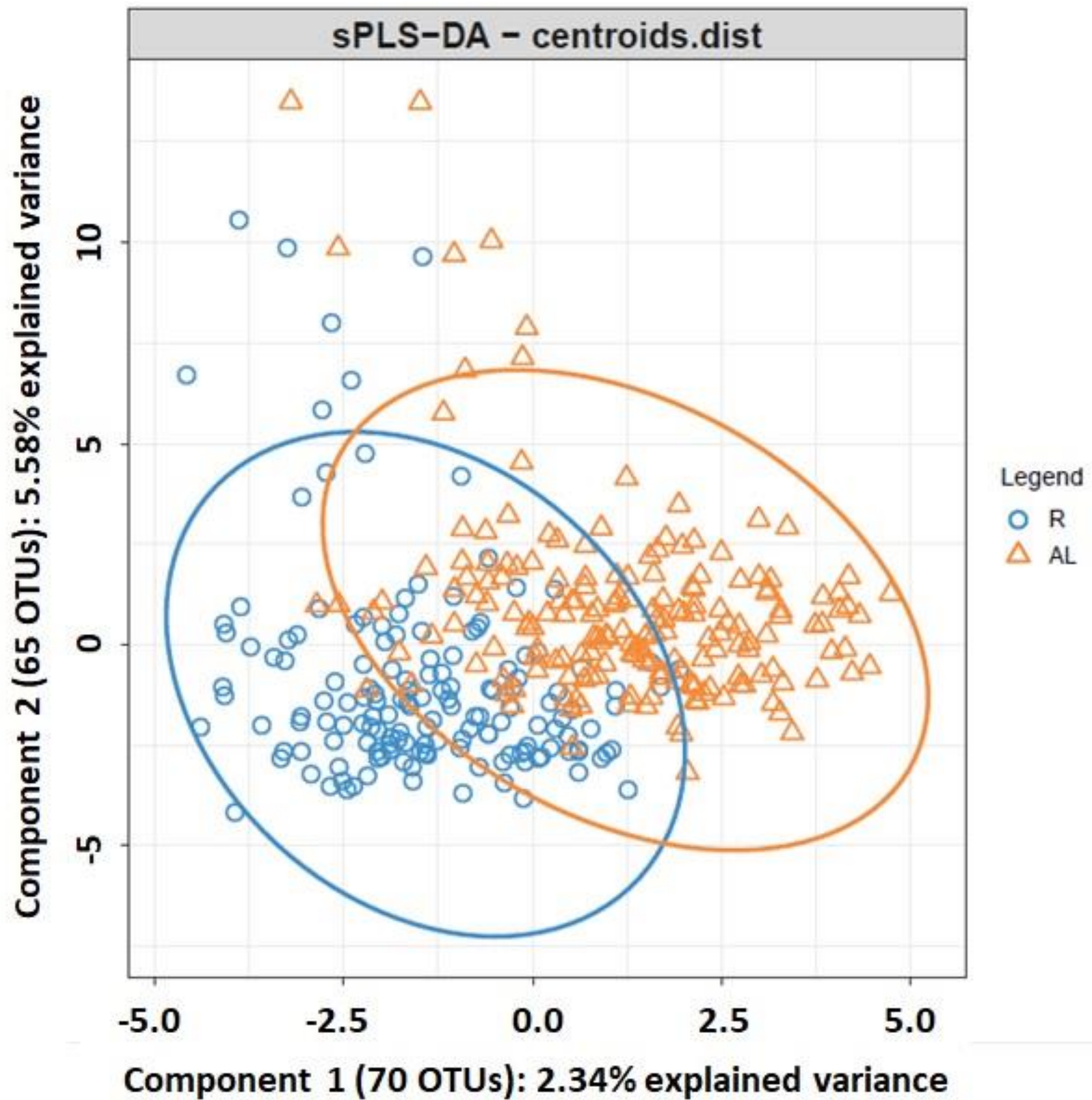

221

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

224

225 Finally, the sPLS-DA conducted to discriminate samples of animals raised within farm B
226 according to the combination of the presence or not of antibiotics in the feed and the feeding
227 regime, selected 9 OTUs for component 1 and 70 OTUs for component 2 (Figure 6).
228 Component 1 explained 3.05% of total variance and defined the discrimination between
229 samples from animals fed withAb and those fed withoutAb. On the other hand, component
230 2 explained 3.05% of total variance and defined the discrimination between samples from
231 animals fed R and those belonging to animals fed AL. The cross-validation assessment of the
232 classification performance of this sPLS-DA showed an overall BER of 0.32 (0.15). The BER
233 per class was 0.34 (0.12) for samples fed R withoutAb, 0.46 (0.14) for samples fedAL
234 withoutAb, 0.29 (0.11) for samples fed R withAb, and 0.20 (0.07) for samples fed AL
235 withAb. The stability of 3 and 11 OTUs selected in components 1 and 2, respectively, across
236 the different cross-validation folds was higher than 0.9.

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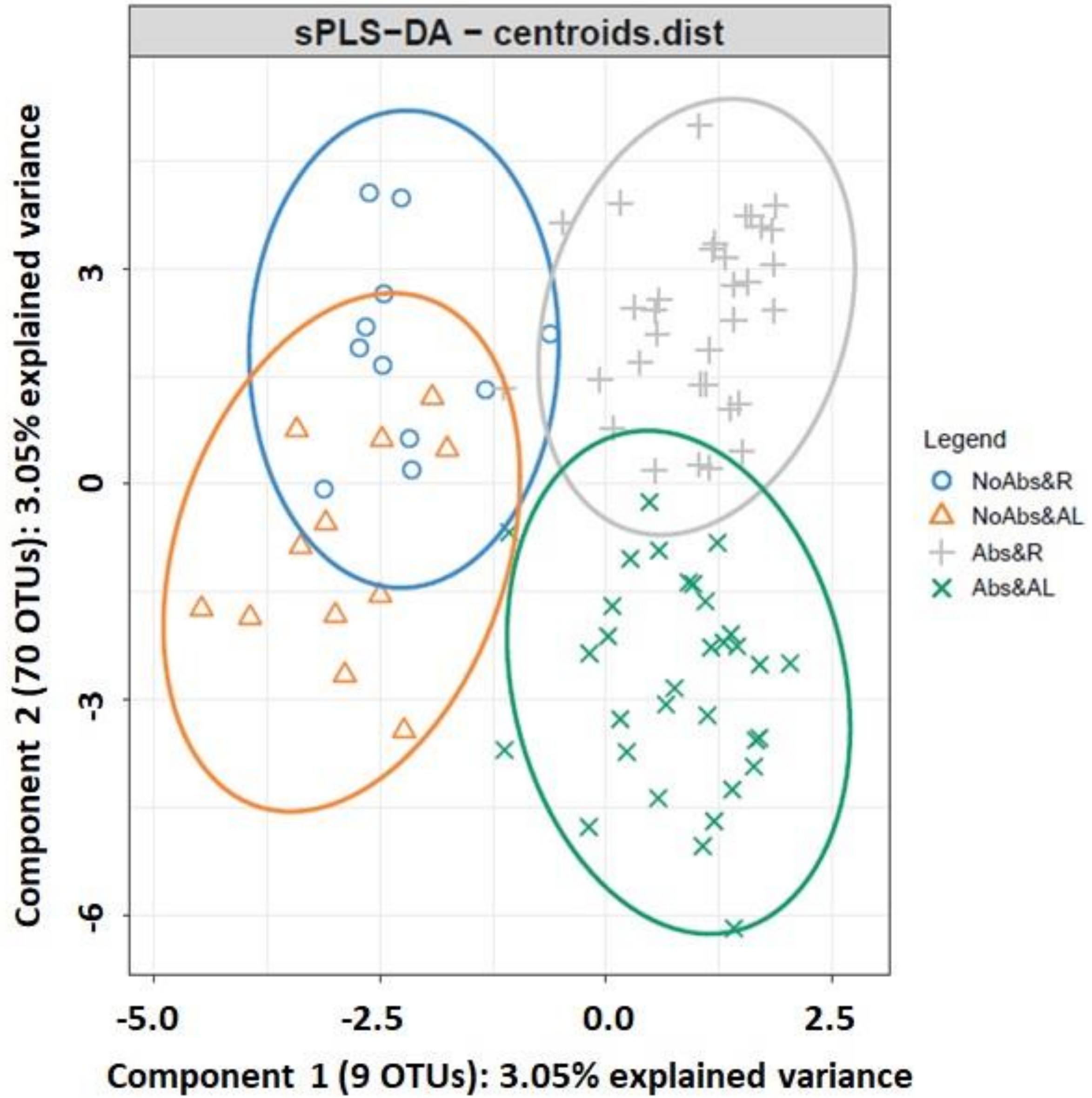

238

239 **Figure 6 Sparse partial least squares discriminant analysis representing cecal samples**

240 **of rabbits raised in farm B and fed R withoutAb (blue), fed AL withoutAb (orange), fed**

241 **R withAb (gray) and fed AL withAb (green).**

242

243

244 ## Discussion

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

252

253 The Illumina MiSeq sequence processing of samples collected from these animals revealed
254 that phyla *Firmicutes*, *Tenericutes* and *Bacteroidetes* dominate the growing meat rabbit cecal
255 ecosystem representing more than 90% of its entire microbial composition. This fact is in
256 accordance with previous studies that have characterized the rabbit cecal microbiota [5-Zou
257 et al., 2016; 7-Chen et al., 2019; 12-Velasco-Galilea et al., 2018] and reported *Firmicutes* as
258 the predominant phylum. However, there are discrepancies between studies in establishing
259 which other phyla are also prevalent in this ecosystem. Whereas we found phyla *Tenericutes*
260 and *Bacteroidetes* representing 7.22 and 5.93% of the cecal microbial composition,
261 respectively, Chen et al. 2019 [7] and Zou et al. 2016 [5] reported *Bacteroidetes* as the second
262 predominant phylum representing 18% and 20% of New Zealand White and Rex rabbit cecal
263 microbial composition, respectively. Conversely, other studies that have previously
264 characterized meat rabbit fecal microbiota identified phyla *Proteobacteria* and
265 *Verrucomicrobia* in higher relative abundances [13-Kylie et al., 2018; 10-Eshar and Weese,
266 2014]. Velasco-Galilea et al. 2018 [12] reported *Firmicutes* (76.42%), *Tenericutes* (7.83) and

267 *Bacteroidetes* (7.42) as the predominant phyla of meat rabbit fecal and cecal microbial
268 communities. These discrepancies found across studies could be attributed to technical issues
269 (e.g., pair of primers, sequencing platform, bioinformatic pipeline employed to process raw
270 sequences or reference database used for the taxonomic assignment of the representative
271 sequences) or to purely biological reasons (e.g., breed, age or section of the GIT sampled).
272 Nonetheless, Kylie et al. (2018) [13] depicted that the relative increase in less beneficial
273 phyla, such as *Proteobacteria*, could be related to seasonal climate changes that impact
274 directly to rabbit health. This impact affects the susceptibility to enteritis and possibly feed
275 conversion efficiency. In any case, this phylum is more prevalent in farm A where the animals
276 were more exposed to changes in climate conditions.

277

278 Regarding the alpha diversity assessment, Shannon and the observed number of OTUs
279 indexes revealed the existence of significant differences between housing conditions (i.e., the
280 experimental farm where the rabbits were raised). Cecal samples collected from rabbits raised
281 in farm B had greater richness and diversity that those belonging to animals raised in farm
282 A. This could be explained by more stable environmental conditions in farm B (i.e., facility
283 better insulated) than in farm A. This combined with the fact that samples of animals raised
284 in farm A were collected from rabbits produced in 4 different batches, could also explain the
285 larger variability in both indexes observed in this farm [13-Kylie et al., 2018]. Despite not
286 having observed significant differences between the presence or not of antibiotic in the feed,
287 nor between feeding regimes, it is noteworthy to mention that samples collected from animals
288 fed AL in both farms had a greater, although not significant, richness than those fed R. This
289 fact is consistent with previous studies in mice that observed a lower alpha diversity in

290 animals with a restricted level of feeding [14-O'Neil et al., 2017; 15-Chen et al., 2016; 16-
291 Zarrinpar et al., 2014]. Surprisingly, but in agreement with our results, studies performed in
292 pigs [17-Soler et al., 2017], chicken [18-Kumar et al., 2018] and Rex rabbits [5] also did not
293 show clear significant differences on alpha diversity indexes between animals fed on diets
294 with antibiotics with respect to those on diets free of antibiotics. Nevertheless, these studies
295 were able to detect differences in the relative abundances of some specific species between
296 diets. For example, Kumar et al. 2018 [18] found that the inclusion of bacitracin in the feed
297 did not affect the chicken bacterial phyla. However, they observed differences between the
298 control and the bacitracin-fed group in the ileal and cecal bacterial populations at lower
299 taxonomic levels.

300

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

306 As it might be expected, analyses of variance confirmed that the largest modification of meat
307 rabbit cecal microbial composition is generated by the housing conditions (in this case
308 represented by the farm factor). Our results revealed that the relative abundances of 6 out of
309 8 phyla are differentially represented between both farms. At genus level, we detected
310 significant differences in the relative abundances of almost all of them. Genera *Bacteroides*,
311 *Parabacteroides*, *Rikenella*, *Anaerofustis*, *Anaerostipes*, *Clostridium*, *Coprobacillus*,
312 *Anaeroplasma* and *Akkermansia* were enriched in cecal samples of rabbits housed in farm A.

313 The first three belong to phylum *Bacteroidetes* and genus *Bacteroides* is the most abundant
314 of them in meat rabbit cecum. Species of this genus are anaerobic Gram-negative members
315 of the family *Bacteroidaceae* that play an important role in the degradation of vegetal
316 polysaccharides and amino acid fermentation in the mammal GIT [19-Fang et al.,2017; 20-
317 Dai et al., 2011]. Moreover, this genus is involved in propionic acid and lactate formation
318 depending on nitrogen organic availability. Nonetheless, some authors showed that great
319 amounts of *Bacteroides* could predict obesity tendency. *Parabacteroides* is also an anaerobic
320 Gram-negative bacterium (family *Porphyromonadaceae*) involved in amino acid transport
321 and metabolism, energy production and conversion, lipid transport and metabolism,
322 recombination and repair, cell cycle control, cell division, and cell motility in the intestinal
323 microbiota of the growing rabbit [21-Sun et al., 2020]. This genus was specifically found in
324 the cecal microbiota of mice raised in conventional conditions and absent in those raised in
325 pathogen-free facilities in a study performed under different housing conditions [22- Müller
326 et al., 2016].

327 Within phylum *Firmicutes*, genus *Clostridium* (family *Clostridiaceae*) is an anaerobic Gram-
328 positive bacterium that inhabits the GIT of many mammals where it acts by degrading
329 cellulose. However, some *Clostridium* species (e.g., *C. perfringens* and *C. difficile*) are
330 pathogenic, and an enrichment of this genus has previously been described in rabbits affected
331 by epizootic rabbit enteropathy [23- Bäuerl et al., 2014]. This genus, together with genus
332 *Bacteroides*, was found enriched in the cecal microbiota of mice housed in open cages
333 compared with those kept in individual ventilated cages [24-Thoene-Reineke et al., 2014].
334 Both genera have been associated with an exacerbation of the intestinal inflammatory
335 response in mammals [25-Terán-Ventura et al., 2010]. Genus *Anaerofustis* (family

336 *Eubacteriaceae*) has been found enriched in cecal samples of rabbits affected by
337 paratuberculosis infection (*Mycobacterium avium*) [26-Arrazuria et al., 2016].

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

342 It is worth mentioning that we have detected genera *Epulopiscium*, *p-75-a5*,
343 *Phascolarctobacterium*, *Campylobacter* and *Desulfovibrio* only in the cecal samples of
344 rabbits housed in farm A. The first three are encompassed within phylum *Firmicutes.* Genus
345 *Epulopiscium* is a large size Gram-positive bacterium that has a nutritional symbiotic
346 relationship with surgeonfish that eats algae and detritus. This bacterium is physically similar
347 to the phylogenetically related *Metabacterium polyspora* which is an endospore-producing
348 bacterium isolated from the cecum of guinea pigs [28-Angert et al., 1996]. On the other hand,
349 genera *Campylobacter* and *Desulfovibrio* are Gram-negative bacteria that belong to phylum
350 *Proteobacteria*. Some species of these genera are pathogens responsible for mammal's
351 infections and diarrheas. The exclusive presence of these genera in farm A could indicate the
352 existence of a potential dysbiosis of the animals raised in that facility that could affect their
353 sanitary status and growth. While farm A was a semi-open-air facility, farm B was artificially
354 ventilated and offered more controlled environmental conditions that favor animal growth.
355 Moreover, the presence of sulfate-reducing bacteria (SRB) such as *Desulfovibrio* could be
356 enhanced by sulfate-secreting bacteria (SSB) such as *Rikenella* in farm A where this genus
357 is significantly more predominant. It is noteworthy to mention that SRB could also obtain

358 sulfate via *cross-feeding* mediated by *Bacteroides*-encoded sulfatases [29-Rey et al., 2013],
359 and interestingly, this phylum is more prevalent in farm A.

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

369

370 Within farm B, the effect of the presence of antibiotics in the feed was assessed by comparing
371 the microbial cecal composition of rabbits fed withAb with that of some animals that received
372 feed withoutAb. As stated above, we did not detect significant differences in alpha diversity
373 between both groups. Nor univariate studies revealed differences in the relative abundances
374 of genera between diets. However, some significant differences were observed at phylum
375 and OTU levels. An overrepresentation of phylum *Cyanobacteria* was found in rabbits fed
376 withoutAb. The detection of this bacterial phylotype, commonly assigned to photosynthetic
377 activity, in the rabbit cecum could suggest contamination during the GIT sampling. However,
378 Zeng et al. 2015 [30] previously reported its presence in rabbit feces. In the present study, all
379 OTUs taxonomically assigned to phylum *Cyanobacteria* are as well encompassed in the
380 order *YS2*. Interestingly, it was demonstrated that this order does not really have

381 photosynthetic capacity and it is currently classified within candidate phylum
382 *Melainabacteria* [31-Di Rienzi et al., 2013]. The non-photosynthetic cyanobacteria *YS2*, now
383 named *Gastranaerophilales*, is a fermenter gut-associated order present in humans and other
384 animals such as squirrels, where its exact role is unknown but it has the capacity to produce
385 hydrogen, fix nitrogen and synthesize vitamins B and K [31-Di Rienzi et al., 2013; 32-
386 Monchamp et al., 2019; 33-Liu et al., 2020]. Our results, in accordance with Kylie et al. 2018
387 [13], revealed that rabbits fed withoutAb exhibited higher abundances of OTUs assigned to
388 phylum *Bacteroidetes* than those fed withAb. In addition, samples of rabbits that received
389 antibiotics had a significant increase of an OTU taxonomically assigned to genus
390 *Coprococcus.* Interestingly, a study that evaluated the differences in bacterial communities
391 of Rex rabbits fed with different antibiotics also found an overrepresentation of this
392 bacterium in animals treated with zinc bacitracin [5-Zou et al., 2016]. *Coprococcus* is an
393 anaerobic bacterium that may protect against colon cancer in humans by producing butyric
394 acid [34-Ai et al., 2019]. We hypothesized that the administration of antibiotics could
395 modulate the abundance of some *Coprococcus* species to provide intestinal protection on
396 meat rabbits. However, it is important to recognize that the reduced sample size of the group
397 of rabbits fed withoutAb may have limited the statistical power to detect microbial
398 composition differences associated with this factor.

399

400 Within this farm, the effect of the feeding regime in microbial composition was also assessed
401 by comparing samples of animals fed R with those fed AL. The main difference found was
402 for phylum *Euryarchaeota* which was overrepresented in animals fed R in farm B. All
403 *Euryarchaeota* species found in the rabbit cecum belong to genus *Methanobrevibacter* that

404 encompasses different hydrogenotrophic methane-producing species. Previous studies in
405 humans [35-Shen and Maitin, 2015] and cattle [36-McCabe et al., 2015; 37-McGovern et al.,
406 2017] found an overrepresentation of *Methanobrevibacter* species in individuals submitted
407 to feed restriction and a negative correlation between the abundance of this bacterium and
408 body mass index. The growth of *Methanobrevibacter* is supported by fermenters such as
409 *Gastranaerophilales* and butyrate-producing bacteria such as *Anaereostipes* via interspecies
410 formate/hydrogen transfer [37-Bui et al., 2019]. A study in mice determined that
411 *Methanobrevibacter smithii* facilitates *Bacteroides thetaiotaomicron* capacity to digest
412 glycans resulting in increased production of short-chain fatty acids [38-Samuel and Gordon,
413 2006]. The same study defined *M. smithii* as a "*power broker*" that regulates polysaccharide
414 fermentation efficiency that influences the fat stores. The lower prevalence of methanogenic
415 archaea in farm A could be explained by the high presence of SRB that outcompetes with
416 methanogens for hydrogen consumption. This fact could favor hydrogen sulfide production
417 and compromise the rabbits' health.

418

419 Regarding the sample classification based on the sPLS-DA study conducted within farm B,
420 component 1 and component 2 discriminated between animals that received or not antibiotics
421 in the feed and between feeding regimes, respectively. It is worth mentioning that 8 out of 9
422 OTUs selected in component 1 were also declared as differentially represented between the
423 presence or the absence of antibiotics in the feed by the univariate analyses. Within farm A,
424 an sPLS-DA was also performed to classify samples according to the feeding regime using
425 microbial information. In this case, the discrimination achieved was quite poor as, despite a
426 large number of OTUs were selected as classifier variables in the tuning process, the

427 classification error rate was high. Nevertheless, bootstrap univariate analyses of variance
428 detected some significant differences at all taxonomic levels analyzed between feeding
429 regimes within farm A. At genus level, *rc4-4* was overrepresented in animals fed AL. This
430 genus belongs to phylum *Firmicutes* and it is known as an obesity-associated bacterium [39-
431 Ziętak et al., 2016] and as a pathogenic candidate identified in mice with multiple sclerosis
432 [40-Gandy et al., 2019]. A potential pro-inflammatory role has been proposed for this genus
433 [40] what could be related to a reduced incidence of enteric disorders when feed restriction
434 is applied. It is worth mentioning that family *Peptococcaceae*, which encompasses genus
435 *rc4-4*, is strongly related to total rabbit weight gain from weaning to 12-week old [41-North
436 et al., 2019]. Although in our study this genus was prevalent in animals fed AL, its association
437 with weight gain is not clear since the greater growth exhibited by these animals was
438 consequence of higher feed intake.

439

440 Different approaches have been applied in this study to evaluate the effect of different
441 environments and management practices, commonly used in rabbit production, in their cecal
442 microbial composition and diversity. Our results confirmed that the most important effect is
443 exerted by the environment provided by the farm where the animals were raised. Those raised
444 in the best insulated facility (farm B) appear to have a microbiota characteristic of healthier
445 animals than those raised in the open-air facility (farm A). It is worth mentioning that the
446 rabbits were housed in cages interspersed with feeding regime. This fact could make possible
447 the exchange of microorganisms between animals of different feeding regimes and therefore
448 have reduced the differences observed between regimes. However, the joint consideration of
449 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 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

472 of healthier animals. Although the level of feeding and the presence of antibiotics in the feed
473 did not modify the global diversity of cecal microbial communities, these factors can increase
474 or decrease the prevalence of specific bacteria which could lead to a microbial composition
475 potentially beneficial for the animal or, at the other extreme, to an origin of future intestinal
476 dysbiosis.

477

478 Methods

479 Animals and experimental design

480 All biological samples used in the study were collected from animals of an experiment
481 conducted at the Institute of Agrifood Research and Technology (IRTA) in different periods
482 and involving two different farms. The objective of that experiment was to estimate the effect
483 of the interaction between the genotype and the feeding regime (i.e., the amount of feed
484 provided during fattening) on growth, feed efficiency, carcass characteristics, and health
485 status of the animals [42-Piles and Sánchez, 2019]. For this particular study, 425 meat rabbits
486 from Caldes line [43-Gómez et al., 2002] of that experiment were randomly selected. Most
487 of them (336) were raised in 4 different batches in a semi-open-air facility (farm A). The
488 remaining animals (89) were produced in a single batch in another facility under better
489 controlled environmental conditions (farm B). Rabbits raised in farm A were housed in
490 collective cages containing 8 kits each one while those raised in farm B were housed in cages
491 with 6 kits each one. All animals were raised under the same management conditions and
492 received the same standard pelleted diet. Some of the rabbits raised in farm B received a diet
493 free of antibiotics and the rest received the same diet but supplemented with antibiotics.

494 Those raised in farm A received oxytetracycline, valnemulin, and colistin while those in farm
495 B received oxytetracycline, valnemulin and neomycin. During the last fattening week all the
496 animals received an antibiotic free diet. Feed was supplied once per day in a feeder with three
497 places for the 4-5 weeks that the fattening lasted. Water was provided *ad libitum* during the
498 whole fattening period. The animals were under two different feeding regimes: (1) *ad libitum*
499 (AL) or (2) restricted (R) to 75% of the AL feed intake. The amount of feed supplied to the
500 animals under R feeding regime in a given week for each batch was computed as 0.75 times
501 the average feed intake of kits on AL from the same batch during the previous week, plus
502 10% to account for a feed intake increase as the animal grows. Kits were randomly assigned
503 to one of these two feeding regimes after weaning (32 days of age). They were categorized
504 into two groups according to their size at weaning (big if their body weight was greater than
505 700 g or small otherwise) aiming to obtain homogenous groups regarding animal size within
506 feeding regime. A maximum of two kits of the same litter were assigned to the same cage in
507 order to remove the possible association between cage and maternal effects on animal growth
508 during the fattening period. The distribution of these animals across the different levels of
509 management factors is shown in Table 3.

510 **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

511

512 Sample processing, DNA extraction and sequencing

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

526

527 A fragment of the 16S rRNA gene including the V4-V5 hypervariable regions was amplified
528 with F515Y/R926 primer combination (5'-GTGYCAGCMGCCGCGGTAA-3', 5'-
529 CCGYCAATTYMTTTRAGTTT-3') [45-Parada et al., 2016] and then re-amplified in a

530 limited-cycle PCR reaction to add sequencing adaptors and 8 nucleotide dual-indexed
531 barcodes of multiplex Nextera® XT kit (Illumina, Inc., San Diego CA, United States)
532 following manufacturer's instructions. The initial PCR reactions were performed for each
533 sample using 12.5 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl forward primer, 5 µl reverse
534 primer and 2.5 µl template DNA (5 ng/ µl). The initial PCR conditions were as follows: initial
535 denaturation for 3 minutes at 95 ºC, 25 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC
536 and 30 seconds at 72 ºC; and final extension for 2 minutes at 72 ºC. The addition of indexes
537 and sequencing adaptors to both ends of the amplified regions took place in a second PCR
538 by using 25 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl index i7, 5 µl index i5, 10 µl PCR
539 Grade water and 5 µl concentrated amplicons of initial PCR. The second PCR conditions
540 were as follows: initial denaturation for 3 minutes at 95 ºC, 8 cycles of 30 seconds at 95 ºC,
541 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 5 minutes at 72 ºC. Final
542 libraries were cleaned up with AMPure XP beads, validated by running 1 µl of a 1:50 dilution
543 on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa Clara, CA, United
544 States) to verify their size, quantified by fluorometry with PicoGreen dsDNA quantification
545 kit (Invitrogen, Life Technologies, Carlsbad, CA, United States), pooled at equimolar
546 concentrations and paired-end sequenced in 5 parallel plates in a Illumina MiSeq 2 x 250
547 platform at the Genomics and Bioinformatics Service (SGB) of the Autonomous University
548 of Barcelona (UAB).

549

550 Bioinformatic pipeline for OTU calling

551 Sequence processing was performed using QIIME software (version 1.9.0) [46- Caporaso et
552 al., 2010]. In a first step, the resulting paired-ended V4-V5 16S rRNA gene reads were

553 assembled into contigs with the python script *multiple_join_paired_ends.py*. Then the
554 contigs were curated using the script *split_libraries.py* with default parameters in order to
555 assign them to samples and to discard those with a low-quality (Q19 was the minimum
556 acceptable quality score). Chimeric sequences generated during the process of DNA
557 amplification were detected with UCHIME algorithm [47- Edgar et al., 2011] and removed.
558 The totality of filtered contigs were clustered into operational taxonomic units (OTUs) with
559 a 97% similarity threshold using the script *pick_open_reference_otus.py* with default
560 parameters [48- Rideout et al., 2014] that grouped, through UCLUST algorithm [49- Edgar,
561 2010], the sequences against Greengenes reference database (version gg_13_5_otus) and also
562 made a *de novo* clustering of those that did not match the database. The generated OTU table
563 was filtered at: (1) sample level: by discarding samples with less than 5,000 final sequence
564 counts and at (2) OTU level: by removing the doubleton ones. The filtered OTU table
565 contained the sequence counts of 963 OTUs for 425 samples. Taxonomic assignment of
566 representative sequences of each OTU defined (963) was conducted by mapping them to the
567 Greengenes reference database gg_13_5_otus with the UCLUST consensus taxonomy
568 assigner (QIIME default parameters). The raw sequence data were deposited in the sequence
569 read archive of NCBI under the BioProject accession number PRJNA524130. Metadata, the
570 prefiltered and normalized OTU tables, and corresponding taxonomic classifications are also
571 included as Additional files 1, 2, 3 and 4, respectively.

572

573 <u>Models and statistical methods</u>

574 In order to study differences in diversity and richness between rabbits grouped according to
575 the farm environment and the management that they received, two alpha diversity indexes

576 (Shannon and the observed number of OTUs) were computed from the OTU table rarified to
577 10,000 sequences per sample with "phyloseq" R package [50-phyloseq]. The statistical
578 method chosen to assess alpha diversity differences between these groups of animals was an
579 analysis of variance that included a factor resulting from the combination of four factors (the
580 farm where the animal was raised, the batch, the presence or the absence of antibiotics in the
581 feed and the feeding regime). The significance threshold was set at 0.05 type I error.

582

583 Different approaches were considered to assess the influence of the environments and
584 management factors on microbial composition. A bootstrap analysis of variance was
585 individually implemented for each OTU to test whether it was differentially represented
586 between the different categories of the factors studied. This univariate analysis was
587 conducted by normalizing the OTU table with the cumulative sum scaling (CSS) method [51-
588 Paulson et al., 2013] and only for those OTUs which were detected in at least 5% of the
589 samples and had a sum of its counts resulting in a frequency greater than 0.01% of the total
590 sum of all OTUs counts across all samples. It was implemented by fitting a model defined
591 by the combination of the four aforementioned factors by using *lm()* function in R [52- R].
592 Then, the differences between the CSS-normalized OTUs counts in the different levels of the
593 studied factors were tested. The significance between the levels of the main factors: farm,
594 presence of antibiotics in the feed and feeding regime was assessed using an F statistic. When
595 the involved interaction terms were significant, the contrasts of interest were studied nested
596 within the levels of other interacting factors, i.e. feeding regime were studied within farm
597 levels. When the interaction terms were not significant, the effects of the different levels were
598 averaged, i.e. the effects of the levels of the batches within farm A were averaged to present

599 the effect associated with this farm. In the performed F tests instead of relying on the
600 theoretical distribution of the statistic under the null hypothesis to define the p-values, they
601 were empirically computed using bootstrap after 1,000 permutations of the dependent
602 variable with respect to the design matrix of factors in the model. The use of bootstrapping
603 enabled the hypothesis test to be done without the need of assuming that data are normally
604 distributed, which is an assumption that fails for OTUs counts. *P*-value was defined as the
605 proportion of bootstrap rounds having an F statistic value equal or greater than that obtained
606 with the original dataset. *P*-values were corrected defining a false discovery rate (FDR) of
607 0.05 [53- Benjamini and Hochberg, 1995]. This bootstrap analysis of variance approach was
608 also implemented to study the effect of the management factors on the relative abundance of
609 bacteria at phylum and genus levels.

610

611 The value of the microbial information to classify samples into the three factors considered
612 in our study was explored using multivariate techniques. In particular, sparse partial least
613 squares-discriminant analysis (sPLS-DA) [54-Le Cao et al., 2008] was used to find the
614 combination of OTUs that allowed the best classification of cecal samples according to: (1)
615 the farm where the animals were raised, (2) the feeding regime within farm A and (3) the
616 combination of feeding regime and the presence or absence of antibiotics in the feed for the
617 animals raised in farm B. This approach was implemented through the R package
618 "mixOmics" [55-mixomics]. In a first step, the function *tune.splsda()* was used to select the
619 optimal sparsity parameters of the sPLS-DA model: the number of components and the
620 number of variables (OTUs) per component. For the tuning process, a 5-fold cross-validation
621 repeated 10 times was performed one component at a time, with a maximum of 4

622 components, on an input grid of values that indicate the number of variables to select on each
623 component. The sparsity parameters were defined, based on the BER and centroids distance,
624 and then included in the final sPLS-DA model. Samples were represented on the first two
625 components and colored according to their class (e.g., R or AL in the case of the feeding
626 regime) in a sample plot with the function *plotIndiv()*. The performance of the sPLS-DA
627 model was assessed with a 5-fold cross-validation repeated 1,000 times that randomly split
628 the data in training and validation sets. In this data partition, it was ensured that 20% of the
629 samples within each level of the discriminant factor were assigned to the validation set. Five
630 different partitions were performed for each replicate to guarantee a different sample
631 distribution in each validation set. The sPLS-DA model with the sparsity parameters
632 previously defined was adjusted in the training set and its classification performance was
633 assessed in the validation set using the overall and BER per class as criteria. The stability of
634 the OTUs selected on each component was also assessed in the cross-validation by computing
635 the selection frequency of each variable across the replicates.

636

637 ## Additional files

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

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

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

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

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

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

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

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

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

655

656 ## List of abbreviations

657 **AL:** *ad libitum*

658 **BER:** balanced error rate

659 **CSS:** cumulative sum scaling

660 **FDR:** false discovery rate

661 **GIT:** gastrointestinal tract

662 **NGS:** next generation sequencing

663 **OTU:** operational taxonomic unit

664 **PCR:** polymerase chain reaction

665 **R:** restricted

666 **sPLS-DA:** sparse partial least squares-discriminant analysis

667 **SRB:** sulfate-reducing bacteria (SRB)

668 **SSB:** sulfate-secreting bacteria (SRB)

669 **WithAb:** feed supplemented with antibiotics

670 **WithoutAb:** feed free of antibiotics

671

672 ## Declarations

673 ***Ethics approval and consent to participate***

674 This study was carried out in accordance with the recommendations of the animal care and
675 use committee of the Institute for Food and Agriculture Research and Technology

676 (IRTA). The protocol was approved by the committee of the Institute for Food and
677 Agriculture Research and Technology (IRTA).

678

679 ***Consent for publication***

680 Not applicable.

681

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

Competing interests

The authors declare that they have no competing interests.

Funding

The experimental design of this work was conducted thanks to funding from INIA project RTA2011-00064-00-00. This study was part of the Feed-a-Gene project and received funding from the European Union’s H2020 program under grant agreement no. 633531. MVG is a recipient of a “Formación de Personal Investigador (FPI)” pre-doctoral fellowship from INIA, associated with the research project RTA2014-00015-C2-01. YRC was funded by Marie Skłodowska-Curie grant (P-Sphere) agreement no. 6655919 (EU).

703 ***Authors' contributions***

704 JS, MP and OR conceived the experimental design. JS, OR, MP and MVG collected
705 biological samples. MVG, OGR, MP, MG and AS processed the samples in the laboratory.
706 MVG processed and analyzed the sequencing data, interpreted data, prepared figures and
707 tables, and wrote the manuscript. JS and YRC helped analyzing the sequencing data. JS, MG,
708 MP, MV and YRC helped interpreting the data, and wrote and revised the manuscript. All
709 authors read and approved the final manuscript.

710

711 ***Acknowledgements***

712 We would like to thank Oscar Perucho, Josep Ramon and Carmen Requena (staff of Unitat
713 de Cunicultura, IRTA) for their contribution to data recording and animal care during the
714 experiment. We also acknowledge Nicolas Boulanger and Joana Ribes (Genomics and NGS
715 Unit, CRAG) for their assistance in massive libraries preparation.

716

717 **References**

718 [1] Gaskins, H. R. Immunological aspects of host/microbiota interactions at the intestinal
719 epithelium. Gastrointestinal microbiology;1997;*2*:537-587.

720

721 [2] Belkaid, Y., Hand, T. W. Role of the microbiota in immunity and inflammation.
722 Cell;2014;*157*(1):121-141.

723

724 [3] Gouet, P. H., Fonty, G. Changes in the digestive microflora of holoxenic* rabbits from
725 birth until adulthood. Annales de biologie animale biochimie biophysique;1979;*19*:553–566.

726

727 [4] Abecia, L., Fondevila, M., Balcells, J., Lobley, G. E., McEwan, N. R. The effect of
728 medicated diets and level of feeding on caecal microbiota of lactating rabbit does. Journal of
729 applied microbiology;2007;*103*(4):787-793.

730

731 [5] Zou, F., Zeng, D., Wen, B., Sun, H., Zhou, Y., Yang, M., et al. Illumina Miseq platform
732 analysis caecum bacterial communities of rex rabbits fed with different antibiotics. AMB
733 express;2016:*6*(1):100.

734

735 [6] Zhu, Y., Sun, Y., Wang, C., Li, F. Impact of dietary fibre: starch ratio in shaping caecal
736 archaea revealed in rabbits. Journal of animal physiology and animal
737 nutrition;2017;*101*(4);635-640.

738

739 [7] Chen, S. Y., Deng, F., Jia, X., Liu, H., Zhang, G. W., Lai, S. J. Gut microbiota profiling
740 with differential tolerance against the reduced dietary fibre level in rabbit. Scientific
741 reports;2019;*9*(1):1-9.

742

743 [8] Combes, S., Michelland, R. J., Monteils, V., Cauquil, L., Soulié, V., Tran, N. U., et al.
744 Postnatal development of the rabbit caecal microbiota composition and activity. FEMS
745 microbiology ecology;2011;77(3):680-689.

746

747 [9] Gidenne, T., García, J., Lebas, F., Licois, D. Nutrition and feeding strategy: interactions
748 with pathology. Nutrition of the rabbit;2010;*20*:179.

749

750 [10] Eshar, D., Weese, J. S. Molecular analysis of the microbiota in hard feces from healthy
751 rabbits (Oryctolagus cuniculus) medicated with long term oral meloxicam. BMC veterinary
752 research;2014;*10*(1):62.

753

754 [11] Gidenne, T., Combes, S., Feugier, A., Jehl, N., Arveux, P., Boisot, P., et al. Feed
755 restriction strategy in the growing rabbit. 2. Impact on digestive health, growth and carcass
756 characteristics. Animal;2009;*3*(4):509-515.

757

758 [12] Velasco-Galilea, M., Piles, M., Viñas, M., Rafel, O., González-Rodríguez, O.,
759 Guivernau, M., et al. Rabbit microbiota changes throughout the intestinal tract. Frontiers in
760 microbiology;2018;*9*:2144.

761

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

[14] O'Neil, D. S., Stewart, C. J., Chu, D. M., Goodspeed, D. M., Gonzalez-Rodriguez, P. J., 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).

782 [18] Kumar, S., Chen, C., Indugu, N., Werlang, G. O., Singh, M., Kim, et al. Effect of
783 antibiotic withdrawal in feed on chicken gut microbial dynamics, immunity, growth
784 performance and prevalence of foodborne pathogens. PLoS One;2018;*13*(2).

785

786 [19] Fang, S., Xiong, X., Su, Y., Huang, L., Chen, C. 16S rRNA gene-based association study
787 identified microbial taxa associated with pork intramuscular fat content in feces and cecum
788 lumen. BMC microbiology;2017;*17*(1):162.

789

790 [20] Dai, Z. L., Wu, G., Zhu, W. Y. Amino acid metabolism in intestinal bacteria: links
791 between gut ecology and host health. Frontiers bioscience;2011;*16*(1):1768-86.

792

793 [21] Sun, X., Shen, J., Liu, C., Li, S., Peng, Y., Chen, C., et al. L-Arginine and N-
794 carbamoylglutamic acid supplementation enhance young rabbit growth and immunity by
795 regulating intestinal microbial community. Asian-Australasian journal of animal
796 sciences;2020;*33*(1):166.

797

798 [22] Müller, V. M., Zietek, T., Rohm, F., Fiamoncini, J., Lagkouvardos, I., Haller, D., et al.
799 Gut barrier impairment by high-fat diet in mice depends on housing conditions. Molecular
800 nutrition & food research;2016;*60*(4):897-908.

801

802 [23] Bäuerl, C., Collado, M. C., Zuniga, M., Blas, E., Martínez, G. P. Changes in cecal
803 microbiota and mucosal gene expression revealed new aspects of epizootic rabbit
804 enteropathy. PLoS One;2014;*9*(8).

805

806 [24] Thoene-Reineke, C., Fischer, A., Friese, C., Briesemeister, D., Göbel, U. B.,
807 Kammertoens, T., et al. Composition of intestinal microbiota in immune-deficient mice kept
808 in three different housing conditions. PloS one;2014;*9*(11).

809

810 [25] Terán-Ventura, E., Roca, M., Martin, M. T., Abarca, M. L., Martinez, V., Vergara, P.
811 Characterization of housing-related spontaneous variations of gut microbiota and expression
812 of toll-like receptors 2 and 4 in rats. Microbial ecology;2010;*60*(3):691-702.

813

814 [26] Arrazuria, R., Elguezabal, N., Juste, R. A., Derakhshani, H., Khafipour, E.
815 Mycobacterium avium subspecies paratuberculosis infection modifies gut microbiota under
816 different dietary conditions in a rabbit model. Frontiers in microbiology;2016;*7*:446.

817

818 [27] Belzer, C., De Vos, W. M. Microbes inside—from diversity to function: the case of
819 Akkermansia. The ISME journal;2012;*6*(8):1449-1458.

820

821 [28] Angert, E. R., Brooks, A. E., Pace, N. R. Phylogenetic analysis of Metabacterium
822 polyspora: clues to the evolutionary origin of daughter cell production in Epulopiscium
823 species, the largest bacteria. Journal of bacteriology;1996;*178*(5:1451-1456.

824

825 [29] Rey, F. E., Gonzalez, M. D., Cheng, J., Wu, M., Ahern, P. P., Gordon, J. I. Metabolic
826 niche of a prominent sulfate-reducing human gut bacterium. Proceedings of the national
827 academy of sciences;2013;*110*(33):13582-13587.

828

829 [30] Zeng, B., Han, S., Wang, P., Wen, B., Jian, W., Guo, W., et al. The bacterial
830 communities associated with fecal types and body weight of rex rabbits. Scientific
831 reports;2015;*5*:9342-9350.

832

833 [31] Di Rienzi, S. C., Sharon, I., Wrighton, K. C., Koren, O., Hug, L. A., Thomas, B. C., et
834 al. The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new
835 candidate phylum sibling to Cyanobacteria. Elife;2013;*2*:e01102.

836

837 [32] Monchamp, M. E., Spaak, P., Pomati, F. Long term diversity and distribution of non-
838 photosynthetic cyanobacteria in peri-alpine lakes. Frontiers in microbiology;2019;*9*:3344.

839

840 [33] Liu, P. Y., Cheng, A. C., Huang, S. W., Lu, H. P., Oshida, T., Liu, W., et al. Body-size
841 Scaling is Related to Gut Microbial Diversity, Metabolism and Dietary niche of Arboreal
842 folivorous flying Squirrels. Scientific reports;2020;*10*(1):1-12.

843

844 [34] Ai, D., Pan, H., Li, X., Gao, Y., Liu, G., Xia, L. C. Identifying gut microbiota associated
845 with colorectal cancer using a zero-inflated lognormal model. Frontiers in
846 microbiology;2019;*10*:826.

847

848 [35] Shen, Q., Maitin, V. Obesity-associated gut microbiota: characterization and dietary
849 modulation. In Diet-Microbe Interactions in the Gut. Academic press;2015:149-171.

850

851 [36] McCabe, M. S., Cormican, P., Keogh, K., O'Connor, A., O'Hara, E., Palladino, R. A.,
852 et al. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an
853 uncharacterised Succinivibrionaceae and an increase of the Methanobrevibacter gottschalkii
854 clade in feed restricted cattle. PloS one;2015;*10*(7).

855

856 [36] McGovern, E., McCabe, M. S., Cormican, P., Popova, M., Keogh, K., Kelly, A. K., et
857 al. Plane of nutrition affects the phylogenetic diversity and relative abundance of
858 transcriptionally active methanogens in the bovine rumen. Scientific reports;2017;*7*(1):1-10.

859

860 [37] Bui, T. P. N., Schols, H. A., Jonathan, M., Stams, A. J., de Vos, W. M., Plugge, C. M.
861 Mutual Metabolic Interactions in Co-cultures of the Intestinal Anaerostipes rhamnosivorans
862 With an Acetogen, Methanogen, or Pectin-Degrader Affecting Butyrate Production.
863 Frontiers in microbiology;2019;*10*:2449.

864

865 [38] Samuel, B. S., Gordon, J. I. A humanized gnotobiotic mouse model of host–archaeal–
866 bacterial mutualism. Proceedings of the national academy of sciences;2006;*103*(26):10011-
867 10016.

868

869 [39] Ziętak, M., Kovatcheva-Datchary, P., Markiewicz, L. H., Ståhlman, M., Kozak, L. P.,
870 Bäckhed, F. Altered microbiota contributes to reduced diet-induced obesity upon cold
871 exposure. Cell metabolism;2016;*23*(6):1216-1223.

872

873 [40] Gandy, K. A. O., Zhang, J., Nagarkatti, P., Nagarkatti, M. The role of gut microbiota in
874 shaping the relapse-remitting and chronic-progressive forms of multiple sclerosis in mouse
875 models. Scientific reports;2019;*9*(1):1-17.

876

877 [41] North, M. K., Dalle Zotte, A., Hoffman, L. C. Composition of rabbit caecal microbiota
878 and the effects of dietary quercetin supplementation and sex thereupon. World rabbit
879 science;2019;*27*(4):185-198.

880

881 [42] Piles, M., Sánchez, J. P. Use of group records of feed intake to select for feed efficiency
882 in rabbit. Journal of animal breeding and genetics;2019;*136*(6):474-483.
883

884 [43] Gómez, E. A., Rafel, O., Ramon, J. The Caldes strain (Spain). Options
885 Méditerranéennes. Série B: Etudes et recherches (CIHEAM);2002.
886

887 [44] Desjardins, P., Conklin, D. NanoDrop microvolume quantitation of nucleic
888 acids. Journal of visualized experiments: JoVE;2010;*45*.
889

890 [45] Parada, A. E., Needham, D. M., Fuhrman, J. A. Every base matters: assessing small
891 subunit rRNA primers for marine microbiomes with mock communities, time series and
892 global field samples. Environmental microbiology;2016;*18*(5):1403-1414.
893

894 [46] Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello,
895 E. K., et al. QIIME allows analysis of high-throughput community sequencing data. Nature
896 methods;2010;*7*:335-336.
897

898 [47] Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., Knight, R. UCHIME improves
899 sensitivity and speed of chimera detection. Bioinformatics;2011;*27*:2194-2200
900

901 [48] Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons,
902 S. M., et al. Subsampled open-reference clustering creates consistent, comprehensive OTU
903 definitions and scales to billions of sequences. PeerJ;2014;*2*:e545.

904

905 [49] Edgar, R. C. Search and clustering orders of magnitude faster than BLAST.
906 Bioinformatics;2010;*26*:2460–2461.

907

908 [50] McMurdie, P. J., Holmes, S. phyloseq: an R package for reproducible interactive
909 analysis and graphics of microbiome census data. PloS one;2013;*8*(4):e61217.

910

911 [51] Paulson, J. N., Stine, O. C., Bravo, H. C., Pop, M. Differential abundance analysis for
912 microbial marker-gene surveys. Nature methods;2013;10(12),1200.

913

914 [52] R Development Core Team. R: A Language and Environment for Statistical Computing.
915 2010[http://cran.r-project.org].

916

917 [53] Benjamini, Y., Hochberg, Y. Controlling the false discovery rate: a practical and
918 powerful approach to multiple testing. Journal of the Royal statistical society: series B
919 (Methodological);1995;57(1):289-300.

920

921 [54] Lê Cao, K. A., Rossouw, D., Robert-Granié, C., Besse, P. A sparse PLS for variable
922 selection when integrating omics data. Statistical applications in genetics and molecular
923 biology;2018;*7*(1).

924

925 [55] Rohart, F., Gautier, B., Singh, A., Le Cao K. A. mixOmics: An R package for 'omics
926 feature selection and multiple data integration. PLoS computational
927 biology;2017;*13*(11):e1005752.