Housing Conditions, Level of Feeding and Presence of Antibiotics in The Feed Shape Rabbit Cecal Microbiota

María Velasco-Galilea (✉ maria.velasco@irta.es)

Institut de Recerca i Tecnologia Agroalimentaries   https://orcid.org/0000-0001-9533-7412

Miriam Guivernau

Institut de Recerca i Tecnologia Agroalimentaries

Miriam Piles

Institut de Recerca i Tecnologia Agroalimentaries

Marc Viñas

Institut de Recerca i Tecnologia Agroalimentaries

Oriol Rafel

Institut de Recerca i Tecnologia Agroalimentaries

Armand Sánchez

Universitat Autonoma de Barcelona

Yuliaxis Ramayo-Caldas

Institut de Recerca i Tecnologia Agroalimentaries

Olga González-Rodríguez

Institut de Recerca i Tecnologia Agroalimentaries

Juan P. Sánchez

Institut de Recerca i Tecnologia Agroalimentaries

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María Velasco-Galilea[1*], Miriam Guivernau[2], Miriam Piles[1], Marc Viñas[2], Oriol Rafel[1], Armand Sánchez[3,4], Yuliaxis Ramayo-Caldas[1], Olga González-Rodríguez[1] and Juan P. Sánchez[1]

[1]Institute for Food and Agriculture Research and Technology (IRTA) – Animal Breeding and Genetics, E08140 Caldes de Montbui, Barcelona, Spain,

[2]Institute for Food and Agriculture Research and Technology (IRTA) - Integral Management of Organic Waste, E08140 Caldes de Montbui, Barcelona, Spain,

[3]Animal Genomics Group, Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Campus UAB, Catalonia, Spain,

[4]Unit of Animal Science, Department of Animal and Food Science, Autonomous University of Barcelona, Barcelona, Spain

*Corresponding author:

María Velasco-Galilea

maria.velasco@irta.es

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 rDNA-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

2

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

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

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

Results

Sequence processing

After the removal of doubletons and samples with low sequence counts, 425 rabbit cecal
samples (Additional file 1) were represented on 14,928,203 sequence counts clustered into
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 presence across

89   samples. Most of the samples had more than 700 different OTUs (mode = 748) and nearly
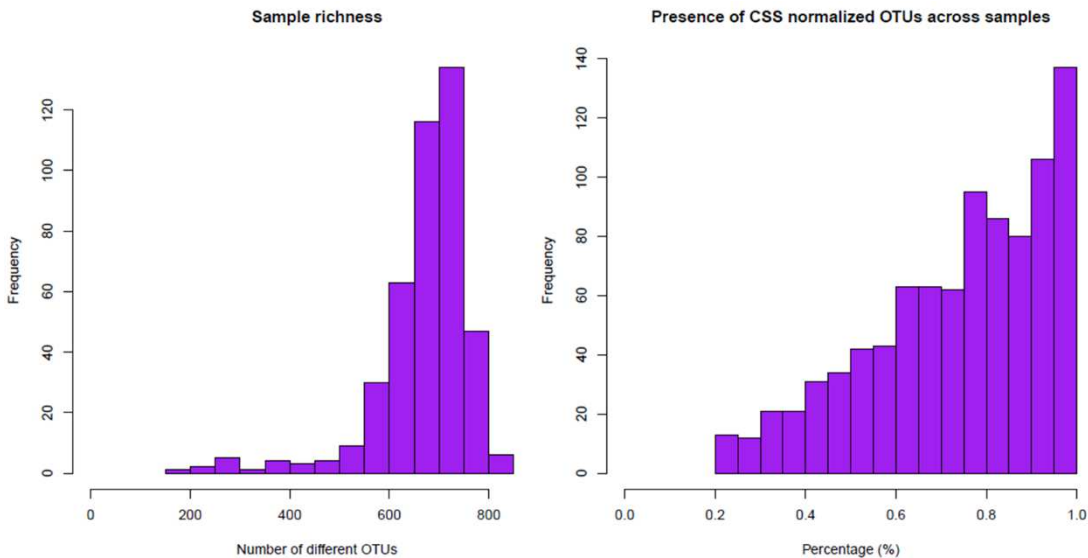
90   140 OTUs were present in all the samples.

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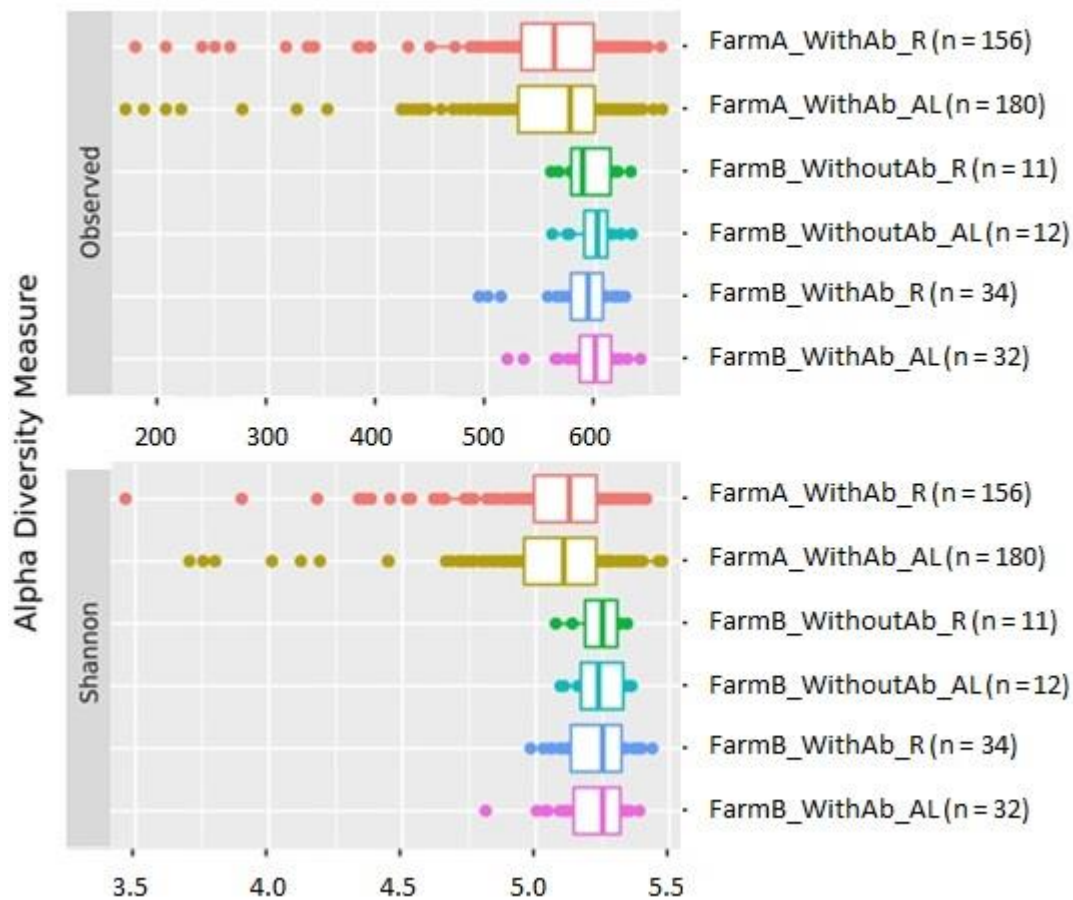
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Figure 2 Microbial richness and diversity between samples grouped according to management that animals received. The cecal microbial richness and diversity were estimated by the observed number of different OTUs and the Shannon indexes, respectively.

Animal management and farm environment shaping cecal microbial composition

According to the taxonomic assignment of representative sequences (Additional file 4) performed with the UCLUST consensus taxonomy assigner on the Greengenes reference 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

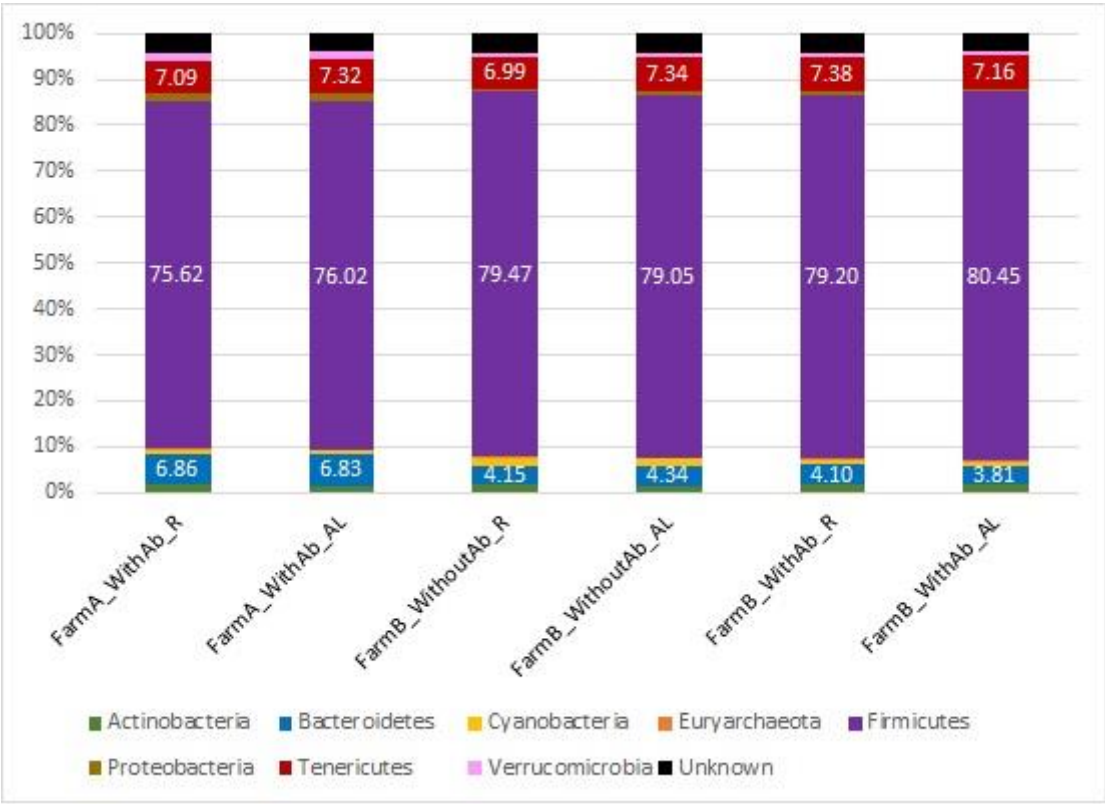123   diversity, in the rabbit cecal samples studied (Figure 3).

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125

Figure 3 Phyla relative abundances of samples grouped according to farm, level of

feeding and presence of antibiotics in the feed.

128

129   <u>Differential cecal microbial composition across farms</u>

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).

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

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

12

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

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 can

be said to be perfect since its overall and balanced error rate (BER) per class across 1000

replicates of 5-folds cross-validation runs was 0.00 (0.00). Furthermore, two OTUs of

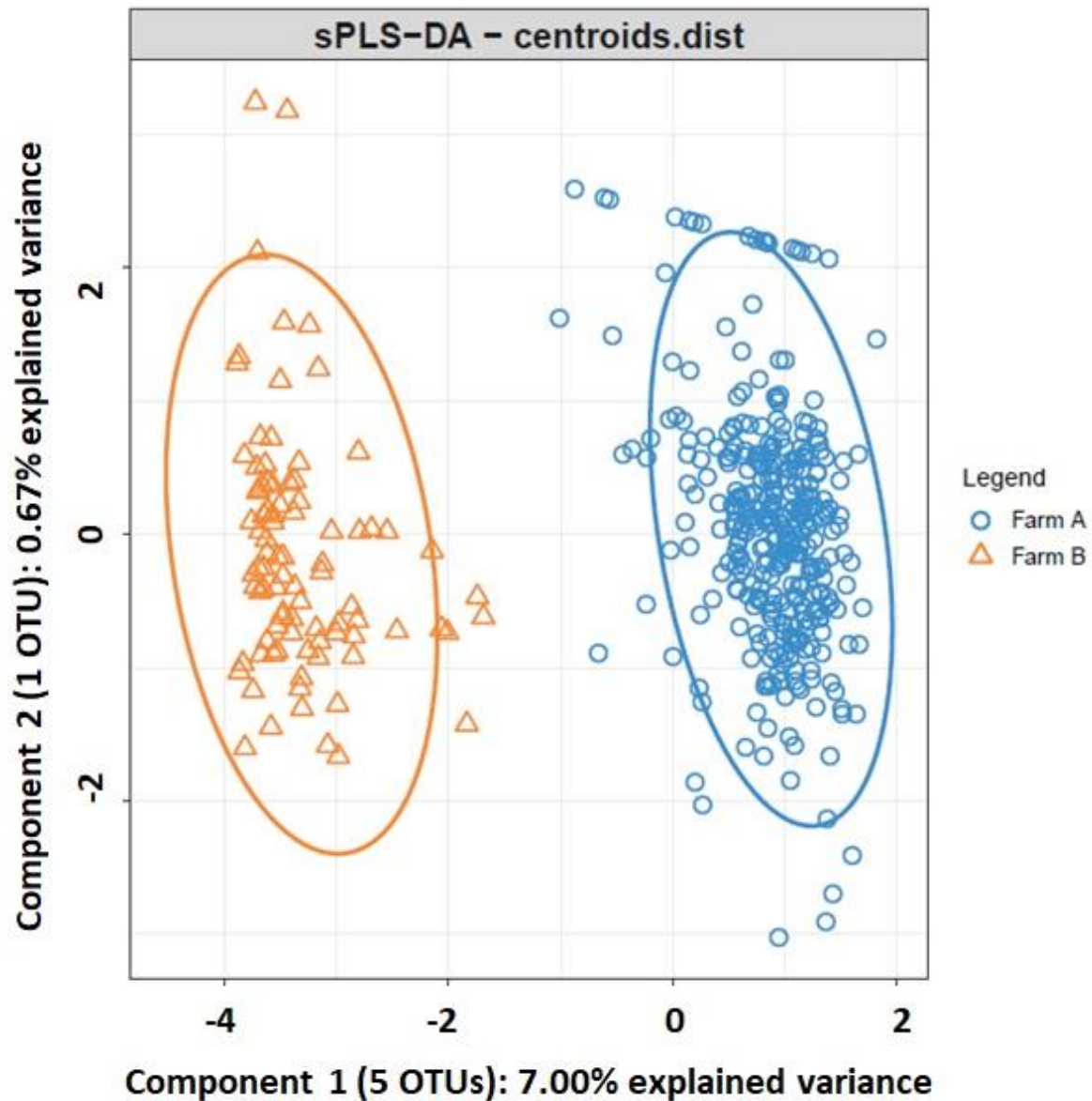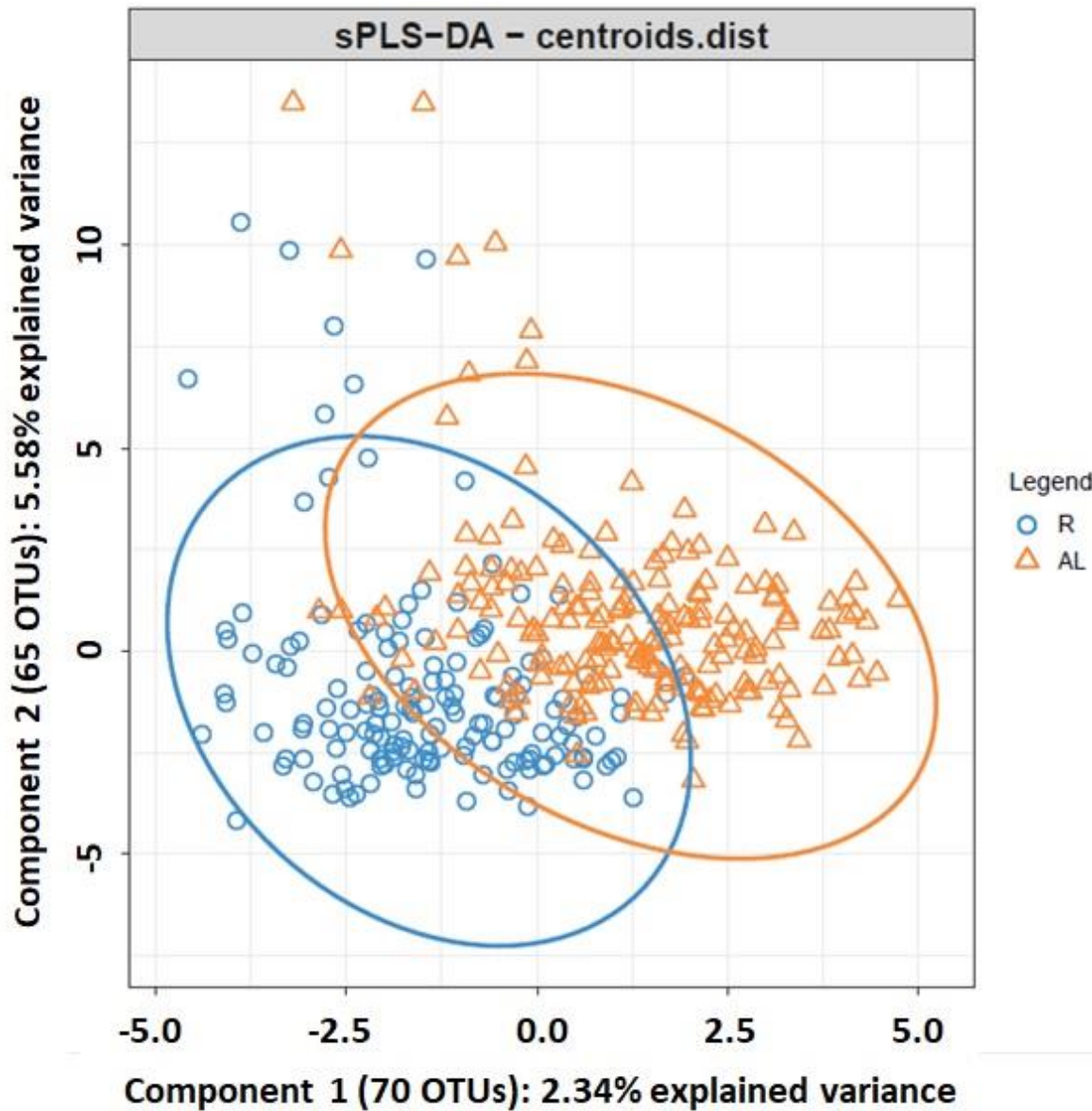component 1 had a stability higher than 0.9.

209

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

The sPLS-DA performed to discriminate samples across feeding regimes within farm A selected 70 OTUs for component 1 and 65 OTUs for component 2 (Figure 5). Component 1 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.

220

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

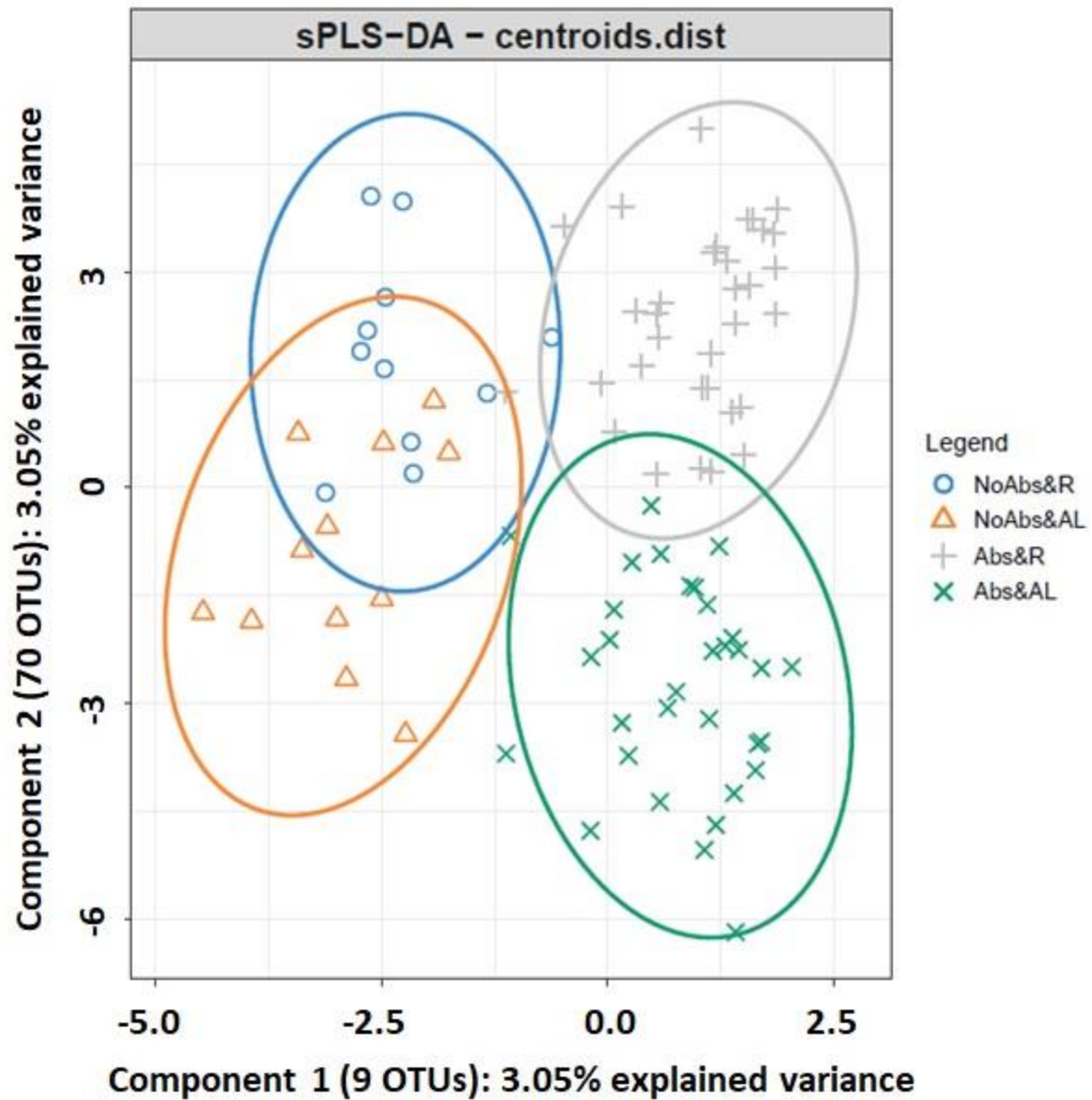236   the different cross-validation folds was higher than 0.9.

237

Figure 6 Sparse partial least squares discriminant analysis representing cecal samples of rabbits raised in farm B and fed R withoutAb (blue), fed AL withoutAb (orange), fed R withAb (gray) and fed AL withAb (green).

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.

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

19

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.

21

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

24

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

450 to the level of feeding received by each animal raised in farm A. It implies the existence of

451 cecal microbiota content patterns characteristic of each regime which could be revealed

452 thanks to the univariate analyses conducted at different taxonomic levels. Similarly, the

453 sPLS-DA performed within farm B also involved the consideration of 70 OTUs to

454 discriminate samples according to the amount of feed consumed. Within this farm, the

455 classification of samples regarding the presence or the absence of antibiotics in the feed

456 needed a smaller number of OTUs than the feeding regime but greater than the farm. This

457 suggests that the effect of the presence of antibiotic in feed is stronger than the feeding level.

458 The implication of the discussed microbial composition and diversity differences originated

459 by the studied management and environmental factors on the animals' performance still

460 needs to be investigated. In future studies the role of specific groups of bacteria in rabbit

461 growth and feed efficiency will be analyzed.

462

463 Conclusions

464 The analysis of a large number of animals from a paternal rabbit line has allowed a deeper

465 comprehension of the role played by different management and environmental factors

466 shaping the composition and diversity of cecal microbial communities. It reveals that the

467 housing conditions offered to the rabbits during their growing play a key role that can result

468 in different microbial alpha diversity and composition of almost all species that inhabit the

469 rabbit GIT. This highlights the importance that a stable and controlled environment could

470 have in the intestinal health and, consequently, in animal performance. It seems clear that the

471 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 <u>Animals and experimental design</u>

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

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 ℃, 25 cycles of 30 seconds at 95 ℃, 30 seconds at 55 ℃

536 and 30 seconds at 72 ℃; and final extension for 2 minutes at 72 ℃. 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 ℃, 8 cycles of 30 seconds at 95 ℃,

541 30 seconds at 55 ℃ and 30 seconds at 72 ℃; and final extension for 5 minutes at 72 ℃. 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 Models and statistical methods

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

33

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

692   *Competing interests*

693   The authors declare that they have no competing interests.

694

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

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

[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.

726

[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.

730

[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.

734

[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.

738

[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.

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

762   [13] Kylie, J., Weese, J. S., Turner, P. V. Comparison of the fecal microbiota of domestic

763   commercial meat, laboratory, companion, and shelter rabbits (Oryctolagus cuniculi). BMC

764   veterinary research;2018;*14*(1):143.

765

766   [14] O'Neil, D. S., Stewart, C. J., Chu, D. M., Goodspeed, D. M., Gonzalez-Rodriguez, P. J.,

767   Shope, C. D., et al. Conditional postnatal deletion of the neonatal murine hepatic circadian

768   gene, Npas2, alters the gut microbiome following restricted feeding. American journal of

769   obstetrics and gynecology;2017;*217*(2):218-e1.

770

771   [15] Chen, J., Toyomasu, Y., Hayashi, Y., Linden, D. R., Szurszewski, J. H., Nelson, H., et

772   al. Altered gut microbiota in female mice with persistent low body weights following

773   removal of post-weaning chronic dietary restriction. Genome medicine;*2016*;8(1):103.

774

775   [16] Zarrinpar, A., Chaix, A., Yooseph, S., Panda, S. Diet and feeding pattern affect the

776   diurnal dynamics of the gut microbiome. Cell metabolism;2014;*20*(6):1006-1017.

777

778   [17] Soler, C., Goossens, T., Bermejo, A., Migura-García, L., Cusco, A., Francino, O., et al.

779   Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during

780   the nursery period. PloS one;2018;*13*(5).

781

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

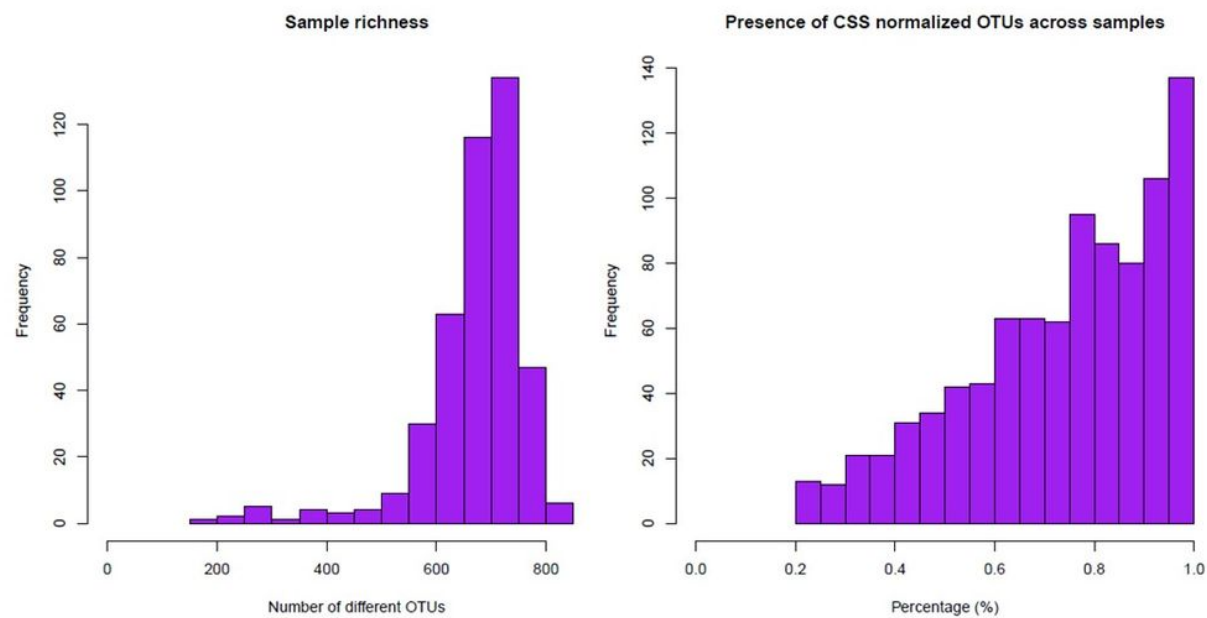927 biology;2017;*13*(11):e1005752.

Figures

Figure 1

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

Figure 2

Microbial richness and diversity between samples grouped according to management that animals received.

Figure 3

Phyla relative abundances of samples grouped according to farm, level of feeding and presence of antibiotics in the feed.
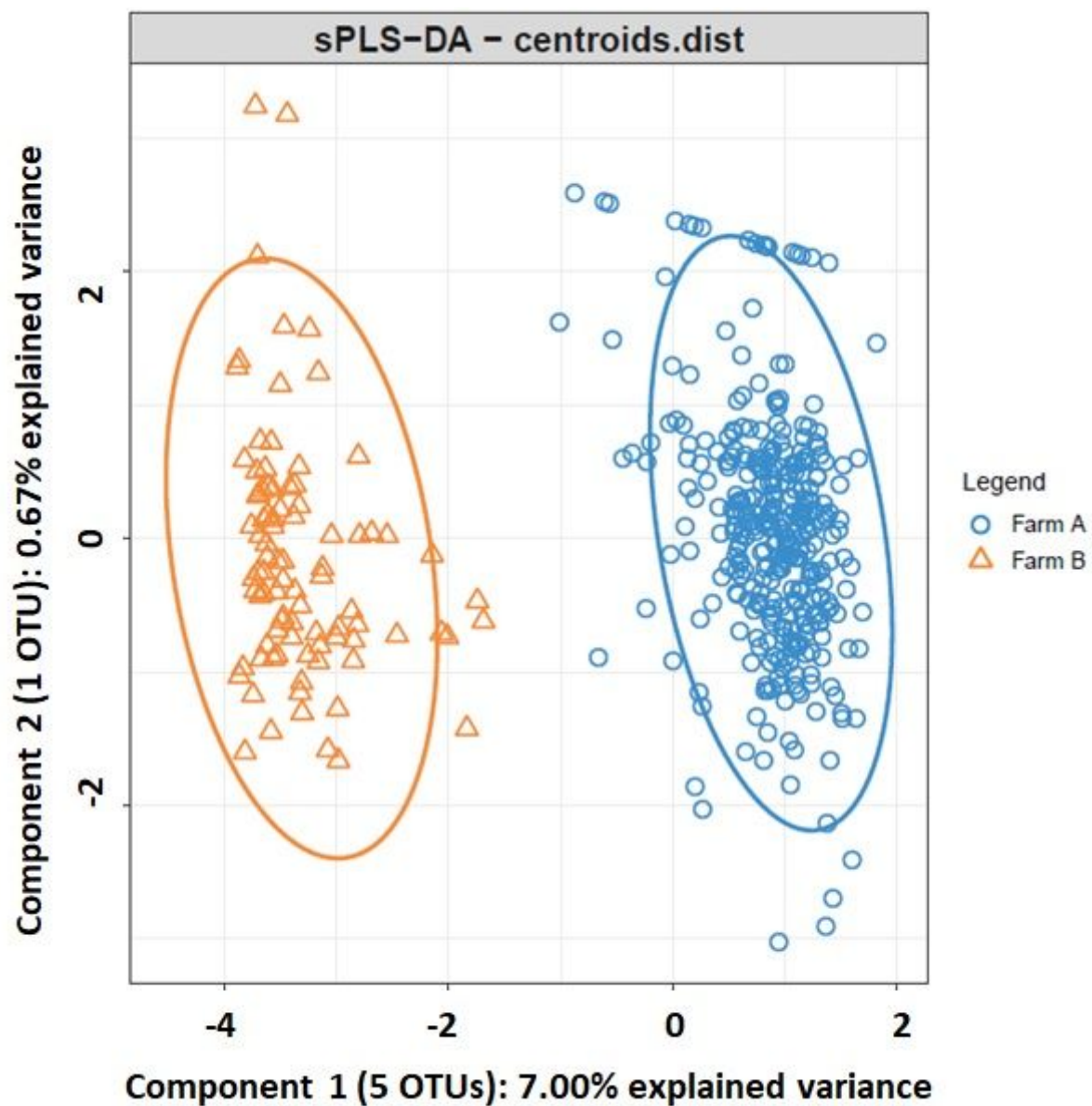
Figure 4

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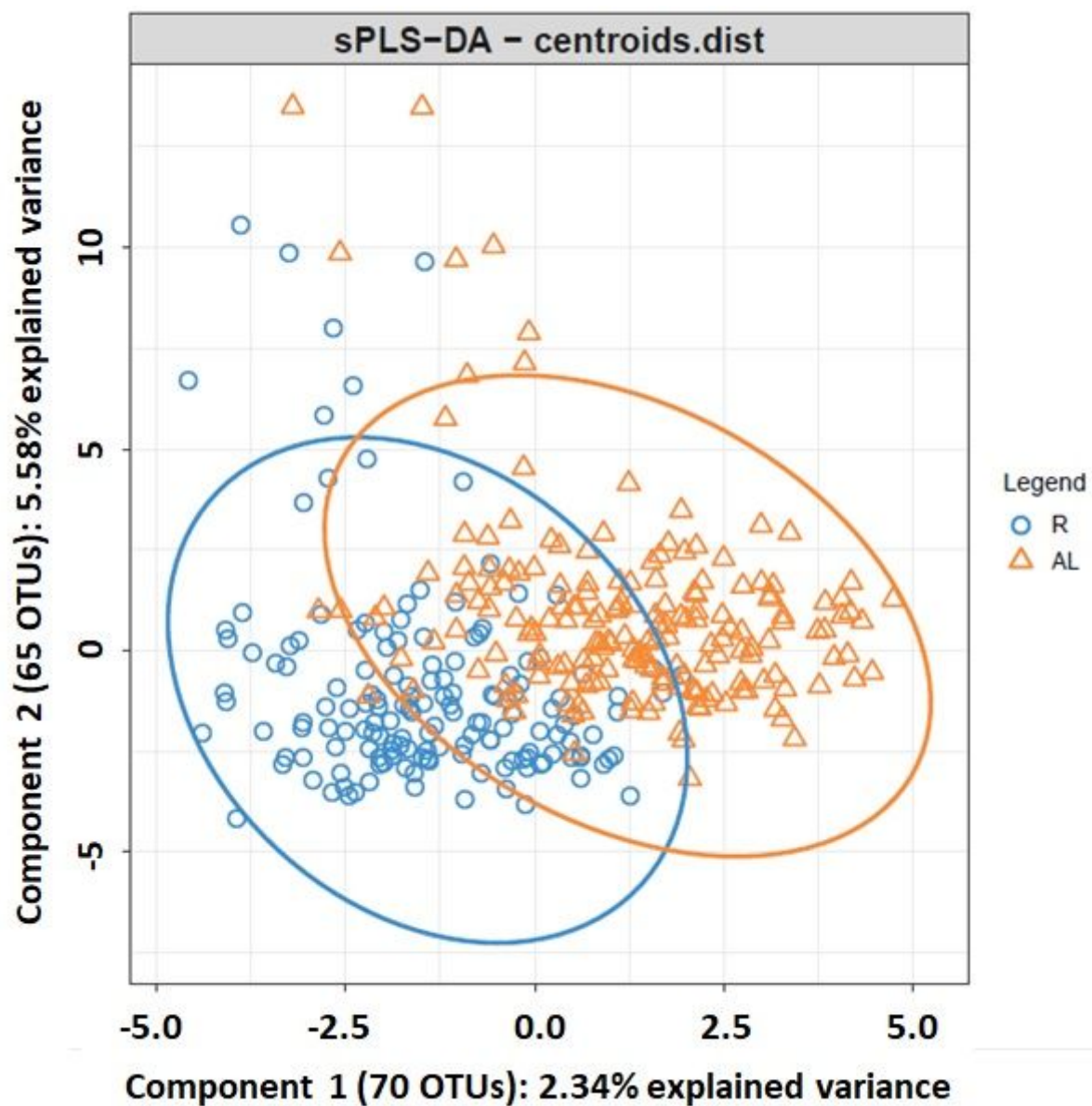
Figure 5

Sparse partial least squares discriminant analysis representing cecal samples of rabbits raised in farm A and fed R (blue) or AL (orange).
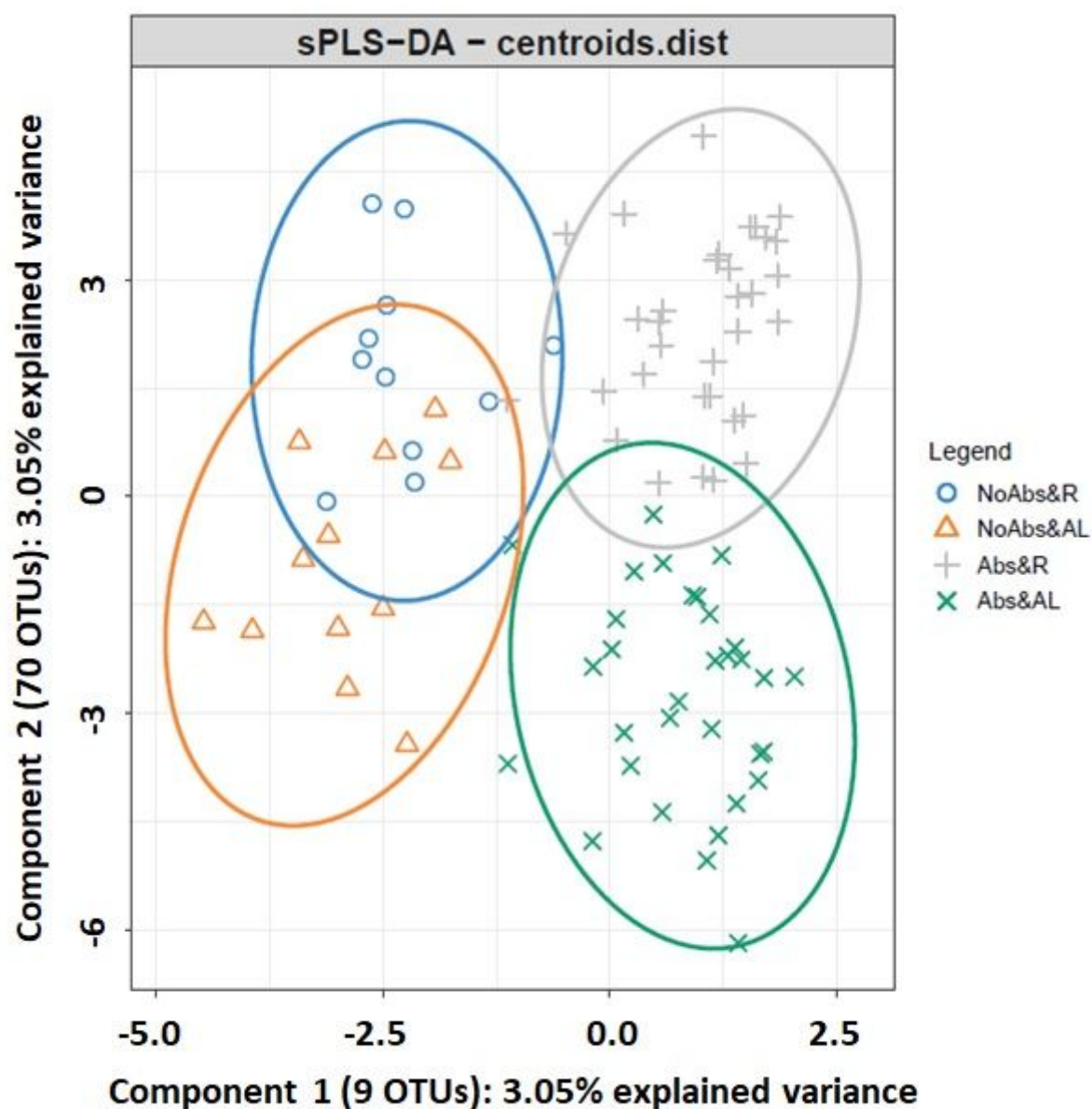
Figure 6

Sparse partial least squares discriminant analysis representing cecal samples of rabbits raised in farm B and fed R withoutAb (blue), fed AL withoutAb (orange), fed R withAb (gray) and fed AL withAb (green).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- repOTUstaxassignments.txt.txt
- phylatable.txt.txt
- otutableprefilteredunnormalized.txt.txt
- otutablefilteredCSSnormalized.txt.txt

- metadata.txt.txt
- generatable.txt.txt
- OTUsdiffsregime.xlsx
- OTUsdiffsfarm.xlsx
- OTUsdiffantibiotics.xlsx