Influence of Genetic Background and Dietary Oleic Acid on Gut Microbiota Composition in Duroc and Iberian Pigs.

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Research

Keywords: gut microbiota, pig, oleic acid

Posted Date: December 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-131496/v1>

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Influence of genetic background and dietary oleic acid on gut microbiota composition in Duroc and Iberian pigs.

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Abstract

Background: Phenotypic variability for productive and meat quality traits has been largely studied in Iberian pigs, especially in genetic selection and nutritional experiments. Complex interactions among genetic background, diet composition and gut microbiota difficult the evaluation of true effects of each factor on phenotypes. In order to disentangle these interactions, we evaluated changes in gut microbiota composition comparing Iberian and Duroc pigs fed diets with different energy source.

Results: Gut microbiota composition was analysed in feces from 48 pigs (Iberian and Duroc) fed a standard diet or a sunflower oil-enriched diet with high oleic acid content (6%). A higher richness was observed for Iberian pigs ($p < 0.05$) and compositional analysis was applied for beta-diversity, differential abundance and pairwise log-ratio analyses. We found significant differences in overall microbiota composition between breeds, and also between diets inside breeds, to a lesser extent. Differential abundance analysis revealed that Duroc animals have more proportion of Actinobacteria and Prevotella, while Iberian replace those microorganisms with other more variable taxa. According to dietary differences, high-oleic fed animals were richer in Prevotella. We also found microbial ratios capable of separating animals by breeds and diets, mostly related to Actinobacteria.

Conclusion: This study reveals that both genetic background and diet composition might have a relevant impact in gut microbiota composition. The application of compositional data analysis has facilitated the identification of microorganisms and ratios as possibly related to metabolic changes due to genetic background and, to a lower extent, to dietary changes. This may lead to a relevant progress in the knowledge of interactions between pig genetics, environment and gut microbiota.

Keywords: gut microbiota; pig; oleic acid

1 Background

2 The contribution of Spanish pig production to worldwide pork market is meaningful. According to FAOSTAT, Spain
3 produces almost 4% of worldwide swine meat, being the fourth producer, and the second one inside EU, with a 19% of
4 communal production, after Germany. One of the most eye-catching contribution of Spanish pig production to
5 international markets comes from Iberian pig, a local breed whose high-quality final products are much appreciated. In
6 fact, Spain ranks as the third country in lard and ham exportations (data from 2018) ¹, with a crucial contribution of dry-
7 cured Iberian ham. The high value of this product is due to the unique organoleptic properties conferred by Iberian pig
8 genetic, phenotypic and production particularities. This rustic and low-prolific local breed has been traditionally raised
9 under a semi-extensive production system at “dehesa” Mediterranean forests, taking advantage of the grass and acorn

resources available ². The combination of their natural high tendency to fat accumulation and the acorn and grass feeding in the late fattening phase (“Montanera”), confers their final products a unique fatty acid (FA) profile composition and a higher intramuscular fat (IMF) content than commercial breeds.

The interest in this high-quality market has led to the search and legal regulation of strategies for the improvement of its efficiency and productivity. In this sense, one of the most common practices nowadays is crossbreeding with Duroc, resulting in a negative impact on final product composition and quality ³. Crossbreeding consequences have been largely studied through different points of view – such as phenotypic traits, meat quality, genetic markers or gene expression ⁴⁻⁶. As the high quality of Iberian products has become the flag for the Iberian pig market to enter international markets, producers keep an interest in improving their products’ organoleptic attributes through FA profile and IMF content optimization ^{7,8}, especially in the recent more intensive production systems. Several strategies such as genetic selection, handling optimization and diet design, are open as key ways of modifying final product composition. Strategies such as dietary FA supplementation are common to enhance these organoleptic traits. Genetic, nutrition and nutrigenomic studies have shown the complexity of the molecular regulation of physiological processes associated to relevant phenotypic traits in Iberian pig genotypes subjected to different diets ^{4,9-11}.

Besides, with the advent of new mass-sequencing techniques, alternative ways have been opened for animal breeding and for in-depth studies of the molecular basis of phenotypic variability. Microbiota studies have hugely benefitted from these NGS techniques, as culture-free sequencing allows obtaining a lot more information from microbiome communities. Gut microbiota has become a key focus in animal breeding industry, as well as in human health, since gut microbiota and host are linked in a bidirectional way; first, diet composition and other environmental elements, as well as animal genetics, might modify microbial population structure ¹²; second, microbiota has been reported to have an impact on animal physiology, on multiple animal productive traits and also on the final products’ quality and composition ^{13,14}. This intricate correlation network makes gut microbiota composition a key factor for understanding the relationships between genotype, phenotype and environment. As a consequence, it might result obvious that different genetic backgrounds can promote different microbiota population structures as it has been proven in other studies ¹⁵. For this reason, the two main breeds employed in Iberian pig production (Iberian and Duroc) might have differences in gut microbiota composition possibly affecting relevant phenotypic traits. Also, dietary modification or supplementation strategies such as FA addition might have an impact on gut microbiota composition, potentially contributing to differences in animal traits, with special relevance on final product composition and quality. Understanding all these relationships and their implications is crucial to comprehend their global effect on animal phenotype.

In this study we compared the gut microbiota composition in pigs from Iberian and Duroc breeds, fed two different isocaloric and isoproteic diets with different energy source (oleic acid vs carbohydrates), in order to dissect the diet and

41 genetic background effects on the gut microbiota composition, as well as to deepen in the metabolic differences between
42 breeds and diet groups, potentially mediated by gut microbiota.

43

44 Methods

45 Experimental design and sampling

46 The experiment was carried out at the facilities of the Instituto Tecnológico Agrario de Castilla y Leon (ITACYL) Pig
47 Test Center (Hontalbilla, Segovia, Spain). A total of forty-eight castrated male purebred pigs, 19 Duroc (DU) and 29
48 Iberian (Torbiscal strain)¹⁶ (IB), were raised in the same commercial farm, weaned at 28 days old and transferred to the
49 experimental facility one month after weaning. The animals were kept under identical management conditions, housed in
50 batches of 4 pigs/pen (1 m² pig⁻¹), with a concrete floor and straw bedding. Temperature was controlled at a mean of 23.8
51 °C throughout the experiment. At 70 days old (± 2 days) and with a mean body weight of 19.7 ± 3.6 kg (17.3 ± 2.8 kg BW
52 in DU; 21.3 ± 3.2 kg BW in IB), both breed groups were distributed in two different experimental groups, with a factorial
53 2x2 design, and fed a control commercial diet with carbohydrates as energy source (C) (9 DU and 13 IB) or a sunflower
54 oil-enriched diet with high oleic acid content (6%) (O) (10 DU and 16 IB). Both diets were formulated according to
55 FEDNA nutritional guidelines (2006) using the Brill Formulation software (Brill Co., Georgia, USA) to be isocaloric and
56 isoproteic (3.3 kcal digestible energy and 15.6% crude protein) (**Additional file 1**) and were provided ad libitum. Diets
57 were provided for 47 days, until animals reached 50.4 ± 7.8 kg BW (51 ± 9 kg BW in DU; 50 ± 7 kg BW in IB). Fresh
58 water was provided ad libitum, with two drinking troughs available in each pen.

59 Animals were slaughtered at the end of treatment, at 117 days old (± 2 days), and individual stool samples were then
60 collected from rectum and rapidly frozen on liquid N₂ and preserved at -80°C until its use for microbiome analysis. All
61 experiments were performed in accordance with the regulations of the Spanish Policy for Protection of Animals employed
62 in Research and other scientific purposes RD53/2013, which meet the European Union Directive 2010/63/EU on the
63 protection of animals used in experimentation. The project was approved on March 20, 2015, by the Comunidad de
64 Madrid animal welfare and protection committee (reference number PROEX-007/15).

65

66 DNA extraction and 16S sequencing

67 Microbiota DNA was extracted from the 48 stool samples using QIAamp PowerFecal® kit (QIAGEN, Hilden, Germany)
68 according to manufacturer's standard protocol. Illumina MiSeq® paired-end sequencing protocol (Illumina, San Diego,
69 CA, USA) was performed by an external service (FISABIO bioinformatics, Valencia, Spain) targeting 16S rRNA gene
70 V3-V4 amplicon. The employed primers were S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21¹⁷, which produce
71 amplicons of 464 bp.

72

73 Sequence processing pipeline

74 Raw reads were pre-processed using Prinseq-lite tool ¹⁸ and in-house software. After trimming low quality bases in each
75 read, sequences shorter than 50 bp and with an average quality score below 30 on a window of 20 bases were discarded.
76 After joining forward and reverse files, a total amount of 11,599,350 reads were processed using Quantitative Insights
77 Into Microbial Ecology (QIIME) version 1.9.1 ¹⁹ into operational taxonomic units (OTUs), following the *de novo* OTU-
78 picking approach, with a clustering threshold of 97% identity. Chimeras were filtered using USEARCH algorithm (v. 6.1)
79 ²⁰. SILVA reference database (release 132) ²¹ was used for taxonomic assignment and chimera filtering. An OTU
80 abundance threshold of 0.005% was established for final quality-filtering, as described in Bokulich *et al.* ²². Processed
81 OTU table was composed by 9,428,777 reads from 1,398 OTUs.
82 Finally, two additional processing steps were performed: a taxonomy filtering, excluding OTUs not assigned to Bacteria
83 at kingdom level in the taxonomic classification, as used primers are designed for bacterial amplicons; and a sample
84 pruning, removing those with sequencing depth lower than 30k total reads, which eliminated one sample for the IB-O
85 group. The final OTU table was composed by 9,162,809 reads from 1,246 OTUs and 47 samples.

86

87 Microbial community analysis

88 All analyses were performed in R, using phyloseq package ²³. Microbiota composition was compared between breed-diet
89 animal pens. Three different approaches were then followed to analyse microbiome composition:
90 Alpha-diversity: For this approach, OTU table was rarefied to minimum sample depth (30,136 reads). Four alpha-
91 diversity measures were calculated: observed richness, Chao1 index, Shannon index and Inverse-Simpson index, using
92 the function estimate_richness from phyloseq. ANOVA for each α -diversity measure was carried out following this
93 model:

$$94 \alpha_{ijk} = \mu + b_i + d_j + bd_{ij} + e_{ijk}$$

95 Being α the α -diversity index for each sample, b the Breed effect (with $i = 2$ levels), d the Diet effect (with $j = 2$ levels)
96 and bd the interaction of both effects.

97 Data transformation: Compositional data analysis: Most of the statistical analysis were made taking into account the
98 compositional nature of microbiome data ²⁴. For the compositional approach our non-rarefied dataset was processed
99 following a number of steps as defined by ²⁵: (1) Zero-counts imputing by Geometric Bayesian Multiplicative
100 replacement; (2) Data closure computed to the total counts per sample; (3) Weighted centred log-ratio (CLR)
101 transformation. This process moves the data to an additive scale, which makes the performance of multivariate hypothesis
102 testing easier. The analysis was repeated glomming the dataset to OTU, genus, family and phylum levels. R packages
103 zCompositions ²⁶ and easyCODA ²⁷ were used for this transformation.

104 **Beta-diversity:** Beta-diversity was computed using a compositional Principal Component Analysis (i.e., PCA after CLR
 105 transformation of the data). For a better visualization of the behaviour of both breed and diet effects, a mixed “Breed-
 106 Diet” factor was considered as PCA grouping variable. The distance matrix was built using Aitchison distance measure,
 107 which is the Euclidean distance after CLR transformation. Individual beta-diversities were calculated as the distance to
 108 centroid for groups from mixed “Breed-Diet” effect (using `vegan::betadisper`). Homogeneity of dispersions respect to the
 109 group centroids was measured through ANOVA-like permutation test (`vegan::permutest`) and post-hoc Tukey HSD test.
 110 For testing the significance of multivariate effects in our *a priori* groups, non-parametric permutational multivariate
 111 analysis of variance (PERMANOVA) ^{28,29} was performed using `adonis2` function from `vegan` package in R ³⁰ with this
 112 model:

$$D_{ij} = \mu + b_i + d_j + bd_{ij} + e_{ij}$$

114 The additive model applied was built sequentially adding Breed (b_i ; $i = [1,2]$) and Diet (d_j ; $j = [1,2]$) factors and their
 115 interactions (bd_{ij}). The Aitchison distance matrix (D) was used as dependent variable.

116

117 Differential abundance analysis

118 Differential abundance analysis was carried out using both DESeq2 ³¹ and ALDEx2 ³² R packages. Default DESeq2
 119 normalization by estimation of size factors as the median ratio of counts ³³ and negative binomial GLM fitting with Wald
 120 significance tests were performed. Differences between breeds and between diets were analysed in separate models
 121 correcting by the other factor. Interactions between factors were also evaluated with an additional model (**Table 1**). P-
 122 values were corrected for multiple testing through Benjamini-Hochberg method. Significantly differentially abundant
 123 (DA) OTUs were considered with a false discovery rate (FDR) cut-off of 0.05 and a fold-change (FC) higher than 1.5 or
 124 lower than -1.5 (i.e., $|\log_2FC| > 0.59$).

125 **Table 1: DESeq2 differential abundance models for each contrast, with R notation.**

Contrast	Model design
Breed contrast	<i>RA ratio ~ Diet + Breed</i>
Diet contrast	<i>RA ratio ~ Breed + Diet</i>
Interaction effects	<i>RA ratio ~ Breed + Diet + Breed:Diet</i>

126

127 Input for ALDEx2 analysis was prepared as the CLR-transformed posterior distribution of the data generated by 128
 128 Monte Carlo samples from the Dirichlet distribution. CLR transformation was made through geometric mean of all
 129 features' abundance. Mono-factorial breed and diet contrasts were performed, using Welch's t-test. P-values were
 130 corrected through Benjamini-Hochberg method. OTUs were considered as significantly differentially abundant between
 131 breeds or diets when corrected p-value was lower than 0.05 and diff.btw (i.e., median difference in CLR values between
 132 factor groups) was higher than 1 or lower than -1. Visualization of coincident DA OTUs between contrasts and pipelines
 133 was carried out with `VennDiagram` package in R ³⁴.

134

135 Pairwise log-ratios analysis

136 Closed data were also used for another approach, using pairwise log-ratios (p-LR) instead of centred log-ratios ²⁷, as a
137 way to evaluate individual relationships among OTUs and features glommed to genus, family and phylum levels.

138 Recursive Partitioning and Regression Trees (RPART) were generated, including the microorganism pairwise log-ratios
139 most suitable to split samples according to the Breed and Diet phenotypic criteria.

140 Pairwise log-ratios analysis is computationally expensive, as feature combinations produce matrices much larger than the
141 original, i.e., a $m \times n$ matrix (*samples* $\times$ *features*) would be transformed in a $m \times n(n - 1)/2$ matrix. For this reason,
142 OTU-grouped dataset was size-reduced by selecting a relevant feature subcomposition generated by sparse Partial Least
143 Square Discriminant Analysis (sPLS-DA), using the 4-leveled mixed trait “Breed-Diet” as classification factor. Five-fold
144 cross-validation with permutation was used to test the optimal component structure for the model. In order to generate
145 the subcomposition, OTUs with a Variable Influence of Projection (VIP) value higher than 1 for each component were
146 selected. As p-LR analysis keeps subcompositional coherence ²⁷, data subsetting has no significant effect on the results.

147

148 Microbiome-phenotype association

149 CLR data were also used to detect associations between microbiome and several phenotypic traits. IMF content was
150 measured in *Biceps femoris* and *Longissimus dorsi*. FA profile was analysed in backfat outer layer (Back) and in ham
151 outer fat layer (Ham). Total FA fractions (saturated, SFA; mono-unsaturated, MUFA; and poly-unsaturated, PUFA) as
152 well as relevant FA contents (oleic, C18:1(9); linoleic, C18:2; palmitic, C16:0; and stearic, C18:0) were also measured,
153 with all quantification methods being described in formerly published studies ¹¹.

154 PERMANOVA controlling both Breed and Diet effects was performed for each phenotypic variable using `vegan::adonis2`
155 function, in order to elucidate linear or nonlinear relationships between samples distribution and each phenotypic trait:

$$156 \quad D_{ij} = \mu + b_i + d_j + P + e_{ij}$$

157 The additive model applied was built sequentially adding Breed (b_i ; $i = [1,2]$) and Diet (d_j ; $j = [1,2]$) factors, and each
158 phenotype (P) was treated as linear regression at the end. The Aitchison distance matrix (D) was used as dependent
159 variable.

160 Additionally, individual OTU association was addressed using Limma linear regression. To avoid the influence of the
161 known breed effects on phenotype which could lead to potential spurious associations, both microbiome and phenotypic
162 datasets were split for carrying out independent DU and IB microbiome-phenotype analyses. To measure diet weight in
163 correlations, two methods were carried out: (1) linear regression of each OTU on each phenotypic variable without diet
164 correction; (2) linear regression of each OTU on the diet regression residuals of each phenotypic variable. P-values were
165 adjusted using Benjamini-Hochberg method (adjusted significance threshold set to FDR = 0.05). Representation of

associations between OTUs and phenotypes was addressed through interaction network in Cytoscape software (v. 3.8.1), setting Kamada-Kawai algorithm (Edge-weighted spring embedded layout) ³⁵ for optimal visualization.

Results

Overall microbiota composition

The overall composition is summarised in **Table 2**. Our final OTU table was composed by 47 samples, as we pruned one sample with less than 30k reads. As mentioned in methods, we applied an additional filter removing those reads not assigned to Bacteria kingdom. After this step, 97.44% of total reads were kept, representing 1,246 OTUs. From this bacteria-classified fraction, 99.93% reads were assigned to phylum level. A total of 17 phyla were identified. Prevalence and abundance of phyla are represented in **Figure 1**.

Table 2: Overall microbiota composition.

	Kingdom	Phylum	Class	Order	Family	Genus
% Sequences ¹	97.44	99.93	99.91	99.91	97.30	86.70
Classified OTUs	1246	1245	1243	1243	1184	988
Identified taxa	1	17	23	33	48	148

Total OTU number, % of sequences assigned to a known taxon at different ranks and unique taxa found at each rank.
¹ Proportion of total reads classified to phylum and lower taxonomic levels are calculated relative to the total reads after filtering OTUs not assigned to Bacteria kingdom.

The 988 annotated OTUs were classified to 148 different genera, covering 86.7% of total reads. 20 genera had an average relative abundance (**RA**) higher than 1%, being *Prevotella_9*, *Lactobacillus*, *Prevotellaceae NK3B31 group*, *Alloprevotella* and *Treponema_2* the most abundant ones (**Additional file 2**). Note that SILVA database subdivides some genera according to sequence clustering, appearing under multiple designations in our database.

On the other hand, 79 genera were present at an average abundance lower than 0.1% (**Additional file 2**). This fact might be taken into account when interpreting the results, as they might be poorly represented across the samples (i.e., low prevalence) and/or have a minimum number of reads per sample (i.e., low coverage). Nonetheless, all the analyses have been performed including these low-abundance OTUs.

Microbiota composition analysis:

Two-way ANOVA for α -diversity measures in rarefied data showed no significant differences between diet groups (C vs O), but all of them (richness: $p = 0.021$; Chao1 index: $p = 0.018$; Shannon index: $p = 0.008$; and Inverse Simpson index: $p = 0.002$) were significantly affected by breed, these measures showing higher values in the IB group (**Figure 2**). Initial average number of OTUs per sample was 1,046 for DU population and 1,097 for IB population. After rarefaction, these numbers were corrected to richness values, with an average of 880 in DU and 944 in IB.

Compositional PCA for β -diversity (**Figure 3**) showed a high dispersion of the data, although breed clustering is observed through PC1. Diet clustering can be observed through PC2, but only when features are analysed at lower taxonomic levels

(i.e., genus and OTU levels). Centroid position also revealed that separation between breeds is higher than separation between diets. At OTU level, centroid distance is maximum for both breed and diet groups. Individual beta-diversities were calculated as the Euclidean distance to centroids for groups from mixed “Breed-Diet” effect. Average individual dispersion between groups was not significantly different, except for OTU-glommed dataset. At OTU level, DU-C samples had an average beta-diversity index significantly lower compared to DU-O, IB-C and IB-O groups, although p-value in DU-C vs IB-C contrast (same diet in different breeds) was close to 0.05. (**Figure 4**).

PERMANOVA analysis confirmed that samples from different breeds are significantly different. Differences between diets (corrected by breed effect) are also significant at OTU, genus and family level, although these differences blur at phylum level (**Table 3**). Nonetheless, the differences between groups are moderate, as roughly 10% of variance from our data can be explained by Breed effect, and only 5% by Diet effect.

Table 3: PERMANOVA statistics.

		F statistic	R ²	p-value
Phylum	<i>Breed</i>	5.05	0.10	0.0017*
	<i>Diet</i>	1.44	0.03	0.1776
	<i>Interaction</i>	1.32	0.03	0.2187
Family	<i>Breed</i>	5.96	0.11	0.0001*
	<i>Diet</i>	2.24	0.04	0.0283*
	<i>Interaction</i>	1.51	0.03	0.1247
Genus	<i>Breed</i>	6.15	0.12	0.0001*
	<i>Diet</i>	2.55	0.05	0.0118*
	<i>Interaction</i>	1.27	0.02	0.1859
OTU	<i>Breed</i>	5.39	0.10	0.0001*
	<i>Diet</i>	3.43	0.07	0.0021*
	<i>Interaction</i>	1.17	0.02	0.2313

F statistic, R² and p-values at different taxonomic levels.* p < 0.05.

Differential abundance (DA) analysis

Two separate contrasts were made, for breed and diet factors, using an FDR cut-off of 0.05 and a fold-change minimum threshold of ± 1.5 ($\log_2 FC \approx \pm 0.59$) for DESeq2 analysis, and a minimum median difference between CLR values of ± 1 ($diff.btw = \pm 1$) for ALDEx2 analysis.

Figure 5 includes volcano plots for both contrasts with DESeq2 and ALDEx2 methods. Both breed and diet contrasts resulted in more restrictive results with ALDEx2 method. Breed contrast shows a higher number of over-abundant (**OA**) OTUs in IB pigs when done with DESeq2, while ALDEx2 breed contrast shows a higher number of DU OA OTUs. On the other hand, O diet presents a higher number of OA OTUs than under-abundant (**UA**) OTUs, compared to C diet, both with DESeq2 and ALDEx2. With DESeq2 pipeline, 468 DA OTUs from 119 different genera were found for DU vs IB contrast, mostly from *Ruminococcaceae*, *Prevotellaceae* and *Lachnospiraceae* families. On the other hand, 185 DA OTUs from 71 genera were found between C and O diet groups, predominated by the same families. With ALDEx2, 207 DA OTUs were found for breed contrast, whereas only 18 DA OTUs were found for diet test, mostly from *Prevotellaceae*

family. Common DA OTUs between pipelines are counted in **Table 4**. For breed contrast, the most representative taxa in overlapping DA OTUs was *Prevotellaceae* (mostly *Prevotella*) (27%), followed by *Lachnospiraceae* (16%), *Ruminococcaceae* (15%) and *Veillonellaceae* (mostly *Megasphaera* and *Anaerovibrio* OTUs) (11%). For diet contrast only 18 OTUs were common to both pipelines, 7 of them from *Prevotellaceae* family. Supplementary material with the complete DA-OTU list for all contrasts is also available (**Additional file 3**).

Table 4: DA OTUs by factor and pipeline.

		DA OTUs	DA genera	DA families	DA phyla
DESeq2	Duroc vs Iberian	468	119	44	13
	Control vs High Oleic	185	71	32	9
ALDEx2	Duroc vs Iberian	207	72	22	6
	Control vs High Oleic	18	13	8	3
Overlap	Duroc vs Iberian	151	63	20	6
	Control vs High Oleic	18	13	8	3

Number of DA OTUs in breed and diet contrasts using DESeq2 or ALDEx2 pipelines, and common DA OTUs between both pipelines. Number of genera, families and phyla grouping these OTUs are also shown.

We evaluated coincident DA OTUs among contrasts and methods, as shown in the Venn diagram (**Figure 6**). When comparing DESeq2 with ALDEx2, 151 DA OTUs were coincident in the breed contrast and 18 coincidences were found in the diet contrast. On the other hand, when comparing the results obtained for breed and diet effects within the same method, we detected some coincident OTUs (75 OTUs in DESeq2 and only 1 OTU in ALDEx2). Only one OTU is common to all four comparisons, the one named as *denovo378129*, from genus *Shuttleworthia*, which was OA in DU and O groups.

Significant interaction between breed and diet was detected for 26 OTUs ($P_{adj} < 0.05$), most of them being qualitative interactions (**Figure 7**). Most of these OTUs were members of *Corynebacterium*, *Lactobacillus* and *Ruminococcaceae* taxa.

Pairwise log-ratio analysis

sPLS-DA for OTU-grouped dataset confirmed a total of 141 relevant OTUs ($VIP \geq 1$) as predictive of the “Breed-Diet” phenotype. This subcomposition has been used to calculate pairwise log-ratios at OTU level. For genus, family and phylum grouped datasets, full table has been used for p-LR calculation. Recursive Partitioning and Regression Trees (**RPART**) were built for each p-LR matrix, as shown in **Figure 8**. We observed that the best classification occurs at family level, being the ratios *Corynebacteriaceae/Spirochaetaceae* (**COR/SPR**) and *Peptostreptococcaceae/Streptococcaceae* (**PEP/STR**) the most relevant to classify between breeds.

At phylum level diet differences were not clear inside each breed, as it was observed in beta-diversity analysis. *Actinobacteria/Epsilonbacteraeota* ratio was able to separate samples by breed, being reliable to classify as IB but not as DU, and was not capable of properly separate diet groups inside each breed.

At family level, three ratios were capable of separating animals in the four Breed-Diet groups with high precision. As mentioned, COR/SPR and PEP/STR ratios mainly clustered Iberian and Duroc separately, but also differentiated dietary groups inside the IB breed, while *Atopobiaceae/Enterobacteriaceae* (ATO/ENT) ratio separated diets within the DU animals. These ratios are closely related with those responsible of group classification at genus level (*Anaerostipes/Corynebacterium_1* and *Family_Peptostreptococcaceae/Peptococcus*), except that these genera ratios can only discriminate between IB-C, IB-O and DU animals, but no ratio was able to differentiate diets within DU breed.

In the case of COR/SPR, both ratio components were responsible of variation. ALDEx2 DA analysis at family level showed that both *Corynebacteriaceae* and *Spirochaetaceae* were significantly DA between DU and IB groups (IB-UA: diff.btw = -3.23 and IB-OA: diff.btw = 1.13, respectively) although *Corynebacteriaceae* reduction in IB is stronger than *Spirochaetaceae* increment. As *Corynebacteriaceae* are *Actinobacteria*, breed separation at phylum level is reflected at lower taxonomic levels. Same occurs at the genus decision tree, whose first ratio *Anaerostipes/Corynebacterium_1* was able to differentiate breeds, with *Corynebacterium* having the highest weight, as DA analysis at genus level stated a *Corynebacterium_1* OA in DU animals genus (diff.btw = -2.78).

The PEP/STR ratio was able to identify IB animals, even separating C and O populations when accounting also COR/SPR ratio. DA analysis revealed that *Peptostreptococcaceae* family was more abundant in DU animals (diff.btw = -1.27), while *Streptococcaceae* family was not differentially abundant between breeds. At genus level, *Peptococcus* abundance played a similar role, as IB pigs had a higher RA (diff.btw = 1.14).

As indicated, ATO/ENT ratio was able to distinguish between C and O diets within DU animals, but only when applied after discriminating IB animals with former ratios (otherwise it would only discriminate between DU-O and the rest of Breed-Diet groups). *Enterobacteriaceae* were OA in DU animals (diff.btw = -1.92) but no abundance differences were detected between diet groups.

Finally, the two representative ratios at OTU level (*denovo219246/denovo273762* and *denovo277739/denovo351253*) were composed by OTUs assigned to *Rickenellaceae/Ruminococcaceae* families and *Peptococcus/Lactobacillus* genera, respectively. Additionally, *denovo219246* and *denovo277739* appeared as DA between IB and DU animals (first one with DESeq2 pipeline and second with both DESeq2 and ALDEx2 pipelines), which gave them a substantial weight in breed differentiation.

271

272 Association with phenotypic traits

PERMANOVA did not show any significant association between overall microbiota composition and the analysed phenotypic traits. When the effect of each individual OTU was evaluated through Limma regression different results were observed for each tested model. Without diet correction, only one OTU was significantly associated to lipidic traits in DU subset, *denovo283234* (*Treponema* 2), which was negatively associated to backfat MUFA and oleic acid content, while,

277 a total of 87 individual OTUs were found as significantly associated to phenotypic traits in IB subset (**Figure 9**). Most of
278 them belonged to *Alloprevotella* and other *Prevotella* groups and were associated to multiple traits. *Ruminococcaceae*
279 OTUs were positively linked to oleic acid and negatively linked to palmitic acid proportions in backfat, while
280 *Rikenellaceae* OTUs mainly appear negatively associated to oleic acid proportion, affecting in both cases overall MUFA
281 and SFA proportions. Some relevant OTUs from *Shuttleworthia*, *Lactobacillus* and *Corynebacterium* genera also
282 appeared associated to FA composition. When diet correction was applied almost every correlation disappeared, and only
283 one OTU was negatively correlated to ham oleic acid proportion in IB subset, *denovo37314* (*Treponema* 2).
284

285 Discussion

286 In this study we explore the composition and disparities of the bacterial fraction of fecal microbiota populations between
287 two different pig breeds (Iberian and Duroc) at 117 days old, fed diets with different energy source (carbohydrates vs.
288 sunflower oil with high oleic acid content). Some important differences were detected between Iberian and Duroc
289 populations, and to a lesser extent also between control and high-oleic diets.

290 Overall taxonomic structure revealed *Bacteroidetes*, *Firmicutes* and *Proteobacteria* as the most abundant phyla, with an
291 important predominance of *Prevotellaceae*, *Lachnospiraceae* and *Ruminococcaceae* families. *Prevotella* genus was the
292 most abundant, with an average RA of 30% in Duroc animals and 22% in Iberian animals when considering all SILVA
293 genus-clustering designations. *Prevotella* are anaerobic saccharolytic bacteria which produce acetate and succinate
294 through fermentation ³⁶ and their abundance is highly related to high-fiber long-term diets ^{37,38}. Their presence in fecal
295 microbiota is highly reported in human ³⁹ and other animals such as ruminants, predominating in rumen microbiota ^{40–42}.
296 Multiple studies have shown the dominance of *Prevotella* in pig gut microbiota ⁴³ as well as their relevance in enterotypes
297 ⁴⁴ or even immune response ⁴⁵. *Alloprevotella* genus is also one of the most representative in our data, with an average
298 RA of 4.2% in DU and 11.6% in IB animals. This genus is also known for its saccharolytic activity ⁴⁶, although it is less
299 studied than *Prevotella* due to its lower abundance in gut communities. *Lachnospiraceae* and *Ruminococcaceae* families
300 also comprehend well-known polysaccharide fermenters, which in addition participate in methanogenesis by producing
301 H₂ and formate as substrates to archaeal communities ⁴⁷. Abundance balance of these three families in gut microbiota,
302 through a number of abundance ratios, might be relevant for host traits related with feed efficiency, metabolism or health.
303 For instance, *Prevotella*-to-*Bacteroides* ratio has been related with weight and fat loss in humans ⁴⁸, and *Firmicutes*-to-
304 *Bacteroidetes* ratio has been repeatedly associated to fiber metabolism or obesity in human populations ^{37,49} and murine
305 models ⁵⁰, although there is controversy about these links ⁵¹. *Prevotellaceae*, *Lachnospiraceae* and *Ruminococcaceae* also
306 compose the core microbiome in rumen, being shared by multiple ruminants in an important abundance ⁵². Our data
307 support that this core microbiota might also be shared by other monogastric species along all digestive tract. Similar
308 findings are reported by Crespo-Piazuelo *et al.* ⁵³ when analysing gut microbiota in different intestinal regions using the

309 same IB-C pig population. However, they reported a range of 35 to 41% of *Prevotella* abundance within all colon regions.
310 These discrepancies must be attributed to different intestinal region, different genotype and diet of the total animal
311 population studied (colon from IB-C animals vs rectum from IB and DU fed C and O diets).
312 Alpha-diversity measures revealed a higher number of different OTUs in IB pigs, which can also explain the higher values
313 of Shannon and Simpson indices. These interesting findings might be related with the higher rusticity and resilience of
314 Iberian pig breed compared with other commercial breeds ⁵⁴, since higher ecological diversity is associated with a better
315 response to environmental disturbances (response diversity) ⁵⁵.
316 Beta-diversity PCA showed us that overall microbiome composition is different between DU and IB animals. Diet effects
317 appear masked by this breed influence, but its impact on microbiome structure is also noticeable. Although no significant
318 interaction between breeds and diets was detected with PERMANOVA, PCA showed a slightly different separation of C
319 and O centroids within each breed, which could suggest a different response to diet between Duroc and Iberian microbial
320 communities. On the other hand, PCA average dispersion within DU-C samples is lower compared to the rest of groups,
321 with this difference being significant ($p < 0.05$) at OTU level. In contrast, average dispersions between IB dietary groups
322 are pretty similar, and DU-O group has a higher dispersion within its samples, although no significant differences were
323 detected with respect to IB groups. This higher dispersion level within DU-O cluster compared to DU-C is coincident
324 with the increment in alpha-diversity variability detected for this group, especially when observing richness or Chao1
325 index values, and opposes to the sample homogeneity within DU-C. In contrast, IB-O animals did not show different
326 alpha-diversity mean or dispersion values compared to IB-C. Thus, it can be hypothesised that Duroc animals might
327 respond in a more heterogeneous way to dietary oleic acid increment.
328 Differential abundance analysis gives a more specific insight about these changes in microbiome population structure
329 among experimental groups. DESeq2 is a RNAseq-based, robust and well documented method broadly used in
330 microbiome DA analyses. However, in recent years the concern about compositional nature of these data has increased,
331 and some authors remark that RNAseq-based tools are not accounting for the compositional nature of microbiome data.
332 For this reason we also performed differential abundance analysis with ALDEx2 method, an ANOVA-based approach
333 which accounts for this intrinsic compositionality and has a similar sensitivity than other methods, also reducing false
334 positive rate near to zero ²⁴. The most conservative strategy would be to focus in the overlapping DA OTU set between
335 DESeq2 and ALDEx2, but due to the restrictive behaviour of ALDEx2 we decided to consider also DESeq2 results for
336 further exploration.
337 Our results comparing diets (C vs O) showed 18 OTUs as DA with both DESeq2 and ALDEx2 methods (i.e. all the OTUs
338 detected by ALDEx2 were included in the DESeq2 output). 12 of them were high-oleic OA OTUs from *Prevotellaceae*,
339 *Lachnospiraceae*, *Muribaculaceae* and *Ruminococcaceae* families, while the remaining 6 UA OTUs were 3
340 *Rikenellaceae* members and 3 OTUs classified as *Clostridium* s.s. 6, *Erysipelotrichaceae* UCG-004 and *Desulfovibrio*,

341 respectively. When exploring DESeq2 results, most of the high-oleic OA OTUs were from the same families, but a high
342 number of OA *Prevotella* and *Alloprevotella* OTUs was detected. Within UA OTUs, a higher variety of families was
343 present, with high representativity of *Prevotellaceae*, *Ruminococcaceae*, *Rikenellaceae* and *Erysipelotrichaceae*. The
344 greater presence of *Prevotella* and kindred taxa in O animals might be related with the higher fiber content in this diet, as
345 it was formulated to be isoenergetic in relation to control diet. Fiber content has already been linked to gut microbiota
346 changes ⁵⁶, and *Prevotella* OTUs have been defined as biomarkers for high-fiber diets ⁵⁷. Generally speaking, high-oleic
347 OA OTUs were rare OTUs (i.e., $RA \leq 0.01\%$ in C group), while high-oleic UA OTUs tended to be more common (i.e.,
348 $RA > 0.01\%$ in C group), meaning that the abundance decrease of common OTUs due to oleic acid and fiber
349 supplementation results in an abundance increment of marginal OTUs. This fact is supported by our Shannon index values
350 showing an upward trend in O group compared to C diet, which, in this case, means a higher overall evenness in O group,
351 as observed richness is not different between diets.

352 Breed differences were more obvious, matching with PCA sample distribution, as centroids were more distant between
353 breeds than between diets. Based on the overlapped results from DESeq2 and ALDEx2, DU animals have a higher
354 abundance of *Actinobacteria*, specifically from *Corynebacterium* and *Bifidobacterium* genera, as well as multiple
355 *Lachnospiraceae* (*Blautia*, *Roseburia*, *Lachnoclostridium*, *Fusicatenibacter*, and *Shuttleworthia*), multiple
356 *Prevotellaceae* OTUs (mostly *Prevotella* and *Alloprevotella*) and other genera such as *Clostridium s.s. 1*, *Holdemanella*,
357 *Megasphaera* or *Succinivibrio*, while IB OA OTUs were scarcer and belonged to more diverse taxa (see **Additional file**
358 **3** for complete OTU list). As far as we know, no former studies have found these differences between Duroc and Iberian
359 pigs, so these findings might be important to define unique microbiome patterns associated to host genotypes. Still, gut
360 microbiota contrasts between Duroc, Landrace and Large White pigs also revealed a higher presence of *Prevotella* in
361 Duroc animals ¹⁵.

362 *Bifidobacterium* are well known anaerobic lactic acid producer bacteria ⁵⁸ whose abundance in fecal microbiota has been
363 reported as negatively correlated with pig age, being more abundant at early life stages ⁵⁹. The transition from weaning to
364 adult cereal-based diet is a key factor in the replacement of these early-colonizers by other microorganisms such as
365 *Prevotella*, *Roseburia* or *Succinivibrio*, more typical of adult fecal microbiota ⁴⁵. Only one *Bifidobacterium* OTU was
366 detected in our data, presenting low abundance (mean $RA = 0.01\%$) and majorly absent in IB animals. This might indicate
367 a slower replacement of weaning to adult microbiota in DU pigs, which might as well be related to their lower alpha-
368 diversity.

369 On its part, *Corynebacterium* includes a wide variety of gram-positive aerobic and facultative species, some of them
370 fermentative organisms with special relevance at amino acid biosynthesis pathways, such as lysine through intermediate
371 diaminopimelate (DAP) from aspartate and pyruvate, or histidine ^{60,61}. Other authors reported lower protein synthesis,
372 degradation and deposition rates in Iberian pigs, compared to other commercial breeds ⁶². *Corynebacterium* might be

373 contributing to this differential protein synthesis and degradation balance, through a differential bioavailability of essential
374 amino acids in the gut, thus, their abundance could be correlated to animal protein deposition.

375 Differential abundance of other groups might be related with richness and resilience of Iberian pigs. In this sense, the
376 lower abundance of multiple *Prevotella* OTUs in IB pigs seem to be related with the previously reported increase of
377 microbial diversity, as it happened when comparing C and O diets. This richness increment might likewise be related with
378 the lower abundance of *Shigella-Escherichia* group, as a higher α -diversity has been associated to the prevention in the
379 establishment of pathogenic microbes such as Enterobacteriaceae³⁷. *Treponema* DA OTUs, which are virtually absent in
380 Duroc animals, appear as moderately abundant in Iberian pigs (RA $\approx$ 1%), thus these fiber-fermenters might have settled
381 the niche that *Prevotella* left. Finally, we detected an abundance opposition between *Prevotella* and *Treponema* taxa. The
382 most abundant OTUs of all the dataset, belonging to *Prevotella* genus, were OA in DU, while several *Treponema* OTUs
383 were OA in IB. As described by Crespo-Piazuelo *et al.*⁵³, this might suggest a competition for dietary fibre.

384 Finally, *denovo378129* (*Shuttleworthia*) was the only OTU differentially abundant between all breed and diet groups.
385 This bacterial group is relatively unknown, although it has been reported as a typical member of animal gut microbiome
386 in several studies^{59,63}. According to our results, *Shuttleworthia* could be a key differentiator between Iberian and Duroc
387 animals, having a relevant link either with fiber or with oleic acid dietary supply.

388 Additionally, DESeq2 allowed us to test interaction effects between breed and diet factors. With this method we were
389 able to find as much as 26 significant interactions, most of them being qualitative. According to DESeq2 DA analysis for
390 diets (C vs O) using DU and IB subpopulations, we were able to classify these interactions based on the different response
391 to diets inside each breed: no response to O diet (NR), abundance increase due to O diet (AI) or abundance decrease due
392 to O diet (AD). 12 OTUs were NR within DU animals (7 IB-AI and 5 IB-AD) while 7 OTUs were NR within IB animals
393 (all of them DU-AI). Only 1 OTU (*denovo347841*) presented an abundance increase in response to O diet within both
394 breeds, being its AI much larger for Iberian animals (quantitative interaction). The remaining 6 interaction-significant
395 OTUs were not significantly DA between diets within each breed. The OTU *denovo347841* has been classified as
396 *Unclassified_Muribaculaceae*, a relatively unknown *Bacteroidales* family which has been observed in multiple gut
397 microbiomes through 16S or metagenomic analyses⁶⁴. Although their main metabolic role remains unknown, their
398 phylogenetic closeness to *Prevotellaceae* and *Bacteroidaceae* could reveal saccharolytic activity. In summary, most of
399 the observed interactions are qualitative interactions in which one of the breeds responds to the diet while the other one
400 does not. Also, no pure qualitative interactions are observed showing opposite responses to the diet in both breeds. The
401 presence of interactions for individual OTU abundances would be a clear indicator of differential effects of diet on the
402 gut microbiota of Duroc and Iberian pigs. This fact can be related with our beta-diversity results, as our PCA showed a
403 tendency to a higher centroid distance within IB-C and IB-O groups than within DU-C and DU-O, although the absence
404 of significant interactions in PERMANOVA test throws some uncertainty about this conclusion. Also, former studies

405 carried out with the same animals have proven the existence of a differential response to O diet for several productive
406 traits and for adipose tissue transcriptome ¹¹. In summary, this differential response may exist for gut microbiome
407 composition and structure, but again might be not clearly noticed due to the low number of animals and to the relatively
408 high resemblance between feed formulations and to the intrinsic difficulty in evaluating and interpreting interaction
409 effects.

410 We also used the log-ratio analysis approach, assuming that ratios between OTUs can also be biologically relevant despite
411 of single association with OTU RAs. Thus, composition differences have been explored more deeply by means of p-LR
412 analysis, which detected those microorganism pairwise ratios whose abundances could separate animals by both breed
413 and diet. In order to check which microbes were mainly responsible of separation between groups at each ratio,
414 supplementary differential abundance analyses were made, using ALDEx2 (as this pipeline uses the same methods as for
415 RPART trees building) with phylum, family and genus-grouped database.

416 With this approach we identified some ratios that could be useful as differentiators between Duroc and Iberian phenotypes.
417 *Actinobacteria* abundance has been observed to be important when compared to *Epsilonbacteraeota* (Phyl. Nov.), a novel
418 phylum proposed by Waite *et al.* ⁶⁵ as a separate group from former *Epsilonproteobacteria* class, which was classified
419 inside *Proteobacteria* phylum. Our dataset contained *Campylobacter* and *Helicobacter* members of *Epsilonbacteraeota*,
420 two genera with diverse metabolisms more known by their pathogenic species affecting human and other animals. One
421 out of our OTUs has been classified as *Campylobacter hyointestinalis*, a species name proposed to *Campylobacter* isolated
422 from pigs with proliferative enteritis ⁶⁶. Interaction relationship of these pathogenic bacteria and *Actinobacteria*
423 (*Corynebacterium*, *Bifidobacterium*, *Collinsella*, *Enterorhabdus* and non-classified *Atopobiaceae*) present in our database
424 remains unknown. However, as *Epsilonbacteraeota* RAs remain fairly constant across samples, differentiation between
425 IB and DU pigs might be due to lower *Actinobacteria* abundances in Iberian animals. *Actinobacteria* relevance in breed
426 separation has been upheld when analysing ratios at lower taxonomic levels. Accordingly,
427 *Corynebacteriaceae/Spirochaetaceae* and *Anaerostipes/ Corynebacterium* were relevant in breed separation at family and
428 genus levels, respectively. In any case, complete separation between breeds and diets, including the discrimination
429 between Duroc diet groups, is only possible at family level making use of three pairwise ratios (COR/SPR, PEP/STR and
430 ATO/ ENT), considering that *Enterobacteriaceae* are more abundant in DU-O group. This fact also reflects the masking
431 effect that breed is causing to differentiate diet effects on microbial populations, previously commented.

432 This type of approach might be interesting to detect microbial biomarkers, given their importance as tools for genetic
433 diagnosis or diet determination. In the case of Iberian pig industry this might be especially useful in order to address
434 frauds due to crossbreeding and dietary manipulation, which can importantly affect final products' quality ^{6,7}.
435 Nevertheless, additional studies including crossbred animals and different production systems are necessary in order to
436 validate these potential biomarkers and establish causal relationships.

437 As an additional approach we related the individual OTU abundances with different fat composition traits, in order to
438 deepen in the potential effects of the gut bacteria on host's metabolism. A former study with the same animals reported
439 significant differences in FA composition between C and O diets ⁶⁷. Our first Limma approach (breed subsetting and no
440 diet correction) was used to globally visualize the correlations between OTU abundances and lipidic composition,
441 independently of their causal relationship. Again, differences were detected between DU and IB, as only one OTU
442 abundance was significantly correlated to FA composition in DU animals. When the second Limma approach was applied
443 (breed subsetting and diet correction) we saw that most of these correlations were caused by the diet influence. In IB
444 subpopulation, *Prevotellaceae* OTUs mostly appear negatively correlated to SFA and/or positively correlated to MUFA
445 (as the variation in one FA alters the proportion of the others). As they have a role in fiber digestion, these correlations
446 might be caused by the higher fiber content in high-oleic diet, as mentioned before. Other correlations might be directly
447 caused by increased supply of dietary oleic acid, such as the negative correlations between OTUs from *Lactobacillus*
448 (*denovo2335*, *denovo274149*) and backfat SFA content, or between *Campylobacter* (*denovo141328*) and both ham and
449 backfat SFA content. *Lactobacillus* are important fermenters with a crucial role in VFA biosynthesis ⁶⁸, so its association
450 with FA availability could be expected. Regarding *Campylobacter*, as it happens with *C. jejuni*, *C. hyointestinalis* might
451 also lack common microbial metabolic pathways related to carbohydrate utilization, replacing them by a rapid utilization
452 of amino acids and SCFA such as lactate and acetate ⁶⁹. Thus, its correlation with FA composition might be related to
453 differential SCFA and amino acid bioavailability between dietary groups. Only one OTU was detected as correlated with
454 FA after correcting by diet (second Limma approach), *denovo37314* (*Treponema* 2), negatively correlated with ham oleic
455 acid content in IB. This suggests that direct (i.e., not caused by diet differences) association between gut microbiota and
456 FA muscle accretion might also exist. Unfortunately, our experiment does not allow to detect these associations, as intra-
457 group variability is very low for lipidic traits and sample size does not provide enough statistical power. Further
458 experimental designs standardizing breed and diet might be adequate to focus on causal relationships between gut
459 microbiota composition and host lipidic traits.

460 As our experiment has focused on the bacterial subset of fecal microbiota, interactions with other microbial clades,
461 especially eukaryotes, must be studied for a better understanding of the real nature of microbial population changes. On
462 the other hand, metabolite variations between breeds and diets must be analyzed in order to stablish the true relationship
463 between microbiome and host phenotype. Mid-gut microbiota populations might also be interesting to observe dietary
464 responses, as multiple digestion processes occur at superior gut sections. Finally, the complexity in analysing crossover
465 designs must be taken into account, and additional experiments focused in a unique effect might help to clarify some of
466 the differences found in this study, although interaction between effects must not be ignored.

467

468 Conclusions

469 Our study reveals that genetic background has an important impact on pig gut microbiota composition, while dietary
470 changes have a smaller and more variable effect, which could be highly dependent on fiber content. It also brings light to
471 the complexity of microbial relationships. We report a higher OTU richness in Iberian pigs, which highlights the
472 importance of genetic background (i.e., animal breed) in overall microbiome composition resemblance, and may suggest
473 a potential relationship between gut microbiota diversity and composition and Iberian pigs' resilience. Dietary
474 modifications had a small effect in microbiome composition, much less important than host genotype. Differential
475 abundance revealed a complex picture of microbe relationships, with a considerable effect of the breed and multiple
476 interactions between breed and diet. In spite of this complexity, we identified relevant DA taxa and taxa ratios as
477 potentially associated to the metabolic differences between breeds and to a lower extent for the diet influence. Finally, an
478 approach to the use of OTU pairwise ratios as a predictive tool has been performed, with some interesting results that
479 should be subject of further researches.

Acknowledgements

The authors are grateful to the staff of the "Pig Test Center" ITACYL (Hontalbilla, Segovia, Spain) for technical assistance with husbandry and sampling. We also thank GABI-INRA for hosting the research stay for the development of bioinformatic pipelines.

Funding

This research was funded by the Comunidad de Madrid (Spain) and European Structural and Investment Funds (project MEDGAN-CM S2013/ABI-2913), and the Spanish Ministry of Science and Innovation (grant PID2019-108695RB-C31). A.L.G. was funded by FPI-INIA grant with reference FPI-SGIT2016-06.

Abbreviations

AD: Abundance decrease
AI: Abundance increase
ATO/ENT: *Atopobiaceae/Enterobacteriaceae*
BW: Body weight
CLR: Centred log-ratio
COR/SPR: *Corynebacteriaceae/Spirochaetaceae*
DA: Differential abundance
FA: Fatty acid
FC: Fold-change
FDR: False discovery rate
IMF: Intramuscular fat
MUFA: Mono-unsaturated fatty acids
NR: no response
OA: Over-abundant
OTU: Operational taxonomic unit
p-LR: Pairwise log-ratio
PCA: Principal Component Analysis
PERMANOVA: Permutational multivariate analysis of variance
PEP/STR: *Peptostreptococcaceae/Streptococcaceae*
PUFA: Poly-unsaturated fatty acids
RA: Relative abundance
RPART: Recursive Partitioning and Regression Trees
SFA: Saturated fatty acids
SCFA: Short-chained fatty acids
sPLS-DA: sparse Partial Least Square Discriminant Analysis
UA: Under-abundant
VIP: Variable Influence of Projection

Availability of data and materials

Raw microbiome data are available upon reasonable request.

Ethics approval and consent to participate

All experiments were performed in accordance with the regulations of the Spanish Policy for Protection of Animals employed in Research and other scientific purposes RD53/2013, which meet the European Union Directive 2010/63/EU on the protection of animals used in experimentation. The project was approved on March 20, 2015, by the Comunidad de Madrid animal welfare and protection committee (reference number PROEX-007/15).

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Contributions

C.Ó. and C.L.B. obtained funding and conceived the experiments. C.Ó., J.G.C. and E.G.I. designed the experiments. R.B., Y.N., E.D.M., E.G.I. and A.L.G. performed the experiments. A.L.G. analysed the data. A.L.G., O.G.R. and J.E. contributed to bioinformatics analysis. A.L.G. and C.Ó. wrote the paper. All authors revised and approved the submitted paper.

References

1. FAO. FAOSTAT Database. Food and Agriculture Organization of the United Nations. <http://www.fao.org/faostat/es/#data/TP/visualize>. Published 2020. Accessed August 17, 2020.
2. López-Bote CJ. Sustained utilization of the Iberian pig breed. *Meat Science*. 1998;49:17-27. doi:10.1016/S0309-1740(98)90036-5
3. Ventanas S, Ventanas J, Ruiz J. Sensory characteristics of Iberian dry-cured loins: Influence of crossbreeding and rearing system. *Meat Science*. 2007;75(2):211-219. doi:10.1016/j.meatsci.2006.07.003
4. Óvilo C, Benítez R, Fernández AI, et al. Longissimus dorsi transcriptome analysis of purebred and crossbred Iberian pigs differing in muscle characteristics. *BMC Genomics*. 2014;15(1):413. doi:10.1186/1471-2164-15-413
5. Robina A, Viguera J, Perez-Palacios T, et al. Carcass and meat quality traits of Iberian pigs as affected by sex and crossbreeding with different Duroc genetic lines. *Spanish Journal of Agricultural Research*. 2013;11(4):1057-1067. doi:10.5424/sjar/2013114-4637
6. Muñoz M, García-Casco JM, Alves E, et al. Development of a 64 SNV panel for breed authentication in Iberian pigs and their derived meat products. *Meat Science*. 2020;167:108152. doi:10.1016/j.meatsci.2020.108152
7. Daza A, Olivares A, Rey AI, Ruiz J, López-Bote CJ. Iberian pig production: The problems of success. *Options Méditerranéennes*. 2008;A(78):163-171. <http://om.ciheam.org/om/pdf/a78/00800259.pdf>. Accessed October 28, 2020.
8. Wood JD, Enser M, Fisher A v., et al. Fat deposition, fatty acid composition and meat quality: A review. *Meat Science*. 2008;78(4):343-358. doi:10.1016/j.meatsci.2007.07.019
9. Ayuso M, Fernández AI, Isabel B, et al. Long term vitamin A restriction improves meat quality parameters and modifies gene expression in Iberian pigs. *Journal of Animal Science*. 2015;93(6):2730-2744. doi:10.2527/jas.2014-8573
10. Ayuso M, Fernández AI, Núñez Y, et al. Developmental stage, muscle and genetic type modify muscle transcriptome in pigs: Effects on gene expression and regulatory factors involved in growth and metabolism. *PLoS ONE*. 2016;11(12):167858. doi:10.1371/journal.pone.0167858
11. Benítez R, Trakooljul N, Núñez Y, et al. Breed, diet, and interaction effects on adipose tissue transcriptome in iberian and duroc pigs fed different energy sources. *Genes*. 2019;10(8):589. doi:10.3390/genes10080589
12. Hanning I, Diaz-Sanchez S. The functionality of the gastrointestinal microbiome in non-human animals. *Microbiome*. 2015;3:51. doi:10.1186/s40168-015-0113-6
13. Khanal P, Maltecca C, Schwab C, Fix J, Tiezzi F. Microbiability of meat quality and carcass composition traits in swine. 2020. doi:10.21203/rs.3.rs-22906/v1

14. Verschuren LMG, Calus MPL, Jansman AJM, et al. Fecal microbial composition associated with variation in feed efficiency in pigs depends on diet and sex. *Journal of Animal Science*. 2018;96(4):1405-1418. doi:10.1093/jas/sky060
15. Bergamaschi M, Tiezzi F, Howard J, et al. Gut microbiome composition differences among breeds impact feed efficiency in swine. *Microbiome*. 2020;8(1):110. doi:10.1186/s40168-020-00888-9
16. Rodríguez-Valdovinos C, García-Casco J, García-Ruiz F, Núñez-Moreno Y, Silió-López L. A further look of the genetic origin and singularity of the Torbiscal Iberian pig line. *Spanish Journal of Agricultural Research*. 2019;17(1):1-13. doi:10.5424/sjar/2019171-13618
17. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*. 2013;41(1):e1-e1. doi:10.1093/nar/gks808
18. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. *Bioinformatics*. 2011;27(6):863-864. doi:10.1093/bioinformatics/btr026
19. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*. 2010;7(5):335-336. doi:10.1038/nmeth.f.303
20. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010;26(19):2460-2461. doi:10.1093/bioinformatics/btq461
21. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*. 2013;41(D1):D590-6. doi:10.1093/nar/gks1219
22. Bokulich NA, Subramanian S, Faith JJ, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods*. 2013;10(1):57-59. doi:10.1038/nmeth.2276
23. McMurdie PJ, Holmes SP. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*. 2013;8(4):e61217. doi:10.1371/journal.pone.0061217
24. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome datasets are compositional: And this is not optional. *Frontiers in Microbiology*. 2017;8(NOV):2224. doi:10.3389/fmicb.2017.02224
25. Greenacre M, Lewi P. Distributional equivalence and subcompositional coherence in the analysis of compositional data, contingency tables and ratio-scale measurements. *Journal of Classification*. 2009;26(1):29-54. doi:10.1007/s00357-009-9027-y
26. Palarea-Albaladejo J, Martín-Fernández JA. ZCompositions - R package for multivariate imputation of left-censored data under a compositional approach. *Chemometrics and Intelligent Laboratory Systems*. 2015;143:85-96. doi:10.1016/j.chemolab.2015.02.019
27. Greenacre M. *Compositional Data Analysis in Practice*. New York: Chapman and Hall/CRC; 2018. doi:10.1201/9780429455537
28. McArdle BH, Anderson MJ. Fitting multivariate models to community data: A comment on distance-based redundancy analysis. *Ecology*. 2001;82(1):290-297. doi:10.1890/0012-9658(2001)082[0290:FMMTCD]2.0.CO;2
29. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*. 2001;26(1):32-46. doi:https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x
30. Oksanen J, Blanchet FG, Friendly M, et al. Vegan: community ecology package. 2018. doi:ISBN 0-387-95457-0
31. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*. 2014;15(12):550. doi:10.1186/s13059-014-0550-8
32. Fernandes AD, Reid JNS, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. Unifying the analysis of high-throughput sequencing datasets: Characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome*. 2014;2(1):15. doi:10.1186/2049-2618-2-15
33. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biology*. 2010;11(10):R106. doi:10.1186/gb-2010-11-10-r106
34. Chen H, Boutros PC. VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics*. 2011;12(1):35. doi:10.1186/1471-2105-12-35
35. Kamada T, Kawai S. An algorithm for drawing general undirected graphs. *Information Processing Letters*. 1989;31(1):7-15. doi:10.1016/0020-0190(89)90102-6
36. Shah HN, Collins DM. Prevotella, a new genus to include Bacteroides melaninogenicus and related species formerly classified in the genus Bacteroides. *International Journal of Systematic Bacteriology*. 1990;40(2):205-208. doi:10.1099/00207713-40-2-205
37. de Filippo C, Cavalieri D, di Paola M, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(33):14691-14696. doi:10.1073/pnas.1005963107
38. Chen T, Long W, Zhang C, Liu S, Zhao L, Hamaker BR. Fiber-utilizing capacity varies in Prevotella- versus Bacteroides-dominated gut microbiota. *Scientific Reports*. 2017;7(1). doi:10.1038/s41598-017-02995-4
39. Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. *Nature*. 2011;473(7346):174-180. doi:10.1038/nature09944
40. Li J, Zhong H, Ramayo-Caldas Y, et al. A catalog of microbial genes from the bovine rumen reveals the determinants of herbivory. *bioRxiv*. 2018:272690. doi:10.1101/272690
41. Lee HJ, Jung JY, Oh YK, Lee SS, Madsen EL, Jeon CO. Comparative survey of rumen microbial communities and metabolites across one caprine and three bovine groups, using bar-coded pyrosequencing and 1H nuclear magnetic resonance spectroscopy. *Applied and Environmental Microbiology*. 2012;78(17):5983-5993. doi:10.1128/AEM.00104-12
42. Lopes LD, de Souza Lima AO, Taketani RG, et al. Exploring the sheep rumen microbiome for carbohydrate-active enzymes. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. 2015;108(1):15-30. doi:10.1007/s10482-015-0459-6
43. Xiao L, Estellé J, Kiilerich P, et al. A reference gene catalogue of the pig gut microbiome. *Nature Microbiology*. 2016;1(12):1-6. doi:10.1038/nmicrobiol.2016.161
44. Ramayo-Caldas Y, Mach N, Lepage P, et al. Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. *ISME Journal*. 2016;10(12):2973-2977. doi:10.1038/ismej.2016.77
45. Mach N, Berri M, Estellé J, et al. Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environmental Microbiology Reports*. 2015;7(3):554-569. doi:10.1111/1758-2229.12285
46. Downes J, Dewhirst FE, Tanner ACR, Wade WG. Description of Alloprevotella rava gen. nov., sp. nov., isolated from the human oral cavity, and reclassification of Prevotella tanneri Moore et al. 1994 as Alloprevotella tanneri gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology*. 2013;63(PART4):1214-1218. doi:10.1099/ijs.0.041376-0
47. Seshadri R, Leahy SC, Attwood GT, et al. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nature Biotechnology*. 2018;36(4):359-367. doi:10.1038/nbt.4110

48. Hjørth MF, Blædel T, Bendtsen LQ, et al. Prevotella-to-Bacteroides ratio predicts body weight and fat loss success on 24-week diets varying in macronutrient composition and dietary fiber: results from a post-hoc analysis. *International Journal of Obesity*. 2019;43(1):149-157. doi:10.1038/s41366-018-0093-2
49. Xu P, Li M, Zhang J, Zhang T. Correlation of intestinal microbiota with overweight and obesity in Kazakh school children. *BMC Microbiology*. 2012;12. doi:10.1186/1471-2180-12-283
50. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(31):11070-11075. doi:10.1073/pnas.0504978102
51. Magne F, Gotteland M, Gauthier L, et al. The firmicutes/bacteroidetes ratio: A relevant marker of gut dysbiosis in obese patients? *Nutrients*. 2020;12(5). doi:10.3390/nu12051474
52. Wallace RJ, Sasson G, Garnsworthy PC, et al. A heritable subset of the core rumen microbiome dictates dairy cow productivity and emissions. *Science Advances*. 2019;5(7). doi:10.1126/sciadv.aav8391
53. Crespo-Piazuelo D, Estellé J, Revilla M, et al. Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. *Scientific Reports*. 2018;8(1). doi:10.1038/s41598-018-30932-6
54. Forero Vizcaíno FJ, Andrés AI. *El Cerdo Ibérico Una Revisión Transversal*. (Forero Vizcaíno FJ, ed.). Sevilla: Consejería de Agricultura y Pesca (Andalucía); 2008.
55. Elmqvist T, Folke C, Nystrom M, et al. Response Diversity, Ecosystem Change, and Resilience. *Frontiers in Ecology and the Environment*. 2003;1(9):488. doi:10.2307/3868116
56. Heinritz SN, Weiss E, Eklund M, et al. Intestinal microbiota and microbial metabolites are changed in a pig model fed a high-fat/low-fiber or a low-fat/high-fiber diet. *PLoS ONE*. 2016;11(4):154329. doi:10.1371/journal.pone.0154329
57. le Sciellour M, Labussière E, Zemb O, Renaudeau D. Effect of dietary fiber content on nutrient digestibility and fecal microbiota composition in growing-finishing pigs. *PLoS ONE*. 2018;13(10). doi:10.1371/journal.pone.0206159
58. Pokusaeva K, Fitzgerald GF, van Sinderen D. Carbohydrate metabolism in Bifidobacteria. *Genes and Nutrition*. 2011;6(3):285-306. doi:10.1007/s12263-010-0206-6
59. Han GG, Lee JY, Jin GD, et al. Tracing of the fecal microbiota of commercial pigs at five growth stages from birth to shipment. *Scientific Reports*. 2018;8(1):6012. doi:10.1038/s41598-018-24508-7
60. Liu Y, White RH, Whitman WB. Methanococci Use the Diaminopimelate Aminotransferase (DapL) Pathway for Lysine Biosynthesis. *Journal of Bacteriology*. 2010;192(13):3304-3310. doi:10.1128/JB.00172-10
61. Oliveira A, Oliveira LC, Aburjaile F, et al. Insight of genus *Corynebacterium*: Ascertaining the role of pathogenic and non-pathogenic species. *Frontiers in Microbiology*. 2017;8(OCT):1937. doi:10.3389/fmicb.2017.01937
62. Rivera-Ferre MG, Aguilera JF, Nieto R. Differences in whole-body protein turnover between Iberian and Landrace pigs fed adequate or lysine-deficient diets. In: *Journal of Animal Science*. Vol 84. Oxford Academic; 2006:3346-3355. doi:10.2527/jas.2005-405
63. Wallace RJ, Rooke JA, McKain N, et al. The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics*. 2015;16(1):839. doi:10.1186/s12864-015-2032-0
64. Lagkouvardos I, Lesker TR, Hitch TCA, et al. Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. *Microbiome*. 2019;7(1):28. doi:10.1186/s40168-019-0637-2
65. Waite DW, Vanwonterghem I, Rinke C, et al. Comparative genomic analysis of the class Epsilonproteobacteria and proposed reclassification to epsilonbacteraeota (phyl. nov.). *Frontiers in Microbiology*. 2017;8(APR). doi:10.3389/fmicb.2017.00682
66. Gebhart CJ, Edmonds P, Ward GE, Kurtz HJ, Brenner DJ. “*Campylobacter hyointestinalis*” sp. nov.: a new species of *Campylobacter* found in the intestines of pigs and other animals. *Journal of Clinical Microbiology*. 1985;21(5):715-720. doi:10.1128/jcm.21.5.715-720.1985
67. Benítez R, Fernández A, Isabel B, et al. Modulatory effects of breed, feeding status, and diet on adipogenic, lipogenic, and lipolytic gene expression in growing iberian and duroc pigs. *International Journal of Molecular Sciences*. 2018;19(1). doi:10.3390/ijms19010022
68. Hammes WP, Vogel RF. The genus *Lactobacillus*. In: *The Genera of Lactic Acid Bacteria*. Springer US; 1995:19-54. doi:10.1007/978-1-4615-5817-0_3
69. Stahl M, Butcher J, Stintzi A. Nutrient acquisition and metabolism by *Campylobacter jejuni*. *Frontiers in cellular and infection microbiology*. 2012;2:5. doi:10.3389/fcimb.2012.00005

Figures

Figure 1 — Phyla prevalence and abundance in dataset. Each dot represents an OTU, being the x-axis its average relative abundance and the y-axis the proportion of samples in which it is present. “Unclassified_Bacteria” is the denomination given to those OTUs with no phylum assignment.

Figure 2 — α -diversity boxplots according to Breed (left) and Diet (right) groups in rarefied data.

Figure 3 — β -diversity plot from PCA of CLR-transformed data, at phylum, family, genus and OTU levels and variance percentage explained by each component. Ellipses show the Normal-theory confidence regions with $\alpha=0.95$. Centroids are represented by diamond-shaped points.

Figure 4 — β -diversity indices by group at different feature glom levels. Tukey HSD test group letters and p-values for significant group comparisons ($p < 0.05$) are represented.

Figure 5 — Volcano plots of differentially abundant OTUs using DESeq2 (A, B) and ALDEx2 (C, D), for breed (left) and diet (right) contrasts. Red dots represent OTUs with an adjusted p-value below the FDR cut-off (0.05) and a FC value above 1.5 or below -1.5 for DESeq2 contrasts, or a median difference between CLR values above 1 or below -1 for ALDEx2 contrasts. OTUs with $\text{Log}_2(\text{FC}) < 0$ or $\text{diff.btw} < 0$ are more abundant in Duroc and Control groups, respectively.

Figure 6 — Venn Diagram comparing DA OTUs in Breed (red) and Diet (blue) contrasts using both DESeq2 and ALDEx2.

Figure 7 — Interaction plots for each OTU with significant interaction between breed and diet factors for DA analysis with DESeq2. Normalized counts from DESeq2 algorithm and standard error of the mean (SEM) are represented in y-axis.

Figure 8 — RPART trees from pairwise log-ratios at phylum, family, genus and OTU levels. Each decision level indicates the threshold value of one p-LR to classify a sample in one phenotypic group or another. Each node box displays the classification, the probability of each class at that node (i.e. the probability of the class conditioned on the node) and the percentage of observations used at that node. Class probabilities at each node are sorted as: DU-C, DU-O, IB-C and IB-O.

Figure 9 — Network representing individual OTU significant correlation with each phenotypic trait within IB subpopulation, from Limma regression analysis, when no diet correction was applied. Edge colour: red = positive correlation; blue = negative correlation. Central nodes have more connections (i.e., significantly associated to more phenotypic traits) than peripheral nodes. Numbered nodes represent OTUs classified as genera with high relevance due to their abundance patterns in our dataset.

Supplementary information

Additional file 1 — Diet composition.

Nutrient and fatty acid composition for Control (C) and High-Oleic (O) diets.

Additional file 2 — Relative abundance of genera

Tables containing overall relative abundance of identified genera and relative abundance by Breed-Diet groups. Mean and standard deviation of RA are shown for each OTU, as well as taxonomic classification. RA_Rank column represents the rareness of each OTU (High_RA: $RA \geq 1\%$; Mid_RA: $1\% > RA \geq 0.1\%$; Low_RA: $RA < 0.1\%$).

Additional file 3 — Differentially abundant OTUs

Tables containing differentially abundant OTUs for each factor (Breed and Diet) with both pipelines (DESeq2 and ALDEx2) and overlapping of both pipelines. Prevalence represents the proportion of samples in which each OTU is found. For DESeq2, normalized counts per group (Ncounts), fold change (log2FC) and adjusted p-value (padj) are shown. For ALDEx2, relative abundance per group (RA), median difference in CLR values between factor groups (diff.btw) and Welch's test adjusted p-value (we.eBH) are shown. In overlapping tables information of both pipelines is shown.

Figures

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Supplementary Files

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