

## 1 Leveraging host-genetics and gut microbiota to determine immunocompetence in pigs

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### 17 18 19 20 **Abstract**

21  
22 The aim of the present work was to identify microbial biomarkers linked to  
23 immunity traits and to characterize the contribution of host-genome and gut microbiota to  
24 the immunocompetence in healthy pigs. To achieve this goal, we undertook a combination  
25 of network, mixed model and microbial-wide association studies (MWAS) for 21  
26 immunity traits and the relative abundance of gut bacterial communities in 389 pigs  
27 genotyped for 70K SNPs. The heritability ( $h^2$ ; proportion of phenotypic variance explained  
28 by the host genetics) and microbiability ( $m^2$ ; proportion of variance explained by the  
29 microbial composition) showed similar values for most of the analyzed immunity traits,  
30 except for both IgM and IgG in plasma that were dominated by the host genetics, and the  
31 haptoglobin in serum which was the trait with larger  $m^2$  (0.275) compared to  $h^2$  (0.138).  
32 Results from the MWAS suggested a polymicrobial nature of the immunocompetence in  
33 pigs and revealed associations between pigs gut microbiota composition and 15 of the  
34 analyzed traits. The lymphocytes phagocytic capacity (quantified as mean fluorescence)  
35 and the total number of monocytes in blood were the traits associated with the largest  
36 number of taxa (6 taxa). Among the associations identified by MWAS, 30% were  
37 confirmed by an information theory network approach. The strongest confirmed

38 associations were between *Fibrobacter* and phagocytic capacity of lymphocytes ( $r=0.37$ ),  
39 followed by correlations between *Streptococcus* and the percentage of phagocytic  
40 lymphocytes ( $r=-0.34$ ) and between *Megasphaera* and serum concentration of haptoglobin  
41 ( $r=0.26$ ). In the interaction network, *Streptococcus* and percentage of phagocytic  
42 lymphocytes were the keystone bacterial and immune-trait, respectively. Overall, our  
43 findings reveal an important connection between immunity traits and gut microbiota in  
44 pigs and highlight the need to consider both sources of information, host genome and  
45 microbial levels, to accurately characterize immunocompetence in pigs.

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47

## 48 **Introduction**

49 The pig industry has a considerable socio-economical value representing around  
50 35% of the total meat produced worldwide [1] and being the most popular meat for  
51 consumption [2]. The intensification of pig production coupled with the ban on in-feed use  
52 of antibiotics has led to a deterioration of the health status of pig farms. In addition, the  
53 current emergence of antibiotic resistance and society demands for healthier products and  
54 environmentally responsible livestock systems, has motivated to explore relevant  
55 approaches for pig and other livestock breeding programs, to improve robustness and  
56 disease resistance [3].

57 The implementation of breeding programs to select animals according to their  
58 robustness presents several challenges and levels of complexity. One of the most relevant  
59 milestones is the identification of selection criteria that combine functional traits with  
60 those of immunocompetence. These complex traits are driven by several physiological and  
61 behavioral mechanisms that in turn are determined by genetic and environmental factors.  
62 Regarding the genetic determinism of immunocompetence, several studies in pigs  
63 acknowledged medium to high heritability estimates [4-9] and reported genomic regions  
64 and candidate genes associated with phenotypic variation of health-related traits [9-15].

65 Over the past few years, multiple studies highlighted the relevant role of the gut  
66 microbiota composition in the homeostasis and function of the mammalian immune system  
67 [16-19]. Gut microbiota can regulate host-immunity through both direct mechanisms like  
68 translocation of bacteria and their components (i.e. metabolites), or mediate indirect process  
69 such as T-cell polarization and the regulation of immune cell trafficking [18]. Commensal  
70 gut populations modulate hosts' immune responses, which in turn can modify the

71 microbiota composition to maintain gut homeostasis [20, 21]. Recently, polymorphisms  
72 located in immune genes associated with the abundance of microbial communities have  
73 been reported [22-25]. Furthermore, it has been suggested that the pattern recognition  
74 receptors, which are proteins capable of recognizing molecules frequently associated with  
75 pathogens, may have evolved to mediate the bidirectional crosstalk between microbial  
76 symbionts and their hosts [26]. This has resulted in a mutualistic and symbiotic partnership  
77 between the immune system and these commensal microorganisms [27]. Therefore, the  
78 immune system not only protects the host from pathogens but can also modulate, and is  
79 itself modulated, by beneficial microbes.

80           Considering the relevant interplay between gut microbiota and host immunity, a  
81 better understanding of the role of gut microbiota in the immunocompetence determination  
82 in pigs could greatly assist in the implementation of selection programs to improve  
83 robustness and disease resistance simultaneously. The present work aimed to identify  
84 microbial biomarkers linked to immunity traits and to estimate the contribution of host-  
85 genome and gut microbial communities to the immunocompetence in healthy pigs.

## 86 **Material and Methods**

### 87 **Ethics Statement**

88           All experimental procedures were performed according to the Spanish Policy for  
89 Animal Protection RD53/2013, which meets the European Union Directive 2010-63-EU  
90 about the protection of animals used in experimentation. The experimental protocol was  
91 approved by the Ethical Committee of the Institut de Recerca i Tecnologia  
92 Agroalimentàries (IRTA).

### 93 **Animal Samples**

94           Samples employed in this study are a subset of pigs reported in Ballester et al. [9]  
95 and Reverter et al. [25]. A total of 405 weaned piglets (204 males and 201 females) from a  
96 commercial Duroc pig line were used. The pigs were distributed in six batches obtained  
97 from 132 sows and 22 boars. All animals were raised on the same farm and fed *ad libitum*  
98 a commercial cereal-based diet.

### 99 **Immunity and hematological traits**

100 Details of the sampling and laboratory processing have been reported [9]. In brief,  
101 blood and saliva samples were collected from all 405 piglets at  $60 \pm 8$  days of age. Blood  
102 samples in 4 ml EDTA tubes were used to measure the hemograms (Laboratory  
103 Echevarne, Spain; Barcelona). Saliva was collected with Salivette tubes (Sarstedt S.A.U.,  
104 Germany) according to the protocols recommended by the manufacturer. Blood samples  
105 for serum were collected in 6 mL tubes with gel serum separator and centrifuged at 1600 g  
106 for 10 min at RT. Plasma was collected from the sampled blood in 6 ml heparinized tubes  
107 and centrifuged at 1300 g for 10 minutes at 4°C. Plasma and serum samples were collected,  
108 aliquoted, and stored at -80°C. The following haematological parameters were included in  
109 this study: total number of eosinophils (EO), leukocytes (LEU), lymphocytes (LYM) and  
110 neutrophils (NEU) in blood. Analyzed immunity parameters included immunoglobulins  
111 (IgA, IgG and IgM) concentrations in plasma; C-reactive protein (CRP), Haptoglobin (HP)  
112 and Nitric Oxide (NO) concentrations in serum; and IgA concentration in saliva (IgAsal).  
113 Gamma-delta T cells ( $\gamma\delta$  T cells) were separated from heparinised peripheral blood by  
114 density-gradient centrifugation with Histopaque-1077 (Sigma, Spain). Phagocytosis assay  
115 was carried out in heparinized whole blood samples incubated with fluorescein (FITC)-  
116 labelled opsonized *Escherichia coli* bacteria using the Phagotest kit (BD Pharmigen,  
117 Spain) as indicated in the manufacturer's protocol. The following phagocytosis traits were  
118 used: percentage of total phagocytic cells (PHAGO\_%); percentage of phagocytic cells  
119 among granulocytes (GRANU\_PHAGO\_%), monocytes (MON\_PHAGO\_%) and  
120 lymphocytes (LYM\_PHAGO\_%); mean fluorescence in FITC among the total phagocytic  
121 cells (PHAGO\_FITC); and mean fluorescence in FITC among the granulocytes (GRANU\_  
122 PHAGO\_FITC), monocytes (MON\_PHAGO\_FITC) and lymphocytes  
123 (LYM\_PHAGO\_FITC) that phagocyte.

#### 124 **DNA extraction, sequencing and bioinformatics analysis**

125 Simultaneous with blood and saliva samples, fecal samples were collected from all  
126 405 piglets. DNA was extracted with the DNeasy PowerSoil Kit (QIAGEN, Hilden,  
127 Germany) following manufacturer's instructions. Extracted DNA was sent to the  
128 University of Illinois Keck Center for Fluidigm sample preparation and paired-end (2 ×  
129 250 nt) sequencing on an Illumina NovaSeq (Illumina, San Diego, CA, USA). The 16S  
130 rRNA gene fragment was amplified using the primers V3\_F357\_N: 5'-  
131 CCTACGGGNGGCWGCAG-3' and V4\_R805: 5'-GACTACHVGGGTATCTAATCC-3'.

134 Sequences were analysed with *Qiime2* [28]; barcode sequences, primers and low-quality  
135 reads (Phred scores of <30) were removed. The quality control also trimmed sequences  
136 based on expected amplicon length and removed chimeras. Afterwards, sequences were  
137 processed into Amplicon Sequences Variants (ASVs) at 99% of identity. Samples with less  
138 than 10,000 reads were excluded and ASVs present in less than three samples and  
139 representing less than 0.001% of the total counts were discarded. ASVs were classified to  
140 the lowest possible taxonomic level based on a primer-specific trained version of  
141 GreenGenes Database [29].

142

### 143 **Genotype data**

144 A total of 390 out of 405 animals were genotyped using the Porcine 70 k GGP  
145 Porcine HD Array (Illumina, San Diego, CA) containing 68,516 single nucleotide  
146 polymorphisms (SNPs). The quality control excluded SNPs with minor allele frequencies  
147 < 5%, rates of missing genotypes above 10%, and SNPs that did not map to the porcine  
148 reference genome (Sscrofa11.1 assembly). Consequently, 42,641 SNPs were retained for  
149 subsequent analysis.

### 150 **Microbiability and heritability estimation**

151 Heritability ( $h^2$ ), i.e. the proportion of variance explained by the host genetics, and  
152 microbiability ( $m^2$ ), i.e. the proportion of variance explained by the microbial composition,  
153 were estimated for each immunity trait based on a mixed-model as follows:

$$154 \quad \mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{u} + \mathbf{W}\mathbf{m} + \mathbf{e}$$

155 where  $\mathbf{y}$  is the  $n$ -dimensional vector containing the individual phenotypes for the immune  
156 trait under consideration;  $\boldsymbol{\beta}$  is the vector of fixed effects, containing the general intercept,  
157 the sex effect (two levels), and batch effect (six levels) for most traits but data of  
158 laboratory analysis (12 levels, two by batch) for phagocytosis-related traits;  $\mathbf{u}$  is the vector  
159 containing the host genetic random effect from each individual;  $\mathbf{m}$  is the vector of the  
160 animal's microbiome random effect;  $\mathbf{X}$ ,  $\mathbf{Z}$  and  $\mathbf{W}$  are, respectively, the incidence matrices  
161 correspondent to  $\boldsymbol{\beta}$ ,  $\mathbf{u}$  and  $\mathbf{m}$ ; and  $\mathbf{e}$  is the vector of residual terms.

162 Assuming independence between random effects, the following distributions were  
163 considered:  $\mathbf{u} \sim \mathcal{N}(0, \mathbf{G}, \sigma_u^2)$ , where  $\sigma_u^2$  is the host genetic effects variance and  $\mathbf{G}$  is the  
164 genomic relationship matrix between individuals, computed following [30], i.e.,

$$165 \quad \mathbf{G} = \frac{\mathbf{S}\mathbf{S}'}{2\sum_j p_j(1-p_j)}$$

being  $\mathbf{S}$  the matrix that contains the centered individual genotype for the

166 42,641 SNPs (columns) of each individual (rows), and  $p_j$  is the frequency of the minimum  
167 allele of the  $j^{\text{th}}$  SNP;  $m \sim N(0, B, \sigma_m^2)$ , where  $\sigma_m^2$  is the microbial effects variance and  $B$  the  
168 microbial relationship matrix computed following [31], i.e.,  $B = \frac{MM'}{n}$ , being  $M$  the matrix  
169 containing the scaled after a previous cumulative sum scaling normalization of the ASV  
170 abundances (columns) for each individual microbiome (rows) and  $n$  the total number of  
171 ASVs; and finally  $e \sim N(0, I\sigma_e^2)$ , where  $\sigma_e^2$  is the error variance.

172

173 The model parameters for each immunity trait were estimated by a Bayesian  
174 approach, using the Bayes Ridge Regression model from BGLR package [32]. We used a  
175 Gibbs sampler with 30,000 iterations and a burn-in of 3,000 rounds. The ‘heritability’

176  $(h^2 = \frac{\sigma_u^2}{(\sigma_u^2 + \sigma_m^2 + \sigma_e^2)})$  and ‘microbiability’  $(m^2 = \frac{\sigma_m^2}{(\sigma_u^2 + \sigma_m^2 + \sigma_e^2)})$  were estimated from the

177 mean of the posterior distributions [33].

178

### 179 Microbial Wide Association Study

180 We performed a Microbial Wide Association Study (MWAS) using a multi-ASV  
181 association method that combines all the ASVs in a single model:

182

$$183 \quad y_i = \beta_0 + \sum_{j \in 1..p} \beta_j x_{ij} + \epsilon_i \quad (2)$$

184

185 Given a trait  $y_i$  measured in  $n$  individuals and a matrix  $X$  containing relative abundances  
186 of  $p$  taxa from a microbial community, here the ASVs effects were treated as draws from  
187 normal distributions as in any Bayesian Ridge Regression approach [32].

188

189 Following the approach of Legarra et al [34], Bayes Factor (BF) for the effect of each taxa  
190 can be derived as the ratio of probabilities  $BF = \frac{P_{H1}(y)}{P_{H0}(y)}$ , where H1 means “the  $j$ -genus has  
191 some effect” and H0 “the  $j$ -genus has no effect”. The calculations from the posterior  
192 distribution are very simple since both probabilities ( $P_{H1}, P_{H0}$ ) are normal density.

193

### 194 Network between microbial and immunity traits

195 To better understand the relationship between microbial communities and  
196 immunity traits we implemented PCIT [35], a network-based approach that combines

197 partial correlation coefficient with information theory to identify significant correlations  
 198 between each possible combination of clr-transformed bacterial abundance and the  
 199 immune-traits [35]. PCIT tests all possible 3-way combinations in the dataset and only  
 200 keeps correlations between traits if they are significant and independent of the association  
 201 of another features. To reduce the complexity of the resulting network, from the PCIT  
 202 significant connections, we kept only the ones involving one immune-trait and one genus  
 203 (i.e. genus-genus and trait-trait interactions were no represented).

204

## 205 Results

206 In this study, 16S rRNA gene sequences, host genotype information and immune  
 207 traits from 389 Duroc pigs were analyzed to estimate both host genomes and gut  
 208 microbiota contribution to the porcine immunocompetence, and to identify microbial  
 209 biomarkers linked to immunity traits. Table 1 summarizes the immunity traits and their  
 210 descriptive statistics used in the present study. Regarding 16S rRNA gene sequences, after  
 211 quality control, a total of 2,055 Amplicon Sequences Variants (ASVs) and 68 genera were  
 212 detected. The dominant bacterial phyla were Bacteroidetes and Firmicutes, and the most  
 213 abundant genera were *Prevotella*, *Lactobacillus*, *Treponema*, *Roseburia* and *Ruminococcus*  
 214 (Supplementary figure 1)

215

216 **Table 1.** Descriptive statistics, mean, standard deviation (SD) and coefficient of variation  
 217 (CV) of the 21 analysed traits.

218

219

Trait	Abrev	Mean	SD	CV
C-reactive protein in serum (µg/ml)	CRP	176.69	128.61	0.73
Eosinophils count n/µL	EO	406.31	191.04	0.47
γδ T-lymphocyte subpopulation (%)	γδ T-cells	7.83	4.94	0.63
Granulocytes phagocytosis FITC	GRANU_PHAGO_FITC	5.15	0.42	0.08
Granulocytes phagocytosis (%)	GRANU_PHAGO_%	91.70	3.85	0.04
Haptoglobin in serum (mg/ml)	HP	1.03	0.67	0.65
IgA in plasma (mg/ml)	IgA	0.65	0.31	0.48
IgA in saliva (mg/dl)	IgAsal	5.13	3.12	0.61
IgG in plasma (mg/ml)	IgG	12.64	4.85	0.38
IgM in plasma (mg/ml)	IgM	2.27	0.79	0.35
Leukocytes count n/µL	LEU	20,350.15	6948.01	0.34
Lymphocytes count n/µL	LYM	12,422.40	4497.03	0.36
Lymphocytes phagocytosis FITC	LYM_PHAGO_FITC	3.16	0.13	0.04

Lymphocytes phagocytosis (%)	LYM_PHAGO_%	6.06	4.02	0.66
Monocytes phagocytosis FITC	MON_PHAGO_FITC	3.92	0.25	0.06
Monocytes phagocytosis (%)	MON_PHAGO_%	49.48	9.72	0.20
Monocytes count n/ $\mu$ L	MONOCITOS_MM	548.42	280.43	0.51
Nitric oxide in serum ( $\mu$ M)	NO	206.45	79.83	0.39
Phagocytosis FITC	PHAGO_FITC	4.71	0.33	0.07
Phagocytosis (% cells)	PHAGO_%	42.94	8.37	0.19
Neutrophils count n/ $\mu$ L	NEU	6985.62	3316.61	0.47

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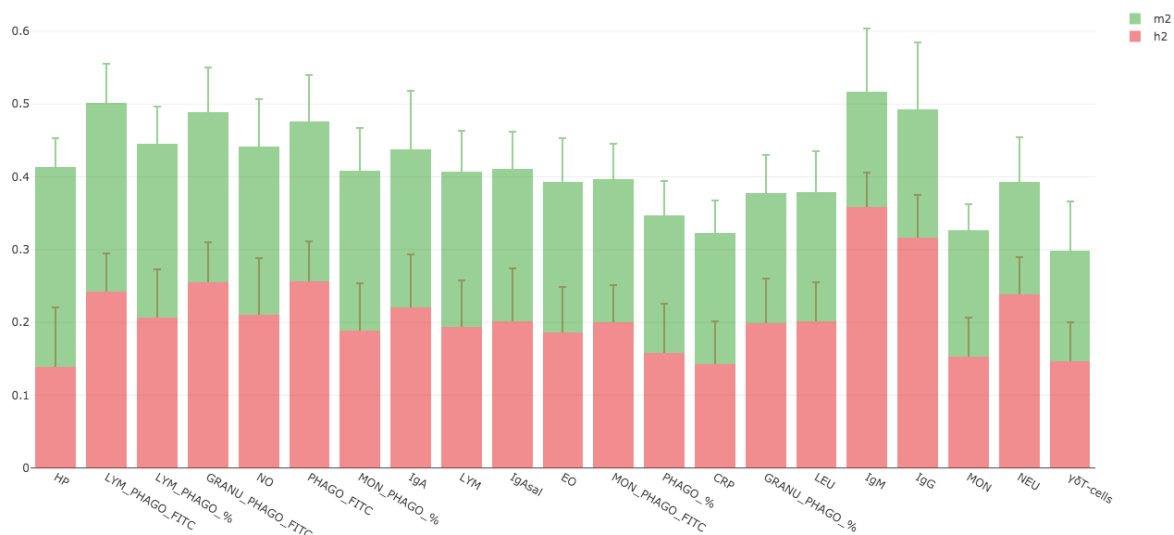
## 222 Heritability and microbiability of immunity traits

223 Posterior estimates of  $h^2$  and  $m^2$  for the 21 health-related traits can be shown in  
224 Figure 1 and Supplementary Table 1. Posterior means of  $h^2$  in the analyses considering  
225 microbiota contribution reached low to medium values (from 0.138 to 0.359), but posterior  
226 probability of  $h^2$  being superior to 0.1 was in all cases above 0.82. Similarly, estimated  $m^2$   
227 reached values between 0.152 and 0.276, and the probability of being above 0.1 was above  
228 0.85 for all immunity and hematological traits (Supplementary Table 1). Among analysed  
229 traits, IgG and IgM in plasma showed the highest genetic determinism ( $h^2= 0.316$  and  
230 0.359), whereas microbiota contribution was below 0.18. Conversely, the Hp concentration  
231 in serum showed the highest microbial effect ( $m^2=0.276$ ), accompanied by the lowest  $h^2$   
232 estimate ( $h^2= 0.138$ ). Considering the joint effects of host-genome and gut microbiota,  
233 these two sources of variation explained from 29.9% to 51.7% phenotypic variance of the  
234 analysed immunity and hematological traits. To be noted, in the 76% (16/21) of these traits  
235 the  $h^2$  and  $m^2$  estimates reaching relatively similar values (Figure 1).

236

237 **Figure 1.** Percentage of phenotypic variance explained by the host-genetic (red points) and  
238 the gut microbial composition (green points) for most relevant immunity traits.





239

240

### 241 Associations between microbial genera and immunity traits

242 Results from the MWAS reported some putative associations between bacterial  
 243 genera abundance and health-related traits (Table 2). In particular, 15 out of the 21  
 244 immunity traits were associated with at least one microbial genus (Table 2). The strongest  
 245 association was observed between the relative abundance of *Chlamydia* and the profile of  
 246 LYM\_PHAGO\_FITC, followed by *Streptococcus* linked to LYM\_PHAGO\_% and  
 247 *Peptococcus* associated with LYM\_PHAGO\_FITC. In addition, several genera showed  
 248 multiple associations with numerous immunity traits: *Desulfovibrio*, *Oribacterium* and  
 249 *Chlamydia* (4 traits) followed by *Oxalobacter* and *Parabacteroides* (3 traits), *Peptococcus*  
 250 and *Streptococcus* (2 traits). As far as the analysed phenotypes, those traits showing the  
 251 highest number of associations with different bacterial taxa were: LYM\_PHAGO\_FITC  
 252 and MON (6 taxa); LYM\_PHAGO\_%, EOS, GRANU\_PHAGO\_FITC (4 taxa) and total  
 253 number of LEU (3 taxa). Meanwhile, only four out of the 15 immunity traits analysed were  
 254 linked with only one genus (Table 2).

255

256 **Table 2.** Results from the microbial-wide association studies

Names	Description_trait	Genus	BPcum
MON	Monocytes count n/μL	<i>Anaerobiospirillum</i>	2.676
LYM_PHAGO_FITC	Lymphocytes phagocytosis FITC	<i>Bacteroides</i>	2.536
IgG	IgG in plasma (mg/ml)	<i>Bulleidia</i>	2.313
GRANU_PHAGO_FITC	Granulocytes phagocytosis FITC	<i>Campylobacter</i>	6.556
MON	Monocytes count n/μL	<i>Catenibacterium</i>	2.952
EO	Eosinophils count n/μL	<i>Chlamydia</i>	5.292
IgAsal	IgA in saliva (mg/dl)	<i>Chlamydia</i>	2.364
LYM_PHAGO_FITC	Lymphocytes phagocytosis FITC	<i>Chlamydia</i>	11.246

LYM_PHAGO_%	Lymphocytes phagocytosis (%)	<i>Chlamydia</i>	3.088
EO	Eosinophils count n/μL	<i>Desulfovibrio</i>	3.369
LEU	Leukocytes count n/μL	<i>Desulfovibrio</i>	2.085
LYM_PHAGO_FITC	Lymphocytes phagocytosis FITC	<i>Desulfovibrio</i>	2.852
MONOCITOS_MM	Monocytes count n/μL	<i>Desulfovibrio</i>	2.427
LYM_PHAGO_FITC	Lymphocytes phagocytosis FITC	<i>Fibrobacter</i>	3.829
HP	Haptoglobin in serum (mg/ml)	<i>Megasphaera</i>	3.582
MON_PHAGO_FITC	Monocytes phagocytosis FITC	<i>Mitsuokella</i>	2.788
CRP	C-Reactive Protein in serum (ug/ml)	<i>Mucispirillum</i>	2.588
LYM_PHAGO_%	Lymphocytes phagocytosis (%)	<i>Oribacterium</i>	3.089
MON_PHAGO_FITC	Monocytes phagocytosis FITC	<i>Oribacterium</i>	2.673
PHAGO_%	% of total phagocytic cells	<i>Oribacterium</i>	2.009
NEU	Neutrophils count n/μL	<i>Oribacterium</i>	2.478
LEU	Leukocytes count n/μL	<i>Oxalobacter</i>	2.816
MON	Monocytes count n/μL	<i>Oxalobacter</i>	2.320
NEU	Neutrophils count n/μL	<i>Oxalobacter</i>	3.216
IgG	IgG in plasma (mg/ml)	<i>Paludibacter</i>	3.018
HP	Haptoglobin in serum (mg/ml)	<i>Parabacteroides</i>	2.018
LYM_PHAGO_FITC	Lymphocytes phagocytosis FITC	<i>Parabacteroides</i>	2.219
MON_PHAGO_FITC	Monocytes phagocytosis FITC	<i>Parabacteroides</i>	5.158
LYM_PHAGO_FITC	Lymphocytes phagocytosis FITC	<i>Peptococcus</i>	7.497
PHAGO_%	% of total phagocytic cells	<i>Peptococcus</i>	2.288
GRANU_PHAGO_FITC	Granulocytes phagocytosis FITC	<i>rc4.4</i>	2.156
LEU	Leukocytes count n/μL	<i>rc4.4</i>	2.619
LYM	Lymphocytes count n/μL	<i>rc4.4</i>	3.621
MON	Monocytes count n/μL	<i>RFN20</i>	3.022
GRANU_PHAGO_FITC	Granulocytes phagocytosis FITC	<i>Sphaerochaeta</i>	2.686
γδ T-cells	γδ T-Lymphocyte subpopulation	<i>Streptococcus</i>	2.444
LYM_PHAGO_%	Lymphocytes phagocytosis (%)	<i>Streptococcus</i>	8.484
EO	Eosinophils count n/μL	<i>Succinivibrio</i>	4.814
MON	Monocytes count n/μL	<i>Succinivibrio</i>	2.439
LYM PHAGO %	Lymphocytes phagocytosis (%)	<i>Treponema</i>	2.187

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## 259 Gut microbial and host-immune interaction network

260 The interplay between microbial and health-related traits was also inferred through  
 261 a network comprised of 63 nodes (42 genera and 21 immunity traits) and 86 edges  
 262 (significant connections) in which only the significant interactions between a bacterial  
 263 genus and an immunity trait were considered (Figure 2). The topological evaluation of the  
 264 network highlights LYM\_PHAGO\_% as the most connected trait, followed by IgAsal,  
 265 NEU and Hp. Meanwhile, at microbial level, *Streptococcus* was the most connected genus  
 266 followed by *Acidaminococcus*, *Desulfovibrio* and *Blautia*. The network approach

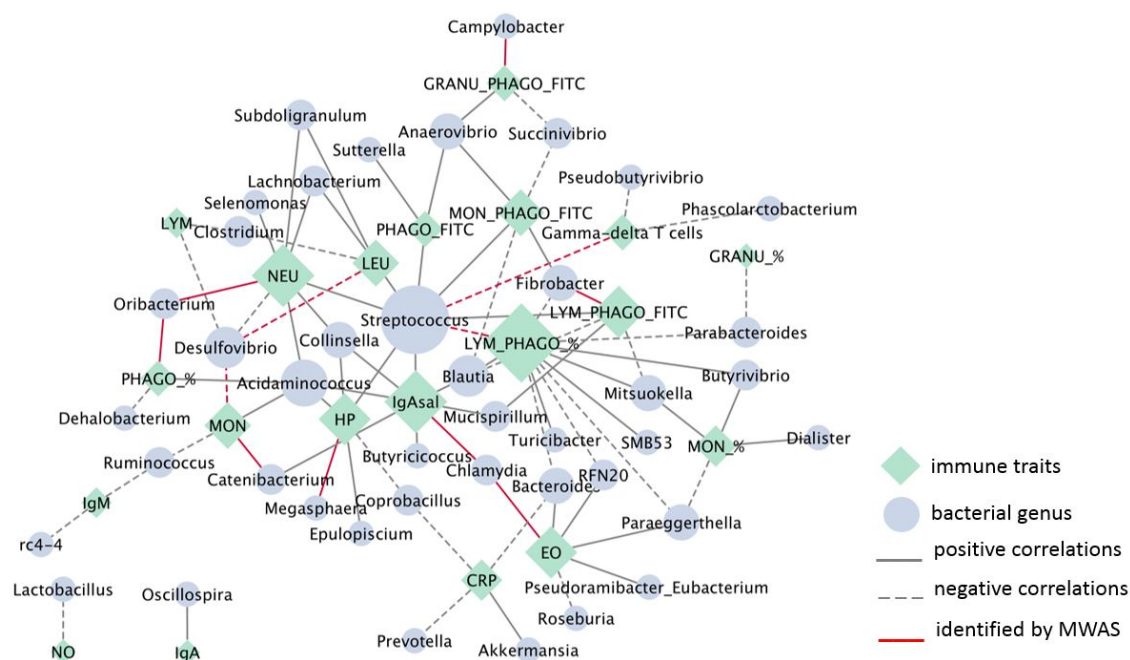
267 confirmed 30% (12/40) of the associations identified by the MWAS (Figure 2). The  
 268 strongest confirmed correlation was between *Fibrobacter* and LYM\_PHAGO\_FITC ( $r=$   
 269 0.37) followed by correlations between *Streptococcus* and LYM\_PHAGO\_% ( $r=-0.34$ ) and  
 270 between *Megasphaera* and Hp ( $r=0.26$ ). To be noted, *Streptococcus* and  
 271 LYM\_PHAGO\_% that showed the strongest confirmed association in the MWAS were  
 272 highly in the interaction-network as the keystone bacterial and immunity trait, respectively.

273

274 **Figure 2.** Microbial - health-related traits network. Green diamond nodes correspond to  
 275 immunity traits (n=21) and blue ellipse nodes correspond to microbial genera (n=42). Node  
 276 sizes are relative to their topological degree (number of connections) and edges are  
 277 continuous or dashed to represent positive or negative correlations, respectively.  
 278 Relationships previously identified by MWAS are highlighted in red.

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## 284 General Discussion

### 285 Host-genome and gut microbiota contribution to porcine immunocompetence

286 We report the first study that aimed to dissect the joint contribution of the host  
 287 genome and the gut microbiota to the immunocompetence in healthy pigs. Estimates of  
 288 microbiability pointed out significant microbial effects on most immunity and  
 289 hematological traits, ranging between 15% and 27% of total phenotypic variance. Effects

290 of microbiota resulted particularly relevant for Hp concentrations in serum, followed by  
291 the parameters related to phagocytosis of lymphocytes. Regarding genomic heritabilities of  
292 these traits, they reached low to moderate values and were substantially lower compared to  
293 the medium to high  $h^2$  previously obtained in the same Duroc population [9] for all traits  
294 but MON and MON\_PHAGO\_%. A dramatic decrease of the estimated host genetic  
295 effects was observed for  $\gamma\delta$  T cells, but also for EOS and NEU counts and  
296 immunoglobulins concentrations in plasma, despite IgM and IgG variability seemed  
297 dominated by host genetics and showed the highest  $h^2$  among analysed traits. These results  
298 would call into question the high genetic determinism of the global immunocompetence in  
299 pigs reported in previous studies [6, 7, 9]. However, it should be considered that the  
300 limited sample size joint with the likely similarity between close relatives (particularly  
301 between full-sibs) in their microbiota profiles makes plausible that the model could not  
302 separate adequately genetic from microbiota effects.

303

#### 304 **Microbial signatures associated with immunity traits**

305 In the present study, we implemented a combination of MWAS and network  
306 approaches to pinpoint microbial signatures associated with immunity traits, revealing  
307 some interesting associations between the composition of the pig gut microbiota and the  
308 host immunity traits. Remarkably, lymphocyte phagocytosis traits were among the most  
309 connected and associated traits to the highest number of taxa and were also central nodes  
310 in the network. The strongest confirmed association involved *Fibrobacter* relative  
311 abundance in gut microbiota and the host phagocytosis capacity of lymphocytes, which  
312 were positively correlated ( $r=0.37$ ). *Fibrobacter* genus is composed of strictly anaerobic  
313 bacteria with cellulolytic capacity capable of degrading complex plant fiber [36] and it has  
314 been associated with better feed efficiency in pigs [37, 38]. Conversely, the relative  
315 abundance of *Streptococcus* showed an opposite association with the percentage of  
316 phagocytic lymphocytes ( $r=-0.34$ ). *Streptococcus* was also the keystone taxa in the  
317 network. In pigs, some *Streptococcus* species are important opportunistic pathogens such  
318 as *Streptococcus suis*, which abundance increased in the stomach and small intestine after  
319 weaning [39]. Piglets with high intestinal concentrations of *S. suis* can serve as a source of  
320 transmission and infection between animals and farms (reviewed in [39]). In general  
321 *Streptococcus* are less abundant in more-feed efficient pigs [37], although there are also  
322 evidences of the immunomodulatory properties of member of *Streptococcus* genus [40,  
323 41].

324           Several studies in mammals have demonstrated that B cells have a significant  
325 phagocytic capacity, being able to phagocytose particles including bacteria [42-44]. Most  
326 important has been the demonstration of the efficient capability of these cells to present  
327 antigen from phagocytosed particles to CD4<sup>+</sup> T cells [42-44], acting as a bridge that link  
328 innate with adaptive immunity. Therefore, considering the inferred high connection of  
329 these phagocytosis phenotypes with gut microbiota, we could hypothesize that, as other  
330 antigen-presenting cells such as dendritic cells or macrophages, the phagocytic  
331 lymphocytes seem to be relevant to maintain immune tolerance to the normal gut  
332 microbiota, being also relevant to control the abundance of opportunistic pathogens. B-  
333 cells also produce secretory IgA, the most abundant secreted isotype in mammals and a  
334 key element to maintain ‘homeostatic immunity’[45]. Secretory IgA was among the most  
335 connected traits in the network being positively associated with several taxa. Among them,  
336 the genus *Blautia* is of particular interest due to its potential role modulating inflammatory  
337 and metabolic diseases, with potential beneficial effects for the host [46]. Therefore,  
338 similar to phagocytic lymphocytes, the interplay between secretory IgA levels and the  
339 abundance of different taxa in our animals may regulate the ecological balance of  
340 commensal bacteria and the development of Ig-A secreting cells. Neutrophils were also  
341 positively correlated with gut microbiota profiles. A systemic immunomodulation of  
342 neutrophils by intestinal microbiota has been demonstrated [47], and a crosstalk between  
343 NEU and gut microbial composition has been also documented [48]. Our results  
344 confirmed a positive association of *Oribacterium* abundance with the quantity of  
345 neutrophils. *Oribacterium* genus belongs to the *Lachnospiraceae* family, and the  
346 abundance of this genus increased in piglets after weaning [49]. Members of the genus  
347 *Oribacterium* produce short-chain fatty acids such as acetate [50], which directly  
348 influences immune system regulation [51], and can contribute to the health of the pig.

349           Among the most connected traits in our network we also found acute-phase protein  
350 Hp, which based on the estimated microbiability appeared preferentially determined by  
351 microbiota effects. The main function of Hp is to facilitate hemoglobin (Hb) clearance.  
352 After the formation of stable Hp-Hb complexes, the macrophage receptor CD163  
353 recognize them and the entire complex is removed from circulation by receptor-mediated  
354 endocytosis [52]. Therefore, Hp favors the reduction of free iron concentrations in the  
355 circulation and tissues [53]. Several bacterial pathogens such as *Staphylococcus*,  
356 *Mycobacteria*, *Salmonella*, *Corynebacterium*, *Haemophilus*, among others, require iron for  
357 growth, thus elaborating different acquisition strategies to uptake heme from the host,

358 particularly from Hb [54-56]. The host immune system has developed antimicrobial  
359 mechanisms, most related to innate pathways, to deplete iron availability for pathogens  
360 [54]. Remarkably, our results indicate a relevant effect of the microbiota composition on  
361 Hp levels which could also modulate the concentration of circulating free iron. We could  
362 hypothesize that the symbiotic microbiota could also modulate the iron levels in these  
363 animals through innate immunity mechanisms to prevent the development of different  
364 opportunistic pathogens. Our results confirmed a positive association between serum  
365 concentration of Hp and the relative abundance of *Megasphaera*, a member of the phylum  
366 Firmicutes. According to this result, an increase in the abundance of *Megasphaera* has  
367 been described in colon content and faeces of pigs fed with iron-deficient diet [57].  
368 Interestingly, this genus was reported as a potential biomarker for immune-mediate  
369 mechanism of protection from diarrhea [58] and positive correlated with luminal IgA  
370 concentration in pigs [49].

371  
372 Finally, it is worth highlighting the negative association between the relative  
373 abundance of *Desulfovibrio* and LEU and MON counts. *Desulfovibrio* is a sulfate-reducing  
374 bacteria (SRB), which can promote the metabolism of sugars [59] and plays also a key role  
375 in intestinal hydrogen and sulfur metabolism [60]. In pigs, *Desulfovibrio* plays a relevant  
376 role during pig gut colonization [49] and was among the dominant genus in healthy pigs  
377 compared with diarrhea-affected piglets [61]. In fact, in weaned piglets, a negative  
378 correlation between *Desulfovibrio* and several inflammatory markers such as IL-1 $\beta$ , IL-2  
379 and IL-6, have been observed [62], which would be in agreement with the negative  
380 correlation observed between *Desulfovibrio* and LEU and MON counts in our piglets.

381  
382 Despite of an inventory of potential gut health biomarkers exists for pigs [63, 64],  
383 our results propose new microbial candidates, and emphasize a polymicrobial nature of  
384 the immunocompetence in pigs. Furthermore, in agreement with previous reports [65], our  
385 results suggest that some immunity traits are influenced by specific microorganisms while  
386 others are determined by interactions between members of the gut microbiome. We also  
387 reveal the joint contribution of the host genome and the gut microbial ecosystem to the  
388 phenotypic variance of immunity parameters and advice that ignoring microbiota effects  
389 could generate an overestimation of genetic parameters. A better understanding of the host  
390 and microbial contribution to immunocompetence will allow to develop holistic breeding

391 strategies to modulate these traits, as well as to improve animal health, robustness and  
392 welfare.

393

## 394 **Conclusions**

395

396 Estimates of heritability and microbiability exposed the joint contribution of both  
397 the host genome and the gut microbial ecosystem to the phenotypic variance of immunity  
398 parameters, and revealed that ignoring microbiota effects on phenotypes could generate an  
399 upward bias in the estimation of genetic parameters. Results from the MWAS suggested a  
400 polymicrobial nature of the immunocompetence in pigs and highlighted associations  
401 between the compositions of pig gut microbiota and 15 of the analyzed immunity traits.  
402 Overall, our findings establish several links between the gut microbiota and the immune  
403 system in pigs, underscoring the importance of considering both sources of information,  
404 host-genome and microbial level, for the genetic evaluation and the modulation of  
405 immunocompetence in pigs.

406

407

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421

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425 **References**

426

- 427 1. OECD, Food, Nations AOotU: **OECD-FAO Agricultural Outlook 2019-2028**;  
428 2019.
- 429 2. Briefs EAM: **World food consumption patterns –trends and drivers**. In.  
430 [http://ec.europa.eu/agriculture/markets-and-prices/market-briefs/index\\_en.htm](http://ec.europa.eu/agriculture/markets-and-prices/market-briefs/index_en.htm);  
431 2015.
- 432 3. Reverter A, Hine BC, Porto-Neto L, Li Y, Duff CJ, Dominik S, Ingham AB:  
433 **ImmuneDEX: a strategy for the genetic improvement of immune competence**  
434 **in Australian Angus cattle. LID - 10.1093/jas/skaa384 [doi] LID - skaa384.**  
435 (1525-3163 (Electronic)).
- 436 4. AH V, LL J, TA N, KH dG: - **Disease incidence and immunological traits for**  
437 **the selection of healthy pigs. A. D - 7909485 (- 0165-2176 (Print)):- 29-34.**
- 438 5. I E-L, E W, U M, C F: - **Genetic variation in parameters reflecting immune**  
439 **competence of swine. D - 8002006 (- 0165-2427 (Print)):- 1-16.**
- 440 6. Clapperton M, Diack AB, Matika O, Glass EJ, Gladney CD, Mellencamp MA,  
441 Hoste A, Bishop SC: **Traits associated with innate and adaptive immunity in**  
442 **pigs: heritability and associations with performance under different health**  
443 **status conditions. Genet Sel Evol 2009, 41(1):54-54.**
- 444 7. L F, Y G, D L, G L, JJ L, A T, AM C, J L, P P, C dV *et al*: - **Immunity traits in**  
445 **pigs: substantial genetic variation and limited covariation. D - 101285081 (-**  
446 **1932-6203 (Electronic)):- e22717.**
- 447 8. I E-L, E W, L M, M M, L A-E, L A, C F: - **Mapping quantitative trait loci for**  
448 **immune capacity in the pig. D - 2985117r (- 0022-1767 (Print)):- 829-835.**
- 449 9. Ballester M, Ramayo-Caldas Y, González-Rodríguez O, Pascual M, Reixach J,  
450 Díaz M, Blanc F, López-Serrano S, Tibau J, Quintanilla R: - **Genetic parameters**  
451 **and associated genomic regions for global immunocompetence and other**  
452 **health-related traits in pigs. 2020, - 10(- 1).**
- 453 10. Luo W, Chen S, Cheng D, Wang L, Li Y, Ma X, Song X, Liu X, Li W, Liang J *et*  
454 *al*: **Genome-wide Association Study of Porcine Hematological Parameters in a**  
455 **Large White &#215; Minzhu F2 Resource Population. International Journal of**  
456 **Biological Sciences 2012, 8(6):870-881.**
- 457 11. Bovo S, Mazzoni G, Bertolini F, Schiavo G, Galimberti G, Gallo M, Dall’Olio S,  
458 Fontanesi L: **Genome-wide association studies for 30 haematological and blood**  
459 **clinical-biochemical traits in Large White pigs reveal genomic regions**  
460 **affecting intermediate phenotypes. Scientific Reports 2019, 9(1):7003.**
- 461 12. Wang JY, Luo Yr Fau - Fu WX, Fu Wx Fau - Lu X, Lu X Fau - Zhou JP, Zhou Jp  
462 Fau - Ding XD, Ding Xd Fau - Liu JF, Liu Jf Fau - Zhang Q, Zhang Q: **Genome-**  
463 **wide association studies for hematological traits in swine. (1365-2052**  
464 (Electronic)).
- 465 13. Jung EJ, Park Hb Fau - Lee JB, Lee Jb Fau - Yoo CK, Yoo Ck Fau - Kim BM, Kim  
466 Bm Fau - Kim HI, Kim Hi Fau - Cho IC, Cho Ic Fau - Lim HT, Lim HT: **Genome-**  
467 **wide association study identifies quantitative trait loci affecting hematological**  
468 **traits in an F2 intercross between Landrace and Korean native pigs. (1365-**  
469 **2052 (Electronic)).**



- 470 14. Ponsuksili S, Reyer H, Trakooljul N, Murani E, Wimmers KA-O: **Single- and**  
471 **Bayesian Multi-Marker Genome-Wide Association for Haematological**  
472 **Parameters in Pigs.** (1932-6203 (Electronic)).
- 473 15. Yan G, Guo T, Xiao S, Zhang F, Xin W, Huang T, Xu W, Li Y, Zhang Z, Huang L:  
474 **Imputation-Based Whole-Genome Sequence Association Study Reveals**  
475 **Constant and Novel Loci for Hematological Traits in a Large-Scale Swine F(2)**  
476 **Resource Population.** (1664-8021 (Print)).
- 477 16. Vernocchi P, Del Chierico F, Putignani L: **Gut Microbiota Profiling:**  
478 **Metabolomics Based Approach to Unravel Compounds Affecting Human**  
479 **Health.** *Frontiers in Microbiology* 2016, **7**:1144.
- 480 17. Schluter J, Peled JU, Taylor BP, Markey KA, Smith M, Taur Y, Niehus R, Staffas  
481 A, Dai A, Fontana E *et al*: **The gut microbiota is associated with immune cell**  
482 **dynamics in humans.** *Nature* 2020, **588**(7837):303-307.
- 483 18. Lo BC, Chen GY, Núñez G, Caruso R: **Gut microbiota and systemic immunity**  
484 **in health and disease.** *International Immunology* 2021, **33**(4):197-209.
- 485 19. Estelle J, Mach N, Ramayo-Caldas Y, Levenez F, Lemonnier G, Denis C, Doré J,  
486 Larzul C, Lepage P, Rogel-Gaillard C: **The influence of host's genetics on the gut**  
487 **microbiota composition in pigs and its links with immunity traits.** In: *10th*  
488 *World Congress of Genetics Applied to Livestock Production.* Vancouver,BC,  
489 Canada; 2014.
- 490 20. Kamada N, Seo Su Fau - Chen GY, Chen Gy Fau - Núñez G, Núñez G: **Role of the**  
491 **gut microbiota in immunity and inflammatory disease.** (1474-1741  
492 (Electronic)).
- 493 21. Gerardo NM, Hoang KL, Stoy KS: **Evolution of animal immunity in the light of**  
494 **beneficial symbioses.** *Philosophical Transactions of the Royal Society B:*  
495 *Biological Sciences* 2020, **375**(1808):20190601.
- 496 22. Khan AA, Yurkovetskiy L, O'Grady K, Pickard JM, de Pooter R, Antonopoulos  
497 DA, Golovkina T, Chervonsky A: **Polymorphic Immune Mechanisms Regulate**  
498 **Commensal Repertoire.** *Cell Reports* 2019, **29**(3):541-550.e544.
- 499 23. J W, Id O, L C, N Z, X X, Y X, B Z: - **Of genes and microbes: solving the**  
500 **intricacies in host genomes.** *D - 101532368* (- 1674-8018 (Electronic)):- 446-461.
- 501 24. Ramayo-Caldas Y, Prenafeta-Boldú F, Zingaretti LM, Gonzalez-Rodriguez O,  
502 Dalmau A, Quintanilla R, Ballester M: - **Gut eukaryotic communities in pigs:**  
503 **diversity, composition and host genetics contribution.** 2020, - 2(- 1).
- 504 25. Reverter A, Ballester M, Alexandre PA, Mármol-Sánchez E, Dalmau A,  
505 Quintanilla R, Ramayo-Caldas Y: **A gene co-association network regulating gut**  
506 **microbial communities in a Duroc pig population.** *Microbiome* 2021, **9**(1):52.
- 507 26. H C, SK M: - **Innate immune recognition of the microbiota promotes host-**  
508 **microbial symbiosis.** *D - 100941354* (- 1529-2916 (Electronic)):- 668-675.
- 509 27. F B, RE L, JL S, DA P, JI G: - **Host-bacterial mutualism in the human intestine.**  
510 *D - 0404511* (- 1095-9203 (Electronic)):- 1915-1920.
- 511 28. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA,  
512 Alexander H, Alm EJ, Arumugam M, Asnicar F *et al*: **Reproducible, interactive,**  
513 **scalable and extensible microbiome data science using QIIME 2.** *Nature*  
514 *Biotechnology* 2019, **37**(8):852-857.
- 515 29. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T,  
516 Dalevi D, Hu P, Andersen GL: **Greengenes, a chimera-checked 16S rRNA gene**  
517 **database and workbench compatible with ARB.** *Appl Environ Microbiol* 2006,  
518 **72**(7):5069-5072.
- 519 30. VanRaden PM: **Efficient Methods to Compute Genomic Predictions.** *Journal of*  
520 *Dairy Science* 2008, **91**(11):4414-4423.

- 521 31. Difford GF, Plichta DR, Løvendahl P, Lassen J, Noel SJ, Højberg O, Wright A-  
522 DG, Zhu Z, Kristensen L, Nielsen HB *et al*: **Host genetics and the rumen**  
523 **microbiome jointly associate with methane emissions in dairy cows.** *PLOS*  
524 *Genetics* 2018, **14**(10):e1007580.
- 525 32. Pérez P, de los Campos G: **Genome-wide regression and prediction with the**  
526 **BGLR statistical package.** *Genetics* 2014, **198**(2):483-495.
- 527 33. de los Campos G, Sorensen D, Gianola D: **Genomic Heritability: What Is It?**  
528 *PLOS Genetics* 2015, **11**(5):e1005048.
- 529 34. Legarra A, Ricard A, Varona L: **GWAS by GBLUP: Single and Multimarker**  
530 **EMMAX and Bayes Factors, with an Example in Detection of a Major Gene**  
531 **for Horse Gait.** *G3: Genes|Genomes|Genetics* 2018, **8**(7):2301.
- 532 35. Reverter A, Chan EK: **Combining partial correlation and an information theory**  
533 **approach to the reversed engineering of gene co-expression networks.** (1367-  
534 4811 (Electronic)).
- 535 36. Neumann AP, McCormick CA, Suen G: **Fibrobacter communities in the**  
536 **gastrointestinal tracts of diverse hindgut-fermenting herbivores are distinct**  
537 **from those of the rumen.** *Environ Microbiol* 2017, **19**(9):3768-3783.
- 538 37. Gardiner GE, Metzler-Zebeli BU, Lawlor PG: **Impact of Intestinal Microbiota on**  
539 **Growth and Feed Efficiency in Pigs: A Review.** *Microorganisms* 2020, **8**(12).
- 540 38. McCormack UM, Curião T, Metzler-Zebeli BU, Wilkinson T, Reyer H, Crispie F,  
541 Cotter PD, Creevey CJ, Gardiner GE, Lawlor PG: **Seeking to improve feed**  
542 **efficiency in pigs through microbial modulation via fecal microbiota**  
543 **transplantation in sows and dietary supplementation of offspring with inulin.**  
544 *Appl Environ Microbiol* 2019:AEM.01255-01219.
- 545 39. Su Y, Yao W, Perez-Gutierrez ON, Smidt H, Zhu W-Y: **Changes in abundance of**  
546 **Lactobacillus spp. and Streptococcus suis in the stomach, jejunum and ileum**  
547 **of piglets after weaning.** *FEMS Microbiology Ecology* 2008, **66**(3):546-555.
- 548 40. van den Bogert B, Meijerink M, Zoetendal EG, Wells JM, Kleerebezem M:  
549 **Immunomodulatory Properties of Streptococcus and Veillonella Isolates from**  
550 **the Human Small Intestine Microbiota.** *PLOS ONE* 2014, **9**(12):e114277.
- 551 41. Perdigon G Fau - Nader de Macias ME, Nader de Macias Me Fau - Alvarez S,  
552 Alvarez S Fau - Oliver G, Oliver G Fau - Pesce de Ruiz Holgado AA, Pesce de  
553 Ruiz Holgado AA: **Enhancement of immune response in mice fed with**  
554 **Streptococcus thermophilus and Lactobacillus acidophilus.** (0022-0302 (Print)).
- 555 42. Parra D, Rieger AM, Li J, Zhang Y-A, Randall LM, Hunter CA, Barreda DR,  
556 Sunyer JO: **Pivotal Advance: Peritoneal cavity B-1 B cells have phagocytic and**  
557 **microbicidal capacities and present phagocytosed antigen to CD4+ T cells.**  
558 *Journal of Leukocyte Biology* 2012, **91**(4):525-536.
- 559 43. Gao J, Ma X, Gu W, Fu M, An J, Xing Y, Gao T, Li W, Liu Y: **Novel functions of**  
560 **murine B1 cells: Active phagocytic and microbicidal abilities.** *European*  
561 *Journal of Immunology* 2012, **42**(4):982-992.
- 562 44. Martínez-Riaño A, Bovolenta ER, Mendoza P, Oeste CL, Martín-Bermejo MJ,  
563 Bovolenta P, Turner M, Martínez-Martín N, Alarcón B: **Antigen phagocytosis by**  
564 **B cells is required for a potent humoral response.** *EMBO reports* 2018,  
565 **19**(9):e46016.
- 566 45. Belkaid Y, Harrison OJ: **Homeostatic Immunity and the Microbiota.** 2017(1097-  
567 4180 (Electronic)).
- 568 46. Liu X, Mao B, Gu J, Wu J, Cui S, Wang G, Zhao J, Zhang H, Chen W: **Blautia—a**  
569 **new functional genus with potential probiotic properties?** *Gut Microbes* 2021,  
570 **13**(1):1-21.

- 571 47. Wu H-J, Wu E: **The role of gut microbiota in immune homeostasis and**  
572 **autoimmunity.** *Gut Microbes* 2012, **3**(1):4-14.
- 573 48. Zhang D, Frenette PS: **Cross talk between neutrophils and the microbiota.**  
574 *Blood* 2019, **133**(20):2168-2177.
- 575 49. Mach N, Berri M, Estellé J, Levenez F, Lemonnier G, Denis C, Leplat J-J,  
576 Chevaleyre C, Billon Y, Doré J *et al.*: **Early-life establishment of the swine gut**  
577 **microbiome and impact on host phenotypes.** *Environmental Microbiology*  
578 *Reports* 2015, **7**(3):554-569.
- 579 50. Sizova MV, Muller PA, Stancyk D, Panikov NS, Mandalakis M, Hazen A,  
580 Hohmann T, Doerfert SN, Fowle W, Earl AM *et al.*: **Oribacterium parvum sp.**  
581 **nov. and Oribacterium asaccharolyticum sp. nov., obligately anaerobic**  
582 **bacteria from the human oral cavity, and emended description of the genus**  
583 **Oribacterium.** *International Journal of Systematic and Evolutionary Microbiology*  
584 2014, **64**(Pt\_8):2642-2649.
- 585 51. Smith PM, Howitt Mr Fau - Panikov N, Panikov N Fau - Michaud M, Michaud M  
586 Fau - Gallini CA, Gallini Ca Fau - Bohlooly-Y M, Bohlooly-Y M Fau - Glickman  
587 JN, Glickman Jn Fau - Garrett WS, Garrett WS: **The microbial metabolites,**  
588 **short-chain fatty acids, regulate colonic Treg cell homeostasis.** 2013(1095-9203  
589 (Electronic)).
- 590 52. MJ N, CB A, SK M: - **CD163 binding to haptoglobin-hemoglobin complexes**  
591 **involves a dual-point.** *D - 2985121r* (- 1083-351X (Electronic)):- 18834-18841.
- 592 53. MacKellar M, Vigerust DJ: **Role of Haptoglobin in Health and Disease: A Focus**  
593 **on Diabetes.** *Clin Diabetes* 2016, **34**(3):148-157.
- 594 54. Johnson EE, Wessling-Resnick M: **Iron metabolism and the innate immune**  
595 **response to infection.** *Microbes and Infection* 2012, **14**(3):207-216.
- 596 55. Choby JE, Skaar EP: **Heme Synthesis and Acquisition in Bacterial Pathogens.**  
597 *Journal of Molecular Biology* 2016, **428**(17):3408-3428.
- 598 56. TW S, DJ M, PW W, R W, SD K, TM V, TL S: - **Complex role of hemoglobin**  
599 **and hemoglobin-haptoglobin binding proteins in.** *D - 0246127* (- 0019-9567  
600 (Print)):- 6213-6225.
- 601 57. Knight LC, Wang M, Donovan SM, Dilger RN: **Early-Life Iron Deficiency and**  
602 **Subsequent Repletion Alters Development of the Colonic Microbiota in the**  
603 **Pig.** *Frontiers in Nutrition* 2019, **6**:120.
- 604 58. Carey MA, Medlock GL, Alam M, Kabir M, Uddin MJ, Nayak U, Papin J, Faruque  
605 ASG, Haque R, Petri WA, Jr. *et al.*: **Megasphaera in the stool microbiota is**  
606 **negatively associated with diarrheal cryptosporidiosis.** *Clinical Infectious*  
607 *Diseases* 2021.
- 608 59. Salazar N, Gueimonde M, Hernández-Barranco AM, Ruas-Madiedo P, de los  
609 Reyes-Gavilán CG: **Exopolysaccharides Produced by Intestinal**  
610 **&lt;em>Bifidobacterium</em> Strains Act as Fermentable**  
611 **Substrates for Human Intestinal Bacteria.** *Appl Environ Microbiol* 2008,  
612 **74**(15):4737.
- 613 60. Ran S, Mu C, Zhu W: **Diversity and community pattern of sulfate-reducing**  
614 **bacteria in piglet gut.** *Journal of Animal Science and Biotechnology* 2019,  
615 **10**(1):40.
- 616 61. Ramon E, Belanche-Muñoz L, Molist F, Quintanilla R, Perez-Enciso M, Ramayo-  
617 Caldas Y: **kernInt: A Kernel Framework for Integrating Supervised and**  
618 **Unsupervised Analyses in Spatio-Temporal Metagenomic Datasets.** *Frontiers*  
619 *in Microbiology* 2021, **12**:60.
- 620 62. Hu R, He Z, Liu M, Tan J, Zhang H, Hou D-X, He J, Wu S: - **Dietary**  
621 **protocatechuic acid ameliorates inflammation and up-regulates intestinal tight**

- 622            **junction proteins by modulating gut microbiota in LPS-challenged piglets.**  
623            2020, - **11**(- 1).
- 624    63.    Wijtten PJ, van der Meulen J Fau - Verstegen MWA, Verstegen MW: **Intestinal**  
625            **barrier function and absorption in pigs after weaning: a review.** (1475-2662  
626            (Electronic)).
- 627    64.    Theo N (ed.): **Intestinal health**: Wageningen Academic Publishers; 2014.
- 628    65.    La Flamme AC, Milling S: **Immunological partners: the gut microbiome in**  
629            **homeostasis and disease.** *Immunology* 2020, **161**(1):1-3.  
630