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1 **Title:**

2 A pilot RNA-seq study in 40 Pietrain ejaculates to characterize the porcine
3 sperm microbiome

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49 **Abstract**

50 The microbiome plays a key role in homeostasis and health and it has been
51 also linked to fertility and semen quality in several animal species including
52 swine. Despite the more than likely importance of sperm bacteria on the boar's
53 reproductive ability and the dissemination of pathogens and antimicrobial
54 resistance genes, the high throughput characterization of the swine sperm
55 microbiome remains scarce. We carried RNA-seq on 40 ejaculates each from
56 a different Pietrain boar and found that a proportion of the sequencing reads
57 did not map to the *Sus scrofa* genome. The current study aimed at using these
58 reads not belonging to pig to carry a pilot study to profile the boar sperm
59 bacterial population and its relation with 7 semen quality traits.

60 We found that the boar sperm contains a broad population of bacteria. The
61 most abundant phyla were *Proteobacteria* (39.1%), *Firmicutes* (27.5%),
62 *Actinobacteria* (14.9%) and *Bacteroidetes* (5.7%). The predominant species
63 contaminated sperm after ejaculation from soil, faeces and water sources
64 (*Bacillus megaterium*, *Brachybacterium faecium*, *Bacillus coagulans*). Some
65 potential pathogens were also found but at relatively low levels (*Escherichia*
66 *coli*, *Clostridioides difficile*, *Clostridium perfringens*, *Clostridium botulinum* and
67 *Mycobacterium tuberculosis*). We also identified 3 potential antibiotic resistant
68 genes from *E. coli* against chloramphenicol, *Neisseria meningitidis* against
69 spectinomycin and *Staphylococcus aureus* against linezolid. None of these
70 genes were highly abundant. Finally, we classified the ejaculates into
71 categories according to their bacterial features and semen quality parameters
72 and identified two categories that significantly differed for 5 semen quality traits
73 and 13 bacterial features including the genera *Acinetobacter*,

74 *Stenotrophomonas* and *Rhodobacter*. Our results show that boar semen
75 contains a bacterial community, including potential pathogens and putative
76 antibiotic resistance genes, and that these bacteria may affect its reproductive
77 performance.

78

79

80 **Keywords**

81 Pig, spermatozoa, microbiome, RNA-seq, sperm quality

82 **1. Introduction**

83 Scientific research has led to the discovery that many compartments of the
84 animal organism contain a rich and complex population of microorganisms
85 known as microbiota, which plays a crucial role in physiological homeostasis
86 and health [1-3] including sperm quality and male fertility [4, 5]. The male's
87 reproductive ability is represented by a set of traits that are important for human
88 health and for the efficiency and sustainability of animal production. In swine,
89 semen quality is regularly measured in the artificial insemination studs as a
90 proxy of the fertilization ability of that sample. Growing research is being
91 devoted to understanding the biological basis and identifying molecular
92 markers linked to semen quality in humans and other animal species. As the
93 presence of bacterial communities in ejaculates is common and the microbiome
94 is popping up as a big contributor of a broad range of phenotypes, several
95 studies have been carried in the field of men fertility [4, 6, 7] and boar sperm
96 quality [8, 9]. Weng et al. [4] identified a complex population of bacteria in
97 human sperm but most interestingly, found that the abundance of some
98 bacteria was related to male fertility. *Lactobacillus crispatus*, *Gardnerella*
99 *vaginalis* and *Lactobacillus acidophilus* were more abundant in the fertile
100 samples whilst *Prevotella vibria* and *Haemophilus parainfluenzae* were present
101 at higher proportion in the unfertile sperm [4]. In a more recent study, a group
102 led by Stephen Krawetz [10] used sperm RNA-seq datasets to identify
103 transcripts of bacterial origin and shed light to the bacterial composition of an
104 ejaculate. They found a diverse bacterial population mostly characterized by
105 members of the phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and
106 *Actinobacteria* [10].

107 In pigs, the presence of bacteria in sperm is well documented and bacterial
108 populations in ejaculates are common [11-13]. In pigs, most of the bacteria
109 present in semen ejaculates have an external origin and have contaminated
110 the sperm after ejaculation. The most abundant sources of contaminations are
111 the prepuce, the prepuce diverticulum and hair [11], the sinks and drains of the stud, the
112 utensils used for ejaculate collection and transfer as well as the laboratory
113 surfaces where the ejaculates are being processed [14]. The presence of
114 bacteria in sperm is of further concern within the One Health concept as
115 commercial sperm doses in the livestock industry can be a major contributor on
116 the dissemination of bacterial pathogens and antibiotic resistance genes
117 (ARGs) [15]. Ubeda and co-authors, using cell culture, concluded that the most
118 abundant bacteria in pig semen were from the Enterobacteriaceae family and
119 included, in order of abundance, *Serratia marcescens*, *Klebsiella oxytoca*,
120 *Providencia stuartii*, *Morganella morganii*, *Proteus mirabilis*, and *Escherichia*
121 *coli*. *S. marcescens*, *K. oxytoca*, *M. morganii*, or *P. mirabilis* were negatively
122 associated with sperm quality [8]. Schulze also recently identified the presence
123 of several species of *Lactobacillus* and an association, *in vitro*, between the
124 abundance of *Lactobacillus buchneri* and sperm motility, mitochondrial activity
125 and membrane integrity and *Lactobacillus animalis* with motility [16]. To control
126 bacterial growth in sperm, antimicrobials are commonly added to semen
127 extenders [13]. Nonetheless, bacteria in these extended ejaculates can be still
128 present due to incomplete efficiency of the antibiotics which could be partially
129 caused by the expression of ARGs by these bacteria. Current high throughput
130 sequencing technologies provide unprecedented capacity to study and expose
131 the complexity of microbial ecosystems. Recently, Even et al. explored for first

132 time the pig sperm microbiome using high throughput sequencing of the 16S
133 bacterial gene. The aim of their study was to identify the factors that influence
134 the sperm microbiome and to assess the adequacy of this technique to routinely
135 monitor the sperm bacterial population [12]. The authors nicely showed that the
136 stud has an effect on the bacterial composition of the porcine semen [12].
137 Although the experimental design did not allow disentangling in detail the stud
138 related factors that shape the seminal microbiota, the flooring type itself
139 (sawdust or slatted floors) showed association with the microbiome
140 composition and diversity [12]. They also found that diluting the ejaculates with
141 extenders, which contain antibiotics, reduces the bacterial diversity in a sample
142 and also contributes reducing the variability in the bacterial diversity between
143 ejaculates [12]. The aim of our study was to characterize the composition of the
144 boar sperm microbiome exploiting a RNA-seq dataset on extended sperm from
145 40 pigs and interrogate the existence of a potential link between the sperm
146 microbiome and semen quality traits.

147

148 **2. Materials and methods**

149 **2.1. Sample collection, purification and phenotyping**

150 Specialized professionals obtained fresh ejaculates from 40 Pietrain boars from
151 three different commercial farms located in Catalonia (~42 °N, ~2 °E), with the
152 gloved-hand method. The farms contained between 114 and 140 boars in 6
153 squared meter pens each harboring 6 boars. All farms had sawdust flooring,
154 did not use air filtration system and employed similar semen collection and
155 processing practices. Ejaculates were collected between March 2015 and
156 January 2017 and boar ages ranged from 9 to 55 months old. After collection,

157 the ejaculates were immediately diluted (1:1) with Androstar® Plus and kept at
158 26 °C for up to 6 hours until they were further diluted (1:2) in Androstar® Plus.
159 Androstar® Plus is a boar semen extender that contains the following
160 antibiotics: apramycin sulphate, cephalosporin – third generation - and
161 gentamicin sulphate. The extended samples were then kept at 16 °C for 6-10
162 additional h until they were processed in our laboratory for phenotyping and
163 spermatozoa purification. Seven sperm phenotypes were measured in the 40
164 samples as previously described by Godia et al. [17]. Phenotypes included the
165 percentage of viable sperm cells after 5 min of incubation at 37 °C (VIAB_5),
166 the percentage of viable sperm cells after 90 min incubation at 37 °C (VIAB_90),
167 percentage of cells with abnormal acrosomes after the 5 min (ACRO_5) and
168 the 90 min (ACRO_90) incubation, the percentage of motile cells after 5 min
169 (MT_5) and 90 min (MT_90) incubation and the percentage of membrane
170 functional spermatozoa after an osmotic stress (ORT, Osmotic Resistance
171 Test). VIAB_5, VIAB_90, ACRO_5 and ACRO_90 were measured by staining
172 the samples with the eosin-negrosin technique after 5 and 90 min incubation at
173 37 °C following the protocol described by Bamba [18]. MT_5 and MT_90 were
174 measured with the computer-assisted semen analysis (CASA) system
175 (Integrated Sperm Analysis System V1.0; Proiser). To calculate ORT the
176 spermatozoa were incubated at 37 °C for 10 min on iso- and hypo-osmotic
177 solutions using the method described by Rodríguez-Gil and Rigau [19].
178 Normal motile spermatozoa were subsequently purified using the BoviPure™
179 colloidal silica particles reagent (Nidacon; Mölndal, Sweden) as detailed by
180 Gòdia et al. [17]. Briefly, the volume of sperm that was used varied according
181 to the sperm concentration, with a maximum of 1 billion cells and not exceeding

182 11 mL. The manufacturer's recommendation of a minimum volume ratio of 25%
183 diluted BoviPure™ / semen was maintained. After centrifugation following the
184 manufacturer's protocol, the cell pellet was washed once with RNase-free
185 phosphate buffer saline (PBS) and then resuspended in 1 mL of RNase-free
186 PBS for optical inspection to confirm the removal of somatic cells. For all
187 samples, aliquots containing ~40 million spermatozoa were then centrifuged
188 and the resulting pellet was stored at -80 °C in 1 mL of Trizol® until further
189 processed for RNA extraction.

190 **2.2. RNA extraction, qPCR validation, library prep, sequencing**

191 RNA was extracted from sperm pellets using a standard Trizol® approach and
192 treated with TURBO DNA-free™ Kit (Invitrogen; Carlsbad, USA) [17]. RNA
193 samples were subjected to RT-qPCR assays to validate the presence of
194 spermatozoa RNA with primers targeting the *PRM1* gene, the absence of RNA
195 from contaminating diploid cells (mainly leukocytes and keratinocytes) using
196 primers against the somatic gene *PTPRC* and the absence of genomic DNA
197 using primers targeting an intergenic region [17]. Total RNA was subjected to
198 mammalian ribosomal RNA (rRNA) depletion with the Ribo-Zero Gold rRNA
199 Removal Kit (Illumina, CA, USA). RNA-seq libraries were prepared with
200 SMARTer Universal Low Input RNA library Prep kit (Clontech, France) and
201 sequenced in an Illumina's HiSeq2000/2500 system to generate 75 base pair
202 long paired end reads. These RNA-seq datasets were initially analyzed to
203 characterize the boar sperm transcriptome [20] and circular RNAome [21]. The
204 RNA-seq data used in this study (total RNA-seq runs) is accessible at the
205 NCBI's under the SRA study accession SRP183646.

206 **2.3. Bioinformatics and statistical analysis**

207 **2.3.1. Identification of RNA molecules of bacterial origin**

208 RNA-seq reads of low quality and adaptor contaminations were removed with
209 Trimmomatic v.0.36 [22]. Filtered reads were then mapped to the *Sus scrofa*
210 genome (Sscrofa11.1) with HISAT2 v.2.1.0 [23] with default parameters except
211 "--max seeds 30" and "-k 2". The reads that did not map to Sscrofa11.1 were
212 screened against the catalogue of porcine Transposable Elements from the
213 Repbase database [24] with HISAT2 v.2.1.0 [23].

214 The reads that remained unmapped were taxonomically classified and
215 quantified with Kraken v.0.10.5 [25] with a threshold score of 0.15 and using the
216 default database that includes NCBI taxonomic information and complete
217 genomes from RefSeq of archaeal, bacteria, phage and viral domains. Only the
218 bacterial-assigned reads were kept for further analysis. The number of reads
219 assigned to a given taxon was normalized by sequencing depth, as counts per
220 million (CPM).

221 For ease of readability, we refer to each bacterial taxon identified in the study,
222 from phyla to species, as bacterial feature. The list of potential pathogens in
223 swine was extracted from the Professional Pig Community pig333 site
224 (www.pig333.com/pig-diseases), The Pig Site (<https://thepigsite.com/disease-and-welfare/managing-disease/bacteria>) and The Swine Health Information
226 Center (<https://www.swinehealth.org/swine-bacterial-disease-matrix/>). The list
227 of bacterial agents and diseases in each of these sources is available at Table
228 S1.

229 **2.3.2. Detection of antimicrobial resistance genes**

230 Unmapped reads were also subjected to identification and relative abundance
231 quantification of ARGs. ARGs were identified using BLASTN v.2.7.1 [26] with

232 100% percentage identity using the Comprehensive Antibiotic Resistance
233 Database (CARD) v.3.0.0 [27]. The number of reads for each ARG was
234 normalized by sequencing depth, as CPM. The read coverage across ARGs of
235 point mutations was individually visualized using R v.3.5.3 [28].

236 The relationship between the abundance of each ARG and the abundance of
237 their corresponding bacteria was calculated with “lm” function from R [28]. The
238 adjusted R-squared was provided by the lm function and corresponds to the
239 Wherry’s formula [29].

240 **2.3.3. Relation between bacterial abundance and semen quality traits**

241 The raw phenotypes were corrected by environmental factors including farm of
242 origin, age of boar when sampled and season-year of sampling using a
243 standard linear model [28]. We assessed the relationship between the semen
244 quality traits and the bacterial features of the sperm microbiome using LINK-
245 HD, an integrative methodology designed to deal with the compositional nature
246 of microbial datasets [30]. The methodology is an extension of Principal
247 Component Analysis (PCA) suitable for analyzing several sources of data that
248 share a common set of observations. It outputs a matrix that is often referred to
249 as the compromise. An eigen-analysis of this matrix not only allows a graphical
250 representation of the samples in a plane, but also allows them to be grouped
251 using a standard cluster methodology like k-means. This analysis included the
252 7 corrected phenotypes and the bacterial features (N=733) with average CPM
253 ≥ 1 and representing more than 0.001% of the total bacterial read counts. We
254 use the cluster classification derived from the compromise structure to perform
255 variable selection through the fitZig function from the metagenomeSeq package
256 v.1.28.2 [31]. fitZig implements an expectation- maximization algorithm (EM) to

257 estimate the differential abundance of taxa using a Zero-Inflated Gaussian (Zig)
258 distribution that takes their sparse nature into account.

259

260 **3. Results**

261 **3.1. RNA-seq statistics**

262 We carried RNA-seq on 40 extended ejaculates each from a different Pietrain
263 pig and obtained an average of 40.7 million reads per sample. In average,
264 98.5% of the reads passed the quality control and 82.7% mapped to the porcine
265 genome (Sscrofa11.1). A tiny proportion (0.012%) of the unmapped reads
266 aligned to Repbase [24] and 25.1% (an average of 1.7 million reads per library)
267 mapped to microbial genomes with Kraken (Table S2).

268 **3.2. Description of the boar sperm microbiome**

269 We identified 733 bacterial features with average abundance ≥ 1 CPM and
270 representing more than 0.001% of the total bacterial read counts. The total
271 bacterial abundance across samples varied between 2,241 and 180,624 CPMs
272 (Fig. 1 and Table S3). The average and median abundances of bacterial reads
273 were 20,149 and 9,785 CPM, respectively and 3 ejaculates had more than
274 70,000 bacterial CPM (Fig. 1). The bacterial features included 15 phyla (Table
275 S3). The most abundant phyla were *Proteobacteria*, with an average of 39.1%
276 of bacterial reads, *Firmicutes* (27.5%), *Actinobacteria* (14.9%) and
277 *Bacteroidetes* (5.7%) (Fig. 2 and Table S3). At the species level, the analysis
278 identified 254 bacterial species (Table S3). The most abundant species were,
279 in this order, *Bacillus megaterium* (868 CPMs and 4.3% of the bacterial reads),
280 *Brachy bacterium faecium* (3.3%), *Bacillus coagulans* (1.2%) and
281 *Campylobacter hominis* (1.0%) (Table 1).

282 **3.3. Boar sperm safety: pathogens and antibiotic resistance genes**

283 We found 12 potentially pathogenic species of bacteria with average
284 abundance ≥ 1 CPM and representing more than 0.001% of the total bacterial
285 read counts but only 7 displayed CPM > 5 . These were, in this order:
286 *Escherichia coli*, *Clostridioides difficile*, *Clostridium perfringens*, *Clostridium*
287 *botulinum*, *Mycobacterium tuberculosis*, *Mycoplasma hyopneumoniae* and
288 *Campylobacter jejuni* (Table 2). With the exception of *E. coli* and *C. difficile*,
289 which ranked 8th and 22nd in the list of most abundant bacterial species, with
290 137 and 50 CPM, respectively, these potential bugs were in general displaying
291 low relative abundance in our samples (Table 2). While nearly all the samples
292 contained at least traces of these bacteria, *M. tuberculosis* was only present in
293 6 samples and it presented moderate abundances (between 28 and 84 CPMs)
294 in all of them (Table S3).

295 We also searched for ARG with average CPM ≥ 1 and found 3 candidates,
296 including ARO:3003497, *Neisseria meningitidis* 16S rRNA mutation conferring
297 resistance to spectinomycin; ARO:3004058, *Staphylococcus aureus* 23S rRNA
298 with mutation conferring resistance to linezolid and ARO:3004150, *E. coli* 23S
299 rRNA with mutation conferring resistance to chloramphenicol. Moreover, all the
300 samples presented CPM ≥ 1 for these 3 ARGs (Table 2).

301 **3.4. Relationship between the sperm microbiome and semen quality**

302 To identify potential relationships between bacterial abundances and semen
303 quality we employed Link-HD [30], a recently developed tool based on STATIS
304 methodology to integrate heterogeneous datasets. This approach analyzes
305 different types of variables measured on the same samples, here bacterial
306 abundance and semen quality phenotypes. To the end, the tool turns each raw

307 data into cross-product matrix, computed on the distances between samples,
308 which are then combined in a common configuration named compromise. A
309 classical Principal Component Analysis (PCA) decomposes the compromise
310 variance into orthogonal components and data structure can be easily
311 recovered using standard clustering techniques. In this study, the samples were
312 clustered into categories according to their microbiome and their semen quality.
313 We included the 733 bacterial features (from phyla to species in Table S3) and
314 7 semen quality traits (Table S4). Link-HD structured the purified ejaculates into
315 2 clusters with 30 (cluster 1) and 10 (cluster 2) samples each (Fig. 3 and Table
316 S5). The analysis also recovers the contribution of each feature into the
317 common structure, which facilitates the interpretability of the results. We found
318 that the 7 semen traits and 67 of the 733 bacterial features associated with the
319 whole-compromise structure (Table S6).

320 We then compared the distribution of these 7 phenotypes and 67 bacterial
321 features in each cluster. The 2 categories showed statistically significant
322 differences for 5 traits. MT_5 and MT_90 did not differ between both groups
323 (Table S7). The feature abundances between the 2 clusters were compared.
324 Thirteen bacterial features resulted in nominal significant differences between
325 clusters (Table 3). These included the genus *Acinetobacter*, *Stenotrophomonas*
326 and *Rhodobacter* (Table 3).

327

328 **4. Discussion**

329 **4.1. Technical considerations**

330 We carried RNA-seq on the extended sperm from 40 pigs with the aim to
331 characterize the boar semen transcriptome in relation to sperm quality. We

332 hypothesized that a proportion of the sequences that did not map to the pig
333 genome (Sscrofa11.1), between 9 and 31% of the reads (Table S2), could
334 correspond to bacterial transcripts. We identified a rich population of bacteria
335 with a diverse abundance profile between the ejaculates. Despite the fact that
336 the processed extended sperm contained antibiotics and that we treated these
337 samples to remove micro-organisms, we found evidences of bacterial presence
338 in their sequenced RNAs. This indicates that the extender did not eliminate or
339 inactivate all the bacteria present in the ejaculate. We can even hypothesize
340 that these bacteria were viable and transcriptionally active at the time that we
341 processed and froze the samples prior to RNA extraction. Dead bacteria would
342 release their RNA content to the extracellular milieu and this would be degraded
343 by action of the ubiquitous extracellular RNases. However, it also seems that
344 the initial bacterial burden in sperm did not experience an exponential growth
345 during the incubation time (12-16 h) in extended sperm. Bacterial growth
346 follows an exponential pattern with a slope that is dependent on the
347 generational interval [32]. Our measure from the RNA-seq datasets, with total
348 bacterial abundances ranging between 180,000 and 2,241 CPM, and a median
349 of 9,785 CPM, suggest that these bacteria did not proliferate at high rates in
350 our samples possibly due to the effect of the antibiotics. In addition, it is even
351 possible that these antibiotics promote a positive selection for the resistant
352 bacteria. In fact, we observed the presence of 3 ARGs that confer resistance to
353 spectinomycin, linezolid and chloramphenicol. However, all this remains
354 speculative and only classical microbiology tests can ascertain the viability of
355 the cells.

356 RNA-seq has several particular characteristics when compared to other high
357 throughput evaluations of bacterial communities. First, it allows exploring gene
358 expression and thus assessing the functional activity of the microbiome. For
359 this reason, RNA-seq based quantification is biased towards the identification
360 of the active bacteria. Second, it allows discriminating between active viable
361 and not-viable or dormant microorganisms as the first have active gene
362 expression. Third, it has higher resolution than the analyses targeting
363 exclusively the 16S gene as RNA-seq targets a larger portion of the bacterial
364 genome [33]. However, we used the Kraken metagenomics tool [25] which was
365 designed to quantify the abundance of bacteria based in their DNA. Kraken has
366 been already previously used to characterize the sperm microbiome using
367 RNA-seq datasets in human [10]. While meta-genomics strictly focuses on the
368 abundance of bacterial specimens, meta-transcriptomics informs on the
369 expression of their genes and thus the function and activity of these micro-
370 organisms in the sample. Our data provides a quantification of each bacterium
371 based in the overall expression of their transcripts which accounts for both the
372 bacterial abundance and their gene expression activity and have the additional
373 advantage to account for active microorganisms. In other words, we cannot
374 state without uncertainty whether one bacterium is more abundant than another
375 in one sample but we can assume that this is the most likely scenario as in part,
376 our measures are reflecting these abundances. For this reason and to ease the
377 message provided in this manuscript, we have referred to bacterial abundance
378 throughout the article.

379 Our experiment is a pilot study based on a small dataset of 40 ejaculates, each
380 from a different boar of the same breed (Pietrain) and representing only 3 studs

381 with similar management conditions and geographic location on the same
382 climatic zone. Although the information is relevant as little is known on the
383 microbiome composition of the boar sperm, our results cannot be extrapolated
384 to other commercial farms, animals and conditions. Further studies involving
385 more animals from different breeds, studs, management conditions and
386 geographic locations will be needed for the accurate characterization of the
387 boar sperm microbiome.

388 **4.2. Sperm microbial composition**

389 According to our data, the boar sperm microbiome differed from the profiles
390 obtained on porcine gut where the most abundant phyla include *Bacteroidetes*
391 and *Firmicutes* and the predominant genus are *Prevotella* and *Roseburia* [34].
392 On the contrary, our data highlights that in the porcine and in human sperm,
393 the 4 most abundant phyla are coincident [10]. Moreover, 11 of the 20 most
394 abundant genera in boar and human sperm were shared in both species. In
395 human sperm, the most abundant bacteria were members of *Actinobacteria*
396 (*Corynebacterium*), *Bacteroidetes* (*Prevotella*), *Firmicutes* (*Lactobacillus*,
397 *Streptococcus*, *Staphylococcus*, *Planococcaceae*, *Finnegoldia*), and
398 *Proteobacteria* (*Haemophilus*, *Burkholderia*) [5]. The differences between the
399 porcine and the human ejaculates could be attributed to multiple technical (e.g.,
400 the selection of antibiotics in extender and the removal of bacteria during the
401 purification of the samples), environmental and biological causes. Although
402 boar studs are kept in high hygienic conditions, pigs are in closer contact with
403 surfaces, soil, faeces and water and are thus more exposed to environmental
404 contaminants than humans.

405 The most abundant bacteria in the boar sperm are mostly environmental (*B.*
406 *megaterium* [35], *B. faecium* [36], *R. pickettii* [37]) and faecal (*C. hominis* [38]
407 and *E. coli*). This suggests that these bacteria have contaminated sperm after
408 ejaculation. *C. acnes* typically colonizes the human skin [39] but can be also
409 found in other compartments including the gastro-intestinal tract [40].
410 Interestingly, *B. subtilis*, a probiotic added in the pig feedstuff and allowed in
411 the European Union Register of Feed Additives, appeared as the 11th most
412 abundant bacteria in the boar sperm (Table 1), again suggesting that it
413 contaminated sperm after ejaculation.

414 Arkfen and co-authors [41] analyzed the airborne microbiome of hog farms and
415 found a similar composition of bacterial phyla as the one described in our study.
416 Moreover, our data is in line with the results obtained in other studies which
417 indicated that the bacteria present in sperm is a result of environmental
418 contamination, mostly attributed to prepuce fluid and hair [42], sinks and drains
419 in the farms, semen collection and processing utensils and the skin flora of
420 working staff [14].

421 Three ejaculates showed a much higher bacterial abundance when compared
422 to the average in all the samples (Fig. 1). Although we don't know the causes,
423 these elevated values of bacterial reads might have been caused by accidental
424 contamination of the ejaculate with particularly large chunks of environmental
425 debris present for example in the boar's prepuce or other surfaces.

426 **4.3. Pathogens and anti-microbial resistances**

427 We found several potential pathogens (Table 2) as included in the Professional
428 Pig Community pig333 site, The Pig Site and the Swine Health Information
429 Center. Some serotypes of these bacteria have been linked to diarrhea (*E. coli*,

430 *C. difficile*, *C. jejuni*), acute enteritis (*C. perfringens*) [43], botulism (*C.*
431 *botulinum*), tuberculosis (*M. tuberculosis*) and enzootic pneumonia (*M.*
432 *hyopneumoniae*) in swine [44]. While 4 of the 5 most abundant potential
433 pathogens showed a continuous pattern of abundance across samples, *M.*
434 *tuberculosis* was only present in 6 samples, all with moderate abundances
435 (CPM between 28 and 84). This quasi bi-modal distribution cannot be explained
436 by factors controlled in our study as these 6 pigs came from different farms,
437 were of varying ages, their ejaculates were collected at different seasons of the
438 year and there was thus no apparent link between these animals. The presence
439 of *M. tuberculosis* complex has been already found in wild boar (*Sus scrofa*) in
440 Eurasia [45]. The pathogenic potential of these bacterial species varies across
441 strains depending on the presence of virulence factors and toxin production.
442 Notwithstanding, our analysis does not allow concluding that any of the
443 specimens identified in this study are pathogenic as the analysis did not have
444 the power and specificity to detect the genes to discriminate between these
445 serotypes.

446 In animal production systems, extended sperm is distributed to multiple farms
447 and geographical locations and despite the fact that it is mixed with antibiotics,
448 some bacteria remain in these ejaculates. Moreover, before they are
449 inseminated into the sow, extended sperm doses will remain at 17 °C in
450 average up to few days, thus potentially allowing the selective growth of
451 bacteria carrying ARGs. Therefore, ejaculates might be an important source
452 and vehicle to disseminate these bacteria to other farms and animals. Hence,
453 the vaginal microbiome in sows inseminated with these doses should be
454 evaluated to determine how the sperm microbiome modulates the female tract,

455 how it impacts on the sow's health and fertility and the extent to which ARGs
456 and pathogens are transmitted through artificial insemination.

457 We identified 3 ARGs that were present at CPM ≥ 1 in all the ejaculates (Table
458 2). These ARGs were point mutation variants in bacterial ribosomal RNA
459 genes. The most abundant ARG potentially conferred resistance to *E. coli* to
460 chloramphenicol, a broad-spectrum antibiotic predominantly active against
461 gram negative bacteria used in human medicine but not authorized by the
462 European Union for use in livestock. However, this antimicrobial can be
463 synthesized by soil bacteria and it may thus be present in farms thereby
464 allowing the generation of ARGs against it. Our results suggest a scarce
465 presence of ARGs in our porcine sperm samples. The ejaculates were diluted
466 with a commercial semen extender that contains the antibiotics apramycin,
467 cephalosporin and gentamicin but no ARGs were found against these 3
468 antibiotics.

469 The 3 bacteria involved in these presumable ARGs (*E. coli*, *N. meningitidis* and
470 *S. aureus*) were detected in our study but their abundances did not relate with
471 the expression levels of their cognate ARGs (Fig. S1). The only exception is for
472 *N. meningitidis* and the ARG for Spectinomycin ($R^2 = 0.74$), but this is largely
473 due to one influential outlier ejaculate for which the abundance of both, these
474 bacteria and ARG were remarkably elevated (Fig. S1). This indicates that not
475 all the bacteria of these species carry the same load of ARG in each sample.

476 These results have to be taken as indicative as in this study we cannot conclude
477 whether these abundances in CPM are large or modest. Moreover, the
478 antimicrobial activity of these ARGs cannot be granted with our study. This
479 activity should be confirmed with a classical microbiological analysis and

480 antimicrobial sensitivity testing with the target antibiotics, according to the
481 Clinical & Laboratory Standards Institute (CLSI) guidelines [46, 47].

482 **4.4. Relationship between the sperm microbiome and semen quality**

483 As the microbiome is a complex system of microbial communities and its
484 genomic characterization generates compositional and sparse data, we used
485 an integrative approach that considers simultaneously the ejaculate bacterial
486 composition and semen quality. This analysis led to the identification of two
487 clusters with 30 and 10 samples each. Five traits and 13 bacterial features
488 showed significant differences between the two clusters (Table S7 and Table
489 3). The fact that this analysis identified two categories based simultaneously on
490 their semen quality and microbiome indicates that the two are related. VIAB_90
491 and ACRO_90 displayed stronger differences between the two groups than
492 VIAB_5 and ACRO_5. This suggests that the long incubation favored the
493 proliferation of bacteria and this led to a stronger bacterial impact on the
494 phenotype. In farm conditions, most doses are used within 48 h after ejaculation
495 but some may be kept up to 6 days. The impact of these conditions in the
496 microbiome could be strong and it should be explored. Bacteria that remains
497 active in the extended sperm despite the presence of antibiotics could alter
498 sperm quality through several mechanisms including the competition for
499 nutrients, the alteration of the microenvironment, the secretion of toxins, or the
500 adhesion to the sperm cell membrane compromising sperm viability or
501 aggregation. The 13 bacterial features showing differences between the two
502 clusters included the genera *Acinetobacter*, *Stenotrophomonas* and
503 *Rhodobacter* (Table 3). One study on human semen from Kiessling et al. [48]
504 identified *Acinetobacter* bacteria in some of the semen samples that they

505 evaluated [48]. An *in vitro* study on rabbit sperm cultured under the presence
506 of *A. baumannii* showed that the motility of the spermatozoa was negatively
507 affected by the presence of this bacterium [49]. A study on boar sperm found
508 *A. iwoffi* in some samples and that the presence of this bacterium was
509 associated to higher production of Reactive Oxidative Species and lipid
510 peroxidation thus potentially altering some semen quality features [50].
511 *Stenotrophomonas* are also typically found in soil and plants and some
512 (including *S. maltophilia*) can be opportunistic pathogens in humans. In swine,
513 it has been previously detected in sperm [13]. A case report on a dog with
514 conception failure and positive for *S. maltophilia*, linked this bacteria with
515 semen quality [51]. Finally, the genus *Rhodobacter* includes several species
516 with a diverse range of energy-based metabolism but has not been previously
517 found in sperm nor linked to sperm quality. This genus can be found in varied
518 habitats including pig manure [52].

519 Semen quality is defined by a set of complex traits that depend on the genetics
520 and age of the boar and on multiple environmental factors including nutrition,
521 photoperiod and heat stress, housing conditions, semen collection frequency
522 and method, sperm dilution rate, storage media and packaging conditions [15].
523 In our study, we could not record most of these parameters. However, while
524 correcting the phenotypes by farm, age and season of the year, we indirectly
525 controlled for a proportion of these factors. First, all the ejaculates were
526 collected at night, stored under the same conditions and processed during the
527 following early afternoon. Moreover, housing conditions, nutrition, collection
528 method as well as storage conditions are farm specific and were thus indirectly
529 corrected when controlling by farm. The photoperiod and heat stress factors

530 were also indirectly considered as we also corrected the phenotypes by the
531 season of the year. Nonetheless, we could not annotate the resting time (the
532 time passed since the previous semen collection), a parameter that is known to
533 affect semen quality [53]. Consequently, our results related to semen quality
534 and the microbiome should be considered as indicative.

535

536 **5. Conclusions**

537 In conclusion, we have identified a large and varied population of bacteria
538 contaminating the boar's extended sperm, including a small proportion of
539 potential pathogens and ARGs. Moreover, some of these bacteria might be
540 related to semen quality. This is of high relevance for two main reasons. First,
541 these bacteria may affect sperm quality and male fertility. Second, since
542 ejaculates are widely distributed across farms, they might be major
543 disseminators of these microbes and ARGs. Thus, the microbial composition in
544 the sperm of swine and other livestock species needs to be studied more
545 profoundly. Moreover, we anticipate that in a not too distant future, the
546 systematic microbiome analysis of semen ejaculates to identify the samples
547 that contain potential pathogens will become common practice. At present, high
548 throughput sequencing is still an expensive technology and this makes its
549 routine application to assess semen quality in swine unfeasible. However,
550 these costs are expected to keep decreasing in the years to come. This drop
551 on sequencing costs should allow the systematic implementation of
552 metagenomics to routinely assess the presence of pathogens and ARGs in the
553 boar sperm.

554

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574

575 **Conflicts of interest**

576 The authors declare no conflict of interest.

577

578 **Availability of data**

579 The datasets generated and analysed are available at NCBI's BioProject
580 PRJNA520978.

581

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712

713 **Tables:**

714 **Table 1.** List of the 20 most abundant bacteria in the sperm from the 40 Pietrain boars.

715

Species	Average abundance	Average percentage over all bacterial reads	Median abundance	CV	Maximum abundance	Minimum abundance
<i>Bacillus megaterium</i>	867.51	4.31	827.93	0.85	4,026.18	0.00
<i>Brachy bacterium faecium</i>	673.39	3.34	98.17	2.06	6,337.78	0.05
<i>Bacillus coagulans</i>	252.71	1.25	7.64	1.81	1,650.99	0.14
<i>Campylobacter hominis</i>	205.28	1.02	8.19	3.56	4,483.10	0.00
<i>Psychrobacter sp PRwf-1</i>	189.58	0.94	16.59	2.28	2,091.32	0.48
<i>Cutibacterium acnes</i>	154.74	0.77	87.77	1.81	1,771.66	8.76

<i>Methylothermobacter mobilis</i>	137.84	0.68	0.92	5.66	4,948.31	0.00
<i>Escherichia coli</i>	136.84	0.68	93.68	0.88	487.26	11.14
<i>Porphyromonas asaccharolytica</i>	136.19	0.68	4.72	3.48	2,614.58	0.00
<i>Ralstonia pickettii</i>	134.52	0.67	67.65	1.61	1,026.71	0.36
<i>Bacillus subtilis</i>	109.63	0.54	87.57	0.81	413.54	6.54
<i>Acinetobacter baumannii</i>	86.29	0.43	8.32	2.93	1,134.84	1.68
<i>Thauera</i> sp MZ1T	80.12	0.40	5.24	3.47	1,675.64	0.05
<i>Saccharomonospora viridis</i>	79.09	0.39	13.27	3.61	1,818.43	0.08
<i>Anaerococcus prevotii</i>	67.92	0.34	12.49	3.18	1,346.78	0.09
<i>Aequorivita sublithicola</i>	67.04	0.33	1.48	5.08	2,139.66	0.34
<i>Advenella kashmirensis</i>	61.93	0.31	3.48	2.87	994.21	0.00
<i>Ornithobacterium rhinotracheale</i>	60.96	0.30	1.44	5.55	2,145.52	0.02

<i>Intrasporangium calvum</i>	58.76	0.29	11.99	3.45	1,266.83	0.05
<i>Pusillimonas sp T7-7</i>	56.98	0.28	1.26	4.67	1,649.63	0.00

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717 CV: Coefficient of variation.

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730 **Table 2.** List of potential pathogens and antimicrobial resistance genes identified in the sperm from the 40 Pietrain boars.

Potential pathogen species	Average abundance	Median abundance	CV	Maximum abundance	Minimum abundance	Disease / health condition
<i>Escherichia coli</i>	136.84	93.68	0.88	487.26	11.14	Diarrhoea and high mortality in piglets
<i>Clostridioides difficile</i>	49.77	16.74	1.76	338.49	0.88	Diarrhoea in piglets
<i>Clostridium perfringens</i>	14.05	5.60	1.47	95.99	0.47	Chronic or acute enteritis in piglets. Sometimes also gangrene and sudden death in adults
<i>Clostridium botulinum</i>	7.67	2.92	1.71	69.73	0.33	Toxins produced by this bacteria cause a progressive flaccid paralysis, but pigs are very resistant to the toxin
<i>Mycobacterium tuberculosis</i>	7.46	0.00	2.60	84.45	0.00	Tuberculosis
<i>Mycoplasma hyopneumoniae</i>	6.24	5.48	0.78	23.39	0.82	Enzootic pneumonia
<i>Campylobacter jejuni</i>	5.86	0.05	3.11	90.93	0.00	Clinical signs are not always present but can cause a watery diarrhea with mucous and blood. Also, food-borne illness in humans
<i>Staphylococcus aureus</i>	4.71	3.23	1.16	32.5	0.2	Occasional cause of abscesses, arthritis, osteomyelitis, mastitis and skin conditions
<i>Erysipelothrix rhusiopathiae</i>	3.91	0.64	2.43	42.22	0.00	Erysipela: skin lesion and arthritis
<i>[Haemophilus] parasuis</i>	3.00	0.12	3.81	67.62	0.00	Glässer disease: polyserosistis and sporadic arthritis

<i>Streptococcus suis</i>	2.42	0.60	2.35	29.64	0.05	Streptococcal infection with pneumonia, septicemia, arthritis, etc. Zoonotic potential
<i>Listeria monocytogenes</i>	2.21	0.97	1.70	21.40	0.02	Rare systemic bacterial septicemia
Potential antibiotic resistant gene						
ARO:3003497_Neisseria_meningitidis_16S_rRNA_mutation_spectinomycin	27.85	21.95	1.11	189.86	2.31	
ARO:3004058_Staphylococcus_aureus_23S_rRNA_with_mutation_linezolid	125.07	103.89	0.78	400.27	5.16	
ARO:3004150_Escherichia_coli_23S_rRNA_with_mutation_chloramphenicol	316.72	198.07	1.01	1634.58	113.99	

731 CV: Coefficient of variation.

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736 **Table 3.** List of bacteria displaying significant differences between clusters.

Bacterial feature	Global average	Average cluster 1	Average cluster 2	Fold change	P-value	Adjusted P-value
d__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhodobacterales	130.18	161.53	36.13	1.13	5.21E-04	3.44E-02
d__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhodobacterales f__Rhodobacteraceae	129.64	160.94	35.72	1.13	5.02E-04	3.44E-02
d__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhodobacterales f__Rhodobacteraceae g__Rhodobacter	4.97	5.70	2.76	0.75	3.90E-04	3.44E-02
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pasteurellales	70.85	82.66	35.44	1.50	5.88E-04	3.44E-02
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pasteurellales f__Pasteurellaceae	70.85	82.66	35.44	1.50	5.88E-04	3.44E-02
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales	977.88	973.87	989.92	1.65	6.08E-04	3.44E-02
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales f__Moraxellaceae g__Acinetobacter	325.35	296.67	411.41	2.21	2.22E-05	5.45E-03
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales f__Moraxellaceae g__Acinetobacter s__Acinetobacter_baumannii	86.29	65.97	147.25	2.21	4.68E-05	8.60E-03
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales f__Moraxellaceae g__Acinetobacter s__Acinetobacter_sp_AD1	48.56	53.47	33.81	1.91	1.66E-04	2.20E-02

d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales	42.32	47.94	25.49	1.24	6.61E-06	2.43E-03
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Xanthomonadaceae	36.39	40.95	22.69	1.27	4.54E-06	2.43E-03
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Xanthomonadaceae g__Stenotrophomonas	6.85	6.93	6.61	1.11	2.10E-04	2.20E-02
d__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Xanthomonadaceae g__Stenotrophomonas s__Stenotrophomonas_maltophilia	6.85	6.93	6.61	1.11	2.10E-04	2.20E-02

737 d: domain; p: phylum; c: class; o: order; f: family; g: genus; s: species.

738 **Figure legends**

739 **Figure 1.** Distribution of overall bacteria abundance for each animal.

740 **Figure 2.** Stackplot of the phyla distribution across the 40 sperm samples. The most
741 abundant phyla were *Proteobacteria* followed by *Firmicutes*, *Actinobacteria* and
742 *Bacteroidetes*.

743 **Figure 3.** Data structure from compromise configuration after applying a clustering
744 using standard k-means with Link-HD. Cluster 1 (red) included 30 samples and cluster
745 2 (blue) 10 samples. Seven semen quality traits and 67 bacterial features were
746 associated to this structure.

747 **Supporting information**

748 **Supplementary Table S1.** List of bacterial agents and diseases available at the
749 Professional Pig Community pig333 site, the Pig Site and the Swine Health Information
750 Center.

751 **Supplementary Table S2.** RNA-seq statistics for each of the 40 Pietrain samples.
752 SD: Standard Deviation.

753 **Supplementary Table S3.** Full list of bacterial features and their abundances in the
754 40 Pietrain samples. CPM: Counts per Million reads; SD: Standard Deviation; d:
755 domain; p: phylum; c: class; o: order; f: family; g: genus; s: species.

756 **Supplementary Table S4.** Phenotypic values for the 7 semen quality traits for each
757 of the 40 samples. VIAB_5: percentage of viable sperm cells after 5 minutes of
758 incubation at 37 °C; VIAB_90: percentage of viable sperm cells after 90 min incubation

759 at 37 °C; ACRO_5: percentage of cells with abnormal acrosomes after the 5 min;
760 ACRO_90: 90 min incubation; ORT, percentage of viable cells after an osmotic stress
761 (Osmotic Resistance Test); MT_5: percentage of motile cells after 5 min; MT_90: 90
762 min incubation.

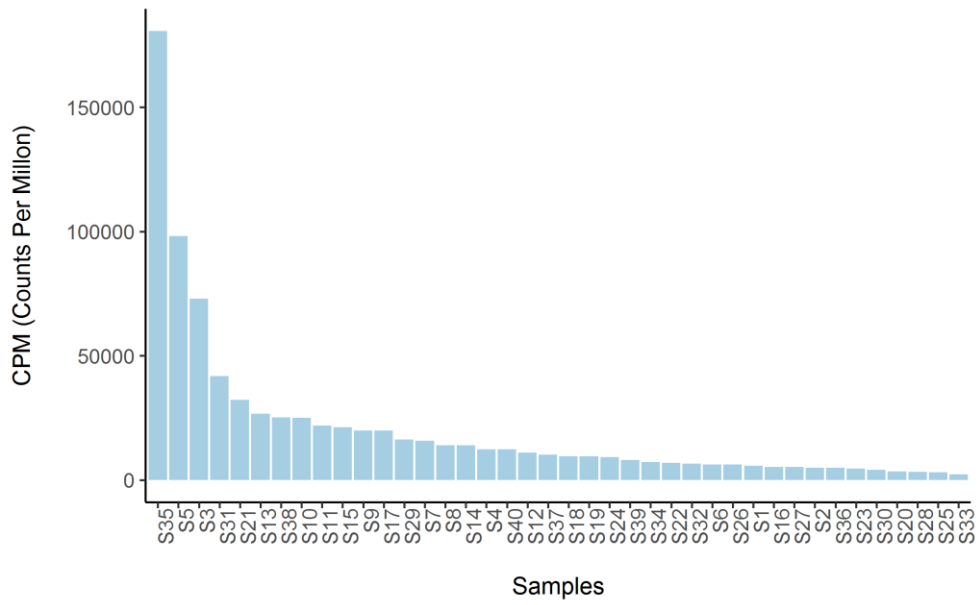
763 **Supplementary Table S5.** Detail of the samples ID belonging to each Link-HD cluster.

764 **Supplementary Table S6.** Detail of the traits and bacterial features contributing to the
765 Link-HD compromise. VIAB_5: percentage of viable sperm cells after 5 minutes of
766 incubation at 37 °C; VIAB_90: percentage of viable sperm cells after 90 min incubation
767 at 37 °C; ACRO_5: percentage of cells with abnormal acrosomes after the 5 min;
768 ACRO_90: 90 min incubation; ORT, percentage of viable cells after an osmotic stress
769 (Osmotic Resistance Test); MT_5: percentage of motile cells after 5 min; MT_90: 90
770 min incubation; d: domain; p: phylum; c: class; o: order; f: family; g: genus; s: species.

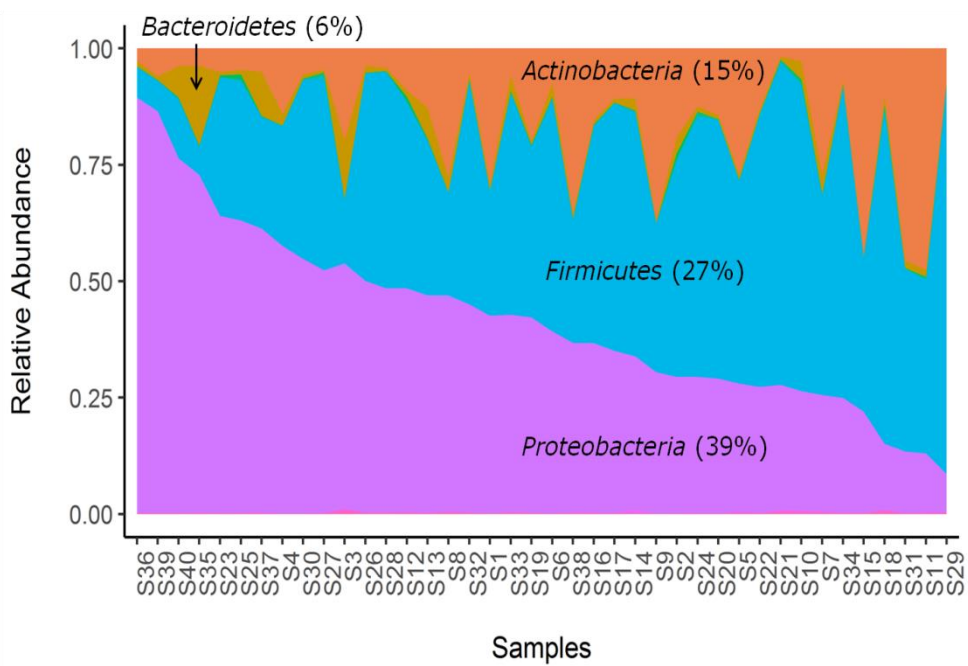
771 **Supplementary Table S7.** List of phenotypes displaying significant differences
772 between the 2 clusters distinguishing both groups. SD: Standard deviation; VIAB_5:
773 percentage of viable sperm cells after 5 minutes of incubation at 37 °C; VIAB_90:
774 percentage of viable sperm cells after 90 min incubation at 37 °C; ACRO_5:
775 percentage of cells with abnormal acrosomes after the 5 min; ACRO_90: 90 min
776 incubation; ORT, percentage of viable cells after an osmotic stress (Osmotic
777 Resistance Test); MT_5: percentage of motile cells after 5 min; MT_90: 90 min
778 incubation.

779 **Supplementary Figure S1.** Linear regression plots (R^2) of the abundance of the
780 antibiotic resistance genes (ARGs) and their related bacterial species.

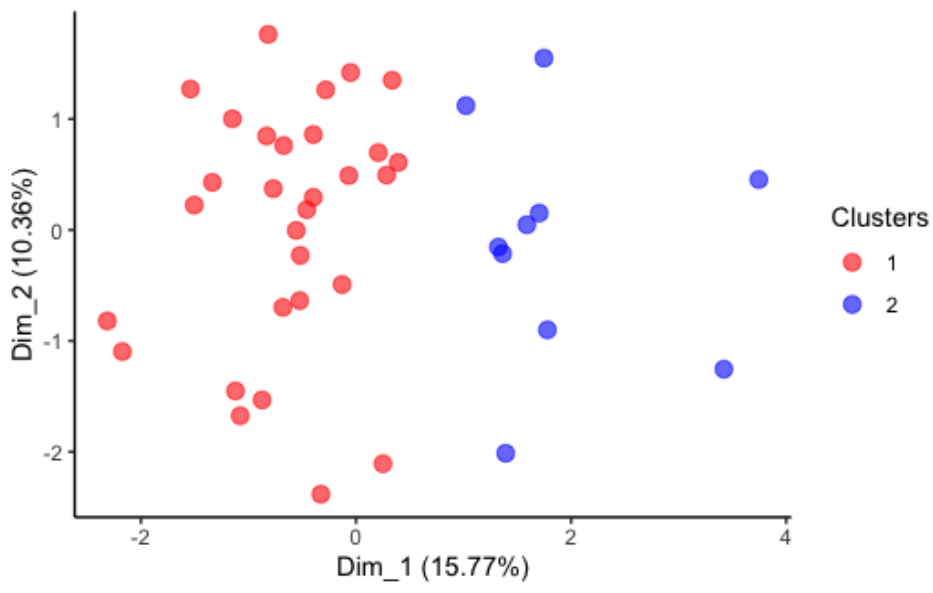
781



788 Figure 1.



789 Figure 2.



790

791 Figure 3.

792