Phylogenetic diversity analysis of shotgun metagenomic reads describes gut microbiome development and treatment effects in the post-weaned pig

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Abstract

Background: Intensive farming practices can increase exposure of animals to infectious agents against which antibiotics are used. Besides leading to antimicrobial resistance (AMR), orally administered antibiotics are well known to cause dysbiosis. To counteract dysbiotic effects, numerous studies in the past two decades sought to understand whether probiotics are a valid tool to help re-establish a healthy gut microbial community after antibiotic treatment. However, although dysbiotic effects of antibiotics are well investigated, little is known about the effects of intramuscular antibiotic treatment on the gut microbiome and a few studies attempted to study treatment effects using phylogenetic diversity analysis techniques. In this study we sought to determine the effects of two probiotic- and one intramuscularly administered antibiotic treatment on the developing gut microbiome of post-weaning piglets between their 3rd and 9th week of life.

Methods: Shotgun metagenomic sequences from over 800 faecal time-series samples derived from 126 piglets and 42 sows were analysed in a phylogenetic framework to characterise the developing gut microbial community composition of post-weaning piglets. We assessed the effects of intramuscular antibiotic treatment and probiotic oral treatment on the diversity of these gut microbial communities using alpha and beta diversity measures.

Results: Differences between individual hosts such as breed, litter, and age, were found to be important contributors to variation in the community composition. Host age was the dominant factor in shaping the gut microbiota of piglets after weaning. The post-weaning pig gut microbiome appeared to follow a highly structured developmental program with characteristic post-weaning changes that can distinguish hosts that were born as little as two days apart in the second month of life. Treatment effects of the antibiotic and probiotic treatments were found but were subtle and included a higher representation of *Mollicutes* associated with intramuscular antibiotic treatment, and an increase of *Lactobacillus* associated with probiotic treatment.

Discussion: The discovery of correlations between experimental factors and microbial community composition is more commonly addressed with OTU-based methods and rarely analysed via phylogenetic diversity measures. The latter method, although less intuitive than the former, suffers less from library size normalization biases, and it proved to be instrumental in this study for the discovery of correlations between microbiome composition and host-, and treatment factors.

Introduction

As the world population grows, there is an accompanying demand for animal-derived products. Intensive animal husbandry and early weaning practices are commonly used to maximize production rates while minimizing costs. In a semi-natural environment, pig weaning occurs between the 12th and the 17th week following birth whereas in intensively farmed pigs it typically occurs at 3-4 weeks of age [1,2]. In intensive pig production early weaning practices increase the risk of enteric infections [3–6], and thereby the need for antimicrobial strategies, which has included the common metaphylactic use of antibiotics [7,8]. Besides contributing to the concerning issue of antimicrobial resistance (AMR) build up, antibiotic usage causes dysbiosis [9–11], a disruption of a balanced state within a gut microbial community, which increases the chance of

60 pathogens gaining a foothold (*i.e.*: colonization of the host or overgrowth) [12,13]. Researchers
61 sought to determine the gut microbial modulatory effects of oral and in-feed antibiotic treatment
62 in the pig [10,11], mouse [14–16], and human [17], and only a few studies report the effects of
63 intramuscular (IM) antibiotic treatment on the gut microbial community of the pig [18,19].
64 Dysbiosis following antibiotic treatment of the young pig via intramuscular (IM) [20] and
65 subcutaneous [9] routes has been documented. It has been reported that a single dose of
66 intramuscularly administered amoxicillin in pigs, immediately after birth, causes long lasting
67 dysbiosis [21], but no studies, to our knowledge, assessed the effects of IM neomycin treatment on
68 the post-weaning pig gut microbiota. IM antibiotic use has a lower risk for inducing AMR than
69 oral antibiotics in livestock production [22–25], and according to a national survey of antibiotic use
70 in 197 large Australian commercial pig herds, 9.6% reported the use of injectable
71 apramycin/neomycin [7]. For these reasons it is relevant to the industry to assess if, and to what
72 extent, IM neomycin causes dysbiosis.
73 Probiotics may play a role in counteracting the dysbiotic effects of antibiotics. Probiotic
74 treatment has been reported to affect the physiology of the host by improving mucosal integrity
75 [26–29], inducing competitive exclusion against pathogenic species [30–35], reducing intestinal
76 inflammation [26,36,37], and pathogen translocation [26,38,39]. In terms of microbial community
77 modulating potential of probiotics, studies in swine using culture based methods reported the
78 reduction of pathogenic species [32,34,40–48] and an increase of *Lactobacillus* species [43,47–49]
79 following the administration of probiotic formulas. However, only in the past decade have
80 advances in and higher accessibility to high-throughput sequencing techniques enabled
81 researchers to better characterize microbial dynamics following probiotic treatment. Several of
82 these studies used the amplification of the bacterial 16S rDNA V4 region, determining the
83 effects of probiotics on the gut microbial community of swine [50–53]. The choice of shotgun
84 metagenomic sequencing over amplicon sequencing, in this study, was dictated by the use of this
85 dataset outside of the scope described in this manuscript. In this study we assessed the effects of
86 two probiotic formulas on the gut microbial community of post-weaning piglets undergoing a
87 two-weeks probiotic treatment immediately after weaning. To assess the effects on the microbial
88 community of the administration of probiotic following antibiotic treatment, two treatment
89 groups were administered IM neomycin treatment followed by two weeks of either of the two
90 probiotic formulae.

The data was analysed from the perspective of the phylogenetic diversity of microbial communities. Firstly, we describe the phylogenetic diversity of microbial communities, and secondly, the role of age, breed and litter in microbial community composition. Finally, we describe the temporal effects on microbial communities, and the associated effects of IM neomycin and probiotic treatments.

Materials & Methods

Animal trial

Metagenomic samples were derived from a pig study conducted at the Elizabeth Macarthur Agricultural Institute (EMAI) NSW, Australia, and approved by the EMAI Ethics Committee (Approval M16/04). Below, we briefly summarise the origin of the samples, with comprehensive details on the animal trial and sample workflow being described previously [54].

Post-weaning piglets (*n*=126) derived from a large commercial pig herd were transferred to the study facility in January 2017. Piglets, aged 22.5±2.5 days at the start of the trial, consisted of 4 main cross-breed types: "Duroc × Landrace" (*n*=46), "Duroc × Large White" (*n*=59), "Landrace × cross bred (LW×D)" (*n*=9), "Large White × Duroc" (*n*=12); and three pig lines (line 319: *n*=9; line 316: *n*=46; line 326: *n*=71) [54]. The study facility consisted of 4 environmentally controlled rooms (Rooms 1–4) with air conditioning, concrete slatted block flooring with underground drainage, and open rung steel pens. The floor was swept daily and the under-floor drainage was flushed twice weekly. A rubber ball was added to each pen for environmental enrichment. Piglets were fed *ad libitum,* a commercial pig grower mix of 17.95% protein, free of antibiotics.

The piglets were distributed over 6 treatment cohorts: a placebo group (Control *n*=29); two probiotic groups (D-Scour *n*=18; ColiGuard *n*=18); one antibiotic group (Neomycin *n*=24) and two antibiotic-then-probiotic treatment groups (Neomycin+D-Scour *n*=18; Neomycin+ColiGuard *n*=18). The commercial probiotic paste preparation D-Scour™ was sourced from International Animal Health, Australasia, and is composed of 1.8 10^8 CFU/g of each of the following: *Lactobacillus acidophilus, Lactobacillus*

120 *delbrueckii* subspecies *bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus,*
121 *Bifidobacterium bifidum, E. faecium*, and *Streptococcus salivarius* subspecies *thermophilus*, with
122 an additional 20mg/g of garlic extract (*Allium sativum*). ColiGuard® is a probiotic formulation
123 developed for the treatment of entero-toxigenic *Escherichia coli* (ETEC) in weaner pigs,
124 developed in collaboration between the NSW DPI and International Animal Health Product, and
125 contains undefined concentrations of: *L. plantarum* and *Lactobacillus salivarius*. In addition, an
126 in-house developed mock community was used as a positive control, composed of *Bacillus*
127 *subtilis* strain 168, *Enterococcus faecium, Staphylococcus aureus* ATCC25923, *Staphylococcus*
128 *epidermidis* ATCC35983, *Enterobacter hormaechei* CP_032842, *Escherichia coli* K-12
129 MG1655, and *Pseudomonas aeruginosa* PAO1, in the following proportions:
130 8.7:13.0:7.7:16.7:38.9:14.5:0.4.
131 Groups of piglets were euthanized at the start (t0: *n*=6), a week after (t2: *n*=12), two weeks
132 after the start of the trial (t4: *n*=36) and at the end of the trial (t10: *n*=72) to obtain biopsy
133 samples that were used in another study. All surviving animals were euthanized at the end of the
134 animal trial. Euthanasia occurred as follows: the piglets were restrained in dorsal recumbency in
135 a bleeding cradle and pentobartitone euthanasia solution (Lethabarb, Virbac Australia, 325 mg
136 pentobarbitone sodium/mL) diluted 1:1 in sterile normal saline was administered via the precava
137 using a 20-21G needle depending on pig weight. The piglets received a dose of approximately 30
138 mg pentobarbitone/Kg to achieve deep anaesthesia, and then immediately exsanguinated.
139 Exsanguination after a non-lethal dose of barbiturate was undertaken to reduce excessive
140 congestion of the visceral blood vessels at the time of intestinal specimen collection (which is
141 likely to occur if euthanasia is undertaken using an overdose of barbiturate alone).
142 Faecal sampling occurred twice weekly for a subset of the piglets (8 per cohort; *n*=48), while
143 it occurred weekly for all the living piglets (*n*=126; see euthanasia groups described above). In
144 addition, 42 faecal samples derived from the piglets' mothers (*n*=42), 18 samples derived from
145 three distinct positive controls (D-Scour™ , ColiGuard®, and mock community), and 20 negative
146 controls were included.
147 A thorough description of the animal trial, and the metadata containing behaviour, weight,
148 and faecal consistency scores recorded over the 6-week period of the trial, is available in our
149 previous work [54].
150

151 ***Metagenomic samples processing***

152 Samples underwent homogenization and storage at -80 ºC. After sample thawing, DNA
153 extraction was performed with the PowerMicrobiome DNA/RNA EP kit (Qiagen), and libraries
154 were prepared using the Hackflex method [55]. Sequencing was performed on three Illumina
155 NovaSeq S4 flow cells, after library normalization and pooling. The data is deposited to the
156 NCBI Short Read Archive under project PRJNA526405 and http://dx.doi.org/10.5524/100890 [54].
157 Samples were assessed for quality using FASTQC
158 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and a combined report of all
159 samples was obtained with MULTIQC [56] (available in our GitHub repository
160 https://github.com/GaioTransposon/metapigs_phylodiv).
161

162 ***Determination of microbial diversity among samples***

163 Phylogenetic diversity of all samples was assessed with PhyloSift [57] using the first 1M read
164 pairs of each sample (parameters: `--chunk-size 1000000 --paired`) (script:
165 *phylosift.nf*). In addition, a separate analysis with PhyloSift [57] was performed using a smaller
166 downsampling, by including only the first 100K read pairs of each sample (parameters: `--`
167 `chunk-size 100000 --paired`). In order to test for associations of phylogenetic
168 diversity with treatment, time of sampling, and differences among hosts at the start of the trial
169 (first post-weaning sample collection time point), analysis of the unrooted phylogenetic diversity
170 (PD) [58], the balance weighted phylogenetic diversity (BWPD) [59] and principal component
171 analysis (PCA) of the Kantorovich-Rubinstein distances [60] (beta diversity analysis) were
172 performed. Collections of phylogenetic placements produced by PhyloSift are compared using
173 guppy [60] to produce the Kantorovich-Rubenstein distances output. The collections of
174 phylogenetic placements are grouped based on the variable of interest (*i.e.*: treatment, time of
175 sampling, and differences among hosts at the start of the trial), and consequently the selection is
176 fed to guppy. Guppy is used in two modalities: 1. `guppy epca` to obtain edge principal
177 components; 2. `guppy fat` to annotate the edges of the phylogenetic tree using the relative
178 abundance of reads placed on each lineage. Alpha-diversity and beta-diversity were analysed and
179 the results were visualized with R [61] and R packages [62–76]. Scripts for the analysis are available at

180 https://github.com/GaioTransposon/metapigs_phylodiv. The data analysis workflow is
181 schematically represented in **Figure 1**.

182 All samples were included in the analysis, except for the analysis of treatment effect, where,
183 in order to minimize the effect of age as a confounding factor, we excluded animals born on
184 2017-01-06 and on 2017-01-07. In this manner the largest age gap between animals was 3 days,
185 with animals born on 2017-01-08 (*n*=24), 2017-01-09 (*n*=24), 2017-01-10 (*n*=23), and 2017-01-
186 11 (*n*=39). As a result, sample sizes at each time point were the following: Control (t0: 26; t2:
187 20; t4: 15; t6: 12; t8: 12; t10: 12), D-Scour™ (t0: 15; t2: 15; t4: 15; t6: 10; t8: 12; t10: 10),
188 ColiGuard® (t0: 16; t2: 16; t4: 16; t6: 12; t8: 12; t10: 12), neomycin (t0: 22; t2: 22; t4: 17; t6: 12;
189 t8: 12; t10: 12), neomycin+D-Scour™ (t0: 17; t2: 17; t4: 17; t6: 12; t8: 12; t10: 12), and
190 neomycin+ColiGuard® (t0: 14; t2: 13; t4: 14; t6: 10; t8: 10; t10: 10).

191 Analysis of raw reads with SortMeRNA[77] (version 4.0.0) was performed as described in
192 Gaio *et al* (2021) [54]. Briefly, raw reads were mapped against the rRNA reference database silva-
193 bac-16s-id90.fasta (parameters: `--fastx --blast 1 --num_alignments 1`) (script:
194 *sortmerna.sh*) and filtered based on e-value (e-value <= 1e-30), identity (identity ≥ 80%), and
195 alignment length (length ≥ 100 bp) (script: *sortmerna_filter.sh*). [54] The output from
196 SortMeRNA[77] was used to compute Principal Component Analysis (PCA) with R [61] (script:
197 *08_sortmerna.R*). Sample counts were normalized for library size by proportions and were tested
198 with the Spearman's Rank correlation coefficient method to find lineages correlating with the
199 weight of the piglets across the trial (script: *08_sortmerna.R*).

200

201 ***Batch effects***

202 A randomized block design was adopted to mitigate batch effects. Because samples were
203 distributed across ten 96-well plates during DNA extraction and library preparation, plate effects
204 were expected. Although samples did not visibly cluster by DNA extraction plate across the first
205 five principal components, a batch effect was found by multiple comparison analysis with
206 ANOVA (alpha diversity: *p* range=0.0001-1; beta diversity: *p* value<0.0001) (**Supplementary**
207 **Figure 1**). Significance of pairwise comparisons was obtained by running Tukey's *post-hoc*
208 analysis (**Supplementary Table 1**). Batch effects were removed with ComBat [78] (script:

209 *02_phylodiv.R*). ComBat relies on robust empirical Bayes regression to remove heterogeneity
210 due to the use of batches (in this study a batch is a 96-well plate) while maintaining the
211 biological variance among samples.[78] The input data used for ComBat in this study is not subject
212 to normalization as it consists of phylogenetic diversity values obtained from a defined number
213 of read-pairs per sample. A parametric adjustment was used in the batch effect correction
214 (`par.prior=TRUE`) and both means and variances were adjusted (`mean.only=FALSE`).
215

216 ***Data availability***

217 The raw sequencing data has been submitted to the NCBI Short Read Archive under project
218 PRJNA526405 and is also available through GigaDB at http://dx.doi.org/10.5524/100890. A
219 thorough description of this dataset and the metadata has been reported previously [54]. Scripts
220 used for the data analysis in this study are available in our GitHub repository
221 https://github.com/GaioTransposon/metapigs_phylodiv.
222

223 **Results**

224 PhyloSift [57] was employed as a means to study microbial community diversity among the
225 samples, and to test for associations with treatment, time of sampling, and differences among
226 hosts during the first week post-weaning. To this end, analysis of the unrooted phylogenetic
227 diversity (PD) [58], the balance weighted phylogenetic diversity (BWPD) [59], and principal
228 component analysis (PCA) of the Kantorovic-Rubinstein distances [60] (beta diversity analysis)
229 were performed.

230

231 ***Phylogenetic diversity of positive controls***

232 Unrooted PD was highest for positive control D-Scour™ (mean±SD=162.2±28.3), slightly lower
233 for positive control ColiGuard® (mean±SD=129.9±50.0) and lowest for the mock community
234 (mean±SD=123.6±28.4). BWPD was highest for positive control D-Scour™ (mean±SD:
235 1.9±0.2), lower for the mock community (mean±SD: 1.6±0.1), and lowest for positive control
236 ColiGuard® (mean±SD: 0.9±0.1). (**Supplementary Table 1**; **Supplementary Figure 2**).

237 Principal component analysis (PCA) of the Kantorovich-Rubenstein distances (beta
238 diversity analysis) was performed on positive control samples. Samples clearly separated in PC1
239 (accounting for 83.62% of the variation), where mock community samples clustered in the lower
240 end of principal component 1 (PC1) showing a higher representation of *Enterobacteriaceae* and
241 *Pseudomonadaceae*, while ColiGuard® and D-Scour™ samples clustered in the higher end of
242 PC1, showing a higher representation of *Lactobacillales*. D-Scour™ samples clustered in the
243 higher end of principal component 2 (PC2) (accounting for 14.52% of the variation), showing a
244 higher representation of *Lactobacillus delbrueckii*, *Enterococcus faecium*, and *Bifidobacterium*,
245 while in the lower end of PC2, mock community samples and ColiGuard® samples, forming two
246 separate clusters, showed a higher representation of *Bacilli*, *Enterobacteriaceae*, and
247 *Gammaproteobacteria*. (**Supplementary Figure 3**).

248

249 ***Phylogenetic diversity of piglet gut communities: host factors***

250 Based on Kruskal-Wallis one-way analysis of variance (Hommel adjusted p values to correct for
251 multiple hypotheses testing), alpha diversity of the piglet samples did not cluster significantly by
252 cross-breed type ($p > 0.05$) or by pig line ($p > 0.05$) in the first week post-weaning (**Figure 2;**
253 **Supplementary Table 1**). A correlation of cross-breed type was found with beta diversity
254 (principal component 3) at t2 (p=0.024). (**Figure 2**; **Supplementary Table 1**)

255 The piglets differed slightly by age, being born between 1 and 5 days apart. Notably, we
256 found a significant correlation between alpha diversity and the age of the piglets at the first
257 sampling time point (unrooted PD: $p < 0.0001$; BWPD: p=0.011) (**Figure 2**; **Supplementary**
258 **Table 1**) and between age and beta diversity at t2 (PC3: p=0.047) and t7 (PC3: p=0.018) (**Figure**
259 **2**; **Supplementary Table 1**). As age groups were confounded with cross-breed types (*i.e.* not all
260 age groups are represented by each of the four cross-breed types), we compared the phylogenetic
261 diversity of age groups within each breed. As cross-breed types "Landrace × cross bred (LW ×
262 D)" and "Large White × Duroc" had only a small number of piglets in each age group (n=9 and
263 n=12, respectively), we tested for an association between phylogenetic diversity and age in
264 cross-breeds "Duroc × Landrace" and "Duroc × Large White" (n=46 and n=59, respectively).
265 Among these cross-breeds, age in the "Duroc × Landrace" piglets (n=46) correlates with alpha
266 diversity during the first week post-weaning (unrooted PD: p=0.006; BWPD: p=0.047) and with

267 beta diversity at t2 (PC2: p=0.048) (**Figure 2**; **Supplementary Figure 4**; **Supplementary Table**
268 **1**).

269 As piglets were derived from 42 distinct sows (maternal sows) and nursed by either the
270 same or a different sow (a nurse sow), a litter effect was expected and determined. Based on
271 Hommel adjusted p values, a similarity of alpha phylogenetic diversity can be seen among
272 piglets from the same maternal sow (unrooted PD: p=0.001; BWPD: p=0.017) and in piglets
273 from the same nurse sow (unrooted PD: p=0.002; BWPD: p=0.027) (**Figure 2**; **Supplementary**
274 **Table 1**). The litter effect described was found significant only in samples from the first week
275 post-weaning as the significance of the correlations did not persist thereafter (**Figure 2**;
276 **Supplementary Table 1**).

277

278 ***A strong effect of aging on phylogenetic diversity***

279 Beta diversity analysis of all samples revealed a distinct and consistent change of the microbial
280 community over time in all piglets, regardless of the treatment. Beta diversity analysis was
281 performed from all reads and from the analysis of 16S rDNA V4 region-containing reads.
282 Samples collected immediately after weaning (t0) were characterized by a higher representation
283 of *Bacteroidetes* chlorobi group and *Clostridia* (PC1, 47.68%), particularly of *Sedimibacterium*
284 and *Desulfosporosinus* (PC2 17.2%), respectively. Between day 0 (t0; immediately after
285 weaning) and day 14 (t4), samples shifted towards a higher representation of *Bifidobacterium*
286 and *Lactobacillus*, as measured from beta diversity analysis (PC1; 47.68% var. explained) and
287 from analysis of 16S rRNA reads (PC1 and PC2; 23.9% and 17.2% var. explained, respectively).
288 During the last two weeks (t6-t10), corresponding to day 21 and 35 of post-weaning, samples
289 shifted towards a higher representation of *Actinobacteria*, (PC2 21.79% var. explained),
290 particularly of *Collinsella* (PC1 23.9%, PC2 17.2%). Lineages of the *Erysipelotrichales*
291 *Catenibacterium* and *Solobacterium* were also found to be representative of samples of this time
292 interval (t6-t10). (**Figure 3**).

293 Beta diversity analysis was performed separately for samples within each time point in order
294 to find lineages associated with variation within each time point. Extent of variation was derived
295 from the product of branch width by the variation explained by the principal component (**Figure**

296 **4**). The lineages *Enterobacteriaceae* (t0=0.05) and *Bacteroides* (t0=0.06) were responsible for
297 variation only during the first week after weaning, and *Methanobrevibacter smithii* during the
298 first and the second week (t0=0.05; t2=0.03). The *Bifidobacterium* lineage was responsible for
299 variation in the second week (t2=0.09). The following lineages were responsible for variation
300 throughout the 6 weeks after weaning: *Bacteroidales* (min=0.04; max=0.20), *Prevotellaceae*
301 (min=0.02; max=0.13), *Coriobacteriaceae* (min=0.01; max=0.09). *Lactobacillus* became
302 variable after the first week post-weaning and remained highly variable throughout the rest of the
303 trial (min=0.03; max=0.30) (**Figure 4**).

304 Taxonomic representation in terms of relative abundance was derived from the branch width
305 of the phylogenetic tree (**Supplementary Figure 5**) and combined with PhyloSift's taxonomic
306 annotation of the marker gene phylogeny. *Lactobacillus acidophilus* increased at the start
307 (t0=0.00; t2=0.07; t4=0.17) then decreased (t6=0.11; t8=0.06; t10=0.08). Among other prevalent
308 lineages during the week after weaning, up to the next week and dropping at later time points, we
309 found *Methanobrevibacter smithii* (t0=0.05; t2=0.03) and *Bacteroidales* (t0=0.01; t2=0.01).
310 Following an opposite trend we found *Ruminococcus* sp. JC304 (t0=0.00; t2=0.00; t4=0.01;
311 t6=0.03; t8=0.03; t10=0.03), *Solobacterium moorei* (t0=0.00; t2=0.01; t4=0.02; t6=0.03;
312 t8=0.02; t10=0.03) and *Prevotella copri* (t0=0.00; t2=0.00; t4=0.05; t6=0.03; t8=0.06; t10=0.03).
313 In modest and stable abundance across the post-weaning period were the following lineages of
314 the order of the *Clostridiales*: *Mogibacterium* sp. CM50 (t0=0.03; t2=0.05; t4=0.03; t6=0.04;
315 t8=0.03; t10=0.04), *Oscillibacter* (t0=0.08; t2=0.06; t4=0.04; t6=0.03; t8=0.04; t10=0.03),
316 *Subdoligranulum variabile* (t0=0.07; t2=0.03; t4=0.05; t6=0.07; t8=0.07; t10=0.08), and
317 *Ruminococcus bromii* (t0=0.03; t2=0.02; t4=0.01; t6=0.01; t8=0.1; t10=0.01). In transient
318 abundance we found *Bifidobacterium thermophilum* RBL67 (t2=0.02; t4=0.03). Gradually
319 increasing from the second week we found *Eubacterium biforme* DSM3989 (t2=0.02; t4=0.02;
320 t6=0.03; t8=0.03; t10=0.02), *Eubacterium rectale* (t4=0.03; t6=0.03; t8=0.03; t10=0.01) and,
321 after the third week, *Faecalibacterium prausnitsii* (t6=0.01; t8=0.01; t10=0.01).
322 (**Supplementary Figure 5**)

323 The effect of time was also clear in alpha diversity, where all the piglet cohorts followed a
324 similar trend over time, independent of the treatment (**Supplementary Figure 6**;
325 **Supplementary Table 1**). Immediately after weaning (t0), the unrooted PD was lower for the

326 piglets than the sows (sows: 328.5±24.0; piglets: 296.5±34.7) and reached a higher unrooted PD
327 to the sows' in the following week (t2) (piglets: 336.18±33.0) (**Supplementary Table 1**). In
328 comparing four timepoints at one-week intervals from the start of the trial, changes in alpha
329 diversity among all the piglets were tested for and significance was determined using the
330 Bonferroni correction. Unrooted phylogenetic diversity increased in the first week following
331 weaning (t0-t2: +9.15%; $p<0.001$) and decreased in the following week (t2-t4: -4.54%; $p<0.001$)
332 amongst the piglets. In contrast, BWPD decreased in the week after weaning (t0-t2: -6.07%;
333 $p<0.001$), to increase in the following week (t2-t4: +4.77%; $p<0.001$) and decrease in the fourth
334 week (t4-t6: -3.67%; $p=0.002$). (**Supplementary Figure 6**; **Supplementary Table 1**).

335

336 ***Effect of antibiotic and probiotic treatment on alpha diversity***

337 We hypothesized that the probiotic treatments, whether alone (D-Scour™ and ColiGuard®) or
338 administered after neomycin (neomycin+D-Scour™ and neomycin+ColiGuard®) would cause a
339 change in the microbial community composition that would be measurable via phylogenetic
340 diversity. We tested whether the treatments correlated with a change in phylogenetic diversity
341 independently of the changes occurring with time. Given the differences in alpha and beta
342 diversity detected among the subjects after weaning, we analyzed the deltas of phylogenetic
343 diversity instead of relying on the absolute means, similar to the procedure applied by Kembel *et*
344 *al* (2012) [79]. Time-point measurements of alpha diversity were taken, and deltas were computed
345 for each piglet. Delta means were compared between cohorts, where the control cohort would
346 serve as a control group for neomycin, D-Scour™ and ColiGuard® cohorts, whereas the
347 neomycin cohort would serve as a control group for the neomycin+D-Scour™ and
348 neomycin+ColiGuard® cohorts.

349 One week after weaning, 90% of the piglets displayed an increase in unrooted PD and 71%
350 displayed a decrease of BWPD. The following week the trend was opposite: 72% of the piglets
351 displayed an increase of BWPD and 76% displayed a decrease of unrooted PD (**Supplementary**
352 **Table 1**). However, the neomycin cohort displayed the smallest BWPD drop in the week
353 following weaning, and the overall trend of neomycin in unrooted PD was the most different
354 from the other cohorts (**Supplementary Figure 6**). Due to the lower drop in BWPD of
355 neomycin, significance was found in BWPD during the first week between neomycin and

356 Neomycin+ColiGuard® (t0-t2; Tukey adjusted *p* value=0.031), and between neomycin and
357 ColiGuard® (t0-t2; Tukey adjusted *p* value=0.041). No other significant differences were found
358 in changes in alpha diversity between time points among the cohorts. (**Supplementary Table 1**)

359

360 ***Effect of antibiotic and probiotic treatment on beta diversity***

361 To investigate the treatment effect on beta diversity, principal component analysis (PCA) of the
362 Kantorovich-Rubenstein distances (beta diversity analysis) was performed on all samples and,
363 additionally, on samples within individual time points. This analysis is conceptually similar to
364 the weighted Unifrac approach for beta diversity analysis, but is designed to work with
365 phylogenetic placement data [60]. When examining all samples together, there was no clear
366 separation of cohorts on any of the five principal component axes. When individual time points
367 were analysed, some clustering by cohort was observed (**Supplementary Figure 7**). D-Scour™
368 separated from the control cohort in PC3 (10.97%) during the first week of probiotic treatment
369 (t2) (**Supplementary Figure 7**). ColiGuard® separated from control during the first week of
370 probiotic treatment (t2) in PC5 (2.82%), and two weeks after the end of probiotic treatment in
371 PC3 (7.87%), showing a higher representation of *Lactobacillus*.

372 Two weeks after the end of neomycin treatment (t6), neomycin separated from control in
373 PC4 (5.65%), with a smaller clustering and a higher representation of *Mollicutes*. Neomycin+D-
374 Scour™ separated from neomycin in PC2 (17.80%) and PC4 (5.65%) at the end of probiotic
375 treatment (t6), as well as a week later (t9) in PC5 (3.93%). In these instances, neomycin clustered
376 towards a higher representation of *Mollicutes*, while Neomycin+D-Scour™ showed a higher
377 representation of *Lactobacillus*. (**Supplementary Figure 7**)

378

379 ***Association between weight and community composition***

380 Weight correlated with the abundance of certain taxa at each time point as it resulted from
381 principal component analysis of 16S rRNA reads. Positively correlating with weight we found
382 among others: *Blautia* (t0), *Cetobacterium* (t0), *Lactobacillus* (t6), *Mycoplasma* (t6),
383 *Anaerostipes* (t8), *Ruminococcus* (t8), *Cerasibacillus* (t10). Negatively correlating with weight
384 we found among others: *Pyramidobacter* (t0), *Odoribacter* (t2), *Schwartzia* (t6), *Streptococcus*

385 (t6), *Dokdonella* (t8). Correlations remained significant after *p* value correction for multiple
386 hypotheses testing (Hommel, and Benjamini & Yekutieli), but only one taxa
387 (*Coprothermobacter*; t0) remained significantly, positively correlated with host weight after
388 applying the more stringent Bonferroni correction (**Supplementary Table 1**).

389

390 ***Effect of treatments on weight gain***

391 Overall weight gain from initial to final weight (**Supplementary Table 1**) was not significantly
392 affected by any treatment. However, the probiotic ColiGuard® was found to have a partial effect
393 on piglet weight gain (**Supplementary Figure 8**; **Supplementary Table 1**). Weight was
394 measured weekly for a total of six measurements. To minimize age as a confounding factor from
395 this analysis, we kept samples from piglets that were born a max of 3 days apart. Based on Tukey
396 adjusted *p* values, a lower weight gain was detected in the ColiGuard® cohort compared to the
397 control cohort between the last day of probiotic treatment and a week after treatment (t4-t6)
398 (p=0.015) (**Supplementary Figure 8**; **Supplementary Table 1**). Similarly, a lower weight gain
399 was detected in the neomycin+ColiGuard® cohort between the 9th day of treatment and the 9th
400 day after probiotic treatment (t4-t8) compared to the neomycin cohort (p=0.011)
401 (**Supplementary Figure 8**; **Supplementary Table 1**). Age and cross-breed differences among
402 piglets were not associated with weight gain (**Supplementary Table 1**).

403

404 ***Estimation of phylogenetic diversity using sample downsampling sizes: 100K***
405 ***versus 1M***

406 We compared unrooted PD and BWPD values obtained using either 100,000 or 1,000,000 reads
407 per sample (corresponding to 0.612% or 6.12% of the average sample). The median unrooted PD
408 was 2.6x higher, and variance improved when the analysis was run from 1M reads per sample,
409 compared to the analysis run using 100K reads per sample (100K: 121±18.67; 1M: 317±50.67;
410 Pearson's r=0.8965), while the median BWPD remained nearly unchanged (100K: 2.11±0.203;
411 1M: 2.11±0.193; Pearson's r=0.9747). Comparing beta diversity values obtained using either
412 100,000 or 1,000,000 reads per sample, we found a high correlation for all five principal
413 components (Pearson's r range=0.9809-0.9956). (**Supplementary Figure 9**)

414

415 **Discussion**

416 The microbial composition of positive control samples was analysed from a taxonomic
417 perspective in our previous study [54], and it was here compared to the phylogenetic diversity
418 obtained for these samples. The unrooted PD reflects the absolute diversity, independently of the
419 relative abundance of each species, within a sample. In fact, low-level contamination (<0.1%)
420 detected in each of the positive controls (mock community: 1 taxon; ColiGuard®: 20 taxa; D-
421 Scour™ : 25 taxa) [54] contributes toward the absolute diversity in the unrooted PD, inflating this
422 value. Unrooted PD values obtained for the positive controls were directly proportional to their
423 respective count of taxa, which include contaminants (D-Scour™ : *n*=33; ColiGuard®: *n*=22;
424 mock community: *n*=8) [54]. On the other hand, the contribution to total diversity of phylogenetic
425 tree edges with uneven quantities of reads placed on either side is down-weighted in BWPD. The
426 fact that ColiGuard®, mock community, and D-Scour™ are mainly composed of 2, 7, and 8 taxa
427 taking up even proportions in the samples, respectively (hence excluding contaminants) [54], was
428 reflected by their lowest, higher, and highest BWPD measured from these samples.

429 The consistent trend in community composition over time, across all the cohorts,
430 indicates that an age-related process of ecological succession is the largest factor shaping the
431 microbial community of post-weaning piglets, as found in this study where animals aged 20-63
432 days were fed the same diet. A peak in unrooted phylogenetic diversity and drop in balance
433 weighted phylogenetic diversity (BWPD) reflects the acquisition of new species with the loss of
434 dominating species. Similarly to Pollock *et al* (2018) [80], we found the relative abundance of
435 *Clostridiales*, and *Lachnospiraceae* to decrease from the start of weaning to 2 weeks into
436 weaning, while the relative abundance of *Lactobacillus*, *Prevotella copri*, *Faecalibacterium*
437 *prausnitsii* and *Erysipelotrichaceae* increased. The increase of *Prevotella* with weaning [80–82] is
438 well documented and it is associated with the increased polysaccharide consumption associated
439 with the start of solid food consumption [81,83]. The relative abundance of *Lactobacillus* goes up
440 with weaning, as other studies suggested [80,81]. However, according to our analysis, this increase
441 concerns *Lactobacillus acidophilus*, while other *Lactobacillus* species, such as *Lactobacillus*
442 *vaginalis* ATCC follow the opposite trend. We also found a clear gradual increase in abundance

443 of *Solobacterium moorei* and *Eubacterium biforme* DSM3989, a transient increase of
444 *Bifidobacterium thermophilum* RBL67 during the second and the third week post weaning, and a
445 sharp decrease in the abundance of *Methanobrevibacter smithii* after the second week post-
446 weaning.

447 The change in phylogenetic diversity detected in the week following the piglets' arrival at
448 the trial site irrespective of the cohort, could be linked to the piglets being subjected to microbial
449 interchange (*e.g.*: new pen mates) and/or to diet transition (peri-weaning transition) to solid food
450 leading to the reshaping of the gut microbial community. [84,85] The week following the drop of
451 BWPD, a significant increase of BWPD was recorded, reflecting the acquisition of a larger
452 proportion of the community by the newly introduced species. The strong changes in
453 phylogenetic diversity detected in the first and the second week could as well be attributable to
454 other post-weaning related physiological changes, as previous studies report [84–87].

455 The highest inter-individual differences among piglets are seen in the first week of life,
456 irrespective of maternal or environmental effects. The microbiota of three week old piglets is still
457 very dynamic, but environmental factors become evident [85]. At six weeks of age, CD8+ T cells
458 infiltrate the intestinal tissue and the mucosa and intestinal lining resemble that of an adult pig [79].

459 The increase of alpha diversity associated with weaning has been measured before [81,82,88], but
460 rarely using metrics that allow the distinction of absolute diversity from evenness. In this study,
461 one week after weaning, piglets reached a comparable absolute diversity to the sows, at which
462 time the piglets were aged between 3.8 and 4.6 weeks. Unrooted PD did not reach higher levels
463 at later sampling time points. The highest BWPD accompanied by a high unrooted PD was
464 reached after the second week post-weaning when piglets were aged between 4.9 and 5.6 weeks.
465 Age-dependent physiological changes could explain i) the major shifts we detected in alpha
466 diversity during the first two weeks after weaning and, ii) the distinct differences in community
467 composition with age, even with a narrow age difference between piglets (1-5 days). We found a
468 significant difference in microbial composition in both absolute diversity as in balance-weighted
469 PD, only in the first week after weaning (piglets aged between 3.8 and 4.6 weeks), between
470 groups of piglets that were separated by up to five days maximum by day of birth. Since age
471 groups were confounded with breeds in our study, we attempted to determine the correlation

472 within single cross-breeds. Unfortunately, although the correlations with age could still be
473 detected, we could not determine the association at later time points due to the introduction of
474 treatment effects.

475 Animal trials are often conducted in controlled environments to minimize environmental
476 effects. However, individual variations such as breed and age are often unavoidable in large
477 animal trials, especially involving animals derived from commercial herds. Previously reported
478 confounding factors include: individual variation, cohabitation, age, maternal effects, hormones,
479 behavioural differences between breeds (*e.g.* coprophagy, mouth to mouth contact) and extent of
480 long-term behavioural adaptation, which can differ between breeds for reasons not attributable to
481 genetics [84–86,89]. A litter effect was found in piglets at the start of the trial (piglets aged between
482 3.8 and 4.6 weeks; samples collected immediately after weaning) and was lost at later time
483 points during the trial. This could be due to either of the aforementioned factors. Co-housing,
484 aging and the splitting of the piglets in separate rooms to receive a different treatment, are
485 possible causes for loss of the litter effect. In this study we confirm the importance of these
486 factors in the contribution to inter-individual variability of gut microbial composition. Motta *et al*
487 (2019) report a correlation of beta diversity with age and no correlation of genotype and litter
488 effect with either alpha or beta diversity [90]. On the contrary, we found the piglet samples to
489 significantly cluster by age and litter immediately after weaning in alpha diversity, and by age
490 and cross-breed in beta diversity during the second week after weaning. Samples also clustered
491 by age groups in beta diversity four weeks after weaning. The groups we tested, based on age,
492 cross-breed, and litter, differed in sample sizes, therefore a non-parametric test was used to test
493 for associations of host factors with phylogenetic diversity. Based on our results we conclude
494 that even small age differences among post- weaning piglets, down to the day, must be
495 accounted for in an experimental set up, but we acknowledge that the method we used may have
496 less power to detect small effects than methods which make assumptions of balance in group
497 sizes.

498 Three groups of piglets (cohorts neomycin, neomycin+D-Scour™ and
499 neomycin+ColiGuard®) underwent five days of treatment with neomycin, via intramuscular
500 administration. Intramuscular neomycin poorly diffuses (<10%) into a healthy gastrointestinal
501 tract [91], therefore a direct effect of neomycin on the gut microbiome may not be expected.

502 However, neomycin showed a different trend in unrooted PD between the second and the third
503 week post-weaning, corresponding to the week following the neomycin treatment period for the
504 neomycin cohort. Taking this time frame into consideration, the neomycin cohort did not
505 increase in BWPD to the extent of the Control cohort. Although statistically significant
506 differences between neomycin and Control in alpha diversity were not reached, possibly due to
507 the effect being too small to detect within a relatively small sample size [92–94], BWPD of the
508 neomycin cohort appears to follow a different trend to the Control cohort from the first week
509 (during neomycin treatment) where neomycin treated piglets show the lowest decrease of BWPD
510 compared to the control cohort and all other cohorts. While all cohorts show an increase in
511 absolute phylogenetic diversity accompanied by a decrease of diversity evenness during this time
512 frame, the neomycin cohort piglets show a lower drop in BWPD, suggesting an increase of
513 species richness, without a corresponding loss of species evenness. Furthermore the neomycin
514 cohort significantly separated from the control cohort in beta diversity two weeks after neomycin
515 treatment, showing a higher representation of *Mollicutes*. Numerous studies report the link of
516 oral antibiotic use with dysbiosis [10,14–17,86,95], as well as with host physiology changes [15]. On the
517 contrary, the effect of intramuscular antibiotic administration on the microbiome is less well
518 investigated. Correlation between intramuscular antibiotic use and dysbiosis has been reported in
519 fish [96], gorillas [19], and pigs [21,97]. In one day old piglets, a single IM injection of amoxicillin
520 (penicillin class) is reported to have an effect on the intestinal microbiota, detectable 40 days
521 post treatment [21]. Zeineldin *et al* (2018) tested the effects of IM administration of several
522 antibiotics of various classes (penicillin, macrolide, cephalosporin and tetracycline), in 8-week
523 old piglets, reporting shifts of the *Firmicutes*/*Bacteroidetes* ratio following treatment (length of
524 the treatment not reported) [97]. The effects of intramuscular administration of neomycin
525 (aminoglycoside class) on the gut microbiota have to our knowledge not been investigated.
526 Based on our results we conclude that a mild effect on phylogenetic diversity is appreciable post
527 IM neomycin treatment, up to two weeks after termination of the treatment. Additional
528 compositional and functional analysis is necessary to determine the source of this mild variation.
529 Differences were not detected at later time points, based on our phylogenetic diversity analyses,
530 suggesting a full recovery of the microbial communities after two weeks from the end of the
531 treatment.

532 It is possible that the large shifts in phylogenetic diversity taking place in the first two weeks
533 irrespective of the treatment (an increase, then decrease of unrooted PD, and an opposite trend of
534 BWPD) have masked the milder effects of the treatment, despite our efforts to control for the
535 effects of aging. This could be the reason why a significantly distinct alpha diversity trend was
536 found in the neomycin+D-Scour™ cohort compared to the neomycin cohort, but not in the D-
537 Scour™ cohort compared to the Control cohort. There are multiple studies reporting beneficial
538 effects of probiotic treatment in sucker and weaner piglets in terms of improved gut mucosal
539 integrity[27,98], growth rate[98–100,] digestibility of proteins and water absorption [98,101] , reduction of
540 pathogen invasion efficiency [38,45,98], and decreased mortality [98,100]. Although the assessment of
541 physiologic changes from probiotic treatments was outside the scope of this study, we found
542 significant separation of neomycin+D-Scour™ cohort samples to neomycin cohort samples in
543 beta diversity 2 and 12 days after D-Scour™ treatment, where neomycin+D-Scour™ samples
544 showed a higher representation of *Lactobacillales* compared to neomycin samples, suggesting a
545 transient establishment of the probiotic strains in the piglet guts.

546 The second probiotic in this study, ColiGuard®, did not have an effect on alpha diversity,
547 but clustering was detected in beta diversity, where ColiGuard® samples separated from Control
548 cohort samples in the first week of probiotic treatment and two weeks after probiotic treatment.
549 Additionally, the ColiGuard® treatment correlated with a lower weight gain, whether or not it
550 was preceded by the antibiotic treatment. However, when comparing the overall weight gain
551 (from the start to the end of the trial, corresponding to the six weeks after weaning) the weight
552 gain in the cohorts receiving ColiGuard® did not differ from the other cohorts.

553 We extracted the 16S rRNA gene hypervariable regions from our dataset, obtained the
554 counts, and ran a correlation analysis to discover lineages that correlated with the weight of the
555 piglets. As a consequence of the library size normalization step, the use of correlation with
556 compositional data can inflate the false discovery rate [102,103]. For this reason it can be expected
557 that some of the lineages we found to correlate with the weight of the piglets (eighty-three
558 distinct species) could be spurious while other correlations may have been missed.

559 As our initial analysis of phylogenetic diversity was obtained using a 100K reads
560 downsampling size per sample [104], we wanted to test to what extent the use of a larger sampling

561 size would affect the diversity obtained from these samples. A 100K downsampling size
562 corresponds to a 0.6% of reads of an average sample. Increasing the sampling size from 0.6% to
563 6%, affected BWPD only slightly, while it increased the unrooted PD and decreased its variance.
564 As expected, a larger sampling size enriches the absolute diversity by increasing the chance to
565 make new read placements, and it lessens the variance in estimates, but it does not affect the core
566 microbiome. However, the small difference between sampling sizes could 1. demonstrate how
567 powerful the method is in describing diversity using a small sampling size, or 2. reflect the limits
568 imposed by the phylogenetic markers database, as it was pointed out by Darling *et al* (2014) [57].

569

570 ## Conclusions

571
572 Our findings stress the importance of confounding factors such as breed, age and maternal
573 effects when assessing the effect of treatment on the gut microbiome. We found that age, even
574 within a narrow age span (1-5 days) can have an impact on microbial shifts and should be
575 accounted for in microbiome studies, either (i). by accounting for it as a confounding variable in
576 the hypothesis-testing model used, or (ii). by avoiding, where possible, the inclusion of subjects
577 of different ages, or (iii). by allowing a sufficiently long period of time prior to the start of the
578 treatment. The latter allows animals to become accustomed to the new environment and
579 researchers to perform additional sampling. Allowing animals a sufficiently long period of time
580 to become accustomed to the new environment (*e.g.*: temperature, humidity, new microbes, etc)
581 is meant to reduce noise derived from external factors, while repeated sampling, likewise, aims
582 to increase the confidence in the signal prior to treatment.

583 Intramuscular neomycin treatment correlated with a clustering in alpha diversity and a
584 higher representation of *Mollicutes* compared to control. D-Scour™ treated piglets displayed a
585 transient establishment of *Lactobacillales*. ColiGuard® treated piglets displayed a clustering in
586 beta diversity and a transient lower weight gain compared to control. Weight correlated with the
587 abundance of a number of lineages. Age was the strongest factor shaping phylogenetic diversity
588 of the piglets.

As previously mentioned, phylogenetic diversity is based on distinct lineages (richness) and their collective structure (proportions reflected by BWPD) and not on a direct assessment of composition and function. These types of analyses will be necessary to further describe the effects of the treatments.

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Conflicts of interest

D-Scour™ was sourced from International Animal Health Products (IAHP). ColiGuard® was developed in a research project with NSW DPI, IAHP and AusIndustry Commonwealth government funding.

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615 **Captions:**

616

617 **Figure 1**. Workflow.

618 Schematic workflow from sample collection to sequencing (orange) and data analysis (green).
619 Scripts (italic) are available in our GitHub repository.

620

621 **Figure 2**. Significant correlations of phylogenetic diversity with specified host factors.

622 The correlation of host factors birth day, cross-breed, birth day within the D × L cross-breed, birth
623 day within the D × LW cross-breed, line, litter (maternal sow and nurse sow) with alpha
624 phylogenetic diversity (**A**) and beta phylogenetic diversity (**B**) from each time point (x-axes) was
625 obtained. Shown in A and B are the significance values of these correlations derived from Kruskal-
626 Wallis analysis, where the symbol shape indicates the phylogenetic diversity measure of alpha
627 (unrooted pd and BWPD) and beta (PC components 1 to 5), while the symbol color indicates the
628 significance before (black) and after (red) Hommel *p* value adjustment. C) For each red symbol in
629 A and B, describing a significant correlation after Hommel *p* value adjustment, a plot of the
630 phylogenetic diversity estimates is shown. Phylogenetic diversity estimates of the litter effect are
631 not shown. All p values are reported in Supplementary Table 1. Abbreviations: D × L = "Duroc ×
632 Landrace" cross breed; D × LW = "Duroc × Large White" cross breed.

633

634 **Figure 3**. Effect of time on beta diversity.

635 Principal component analysis (PCA) of samples. PCA from edge component analysis with
636 PhyloSift (top) and PCA from 20 most abundant 16S reads extracted with SortMeRNA (bottom).
637 In the top, distribution of samples on either side of the plot (left *versus* right; top *versus* bottom)
638 reflect the lineages that were found to explain the variation. Samples are color coded by time post-
639 weaning (days). In the lower plot, arrows indicate which of the 20 lineages contributed to the
640 variation of samples across time, where arrows thickness represents a higher (thicker) or lower
641 (thinner) contribution. Samples are color coded by time point during the trial (t0: start of the
642 trial/first day after weaning; t10: last day of the trial/40th day after weaning).

643

644 **Figure 4**. Lineages displaying the highest variation in beta diversity across time.

645 Heatmap of lineages explaining the community composition of samples from separate time points
646 of the trial (1 week interval between time points) derived from edge principal component analysis.
647 Intensity is derived from branch width by the percentage of variability explained by the principal
648 components.

649

650 **Supplementary Figure 1**. Batch effect on alpha and beta diversity before batch effect removal.

651 Batch effect by alpha (top two plots) and beta diversity (bottom five plots) before batch effect
652 removal. Samples are grouped by DNA extraction plate. The *p* values are derived from multiple
653 comparison analysis with ANOVA, indicating equality of the means. *Post hoc* corrected *p* values
654 for pairwise comparisons are provided in Supplementary Table 1.

655

656 **Supplementary Figure 2**. Alpha phylogenetic diversity of cohorts.

657 Alpha phylogenetic diversity per cohort from samples across all time points. Balance-weighted
658 phylogenetic diversity (BWPD) (top) (mean±SD: Positive control Mock community: 1.59±0.07;
659 Positive control D-Scour™: 1.94±0.21; Positive control ColiGuard®: 0.86±0.14; Control:
660 2.13±0.13; D-Scour™: 2.16±0.12; ColiGuard®: 2.12±0.12; neomycin: 2.12±0.16; neomycin+D-
661 Scour™: 2.13±0.13; neomycin+ColiGuard®: 2.10±0.14; sows: 2.12±0.15; all piglet cohorts:
662 2.13±0.13); Unrooted phylogenetic diversity (bottom) (mean±SD: Positive control Mock
663 community: 123.62±24.41; Positive control D-Scour™: 162.14±28.27; Positive control
664 ColiGuard®: 129.88±50.00; Control: 311.23±29.23; D-Scour™: 316.35±24.99; ColiGuard®:
665 311.98±42.51; neomycin: 314.81±44.01; neomycin+D-Scour™: 312.66±40.18;
666 neomycin+ColiGuard®: 316.29±31.20; sows: 328.51±24.00; all piglet cohorts: 313.86±36.00).

667

668 **Supplementary Figure 3**. Beta diversity of positive controls.

669 Principal component analysis (PCA) of positive control samples. PCA from edge component
670 analysis with PhyloSift. Distribution of samples on either side of the plot (left *versus* right; top
671 *versus* bottom) reflect the lineages that were found to explain the variation.

672

673 **Supplementary Figure 4**. Alpha phylogenetic diversity by age breed.

674 Alpha diversity of samples from the start of the trial (immediately after weaning) grouped by breed
675 and by date of birth. Unrooted phylogenetic diversity (top) and balance-weighted phylogenetic
676 diversity (bottom). P values are derived from Kruskal-Wallis analysis of variance. Piglets of the
677 Duroc × Landrace breed (n=46) separated significantly by age in unrooted phylogenetic diversity
678 and in BWPD at the start of the trial (t0) (Hommel adjusted p value: unrooted pd=0.006;
679 BWPD=0.047). All post hoc corrected p values are provided in Supplementary Table 1.

680

681 **Supplementary Figure 5**. Relative abundance heatmap.

682 Most abundant lineages within each time point (columns) are obtained from analysis with guppy
683 fat. Guppy fat outputs trees with fattened edges in proportion to the relative abundance of reads
684 place in each lineage. The branch width of the trees, each corresponding to samples from distinct
685 time points, are the entries for this heat map. The distance between each time point is one week.

686

687 **Supplementary Figure 6**. Time trend of alpha diversity by cohort.

688 Unrooted phylogenetic diversity (**A**) and balance-weighted phylogenetic diversity (**B**) describe
689 richness and evenness, respectively, of alpha phylogenetic diversity for all samples across time,
690 grouped and color coded by cohort. The p values derive from pairwise comparisons of time points
691 of all treatment cohorts. The p values and *post hoc* corrected p values of time points comparisons
692 for each separate treatment cohort are provided in Supplementary Table 1.

693

694 **Supplementary Figure 7**. Significant differences in beta diversity between cohorts at

695 specific time points.

696 Significance was determined by comparing groups by pairwise t-test and the resulting *p* values
697 were adjusted with the Bonferroni method. Significance values are provided in Supplementary
698 Table 1. The x-axes represent the principal component. As plots are derived from distinct guppy
699 runs, each principal component explains variation to a different extent (percentage specified in
700 parentheses). The number of samples is specified on the y-axis. Distribution of the samples on
701 either side of a plot (left *versus* right) reflects the lineages that were found to explain the variation.
702 Distributions are color coded by cohort.

703

704 **Supplementary Figure 8**. Change in weight gain of piglets by cohort across the trial.

705 On the y-axis of plots the change in weight gain between time points is provided in percentage.
706 Letters on the top left of each plot indicate the time points compared with one week interval (t0-
707 t2, t2-t4, t6-t8, t8-t10) and with two weeks interval (t2-t6, t4-t8, t0-t8). Pairwise t-test comparisons
708 between cohorts were computed. A significant difference was found between Control and
709 ColiGuard (t4-t6, Tukey adjusted *p* value=0.0084), between neomycin and neomycin+ColiGuard
710 (t4-t6, Tukey adjusted *p* value=0.0152) and between neomycin and neomycin+ColiGuard (t4-t8,
711 Tukey adjusted *p* value=0.011).

712

713 **Supplementary Figure 9**. Correlation of diversity indices between the analysis run using 100,000
714 (100K) and 1,000,000 (1M) reads per sample.

715 Phylogenetic diversity analysis (alpha and beta) was run using either 100K or 1M reads per sample,
716 corresponding to a 0.6% or 6% of the average sample. We show the Pearson's correlation of these
717 analyses run using distinct downsampling sizes. Significance of correlations is reported within
718 each plot. All the diversity indices are provided in Supplementary Table 1.

719

720 **Supplementary Table 1**. Statistical analysis output.

721 Statistical analysis output is split across the following sheets. **all_padj_Hommel** : list of *p* values
722 obtained from running Kruskal-Wallis test, adjusted using the Hommel correction for
723 phylogenetic diversity values: unrooted pd, BWPD, and five principal components. Grouping of

samples is based on: cross-breed, line, date of birth, date of birth within distinct cross-breeds, maternal sow and nurse sow. Sample size and sample collection date is reported. **all_pvalues** : reports the *p* values of "all_padj_Hommel" sheet, prior to Hommel *p* value adjustment. **alpha_delta_cohorts** : deltas between time points are obtained per cohort and these deltas are compared using ANOVA, adjusting *p* values using the TukeyHSD method. **alpha_means** : the means and standard deviations of alpha diversity (unrooted pd and BWPD) obtained per time point and cohort. **alpha_time** : results of comparison between time points of alpha diversity (unrooted pd and BWPD) using the t-test and Bonferroni *p* value correction. **batch_post_process** : results of comparison of alpha diversity values by DNA extraction plate (1-10) after batch effect removal, run using ANOVA and TukeyHSD *p* value adjustment method. *batch_pre_process* : results of comparison of alpha diversity values by DNA extraction plate (1-10) before batch effect removal, run using ANOVA and TukeyHSD *p* value adjustment method. **deltas_percent_change** : comparison of alpha diversity values (unrooted pd and BWPD) between time points within distinct cohorts and within all cohorts. Sample size (*n*), deltas (percentage) are shown. **guppy_padj** : *p* values from "guppy_pvalues" are adjusted using the Bonferroni method and results are reported. **guppy_pvalues** : output of single guppy runs is analyzed by comparing beta diversity values by cohort at each time point using the pairwise t test; results of the tests are here shown. **PD_100K_vs_1M_stats** : alpha and beta diversity values obtained with PhyloSift using either 100,000 reads or 1,000,000 reads downsampling size. **weight_cohort_stats** : piglets were weighted at each time point. Deltas are obtained per piglet and means were compared between cohorts using ANOVA and TukeyHSD *p* value correction, results are reported. **weight_taxa** : Spearman's rank correlation was assessed between pig weight and abundance of lineages obtained from 16S rRNA containing reads. Significance values are adjusted using the distinct methods listed.

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754
755
756

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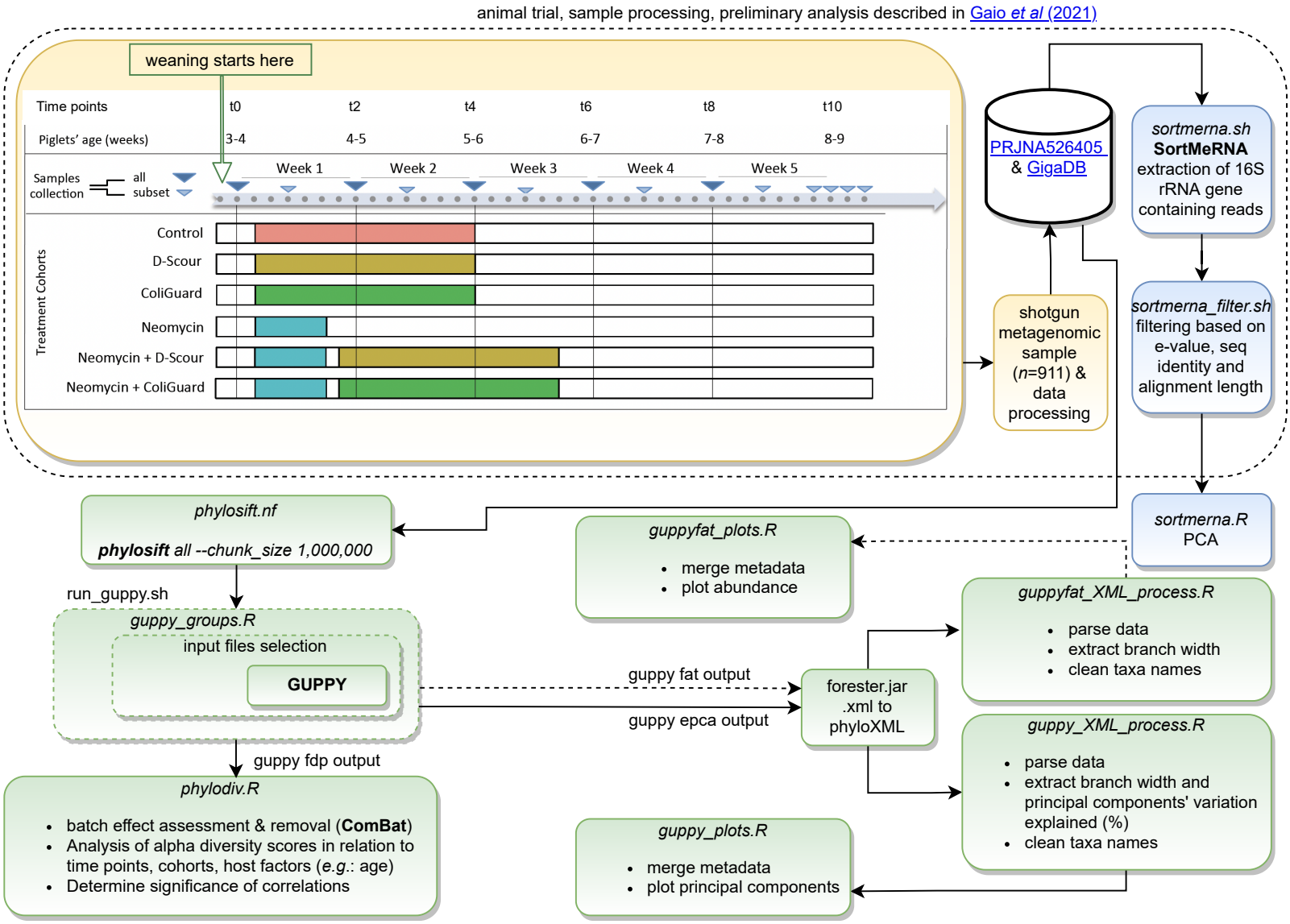
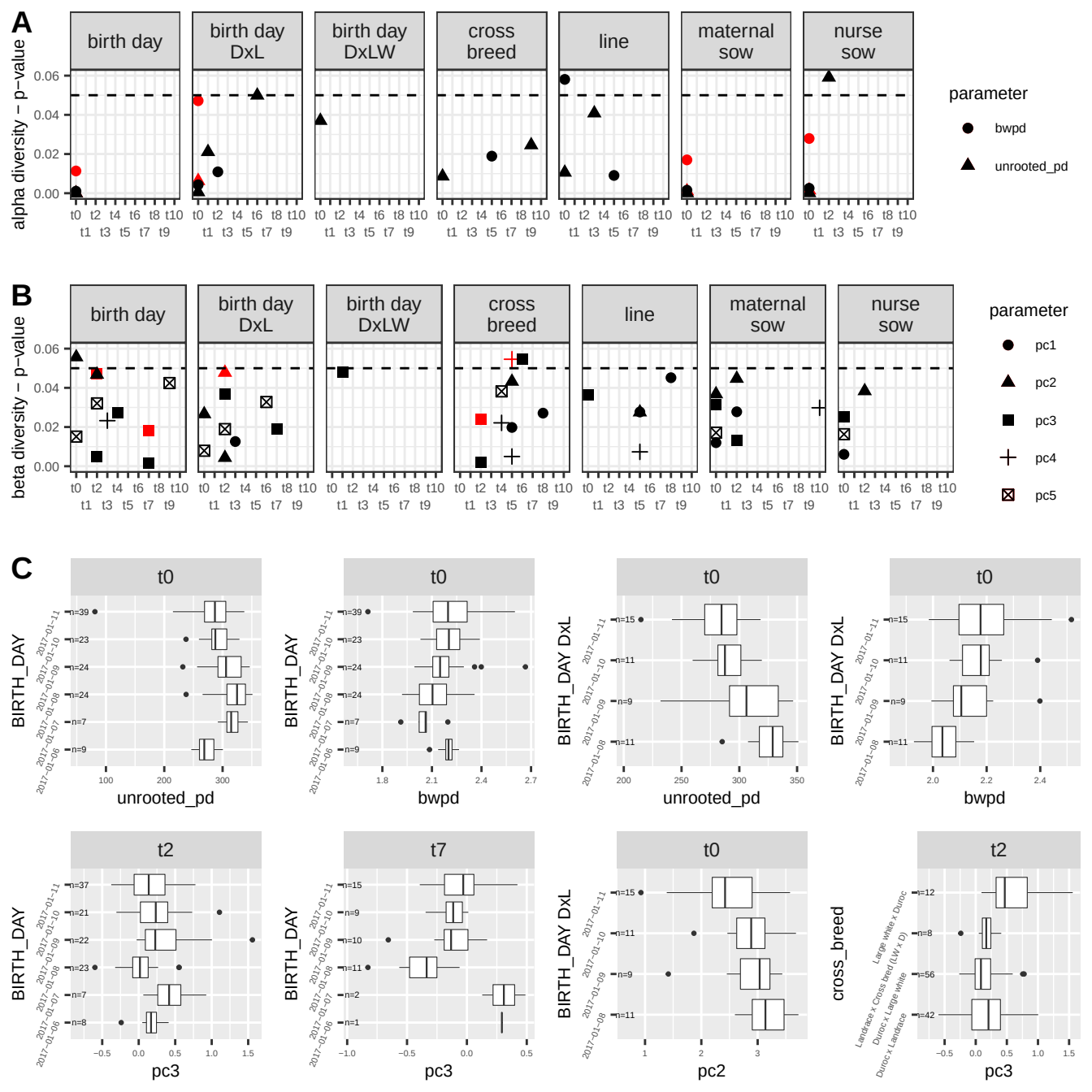
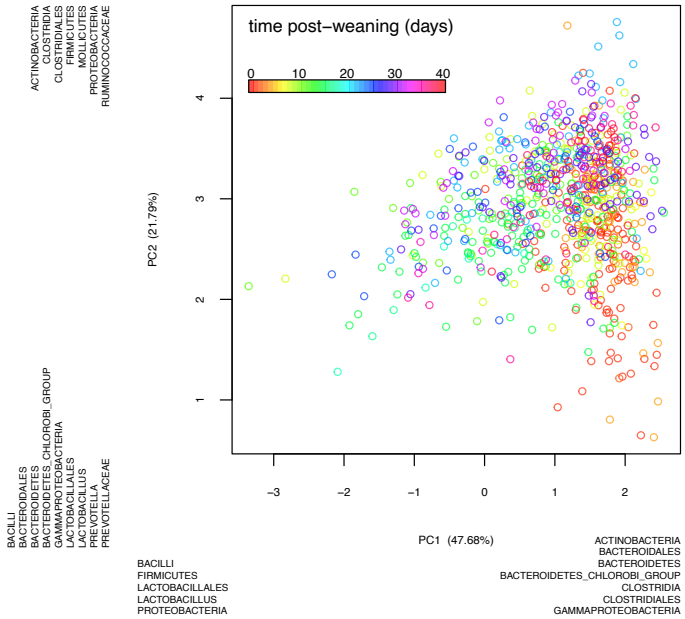
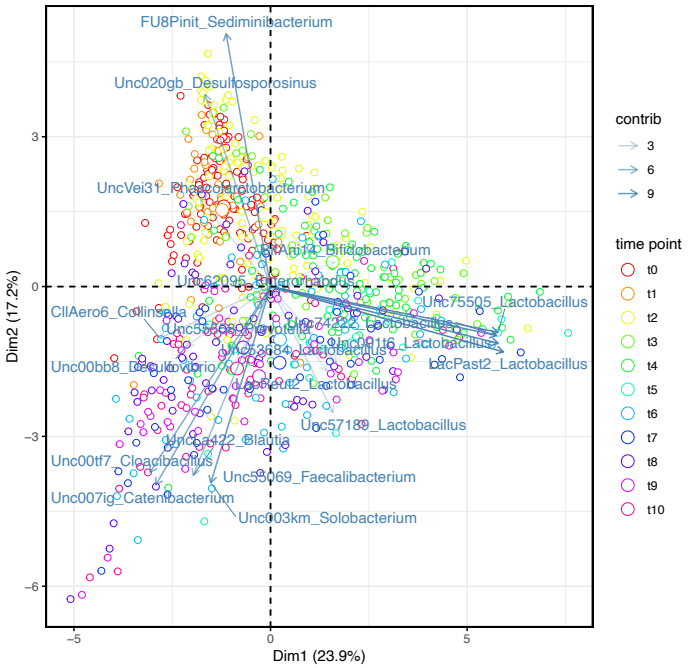

animal trial, sample processing, preliminary analysis described in Gaio et al (2021)
weaning starts here
Time points
t0
t2
t4
t6
t8
t10
Piglets' age (weeks)
3-4
4-5
5-6
6-7
7-8
8-9
Samples collection
all
subset
Week 1
Week 2
Week 3
Week 4
Week 5
Treatment Cohorts
Control
D-Scour
ColiGuard
Neomycin
Neomycin + D-Scour
Neomycin + ColiGuard
PRJNA526405 & GigaDB
sortmerna.sh
SortMeRNA extraction of 16S rRNA gene containing reads
sortmerna_filter.sh
filtering based on e-value, seq identity and alignment length
shotgun metagenomic sample (n=911) & data processing
sortmerna.R
PCA
phylosift.nf
phylosift all --chunk_size 1,000,000
run_guppy.sh
guppy_groups.R
input files selection
GUPPY
guppy fat output
guppy epca output
guppy fdp output
forester.jar .xml to phyloXML
guppyfat_plots.R
merge metadata
plot abundance
guppyfat_XML_process.R
parse data
extract branch width
clean taxa names
guppy_XML_process.R
parse data
extract branch width and principal components' variation explained (%)
clean taxa names
phylodiv.R
batch effect assessment & removal (ComBat)
Analysis of alpha diversity scores in relation to time points, cohorts, host factors (e.g.: age)
Determine significance of correlations
guppy_plots.R
merge metadata
plot principal components


A
birth day
birth day DxL
birth day DxLW
cross breed
line
maternal sow
nurse sow
alpha diversity – p−value
parameter
bwpd
unrooted_pd
B
beta diversity – p−value
pc1
pc2
pc3
pc4
pc5
C
t0
t2
t7
BIRTH_DAY
BIRTH_DAY DxL
cross_breed
unrooted_pd
bwpd
pc3
pc2
Landrace x Cross breed (LW x D)
Large white x Duroc
Duroc x Large white
Duroc x Landrace


time post−weaning (days)
0
10
20
30
40
PC2 (21.79%)
PC1 (47.68%)
ACTINOBACTERIA
CLOSTRIDIA
CLOSTRIDIALES
FIRMICUTES
MOLLICUTES
PROTEOBACTERIA
RUMINOCOCCACEAE
BACILLI
BACTEROIDALES
BACTEROIDETES
BACTEROIDETES_CHLOROBI_GROUP
GAMMAPROTEOBACTERIA
LACTOBACILLALES
LACTOBACILLUS
PREVOTELLA
PREVOTELLACEAE


FU8Pinit_Sedimini bacterium
Unc020gb_Desulfosporosinus
UncVei31_Phascolarctobacterium
Unc75505_Lactobacillus
CllAero6_Collinsella
Unc00116_Lactobacillus
lacPast2_Lactobacillus
Unc57189_Lactobacillus
Unc00tf7_Cloacibacillus
Unc55069_Faecalibacterium
Unc007ig_Catenibacterium
Unc003km_Solobacterium
contrib
3
6
9
time point
t0
t1
t2
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t4
t5
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t7
t8
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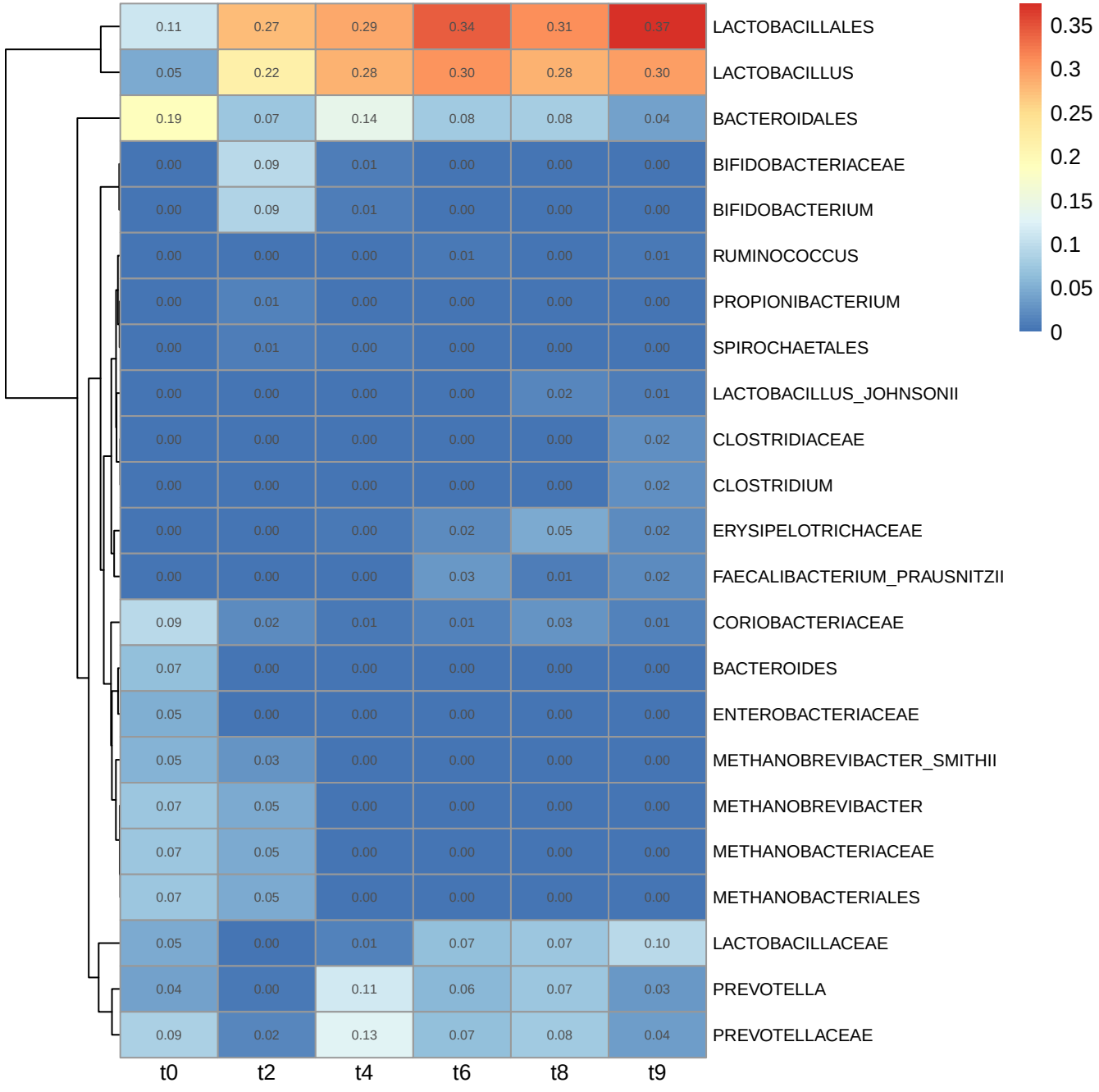
t10
Dim2 (17.2%)
Dim1 (23.9%)


0.11 0.27 0.29 0.34 0.31 0.37 LACTOBACILLALES
0.05 0.22 0.28 0.30 0.28 0.30 LACTOBACILLUS
0.19 0.07 0.14 0.08 0.08 0.04 BACTEROIDALES
0.00 0.09 0.01 0.00 0.00 0.00 BIFIDOBACTERIACEAE
0.00 0.09 0.01 0.00 0.00 0.00 BIFIDOBACTERIUM
0.00 0.00 0.00 0.01 0.00 0.01 RUMINOCOCCUS
0.00 0.01 0.00 0.00 0.00 0.00 PROPIONIBACTERIUM
0.00 0.01 0.00 0.00 0.00 0.00 SPIROCHAETALES
0.00 0.00 0.00 0.00 0.02 0.01 LACTOBACILLUS_JOHNSONII
0.00 0.00 0.00 0.00 0.00 0.02 CLOSTRIDIACEAE
0.00 0.00 0.00 0.00 0.00 0.02 CLOSTRIDIUM
0.00 0.00 0.00 0.02 0.05 0.02 ERYSIPELOTRICHACEAE
0.00 0.00 0.00 0.03 0.01 0.02 FAECALIBACTERIUM_PRAUSNITZII
0.09 0.02 0.01 0.01 0.03 0.01 CORIOBACTERIACEAE
0.07 0.00 0.00 0.00 0.00 0.00 BACTEROIDES
0.05 0.00 0.00 0.00 0.00 0.00 ENTEROBACTERIACEAE
0.05 0.03 0.00 0.00 0.00 0.00 METHANOBREVIBACTER_SMITHII
0.07 0.05 0.00 0.00 0.00 0.00 METHANOBREVIBACTER
0.07 0.05 0.00 0.00 0.00 0.00 METHANOBACTERIACEAE
0.07 0.05 0.00 0.00 0.00 0.00 METHANOBACTERIALES
0.05 0.00 0.01 0.07 0.07 0.10 LACTOBACILLACEAE
0.04 0.00 0.11 0.06 0.07 0.03 PREVOTELLA
0.09 0.02 0.13 0.07 0.08 0.04 PREVOTELLACEAE
t0 t2 t4 t6 t8 t9
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0