Community composition and development of the post-weaning piglet gut microbiome

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ABSTRACT

We report on the largest metagenomic analysis of the pig gut microbiome to date. By processing over 800 faecal time-series samples from 126 piglets and 42 sows, we generated over 8Tbp of metagenomic shotgun sequence data. Here we describe the animal trial procedures, the generation of our metagenomic dataset and the analysis of the microbial community composition using a phylogenetic framework. We assess the effects of intramuscular antibiotic treatment and probiotic oral treatment on the diversity of gut microbial communities. We found differences between individual hosts such as breed, litter, and age, to be important contributors to variation in the community composition. Treatment effects of the antibiotic and probiotic treatments were found but were subtle, while host age was the dominant factor in shaping the gut microbiota of piglets after weaning. The post-weaning pig gut microbiome appears 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.

INTRODUCTION

As the world population grows, there is an accompanying demand for animal derived products. In a semi-natural environment pig weaning occurs between the 12th and the 17th weeks from birth, whereas in the farm this typically occurs at 4 weeks of age[1]. Intensive animal husbandry and early weaning practices are commonly used to maximise production rates while minimizing costs. Both practices increase the risk of infections with pathogenic organisms, and thereby the need for antimicrobial strategies, which has included the common use of antibiotics. [2–12] Although antibiotics may kill some pathogens, the surviving bacteria can develop antimicrobial resistance (AMR) against the class of antibiotic used [13–20], as well as against other antibiotic classes [21–31]. A retrospective U.S. livestock study found evidence of multidrug resistance (resistance to >3 antimicrobial drug classes) in *Escherichia coli* that increased from 7.2% to 63.6% between the 1950s and the 2000s [32]. As the incidence of multidrug resistant (MDR) pathogens expands, the World Health Organization (WHO) has recognized AMR as one of the top health challenges of the current century [33,34].

Besides leading to AMR development, antibiotic usage is known to cause dysbiosis [34–37], the disruption of a balanced state within a gut microbial community. A balanced state decreases the chance of pathogens gaining a foothold, as there is niche and nutrient competition in play [39,40]. In livestock production, intramuscular (IM) antibiotic treatment is preferred over oral antibiotic treatment, as it is thought to contribute less to AMR than oral antibiotic treatment [41–43]. Extensive evidence exists on dysbiosis as a consequence of oral antibiotic treatment on the gut microbiome [22,35–38,44,45], whereas only a handful of studies report the effects of intramuscular (IM) antibiotic treatment on the gut microbial community [46–48], and none, to our knowledge have studied the use of IM neomycin treatment.

A number of non-antibiotic strategies to increase resistance to disease have been studied [49]. Strategies for which beneficial effects have been reported consist of hydrolases [50], fiber intake [51], β-lactamase enzymes [52], non-resistance inducing antimicrobial drugs [53,54], vaccination [55–58], phage therapy [59], in-feed organic acids [60], starches [61], and liquid feed [62–64]. Extensive evidence exists on the use of probiotics. Probiotics are reported to promote intestinal health in multiple ways: improvement of mucosal integrity [65–68], competitive exclusion against pathogenic species [50,69–73], reduction of intestinal inflammation [65,74,75] and of pathogen translocation [65,76,77]. Their efficacy has been determined, among others, in cell cultures [70,74,75,78], mice [50,67,68,73], and swine [65,66,74,76,79–83]. However, while most evidence of beneficial effects from probiotic treatment is derived from host immunity responses [65–68,74–

[76,80], host physiology [74,80–82], and pathogen relocation [50,65,69,70,72,77–79,84], fewer studies exist on the effects of probiotics on the whole gut microbial community [83,85].

This study was conducted to: 1) generate a public metagenomic databank of the gut microbiome of weaner piglets aged between 3 and 9 weeks old; 2) assess the effects of IM neomycin antibiotic use on the gut community; and 3) assess the effects of two probiotic formulations on the gut community. The data we present here is analysed from the perspective of the phylogenetic diversity of the microbial communities.

In the first results section we describe the phylogenetic diversity of the intestinal microbiome of the piglets and how it compares to the known composition of positive controls. Then, we describe evidence that highlights the importance of age, breed and litter as strongly associated factors with changes in community composition. Lastly, we describe the strong effect of time on community composition and the milder effect of antibiotic and probiotic treatments on community composition.

METHODS

Pig trial

Animal studies were conducted at the Elizabeth Macarthur Agricultural Institute (EMAI) NSW, Australia and were approved by the EMAI Ethics Committee (Approval M16/04). The trial animals comprised 4-week old male weaner pigs (*n*=126) derived from a commercial swine farm and transferred to the study facility in January 2017. These were cross-bred animals of Landrace, Duroc and Large White breeds and had been weaned at approximately 3 weeks of age.

The pig facility consisted of four environmentally controlled rooms (Rooms 1-4) with air conditioning, concrete slatted block flooring with underground drainage and open rung steel pens (**S1**). Each room had nine pens, consisting of a set of six and a set of three pens, designated a-f and g-i respectively, with the two sets of pens being physically separate, *i.e.* animals could come in contact with each other through the pen's bars within each set of pens, but not between sets. The rooms were physically separated by concrete walls and contamination between rooms was minimized by using separate equipment (boots, gloves, coveralls) for each room. In addition, under-floor drainage was flushed twice weekly and the

93 flushed faeces/urine was retained in under-floor channels that ran the length of the facility,
94 so that Rooms 1, 2 were separate from Rooms 3, 4 and flushing was in the direction 1 to 2
95 and 3 to 4.

96 The pigs were fed *ad libitum* a commercial pig grower mix of 17.95% protein free of
97 antibiotics, via self-feeders. On the day of arrival (day 1) 30, 18, 18, and 60 pigs were
98 allocated randomly to Rooms 1, 2, 3 and 4 respectively in groups of 6, 6, 6 and 6-7 pigs per
99 pen respectively (**S1A**). Pigs were initially weighed on day 2, and some pigs were moved
100 between pens to achieve an initial mean pig weight per treatment of approximately 6.5 kg
101 (range: 6.48-6.70; mean±SD: 6.53±0.08). Pigs were weighed weekly throughout the trial
102 (**Supplementary file 1**). Behaviour and faecal consistency scores were taken daily over the
103 6-week period of the trial (**Supplementary file 2**). Developmental and commercial probiotic
104 paste preparations ColiGuard® and D-Scour™ from International Animal Health,
105 Australasia, were used in some treatment groups.

106 The animals were acclimatised for 2 days before the following treatments were
107 administered: Room 1 - oral 1 g/pig of placebo paste daily for 14 d; Room 2 - oral 1 g/pig of
108 D-Scour™ paste daily for 14 d; Room 3 - oral 1 g/pig of ColiGuard® paste daily for 14 d;
109 Room 4 - intramuscular (IM) injection of antibiotic administered at 0.1 mL per pig daily
110 from a 200 mg/mL solution for a total treatment duration of 5 d.

111 On the day following the final neomycin treatment (day 8), 36 pigs were moved from
112 Room 4 to Room 2 (*n*=18, 6 in each pen, pens g-i), and to Room 3 (*n*=18, 6 in each pen, pens
113 g-i) (**S1**). The following day (day 9), oral administration of D-Scour™ (1 g/pig) and of
114 ColiGuard® (1 g/pig) commenced for pigs in Room 2 pens g-i and in Room 3 pens g-i,
115 respectively, and continued for a period of 14 days. Assignment of the 36 neomycin-treated
116 pigs to the treatment groups neomycin+D-Scour™ (*n*=18; Room 2 pens g-i) and
117 neomycin+ColiGuard® (*n*=18; Room 3 pens g-i), was carried out by distributing them so
118 that the mean weight of the animals distributed across pens and rooms was similar
119 (**Supplementary file 1**). By this time point, each occupied pen in the trial housed six pigs.
120 (**S1B**) From that time, twelve piglets from the original 126 were no longer present, as they
121 had been euthanised as pre-treatment controls at the start of the trial.

122 Faecal samples were collected from all piglets once per week and from a subset (*n*=48
123 pigs; 8 from each of the six cohorts) twice per week over the 6-week study period (**Figure**

124 **1**). From each piglet, faeces were collected per rectum with new disposable gloves; where
125 minimal or no faeces could be collected on a collection day, sampling was performed the
126 following morning. Samples were placed in 50 mL Falcon tubes and stored at 4ºC within 30
127 mins of sample collection for a minimum of 30 mins and a maximum period of 6 h.

128 *Faecal sample processing*

129 Samples (3g/pig) were mixed with 15 mL PBS (200 mg/mL), in sterile stomacher bags
130 and homogenized with a Bio-Rad stomacher. The homogenised samples were divided in
131 replicates: one replicate was stored directly at -80ºC and one replicate was supplemented
132 with glycerol (20% v/v) (Sigma-Aldrich) then stored at -80º C. In addition, single time-point
133 faecal samples from the dams of the trial pigs (*n*=42) were obtained from the commercial
134 facility of origin and were pre-processed at EMAI as described above. Thus, a total of 911
135 unique samples, between one and ten samples per subject (mean: 4.8; median: 3) (**S2**), were
136 obtained throughout this study. At the end of the trial period, all samples were transported
137 from EMAI to the University of Technology Sydney (UTS) for further processing.

138 *Positive controls*

139 As a positive control "mock community" for this study, four Gram positive (*Bacillus*
140 *subtilis* strain 168, *Enterococcus faecium*, *Staphylococcus aureus* ATCC25923,
141 *Staphylococcus epidermidis* ATCC35983) and three Gram negative (*Enterobacter*
142 *hormaechei* CP_032842, *Escherchia coli* K-12 MG1655, *Pseudomonas aeruginosa* PAO1)
143 bacterial strains from -80°C stocks were cultured at 37ºC for 16 h in LB (Luria-Bertani) then
144 centrifuged at 14,000 rpm for 10 mins. From the resulting pellets, 1 g was transferred to 1
145 mL of LB and homogenised and a 1:10 dilution of this was made for each bacterial culture.
146 Ten microliters of bacterial suspension from each of the cultures was used to determine the
147 number of colony forming units (CFU) in the original suspension in the following manner:
148 by further diluting tenfold in LB and by plating onto 1.6% LB agar plates and incubacted
149 overnight. The remaining suspensions (990 µL from each bacterial culture) were pooled into
150 a sterile tube, then aliquoted into Eppendorf tubes in 500 µL volumes/tube. As a washing
151 step, Eppendorf tubes were centrifuged at 14,000 rpm for 10 mins, 500 µL PBS was added to
152 the pellet and subsequently resuspended. These tubes constituted the mock community
153 samples and were stored at -80ºC. Expected proportions of the mock community members
154 were determined from the estimated colony forming units (CFU) multiplied by the genome

155 size and were as follows: 8.7:13.0:7.7:16.7:38.9:14.5:0.4 for *S. aureus*, *B. subtilis*, *E.*
156 *faecium, S. epidermidis*, *P. aeruginosa*, *E. cloacae, and E.coli* respectively.

157 The two probiotic formulations used in this study were used as two additional positive
158 controls. D-Scour™ is a commercially available probiotic formulation for livestock, with
159 each gram containing 180 million CFU of the following: *Lactobacillus acidophilus*,
160 *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus*
161 *rhamnosus*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus salivarius*
162 subspecies *thermophilus*, with an additional 20 mg of garlic extract (*Allium sativum*). The
163 probiotic ColiGuard is a probiotic formulation developed for the treatment of entero-
164 toxigenic *Escherichia coli* (ETEC) in weaner pigs, developed in collaboration between the
165 NSW DPI and International Animal Health Products, containing undefined concentrations of
166 *Lactobacillus plantarum* and *Lactobacillus salivarius*.

167 *DNA extraction*

168 Piglet and sow faecal samples, mock community samples, negative controls and
169 probiotic samples (D-Scour™ and ColiGuard® paste) were allocated to a randomized block
170 design to control for batch effects in DNA extraction and library preparation. The faecal
171 samples were thawed on ice first, followed by the probiotics and mock community samples.
172 MetaPolyzyme (Sigma-Aldrich) treatment was performed according to the manufacturer's
173 instructions except for the dilution factor, which we allowed to be 4.6 times higher.
174 Immediately after incubation, DNA extraction was performed with the MagAttract
175 PowerMicrobiome DNA/RNA EP kit (Qiagen) according to the manufacturer's instructions.
176 Quantification of DNA was performed using PicoGreen (Thermofisher) and measurements
177 were performed with a plate reader (Tecan, Life Sciences) using 50 and 80 gain settings. All
178 samples were diluted to 10 ng/μL.

179 *Library preparation*

180 Sample index barcode design using a previously introduced method [86] yielded a set of 96
181 x 8nt sequences with a 0.5 mean GC content and none of the barcodes containing 3 or more
182 identical bases in a row. Nine hundred-sixty different combinations of i5 and i7 primers were
183 used to create a uniquely barcoded library for each sample. The detailed sample-to-barcode
184 assignment is given in **Supplementary file 3**. Library preparation was carried out using a
185 modification of the Nextera Flex protocol to produce low bias, low cost shotgun libraries, as

186 described in a previous manuscript [86]. Following the amplification step, samples were
187 centrifuged at 280 x *g* for 1 min and stored between 1 and 5 days at 4°C.

188 *Size selection and purification*

189 Samples from the same 96-well plates were pooled into one tube by taking 5 µL from
190 each library. This generated 10 pooled samples, one for each plate. A master pool was
191 created by pooling 5 µL from the pool of each plate into a single pool. Forty microliters from
192 each of the 10 plate pools and 40 µL from the master pool underwent library size selection
193 and purification using equal volumes of SPRIselect beads (Beckman Coulter) and ultrapure
194 water (Invitrogen). Sample cleaning with SPRI-beads was performed as described previously
195 [86]. A purified master pool comprising samples from all plates, and purified pools of
196 individual plates to check for plate-specific anomalies, were diluted to 4 nM and fragment
197 size distribution was assessed using the High Sensitivity DNA kit on the Bioanalyzer
198 (Agilent Technologies, USA).

199 *Normalisation and sequencing*

200 The master pool was sequenced on an Illumina MiSeq v2 300 cycle nano flow cell
201 (Illumina, USA). Read counts were obtained and used to normalise libraries. The liquid
202 handling robot OT-One (Opentrons) was programmed to re-pool libraries based on read
203 counts obtained from the previous MiSeq run. The script used to achieve the normalization is
204 available through our Github repository.

205 The read count distribution after normalisation is displayed in supplementary figure
206 (**S3**). The normalized and purified pooled library was sequenced on an Illumina NovaSeq S4
207 flow cell at the Ramaciotti Centre for Genomics (Sydney, NSW, Australia), generating a
208 total of 27 billion read pairs from 911 samples.

209 *Sequence data processing*

210 Adapter trimming (parameters: k=23 hdist=1 tpe tbo mink=11), PhiX DNA removal
211 (parameters: k=31 hdist=1), and quality filtering (parameters: ftm=0 qtrim=r trimq=20), were
212 performed using bbduk.sh (http://jgi.doe.gov/data-and-tools/bbtools). Quality assessment of
213 raw reads was carried out using FASTQC
214 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and a combined report of all
215 samples was obtained with MULTIQC [87] (**Supplementary File 4**). The presence of PCR

duplicates was assessed by feeding read pairs to dedupe.sh (http://jgi.doe.gov/data-and-tools/bbtools) (parameters: ac=f). Nextflow [88] was used to manage processing of the data on the HPC.

Determination of microbial diversity among samples

Phylogenetic diversity of all samples was assessed with PhyloSift [89] using the first 100k read pairs of each sample (parameters: --chunk-size 100000 --paired). In order to test for associations of phylogenetic diversity with treatment, time of sampling, and differences among hosts at the start of the trial, analysis of the unrooted phylogenetic diversity (PD) [90], the balance weighted phylogenetic diversity (BWPD) [91] and principal component analysis (PCA) of the Kantorovich-Rubinstein distances [92] (beta diversity analysis) were performed. Alpha-diversity and beta-diversity were analyzed and the results were visualized with R [93] and R packages [91–107]. The data analysis is schematically represented in **Supplementary Figure 4** (**S4**).

Additionally, SortmeRNA [111] (version 2.1) was applied to extract 16S ribosomal RNA genes from raw reads. The rRNA reference database silva-bac-16s-id90 was used. Hits were filtered (e-value <= 1e-30) and PCA was computed with R [93]. Sample counts were normalized for library size by proportions and were tested with the Spearman's Rank correlation coefficient method to find taxa correlating with the weight of the piglets across the trial.

*Batch effect*s

A randomized block design was adopted to mitigate batch effects. Because samples were distributed across ten 96-well plates during DNA extraction and library preparation, plate effects were expected. Although samples did not visibly cluster by DNA extraction plate across the first five principal components (**S5**), a batch effect was found by multiple comparison analysis with ANOVA and by applying Tukey post-hoc correction to pairwise comparisons. Batch effects were detected (ANOVA, alpha diversity: p range=4.8e-12-0.00011; beta diversity: p value<0.0023) (**S6**; **Supplementary file 5**) and removed with COMBAT[7] (ANOVA p value=1) (**S7**; **Supplementary file 5**).

Technical analysis of positive controls

245 As a quality control step, taxonomic analysis of the positive control samples and their
246 technical replicates was performed with MetaPhlAn2 [112]. The positive controls included the
247 mock community (*n*=8), D-Scour™ (*n*=8), and ColiGuard® (*n*=8) samples.

248 *Data availability*

249 The raw sequencing data has been submitted to the NCBI Short Read Archive under
250 project PRJNA526405. Metadata and scripts are available in this article, its Supplementary
251 material and in our Github repository https://github.com/GaioDany/metapigs_base

252

253 **RESULTS**

254 PhyloSift [89] was employed as a means to study microbial community diversity among
255 the samples, and to test for associations with treatment, time of sampling, and differences
256 among hosts at the start of the trial. To this end, analysis of the unrooted phylogenetic
257 diversity (PD) [90], the balance weighted phylogenetic diversity (BWPD) [91] and principal
258 component analysis (PCA) of the Kantorovich-Rubinstein distances [92] (beta diversity
259 analysis) were performed.

260 *Comparison of the expected and the observed taxonomic profile of the positive controls*

261 All the mock community members, in seven of the eight technical replicates, were
262 detected by MetaPhlAn2 (**S8**). One sample failed to sequence, reporting zero counts for any
263 species. The observed mean relative abundances were as follows: *B. subtilis* (mean±SD:
264 2.92±0.994), *E. cloacae* (mean±SD: 38.0±6.404), *E. faecium* (mean±SD: 0.97±0.081), *E.coli*
265 (mean±SD: 10.12±1.480), *E.coli* unclassified (mean±SD: 7.83± 1.755), *P. aeruginosa*
266 (mean±SD: 26.72±3.026), *S. aureus* (mean±SD: 9.90±3.613), *S. epidermidis* (mean±SD:
267 3.54±1.435). Isolate *E. cloacae* C15117, used in this study for the make-up of the mock
268 community, was recently found to be most closely related to the *Enterobacter hormaechei*
269 phylogenomic group C type strain DSM 16687 and therefore re-identified as *Enterobacter*
270 *hormaechei* subsp. *oharae*[113] . For this reason, taxonomic assignment by MetaPhlAn2
271 attributed the reads to *E. cloacae* instead. The expected proportions of the mock community
272 members were derived from the CFU by the genome size. Based on the expected and the
273 observed relative abundance, we found, with the exception of *S. aureus* (exp: 8.7% obs:
274 9.9%), three Gram positive members to be under-represented (*B. subtilis*: exp: 13.0% obs:

275 2.9%; *E. faecium:* exp: 7.7% obs: 1.0%; *S. epidermidis*: exp: 16.7% obs: 3.5%) and, with the
276 exception of *P. aeruginosa* (exp: 38.9% obs: 26.8%), two Gram negative members to be
277 over-represented (*E. cloacae:* exp: 14.5% obs: 38.0%; *E.coli*: exp: 0.4% obs: 7.8-10.1%)
278 (**S9**). Taxonomic assignment of the mock community samples reported one contaminating
279 species in one of the eight replicates: *Lactobacillus salivarius* (mean: 0.008) (**S10**).

280 The probiotic D-Scour™ is expected to contain, per gram, a total of 180 million CFU of
281 *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Lactobacillus*
282 *plantarum*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Enterococcus faecium*,
283 *Streptococcus salivarius* subspecies *thermophilus* in unknown proportions. From taxonomic
284 analysis with MetaPhlAn2, we can conclude that 6 of the 7 expected species were
285 determined to be present in the replicates in the following mean relative abundances:
286 *Bifidobacterium bifidum*: mean±SD: 40.01±12.558; *Enterococcus faecium*: mean±SD:
287 30.98±13.472; *Lactobacillus delbrueckii*: mean±SD: 11.56±7.148; *Lactobacillus plantarum*:
288 mean±SD: 6.23±7.863; *Lactobacillus rhamnosus*: mean±SD: 2.08±1.226; *Streptococcus*
289 *thermophilus*: mean±SD: 4.28±1.523. *Lactobacillus acidophilus* was not detected and
290 *Lactobacillus helveticus* was detected instead (*Lactobacillus helveticus*: mean±SD:
291 4.75±2.431) (**S8**). An additional 25 taxa were detected, of which 18 and 7 were identified at
292 the species and at the genus level, respectively. Contaminants were present at a higher
293 concentration in three technical replicates (R3, R7, R8) with the most frequent contaminant
294 (*Methanobrevibacter* spp.) being present in 5 of the 8 replicates (**S11**).

295 Taxonomic analysis of the technical replicates of the probiotic ColiGuard® also showed
296 a species profile consistent with the expected profile, with *Lactobacillus salivarius* and
297 *Lactobacillus plantarum* in a 9:1 ratio (*Lactobacillus salivarius*: mean±SD: 93.52±1.617;
298 *Lactobacillus plantarum*: mean±SD: 6.10±1.134) across the replicates (**S8**). ColiGuard®
299 contained a total of 20 contaminants, of which 16 and 4 were identified at the species and the
300 genus level, respectively. Contaminants were present at a higher level in two technical
301 replicates (R5, R7), with R7 displaying the most diverse and highest contamination rate (R7:
302 14 taxa; total contaminating reads: 2.67%; R5: 9 taxa; total contaminating reads: 0.30%).
303 (**S12**).

304 *Phylogenetic diversity of positive controls and how it compares to the taxonomic profile*

305 Alpha diversity of the positive controls reflects the expected alpha diversity, with the
306 mock community, ColiGuard® and D-Scour™ positive controls, displaying a progressively
307 higher unrooted PD (mean±SD: Mock community: 31.53±29.50; ColiGuard®: 58.52±21.70;
308 D-Scour™ : 64.84±21.30). BWPD for the positive control ColiGuard® appears on the far left
309 of the plot (mean±SD: 0.82±0.15), distant from the mock community (mean±SD: 1.58±0.12)
310 and the positive control D-Scour™ (mean±SD: 1.89±0.26), which contains 2, 7 and 8 main
311 species, respectively (**S13**; **Supplementary file 1**). The unrooted PD of the positive control
312 ColiGuard® (mean±SD: 58.52±21.70) is closer to the unrooted PD of the Mock Community
313 (mean±SD: 31.53±29.50) and to that of the positive control D-Scour™ (mean±SD:
314 64.84±21.30), than the BWPD.

315 The contribution to total diversity of phylogenetic tree edges with uneven quantities of
316 reads placed on either side is down-weighted in BWPD, which is reflected in the low
317 reported BWPD of the positive control ColiGuard®. ColiGuard® is mainly composed of two
318 species in an uneven ratio (1:9) as by taxonomic analysis with MetaPhlAn2 (**S8**), in contrast
319 with the mock community (0.01:1:3:4:8:10:10:27:38) and the positive control D-Scour™
320 (2.08:4.28:4.75:6.23:11.56:30.98:40.01) (**S8**). On the other hand, the unrooted PD reflects
321 the absolute diversity, independently of the relative abundance of each species, within a
322 sample. In fact, low-level contamination (<0.1%) detected in each of the positive controls
323 (Mock Community: 1 taxon; ColiGuard®: 20 taxa; D-Scour™ : 25 taxa) (**S11-13**) contributes
324 toward the absolute diversity in the unrooted PD, erroneously inflating this value (mean±SD:
325 Mock Community: 31.53±29.50; ColiGuard®: 58.52±21.70; D-Scour™ : 64.84±21.30)
326 (**Figure 2**).

327 *Phylogenetic diversity of piglet gut communities immediately after weaning*

328 The 126 piglets used in this study were derived from 4 main breed cross types "Duroc ×
329 Landrace" (n=46), "Duroc × Large White" (n=59), "Landrace × cross bred (LWxD)" (n=9),
330 "Large White × Duroc" (n=12), and three pig lines (line 319: n=9; line 316: n=46; line 326:
331 n=71). The piglets also differed slightly by age, being born between 1 and 6 days apart.

332 Based on Kruskal-Wallis one-way analysis of variance (Hommel adjusted p values to
333 correct for multiple testing), phylogenetic diversity of the piglet samples did not cluster
334 significantly by breed cross type ($p > 0.05$) or by pig line ($p > 0.05$) at the start of the trial,
335 but a significant difference was found with breed and line in the fourth week of the trial

336 (breed: p=0.04; pig line: p=0.02) (**Figure 2**). Additionally a correlation of breed cross type
337 was found with beta diversity principal components (PC) in the second week of the trial
338 (PC3: p=0.0357) and in the fifth week of the trial (PC1: p=0.027). Alpha diversity
339 significantly correlated with pig line in the fourth week of the trial (BWPD: p=0.017).
340 (**Figure 2**; **Supplementary file 5**)

341 Notably, we found a significant correlation between alpha diversity and the age of the
342 piglets at the first sampling time point (unrooted PD: p=0.0016; BWPD: p=0.0355) (**Figure**
343 **2**; **Supplementary file 5**). While a correlation with age was found for alpha diversity only at
344 the start of the trial, age of the piglet was observed to be weakly associated with differences
345 in community composition in week two (PC3: p=0.0507) (**S14**; **Supplementary file 5**).

346 As age groups were confounded with cross-breed types (*i.e.* not all age groups are
347 represented by each of the four cross-breed types), we compared the phylogenetic diversity
348 of age groups within each breed. As cross-breed types "Landrace × cross bred (LW × D)"
349 and "Large White × Duroc" had only a small number of piglets in each age group, we tested
350 for an association between phylogenetic diversity and age in cross-breeds "Duroc ×
351 Landrace" and "Duroc × Large White". In these cross-breeds, alpha and beta diversity are
352 correlated with age in the "Duroc × Landrace" piglets (unrooted PD: p=0.0059; BWPD:
353 p=0.0226; PC2: p=0.0263; PC5: p=0.0063) and to a lesser extent in the "Duroc × Large
354 White" piglets (unrooted PD: p=0.0529; PC5: p=0.0310) during the first week of the trial. In
355 the "Duroc × Landrace" piglets, a correlation between age and beta diversity was detected in
356 week two (PC2: p=0.0347) (**Figure 2**; **S14**; **Supplementary file 5**). Differences in beta
357 diversity by date of birth, within the same cross-breed (Duroc × Large white), were found to
358 be significant (TukeyHSD adjusted p value: 1 day difference: 0.000782; 2 days difference:
359 0.018603) (**Supplementary file 5**).

360 *Maternal effect on phylogenetic diversity*

361 As piglets were derived from 42 distinct sows (maternal sows), and nursed by either the
362 same or a different sow (a nurse sow) (**Supplementary File 5**), a litter effect was expected
363 and determined. Based on Hommel adjusted p values, a similarity of alpha and beta
364 phylogenetic diversity can be seen among piglets from the same maternal sow (unrooted PD:
365 p=0.0096; BWPD: p=0.0467) and in piglets from the same nurse sow (unrooted PD:
366 p=0.0320; PC1: p=0.0391) (**Figure 2**; **S16-19**; **Supplementary file 5**). The litter effect

367 described was found in samples at the start of the trial. Significance of the correlations did
368 not persist thereafter (**Figure 2**; **Supplementary file 5**).

369 *A strong effect of aging on phylogenetic diversity*

370 Beta diversity analysis revealed a distinct and consistent change of the microbial
371 community over time in all piglets, regardless of the treatment. Samples clustered in PC2
372 (accounting for 21.68% of the variation), showing a higher representation of *Bacteroidetes*,
373 *Gammaproteobacteria* and *Prevotellaceae* from day 0-20, and a higher representation of
374 *Firmicutes*, *Mollicutes* and *Ruminococcaceae* from day 20-40 (**Figure 3**; **S20**). In PC1
375 (accounting for 46.99% of the variation) samples shifted towards a higher representation of
376 *Lactobacilli* from day 0 to day 20, and towards a higher representation of *Actinobacteria*,
377 *Clostridiales* and *Mollicutes* from day 20 to day 40 (**Figure 3**; **Supplementary file 5**). A
378 temporary shift towards a higher representation of *Bifidobacteriaceae* (*Bifidobacterium*
379 being a component of the probiotic D-Scour™) is seen in PC5 (3.04% of the variation
380 explained) one and two weeks from the start of the trial (**S21**).

381 Beta diversity analysis was performed separately for samples within each time point in
382 order to find taxa associated with variation within each time point. Extent of variation was
383 derived from the product of branch width by the variation explained by the principal
384 component (**S22**). The following taxa were responsible for variation only at the start of the
385 trial: *Gammaproteobacteria* (t0=0.12), *Enterobacteriaceae* (t0=0.08) and *Archaea* (t0=0.03).
386 The following taxa were responsible for variation throughout the trial: *Clostridiales*
387 (min=0.11; max=0.25), *Bacteroidetes* (min=0.06; max=0.11), *Bacteroidetes chlorobi* group
388 (min=0.05; max=0.10). *Lactobacillus* became variable after the first week of the trial and
389 remained highly variable throughout the trial (min=0.13; max=0.22). Some taxa contributed
390 less to the variability among the piglets' faecal population, but they nonetheless consistently
391 contributed to variability: *Bacteroidales* (min=0.01; max=0.04); *Firmicutes* (min=0.05;
392 max=0.08); *Bacilli* (min=0.06; max=0.09); *Lactobacillales* (min=0.04; max=0.08);
393 *Actinobacteria* (min=0.02; max=0.09); *Proteobacteria* (min=0.02; max=0.07). The
394 variability of *Prevotella* (min=0.01; max=0.04) increased from the second week of the trial.
395 (**S22**)

396 Taxonomic representation in terms of abundance was derived from analysis of samples
397 with guppy fat, where abundance was derived from the branch width (**Figure 4**) and

398 combined with PhyloSift's taxonomic annotation of the marker gene phylogeny. Overall,
399 *Clostridiales* were the most abundant throughout the trial (t0=0.18; t2=0.20; t4=0.11;
400 t6=0.09; t8=0.10; t10=0.08). *Lactobacillus acidophilus* increased at the start (t0=0.00;
401 t2=0.05; t4=0.13) then decreased (t6=0.09; t8=0.05; t10=0.07). *Subdoligranulum variabile*
402 decreased after the first week (t0=0.05; t2=0.02) then gradually increased (t4=0.04; t6=0.05;
403 t8=0.06; t10=0.07). Among other prevalent taxa at the start of the trial up to the second week
404 and dropping at later time points we found *Metahobrevibacter smithii* (t0=0.06; t2=0.03) and
405 *Bacteroidales* (t0=0.02; t2=0.01). Following an opposite trend we found *Ruminococcus* sp.
406 JC304 (t0=0.00; t2=0.00; t4=0.01; t6=0.02; t8=0.02; t10=0.03), *Solobacterium moorei*
407 (t0=0.00; t2=0.01; t4=0.02; t6=0.02; t8=0.02; t10=0.02) and *Prevotella copri* (t0=0.00;
408 t2=0.00; t4=0.04; t6=0.03; t8=0.05; t10=0.02). In modest and stable abundance across the
409 trial were *Mogibacterium* sp. CM50 (t0=0.02; t2=0.03; t4=0.02; t6=0.03; t8=0.02; t10=0.04)
410 and *Oscillibacter* (t0=0.06; t2=0.04; t4=0.03; t6=0.03; t8=0.03; t10=0.03). In transient
411 abundance we found *Bifidobacterium thermophilum* RBL67 (t2=0.01; t4=0.02). Gradually
412 increasing from the second week we found *Eubacterium biforme* DSM3989 (t2=0.01;
413 t4=0.02; t6=0.02; t8=0.02; t10=0.02), *Eubacterium rectale* (t4=0.02; t6=0.02; t8=0.03;
414 t10=0.01) and, after the third week, *Faecalibacterium prausnitsii* (t6=0.01; t8=0.02;
415 t10=0.02).

416 The effect of time was also clear in alpha diversity, where all the piglet cohorts followed
417 a similar trend over time, independent of the treatment (**S23**; **Supplementary file 5**). Upon
418 arrival on the trial site, the piglets' unrooted PD was lower than the sows (sows: 125.39;
419 piglets range: 104.6-112.7) and reached a similar unrooted PD to the sows' in the second
420 week (range: 123.4-130.7) (**Supplementary file 5**).

421 In comparing four timepoints at one week intervals from the start of the trial (intervals
422 labeled as A, B, C, D), changes in alpha diversity among all the piglets were tested for and
423 significance was determined using the Bonferroni correction. Unrooted phylogenetic
424 diversity increased after the start of the trial (A mean: 107.86; B mean: 127.21; $p<0.001$),
425 decreased in the following week (C mean: 122.92; p=ns) and the fourth week (D mean:
426 118.74; p=ns) amongst the piglets cohorts. In contrast, BWPD decreased after the start of the
427 trial (A mean: 2.17; B mean: 2.05; $p<0.0001$), to increase in the following week (C mean:
428 2.14; $p<0.0001$) and decrease in the fourth week (D mean: 2.07; $p<0.01$). (**S23**;
429 **Supplementary file 5**). The increase in unrooted PD in the first week was significant for all

430 treatment cohorts (*p* range: 0.0015-0.03) except ColiGuard® and neomycin (*p*=ns) cohorts.
431 The decrease in BWPD in the first week was only significant for the control (*p*=0.04),
432 ColiGuard® (*p*=0.0015) and neomycin+ColiGuard® (*p*=0.036) cohorts. In week two only
433 the increase of BWPD of the control cohort reached statistical significance (*p*=0.0121). (**S24**;
434 **Supplementary file 5**)

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

436 We hypothesized that the probiotic treatments, whether alone (D-Scour™ and
437 ColiGuard®) or administered after neomycin (neomycin+D-Scour™ and
438 neomycin+ColiGuard®) would cause a change in the microbial community composition that
439 would be measurable via phylogenetic diversity. We tested whether the treatments correlated
440 with a change in phylogenetic diversity independently of the changes occurring with
441 time. Given the differences in alpha and beta diversity detected among the subjects at the
442 start of the trial, we analyzed the deltas of phylogenetic diversity instead of relying on the
443 absolute means, similar to the procedure applied by Kembel *et al* (2012) [114]. Time-point
444 measurements of alpha diversity were taken and deltas were computed for each piglet. Delta
445 means were compared between cohorts, where the control cohort would serve as a control
446 group for neomycin, D-Scour™ and ColiGuard® cohorts, whereas the neomycin cohort
447 would serve as a control group for the neomycin+D-Scour™ and neomycin+ColiGuard®
448 cohorts.

449 After the first week of the trial, the majority of the piglets displayed an increase in
450 unrooted PD (85%) and a decrease of BWPD (75%). The following week the trend was
451 opposite: an increase of BWPD (78%) and a decrease of unrooted PD (60%)
452 (**Supplementary file 5**). However, the neomycin cohort displayed the smallest BWPD drop
453 in the week following the start of the trial and the overall trend of neomycin in unrooted PD
454 was the most different from the other cohorts (**S25**). Due to the delayed rise in unrooted PD
455 of neomycin, significance was found in unrooted PD between neomycin and neomycin+D-
456 Scour™ during the first week of D-Scour™ treatment (neomycin mean: 1.59; neomycin+D-
457 Scour™ mean: -11.79; *p*= 0.012188) (**S25 B-C**) and in unrooted PD between neomycin and
458 neomycin+ColiGuard® (neomycin mean: -5.53; neomycin+ColiGuard®: -6.70; *p*=0.043138)
459 (**S25 C-D**). The change was opposite in BWPD (*p*=0.049263) (**S26 B-C**) and persisted
460 during the following week, up to 3 days post- D-Scour™ treatment (neomycin mean: -6.34;
461 neomycin+D-Scour™ mean: 4.52; BH adjusted *p*=0.024092) (**S26 B-D**).

462 The only significant difference between the neomycin and the control cohort was
463 detected in BWPD in the second week of the trial (control mean: 7.10; neomycin mean:
464 0.28) p=0.033751) (**S26 B-C**).

465 No significant differences were detected between the control and the D-Scour™ cohort
466 (p<0.05) (**Supplementary file 5**). Instead, a significant decrease in BWPD was detected in
467 the ColiGuard® cohort between the start of the trial and the end of the ColiGuard®
468 treatment, where nearly all piglets (88%) in the ColiGuard® cohort displayed a decrease in
469 BWPD, whereas the control cohort was split between piglets increasing (53%) and
470 decreasing (47%) in BWPD (control mean: 0.85; ColiGuard® mean: -5.49; p=0.040041)
471 (**S26 A-C**).

472 *Effects of antibiotic and probiotic treatment on beta diversity*

473 To investigate the treatment effect on beta diversity, principal component analysis
474 (PCA) of the Kantorovich-Rubenstein distances (beta diversity analysis) was performed on
475 all samples and, additionally, on samples within individual time points. This analysis is
476 conceptually similar to the weighted Unifrac approach for beta diversity analysis, but is
477 designed to work with phylogenetic placement data [92]. When examining all samples together,
478 there was no clear separation of cohorts on any of the five principal component axes. When
479 individual time points were analysed, some clustering by cohort was observed (**S27**). D-
480 Scour™ separated from the control cohort in PC2 (17.95%) and in PC3 (8.06%) one week
481 and two weeks after probiotic treatment, respectively (**S27**). ColiGuard® separated from
482 control two weeks post probiotic treatment in PC3 (7.88%). Two weeks after the end of
483 neomycin treatment, neomycin separated from control in PC4 (5.32%), with a smaller
484 clustering and a higher representation of *Mollicutes*. Two and four weeks after probiotic
485 treatment, neomycin+D-Scour™ separated from neomycin in PC2 (17.74%) and PC5
486 (3.81%), respectively. In both timepoints and principal components, neomycin+D-Scour™
487 showed a higher representation of *Lactobacillales*. (**S27**)

488 *Association between weight and community composition*

489 Weight correlated with the abundance of certain species (n=83) at each time point as it
490 resulted from principal component analysis of 16S rRNA reads. Positively correlating with
491 weight we found among others: *Blautia* (t0), *Cetobacterium* (t0), *Ruminibacter* (t0),
492 *Rickettsia* (t2), *Lactobacillus* (t6), *Mycoplasma* (t6), *Anaerostipes* (t8), *Ruminococcus* (t8-

493 t10), *Cerasibacillus* (t10). Negatively correlating with weight we found among others:
494 *Pyramidobacter* (t0), *Odoribacter* (t2), *Schwartzia* (t6), *Streptococcus* (t6), *Dokdonella* (t8).
495 (**S28-S33**) Significance values and correlation estimates are given in **Supplementary file 5**.

496 *Effect of treatments on weight gain*

497 Overall weight gain from initial to final weight (**S34 A-E**; **Supplementary file 5**) was
498 not significantly affected by any treatment. However, the probiotic ColiGuard® was found to
499 have a partial effect on piglet weight gain (**S34**; **Supplementary file 5**). Weight was
500 measured weekly for a total of six measurements. Based on Tukey adjusted *p* values, a lower
501 weight gain was detected in the ColiGuard® cohort in week 3 compared to the control cohort
502 (p=0.0008) (**S34 C-D**; **Supplementary file 5**). Similarly, a lower weight gain was detected
503 in the neomycin+ColiGuard® cohort in C-E compared to the neomycin cohort (p=0.0393)
504 (**S34 C-E**; **Supplementary file 5**). Breed and age differences among piglets were not
505 associated with weight gain (**Supplementary file 5**).

506

507 **DISCUSSION**

508 The consistent trend in community composition over time, across all the cohorts,
509 indicates that an age-related process of ecological succession is the largest factor shaping the
510 microbial community of post-weaning piglets, as found in this study where animals aged 20-
511 63 days and were fed the same diet. A peak in unrooted phylogenetic diversity and drop in
512 balance weighted phylogenetic diversity (BWPD) reflects the acquisition of new species
513 with the loss of dominating species. This change, detected in the week following the piglets'
514 arrival at the trial site irrespective of the cohort, could be linked to the piglets being
515 subjected to microbial interchange (e.g.: new pen mates [115,116]) and/or to diet transition (peri-
516 weaning transition to solid food [115,116]) leading to the reshaping of the gut microbial
517 community. The week following the drop of BWPD, a significant increase of BWPD was
518 recorded, reflecting the acquisition of a larger proportion of the community by the newly
519 introduced species. The strong changes in phylogenetic diversity detected in the first and the
520 second week could as well be attributable to other post-weaning related physiological
521 changes, as previous studies report [44,115–117].

522 The highest inter-individual differences among piglets are seen in the first week of life,
523 irrespective of maternal or environmental effects. The microbiota of 3 week old piglets is

524 still very dynamic, but environmental factors become evident [116]. At 6 weeks of age, CD8+ T
525 cells infiltrate the intestinal tissue and the mucosa and intestinal lining resemble that of an
526 adult pig [115] . In this study, piglets reached a comparable alpha diversity to the sows after the
527 first week of the trial, at which time the piglets were aged between 3.8 and 4.6 weeks.
528 Unrooted PD did not reach higher levels at later sampling time points. The highest BWPD
529 accompanied by a high unrooted PD was reached after the second week of the trial when
530 piglets were aged between 4.9 and 5.6 weeks. Age-dependent physiological changes could
531 explain i) the major shifts we detected in alpha diversity during the first two weeks of the
532 trial and, ii) the distinct differences in community composition with age, even with a narrow
533 age difference between piglets (1-6 days). We were able to appreciate a significant trend of
534 increasing unrooted PD and decreasing BWPD with age in piglets that separated a 6 days
535 maximum by day of birth from each other. Since age groups were confounded with breeds in
536 our study, we attempted to determine the correlation within single breeds. Unfortunately,
537 although the correlations with age could still be detected, we could not determine the
538 association at later time points due to the introduction of treatment effects.

539 Animal trials are often conducted in controlled environments so as to minimize
540 environmental effects. However, individual variations such as breed and age are often
541 unavoidable in large animal trials. Previously reported confounding factors include:
542 individual variation [44], cohabitation [115,116], age [44,115,116], maternal effects [115,116,118], hormones [44],
543 behavioural differences between breeds (e.g. coprophagy, mouth to mouth contact) and
544 extent of long-term behavioural adaptation, which can differ between breeds for reasons not
545 attributable to genetics [44,115,116]. A litter effect was found in piglets at the start of the trial and
546 was lost at later time points during the trial. This could be due to either of the
547 aforementioned factors. Co-housing, aging and the splitting of the piglets in separate rooms
548 to receive a different treatment, are possible causes for loss of the litter effect. In this study
549 we confirm the importance of these factors in the contribution to inter-individual variability
550 of gut microbial composition. Motta *et al* (2019) report a correlation of beta diversity with
551 age and no correlation of genotype and litter effect with either alpha or beta diversity [9] . On
552 the contrary, we found the piglet samples to significantly cluster by litter, breed and by age
553 up to the second and the fourth week post weaning, in alpha diversity and beta diversity,
554 respectively. We conclude that even small age differences among post- weaning piglets,
555 down to the day, must be accounted for in an experimental set up.

556 Three groups of piglets (cohorts neomycin, neomycin+D-Scour™ and
557 neomycin+ColiGuard®) underwent 5 days of treatment with the broad-spectrum antibiotic
558 neomycin, via intramuscular administration. Intramuscular neomycin poorly diffuses (<10%)
559 into a healthy gastrointestinal tract [119] , therefore a direct effect of neomycin on the gut
560 microbiome may not be expected. However, neomycin showed a different trend in unrooted
561 PD between the second and the third week of the trial, corresponding to the week following
562 the neomycin treatment period for the neomycin cohort. Taking this time frame into
563 consideration, the neomycin cohort did not increase in BWPD to the extent of the Control
564 cohort. Although statistically significant differences between neomycin and Control in alpha
565 diversity were not reached, the BWPD of the neomycin cohort appears to follow a different
566 trend to the Control from the first week (during neomycin treatment) where neomycin treated
567 piglets show the lowest decrease of BWPD compared to the control cohort and all other
568 cohorts. While all cohorts show an increase in absolute phylogenetic diversity accompanied
569 by a decrease of diversity evenness during this time frame, the neomycin cohort piglets show
570 a lower drop in BWPD, suggesting an increase of species richness, without a corresponding
571 loss of species evenness. Furthermore the neomycin cohort significantly separated from the
572 control cohort in beta diversity in the third week of the trial, showing a higher representation
573 of *Mollicutes*. Numerous studies report the link of oral antibiotic use with dysbiosis [22,35–
574 38,44,45], as well as with host physiology changes [37]. On the contrary, the effect of intramuscular
575 antibiotic administration on the microbiome is less well investigated. Correlation between
576 intramuscular antibiotic use and dysbiosis has been reported in fish [46], gorillas [47], humans [120],
577 and pigs [48,121]. In 1 day old piglets, a single IM injection of amoxicillin (penicillin class) is
578 reported to have an effect on the intestinal microbiota, detectable 40 days post treatment [48].
579 Zeineldin *et al* (2018) tested the effects of IM administration of several antibiotics of various
580 classes (penicillin, macrolide, cephalosporin and tetracycline), in 8-week old piglets,
581 reporting shifts of the *Firmicutes*/*Bacteroidetes* ratio following treatment (length of the
582 treatment not reported)[121]. The effects of intramuscular administration of neomycin
583 (aminoglycoside class) on the gut microbiota have to our knowledge not been investigated.
584 Based on our results we conclude that a mild effect on phylogenetic diversity is appreciable
585 post IM neomycin treatment, up to two weeks after termination of the treatment. Additional
586 compositional and functional analysis is necessary to determine the source of this mild
587 variation. Differences were not detected at later time points, based on our phylogenetic

588 diversity analyses, suggesting a full recovery of the microbial communities after two week
589 from the end of the treatment.

590 It is possible that the large shifts in phylogenetic diversity taking place in the first two
591 weeks irrespective of the treatment (an increase, then decrease of unrooted PD, and an
592 opposite trend of BWPD) have masked the milder effects of the treatment, despite our efforts
593 to control for the effects of aging. This could be the reason why a significantly distinct alpha
594 diversity trend was found in the neomycin+D-Scour™ cohort compared to the neomycin
595 cohort, but not in the D-Scour™ cohort compared to the Control cohort. The neomycin+D-
596 Scour™ cohort underwent 5 days of neomycin treatment followed by 2 weeks of D-Scour™
597 treatment. A significant increase of BWPD was detected in the two-week period of D-
598 Scour™ treatment, indicating a possible enhancement of microbiome evenness following
599 neomycin treatment. To our knowledge there are no studies reporting an increased evenness
600 in piglet gut community composition following a specific probiotic treatment. There are
601 instead multiple studies reporting beneficial effects of probiotic treatment in sucker and
602 weaner piglets in terms of improved gut mucosal integrity [66,80], growth rate [80–83], digestibility
603 of proteins and water absorption [80,83], reduction of pathogen invasion efficiency [76,79,80], and
604 decreased mortality [80,82]. Although the assessment of physiologic changes from probiotic
605 treatments was outside the scope of this study, we found significant separation of
606 neomycin+D-Scour™ cohort samples to neomycin cohort samples in beta diversity 3 and 10
607 days after D-Scour™ treatment, where neomycin+D-Scour™ samples showed a higher
608 representation of *Lactobacillales* compared to neomycin samples, suggesting a transient
609 establishment of the probiotic strains in the piglet guts.

610 The second probiotic in this study, ColiGuard®, did not have an effect on alpha
611 diversity, but clustering was detected in beta diversity, where ColiGuard® samples separated
612 from Control cohort samples in the third principal component (explaining 7.88% of the
613 variation) two weeks post probiotic treatment. Additionally, the ColiGuard® treatment
614 correlated with a lower weight gain, whether or not it was preceded by the antibiotic
615 treatment. However, when comparing the overall weight gain (from the start to the end of the
616 trial) the weight gain in the cohorts receiving ColiGuard® did not differ from the other
617 cohorts.

618 We extracted the 16S rRNA gene hypervariable regions from our dataset, obtained the
619 counts, and ran a correlation analysis to discover taxa that correlated with the weight of the

620 piglets. As a consequence of the library size normalization step, the use of correlation with
621 compositional data can inflate the false discovery rate [122,123]. For this reason it can be
622 expected that some of the taxa we found to correlate with the weight of the piglets (eighty-
623 three distinct species) could be spurious while other correlations may have been missed.

624 *Technical controls in metagenomic studies and methodological limitations*

625 Taxonomic assignment of the raw reads from the positive controls was performed with
626 MetaPhlAn2[112] which relies on a ca. 1M unique clade-specific markers derived from 17,000
627 reference genomes. Such a database to map against the positive controls suffices as these
628 organisms are cultivable, and for this reason they are widely studied hence the sequences are
629 known. This is not the case for real-world samples where mapping against a database (which
630 completeness relies on studied and often cultivable organisms) would narrow the view on the
631 true diversity within the sample.

632 Positive controls with well-studied members and known ratios within the samples, has
633 proven to be a valuable tool to assess consistency among technical replicates across batches
634 and to detect possible biases derived from the DNA extraction method.

635 Systematic taxonomic bias in microbiome studies, resulting from differences in cell wall
636 structures between Gram positive and Gram negative bacteria, have previously been
637 reported; sample treatment with enzymatic cocktails can modestly reduce this bias [124–126].
638 Although we implemented this step in our workflow, it seems that, from the read abundance
639 of our mock community, which contained three Gram negative and four Gram positive
640 strains, a bias towards Gram negative taxa may still be present.

641 In terms of contamination we concluded that: a) contamination in our study was not
642 batch specific; b) a problem of sample cross-contamination existed at the DNA extraction
643 step between neighbouring wells. During the bead beating step of DNA extraction, the deep-
644 well plate is sealed with a sealing mat, rotated and placed in a plate shaker for the bead
645 beating to take place. We consider that sample cross-contamination is most likely to occur
646 during this step.

647

648 **Conclusion**

649 Our study provides a publicly-available databank of the pig gut metagenome. Our findings
650 further stress the importance of confounding factors such as breed, age and maternal effects
651 when assessing the effect of treatment on the gut microbiome. We found that age, even
652 within a narrow age span (1-6 days) can have an impact on microbial shifts and should be
653 accounted for in microbiome studies. Intramuscular neomycin treatment correlated with a
654 clustering in alpha diversity and a higher representation of *Mollicutes* compared to control.
655 D-Scour™ treated piglets displayed a mild shift in alpha diversity compared to control, and a
656 transient establishment of *Lactobacillales*. ColiGuard® treated piglets displayed a clustering
657 in beta diversity and a transient lower weight gain compared to control. Weight correlated
658 with the abundance of a number of taxa. Age was the strongest factor shaping phylogenetic
659 diversity of the piglets.

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

664

665 **Declarations**

666

667 *Competing Interests*

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

671

672 *Authors Contributions*

673 Pig Trial: TC, LF, DG, TZ, GJE, AED, SPD
674 DNA extraction: DG, ML
675 Library prep, robot pooling: DG, ML, KA, AED
676 Sequencing data processing: MZD, DG, AED

677 Data analysis: DG, AED

678 Manuscript: DG

679 Manuscript editing: DG, AED, GJE, John Webster

680

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698

699 **Supplementary Files**

700 **Supplementary File 1**. (animal details)

701 **Supplementary File 2**. (metadata)

702 **Supplementary File 3**. (barcodes)

703 **Supplementary File 4**. (multiqc report)

704 **Supplementary file 5**. (stats output)

705

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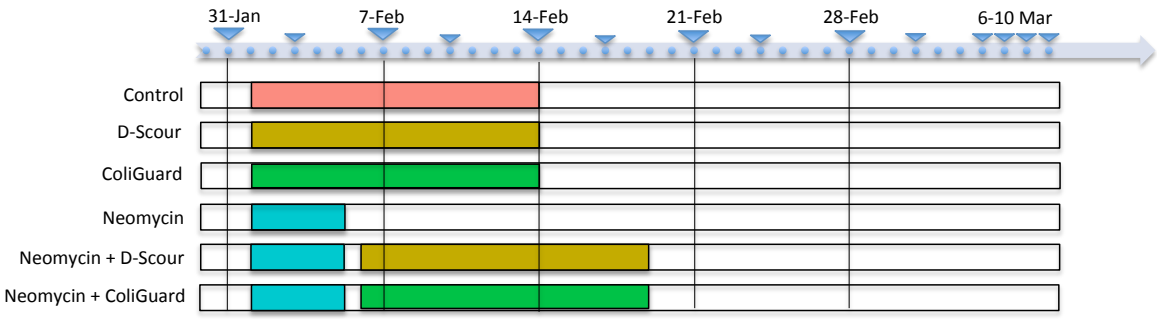


Figure 1. Timeline.

Timeline of the trial indicating the start and the length of the treatment for each cohort. Piglets arrived on the site of the trial on January 30th and were allowed 2 days of acclimatisation before the start of the treatments (pink: placebo paste; yellow: probiotic D-Scour™ formulation; green: probiotic ColiGuard® formulation; blue: antibiotic neomycin intramuscular injection). Vertical lines indicate main days of sampling where all piglets where sampled (*n*=126). Small arrows indicate days of sampling for a subset of the piglets (8 per cohort; *n*=48).

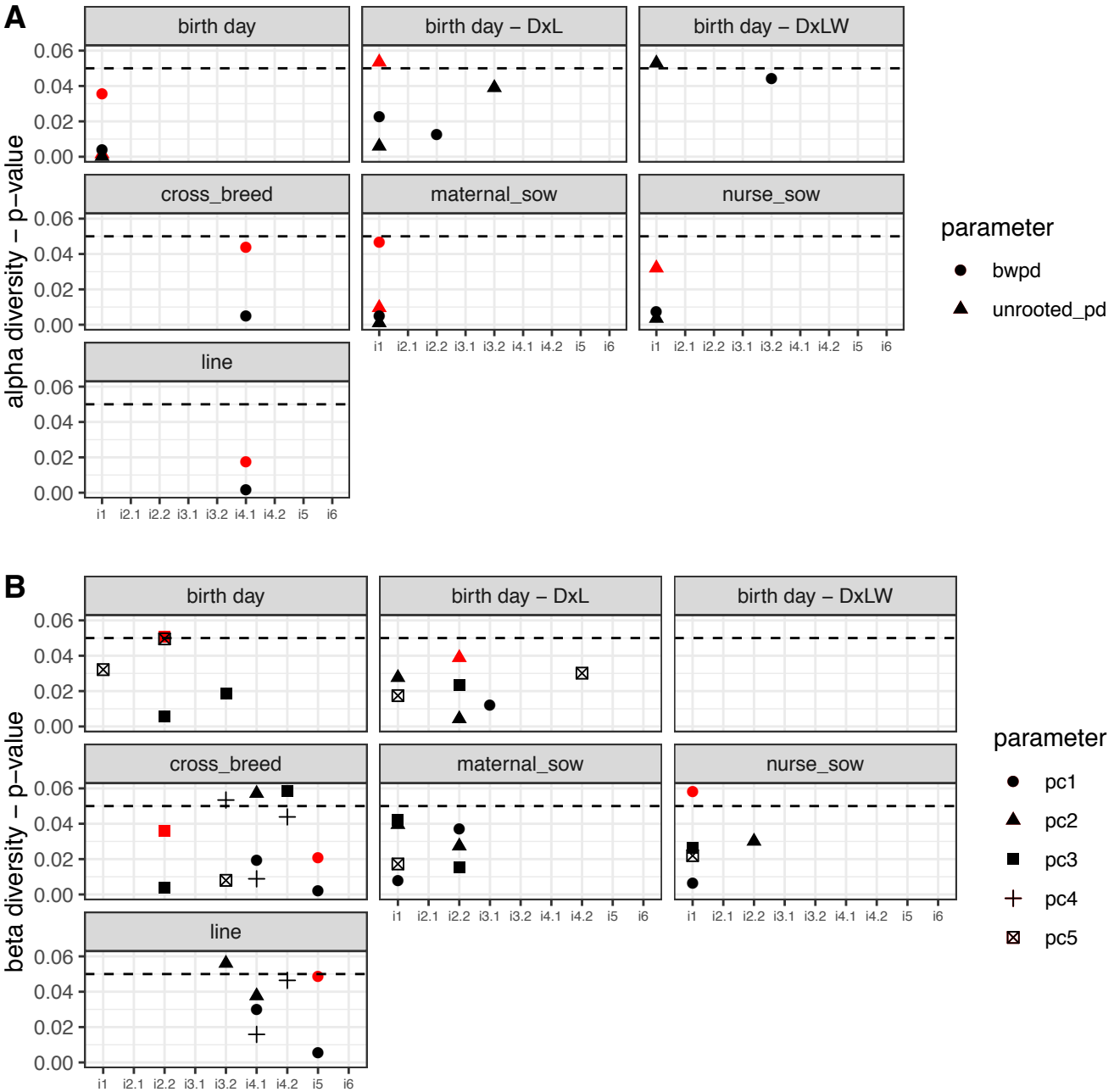


Figure 2. Significance of correlations between phylogenetic diversity and specified factors.

Plots of *p* values derived from Kruskal-Wallis analysis of variance (black) and adjusted *p* values by Benjamini-Hochberg method (red). x-axis displays distinct sampling time points in chronological order. Correlation of specified factors of samples are shown with (**A**) alpha diversity and (**B**) beta diversity. Abbreviations: DxL = "Duroc x Landrace" cross breed; DxLW = "Duroc x Large White" cross breed.

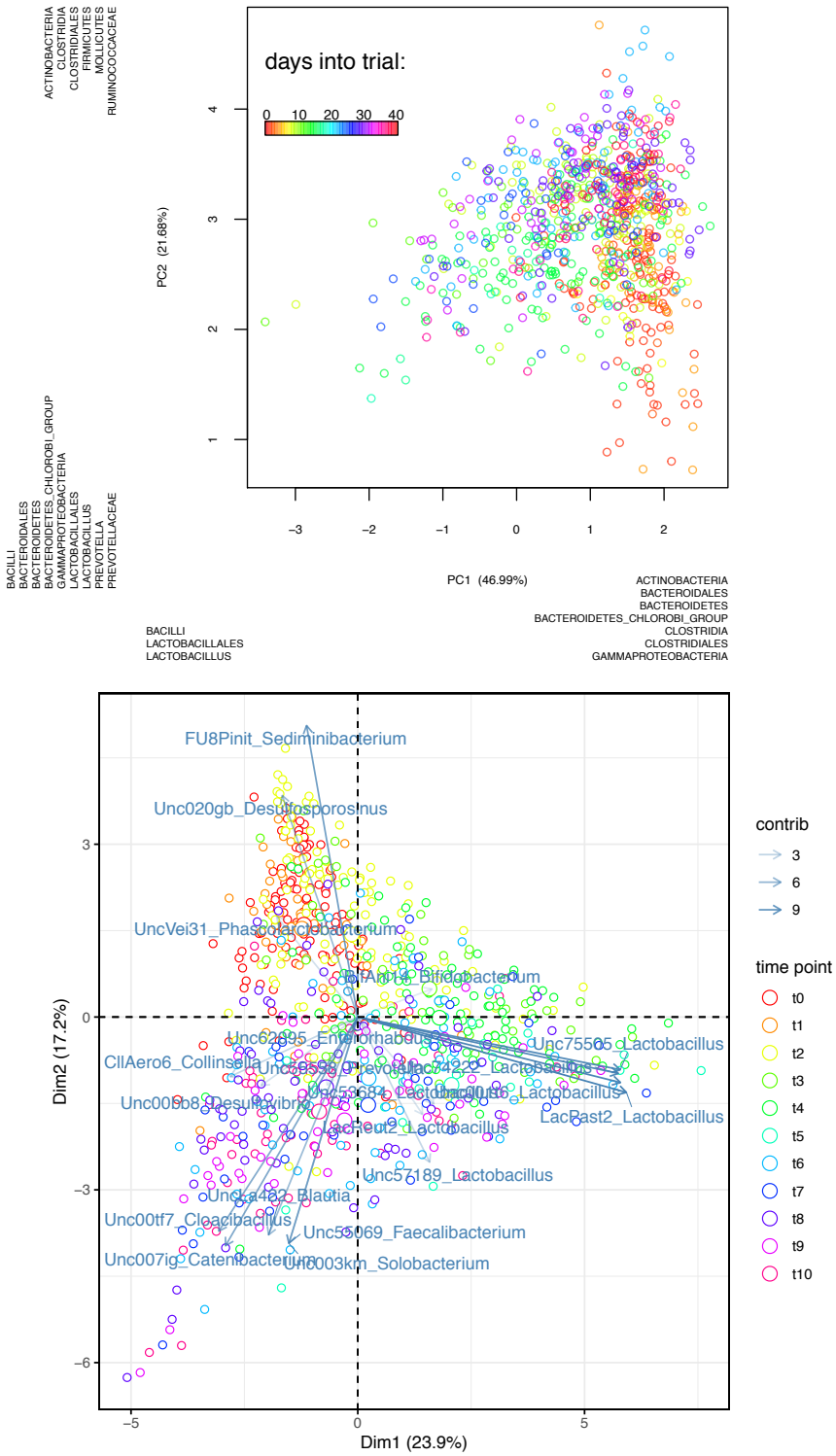


Figure 3. Effect of time on beta diversity.

Principal component analysis (PCA) of samples. PCA from edge component analysis with PhyloSift (top) and PCA from 20 most abundant 16S reads extracted with SortMeRNA (bottom). In the top, distribution of samples on either side of the plot (left *versus* right; top *versus* bottom) reflect the taxa that were found to explain the variation. Samples are colour coded by days into the trial. In the lower plot, arrows indicate which of the 20 taxa read contributed to the variation of samples, where arrows thickness represents a higher (thicker) or lower (thinner) contribution. Samples are colour coded by time point during the trial.

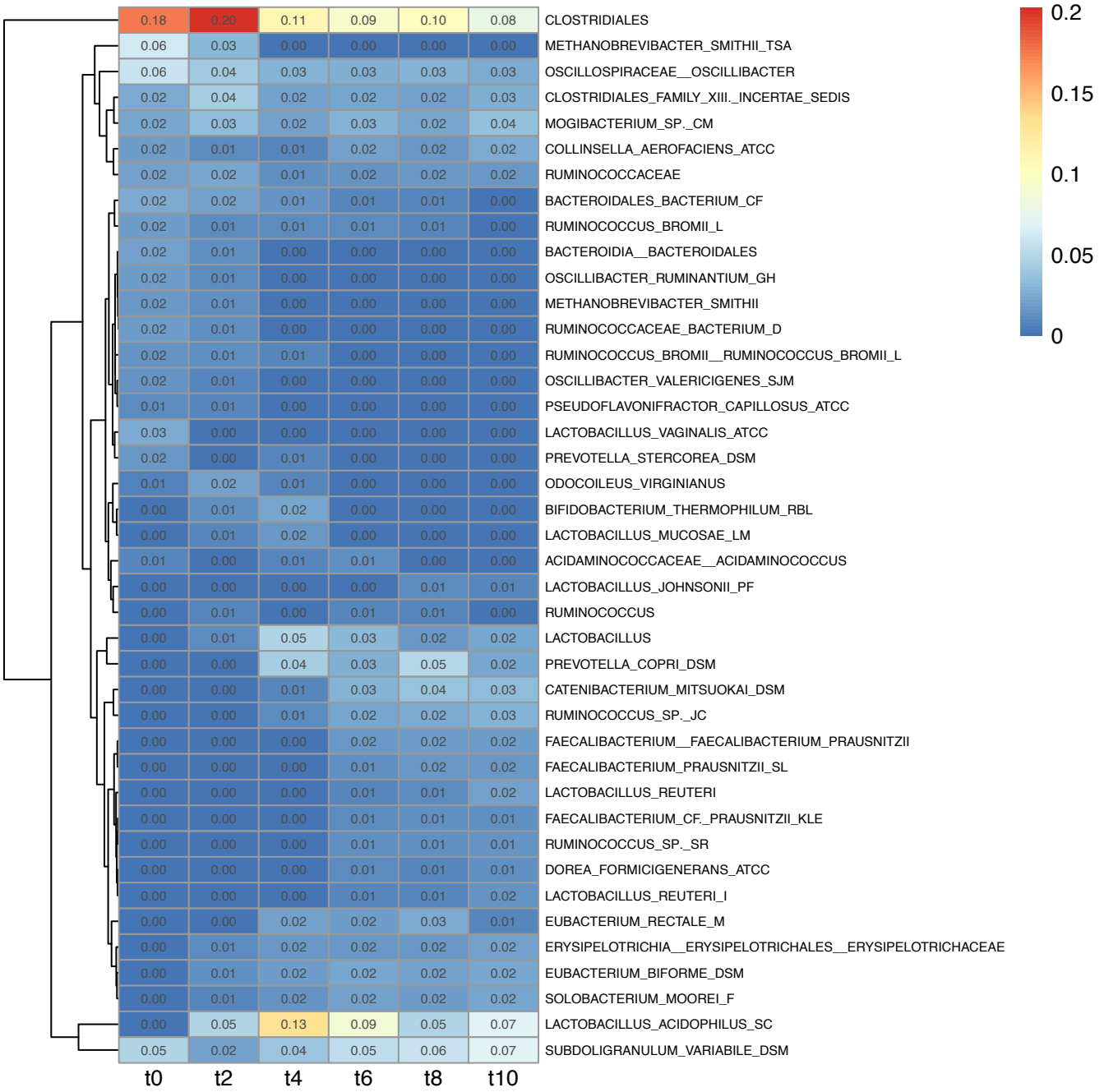
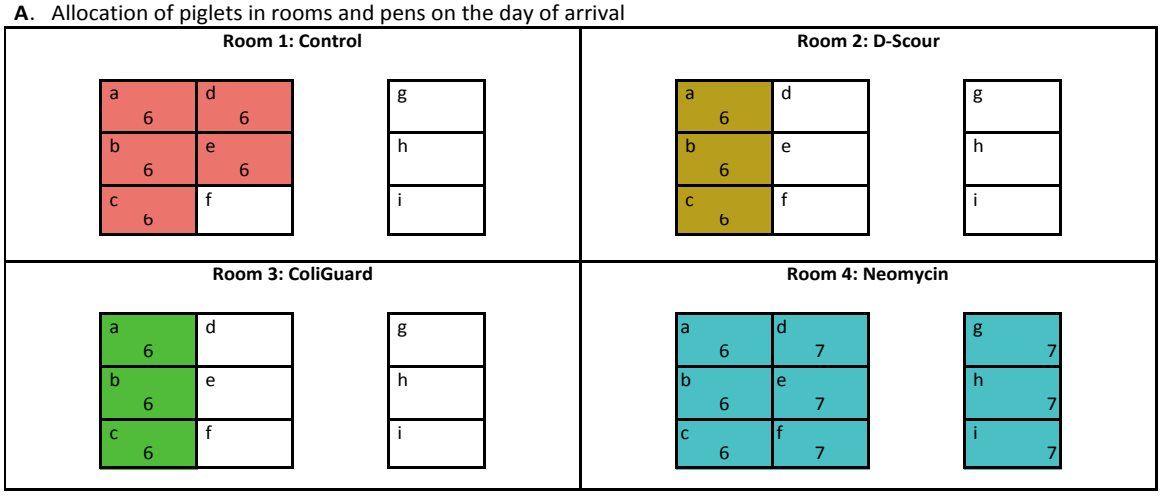


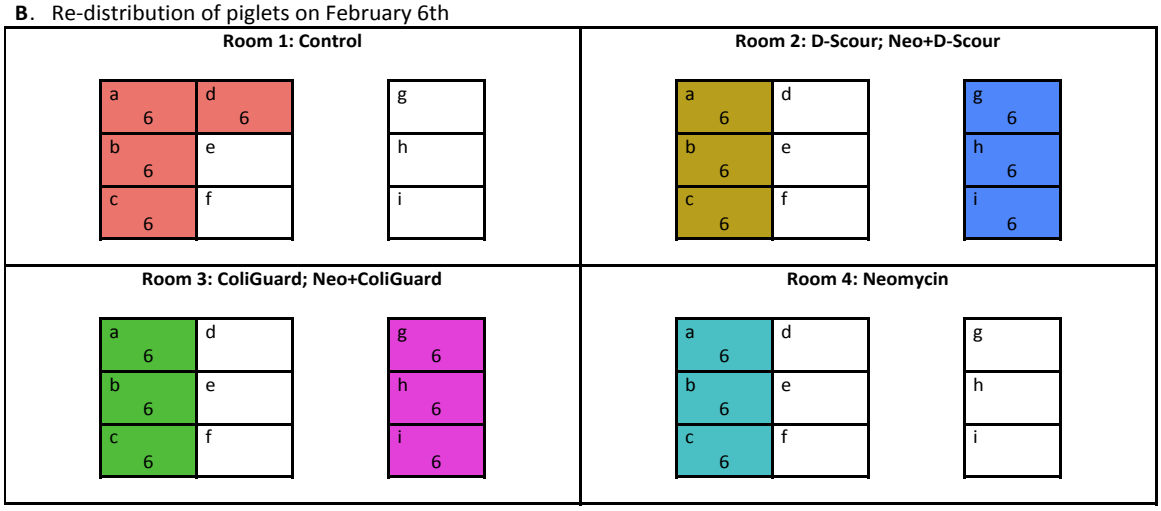
Figure 4. Abundance heatmap.

Most abundant taxa within each time point are obtained from analysis with guppy fat. Abundance is derived from the branch width. The distance between each time point is of one week.

A. Allocation of piglets in rooms and pens on the day of arrival

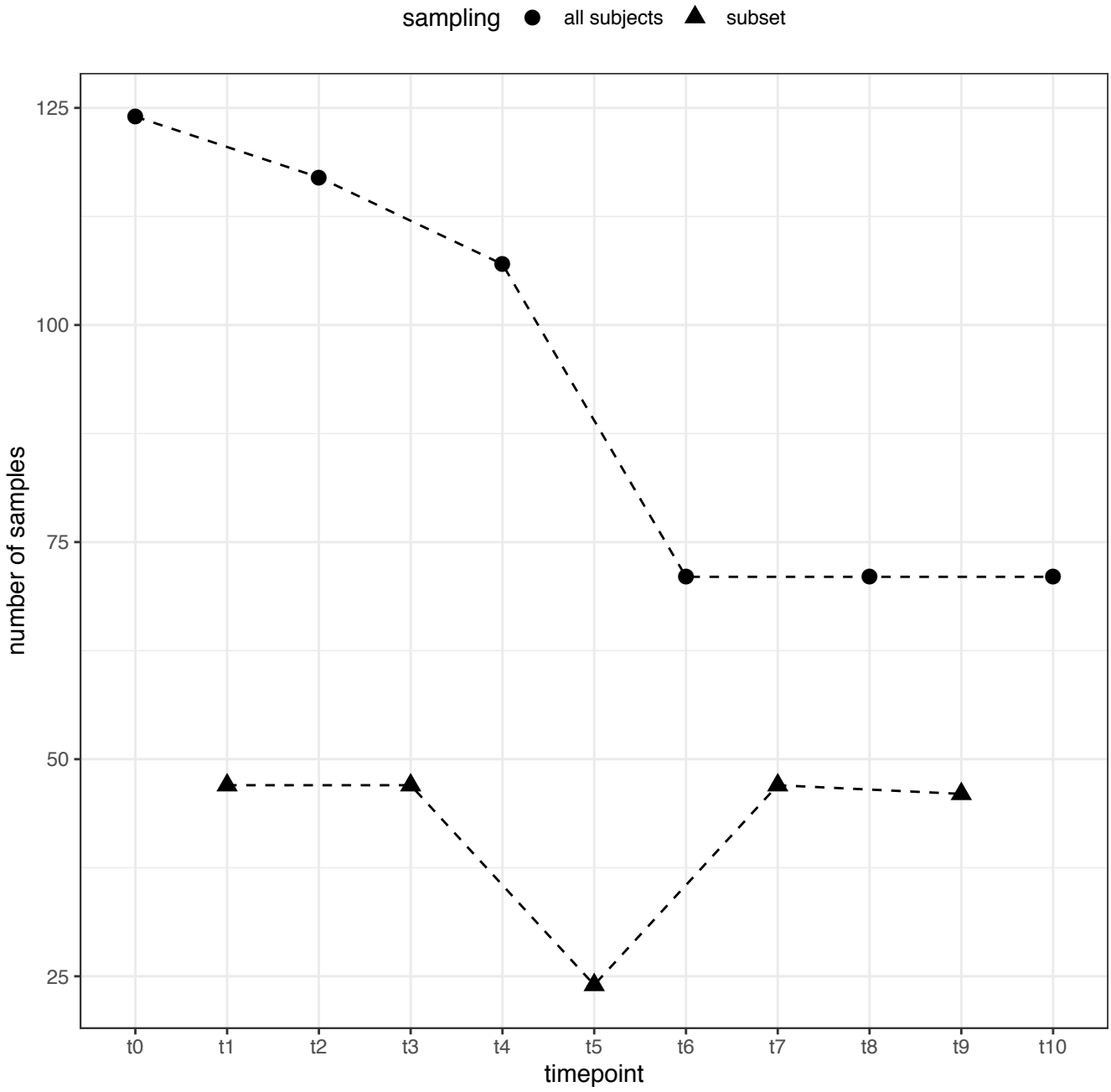


B. Re-distribution of piglets on February 6th



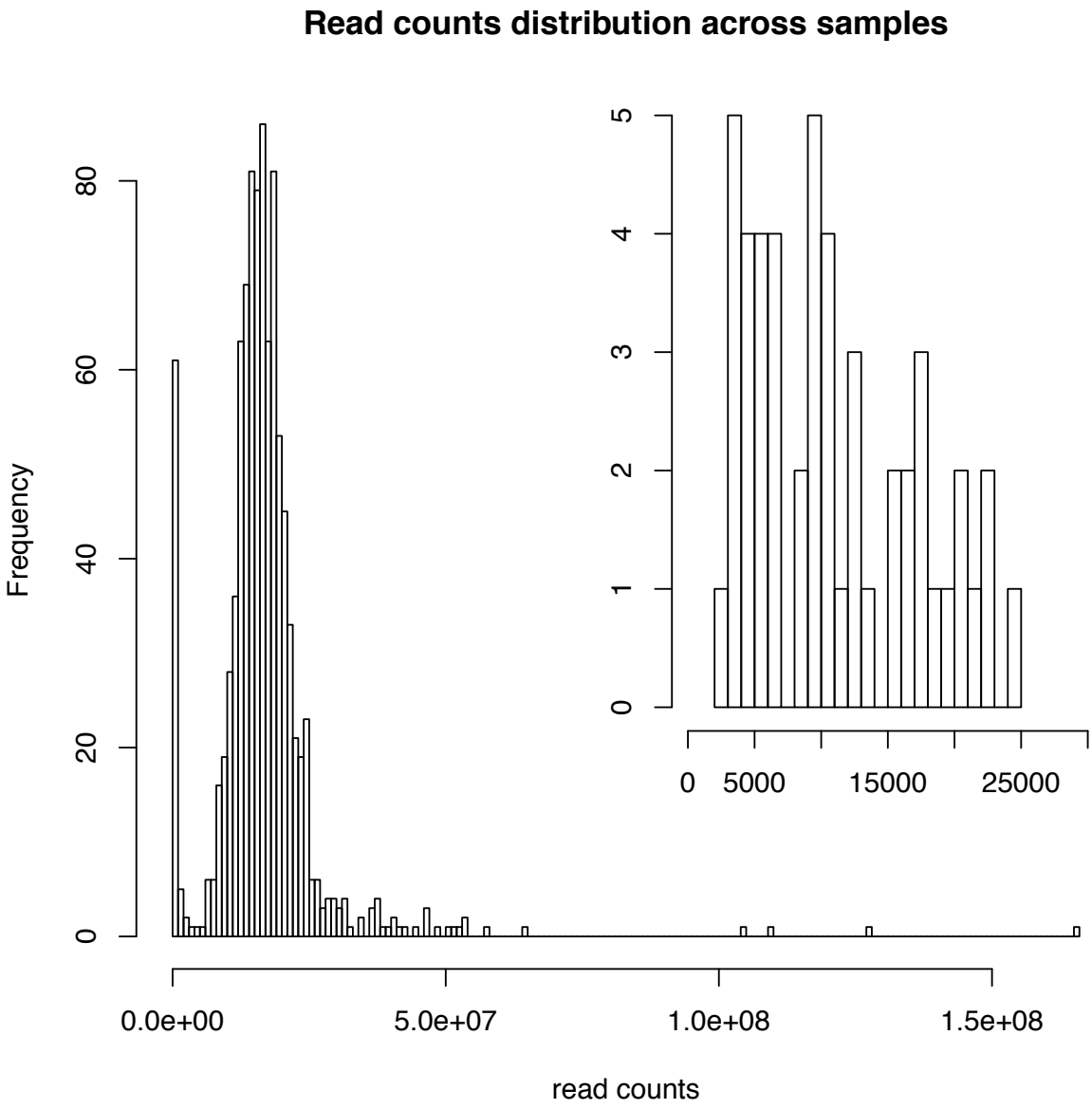
Supplementary Figure 1. Piglets placements across rooms and pens.

Disposition of piglets in rooms (1-4) and pens (a-i) at the start of the trial (**A**) and re-arrangement of a subset of neomycin treated piglets from room 4 to room 2 and room 3, for D-Scour™ and ColiGuard® treatment, respectively (**B**).



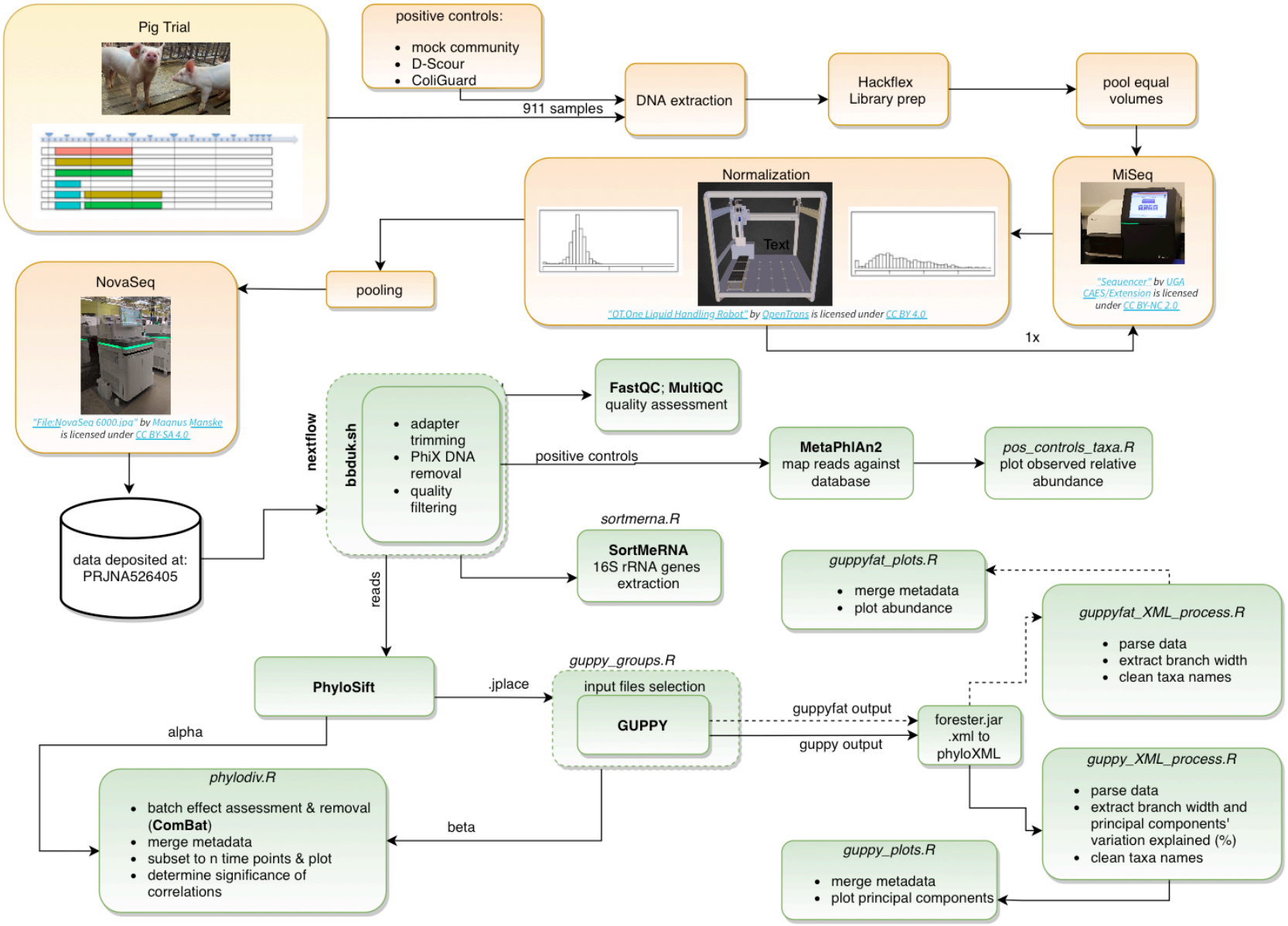
Supplementary Figure 2. Sample collection.

Samples were collected from all piglets once weekly, and from a subset group (8 per cohort; *n*=48) twice weekly. Overall, piglets were sampled between 1 and 10 times.



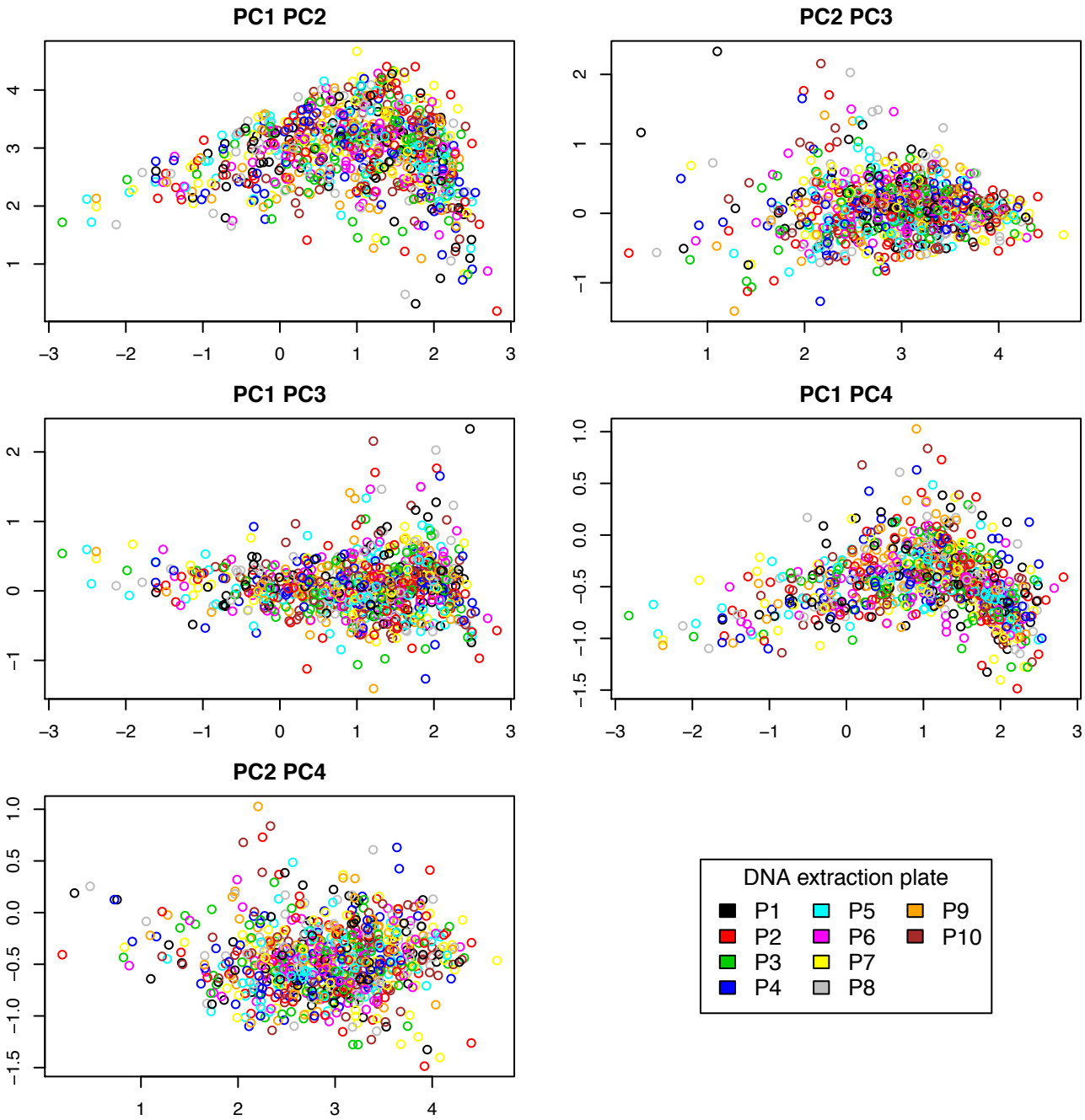
Supplementary Figure 3. Read counts distribution.

Read counts distribution of all samples (main histogram) and low read counts samples (top right histogram).



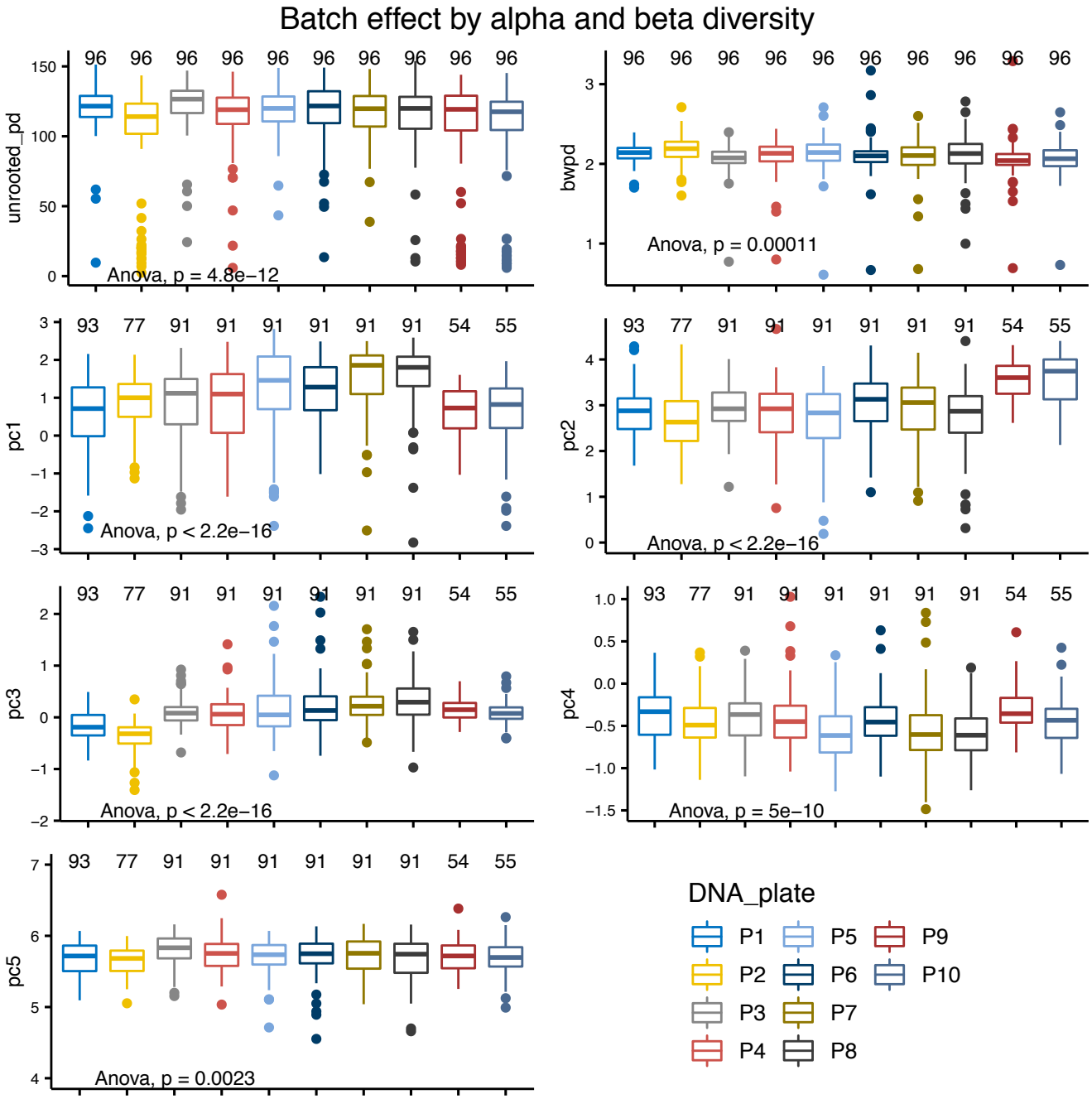
Supplementary Figure 4. Summarised workflow.

Schematic workflow from sample collection to sequencing (orange) and data analysis (green). R scripts (italic) are available in our Github repository.



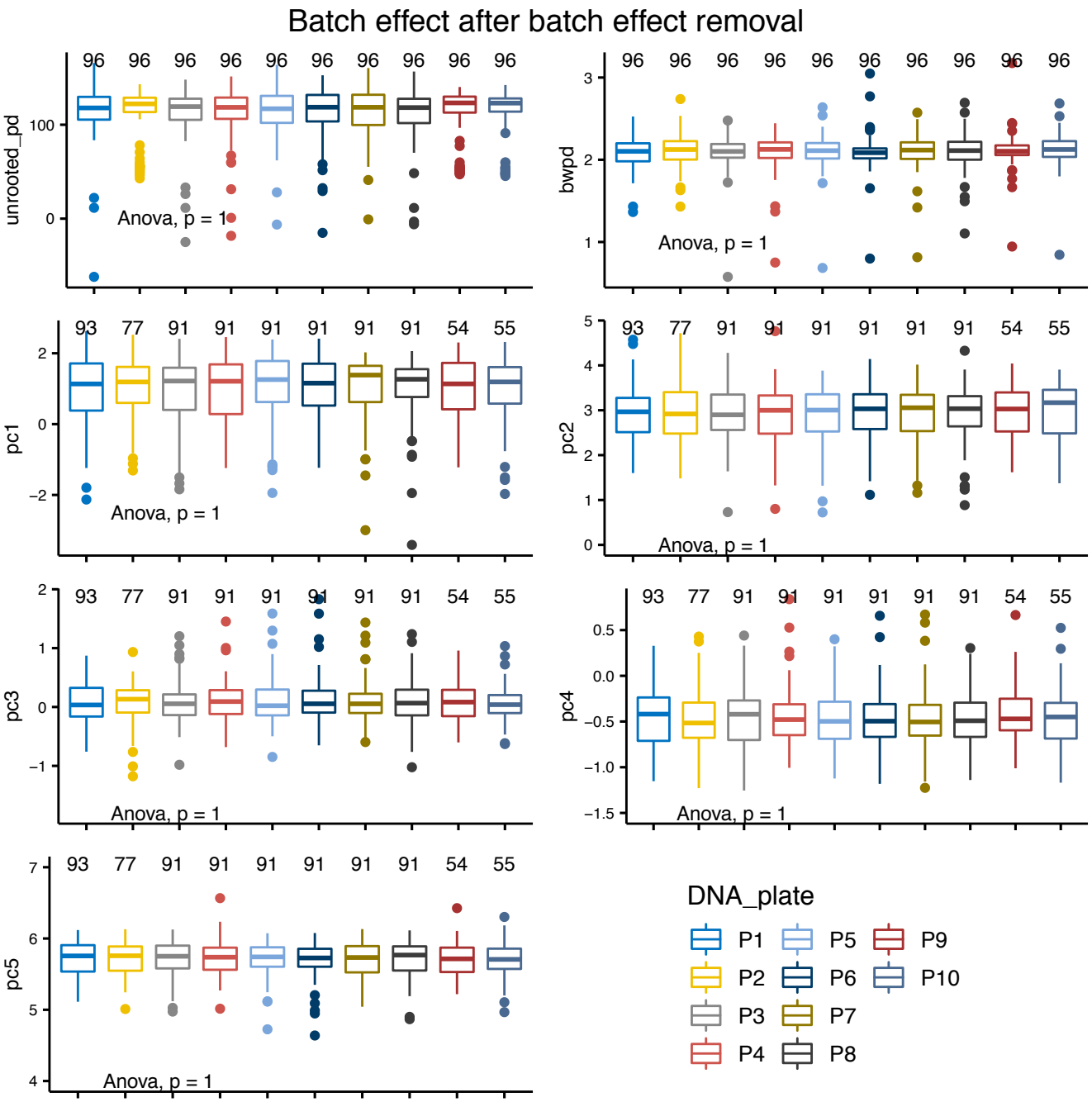
Supplementary Figure 5. Principal component analysis of batch effect before batch effect removal.

Principal components 1 to 4 from beta diversity analysis of all samples before batch effect removal. Samples are coloured by DNA extraction plate. No clustering of samples by DNA extraction plate is visible.



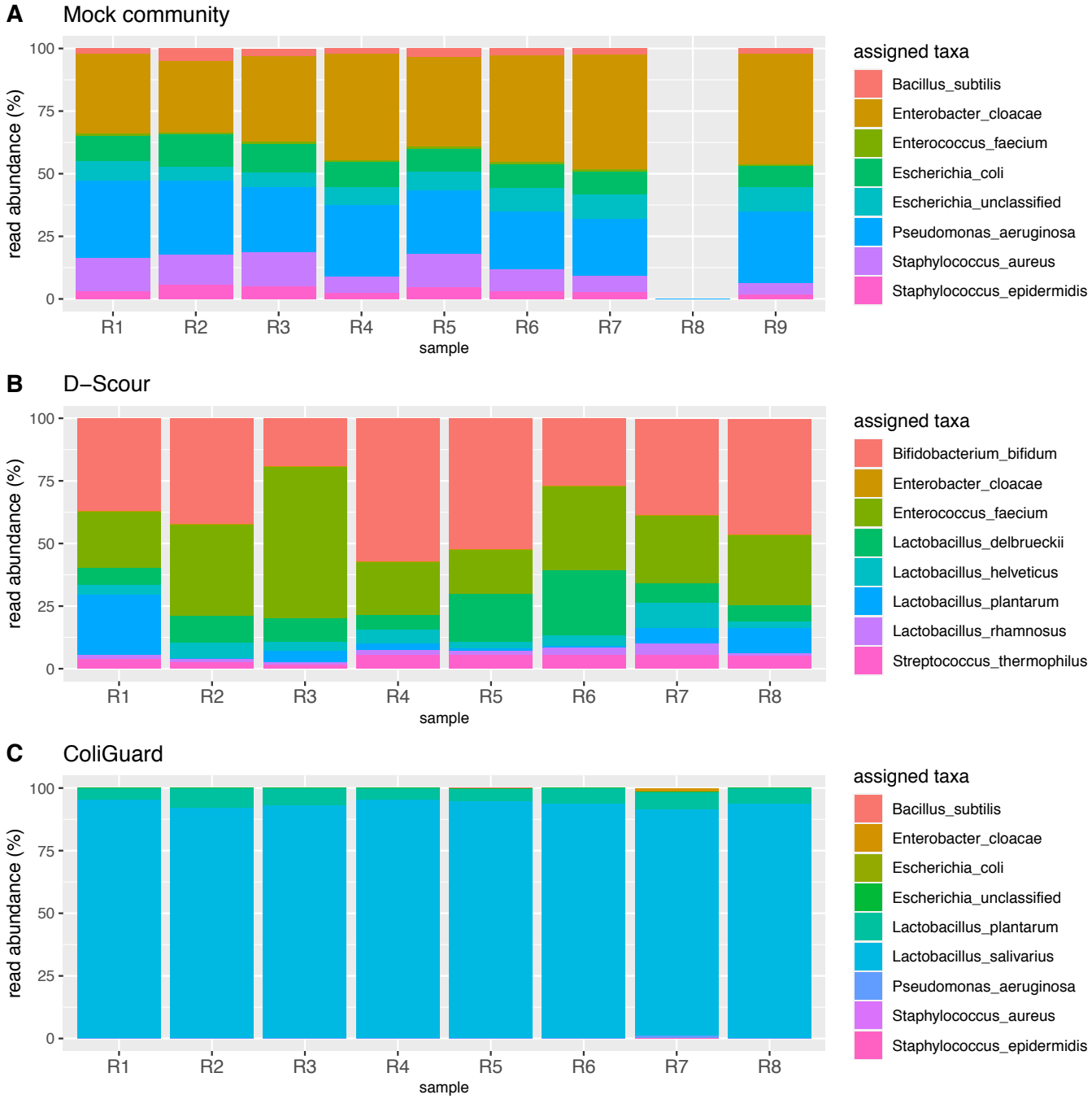
Supplementary Figure 6. Batch effect on alpha and beta diversity before batch effect removal.

Batch effect by alpha (top two plots) and beta diversity (bottom five plots) before batch effect removal. Samples are grouped by DNA extraction plate. The *p* values are derived from multiple comparison analysis with ANOVA, indicating equality of the means. *Post hoc* corrected *p* values for pairwise comparisons are provided in Supplementary File 5.



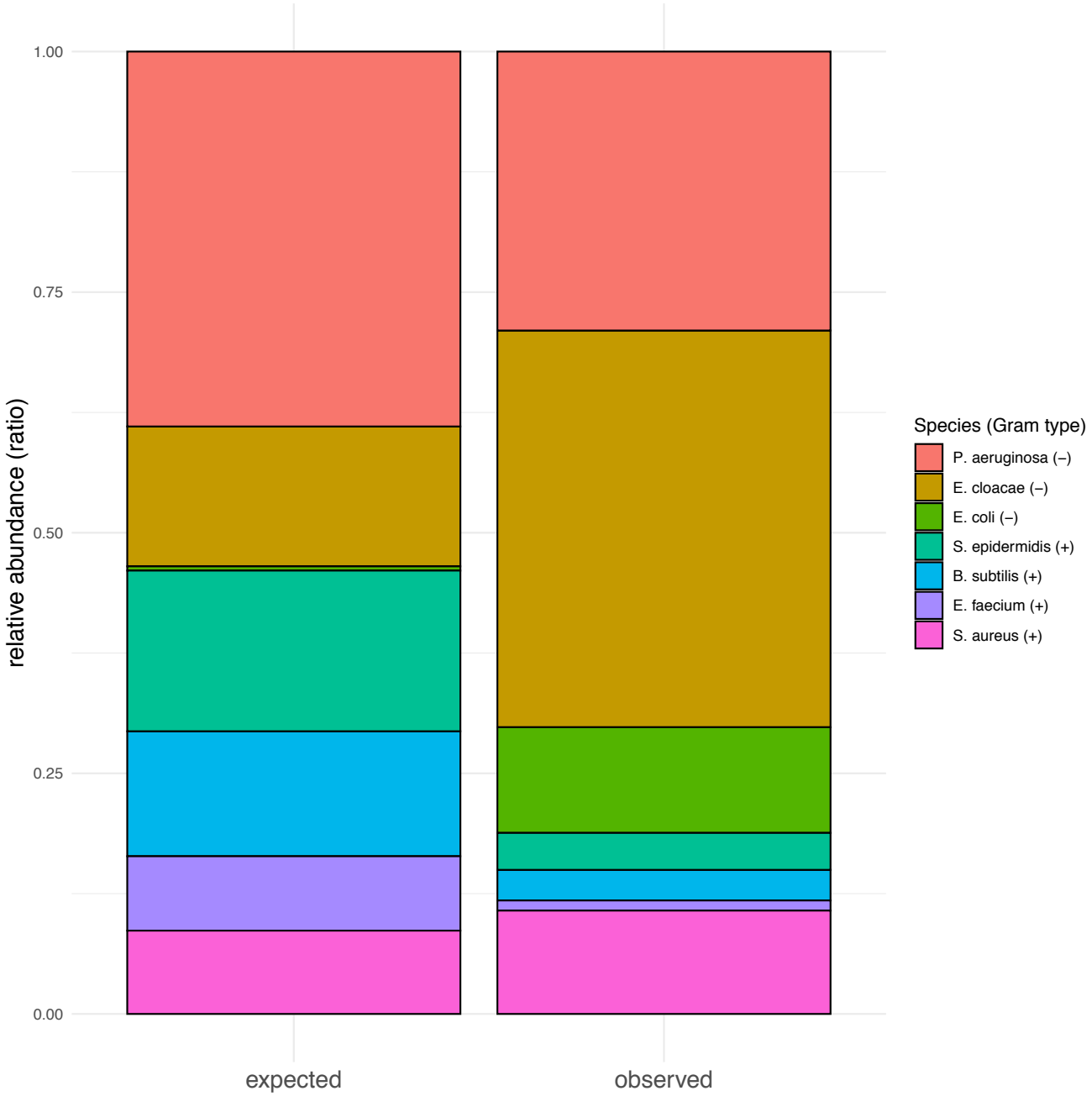
Supplementary Figure 7. Batch effect on alpha and beta diversity after batch effect removal.

Batch effect by alpha (top two plots) and beta diversity (bottom five plots) after batch effect removal. Samples are grouped by DNA extraction plate. The *p* values are derived from multiple comparison analysis with ANOVA, indicating equality of the means. *Post hoc* corrected *p* values for pairwise comparisons are provided in Supplementary File 5.



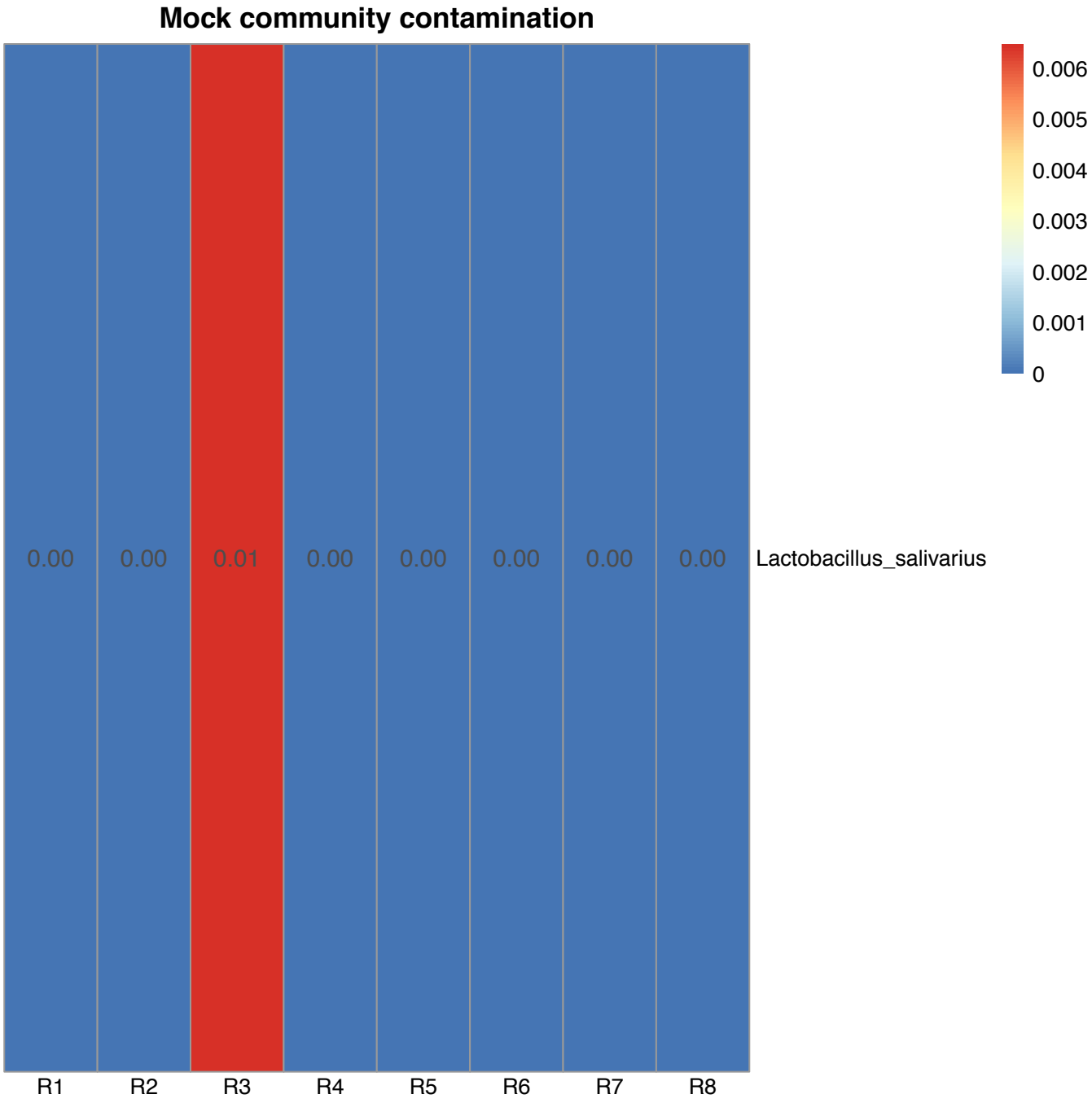
Supplementary Figure 8. Taxonomic assignment of reads from positive control samples.

Taxonomic profile of the positive controls used in this study obtained by mapping the reads against a ~1M bacterial genomes database with MetaPhlAn2. Each stacked barplot represents a technical replicate. Taxa of which reads are present in >0.1% are displayed. **A**. In-house made mock community; **B**. commercially available livestock probiotic D-Scour™ ; **C**. ColiGuard®.



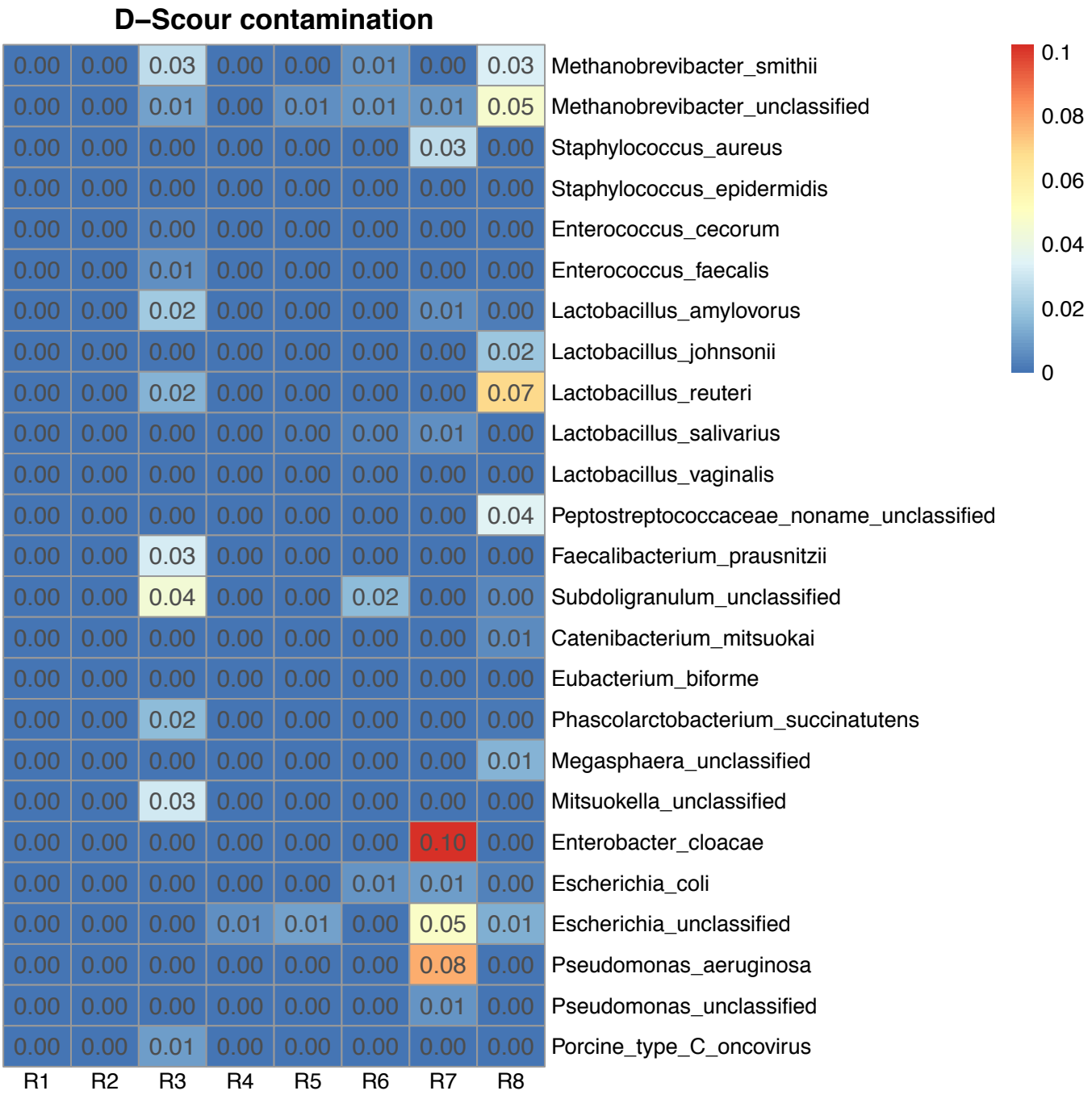
Supplementary Figure 9. Expected and observed relative abundance of mock community members.

Expected relative abundance is derived by multiplying CFU count by genome size. Observed relative abundance is derived by multiplying reads mapping with MetaPhlAn2. A higher observed/expected ratio is observed in two of the three Gram negative species and a lower observed/expected ratio is observed in three of the four Gram negative species.



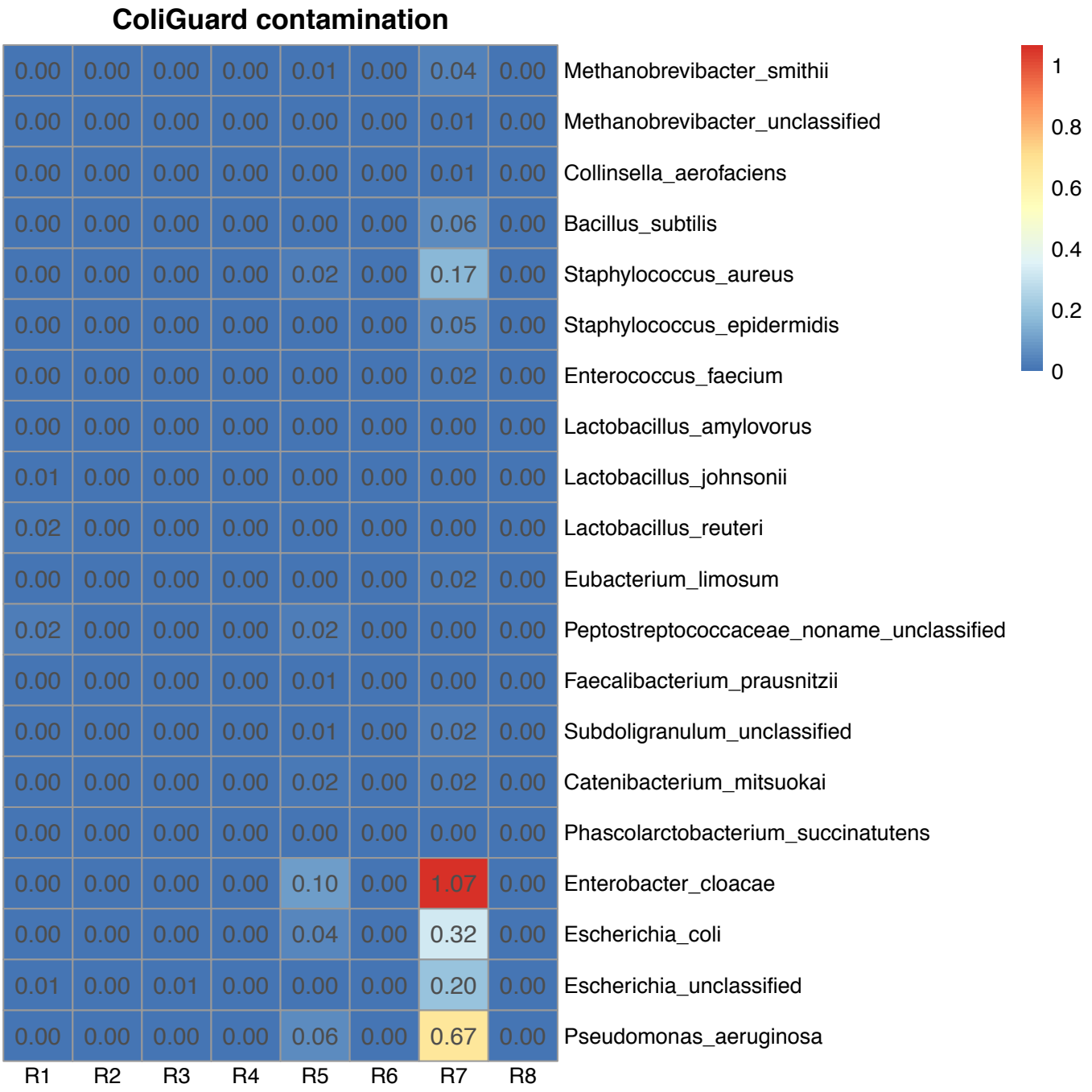
Supplementary Figure 10. Contamination rate in mock community samples.

Heatmap of contaminating species present in the positive control mock community technical replicates. *Lactobacillus salivarius* is found in one replicate at 0.01% of the total reads.



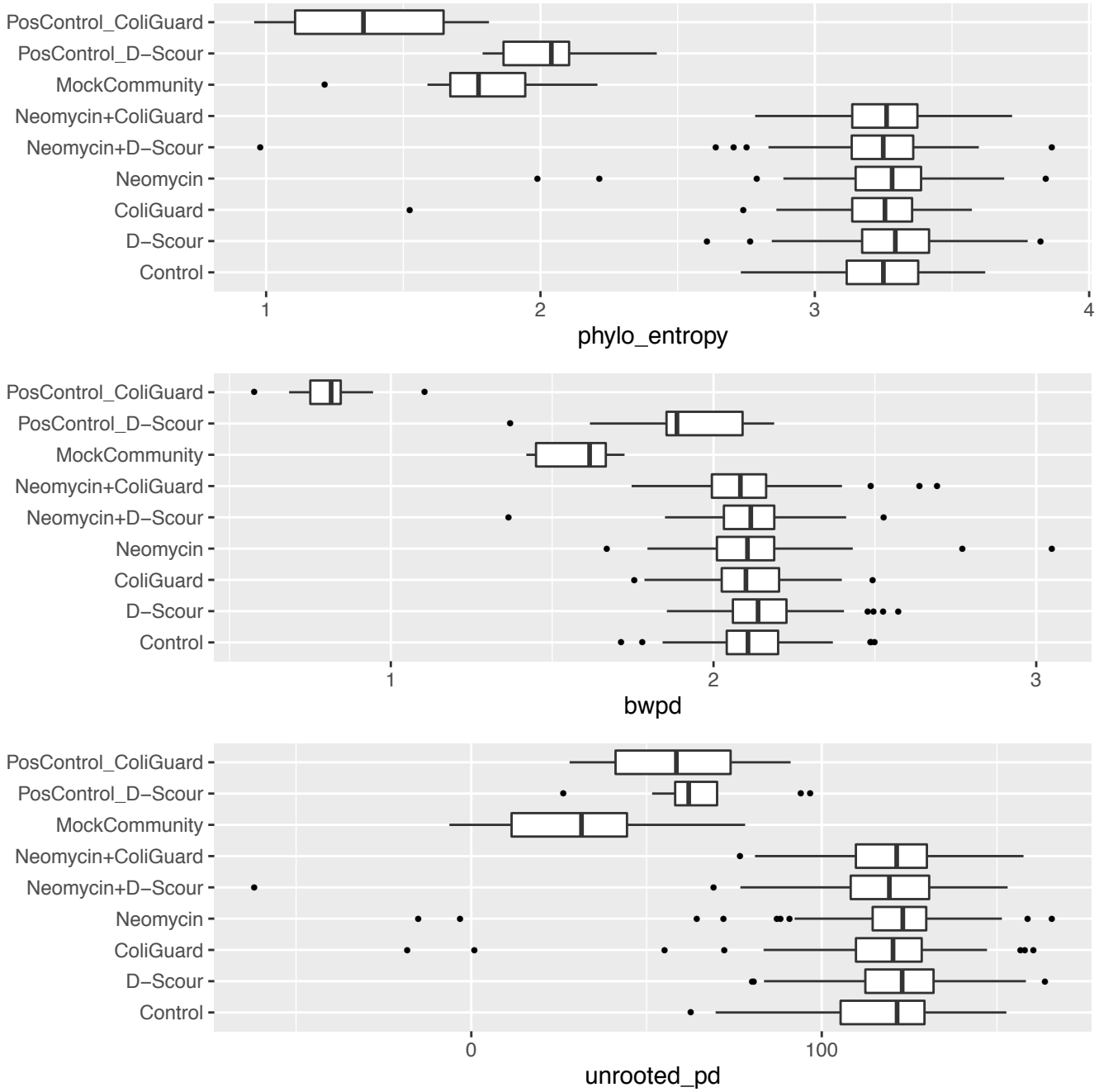
Supplementary Figure 11. Contamination rate in D-Scour™ samples.

Heatmap of contaminating species present in the positive control D-Scour™ technical replicates. The D-Scour™ technical replicates contained 25 contaminants, of which 18 and 7 were identified at the species and at genus level, respectively. Contaminants are majorly present in three technical replicates (R3, R7, R8) and the most frequent contaminant (*Metahobrevibacter*) was present in 5 of the 8 replicates.



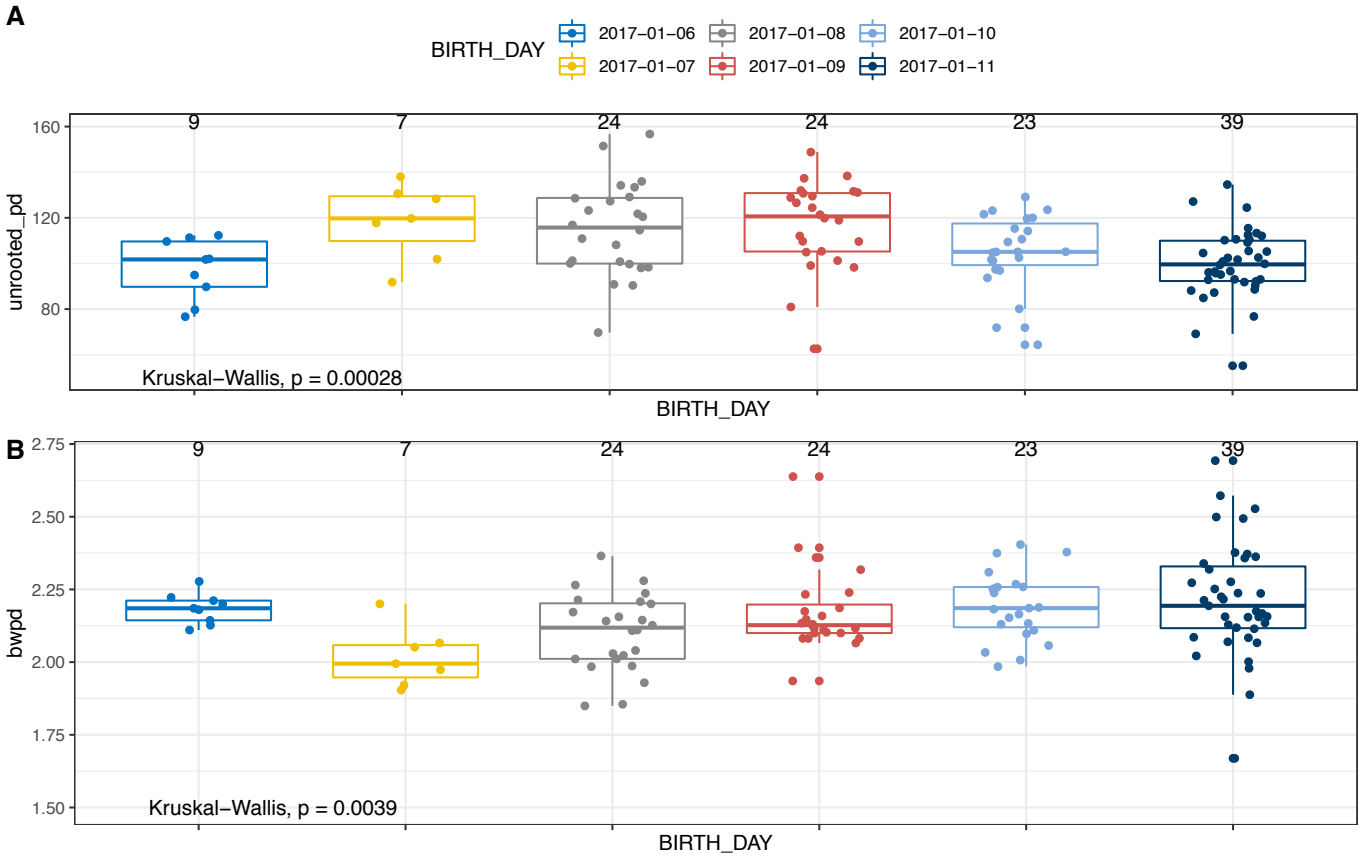
Supplementary Figure 12. Contamination rate in ColiGuard® samples.

Heatmap of contaminating species present in the positive control ColiGuard® technical replicates. ColiGuard® contained 20 contaminants, of which 16 and 4 were identified at the species and at genus level, respectively. Contaminants were present majorly in two technical replicates (R5, R7).



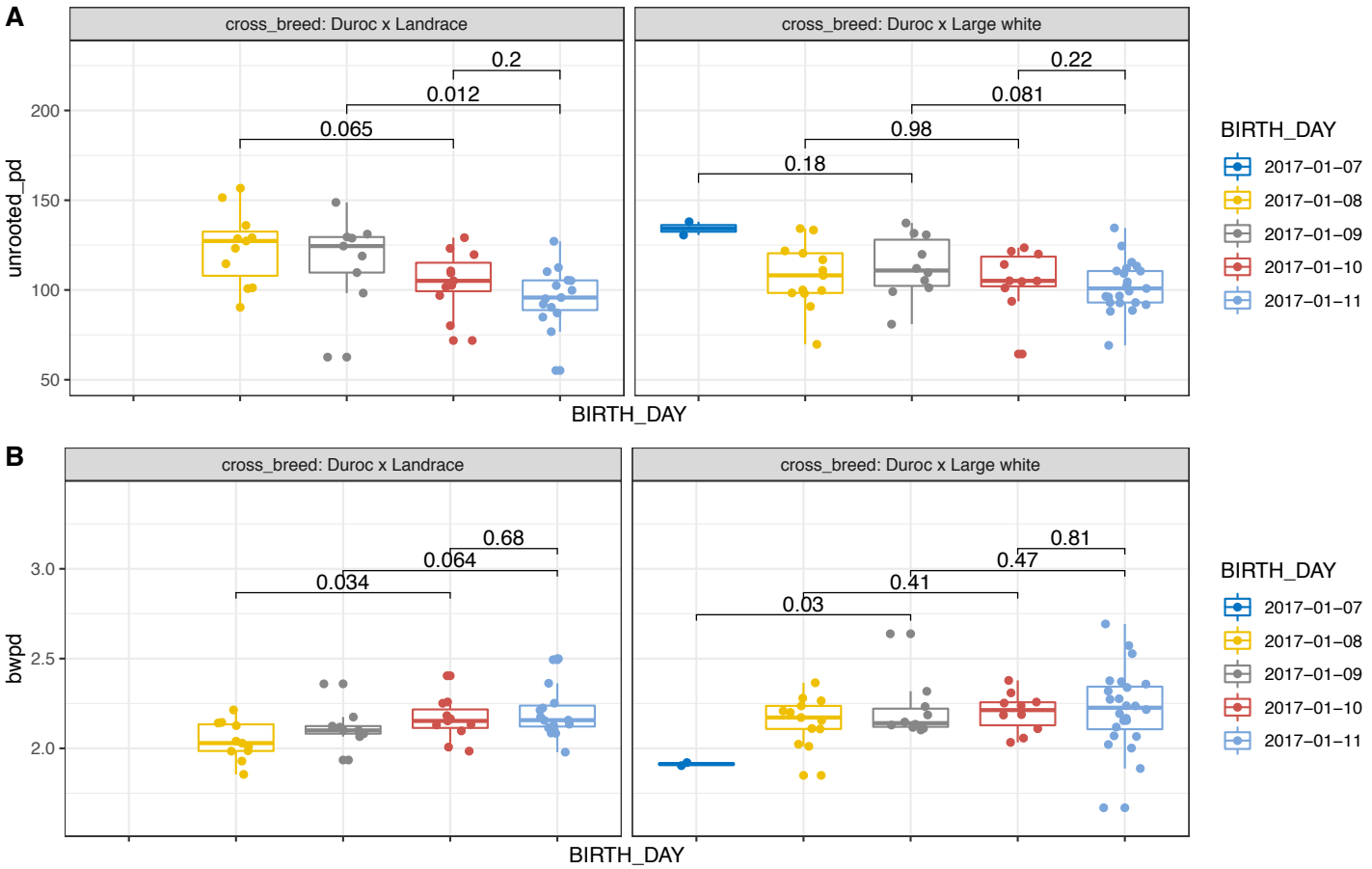
Supplementary Figure 13. Alpha phylogenetic diversity of cohorts.

Alpha phylogenetic diversity per cohort from samples across all time points. Phylogenetic entropy (top); Balance-weighted phylogenetic diversity (BWPD) (mean±SD: Positive control Mock community: 1.58±0.12; Positive control D-Scour™: 1.89±0.26; Positive control ColiGuard®: 0.82±0.15; Control: 2.12±0.14; D-Scour™: 2.14±0.14; ColiGuard®: 2.11±0.13; neomycin: 2.12±0.17; neomycin+D-Scour™: 2.12±0.14; neomycin+ColiGuard®: 2.10±0.15; sows: 2.13±0.14; all piglet cohorts: 2.12±0.14); (middle); Unrooted phylogenetic diversity (Positive control Mock community: 31.53±29.50; Positive control D-Scour™: 64.84±21.30; Positive control ColiGuard®: 58.52±21.70; Control: 117±16.8; D-Scour™: 121±14.7; ColiGuard®: 118±22.3; neomycin: 119±22.6; neomycin+D-Scour™: 116±23.4; neomycin+ColiGuard®: 121±15.8; sows: 125.39±7.56; all piglet cohorts: 118.54±19.70) (bottom).



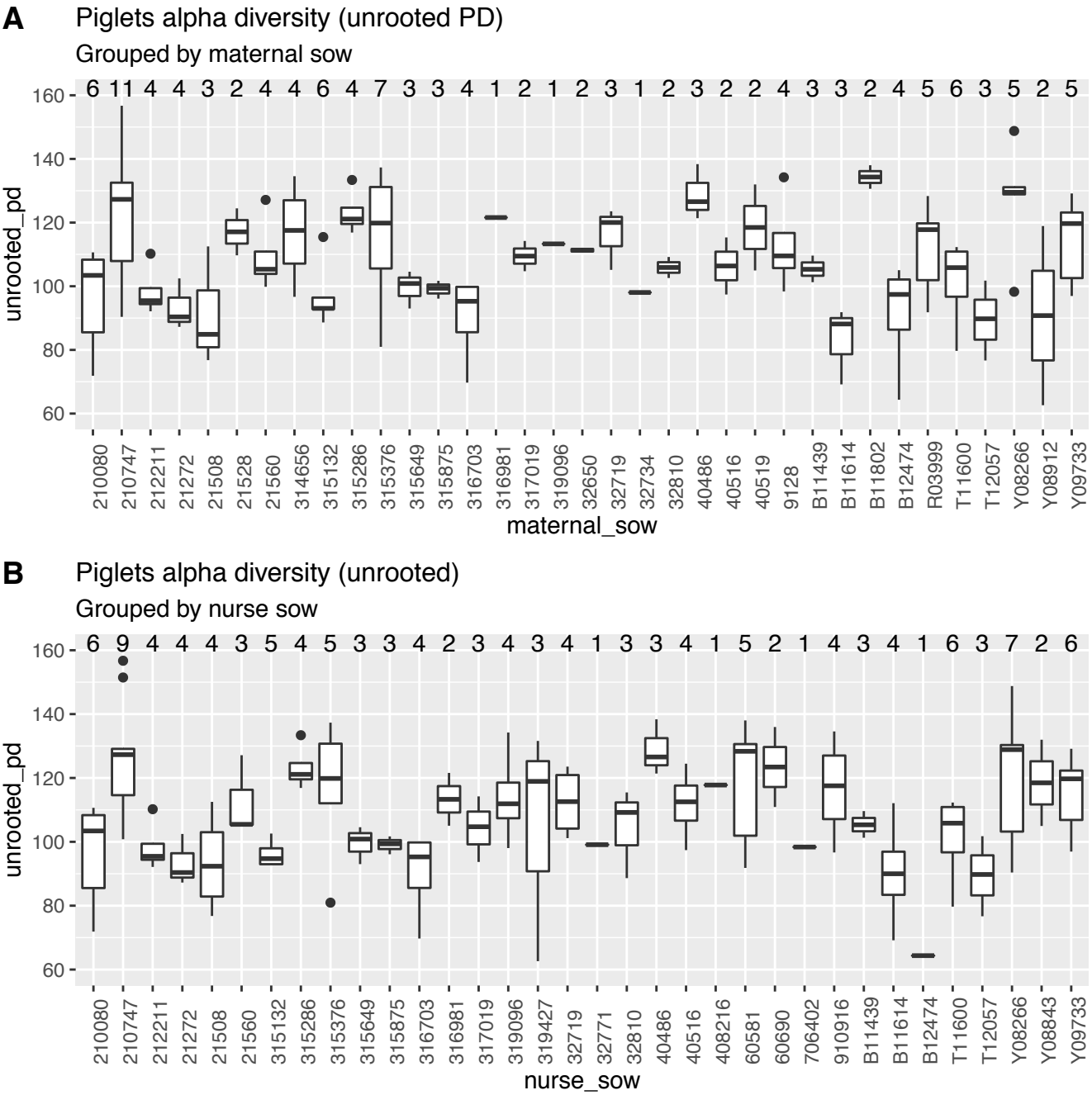
Supplementary Figure 14. Alpha phylogenetic diversity by age group.

Alpha diversity of samples from the start of the trial grouped by the date of birth of the piglet. Unrooted phylogenetic diversity (top) and balance weighted phylogenetic diversity (bottom). *P* values are derived from Kruskal-Wallis analysis of variance. Pairwise comparisons and *post hoc* corrected *p* values are provided in Supplementary File 5.



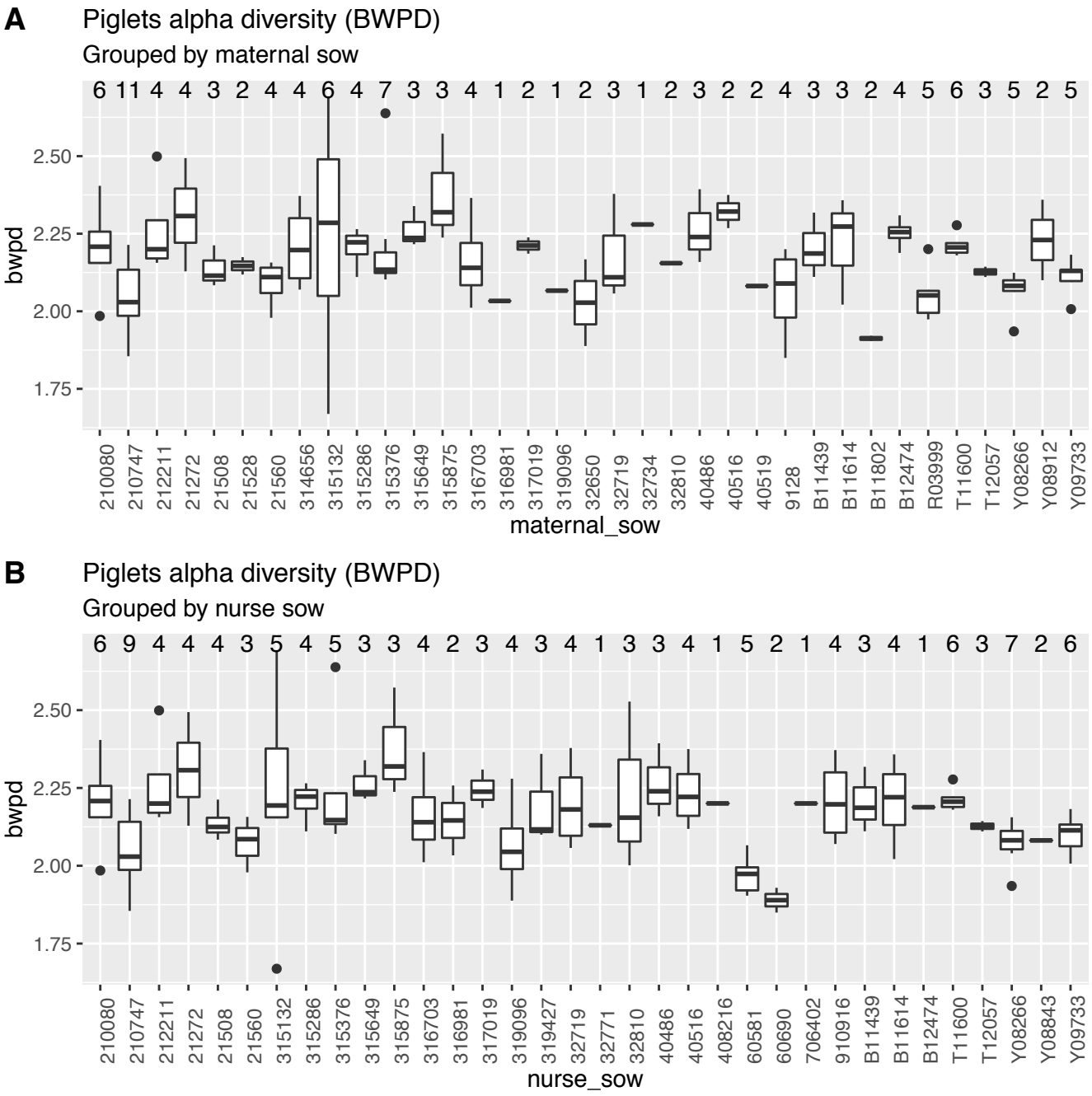
Supplementary Figure 15. Alpha phylogenetic diversity by age breed.

Alpha diversity of samples from the start of the trial grouped by breed and by date of birth. Unrooted phylogenetic diversity (top) and balance weighted phylogenetic diversity (bottom). *P* values are derived from Kruskal-Wallis analysis of variance. Pairwise comparisons and *post hoc* corrected *p* values are provided in Supplementary File 5.



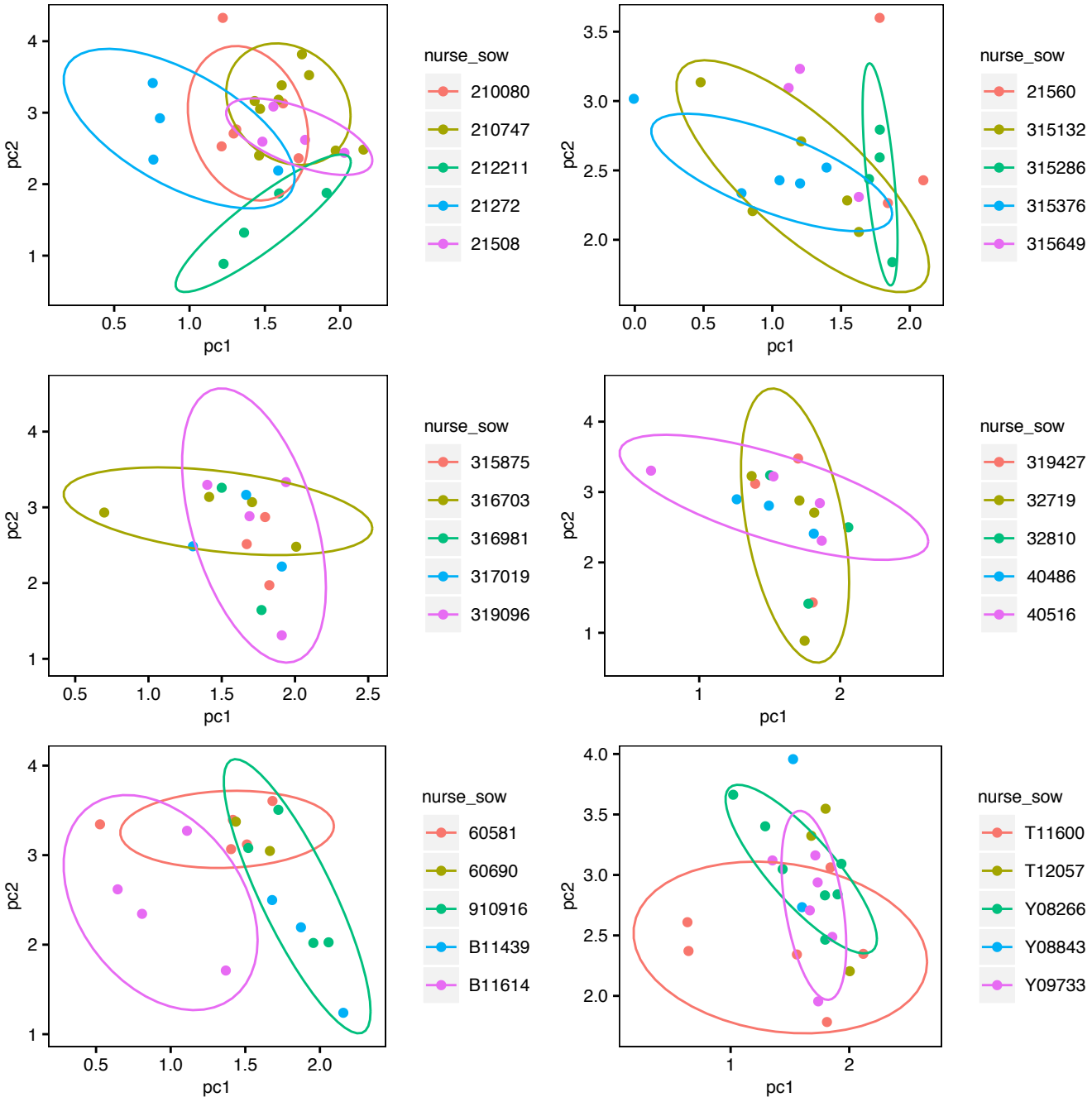
Supplementary Figure 16. Unrooted alpha phylogenetic diversity by maternal and nurse sow.

Alpha phylogenetic diversity (unrooted) of samples from piglets at the start of the trial (one time point -sample per piglet). The grouping is based on the maternal sow (**A**) or nurse sow (**B**). *P* values from multiple groups comparison by maternal sow: Kruskal-Wallis *p* values: 0.0011; BWPD: 0.0049. *P* values from multiple groups comparison by nurse sow: Kruskal-Wallis *p* values: 0.0036; BWPD: 0.0071. *Post-hoc* pairwise comparisons and adjusted *p* values are provided in Supplementary File 5.



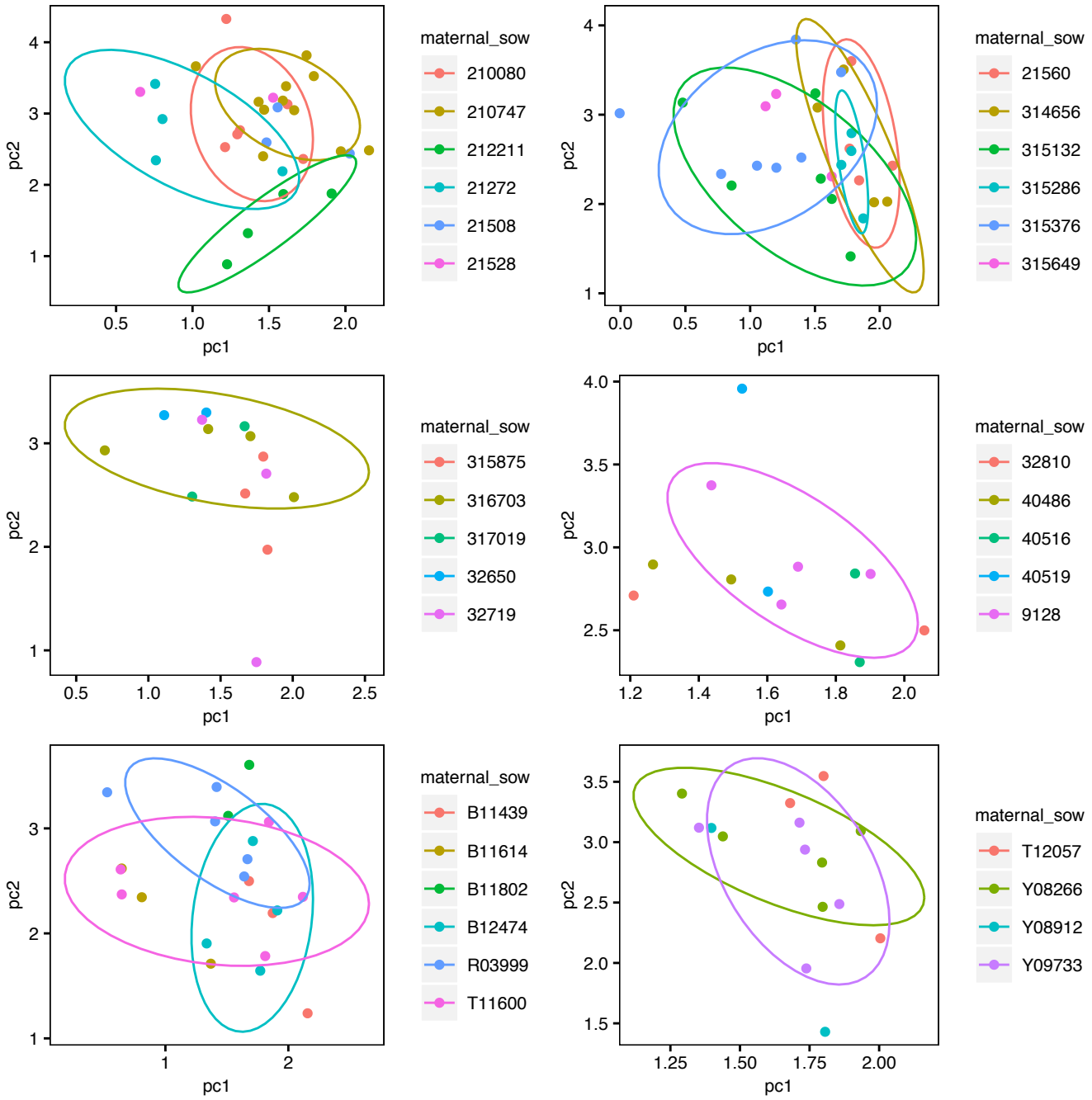
Supplementary Figure 17. Balance weighted alpha phylogenetic diversity by maternal and nurse sow.

Alpha phylogenetic diversity (unrooted) of samples from piglets at the start of the trial (one time point -sample per piglet). The grouping is based on the maternal sow (**A**) or nurse sow (**B**). *P* values from multiple groups comparison by maternal sow: Kruskal-Wallis p=0.0049. *P* values from multiple groups comparison by nurse sow: Kruskal-Wallis p=0.0071. *Post-hoc* pairwise comparisons and adjusted p values are provided in Supplementary File 5.



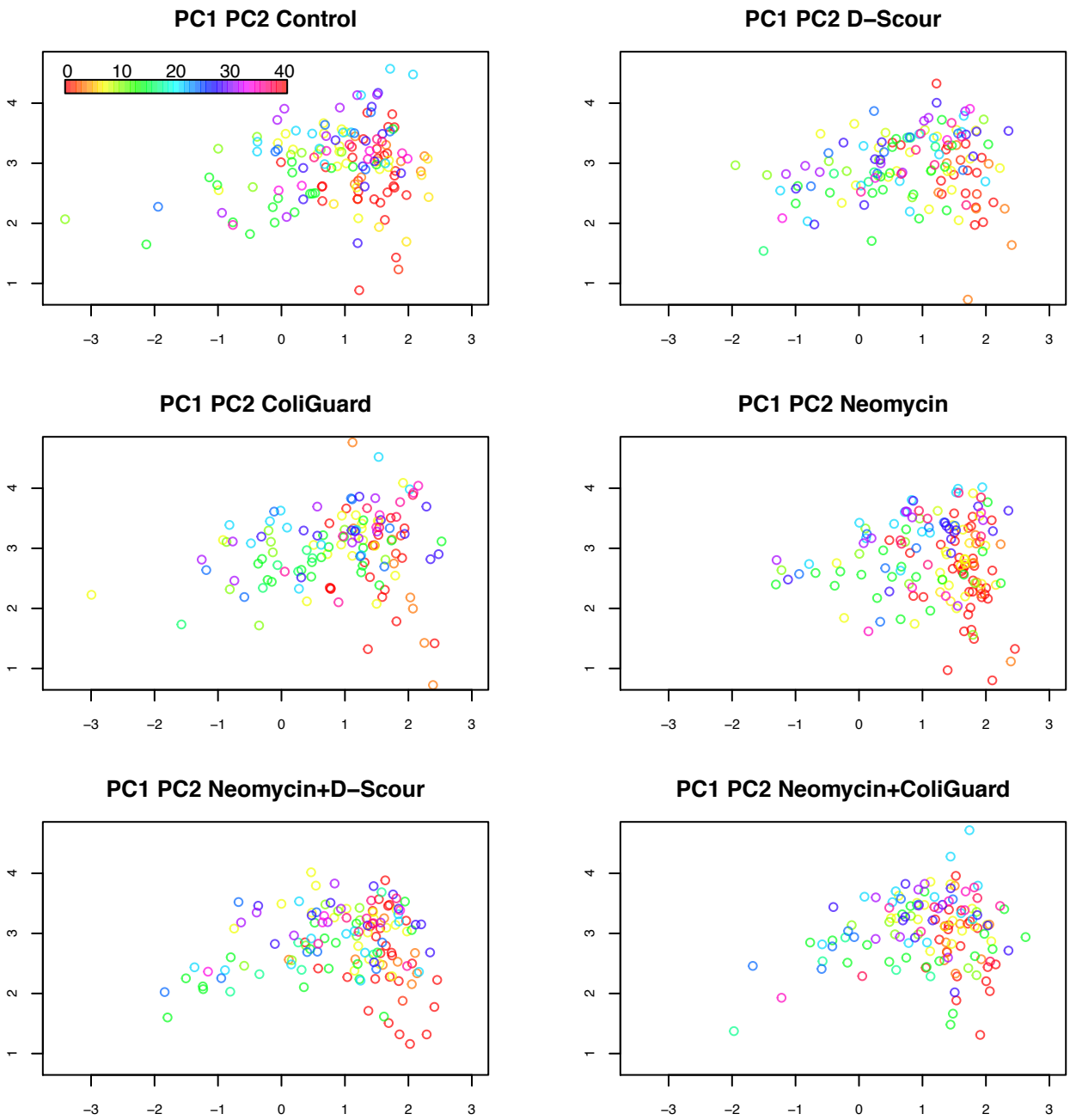
Supplementary Figure 18. Principal component analysis of piglet samples by nurse sow.

First two principal components of beta phylogenetic diversity. Principal component 1 (PC1) and principal component 2 (PC2) explaining 46.99% and 21.68% of the variation, respectively.
Samples from piglets at the start of the trial (one time point -sample per piglet) coloured by nurse sow (n=30). Ellipse is drawn at 0.80 confidence level.



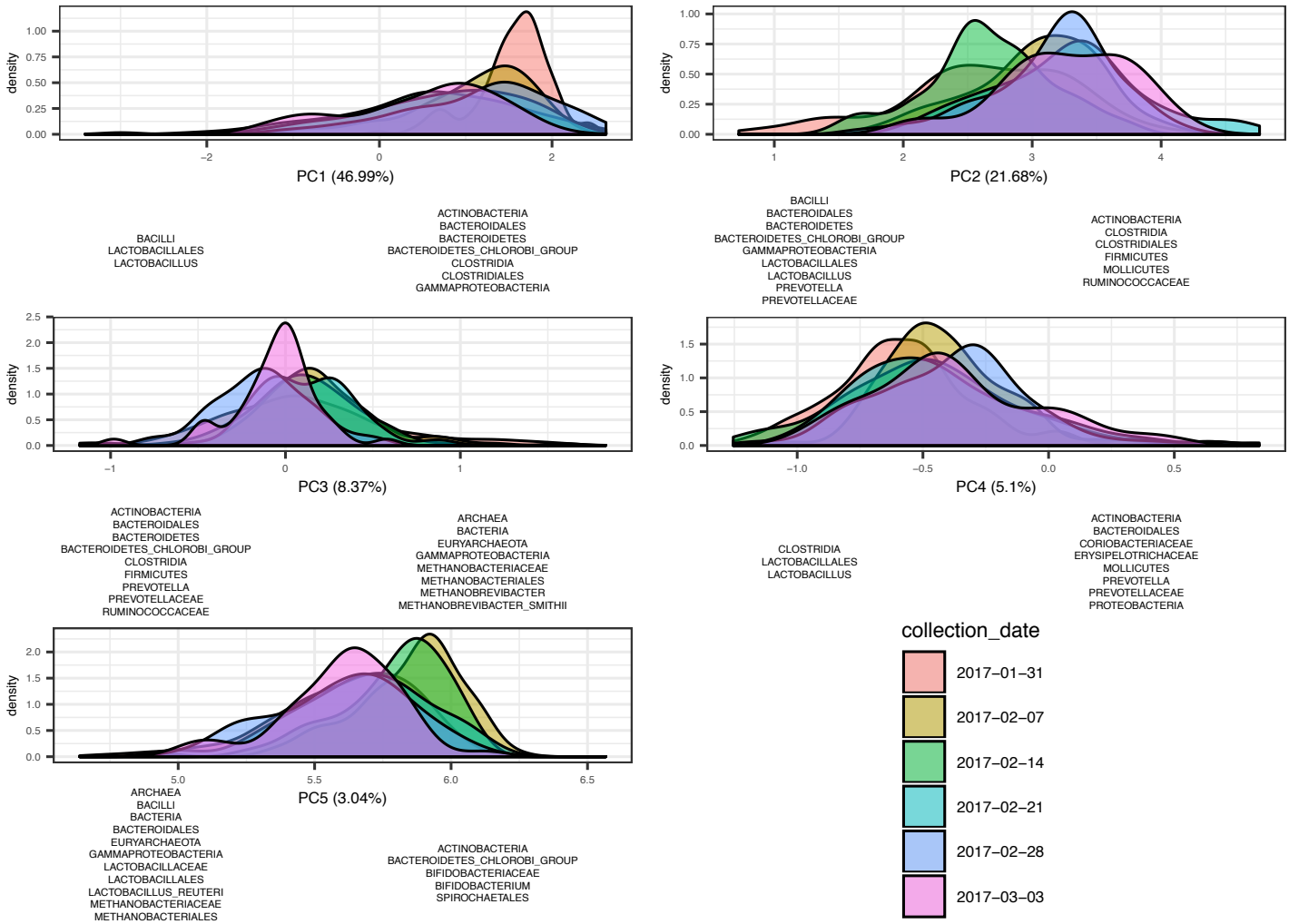
Supplementary Figure 19. Principal component analysis of piglet samples by maternal sow.

First two principal components of beta phylogenetic diversity. Principal component 1 (PC1) and principal component 2 (PC2) explaining 46.99% and 21.68% of the variation, respectively. Samples from piglets at the start of the trial (one time point -sample per piglet) colored by maternal sow (n=32). Ellipse is drawn at 0.80 confidence level.



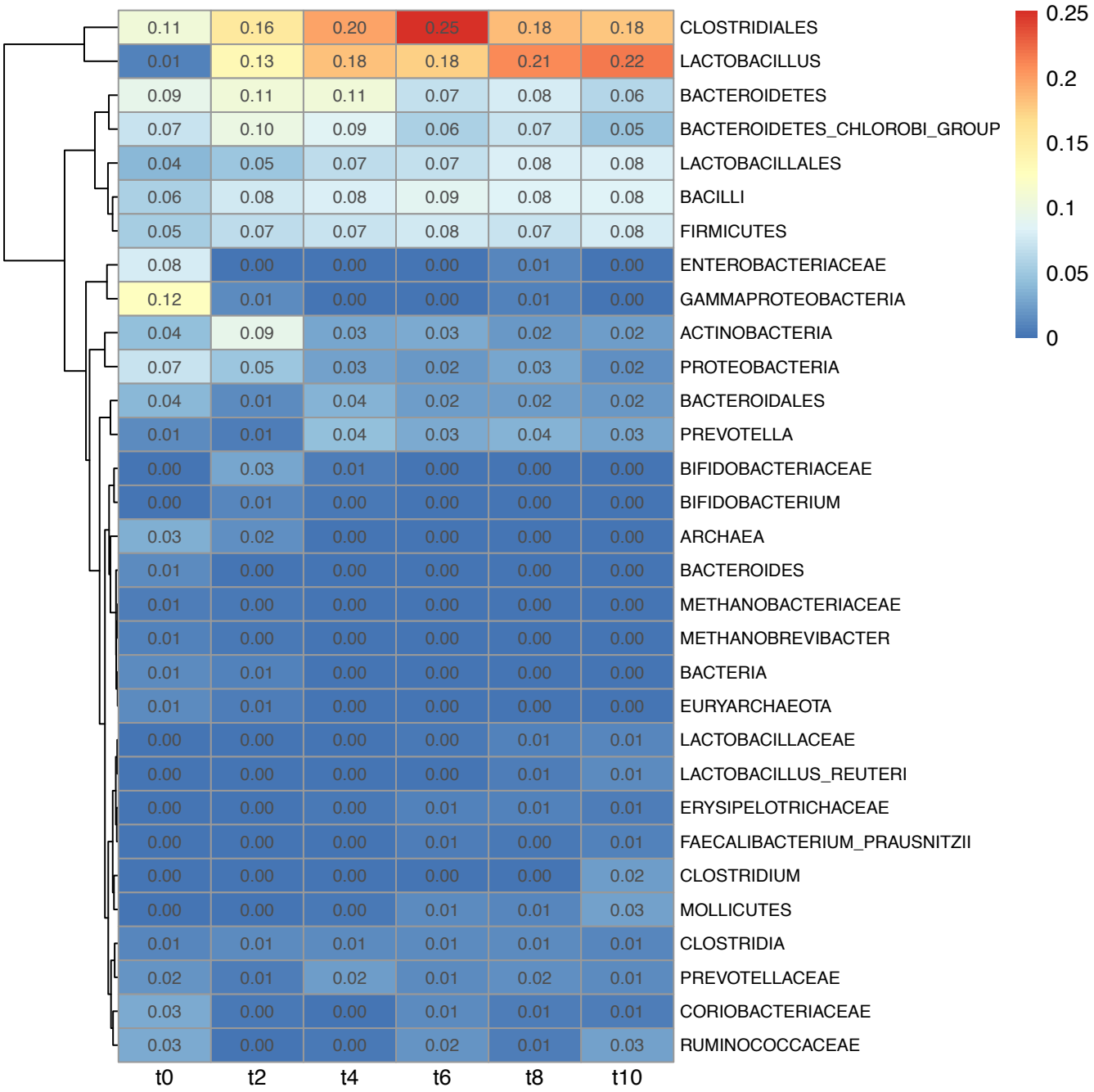
Supplementary Figure 20. Time effect on beta diversity per cohort.

Edge principal component analysis of all samples coloured by sample collection time across the trial (day 0: red; day 10: yellow; day 20: green; day 30: light blue; day 40: dark blue) where principal component 1 (PC1) explains 46.99% of the variation and principal component 2 (PC2) explains 21.68% of the variation. Each plot displays all samples within each cohort.



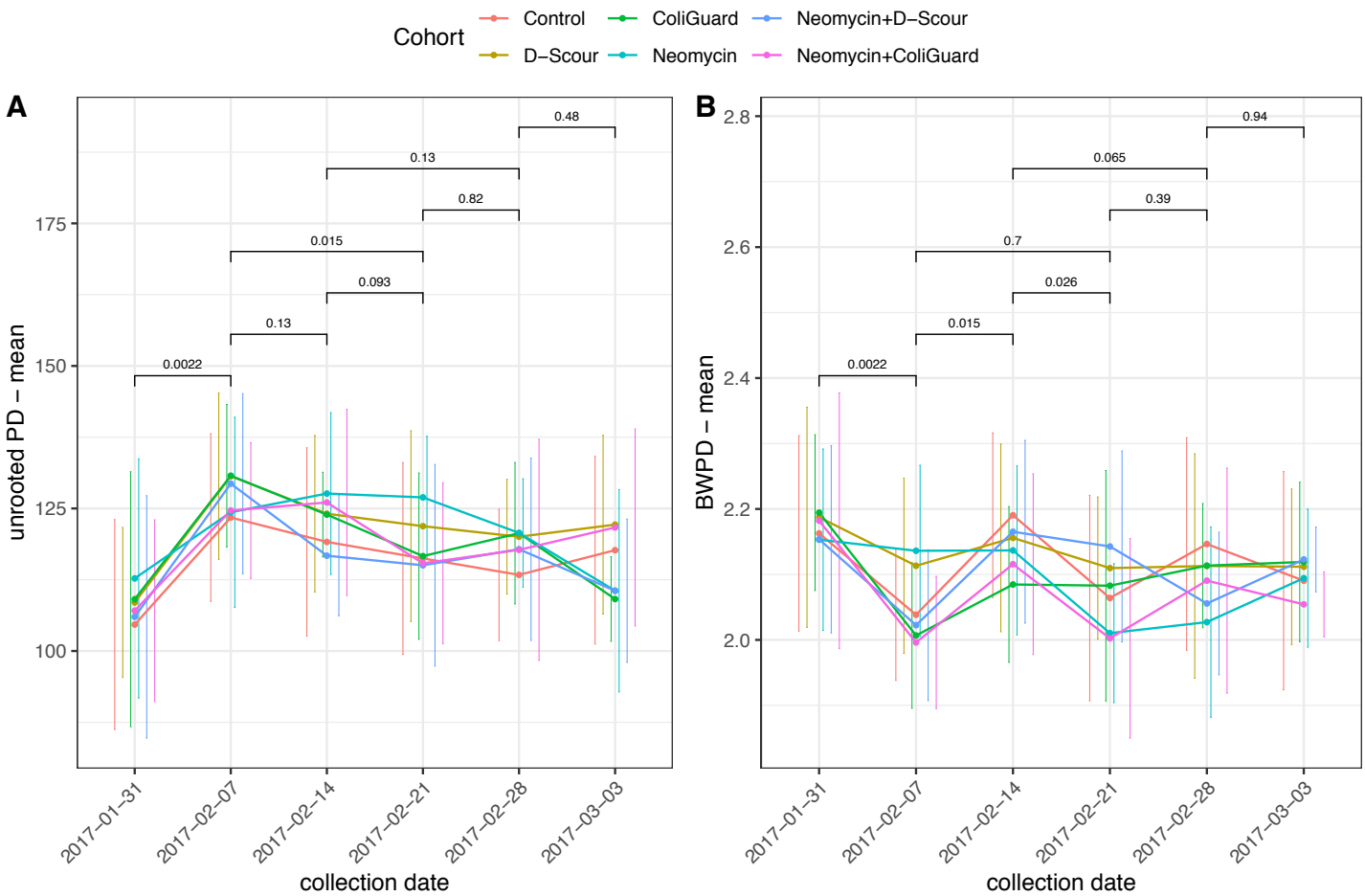
Supplementary Figure 21. Time effect on beta diversity.

Each plot represents the samples distribution along one of the five principal components across time. Most variation is explained by PC1 (46.99%), followed by PC2 (21.68%), PC3 (8.37%), PC4 (5.1%) and PC5 (3.04%). Distribution of the samples on either side of a plot (left *versus* right) reflects the taxa that were found to explain the variation. The distributions are colour coded by time point during the trial.



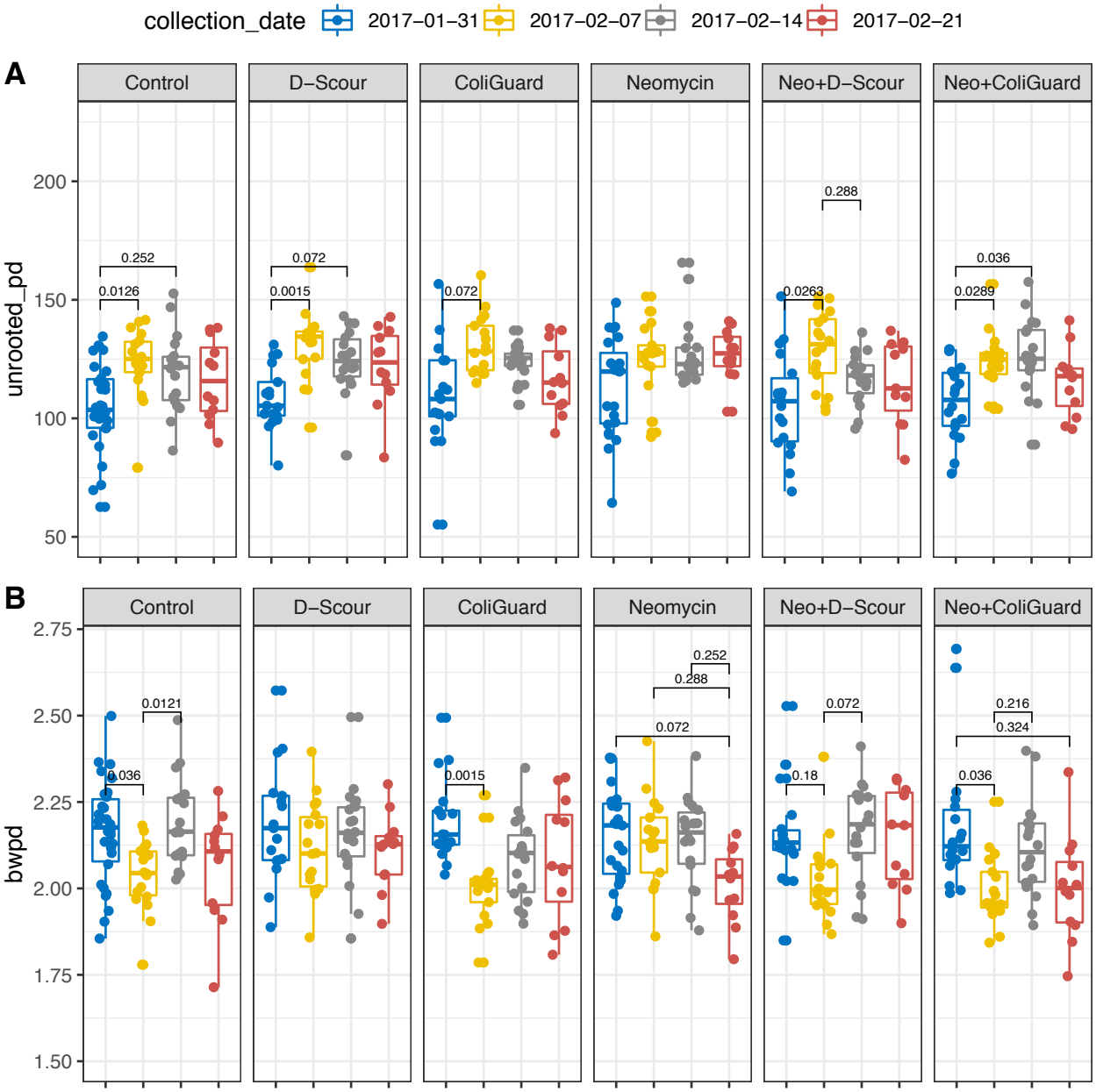
Supplementary Figure 22. Taxa displaying the highest variation in beta diversity across time.

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



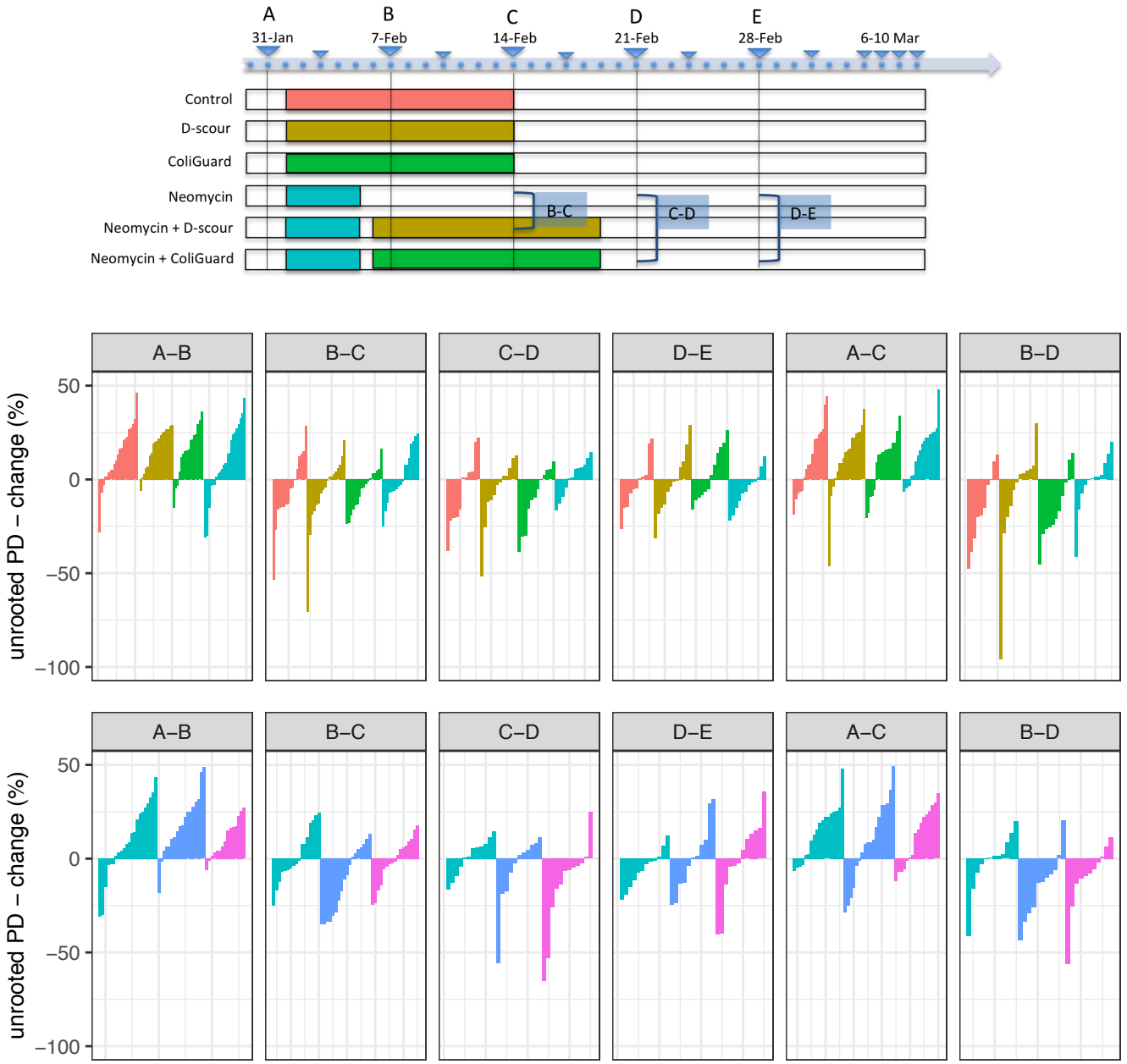
Supplementary Figure 23. Time trend of alpha diversity by cohort.

Unrooted PD (**A**) and BWPD (**B**) describe richness and evenness, respectively, of alpha phylogenetic diversity for all samples across time, grouped and colour coded by cohort. The *p* values derived from pairwise comparisons of time points within each of the cohorts. *P* values and *post-hoc* corrected *p* values of time points comparisons for each separate cohort are provided in Supplementary File 5.



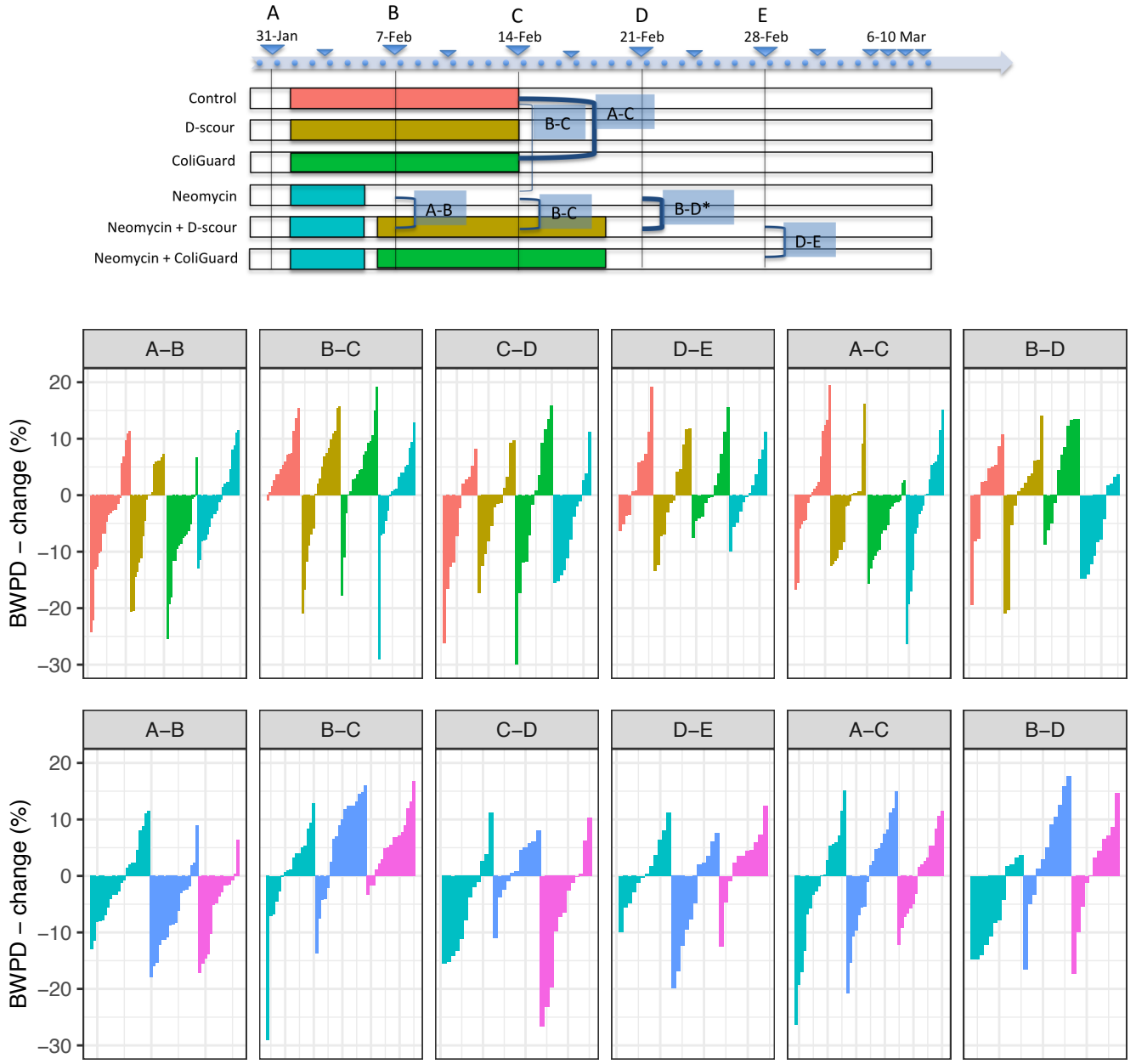
Supplementary Figure 24 Alpha diversity by cohort across four time points.

Alpha phylogenetic diversity for each cohort for a subset of 4 time points. *P* values are derived from pairwise comparisons of the means of each time point of (**A**) unrooted phylogenetic diversity and (**B**) balance weighted phylogenetic diversity. Bonferroni correction of *p* values is applied. More time points were tested and significance was determined; reported in Supplementary File 5.



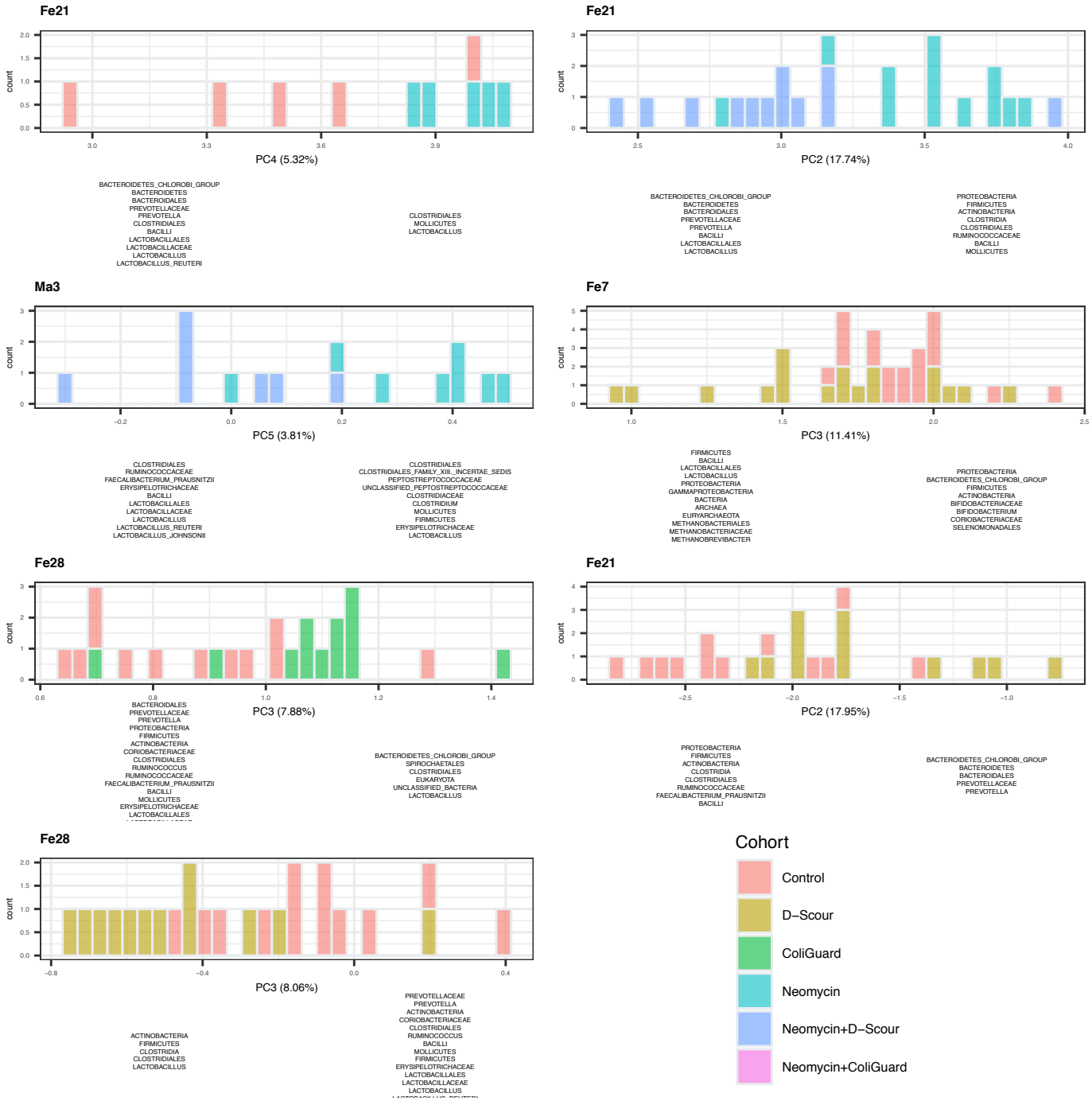
Supplementary Figure 25. Deltas of unrooted alpha phylogenetic diversity between time points.

Change of alpha unrooted phylogenetic diversity in samples between time points across the trial. On the y-axis of plots the percentage difference in unrooted PD between time points per cohort. Letters on the top left of each plot indicate the time points compared with one week interval (A-B, B-C, C-D, D-E) and two weeks interval (A-C, B-D). Pairwise t-test comparisons between cohorts were computed. Brackets in the timeline (top) indicate a significant *p* value ($p<0.05$) between cohorts (1-week interval); bold brackets indicate a significant *p* value ($p<0.05$) between cohorts (2-weeks interval). * indicate significance reached when applying Benjamini-Hochberg *post-hoc* correction.



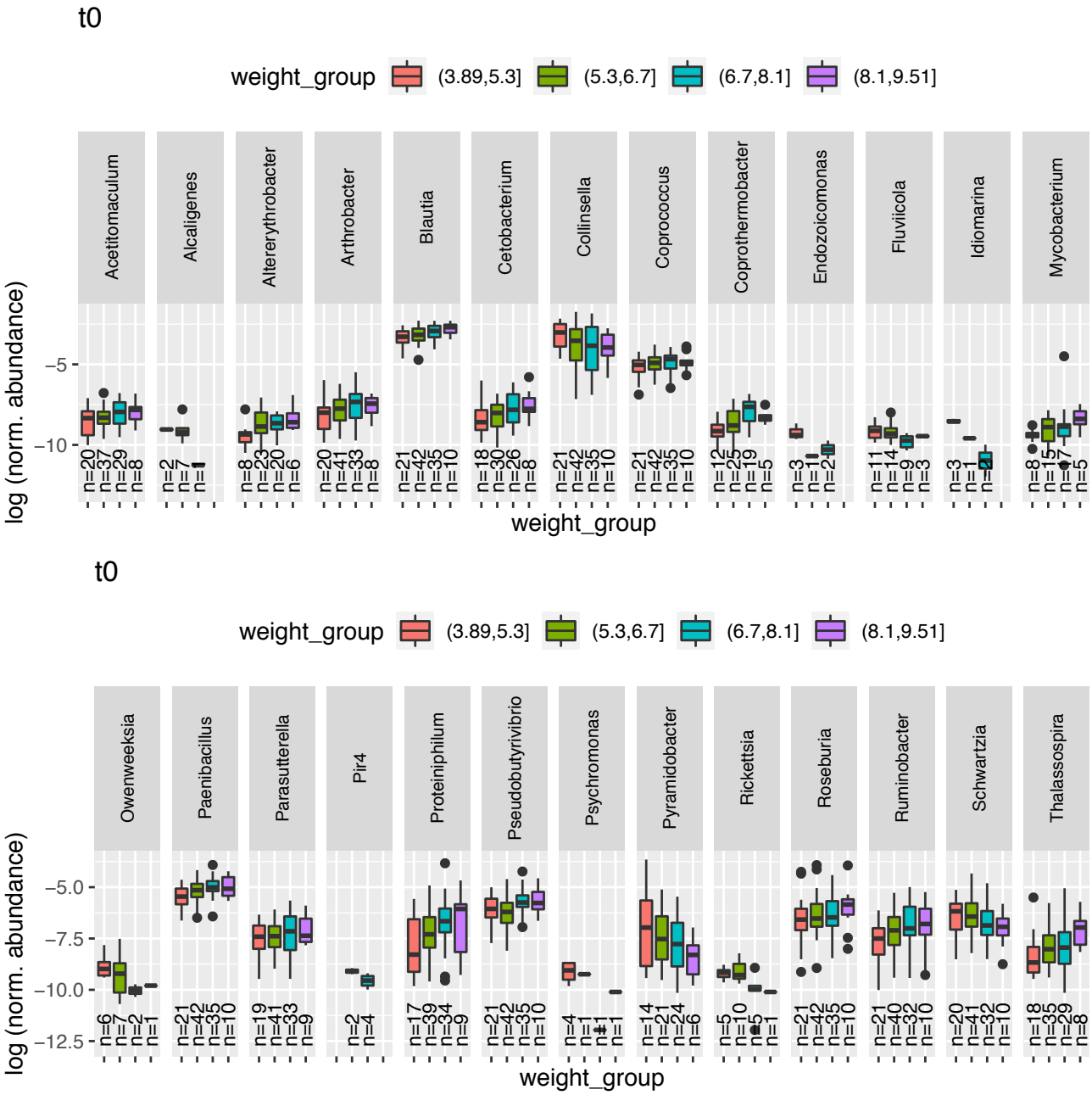
Supplementary Figure 26. Deltas of balance weighted alpha phylogenetic diversity between time points.

Change of alpha balance weighted phylogenetic diversity in samples between time points across the trial. On the y-axis of plots the percentage difference in balance weighted phylogenetic diversity between time points per cohort. Letters on the top left of each plot indicate the time points compared with one week interval (A-B, B-C, C-D, D-E) and two weeks interval (A-C, B-D). Pairwise t-test comparisons between cohorts were computed. Brackets in the timeline (top) indicate a significant *p* value ($p<0.05$) between cohorts (1-week interval); bold brackets indicate a significant *p* value ($p<0.05$) between cohorts (2-weeks interval). * indicate significance reached when applying Benjamini-Hochberg *post-hoc* correction.



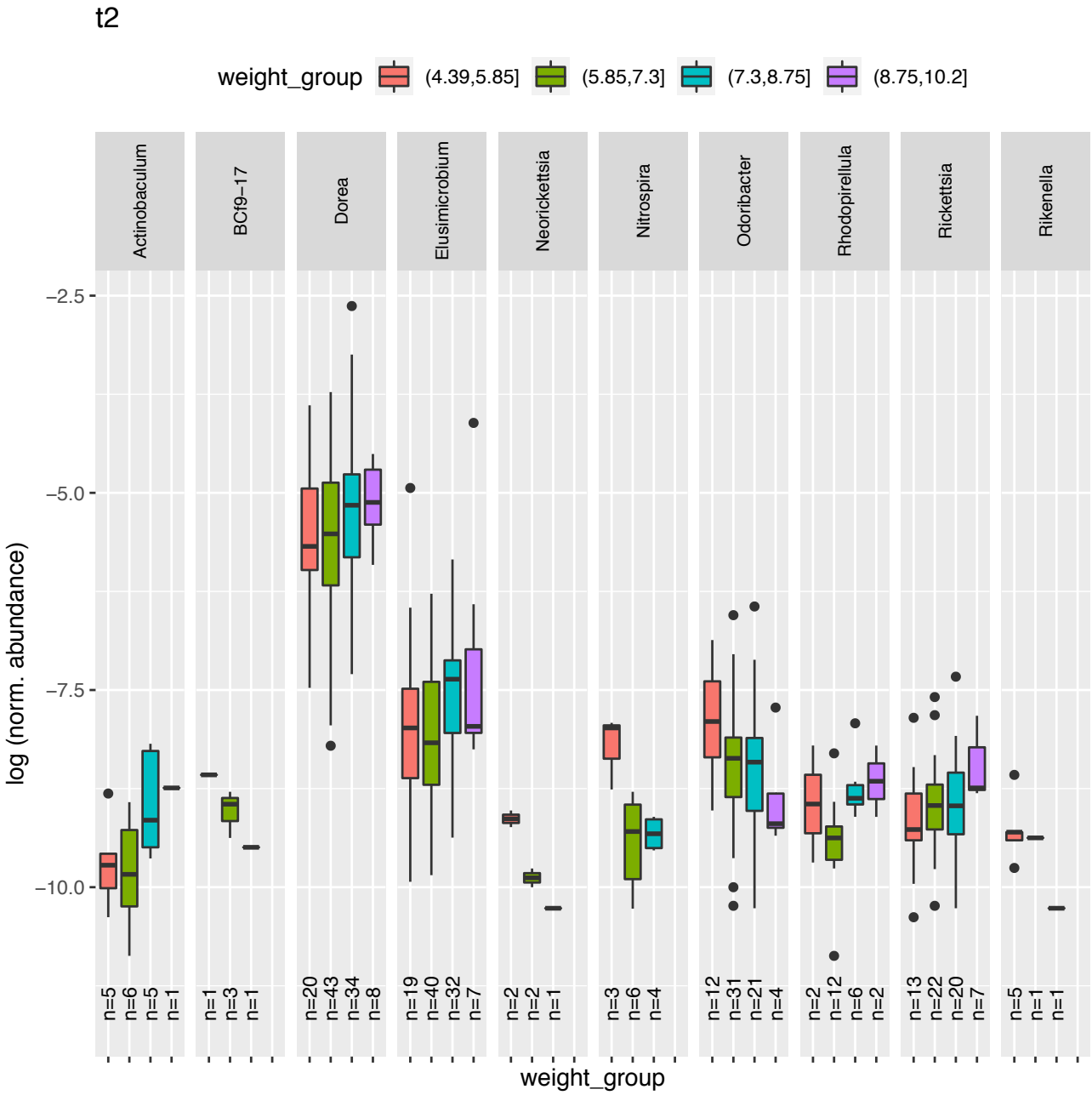
Supplementary Figure 27. Significant differences in beta diversity between cohorts at specific time points.

Significance was determined by comparing groups by pairwise t-test and the resulting *p* values where adjusted with the Bonferroni method. Significance values are provided in Supplementary File 5. The x-axes represent the principal component. As plots are derived from distinct guppy runs, each principal component explains variation to a different extent (percentage specified in parentheses). The number of samples is specified on the y-axis. Distribution of the samples on either side of a plot (left *versus* right) reflects the taxa that were found to explain the variation. Distributions are colour coded by cohort.



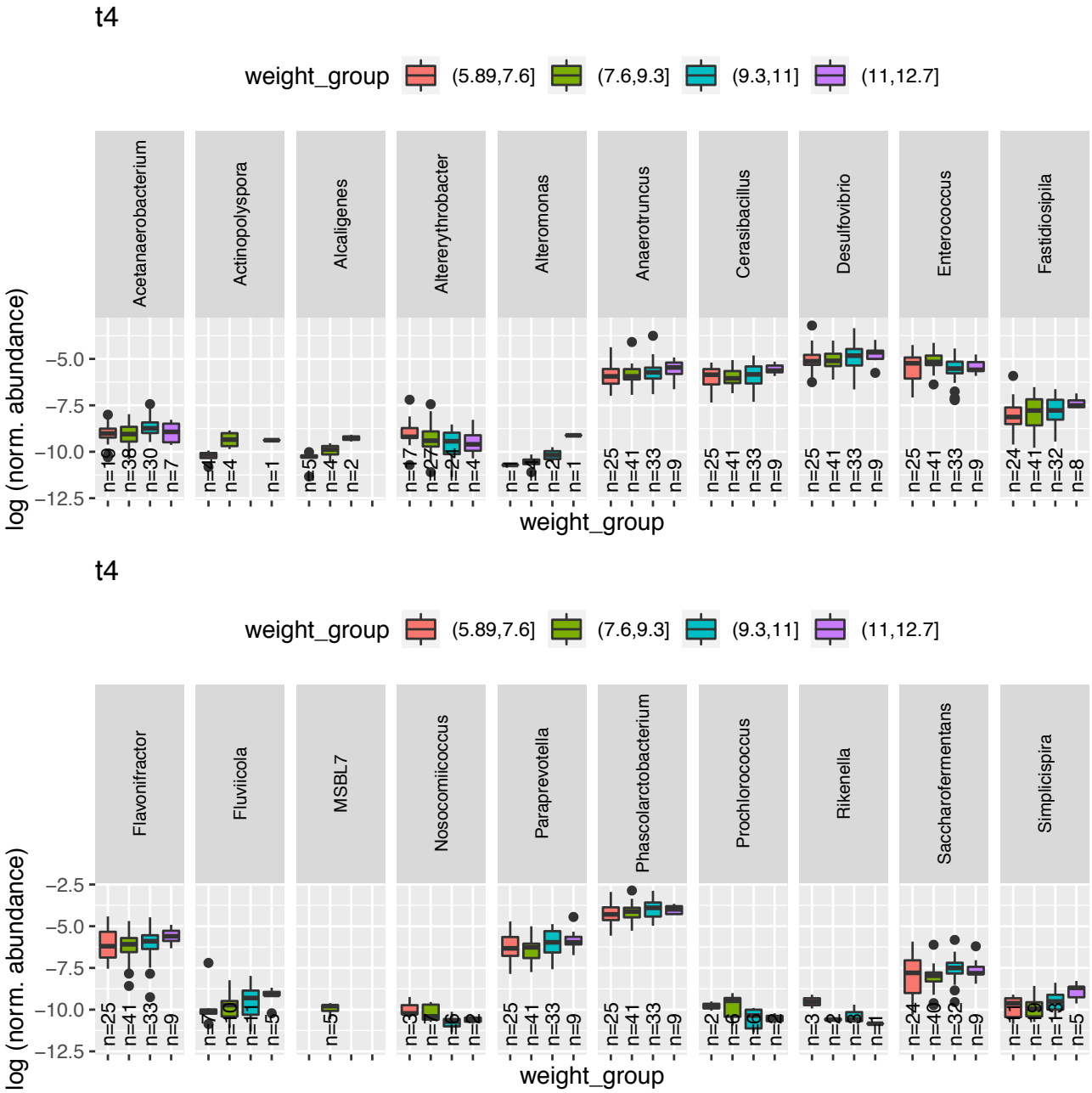
Supplementary Figure 28. Correlation of weight with taxonomy at time point 0.

16S rRNA reads were extracted with SortMeRNA. Abundance was estimated and correlation with weight was assessed with the Spearman method. Significance values are provided in Supplementary File 5. Plotted are the correlations with p value < 0.05. For visualization purposes, subjects are binned into weight groups (x-axis). Bins are formed with subjects whose weight falls below the first quartile (red), above the first quartile (green), below the third quartile (blue), above the third quartile (purple). At the bottom of each whisker plot, the samples size for each bin is provided.



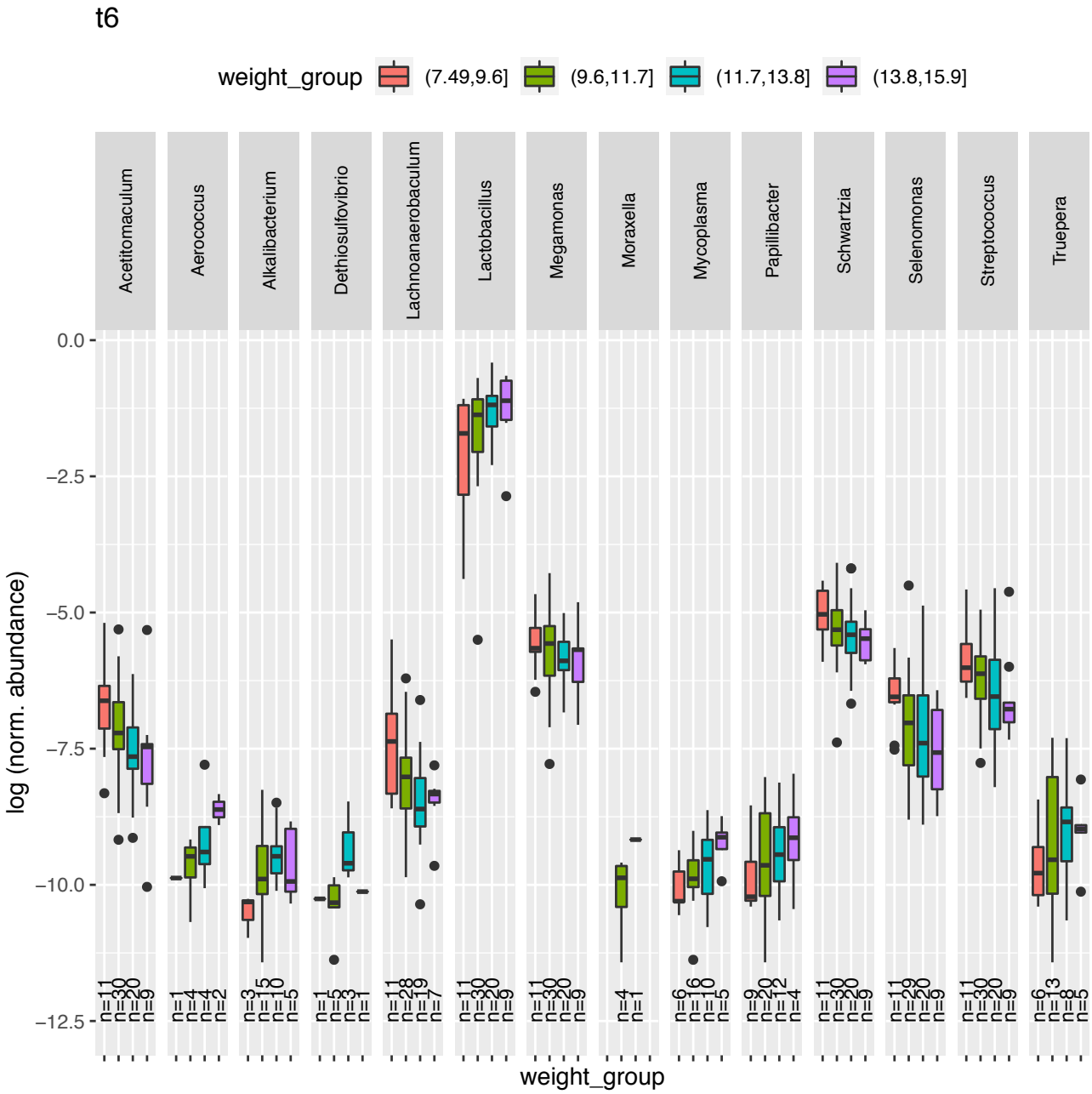
Supplementary Figure 29. Correlation of weight with taxonomy at time point 2.

16S rRNA reads were extracted with SortMeRNA. Abundance was estimated and correlation with weight was assessed with the Spearman method. Significance values are provided in Supplementary File 5. Plotted are the correlations with p value < 0.05. For visualization purposes, subjects are binned into weight groups (x-axis). Bins are formed with subjects whose weight falls below the first quartile (red), above the first quartile (green), below the third quartile (blue), above the third quartile (purple). At the bottom of each whisker plot, the samples size for each bin is provided.



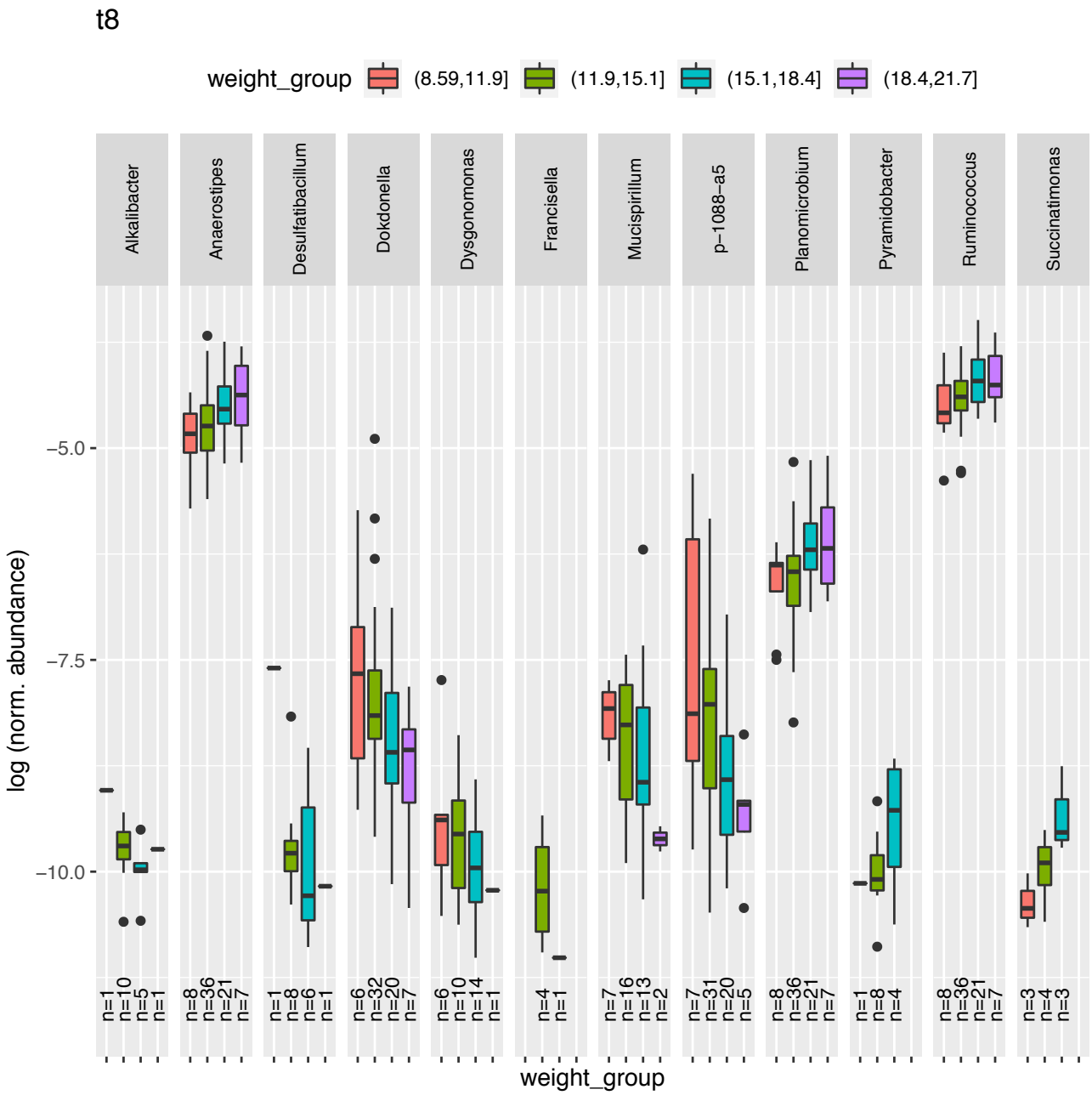
Supplementary Figure 30. Correlation of weight with taxonomy at time point 4.

16S rRNA reads were extracted with SortMeRNA. Abundance was estimated and correlation with weight was assessed with the Spearman method. Significance values are provided in Supplementary File 5. Plotted are the correlations with p value < 0.05. For visualization purposes, subjects are binned into weight groups (x-axis). Bins are formed with subjects whose weight falls below the first quartile (red), above the first quartile (green), below the third quartile (blue), above the third quartile (purple). At the bottom of each whisker plot, the samples size for each bin is provided.



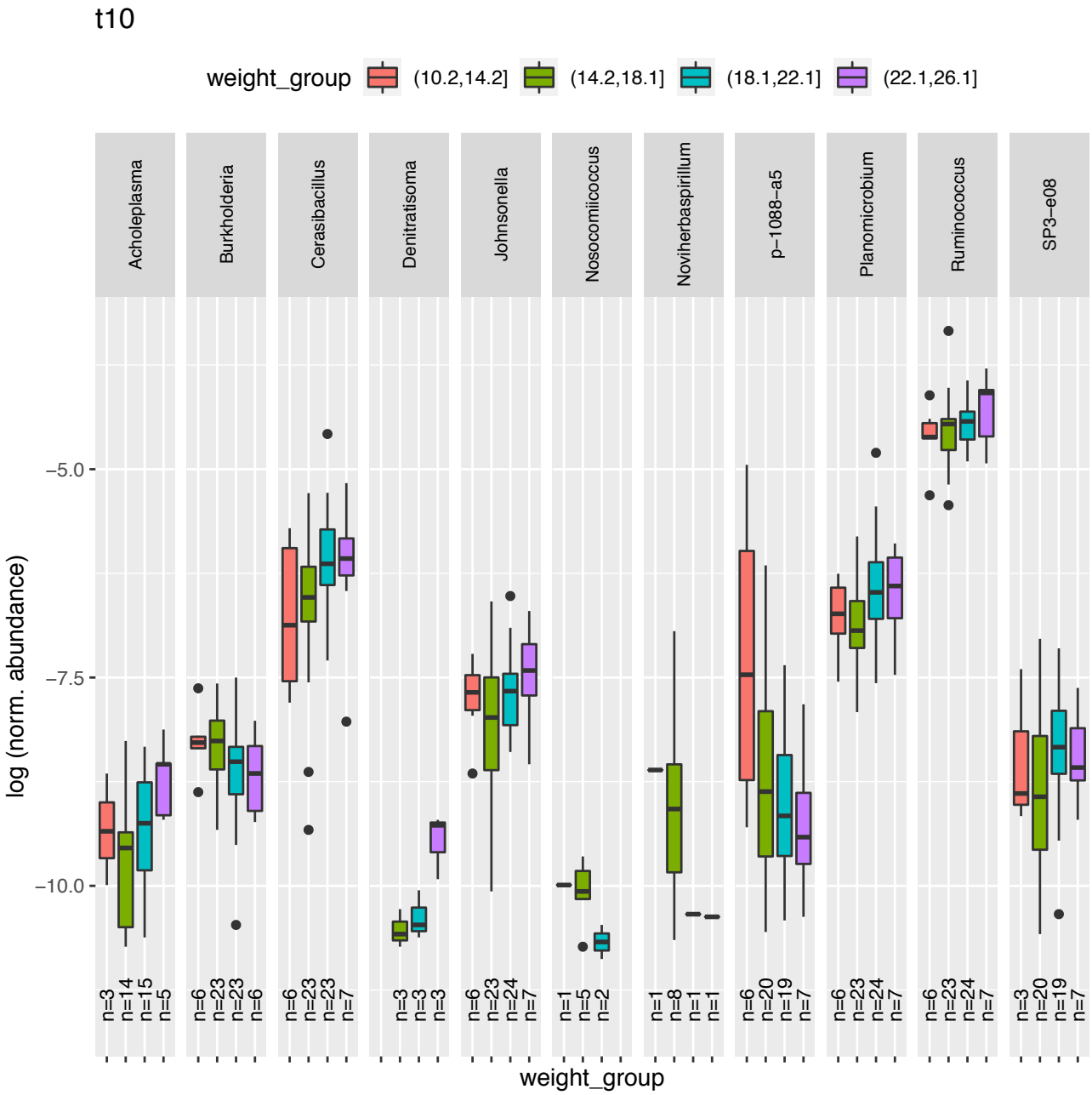
Supplementary Figure 31. Correlation of weight with taxonomy at time point 6.

16S rRNA reads were extracted with SortMeRNA. Abundance was estimated and correlation with weight was assessed with the Spearman method. Significance values are provided in Supplementary File 5. Plotted are the correlations with p value < 0.05. For visualization purposes, subjects are binned into weight groups (x-axis). Bins are formed with subjects whose weight falls below the first quartile (red), above the first quartile (green), below the third quartile (blue), above the third quartile (purple). At the bottom of each whisker plot, the samples size for each bin is provided.



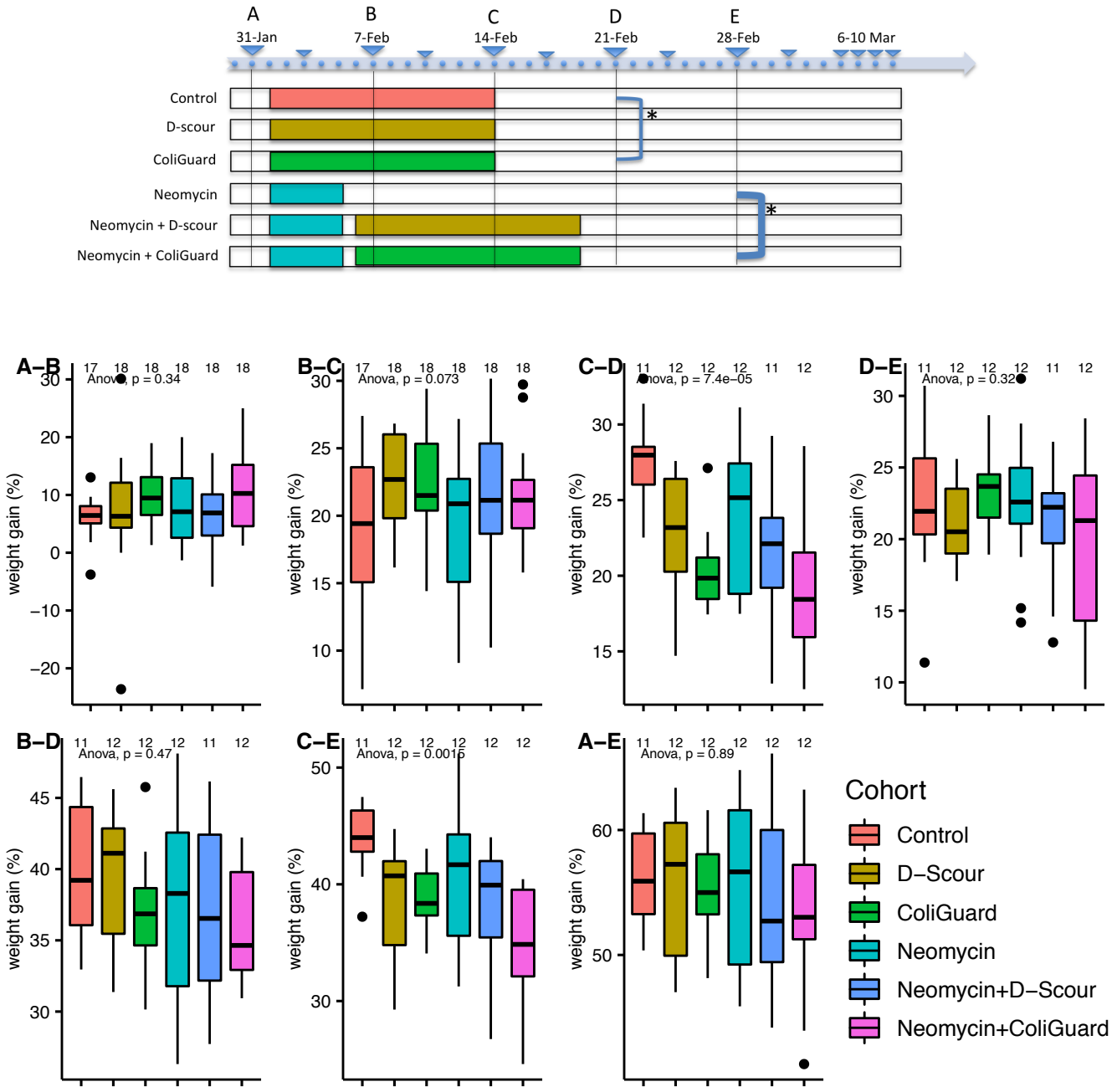
Supplementary Figure 32. Correlation of weight with taxonomy at time point 8.

16S rRNA reads were extracted with SortMeRNA. Abundance was estimated and correlation with weight was assessed with the Spearman method. Significance values are provided in Supplementary File 5. Plotted are the correlations with p value < 0.05. For visualization purposes, subjects are binned into weight groups (x-axis). Bins are formed with subjects whose weight falls below the first quartile (red), above the first quartile (green), below the third quartile (blue), above the third quartile (purple). At the bottom of each whisker plot, the samples size for each bin is provided.



Supplementary Figure 33. Correlation of weight with taxonomy at time point 10.

16S rRNA reads were extracted with SortMeRNA. Abundance was estimated and correlation with weight was assessed with the Spearman method. Significance values are provided in Supplementary File 5. Plotted are the correlations with p value < 0.05. For visualization purposes, subjects are binned into weight groups (x-axis). Bins are formed with subjects whose weight falls below the first quartile (red), above the first quartile (green), below the third quartile (blue), above the third quartile (purple). At the bottom of each whisker plot, the samples size for each bin is provided.



Supplementary Figure 34. Weight gain of piglets by cohort across the trial.

On the y-axis of plots the weight gain between time points is provided in percentage. Letters on the top left of each plot indicate the time points compared with one week interval (A-B, B-C, C-D, D-E) and with two weeks interval (B-D, C-E, A-E). Pairwise t-test comparisons between cohorts were computed. Brackets in the timeline (top) indicate a significant *p* value between cohorts (1-week interval); bold brackets indicate a significant *p* value between cohorts (2-weeks interval). A significant difference was found between Control and ColiGuard® (C-D, Tukey adjusted *p* value=0.000747) and between neomycin and neomycin+ColiGuard® (C-E, Tukey adjusted *p* value=0.039286). *in the timeline indicate that the significance was maintained after applying Tukey HSD *post-hoc* correction.