1   **Weaning age and its effect on the development of the swine gut microbiome and resistome**

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Abstract

Piglets are often weaned between 19 and 22 d of age in North America although in some swine operations this may occur at 14 d or less. Piglets are abruptly separated from their sow at weaning and are quickly transitioned from sow's milk to a plant-based diet. The effect of weaning age on the long-term development of the pig gut microbiome is largely unknown. Here, pigs were weaned at either 14, 21, or 28 d of age and fecal samples collected 20 times from d 4 (neonatal) through to marketing at d 140. The fecal microbiome was characterized using 16S rRNA gene and shotgun metagenomic sequencing. The fecal microbiome of all piglets shifted significantly three to seven days post-weaning with an increase in microbial diversity. Several *Prevotella* spp. increased in relative abundance immediately after weaning as did butyrate-producing species such as *Butyricicoccus porcorum*, *Faecalibacterium prausnitzii*, and *Megasphaera elsdenii*. Within 7 days of weaning, the gut microbiome of pigs weaned at 21 and 28 days of age resembled that of pigs weaned at 14 d. Resistance genes to most antimicrobial classes decreased in relative abundance post-weaning with the exception of those conferring resistance to tetracyclines and macrolides-lincosamides-streptogramin B. The relative abundance of microbial carbohydrate-active enzymes (CAZymes) changed significantly in the post-weaning period with an enrichment of CAZymes involved in degradation of plant-derived polysaccharides. These results demonstrate that the pig gut microbiome tends change in a predictable manner post-weaning and that weaning age has only a temporary effect on this microbiome.

Importance

Piglets are abruptly separated from their sow at weaning and are quickly transitioned from sow's milk to a plant-based diet. This is the most important period in commercial swine production yet

49 the effect of weaning age on the long-term development of the pig gut microbiome is largely

50 unknown. Metagenomic sequencing allows for a higher resolution assessment of the pig gut

51 microbiome and enables characterization of the resistome. Here we used metagenomic

52 sequencing to identify bacterial species that were enriched post-weaning and therefore may

53 provide targets for future manipulation studies. In addition, functional profiling of the

54 microbiome indicated that many carbohydrate and metabolic enzymes decrease in relative

55 abundance of after weaning. This study also highlights the challenges faced in reducing

56 antimicrobial resistance in pigs as genes conferring tetracycline and macrolide resistance

57 remained relatively stable from 7 days of age through to market weight at 140 d despite no

58 exposure to antimicrobials.

59 **Introduction**

60 In commercial swine production, the suckling-weaning transition is the most critical period for

61 piglet health. When piglets are weaned they are abruptly separated from their sow and their diet

62 is changed from an easily digestible milk-based one to a more complex plant-based diet. The risk

63 of developing health problems is increased as piglets are subjected to stress as a result of mixing

64 with unfamiliar piglets, handling, and separation from the sow (1). This stress frequently leads to

65 reduced feed intake immediately following weaning which negatively affects growth

66 performance (2). Consequently, newly weaned piglets frequently develop post-weaning diarrhea,

67 resulting in significant economic losses due to associated piglet morbidity, mortality, and

68 treatment (3). Weaning times vary but piglets can be weaned as young as 14 d or less in some in

69 North American commercial swine operations. Earlier weaning ages allow for a greater number

70 of piglets weaned per sow per year and may also decrease the risk of transmission of certain

71 pathogens from the sow to piglets. However, piglets that are weaned relatively early may be

72 more susceptible to disease and other complications (4).

73   As with humans and other mammals, the gut microbiome is an important factor affecting

74 swine health. There are an estimated 17 million plus microbial genes in the pig gut microbiome

75 (5) compared to 20,000 to 25,000 genes in the swine genome (6). This greatly expands the

76 genetic potential of the host, particularly as certain microbes can metabolize otherwise non-

77 digestible dietary carbohydrates into a usable energy source. It has been well documented that

78 the pig gut microbiome undergoes a rapid shift following weaning including a decrease in

79 members of the Proteobacteria phylum and *Bacteroides* genus and an increase in genera such as

80 *Prevotella*, *Roseburia*, and *Succinivibrio* (7-10). However, relatively little is known about how

81 weaning age affects the short- and long-term development of the pig gut microbiome. In this

82 study, we weaned pigs at three different ages, 14, 21, and 28 days, and collected fecal samples 20

83 times from the neonatal stage until they reached market weight. The fecal microbiome and

84 resistome were assessed using 16S rRNA gene and shotgun metagenomic sequencing to

85 determine how weaning age affected both over the course of the swine production cycle.

86 **Results**

87 ***Effect of weaning age on pig performance***

88   As expected, all pigs gained less weight in the 7 d post-weaning period compared to pigs

89 that were either still nursing or had already been on solid feed for longer than 7 d (Fig. 1). From

90 d 35 onward pigs from all weaning age groups grew at the same rate. There was also no

91 association with weaning age and a pig being removed from the study due to antimicrobial

92 treatment or death (P > 0.05).

93 ***Sequencing***

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94   The 16S rRNA gene sequencing of the mock community reflected the expected

95   composition with minor exceptions. There was a larger than expected relative abundance of

96   *Clostridium* (Table S1) and an absence of *Cutibacterium acnes* (formerly *Propionibacterium*

97   *acnes*); however, this species is known to be poorly amplified by the primers used in this study

98   (11). After processing, there were 35,448 ± 1,247 SEM 16S rRNA gene sequences and

99   16,699,263 ± 680,292 shotgun metagenomic paired-end sequences per sample. For the

100 metagenomic samples, host contamination accounted for 42.3% ± 2.0% of the sequences.

101 ***Weaning age and the development of the gut microbiome***

102 Weaning age had a strong but temporary effect on the gut microbial community structure

103 (Fig. 2; S2; Fig. S1). Within three days of weaning (d 18), the d 14-weaned pigs had a gut

104 microbiota that was significantly different from that of the pigs that were still nursing

105 (PERMANOVA: $R^2 > 0.25$. P < 0.001). By 25 days of age, the gut microbiota of piglets weaned

106 on d 21 was significantly different from that of both the d 14- and d 28-weaned groups

107 (PERMANOVA: $R^2 \geq 0.13$. P < 0.001). However, on d 28, the d 14- and d 21-weaned piglets

108 largely clustered together and separately from the d 28-weaned piglets which were still nursing

109 up to that point. Interestingly, the gut microbial community structure of piglets weaned at 28

110 days of age remained significantly different from that of the d 14-weaned pigs at d 35 and from

111 the d 21-weaned pigs until and including d 42.

112 There was an increase in richness (number of OTUs) and diversity (Shannon diversity

113 index) four days post-weaning in the d 14-weaned piglets compared to the still-nursing piglets

114 (Fig. 3A, B). Similarly, from d 25 to 29, both the d 14- and d 21-weaned piglets had greater

115 diversity and richness than the still-nursing d 28-weaned group. These differences had

116 disappeared by d 32, and with the exception of d 42 when the d 28-weaned piglets had a richer

117   microbiota than the other two groups, the diversity of the piglet gut microbiota was not affected

118   by age at weaning. Based on the shotgun metagenomic sequencing analysis, the shifts observed

119   in the gut microbiome post-weaning were associated with a number of different bacterial species

120   (Fig. 3C; see tables S3 and S4 at https://doi.org/10.6084/m9.figshare.c.5619817.v1). Among

121   those that increased in relative abundance post-weaning were several *Prevotella* spp. including

122   *Prevotella copri*, *Prevotella pectinovora*, *Prevotella* sp. P2-180, *Prevotella* sp. P3-122, and

123   *Prevotella stercorea*. *Butyricicoccus porcorum*, *Faecalibacterium prausnitzii*, *Selenomonas*

124   *bovis*, and *Treponema porcinum* were also among those significantly enriched in pigs that had

125   been weaned at either d 14 or 21 compared to piglets that were not weaned until d 28 (P < 0.05).

126   Bacterial species that were consistently associated with nursing pigs included

127   *Anaeromassilibacillus senegalensis*, *Bacteroides fragilis*, *Clostridioides difficile*, *Clostridium*

128   *porci*, *Clostridium scindens*, *Desulfovibrio piger*, *Escherichia coli*, *Phocaeicola vulgatus*, and

129   *Shigella sonnei* (Table S4). At 35 days of age, only three bacterial species were differentially

130   relatively abundant between the d 14- and d 21-weaned pigs and those weaned on d 28:

131   *Bariatricus massiliensis*, *B. porcorum*, and *D. piger*, all of which were enriched in the d 14-

132   weaned pigs (Table S4). Once the pigs had reached 70 days of age, there were no bacterial

133   species with a relative abundance greater than 0.1% that differed among the groups (P > 0.05).

134   ***Functional changes in the microbiome post-weaning***

135   Functional profiling of the gut microbiome was carried out using the MetaCyc metabolic

136   pathway database and the CAZy database of carbohydrate-active enzymes (CAZymes). The

137   relative abundance of the CAZymes and MetaCyc pathways shifted in a similar way to the

138   microbial taxa post-weaning (Fig. 4A, B). The CAZymes are grouped into the following classes:

139   auxiliary activities (AAs), carbohydrate esterases (CEs), glycoside hydrolases (GHs),

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140   glycosyltransferases (GTs), polysaccharide lyases (PLs), and carbohydrate-binding modules

141   (CBMs) which have no enzymatic activity but aid and enhance the catalytic activity of other

142   CAZymes. In total, 237 CAZy families were detected among all samples (Table S5) in

143   comparison with only 61 found within the pig genome (Table S6). All of the CAZyme classes

144   decreased in relative abundance after weaning (Fig. 4C). Overall, 61.5% of the CAZymes were

145   classified as glycoside hydrolases and 24.7% as glycosyltransferases. However, there were still a

146   number of CAZy families that were enriched in the gut microbiomes of post-weaned pigs

147   compared to those still nursing (Table S7). The only AA identified was AA10 (copper-dependent

148   lytic polysaccharide monooxygenases) and in only 35 of the samples (Table S5).

149   At 21 days of age, there were 141 unique CAZy families that were differentially

150   abundant between the d 14-weaned pigs and the d 21- and 28-weaned piglets that were still

151   nursing ($P < 0.05$; Table S7). Similarly, at 28 days of age, 134 CAZy families were differentially

152   abundant between the still nursing d 28-weaned piglets and the post-weaned d 14- and d 21-

153   weaned pigs ($p < 0.05$; Table S7). There were no differences in CAZy family relative abundance

154   among the three weaning age groups by d 35 ($P > 0.05$). Many of the alterations in the CAZyme

155   profiles post-weaning reflect the change in diet with CAZy families with lactose-degradation

156   activity (GH2 and GH42) and activity against other components of porcine milk oligosaccharides

157   (PMOs) (GH16, GH18, GH20, GH29, GH30, GH35, GH95, GH139, and GH141) enriched in

158   pigs that were nursing compared to those that had been weaned. Meanwhile, CAZy families

159   including CBMs with mannan- pectin-, starch-, and xylan-binding functions (CBM23, CBM25

160   CBM26, and CBM77) and GHs with activity against plant cell carbohydrates (GH5, GH39,

161   GH48, GH53, GH93, and GH94) (12, 13), were more relatively abundant in post-weaned pigs

162   that were consuming only a plant-based solid feed.

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163   A large number of MetaCyc metabolic pathways were also differentially abundant

164   between weaned and nursing piglets at d 21 (196 unique pathways) and d 28 (231 unique

165   pathways) with the majority enriched in the gut microbiome of nursing piglets (see Tables S8

166   and S9 at https://doi.org/10.6084/m9.figshare.c.5619817.v1). As with the CAZymes there was an

167   enrichment of MetaCyc pathways involved in fucose and lactose degradation in the nursing

168   piglets and an increased relative abundance of certain starch degradation pathways post-weaning.

169   *Weaning age and the gut resistome*

170   Antimicrobial resistance remains a serious challenge to the swine industry and therefore

171   we also characterized the antimicrobial resistome of the pigs longitudinally and in response to

172   weaning age. Similar to the functional analysis, samples clustered by weaning age on d 21 and d

173   28 when assessed using the relative abundance of antimicrobial resistance genes (ARGs) (Fig.

174   5A). The large majority of ARGs that were differentially abundant were enriched in the nursing

175   piglets compared to the weaned pigs (see Table S10 at

176   https://doi.org/10.6084/m9.figshare.c.5619817.v1). Notable ARGs that were more relatively

177   abundant in the weaned pigs included bla_{ACI-1}, *cfxA6*, *erm*(Q), *tet*(44), and *tet*(L). The relative

178   abundance of ARGs conferring resistance to multiple drugs, aminoglycosides, polypeptides, and

179   quinolones as well as several other drug classes decreased post-weaning in all weaning age

180   groups (Fig. 5B). However, tetracycline resistance genes remained relatively stable throughout

181   the pig production cycle. Of the 250 unique ARGs detected, *tet*(Q), *tet*(W), *tet*(O), *aph(3')-IIIa*,

182   *mel*, *tet*(W/N/W), *tet*(40), and *tet*(44) were the most relatively abundant among all samples (see

183   Table S11 at https://doi.org/10.6084/m9.figshare.c.5619817.v1).

184   **Discussion**

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185 As expected, there was a substantial shift in the pig gut microbiome within three days of

186 weaning. The sudden change from a milk-based diet to one that is plant-based and less digestible

187 by the pig is largely responsible for this shift immediately post-weaning (1, 14). However,

188 weaning age had no apparent long-term effects on the gut microbiome or the average daily gain

189 of the pigs. A recent study by Massacci et al. (15) that also weaned pigs at different ages (14, 21,

190 28, and 42 d) with sampling up to 60 d of age also reported no weaning age effect on the

191 microbial community structure at 60 d. Therefore, it appears that a later weaning age only delays

192 post-weaning changes in the gut microbiome rather than affecting the assembly and stability of

193 the microbial community.

194 Several short-chain fatty acid-producing bacterial species were prevalent among those

195 that were more relatively abundant in pigs that had been weaned. These included *Anaerovibrio*

196 *slackiae* (acetate, propionate), *B. porcorum* (butyrate), *Coprococcus catus* (butyrate, propionate),

197 *F. prausnitzii* (butyrate), *Megasphaera elsdenii* (acetate, butyrate, propionate),

198 *Phascolarctobacterium succinatutens* (propionate), *P. copri* (acetate), *Prevotella mizrahii*

199 (acetate), *P. pectinovora* (acetate), and *S. bovis* (acetate, propionate) (16-21). Short-chain fatty

200 acid-production occurs mostly in the lower gastrointestinal tract of pigs as a result of bacterial

201 fermentation of undigested carbohydrates (22). Acetate, butyrate, and propionate have anti-

202 inflammatory effects on the host (23) and provide up to 25% of daily energy requirements in pigs

203 (24). Butyrate in particular is the primary energy source of colonocytes and regulates apoptosis

204 (25).

205 Interestingly, *F. prausnitzii* has also been reported to be more relatively abundant at 60

206 days of age in pigs weaned at 21, 28, and 42 d vs. 14 d (15) and in healthy pigs vs. those with

207 post-weaning diarrhea (26). *Butyricicoccus porcorum* has been associated with higher feed

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208 efficiency as have *Treponema porcinum* and *Treponema succinifaciens* which were also more

209 relatively abundant in weaned pigs here (27). In-feed supplementation with *Butyricicoccus*

210 *pullicaecorum* has been shown to improve health and feed efficiency in broiler chickens (28) and

211 *F. prausnitzii* reduced intestinal permeability and cytokine expression in a mouse colitis model

212 (29). Therefore, *B. porcorum* and *F. prausnitzii*, as well as potentially other bacterial species that

213 were more relatively abundant in weaned pigs, are attractive targets for microbiome

214 manipulation and further study into their role in pig gut health.

215 The bacterial species that were enriched in the microbial communities of pigs that were

216 still nursing at d 21 and d 28 include several potentially pathogenic species such as *C. difficile*, *E.*

217 *coli*, *S. sonnei*, and *Streptococcus suis*. It is difficult to assess virulence of these species here,

218 however, the presence of potentially pathogenic bacteria pre-weaning may be a risk factor for

219 post-weaning morbidity and mortality (30). Although, many of the more relatively abundant

220 bacterial species were differentially abundant pre- and post-weaning, several remained relatively

221 stable throughout the pig production cycle. In particular, *Lactobacillus johnsonii*, *Mogibacterium*

222 *kristiansenii*, and *Subdoligranulum variabile* were not affected by weaning. Among these

223 bacterial species *L. johnsonii* is the best described and has been reported to improve sow

224 reproductive performance (31) and average daily gain in piglets during the first 35 days of life

225 (32) when delivered in feed. *S. variabile*, a butyrate-producing bacterial species, is the only

226 member of its genus and has been previously reported to be a member of the "core microbiota"

227 of the pig gastrointestinal tract (33). *M. kristiansenii* has only recently been described and was

228 originally isolated from pig feces (18).

229 The functional profile of the gut microbiome also shifted after weaning in all weaning

230 age groups similar to that of the taxonomic profiles. This included a decrease in the relative

231 abundance of all CAZy families post-weaning. The CAZymes encoded by the pig genome are

232 greatly outnumbered by those in the gut microbiome thereby providing the host with an

233 additional source of energy as discussed earlier. Sow's milk contains not only lactose but at least

234 119 PMOs (34) which are composed of the monosaccharides fucose, galactose, glucose, N-

235 acetylglucosamine, N-acetylgalactosamine, and sialic acid bound to a lactose or N-

236 actelyllactosamine core (35). These PMOs are generally resistant to host digestive enzymes in

237 the small intestine and are instead fermented by the colonic microbiome into SCFAs (36, 37).

238   In humans, *Bifidobacterium* and *Bacteroides* spp., including *B. fragilis and P. vulgatus*

239 (formerly *Bacteroides vulgatus*), have been shown to metabolize human milk oligosaccharides

240 (38). *Bacteroides fragilis*, which was among the most relatively abundant species in nursing

241 piglets here, carries a number of glycoside hydrolase family genes that facilitate breakdown of

242 milk oligosaccharides (39). Metabolites from the degradation of sialylated bovine milk

243 oligosaccharides by *B. fragilis* has also been shown to enhance the growth of *E. coli* in vitro (40).

244 All of the GH families found in *B. fragilis*, i.e., GH2, GH16, GH18, GH20, GH29, GH33, and

245 GH95, were enriched in the gut microbiomes of nursing piglets. Similarly, GH families and

246 CBMs associated with degradation of plant polysaccharides were more relatively abundant in

247 fecal samples from pigs that had been weaned and consuming a solid plant-based diet for at least

248 7 d.

249   The relative abundance of ARGs within several antimicrobial classes decreased post-

250 weaning. However, ARGs conferring resistance to the tetracycline and MLS_B classes remained

251 relatively stable throughout the study despite the fact that none of the pigs were exposed to any

252 antimicrobials. Not surprisingly, these are the antimicrobial classes with the longest history of

253 use in swine production and are still among the most frequently administered antimicrobials in

254   North American pigs (41, 42). This background level of tetracycline and MLS_B resistance

255   probably also explains why several studies have reported limited or only temporary effects on the

256   pig gut microbiome following exposure to drugs of these antimicrobial classes (8, 43, 44). The

257   reason for the significant decrease in other ARGs after weaning is likely due to the post-weaning

258   shift in bacterial taxa carrying these ARGs. For example, many of the relatively abundant

259   multidrug ARGs such as *mdtF*, *acrF*, *evgS*, *acrB*, *mdtO*, *mdtP*, and *cpxA*, are found in the

260   majority of *E. coli* and *S. sonnei* genomes and both of these species decreased in relative

261   abundance post-weaning. In contrast, relatively abundant tetracycline resistance genes such as

262   *tet*(Q), *tet*(W), and *tet*(O) have a much wider host range (45).

263   Two of the ARGs that were more relatively abundant in weaned piglets compared to

264   those still nursing were the Ambler class A beta-lactamase genes bla_{CfxA6} and bla_{ACI-1}.

265   Additionally, bla_{CfxA2} was enriched in piglets weaned at d 14 and 21 compared to those still

266   nursing on d 28. Both bla_{CfxA2} and bla_{CfxA6} have been identified in several *Prevotella* spp. (46)

267   which likely accounts for the post-weaning enrichment of these ARGs. In *Prevotella* spp. the

268   bla_{CfxA} genes have been shown to confer resistance to ampicillin but not cefmetazole (47). The

269   bla_{ACI-1} gene may be associated with *M. elsdenii* as has been demonstrated in human gut

270   metagenomes (48). Overall, these results again demonstrate the challenges faced when it comes

271   to reducing antimicrobial resistance in swine as none of the pigs in this study were exposed to

272   antimicrobials.

273   In conclusion, this study shows that weaning age has little effect on the long-term

274   development and composition of the pig gut microbiome and resistome. Instead, the pig gut

275   microbiome tends to change in a rather predictable manner post-weaning in a swine production

276   environment. Many ARGs also persisted in the feces of the pigs throughout the study likely

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277   reflecting the long history of use of certain antimicrobial classes in swine production. Several

278   bacterial species with potential beneficial properties such as SCFA production were found to be

279   enriched post-weaning and are attractive targets for future microbiome manipulation and culture-

280   based studies.

281   **Experimental Procedures**

282   *Animals and experimental design*

283   All pig experiments were carried out at the swine unit of the Lacombe Research and

284   Development Centre. Seven pregnant sows that farrowed within 24 h of each other were used in

285   the study. A total of 45 piglets (n = 15 per weaning age group) were randomly selected for

286   inclusion in the study based on litter, weight, and sex, with low-weight piglets excluded.

287   Following weaning, all pigs were fed the same starter diet that was free of antibiotics, prebiotics,

288   and probiotics (see Table S12 at https://doi.org/10.6084/m9.figshare.c.5619817.v1). Any pig that

289   required an antibiotic treatment was removed from the study. Animals in this experiment were

290   cared for in agreement with the Canadian Council for Animal Care (2009) guidelines. The

291   Lacombe Research and Development Centre Animal Care Committee reviewed and approved all

292   procedures and protocols involving animals.

293   On d 4 prior to sampling, 15 piglets were randomly chosen from among the 7 litters and

294   designated to be weaned at 14, 21, or 28 days of age (Fig. S2). Piglets were sampled using fecal

295   swabs (FLOQSwabs, Copan, Murrieta, CA, USA) beginning at 4 days of age and repeated on

296   days 7 and 11. At 14 days of age, piglets assigned to the d 14 weaning group were removed from

297   their sow after sampling and transferred to a nursery room within the swine barn. Fecal sampling

298   continued for all piglets at 15, 18, and 21 d of age. On d 21, piglets in the d 21 weaned group

299   were removed from their sow and placed in a nursery room. Fecal samples were taken from all

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300 pigs on d 22, 25, and 28, and on d 28 the d 28-weaned piglets were weaned from their sow and

301 placed in the nursery room. Piglets were then sampled on d 29, 32, 35, 42, 49, 56, 70, 84, 112,

302 and 140. All fecal swabs were immediately placed on ice, transported to the laboratory, and

303 stored at -80°C until DNA extraction.

304 ***DNA extraction and 16S rRNA gene and shotgun metagenomic sequencing***

305 DNA was extracted from fecal material collected on FLOQSwabs with the QIAamp

306 BiOstic Bacteremia DNA Kit (Qiagen, Mississauga, ON, Canada) as per manufacturer's

307 instructions with the following modifications. Sterile scissors were used to remove the swab

308 which was then placed into a PowerBead tube with MBL solution and agitated at 70°C and 400

309 rpm for 15 min. After heating, the tubes were shaken in a FastPrep-24 (MP Biomedicals, Solon,

310 OH, USA) at 4.0 m/s for 45 s. Tubes were allowed to rest in the MP FastPrep-24 for 5 min.

311 Using sterile forceps, swabs were removed from PowerBead tubes prior to pelleting debris at

312 10,000 x g for 2 min. All remaining steps were followed as per the manufacturer's protocol.

313 Extracted bacterial DNA was loaded onto nine 96-well plates and two wells on each plate

314 included a positive control (MSA-1002, 20 Strain Even Mix Genomic Material, ATCC,

315 Manassas, VA, USA) and negative control (water). Negative extraction controls were also

316 included. DNA was quantified and analyzed using the Qubit dsDNA HS Assay Kit (Thermo

317 Fisher Scientific, Waltham, MA, USA) and Agilent High Sensitivity D1000 ScreenTape System

318 (Santa Clara, CA, USA). The V4 hypervariable region of the 16S rRNA gene was amplified as

319 per Kozich et al. (49). To prepare each 16S rRNA gene library, 5 μl of each sample from three

320 96-well plates were pooled at a time. The pooled library was normalized to 0.4 nM and

321 submitted to the Genomics Facility in the Infectious Bacterial Diseases Research Unit at USDA-

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322   ARS-NADC in Ames, IA for 250 bp paired-end sequencing on a MiSeq instrument (Illumina,

323   San Diego, CA) using v2 chemistry.

324   DNA from d 7, 14, 21, 28, 35, 70, and 140 of all pigs that remained in the study through

325   to d 140 was also subjected to shotgun metagenomic sequencing. Metagenomic libraries were

326   prepared using 700 ng of DNA and the TruSeq DNA PCR-Free Library Prep Kit (Illumina Inc.)

327   following the manufacturer's recommended protocol. Briefly, DNA was fragmented to an

328   average length of 400 bp with a Covaris LE220 instrument, end-repaired, A-tailed, and indexed

329   with TruSeq Illumina adapters. Libraries were then validated on a Fragment Analyzer system

330   with a High Sensitivity NGS Fragment Kit (Agilent Technologies, Mississauga, ON, Canada) to

331   check for size and quantified by qPCR using the Kapa Library Quantification Illumina/ABI

332   Prism Kit protocol (KAPA Biosystems, Wilmington, MA, USA). Equimolar quantities of each

333   library were then pooled and sequenced on the Illumina NovaSeq 6000 instrument with a SP

334   flowcell (2 x 250 bp) following manufacturer's instructions.

335   ***16S rRNA gene sequence analysis***

336   The 16S rRNA were processed using DADA2 v. 1.14 (50) in R v. 3.6.3. Briefly, the

337   forward and reverse reads were trimmed to 200 and 210 bp, respectively, merged with a

338   minimum overlap of 75 bp, and chimeras removed. The RDP naive Bayesian classifier (51) and

339   the SILVA SSU database release 138 (52) were then used to assign taxonomy to each merged

340   sequence, referred to here as operational taxonomic units (OTUs) with 100% similarity. OTUs

341   that were classified as chloroplasts, mitochondria, or eukaryotic in origin, and those that were

342   identified in the extraction control samples at an equal or higher abundance than the biological

343   samples were removed prior to analyses. The number of OTUs, Shannon diversity index, inverse

344   Simpson's diversity index, and the Bray-Curtis dissimilarities were calculated in R v. 4.0.0 using

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345   Phyloseq 1.32.0 (53) and vegan v. 2-5.6 (54). To account for uneven sequencing depth all

346   samples were randomly subsampled to 6,900 sequences per sample prior to analyses.

Metagenomic sequence analysis

348   Metagenomic sequences were trimmed (quality score < 15 over a sliding window of 4 bp;

349   minimum length of 50 bp) and sequencing adapters removed using Trimmomatic v. 0.38 (55).

350   Bowtie2 v. 2.4.2-1 (56) was used to align host sequences to the *Sus scrofa* genome (Sscrofa11.1)

351   for removal. Taxonomy was assigned to the filtered metagenomic sequences using Kaiju v. 1.7.3

352   (57) and the NCBI non-redundant protein database (October 13, 2020). For functional profiling

353   of the metagenomic samples, HUMAnN v. 3.0.0.alpha.1 (58) was used to align reads to the

354   UniRef90 database which were then collapsed into MetaCyc metabolic and enzyme pathways

355   (59). Reads were aligned to the Comprehensive Antibiotic Resistance Database (CARD) v. 3.0.8

356   (60) and the Carbohydrate-Active enZYmes (CAZy) Database (dbCAN2) v. 07312020 (61)

357   using DIAMOND v. 0.9.28 (62) ($\geq$ 90% amino acid identify and $\geq$ 90% coverage).

Statistical analysis

359   Fisher's exact test was used to determine if weaning age was associated with removal

360   from the study post-weaning due to antimicrobial treatment or death. The effect of weaning age

361   on the microbial community structure was assessed using the Bray-Curtis dissimilarities and

362   PERMANOVA (adonis2 function). The R package pairwiseAdonis (63) was used to compare the

363   Bray-Curtis dissimilarities within each sampling time and the Benjamini-Hochberg procedure

364   was used to correct P-values for multiple comparisons. The effect of weaning age on the relative

365   abundance of microbial species, CAZy families, MetaCyc pathways, and ARGs was determined

366   using MaAsLin2 (microbiome multivariable associations with linear models) v. 1.5.1 (64) in R.

367   Only those microbial species with an average relative abundance of at least 0.1% and CAZy

16

368   families, MetaCyc pathways, and ARGs identified in at least 25% of samples were included in

369   these analyses.

370   *Data Availability*

371   All 16S rRNA gene and metagenomic sequencing data are available at the NCBI sequence read

372   archive under BioProject PRJNA629856.

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611 **Figure Legends**

612 **Figure 1.** Average daily gain in kg of pigs by weaning age within each weighing period.

613 Different lowercase letters indicate significantly different means (P < 0.05).

614 **Figure 2.** Non-metric multidimensional scaling plot of the Bray-Curtis dissimilarities for the pig

615 fecal microbiota by weaning age and sampling day based on 16S rRNA gene sequencing.

616 **Figure 3.** The A) number of OTUs and B) Shannon diversity index values based on 16S rRNA

617 gene sequencing and C) the 15 most relatively-abundant bacterial species based on shotgun

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618   metagenomic sequencing for the pig fecal microbiome by weaning age and sampling day . In A

619   and B, different lowercase letters indicate significantly different means (P < 0.05). In C, species

620   are ordered by overall percent relative abundance. *Butyricicoccus porcorum* and

621   *Faecalibacterium prausnitzii* are also included based on their enrichment post-weaning and

622   butyrate-producing activities.

623   **Figure 4.** Non-metric multidimensional scaling plot of the Bray-Curtis dissimilarities for the A)

624   CAZymes and B) MetaCyc metabolic pathways of the pig fecal microbiome and C) percent

625   relative abundance of CAZyme classes by weaning age and sampling day.

626   **Figure 5.** Non-metric multidimensional scaling plot of the Bray-Curtis dissimilarities for the A)

627   antimicrobial resistance genes and B) percent relative abundance of antimicrobial resistance

628   genes by antimicrobial class by weaning age and sampling day.

629   **Supplemental Material**

630   **Figure S1.** Non-metric multidimensional scaling plot of the Bray-Curtis dissimilarities for the

631   fecal microbiota by weaning age and age of piglets.

632   **Figure S2.** Frequency of fecal sampling of the pigs in this study.

633   **Table S1.** Percent relative abundance of genera identified in the mock communities (ATCC

634   MSA-1002) via 16S rRNA gene sequencing. Note: *Schaalia* spp. are classified as *Actinomyces*

635   spp. within the SILVA SSU database.

636   **Table S2.** Pairwise PERMANOVA of the Bray-Curtis dissimilarities by weaning age within

637   each sampling time.

638   **Table S3.** Percent relative abundance (± SEM) of microbial species by weaning age and

639   sampling time based on metagenomic sequencing. Only those microbial species with a relative

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640   abundance greater than 0.01% are included. Species are listed by overall percent relative
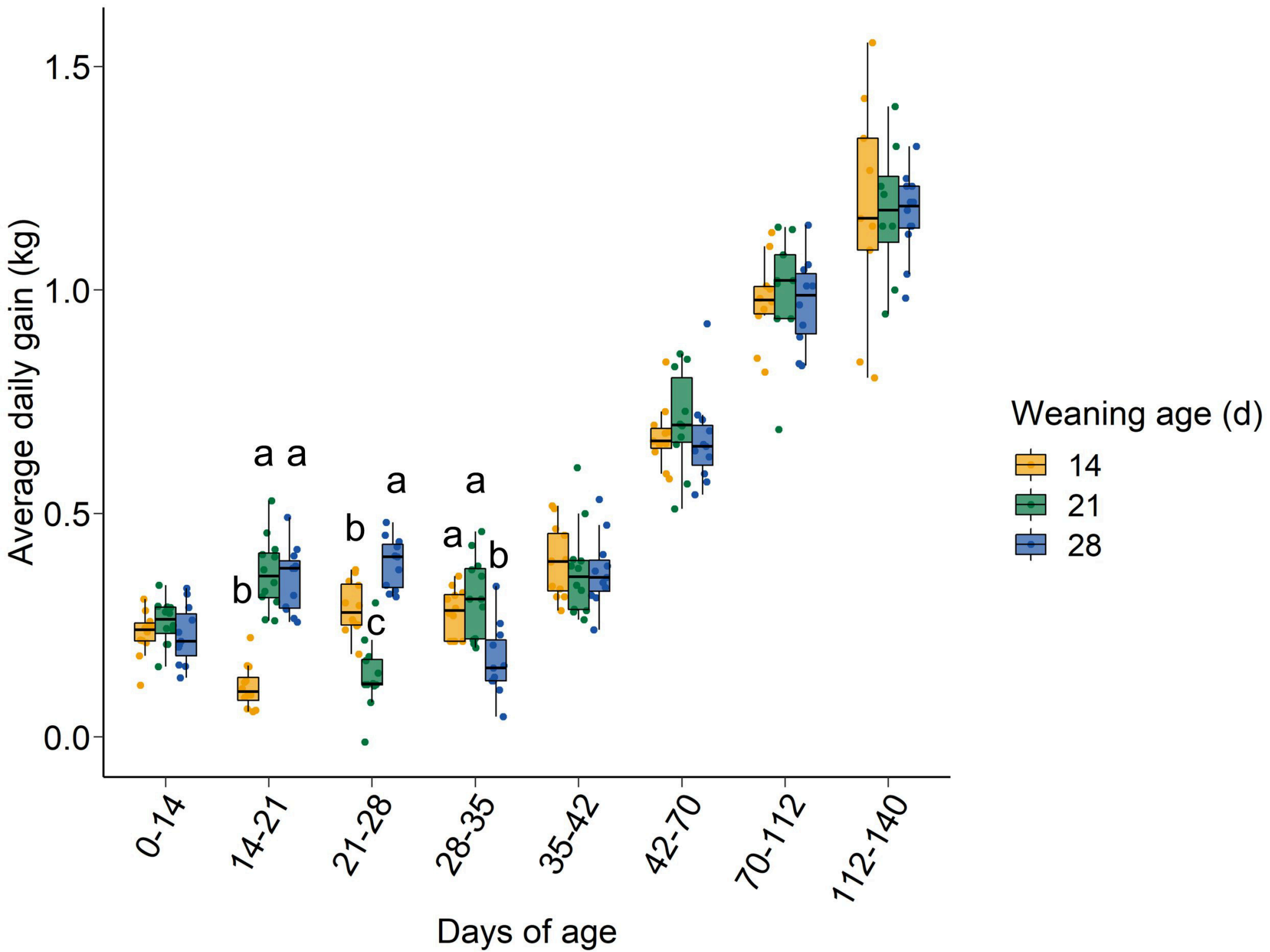
641   abundance.

642   **Table S4.** Differentially abundant microbial species based on metagenomic sequencing.

643   Negative coefficient values (highlighted) at 21 d of age indicate that the microbial species was

644   more relatively abundant in the d 14-weaned pigs vs. the d 21- and 28-weaned pigs. Negative

645   coefficient values (not highlighted) at 28 and 35 d of age indicate that the microbial species was

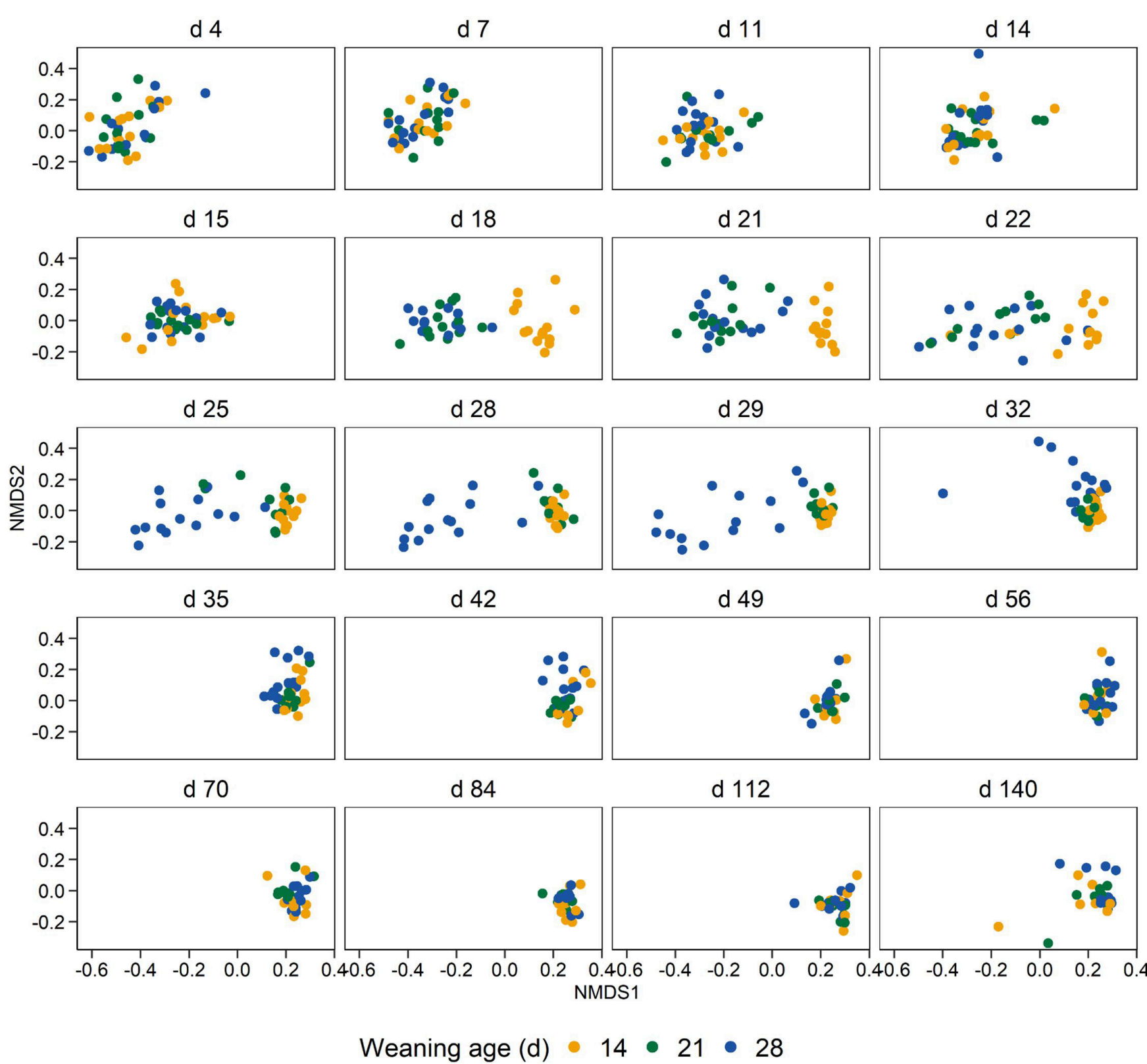646   more relatively abundant in the d 28-weaned pigs vs. the d 14- and 21-weaned pigs.

647   **Table S5**. Percent relative abundance (± SEM) of CAZy families detected in at least one sample

648   by weaning age and sampling time.

649   **Table S6**. CAZy families detected in the *Sus scrofa* genome at 90% identity.

650   **Table S7**. Differentially abundant CAZy families on d 21 and 28 between weaned vs. nursing

651   piglets. Negative coefficient values (highlighted) at 21 d of age indicate that the CAZy family

652   was more relatively abundant in the d 14-weaned pigs vs. the d 21- and 28-weaned pigs.

653   Negative coefficient values (not highlighted) at 28 d of age indicate that the CAZy family was

654   more relatively abundant in the d 28-weaned pigs vs. the d 14- and 21-weaned pigs.
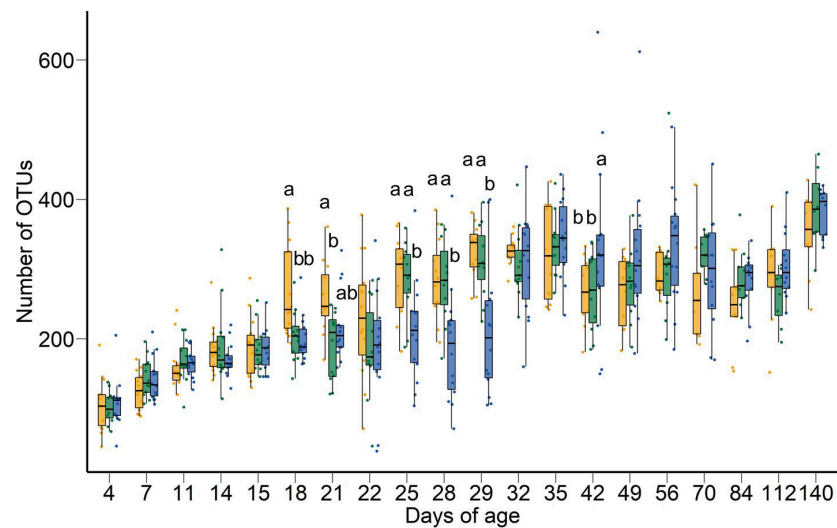
655   **Table S8.** Percent relative abundance (± SEM) of MetaCyc pathways detected in at least one

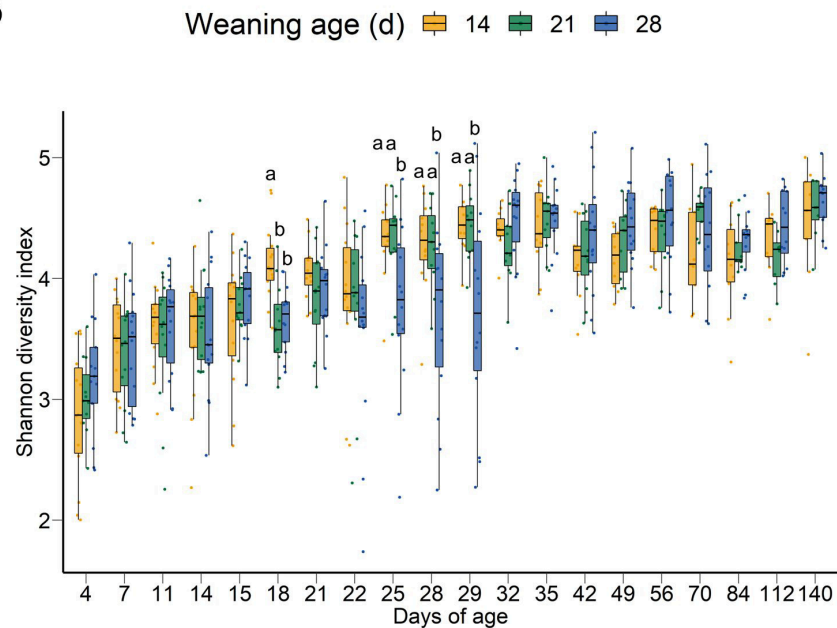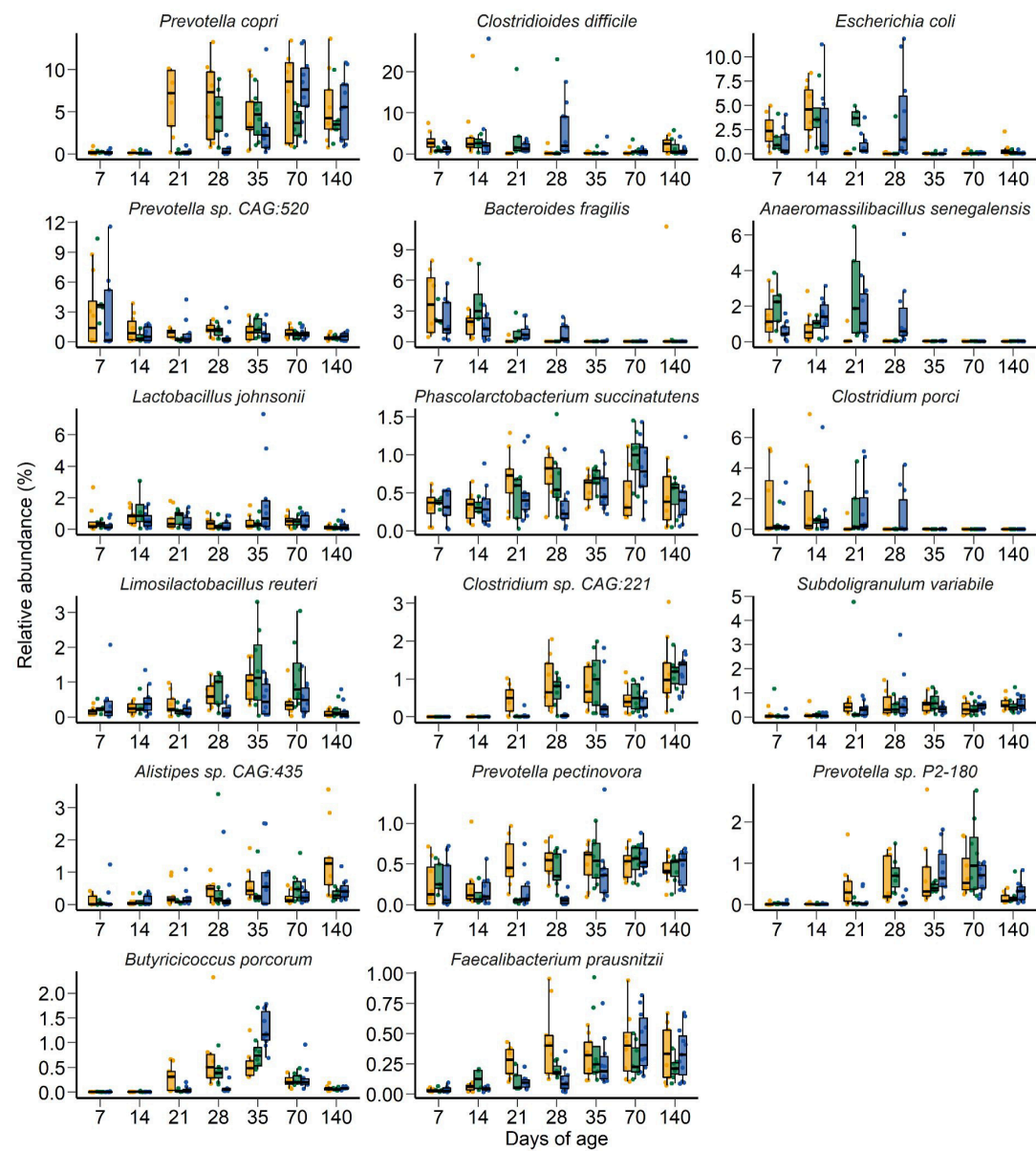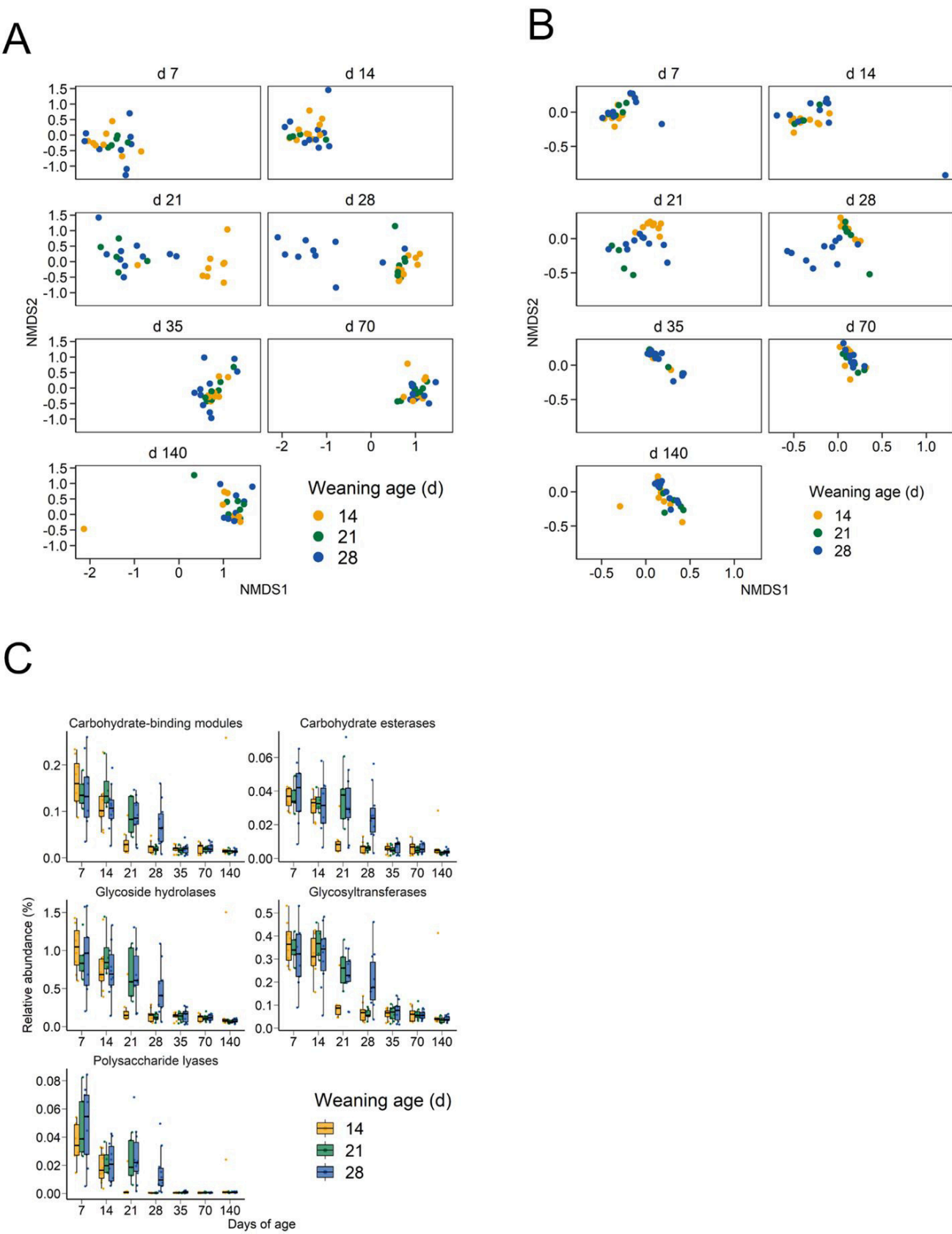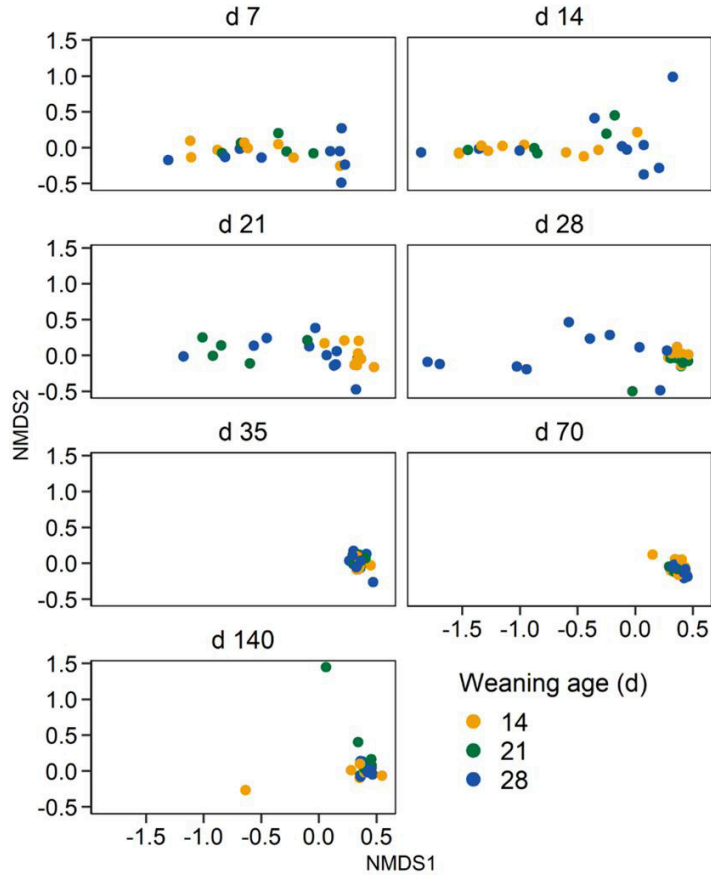656   metagenomic sample by weaning age and sampling time.

657

Weaning age (d) ● 14 ● 21 ● 28

A

B

Weaning age (d) 14 21 28

C

Prevotella copri *Clostridioides difficile* *Escherichia coli*

Prevotella sp. CAG:520 *Bacteroides fragilis* *Anaeromassilibacillus senegalensis*

Lactobacillus johnsonii *Phascolarctobacterium succinatutens* *Clostridium porci*

Limosilactobacillus reuteri *Clostridium sp. CAG:221* *Subdoligranulum variabile*

Alistipes sp. CAG:435 *Prevotella pectinovora* *Prevotella sp. P2-180*

Butyricicoccus porcorum *Faecalibacterium prausnitzii*

Figure 4.

Figure 5.