

1 **Impact of diet on jejunal microbiota composition during broiler development**
2 **with special focus on *Enterococcus hirae* and *Enterococcus faecium***

3

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17

18 **Abstract**

19 Modern broiler breeds allow for high feed efficiency and rapid growth, but come at a
20 cost of increased susceptibility to pathogens and disease. Broiler growth rate, feed
21 efficiency, and health are furthermore affected by the composition of the gut microbiota,
22 which in turn is influenced by diet composition. In this study we therefore assessed
23 how diet composition alters the broiler jejunal gut microbiota. A total of 96 broiler
24 chickens were divided into four diet groups: control, coated butyrate supplementation,
25 medium chain fatty acid supplementation, or a high-fibre low-protein content. Diet
26 groups were sub-divided into age groups (4, 12 and 33 days of age) resulting in groups
27 of 8 broilers per diet per age. The jejunum content jejunum was used for metagenomic
28 shotgun sequencing to determine the microbiota composition on species level. Among
29 all diet groups, a total of 104 differential abundant bacterial species were detected.
30 Most notably were the changes in the jejunal microbiota induced by butyrate
31 supplementation when compared to the control diet, resulting in the reduced relative
32 abundance of mainly *Enterococcus faecium* and the opportunistic pathogen
33 *Enterococcus hirae* in broilers 4 days post-hatch. At this early stage of development,
34 the immune system is still immature thereby highlighting the importance to study the
35 relation of diet and the jejunal microbiota. Future studies should furthermore elucidate
36 how diet can be used to promote a beneficial microbiota in the early stages of broiler
37 development.

38

39 **Introduction**

40 The continuous expansion of the poultry industry comes with the demand to improve
41 sustainability of production. Modern broiler breeds offer the advantage of rapid growth
42 and increased feed efficiency, but come with the disadvantage of increased
43 susceptibility to physiological and metabolic disorders, and have indications of inferior
44 immunity [1-6]. Although diet composition has also been optimized for sustainability in
45 terms of growth rate and feed efficiency, the effect of diet on the composition of the
46 intestinal microbiota has not been fully explored. Namely the composition of the jejunal
47 microbiota and the potential diet-induced effects thereof are currently unknown, which
48 is of specific interest as it is one of the principal sites of nutrient absorption [21].

49

50 The gastrointestinal tract and corresponding intestinal microbiota together contribute
51 to feed efficiency, the development of the immune system and ultimately to the state
52 of health and disease [7-10]. In turn, diet composition is known to alter both intestinal
53 physiology and microbiota composition and is thus proposed as a tool to facilitate
54 sustainability in terms of feed efficiency, animal health and reduced mortality. The
55 beneficial effects of diet composition were first observed when growth promoters in the
56 form of antibiotics were established to affect the intestinal microbiota, resulting in
57 beneficial effects on the general health and feed efficiency of chickens [11-14].
58 However, this sub-therapeutic use of antibiotics has the added effect to enrich for
59 antibiotic resistant bacteria and its use is now prohibited globally [15-20]. As a result,
60 the impact of diet composition and additives on the intestinal microbiota are
61 investigated in search for alternatives to mimic mostly the beneficial effects introduced
62 by antibiotics. Although 16S rRNA gene sequencing remains the most common
63 approach to determine diet-induced effects in the bacterial community composition, its

64 resolution is surpassed by that of metagenomic shotgun sequencing (MSS). By
65 sequencing the full microbiome, MSS is not only able to detect bacteria on the genera
66 taxonomic-level but can additionally determine bacterial species. Moreover, MSS can
67 be used to study gene composition and their corresponding gene pathways [21].

68

69 The small intestine is specialized for nutrient absorption, where amino acids are mainly
70 absorbed in the proximal part of the jejunum, while fatty acids are utilized in the distal
71 parts of the jejunum [22, 23]. Both parts are densely colonized with bacteria and in the
72 case of broiler chickens, the most abundant bacteria in the small intestine include lactic
73 acid-producing bacteria *Lactobacillus*, *Enterococcus* and *Streptococcus*, from which
74 *Lactobacillus* is overall the most abundant genera [24-28]. The high abundance of
75 *Lactobacillus* suggests that these bacteria play a prominent role in the intestines and
76 is one of the reasons why *Lactobacillus* is commonly applied in probiotics [29, 30]. Diet
77 composition is explored as an approach to induce shifts in the intestinal microbiota, for
78 instance by altering the ratio of fatty acids and fibres in feed. When animal fat and
79 soybean oil were supplemented with medium-chain fatty acids (MCFA; 0.3% C10 and
80 2.7% C12) for 34 days, the broiler ileum microbiota showed a reduction of
81 *Lactobacillus*, *Enterococcaceae*, *Micrococcaeae* and an increase in
82 *Enterobacteriaceae* [31]. MCFAs have been observed to have antibacterial properties
83 against opportunistic pathogens like *Clostridium perfringens* and *Escherichia coli* when
84 applied in *in vitro* experiments, but it is unknown if the antibacterial properties persist
85 in a complex system as the intestinal microbiota [32-34]. Another example is butyrate,
86 which is a short chain fatty acid (SCFA) and is the preferred energy-providing substrate
87 of colonocytes [35]. When broiler feed was supplemented with butyrate for 42 days,
88 both feed efficiency and villi size were increased [36]. Butyrate can be rapidly absorbed

89 by the microbiota and intestinal cells located in the proximal sites of the intestine. In
90 order to slowly release butyrate over the full length of the intestine, Mallo et al., 2021,
91 supplemented coated butyrate for 42 days and observed similar results to uncoated
92 butyrate [37]. Supplementation of fibre in feed is known to induce changes in the
93 intestinal microbiota of broilers. Mainly the bacteria located in the caecal microbiota
94 can ferment fibre, generating components including SCFAs [38]. While low level fibre
95 supplementation can increase the amount of butyric acid in the cecum of 21-day-old
96 broilers and increased the abundance of *Helicobacter pullorum* and *Megamonas*
97 *hypermegale*, high levels of fibre supplementation increased the abundance of taxa
98 that may include pathogens, namely *Selenomonadales*, *Enterobacteriales*, and
99 *Campylobacteriales* [39].

100

101 The majority of previously discussed studies analyse diet-induced effects on the
102 genera taxonomic-level of bacteria, preventing the observation of for instance species-
103 specific effects. Only a subset of bacteria has been used for species-specific
104 comparisons, thereby potentially neglecting other important bacteria including
105 opportunistic pathogens. Moreover, while the jejunum is one of the principle sites of
106 nutrient absorption, the effects of diet on the jejunal microbiota are unknown. In this
107 study, we therefore assessed how different diets impacts the composition of the jejunal
108 microbiota on the species level by performing MSS of the microbiota in broilers during
109 the first 33 days post-hatch.

110

111 Results

112 *Diet-associated differences in the jejunal microbiota*

113 A total of 96 Ross 308 broilers were housed in ground cages. They were divided into
114 four diet groups, to study the effect of diet: (1) control diet (CON); (2) control diet
115 supplemented with butyrate (BUT), (3) control diet supplemented with medium-chain
116 fatty acids (MCFA) and (4) a diet with high-fibre low-protein composition (HFLP). The
117 jejunal microbiota was studied by taking chyme samples after either 4, 12 or 33 days
118 post-hatch, thus studying groups of 8 broilers per diet per timepoint. Samples were
119 used for metagenomic shotgun sequencing, resulting in 137.6M [SE 67.4] reads per
120 sample and 32.7M [SE 2.5] assigned read pairs per sample after taxonomic
121 classification. Sample s2229 contained the lowest number of assigned read pairs
122 (1.7M) and was therefore excluded from downstream analysis. This sample was part
123 of the BUT group 12 days post-hatch. There was no systemic difference in the top 10
124 abundant bacterial species, between the diet groups. In all diet groups, the most
125 abundant 10 species included: *Lactobacillus johnsonii*, *Limosilactobacillus reuteri*,
126 *Ligilactobacillus salivarius*, *Enterococcus hirae*, *Lactobacillus crispatus*, *Pediococcus*
127 *acidilactici*, *Enterococcus faecium*, *Corynebacterium stationis*, *Limosilactobacillus*
128 *vaginalis* and *Enterococcus faecalis*. These are all lactic acid bacteria, except for
129 *C. stationis* [40, 41]. The jejunal microbiota displays an overall age dependent effect,
130 independent of diet. This includes an overall high abundance of *E. hirae* 4 days post-
131 hatch (relative abundance 12.95% [SE 0.04]), which is reduced at 12 days post-hatch
132 (0.83% [SE 0.01]). In a similar way, the jejunal microbiota showed a steep decrease of
133 *L. johnsonii* from 12 days post-hatch (58.99% [SE 0.03]), compared to 33 days post-
134 hatch (18.16% [SE 0.04]). In contrast, the abundance of *L. salivarius* and *L. crispatus*

135 increased over the course of 12 days post-hatch (0.00% [SE 0.00]; 0.31% [SE 0.00])
136 to 33 days post-hatch (20.05% [SE 0.28]; 6.86% [SE 0.04]).
137

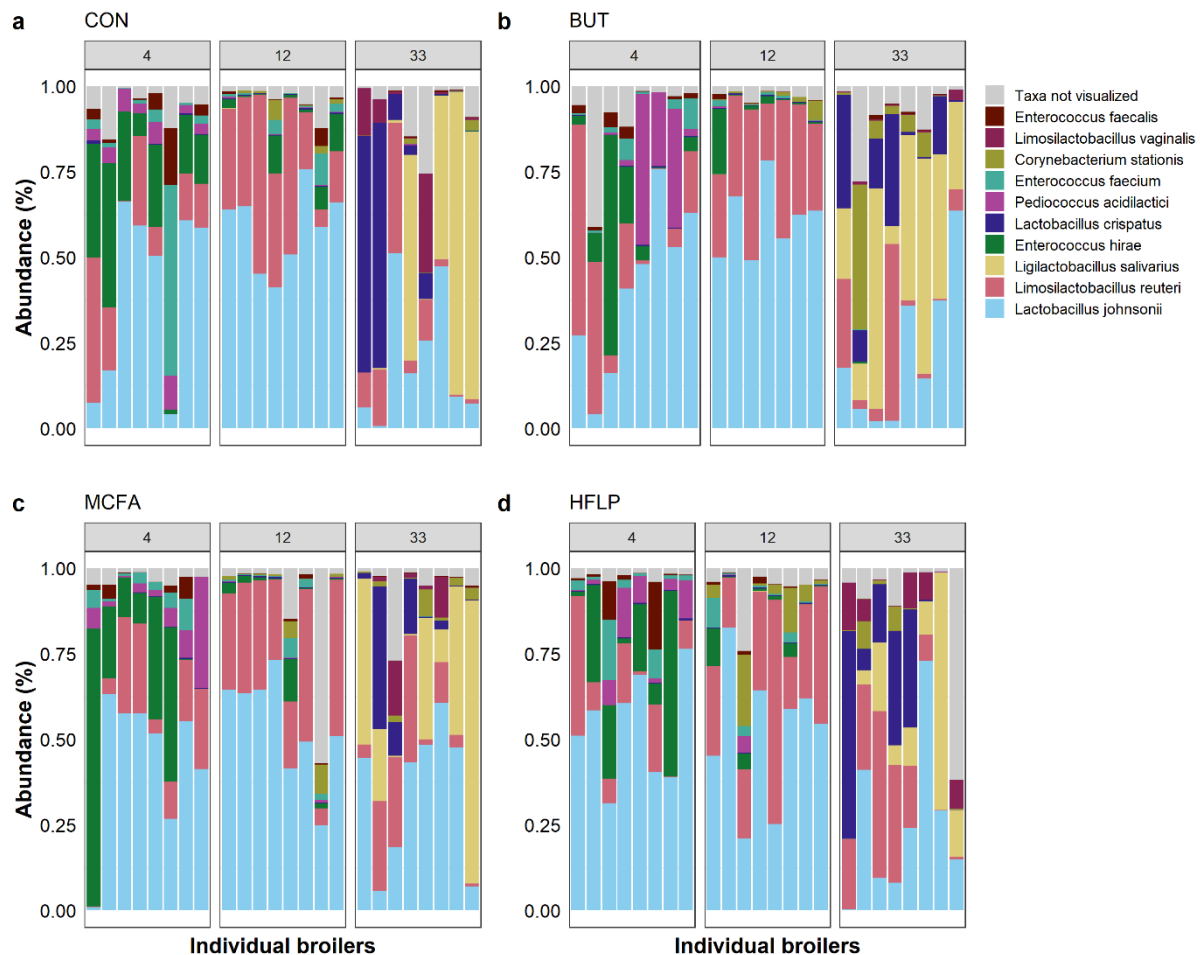
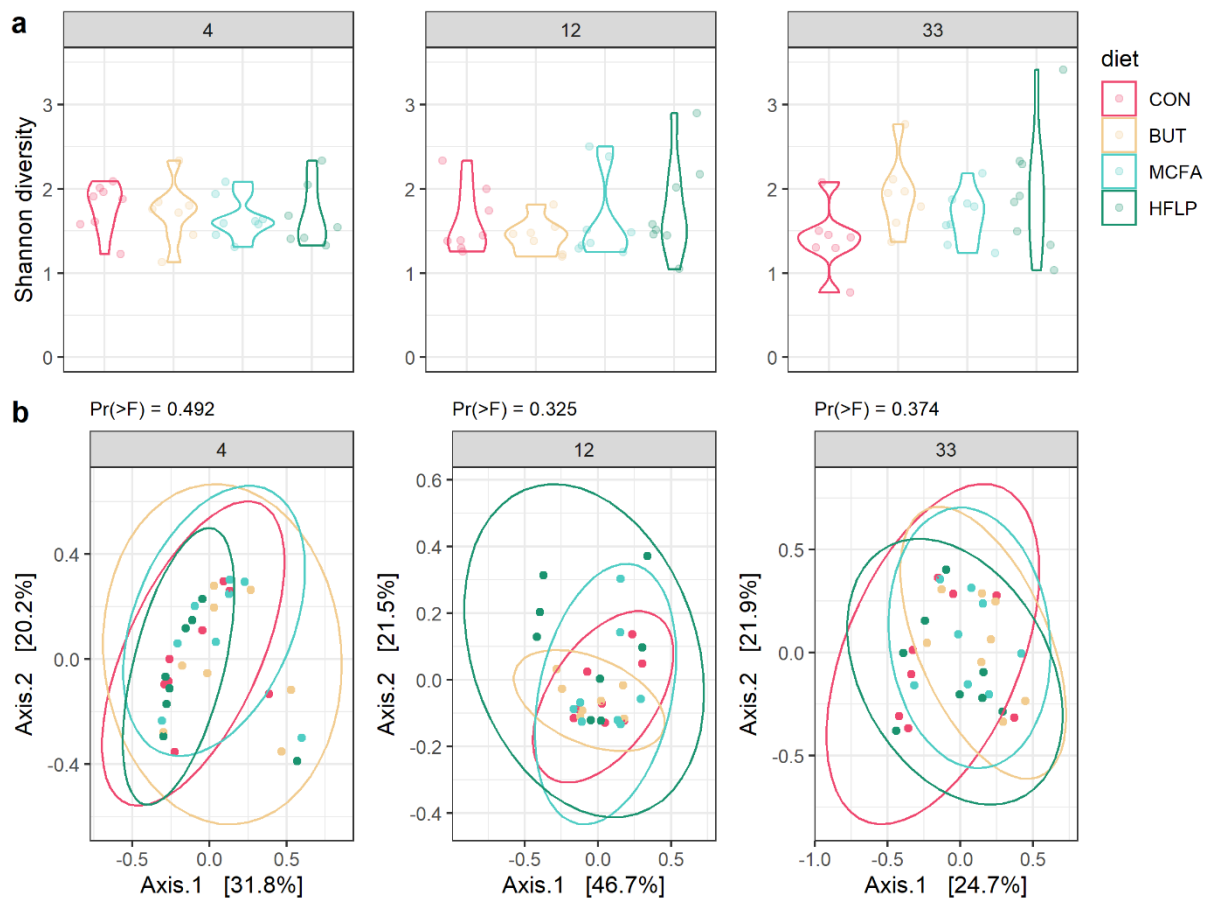


Figure 1 | Association of diet and the composition of the jejunal microbiota at different ages (d4, d12, and d33). Relative abundance of the 10 most abundant bacterial species per diet group. (a) Control diet (CON), (b) control diet plus butyrate (BUT), (c) control diet plus medium-chain fatty acids (MCFA) and (d) a high-fibre low-protein diet (HFLP). Broilers are grouped by columns, representing the number of days post-hatch. Abundance was plotted on the relative abundance scale from 0.00 to 1.00. The jejunal microbiota diversity expressed as Shannon index and the microbiota evenness expressed as Pielou index, were not significantly different between diet

148 groups (figure 2a, figure s1). Overall, the total species diversity is highly similar among
149 diet groups. Multidimensional scaling (MDS) was applied on Bray-Curtis distance
150 matrices and revealed that diet was not a main driver of the observed variance in
151 microbiota composition between samples at either 4, 12 or 33 days post-hatch (figure
152 2b).
153



154
155 **Figure 2 | Diversity indices of jejunal microbiota per diet group at different ages**
156 **(d4, d12, and d33).** (a) Alpha diversity per diet group expressed by Shannon diversity
157 index on OTU level. Diet groups did not differ in terms of alpha diversity when
158 compared with Wilcoxon rank-sum tests. (b) Beta diversity of bacterial species, using
159 MDS ordination based on Bray-Curtis dissimilarity on OTU level. Individual broilers and
160 corresponding ellipses are coloured according to diet group. Plot panels represent
161 broiler groups of 4, 12 and 33 days post-hatch. Permutational multivariate analysis of

162 variance and testing for homogeneity of multivariate dispersions revealed no significant
163 differences between groups.

164

165 Differential abundance analysis revealed a total of 104 bacterial species that were
166 significantly different in terms of abundance when comparing diet groups to the control
167 group (figure 4, table s1-9). At 4 days post-hatch, the jejunal microbiota of the **BUT** diet
168 resulted in 43 differentially abundant bacteria, compared to the control group. Bacteria
169 with a relative abundance above 0.01% and that changed in terms of relative
170 abundance compared to the control group, expressed as log₂ fold changes (l2fc),
171 included: a reduction of *E. hirae* (-2.9 l2fc, 4.2% abundance), *Enterococcus faecium* (-
172 1.8 l2fc, 1.2% abundance), *Enterococcus durans* (-2.6 l2fc, 0.04% abundance),
173 *Erysipelatoclostridium ramosum* (-2.4 l2fc, 0.03% abundance), *Enterococcus avium* (-
174 1.8 l2fc, 0.02% abundance), *Lacrimispora saccharolytica* (-1.5 l2fc, 0.01%
175 abundance), *Massilistercora timonensis* (-1.5 l2fc, 0.01% abundance),
176 *Lachnoclostridium phocaeense* (-1.4 l2fc, 8.3e-3% abundance) and of *Weissella*
177 *paramesenteroides* (-5.1 l2fc, 2.2e-4% abundance). The **MCFA** diet resulted in 6
178 differentially abundant bacteria that are present in low abundance, including a
179 reduction of *Pediococcus pentosaceus* (-1.6 l2fc, 0.04% abundance) and of
180 *W. paramesenteroides* (-4.8 l2fc, 2.8e-4% abundance). The **HFLP** diet resulted in 7
181 differentially abundant bacteria that are present in low abundance, including a
182 reduction of *P. pentosaceus* (-1.3 l2fc, 0.043% abundance) and of
183 *W. paramesenteroides* (-5.1 l2fc, 7.3e-5% abundance).

184

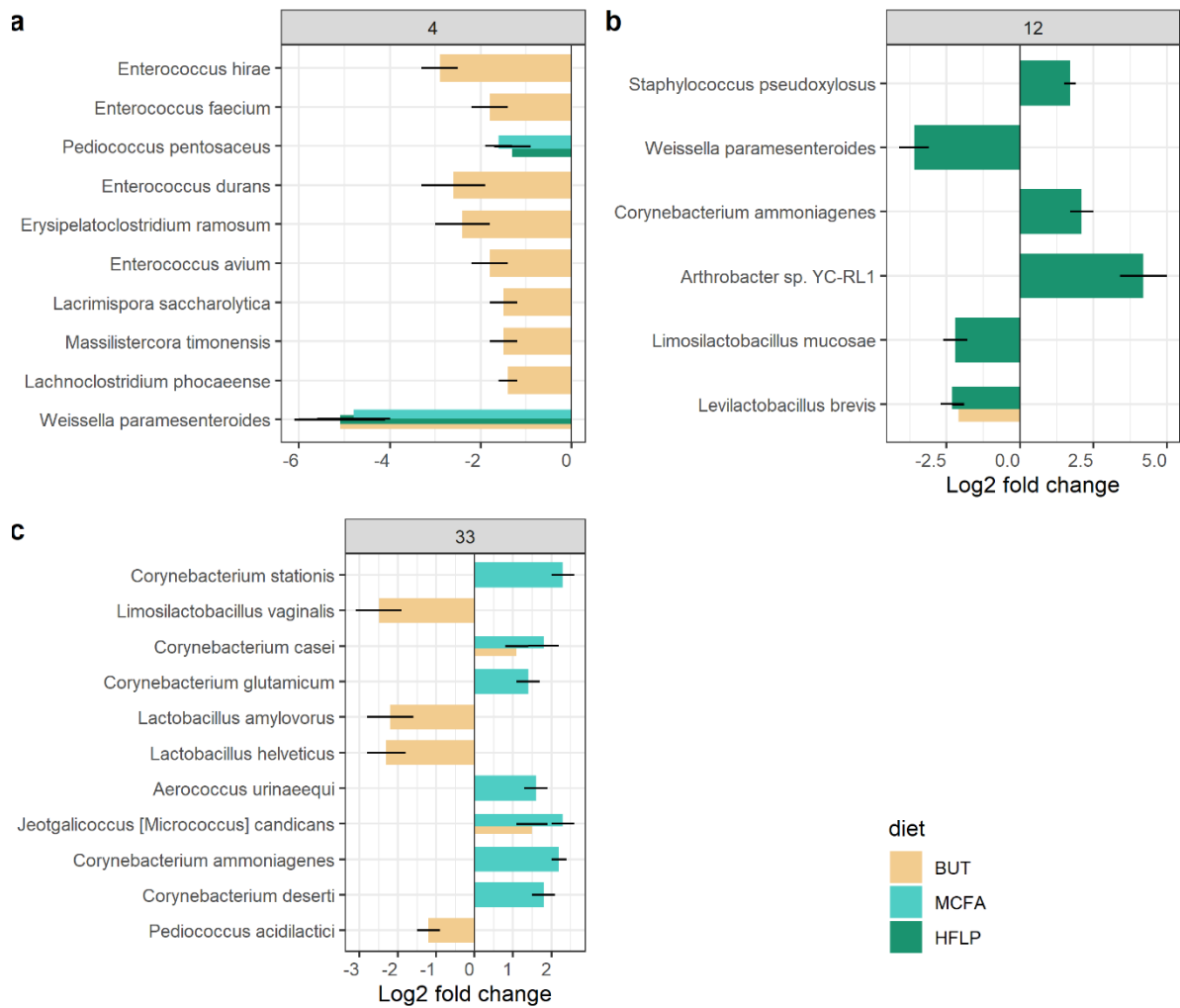
185 At 12 days post-hatch, the **BUT** diet resulted in 17 differentially abundant bacteria that
186 are present in low abundance, including a reduction of *L. brevis* (-2.1 l2fc, 1.1e-3%

187 abundance). The **MCFA** diet resulted in 3 differentially abundant bacteria that have a
188 low relative abundance in the MCFA and control diet groups (all below 1e-2%
189 abundance, table s5). The **HFLP** diet resulted in 31 differentially abundant bacteria,
190 including the increase of *Staphylococcus pseudoxylosus* (1.7 l2fc, 0.71% abundance),
191 *Corynebacterium ammoniagenes* (2.1 l2fc, 0.03% abundance), *Arthrobacter* sp. YC-
192 RL1 (4.2 l2fc, 0.018% abundance and the decrease of *W. paramesenteroides* (-3.6
193 l2fc, 4.2e-4% abundance), *Limosilactobacillus mucosae* (-2.2 l2fc, 9.7e-5%
194 abundance) and *L. brevis* (-2.3 l2fc, 1.4e-3% abundance).

195

196 At 33 days post-hatch, the **BUT** diet resulted in 18 differentially abundant bacteria,
197 including a reduction of *L. vaginalis* (-2.5 l2fc, 0.42% abundance), *Lactobacillus*
198 *amylovorus* (-2.2 l2fc, 0.07% abundance, *Lactobacillus helveticus* (-2.3 l2fc, 0.05%
199 abundance), *P. acidilactici* (-1.2 l2fc, 0.01% abundance) and the increase of
200 *Corynebacterium casei* (1.1 l2fc, 0.26% abundance) and *Jeotgalicoccus (Micrococcus)*
201 *candicans* (1.5 l2fc, 0.03% abundance). The **MCFA** diet resulted in 33 differentially
202 abundant bacteria, including the increased abundance of *Corynebacterium stationis*
203 (2.3 l2fc, 1.7% abundance), *C. casei* (1.8 l2fc, 0.16% abundance), *Corynebacterium*
204 *glutamicum* (1.3 l2fc, 0.12% abundance), *Aerococcus urinaeequi* (1.6 l2fc, 0.04%
205 abundance), *J. (M.) candicans* (2.3 l2fc, 0.02% abundance), *C. ammoniagenes* (2.1
206 l2fc, 0.02% abundance) and *Corynebacterium deserti* (1.8 l2fc, 0.01% abundance).
207 The **HFLP** resulted in 23 differentially abundant bacteria that have a low relative
208 abundance in the HFLP and control diet groups (all below 1e-2% abundance, table
209 s9).

210



211

212 **Figure 3| Diet-induced differentially abundant bacteria in jejunal microbiota at**
 213 **different ages (d4, d12, and d33).** Differential abundance analysis on BUT, MCFA
 214 and HFLP diet groups as a function of the control diet group at (a) 4 days post-hatch,
 215 (b) 12 days post-hatch and (c) 33 days post-hatch. Log2 fold change (l2fc) differences
 216 are visualized by bars, coloured according to diet group and the standard error by error
 217 bars. Differentially abundant bacteria are visualized (abundance > 0.001% ; p-value <
 218 0.05; l2fc > |1|) and ordered from most abundant (top) to least abundant (bottom).

219

220 *Diet-associated differences in the abundance of opportunistic pathogenic and potential*
221 *beneficial bacteria.*

222 The observed diet-induced differentially abundant bacteria include the opportunistic
223 pathogens *E. hirae*, *E. faecium*, *E. durans* and *S. pseudoxylosus*. From this selection,
224 *E. hirae*, *E. faecium* and *E. durans* are present in high relative abundance in broilers
225 in the control group at an early stage of broiler development (20.53% [SE 0.14], 1.86%
226 [SE 0.19], 0.11% [SE 0.00] at 4 days post-hatch) compared to broilers of 12 days post-
227 hatch (1.75% [SE 0.05], 0.66% [SE 0.03], 0.01% [SE 0.00], figure 4). *E. faecium*,
228 *E. durans* and *E. hirae* were all shown to decrease in abundance at 4 days post-hatch
229 as a result of butyrate supplementation compared to the control diet. In order to confirm
230 that presence of these closely related species and exclude the possibility of
231 misannotation by the aligner tool, sequencing data of the control diet group 4 days
232 post-hatch was mapped to the genome of all detected enterococcal species (105.1M
233 [SE 33.0] reads per sample). This resulted in a high number of reads per sample
234 mapping to *E. hirae* (10.0M [SE 6.0] reads, 90.9% [SE 5.3] coverage, 415.7 [SE 286.1]
235 depth), *E. faecium* (0.8M [SE 0.3] reads, 88.0% [SE 2.1] coverage, 31.4 [SE 15.8]
236 depth), *E. faecalis* (0.3M [SE 0.3] reads, 88.6% [SE 6.6] coverage, 12.4 [SE 14.6]
237 depth) and in lesser extend to other enterococcal species (all below 70% coverage),
238 leading us to conclude that at least *E. hirae* and *E. faecium* are present in the jejunal
239 microbiota of these broilers and affected by butyrate supplementation (table s10).

240

241 This analysis was repeated for the bacteria *L. mucosae*, *L. vaginalis*, *L. brevis*,
242 *L. amylovarus*, *L. helveticus*, *P. pentosaceus* and *W. paramesenteroides* since these
243 are considered beneficial to gut health and applied in probiotics [42-47]. *L. mucosae*
244 and *L. brevis* were shown to decrease in abundance at 12 days post-hatch as a result

245 of the HFLP diet compared to the control diet. At 12 days post-hatch butyrate
246 supplementation results in a decreased abundance of *L. brevis*. Sequencing data of
247 the control diet group 12 days post-hatch was mapped to the genome of all detected
248 *Limosilactobacillus* and *Levilactobacillus* species (106.9M [SE 4.5] reads per sample).
249 This resulted in a high number of reads per sample mapping to *L. reuteri* (7.3M [SE
250 1.6] reads, 82.1% [SE 0.4] coverage, 403.1 [SE 100.2] depth) while other detected
251 *Limosilactobacillus* and *Levilactobacillus* species had a coverage below 70%. Due to
252 the low number of reads and coverage, the differential abundance of *L. mucosae* and
253 *L. brevis* at 12 days post-hatch could therefore not be confirmed. *L. vaginalis*,
254 *L. amylovarus* and *L. helveticus* were shown to decrease in abundance at 33 days
255 post-hatch as a result of the BUT diet compared to the control diet. Sequencing data
256 of the control diet group 33 days post-hatch was mapped to the genome of all detected
257 *Limosilactobacillus* and *Lactobacillus* species (113.9M [SE 35.0] reads per sample).
258 This resulted in a high number of reads per sample mapping to *L. reuteri*, (1.8M [SE
259 2.0] reads, 83.6% [SE 5.9] coverage, 103.7 [SE 128.5] depth), *L. vaginalis*, (0.4M [SE
260 2.2] reads, 86.8% [SE 10.9] coverage, 26.2 [SE 140.5] depth) and to *L. crispatus* (2.7M
261 [SE 14.4] reads, 87.1% [SE 8.3] coverage, 154.1 [SE 934.3] depth), while other
262 detected *Limosilactobacillus* and *Lactobacillus* species had a coverage below 70%.
263 Due to the low number of reads and coverage, the differential abundance of
264 *L. vaginalis*, *L. amylovarus* and *L. helveticus* at 33 days post-hatch could therefore not
265 be confirmed.

266

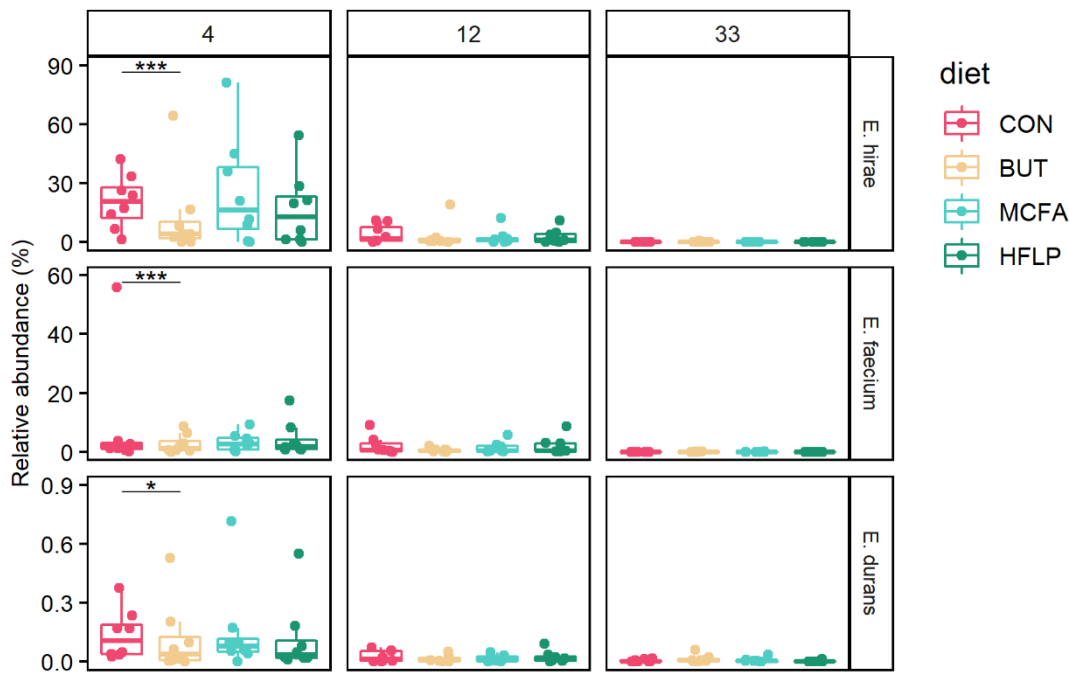
267 *P. pentosaceus* was shown to decrease in abundance at 4 days post-hatch as a result
268 of MCFA and HFLP diet. *P. acidilactici* on the other hand, decreased in abundance at
269 33 days post hatch as a result of the BUT diet. When mapping the sequencing data of

270 the control diet group at 4 and 33 days post-hatch to all detected *Pediococcus* species
271 (105.1M [SE 33.0] and 106.9M [SE 4.5] reads per sample, 4 and 33 days post-hatch
272 respectively). This resulted in a high number of reads per sample mapping to
273 *P. acidilactici* 4 days post-hatch (1.3M [SE 1.4] reads, 87.2% [SE 1.0] coverage , 76.3
274 [SE 92.5] depth), while other detected *Pediococcus* species at 4 or 33 days post-hatch
275 had a coverage below 70%. Due to the low number of reads and coverage, the
276 differential abundance of *P. pentosaceus* at 4 days post-hatch and *P. acidilactici* 33
277 days post-hatch could therefore not be confirmed.

278

279 Finally, *W. paramesenteroides* was shown to decrease in abundance at 4 days post
280 hatch for the BUT, MCFA and HFLP diet and at 12 days post hatch for the HFLP diet.
281 When mapping the sequencing data of the control diet group at 4 and 12 days post-
282 hatch to all detected *Weisella* species (105.1M [SE 33.0] and 106.9M [SE 4.5] reads
283 per sample, 4 and 12 days post-hatch respectively). The detected *Weisella* species at
284 4 or 12 days post-hatch had a coverage below 70% and therefore the differential
285 abundance of *W. paramesenteroides* at these timepoints cannot be confirmed.

286



287

288

289 **Figure 4| Diet-induced differentially abundant opportunistic pathogens.** Relative
290 abundance of opportunistic pathogens *E. hirae*, *E. faecium* and *E. durans*, indicated
291 by rows. Columns represent broiler groups of 4, 12 and 33 days post-hatch. Individual
292 broilers are coloured according to diet group. Adjusted p-values are calculated as part
293 of ANCOMBC as function of the control diet and indicated by * < 0.05, ** < 0.01 and *** <
294 0.001.

295

296 Discussion

297 In this study, we determined the jejunal bacterial microbiota of broilers 4, 12 and 33
298 days post-hatch using metagenomic shotgun sequencing (MSS) and evaluated to what
299 extent diet modulates the jejunal microbiota composition. The results confirmed that
300 diet supplemented with either butyrate (BUT), medium chain fatty acids (MCFA) or diet
301 with high-fibre low-protein (HFLP), can induce significant differences in the relative
302 abundance of a total of 104 bacterial species. The results of butyrate supplementation
303 are of specific interest as they reduced the relative abundance of highly abundant
304 enterococci in the jejunal microbiota 4 days post-hatch, a critical stage for broiler
305 health. Specifically, MSS allowed to differentiate between bacteria on species level
306 and revealed that butyrate supplementation greatly reduces the relative abundance of
307 *Enterococcus hirae* and *Enterococcus faecium*.

308
309 Regardless of the fluctuations in microbiota composition in the first weeks of life, we
310 observed that the most abundant species are mainly part of the genera *Lactobacillus*.
311 This is in concordance with previous studies that analysed the small intestines of
312 broilers [24, 27, 48]. MSS allowed us to surpass the level resolution of 16S rRNA gene
313 sequencing. To our knowledge, this is the first time that the composition of broiler
314 jejunal microbiota has been determined at the bacterial taxonomic species level. When
315 comparing the ten most abundant bacterial species at 4, 12 and 33 days post-hatch,
316 we observed fluctuations in the jejunal microbiota during broiler development. This
317 included a transition from high relative abundant *Lactobacillus johnsonii* (58.99% [SE
318 0.15]) at 12 days post-hatch to *Ligilactobacillus salivarius* and *Lactobacillus crispatus*
319 20.35% [SE 0.28]; 6.86% [SE 0.21]) at 33 days post-hatch. This is similar to the
320 findings of Lu et al., 2023, when studying the broiler ileum microbiota. They observed

321 a transition of the most dominant species, switching from *Lactobacillus acidophilus* at
322 14 days post-hatch (53% abundance) to *L. crispatus* at 28 days post-hatch (75%
323 abundance) [24]. The ileum is the small intestinal region located directly downstream
324 of the jejunum and the corresponding microbiotas are known to share similarities in
325 their composition, potentially explaining these similar findings [49].

326

327 We observed *E. hirae* to be the second most abundant bacterial species in the jejunal
328 microbiota 4 days post-hatch among diet groups (12.95% [SE 0.21]) and observed a
329 much lower relative abundance of *E. hirae* 12 days post-hatch (0.83% [SE 0.05]). This
330 matches with the findings of Schokker et al., 2017, where a decrease in overall
331 enterococcal abundance was observed from 21.7% 4 days post-hatch to 4.9% 14 days
332 post-hatch [48]. We determined that supplementation of butyrate to broiler feed
333 resulted in a 2.9 log₂ fold change decrease in abundance of *E. hirae* in broilers 4 days
334 post-hatch. Additionally, the butyrate supplemented group showed a decrease of
335 several enterococci species at 4 days post-hatch, including *E. faecium* and
336 *Enterococcus durans* and *Enterococcus avium*. From these, only *E. hirae* and
337 *E. faecium* were present in sufficient abundance to ensure that this species is present
338 with at least 70% genome coverage. The butyrate applied in this study is coated, which
339 was previously found to result in the slow release of butyrate along the length of the
340 intestinal tract [50, 51]. Sun et al., 2022, has shown that coated butyrate can reduce
341 the abundance of enterococci in the ileum microbiota of squabs [52], but this is the first
342 time this function is demonstrated in broilers. *E. hirae* is an opportunistic pathogen that
343 can cause locomotion problems, endocarditis and septicaemia in broiler chickens [53-
344 56]. The effect of butyrate on the abundance of *E. hirae* 4 days post-hatch is of specific
345 interest, since this early stage represents the most critical period during broiler

346 development. In this stage, the immune and digestive system are still immature,
347 thereby increasing the susceptibility to disease [57]. This period is furthermore marked
348 by the transition from aerial breathing, initiation of thermal regulation and changes in
349 diet composition, from yolk to solid feed, contributing to the overall high stress load
350 during early broiler development [58]. While some short chain fatty acids directly inhibit
351 bacterial growth, butyrate supplementation only resulted in limited growth inhibition of
352 *E. faecium* and *E. hirae*, when tested *in vitro* [32-34, 59]. Therefore, this effect is not
353 likely to be caused by butyrate directly, but rather indirect, i.e. by changes in the jejunal
354 microbiota as a result of the butyrate supplementation.

355

356 *E. faecium* is known as a gut commensal, but also as an opportunistic pathogen in
357 humans and animals [60, 61]. Moreover, specific isolates of *E. faecium* are applied as
358 probiotics in broilers [62, 63]. The distinction between these groups lies in differences
359 in their genetic makeup [64, 65]. The detected *E. faecium* should therefore first be
360 classified in order to conclude about its impact on broiler health. *L. mucosae*,
361 *L. vaginalis*, *L. brevis*, *L. amylovarus*, *L. helveticus*, *P. pentosaceus* and
362 *W. paramesenteroides* are considered beneficial for gut health and were found
363 differentially abundant as a result of diet. Due to the low relative abundance, these
364 species did not reach the threshold of 70% genome coverage and their presence could
365 therefore not be validated.

366

367 Modern broilers breeds have been primarily selected for rapid growth rate and feed
368 efficiency. While diet composition has additionally been optimized for broiler growth
369 rate, feed efficiency and health benefits, the relation between diet and the jejunal
370 microbiota is not fully understood. In this study we showed that supplementation of diet

371 with either BUT, MCFA or HFLP induced changes in the jejunal microbiota composition
372 at bacterial species level of broilers 4, 12 or 33 days post-hatch. Most notably was the
373 observed reduction in the abundance of *E. faecium* and of the opportunistic pathogen
374 *E. hirae* in the BUT diet, compared to the control diet. The impact of diet composition
375 on microbiota composition of opportunistic pathogens during early broiler
376 development, highlights the importance to study the relation of diet and the jejunal
377 microbiota. Future studies should furthermore elucidate how diet can be used to
378 promote a beneficial microbiota in the early stages of broiler development and could
379 be supported by metatranscriptomics.

380

381 **Methods**

382 The experiment was conducted at the experimental research facility of Wageningen
383 University and Research. All procedures complied with the Dutch law on animal
384 experiments; the project was approved by the Central Commission on Animal
385 Experiments (license number AVD4010020197985) and the experiment by the Ethical
386 Committee of Wageningen University & Research, the Netherlands; experiment no.
387 2019.D-0009.001.

388

389 *Classification of broiler groups*

390 Day-old Ross 308 male broiler chickens were obtained from a commercial hatchery
391 (Probroed & Slood, Groenlo, The Netherlands) and housed floor pens with wood
392 shavings as substrate *ad libitum* access to feed and water as described by Perricone
393 et al., 2023 (REF PENDING). Broilers were divided into age groups of 4, 12, and 33
394 days post-hatch, which were subdivided into four diet groups for microbiota analysis,
395 as described in Perricone et al., 2023 (REF PENDING). In summary, these groups are:
396 (1) control diet without any supplementation (CON); (2) control diet supplemented with
397 sodium butyrate (BUT, Excential Butycoat®, Orffa, Werkendam, the Netherlands), (3)
398 control diet supplemented with a mixture of medium-chain fatty acids (MCFA,
399 Aromabiotic®, Nuscience, Belgium) and (4) a diet with a high-fibre low-protein
400 composition compared to the control diet (HFLP). While the BUT and MCFA diet
401 involve supplementation of components, the HFLP diet involved substitutions of
402 several components of the control diet, including the substitution of rapeseed meal by
403 potato protein and an increase of sunflower seed meal and corn, and a reduction in
404 soybean meal. To summarize, 8 broilers were studied per diet per timepoint. Cages
405 were separated into 8 blocks (1 broiler/diet group/age/block), in order to prevent the

406 exchange of manure and or litter, as described by Perricone et al., 2023 (REF
407 PENDING). Feed was provided via a round feeder (diameter: 35 cm) hanging in the
408 pen. Water was provided via seven nipples along the side wall of a pen. Broilers were
409 vaccinated against infectious bronchitis before arrival at the experimental facility and
410 on d25, and against Newcastle disease at d15.

411

412 *Sample collection, storage and DNA extraction*

413 Chyme samples were collected from the jejunum of broilers by squeezing the jejunum
414 content in a collection tube. Samples were snap-frozen and transferred to storage at -
415 80°C. One freeze-thaw cycle was introduced when dividing samples into aliquots of
416 0.2 g. Aliquoted samples were used for DNA extraction with the Invitrogen PureLink
417 Genomic DNA Mini Kit according to the manufacturer's instructions. Total DNA was
418 quantified by using an Agilent 2200 Tapestation.

419

420 *Metagenomic shotgun sequencing and data processing*

421 DNA samples were sent to GenomeScan B.V. (Leiden, the Netherlands) for
422 Metagenomic shotgun sequencing. The NovaSeq 6000 (Illumina, San Diego, USA)
423 was applied with S2 flow cells and the 2 x 150 bp paired-end kit (Illumina) according
424 to company protocols. Samples contained on average 137.6M [SE 67.4] reads.
425 Sequencing reads were adapter-clipped, erroneous-tile filtered, and quality-trimmed at
426 $\geq Q20$ (PHRED score) using BBduk v38.96 and subsequently filtered for host DNA
427 using the global-alignment algorithm of BBmap v38.96 with default settings and *fast=t*
428 (broiler genome version 2021/01/19, accession number GCF_016699485.2) [66].
429 Read pairs were then used for taxonomic classification by Kraken v2.1.2 using the
430 premade standard Kraken RefSeq nucleotide database and applying a confidence cut-

431 off of 0.3 (database version 5/17/2021) [67]. This resulted into 32.7M [SE 2.5] assigned
432 read pairs per sample. The sample with the lowest number of assigned read pairs
433 (1.7M) was excluded from downstream analysis (s2229, BUT group 12 days post-
434 hatch). Kraken2 results were converted into a biom-file using kraken-biom v1.0.1 at
435 standard settings in order to export counts on strain level, here referred to as OTU
436 level [68].

437

438 *Data analysis*

439 Analysis of sequencing data was performed in R version 4.0, RStudio v2022.02.2+485
440 and functions of R packages phyloseq (version 1.4) and ggplot2 [69, 70]. The top 10
441 abundant bacteria in the jejunal microbiota were plotted using the tax_glom function of
442 the phyloseq package (while removing unassigned reads), aggregate function of
443 microbiomeutilities and plotting functions of the microbiome package [71, 72]. The
444 Shannon diversity and Pielou evenness index were calculated on OTU level by first
445 applying rarefaction to an equal library size (720,000 reads, matching the sample with
446 the lowest number of reads), using the rarefy_even_depth function of phyloseq
447 (set.seed=194175, replace=FALSE) [69]. Consequently, the alpha diversity function of
448 the microbiome package was applied [72]. The beta diversity analysis using MDS
449 ordination and Bray-Curtis dissimilarity was calculated on OTU level using the distance
450 and ordinate functions of the phyloseq package [69]. Permutational Multivariate
451 Analysis of Variance (PERMANOVA) and tests on homogeneity of dispersion were
452 employed using the adonis2 function (999 permutations, seed of 194175) and
453 betadisper function of the vegan package [73]. Differential abundance analysis was
454 performed by first selecting bacterial species for 10% prevalence and 0.001%
455 abundance and subsequent analysis using ANCOM-BC version 1.6.0. ANCOM-BC

456 was applied with standard settings, including Bonferroni correction for false discovery
457 rate, batch correction for cage blocks and an alpha of 0.05 [74]. Structural zeros were
458 included in the analysis (*struc_zero=TRUE*) and are indicated in tables s1-s9. A
459 subsequent cut-off of 0.01% abundance per bacterial species per diet group and an
460 absolute fold change cut-off of 2 were applied to generate the differential abundance
461 plot (figure 3). The validation of detected bacterial species was performed by listing the
462 reference genomes from all enterococcal species detected by kraken and downloading
463 the corresponding RefSeq sequence from the NCBI database, filtering on full genomes
464 and selecting the top hit when sorting by significance. Potential plasmids were
465 excluded from the reference genomes and the resulting genomes were used to create
466 a database using KMA version 1.4.3 and the index function with settings *-sparse TG*.
467 Sequencing reads were subsequently aligned to this database using KMA and settings
468 *-1t1, -ca, -apm p* and *-ef* [75].

469

470 *Data availability*

471 Sequencing files have been submitted into the Sequence Read Archive (SRA) at the
472 NCBI under accession number PRJNA952340. The phyloseq object is available at
473 [10.5281/zenodo.7744071](https://doi.org/10.5281/zenodo.7744071).

474

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478

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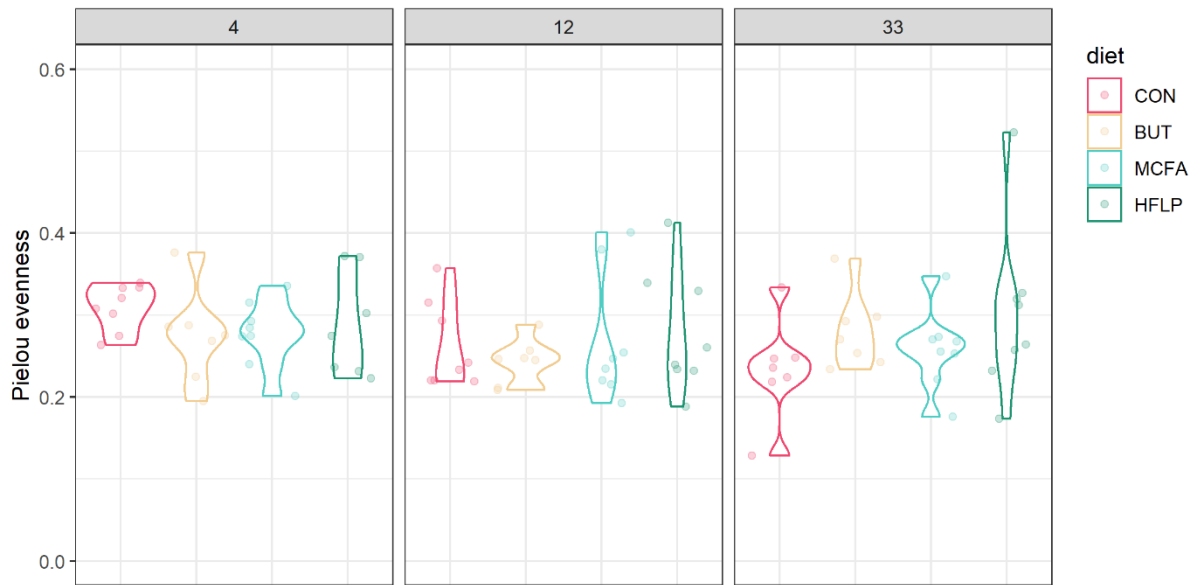
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652 **Supplementary information**

653



655 **Figure s1 | Microbiota evenness per diet group.** Alpha diversity per diet group
656 expressed by Pielou evenness index on OTU level. Diet groups did not differ in terms
657 of alpha diversity when compared with Wilcoxon rank-sum tests.