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Journal Name:	Frontiers in Physiology
ISSN:	1664-042X
Article type:	Original Research Article
Received on:	05 Dec 2014
Accepted on:	14 Apr 2015
Provisional PDF published on:	14 Apr 2015
Frontiers website link:	<a href="http://www.frontiersin.org">www.frontiersin.org</a>
Citation:	Rurangwa E, Sipkema D, Kals J, Ter_veld M, Forlenza M, Bacanu GM, Smidt H and Palstra AP(2015) Impact of a novel protein meal on the gastrointestinal microbiota and host transcriptome of larval zebrafish <i>Danio rerio</i> . <i>Front. Physiol.</i> 6:133. doi:10.3389/fphys.2015.00133
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# Impact of a novel protein meal on the gastrointestinal microbiota and the host transcriptome of larval zebrafish *Danio rerio*

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Running head: Gastrointestinal microbiota and host transcriptome of larval zebrafish

## Abstract

Larval zebrafish was subjected to a methodological exploration of the gastrointestinal microbiota and transcriptome. Assessed was the impact of two dietary inclusion levels of a novel protein meal (NPM) of animal origin (ragworm *Nereis virens*) on the gastrointestinal tract (GIT). Microbial development was assessed over the first 21 days post egg fertilisation (dpf) through 16S rRNA gene-based microbial composition profiling by pyrosequencing. Differentially expressed genes in the GIT were demonstrated at 21 dpf by whole transcriptome sequencing (mRNAseq). Larval zebrafish showed rapid temporal changes in microbial colonization but domination occurred by one to three bacterial species generally belonging to *Proteobacteria* and *Firmicutes*. The high iron content of NPM may have led to an increased relative abundance of bacteria that were related to potential pathogens and bacteria with an increased iron metabolism. Functional classification of the 328 differentially expressed genes indicated that the GIT of larvae fed at higher NPM level was more active in *transmembrane ion transport* and *protein synthesis*. mRNAseq analysis did not reveal a major activation of genes involved in the immune response or indicating differences in iron uptake and homeostasis in zebrafish fed at the high inclusion level of NPM.

**Keywords:** zebrafish nutrition, 16S rRNA-based microbial composition, pyrosequencing, mRNA sequencing, gastrointestinal tract transcriptome, iron metabolism, aquaculture

## 1. Introduction

The diet has profound effects on the microbial composition and on the nutrient uptake by the enterocytes in the GIT. Moreover, the diet has effects on the interactions between host and

52 microbes, aspects of which can be very specific (Rawls et al., 2004) but which is also  
53 surprisingly conserved across all vertebrates (Rawls et al., 2006). During long-lasting  
54 interactions, coevolution between hosts and microbes has resulted in a microbial ecosystem that  
55 is monitored and controlled by the host while the microbiota influence their host to maintain a  
56 stable niche for its continued presence (Neish, 2009).

57 Commensal microbial communities play an important role in the host's GIT development,  
58 nutrition and protection against pathogens (Verschuere et al., 2000; Bates et al., 2006; Nayak,  
59 2010; Ringø et al., 2010). In the absence of microbes (germ-free fish), specific aspects of GIT  
60 differentiation and functions are arrested or altered (Bates et al., 2006). GIT microbiota are  
61 involved in the host's feed digestion and physiological processes by producing vitamins,  
62 digestive enzymes, amino acids, essential growth factors and metabolites (Nayak, 2010). They  
63 affect a wide range of biological processes including nutrient processing and absorption,  
64 regulation of intestinal glycan expression, development of the mucosal immune system and  
65 fortification of the innate immune defences, angiogenesis, and epithelial renewal (reviewed by  
66 Kanther and Rawls, 2010, and Rawls et al., 2004).

67  
68 Zebrafish offers interesting features as model organism to study the nutritional impact of  
69 alternative protein sources on the GIT functions, development of the microbial community and  
70 host-microbe interactions by combining several molecular based approaches. Key features of the  
71 zebrafish model include a characterized genome, a wide variety of molecular and bioinformatic  
72 tools and a well-characterized rapid embryonic development (Westerfield, 1993; Kimmel et al.,  
73 1995; Ulloa et al., 2011). With these advantages related to the use of zebrafish as an  
74 experimental fish model, nutritional research in aquaculture can be conducted at reduced cost,  
75 time and space needed in research facilities (Gomez-Requeni et al., 2010; Ribas and Piferrer,  
76 2013). Zebrafish thereby offers an opportunity to gain mechanistic insights but, as any model,  
77 does not replace the commercial species of interest that has its own GIT characteristics.

78 When kept at 28 degrees, zebrafish larvae hatch from their chorions within 3 days post-  
79 fertilization (dpf), and the mouth opens around 74 hours post-fertilization (hpf). The GIT is  
80 colonized by microbiota from the environment after hatching within 12–24 h, concurrent with  
81 digestive tract differentiation (Hansen and Olafsen, 1999; Bates et al., 2006; Rawls et al., 2007;  
82 Kanther and Rawls, 2007; Nayak, 2010). By 4 dpf, within a day after mouth opening, the  
83 digestive tract is colonized by a small number of bacteria and their number increases after  
84 swallowing has started (Bates et al., 2006). At 5 dpf, the GIT is fully functional from a  
85 nutritional point of view when lipid and protein macromolecule uptake is apparent (Farber et al.,  
86 2001; Wallace et al., 2005), a regular pattern of spontaneous movement is visible and exogenous  
87 feeding commences (Holmberg et al., 2004). The yolk is largely absorbed and GIT  
88 morphogenesis has proceeded to a stage that supports feeding and digestion (Farber et al., 2001;  
89 Rawls et al., 2004).

90  
91 This study aimed to determine the impact of a novel protein meal (NPM) on the  
92 gastrointestinal microbiota and the host transcriptome of larval zebrafish. The NPM that was  
93 tested is of animal origin (ragworm *Nereis virens*) and has been demonstrated to be beneficial for  
94 hematocrit levels and general physiological performance as suggested by improved growth in  
95 common sole *Solea solea* when compared to fish fed with commercial pelleted feeds (Kals,  
96 2014). Therewith it potentially has considerable importance for aquaculture nutrition. The  
97 present study was undertaken to assess the impact of two dietary inclusion levels of the NPM on  
98 the GIT of the developing zebrafish, specifically on 1) microbial development over the first 21  
99 dpf, and 2) molecular differentiation in physiological processes in the host by differentially  
100 expressed genes at 21 dpf as determined by mRNAseq. These investigations are performed with  
101 an unbiased approach.

102 Pathogens residing in the GIT are known to be stimulated in their pathogenic potential by  
103 increased iron availability (Kortman et al., 2012). As feed ingredients of animal origin are

104 expected to be rich iron sources, a bias was introduced to particularly assess (1) changes in the  
105 abundance of potential pathogens and consequences for the expression of GIT genes involved in  
106 immune response, and (2) changes in the abundance of bacteria with increased iron metabolism  
107 and consequences for the expression of GIT genes involved in iron uptake and homeostasis. It is  
108 hypothesized that increasing the dietary inclusion level of the NPM will lead to (1) a higher  
109 abundance of potential pathogens and bacteria with an increased iron metabolism, and (2)  
110 differential expression of genes indicating an activation of the immune response, lower iron  
111 uptake and increased attention for maintaining homeostasis.

112

113

## 114 **2. Methods**

115

### 116 **2.1 Zebrafish husbandry**

117

118 Zebrafish (*Danio rerio* Hamilton 1822) embryos were obtained from breeders of the Zod2F7  
119 strain. The ancestral diet consisted of live nauplii of *Artemia* (brine shrimp) and commercial  
120 flake diet for ornamental fish (Tetra). At 5 dpf, 1,800 larvae from a single batch were randomly  
121 distributed over 6 experimental aquaria (each 6 L with 50 individuals per liter) installed in a  
122 thermo-regulated water bath and with individual inflow water connected to a flow-through  
123 system. Larvae were reared at a photoperiod of 14/10 h light/dark and under optimal water  
124 quality conditions (Temperature  $25.9 \pm 0.3$  °C; pH  $8.1 \pm 0.1$ ; dissolved oxygen  $7.6 \pm 0.3$  mg/L;  
125 ammonium, nitrogen and nitrate null; nitrite concentration 0.00-0.05 mg/L). Larvae were fed  
126 *Paramecium* (diet p) at 4 and 5 dpf, then gradually weaned to experimental diets between 6 and  
127 9 dpf (diets pB and pE; Fig. 1) and, from 10 dpf onwards, fed with experimental diets (diets B  
128 and E) until 21 dpf. During the transitional feeding period from live to inert dry feed, larvae were  
129 fed a daily ration of paramecium decreasing with 20% per day. Before live prey had disappeared  
130 in the tanks, feeding was completed with an increasing amount of experimental dry feeds.

131

### 132 **2.2 Experimental diets**

133

134 The experimental diets were isonitrogenous, isoenergetic, equal in amino acids composition,  
135 calcium and phosphates, but differed in concentration of the NPM ragworm (*Nereis virens*) meal  
136 (Seabait Ltd, Woodhorn Village, UK). Diet B (10% NPM) and E (75% NPM) were fed by hand  
137 till satiation 4-7 times per day. The dry micro-particulate lyophilised diets were prepared through  
138 cold extrusion (Research Diet Services, Wijk bij Duurstede, the Netherlands). 200 µm micro-  
139 particulates were fed between 6 and 10 dpf and 200-300 µm between 11 and 20 dpf for both  
140 experimental diets. The crude composition (Table 1) was analysed at Nutrilab bv (Giessen, the  
141 Netherlands) and the iron content was analysed at the Chemical Biological Soil Laboratory  
142 (Wageningen, the Netherlands) using Inductively Coupled Plasma Atomic Emission  
143 Spectroscopy.

144

### 145 **2.3 Larval sample collection and storage**

146

147 Larvae were sacrificed by an overdose of the anaesthetic 1.0% tricaine methane sulfonate  
148 buffered with 1.5% NaHCO<sub>3</sub>. Triplicate pools of ten larvae per diet were collected in sterile  
149 condition at 5, 7, 14 and 21 dpf. External surfaces of larvae were disinfected by rinsing with 70%  
150 ethanol for 2 min and then several times with sterile filtered (0.2 micron) Milli-Q water. In  
151 addition, at 21 dpf, extra triplicate pools of ten larvae per diet were collected for GIT sampling.  
152 Larvae were anaesthetized, disinfected, and dissected on ice in sterile conditions using flamed  
153 instruments between two different samples. Whole larvae were kept frozen in sterile Eppendorff  
154 tubes at -20°C directly upon sampling and were then stored at -80°C, while extra GIT samples

155 from day 21 were stored in RNeasy lysis buffer (Applied Biosystems, Nieuwerkerk a/d IJssel, the  
156 Netherlands) at -20°C.

157

#### 158 **2.4 Microbiological analyses: DNA isolation**

159

160 Microbial DNA was isolated using the protocol described by Roeselers et al. (2011) with  
161 some modifications: Ten larvae were combined in 2.0 ml screw-cap tubes containing 0.1mm  
162 Zirconia/silica beads and 2.5 mm Glass beads (Biospec Products). 800 µl 120mM Na-phosphate  
163 buffer (pH 8.0) and 400 µl of lysis solution containing 10% sodium dodecyl sulfate, 0.5M Tris-  
164 HCl (pH 8.0) and 0.1M NaCl was added homogenisation in a Mini-beadbeater (Biospec  
165 Products) for 6 min at 5500 rpm. The supernatants were transferred to new tubes and lysozyme  
166 was added to a final concentration of 10 mg/ml followed by incubation at 42°C for 30 min.  
167 Ammonium acetate (7.5M) was added to the supernatant (2:5 v/v) and samples were incubated at  
168 -20°C for 5 min. Samples were centrifuged for 5 min at 12,000 g and the supernatants were  
169 transferred to new tubes. DNA was precipitated at room-temperature with isopropyl alcohol (500  
170 µl) and pelleted by centrifugation at 12,000 rpm for 30 min at 4°C. Pellets were washed with -  
171 20°C 70% ethanol and air-dried for 45 min before resuspension in 50 µl nuclease free water.

172

#### 173 **2.5 Microbiological analyses: RT-PCR**

174

175 RT-PCR targeting 16S rRNA was performed with the primers 27F and 1492R (Lane, 1991)  
176 for total RNA extracted from two pools of GIT for both diet B and diet E (for RNA extraction  
177 procedure see section “*mRNAseq: Total RNA isolation*”). The RT reaction (20 µl) contained 50  
178 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 5 µM DTT, 0.5 mM of each dNTP, 2 pmol of primers  
179 27F and 1492R, 200 U of SuperScript™ Reverse Transcriptase (Invitrogen), 40 U of  
180 RNasin® Plus RNase Inhibitor (Promega) and 1 µg of RNA extracted from 10 pooled larvae or  
181 GITs for each of the two diets. Reactions were incubated at 55 °C for 60 minutes, followed by 15  
182 minutes at 70 °C to denature the reverse-transcriptase.

183

#### 184 **2.6 Microbiological analyses: PCR and Sequencing**

185

186 For 16S rRNA gene-based microbial composition profiling, barcoded amplicons from the  
187 V1-V2 region of 16S rRNA genes were generated from all DNA and reverse transcribed RNA  
188 samples by PCR using the 27F-DegS primer that was appended with the titanium sequencing  
189 adaptor A and an 8 nt sample-specific barcode at the 5' end, and an equimolar mix of two  
190 reverse primers (338R I and II), that carried the titanium adaptor B at the 5' end<sup>58</sup>.

191

192 PCRs were performed using a thermocycler GS0001 (Gene Technologies, Braintree, U.K.) in  
193 a total volume of 100 µl containing 1× HF buffer (Finnzymes, Vantaa, Finland), 2 µl 10 mM  
194 (each nucleotide) PCR Grade Nucleotide Mix (Roche, Diagnostics GmbH, Mannheim,  
195 Germany), 2 units of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a  
196 forward and reverse primer mix (Biolegio BV, Nijmegen, the Netherlands), and 0.2-0.4 ng/µl of  
197 template DNA (or cDNA). The amplification program consisted of an initial denaturation at  
198 98°C for 30 s, 35 cycles of: denaturation at 98°C for 10 s, annealing at 56°C for 20 s and  
199 elongation at 72°C for 20 s, and a final extension at 72°C for 10 min. PCR products were  
200 purified with the High Pure Cleanup Micro Kit (Roche) using 10 µl nuclease-free water for  
201 elution, and quantified using a NanoDrop ND-1000 spectrophotometer. Purified PCR products  
202 were mixed in equimolar amounts and run on an agarose gel, followed by excision and  
203 purification by the DNA gel extraction kit (Millipore, Billerica, MA, USA). Purified amplicon  
204 pools were pyrosequenced using a Genome Sequencer FLX in combination with titanium  
205 chemistry (GATC-Biotech, Konstanz, Germany). Pyrosequencing data were deposited at the  
206 European Bioinformatics Institute in the sequence read archive under study accession number

207 PRJEB4784 and sample accession numbers ERS362581 – ERS362592 and ERS362595 -  
208 ERS362598.

209

## 210 ***2.7 Microbiological analyses: Sequence analysis***

211

212 Pyrosequencing data were analysed using the QIIME 1.5.0 pipeline (Caporaso et al., 2010).  
213 Low quality sequences were removed using default parameters. Operational taxonomic units  
214 (OTUs) were identified at the 97% identity level. Representative sequences from the OTUs were  
215 aligned using PyNAST (DeSantis et al., 2006). The taxonomic affiliation of each OTU was  
216 determined using the RDP Classifier at a confidence threshold of 80% against the 12\_10  
217 Greengenes core set (Wang et al., 2007). Possible chimeric OTUs were identified using QIIME's  
218 ChimeraSlayer and removed from the initially generated OTU list, producing a final set of non-  
219 chimeric OTUs.

220

## 221 ***2.8 Microbiological analyses: Statistical analysis***

222

223 OTU singletons and OTUs related to chloroplasts were removed prior to analyses. The  
224 relationship between microbial community composition, diet and time was analyzed by  
225 canonical correspondence analysis (CCA) using CANOCO 5 (Ter Braak, C.J.F., Šmilauer, P.  
226 *Canoco Reference Manual And User's Guide: Software For Ordination, Version 5.0*. Ithaca:  
227 Microcomputer Power, USA. pp. 496 (2012)). Rare OTUs were down-weighted using the default  
228 option. The different diets (p, pB, pE, B and E) were tested for significant contribution to the  
229 explanation of the variation in the OTU distribution with the Monte Carlo permutation test  
230 associated with the forward selection subroutine. The OTUs that contributed most to different  
231 microbial profiles between diet B and E were calculated using SIMPER in the software package  
232 PRIMER 6 v6.1.9 (PRIMER-E Ltd, Plymouth, UK) using normalised OTU tables (square root)  
233 of day 14 and day 21.

234

## 235 ***2.9 mRNAseq: Total RNA isolation***

236

237 GITs of two pools of ten larvae per diet which had been stored in RNAlater were lysed in  
238 QIAzol Lysis Reagent, a Qiagen TissueRuptor was used to cut up the tissue samples and RNA  
239 was extracted using the Qiagen miRNeasy Mini Kit according to the manufacturer's description  
240 (Qiagen Benelux BV, Venlo, the Netherlands). RNA was eluted in 50 µl and quantified by  
241 Nanodrop (Thermo Fisher Scientific, Amsterdam, the Netherlands). Integrity of the RNA was  
242 confirmed using an Agilent bioanalyzer2100.

243

## 244 ***2.10 mRNAseq: Library preparation and sequencing***

245

246 For each sample a RNA-seq library was prepared with the Illumina Truseq mRNASeq  
247 Sample Preparation Kit according to the manufacturer's description (Illumina, San Diego CA,  
248 USA). Each library was sequenced twice in a paired-end sequencing run with a read length of 50  
249 nucleotides on a Illumina HiSeq2000 with version 2 sequencing chemistry. For each library  
250 approximately 20 to 30 million read pairs were obtained.

251

## 252 ***2.11 mRNAseq: Data analysis***

253

254 Raw reads were quality trimmed using the quality\_trim module in the CLCBio assembly cell  
255 version 4.01. Reads were mapped to the annotated cDNA's in the ZV9 zebrafish genome  
256 assembly using the ref\_assembly\_short module in the CLCBio assembly cell version 4.01. The  
257 data were converted to a table using the assembly\_table module in the CLCBio assembly cell  
258 version 4.01. A custom perl script was used to convert this table to a tab separated value table.

259 This table was used in R package DESeq v1.0.6 to analyse expression in the different samples  
 260 (Anders and Huber, 2010). Raw RNA-seq data (reads) have been submitted to the NCBI project  
 261 data archive under Bioproject number 229446 (Biosample numbers SRS506058 for B1,  
 262 SRS506087 for B2, SRS506089 for E1, SRS506092 for E2).

263 Gene expression of differentially expressed genes at  $P < 0.05$ , both up- or down-regulated, was  
 264 functionally characterised and classified using DAVID 6.7 (The Database for Annotation,  
 265 Visualization and Integrated Discovery, Huang et al., 2009ab).

266

## 267 **2.12 Ethics**

268

269 All experiments were performed in accordance with relevant guidelines and regulations.  
 270 Protocols used complied with the current laws of the Netherlands and were approved by the  
 271 Animal Experimental Committee (DEC) of the Wageningen UR in Lelystad (The Netherlands)  
 272 under number 2011102.

273

274

## 275 **3. Results**

276

### 277 **3.1 Impact of diets on GIT microbial community**

278

279 The development of the microbial community was assessed over the first 21 days post egg  
 280 fertilisation (dpf). At a confidence threshold of 80%, 97,675 out of 97,894 qualified non-  
 281 chimeric reads could be assigned to a known phylum using the RDP classifier. Qualified non-  
 282 chimeric read numbers ranged from 1,740 to 10,685 reads per sample (average: 6,118 reads per  
 283 sample), and the rarefaction curves showed that samples were sufficiently deep sequenced to  
 284 discuss similarity and differences for the more abundant OTUs (supplementary Fig. S1).

285 All 16S rRNA sequences found in zebrafish 5 dpf were classified as either *Proteobacteria* (48%)  
 286 or *Firmicutes* (52%). The bacterial composition changed at 7 dpf with the appearance of  
 287 *Actinobacteria* for both diets tested (paramecium plus diet B: pB, and paramecium plus diet E:  
 288 pE) and *Bacteroidetes* for zebrafish fed with pE (Fig. 1). This was followed by a further increase  
 289 of diversity at 14 dpf for both diets (B and E without paramecium). The trend of diversifying  
 290 microbiota was halted at 21 dpf due to the increased dominance of *Proteobacteria*, especially on  
 291 diet B where they made up for 99% of all reads. Although for day 21, we only had one zebrafish  
 292 larvae-derived sample per diet, 16S rRNA gene analysis based on RNA-extracted from 4  
 293 additional samples (2 for diet B and 2 for diet E) from 21 dpf confirmed the relative abundance  
 294 of 99% at this day.

295

296 The bacterial colonisation of zebrafish and the impact of diet on the colonisation were  
 297 analysed at the approximate species level (97% identity based on rRNA gene sequence) by  
 298 canonical correspondence analysis (CCA). The first two CCA axes had eigenvalues of 0.89 and  
 299 0.80, respectively and explained 27% of the variation in species data and 72% of the variation in  
 300 the microbial taxa-time interactions (Fig. 2). The microbiota changed dramatically during the  
 301 first 21 dpf and the high impact of time ( $P = 0.002$ ) masked treatment (diet) effects. Therefore, the  
 302 impact of diet on zebrafish-associated microbiota was based on the zebrafish gut-derived RNA  
 303 samples on day 21 for diet B and diet E for which replicate samples were available. These RNA-  
 304 based samples were similar to the DNA-based samples for 21 dpf with respect to their microbial  
 305 profiles at the OTU-level (Fig. S2), which indicates that the approach used to obtain 16S rRNA  
 306 gene amplicons (whole fish + DNA extraction vs. fish gut + RNA extraction and reverse  
 307 transcription) did not have a major impact on the data obtained. The dominant early bacterial  
 308 colonisers of zebrafish included members of the *Clostridia* (*Firmicutes*) and *Procabacteriaceae*,  
 309 *Trabulsiella*, and *Xanthomonadaceae* (all *Proteobacteria*) (Fig. 3). These OTUs were mostly  
 310 absent at day 7 and were replaced by OTUs most closely related to *Propionibacterium acnes*

311 (*Actinobacteria*), *Rhodanobacter* and *Rhizobium* (both *Proteobacteria*). *Propionibacterium*  
 312 *acnes* was still highly abundant at day 14 as was *Rhodanobacter* albeit at a lower percentage.  
 313 Populations within the *Rhodobacteraceae*, and *Methylobacteraceae* (both *Proteobacteria*) were  
 314 newly appearing dominant OTUs at day 14. Although OTUs most closely related to  
 315 *Propionibacterium acnes* and *Rhodobacteraceae* were still found at low levels at day 21, those  
 316 falling within *Rhodanobacter* and *Methylobacteraceae* had disappeared. Instead, populations  
 317 within the *Comamonadaceae*, *Aeromonadaceae*, *Acidovorax* and *Pseudomonas* (all  
 318 *Proteobacteria*) dominated the zebrafish-associated microbiota.

319

320 The OTUs that contributed most (1% or more) to the differences in microbial profiles in  
 321 zebrafish fed with diet B or diet E at day 21 were identified by SIMPER (Table 2). OTUs with a  
 322 higher relative abundance for diet E that mainly contributed to the differences in microbial  
 323 profiles between the diets were OTU45 (*Plesiomonas shigelloides*), OTU286 (*Acidovorax* sp.),  
 324 OTU1407 (family *Aeromonadaceae*), OTU 441 (*Trabulsiella* sp.), OTU552 (order  
 325 *Legionellales*), OTU1459 (*Novospirillum itersonii*), OTU1414 (*Rheinheimera* sp.), OTU832  
 326 (*Propionibacterium acnes*), OTU463 (family *Alcaligenaceae*), OTU16 (*Burkholderia* sp.) and  
 327 OTU 1182 (*Halomonas* sp.). Other OTUs that contributed less to the difference observed  
 328 between diet E and B, but which were completely absent in zebrafish fed with diet B were  
 329 OTU1278 (*Achromobacter* sp.), OTU568 (*Janibacter* sp.) and OTU1260 (*Cupriavidus* sp.). The  
 330 OTUs with a higher relative abundance with diet B were *Pseudomonas* spp. (OTU1214 and  
 331 OTU1306), which were among the most dominant OTUs found in zebrafish at day 21 and had an  
 332 even higher relative abundance for diet B than for diet E.

333

### 334 3.2 *mRNAseq gene expression analyses*

335

336 In order to assess potential differences in how the zebrafish host responds to the different  
 337 dietary treatments, intestinal tissue-associated gene expression was measured at 21 dpf. Reads  
 338 were mapped to the 27,882 annotated cDNA's of the ZV9 zebrafish genome assembly that were  
 339 used for further analysis. In total, 328 genes were differentially expressed corresponding to  
 340 1.18% of the total number of genes (Table S1). Of these, expression of 214 genes was up-  
 341 regulated and expression of 114 genes was down-regulated in larvae fed with diet E vs B.  
 342 Among these, expression of 16 differentially expressed genes were detected exclusively for  
 343 larvae fed with diet E and were below detection thresholds for larvae of diet B (fold change - fc  
 344 "inf"), whereas for 10 other differentially expressed genes the opposite was true (fc "0").

345

346 Of the total number of 27,882 genes that were analysed, 19,990 genes could be converted to  
 347 a DAVID id, and of the total number of 328 differentially regulated genes, 264 genes could be  
 348 converted as such. Genes with a DAVID id were used for unbiased functional annotation.

349

350 Functional annotation clustering revealed that the differentially expressed genes represented  
 351 27 annotation clusters. 24 terms representing 7 of these clusters were significantly enriched at  
 352  $P < 0.05$  (Table S2). 52 terms were not clustered. The functional annotation clusters were  
 353 associated with *ribosome components and activity* (enrichment score - es 14.61) and *transport*  
 354 (es 1.4) as the dominant clusters. Other clusters involved *other glycan degradation* (es 1.25);  
 355 *glycosaminoglycan metabolic process* (es 1.13); *extracellular matrix structural constituent* (es  
 356 0.66); *keratin type-1* (es 0.61) and *hydrogen ion transmembrane transporter activity* (es 0.60).  
 357 The functional annotation chart revealed 65 records of which 40 were significantly enriched  
 358 (Table S3). In addition to many terms that were associated with *ribosome*, *transport* and the  
 359 other mentioned clusters, enriched records were associated with: *interferon-induced 6-16*,  
 360 *interferon binding* and *interferon receptor activity*; *glycoside hydrolase* and *oxidative*  
 361 *phosphorylation*.

362



363 Gene functional classification showed the presence of 7 gene groups as determined by 73  
 364 differentially expressed genes (Table 3; S4) agreeing with the existence of 7 enriched  
 365 functionally annotated clusters. Also here the dominant gene groups represented *ribosome*  
 366 *components and activity* and *transport*. The gene group *ribosome components and activity*  
 367 consisted of 27 genes that were all significantly up-regulated at fc 1.47-1.85 in larvae fed with  
 368 diet E vs those fed with diet B (75% vs 10% NPM). Also in the gene group *transport* all four  
 369 genes were up-regulated at fc 1.56 up to 24.20 for *solute carrier family 12 (sodium/chloride*  
 370 *transporters), member 3*. Other groups involved *WD40 repeats acting as protein-protein*  
 371 *interaction sites; nucleotide binding; transcription; metal binding/zinc fingers* and *membrane*. In  
 372 gene groups *WD40 repeats* (4 genes) and *metal binding/zinc fingers* (13 genes) all genes were  
 373 up-regulated while groups *nucleotide binding, transcription* and *membrane* contained both genes  
 374 that were up-regulated as well as genes that were down-regulated. Among them were genes that  
 375 were specific for larvae fed with diet E (*zgc:110560* or *hypothetical protein LOC100150958*;  
 376 *similar to Serine/threonine-protein kinase Pim-3* or *si:dkey-108d22.5*; *forkhead box G1* and  
 377 *mediator of RNA polymerase II transcription subunit 11*) and genes specific for larvae fed with  
 378 diet B (*zgc:172065* or *hypothetical LOC100001153*).

379

380

#### 381 4. Discussion

382

383 This study represents a methodological exploration assessing the development of the  
 384 gastrointestinal microbiota through 16S rRNA gene-based microbial composition profiling by  
 385 pyrosequencing and the functional response of the transcriptome by mRNAseq. Both approaches  
 386 provided complementary information on the nutritional impact of a novel protein source relevant  
 387 for aquaculture. The impact of the novel protein meal concerned the impact on the GIT  
 388 microbiota and, either directly or indirectly through the microbiota, on the host transcriptome.

389

390 The two experimental diets especially differed in the level of iron (Table 1). This difference  
 391 originated from the difference in NPM as it is a rich iron source, consisting mostly of heme iron.  
 392 Feed ingredients of animal origin, like the NPM in this study, are rich in iron. Iron can be taken  
 393 up in two forms; as heme (e.g. hemoglobin), and as non-heme (e.g. iron sulfate). The uptake of  
 394 heme differs greatly from the absorption of inorganic iron as the uptake of heme iron is not  
 395 physiologically regulated and independent of the intestinal pH in contrast to the uptake of  
 396 inorganic iron (Kraemer and Zimmermann, 2007). Uptake of inorganic iron is more complex and  
 397 requires reduction of  $Fe^{3+}$  to  $Fe^{2+}$  which, in turn, requires an acidic environment as provided by  
 398 the gut in monogastric animals. Despite very few mechanistic studies of piscine intestinal iron  
 399 uptake, zebrafish is supposed to take up iron from the diet in the intestinal enterocytes not any  
 400 different than by the mechanism that all vertebrates apply (reviewed by Bury et al., 2003). Iron  
 401 homeostasis is crucial since, in excess, iron can be detrimental to health because of its production  
 402 of oxygen free radicals, and, when too low, loss of energy due to the decrease of haemoglobin  
 403 concentrations and cytochrome capacity in aerobic metabolism. In diets B and E, calculated iron  
 404 content was 312 and 1486 mg kg<sup>-1</sup>, respectively (Table 1). These levels exceed the daily iron  
 405 requirements in fish that ranges between 30 and 170 mg kg<sup>-1</sup> DM food (Watanabe et al., 1997).  
 406 Also experimental studies on dietary iron supplementation in fish report on findings that indicate  
 407 that such levels have already reached a plateau for physiological effects (tilapia: Shiau and Su,  
 408 2003; rainbow trout: Carriquiriborde et al., 2004). So most probably any difference in GIT  
 409 response to the experimental diets does not relate to an iron shortage in fish fed with diet B but  
 410 may reflect effects of iron overloading, especially in fish fed with diet E.

411

412 Of 97,894 representative sequences of GIT microbiota in the present study, the dominant  
 413 phylum was *Proteobacteria*. Other main phyla encountered and ranked according to average  
 414 relative abundance were *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. The rapid temporal

415 changes in GIT microbiota make it difficult to compare our results directly to other studies that  
 416 have been done on the identification of zebrafish GIT microbiota (Bates et al., 2006; Roeselers et  
 417 al., 2011; Semanova et al., 2012; Lan and Love, 2012). However, our data are similar to  
 418 published data for two important aspects: (i) larval zebrafish GITs are dominated by one to three  
 419 bacterial species, (ii) these dominant species generally belong to *Proteobacteria* and *Firmicutes*.  
 420

421 Firstly, many larval and juvenile animals still have immature gut microbiota that are not yet  
 422 fully functional and may be dominated by a few early colonisers. For human gut microbiota it  
 423 has been shown that infant gut bacterial species have faster growth rates than adult gut bacterial  
 424 species, which favours early colonisation (De Muinck et al., 2013). With respect to the second  
 425 point, dominant bacteria in the GITs of fish juveniles have been identified as *Pseudomonas*  
 426 (*Proteobacteria*) in zebrafish and salmon (Bates et al., 2006; Navarette et al., 2009), and an  
 427 unidentified gammaproteobacterium in juvenile pinfish (Givens, 2012). Similarly, the GIT  
 428 microbiota of juvenile Siberian sturgeon was shown to be mono-dominated, only by  
 429 *Cetobacterium somerae* (*Fusobacteria*) (Geraylou et al., 2013). Thereby it should be noted that  
 430 the herbivorous pinfish and the carnivorous salmon and sturgeon have stomachs and GIT  
 431 morphologies that are different from the omnivorous stomachless zebrafish.  
 432

433 At 5 dpf, zebrafish GIT in our study was dominated by members of the genus *Clostridium*  
 434 (*Firmicutes*) with  $40 \pm 11\%$  of the reads and the family *Procabacteriaceae* (*Proteobacteria*) with  
 435  $22 \pm 21\%$  of the reads. *Clostridium* is a well-known inhabitant of the animal gut. Although the  
 436 genus *Clostridium* has been related to animal diseases, commensal *Clostridium* spp. are  
 437 dominant players in the maintenance of gut homeostasis in man and other animals including  
 438 many fish species (Sullam et al., 2012; Lopetuso et al., 2013). *Clostridium* spp. were previously  
 439 found to be abundant in the GIT of fed juvenile zebrafish (Semanova et al., 2012), but absent or  
 440 not abundant in starved juveniles and in the adult zebrafish GIT (Roeselers et al., 2011;  
 441 Semanova et al., 2012), which may indicate that it is an early coloniser of the zebrafish GIT that  
 442 disappears with gut maturation. This is also confirmed by our data as *Clostridium* spp. were  
 443 detected only in a few samples at later time points. Little information exists on the family  
 444 *Procabacteriaceae* (*Proteobacteria*) and they are not frequently encountered in animal gut.  
 445 Candidatus *Procabacter acanthamoebae* was identified as an *Acanthamoeba* endosymbiont  
 446 (Horn et al., 2002). *Acanthamoeba* is related to a number of animal diseases (Paterson et al.,  
 447 2011), however, no visible signs of distress were recorded at 5 dpf and onwards no more reads  
 448 were detected that were affiliated to *Procabacteriaceae*. The zebrafish-associated microbiota  
 449 shifted remarkably between 5 and 7 dpf (Fig. 2) and only few of the OTUs present at 5 dpf were  
 450 also found at 7 dpf. One OTU that was recovered at both days was a *Rhodanobacter* sp.  
 451 (*Proteobacteria*) that accounted for  $37 \pm 34\%$  of the reads at 7 dpf. *Rhodanobacter* spp. are  
 452 typically known for their potential for partial or complete denitrification (Kostka et al., 2012)  
 453 and may have been derived from the fish tank water filtration system. The other dominant OTU  
 454 at 7dpf was closely related to *Propionibacterium acnes* (*Actinobacteria*) and this OTU remained  
 455 traceable at 14 and 21 dpf albeit at lower relative abundance. A large number of new OTUs was  
 456 found at 14 dpf of which many were lost again at 21 dpf. However, some, such as a number of  
 457 OTUs belonging to the family *Rhodobacteraceae*, appeared at 14 dpf and remained.  
 458 *Rhodobacteraceae* are commonly found in the aquatic habitat, but are not typical gut-associated  
 459 bacteria (Elifantz et al., 2013). Despite the large fluctuations of zebrafish-associated microbiota  
 460 over time, it is apparent that the inter-individual variation within time points decreases (Fig. 2,  
 461 Fig. S2), which indicates that a more stable and homogenous microbiota becomes associated  
 462 with the zebrafish population at 21 dpf. In addition, the dominant OTU at 21 dpf, a *Pseudomonas*  
 463 sp., is in line with previous studies in zebrafish (Bates et al., 2006; Roeselers et al., 2011;  
 464 Semanova et al., 2012; Lan and Love, 2012), which could indicate gut maturation.  
 465

466 The OTUs that were present at a higher relative abundance in fish fed with the experimental  
467 diet E as compared to animals fed with the control diet B and contributed most to the difference  
468 between gut microbiota for the different diets can roughly be divided into three groups based on  
469 comparison to their near neighbours: (1) potential pathogens, (2) bacteria with an increased iron  
470 metabolism, and (3) common aquatic bacteria. From the first group, *Plesiomonas shigelloides*  
471 (OTU45) contributed most to the difference in microbiota between diet E and diet B. It is an  
472 emerging pathogen that is widespread in the aquatic environment and has been related to  
473 gastrointestinal infections and other diseases in a wide range of animal hosts including fish  
474 (Chen et al., 2013; Joh et al., 2013). Also the family *Aeromonadaceae* (to which OTU1407  
475 belongs) harbours many fish pathogens that are associated to gastroenteritis and wound  
476 infections (Tomás, 2012). The genus *Trabulsiella* was proposed in 1991 (McWhorter et al.,  
477 1991) as a genus that is highly related to pathogenic *Salmonella* sp. Currently two species  
478 belonging to this genus have been described and were isolated from human and termite gut.  
479 Although *T. guamensis* can occur in human diarrheal stools, there still is no evidence that it  
480 actually causes diarrhea (McWhorter et al., 1991; Chou et al., 2007). The order *Legionellales*  
481 (OTU 552) comprises the families *Legionellaceae* and *Coxiellaceae* that are both known to  
482 represent common animal pathogens (Garrity et al., 2005). The genus *Burkholderia* (OTU16)  
483 represents both pathogenic (animals and plants) and non-pathogenic species (Estrada-de los  
484 Santos et al., 2013). *Propionibacterium acnes* (OTU832) and *Cupriavidus* (OTU1260) spp. are  
485 commensal inhabitants of the skin and GIT of animals, but are also related to infections,  
486 especially in immuno-compromised individuals (Perry and Lambert, 2006; Balada-Llasat et al.,  
487 2010). In addition, some *Cupriavidus* spp., such as *C. gilardii* and *C. metallidurans* are  
488 particularly resistant to high metal concentrations (Kirsten et al., 2011). The *Halomonas* sp.  
489 (OTU1182) that was markedly increased in zebrafish fed at high NPM inclusion level shared 96  
490 to 98% percent identity with *Halomonas titanicae* strains SSA831, SSA728 and SSA637 based  
491 on the 16S rRNA gene. *Halomonas titanicae* was isolated from corroded parts of the RMS  
492 Titanic wreck and possesses an unusual high number of iron reductases, iron uptake regulators,  
493 ferrocyclase, iron transporters, and iron-binding periplasmic protein-encoding genes (Sánchez-  
494 Porro et al., 2013). In addition, also *Novospirillum itersonii* (OTU1459), previously named  
495 *Aquaspirillum itersonii*, is best known for its iron reduction capacities (Dailey and Lascelles,  
496 1977) and may have been selected for by the high iron content of diet E. In future studies, a tank  
497 water control should be included, in this case to be able to confirm that these iron reducers  
498 originated from the tank water.  
499

500 In this study, we have applied mRNAseq in an unbiased approach to investigate the  
501 molecular differentiation of physiological processes in the GIT as indicated by differentially  
502 expressed genes. In other recent studies that we found in literature, only whole-body mRNAseq  
503 was performed at such young stages of developing zebrafish, or microarray studies specifically  
504 on the GIT. Note that because in this study the whole GIT was analyzed, any variation in  
505 physiological processes occurring along the GIT was thereby discarded (Clements et al., 2014).  
506 Although zebrafish belongs to the cyprinid family, a family of fishes that do not possess a  
507 stomach, also stomachless fish show regional differentiation in GIT function (German, 2009)  
508 and microbial communities (Clements et al., 2014). In our study only approximately 1% of the  
509 total number of genes was differentially expressed. Functional classification of genes revealed  
510 that by far the most dominant gene groups represented *ribosome components and activity* and  
511 *transport* that were enriched in their expression in the larvae fed at high inclusion levels vs. those  
512 fed at low inclusion levels of the NPM. These gene groups included 23 ribosomal proteins and  
513 several solute carrier families of sodium, potassium, chloride, dicarboxylate, and aminoacid and  
514 glucose transporters. These data would suggest that the GIT of larvae fed at higher inclusion of  
515 the protein meal is much more active in transmembrane ion transport and protein synthesis,  
516 perhaps for making the machinery to perform this transport.  
517

518 Among the individual genes, we have found 10 uniquely expressed genes for fish fed at low  
 519 inclusion levels and 16 genes for fish fed at high inclusion levels. The 10 uniquely expressed  
 520 genes for fish fed at low inclusion levels were all uncharacterised genes except for one:  
 521 *secretogranin V (7B2 protein)*, a gene required for the production of an active Proprotein  
 522 convertase 2 (PC2) enzyme (also known as prohormone convertase 2 or neuroendocrine  
 523 convertase 2 enzyme) that is responsible for the first step in the maturation of many  
 524 neuroendocrine peptides from their precursors, such as the conversion of proinsulin to insulin  
 525 intermediates (Mbikay et al., 2001; Portela-Gomes et al., 2008). The 16 uniquely expressed  
 526 genes for fish fed at high inclusion levels were all, except for four genes, characterized. Among  
 527 them were *cadherin 16*, *KSP-cadherin*, a calcium-dependent, membrane-associated glycoprotein,  
 528 and *claudin 19*, involved in magnesium transport. These genes also have a clear role in *transport*  
 529 and so has the highest up-regulated expressed gene in fish fed at high inclusion level at fc 768:  
 530 *stanniocalcin 1, like*. Stanniocalcin 1 is involved in calcium homeostasis. It has been found to  
 531 reduce  $\text{Ca}^{2+}$  uptake via the inhibition of epithelial  $\text{Ca}^{2+}$  channel mRNA expression in zebrafish  
 532 embryos (Tseng et al., 2009). As such it would fit well with a role in the GIT, with the dominant  
 533 gene group *transport* and with other strongly up-regulated genes expressing channels and  
 534 transporters (*purinergic receptor P2X, ligand-gated ion channel, 3b* at fc 102; *solute carrier*  
 535 *family 12 (sodium/chloride transporters), member 3* at fc 24; *chloride channel accessory 2* at fc  
 536 4.93; *slc12a10.3 solute carrier family 12 (sodium/potassium/chloride transporters), member*  
 537 *10.3* at fc 3.24; *solute carrier family 16 (monocarboxylic acid transporters), member 9a* at fc  
 538 2.86; *solute carrier family 12, member 10.1* at fc 2.48; *solute carrier family 25, member 38a* at fc  
 539 2.06; *solute carrier family 2 (facilitated glucose transporter), member 13* at fc 2.06). However,  
 540 such high level of difference in expression is often indicative for immune-related genes.  
 541 Stanniocalcin 1 also has an immune-related function. It is an inhibitor of macrophage chemotaxis  
 542 and chemokinesis (Kanellis et al., 2004) and modulates transendothelial migration of leukocytes  
 543 (Chakraborty et al., 2007) in humans. Thus a role in modulating the immune/inflammatory  
 544 response could be expected. There are more signs for an immune response in fish fed at high  
 545 NPM levels given the roles of other up-regulated immune-related genes such as *interlectin 2* at fc  
 546 8.64; *radical S-adenosyl methionine domain containing 2* at fc 6.29 (see also later); *ISG15*  
 547 *ubiquitin-like modifier* at fc 4.99 and *B-cell CLL/lymphoma 6a (zinc finger protein 51)* at fc 2.83.  
 548 Rawls et al. (2004) performed DNA microarray comparisons of gene expression in the digestive  
 549 tracts of 6 dpf zebrafish and revealed that 212 genes were regulated by the microbiota, including  
 550 genes involved in innate immune responses. Thus we cannot rule out that fish fed at high NPM  
 551 levels display an gastrointestinal immune response.

552  
 553 Because the two experimental diets especially differed in the level of iron, in a biased  
 554 approach, we have analyzed the expression profiles of genes functionally involved in iron uptake  
 555 and homeostasis. Twenty seven genes were identified as involved in iron homeostasis but were  
 556 non-differentially expressed at a fold change 0.27 – 1.54 (Table S5). Among them were genes  
 557 encoding for *ferritin*, *transferrin receptors*, *hephaestin*, *ferrochelatase*, an *iron-responsive*  
 558 *element binding protein*, an *iron-regulated transporter* and *ceruloplasmin*; but also *heme*  
 559 *oxygenase*, *heme binding protein*, a *heme transporter*, and finally *hepcidin* and the *interleukin 6*  
 560 *receptor*. In a parallel study we have investigated the effects on adult zebrafish fed with the same  
 561 experimental diets for one month (Palstra et al., unpublished data). In a biased approach,  
 562 quantitative real-time PCR was performed on individual GIT and liver of these fish. Here we did  
 563 find significant differential expression of several of these genes: GIT expression of marker gene  
 564 *hepcidin antimicrobial peptide 1 (hamp1)* was significantly higher, and of *hephaestin-like 1*  
 565 significantly lower in fish fed at high vs. low iron level. Liver expression of marker genes  
 566 *transferrin a* and *hamp1* was significantly higher, and of *ferritin heavy polypeptide 1a*  
 567 significantly lower in fish fed at high vs. low iron level. These expression profiles, supported by  
 568 data on body composition, suggest that in adult fish fed at higher iron level, less dietary iron  
 569 uptake occurs, less iron is released in the circulation, and less iron is taken up and stored in the

570 liver. This suggests a metabolic defense mechanism against iron overload. Indeed, metal  
571 absorption is lower when metal concentrations are elevated (reviewed by Karasov and Douglas,  
572 2013). However, as based on the absence of differential expression of such genes for the larval  
573 zebrafish in this study, we cannot conclude that major changes occur in iron uptake and  
574 homeostasis during the earliest stages of development. Although diets especially differed in iron  
575 content, no data have been collected in this study that show that this difference also leads to a  
576 difference in iron availability to the GIT. The competitive activity of the microbiota may result  
577 in an alteration of the iron availability for the gastrointestinal functions. Some genes that were  
578 differentially expressed may have a relation with iron homeostasis. Among them was *wdr45 like*  
579 that was up-regulated at fold change 1.6 in larvae fed at higher iron level. *Wdr45* is associated  
580 with human brain iron accumulation (Haack et al., 2012). *Radical S-adenosyl methionine domain*  
581 *containing 2 (rsad2)* is an interferon-inducible iron-sulfur cluster-binding antiviral protein that  
582 was up-regulated at fold change 6.29 at higher iron levels. Other genes that may be involved  
583 could be many of the unknown differentially expressed genes belonging to the cluster metal  
584 binding/zinc fingers.

585

586 The developing gastrointestinal microbiota of larval zebrafish showed rapid temporal  
587 changes until a suspected stable and mature state at 21 dpf. At all times, the larval zebrafish GITs  
588 were dominated by one to three bacterial species generally belonging to *Proteobacteria* and  
589 *Firmicutes*. The OTUs that contributed most to the difference between gastrointestinal  
590 microbiota for the different diets represented common aquatic bacteria but also bacteria related  
591 to potential pathogens and bacteria with an increased iron metabolism. As for the gastrointestinal  
592 transcriptome at 21 dpf, the GIT of larvae fed at higher NPM inclusion is more active in  
593 transmembrane ion transport and protein synthesis. Although some indications existed,  
594 transcriptomic analysis did not reveal signs for the occurrence of a major immune/inflammatory  
595 activation and/or iron overload response.

596

597 The gained insights on the impact of the NPM on larval zebrafish GIT microbiology and  
598 physiology are valuable information for fish specifically, and vertebrates in general. Caution is  
599 required with the transfer of knowledge towards commercially produced species in aquaculture,  
600 particularly because many of them are carnivorous. Carnivorous fishes like salmon (Navarette et  
601 al., 2009) and sole (Martin-Antonio et al., 2007; Tapia-Paniagua et al., 2010) possess a  
602 specialized GIT region with an acidic environment, or a stomach, while the omnivorous cyprinid  
603 zebrafish is stomachless. Such interspecific differences in GIT morphology have important  
604 consequences for the GIT microbial composition and physiology (Clements et al., 2014), and  
605 thus for the dietary impact of the NPM.

606

607

## 608 **Acknowledgements**

609

610 The authors wish to thank W. Nusselder, S. Visser and T. Wieggers (Aquatic Research  
611 Facility of Wageningen University, Wageningen, the Netherlands) for feeding the fish during the  
612 experiment and assistance with sampling, M. Naim (Wageningen University, Wageningen, the  
613 Netherlands) for performing the reverse transcription reaction with the RNA samples and  
614 preparing those samples for pyrosequencing and H. Jansen (ZF-screens B.V., Leiden, the  
615 Netherlands) for performing mRNAseq and providing an initial data analysis. The research was  
616 supported by a grant from the Dutch Ministry of Economic Affairs, Agriculture and Innovation  
617 (project “Marine Aquaculture: Customized Nutrition”, KB-14-005-014 to A.P. Palstra).

618

619

## 620 **Author Contributions**

621

622 Conceived and designed the experiments: ER, DS, JK, APP. Performed the experiments: ER,  
623 DS, MtV, MF, GMB. Analyzed the data: ER, DS, APP. Wrote the paper: ER, DS, JK, HS, APP.

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## 626 **Competing Financial Interests statement**

627

628 The authors declare no competing financial interests.

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809 **Figure legends**

810

811 **Fig. 1: Taxonomic distribution of bacterial 16S rRNA gene reads retrieved from zebrafish**  
812 **raised with different diets during the first 21 dpf.** Only phyla that represent more than 1% of  
813 the reads in at least one of the samples are shown and chloroplasts-affiliated reads were removed  
814 prior to analysis. The pie diagrams shown are averages of replicate samples with the number of  
815 samples and total number of reads below each pie chart. Not all replicate samples that were  
816 initially taken resulted in successful DNA extraction due to the small sample size. The step-wise  
817 decrease of *Paramecium* in the diet is indicated below the pie charts, with the percentage of  
818 *Paramecium* in yellow and the percentage of Diet B/E in green.

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820 **Fig. 2: CCA-ordination plot of the zebrafish microbiota.** The red triangles represent the  
821 centroids of the datasets belonging to different time points indicated with the number in red.  
822 Each data point refers to DNA extracted from 10 pooled zebrafish. Sample names are build up as  
823 follows: ZF= zebrafish; dpf (5, 7, 14 or 21); diet (p, pB, pE, B, E); replicate (1, 2).

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825 **Fig. 3: Heatmap of the operational taxonomic units (OTUs) (97% similarity) that**  
826 **represented more than 1% of the reads in at least one of the zebrafish samples.** These OTUs  
827 represented 85-100% of the reads in the different samples. Relative abundance of OTUs is  
828 marked according to the legend in the figure. Samples are organised per day and according to  
829 diet B or E within day 14 and day 21. OTUs were classified up to the phylum (p), class (c), order  
830 (o), family (f), genus (g) or species (s) level. “Bact” refers to the phylum *Bacteroidetes*, “Planct”  
831 refers to the phylum *Planctomycetes*. Sample names are built up as follows: ZF= zebrafish; dpf  
832 (5, 7, 14 or 21); diet (p, pB, pE, B, E); replicate (1, 2); (R) if a sample is derived from RNA.

833 **Tables**834 **Table 1: Experimental diets.**

Amount of novel protein meal (%)	Dietary treatment	
	10 <sup>l</sup>	75 <sup>l</sup>
Code	B	E
<b>Ingredients in %</b>		
Novel protein meal <sup>a</sup>	10.00	75.00
Pea protein <sup>b</sup>	21.88	7.94
Casein <sup>c</sup>	19.42	3.66
Soy Protein Concentrate <sup>d</sup>	15.20	0.00
Fish Oil <sup>e</sup>	10.24	0.00
Diamol <sup>f</sup>	8.33	0.00
Sugar <sup>g</sup>	1.32	0.00
Lime <sup>h</sup>	0.20	0.00
Wheat gluten <sup>i</sup>	5.00	5.00
Binder 1 <sup>j</sup>	2.00	2.00
Binder 2 <sup>k</sup>	2.00	2.00
Salt <sup>l</sup>	2.00	2.00
Binder 3 <sup>m</sup>	1.00	1.00
Mineral and vitamin premix <sup>n</sup>	1.36	1.36
Betaine <sup>o</sup>	0.05	0.05
<b>Calculated (composition)</b>	<b>B</b>	<b>E</b>
DM (g.kg <sup>-1</sup> )	921.7	941.9
ASH (g.kg <sup>-1</sup> )	144.5	157.4
CP (g.kg <sup>-1</sup> )	533.2	533.1
EE (g.kg <sup>-1</sup> )	134.8	134.8
Ca (g.kg <sup>-1</sup> )	2.4	2.3
P (g.kg <sup>-1</sup> )	5.6	6.1
GE	21.4	21.2
CP/GE	25.9	26.2
Iron (mg.kg <sup>-1</sup> )	312	1486
<b>Calculated amino acids (g.kg<sup>-1</sup>)</b>		
Lysine <sup>*</sup>	37.1	34.1
Methionine <sup>*</sup>	10.3	10.3
Cysteine <sup>**</sup>	5.6	5.7
Threonine <sup>*</sup>	21.2	19.7
Tryptophan <sup>*</sup>	6.3	6.1
Isoleucine <sup>*</sup>	26.0	21.8
Arginine <sup>*</sup>	32.9	32.8
Phenylalanine <sup>*</sup>	27.6	22.0
Histidine <sup>*</sup>	14.5	13.4
Leucine <sup>*</sup>	45.2	37.0
Tyrosine <sup>**</sup>	21.6	19.1
Valine <sup>*</sup>	29.7	25.5

Alanine	22.2	34.1		
Asparagine	50.8	44.2		
Glutamate	106.0	78.7		
Glycine	18.9	26.3		
Proline	40.1	38.3		
Serine	27.0	21.0		
* essential, ** conditionally essential				
<b>Analysed (composition)</b>				
Size of feed	<b>200µm</b>	<b>200-300µm</b>	<b>200µm</b>	<b>200-300µm</b>
DM (g.kg <sup>-1</sup> )	960	956	961	948
ASH (g.kg <sup>-1</sup> .dm)	164	159	185	184
CP (g.kg <sup>-1</sup> .dm)	573	586	556	572
EE (g.kg <sup>-1</sup> .dm)	122	120	103	156
GE	20.9	21.0	20.0	21.3
CP/GE	27.4	27.9	27.8	26.9

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<sup>1</sup>Percentage of novel protein meal: Recipes are isoenergetic. Composition of diet B and E are equal in macronutrients, amino acids, calcium and phosphates. The novel protein meal contains 17 percent of fat (ether extract) of which the composition is comparable to that of fish oil as the novel protein meal is made of a marine invertebrate. Calculated omega 3 content of diet B and E were 21.3 and 19.5 g.kg<sup>-1</sup>.dm<sup>-1</sup> respectively. <sup>a</sup> Ingredient is not specified because of confidentiality reasons of ongoing research; <sup>b</sup> Roquette Freres, Lestrem, France; <sup>c</sup> Acid casein 30/60 mesh, Lactalis, Bourgbarré, France; <sup>d</sup> Soycomil R ADM Eurpoort BV, the Netherlands; <sup>e</sup> Coppens International, the Netherlands; <sup>f</sup> Damolin A/S, Hamburg, Germany; <sup>g</sup> Melis Suikerunie, Dinteloord, the Netherlands; <sup>h</sup> Inducal 250, Sibelco/Ankerpoort, Maastricht, the Netherlands; <sup>i</sup> Gluvital 21000, Cargill, Bergen op Zoom, the Netherlands; <sup>j</sup> Binder1, <sup>k</sup> Binder2 and <sup>m</sup> Binder3: Ingredients are not specified because of confidentiality reasons of ongoing research; <sup>l</sup> Animalfeed salt, Kloek zout, the Netherlands, <sup>n</sup> Betafin, Danisco Animal Nutrition Marlborough UK; <sup>†</sup> vitamins (mg or IU kg<sup>-1</sup> diet) include: vitamin A (retinyl acetate), 2.4 mg, 8000 IU; vitamin D3 (cholecalciferol), 0.04 mg, 1700 IU; vitamin K3 (menadione sodium bisulfite), 10 mg; vitamin B1 (thiamine), 8 mg; vitamin B2 (riboflavin), 20 mg; vitamin B6, <sup>2</sup> vitamin B<sub>12</sub> (cyanocobalamin) 0.02mg (pyridoxine hydrochloride), 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; niacin, 70 mg; pantothenic acid, 30 mg, choline, 1500 mg; vitamin C, 500 mg; vitamin E, 300 mg; Minerals (g or mg kg<sup>-1</sup> diet): Mn (manganese oxide), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc oxide), 30 mg; Se (sodium selenite), 0.3 mg; Fe (Iron Sulfate), 60 mg; Calcium carbonate, 2150 mg; Dicalcium phosphate, 5000 mg; Potassium Chloride, 1000mg; Antioxidant BHT (E300-321), 100 mg; Anti-fungal Calcium propionate, 1000 mg. Abbreviations: DM= dry matter; CP= crude protein; EE= ether extract; C= calcium; P= phosphate; GE= gross energy.

860 **Table 2: Bacterial OTUs that contribute most to the difference between diet E and diet B at**  
 861 **day 21.**

#OTU ID	ZF21BR [%]	ZF21ER [%]	ZF21ER-ZF21BR	Av.Diss	Diss/SD	Contrib [%]	Cum [%]	taxon ID
11	0.76	3.24	2.48	4.2	3.69	10.95	10.95	(s) <i>Plesiomonas shigelloides</i>
1214	2.62	2.06	-0.56	2.9	1.33	7.55	18.5	(s) <i>Pseudomonas alcaligenes</i>
286	1.13	2.43	1.3	2.28	1.16	5.93	24.43	(g) <i>Acidovorax</i>
1407	1.66	1.96	0.3	2.22	1.68	5.8	30.23	(f) <i>Aeromonadaceae</i>
1306	8.68	7.96	-0.72	1.52	1.49	3.96	34.19	(g) <i>Pseudomonas</i>
1394	0.99	0.45	-0.54	0.96	1.3	2.49	36.69	(f) <i>Aeromonadaceae</i>
1392	2.22	2.06	-0.16	0.94	1.46	2.45	39.13	(f) <i>Comamonadaceae</i>
1538	0.51	0.37	-0.14	0.88	2.37	2.28	41.41	(g) <i>Vogesella</i>
441	0.05	0.48	0.43	0.74	2.32	1.93	43.34	(g) <i>Trabulsiella</i>
552	0.34	0.42	0.08	0.73	3.68	1.89	45.23	(o) <i>Legionellales</i>
1459	0.21	0.42	0.21	0.56	1.42	1.46	46.69	(s) <i>Novospirillum itersonii</i>
1381	0.29	0.14	-0.15	0.51	1.27	1.32	48.01	(f) <i>Comamonadaceae</i>
1414	0.5	0.58	0.08	0.5	5.57	1.32	49.33	(g) <i>Rheinheimera</i>
370	0.37	0.12	-0.25	0.5	1.27	1.31	50.64	(g) <i>Pseudomonas</i>
832	0.08	0.37	0.29	0.5	1.26	1.3	51.94	(s) <i>Propionibacterium acnes</i>
422	0.27	0	-0.27	0.46	1.75	1.2	53.14	(c) <i>Gammaproteobacteria</i>
463	0	0.26	0.26	0.45	6.56	1.18	54.32	(f) <i>Alcaligenaceae</i>
1086	0.25	0.07	-0.18	0.42	1.09	1.1	55.42	(f) <i>Rhodobacteraceae</i>
8	0	0.24	0.24	0.42	3.77	1.09	56.51	(f) <i>Sphingobacteriaceae</i>
859	0.32	0.14	-0.18	0.4	1.4	1.05	57.56	(o) <i>Aeromonadales</i>
16	0.13	0.36	0.23	0.39	5.99	1.02	58.58	(g) <i>Burkholderia</i>
1182	0.15	0.37	0.22	0.38	4.28	1	59.58	(g) <i>Halomonas</i>
1127	0.31	0.1	-0.21	0.37	1.73	0.96	60.54	(p) <i>Cyanobacteria</i>
576	0.2	0	-0.2	0.34	0.86	0.9	61.43	(f) <i>Comamonadaceae</i>
1278	0	0.2	0.2	0.34	14.24	0.89	62.33	(g) <i>Achromobacter</i>
1167	0.18	0	-0.18	0.32	3.31	0.82	63.15	(f) <i>Pseudomonadaceae</i>
568	0	0.18	0.18	0.31	0.87	0.82	63.97	(g) <i>Janibacter</i>
1117	0.18	0	-0.18	0.31	0.86	0.82	64.79	(p) <i>Proteobacteria</i>
152	0.05	0.18	0.13	0.3	1.13	0.79	65.58	(c) <i>Gammaproteobacteria</i>
1260	0	0.17	0.17	0.3	3.77	0.77	66.35	(g) <i>Cupriavidus</i>
1016	0.05	0.21	0.16	0.29	1.53	0.75	67.1	(f) <i>Halomonadaceae</i>
1015	0.17	0	-0.17	0.28	21.34	0.74	67.84	(f) <i>Comamonadaceae</i>
1133	0.17	0	-0.17	0.28	21.34	0.74	68.57	(f) <i>Pseudomonadaceae</i>
414	0.07	0.17	0.1	0.28	1.23	0.74	69.31	(c) <i>Gammaproteobacteria</i>
288	0.16	0.07	-0.09	0.27	1.17	0.72	70.03	(f) <i>Pseudomonadaceae</i>

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Only OTUs that cumulatively contribute to 50% of the difference between bacterial profiles between diet B and E are shown based on SIMPER analysis. Average relative abundances of square root-transformed data for diet B and E is indicated in the columns ZF21B and ZF21E, respectively. “Av.Diss.” indicates the average dissimilarity between the diets for each OTU; “Diss/SD” indicates the dissimilarity divided by the standard deviation; “Contrib” is the relative contribution to the difference between diet B and E; and “Cum” represents the cumulative relative contribution to the difference starting from the top with the OTUs that contribute most to the difference. OTUs in grey refer to OTUs that were more abundant for diet E. OTUs were classified up to the phylum (p), class (c), order (o), family (f), genus (g) or species (s) level.

872 **Table 3: Functional gene groups and their differentially expressed genes.**  
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<b>Gene Group 1</b>		<i>ribosome components and activity</i>	
<b>Enrichment Score: 14.61</b>			
<b>ENSEMBL_GENE_ID</b>	<b>Gene Name</b>	<b>fc</b>	<b>pval</b>
ENSDARG00000034291	ribosomal protein L37; hypothetical LOC100000999	1.85	0.002
ENSDARG00000043509	ribosomal protein L11	1.80	0.003
ENSDARG00000051783	ribosomal protein, large, P0	1.70	0.006
ENSDARG00000077291	ribosomal protein S2	1.69	0.006
ENSDARG00000030602	ribosomal protein S19	1.67	0.008
ENSDARG00000009285	ribosomal protein L15	1.64	0.011
ENSDARG00000034897	ribosomal protein S10	1.64	0.012
ENSDARG00000035871	ribosomal protein L30	1.62	0.013
ENSDARG00000042389	zgc:171772	1.58	0.019
ENSDARG00000036875	ribosomal protein S12	1.58	0.018
ENSDARG00000070849	ribosomal protein S15	1.57	0.021
ENSDARG00000046119	ribosomal protein S3	1.55	0.023
ENSDARG00000030408	ribosomal protein S26, like	1.54	0.027
ENSDARG00000037071	ribosomal protein S26	1.54	0.028
ENSDARG00000057556	zgc:65996	1.53	0.028
ENSDARG00000053058	ribosomal protein S11	1.51	0.033
ENSDARG00000035692	ribosomal protein S3A	1.51	0.034
ENSDARG00000055475	zgc:73262	1.50	0.043
ENSDARG00000020197	ribosomal protein L5a	1.50	0.037
ENSDARG00000011201	ribosomal protein, large P2, like	1.50	0.044
ENSDARG00000053457	similar to ribosomal protein L23; ribosomal protein L23	1.49	0.043
ENSDARG00000037350	similar to ribosomal protein L9; ribosomal protein L9	1.49	0.040
ENSDARG00000041435	ubiquitin A-52 residue ribosomal protein fusion product 1	1.49	0.043
ENSDARG00000023298	zgc:109888	1.49	0.042
ENSDARG00000014867	ribosomal protein L8	1.48	0.041
ENSDARG00000015490	ribosomal protein L24	1.48	0.050
ENSDARG00000025073	ribosomal protein L18a	1.47	0.044
ENSDARG00000013012	ribosomal protein L36	1.47	0.045
<b>Gene Group 2</b>		<i>transport</i>	
<b>Enrichment Score: 1.31</b>			
<b>ENSEMBL_GENE_ID</b>	<b>Gene Name</b>	<b>fc</b>	<b>pval</b>
ENSDARG00000013855	solute carrier family 12 (sodium/chloride transporters), member 3	24.20	0.000
ENSDARG00000055253	similar to Solute carrier family 12 member 3 (Thiazide-sensitive sodium-chloride cotransporter)	3.24	0.020
ENSDARG00000013743	(Na-Cl symporter); slc12a10.3 solute carrier family 12 (sodium/potassium/chloride transporters), member 10.3 solute carrier family 12	2.48	0.033
ENSDARG00000053853	(sodium/potassium/chloride transporters), member 10.1; solute carrier family 12, member 2-like solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2	1.56	0.024
<b>Gene Group 3</b>		<i>WD40 repeats acting as protein-protein interaction sites</i>	
<b>Enrichment Score: 0.22</b>			
<b>ENSEMBL_GENE_ID</b>	<b>Gene Name</b>	<b>fc</b>	<b>pval</b>
ENSDARG00000075883	BUB3 budding uninhibited by benzimidazoles 3 homolog (yeast)	1.97	0.027
ENSDARG00000045019	zgc:85939	1.63	0.026
ENSDARG00000021557	wdr45 like	1.60	0.026
ENSDARG00000041619	guanine nucleotide binding protein (G protein), beta polypeptide 2- like 1	1.48	0.043

<b>Gene Group 4</b>		<i>nucleotide binding</i>		
<b>Enrichment Score: 0.09</b>				
<b>ENSEMBL_GENE_ID</b>	<b>Gene Name</b>	<b>fc</b>	<b>pval</b>	
ENSDARG00000055385	zgc:110560; hypothetical protein LOC100150958	Inf	0.021	
ENSDARG00000074873	similar to Serine/threonine-protein kinase Pim-3; si:dkey-108d22.5	Inf	0.001	
ENSDARG00000052900	zgc:153642	7.94	0.000	
ENSDARG00000002210	zgc:92836	2.29	0.005	
ENSDARG00000041411	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	2.05	0.040	
ENSDARG00000002344	zgc:55461; zgc:123194; zgc:153264; zgc:123292; tubulin, beta 2c; zgc:153426	1.61	0.019	
ENSDARG00000031164	tubulin, alpha 8 like 2	1.49	0.040	
ENSDARG00000040984	heat shock protein 13	0.56	0.038	
ENSDARG00000056443	zgc:152753	0.41	0.038	
ENSDARG00000015134	similar to calcium/calmodulin-dependent protein kinase kinase 1, alpha; zgc:194737	0.27	0.049	
ENSDARG00000035178	guanine nucleotide binding protein (G protein), alpha 14	0.19	0.035	
ENSDARG00000014373	vasa homolog	0.18	0.022	
ENSDARG00000030644	guanine nucleotide binding protein (G protein), alpha inhibiting activity	0.07	0.049	
ENSDARG00000078093	polypeptide 3 zgc:172065; hypothetical LOC100001153	0.00	0.012	
<b>Gene Group 5</b>		<i>transcription</i>		
<b>Enrichment Score: 0.02</b>				
<b>ENSEMBL_GENE_ID</b>	<b>Gene Name</b>	<b>fc</b>	<b>pval</b>	
ENSDARG00000070769	forkhead box G1	Inf	0.042	
ENSDARG00000069337	Mediator of RNA polymerase II transcription subunit 11	Inf	0.000	
ENSDARG00000079406	homeo box C11a; homeo box C11b	14.81	0.041	
ENSDARG00000058133	forkhead box D1	2.22	0.024	
ENSDARG00000043531	v-jun sarcoma virus 17 oncogene homolog (avian)	1.81	0.033	
ENSDARG00000040253	one cut domain, family member, like	0.48	0.024	
ENSDARG00000056407	interferon regulatory factor 8	0.35	0.034	
<b>Gene Group 6</b>		<i>metal binding/zinc fingers</i>		
<b>Enrichment Score: 0.02</b>				
<b>ENSEMBL_GENE_ID</b>	<b>Gene Name</b>	<b>fc</b>	<b>pval</b>	
ENSDARG00000056907	zgc:173949; hypothetical protein LOC100006493; similar to retinoblastoma-binding protein 6	26.61	0.026	
ENSDARG00000070864	B-cell CLL/lymphoma 6 (zinc finger protein 51)	2.83	0.005	
ENSDARG00000021677	similar to PHD finger protein 6	2.37	0.016	
ENSDARG00000013279	zgc:153635	2.02	0.023	
ENSDARG00000028476	zgc:65779; hypothetical LOC791614	1.90	0.003	
ENSDARG00000001897	zgc:110815	1.76	0.032	
ENSDARG00000043323	ligand of numb-protein X 1	1.73	0.041	
ENSDARG00000071558	zgc:154176	1.71	0.012	
ENSDARG00000008218	zgc:77303	1.69	0.029	
ENSDARG00000038006	odd-skipped related 2 (Drosophila)	1.67	0.048	
ENSDARG00000014794	zgc:92453	1.52	0.045	
ENSDARG00000055475	zgc:73262	1.50	0.043	
ENSDARG00000023298	zgc:109888	1.49	0.042	
<b>Gene Group 7</b>		<i>membrane</i>		
<b>Enrichment Score: 0.006</b>				
<b>ENSEMBL_GENE_ID</b>	<b>Gene Name</b>	<b>fc</b>	<b>pval</b>	
ENSDARG00000044990	zgc:65811	2.54	0.000	

ENSDARG00000076899	solute carrier family 2 (facilitated glucose transporter), member 13a	2.06	0.024
ENSDARG00000055307	synaptophysin-like 2a	0.63	0.043
ENSDARG00000019137	translocating chain-associating membrane protein 1	0.62	0.027
ENSDARG00000059824	hypothetical LOC564868; zgc:153102	0.44	0.019
ENSDARG00000027065	zgc:165543; similar to Sodium-coupled neutral amino acid transporter 3 (Na(+)-coupled neutral amino acid transporter 3) (System N amino acid transporter 1) (N-system amino acid transporter 1) (Solute carrier family 38 member 3)	0.34	0.008

874

875 Shown are each of the functional gene groups, enrichment score and name; the Ensemble gene  
 876 ID and gene name; fold change and P-values for differentially expressed genes ( $P \leq 0.05$ ).



Figure 1.TIF

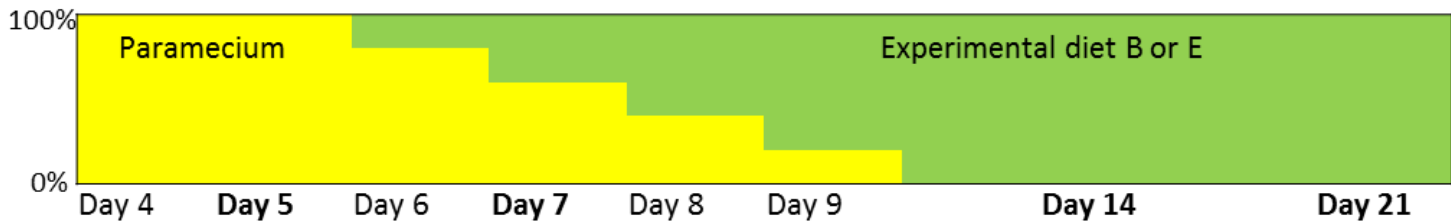
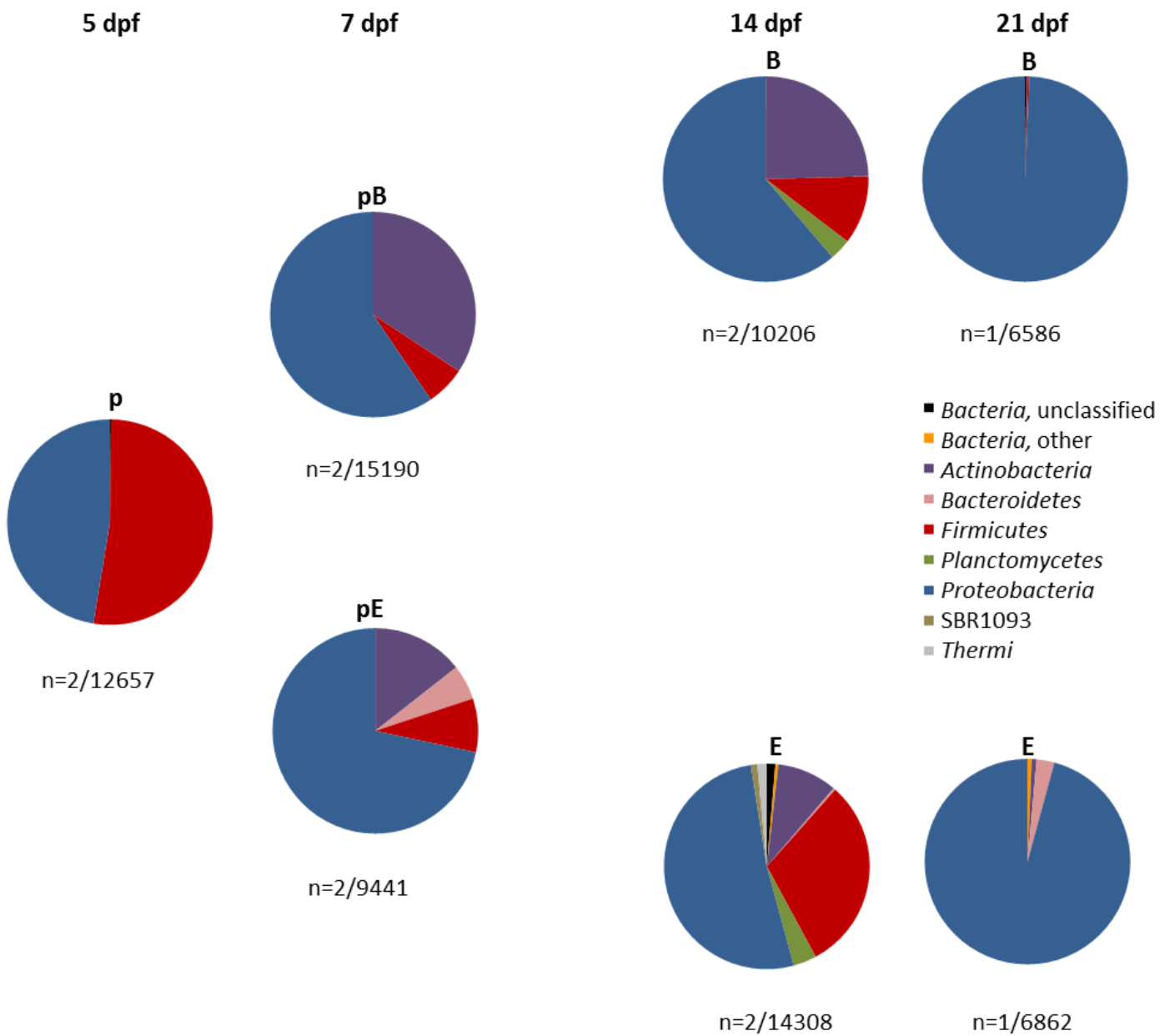


Figure 2.TIF

