Dietary supplementation with probiotics regulates gut microbiota structure and function in Nile tilapia exposed to aluminum

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Backgrounds and aims: Aluminum contamination of water is becoming increasingly serious and threatens the health status of fish. *Lactobacillus plantarum* CCFM639 was previously shown to be a potential probiotic for alleviation aluminum toxicity in Nile tilapia. Considering the significant role of the gut microbiota on fish health, it seems appropriate to explore the relationships among aluminum exposure, probiotic supplementation, and the gut microbiota in Nile tilapia and to determine whether regulation of the gut microbiota is related to alleviation of aluminum toxicity by a probiotic in Nile tilapia. Methods and results: The tilapia were assigned into four groups, control, CCFM639 only, aluminum only, and aluminum + CCFM639 groups for an experimental period of 4 weeks. The tilapia in the aluminum only group were grown in water with an aluminum ion concentration of 2.73 mg/L. The final concentration of CCFM639 in the diet was 10^8 CFU/g. The results show that environmental aluminum exposure reduced the numbers of *L. plantarum* in tilapia feces and altered the gut microbiota. As the predominant bacterial phyla in the gut, the abundances of Bacteroidetes and Proteobacteria in aluminum-exposed fish were significantly elevated and lowered, respectively. At the genus level, fish exposed to aluminum had a significantly lower abundance of *Deefgea*, *Plesiomonas,* and *Pseudomonas* and a greater abundance of *Flavobacterium*, *Enterovibrio*, *Porphyromonadaceae_uncultured*, and *Comamonadaceae*. When tilapia were exposed to aluminum, the administration of a probiotic promoted aluminum excretion through the feces and led to a decrease in the abundance of *Comamonadaceae, Enterovibrio* and *Porphyromonadaceae*. Notably, supplementation with a probiotic only greatly decreased

the abundance of *Aeromonas* and *Pseudomonas*. Conclusion: Aluminum exposure altered the diversity of the gut microbiota in Nile tilapia, and probiotic supplementation allowed the recovery of some of the diversity. Therefore, regulation of gut microbiota with a probiotic is a possible mechanism for the alleviation of aluminum toxicity in Nile tilapia.

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Abstract

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Methods and results: The tilapia were assigned into four groups, control, CCFM639 only, aluminum only, and aluminum + CCFM639 groups for an experimental period of 4 weeks. The tilapia in the aluminum only group were grown in water with an aluminum ion concentration of 2.73 mg/L. The final concentration of CCFM639 in the diet was 10^8 CFU/g. The results show that environmental aluminum exposure reduced the numbers of *L. plantarum* in tilapia feces and altered the gut microbiota. As the predominant bacterial phyla in the gut, the abundances of Bacteroidetes and Proteobacteria in aluminum-exposed fish were significantly elevated and lowered, respectively. At the genus level, fish exposed to aluminum had a significantly lower abundance of *Deefgea*, *Plesiomonas,* and *Pseudomonas* and a greater abundance of *Flavobacterium*, *Enterovibrio*, *Porphyromonadaceae_uncultured*, and *Comamonadaceae*. When tilapia were exposed to aluminum, the administration of a probiotic promoted aluminum excretion through the feces and led to a decrease in the abundance of *Comamonadaceae, Enterovibrio* and *Porphyromonadaceae*. Notably, supplementation with a probiotic only greatly decreased the abundance of *Aeromonas* and *Pseudomonas*.

Conclusion: Aluminum exposure altered the diversity of the gut microbiota in Nile tilapia, and probiotic supplementation allowed the recovery of some of the diversity. Therefore, regulation of gut microbiota with a probiotic is a possible mechanism for the alleviation of aluminum toxicity in Nile tilapia.

1. Introduction

Aluminum, the third commonest chemical element and the most abundant metal on earth, is ubiquitous in the environment. In recent years, environmental aluminum levels have increased due to diverse anthropogenic activities such as water treatment, eutrophic lakes control, mining operations, and industrial landfill (Fernandez-Davila et al. 2012; Garcia-Medina et al. 2011).The aluminum level reaches 5.7 mg/L in certain rivers and lakes in England, the United States, China, and Brazil (Camargo et al. 2009; da Cruz et al. 2015), research has been shown that concentration of aluminum ranging from 0.1 to 0.2 mg/L can be harmful to fish (Baker & Schofield 1982; Wang et al. 2013). Excessive aluminum can accumulate in multiple fish tissues and organs and exert adverse effects on the blood circulation and on endocrine, metabolic and reproductive function (Azmat et al. 2012). Excess aluminum ions in water were reported to cause mortalities and decreasing population of *Atlantic salmon* in Norway, southeast Canada, and the northeastern United States (Monette & McCormick 2008). Aluminum contamination causes economic losses in aquaculture and poses potential human health risks from consumption of aquatic products.

Microorganisms produce a variety of metabolites that can have remarkable effects on the external environment and on the host, including changes in pH, suppression of inflammation, and detoxification (Berdy 2005; Louis et al. 2014). Hence, the gut microbiota can significantly alter the host's physiology and its metabolism of nutrients and exogenous toxic substances, and it can shape the microbiome and immune systems (Ni et al. 2014). They may be important mediators of the bioavailability and toxicity of toxic metals. Indeed, long-term toxic metal exposure, including aluminum, lead and chromium, altered the composition of intestinal microbiota (Wu et al. 2017; Zhai et al. 2017).

Lactic acid bacteria probiotics, which are generally derived from humans or food products, are generally recognized as safe (GRAS) strains (Farnworth 2008) and have been widely applied in various situations, including aquaculture, to improve food safety (Sihag & Sharma 2012). Some probiotics have been used in the culture of some aquatic organisms to promote growth and control infectious disease. They can improve the host's intestinal microflora balance and increase the protective effect against pathogenic bacteria (Lahti et al. 2013; Pirarat et al. 2015). For example, *L. johnsonii* La1*, L. rhamnosus* LC705*, B. lactis* Bb12*, L. casei* Shirota and others can effectively control infection with *Vibrio anguillarum*, *Flavobacterium psychrophilum* and *Aeromonas salmonicida,* to prevent furunculosis in rainbow trout (Nikoskelainen et al. 2001), and *L. rhamnosus* GG can control infection of tilapia by *Edwardsiella tarda* and *Streptococcus agalactiae* (Pirarat et al. 2006; Pirarat et al. 2015).

83   Our previous study showed that supplementation with probiotic *L. plantarum* CCFM639, a
84   strain with superb aluminum binding and tolerance abilities, decreased the aluminum level in
85   tissues and alleviated aluminum toxicity by preventing oxidative stress and histopathological
86   changes in tilapia (Yu et al. 2017). However, it remains unclear whether the mechanisms of
87   recovery and alleviation are related to the gut microbiota. Therefore, we investigated alterations
88   in the composition and structure of the intestinal microbiota in tilapia after aluminum exposure
89   and the addition *L. plantarum* CCFM639 to their daily feed.

90   Tilapia is one of the most important aquatic species in aquaculture worldwide and is farmed
91   in more than 120 countries and territories (Junning et al. 2018). In 2015, its production accounted
92   for more than 10% of all farmed fish worldwide (FAO 2015). Moreover, tilapia are recognized
93   as a good biological model because they are easy to handle, culture, and maintain in the
94   laboratory (Korkmaz et al. 2009), and because they display excellent stress sensitivity (Zheng et
95   al. 2016). The aim of the study was to explore whether the regulation of gut microbiota is an
96   aluminum toxicity alleviation mechanism exerted by probiotics in tilapia.

97   **2. Materials and methods**

98   *2.1. Probiotic and fish diet preparation*

99   *L. plantarum* CCFM639, kindly provided by the in-house Culture Collections of Food
100 Microbiology (CCFM), Jiangnan University (Wuxi, China), was inoculated in MRS broth
101 (Qingdao Hopebio, China) and in a static condition at 37 °C for 18 h. After centrifugation at
102 8000 g at 4°C for 5 min, the medium was removed and the cell pellets were suspended with
103 sterile normal saline solution(0.85%) to one-hundredth of the original medium volume. The
104 pellets were mixed with the fish basal diet using a sterile spreader to distribute the bacterial cells
105 evenly and to achieve a final probiotic concentration of 10^8 CFU/g in the feed. The formula and
106 nutrient levels of the basal fish diet were consistent with those in previous studies (Yu et al.
107 2017). The dose of the *L. plantarum* strain was selected on the basis of previous reports (Heo et
108 al. 2013; Ridha & Azad 2012; Ridha & Azad 2016). The bacterial concentration in the fish diet
109 was also confirmed by colony counting. The bacteria-containing feed was prepared weekly and
110 stored at 4°C before use.

111 *2.2. Experimental design*

112   One hundred ninety-two male tilapia were purchased from the Freshwater Fisheries
113 Research Centre of the Chinese Academy of Fishery Sciences in Wuxi and stocked in a
114 cylindrical aquarium (0.6 m^2 × 0.85 m) at a fish loading ratio of 1.09 g/L for 3 weeks. The
115 average (± SEM) weight of the tilapia was 34.01 ± 0.19 g. The amount of feed consumed each
116 day was 3% of the average body weight of the fish. The fish were fed manually at 9 am and 5 pm

117 each day. After a 3-week adaptation period, the fish were fasted for 1 day. The four groups are
118 listed in Table 1, including the control group, the probiotic group (639 only), the aluminum
119 exposure group (Al only) and the probiotic intervention group (Al + 639) randomly. The fish in
120 each group were randomly assigned to three tanks with 16 tilapias in each tank. In the aluminum
121 exposure group, the tilapia were grown in water with an aluminum ion concentration
122 ($AlCl_3 \cdot 6H_2O$) of 2.73 mg/L. The selection of this dose was based mainly on the aluminum
123 exposure doses reported in drinking water, rivers and lakes in previous studies (Yu et al. 2017).
124 The test period was 4 weeks, the freshwater was changed and the aluminum level in the water
125 was checked every 2 days. Inductively coupled plasma mass spectrometry (ICP-MS; NexIon-
126 300X, PerkinElmer) was used to determine the amount of aluminum in the water.

127 To ensure that fecal samples were not affected by the high level of waterborne aluminum,
128 they were collected quickly after the water was changed to normal water (without aluminum).
129 After collection of the fecal samples, the aluminum ions were added into the water. At the end of
130 the assay, the tilapia were sacrificed under anesthesia after 24 h of fasting. The entire intestinal
131 tract was removed under aseptic conditions and 0.2 g of intestinal contents was squeezed and
132 collected in sterile tubes for analysis of the intestinal microbiota.

133 The animal experiments was approved by the Ethics Committee of Jiangnan Universi
134 ty, China (JN No. 20151027-1129-3), and all procedures about the care and use of exper
135 imental animals followed the guidelines set by the European Community (directive 2010/
136 63/EU).

137 *2.3. Determination of fecal aluminum levels*

138 One gram sample of feces was transferred to a microwave digestion tank (OMNI; CEM,
139 UK) with 70% concentrated nitric acid. The microwave digestion system (MARS; CEM, UK)
140 was used. The heating procedure of digestion included three stages: stage 1—power 2000 W,
141 ramp 3:00, temperature 120°C, hold 3:00; stage 2—power 2000 W, ramp 3:00, temperature
142 150°C, hold 10:00; stage 3—power 2000 W, ramp 5:00, temperature 190°C, hold 16:00. After
143 the temperature fell below 50°C, the samples were removed and diluted to 50 mL with deionized
144 water. ICP-MS (NexIon-300X, PerkinElmer) was used to determine the amount of aluminum in
145 the samples (Ciavardelli et al. 2012).

146 *2.4. RT-qPCR analyses for mRNA expression of L. plantarum in feces*

147 The 0.2 g of fecal samples were collected to extract the total genomic DNA following the
148 instructions of the FastDNA Spin Kit (MP Biomedicals, California,). The fecal genomic DNA
149 was used as a template, and real-time quantitative polymerase chain reaction (RT-qPCR; CFX96,

150 Bio-Rad) was carried out using the specific primer. The primer sequences (5′-3′) referred to

151 previous study (Wang 2018) and as follows, LP-F   GGAGCCGCTATTAGTATTTTCAT   and

152 LP-R   AATACAAGCAAGTCTT185GGACCAG. The Ct value of the fecal sample

153 fluorescence quantification was brought into the corresponding standard curve to calculate the

154 copy number, which was converted into the amount per gram of feces. The standard curve of

155 RT-qPCR was as follows: Ct=-3.1434*lg (copies) + 36.977 (R^2=0.9913) (Wang 2018).

156 *2.5. Analysis of gut microbiota*

157 DNA was extracted from 0.2 g of the contents of the whole intestinal tract using an EZNA

158 DNA Kit (Omega Bio-tek, Georgia, USA) and stored at -20°C. PCR amplification of the 16S

159 rRNA gene was conducted using the forward primer 515F (5′-barcode-

160 GTGCCAGCMGCCGCGG-3′) and the reverse primer 907R (5′-

161 CCGTCAATTCMTTTRAGTTT-3′) (Zhai et al. 2016). Different samples were distinguished

162 with an 8-base barcode and sequenced using a miseq sequencer (Illumina, Inc., California, USA;

163 illumina miseq PE250). The raw data were quality-filtered and aligned using Trimmomatic and

164 FLASH. The operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff

165 using UPARSE (http://drive5.com/uparse/). The chimeric sequences were identified and

166 removed using UCHIME (http://www.drive5.com/uchime/). The OTU germline type was

167 identified by the RDP classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU115) 16S

168 rRNA database using a confidence threshold of 70% (Gajardo et al. 2016; Huyben et al. 2018).

169 *2.6. Data analysis*

170 The experimental results were analyzed and tested using analysis of variance and non-

171 parametric tests. The alpha diversity (Chao and Shannon indices) and the beta diversity

172 (PERMANOVA) of the microbiome were calculated based on the OTU level. Tukey's post hoc

173 test of one-way analysis of variance (ANOVA) were performed in aluminum and *L. plantarum*

174 levels of feces. A P value of less than 0.05 was used as a cutoff to indicate a statistically

175 significant difference. The results were plotted using Origin 8.6 software (Originlab,

176 Massachusetts, USA).

177 **3. Results**

178 *3.1. Aluminum level in feces*

179 Table 2 (DatasetS1) shows the weekly changes in the fecal aluminum content in tilapia. The

180 fecal aluminum content was very low in the control and CCFM639 only groups, and was

181 markedly elevated after aluminum exposure (P <0.05). Moreover, the aluminum level in feces

182   also increased as the duration of aluminum exposure increased. CCFM639 treatment promoted
183   the elimination of aluminum in the feces. At the fourth week, the fecal aluminum level in the
184   aluminum only group was 25.67 mg/kg. With the continuous administration of CCFM639, the
185   fecal aluminum levels were significantly greater than those in the Al-only group at each test
186   point (P<0.05), up to 35.46 mg/kg at the third week.

187   *3.2. Quantification of Lactobacillus in feces*

188   Table 3 (DatasetS2) shows that the amount of *L. plantarum* in tilapia feces in the CCFM639-
189   only group was higher than control group by two orders of magnitude. By week 4, the content of
190   *L. plantarum* in tilapia feces had been significantly decreased by aluminum exposure, from $10^{5.4}$
191   copies per gram of feces to $10^{5.0}$ copies per gram of feces (P<0.05), whereas the addition of
192   CCFM639 led to a significant increase in the *L. plantarum* content in feces to that in the   639-
193   only group.

194   *3.3. Intestinal microbial diversity and composition*

195   The numbers of OUTs of each group were 147.3, 172.0, 243.7, 206.3, respectively.
196   Concerning alpha diversity microbiome analysis, Shannon and Chao1 indices were found higher
197   in the 639 only and the Al+639 treatment groups compared to the control (Figure 1, DatasetS3).
198   As shown in Figure 2A (DatasetS3), Principal Coordinate Analysis based on Unweighted unifrac
199   distance indicated a overall significant clustering on the fecal microbiota composition (P=0.002),
200   in which PC1 explained 65.75% of the difference. Moreover, the results of PC1 analysis show
201   that aluminum treatment had the greatest effect on the composition of the gut microbiota in
202   tilapia (Figure 2B, DatasetS3, P<0.05).

203   Aluminum exposure changed the composition of the gut microbiota in Nile tilapia. The four
204   predominant bacterial phyla Fusobacteria, Proteobacteria, Bacteroidetes, and Firmicutes,
205   accounted for 53.74%, 38.21%, 7.54%, and 0.38%, respectively, in control group (Figure 3,
206   DatasetS4). Fish exposed to aluminum had a significantly greater abundance of Bacteroidetes
207   and fewer Firmicutes, whereas administration of CCFM639 had the opposite effect. Interestingly,
208   CCFM639 administration led to an increase in the abundance of Fusobacteria and Firmicutes and
209   a decrease in the abundance of Proteobacteria.

210   As shown in Figure 4 (DatasetS5), 17 dominant genera were detected, the five most
211   dominant were *Cetobacterium, Deefgea, Plesiomonas, Flavobacterium* and *Cytophagales*.
212   Aluminum exposure significantly reduced the abundance of *Plesiomonas*, *Deefgea,* and
213   *Pseudomonas* and drastically increased the abundance of *Flavobacterium*, *Enterovibrio*, and the

214 families *Porphyromonadaceae* and *Comamonadaceae* (Figure 5 and Table 4, DatasetS5; P<0.05).
215 In the tilapia exposed to aluminum, the administration of *L. plantarum* CCFM639 further led to a
216 decrease in the abundance of *Enterovibrio, Comamonadaceae* and *Porphyromonadaceae*
217 (P<0.05). Unlike the results in the control group, supplementation with *L. plantarum* CCFM639
218 greatly reduced the abundance of *Aeromonas* and *Pseudomonas* (P<0.05).

219 **4. Discussion**

220 Aquatic animals, especially fish, have direct contact with the water environment, which
221 contains various pollutants and ever-changing microbiota (Egerton et al. 2018). An excessive
222 aluminum concentration in the aquatic ecosystem can be harmful to the physiological functions
223 of fish, and even threaten their survival (Egerton et al. 2018). In addition, the gut microbiota,
224 which has a close association with fish health, can affect its metabolism, physiology, and
225 immune function, and great inter-individual microbial diversity exists (Sullam et al. 2012). In
226 this study, we adopted a culture-independent technology, next-generation sequencing, which is
227 an up-to-date analytical method that can be widely used for analysis of host microbiota,
228 including that of fish. More specifically, next-generation sequencing can be used to provide
229 detailed information of low abundance microbiota can be provided and the genetic potential of
230 species can even be predicted (Ghanbari et al. 2015). Our results show that the dominant four
231 phyla in Nile tilapia were Fusobacteria, Proteobacteria, Bacteroidetes, and Firmicutes.
232 Proteobacteria, Bacteroidetes, and Firmicutes accounted for 90% of the gut microbiota in a
233 previous study of the teleost fishes (Giatsis et al. 2015; Liu et al. 2013; Ran et al. 2015). The
234 intestinal microbiota of fish species including tilapia is characterized by various levels of
235 Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria and Firmicutes (Adeoye et al. 2016;
236 Wu et al. 2013). At the genus level, the relative abundance of dominant *Cetobacterium* (affiliated
237 with *Fusobacteria*) and *Plesiomonas* were also consistent with the results of a previous study in
238 zebrafish (Ma et al. 2018).

239 Despite various studies focusing on aluminum exposure and its effects on various host
240 organs, including the liver, kidneys, and brain (Park et al. 2015), the link between aluminum
241 exposure and the host intestinal microbiota remains unclear. The fish gut microbiota is
242 commonly treated as an organ with a significant role in essential physiological functions and
243 overall health. Consistent with our hypothesis, the fecal aluminum level of tilapia increased
244 significantly when the fish were exposed to aluminum, and CCFM639 supplementation
245 promoted aluminum excretion in feces as its excellent aluminum binding ability (Table 1, Figure
246 6). These results are consistent with our previous study in a mouse model (Yu et al. 2016). In
247 addition, the amount of *L. plantarum* in the tilapia feces increased markedly after the fish

248 ingested feed mixed with a probiotic (Table 2). It is notable that aluminum exposure reduced the
249 numbers of *L. plantarum* in tilapia feces at week 4 (Table 3), possibly due to a decrease in
250 aluminum-intolerant *L. plantarum* in the gut caused by aluminum exposure.

251 Environmental exposure to aluminum altered the structure and relative abundance of the
252 intestinal microbiota of Nile tilapia, resulting in a significant decrease in the relative abundance
253 of the phyla Firmicutes and Proteobacteria and the genera *Deefgea*, *Plesiomonas* and
254 *Pseudomonas* and elevated the relative abundance of the phylum Bacteroidetes, and the genera
255 *Comamonadaceae* and *Porphyromonadaceae*, *Flavobacterium* and *Enterovibrio* (P<0.05;
256 Figures 3, 4, and 5). The alpha diversity results show no significant difference between the
257 control and 639 groups, and between the Al only and Al + 639 group. Accroding to previous
258 studies (Qin et al. 2018; Uronis et al. 2011), we hypothesized that administration of a single
259 probiotic CCFM639 may not exert a dramatic effect on the richness of the whole gut microbiota,
260 but it may induce alterations in the abundance of specific genera. A previous study demonstrated
261 that toxic metal exposure can influence the gut microbiota composition in mice (Zhai et al. 2017).
262 Ingestion of aluminum damage the intestinal mucosa and reduce the intestinal barrier function
263 and immune function (Vignal et al. 2016). Pathogens and opportunistic pathogens in the
264 intestinal tract can take the opportunity to multiply, causing disordered gut microbiota (Berg
265 1996). A lower relative abundance of phylum Proteobacteria and a higher relative abundance of
266 genus *Cetobacterium* were found after aluminum exposure. Exposure to silver led to a similar
267 alteration in male zebrafish (Ma et al. 2018). The pathogen *Flavobacterium* is considered
268 responsible for several fish diseases, including fry syndrome and bacterial cold water disease,
269 which can cause high mortality levels in young fish (Leal et al. 2010; Nematollahi et al. 2003).
270 The increase in the relative abundance of *Flavobacterium* may be due to its good aluminum
271 tolerance capacity (more than 2000 ppm) (Konishi et al. 1994). Similar speculation can be used
272 to explain the increase in the relative abundance of *Comamonadaceae*. A series of metal-resistant
273 genes and gene clusters was found in the whole-genome sequencing of the *Comamonas* strain,
274 including arsenic, stibium, copper and so forth (Li et al. 2013; Lin et al. 2015). Wu et al. studied
275 the virulence of several *Enterovibrio* and *Vibrio* strains for zebrafish; the results showed that
276 *Enterovibrio* had a high level of virulence (LD_{50} values around 10^4 CFU/g) , and that *Vibrio* had
277 a moderate level of virulence (LD_{50} values around 10^6 CFU/g)   (Wu et al. 2015). *Enterovibrio*
278 promotes the production of indole, which is a toxin that is harmful to intestinal lactic acid
279 bacteria in excess quantities (Nowak & Libudzisz 2006; Pascual et al. 2009).
280 *Porphyromonadaceae* is a family in the of Bacteroidetes phylum that can also exert negative
281 effects on the host (Russell et al. 2015). Therefore, aluminum exposure led to an increase in the
282 relative abundance of some harmful bacteria. The dynamic changes in the gut microbiota can

283   directly affect the intestinal mucosa and indirectly affect the health of the fish (Perez et al. 2010).
284   The results help to further explain the harmful effects of aluminum exposure on the host's
285   growth and antioxidant system in previous study (Yu et al. 2017).

286   Probiotics have shown an excellent ability to resist disease and prevent pathogens (Hai
287   2015). Probiotic supplementation results in improvements in microvilli density and length, which
288   can increase the absorptive surface of the fish intestines and ultimately enhance the host's
289   physical barrier against potential pathogens (Standen et al. 2016). The use of probiotics
290   stimulates the proliferation of a few probiotic bacteria and decreases the potential pathogens in
291   fish. Numerous studies have demonstrated the significant effects of probiotics in protecting
292   aquatic animals against infection by pathogens, such as the effects of *Bacillus* spp. against
293   *Streptococcus iniae* (Cha et al. 2013), and the effects of *Pseudomonas* spp. against *F.*
294   *psychrophilum* (Korkea-aho et al. 2012). Compared with the Al-only group, the administration of
295   CCFM639 significantly reduced the abundance of *Enterovibrio, Comamonadaceae* and
296   *Porphyromonadaceae*, indicating the potential protective effect of CCFM639 treatment against
297   aluminum-induced increases in pathogen. *Aeromonas* is one of the fish pathogens that causes
298   several fish diseases, including motile aeromonad septicemia, furunculosis, ulcer disease, and
299   carp erythrodermatitis (Miller & Harbottle 2018). In 639 only group, the abundance of
300   *Aeromonas*.was decreased, which indicates modification of the bacterial community composition
301   by administration of the probiotic CCFM639. Studies in tilapia have also demonstrated an
302   improvement in gut microbiota with the addition of probiotics (Standen et al. 2015).

303   **Conclusions**

304   In conclusion, the accumulated aluminum in Nile tilapia can be excreted through feces, and
305   oral administration of *L. plantarum* CCFM639 increased aluminum excretion (Figure 6).
306   Moreover, environmental exposure to aluminum altered the composition of the gut microbiota,
307   and alteration in the levels of *Enterovibrio, Comamonadaceae,* and *Porphyromonadaceae* can be
308   recovered by administration of CCFM639. Therefore, this study provides a further explanation of
309   the protective mechanisms of CCFM639 against aluminum toxicity. Apart from promotion of
310   aluminum discharge through feces, regulation of the gut microbiota with *L. plantarum* CCFM639
311   may be an underlying mechanism by which aluminum toxicity is alleviated.

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Figure 1

Figure 1 Alpha diversity results for the gut microbiota of Nile tilapia

The data shown are the means ±SEM for each group. Asterisks represent significant differences compared to the control group, P=0.007 and 0.042 for Chao (A), P=0.006 and 0.041 for Shannon (B).

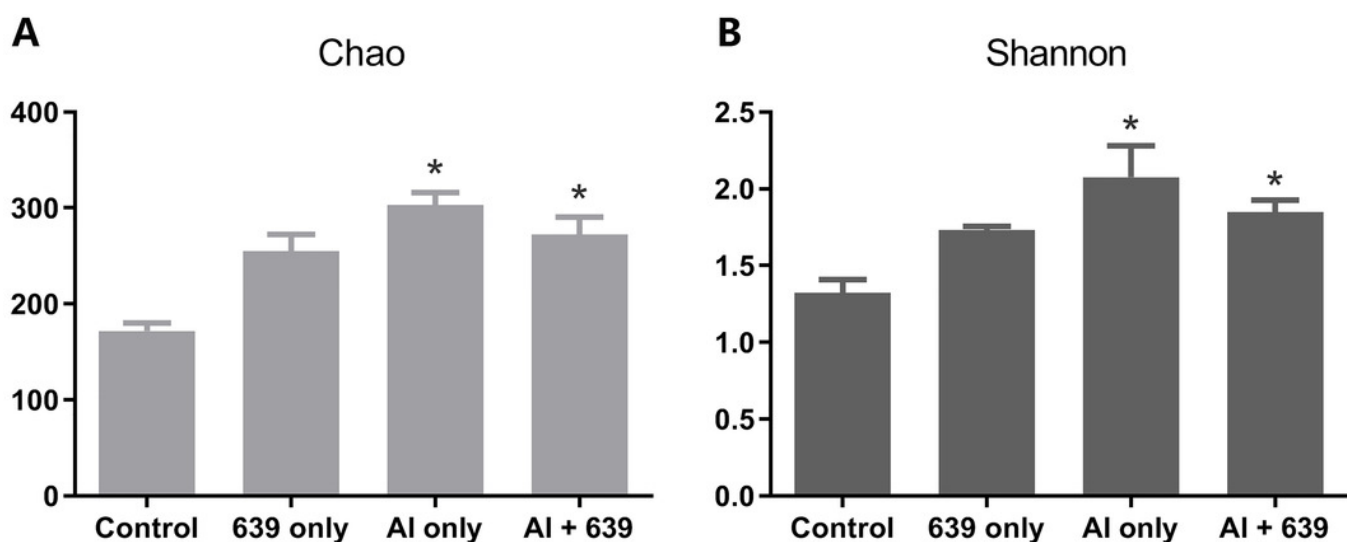



Figure 2

Principal coordinate score plots for the gut microbiota of Nile tilapia

(A) Unweighted unifrac distance. (B) PC1 values. The asterisk indicates the statistically significant differences (P < 0.05) between different groups, ns indicates no statistically significant differences.

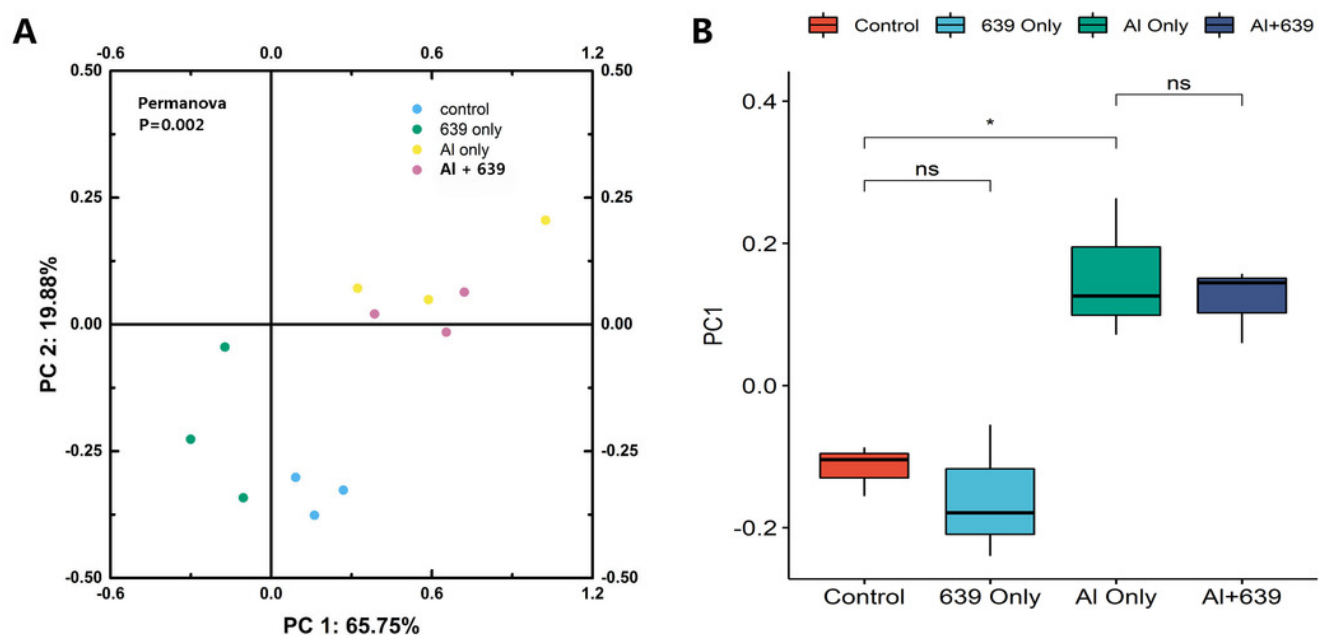



Figure 3

Effect of *L. plantarum* CCFM639 on the relative abundance (relative OTU composition) of the components of gut microbiota in Nile tilapia at the phylum level

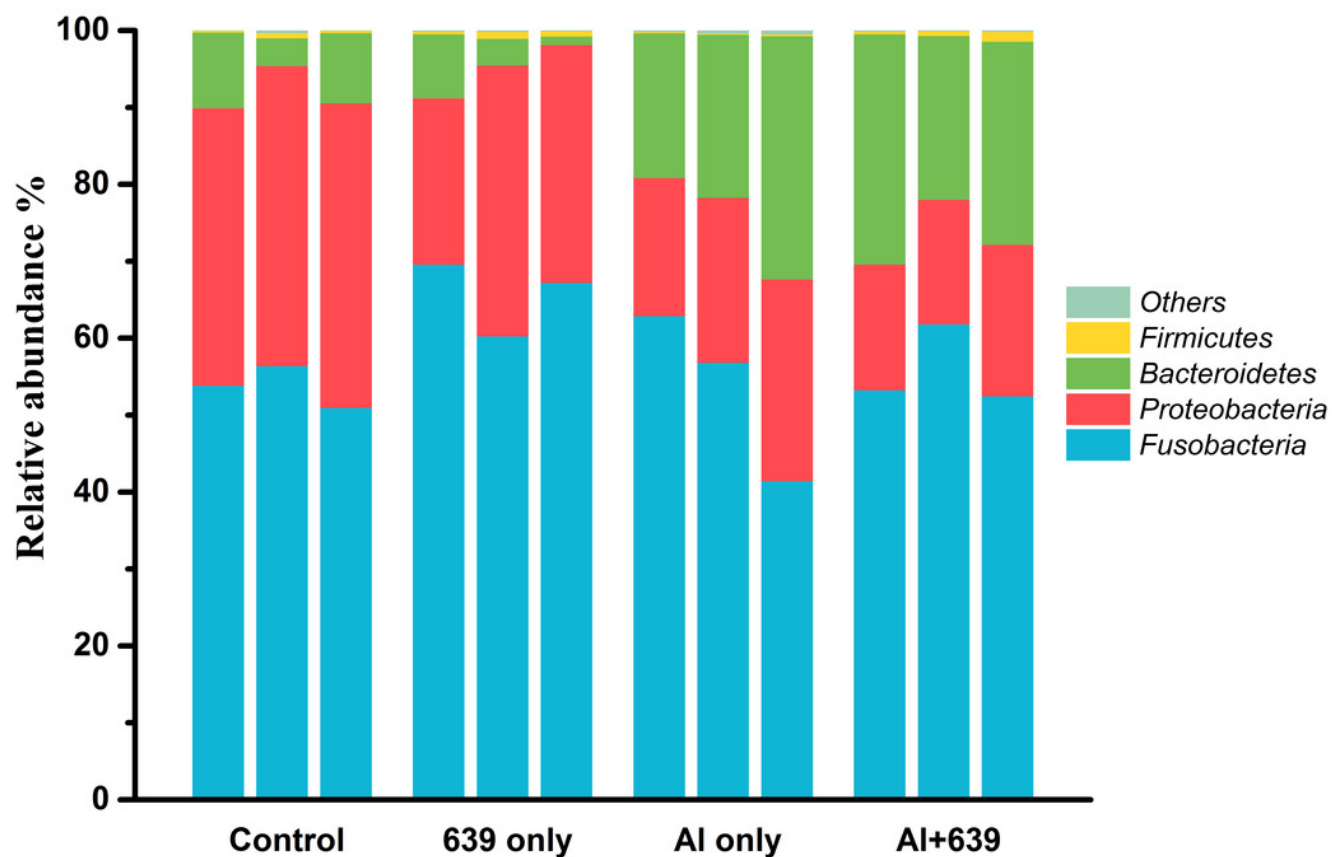



Figure 4

Effects of *L. plantarum* CCFM639 on the relative abundance (relative OTU composition) of the gut microbiota in Nile tilapia at the genus level

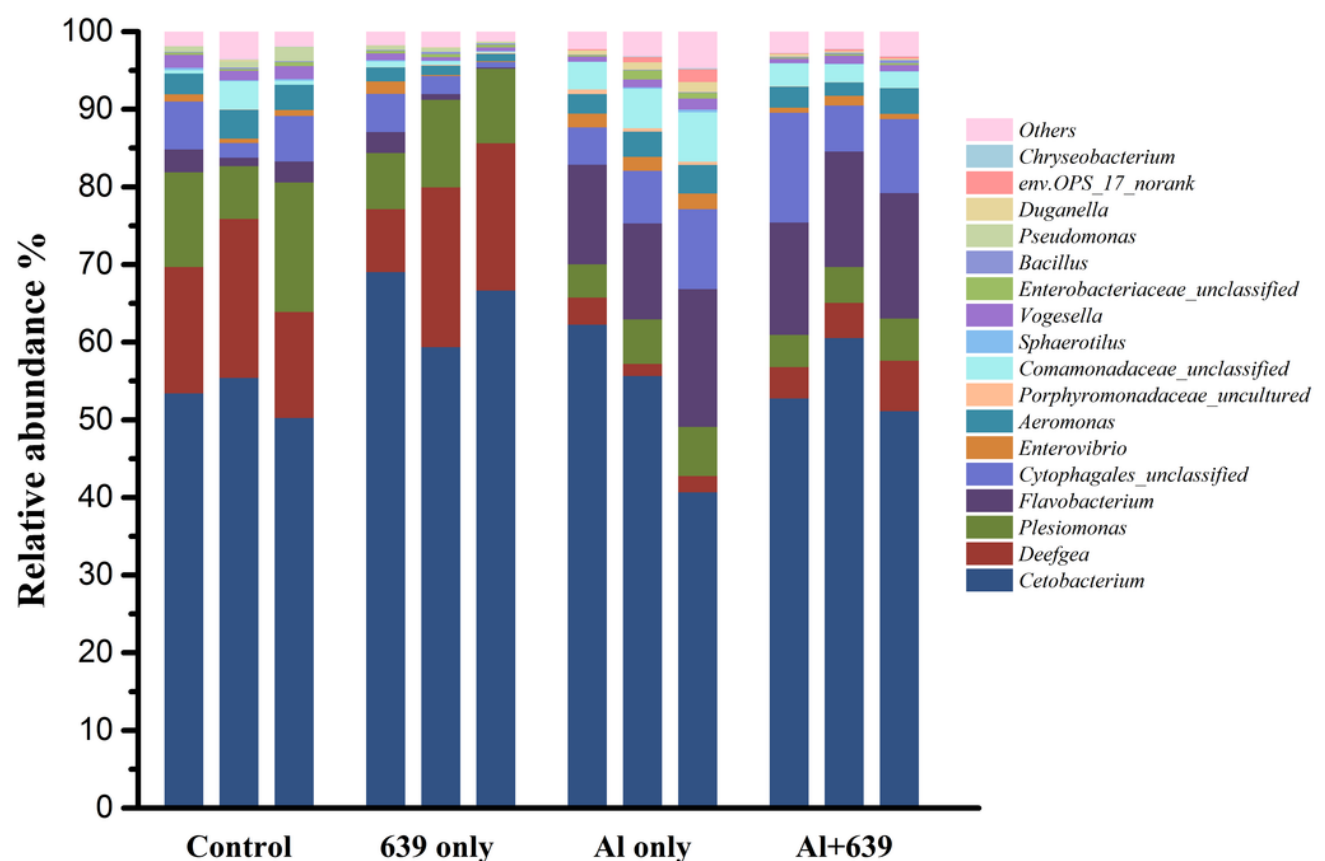



Figure 5

Relative abundance (relative OTU composition, % ±SEM) of the gut microbiota in Nile tilapia at the genus level

Relative abundance of Cetobacterium, Deefgea, Plesiomonas *and Flavobacterium; (B)*

Relative abundance of Cytophagales, Enterovibrio, Aeromonas *and Porphyromonadaceae; (C)*

Relative abundance of Comamonadaceae , Sphaerotilus, Vogesella *and Enterobacteriaceae;*

(D) Relative abundance of Bacillus, Pseudomonas, Duganella, *env.OPS_17-norank and*

Chryseobacterium[i]. Data are expressed as mean ± SEM.

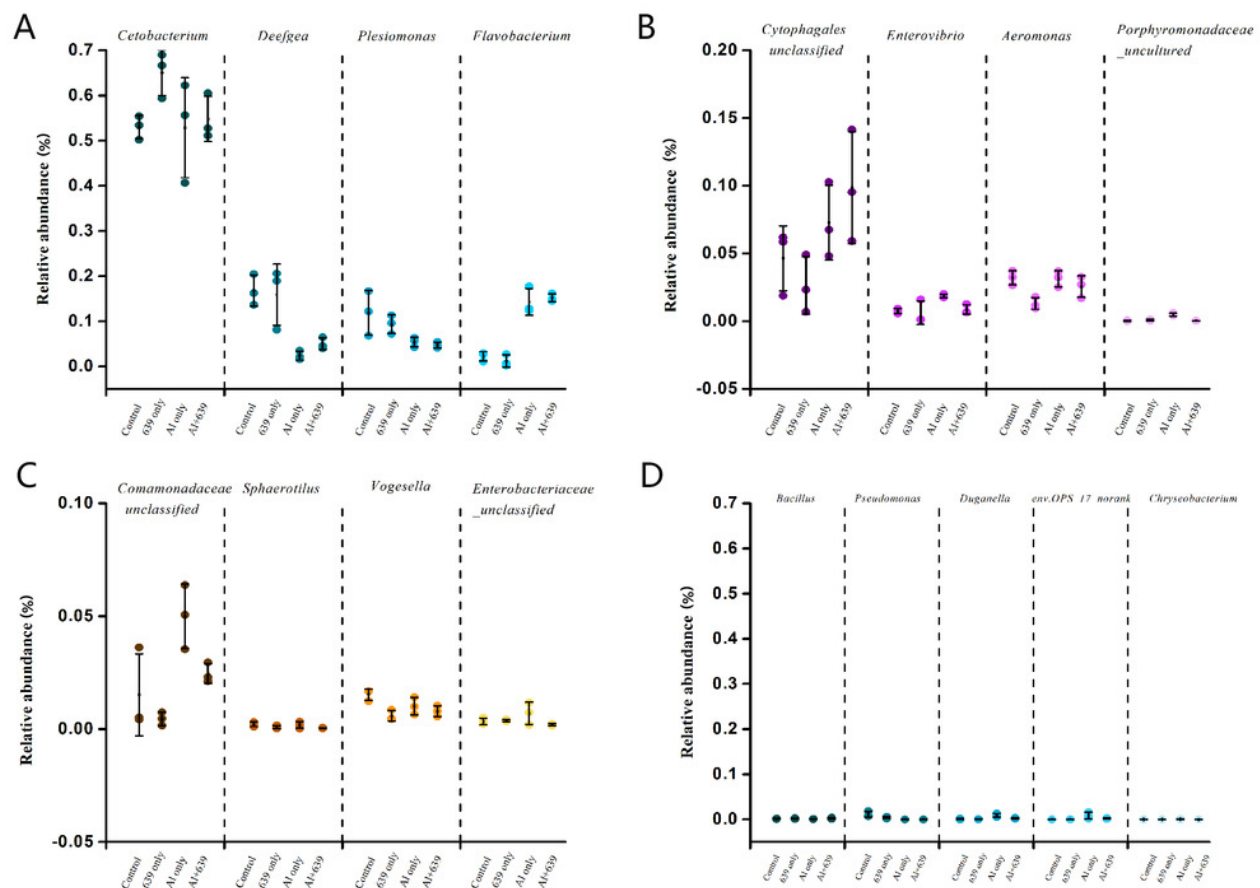
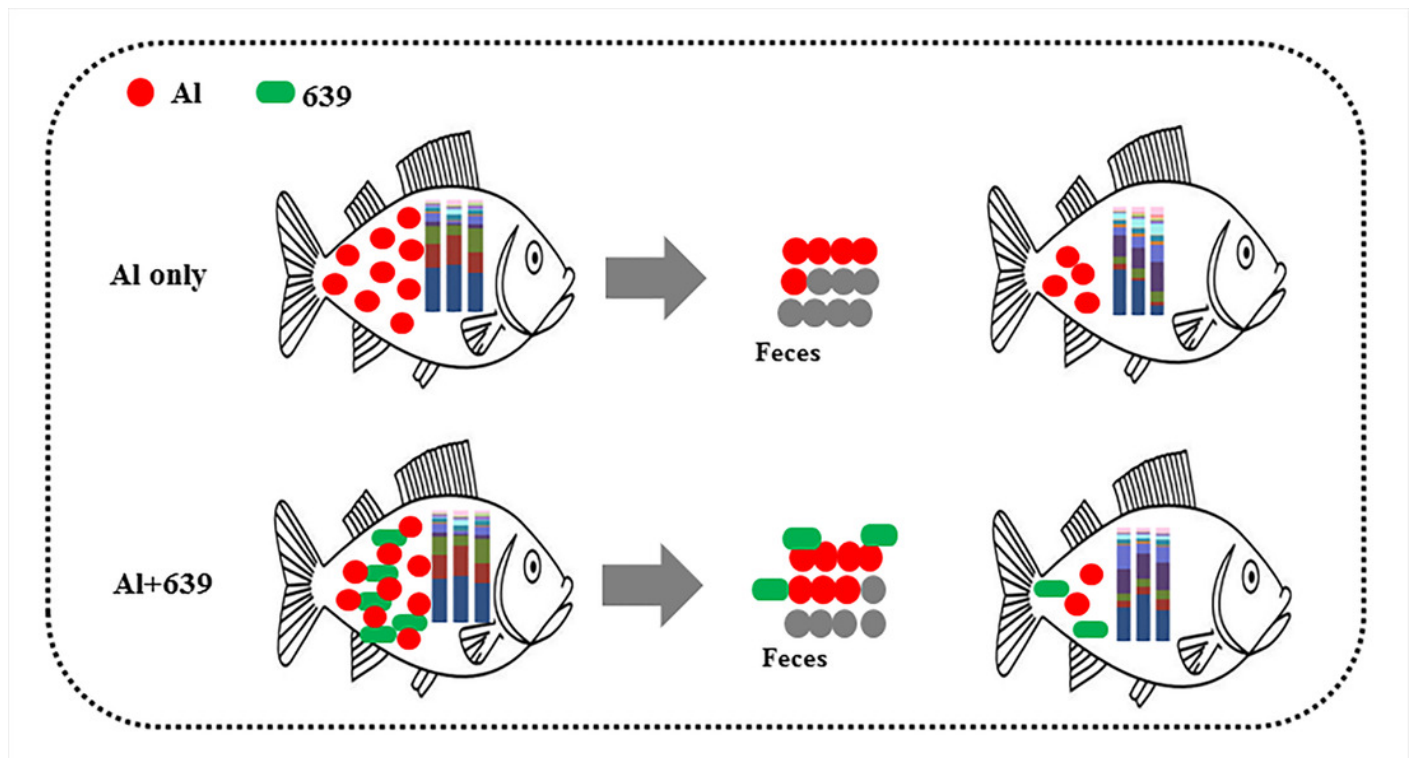



Figure 6

Potential protective mechanism of CCFM639 against aluminum induced gut injuries in Nile tilapia

Experimental groups for tilapia with and without Al exposure and CCFM639 feed

1   Table 1. Experimental groups of tilapia with and without aluminum exposure and probiotic feed

Group	Experiment time (4 weeks)
Control	Basic feed + normal water
639 only	probiotic feed + normal water
Al only	Basic feed + aluminum water
Al + 639	probiotic feed + aluminum water

2   CCFM639 feed, feed containing *L. plantarum* CCFM639 at a concentration of 10^8 CFU/g;

3   aluminum water, an aqueous environment containing 2.73 mg/L of aluminum ions.

Effect of dietary supplementation with CCFM639 on Al contents in Nile tilapia feces

1   Table 2. Effects of dietary supplementation with CCFM639 on aluminum contents in Nile

2   tilapia feces

Group	Aluminum level (mg/kg)				
	0 week	Week 1	Week 2	Week 3	Week 4
Control	1.13 ± 0.06^{aA}	1.54 ± 0.07^{aA}	1.83 ± 0.14^{aA}	1.80 ± 0.10^{aA}	1.54 ± 0.04^{aA}
639 only	1.19 ± 0.04^{aA}	1.55 ± 0.05^{aA}	1.68 ± 0.13^{aA}	1.74 ± 0.10^{aA}	1.58 ± 0.06^{aA}
Al only	1.08 ± 0.13^{aA}	22.33 ± 1.31^{bB}	23.67 ± 1.54^{bB}	25.22 ± 1.32^{bB}	25.67 ± 1.01^{bB}
Al + 639	1.05 ± 0.08^{aA}	25.67 ± 1.23^{cB}	33.14 ± 2.53^{cC}	35.46 ± 2.05^{cC}	33.79 ± 2.37^{cC}

3   The data shown are the mean ± SEM for each group. The means with different superscript

4   lowercase letters differ significantly among groups, and the superscript capital letters indicate a

5   significant difference among time-points ($P<0.05$).

Effect of dietary supplementation with CCFM639 on *L. plantarum* quantification in Nile tilapia feces

1   Table 3. Effects of dietary supplementation with CCFM639 on *L. plantarum* quantification in

2   Nile tilapia feces

Group	Number of *L. plantarum* (log copies/g feces)		
	Week 0	Week 2	Week 4
Control	5.49 ± 0.19[aA]	5.47 ± 0.09[aA]	5.40 ± 0.04[aA]
639 only	5.52 ± 0.01[aA]	7.83 ± 0.12[bB]	7.73 ± 0.02[bB]
Al only	5.49 ± 0.01[aA]	5.24 ± 0.08[aB]	4.99 ± 0.05[cC]
Al + 639	5.54 ± 0.13[aA]	7.46 ± 0.01[cB]	7.31 ± 0.13[dB]

3   The data shown are the mean ± SEM for each group. The means with different superscript

4   lowercase letters differ significantly among groups, and the superscript capital letters indicate a

5   significant difference among time-points (P<0.05).

Effects of CCFM639 on aluminum-induced changes in relative abundance of *Aeromonas*, *Enterovibrio, Comamonadaceae* and *Porphyromonadaceae* of Nile tilapia

1   Table 4. Effects of CCFM639 on aluminum-induced changes in relative abundance of

2   *Aeromonas, Enterovibrio, Comamonadaceae* and *Porphyromonadaceae* of Nile tilapia

Group	*Aeromonas*	*Comamonad aceae*	*Enterovibrio*	*Porphyromonadaceae*
Control	3.20 ± 0.31^a	1.52 ± 1.05^a	0.76 ± 0.10^a	0.03 ± 0.02^a
639 only	1.31 ± 0.25^b	0.45 ± 0.17^a	0.62 ± 0.50^a	0.09 ± 0.03^a
Al only	3.14 ± 0.34^a	5.00 ± 0.82^b	1.86 ± 0.08^b	0.49 ± 0.06^b
Al + 639	2.57 ± 0.50^a	2.46 ± 0.25^a	0.87 ± 0.20^a	0.04 ± 0.004^a

3   The data shown are the means $\pm$ SEM for each group. The different superscript letters represent

4   significant differences between groups ($P<0.05$).