The intestinal microbiota-driven dopamine level influences social interaction and innate color preference in zebrafish larvae

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Abstract

Gut microbiota influence neurodevelopment of brain and programing of behaviors. However, the mechanism underlining the relationship between shoals' behaviors and intestinal microbiota remain controversial and the roles of neurotransmitters are still unclear. Here we show that, shoaling behavior affected the innate color preference of shoals, indicating that shoals tended to choose a favorable color environment that benefits social contact. Meanwhile, administration of D1-R antagonist disrupted the social interaction which led to the deficits of color preference. More importantly, the altered microbiota caused by an antibiotic OTC decreased the sociability and weakened shoals' color preference. When given a supplement of LGG after OTC exposure, fish exhibited an unexpectedly recovery capability in social cohesion and color preference. Our findings show that dopamine level of brain could mediate both social recognition and color preference, and highlight the pathway of microbial metabolites through the microbiota-gut-brain axis that coordinate the production of dopamine.

Keywords: Intestinal microbiota, Social interaction, Innate color preference, Dopamine, Zebrafish

Introduction

Microorganisms, especially the microbiota which reside in the gastrointestinal tract, may influence brain physiology and social behaviors across diverse animal species (**Sherwin et al., 2019**). Social behavior is observed only when animals gather into a group. Some of these behaviors are beneficial for animals, such as division of labor, cooperative care, shared risk and increased immunity, and others are deleterious for groups, such as conflict, dominance and coercion (**Mason and Shan, 2017**). Microbes are implicated in determining social cues in animals, and the altered gut microbiota may affect the social interactions between animals (**Buffington et al., 2016**). For example, germ-free mice which is absence of the microbiota displays deficits in social cognition and social recognition (**Sgritta et al., 2019; Desbonnet et al., 2014**). A successful strategy in unraveling the role of the microbiota in social behavior is the use of antibiotics to deplete the microbiota. A mixture of antibiotics can reduce the social cohesion of zebrafish (**Wang et al., 2016**). Although the perturbations of the microbiota often negatively affect social interaction, modulation of gut microbiota through probiotic supplement can have a beneficial effect. For instance, the social deficit can be reversed during treatment with *Lactobacillus reuteri* (**Buffington et al., 2016**), and zebrafish with the probiotic strain *Lactobacillus rhamnosus* IMC 501 increase shoaling behavior (**Borrelli et al., 2016**). Because social

54 interaction is such a vital component of human and animals' mental health,

55 the change of this behavior may have negative effects on the whole fitness

56 (**Holt-Lunstad et al., 2015**). Deficits in social interaction manifest in

57 several neuropsychiatric diseases such as schizophrenia, social anxiety,

58 and depression (**Sherwin et al., 2016**).

59 As is well known, zebrafish are social animals and tend to travel in

60 shoals (**Miller and Gerlai, 2007**). Meanwhile, there is a vital necessity for

61 fish to sense danger and stay away from predators, thus it is beneficial for

62 a fish to stay tight within a shoal and to assess the social interaction

63 efficiently. Furthermore, animals in groups are often better able to perceive

64 colors in their environment (**Endler, 1992; Kelber and Osorio, 2010;**

65 **Skorupski and Chittka, 2011**), and their color preferences are either most

66 represented in the environment (**Lunau et al., 2011**) or contrast with the

67 background (**Endler et al., 2005**). Plenty of researches have proven that

68 zebrafish have preference towards different colors (**Avdesh et al., 2012;**

69 **Bault et al., 2015; Oliveira et al., 2015; Fleisch and Neuhauss, 2006; Li**

70 **et al., 2013; Colwill et al., 2005**). Surprising, the color preference of

71 zebrafish has been extensively studied but still remains controversial. For

72 instance, some researches show a strong preference for blue (**Avdesh et al.,**

73 **2012; Fleisch and Neuhauss, 2006**), whereas others report a clear

74 aversion for this color (**Bault et al., 2015; Oliveiraet al., 2015; Li et al.,**

75 **2013; Colwill et al., 2005**). It is still not clear whether the shoaling

4

76 behavior affect the color preference. We make a hypothesis that the

77 characteristic of background color may influence the efficiency of social

78 contact within a shoal and consequently shoal may prefer certain

79 background colors in order to keep the social contact, which can be called

80 as innate color preference of shoals. Recently, Park *et al*. (**2016**) used

81 zebrafish larvae (5 days post fertilization (dpf)) to test the innate color

82 preference in shoals. However, shoaling behavior usually starts to develop

83 after 7 dpf, becoming progressively stronger for the mature (**Buske and**

84 **Gerlai, 2011; Dreosti, 2015; Hinz, 2017**). As far as we know, no research

85 has been published that investigate the innate color preference of shoals,

86 although shoaling and social behavior in general has received considerable

87 critical attentions.

88   The cohesion of shoals has been found to be associated with the whole

89 brain dopamine level (**Buske and Gerlai, 2012**). Dopamine is one of the

90 major neurotransmitters in the central nervous system of the vertebrate

91 brain which plays important roles in a variety of cerebral functions, such

92 as mood, attention, reward, and memory (**Ma and Lopez, 2003; Girault**

93 **and Greengard, 2004; Vidal-Gadea et al., 2011**). Abundant evidence

94 shows that dopamine is associated with the neurobehavioral functions in

95 zebrafish (**Saif et al., 2013; Shams et al., 2018**). Saif *et al.* (**2013**) found

96 that strong social stimuli will increase the dopamine and its metabolite 3,4-

97 dihydroxyphenylacetic acid (DOPAC) levels in the brain of the adult

98 zebrafish. The short-term isolated zebrafish could reduce the level of
99 DOPAC (**Shams et al., 2018**). The social interaction of shoals in zebrafish
100 can be affected by the dopaminergic system through influence of the
101 dopamine level (**Scerbina et al., 2012**). D1 dopamine receptor antagonist
102 (SCH23390) is most abundantly expressed dopamine receptor subtypes in
103 the brain of zebrafish (**Li et al., 2007**). And SCH23390 disrupts social
104 preference of zebrafish by decreasing the level of dopamine in
105 dopaminergic system (**Steketee, 1998; Kurata and Shibata, 1991**).

106   In the present study, we evaluated the influence of antibiotic and
107 probiotic which affected gut microbiota of zebrafish on the social
108 interaction and innate color preference. Meanwhile, we assessed the
109 influence of SCH23390 which could affect dopamine level of brains on the
110 social interaction and innate color preference. To analyze the dopamine
111 levels and gut microbial communities, we established the correlation
112 between intestinal microbiota and behaviors through neurotransmitters in
113 brains. Further understanding of how intestinal microbiota influence the
114 brain may be helpful for elucidating the causal mechanisms underlying
115 behavior of shoals and for generating new behavioral paradigms for social
116 disorders, such as social anxiety, and depression.

117 **Results**

118 **OTC or LGG affects social interaction and color preference in larval**
119 **zebrafish**

120   In this study, in order to assess the influence of oxytetracycline (OTC)

121   or *Lactobacillus rhamnosus* GG (LGG) on shoal interaction and color

122   preference, the larval zebrafish (5 dpf) were exposed by OTC (500 µg/L)

123   and LGG (10^6 CFU/mL) for 21 days, respectively (Figure 1A). To

124   investigate the locomotor behavior of larvae which may affect shoaling

125   behavior and color selection, the larvae were transferred into 12-well plates

126   to measure locomotion. the behavioral trajectory and the results of average

127   movement speed (velocity) were not significantly changed by OTC and

128   LGG exposure (Figure 1B-C). The results showed that OTC or LGG may

129   not cause the locomotor change in zebrafish larvae.

130   The larvae shoaling behavior was analyzed for shoal size area (SA),

131   nearest distance (ND), farthest distance (FD) and average distance (AD) in

132   control (Ctrl), OTC and LGG group (Figure 1D). As demonstrated in

133   Figure 1E, in OTC group, compared to Ctrl group, although FD was not

134   significantly different between the two groups, SA ($t_{23} = 4.20$, $P < 0.001$),

135   ND ($t_{23} = 3.41$, $P < 0.01$) and AD ($t_{23} = 2.78$, $P < 0.05$) significantly

136   increased. Additionally, shoaling behavior of larvae was significantly

137   different between the Ctrl and LGG group. Although ND did not change

138   between the two treatments groups ($t_{23} = 0.79$, $P > 0.05$), SA ($t_{23} = 3.47$, P

139   < 0.01), FD ($t_{23} = 2.22$, $P < 0.05$) and AD ($t_{23} = 2.30$, $P < 0.05$) were

140   significantly decreased in the LGG group. Together, these data indicated

141   that the shoal cohesion in larvae was decreased through the OTC exposure,

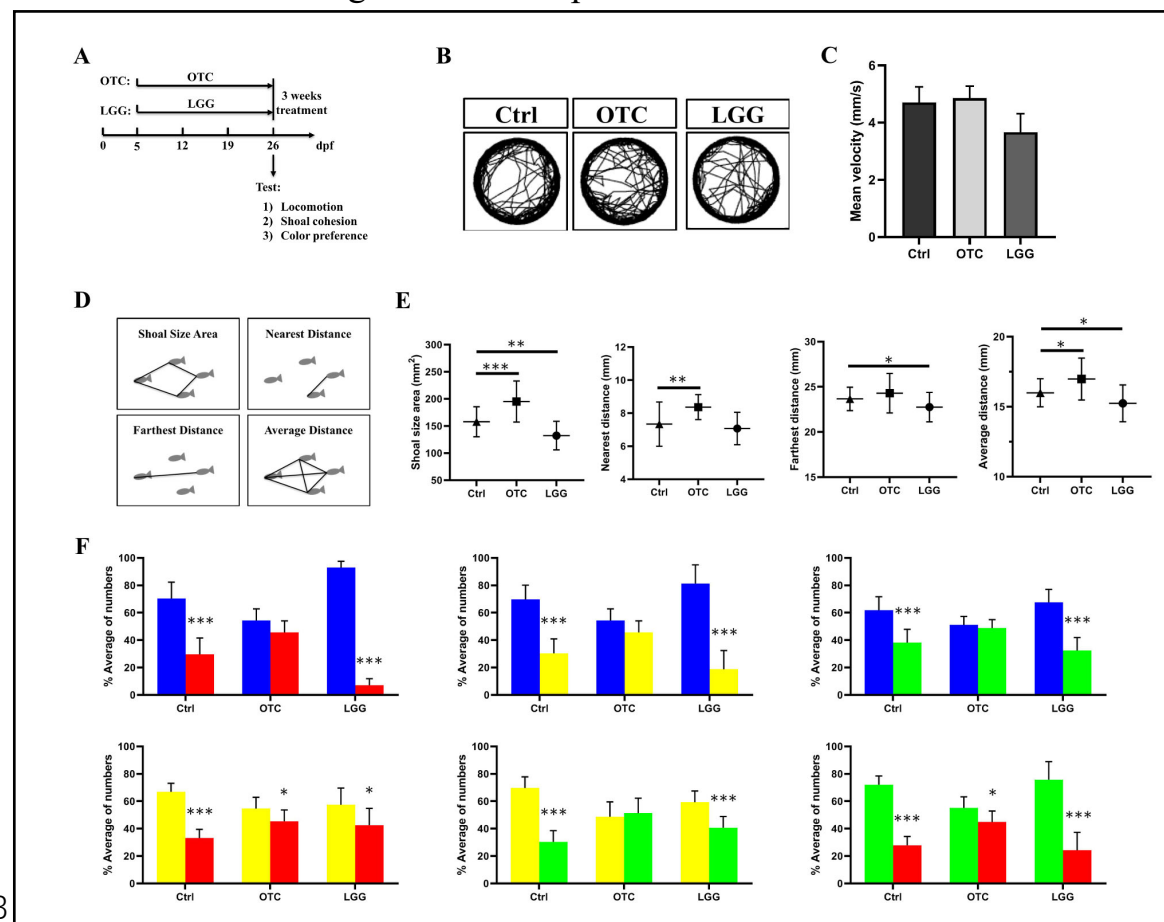142 and increased through the LGG exposure.

Figure 1. OTC or LGG affects social interaction and color preference in larval zebrafish.

(A) Schematic describing the experimental procedure for OTC or LGG treatment. The

behavioral trajectory (B) and the mean velocity (C) of the zebrafish larvae from control

group (Ctrl) and exposure groups (OTC or LGG) were tested during 10-min period

(n=12/ group, 4 groups per treatment). (D) Schematic describing the testing index of

social interaction. (F) The shoaling behavior of zebrafish larvae through OTC or LGG

treatment (n=6/group, 4 groups per treatment). (G) The shoals of zebrafish larvae

exhibit the color preference with 6 color combinations (B-R, B-Y, B-G, Y-R, Y-G, G-

R) for OTC or LGG treatment. The percentage of numbers of 20-larval zebrafish spent

in each colored zone was counted every 20 s for a total of 10 min (n=10 for Ctrl group,

154   n=8 for OTC group, n=9 for LGG group). * p<0.01, ** p<0.01, *** p<0.001.

155   To investigate color preference in shoals with different treatments, a

156   shoal was introduced and allowed to swim freely in color-enriched CPP

157   tank. After 5-min acclimation to the treatments, the location of each larva

158   in each colored zone was counted every 20 s for 10 min total of video

159   recording. As showed in Figure 1F, in Ctrl group, there was a distinct color

160   preference among two color combination (B-R, B-Y, B-G, Y-R, Y-G, G-R),

161   and the order of BYGR preference was B > Y > G > R. However, the OTC

162   treatment group lost their color preference (B-R, B-Y, B-G, Y-G) or

163   attenuated their color selection (Y-R, G-R). Unlike the OTC group, the

164   LGG treatment group maintain the inherent color preference except the

165   preference of Y-R was a little bit decreased, and the order of BYGR

166   preference was the same with Ctrl group (B > Y > G > R). Hence, our

167   results showed that the color preference of shoals was lost or weakened

168   through the OTC treatment, and the LGG exposure group can maintain the

169   existence of the innate color preference in shoals.

170   **LGG improves the recovery of social interaction and color preference**

171   **after OTC treatment**

172   The natural microbiota has small amounts of probiotic lactic acid

173   bacteria, and probiotic supplement can reverse the social deficits

174   (**Buffington et al., 2016**). However, whether probiotic can confer

175   neuroprotection or ameliorate deficiency of color preference caused by

176   antibiotic treatment has not been tested. To address this issue, zebrafish

177   larvae received probiotic LGG (10^6 CFU/ mL) starting immediately after

178   21 days OTC (500 µg/L) treatment and then again daily until 21 days
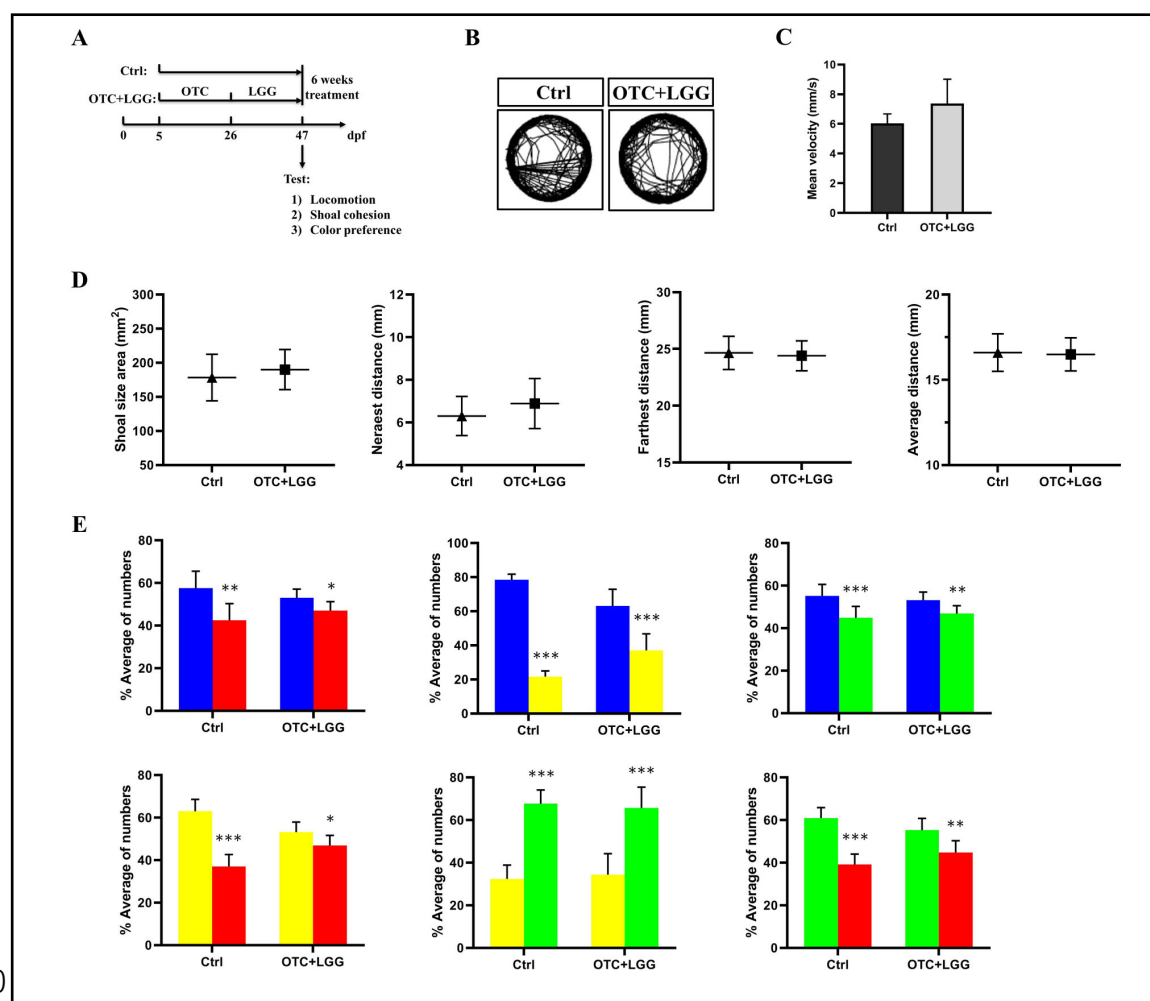
179   (Figure 2A).

Figure 2. LGG improves the recovery of social interaction and color preference after OTC treatment. (A) Schematic describing the experimental procedure for OTC and LGG treatment. The behavioral trajectory (B) and the mean velocity (C) of the zebrafish larvae from control group (Ctrl) and exposure groups (OTC+LGG) were tested during 10-min period (n=12/ group, 4 groups per treatment). (D) The shoaling behavior of zebrafish larvae through OTC and LGG treatment (n=6/group, 4 groups per treatment).

187   (G) The shoals of zebrafish larvae exhibit the color preference with 6 color

188   combinations (B-R, B-Y, B-G, Y-R, Y-G, G-R) for OTC and LGG treatment. The

189   percentage of numbers of 20-larval zebrafish spent in each colored zone was counted

190   every 20 s for a total of 10 min (n=8 for Ctrl or OTC+LGG group, respectively). *

191   p<0.01, ** p<0.01, *** p<0.001.

192   From Figure 2B-C, the results showed that the LGG strain could not

193   change the behavioral trajectory and mean velocity after OTC exposure.

194   We next set out to identify the recovery of social cohesion, as showed in

195   Figure 2D, there were no significant difference between Ctrl and

196   OTC+LGG group among shoaling behavior. These data indicated that

197   probiotic LGG could promote the recovery of shoal cohesion through the

198   damage caused by OTC. More importantly, we focused on the variation of

199   color preference, from Figure 2E, although the selections of colors among

200   B-R, B-G, Y-R, and G-R were a little weakened, the order of BYGR

201   preference was the same with Ctrl group (B > G > Y > R). These data raised

202   the possibility that LGG strain could promote the recovery of social

203   cohesion and color selection through the damage caused by OTC.

204   **SCH23390 affects shoal interaction and color preference in larval**

205   **zebrafish**

206   From the above results in Figure 1E-F and Figure 2D-E, the strength

207   of social cohesion may drive the color discrimination in shoals of zebrafish.

208   To identify the role of shoaling behavior in color preference, we selected a

209   D1-receptor antagonist SCH23390, which could disrupt social preference

210   and decrease social interaction in zebrafish. To start the experiment, the

211   SCH23390 (SCH) group (a shoal of 20 larvae at 26 dpf) remained in the

212   SCH23390 solution for 1 h (Figure 3A). Because the dopaminergic system

213   is associated with a number of brain functions (e.g., motor function). Thus,

214   D1-receptor antagonist may potentially lead to the altered shoaling

215   behavior as a result of abnormal motor or activity level. However, our

216   results demonstrated that the treat fish did not display abnormal

217   locomotory activity or posture patterns and their mean velocity was also
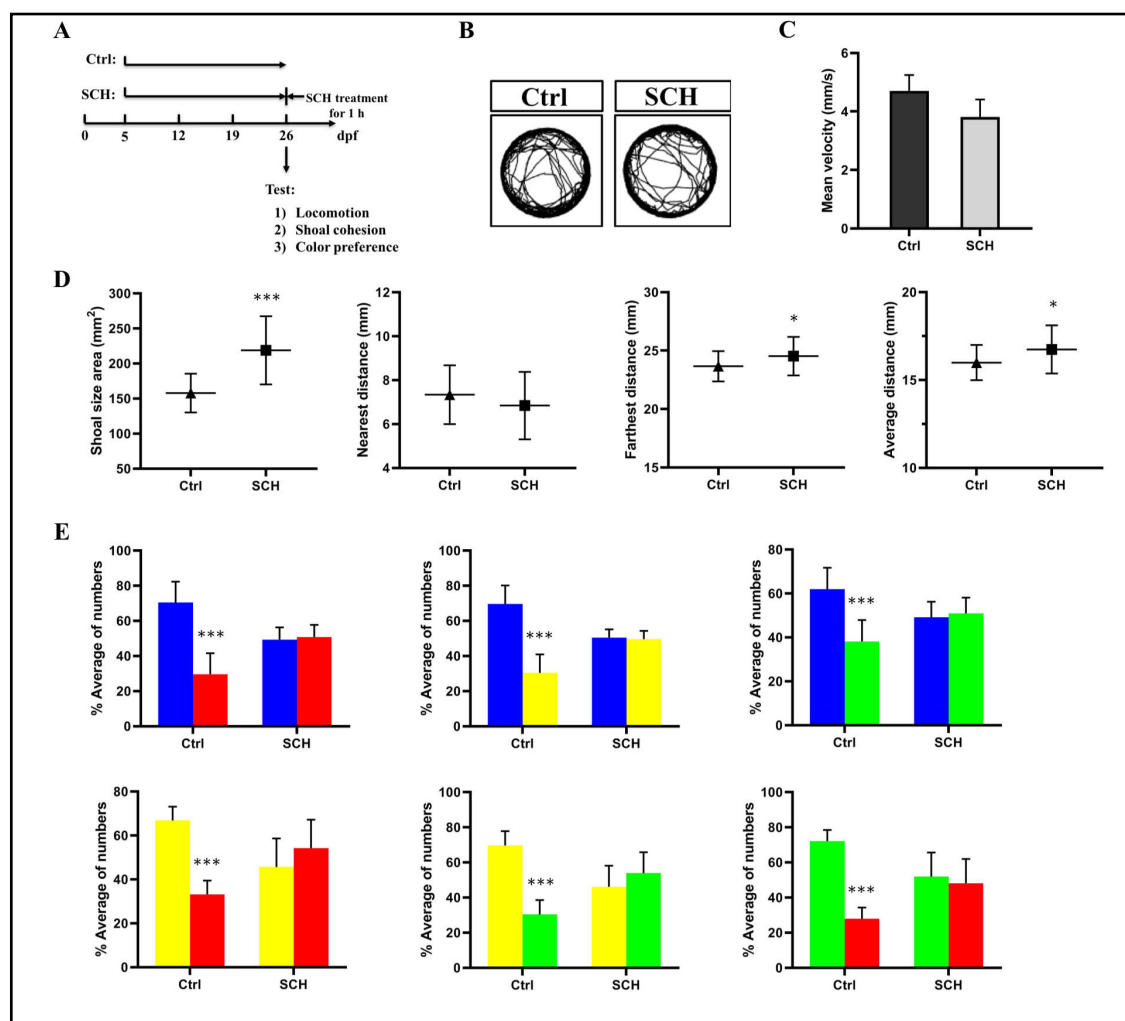
218   unaltered (Figure 3B-C).

219

12

220   **Figure 3.** SCH23390 affects shoal interaction and color preference in larval zebrafish.

221   (A) Schematic describing the experimental procedure for SCH23390 treatment. The

222   behavioral trajectory (B) and the mean velocity (C) of the zebrafish larvae from control

223   group (Ctrl) and SCH23390 treatment groups (SCH) were tested during 10-min period

224   (n=12/group, 4 groups per treatment). (D) The shoaling behavior of zebrafish larvae

225   through SCH23390 treatment (n=6/group, 4 groups per treatment). (G) The color

226   preference of shoals with SCH23390 treatment in 6 color combinations (B-R, B-Y, B-

227   G, Y-R, Y-G, G-R). The percentage of numbers of 20-larval zebrafish spent in each

228   colored zone was counted every 20 s for a total of 10 min (n=9 for Ctrl or SCH group,

229   respectively). * p<0.01, ** p<0.01, *** p<0.001.

230   From the results in Figure 3D, shoal cohesion of larvae treated with

231   SCH23390 was significantly different from Ctrl group. SA (t_{23} = 5.91, P <

232   0.001), FD (t_{23} = 2.08, P < 0.05), and AD (t_{23} = 2.22, P < 0.05) were

233   significantly decreased in the SCH group, but ND did not alter between the

234   two treatments group (t_{23} = 1.21, P > 0.05). These data suggested that

235   SCH23390 significantly decreased social and explorative behavior in

236   zebrafish larvae. From the results of color preference, by contrast with the

237   shoals without SCH23390 treatment, the shoals lost their interest in color

238   combinations in color-enriched CPP tank (Figure 3E). These results

239   suggested that the color preference of shoals required the participation of

240   social interaction.

241   **Dopamine level analysis of the treatment groups**

13

242   As D1-receptor antagonist mediated the social interaction and

243   affected color selection in shoals of larval zebrafish (Figure 3D-E), we

244   hypothesized that the altered social interaction and color preference of the

245   exposed larvae may associated with the dopamine level in the brain. To

246   target the dopamine level, we tested the dopamine concentration of the

247   exposed groups using ELISA. As showed in Figure 4A, OTC was

248   significantly decreased the dopamine level ($t_2 = 6.27$, $P < 0.05$), while LGG

249   was significantly increased the dopamine concentration ($t_2 = 4.82$, $P <$

250   0.05). From Figure 4B, LGG recover the dopamine level after the OTC

251   treatment ($t_2 = 0.34$, $P > 0.05$). In addition, our data verified the results that

252   SCH23390 can significantly decreased the dopamine level ($t_2 = 37.16$, $P <$

253   0.001) in the larval brains (Figure 4C). These results suggest that the

254   dopamine which is associated with social cohesion is primary responsible

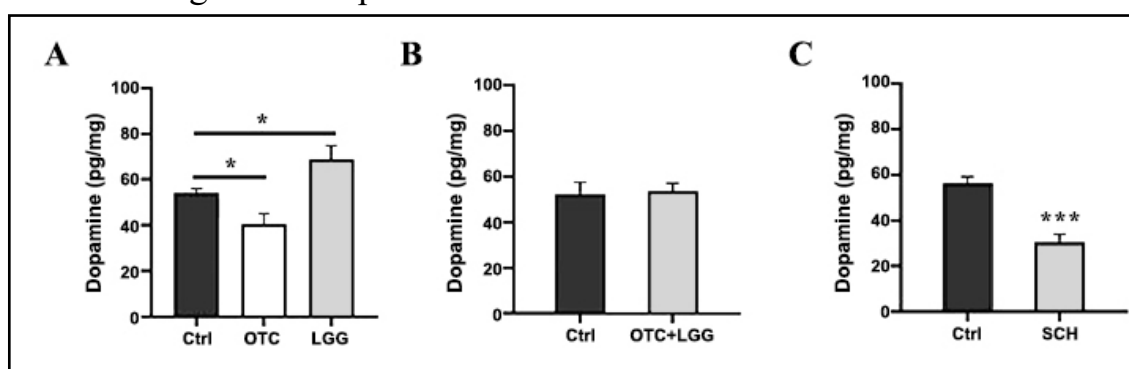255   for eliciting the color preference.

257   **Figure 4.** Dopamine level analysis of the treatment groups. (A) The dopamine levels in

258   the brain of larvae through OTC or LGG treatment. (B) The dopamine levels in the

259   brain of larvae through OTC and LGG treatment. (C) The dopamine levels in the brain

260   of larvae through SCH23390 treatment. Error bars represents the standard error of the

261   mean (SEM) (n=30/group, 3 groups). Statistical significance was set to P<0.05 (∗),

262   P<0.01(∗∗), P<0.001 (∗∗∗).

Gut microbial communities of zebrafish in OTC or LGG treatment group

265   Considering that the intestine is the main area of antibiotic or

266   probiotic absorption and that intestinal health is very important to fish. The

267   gut microorganisms of the OTC or LGG group were identified using 16S

268   rRNA gene sequencing (Miseq) of the total DNA extracted directly from

269   the two treatment groups. A Venn chart at the OTU level showed the

270   similarity of each group with 152 shared members, and the OTC group

271   showed low similarity compared to the Ctrl (Figure 5A). The community

272   diversity (Shannon) and richness (Chao) were evaluated in Figure 5B-C.

273   Compared with Ctrl group, OTC group had lower bacterial diversity (P <

274   0.05), and LGG had higher bacterial diversity (P < 0.01) (Figure 5B). The

275   microbial richness from OTC group had a significant decrease compared

276   with the Ctrl group (P < 0.05), and the LGG group showed an increasing

277   trend with the Ctrl group but this was not statistically significant (P > 0.05).

278   PCoA was used to characterize the distribution of variations in community

279   composition among the three treatments, with most of the variation

280   explained by the first two coordinates (22.5% and 36.7%, respectively,

281   Figure 5D). Samples from the OTC and the LGG treatment group were

282   distinctly separated from the Ctrl group, this result indicated that the OTC

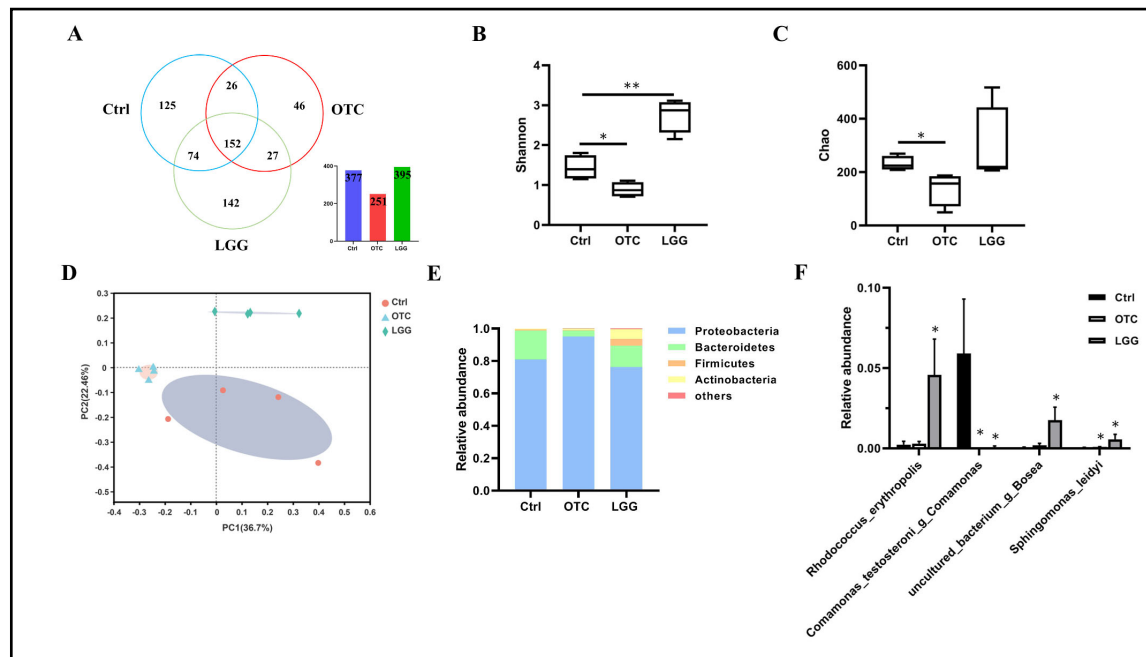283   or LGG had a greater influence on gut bacterial communities.

Figure 5. Effect of OTC or LGG exposure on the intestinal microbial composition. (A) The Venn diagram of the three groups. Shannon (B) and Chao (C) index of gut microbiota in the treatment groups. (D) Principal co-ordinates analysis (PCoA) of intestinal bacteria in the treatment groups. Gut microbiota composition at the phylum level (E) and at the species level (F). Error bars represents the standard error of the mean (SEM). Statistical significance was set to $P<0.05$ (∗), $P<0.01$(∗∗), $P<0.001$ (∗∗∗).

291   The massive sequencing of the gut microbiota revealed that Proteobacteria was the predominant phylum. In Ctrl group, the relative abundances of the four phyla (Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria) were 81.0%, 17.7%, 0.8%, 0.4%, respectively. In the OTC treatment group, the relative abundances of the four phyla were 95.0%, 3.8%, 0.3% and 0.9%, respectively. In the LGG treatment group, the relative abundances of the four phyla were 76%, 6.4%, 6.6% and 9.8%,

16

298   respectively. To assess the relative abundance of species level, the relative

299   abundance of the four species is depicted in Figure 5F.

300   *Rhodococcus_erythropolis* was the most abundant bacterium in LGG

301   group, and had a significant increase compared with control group.

302   *Comamonas_testosteroni_g_Comamonas* was found a significant decrease

303   in OTC or LGG group compared with the control. While a significant

304   increase of relative abundance in *uncultured_bacterium_g_Bosea* was

305   observed in LGG group compared to control group. In addition,

306   *Sphingomonas_leidyi* was also found a significant increase in OTC or LGG

307   group compared to the control. Hence, these data indicated that OTC or

308   LGG exposure could affect the intestinal bacterial communities.

309   **Gut microbial communities of zebrafish in OTC and LGG treatment**

310   **group**

311   To investigate the role of gut microbiota in promoting the recovery of

312   social cohesion and color selection, we analyzed the composition of

313   zebrafish intestinal microbiota by LGG strain supplementary after OTC

314   treatment. As shown in Figure 6A, the similarity between Ctrl group and

315   OTC+LGG group were 162 shared members in a Venn diagram at the OUT

316   level. From Figure 6B-C, the microbial richness (Chao) and community

317   diversity (Shannon) from the OTC+LGG group showed a decreasing trend

318   with the control group but this was not statistically significant (P > 0.05).

319   Samples from Ctrl and OTC+LGG group clustered together in PCoA

320 (Figure 6D), the data indicated that the OTC+LGG group had a lower

321 influence on gut bacterial communities compared with Ctrl group.
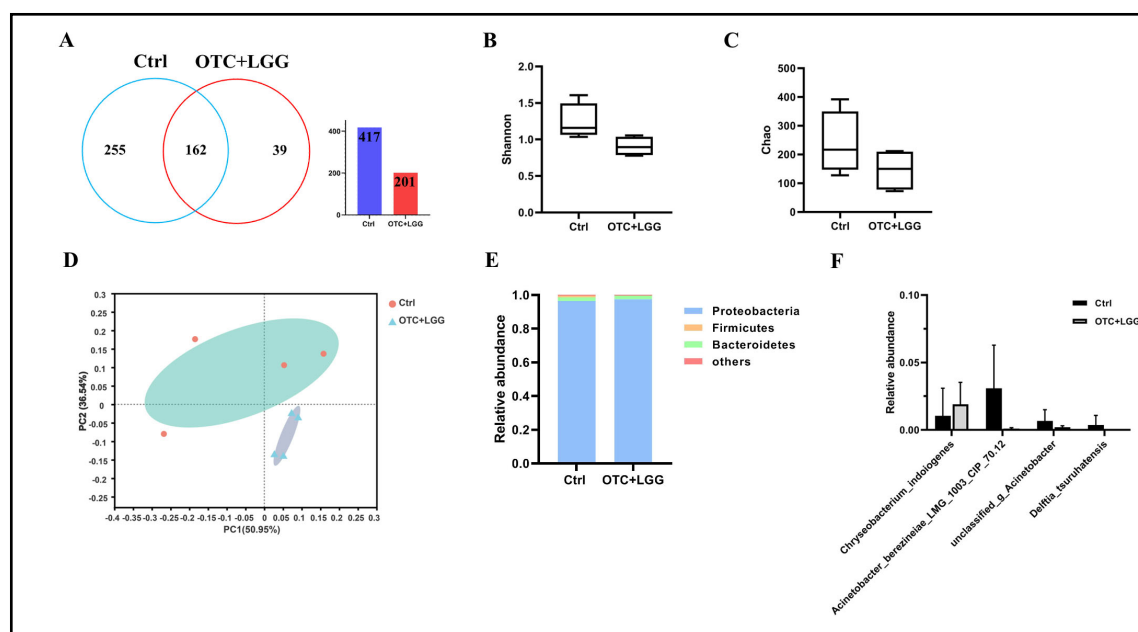

323 **Figure 6.** Effect of OTC and LGG exposure on the intestinal microbial composition.

324 (A) The Venn diagram of the two groups. Shannon (B) and Chao (C) index of gut

325 microbiota in the treatment groups. (D) Principal co-ordinates analysis (PCoA) of

326 intestinal bacteria in the treatment groups. Gut microbiota composition at the phylum

327 level (E) and at the species level (F). Error bars represents the standard error of the

328 mean (SEM). Statistical significance was set to $P<0.05$ ($*$), $P<0.01$($**$), $P<0.001$ ($***$).

329   The relative abundance of gut microbiota between Ctrl group and

330 OTC+LGG group at phylum level is shown in Figure 6E. The dominant

331 phyla in the two groups were Proteobacteria, Bacteroidetes and Firmicutes.

332 In the control group, the relative abundances of the three phyla were 96.5%,

333 2.2% and 2.3%, respectively. The relative abundances of the three phyla in

334 OTC+LGG group were 97.4%, 2.1% and < 0.1%, respectively.

18

335   Additionally, the relative abundance of four species in control and

336   OTC+LGG group was assessed in Figure 6F. The results showed that the

337   relative abundance of four species did not have a significant difference

338   between Ctrl and OTC+LGG group. Therefore, these results suggested that

339   probiotic LGG strain could recovery the gut bacterial composition of

340   zebrafish larvae after the damage by OTC treatment.

341   **Discussion**

342   The shoaling behavior is one of the most robust and consistent

343   behavioral features in zebrafish, which has been observed both in nature

344   (**Engeszer et al., 2007**) and in the laboratory (**Gerlai, 2014**). Fish often

345   forms aggregations and is found mostly in lakes, puddles, ponds, rice fields,

346   ditches and small watercourses (**Spence et al., 2007**). Sociability varies

347   markedly among different living conditions and associates with gut

348   microbiota. OTC is a commonly used antibiotics in human and veterinary

349   medicine (**Rigos and Troisi, 2005**) which has been frequently detected in

350   surface water, groundwater and seawater (**Nie et al., 2013**). Probiotic as a

351   living and supplementary microorganism, are found in foods (e.g., yogurt,

352   snacks, breakfast cereals and infant formulas) (**Suez et al., 2019**). From

353   our results, the social cohesion of larvae was decreased through OTC

354   treatment, and increased during the LGG treatment (Figure 1E). More

355   importantly, the strain LGG could reversed the social deficits after the

356   treatment with OTC (Figure 2D). Our data of the decreased shoaling

357 behavior were consistent with the study by the antibiotic exposure (β-

358 diketone) (**Wang et al., 2016**). Moreover, studies using probiotics (such as

359 *Lactobacillus reuteri, Lactobacillus rhamnosus* IMC 501) have been

360 beneficial in demonstrating a potential role in regulating social behavior

361 (**Buffington et al., 2016; Borrelli et al., 2016**). Therefore, the changes of

362 living conditions through OTC or LGG exposure may influence sociability

363 and social cognition in zebrafish larvae, and probiotic LGG supplementary

364 could recover the social cohesion after the OTC exposure.

365   The intricate living conditions which contain abundant colors and

366 shoaling behavior may affect foraging, predator avoidance and

367 reproductive success. The innate color preference would spur shoal on to

368 emigrate to the environment with a favorable color background that

369 benefits social contact. To investigate color preference of zebrafish shoals

370 in different exposure conditions, we measured the color selection of larval

371 shoals with different treatments. According to our studies in Figure 1F, the

372 control group show a clear and strong innate color preference in shoals of

373 larvae, and the order of RYGB preference was B > Y > G > R. In addition,

374 we found that OTC treatment group lost or weaken their color preference.

375 By contrast, the LGG exposure group could maintain the existence of the

376 innate color preference in shoals. To our surprise, probiotic LGG

377 supplementary could recover color selection after the deficiency caused by

378 OTC (Figure 2E). Although the selections of colors among some color

combinations were a little weakened, the order of BYGR preference was the same with control group (B > G > Y > R). Some results of color preference were consistent with the findings which were reported by Park *et al*. (**2016**) and Peeters *et al.* (**2016**). They used zebrafish larvae (5 dpf) to test the innate color preference in shoals, and found that zebrafish preferred B over R, and B over Y, and B over G. However, literature has emerged that offers contradictory findings about the innate color preference in shoals. The larvae were employed by different treatments did not exhibit abnormal locomotor behavior or behavioral trajectory (Figure 2B-C, Figure 3B-C). A possible explanation for the contradictory conclusions is that the 5 dpf larvae did not develop shoaling behavior (**Buske and Gerlai, 2011; Dreosti et al., 2015; Hinz and de Polavieja, 2017**). Besides, from our results, the order of color preference in Y-G combination was a little difference between the two control groups at different developmental days. Color is perceived primarily through cones in the retia of zebrafish, and fish do not possess yellow cones which can sense yellow light (**Neitz and Neitz, 2011**), so we hypothesized that the deficits of yellow cones may cause the difference during color selection.

As shown in Figure 1E-F and Figure 2D-E, there was a correlation between social cohesion and color discrimination in shoals of larval zebrafish. To investigate the role of social interaction in the innate color preference in shoals, we employed a SCH23390 and analyzed its effects on

401 behavior of color preference. The drug was chosen because D1-R

402 antagonist, SCH23390, is the most abundantly expressed dopamine

403 receptor subtypes in the brain of zebrafish (**Li et al., 2007**). Second,

404 SCH23390 disrupts social preference of zebrafish by decreasing the level

405 of dopamine in dopaminergic system (**Steketee, 1998; Kurata and**

406 **Shibata, 1991**). Finally, the drug is water soluble, and zebrafish can be

407 administered by simple immersion in the drug solution. From our results

408 in Figure 3, the D1-R antagonist treatment which can disrupt the social

409 interaction led to the deficits of color preference in shoals. The fish were

410 employed by SCH23390 did not exhibit abnormal motor or posture (Figure

411 3B-C), and the results of locomotor behavior were consistent with the study

412 by Scerbina *et al.* (**2012**). The dopaminergic system is involved in several

413 brain functions which has been found to be associated with the shoaling

414 tendencies (**Suriyampola et al., 2016**). Dopamine receptors distribute in

415 different brain regions. Clearly, the specific areas are involved in cognition,

416 including hippocampus, the prefrontal cortex, the amygdale, and the

417 ventral and dorsal parts of the striatum. There are four different dopamine

418 receptor subtypes (D1, D2, D3, and D4) in the brain of zebrafish (**Li et al.,**

419 **2007; Boehmler et al., 2004; Boehmler et al., 2007**). Among the different

420 types of dopaminergic receptors, the excitatory D1 receptor (D1-R)

421 subtype is the most predominately expressed in the brain regions

422 (**Fremeau et al., 1991**). D1-R activate the production of intracellular 3'-

423 5'-cyclic adenosine monophosphate (cAMP) through adenylyl cyclase

424 induction and regulate intercellular calcium signaling or protein kinase

425 activity (**Naderi et al., 2016**). Therefore, these results suggested that social

426 interaction can be affected by dopaminergic system, which could be

427 responsible for eliciting the preference for colors.

428 We next set out to identify the dopamine level of different treatments

429 that regulate social cohesion and color discrimination in zebrafish larvae.

430 From our results, we found that OTC decreased the dopamine level in

431 brains of larvae, while LGG increased the dopamine concentration (Figure

432 4A). Meanwhile, LGG recovered the dopamine level after the OTC

433 treatment (Figure 4B). In addition, our data verified the results that

434 SCH23390 severely reduced the dopamine level in the larval brains (Figure

435 4C). These data raised the possibility that OTC or LGG through influence

436 of dopamine level of larval brains was primary responsible for social

437 cohesion and color preference. The dopamine plays key roles in the

438 neurobehavioral functions in zebrafish (**Saif et al., 2013; Shams et al.,**

439 **2018**). For instance, the strong social stimuli will increase the dopamine

440 and DOPAC levels in the brain of the adult zebrafish (**Saif et al., 2013**),

441 and the short-term isolated zebrafish could reduce the level of DOPAC

442 (**Shams et al., 2018**). Several potential mechanisms may be responsible for

443 the decreased the level of dopamine through exposure to the SCH23390.

444 The decreased of dopamine levels imply the reduced dopamine production

445 and/or increased dopamine degradation in response to the employed

446 SCH23390 (**Diop et al., 1988**). Yung *et al.* (**1995**) have shown that D1-R

447 localized in the post-synaptic terminals of neurons in the basal ganglia. The

448 blockade of post-synaptic neurotransmitter receptors may impair signaling

449 downstream and reduce neurotransmitter release. The antagonist of D1

450 receptor could increase the concentration of dopamine in the synaptic cleft

451 which lead to reuptake and leakage to extra-synaptic areas. The increased

452 extra-synaptic dopamine could activate dopaminergic autoreceptors on the

453 pre-synaptic neuron and inhibit the dopamine synthesis (**Tissari and**

454 **Lillgals, 1993**). Taken together, these studies and our own suggest the role

455 of dopamine level in social interaction and innate color preference of

456 shoals, with the deficits of sociability and color discrimination linked to

457 the decreased dopamine level in the brain of zebrafish.

458   The altered microbiota, specifically the changes of microbiota that

459 resides in the gastrointestinal system, may influence neurodevelopment

460 and programming of social behaviors (**Sherwin et al., 2019**). Indeed, the

461 antibiotic OTC treatment was associated with a reduction in gut microbiota

462 diversity (Shannon) and richness (Chao) (Figure 5B-C). Moreover, under

463 conditions of OTC exposures, the relative abundance of microbiota on

464 phylum level or in species level was significantly influenced by antibiotic

465 (Figure 5E-F). By contrast, probiotic LGG could promote the increase of

466 gut microbiota diversity and richness (Figure 5B-C), and the relative

467   abundance of microbiota on phylum level (Bacteroidetes, Firmicutes,

468   Actinobacteria) or on species level was increased by LGG treatment

469   (Figure 5E-F). Although antibiotic administration often perturbs

470   microbiota, modulation of gut bacteria through probiotic strain LGG

471   supplementary can have an advantageous result (Figure 6). The intestinal

472   microbiota can signal to the brain which may affect behavioral processes

473   of sociability via numerous pathways (the microbiota-gut-brain axis),

474   including production of microbial metabolites, immune activation,

475   activation of the vagus nerve, and production of various neurotransmitters

476   (**Sherwin et al., 2019**). Gut microbiota produced vast metabolites through

477   the pathway of microbial metabolites, such as volatile carboxylic acids,

478   esters, neurotransmitters (e.g., dopamine), and fatty acids, some of which

479   may influence brain physiology and behavior (**Roshchina, 2016**).

480   Additionally, in vitro studies indicated that certain bacteria had the capacity

481   to produce neurotransmitters such as noradrenaline, dopamine, and -

482   aminobutyric acid (**Taj and Jamil, 2018; Marques et al., 2016**). Some

483   researches indicated that the gut microbiota could influence serotonergic

484   neurotransmission by regulating the availability of its precursor,

485   tryptophan (**Clarke et al., 2013**). Thus, these data raised the possibility that

486   microbiota-driven neurotransmitters (dopamine) mediated both social

487   recognition and color discrimination, with the pathway of microbial

488   metabolites having an important role in the production of dopamine.

Materials and methods

Zebrafish husbandry and embryo collection

Zebrafish (AB strain) used in this study were obtained from breeding center at University of Science and Technology of China. Zebrafish were maintained at 28 ± 0.5 ℃ with a 14 h light/10 h dark cycle (room fluorescent light, 08:00 am-22:00 pm). The pH and conductivity in circulating water of the aquarium were 7.0-7.4 and 1500-1600 μs/cm, respectively. All adult zebrafish were fed fresh brine shrimps twice a day, at 09:00 am and 14:00 pm. Prior to the experiment, the embryos were collected from spawning healthy adults. To obtain the normal fertilized embryos for tests, the dead embryos and sundries were cleared.

Antibiotic and SCH23390 exposure, probiotic culture and exposure

The OTC as a common antibiotic, was purchased from Aladdin (Shanghai, China). OTC was dissolved in water with a concentration at 100 mg/L as a stock solution. Based on the available literature on the concentration of antibiotic which can affect shoaling behavior, the concentration of OTC used in the present study was set at 500 μg/L (**Wang et al., 2016**). The D1-receptor antagonist SCH23390 (R-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-1-ol; Cat # D054; Sigma-Aldrich) was applied to treat individuals which significantly reduced the amount of dopamine in the brain of zebrafish (**Scerbina et al.,**

510   **2012**). Before experiment, fish were placed in drug exposure beaker (0.1

511   mg/L, 500 mL in volume) and remained in D1-receptor antagonist solution

512   for 1 h. Because the 1 h exposure period is sufficiently long for the drug to

513   reach the zebrafish brain through the vasculature of their gills and skin

514   (**Scerbina et al., 2012**). All the animals in the beaker were offered the same

515   conditions (including illumination, temperature and dissolved oxygen)

516   which were identical to the standard aquarium.

517   The probiotic strain LGG was obtained from Bnbio (Beijing, China),

518   and was cultured in MRS medium at 37 °C for 24 h. The fermentation broth

519   was centrifuged at 1000 rpm for 2 min at 4 °C. The strain was obtained and

520   washed twice with sterile PBS buffer and resuspended. The final

521   concentration of LGG was 10^8 CFU/mL and stored at 4 °C. In order to

522   maintain constant OTC or LGG concentration for the period of exposure,

523   the OTC or LGG solutions were renewed with freshly prepared solutions

524   every day.

525   **Locomotor activity test**

526   Locomotor behavior was assessed according to the published protocol

527   (**Zhou et al., 2014**). The larval zebrafish were transferred into 12-well

528   plates with one larva and 2 mL system water for each well. The individuals

529   were acclimated to the recording condition for 5 min. After the acclimation,

530   locomotor behavior was tested in a 10-min period. The trajectory of the

531   larva was recorded by industrial camera (Guangdong, China) and the

532 videos were analyzed using LSRtrack and LSRanalyze software which was

533 described by Zhou *et al.* (**2014**). The velocity was recorded to assess motor

534 function.

535 **Shoaling behavioral assay**

536 To test the social behavior, a four-fish assay was used to measure the

537 social interaction of zebrafish larvae in a 6-well plate according to Kyzer

538 *et al.* (**2012**). Briefly, 24 normal larvae were randomly selected from each

539 treatment and transferred to the 6-well plate with each well containing 4

540 larvae and 5 mL water. After the acclimation of 5 min, the shoaling

541 behavior was video-recorded for 10 min, and analyzed using 10

542 screenshots made every 1 min during the recording period. Each screenshot

543 was calibrated to the size of the 6-well plate and measured the distances

544 between each fish in the group using Image J software. The shoal size area,

545 nearest distance, farthest distance, and average distance were calculated to

546 assess the shoaling behavior (**Shen et al., 2020; Borrelli et al., 2016**).

547 **Color preference test**

548 The color-enriched conditional place preference (CPP) apparatus, is a

549 customized fish tank (20 cm length × 10 cm width × 10 cm height), colored

550 with four color combinations (red (R), green (G), yellow (Y) and blue (B)).

551 To create the preference for two colors, the CPP tank was divided into two

552 compartments which were covered with the corresponding colors on all

553 side except the top. A video camera (NVH-589MW; Wang Shi Wu You

554   Corporation; China) was placed above the CPP tank for vertical video

555   tracking. The color preference apparatus was placed over the LED light

556   panel to ensure the light source could cover the whole tank. The detailed

557   apparatus has been described in our previous work (**Wang et al., 2014**). To

558   test the color preference of shoals in zebrafish larvae, the experimental

559   tanks were poured into 500 mL fresh fish water. There was no physical

560   barrier between the two compartments and the 20-larval zebrafish (a shoal)

561   could swim freely in the entire tank. After 5-min adaption, the proportion

562   of numbers stayed in each colored zone was recorded every 20 s for 10-

563   min experiment. The outside of color-enriched CPP tank was opaque to

564   prevent external visual interference from all direction. To minimize the

565   effect of noise, the experimental room was closed and kept quiet, and the

566   experimenter was not visible to the fish during the recording.

567   **Dopamine content analysis**

568   Fish were decapitated and the brains of each treatment groups were

569   dissected on ice, and frozen in a microcentrifuge tube (30 brain per tube)

570   at -80 °C. The level of dopamine was measured by ELISA according to the

571   manufacturer's (Wuhan Xinqidi Biological Technology Co. Ltd., China)

572   instructions. Briefly, the pre-cooled phosphate buffered saline (PBS, 1:9,

573   m/v) was added to the brain tissues of larvae, the tissues were ground and

574   centrifuged for 2 min at 5000 g to obtain the supernatant. The standards

575   and the supernatant of each sample were added to each well of 48-well

576   plates precoated with primary antibodies. The plates were incubated at 37 ℃

577   for 60 min after adding enzymeconjugate, and then was rinsed with

578   distilled water. During 30 min of the chromogenic reaction, the Optical

579   Density (O.D.) was measured at 450 nm by a microtiter plate reader

580   (Thermo, USA). The concentration of dopamine (pg/mg brain tissue) is

581   calculated by comparing the O.D. of the samples to the standard curve.

582   **Bacterial genomic DNA extraction**

583   In order to study the bacterial composition analysis, total bacterial

584   DNA was isolated from 30 larvae per treatment group by using the E.Z.N.A.

585   ® Soil DNA Kit (OMEGA, USA). DNA yield was measured in a NaNo

586   DROP 2000 Spectrophotometer (Thermo, USA).

587   **Illumina high-throughput sequencing of barcoded 16S rRNA genes**

588   The V3-V4 region of the 16S rRNA gene was amplified with the

589   primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-

590   GGACTACHVGGGTWTCTAAT-3'). PCR reactions were performed in a

591   reaction mixture containing (20 µL), 4 µL of 5 ×Fast Pfu Buffer, 2 µL of

592   2.5 mM dNTP, 0.8 µL of 5 µM Forward Primer, 0.8 µL of 5 µM Reverse

593   Primer, 0.4 µL of FastPfu Polymerase and 10 ng of template DNA. The

594   PCR consisted of an initial denaturation time of 3 min at 95 ℃ followed

595   by 29 cycles (95 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 45 s), and the

596   final extension period lasted for 10 min at 72 ℃. PCR products were

597   purified and were subjected to Illumina based high-throughput sequencing

598   (Majorbio Bio-Pharm Technology, Co., Ltd., Shanghai, China).

599   **Bioinformatics analysis**

600   Alpha diversity was calculated by using Phylogenetic Distance over

601   OTU counts (**Faith, 1992**), and the beta diversity was estimated by using

602   Unifrac metric (**Lozupone et al., 2006**). Taxonomic richness and diversity

603   estimators were determined based on OTU abundance matrices in Mothur

604   (Version v.1.30.1), Chao was used to assess the community richness, and

605   the diversity was reflected by Shannon. Principal co-ordinates analysis

606   (PCoA) was the distance matrix and performed by using a MATLAB

607   R2016a environment. The data in taxonomy of intestinal bacterial

608   communities were presented as mean ± standard error of the mean (SEM).

609   Statistical analysis was performed by the two-tailed t test.

610   **Statistical analysis**

611   All experimental results were expressed as the means ± standard error

612   of the mean (SEM) and analyzed by an independent t-test using the SPSS

613   statistics program. Significance was set at $p < 0.05$ for all the experiments.

614

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