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1 **Anti-diarrheal drug loperamide induces dysbiosis in zebrafish microbiota**  
2 **via bacterial inhibition**

3

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14

15 **Keywords:** zebrafish, microbiota, host-microbe system, dysbiosis, loperamide, colonization,  
16 antibacterial activity

17 **Abstract**

18 ***Background***

19 Perturbations of animal-associated microbiomes from chemical stress can affect host  
20 physiology and health. While dysbiosis induced by antibiotic treatments and disease are well  
21 known, chemical, non-antibiotic drugs have recently been shown to induce changes in  
22 microbiome composition, warranting further exploration. Loperamide is an opioid-receptor  
23 agonist drug widely prescribed drug for treating acute diarrhea in humans. Loperamide is also  
24 used as a tool to study the impact of bowel dysfunction in animal models by inducing  
25 constipation, but its effect on host-associated microbiota is poorly characterized.

26 ***Results***

27 We used conventional and gnotobiotic larval zebrafish models to show that in addition to host-  
28 specific effects, loperamide also has anti-bacterial activities that directly induce changes in  
29 microbiota diversity. This dysbiosis is due to changes in bacterial colonization, since germ-free  
30 zebrafish mono-reconventionalized with bacterial strains sensitive to loperamide are colonized  
31 up to 100-fold lower when treated with loperamide. Consistently, the bacterial diversity of  
32 gnotobiotic zebrafish colonized by a mix of representative bacterial strains is affected by  
33 loperamide treatment.

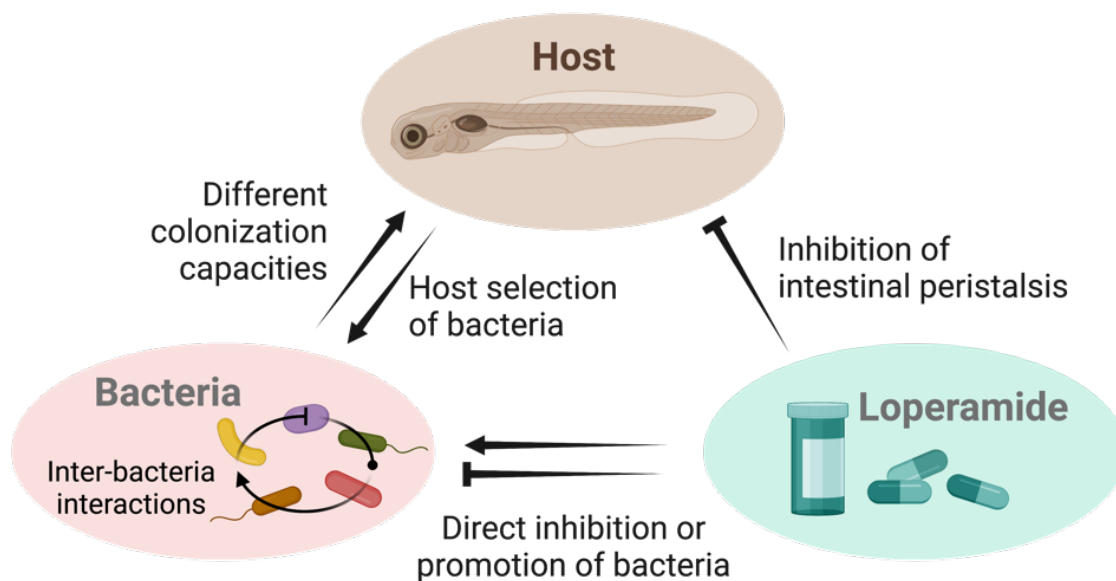
34 ***Conclusion***

35 Our results demonstrate that loperamide, in addition to host effects, also induces dysbiosis in a  
36 vertebrate model, highlighting that established treatments can have overlooked secondary  
37 effects on microbiota structure and function. This study further provides a insights for future  
38 studies exploring how common medications directly induce changes in host-associated  
39 microbiota.

40

41 **Graphical Abstract**

42



43

44

## 45 **Background**

46

47 Animal-associated microbiomes are dynamic communities that play essential roles in the  
48 physiology, health, and evolution of their hosts [1]. Numerous studies have explored the impact  
49 of different phenomena on microbiota stability, including antibiotic treatments, gut health, or  
50 environmental factors, in order to understand the consequences of microbiota perturbations on  
51 host functions [2–5]. These perturbations may lead to dysbiosis or a change in microbial  
52 community composition and/or function, relative to the steady state, with potential implications  
53 for host health [6, 7].

54

55 While the complexity of microbiota in humans and animal models limits functional and  
56 mechanistic studies, germ-free and gnotobiotic animal models with controlled, tractable  
57 microbiota are widely used to study host-microbiota interactions [8]. Compared to conventional  
58 animals with relatively variable microbiota [9], gnotobiotic animals with host-specific bacterial  
59 consortia can mimic key phenotypes for mechanistic studies, and are powerful tools to simplify  
60 microbiota and increase experimental reproducibility [10]. In particular, zebrafish (*Danio*  
61 *rerio*), which possesses both an innate and adaptive immune system and a mammal-like  
62 intestinal epithelium, has emerged as an established gnotobiotic model to study vertebrate host-  
63 microbiota interactions [11, 12]. Gnotobiotic larval zebrafish can indeed be easily reared to  
64 study simplified host-microbial systems in the context of developmental biology, immunology,  
65 and disease [13, 14].

66

67 Loperamide is a prevalent medication for treating diarrhea in humans and animals that acts on  
68  $\mu$ -opioid receptors in the large intestine, decreasing intestinal peristaltic activity and increasing  
69 the absorption of fluids [15–18]. Loperamide is also used to study bowel dysfunction and  
70 constipation in animal models, including rats, mice, and zebrafish, generating a relevant model  
71 of irritable bowel syndrome or opioid-induced bowel dysfunction disorder [19–21]. In  
72 zebrafish, loperamide treatment was shown to cause a significant decrease in intestinal  
73 peristaltic frequency that can be restored by the presence of specific bacteria or acetylcholine  
74 [21, 22].

75

76 Despite its pervasive use in humans and animal models, the potential effects of loperamide on  
77 host-associated microbiota *in vitro* and *in vivo* are poorly characterized. It has been suggested  
78 that slow transit time and constipation induced by loperamide could be responsible for changes

79 in bacterial composition and decreased diversity observed in rats and mice [23–26]. Although  
80 publications using loperamide to investigate host-associated microbiota establish the link  
81 between constipation and microbial dysbiosis, recent studies have identified loperamide  
82 hydrochloride and its derivatives as molecules displaying bactericidal activity [27–29]. Hence  
83 the extent of microbial dysbiosis directly caused by this compound versus its impact on the  
84 alteration of host function are poorly understood and ignored in animal models.

85

86 In this study, we used conventional and gnotobiotic larval zebrafish to reproduce *in vivo*  
87 loperamide-induced dysbiosis based on *in vitro* bacterial sensitivity to loperamide. We found  
88 that loperamide leads to recoverable dysbiosis in conventional larval zebrafish according to  
89 strain-specific inhibition or promotion of bacteria. Our results demonstrate how a relevant  
90 chemical perturbation induces dysbiosis in a vertebrate microbiome model. These findings  
91 should be considered in the context of secondary effects of established treatments, assumed  
92 mode of action in animal models, and microbiota community recovery.

## 93 **Methods**

94

### 95 ***General zebrafish husbandry***

96 Wild-type AB/AB zebrafish (*Danio rerio*) fertilized eggs at 0 days post fertilization (dpf) were  
97 obtained from the Zorngl'hub platform at Institut Pasteur. All procedures were performed at  
98 28°C under a laminar microbiological hood with single-use disposable plastic ware according  
99 to European Union guidelines for handling of laboratory animals and were approved by the  
100 relevant institutional Animal Health and Care Committees. Eggs were kept in 25 cm<sup>3</sup> vented  
101 flasks (Corning 430639) with 20 mL of autoclaved mineral water (Volvic) until 4 dpf (30 – 33  
102 eggs/flask) and transferred to new flasks after hatching at 4 dpf (10 – 15 fish/flask). At 6 dpf,  
103 each fish was transferred to an individual well of a 24-well plate (TPP 92024) in 2 mL of  
104 autoclaved mineral water and maintained until the end of the experiment (11 dpf). Conventional  
105 zebrafish embryos were transferred to flasks at 1 dpf and maintained as described. At the end  
106 of the experiment, zebrafish were euthanized with an overdose of tricaine (MS-222, Sigma-  
107 Aldrich E10521) at 0.3 mg/mL for 10 minutes.

108

109 Fish were fed with sterile *Tetrahymena thermophila* every 48 hours starting at 4 dpf. Germ-free  
110 *T. thermophila* stocks were kept in 15 mL of PPYE broth (0.25% protease peptone BD Bacto  
111 #211684, 0.25% yeast extract BD Bacto #212750) supplemented with penicillin G (10 unit/mL)  
112 and streptomycin (10 µg/mL) at 28°C. Every week, a new stock was inoculated with 100 µL of  
113 the previous stock and tested for sterility on LB, TYES, and YPD agar media plates. To prepare  
114 food for the zebrafish, *T. thermophila* was inoculated at a 1:50 ratio from the stock into 20 mL  
115 MYE broth (1% milk powder, 1% yeast extract) and grown for 2 days. On feeding day, the *T.*  
116 *thermophila* was transferred to a 50 mL Falcon tube and washed 3 times (4400 rpm, 3 min at  
117 25 °C) with sterile mineral water. Resuspended *T. thermophila* was added to the fish in culture  
118 flasks (500 µL in 20 mL) or 24-well plates (50 µL in 2 mL).

119

### 120 ***Germ-free zebrafish sterilization***

121 The zebrafish embryos were sterilized as previously described with the following modifications  
122 [13, 30]. Recently fertilized zebrafish eggs (0 dpf) were bleached (0.000005 % final v/v) for 5  
123 minutes, then washed 2 times in sterile mineral water. Eggs were then maintained in 50 mL  
124 Falcon tubes (100 eggs/tube) overnight in 35 mL of sterile mineral water supplemented with  
125 0.4 µg/mL methylene blue solution (Sigma Aldrich 50484). At 1 dpf, the volume of each tube  
126 was adjusted to 50 mL and the eggs were treated with an antibiotic cocktail for 2 hours with

127 gentle agitation at 10 rev/min: penicillin G: streptomycin at 100 µg/mL (GIBCO 15140148),  
128 kanamycin sulfate at 400 µg/mL (PAN BIOTECH P06-04010P) and amphotericin B solution  
129 at 250 µg/mL (Sigma-Aldrich A2942). Then, the eggs were washed 3 times with sterile mineral  
130 water and resuspended in 50 mL water. The eggs were bleached (0.000005 % final v/v) for 15  
131 minutes with inversion every 3 minutes, then washed 3 times in sterile mineral water and  
132 resuspended in 50 mL water. Finally, the eggs were treated with 1 % Romeoid solution (COFA,  
133 France) for 10 minutes, then washed 3 times in sterile mineral water. Eggs were then transferred  
134 to 25 cm<sup>3</sup> vented flasks and maintained as described above.

135

136 Sterility was confirmed at 3 dpf by spotting 50 µL of water from each flask on LB, TYES and  
137 YPD agar plates and incubated at 28 °C under aerobic conditions for at least 3 days. In addition,  
138 monthly checks of bacterial contamination were done by PCR amplification of water samples  
139 with 16S rRNA gene primers as described below in the characterization section. Contaminated  
140 flasks were immediately removed from the experiment and not included in the results.

141

#### 142 ***Germ-free zebrafish re-conventionalization***

143 Germ-free zebrafish larvae were re-conventionalized at 4 dpf, as follows. Overnight cultures of  
144 a single bacterial colony in 5 mL of liquid media were washed twice with sterile Volvic water  
145 and normalized to OD-0.1 in water. For mono-reconventionalization, 200 µL of bacterial  
146 suspension was added into flasks of germ-free zebrafish in 20 mL of Volvic water at a final  
147 concentration of 5 x 10<sup>5</sup> CFU/mL. For mix-reconventionalization, 200 µL of each strain was  
148 added per flask at a final concentration of 5 x 10<sup>5</sup> CFU/mL per strain. Water samples were  
149 plated in serial dilutions to confirm final bacterial concentration and sterility. Re-  
150 conventionalization was performed for 48 hours until fish were transferred to sterile water in  
151 24-well plates.

152

#### 153 ***Zebrafish loperamide treatment***

154 Loperamide hydrochloride (Sigma-Aldrich 34014) was dissolved in pure dimethyl sulfoxide  
155 (DMSO, Sigma-Aldrich D8418) at a stock concentration of 100 mg/mL. Larval zebrafish were  
156 treated at 5 dpf with loperamide at a final concentration of 10 mg/L in 20 mL vented flasks for  
157 24 hours, which has been previously shown to significantly reduce peristaltic movement in  
158 larval zebrafish at 4 - 6 dpf [21]. Sterile DMSO added at a final concentration of 1:10000 was  
159 used as the control. After 24 hours of treatment, all 6 dpf fish were transferred to water and  
160 maintained until sampling.



161

162 ***Conventional zebrafish sampling and DNA extraction***

163 Zebrafish larvae were sampled at each of 3 timepoints (6 dpf, 7 dpf, 11 dpf) with 3 treatment  
164 conditions (control water, DMSO 1:10000, Loperamide 10 mg/L) as follows. At each  
165 timepoint, 5 larval fish per condition (15 total) were washed twice by transfers to clean, sterile  
166 water to remove environmental and residual bacteria. Each fish was then added to a sterile 2-  
167 mL microcentrifuge tube in 200  $\mu$ L of water and euthanized with tricaine at 0.3 mg/mL. All  
168 liquid was removed from the tissue, then the samples (45 total) were immediately frozen at -  
169 80°C and stored until DNA extraction.

170

171 DNA extraction was performed from single larval zebrafish using the DNeasy Blood & Tissue  
172 kit (Qiagen 69504) with modifications as follows. Tissue samples were thawed at room  
173 temperature, then 380  $\mu$ L Buffer ATL and 20  $\mu$ L proteinase K were added directly to each  
174 individual larva in a 2 mL tube. Samples were vortexed for 15 seconds, then incubated  
175 overnight (15-18 hours) at 56°C and 300 rpm until fully lysed. After lysis, 4  $\mu$ L of RNase A  
176 solution was added and the samples were incubated for 5 minutes at room temperature to  
177 remove residual RNA. Next, 400  $\mu$ L Buffer AL and 400  $\mu$ L 100 % ethanol were added and  
178 mixed by vortexing before loading the lysate onto the DNeasy mini spin column in 2 x 600  $\mu$ L  
179 loads. DNA purification and cleanup proceeded according to the manufacturer's  
180 recommendations with a final elution volume of 50  $\mu$ L in Buffer AE. Purified DNA was  
181 quantified using the Qubit HS DNA fluorometer kit (ThermoFisher Q32851) and purity was  
182 assessed with the Nanodrop spectrophotometer (ThermoFisher). DNA yields per single fish  
183 sample ranged from 10-15 ng/ $\mu$ L in 50  $\mu$ L with purity ratios >1.8. Negative controls for the  
184 extraction kit were prepared alongside zebrafish samples, but with no tissue input.

185

186 ***Conventional fish 16S rRNA gene amplicon sequencing and analysis***

187 16S rRNA gene amplicons of the V6 region for the 45 conventional zebrafish samples, 2 mock  
188 community samples (Zymo Research DNA standard I D6305), 2 negative DNA extraction  
189 samples, and blank PCR control were prepared using 967F/1064R primers. The DNA extraction  
190 negative control samples were pooled and concentrated prior to PCR to obtain enough product  
191 for sequencing. A two-step PCR reaction using 200 ng of zebrafish DNA was performed in  
192 duplicate 50  $\mu$ L reactions as previously described [31, 32]. Each first step reaction included 25  
193  $\mu$ L 2X Phusion Mastermix (Thermo Scientific F531S), 1.5  $\mu$ L of 10  $\mu$ M F/R primer mix (967F:  
194 CTAACCGANGAACCTYACC, CNACGCGAAGAACCTTANC,

195 CAACGCGMARAACCTTACC, ATACGCGARGAACCTTACC (equimolar mix) / 1064R:  
196 CGACRRCCATGCANCACCT), 13 - 20  $\mu$ L template DNA (200 ng), and 3.5 – 10.5  $\mu$ L  
197 nuclease-free water (up to 50  $\mu$ L). PCR amplification (step 1) conditions were denaturing at  
198 98°C for 3 min followed by 30 cycles of denaturation at 98°C for 10 s, primer annealing at  
199 56°C for 30 s, and extension at 72°C for 20 s, then a final extension at 72°C for 20 s. Negative  
200 controls for the PCR reagents were prepared alongside zebrafish DNA samples, but with  
201 additional nuclease-free water input. PCR products were assed for concentration (Qubit DNA  
202 HS reagents) and expected size using agarose gel electrophoresis. A second PCR step was  
203 performed to attach sequencing barcodes and adaptors according to Illumina protocols. The  
204 PCR products were analyzed with 250 bp paired-end sequencing to obtain overlapping reads  
205 on an Illumina MiSeq at the Institut Pasteur Biomics platform.

206  
207 The resulting 16S rRNA gene amplicon sequences were demultiplexed and quality filtered  
208 using DADA2 (v1.6.0) implemented in QIIME2 (v2020.11.1) with additional parameters --p-  
209 trunc-len-r 80 --p-trunc-len-f 80 --p-trim-left-r 19 --p-trim-left-f 19 to determine amplicon  
210 sequence variants (ASVs) [33, 34]. All ASVs were summarized with the QIIME2 pipeline  
211 (v2020.11.1) and classified directly using the SILVA database (99 % similarity, release #134)  
212 [35, 36]. Processed ASV and associated taxonomy data was exported as a count matrix for  
213 analysis in R (v4.1.3). The positive and negative controls were checked to ensure sequencing  
214 quality and expected relative abundances. Non-bacterial and chloroplast sequences were then  
215 removed, and the data was normalized by percentage to the total ASVs in each sample for  
216 further dissimilarity metric analysis.

217  
218 All descriptive and statistical analyses were performed in the R statistical computing  
219 environment with the *tidyverse* v1.3.1, *vegan* v2.5.7 and *phyloseq* v1.38.0 packages [37–39].  
220 Rarefaction curves and sequencing coverage estimates were generated using the `rarecurve()`  
221 commands with `sample=[number of reads in smallest sample]` in *vegan* v2.5.7 [40]. Non-metric  
222 dimensional analysis (NMDS) was used to determine the influence of timepoint or loperamide  
223 treatment on the ASV-level composition. The Bray-Curtis dissimilarity metric was calculated  
224 with `k = 2` for max 50 iterations and 95 % confidence intervals (standard deviation) were  
225 plotted. Statistical testing of the beta-diversity was done using the PERMANOVA *adonis2* test  
226 implemented in *vegan* (`method = "bray", k = 2`) [41, 42]. Within-condition variability was  
227 calculated using the command `vegdist(method = "bray", k = 2)` and the matrix was simplified  
228 to include samples compared within each timepoint.

229

230 Significant differences in genera between DMSO (reference) and loperamide-treated (test) at  
231 each timepoint were calculated using *limma* implemented in the *microbiomeMarker* v1.1.2  
232 package using the following conditions: norm = "RLE", pvalue\_cutoff = 0.05, taxa\_rank =  
233 "Genus", p\_adjust = "fdr" [43–45]. Simpson's diversity values were calculated for each sample  
234 at the ASV level using the *vegan* package and analyzed using the non-parametric Kruskal–  
235 Wallis rank sum test in R. Additional visualizations were computed using the *ComplexHeatmap*  
236 v2.10.0 and *UpSetR* v1.4.0 packages [46, 47]. All processed sequencing files, bash scripts,  
237 QIIME2 artifacts, and Rmd scripts to reproduce the figures in the manuscript are available on  
238 Zenodo [48].

239

### 240 ***Measurement of zebrafish growth and development***

241 In order to determine the effect of loperamide growth on larval fish growth and development,  
242 9-10 fish were sampled at each timepoint (6, 7, 11 dpf) for each condition (control water,  
243 DMSO, loperamide) = 85 fish total. After euthanasia, the samples were fixed in 1 %  
244 paraformaldehyde (PFA) and stored at 4°C. After fixation, the samples were rinsed 3 times with  
245 PBS then placed into individual wells in a plate. Microscopy images were taken with a Leica  
246 M80 10X with a Leica IC80 HD camera. Four images were captured per sample: whole at 2.5X,  
247 caudal at 5X, lateral at 5X and head at 5X for a total of 337 images for 85 samples. Relevant  
248 measurements of each fish sample were performed using ImageJ [49]. Four measurements in  
249 millimeters were taken per fish: eye diameter, rump-anus length, standard length, and tail width  
250 according to methods previously described [50–52].

251

### 252 ***Bacterial strains and growth conditions***

253 Bacterial strains are listed in Supplementary Table S1. Zebrafish-associated strains were grown  
254 in Tryptone Yeast Extract Salts (TYES) or Miller's Lysogeny Broth (LB) (Corning) and  
255 incubated at 28°C with rotation. Cultures on solid media were on LB or TYES with 1.5 % agar.  
256 Bacteria were always streaked from glycerol stocks on LB- or TYES-agar before inoculation  
257 with a single colony in liquid cultures. All media and chemicals were purchased from Sigma-  
258 Aldrich.

259

### 260 ***Isolation and 16S characterization of bacteria from conventional zebrafish***

261 Five of the zebrafish-associated strains were previously isolated and characterized from the  
262 zebrafish environment [53]. The following strains were isolated and identified in the same way

263 in this study: S2, S4, S8, S9. Zebrafish lysates and tank water were serially diluted and plated  
264 on R2A, TYES, and LB agar and incubated at 28°C for up to 3 days. Each colony morphotype  
265 per media was catalogued and re-streaked on the same agar. The morphotype identification was  
266 done as previously described [53, 54]. Individual colonies were picked for each morphotype  
267 from each agar plates, vortexed in 200 µl DNA-free water and boiled for 10 min at 90°C. Five  
268 µl of this bacterial suspension was used as template for colony PCR to amplify the 16S rRNA  
269 gene with the universal primer pair 27f and 1492R. 16S rRNA gene PCR products were verified  
270 on 1% agarose gels, purified with the QIAquick PCR purification kit (Qiagen) and two PCR  
271 products for each morphotype were sent for sequencing (Eurofins, Ebersberg, Germany).  
272 Individual 16S rRNA- gene sequences were compared with those available in the EzBioCloud  
273 database. Species-level identification was performed based on the 16S rRNA gene sequence  
274 similarity was >99%. The zebrafish-associated strains used in this study (Table S1) were chosen  
275 from this catalogue based on their sensitivity to loperamide and match with significant changes  
276 in the conventional 16S rRNA gene amplicon data.

277

### 278 ***Bacterial growth curves and survival assays***

279 Overnight cultures of a single bacterial colony in 5 mL of liquid media were measured and  
280 normalized to OD-0.5. Liquid media supplemented with 10 mg/L loperamide in DMSO or  
281 1:10000 DMSO or control was added to a TPP flat-bottom polystyrene 96-well plate. Bacterial  
282 cultures were added to each condition in triplicate at a final starting concentration of OD-0.05  
283 in 100 µL. Negative control wells were included for each media and condition. A plastic  
284 adhesive film (adhesive sealing sheet, Thermo Scientific, AB0558) was used to seal the wells,  
285 and the plates were then incubated in a TECAN Infinite M200 Pro spectrophotometer for 20  
286 hours at 28°C. OD600 was measured every 30 minutes, after a 30-second orbital shaking of 2  
287 mm amplitude.

288

289 Bacterial survival in water was tested using the *in vivo* re-conventionalization conditions  
290 described above. Overnight cultures of a single bacterial colony in 5 mL of liquid media were  
291 washed twice with autoclaved Volvic water, measured and normalized to OD-0.1 in water.  
292 Bacteria were inoculated at a final concentration of  $5 \times 10^5$  CFU/mL into 10 mL of Volvic water  
293 supplemented with loperamide in DMSO at 10 mg/L or 1:10000 DMSO or control. Viable  
294 colony forming units (CFUs) were counted from each flask at 0, 6, 24, 48, and 72 hours as  
295 follows. Three x 200 µL aliquots were sampled and dilutions were made, then 10 µL drops  
296 were plated on LB or TYES and grown at 28°C for 2 days. CFUs were then counted for each

297 strain and CFUs/mL were calculated by  $1000 \mu\text{L}/\text{mL} / 10 \mu\text{L}$  plated \* dilution factor \* (average  
298 of replicate CFUs per strain). Survival of each strain was repeated at least two independent  
299 times.

300

### 301 ***Quantification of gnotobiotic zebrafish bacterial load by CFU counts***

302 Zebrafish were sampled at each of 3 timepoints (6 dpf, 7 dpf, 11 dpf) with 3 treatment  
303 conditions (control water, DMSO 1:10000, Loperamide 10 mg/L). At each timepoint, 3-4 larval  
304 fish per condition were washed twice by 2 transfers to clean, sterile water in petri dishes to  
305 remove environmental and residual bacteria. The larvae were then euthanized with tricaine at  
306 0.3 mg/mL and added in 500  $\mu\text{L}$  of sterile water to 2 mL tubes containing 1.4 mm ceramic  
307 beads (Fischer Scientific 15555799). Fish were homogenized for 2 x 45 seconds at 6000 rpm  
308 using a 24 Touch Homogenizer (Bertin Instruments). These homogenization conditions are  
309 sufficient to lyse zebrafish tissue, but not harmful to the bacteria. The lysate was then diluted  
310 from 10- 100-fold. For the mono-reconventionalized fish, 10  $\mu\text{L}$  drops were plated in triplicate  
311 for each dilution on media. After 2 days of incubation at 28°C, CFUs were counted and CFUs  
312 per fish were calculated by  $500 \mu\text{L}$  lysate /  $10 \mu\text{L}$  plated \* dilution factor \* average of replicate  
313 CFUs. For the mix-reconventionalized fish, 3 x 100  $\mu\text{L}$  from each dilution was spread on media  
314 using sterile glass beads to differentiate the colonies. After 2 days of incubation at 28°C, CFUs  
315 were counted for each strain and CFUs per strain per fish were calculated by  $500 \mu\text{L}$  lysate /  
316  $100 \mu\text{L}$  plated \* dilution factor \* (average of replicate CFUs per strain).

317

### 318 ***Statistical Analyses***

319 All plotting and statistical analyses were performed in the R statistical computing environment  
320 (4.1.3) using RStudio (v.2022.02.1) with the *tidyverse* v1.3.1, *ggpubr* v0.4.0, *ggtext* v0.1.1 and  
321 *patchwork* v1.1.1 packages [39, 55–57]. Non-parametric global Kruskal-Wallis tests and  
322 subsequent Wilcoxon pairwise tests were performed to compare loperamide-treated condition  
323 to the DMSO control using `compare_means()` or `stat_compare_means()` when  $p < 0.05$  is  
324 significant. For the comparison of zebrafish colonization and water survival, mean CFUs/mL  
325 or CFUs/fish of each strain S1 – S10 were calculated for control conditions at 48 h or T0,  
326 respectively. The colonization efficiency for each strain was calculated by *Colonization*  
327 *efficiency* =  $\text{mean CFUs per Fish} / \text{mean Water CFUs per mL} * 100$ . The correlation between  
328 the variables was fit with `geom_smooth(method = "lm")` and the fit was indicated with  
329 correlations using `stat_cor()` and `stat_regline_equation()`. Hypothetical bacterial composition  
330 comparison of mono-reconventionalized fish was calculated by the mean CFUs per fish per

331 strain / the sum of mean CFUs per fish of S1, S3, S5, S6, S7. Bacterial composition comparison  
332 of mix-reconventionalized fish was calculated by the mean CFUs of each strain / the total CFUs  
333 of all strains in each fish. Simpson's diversity values were calculated for each mix-  
334 reconventionalized fish based on percent abundance per strain using the *vegan* package and  
335 analyzed using the non-parametric Kruskal–Wallis rank sum test in R. All raw data and Rmd  
336 scripts to reproduce the figures and statistical tests in the manuscript are available on Zenodo  
337 [48].

338

## 339 **Results**

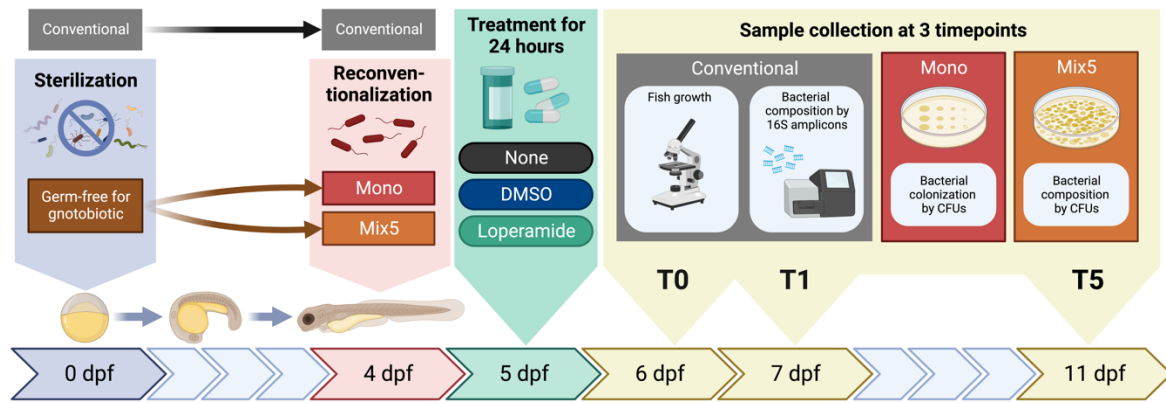
340

### 341 *Loperamide treatment induces recoverable dysbiosis in conventional larval zebrafish* 342 *microbiota*

343 Using the experimental procedure described in Figure 1, we determined the impact of  
344 loperamide treatment on conventional larval zebrafish microbiota using 16S rRNA gene  
345 amplicons sequenced from whole fish samples after 24 hours of treatment (T0), 24 hours of  
346 recovery (T1), and 5 days of recovery (T5). A total of 2,161,882 quality-controlled, bacterial  
347 16S rRNA gene amplicon sequences were analyzed from 45 larval zebrafish samples (Fig.  
348 S1A). Sequence variant analysis using QIIME2 and taxonomic classification resulted in the  
349 detection of 1,186 bacterial genera across 39 phyla, to sufficiently cover the estimated high  
350 diversity in the samples (Fig. S1B). Blank negative control samples were analyzed to confirm  
351 the absence of contamination relative to the zebrafish samples and a sequenced mock  
352 community yielded the expected sequencing proportions (Fig. S2). Proteobacteria was the  
353 dominant phylum in the larval zebrafish microbiota comprising  $75 \pm 17\%$  of the samples,  
354 followed by Bacteroidota ( $9.6 \pm 9.2\%$ ) and Firmicutes ( $5.0 \pm 13\%$ ) (Fig. S3; values averaged  
355 across all samples). The largest group of 100 shared genera was common to all DMSO and  
356 loperamide samples, regardless of timepoint or treatment (Fig. S4B; black bar).

357





**Figure 1. Experimental scheme of the larval zebrafish assays and sample collection.**

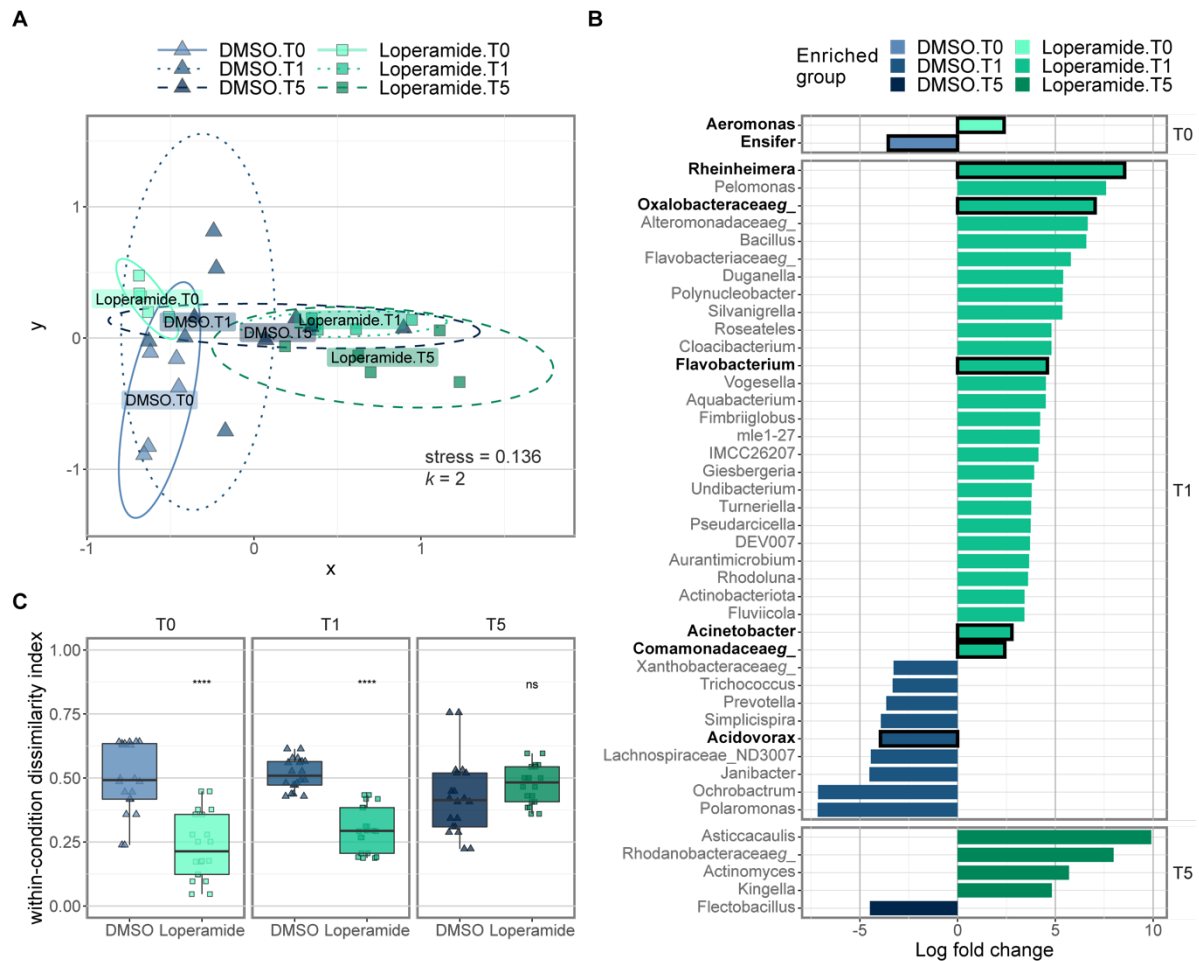
Conventional, mono-conventionalized, or Mix5-conventionalized larval zebrafish were exposed at 5 dpf to water (control), DMSO (control) or 10 mg/L loperamide hydrochloride (treated) for 24 hours, then transferred to water at 6 dpf. Samples were collected at 6 dpf, 7 dpf, and 11 dpf (T0, T1, T5) to measure fish growth and quantify bacterial community composition in all conditions (dpf = days post fertilization; CFUs = colony forming units).

358

359

360 Differences in the conventional zebrafish bacterial community composition were observed  
361 between the timepoints and treatment (Figs. 2, S3, S4, and S5). At T0 and T1, the beta-diversity  
362 of loperamide-treated fish microbiota was significantly different from the DMSO control (Figs.  
363 2A, S5BC; adonis2 PERMANOVA  $R^2 = 0.43, 0.51$ ;  $p < 0.01$ ). However, after 5 days of  
364 recovery (T5), the DMSO and loperamide-treated fish microbiota composition was not  
365 significantly different, indicating a recovery of microbiota composition once the treatment  
366 ended (Fig. 2A, S5D; adonis2 PERMANOVA  $R^2 = 0.22$ ;  $p > 0.05$ ). This recoverable dysbiosis  
367 in microbiota composition induced by loperamide treatment was driven by a decrease in genus  
368 *Ensifer* and an increase in genus *Aeromonas* at T0 (Figs. 2B, S4A). At T1, there were major  
369 significant differences, affecting 37 different taxonomic groups, 6 of them with  $>1\%$   
370 abundance: a 4-log decrease in *Acidovorax* and significant enrichment of *Comamonadaceae*,  
371 *Acinetobacter*, *Flavobacterium*, *Oxalobacteraceae*, and *Rheinheimera* taxa (Fig. 2B). After 5  
372 days of recovery at T5, there were no significantly different genera that were  $>1\%$  abundant in  
373 the conventional zebrafish (Fig. 2B). Loperamide treatment also resulted in significantly  
374 decreased within-group beta-diversity compared to the DMSO control at T0 and T1, but not T5  
375 (Fig. 2C). Despite the differences in bacterial composition and treatment regimen, growth and  
376 development of conventional zebrafish at all three timepoints was not affected by loperamide  
377 treatment (Fig. S6).

378



**Figure 2. Loperamide affects conventional zebrafish microbiota as measured by 16S rRNA gene amplicons. (A)** NMDS plot calculated using Bray-Curtis beta-diversity ( $k = 2$ ) of percent normalized ASVs from 16S rRNA gene amplicons. Ellipse lines show the 95 % confidence interval (standard deviation). Stress = 0.136; adonis2 PERMANOVA  $R^2 = 0.104$ ;  $p = 0.029^*$ . Only DMSO and loperamide-treated samples are shown. **(B)** Significant differentially abundant genera in loperamide-treated fish, compared to DMSO controls at each timepoint calculated using Limma (one-against-one) with conditions: relative log expression (RLE) normalized, effect log fold change  $>2$ , Benjamini & Hochberg adjusted  $p$ -value  $< 0.05$  ( $n = 5$  per condition). Genera that occur at mean percent abundance  $>1\%$  are outlined in black and bold. **(C)** Beta-dispersion or within-condition dissimilarity index calculated using Bray-Curtis beta-diversity ( $n = 20$ ). \*\*\*\*  $p < 0.001$  for Loperamide treatment, compared to DMSO. Wilcoxon test.

379

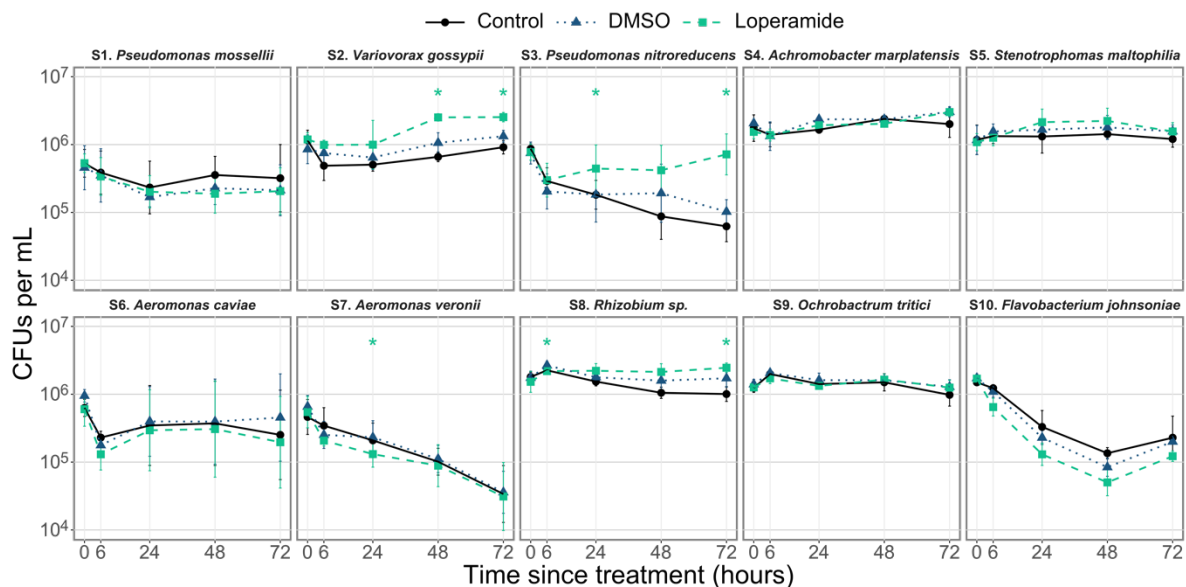
380

381 ***Members of the conventional zebrafish community are inhibited or promoted by loperamide***  
382 ***in vitro***

383 Based on the changes observed in the conventional zebrafish bacterial community, 9 strains  
384 isolated from the zebrafish environment (conventional larvae or rearing water) and a  
385 *Flavobacterium* spp. were tested for their sensitivity to loperamide *in vitro* (Table S1). When



386 grown in rich media in the presence of loperamide, the growth of 8/10 strains was significantly  
387 affected, while S2 *Variovorax gossypii* and S3 *Pseudomonas nitroreducens* were not affected  
388 (Fig. S7). One strain (S1 *Pseudomonas mosselii*) showed increased growth rate and carrying  
389 capacity in the presence of loperamide, compared to DMSO control. All other affected strains  
390 (7/10) showed no growth, delayed growth, slower growth rates or reduced carrying capacity  
391 when grown in media supplemented with loperamide (Fig. S7). In addition to growth, survival  
392 in water according to *in vivo* conditions was tested for the 10 strains by counting daily CFUs  
393 for three days of incubation. Survival of 6 out of 10 strains was not significantly affected by  
394 loperamide in these conditions: S1, S4, S5, S6, S9, and S10 (Fig. 3). Three strains (S2, S3, S8)  
395 showed increased survival in the presence of loperamide, while S7 *Aeromonas veronii* was the  
396 only strain with significantly inhibited survival at 24h.  
397



**Figure 3. *In vitro* survival in water of zebrafish-associated bacterial strains is affected by loperamide.** Survival in water for 72 hours after inoculation at 10<sup>6</sup> CFUs/mL (mean ± standard deviation per condition is shown, n = 6-12; 2-4 independent assays of 3 biological replicates). \* p<0.05 for loperamide treatment, compared to DMSO. Wilcoxon test. Note log scale on y-axis.

398

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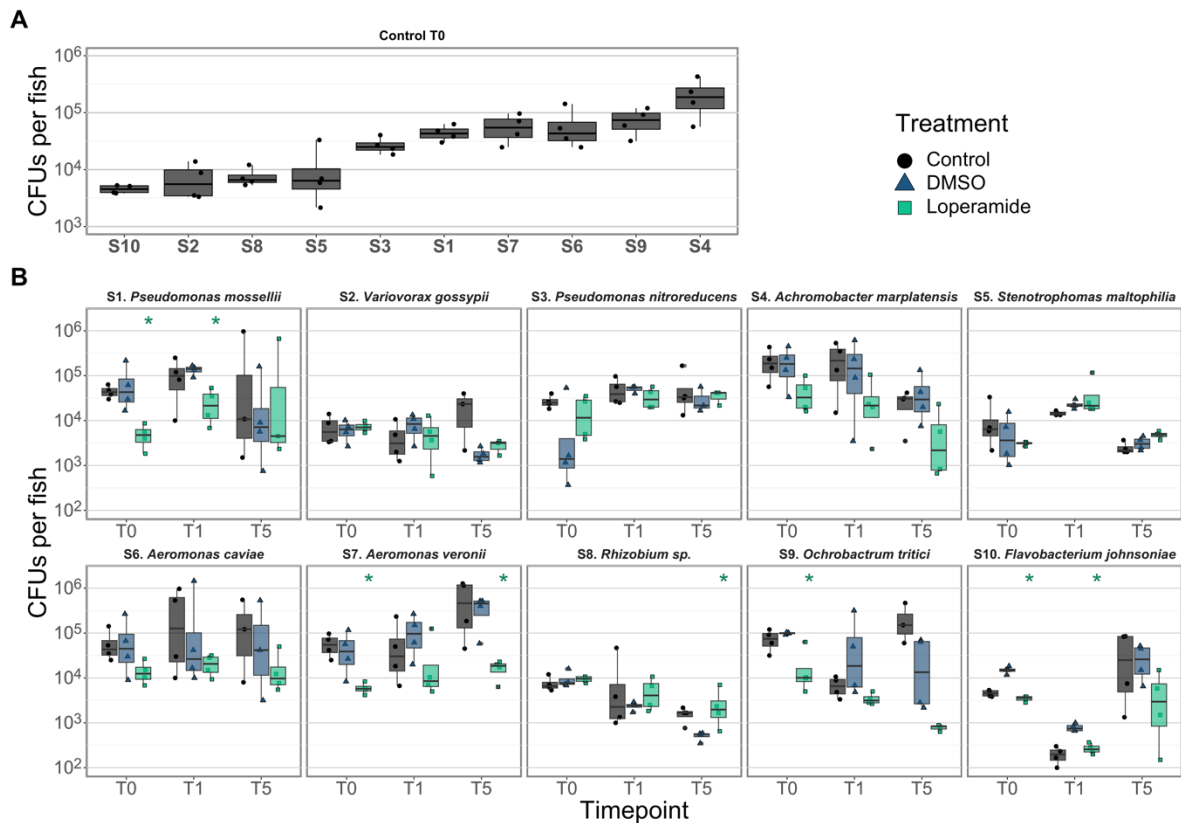
400 ***Individual bacterial colonization of mono-reconventionalized larval zebrafish is strain-***  
401 ***specific and affected by loperamide***

402 In order to test the zebrafish colonization capacity of bacteria and the loperamide effects *in*  
403 *vivo*, 10 bacterial strains were individually added to reconventionalize GF fish and then sampled  
404 at T0, T1, and T5 for whole fish CFU counts. All bacterial strains colonized the zebrafish in

405 control conditions at 6 dpf after 2 days of re-conventionalization at  $10^3$  to  $10^6$  CFUs per larvae  
406 (Fig. 4A). S4 *Achromobacter marplatensis* had the highest bacterial colonization capacity at a  
407 mean of  $2.2 \times 10^5$  CFU/fish, while the bacterial load of larvae reconventionalized with non-  
408 autochthonous S10 *Flavobacterium johnsoniae* was only  $4.6 \times 10^3$  CFU/fish. Overall bacterial  
409 colonization of the zebrafish was on average 10- to 100-fold lower than the number of CFUs  
410 per mL in the water at this time with colonization efficiencies of 0.7 – 52 % (Fig. S8A). Strain  
411 S7 *A. veronii* displayed the highest colonization efficiency with a mean of  $1.1 \times 10^5$  CFUs/mL  
412 in the water, compared to  $5.9 \times 10^4$  CFUs per fish (efficiency = 52.9 %). Conversely, strains  
413 S8, S5, and S2 had colonization efficiencies of ~1% with  $\sim 10^6$  CFUs/mL in the water, compared  
414 to  $\sim 10^4$  CFUs per fish (Fig. S8A). Despite these large strain-specific differences in colonization  
415 efficiency, overall bacterial colonization per fish correlated with number of bacteria in the water  
416 at the time of sampling (Fig. S8B;  $R^2 = 0.69$ ,  $p = 0.03^*$ ).

417  
418 The addition of loperamide led to a reduction or increase in larval zebrafish bacterial load for  
419 half of the assayed strains. Five strains were not significantly affected by loperamide in mono-  
420 reconventionalized zebrafish: S2, S3, S4, S5, and S6. Colonization of larvae exposed to S1 *P.*  
421 *mosselii* or S10 *F. johnsoniae* was significantly reduced in the presence of loperamide at T0  
422 and T1, but recovered to match DMSO-level colonization by T5 (Fig. 4B;  $p < 0.05$ ). S7 *A.*  
423 *veronii* and S9 *Ochrobactrum tritici* bacterial load was reduced at all timepoints with  
424 loperamide treatment. One strain (S8 *Rhizobium* sp.) showed higher colonization only at T5  
425 after loperamide treatment (Fig. 4B;  $p < 0.05$ ). These strain-specific colonization changes due  
426 to loperamide confirm inhibition or promotion of bacteria *in vivo*, in addition to the host-  
427 exclusive effects of the molecule. A summary of how loperamide affects *in vitro* growth and  
428 survival, and *in vivo* mono-colonization of all strains is detailed in Table 1.

429



**Figure 4. Loperamide can increase or reduce mono-reconventionalized zebrafish colonization.** CFUs per fish of mono-reconventionalized fish in (A) control conditions at T0 (6 dpf) ordered by colonization capacity and (B) after exposure to loperamide at 3 timepoints (n = 4 fish). Each point represents a single zebrafish (mean of 3 technical replicates). \* p<0.05 for loperamide treatment, compared to DMSO. Wilcoxon test. Note log scale for y-axis.

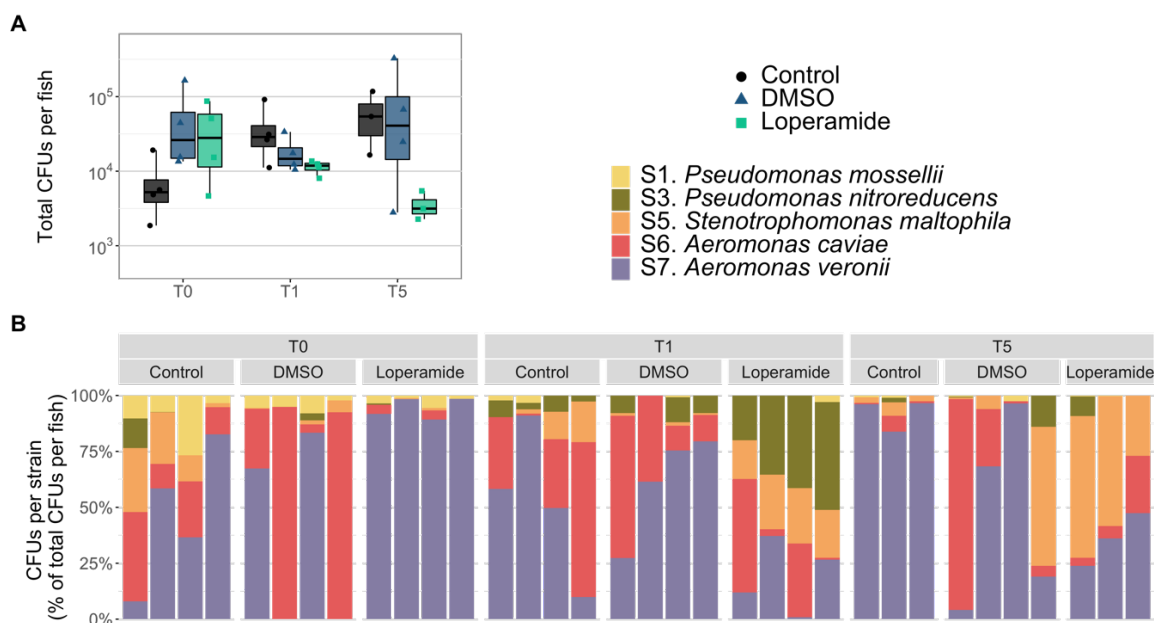
430

**Table 1. Summary of *in vitro* effects of loperamide on zebrafish strains.** Significant changes in growth in media, survival in water, and *in vivo* zebrafish colonization for loperamide-treated, compared to DMSO control.

Strain	Growth in media	Survival in water	Zebrafish mono colonization
S1. <i>Pseudomonas mossellii</i>	Promoted	No effect	Reduced at T0 and T1, then recovery
S2. <i>Variovorax gossypii</i>	No effect	Increased from 6 hours	No effect
S3. <i>Pseudomonas nitroreducens</i>	No effect	Increased from 24 hours	No effect
S4. <i>Achromobacter marplatensis</i>	Reduced	No effect	No effect
S5. <i>Stenotrophomas maltophilia</i>	Reduced	No effect	No effect
S6. <i>Aeromonas caviae</i>	Inhibited	No effect	No effect
S7. <i>Aeromonas veronii</i>	Inhibited	Decreased at 24 hours	Reduced at T0 and T5

<b>S8. <i>Rhizobium</i> sp.</b>	Inhibited	Increased from 24 hours	Increased at T5
<b>S9. <i>Ochrobactrum tritici</i></b>	Inhibited	No effect	Reduced at T0
<b>S10. <i>Flavobacterium johnsoniae</i></b>	Inhibited	No effect	Reduced at T0 and T1, then recovery

431  
 432 ***Loperamide treatment induces expected dysbiosis in mix-reconventionalized gnotobiotic***  
 433 ***larval zebrafish***  
 434 In order to evaluate how loperamide affects a multi-species bacterial community *in vivo*, germ-  
 435 free zebrafish were reconventionalized with an equal mix of strains S1, S3, S5, S6, and S7.  
 436 These strains were selected according to their varying sensitivities to loperamide *in vitro* and  
 437 *in vivo*. Loperamide treatment did not significantly impact the total number of CFUs per mix-  
 438 reconventionalized fish (Fig. 5A). However, the addition of loperamide induced an increase in  
 439 S7 *A. veronii* and a decrease in S6 *A. caviae* at T0, relative to the DMSO control (Fig. 5B).  
 440 Meanwhile, S3, S5, and S6 increased in loperamide-treated samples at T1. Finally, at T5 after  
 441 5 days of recovery, S5 *S. maltophila* and S7 *A. veronii* were the most abundant strains. (Fig.  
 442 5B). These changes in the proportion of each strain per fish reflect *in vitro* sensitivity to  
 443 loperamide and changes measured in conventional fish during loperamide treatment.  
 444



**Figure 5. Loperamide affects mix-reconventionalized gnotobiotic zebrafish bacterial load and composition. (A)** Total CFUs per fish of mix-reconventionalized fish after exposure to loperamide at 3 timepoints (n = 3-4 fish). Each point represents a single zebrafish (mean of 3 technical replicates). No significant changes were found for loperamide treatment,

compared to DMSO. Wilcoxon test. Note log scale for CFUs. **(B)** Percent abundance of each strain per mix-reconventionalized fish. Each bar is an individual fish sample.

445

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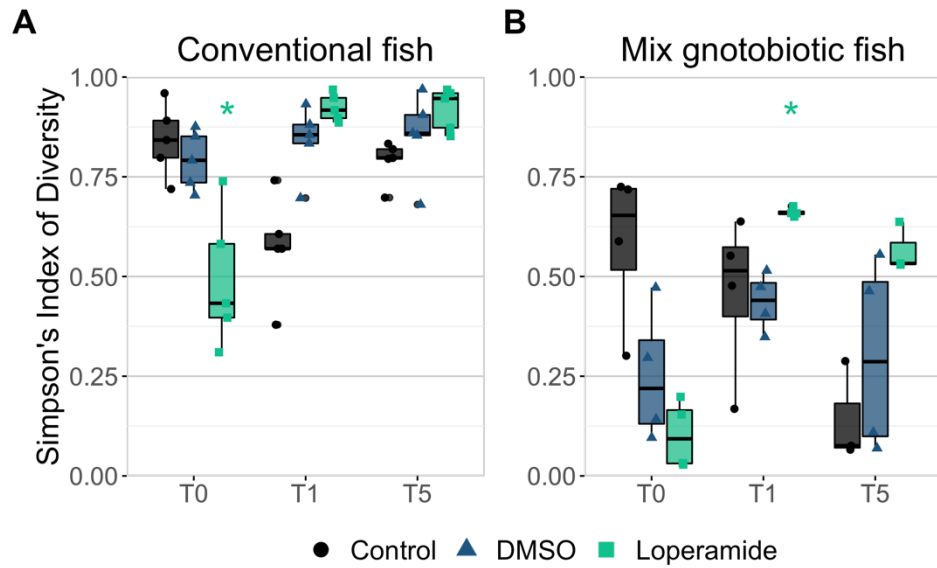
447 Differences in strain-specific colonization efficiency in zebrafish individually  
448 reconventionalized with these 5 strains may have contributed to loperamide-independent effects  
449 on the mix-reconventionalized bacterial colonization (Fig. S8). We compared the mix-  
450 reconventionalized bacterial composition with the sum of mono-reconventionalized bacterial  
451 abundances for S1, S3, S5, S6, and S7 (Fig. S9). This comparison of the mono means to the  
452 mix showed that the composition of the mix-reconventionalized fish was different from the sum  
453 of the mono-conventionalized fish in all conditions (Fig. S9AB). Therefore, inter-bacterial  
454 competition in the mix-reconventionalized fish also contributed to changes in community  
455 composition, in addition to host selection and bacterial inhibition by loperamide. Comparison  
456 of the CFUs per strain in mono-reconventionalized fish to mix-reconventionalized fish also  
457 showed increased colonization for each strain in mono- than when part of a mix, regardless of  
458 timepoint or treatment and despite the increased number of bacteria added (Fig. S9CD). Even  
459 in control conditions, each strain colonized 10-10000 times higher when added alone than when  
460 added as part of a mix (Fig. S9CD).

461

462 Further comparison of the bacterial composition in conventional and gnotobiotic zebrafish  
463 focused on changes in alpha-diversity after loperamide treatment and during recovery.  
464 Loperamide-treated conventional fish alpha-diversity measured by Simpson's Index  
465 significantly decreased after 24 hours of loperamide treatment (T0;  $p < 0.05$ ), then increased  
466 after 24 hours of recovery (T1) and stayed similar to control diversity at T5 days post-treatment  
467 (Fig. 6A). This decrease in diversity was confirmed by the lower number of ASVs (richness)  
468 detected in the loperamide-treated samples at T0 (Fig. S1B, first panel). Similarly, the alpha-  
469 diversity of loperamide-treated mix-reconventionalized gnotobiotic zebrafish decreased at T0,  
470 significantly increased at T1 ( $p < 0.05$ ), and recovered to match the control at T5 (Fig. 6B). These  
471 results show that in both natural and synthetic zebrafish bacterial communities, loperamide  
472 induced a significant, but recoverable, dysbiosis and associated loss in diversity.

473

474



**Figure 6. Alpha-diversity of conventional and gnotobiotic zebrafish decreases but recovers after loperamide treatment.** Simpson's index of diversity calculated at each timepoint for control water, DMSO, and loperamide-treated samples for (A) 16S rRNA gene amplicon data at the ASV level in conventional fish (n = 5 fish) and (B) CFUs per strain in mix-reconventionalized fish (n = 3-4 fish). Each point represents a single zebrafish with boxplots shown per condition. \* p<0.05 for loperamide treatment, compared to DMSO. Wilcoxon test.

475

## 476 Discussion

477

478 Understanding the impact of non-antibiotic drugs on host-associated microbiota is critical for  
479 sustaining health in humans as well as animal models. In this study, we evaluated the effects of  
480 loperamide, a widely prescribed anti-diarrheal compound also used as a tool to study the impact  
481 of bowel dysfunctions in animal models. Using conventional and gnotobiotic zebrafish, we  
482 showed that loperamide directly induced significant but recoverable dysbiosis by broad-range  
483 inhibition. The effects of loperamide on zebrafish-associated bacteria characterized by growth,  
484 survival, and colonization capacity were strain-specific and changed in the presence of other  
485 bacteria or the zebrafish host.

486

487 Loperamide-induced decreases in microbiota alpha-diversity and beta-dispersion immediately  
488 after loperamide treatment. These changes were not permanent and initial alpha-diversity  
489 recovered within 24 hours after loperamide exposition, and within 5 days for beta-diversity.  
490 These results were consistent with a previous study in mice, in which loperamide was used to  
491 increase gastrointestinal transit time, but also led to alterations in the gut microbial community  
492 that were reversible after treatment interruption [58]. This dysbiosis was presumed to result  
493 from a reduction of peristaltic movement, but our results suggest that it could also be explained  
494 by the loperamide bactericidal activity [27, 28].

495

496 We found that the effects of loperamide treatment on zebrafish microbiota composition  
497 depended on a strain's survival in water and colonization capacity. In conventional zebrafish,  
498 loperamide induced a significant increase in the *Aeromonas* genus at T0, but not at T1 or T5.  
499 In mono-reconventionalized fish, S6 *A. caviae* was not affected by loperamide, but S7 *A.*  
500 *veronii* showed impaired colonization despite its high colonization efficiency. S7 *A. veronii*  
501 was the only strain with inhibited growth and decreased survival in water over time, which may  
502 have contributed to its inability to recover colonization capacity after loperamide treatment. In  
503 the mix-reconventionalized fish, S7 *A. veronii* was the most abundant strain in the loperamide-  
504 treated zebrafish at T0, but significantly decreased at T1 and T5, consistent with its colonization  
505 in the mono-reconventionalized larvae and the conventional zebrafish composition. Other  
506 bacteria- or host-related factors induced by the presence of loperamide could explain reduced  
507 S7 *A. veronii* abundance, such as reduced feeding, chemokinesis, or motility [22, 59, 60].  
508 Previous studies of gnotobiotic zebrafish colonization have demonstrated the strain-specific  
509 importance of chemotaxis and host gut motility for intestinal colonization [61, 62], bacterial



510 motility and host cues with *A. veronii*. [60], and general induction of host immune responses or  
511 locomotive behavior [63–65]. In a mix-reconventionalized community, bacteria-bacteria  
512 interactions also contribute to changes in relative abundance, regardless of host factors. For  
513 example, the ecological niche left by S7 *A. veronii* due to direct inhibition or decreased  
514 intestinal peristalsis from loperamide treatment, could explain why S3 *P. nitroreducens* showed  
515 a significant increase at T1 only in loperamide-treated samples.

516  
517 Interestingly, loperamide did not increase bacterial load at the measured timepoints in our study.  
518 Similar results were also obtained in loperamide-induced constipation model in rats [66]. This  
519 may be due to colonization constraints imposed by loperamide toxicity, the larval fish size, or  
520 nutrient limitations, since previous studies of gnotobiotic zebrafish have also not detected more  
521 than  $10^6$  CFUs/larvae [22, 63, 64]. Our study is limited to bulk culturable CFUs per fish  
522 associated with 10 bacterial strains at 3 timepoints. Future studies should investigate the  
523 localization and quantification of transit time of fluorescently tagged bacteria to further  
524 understand intestinal-specific changes upon loperamide treatment.

525  
526 In all previous studies where loperamide-induced constipation has been considered to affect the  
527 host microbiome, these changes have been attributed to decreased stool frequency and increased  
528 colonic contractions by inhibition of intestinal water secretion and colonic peristalsis, which  
529 extends the fecal evacuation time and delays the intestinal luminal transit rate [15, 67].  
530 However, our results demonstrated that the changes in microbiota composition and diversity  
531 are also partially due to strain-specific bacterial inhibition or promotion by the loperamide  
532 exposure. In addition to the zebrafish-associated strains studied here, loperamide exhibits  
533 bactericidal activity against diverse host-associated microbes including mycobacterial strains  
534 (e.g. *Mycobacterium tuberculosis*) and *Staphylococcus aureus*, but not *Escherichia coli* [27,  
535 68]. These microbes are members of the human and vertebrate microbiome that may be directly  
536 affected by loperamide treatment, resulting in unforeseen microbiota modulation [69].

537  
538 Prior studies of loperamide-induced gastro-intestinal disorders determined that various  
539 treatments restore host health and improve the associated symptoms (i.e. constipation or gut  
540 transit time). For example, konjac oligo-glucomannan alleviates defecation infrequency and  
541 suppressed the growth of *Bacteroides* in mice [70], raffinose-oligosaccharide improved gastro-  
542 intestinal transit rate and reduced the serum levels of vasoactive intestinal peptide in mice [71],  
543 and probiotics improved constipation by altering metabolite, amino acid, inflammatory



544 cytokines, and/or neurotransmitter abundances in rats [20, 72, 73]. In all of these studies, the  
545 effect of treatment and changes in host physiology were inferred to constipation or the relevant  
546 model phenotype. However, all of these described effects may also be attributed to ancillary  
547 microbiota modulations. The perturbation of host microbiomes is frequently described to cause  
548 significant changes in host metabolite and peptide abundances, immune response, and  
549 physiology and health [1, 74, 75]. Our results indicate that animal models using loperamide to  
550 study bowel dysfunction and constipation cannot distinguish the effects of loperamide on host  
551 function from the effects of microbiota modulation by loperamide.

552

### 553 **Conclusions**

554 In summary, our results demonstrate that loperamide induces significant changes in the  
555 microbiota, which may influence experimental outcomes especially if the host immune system  
556 or behavior are considered. As a common medication used to alleviate diarrhea and bowel  
557 disorders in humans, loperamide is also likely to produce under-studied antibiotic effects on  
558 intestinal microbiota. This emphasizes the need to better characterize relationships between  
559 host physiological changes, microbial community structure, and disease or dysbiosis states.

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570 BioRender.com.

571

## 572 **Authors' Contributions**

573 RJS, JMG, and DPP contributed conception and design of the study. RJS and DPP performed  
574 the experiments. SB and ND performed the zebrafish imaging assays. RJS analyzed the data  
575 and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read  
576 and approved the submitted version.

577

## 578 **Competing Interests**

579 The authors declare no competing interests.

580

## 581 **Ethics Statement**

582 All animal experiments described in the present study were conducted at the Institut Pasteur  
583 according to European Union guidelines for handling of laboratory animals  
584 ([http://ec.europa.eu/environment/chemicals/lab\\_animals/home\\_en.htm](http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm)) and authorized by the  
585 Institut Pasteur Animal Health and Care Committees under permit #dap220109.

586

## 587 **Availability of Data and Material**

588 The raw 16S rRNA gene amplicon sequences generated for this study can be found in the NCBI  
589 Sequencing Read Archive in [BioProject no. PRJNA908751](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA908751). All other raw data, processed  
590 sequencing files, QIIME2 artifacts, and scripts to reproduce the figures in the manuscript are  
591 available in the Zenodo repository, <https://doi.org/10.5281/zenodo.7415697> [48].

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