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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.

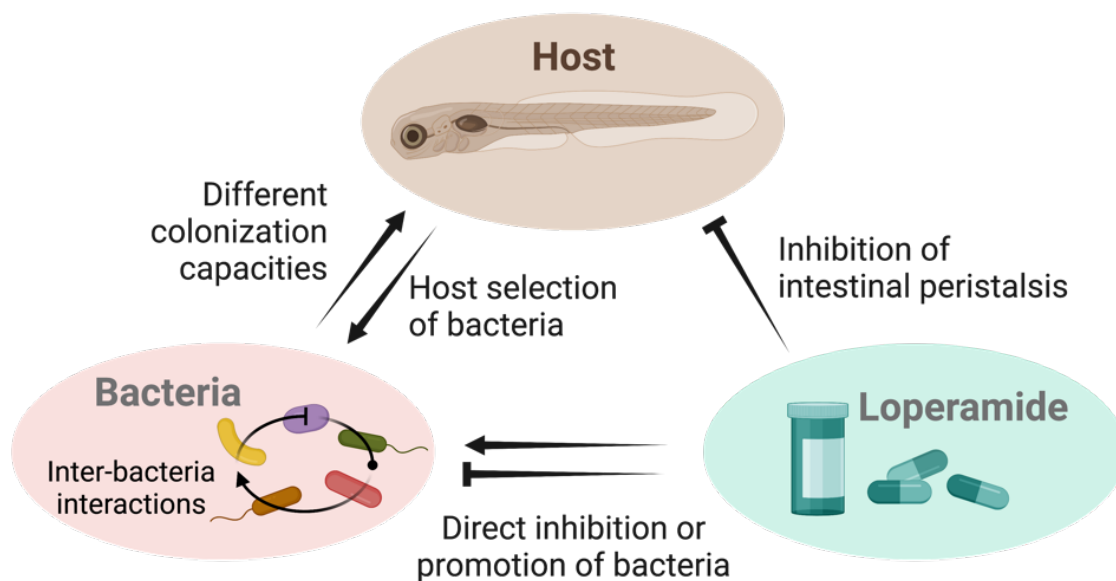
34 ***Conclusion***

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 μ -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 cm³ 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 cm³ 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 10⁵ CFU/mL. For mix-reconventionalization, 200 µL of each strain was
148 added per flask at a final concentration of 5 x 10⁵ 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 μ 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 μ L Buffer ATL and 20 μ 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 μ 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 μ L Buffer AL and 400 μ L 100 % ethanol were added and
178 mixed by vortexing before loading the lysate onto the DNeasy mini spin column in 2 x 600 μ L
179 loads. DNA purification and cleanup proceeded according to the manufacturer's
180 recommendations with a final elution volume of 50 μ 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/ μ L in 50 μ 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 μ L reactions as previously described [31, 32]. Each first step reaction included 25
193 μ L 2X Phusion Mastermix (Thermo Scientific F531S), 1.5 μ L of 10 μ M F/R primer mix (967F:
194 CTAACCGANGAACCTYACC, CNACGCGAAGAACCTTANC,

195 CAACGCGMARAACCTTACC, ATACGCGARGAACCTTACC (equimolar mix) / 1064R:
196 CGACRRCCATGCANCACCT), 13 - 20 μ L template DNA (200 ng), and 3.5 – 10.5 μ L
197 nuclease-free water (up to 50 μ 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 5×10^5 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 μL 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 μL 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 μL 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

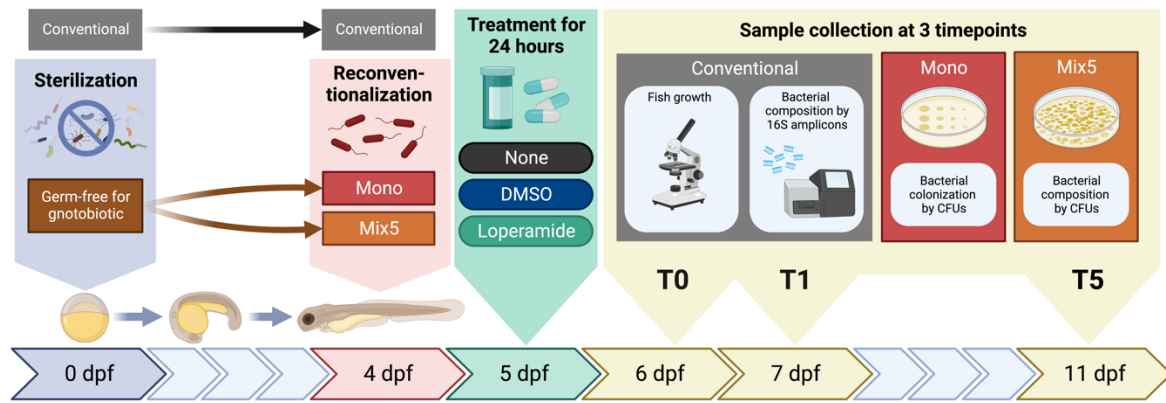


Figure 1. Experimental scheme of the larval zebrafish assays and sample collection.

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

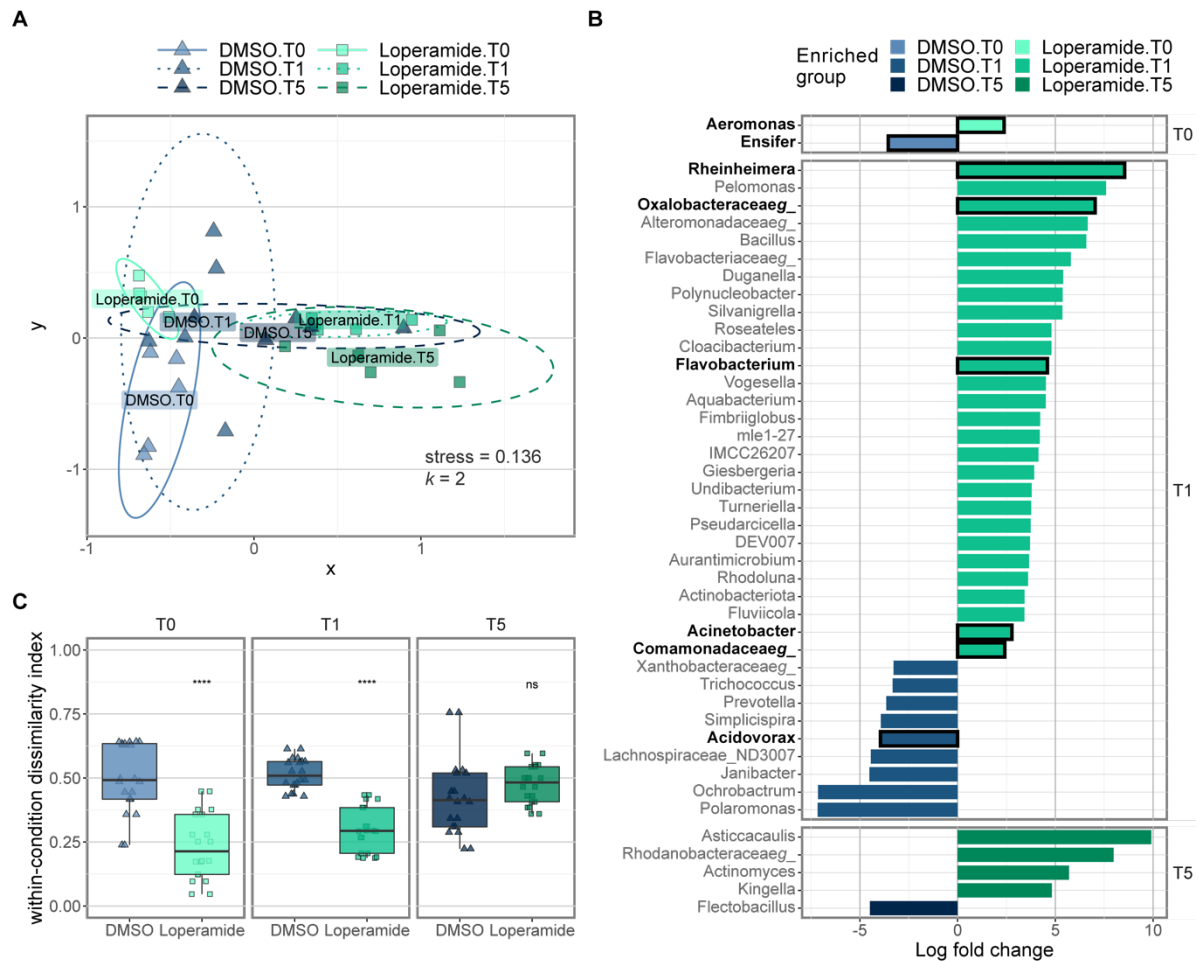


Figure 2. Loperamide affects conventional zebrafish microbiota as measured by 16S rRNA gene amplicons. (A) NMDS plot calculated using Bray-Curtis beta-diversity ($k = 2$) of percent normalized ASVs from 16S rRNA gene amplicons. Ellipse lines show the 95 % confidence interval (standard deviation). Stress = 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

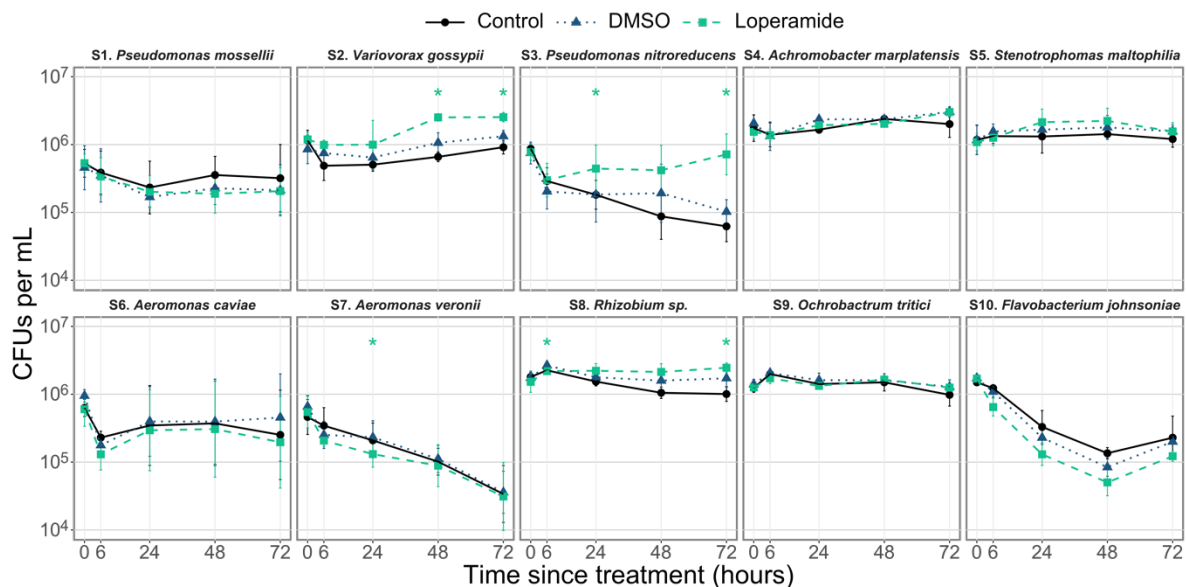


Figure 3. *In vitro* survival in water of zebrafish-associated bacterial strains is affected by loperamide. Survival in water for 72 hours after inoculation at 10⁶ CFUs/mL (mean ± standard deviation per condition is shown, n = 6-12; 2-4 independent assays of 3 biological replicates). * p<0.05 for loperamide treatment, compared to DMSO. Wilcoxon test. Note log scale on y-axis.

398

399

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 2.2×10^5 CFU/fish, while the bacterial load of larvae reconventionalized with non-
408 autochthonous S10 *Flavobacterium johnsoniae* was only 4.6×10^3 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 1.1×10^5 CFUs/mL
412 in the water, compared to 5.9×10^4 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

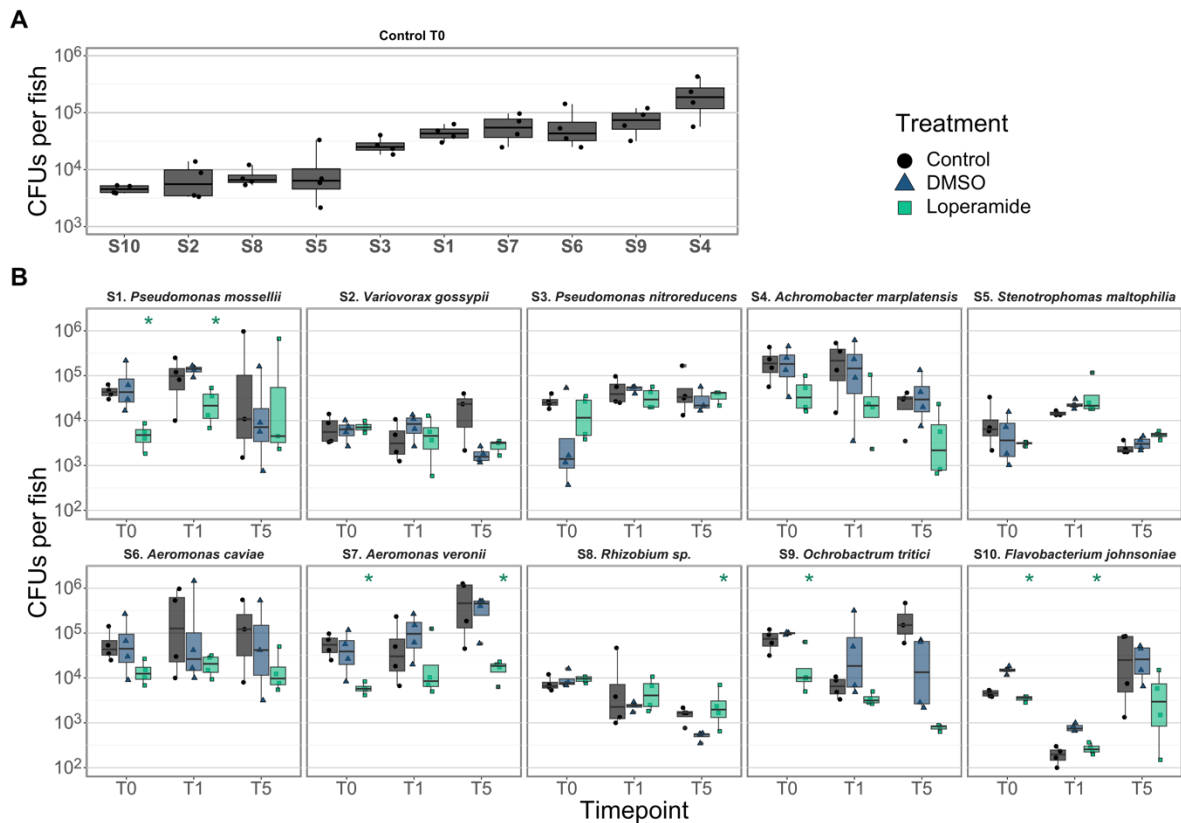


Figure 4. Loperamide can increase or reduce mono-reconventionalized zebrafish colonization. CFUs per fish of mono-reconventionalized fish in (A) control conditions at T0 (6 dpf) ordered by colonization capacity and (B) after exposure to loperamide at 3 timepoints (n = 4 fish). Each point represents a 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

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

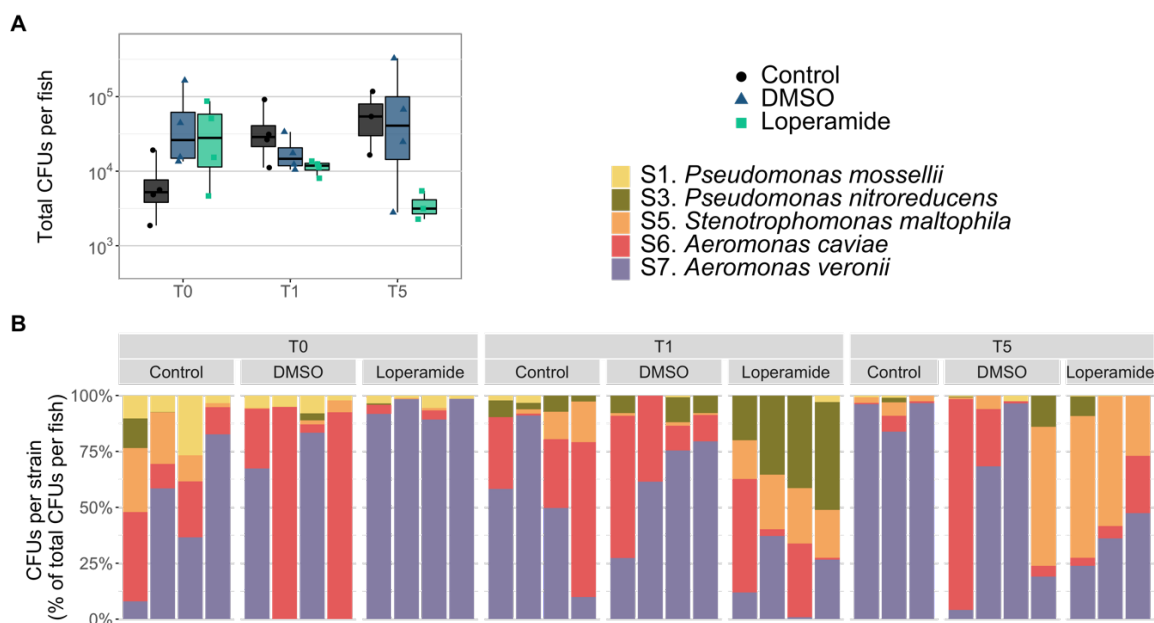


Figure 5. Loperamide affects mix-reconventionalized gnotobiotic zebrafish bacterial load and composition. (A) Total CFUs per fish of mix-reconventionalized fish after exposure to loperamide at 3 timepoints (n = 3-4 fish). Each point represents a single zebrafish (mean of 3 technical replicates). No significant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

446

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

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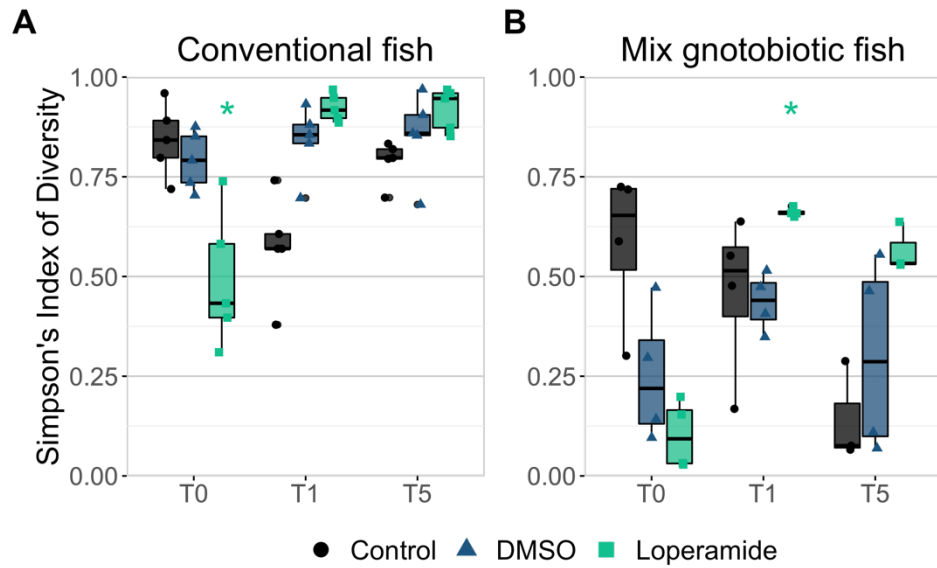


Figure 6. Alpha-diversity of conventional and gnotobiotic zebrafish decreases but recovers after loperamide treatment. Simpson's index of diversity calculated at each timepoint for control water, DMSO, and loperamide-treated samples for (A) 16S rRNA gene amplicon data at the ASV level in conventional 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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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) 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](#). 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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