Mining zebrafish microbiota reveals key community-level resistance against fish pathogen infection.

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ABSTRACT

The long-known resistance to pathogens provided by host-associated microbiota fostered the notion that adding protective bacteria could prevent or attenuate infection. However, the identification of endogenous or exogenous bacteria conferring such protection is often hindered by the complexity of host microbial communities. Here, we used zebrafish and the fish pathogen *Flavobacterium columnare* as a model system to study the determinants of microbiota-associated colonization resistance. We compared infection susceptibility in germ-free, conventional and re-conventionalized larvae and showed that a consortium of 10 culturable bacterial species are sufficient to protect zebrafish. Whereas survival to *F. columnare* infection does not rely on host innate immunity, we used antibiotic dysbiosis to alter zebrafish microbiota composition, leading to the identification of two different protection strategies. We first identified that the bacterium *Chryseobacterium massiliae* individually protects both larvae and adult zebrafish. We also showed that an assembly of 9 endogenous zebrafish species that do not otherwise protect individually confer a community-level resistance to infection. Our study therefore provides a rational approach to identify key endogenous protecting bacteria and promising candidates to engineer resilient microbial communities. It also shows how direct experimental analysis of colonization resistance in low-complexity *in vivo* models can reveal unsuspected ecological strategies at play in microbiota-based protection against pathogens.

INTRODUCTION

Animal resident microbial consortia form complex and long-term associations with important community-level functions essential for host development and physiology (1, 2). Microbial ecosystems also provide protection against exogenous pathogens by inhibition of pathogen settlement and growth and/or stimulation of the host immune system (3-8). From the perspective of microbial community composition, a shift or reduction in resident microbial diversity, a phenomenon generally referred to as dysbiosis, is often associated with increased susceptibility to infection due to the loss or change in abundance of key microbial community members (3, 9). These observations early supported the notion that addition or promotion of individually or communally protective bacteria (such as probiotics) could minimize microbiota dysbiosis or directly prevent infection to restore host health (10-12).

Although the efficacy of probiotics has been shown in animal and humans, their mechanisms of action are poorly understood and low throughput experimental models often offer limited information on the individual contribution of probiotic species to community functions (1, 6, 7, 13, 14). Moreover, characterization of bacterial strains improving colonization resistance is still hindered by the complexity of host-commensal ecosystems. Zebrafish have recently emerged as a powerful tool to study microbe-microbe and host-microbe interactions (15-19). Zebrafish can be easily reared germ-free or gnotobiotically in association with specific bacterial species (15, 20). Moreover, zebrafish bacterial communities are increasingly well characterized and a number of phylogenetically distinct zebrafish gut bacteria can be cultured, making this model system directly amenable to microbiota manipulation and assessment of probiotic effect on host infection resistance (21-24). Several studies have used zebrafish to evaluate the effect of

92 exogenous addition of potential probiotics on host resistance to infection by various
93 pathogens (22-29). However, the role of the endogenous microbial community in
94 protecting against invasive pathogen was rarely assessed and the reported protections
95 were often partial, illustrating the difficulty in identifying fully protective exogenous
96 probiotics.
97 Here we used germ-free and conventional zebrafish larvae to mine the indigenous
98 commensal microbiota for bacterial species protecting against *Flavobacterium*
99 *columnare*, a bacterial pathogen affecting wild and cultured fish species. We identified
100 two distinct infection resistance strategies preventing mortality caused by *F. columnare*,
101 mediated either by an individual member of the microbiota, the *Bacteroidetes*
102 *Chryseobacterium massiliae* or by an assembly of 9 individually non-protecting bacterial
103 species that formed a protective community. Our results demonstrated that mining host
104 microbiota constitutes a powerful approach to identify key mediators of intrinsic
105 colonization resistance, providing insight into how to engineer ecologically resilient and
106 protective microbial communities.
107

108 **MATERIALS AND METHODS**

109 **Bacterial strains and growth conditions.**

110 Bacterial strains isolated from zebrafish microbiota are listed in Table 1. *F. columnare*
111 strains (Suppl. Table S3) were grown at 28°C in tryptone yeast extract salts (TYES) broth
112 [0.4 % (w/v) tryptone, 0.04 % yeast extract, 0.05 % (w/v) $MgSO_4$ $7H_2O$, 0.02 % (w/v)
113 $CaCl_2$ $2H_2O$, 0.05 % (w/v) D-glucose, pH 7.2]. *F. columnare* strains were assigned into
114 four genomovar groups using 16S rRNA restriction fragment length polymorphism
115 analysis, including genomovar I, I/II, II, and III (30). All 10 strains of the core zebrafish
116 microbiota species were grown in TYES or LB at 28°C.

117

118 **Ethics statement**

119 All animal experiments described in the present study were conducted at the Institut
120 Pasteur (larvae) or at INRA Jouy-en-Josas (adults) according to European Union
121 guidelines for handling of laboratory animals
122 (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were
123 approved by the relevant institutional Animal Health and Care Committees.

124

125 **General handling of zebrafish**.

126 Wild-type AB fish, originally purchased from the Zebrafish International Resource
127 Center (Eugene, OR, USA), or *myd88*-null mutants (*myd88*$^{hu3568/hu3568}$) (31), kindly
128 provided by A.H Meijer, (Leiden University, the Netherlands), were raised in our facility.
129 A few hours after spawning, eggs were collected, rinsed, and sorted under a dissecting
130 scope to remove faeces and unfertilized eggs. All following procedures were performed
131 in a laminar microbiological cabinet with single-use disposable plasticware. Fish were
132 kept in sterile 25 cm^3 vented cap culture flasks containing 20 mL of water (0-6 dpf- 15

133 fish per flasks) or 24-well microtiter plates (6-15 dpf-1 fish per 2 mL well) in autoclaved
134 mineral water (Volvic) at 28°C. Fish were fed 3 times a week from 4 dpf with germ-free
135 *Tetrahymena thermophila* protozoans (22). Germ-free zebrafish were produced after
136 sterilizing the egg chorion protecting the otherwise sterile egg, with antibiotic and
137 chemical treatments (see below), whereas conventional larvae (with facility-innate
138 microbiota) were directly reared from non-sterilized eggs and then handled exactly as the
139 germ-free larvae.
140
141 **Sterilization of zebrafish eggs.**
142 Egg sterilization was performed as previously described with some modifications (22).
143 Freshly fertilized zebrafish eggs were first bleached (0.003%) for 5 min, washed 3 times
144 in sterile water under gentle agitation and maintained overnight in groups of 100 eggs per
145 75 cm^3 culture flasks with vented caps containing 100 mL of autoclaved Volvic mineral
146 water supplemented with methylene blue solution (0.3 µg/mL). Afterwards, eggs were
147 transferred into 50 mL Falcon tubes (100 eggs per tube) and treated with a mixture of
148 antibiotics (500 µL of penicillin G: streptomycin, 10,000 U/ml: 10 mg/mL GIBCO
149 #P4333), 200 µL of filtered kanamycin sulfate (100 mg/mL, SERVA Electrophoresis
150 #26899) and antifungal drug (50 µL of amphotericin B solution Sigma-Aldrich (250
151 µg/mL) #A2942) for 2 h under agitation at 28°C. Eggs were then washed 3 times in sterile
152 water under gentle agitation and bleached (0.003%) for 15 min, resuspending the eggs
153 every 3 min by inversion. Eggs were washed again 3 times in water and incubated 10 min
154 with 0.01% Romeiod (COFA, Coopérative Française de l'Aquaculture). Finally, eggs
155 were washed 3 times in water and transferred into 25 cm^3 culture flasks with vented caps
156 containing 20 mL of water. After sterilization, eggs were transferred with approximately

157 30 to 35 eggs / flasks and were transferred into new flasks at 4 dpf before re-
158 conventionalization with 10 to 15 fish / flask. We monitored sterility at several points
159 during the experiment by spotting 50 µL of water from each flask on LB, TYES and on
160 YPD agar plates, all incubated at 28°C under aerobic conditions. Plates were left for at
161 least 3 days to allow slow-growing organisms to multiply. Spot checks for bacterial
162 contamination were also carried out by PCR amplification of water samples with the 16S
163 rRNA gene primers and procedure detailed further below. If a particular flask was
164 contaminated, those fish were removed from the experiment.
165
166 **Procedure for raising germ-free zebrafish.**
167 After hatching, fish were fed with germ-free *T. thermophila* 3 times per week from 4 dpf
168 onwards. *(i) T. thermophila stock.* A germ-free line of *T. thermophila* was maintained at
169 28°C in 20 mL of PPYE (0.25% proteose peptone BD Bacto #211684, 0.25% yeast
170 extract BD Bacto #212750) supplemented with penicillin G (10 unit/mL) and
171 streptomycin (10µg/mL). Medium was inoculated with 100 µL of the preceding *T.*
172 *thermophila* stock. After one week of growth, samples were taken, tested for sterility on
173 LB, TYES and YPD plates and restocked again. *(ii) Growth. T. thermophila* were
174 incubated at 28°C in MYE broth (1% milk powder, 1% yeast extract) inoculated from
175 stock suspension at a 1:50 ratio. After 24 h of growth, *T. thermophila* were transferred to
176 Falcon tubes and washed (4400 rpm, 3 min at 25°C) 3 times in 50 mL of autoclaved
177 Volvic water. Finally, *T. thermophila* were resuspended in sterile water and added to
178 culture flasks (500 µL in 20 mL) or 24-well plates (50 µL / well). Sterility of *T.*
179 *thermophila* was tested by plating and 16S rRNA PCR as described in the section above.

180 (iii) *Fine-powder feeding*. When indicated, fish were fed with previously γ-ray-sterilized
181 fine-powdered food suitable for an early first feeding gape size (ZM-000 fish feed, ZM
182 Ltd) every 48 hours (32).
183
184 **Re-conventionalization of germ-free zebrafish**.
185 At 4 dpf, just after hatching, zebrafish larvae were re-conventionalized with a single
186 bacterial population or a mix of several. The 10 bacterial strains constituting the core
187 protective microbiota were grown for 24h in suitable media (TYES or LB) at 28°C.
188 Bacteria were then pelleted and washed twice in sterile water, and all adjusted to the same
189 cell density (OD_{600} = 1 or 5.10^7 cfu/mL) (i) *Re-conventionalization with individual*
190 *species.* Bacteria were resuspended and transferred to culture flasks containing germ-free
191 fish at a final concentration of 5.10^5 cfu/mL. (ii) *Re-conventionalization with bacterial*
192 *mixtures*. For the preparation of Mix10, Mix9, Mix8 and all other mixes used, equimolar
193 mixtures were prepared by adding each bacterial species at initial concentration to 5.10^7
194 cfu/mL. Each bacterial mixture suspension was added to culture flasks containing germ-
195 free fish at a final concentration of 5.10^5 cfu/mL.
196
197 **Infection challenges.**
198 *F. columnare* strains (Suppl. Table S3) were grown overnight in TYES broth at 28°C.
199 Then, 2 mL of culture were pelleted (10,000 rpm for 5 min) and washed once in sterile
200 water. GF zebrafish were brought in contact with the tested pathogens at 6 dpf for 3h by
201 immersion in culture flasks with bacterial doses ranging from 5.10^2 to 5.10^7 cfu/mL. Fish
202 were then transferred to individual wells of 24-well plates, containing 2 mL of water and
203 50 µL of freshly prepared GF *T. thermophila* per well. Mortality was monitored daily as
204 described in (22) and as few as 54±9 cfu/larva of *F. columnare* were recovered from

205 infected larvae. All zebrafish experiments were stopped at day 9 post-infection and
206 zebrafish were euthanized with tricaine (MS-222) (Sigma-Aldrich #E10521). Each
207 experiment was repeated at least 3 times and between 10 and 15 larvae were used per
208 condition and per experiment.
209
210 **Collection of eggs from other zebrafish facilities.**
211 Conventional zebrafish eggs were collected in 50 mL Falcon tubes from the following
212 facilities: Facility 1 - zebrafish facility in Hospital Robert Debré, Paris; Facility 2 - Jussieu
213 zebrafish facility A2, University Paris 6; Facility 3 - Jussieu – zebrafish facility C8
214 (UMR7622), University Paris 6; Facility 4: AMAGEN commercial facility, Gif sur
215 Yvette; Larvae were treated with the same rearing conditions, sterilization and infection
216 procedures used in the Institut Pasteur facility.
217
218 **Determination of fish bacterial load using cfu count.** Zebrafish were euthanized with
219 tricaine (MS-222) (Sigma-Aldrich #E10521) at 0.3 mg/mL for 10 minutes. Then they
220 were washed in 3 different baths of sterile PBS-0.1% Tween to remove bacteria loosely
221 attached to the skin. Finally, they were transferred to tubes containing calibrated glass
222 beads (acid-washed, 425 µm to 600 µm, SIGMA-ALDRICH #G8772) and 500 µL of
223 autoclaved PBS. They were homogenized using FastPrep Cell Disrupter (BIO101/FP120
224 QBioGene) for 45 s at maximum speed (6.5 m/s). Finally**,** serial dilutions of recovered
225 suspension were spotted on TYES agar and cfu were counted after 48h of incubation at
226 28°C.
227
228 **Characterization of zebrafish microbial content**.
229 Over 3 months, the experiment was run independently 3 times and 3 different batches of

230 eggs were collected from different fish couples in different tanks. Larvae were reared as
231 described above. GF and Conv larvae were collected at 6 dpf and 11 dpf for each batch.
232 Infected Conv larvae were exposed to *F. columnare*^{ALG} for 3h by immersion as described
233 above. For each experimental group, triplicate pools of 10 larvae (one in each
234 experimental batch) were euthanized, washed and lysed as above. Lysates were split into
235 3 aliquots, one for culture followed by 16S rRNA gene sequencing (A), one for 16S rRNA
236 gene clone library generation and Sanger sequencing (B), and one for Illumina
237 metabarcoding-based sequencing (C).
238
239 *A) Bacterial culture followed by 16S rRNA gene-based identification*
240 Lysates were serially diluted and immediately plated on R2A, TYES, LB, MacConkey,
241 BHI, BCYE, TCBS and TSB agars and incubated at 28ºC for 24-72h. For each agar,
242 colony morphotypes were documented, and colonies were picked and re-streaked on the
243 same agar in duplicate. In order to identify the individual morphotypes, individual
244 colonies were picked for each identified morphotype from each agar, vortexed in 200 µL
245 DNA-free water and boiled for 20 min at 90ºC. Five µL of this bacterial suspension were
246 used as template for colony PCR to amplify the 16S rRNA gene with the universal primer
247 pair for the Domain bacteria 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r
248 (5'-GGT TAC CTT GTT ACG ACT T-3'). Each primer was used at a final concentration
249 of 0.2 µM in 50 µL reactions. PCR cycling conditions were - initial denaturation at 94°C
250 for 2 min, followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 56°C for
251 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min.
252 16S rRNA gene PCR products were verified on 1% agarose gels, purified with the
253 QIAquick® PCR purification kit and two PCR products for each morphotype were sent
254 for sequencing (Eurofins, Ebersberg, Germany). 16S rRNA sequences were manually

255 proofread, and sequences of low quality were removed from the analysis. Primer
256 sequences were trimmed, and sequences were compared to GenBank (NCBI) with
257 BLAST, and to the Ribosomal Database Project with SeqMatch. For genus determination
258 a 95% similarity cut-off was used, for Operational Taxonomic Unit determination, a 98%
259 cut-off was used.
260
261 *B) 16S rRNA gene clone library generation*
262 Total DNA was extracted from the lysates with the Mobio PowerLyzer® Ultraclean® kit
263 according to manufacturer's instructions. Germ-free larvae and DNA-free water were
264 also extracted as control samples. Extracted genomic DNA was verified by Tris-acetate-
265 EDTA-agarose gel electrophoresis (1%) stained with GelRed and quantified by applying
266 2.5 µL directly to a NanoDrop® ND-1000 Spectrophotometer. The 16S rRNA gene was
267 amplified by PCR with the primers 8f and 1492r, and products checked and purified as
268 described in section A. Here, we added 25-50 ng of DNA as template to 50 µL reactions.
269 Clone libraries were generated with the pGEM®-T Easy Vector system (Promega)
270 according to manufacturer's instructions. Presence of the cloned insert was confirmed by
271 colony PCR with vector primers gemsp6 (5'-GCT GCG ACT TCA CTA GTG AT-3')
272 and gemt7 (5'-GTG GCA GCG GGA ATT CGA T-3'). Clones with an insert of the
273 correct size were purified as above and sent for sequencing (Eurofins, Ebersberg,
274 Germany). Blanks using DNA-free water as template were run for all procedures as
275 controls. For the three independent runs of the experiment, 10 Conv fish per condition (6
276 and 11 dpf, exposed or not to *F. columnare*) and per repeat were pooled. Each pool of 10
277 fish was sequenced separately, generating 3 replicates for each condition (n=12), resulting
278 in a total of 857 clones. Clone library coverage was calculated with the following formula
279 $[1-(n_1/N_2)] \times 100$, where n_1 is the number of singletons detected in the clone library, and

280 N_2 is the total number of clones generated for this sample. Clone libraries were generated
281 to a minimum coverage of 95%. Sequence analysis and identification was carried out as
282 in section A.

283 *C) by 16S rRNA V3V4 Amplicon Illumina sequencing*

284 To identify the 16S rRNA gene diversity in our facility and fish collected from 4 other
285 zebrafish facilities, fish were reared as described above. GF fish were sterilised as above,
286 and uninfected germ-free and conventional fish were collected at 6 dpf and 11 dpf.
287 Infection was carried out as above with *F. columnare*^{ALG} for 3h by bath immersion,
288 followed by transfer to clean water. Infected conventional fish were collected at 6 dpf 6h
289 after infection with *F. columnare* and at 11 dpf, the same as uninfected fish. GF infected
290 larvae were only collected at 6 dpf 6h post infection, as at 11 dpf all larvae had succumbed
291 to infection. Triplicate pools of 10 larvae were euthanized, washed and lysed as above.
292 Total DNA was extracted with the Mobio PowerLyzer® Ultraclean® kit as described
293 above and quantified with a NanoDrop® ND-1000 Spectrophotometer and sent to IMGM
294 Laboratories GmbH (Germany) for Illumina sequencing. Primers Bakt_341F (5'-
295 CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-
296 GACTACHVGGGTATCTAATCC-3'), amplifying variable regions 3 and 4 of the 16S
297 gene were used for amplification (33). Each amplicon was purified with solid phase
298 reversible immobilization (SPRI) paramagnetic bead-based technology (AMPure XP
299 beads, Beckman Coulter) with a Bead:DNA ratio of 0.7:1 (v/v) following manufacturer´s
300 instructions. Amplicons were normalized with the Sequal-Prep Kit (Life Technologies),
301 so each sample contained approximately 1 ng/µl DNA. Samples, positive and negative
302 controls were generated in one library. The High Sensitivity DNA LabChip Kit (was used
303 on the 2100 Bioanalyzer system (both Agilent Technologies) to check the quality of the

304 purified amplicon library. For cluster generation and sequencing, MiSeq® reagents kit
305 500 cycles Nano v2 (Illumina Inc.) was used. Before sequencing, cluster generation by
306 two-dimensional bridge amplification was performed, followed by bidirectional
307 sequencing, producing 2 x 250 bp paired-end (PE) reads.
308 MiSeq® Reporter 2.5.1.3 software was used for primary data analysis (signal processing,
309 de-multiplexing, trimming of adapter sequences). CLC Genomics Workbench 8.5.1
310 (Qiagen) was used for read-merging, quality trimming and QC reports and OTU
311 definition were carried out in the CLC plugin Microbial Genomics module.
312
313 **Comparison of whole larvae vs intestinal bacterial content.**
314 Larvae re-conventionalized with Mix10 and infected with *F. columnare*^{ALG} at 6 dpf for
315 3h were euthanized and washed. DNA was extracted from pools of 10 whole larvae or of
316 pools of 10 intestinal tubes dissected with sterile surgical tweezer and subjected to
317 Illumina 16S rRNA gene sequencing. GF larvae and dissected GF intestines were
318 sampled as controls. As dissection of the larval guts involved high animal loss and was a
319 potential important contamination source, we proceeded with using entire larvae for the
320 rest of the study.
321
322 **Whole genome sequencing**.
323 Chromosomal DNA of the ten species composing the core of zebrafish larvae microbiota
324 was extracted using the DNeasy Blood & Tissue kit (QIAGEN) including RNase
325 treatment. DNA quality and quantity were assessed on a NanoDrop ND-1000
326 spectrophotometer (Thermo Scientific).
327 DNA sequencing libraries were made using the Nextera DNA Library Preparation Kit
328 (Illumina Inc.) and library quality was checked using the High Sensitivity DNA LabChip

329 Kit on the Bioanalyzer 2100 (Agilent Technologies). Sequencing clusters were generated
330 using the MiSeq reagents kit v2 500 cycles (Illumina Inc.) according to manufacturer's
331 instructions. DNA was sequenced at the Helmholtz Centre for Infection Research by
332 bidirectional sequencing, producing 2 x 250 bp paired-end (PE) reads. Between 1,108,578
333 and 2,914,480 reads per sample were retrieved with a median of 1,528,402. Reads were
334 quality filtered, trimmed and adapters removed with trimmomatic 0.39 (34) and genomes
335 assembled using SPAdes 3.14 (35).
336
337 **Bacterial species identification**.
338 Whole genome-based bacterial species identification was performed by the TrueBac ID
339 system (v1.92, DB:20190603) [https://www.truebacid.com/; (36). Species-level
340 identification was performed based on the algorithmic cut-off set at 95% ANI when
341 possible or when the 16S rRNA gene sequence similarity was >99 %.
342
343 **Monitoring of bacterial dynamics**. T
344 hree independent experiments were run over 6 weeks with eggs collected from different
345 fish couples from different tanks to monitor establishment and recovery. Larvae were
346 reared, sterilized and infected as above with the only difference that 75 cm^3 culture flasks
347 with vented caps (filled with 50 mL of sterile Volvic) were used to accommodate the
348 larger number of larvae required, as in each experiment. Larvae for time course Illumina
349 sequencing were removed sequentially from the experiment that monitored the survival
350 of the larvae. Animals were pooled (10 larvae for each time point/condition), euthanized,
351 washed and lysed as described above and stored at -20°C until the end of the survival
352 monitoring, and until all triplicates had been collected.
353

354 *A) Community establishment*

355 In order to follow the establishment of the 10 core strains in the larvae, GF larvae were
356 re-conventionalized with an equiratio Mix10 as above. Re-con^{Mix10} larvae were sampled
357 at 4 dpf immediately after addition of the 10 core species and then 20 min, 2h, 4h and 8h
358 after. Germ-free, conventional larvae and the inoculum were also sampled as controls.
359

360 *B) Induction of dysbiosis*

361 Different doses of kanamycin (dose 1= 200 µg/mL; dose 2= 50 µg/mL; dose 3= 25
362 µg/mL) and a penicillin/streptomycin antibiotic mix (dose 1= 250 µg/mL; dose 2= 15.6
363 µg/mL were tested on re-con^{Mix10} 4 dpf zebrafish larvae by adding them to the flask water
364 to identify antibiotic treatments that were non-toxic to larvae but that caused dysbiosis.
365

366 After 16 hours of treatment, antibiotics were extensively washed off with sterile water
367 and larvae were challenged with *F. columnare*^{ALG}, leading to the death of all larvae – e.g.
368 successful abolition of colonization resistance with best results in all repeats with 250
369 µg/mL penicillin/streptomycin and 50 µg/mL kanamycin as antibiotic treatment.
370

371 *C) Community recovery*

372 As in B) after 8h of incubation, 4 dpf recon^{Mix10} larvae were treated with 250 µg/mL
373 penicillin/streptomycin and 50 µg/mL kanamycin for 16h. Antibiotics were extensively
374 washed off and larvae were now left to recover in sterile water for 24h to assess resilience
375 of the bacterial community. Samples (pools of 10 larvae) were taken at 3h, 6h, 12h, 18h
376 and 24h during recovery and sent for 16S rRNA Illumina sequencing. Larvae were then
377 challenged at 6 dpf with *F. columnare*^{ALG} for 3h and survival was monitored daily for 9
378 days post-infection. All time course samples were sequenced by IMGM Laboratories

379 GmbH, as described above.

380

381 **Statistical analysis of metataxonomic data**. 16S RNA analysis was performed with
382 SHAMAN (37)]. Library adapters, primer sequences, and base pairs occurring at 5' and
383 3'ends with a Phred quality score <20 were trimmed off by using Alientrimmer (v0.4.0).
384 Reads with a positive match against zebrafish genome (mm10) were removed. Filtered
385 high-quality reads were merged into amplicons with Flash (v1.2.11). Resulting amplicons
386 were clustered into operational taxonomic units (OTU) with VSEARCH (v2.3.4) (38).
387 The process includes several steps for de-replication, singletons removal, and chimera
388 detection. The clustering was performed at 97% sequence identity threshold, producing
389 459 OTUs. The OTU taxonomic annotation was performed against the SILVA SSU
390 (v132) database (39) completed with 16S sequence of 10 bacterial communities using
391 VSEARCH and filtered according to their identity with the reference (40). Annotations
392 were kept when the identity between the OTU sequence and reference sequence is ≥
393 78.5% for taxonomic Classes, ≥ 82% for Orders, ≥ 86.5% for Families, ≥ 94.5% for
394 Genera and ≥ 98% for species. Here, 73.2% of the OTUs set was annotated and 91.69 %
395 of them were annotated at genus level.
396 The input amplicons were then aligned against the OTU set to get an OTU contingency
397 table containing the number of amplicon associated with each OTU using VSEARCH
398 global alignment. The matrix of OTU count data was normalized for library size at the
399 OTU level using a weighted non-null count normalization. Normalized counts were then
400 summed within genera. The generalized linear model (GLM) implemented in the DESeq2
401 R package (41) was then applied to detect differences in abundance of genera between
402 each group. We defined a GLM that included the treatment (condition) and the time

403 (variable) as main effects and an interaction between the treatment and the time. Resulting
404 P values were adjusted according to the Benjamini and Hochberg procedure.
405 The statistical analysis can be reproduced on shaman by loading the count table, the
406 taxonomic results with the target and contrast files that are available on figshare
407 https://doi.org/10.6084/m9.figshare.11417082.v2.
408 **Determination of cytokine levels**. Total RNAs from individual zebrafish larvae were
409 extracted using RNeasy kit (Qiagen), 18h post pathogen exposure (12h post-wash).
410 Oligo(dT17)-primed reverse transcriptions were carried out using M-MLV H- reverse-
411 transcriptase (Promega). Quantitative PCRs were performed using Takyon SYBR Green
412 PCR Mastermix (Eurogentec) on a StepOne thermocycler (Applied Biosystems). Primers
413 for *ef1a* (housekeeping gene, used for cDNA amount normalization), *il1b*, *il10* and *il22*
414 are described in (22). Data were analysed using the ΔΔCt method. Four larvae were
415 analysed per condition. Zebrafish genes and proteins mentioned in the text: *ef1a*
416 NM_131263*; il1b* BC098597*; il22* NM_001020792*; il10* NM_001020785*; myd88*
417 NM_212814.
418
419 **Histological comparisons of GF, Conv and Re-Conv fish GF infected or not with *F.***
420 ***columnare***
421 Fish were collected 24h after infection (7 dpf) and were fixed for 24h at 4°C in Trump
422 fixative (4% methanol-free formaldehyde, 1% glutaraldehyde in 0.1 M PBS, pH 7.2) and
423 sent to the PIBiSA Microscopy facility services (https://microscopies.med.univ-tours.fr/)
424 in the Faculté de Médecine de Tours (France), where whole fixed animals were processed,
425 embedded in Epon. Semi-thin sections (1µm) and cut using a X ultra-microtome and then
426 either dyed with toluidine blue for observation by light microscopy and imaging or
427 processed for Transmission electron microscopy.

428 **Adult zebrafish pre-treatment with *C. massiliae***

429 The zebrafish line AB was used. Fish were reared at 28°C in dechlorinated recirculated
430 water, then transferred into continuous flow aquaria when aging 3-4 months for infection
431 experiments. *C. massiliae* was grown in TYES broth at 150 rpm and 28°C until stationary
432 phase. This bacterial culture was washed twice in sterile water and adjusted to OD_{600nm} =
433 1. Adult fish re-conventionalization was performed by adding *C. massiliae* bacterial
434 suspension directly into the fish water (1L) at a final concentration of 2.10^6 cfu/mL.
435 Bacteria were maintained in contact with fish for 24 h by stopping the water flow then
436 subsequently removed by restoring the water flow. *C. massiliae* administration was
437 performed twice after water renewal. In the control group, the same volume of sterile
438 water was added.

439

440 **Adult zebrafish infection challenge**

441 *F. columnare* infection was performed just after fish re-conventionalization with *C.*
442 *massiliae*. The infection was performed as previously described by Li and co-workers
443 with few modifications [Li et al., 2017]. Briefly, *F. columnare* strain ALG-0530 was
444 grown in TYES broth at 150 rpm and 28 °C until late-exponential phase. Then, bacterial
445 cultures were diluted directly into the water of aquaria (200 mL) at a final concentration
446 of 5.10^6 cfu/mL. Bacteria were maintained in contact with fish for 1 h by stopping the
447 water flow then subsequently removed by restoring the water flow. Sterile TYES broth
448 was used for the control group. Bacterial counts were determined at the beginning of the
449 immersion challenge by plating serial dilutions of water samples on TYES agar. Water
450 was maintained at 28°C and under continuous oxygenation for the duration of the

immersion. Groups were composed of 10 fish. Virulence was evaluated according to fish mortality 10 days post-infection.

Statistical methods.

Statistical analyses were performed using unpaired, non-parametric Mann-Whitney test or unpaired t-tests. Analyses were performed using Prism v8.2 (GraphPad Software). Evenness: The Shannon diversity index was calculated with the formula ($H_S = -\Sigma[P(\ln(P)])$ where P is the relative species abundance. Total evenness was calculated for the Shannon index as $E = H_S/H_{max.}$ The less evenness in communities between the species (and the presence of a dominant species), the lower this index is.

Data availability

Bacterial genome sequences obtained in the present study are available at the European Nucleotide Archive with the project number PRJEB36872, under accession numbers numbers ERS4385993 (*Aeromonas veronii 1*); ERS4386000 (*Aeromonas veronii 2*); ERS4385996 (*Aeromonas caviae*); ERS4385998 (*Chryseobacterium massiliae*); ERS4385999 (*Phyllobacterium myrsinacearum*); ERS4406247 (*Pseudomonas sediminis*); ERS4385994 (*Pseudomonas mossellii*) ERS4386001 (*Pseudomonas nitroreducens*); ERS4385997 (*Pseudomonas peli*); ERS4385995 (*Stenotrophomas maltophilia*);

474 **RESULTS**

475

476 ***Flavobacterium columnare* kills germ-free but not conventional zebrafish**

477 To investigate microbiota-based resistance to infection in zebrafish, we compared the
478 sensitivity of germ-free (GF) and conventional (Conv) zebrafish larvae to *F. columnare*,
479 an important fish pathogen affecting carp, channel catfish, goldfish, eel, salmonids and
480 tilapia and previously shown to infect and kill adult zebrafish (12, 42-45). We used bath
481 immersion to expose GF and Conv zebrafish larvae at 6 days post fertilization (dpf), to a
482 collection of 28 *F. columnare* strains, belonging to four different genomovars for 3h at
483 5.10^5 colony forming units (cfu)/mL. Daily monitoring showed that 16 out of 28 *F.*
484 *columnare* strains killed GF larvae in less than 48h (Supplementary Fig. S1A), whereas
485 Conv larvae survived exposure to all tested virulent *F. columnare* strains (Supplementary
486 Fig. S1B). Exposure to the highly virulent strain ALG-00-530 (hereafter *F.*
487 *columnare*^{ALG}) also showed that GF mortality was fast (1 day) and dose-dependent and
488 that Conv zebrafish survived all but the highest dose (10^7 cfu/mL) (Fig. 1). Similar
489 survival of infected Conv larvae was obtained with zebrafish AB strain eggs obtained
490 from 4 different zebrafish facilities (Supplementary Fig. S2), suggesting that
491 conventional zebrafish microbiota could provide protection against *F. columnare*
492 infection.

493

494

495 **A community of 10 culturable bacterial strains protect against *F. columnare***
496 **infection**

497 In our rearing conditions, the conventional larval microbiota is acquired after hatching
498 from microorganisms present on the egg chorion and in fish facility water. To test the
499 hypothesis that microorganisms associated with conventional eggs provided protection
500 against *F. columnare*^{ALG}, we exposed sterilized eggs to either fish facility tank water or
501 to non-sterilized conventional eggs at 0 or 4 dpf (before or after hatching, respectively).
502 In both cases, these re-conventionalized (re-Conv) zebrafish survived *F. columnare*^{ALG}
503 infection as well as Conv zebrafish (Supplementary Fig. S3). To determine the
504 composition of conventional zebrafish microbiota, we generated 16S rRNA gene clone
505 libraries from homogenate pools of Conv larvae aged 6 and 11 dpf exposed or not to *F.*
506 *columnare*^{ALG}, sampled over 3 months from 3 different batches of larvae (n=10). A total
507 of 857 clones were generated for all samples. We identified 15 operational taxonomical
508 units (OTUs), 10 of which were identified in all experiments (Table 1, Suppl. Table S1).
509 Two OTUs (belonging to an *Ensifer* sp. and a *Hydrogenophaga* sp.) were only detected
510 once, and a *Delftia* sp., a *Limnobacter* sp. and a *Novosphingobium* sp. were detected more
511 than once (2, 3 and 2 times, respectively), but not consistently in all batches of fish (Table
512 1, Suppl. Table S1). Moreover, deep-sequencing of the 16S rRNA V3-V4 region of
513 gDNA retrieved from larvae originating from the other four zebrafish facilities described
514 above, revealed that OTUs for all of these 10 strains were also detected in Conv larvae,
515 with the exception of *A. veronii* strain 2 that was not detected in all samples (Suppl. Table
516 S2).

517 To isolate culturable zebrafish microbiota bacteria, we plated dilutions of homogenized
518 6 dpf and 11 dpf larvae pools on various growth media and we identified 10 different

519 bacterial morphotypes. 16S-based analysis followed by full genome sequencing
520 identified 10 bacteria corresponding to 10 strains of 9 different species that were also
521 consistently detected by culture-free approaches (Table 1). To assess the potential
522 protective role of these 10 strains, we re-conventionalized GF zebrafish at 4 dpf with a
523 mix of all 10 identified culturable bacterial species (hereafter called Mix10), each at a
524 concentration of 5.10^5 cfu/mL and we monitored zebrafish survival after exposure to *F.*
525 *columnare*^{ALG} at 6 dpf. We showed that zebrafish re-conventionalized with the Mix10
526 (Re-Conv^{Mix10}) displayed a strong level of protection against all identified highly virulent
527 *F. columnare* strains (Supplementary Fig. S4). These results demonstrated that the Mix10
528 constitutes a core protective bacterial community providing full protection of zebrafish
529 larvae against *F. columnare* infection.
530

531 **Community dynamics under antibiotic-induced dysbiosis reveal a key contributor**
532 **to resistance to *F. columnare* infection**

533 To further analyze the determinants of Mix10 protection against *F. columnare*^{ALG}
534 infection, we inoculated 4 dpf larvae with an equal-ratio mix of the 10 bacteria (at 5.10^5
535 cfu/mL each) and monitored their establishment over 8 hours. We first verified that whole
536 larvae bacterial content (OTU abundance) was not significantly different from content of
537 dissected intestinal tubes (p=0.99, two-tailed t-test) (Supplementary Fig. S5) and
538 proceeded to use entire larvae to monitor bacterial establishment and recovery in the rest
539 of the study. We then collected pools of 10 larvae immediately after re-
540 conventionalization (t_0), and then at 20 min, 2 hours, 4 hours and 8 hours in three
541 independent experiments. Illumina sequencing of the 16S rRNA gene was used to follow
542 bacterial relative abundance. At t_0, all species were present at > 4% in the zebrafish, apart

543 from *A. veronii* strains 1 (0.2%) and 2 (not detected) (Supplementary Fig. S6). *Aeromonas*
544 *caviae* was detected as the most abundant species (33%), followed by *Stenotrophomonas*
545 *maltophilia* (23%) and *Chryseobacterium massiliae* (12%), altogether composing 68% of
546 the community (Supplementary Fig. S6). The relative species abundance, possibly
547 reflecting initial colonization ability, was relatively stable for most species during
548 community establishment, with similar species evenness at t_0 (E = 0.84) and t_{8h} (E = 0.85).
549 Whereas both Conv and Re-Conv^{Mix10} larvae were protected against *F. columnare*^{ALG}
550 infection, the global structure of the reconstituted Mix10 population was different from
551 the conventional one at 4 dpf (Supplementary Fig. S6).
552 To test the sensitivity to disturbance and the resilience of the protection provided by
553 Mix10 bacterial community, we subjected Re-Conv^{Mix10} zebrafish to non-toxic antibiotic
554 treatment at 4dpf using either 250 µg/mL penicillin/streptomycin combination (all
555 members of the Mix10 bacteria are sensitive to penicillin/streptomycin) or 50 µg/mL
556 kanamycin (affecting all members of the Mix10 bacteria except *C. massiliae*, *P.*
557 *myrsinacearum* and *S. maltophilia*) (Suppl. Fig. S7). At 5 dpf, after 16 hours of exposure,
558 antibiotics were washed off and zebrafish were immediately exposed to *F. columnare*^{ALG}.
559 Both antibiotic treatments resulted in complete loss of the protection against *F.*
560 *columnare*^{ALG} infection observed in Re-Conv^{Mix10} (Fig. 2A). We then used the same
561 antibiotic treatments but followed by a 24h recovery period after washing off the
562 antibiotics at 5 dpf, therefore only performing the infection at 6 dpf (Fig. 2B). Whilst Re-
563 Conv^{Mix10} larvae treated with penicillin/streptomycin showed similar survival to infected
564 GF larvae, kanamycin-treated Re-Conv^{Mix10} zebrafish displayed restored protection after
565 24h recovery and survived similarly to untreated conventionalized fish (Fig. 2B).
566 Sampling and 16S gene analysis during recovery experiments at different time points

567 showed that bacterial community evenness decreased after antibiotic administration for
568 both treatments (E = 0.85 for 4 dpf control, E = 0.72 for t_0 kanamycin and E = 0.7 for t_0
569 penicillin/streptomycin), and continued to decrease during recovery (E = 0.6 and 0.64 for
570 kanamycin and penicillin/streptomycin treatment after 24h recovery, respectively).
571 Although *C. massiliae* remained detectable immediately after both antibiotic treatments,
572 penicillin/streptomycin treatment led a significant reduction in its relative abundance
573 (0.21%) (Fig. 2C). By contrast, *C. massiliae* relative abundance rebounded 6h after
574 cessation of kanamycin treatment and was the dominant member (52%) of the
575 reconstituted microbiota after 24h recovery period (Fig. 2D), suggesting that the
576 protective effect observed in the kanamycin-treated larvae might be due to the recovery
577 of *C. massiliae*.
578

579 **Resistance to *F. columnare* infection is provided by both individual and**
580 **community-level protection**

581 To test the potential key role played by *C. massiliae* in protection against *F. columnare*^{ALG}
582 infection, we exposed 4 dpf GF zebrafish to *C. massiliae* only and showed that it
583 conferred individual protection at doses as low as 5.10^2 cfu/mL (Fig. 3). Whereas none
584 of the 9 other species composing the Mix10 were protective individually (Fig. 3A), their
585 equiratio combination (designated as Mix9) conferred protection to zebrafish, although
586 not at doses lower than 5.10^4 cfu/mL and not as reproducibly as with *C. massiliae* (Fig.
587 3B). To identify which association of species protected Re-Conv^{Mix9} zebrafish against *F.*
588 *columnare*^{ALG} infection, we tested all 9 combinations of 8 species (Mix8), as well as
589 several combinations of 7, 6, 4 or 3 species and showed no protection (Suppl. Fig. S8A
590 and Suppl. Table S4). We then tested whether lack of protection of Mix8 compared to

591 Mix9 could rely on a density effect by doubling the concentration of any of the species
592 within the non-protective Mix8a (Suppl. Fig. S8B) and showed no protection. These
593 results therefore indicated that microbiota-based protection against *F. columnare*^{ALG}
594 infection relied on either *C. massiliae*-dependent membership effect or on a community-
595 dependent effect mediated by the Mix9 consortium.
596
597 **Pro- and anti- inflammatory cytokine production does not contribute to microbiota-**
598 **mediated protection against *F. columnare*^{ALG} infection.**
599 To test the contribution of the immune response of zebrafish larvae to resistance to *F.*
600 *columnare* infection, we used qRT-PCR to measure cytokine mRNA expression in GF
601 and Conv zebrafish exposed or not to *F. columnare*^{ALG}. We also tested the impact of re-
602 conventionalization with *C. massiliae* (re-Conv^{Cm}), Mix10 (re-Conv^{Mix10}) or with Mix4
603 (*A. caviae*, both *A. veronii* spp., *P. mossellii*) as a non-protective control (Suppl. Table
604 S4). We tested genes encoding IL1β (pro-inflammatory), IL22 (promoting gut repair),
605 and IL10 (anti-inflammatory) cytokines. While we observed some variation in *il10*
606 expression among non-infected re-conventionalized larvae, this did not correlate with
607 protection. Furthermore, *il10* expression was not modulated by infection in any of the
608 tested conditions (Fig. 4A). By contrast, we observed a strong induction of *il1b* and *il22*
609 in GF zebrafish exposed to *F. columnare*^{ALG} (Fig. 4BC). However, although this
610 induction was reduced in protected Conv, Re-Conv^{Cm} and Re-Conv^{Mix10}, it was also
611 observed in non-protective Re-Conv^{Mix4} larvae, indicating that down-modulation of the
612 inflammatory response induced by *F. columnare* does not correlate with resistance to
613 infection. Consistently, the use of a *myd88* mutant, a key adaptor of IL-1 and toll-like
614 receptor signalling deficient in innate immunity(31, 46), showed that Conv or Re-

615 Conv^{Mix10}, but not GF *myd88* mutants survived *F. columnare* as well as wild-type
616 zebrafish (Figure 4D). Moreover, *il1b* induction by *F. columnare* infection was observed
617 only in GF larvae and was *myd88*-independent (Suppl. Figure S9). These results therefore
618 indicated that the tested cytokine responses do not play a significant role in the
619 microbiota-mediated protection against *F. columnare* infection.
620

621 ***C. massiliae* and Mix9 protect zebrafish from intestinal damages upon *F.***
622 ***columnare*^{ALG} infection**

623 Histological analysis of GF larvae fixed 24h after exposure to *F. columnare*^{ALG} revealed
624 extensive intestinal damage (Fig. 5A) prior to noticeable signs in other potential target
625 organs such as gills or skin. To test the requirement for gut access in *F. columnare*^{ALG}
626 infection process, we modified our standard rearing protocol of GF fish, which involves
627 feeding with live germ-free *T. thermophila*. We found that, if left unfed, GF zebrafish did
628 not die after *F. columnare*^{ALG} exposure, while feeding with either *T. thermophila* or
629 another food source such as sterile fish food powder, restored sensitivity to *F.*
630 *columnare*^{ALG} infection (Suppl. Fig S10), suggesting that successful infection requires
631 feeding and ingestion.
632 Histological sections consistently showed severe disorganization of the intestine with
633 blebbing in the microvilli and vacuole formation in *F. columnare*^{ALG}-infected GF larvae
634 (Fig. 5). In contrast, zebrafish pre-incubated with either *C. massiliae* or Mix9 consortium
635 at 4 dpf, and then exposed to *F. columnare*^{ALG} at 6 dpf showed no difference compared
636 to non-infected larvae or conventional infected larvae (Fig. 5), confirming full protection
637 against *F. columnare*^{ALG} at the intestinal level.
638

639 ***C. massiliae* protects larvae and adult zebrafish against *F. columnare***

640 The clear protection provided by *C. massiliae* against *F. columnare*^{ALG} infection
641 prompted us to test whether exogenous addition of this bacterium could improve
642 microbiota-based protection towards this widespread fish pathogen. We first showed that
643 zebrafish larvae colonized with *C. massiliae* were fully protected against all virulent *F.*
644 *columnare* strains identified in this study (Fig. 6A). To test whether *C. massiliae* could
645 also protect adult zebrafish from *F. columnare* infection, we pre-treated conventional 3-
646 4-month-old Conv adult zebrafish with *C. massiliae* for 48h before challenging them with
647 a high dose (5.10^6 cfu/mL) *F. columnare*^{ALG}. Monitoring of mortality rate showed that
648 pre-treatment with *C. massiliae* significantly increased the survival rate of adult zebrafish
649 upon *F. columnare*^{ALG} infection compared to non-treated conventional fish ($p=0.0084$
650 Mann-Whitney test, Figure 6B). Taken together, these results show that *C. massiliae* is a
651 putative broad-spectrum probiotic protecting zebrafish against columnaris disease caused
652 by *F. columnare*.

653

654

655 **DISCUSSION**

656

657 In this study, we used re-conventionalization of otherwise germ-free zebrafish larvae to
658 show that conventional-level protection against infection by a broad range of highly
659 virulent *F. columnare* strains is provided by a set of 10 culturable bacterial strains,
660 belonging to 9 different species, isolated from the indigenous standard laboratory
661 zebrafish microbiota. With the exception of the Bacteroidetes *C. massiliae*, this protective
662 consortium was dominated by Proteobacteria such as *Pseudomonas* and *Aeromonas* spp.,
663 bacteria commonly found in aquatic environments (47, 48). Despite the relative
664 permissiveness of zebrafish larvae microbiota to environmental variations and inherent
665 variability between samples (49), we showed that these ten bacteria were consistently
666 identified in four different zebrafish facilities, suggesting the existence of a core
667 microbiota with important colonization resistance functionality. Use of controlled
668 combinations of these 10 bacterial species enabled us to show a very robust species-
669 specific protection effect in larvae mono-associated with *C. massiliae.* We also identified
670 a community-level protection provided by the combination of the 9 other species that
671 were otherwise unable to protect against *F. columnare* when provided individually. This
672 protection was however less reproducible and required a minimum inoculum of 5.10^4
673 cfu/mL, compared to 5.10^2 cfu/mL with *C. massiliae*. These results therefore suggest the
674 existence of two distinct microbiota-based protection scenarios: one based on a
675 membership effect provided by *C. massiliae*, and the other mediated by the higher-order
676 activity of the Mix9 bacterial community.

677

678 Although protection against *F. columnare* infection does not seem to rely on microbiota-
679 based immuno-modulation, we cannot exclude that, individually, some members of the
680 studied core zebrafish microbiota could induce pro- or anti-inflammatory responses
681 masked in presence of the full Mix10 consortium (1). We also acknowledge that the
682 mechanistic aspects of the two identified colonization resistance scenarios are still unclear
683 and further studies are required to determine whether they involve nutrient depletion,
684 adhesion inhibition, direct or indirect growth inhibition leading to niche exclusion or any
685 other process (6, 13). In the case of the protection provided by *C. massiliae* against *F.*
686 *columnare,* these two phylogenetically close bacteria could compete for similar resources
687 and directly antagonize each other, as shown to occur between *Bacteroidetes* species (50-
688 53). Interestingly, infected larvae re-conventionalized with either C. *massiliae* or Mix9
689 showed no signs of the intestinal damage displayed by germ-free larvae, suggesting that
690 both *C. massiliae* and Mix9 provide similar intestinal resistance to *F. columnare*
691 infection. Whereas microbial colonization contributes to gut maturation and stimulates
692 the production of epithelial passive defences such as mucus (54, 55), lack of intestinal
693 maturation is unlikely to be contributing to *F. columnare*-induced mortality, as mono-
694 colonized larvae or larvae re-conventionalized with non-protective mixes died as rapidly
695 as germ-free larvae.
696 Several studies have monitored the long-term assembly and development of the zebrafish
697 microbiota from larvae to sexually mature adults, however little is known about the initial
698 colonization establishment of the larvae after hatching (56, 57). Neutral (stochastic) and
699 deterministic (host niche-based) processes (58-60) lead to microbial communities that are
700 mostly represented by a limited number of highly abundant species with highly diverse
701 low-abundant populations. In our experiments, the Mix10 species inoculum corresponded

702 to an equiratio bacterial mix, thus starting from an engineered and assumed total evenness
703 (E = 1) (61, 62). Evenness was still relatively high (0.84) and remained similar up until
704 8h in our study, indicating that most of the ten species were able to colonize the larvae.
705 From the perspective of community composition, a loss of diversity is often associated
706 with decreased colonization resistance, but it remains unclear whether this increased
707 susceptibility is due to the loss of certain key member species of the microbial community
708 and/or a change in their prevalence (3, 9).
709 We further investigated resistance to infection by exposing established bacterial
710 communities to different antibiotic perturbations, followed by direct challenge with *F.*
711 *columnare* (to study core microbiota sensitivity to disturbance) or after recovery (to study
712 its resilience) (12, 63). Antibiotics are known to shift the composition and relative
713 abundances of the microbiota according to their spectrum (13, 64). We observed that
714 penicillin/streptomycin treatment that would affect most of the core species, reduced the
715 abundance of all but two species (*A. veronii* 1 and *P. myrsinacearum)* that became
716 relatively dominant during recovery, but failed to provide protection against *F.*
717 *columnare*. With the kanamycin treatment, colonization resistance was fully restored at
718 the end of the 24h recovery period, indicative of a resilience that could result from species
719 recovering quickly to their pre-perturbation levels due to fast growth rates, physiological
720 flexibility or mutations (65). Interestingly, even taking into account potential biases
721 associated with the use of the 16S rRNA as a proxy index to determine relative abundance
722 (66, 67), evenness was similarly reduced during recovery for both treatments, but
723 abundance at phylum level changed to 48% for Proteobacteria, and 52% for Bacteroidetes
724 compared to the >98% of Proteobacteria with the penicillin/streptomycin treatment.
725 Furthermore, *C. massiliae* was detected as rare (<1%) in conventional larvae, suggesting

726 that it could have a disproportionate effect on the community or that community-level
727 protection provided by the nine other bacteria was also responsible for the protection of
728 conventional larvae to *F. columnare* infection.
729 We showed that germ-free zebrafish larvae are highly susceptible to a variety of different
730 *F. columnare* genomovars isolated from different hosts, demonstrating that they are a
731 robust animal model for the study of its pathogenicity. Recently, *F. columnare* mutants
732 in Type 9 secretion system (T9SS) were shown to be avirulent in adult zebrafish,
733 suggesting that proteins secreted by the T9SS are likely to be key, but still largely
734 unidentified, *F. columnare* virulence determinants (45). Body skin, gills, fins and tail are
735 also frequently damaged in salmonid fish, whereas severe infection cases are associated
736 with septicemia (68). We could not identify such clear *F. columnare* infection sites in
737 zebrafish larvae, perhaps due to the very low dose of infection used, with less than 100
738 cfu recovered from infected moribund larvae. However, several lines of evidence suggest
739 that the gut is the main target of *F. columnare* infection in our model: (i) unfed germ-free
740 larvae survived exposure, (ii) histology analysis showing severe disruption of the
741 intestinal region just hours after infection in germ-free larvae, and (iii) induction of *il22*
742 in germ-free larvae exposed to *F. columnare*, since a major function of IL-22 is to
743 promote gut repair (69). This induction appears to be a consequence of the pathogen-
744 mediated damage, as there was no observed induction in conventional or re-
745 conventionalized larvae. The very rapid death of larvae likely caused by this severe
746 intestinal damage may explain why other common target organs of columnaris disease
747 showed little damage.
748
749 In this study, we showed that *C. massiliae* is a promising probiotic candidate that could

750 contribute to reduce the use of antibiotic to prevent columnaris diseases in research and
751 aquaculture settings. Whereas *C. massiliae* provided full and robust protection against all
752 tested virulent *F. columnare* genomovars and significantly increased survival of exposed
753 adult conventional zebrafish, further studies are needed to elucidate *C. massiliae*
754 protection potential in other teleost fish. However, the endogenous nature of *C. massiliae*
755 suggests that it could establish itself as a long-term resident of the zebrafish larval and
756 adult microbiota, an advantageous trait when seeking a stable modulation of the bacterial
757 community over long periods (33).
758
759 In conclusion, the use of a simple and tractable zebrafish larval model to mine indigenous
760 host microbial communities allowed us to identify two independent protection pathways
761 against the same pathogen. Whereas further study will determine how these pathways
762 may contribute to protection against a wider range of pathogens, this work also provides
763 insights into how to the engineering of stable protective microbial communities with
764 controlled colonization resistance functions.
765
766
767
768

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CONFLICT OF INTEREST

The authors of this manuscript have the following conflict of interest: a provisional patent application has been filed: "*bacterial strains for use as probiotics, compositions thereof, deposited strains and method to identify probiotic bacterial strains*" by J.-M.G, F. A. S., D.P.-P. and J. B. B. The other authors declare no conflict of interest in relation to the submitted work.

AUTHOR CONTRIBUTIONS:

F. A. S., J. B. B., D.P.-P., J.-P. L. and J.-M.G. designed the experiments. O.R. contributed to the initial experiments. V.B. and J.-P. L. provided zebrafish material and advice. F. A. S, J. B. B., D.P.-P., B.A., V.B. and J.-P. L. performed the experiments. S.B., S.H. performed bacterial genome sequencing and analysis, A.G., S.V., F. A. S and D.P.-P. performed the bioinformatic and sequence analyses. F. A. S, J. B. B., D.P.-P., J.-P. L. and J.-M.G. analysed the data and wrote the paper with significant contribution from O.R. and E.D..

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1011 **TABLES**

1012

1013 **Table 1. The 10 strains composing the core of zebrafish larvae microbiota**
1014 Bacterial strains consistently detected at all time points (6 and 11 dpf) in all experiment
1015 runs and constituting the core of conventional zebrafish larval microbiota and their
1016 taxonomic affiliation.
1017

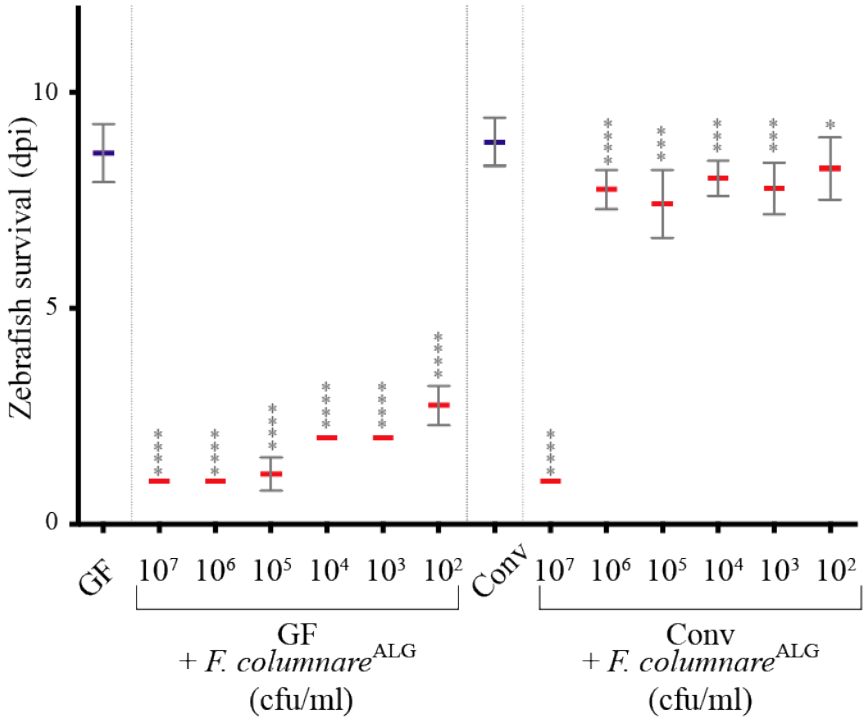
Bacterial species of the core zebrafish microbiota	ANI %[a]	16S rRNA (%)[b]	*recA* (%)[c]	*rplC* (%)[d]
Aeromonas veronii 1	*96.52*	*98.27*	*97.00*	*99.84*
Aeromonas veronii 2	*96.58*	*99.53*	*98.31*	*99.68*
Aeromonas caviae	*97.97*	*99.94*	*98.78*	*99.84*
Chryseobacterium massiliae	*95.85*	*99.86*	*96.61*	*99.84*
Phyllobacterium myrsinacearum	*98.58*	*99.86*	*99.72*	*100*
Pseudomonas sediminis	*96.12*	*99.73*	*97.70*	*99.84*
Pseudomonas mossellii	*99.39*	*98.27*	*100*	*99.84*
*Pseudomonas nitroreducens**	*92.14*	*99.80*	*94.95*	*99.06*
*Pseudomonas peli**	*88.84*	*99.20*	*91.51*	*95.44*
*Stenotrophomas maltophilia**	*90.94*	*97.85*	*95.38*	*99.08*

1018 [a] Average Nucleotide Identity value
1019 [b] 16S rRNA gene sequence similarity
1020 [c] *recA* gene sequence similarity
1021 [d] *rplC* gene sequence similarity
1022 *Species ambiguously identified
1023

1024

1025 **FIGURES**

1026



1027

1028 **Figure 1. *Flavobacterium columnare* kills germ-free but not conventional zebrafish.**
1029 6 dpf (corresponding to 0 dpi) GF or Conv zebrafish larvae were exposed to different
1030 doses of *F. columnare*^{ALG} by bath immersion and transferred after 3h into sterile water.
1031 Mean survival is represented by a thick horizontal bar with standard deviation. For each
1032 condition, n = 12 zebrafish larvae. Larvae mortality rate was monitored daily and
1033 surviving fish were euthanized at day 9 post infection. Statistics correspond to unpaired,
1034 non-parametric Mann-Whitney test comparing all conditions to non-infected GF (left) or
1035 Conv (right). ****: $p<0.0001$; ***: $p<0.001$; *: $p<0.05$, absence of *: non-significant.
1036 Blue mean bars correspond to non-exposed larvae and red mean bars correspond to larvae
1037 exposed to *F. columnare*.

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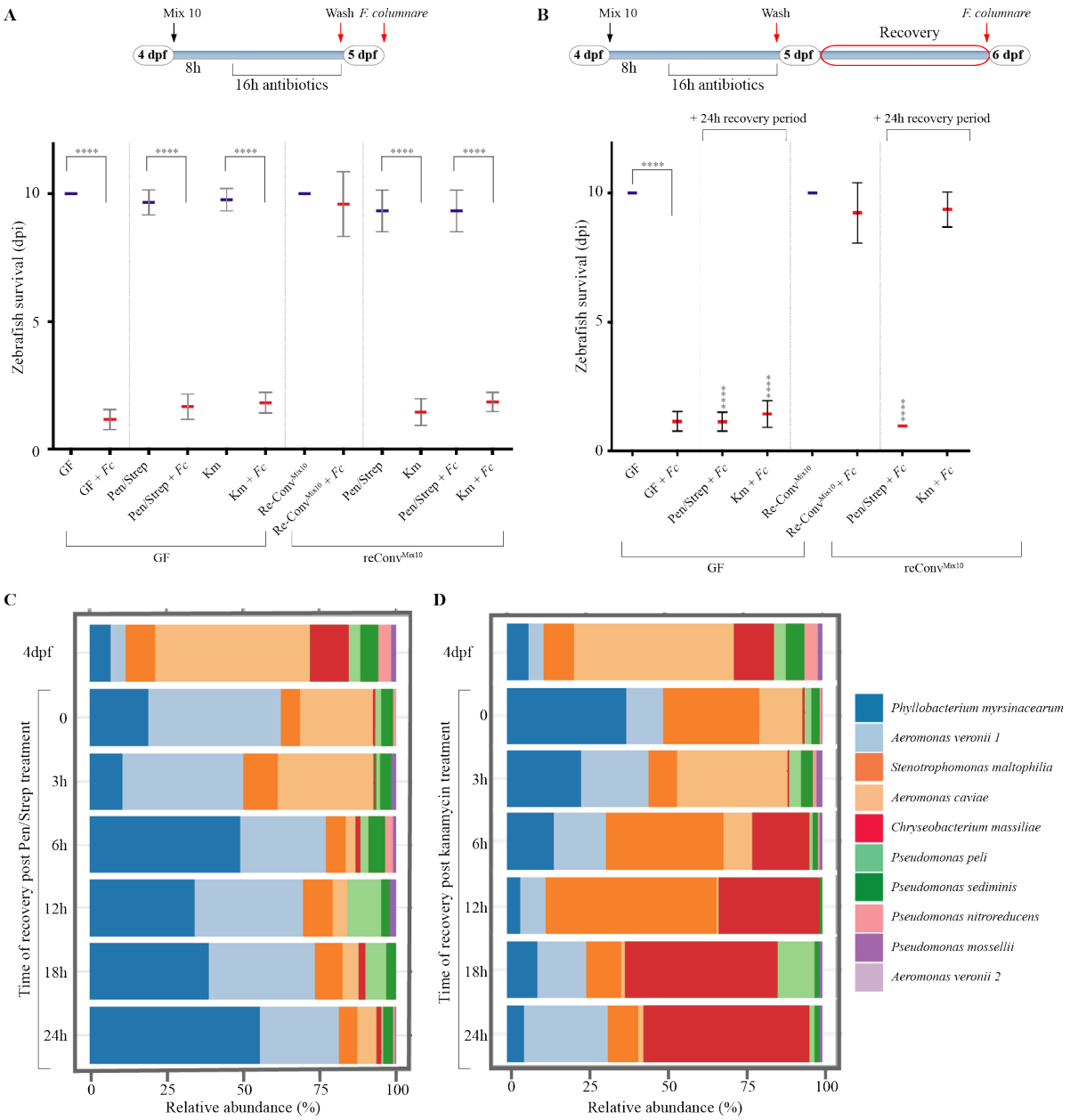

Figure 2 Analysis of protection against *F. columnare* infection after antibiotic dysbiosis. A: Response of zebrafish larvae to exposure to *F. columnare*^{ALG} after antibiotic-induced dysbiosis with a diagram showing timing and treatments of the experiment. **B:** A 24h period after antibiotic treatment allows the recovery of protection in kanamycin-treated zebrafish larvae with a diagram showing timing and treatments. Mean survival is represented by a thick horizontal bar with standard deviation. For each condition, n = 12 zebrafish larvae. Blue mean bars correspond to larvae not exposed to the pathogen and red mean bars correspond to exposed larvae. Larvae mortality rate was monitored daily and surviving fish were euthanized at day 9 post exposition to the pathogen. Indicated statistics correspond to unpaired, non-parametric Mann-Whitney test. ****: $p<0.0001$; absence of *: non-significant. **C:** Community recovery profile with streptomycin/penicillin treatment. **D:** Community recovery profile with kanamycin treatment. Pools of 10 larvae were collected for 16S rRNA sequencing.

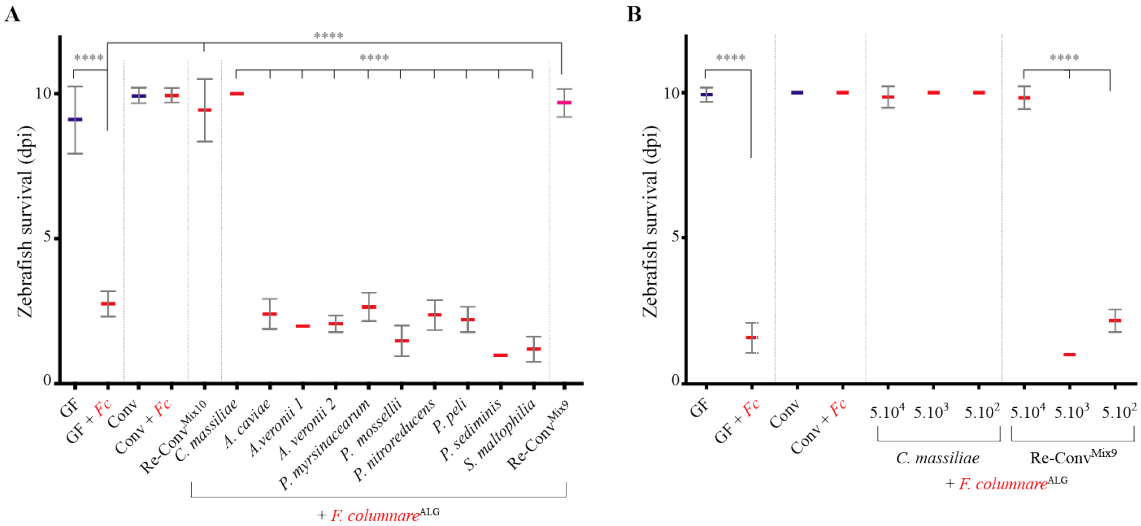

Figure 3. Protection against *F. columnare* in zebrafish re-conventionalized with individual or mixed bacterial strains isolated from zebrafish. A: Determination of the level of protection provided by each of the 10 bacterial strains composing the core protective zebrafish microbiota. Bacteria were added individually to the water on hatching day (dose 5.10^5 cfu/mL). **B**: Level of protection provided by different amount of *C. massiliae* and Mix9. Mix9 only protected at the highest inoculum doses. Mean survival is represented by a thick horizontal bar with standard deviation. For each condition, n = 12 zebrafish larvae. Blue mean bars correspond to larvae not exposed to the pathogen and red mean bars correspond to exposed larvae. Larvae mortality rate was monitored daily and surviving fish were euthanized at day 9 post exposition to the pathogen. Indicated statistics correspond to unpaired, non-parametric Mann-Whitney test. ****: $p<0.0001$; absence of *: non-significant.

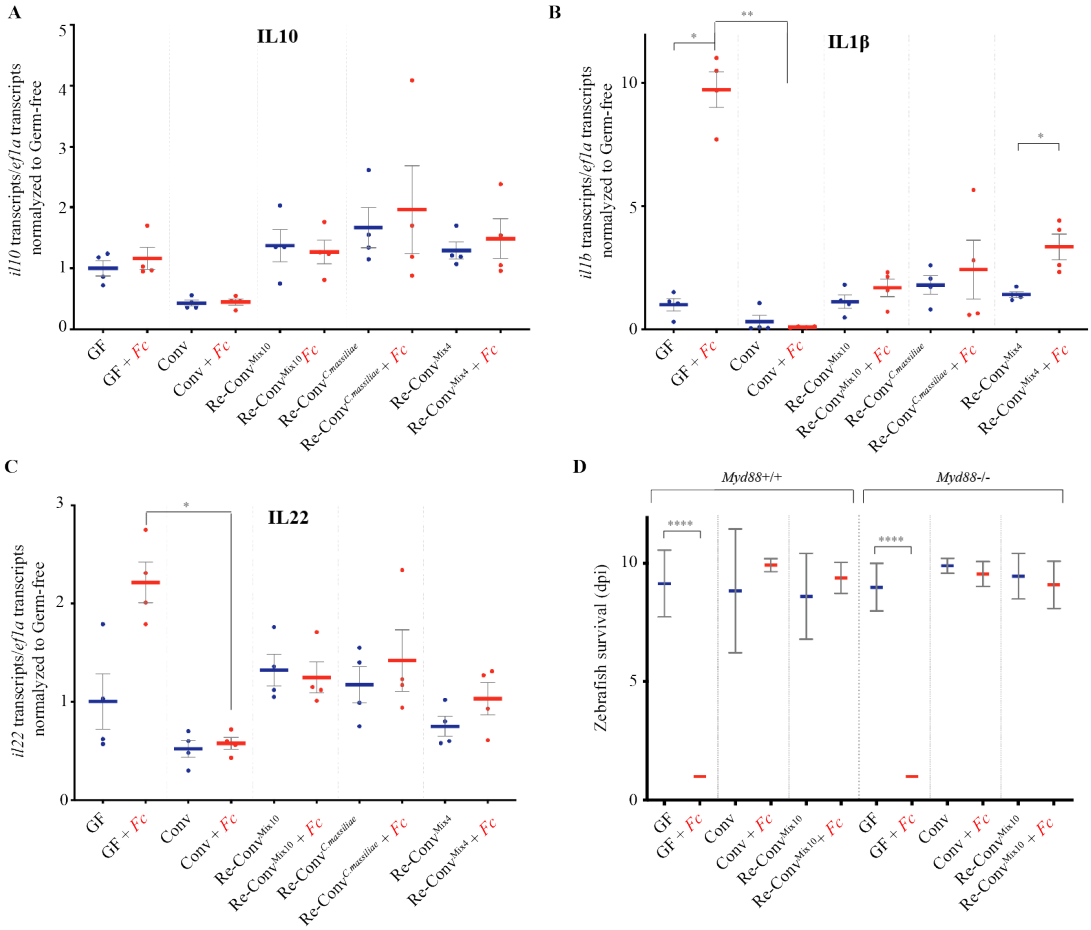

Figure 4. Zebrafish immune response to *F.columnare* infection. A-C: qRT-PCR analysis of host gene expression, 18 hours after exposure to *F. columnare,* in larvae re-conventionalized with indicated bacteria or bacterial mixes; each point corresponds to an individual larva. Expression of *il10* (**A**), *il1b* (**B**), and *il22* (**C**), by wild-type AB zebrafish; **D**: Comparison of the survival of *myd88-/-* and background-matched *myd88+/+* zebrafish after re-conventionalization and exposure to *F. columnare*^{ALG}. Mean survival is represented by a thick horizontal bar with standard deviation. For each condition, n = 12 zebrafish larvae. Larvae mortality rate was monitored daily and surviving fish were euthanized at day 9 post exposition to the pathogen. A-D: Blue bars correspond to larvae not exposed to the pathogen and red mean bars correspond to exposed larvae. Indicated statistics correspond to unpaired, non-parametric Mann-Whitney test. ****: p<0.0001; **: p<0.005*: p<0.05, absence of *: non-significant.

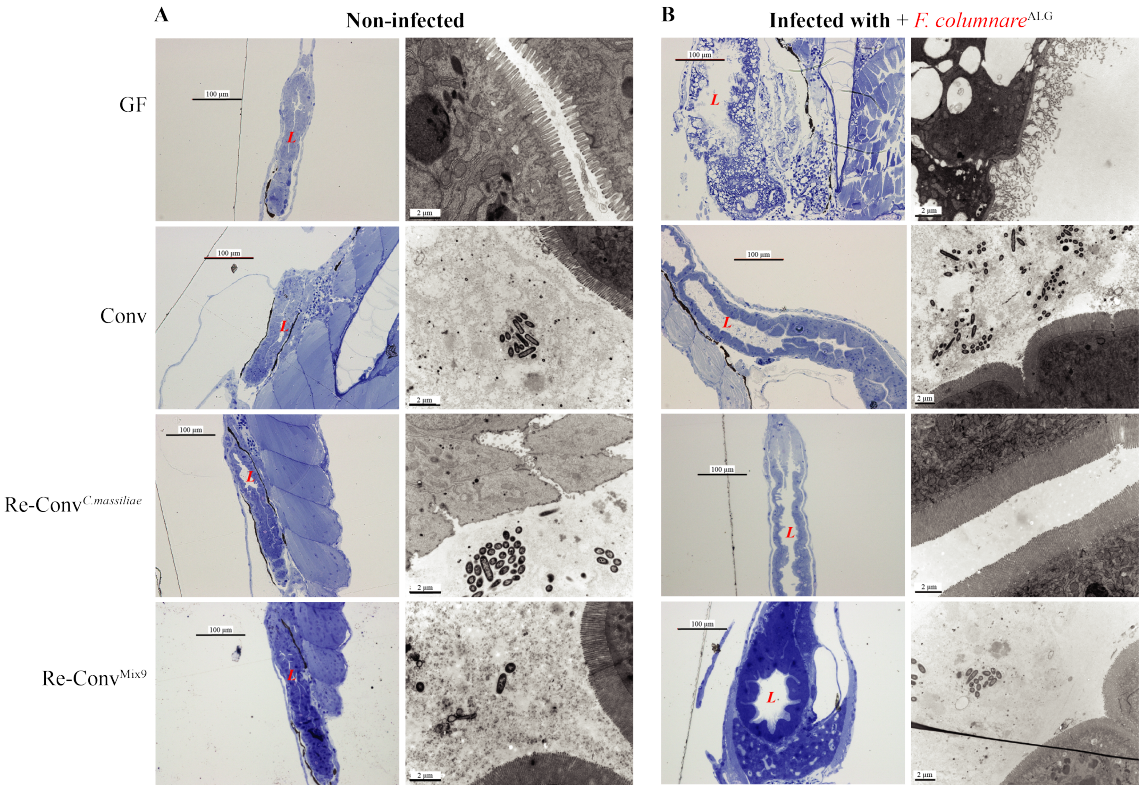

Figure 5. Intestine of infected germ-free zebrafish displays severe disorganization. Germ-free, conventional and re-conventionalized zebrafish larvae. Re-conventionalized zebrafish were inoculated at 4 dpf with Mix9 or *C. massiliae.* **A**: Representative picture of intestines of non-infected larvae. Fish were fixed for histology analysis or electron microscopy at 7 dpf. **B**: Representative picture of intestines of infected larvae exposed at 7 dpf to *F.columnare*^{ALG}. In **A** and **B**: *Left column:* Toluidine blue staining of Epon-embedded zebrafish larvae for Light microscopy. *Right column*: Transmission electron microscopy. at 7 dpf (*right*). L= intestinal lumen.

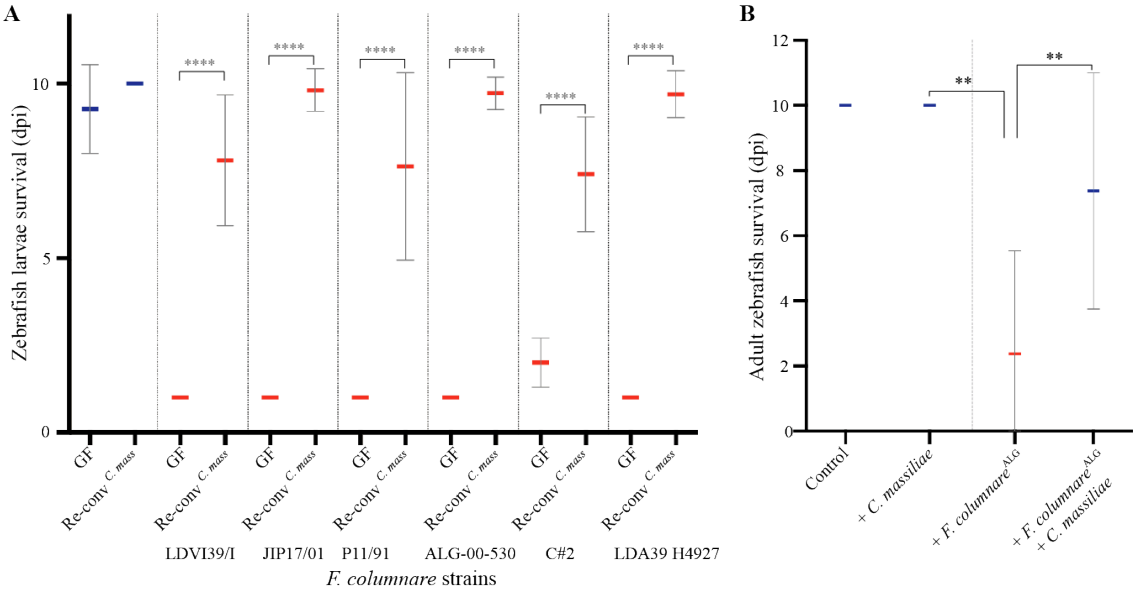

Figure 6. Pre-exposure to *C. massiliae* protects larval and adult zebrafish against *F. columnare* infection

A: Zebrafish larvae were inoculated at 4 dpf with 5.10^5 cfu/mL of *C. massiliae* for 48h before infection at 6 dpf with virulent *F. columnare* strains. **B:** Survival of adult zebrafish with or without pre-exposure to *C. massiliae* (2.10^6 cfu/mL for 48h) followed by exposure to *F. columnare*^{ALG} (5.10^6 cfu/mL for 1h) Mean survival is represented by a thick horizontal bar with standard deviation. For each condition, n = 12 zebrafish larvae or 10 adult. Zebrafish mortality rate was monitored daily and surviving fish were euthanized at day 9 post exposition to the pathogens. Blue bars correspond to larvae not exposed to the pathogen and red mean bars correspond to exposed larvae. Indicated statistics correspond to unpaired, non-parametric Mann-Whitney test. ****: $p<0.0001$; **: $p<0.005$; absence of *: non-significant.