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

Franziska A. STRESSMANN[1,2†], Joaquin BERNAL-BAYARD[1,3†], David PEREZ-PASCUAL[1], Bianca AUDRAIN[1], Olaya RENDUELES[1,4], Valérie BRIOLAT[5], Sebastian BRUCHMANN[6,7], Stevenn VOLANT[8], Amine GHOZLANE[8], Susanne HAUSSLER[6,9], Eric DUCHAUD[10] Jean-Pierre LEVRAUD[5] and Jean-Marc GHIGO[1]*

[1] *Genetics of Biofilms Laboratory, Institut Pasteur,* UMR CNRS2001, *Paris 75015, France*

[2] *current address: Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Department of Chemical Analytics and Biogeochemistry, Müggelseedamm 310, 12587, Berlin, Germany*

[3] *Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain*

[4] *current address: Microbial Evolutionary Genomics Laboratory, Institut Pasteur, UMR3525, Paris 75015, France*

[5] *Macrophages and Development of Immunity Laboratory, Institut Pasteur, UMR3738 CNRS, Paris 75015, France*

[6] *Department of Molecular Bacteriology, Helmholtz Centre for Infection Research, Braunschweig, Germany.*

[7] *current address: University of Cambridge, Department of Veterinary Medicine, Madingley Road Cambridge CB3 0ES, UK*

[8] *Hub de Bioinformatique et Biostatistique – Département Biologie Computationnelle, Institut Pasteur, USR 3756 CNRS, Paris, France*

[9] *current address:Department of Clinical Microbiology, Rigshospitalet; Copenhagen, 2100, Denmark*

[10] *Unité VIM, INRAE, Université Paris-Saclay, 78350 Jouy-en-Josas, France.*

† FS and JBB equally contributed to this work.

*** Corresponding author:** Jean-Marc Ghigo (jmghigo@pasteur.fr).

**Running title:** Microbiota-based colonization resistance in zebrafish

**Keywords:** Zebrafish; germ-free; probiotic; microbiota; colonization resistance, infection, *Flavobacterium columnare.*

**ABSTRACT**

The identification of commensal bacteria conferring resistance against pathogens is often hindered by the complexity of host microbial communities. We used germ-free, conventional and re-conventionalized zebrafish as a model to study the determinant of microbiota-associated colonization resistance against the fish pathogen *Flavobacterium columnare*. We showed that a consortium of 10 culturable endogenous bacterial species protects zebrafish from lethal intestinal damages caused by *F. columnare* infection using two different scenarios that do not rely on host innate immunity. Alteration of microbiota composition upon antibiotic dysbiosis first identified *Chryseobacterium massilliae* as a key bacterium protecting both larvae and adult zebrafish. We also showed that an assembly of 9 species that do not otherwise protect individually confer a community-level resistance to infection. Our study reveals the ecological strategies at play in microbiota-based protection against pathogens in a low-complexity *in vivo* model, opening perspectives for the rational engineering of resilient microbial communities.

## INTRODUCTION

Animal resident microbial consortia form complex and long-term associations with important community-level functions essential for host development and physiology (Rolig et al. 2015, McFall-Ngai 2002). Microbial ecosystems also provide protection against exogenous pathogens by inhibition of pathogen settlement and growth and/or stimulation of the host immune system (van der Waaij, Berghuis-de Vries, and Lekkerkerk-van der Wees 1972, Cani and Delzenne 2009, Stecher and Hardt 2008, 2011, Falcinelli et al. 2016, Olsan et al. 2017). 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 (Littman and Pamer 2011, van der Waaij, Berghuis-de Vries, and Lekkerkerk-van der Wees 1972). 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 (Heselmans et al. 2005, Boirivant and Strober 2007, Robinson, Bohannan, and Young 2010).

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 (Vollaard and Clasener 1994, Gill 2003, Stecher and Hardt 2011, Rolig et al. 2015, Falcinelli et al. 2016). 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 (Rawls, Samuel, and Gordon 2004, Milligan-Myhre et al. 2011, Burns and Guillemin 2017, Douglas 2019, Flores et al. 2020). Zebrafish can be easily reared germ-free or gnotobiotically in association with specific bacterial species (Rawls, Samuel, and Gordon 2004, Melancon et al. 2017). 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 (Cantas et al. 2012, Rendueles et al. 2012, Caruffo et al. 2016, Perez-

Ramos et al. 2018). Several studies have used zebrafish to evaluate the effect of exogenous addition of potential probiotics on host resistance to infection by various pathogens (Rendueles et al. 2012, Chu et al. 2014, Caruffo et al. 2016, Wang et al. 2016, Qin et al. 2017, Perez-Ramos et al. 2018, Girija et al. 2018, Lin et al. 2019). However, the role of the endogenous microbial community in protecting against invasive pathogen was rarely assessed and the reported protections were often partial, illustrating the difficulty in identifying fully protective exogenous probiotics.

Here we used germ-free and conventional zebrafish larvae to mine the indigenous commensal microbiota for bacterial species protecting against *Flavobacterium columnare*, a bacterial pathogen affecting wild and cultured fish species. We identified two distinct infection resistance strategies preventing mortality caused by *F. columnare*, mediated either by an individual member of the microbiota, the *Bacteroidetes Chryseobacterium massiliae* or by an assembly of 9 individually non-protecting bacterial species that formed a protective community. Our results demonstrated that mining host microbiota constitutes a powerful approach to identify key mediators of intrinsic colonization resistance, providing insight into how to engineer ecologically resilient and protective microbial communities.

114 **RESULTS**

115

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

117 To investigate microbiota-based resistance to infection in zebrafish, we compared the
118 sensitivity of germ-free (GF) and conventional (Conv) zebrafish larvae to *F. columnare*,
119 an important fish pathogen affecting carp, channel catfish, goldfish, eel, salmonids and
120 tilapia and previously shown to infect and kill adult zebrafish (Soto et al. 2008, Olivares-
121 Fuster et al. 2011, Declercq et al. 2013, Li et al. 2017, Robinson, Bohannan, and Young
122 2010). We used bath immersion to expose GF and Conv zebrafish larvae at 6 days post
123 fertilization (dpf), to a collection of 28 *F. columnare* strains, belonging to four different
124 genomovars for 3h at $5.10^5$ colony forming units (cfu)/mL. Daily monitoring showed that
125 16 out of 28 *F. columnare* strains killed GF larvae in less than 48h (Supplementary Fig.
126 S1A), whereas Conv larvae survived exposure to all tested virulent *F. columnare* strains
127 (Supplementary Fig. S1B). Exposure to the highly virulent strain ALG-00-530 (hereafter
128 *F. columnare*$^{ALG}$) also showed that GF mortality was fast (1 day) and dose-dependent and
129 that Conv zebrafish survived all but the highest dose ($10^7$ cfu/mL) (Fig. 1). Similar
130 survival of infected Conv larvae was obtained with zebrafish AB strain eggs obtained
131 from 4 different zebrafish facilities (Supplementary Fig. S2), suggesting that
132 conventional zebrafish microbiota could provide protection against *F. columnare*
133 infection.

134

135 **A community of 10 culturable bacterial strains protect against *F. columnare***
136 **infection**

137 In our rearing conditions, the conventional larval microbiota is acquired after hatching
138 from microorganisms present on the egg chorion and in fish facility water. To test the
139 hypothesis that microorganisms associated with conventional eggs provided protection
140 against *F. columnare*$^{ALG}$, we exposed sterilized eggs to either fish facility tank water or
141 to non-sterilized conventional eggs at 0 or 4 dpf (before or after hatching, respectively).
142 In both cases, these re-conventionalized (re-Conv) zebrafish survived *F. columnare*$^{ALG}$
143 infection as well as Conv zebrafish (Supplementary Fig. S3). To determine the
144 composition of conventional zebrafish microbiota, we generated 16S rRNA gene clone
145 libraries from homogenate pools of Conv larvae aged 6 and 11 dpf exposed or not to *F.*

146 *columnare*$^{ALG}$, sampled over 3 months from 3 different batches of larvae (n=10). A total
147 of 857 clones were generated for all samples. We identified 15 operational taxonomical
148 units (OTUs), 10 of which were identified in all experiments (Table 1, Suppl. Table S1).
149 Two OTUs (belonging to an *Ensifer* sp. and a *Hydrogenophaga* sp.) were only detected
150 once, and a *Delftia* sp., a *Limnobacter* sp. and a *Novosphingobium* sp. were detected more
151 than once (2, 3 and 2 times, respectively), but not consistently in all batches of fish (Table
152 1, Suppl. Table S1). Moreover, deep-sequencing of the 16S rRNA V3-V4 region of
153 gDNA retrieved from larvae originating from the other four zebrafish facilities described
154 above, revealed that OTUs for all of these 10 strains were also detected in Conv larvae,
155 with the exception of *A. veronii* strain 2 that was not detected in all samples (Suppl. Table
156 S2).
157 To isolate culturable zebrafish microbiota bacteria, we plated dilutions of homogenized
158 6 dpf and 11 dpf larvae pools on various growth media and we identified 10 different
159 bacterial morphotypes. 16S-based analysis followed by full genome sequencing
160 identified 10 bacteria corresponding to 10 strains of 9 different species that were also
161 consistently detected by culture-free approaches (Table 1). To assess the potential
162 protective role of these 10 strains, we re-conventionalized GF zebrafish at 4 dpf with a
163 mix of all 10 identified culturable bacterial species (hereafter called Mix10), each at a
164 concentration of $5.10^5$ cfu/mL and we monitored zebrafish survival after exposure to *F.*
165 *columnare*$^{ALG}$ at 6 dpf. We showed that zebrafish re-conventionalized with the Mix10
166 (Re-Conv$^{Mix10}$) displayed a strong level of protection against all identified highly virulent
167 *F. columnare* strains (Supplementary Fig. S4). These results demonstrated that the Mix10
168 constitutes a core protective bacterial community providing full protection of zebrafish
169 larvae against *F. columnare* infection.
170
171 **Community dynamics under antibiotic-induced dysbiosis reveal a key contributor**
172 **to resistance to *F. columnare* infection**
173 To further analyze the determinants of Mix10 protection against *F. columnare*$^{ALG}$
174 infection, we inoculated 4 dpf larvae with an equal-ratio mix of the 10 bacteria (at $5.10^5$
175 cfu/mL each) and monitored their establishment over 8 hours. We first verified that whole
176 larvae bacterial content (OTU abundance) was not significantly different from content of
177 dissected intestinal tubes (p=0.99, two-tailed t-test) (Supplementary Fig. S5) and

178 proceeded to use entire larvae to monitor bacterial establishment and recovery in the rest
179 of the study. We then collected pools of 10 larvae immediately after re-
180 conventionalization ($t_0$), and then at 20 min, 2 hours, 4 hours and 8 hours in three
181 independent experiments. Illumina sequencing of the 16S rRNA gene was used to follow
182 bacterial relative abundance. At $t_0$, all species were present at > 4% in the zebrafish, apart
183 from *A. veronii* strains 1 (0.2%) and 2 (not detected) (Supplementary Fig. S6). *Aeromonas*
184 *caviae* was detected as the most abundant species (33%), followed by *Stenotrophomonas*
185 *maltophilia* (23%) and *Chryseobacterium massiliae* (12%), altogether composing 68% of
186 the community (Supplementary Fig. S6). The relative species abundance, possibly
187 reflecting initial colonization ability, was relatively stable for most species during
188 community establishment, with similar species evenness at $t_0$ (E = 0.84) and $t_{8h}$ (E = 0.85).
189 Whereas both Conv and Re-Conv$^{Mix10}$ larvae were protected against *F. columnare*$^{ALG}$
190 infection, the global structure of the reconstituted Mix10 population was different from
191 the conventional one at 4 dpf (Supplementary Fig. S6).
192 To test the sensitivity to disturbance and the resilience of the protection provided by
193 Mix10 bacterial community, we subjected Re-Conv$^{Mix10}$ zebrafish to non-toxic antibiotic
194 treatment at 4dpf using either 250 µg/mL penicillin/streptomycin combination (all
195 members of the Mix10 bacteria are sensitive to penicillin/streptomycin) or 50 µg/mL
196 kanamycin (affecting all members of the Mix10 bacteria except *C. massiliae*, *P.*
197 *myrsinacearum* and *S. maltophilia*) (Suppl. Fig. S7). At 5 dpf, after 16 hours of exposure,
198 antibiotics were washed off and zebrafish were immediately exposed to *F. columnare*$^{ALG}$.
199 Both antibiotic treatments resulted in complete loss of the protection against *F.*
200 *columnare*$^{ALG}$ infection observed in Re-Conv$^{Mix10}$ (Fig. 2A). We then used the same
201 antibiotic treatments but followed by a 24h recovery period after washing off the
202 antibiotics at 5 dpf, therefore only performing the infection at 6 dpf (Fig. 2B). Whilst Re-
203 Conv$^{Mix10}$ larvae treated with penicillin/streptomycin showed similar survival to infected
204 GF larvae, kanamycin-treated Re-Conv$^{Mix10}$ zebrafish displayed restored protection after
205 24h recovery and survived similarly to untreated conventionalized fish (Fig. 2B).
206 Sampling and 16S gene analysis during recovery experiments at different time points
207 showed that bacterial community evenness decreased after antibiotic administration for
208 both treatments (E = 0.85 for 4 dpf control, E = 0.72 for $t_0$ kanamycin and E = 0.7 for $t_0$
209 penicillin/streptomycin), and continued to decrease during recovery (E = 0.6 and 0.64 for

210 kanamycin and penicillin/streptomycin treatment after 24h recovery, respectively).
211 Although *C. massiliae* remained detectable immediately after both antibiotic treatments,
212 penicillin/streptomycin treatment led a significant reduction in its relative abundance
213 (0.21%) (Fig. 2C). By contrast, *C. massiliae* relative abundance rebounded 6h after
214 cessation of kanamycin treatment and was the dominant member (52%) of the
215 reconstituted microbiota after 24h recovery period (Fig. 2D), suggesting that the
216 protective effect observed in the kanamycin-treated larvae might be due to the recovery
217 of *C. massiliae*.
218

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

221 To test the potential key role played by *C. massiliae* in protection against *F. columnare*$^{ALG}$
222 infection, we exposed 4 dpf GF zebrafish to *C. massiliae* only and showed that it
223 conferred individual protection at doses as low as $5.10^2$ cfu/mL (Fig. 3). Whereas none
224 of the 9 other species composing the Mix10 were protective individually (Fig. 3A), their
225 equiratio combination (designated as Mix9) conferred protection to zebrafish, although
226 not at doses lower than $5.10^4$ cfu/mL and not as reproducibly as with *C. massiliae* (Fig.
227 3B). To identify which association of species protected Re-Conv$^{Mix9}$ zebrafish against *F.*
228 *columnare*$^{ALG}$ infection, we tested all 9 combinations of 8 species (Mix8), as well as
229 several combinations of 7, 6, 4 or 3 species and showed no protection (Suppl. Fig. S8A
230 and Suppl. Table S4). We then tested whether lack of protection of Mix8 compared to
231 Mix9 could rely on a density effect by doubling the concentration of any of the species
232 within the non-protective Mix8a (Suppl. Fig. S8B) and showed no protection. These
233 results therefore indicated that microbiota-based protection against *F. columnare*$^{ALG}$
234 infection relied on either *C. massiliae*-dependent membership effect or on a community-
235 dependent effect mediated by the Mix9 consortium.
236

237 **Pro- and anti- inflammatory cytokine production does not contribute to microbiota-**
238 **mediated protection against *F. columnare*$^{ALG}$ infection.**

239 To test the contribution of the immune response of zebrafish larvae to resistance to *F.*
240 *columnare* infection, we used qRT-PCR to measure cytokine mRNA expression in GF
241 and Conv zebrafish exposed or not to *F. columnare*$^{ALG}$. We also tested the impact of re-

242 conventionalization with *C. massiliae* (re-Conv$^{Cm}$), Mix10 (re-Conv$^{Mix10}$) or with Mix4
243 (*A. caviae*, both *A. veronii* spp., *P. mossellii*) as a non-protective control (Suppl. Table
244 S4). We tested genes encoding IL1β (pro-inflammatory), IL22 (promoting gut repair),
245 and IL10 (anti-inflammatory) cytokines. While we observed some variation in *il10*
246 expression among non-infected re-conventionalized larvae, this did not correlate with
247 protection. Furthermore, *il10* expression was not modulated by infection in any of the
248 tested conditions (Fig. 4A). By contrast, we observed a strong induction of *il1b* and *il22*
249 in GF zebrafish exposed to *F. columnare*$^{ALG}$ (Fig. 4BC). However, although this
250 induction was reduced in protected Conv, Re-Conv$^{Cm}$ and Re-Conv$^{Mix10}$, it was also
251 observed in non-protective Re-Conv$^{Mix4}$ larvae, indicating that down-modulation of the
252 inflammatory response induced by *F. columnare* does not correlate with resistance to
253 infection. Consistently, the use of a *myd88* mutant, a key adaptor of IL-1 and toll-like
254 receptor signalling deficient in innate immunity(Cheesman et al. 2011, van der Vaart et
255 al. 2013), showed that Conv or Re-Conv$^{Mix10}$, but not GF *myd88* mutants survived *F.*
256 *columnare* as well as wild-type zebrafish (Figure 4D). Moreover, *il1b* induction by *F.*
257 *columnare* infection was observed only in GF larvae and was *myd88*-independent (Suppl.
258 Figure S9). These results therefore indicated that the tested cytokine responses do not
259 play a significant role in the microbiota-mediated protection against *F. columnare*
260 infection.
261
262 ***C. massiliae* and Mix9 protect zebrafish from intestinal damages upon *F.***
263 ***columnare*$^{ALG}$ infection**
264 Histological analysis of GF larvae fixed 24h after exposure to *F. columnare*$^{ALG}$ revealed
265 extensive intestinal damage (Fig. 5A) prior to noticeable signs in other potential target
266 organs such as gills or skin. To test the requirement for gut access in *F. columnare*$^{ALG}$
267 infection process, we modified our standard rearing protocol of GF fish, which involves
268 feeding with live germ-free *T. thermophila*. We found that, if left unfed, GF zebrafish did
269 not die after *F. columnare*$^{ALG}$ exposure, while feeding with either *T. thermophila* or
270 another food source such as sterile fish food powder, restored sensitivity to *F.*
271 *columnare*$^{ALG}$ infection (Suppl. Fig S10), suggesting that successful infection requires
272 feeding and ingestion.

273 Histological sections consistently showed severe disorganization of the intestine with
274 blebbing in the microvilli and vacuole formation in *F. columnare*$^{ALG}$-infected GF larvae
275 (Fig. 5). In contrast, zebrafish pre-incubated with either *C. massiliae* or Mix9 consortium
276 at 4 dpf, and then exposed to *F. columnare*$^{ALG}$ at 6 dpf showed no difference compared
277 to non-infected larvae or conventional infected larvae (Fig. 5), confirming full protection
278 against *F. columnare*$^{ALG}$ at the intestinal level.
279
280 ***C. massiliae* protects larvae and adult zebrafish against *F. columnare***
281 The clear protection provided by *C. massiliae* against *F. columnare*$^{ALG}$ infection
282 prompted us to test whether exogenous addition of this bacterium could improve
283 microbiota-based protection towards this widespread fish pathogen. We first showed that
284 zebrafish larvae colonized with *C. massiliae* were fully protected against all virulent *F.*
285 *columnare* strains identified in this study (Fig. 6A). To test whether *C. massiliae* could
286 also protect adult zebrafish from *F. columnare* infection, we pre-treated conventional 3-
287 4-month-old Conv adult zebrafish with *C. massiliae* for 48h before challenging them with
288 a high dose ($5.10^6$ cfu/mL) *F. columnare*$^{ALG}$. Monitoring of mortality rate showed that
289 pre-treatment with *C. massiliae* significantly increased the survival rate of adult zebrafish
290 upon *F. columnare*$^{ALG}$ infection compared to non-treated conventional fish ($p=0.0084$
291 Mann-Whitney test, Figure 6B). Taken together, these results show that *C. massiliae* is a
292 putative broad-spectrum probiotic protecting zebrafish against columnaris disease caused
293 by *F. columnare*.
294
295

296 **DISCUSSION**

297

298 In this study, we used re-conventionalization of otherwise germ-free zebrafish larvae to
299 show that conventional-level protection against infection by a broad range of highly
300 virulent *F. columnare* strains is provided by a set of 10 culturable bacterial strains,
301 belonging to 9 different species, isolated from the indigenous standard laboratory
302 zebrafish microbiota. With the exception of the Bacteroidetes *C. massiliae*, this protective
303 consortium was dominated by Proteobacteria such as *Pseudomonas* and *Aeromonas* spp.,
304 bacteria commonly found in aquatic environments (Mena and Gerba 2009, Goncalves
305 Pessoa et al. 2019). Despite the relative permissiveness of zebrafish larvae microbiota to
306 environmental variations and inherent variability between samples (Stephens et al. 2016),
307 we showed that these ten bacteria were consistently identified in four different zebrafish
308 facilities, suggesting the existence of a core microbiota with important colonization
309 resistance functionality. Use of controlled combinations of these 10 bacterial species
310 enabled us to show a very robust species-specific protection effect in larvae mono-
311 associated with *C. massiliae.* We also identified a community-level protection provided
312 by the combination of the 9 other species that were otherwise unable to protect against *F.*
313 *columnare* when provided individually. This protection was however less reproducible
314 and required a minimum inoculum of $5.10^4$ cfu/mL, compared to $5.10^2$ cfu/mL with *C.*
315 *massiliae*. These results therefore suggest the existence of two distinct microbiota-based
316 protection scenarios: one based on a membership effect provided by *C. massiliae*, and the
317 other mediated by the higher-order activity of the Mix9 bacterial community.

318

319 Although protection against *F. columnare* infection does not seem to rely on
320 microbiota-based immuno-modulation, we cannot exclude that, individually, some
321 members of the studied core zebrafish microbiota could induce pro- or anti-inflammatory
322 responses masked in presence of the full Mix10 consortium (Rolig et al. 2015).Further
323 studies will be required to characterize the different mechanisms of the two identified
324 colonization resistance scenarios, which could include nutrient depletion, adhesion
325 inhibition, direct contact-growth inhibition or release of inhibitory molecules leading to
326 niche exclusion (Vollaard and Clasener 1994, Stecher and Hardt 2011). In the case of the
327 protection provided by *C. massilliae* against *F. columnare,* these two phylogenetically
328 close bacteria could compete for similar resources and directly antagonize each other, as

329 shown to occur between *Bacteroidetes* species (Chatzidaki-Livanis, Coyne, and
330 Comstock 2014, Russell et al. 2014, Roelofs et al. 2016, Jacobson et al. 2018).
331 Interestingly, infected larvae re-conventionalized with either C. *massiliae* or Mix9
332 showed no signs of the intestinal damage displayed by germ-free larvae, suggesting that
333 both *C. massiliae* and Mix9 provide similar intestinal resistance to *F. columnare*
334 infection. Whereas microbial colonization contributes to gut maturation and stimulates
335 the production of epithelial passive defences such as mucus (Kamada et al. 2013, Ganz,
336 Melancon, and Eisen 2016), lack of intestinal maturation is unlikely to be contributing to
337 *F. columnare*-induced mortality, as mono-colonized larvae or larvae re-conventionalized
338 with non-protective mixes died as rapidly as germ-free larvae.

339 Several studies have monitored the long-term assembly and development of the
340 zebrafish microbiota from larvae to sexually mature adults, however little is known about
341 the initial colonization establishment of the larvae after hatching (Bates et al. 2006, Yan,
342 van der Gast, and Yu 2012). Neutral (stochastic) and deterministic (host niche-based)
343 processes (Costello et al. 2012, Rogers et al. 2013, Burns et al. 2016) lead to microbial
344 communities that are mostly represented by a limited number of highly abundant species
345 with highly diverse low-abundant populations. In our experiments, the Mix10 species
346 inoculum corresponded to an equiratio bacterial mix, thus starting from an engineered
347 and assumed total evenness (E = 1) (Hibbing et al. 2010, Shafquat et al. 2014). Evenness
348 was still relatively high (0.84) and remained similar up until 8h in our study, indicating
349 that most of the ten species were able to colonize the larvae. From the perspective of
350 community composition, a loss of diversity is often associated with decreased
351 colonization resistance, but it remains unclear whether this increased susceptibility is due
352 to the loss of certain key member species of the microbial community and/or a change in
353 their prevalence (van der Waaij, Berghuis-de Vries, and Lekkerkerk-van der Wees 1972,
354 Littman and Pamer 2011).

355 We further investigated resistance to infection by exposing established bacterial
356 communities to different antibiotic perturbations, followed by direct challenge with *F.*
357 *columnare* (to study core microbiota sensitivity to disturbance) or after recovery (to study
358 its resilience) (Robinson, Bohannan, and Young 2010, Shade et al. 2012). Antibiotics are
359 known to shift the composition and relative abundances of the microbiota according to
360 their spectrum (Vollaard and Clasener 1994, Willing, Russell, and Finlay 2011). We

361 observed that penicillin/streptomycin treatment that would affect most of the core species,
362 reduced the abundance of all but two species (*A. veronii* 1 and *P. myrsinacearum)* that
363 became relatively dominant during recovery, but failed to provide protection against *F.*
364 *columnare*. With the kanamycin treatment, colonization resistance was fully restored at
365 the end of the 24h recovery period, indicative of a resilience that could result from species
366 recovering quickly to their pre-perturbation levels due to fast growth rates, physiological
367 flexibility or mutations (Brugman et al. 2009). Interestingly, even taking into account
368 potential biases associated with the use of the 16S rRNA as a proxy index to determine
369 relative abundance (Salonen et al. 2010, Kunin et al. 2010), evenness was similarly
370 reduced during recovery for both treatments, but abundance at phylum level changed to
371 48% for Proteobacteria, and 52% for Bacteroidetes compared to the >98% of
372 Proteobacteria with the penicillin/streptomycin treatment. Furthermore, *C. massiliae* was
373 detected as rare (<1%) in conventional larvae, suggesting that it could have a
374 disproportionate effect on the community or that community-level protection provided
375 by the nine other bacteria was also responsible for the protection of conventional larvae
376 to *F. columnare* infection.

377 Our study showed that germ-free zebrafish larvae are highly susceptible to a
378 variety of different *F. columnare* genomovars isolated from different hosts,
379 demonstrating that they are a robust animal model for the study of its pathogenicity.
380 Recently, *F. columnare* mutants in Type 9 secretion system (T9SS) were shown to be
381 avirulent in adult zebrafish, suggesting that proteins secreted by the T9SS are likely to be
382 key, but still largely unidentified, *F. columnare* virulence determinants (Li et al. 2017).
383 Body skin, gills, fins and tail are also frequently damaged in salmonid fish, whereas
384 severe infection cases are associated with septicemia (Bernardet and Bowman 2006). We
385 could not identify such clear *F. columnare* infection sites in zebrafish larvae, perhaps due
386 to the very low dose of infection used, with less than 100 cfu recovered from infected
387 moribund larvae. However, several lines of evidence suggest that the gut is the main target
388 of *F. columnare* infection in our model: (i) unfed germ-free larvae survived exposure, (ii)
389 histology analysis showing severe disruption of the intestinal region just hours after
390 infection in germ-free larvae, and (iii) induction of *il22* in germ-free larvae exposed to *F.*
391 *columnare*, since a major function of IL-22 is to promote gut repair (Shabgah et al. 2017).
392 This induction appears to be a consequence of the pathogen-mediated damage, as there

was no observed induction in conventional or re-conventionalized larvae. The very rapid death of larvae likely caused by this severe intestinal damage may explain why other common target organs of columnaris disease showed little damage.

In this study, we showed that *C. massiliae* is a promising probiotic candidate that could contribute to reduce the use of antibiotic to prevent columnaris diseases in research and aquaculture settings. Whereas *C. massiliae* provided full and robust protection against all tested virulent *F. columnare* genomovars and significantly increased survival of exposed adult conventional zebrafish, further studies are needed to elucidate *C. massiliae* protection potential in other teleost fish. However, the endogenous nature of *C. massiliae* suggests that it could establish itself as a long-term resident of the zebrafish larval and adult microbiota, an advantageous trait when seeking a stable modulation of the bacterial community over long periods (Lemon et al. 2012).

In conclusion, the use of a simple and tractable zebrafish larval model to mine indigenous host microbial communities allowed us to identify two independent protection pathways against the same pathogen. Whereas further study will determine how these pathways may contribute to protection against a wider range of pathogens, this work also provides insights into how to the engineering of stable protective microbial communities with controlled colonization resistance functions.

417 **MATERIALS AND METHODS**

418

419 **Bacterial strains and growth conditions.** Bacterial strains isolated from zebrafish
420 microbiota are listed in Table 1. *F. columnare* strains (Suppl. Table S3) were grown at
421 28°C in tryptone yeast extract salts (TYES) broth [0.4 % (w/v) tryptone, 0.04 % yeast
422 extract, 0.05 % (w/v) $MgSO_4$ $7H_2O$, 0.02 % (w/v) $CaCl_2$ $2H_2O$, 0.05 % (w/v) D-glucose,
423 pH 7.2]. *F. columnare* strains were assigned into four genomovar groups using 16S rRNA
424 restriction fragment length polymorphism analysis, including genomovar I, I/II, II, and
425 III (Garcia et al. 2018). All 10 strains of the core zebrafish microbiota species were grown
426 in TYES or LB at 28°C.

427

428 **Ethics statement**

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

434

435 **General handling of zebrafish**. Wild-type AB fish, originally purchased from the
436 Zebrafish International Resource Center (Eugene, OR, USA), or *myd88*-null mutants
437 (*myd88*$^{hu3568/hu3568}$) (van der Vaart et al. 2013), kindly provided by A.H Meijer, (Leiden
438 University, the Netherlands), were raised in our facility. A few hours after spawning, eggs
439 were collected, rinsed, and sorted under a dissecting scope to remove faeces and
440 unfertilized eggs. All following procedures were performed in a laminar microbiological
441 cabinet with single-use disposable plasticware. Fish were kept in sterile 25 $cm^3$ vented
442 cap culture flasks containing 20 mL of water (0-6 dpf- 15 fish per flasks) or 24-well
443 microtiter plates (6-15 dpf-1 fish per 2 mL well) in autoclaved mineral water (Volvic) at
444 28°C. Fish were fed 3 times a week from 4 dpf with germ-free *Tetrahymena thermophila*
445 protozoans (Rendueles et al. 2012). Germ-free zebrafish were produced after sterilizing
446 the egg chorion protecting the otherwise sterile egg, with antibiotic and chemical
447 treatments (see below), whereas conventional larvae (with facility-innate microbiota)
448 were directly reared from non-sterilized eggs and then handled exactly as the germ-free
449 larvae.

450

451 **Sterilization of zebrafish eggs.** Egg sterilization was performed as previously described
452 with some modifications (Rendueles et al. 2012). Freshly fertilized zebrafish eggs were
453 first bleached (0.003%) for 5 min, washed 3 times in sterile water under gentle agitation
454 and maintained overnight in groups of 100 eggs per 75 $cm^3$ culture flasks with vented
455 caps containing 100 mL of autoclaved Volvic mineral water supplemented with
456 methylene blue solution (0.3 µg/mL). Afterwards, eggs were transferred into 50 mL
457 Falcon tubes (100 eggs per tube) and treated with a mixture of antibiotics (500 µL of
458 penicillin G: streptomycin, 10,000 U/ml: 10 mg/mL GIBCO #P4333), 200 µL of filtered
459 kanamycin sulfate (100 mg/mL, SERVA Electrophoresis #26899) and antifungal drug
460 (50 µL of amphotericin B solution Sigma-Aldrich (250 µg/mL) #A2942) for 2 h under
461 agitation at 28°C. Eggs were then washed 3 times in sterile water under gentle agitation
462 and bleached (0.003%) for 15 min, resuspending the eggs every 3 min by inversion. Eggs
463 were washed again 3 times in water and incubated 10 min with 0.01% Romeiod (COFA,
464 Coopérative Française de l'Aquaculture). Finally, eggs were washed 3 times in water and
465 transferred into 25 $cm^3$ culture flasks with vented caps containing 20 mL of water. After
466 sterilization, eggs were transferred with approximately 30 to 35 eggs / flasks and were
467 transferred into new flasks at 4 dpf before re-conventionalization with 10 to 15 fish /
468 flask. We monitored sterility at several points during the experiment by spotting 50 µL of
469 water from each flask on LB, TYES and on YPD agar plates, all incubated at 28°C under
470 aerobic conditions. Plates were left for at least 3 days to allow slow-growing organisms
471 to multiply. Spot checks for bacterial contamination were also carried out by PCR
472 amplification of water samples with the 16S rRNA gene primers and procedure detailed
473 further below. If a particular flask was contaminated, those fish were removed from the
474 experiment.
475

476 **Procedure for raising germ-free zebrafish.** After hatching, fish were fed with germ-
477 free *T. thermophila* 3 times per week from 4 dpf onwards. *(i) T. thermophila stock.* A
478 germ-free line of *T. thermophila* was maintained at 28°C in 20 mL of PPYE (0.25%
479 proteose peptone BD Bacto #211684, 0.25% yeast extract BD Bacto #212750)
480 supplemented with penicillin G (10 unit/mL) and streptomycin (10µg/mL). Medium was
481 inoculated with 100 µL of the preceding *T. thermophila* stock. After one week of growth,
482 samples were taken, tested for sterility on LB, TYES and YPD plates and restocked again.

483 *(ii) Growth. T. thermophila* were incubated at 28°C in MYE broth (1% milk powder, 1%
484 yeast extract) inoculated from stock suspension at a 1:50 ratio. After 24 h of growth, *T.*
485 *thermophila* were transferred to Falcon tubes and washed (4400 rpm, 3 min at 25°C) 3
486 times in 50 mL of autoclaved Volvic water. Finally, *T. thermophila* were resuspended in
487 sterile water and added to culture flasks (500 µL in 20 mL) or 24-well plates (50 µL /
488 well). Sterility of *T. thermophila* was tested by plating and 16S rRNA PCR as described
489 in the section above. (iii) *Fine-powder feeding*. When indicated, fish were fed with
490 previously γ-ray-sterilized fine-powdered food suitable for an early first feeding gape size
491 (ZM-000 fish feed, ZM Ltd) every 48 hours (Pham et al. 2008).
492
493 **Re-conventionalization of germ-free zebrafish**. At 4 dpf, just after hatching, zebrafish
494 larvae were re-conventionalized with a single bacterial population or a mix of several.
495 The 10 bacterial strains constituting the core protective microbiota were grown for 24h
496 in suitable media (TYES or LB) at 28°C. Bacteria were then pelleted and washed twice
497 in sterile water, and all adjusted to the same cell density ($OD_{600}$ = 1 or $5.10^7$ cfu/mL) (i)
498 *Re-conventionalization with individual species.* Bacteria were resuspended and
499 transferred to culture flasks containing germ-free fish at a final concentration of $5.10^5$
500 cfu/mL. (ii) *Re-conventionalization with bacterial mixtures*. For the preparation of
501 Mix10, Mix9, Mix8 and all other mixes used, equimolar mixtures were prepared by
502 adding each bacterial species at initial concentration to $5.10^7$ cfu/mL. Each bacterial
503 mixture suspension was added to culture flasks containing germ-free fish at a final
504 concentration of $5.10^5$ cfu/mL.
505
506 **Infection challenges.** *F. columnare* strains (Suppl. Table S3) were grown overnight in
507 TYES broth at 28°C. Then, 2 mL of culture were pelleted (10,000 rpm for 5 min) and
508 washed once in sterile water. GF zebrafish were brought in contact with the tested
509 pathogens at 6 dpf for 3h by immersion in culture flasks with bacterial doses ranging from
510 $5.10^2$ to $5.10^7$ cfu/mL. Fish were then transferred to individual wells of 24-well plates,
511 containing 2 mL of water and 50 µL of freshly prepared GF *T. thermophila* per well.
512 Mortality was monitored daily as described in (Rendueles et al. 2012) and as few as 54±9
513 cfu/larva of *F. columnare* were recovered from infected larvae. All zebrafish experiments
514 were stopped at day 9 post-infection and zebrafish were euthanized with tricaine (MS-
515 222) (Sigma-Aldrich #E10521). Each experiment was repeated at least 3 times and

516 between 10 and 15 larvae were used per condition and per experiment.
517
518 **Collection of eggs from other zebrafish facilities.** Conventional zebrafish eggs were
519 collected in 50 mL Falcon tubes from the following facilities: Facility 1 - zebrafish facility
520 in Hospital Robert Debré, Paris; Facility 2 - Jussieu zebrafish facility A2, University Paris
521 6; Facility 3 - Jussieu – zebrafish facility C8 (UMR7622), University Paris 6; Facility 4:
522 AMAGEN commercial facility, Gif sur Yvette; Larvae were treated with the same rearing
523 conditions, sterilization and infection procedures used in the Institut Pasteur facility.
524
525 **Determination of fish bacterial load using cfu count.** Zebrafish were euthanized with
526 tricaine (MS-222) (Sigma-Aldrich #E10521) at 0.3 mg/mL for 10 minutes. Then they
527 were washed in 3 different baths of sterile PBS-0.1% Tween to remove bacteria loosely
528 attached to the skin. Finally, they were transferred to tubes containing calibrated glass
529 beads (acid-washed, 425 µm to 600 µm, SIGMA-ALDRICH #G8772) and 500 µL of
530 autoclaved PBS. They were homogenized using FastPrep Cell Disrupter (BIO101/FP120
531 QBioGene) for 45 s at maximum speed (6.5 m/s). Finally**,** serial dilutions of recovered
532 suspension were spotted on TYES agar and cfu were counted after 48h of incubation at
533 28°C.
534
535 **Characterization of zebrafish microbial content**. Over 3 months, the experiment was
536 run independently 3 times and 3 different batches of eggs were collected from different
537 fish couples in different tanks. Larvae were reared as described above. GF and Conv
538 larvae were collected at 6 dpf and 11 dpf for each batch. Infected Conv larvae were
539 exposed to *F. columnare*$^{ALG}$ for 3h by immersion as described above. For each
540 experimental group, triplicate pools of 10 larvae (one in each experimental batch) were
541 euthanized, washed and lysed as above. Lysates were split into 3 aliquots, one for culture
542 followed by 16S rRNA gene sequencing (A), one for 16S rRNA gene clone library
543 generation and Sanger sequencing (B), and one for Illumina metabarcoding-based
544 sequencing (C).
545
546 *A) Bacterial culture followed by 16S rRNA gene-based identification*
547 Lysates were serially diluted and immediately plated on R2A, TYES, LB, MacConkey,
548 BHI, BCYE, TCBS and TSB agars and incubated at 28ºC for 24-72h. For each agar,

549 colony morphotypes were documented, and colonies were picked and re-streaked on the
550 same agar in duplicate. In order to identify the individual morphotypes, individual
551 colonies were picked for each identified morphotype from each agar, vortexed in 200 µL
552 DNA-free water and boiled for 20 min at 90ºC. Five µL of this bacterial suspension were
553 used as template for colony PCR to amplify the 16S rRNA gene with the universal primer
554 pair for the Domain bacteria 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r
555 (5'-GGT TAC CTT GTT ACG ACT T-3'). Each primer was used at a final concentration
556 of 0.2 µM in 50 µL reactions. PCR cycling conditions were - initial denaturation at 94°C
557 for 2 min, followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 56°C for
558 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min.
559 16S rRNA gene PCR products were verified on 1% agarose gels, purified with the
560 QIAquick® PCR purification kit and two PCR products for each morphotype were sent
561 for sequencing (Eurofins, Ebersberg, Germany). 16S rRNA sequences were manually
562 proofread, and sequences of low quality were removed from the analysis. Primer
563 sequences were trimmed, and sequences were compared to GenBank (NCBI) with
564 BLAST, and to the Ribosomal Database Project with SeqMatch. For genus determination
565 a 95% similarity cut-off was used, for Operational Taxonomic Unit determination, a 98%
566 cut-off was used.
567
568 *B) 16S rRNA gene clone library generation*
569 Total DNA was extracted from the lysates with the Mobio PowerLyzer® Ultraclean® kit
570 according to manufacturer's instructions. Germ-free larvae and DNA-free water were
571 also extracted as control samples. Extracted genomic DNA was verified by Tris-acetate-
572 EDTA-agarose gel electrophoresis (1%) stained with GelRed and quantified by applying
573 2.5 µL directly to a NanoDrop® ND-1000 Spectrophotometer. The 16S rRNA gene was
574 amplified by PCR with the primers 8f and 1492r, and products checked and purified as
575 described in section A. Here, we added 25-50 ng of DNA as template to 50 µL reactions.
576 Clone libraries were generated with the pGEM®-T Easy Vector system (Promega)
577 according to manufacturer's instructions. Presence of the cloned insert was confirmed by
578 colony PCR with vector primers gemsp6 (5'-GCT GCG ACT TCA CTA GTG AT-3')
579 and gemt7 (5'-GTG GCA GCG GGA ATT CGA T-3'). Clones with an insert of the
580 correct size were purified as above and sent for sequencing (Eurofins, Ebersberg,
581 Germany). Blanks using DNA-free water as template were run for all procedures as

controls. For the three independent runs of the experiment, 10 Conv fish per condition (6 and 11 dpf, exposed or not to *F. columnare*) and per repeat were pooled. Each pool of 10 fish was sequenced separately, generating 3 replicates for each condition (n=12), resulting in a total of 857 clones. Clone library coverage was calculated with the following formula $[1-(n_1/N_2)] \times 100$, where $n_1$ is the number of singletons detected in the clone library, and $N_2$ is the total number of clones generated for this sample. Clone libraries were generated to a minimum coverage of 95%. Sequence analysis and identification was carried out as in section A.

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

To identify the 16S rRNA gene diversity in our facility and fish collected from 4 other zebrafish facilities, fish were reared as described above. GF fish were sterilised as above, and uninfected germ-free and conventional fish were collected at 6 dpf and 11 dpf. Infection was carried out as above with *F. columnare*$^{ALG}$ for 3h by bath immersion, followed by transfer to clean water. Infected conventional fish were collected at 6 dpf 6h after infection with *F. columnare* and at 11 dpf, the same as uninfected fish. GF infected larvae were only collected at 6 dpf 6h post infection, as at 11 dpf all larvae had succumbed to infection. Triplicate pools of 10 larvae were euthanized, washed and lysed as above. Total DNA was extracted with the Mobio PowerLyzer® Ultraclean® kit as described above and quantified with a NanoDrop® ND-1000 Spectrophotometer and sent to IMGM Laboratories GmbH (Germany) for Illumina sequencing. Primers Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3'), amplifying variable regions 3 and 4 of the 16S gene were used for amplification (Lemon et al. 2012).

Each amplicon was purified with solid phase reversible immobilization (SPRI) paramagnetic bead-based technology (AMPure XP beads, Beckman Coulter) with a Bead:DNA ratio of 0.7:1 (v/v) following manufacturer´s instructions. Amplicons were normalized with the Sequal-Prep Kit (Life Technologies), so each sample contained approximately 1 ng/µl DNA. Samples, positive and negative controls were generated in one library. The High Sensitivity DNA LabChip Kit (was used on the 2100 Bioanalyzer system (both Agilent Technologies) to check the quality of the purified amplicon library. For cluster generation and sequencing, MiSeq® reagents kit 500 cycles Nano v2 (Illumina Inc.) was used. Before sequencing, cluster generation by two-dimensional

615 bridge amplification was performed, followed by bidirectional sequencing, producing 2
616 x 250 bp paired-end (PE) reads.
617 MiSeq® Reporter 2.5.1.3 software was used for primary data analysis (signal processing,
618 de-multiplexing, trimming of adapter sequences). CLC Genomics Workbench 8.5.1
619 (Qiagen) was used for read-merging, quality trimming and QC reports and OTU
620 definition were carried out in the CLC plugin Microbial Genomics module.
621
622 **Comparison of whole larvae vs intestinal bacterial content.** Larvae re-
623 conventionalized with Mix10 and infected with *F. columnare*$^{ALG}$ at 6 dpf for 3h were
624 euthanized and washed. DNA was extracted from pools of 10 whole larvae or of pools of
625 10 intestinal tubes dissected with sterile surgical tweezer and subjected to Illumina 16S
626 rRNA gene sequencing. GF larvae and dissected GF intestines were sampled as controls.
627 As dissection of the larval guts involved high animal loss and was a potential important
628 contamination source, we proceeded with using entire larvae for the rest of the study.
629
630 **Whole genome sequencing**. Chromosomal DNA of the ten species composing the core
631 of zebrafish larvae microbiota was extracted using the DNeasy Blood & Tissue kit
632 (QIAGEN) including RNase treatment. DNA quality and quantity were assessed on a
633 NanoDrop ND-1000 spectrophotometer (Thermo Scientific).
634 DNA sequencing libraries were made using the Nextera DNA Library Preparation Kit
635 (Illumina Inc.) and library quality was checked using the High Sensitivity DNA LabChip
636 Kit on the Bioanalyzer 2100 (Agilent Technologies). Sequencing clusters were generated
637 using the MiSeq reagents kit v2 500 cycles (Illumina Inc.) according to manufacturer's
638 instructions. DNA was sequenced at the Helmholtz Centre for Infection Research by
639 bidirectional sequencing, producing 2 x 250 bp paired-end (PE) reads. Between 1,108,578
640 and 2,914,480 reads per sample were retrieved with a median of 1,528,402. Reads were
641 quality filtered, trimmed and adapters removed with trimmomatic 0.39 (Bolger, Lohse,
642 and Usadel 2014) and genomes assembled using SPAdes 3.14 (Bankevich et al. 2012).
643
644 **Bacterial species identification**. Whole genome-based bacterial species identification
645 was performed by the TrueBac ID system (v1.92, DB:20190603)
646 [https://www.truebacid.com/; (Ha et al. 2019). Species-level identification was

647 performed based on the algorithmic cut-off set at 95% ANI when possible or when the
648 16S rRNA gene sequence similarity was >99 %.
649
650 **Monitoring of bacterial dynamics**. Three independent experiments were run over 6
651 weeks with eggs collected from different fish couples from different tanks to monitor
652 establishment and recovery. Larvae were reared, sterilized and infected as above with the
653 only difference that 75 $cm^3$ culture flasks with vented caps (filled with 50 mL of sterile
654 Volvic) were used to accommodate the larger number of larvae required, as in each
655 experiment. Larvae for time course Illumina sequencing were removed sequentially from
656 the experiment that monitored the survival of the larvae. Animals were pooled (10 larvae
657 for each time point/condition), euthanized, washed and lysed as described above and
658 stored at -20ºC until the end of the survival monitoring, and until all triplicates had been
659 collected.
660
661 *A) Community establishment*
662 In order to follow the establishment of the 10 core strains in the larvae, GF larvae were
663 re-conventionalized with an equiratio Mix10 as above. Re-con$^{Mix10}$ larvae were sampled
664 at 4 dpf immediately after addition of the 10 core species and then 20 min, 2h, 4h and 8h
665 after. Germ-free, conventional larvae and the inoculum were also sampled as controls.
666
667 *B) Induction of dysbiosis*
668 Different doses of kanamycin (dose 1= 200 µg/mL; dose 2= 50 µg/mL; dose 3= 25
669 µg/mL) and a penicillin/streptomycin antibiotic mix (dose 1= 250 µg/mL; dose 2= 15.6
670 µg/mL were tested on re-con$^{Mix10}$ 4 dpf zebrafish larvae by adding them to the flask water
671 to identify antibiotic treatments that were non-toxic to larvae but that caused dysbiosis.
672
673 After 16 hours of treatment, antibiotics were extensively washed off with sterile water
674 and larvae were challenged with *F. columnare*$^{ALG}$, leading to the death of all larvae – e.g.
675 successful abolition of colonization resistance with best results in all repeats with 250
676 µg/mL penicillin/streptomycin and 50 µg/mL kanamycin as antibiotic treatment.
677
678 *C) Community recovery*
679 As in B) after 8h of incubation, 4 dpf recon$^{Mix10}$ larvae were treated with 250 µg/mL
680 penicillin/streptomycin and 50 µg/mL kanamycin for 16h. Antibiotics were extensively

681 washed off and larvae were now left to recover in sterile water for 24h to assess resilience
682 of the bacterial community. Samples (pools of 10 larvae) were taken at 3h, 6h, 12h, 18h
683 and 24h during recovery and sent for 16S rRNA Illumina sequencing. Larvae were then
684 challenged at 6 dpf with *F. columnare*$^{ALG}$ for 3h and survival was monitored daily for 9
685 days post-infection. All time course samples were sequenced by IMGM Laboratories
686 GmbH, as described above.
687
688 **Statistical analysis of metataxonomic data**. 16S RNA analysis was performed with
689 SHAMAN (Volant et al. 2019)]. Library adapters, primer sequences, and base pairs
690 occurring at 5' and 3'ends with a Phred quality score <20 were trimmed off by using
691 Alientrimmer (v0.4.0). Reads with a positive match against zebrafish genome (mm10)
692 were removed. Filtered high-quality reads were merged into amplicons with Flash
693 (v1.2.11). Resulting amplicons were clustered into operational taxonomic units (OTU)
694 with VSEARCH (v2.3.4) (Rognes et al. 2016). The process includes several steps for de-
695 replication, singletons removal, and chimera detection. The clustering was performed at
696 97% sequence identity threshold, producing 459 OTUs. The OTU taxonomic annotation
697 was performed against the SILVA SSU (v132) database (Quast et al. 2012) completed
698 with 16S sequence of 10 bacterial communities using VSEARCH and filtered according
699 to their identity with the reference (Yarza et al. 2014). Annotations were kept when the
700 identity between the OTU sequence and reference sequence is ≥ 78.5% for taxonomic
701 Classes, ≥ 82% for Orders, ≥ 86.5% for Families, ≥ 94.5% for Genera and ≥ 98% for
702 species. Here, 73.2% of the OTUs set was annotated and 91.69 % of them were annotated
703 at genus level.
704 The input amplicons were then aligned against the OTU set to get an OTU contingency
705 table containing the number of amplicon associated with each OTU using VSEARCH
706 global alignment. The matrix of OTU count data was normalized for library size at the
707 OTU level using a weighted non-null count normalization. Normalized counts were then
708 summed within genera. The generalized linear model (GLM) implemented in the DESeq2
709 R package (Love, Huber, and Anders 2014) was then applied to detect differences in
710 abundance of genera between each group. We defined a GLM that included the treatment
711 (condition) and the time (variable) as main effects and an interaction between the
712 treatment and the time. Resulting P values were adjusted according to the Benjamini and
713 Hochberg procedure.

714 The statistical analysis can be reproduced on shaman by loading the count table, the
715 taxonomic results with the target and contrast files that are available on figshare
716 https://doi.org/10.6084/m9.figshare.11417082.v2.
717
718 **Determination of cytokine levels**. Total RNAs from individual zebrafish larvae were
719 extracted using RNeasy kit (Qiagen), 18h post pathogen exposure (12h post-wash).
720 Oligo(dT17)-primed reverse transcriptions were carried out using M-MLV H- reverse-
721 transcriptase (Promega). Quantitative PCRs were performed using Takyon SYBR Green
722 PCR Mastermix (Eurogentec) on a StepOne thermocycler (Applied Biosystems). Primers
723 for *ef1a* (housekeeping gene, used for cDNA amount normalization), *il1b*, *il10* and *il22*
724 are described in (Rendueles et al. 2012). Data were analysed using the ΔΔCt method. Four
725 larvae were analysed per condition. Zebrafish genes and proteins mentioned in the text:
726 *ef1a* NM_131263*; il1b* BC098597*; il22* NM_001020792*; il10* NM_001020785*; myd88*
727 NM_212814.
728
729 **Histological comparisons of GF, Conv and Re-Conv fish GF infected or not with *F.***
730 ***columnare***
731 Fish were collected 24h after infection (7 dpf) and were fixed for 24h at 4°C in Trump
732 fixative (4% methanol-free formaldehyde, 1% glutaraldehyde in 0.1 M PBS, pH 7.2) and
733 sent to the PIBiSA Microscopy facility services (https://microscopies.med.univ-tours.fr/ )
734 in the Faculté de Médecine de Tours (France), where whole fixed animals were processed,
735 embedded in Epon. Semi-thin sections (1µm) and cut using a X ultra-microtome and then
736 either dyed with toluidine blue for observation by light microscopy and imaging or
737 processed for Transmission electron microscopy.
738
739 **Adult zebrafish pre-treatment with *C. massiliae***
740 The zebrafish line AB was used. Fish were reared at 28°C in dechlorinated recirculated
741 water, then transferred into continuous flow aquaria when aging 3-4 months for infection
742 experiments. *C. massiliae* was grown in TYES broth at 150 rpm and 28°C until stationary
743 phase. This bacterial culture was washed twice in sterile water and adjusted to $OD_{600nm}$ =
744 1. Adult fish re-conventionalization was performed by adding *C. massiliae* bacterial
745 suspension directly into the fish water (1L) at a final concentration of $2.10^6$ cfu/mL.
746 Bacteria were maintained in contact with fish for 24 h by stopping the water flow then

747 subsequently removed by restoring the water flow. *C. massiliae* administration was
748 performed twice after water renewal. In the control group, the same volume of sterile
749 water was added.
750
751 **Adult zebrafish infection challenge**
752 *F. columnare* infection was performed just after fish re-conventionalization with *C.*
753 *massiliae*. The infection was performed as previously described by Li and co-workers
754 with few modifications [Li et al., 2017]. Briefly, *F. columnare* strain ALG-0530 was
755 grown in TYES broth at 150 rpm and 28 °C until late-exponential phase. Then, bacterial
756 cultures were diluted directly into the water of aquaria (200 mL) at a final concentration
757 of $5.10^6$ cfu/mL. Bacteria were maintained in contact with fish for 1 h by stopping the
758 water flow then subsequently removed by restoring the water flow. Sterile TYES broth
759 was used for the control group. Bacterial counts were determined at the beginning of the
760 immersion challenge by plating serial dilutions of water samples on TYES agar. Water
761 was maintained at 28°C and under continuous oxygenation for the duration of the
762 immersion. Groups were composed of 10 fish. Virulence was evaluated according to fish
763 mortality 10 days post-infection.
764
765 **Statistical methods.** Statistical analyses were performed using unpaired, non-parametric
766 Mann-Whitney test or unpaired t-tests. Analyses were performed using Prism v8.2
767 (GraphPad Software).
768 Evenness: The Shannon diversity index was calculated with the formula ($H_S$ = -
769 $\Sigma[P(\ln(P)])$ where P is the relative species abundance. Total evenness was calculated for
770 the Shannon index as $E = H_S/H_{max.}$ The less evenness in communities between the species
771 (and the presence of a dominant species), the lower this index is.
772

**ACKNOWLEDGEMENTS**

We thank Mark McBride, Pierre Boudinot and Rebecca Stevick for critical reading of the manuscript. We are grateful to the late and regretted Covadonga Arias for the gift of *F. columnare* ALG 00-530, to Mark McBride for *F. columnare* C#2 strain and to Jean-François Bernardet for all other *F. columnare* strains. Prof. Annemarie Meijer (Leiden University) kindly provided the *myd88* mutant zebrafish line. We thank Julien Burlaud-Gaillard and Rustem Uzkebov the IBiSA Microscopy facility, Tours University, France for their reactivity and the following zebrafish facility teams for providing eggs: José Perez and Yohann Rolin(Institut Pasteur), Nadia Soussi-Yanicostas (INSERM Robert Debré), Sylvie Schneider-Manoury and Isabelle Anselme (UMR7622, University Paris 6) and Frédéric Sohm (AMAGEN Gif-sur-Yvette). This work was supported by the Institut Pasteur, the French Government's *Investissement d'Avenir* program: *Laboratoire d'Excellence* 'Integrative Biology of Emerging Infectious Diseases' (grant no. ANR-10-LABX-62-IBEID to J.M.G.), the Fondation pour la Recherche Médicale (grant no. DEQ20180339185 to J.M.G.). F.S. was the recipient of a post-doctoral Marie Curie fellowship from the EU-FP7 programme, J.B.B. was the recipient of a long-term post-doctoral fellowship from the Federation of European Biochemical Societies(FEBS) and by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 842629 D.P-P was supported by an Institut Carnot MS Postdoctoral fellowship.

**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..

## COMPETING FINANCIAL INTERESTS

The authors of this manuscript have the following competing interests: 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.

## 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*);

824 **REFERENCES**

825

826 Bankevich, A., S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M.
827 Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, A. V. Pyshkin, A. V. Sirotkin, N.
828 Vyahhi, G. Tesler, M. A. Alekseyev, and P. A. Pevzner. 2012. "SPAdes: a new
829 genome assembly algorithm and its applications to single-cell sequencing." *J Comput*
830 *Biol* 19 (5):455-77. doi: 10.1089/cmb.2012.0021.
831 Bates, J. M., E. Mittge, J. Kuhlman, K. N. Baden, S. E. Cheesman, and K. Guillemin.
832 2006. "Distinct signals from the microbiota promote different aspects of zebrafish gut
833 differentiation." *Dev Biol* 297 (2):374-86. doi: 10.1016/j.ydbio.2006.05.006.
834 Bernardet, J.-F., and J. P. Bowman. 2006. "The Genus Flavobacterium." *Prokaryotes*
835 7:481-531.
836 Boirivant, M., and W. Strober. 2007. "The mechanism of action of probiotics." *Curr*
837 *Opin Gastroenterol* 23 (6):679-92. doi: 10.1097/MOG.0b013e3282f0cffc.
838 Bolger, A. M., M. Lohse, and B. Usadel. 2014. "Trimmomatic: a flexible trimmer for
839 Illumina sequence data." *Bioinformatics* 30 (15):2114-20. doi:
840 10.1093/bioinformatics/btu170.
841 Brugman, S., K. Y. Liu, D. Lindenbergh-Kortleve, J. N. Samsom, G. T. Furuta, S. A.
842 Renshaw, R. Willemsen, and E. E. Nieuwenhuis. 2009. "Oxazolone-induced
843 enterocolitis in zebrafish depends on the composition of the intestinal microbiota."
844 *Gastroenterology* 137 (5):1757-67 e1. doi: 10.1053/j.gastro.2009.07.069.
845 Burns, A. R., and K. Guillemin. 2017. "The scales of the zebrafish: host-microbiota
846 interactions from proteins to populations." *Curr Opin Microbiol* 38:137-141. doi:
847 10.1016/j.mib.2017.05.011.
848 Burns, A. R., W. Z. Stephens, K. Stagaman, S. Wong, J. F. Rawls, K. Guillemin, and B.
849 J. Bohannan. 2016. "Contribution of neutral processes to the assembly of gut microbial
850 communities in the zebrafish over host development." *ISME J* 10 (3):655-64. doi:
851 10.1038/ismej.2015.142.
852 Cani, P. D., and N. M. Delzenne. 2009. "The role of the gut microbiota in energy
853 metabolism and metabolic disease." *Curr Pharm Des* 15 (13):1546-58.
854 Cantas, L., J. R. Sorby, P. Alestrom, and H. Sorum. 2012. "Culturable gut microbiota
855 diversity in zebrafish." *Zebrafish* 9 (1):26-37. doi: 10.1089/zeb.2011.0712.
856 Caruffo, M., N. C. Navarrete, O. A. Salgado, N. B. Faundez, M. C. Gajardo, C. G.
857 Feijoo, A. Reyes-Jara, K. Garcia, and P. Navarrete. 2016. "Protective Yeasts Control
858 V. anguillarum Pathogenicity and Modulate the Innate Immune Response of
859 Challenged Zebrafish (Danio rerio) Larvae." *Front Cell Infect Microbiol* 6:127. doi:
860 10.3389/fcimb.2016.00127.
861 Chatzidaki-Livanis, M., M. J. Coyne, and L. E. Comstock. 2014. "An antimicrobial
862 protein of the gut symbiont Bacteroides fragilis with a MACPF domain of host
863 immune proteins." *Mol Microbiol* 94 (6):1361-74. doi: 10.1111/mmi.12839.
864 Cheesman, S. E., J. T. Neal, E. Mittge, B. M. Seredick, and K. Guillemin. 2011.
865 "Epithelial cell proliferation in the developing zebrafish intestine is regulated by the
866 Wnt pathway and microbial signaling via Myd88." *Proc Natl Acad Sci U S A* 108
867 Suppl 1:4570-7. doi: 10.1073/pnas.1000072107.
868 Chu, W., S. Zhou, W. Zhu, and X. Zhuang. 2014. "Quorum quenching bacteria Bacillus
869 sp. QSI-1 protect zebrafish (Danio rerio) from Aeromonas hydrophila infection." *Sci*
870 *Rep* 4:5446. doi: 10.1038/srep05446.

871 Costello, E. K., K. Stagaman, L. Dethlefsen, B. J. Bohannan, and D. A. Relman. 2012.
872 "The application of ecological theory toward an understanding of the human
873 microbiome." *Science* 336 (6086):1255-62. doi: 10.1126/science.1224203.
874 Declercq, A. M., F. Haesebrouck, W. Van den Broeck, P. Bossier, and A. Decostere.
875 2013. "Columnaris disease in fish: a review with emphasis on bacterium-host
876 interactions." *Vet Res* 44:27. doi: 10.1186/1297-9716-44-27.
877 Douglas, A. E. 2019. "Simple animal models for microbiome research." *Nat Rev*
878 *Microbiol* 17 (12):764-775. doi: 10.1038/s41579-019-0242-1.
879 Falcinelli, S., A. Rodiles, S. Unniappan, S. Picchietti, G. Gioacchini, D. L. Merrifield,
880 and O. Carnevali. 2016. "Probiotic treatment reduces appetite and glucose level in the
881 zebrafish model." *Sci Rep* 6:18061. doi: 10.1038/srep18061.
882 Flores, E. M., A. T. Nguyen, M. A. Odem, G. T. Eisenhoffer, and A. M. Krachler. 2020.
883 "The zebrafish as a model for gastrointestinal tract-microbe interactions." *Cell*
884 *Microbiol* 22 (3):e13152. doi: 10.1111/cmi.13152.
885 Ganz, J., E. Melancon, and J. S. Eisen. 2016. "Zebrafish as a model for understanding
886 enteric nervous system interactions in the developing intestinal tract." *Methods Cell*
887 *Biol* 134:139-64. doi: 10.1016/bs.mcb.2016.02.003.
888 Garcia, J. C., B. R. LaFrentz, G. C. Waldbieser, F. S. Wong, and S. F. Chang. 2018.
889 "Characterization of atypical Flavobacterium columnare and identification of a new
890 genomovar." *J Fish Dis* 41 (7):1159-1164. doi: 10.1111/jfd.12778.
891 Gill, H. S. 2003. "Probiotics to enhance anti-infective defences in the gastrointestinal
892 tract." *Best Pract Res Clin Gastroenterol* 17 (5):755-73.
893 Girija, V., B. Malaikozhundan, B. Vaseeharan, S. Vijayakumar, N. Gobi, M. Del Valle
894 Herrera, J. C. Chen, and P. Santhanam. 2018. "In vitro antagonistic activity and the
895 protective effect of probiotic Bacillus licheniformis Dahb1 in zebrafish challenged
896 with GFP tagged Vibrio parahaemolyticus Dahv2." *Microb Pathog* 114:274-280. doi:
897 10.1016/j.micpath.2017.11.058.
898 Goncalves Pessoa, R. B., W. F. de Oliveira, D. S. C. Marques, M. T. Dos Santos
899 Correia, Evmm de Carvalho, and Lcbb Coelho. 2019. "The genus Aeromonas: A
900 general approach." *Microb Pathog* 130:81-94. doi: 10.1016/j.micpath.2019.02.036.
901 Ha, S. M., C. K. Kim, J. Roh, J. H. Byun, S. J. Yang, S. B. Choi, J. Chun, and D. Yong.
902 2019. "Application of the Whole Genome-Based Bacterial Identification System,
903 TrueBac ID, Using Clinical Isolates That Were Not Identified With Three Matrix-
904 Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-
905 TOF MS) Systems." *Ann Lab Med* 39 (6):530-536. doi: 10.3343/alm.2019.39.6.530.
906 Heselmans, M., G. Reid, L. M. Akkermans, H. Savelkoul, H. Timmerman, and F. M.
907 Rombouts. 2005. "Gut flora in health and disease: potential role of probiotics." *Curr*
908 *Issues Intest Microbiol* 6 (1):1-7.
909 Hibbing, M. E., C. Fuqua, M. R. Parsek, and S. B. Peterson. 2010. "Bacterial
910 competition: surviving and thriving in the microbial jungle." *Nat Rev Microbiol* 8
911 (1):15-25. doi: 10.1038/nrmicro2259.
912 Jacobson, A., L. Lam, M. Rajendram, F. Tamburini, J. Honeycutt, T. Pham, W. Van
913 Treuren, K. Pruss, S. R. Stabler, K. Lugo, D. M. Bouley, J. G. Vilches-Moure, M.
914 Smith, J. L. Sonnenburg, A. S. Bhatt, K. C. Huang, and D. Monack. 2018. "A Gut
915 Commensal-Produced Metabolite Mediates Colonization Resistance to Salmonella
916 Infection." *Cell Host Microbe* 24 (2):296-307.e7. doi: 10.1016/j.chom.2018.07.002.

Kamada, N., S. U. Seo, G. Y. Chen, and G. Nunez. 2013. "Role of the gut microbiota in immunity and inflammatory disease." *Nat Rev Immunol* 13 (5):321-35. doi: 10.1038/nri3430.

Kunin, V., A. Engelbrektson, H. Ochman, and P. Hugenholtz. 2010. "Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates." *Environ Microbiol* 12 (1):118-23. doi: 10.1111/j.1462-2920.2009.02051.x.

Lemon, K. P., G. C. Armitage, D. A. Relman, and M. A. Fischbach. 2012. "Microbiota-targeted therapies: an ecological perspective." *Sci Transl Med* 4 (137):137rv5. doi: 10.1126/scitranslmed.3004183.

Li, N., Y. Zhu, B. R. LaFrentz, J. P. Evenhuis, D. W. Hunnicutt, R. A. Conrad, P. Barbier, C. W. Gullstrand, J. E. Roets, J. L. Powers, S. S. Kulkarni, D. H. Erbes, J. C. Garcia, P. Nie, and M. J. McBride. 2017. "The Type IX Secretion System Is Required for Virulence of the Fish Pathogen Flavobacterium columnare." *Appl Environ Microbiol* 83 (23). doi: 10.1128/AEM.01769-17.

Lin, Y. S., F. Saputra, Y. C. Chen, and S. Y. Hu. 2019. "Dietary administration of Bacillus amyloliquefaciens R8 reduces hepatic oxidative stress and enhances nutrient metabolism and immunity against Aeromonas hydrophila and Streptococcus agalactiae in zebrafish (Danio rerio)." *Fish Shellfish Immunol* 86:410-419. doi: 10.1016/j.fsi.2018.11.047.

Littman, D. R., and E. G. Pamer. 2011. "Role of the commensal microbiota in normal and pathogenic host immune responses." *Cell Host Microbe* 10 (4):311-23. doi: 10.1016/j.chom.2011.10.004.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." *Genome Biology* 15 (12):550. doi: 10.1186/s13059-014-0550-8.

McFall-Ngai, M. J. 2002. "Unseen forces: the influence of bacteria on animal development." *Dev Biol* 242 (1):1-14. doi: 10.1006/dbio.2001.0522.

Melancon, E., S. Gomez De La Torre Canny, S. Sichel, M. Kelly, T. J. Wiles, J. F. Rawls, J. S. Eisen, and K. Guillemin. 2017. "Best practices for germ-free derivation and gnotobiotic zebrafish husbandry." *Methods Cell Biol* 138:61-100. doi: 10.1016/bs.mcb.2016.11.005.

Mena, K. D., and C. P. Gerba. 2009. "Risk assessment of Pseudomonas aeruginosa in water." *Rev Environ Contam Toxicol* 201:71-115. doi: 10.1007/978-1-4419-0032-6_3.

Milligan-Myhre, K., J. R. Charette, R. T. Phennicie, W. Z. Stephens, J. F. Rawls, K. Guillemin, and C. H. Kim. 2011. "Study of host-microbe interactions in zebrafish." *Methods Cell Biol* 105:87-116. doi: 10.1016/B978-0-12-381320-6.00004-7.

Olivares-Fuster, O., S. A. Bullard, A. McElwain, M. J. Llosa, and C. R. Arias. 2011. "Adhesion dynamics of Flavobacterium columnare to channel catfish Ictalurus punctatus and zebrafish Danio rerio after immersion challenge." *Dis Aquat Organ* 96 (3):221-7. doi: 10.3354/dao02371.

Olsan, E. E., M. X. Byndloss, F. Faber, F. Rivera-Chavez, R. M. Tsolis, and A. J. Baumler. 2017. "Colonization resistance: The deconvolution of a complex trait." *J Biol Chem* 292 (21):8577-8581. doi: 10.1074/jbc.R116.752295.

Perez-Ramos, A., M. L. Mohedano, M. A. Pardo, and P. Lopez. 2018. "beta-Glucan-Producing Pediococcus parvulus 2.6: Test of Probiotic and Immunomodulatory Properties in Zebrafish Models." *Front Microbiol* 9:1684. doi: 10.3389/fmicb.2018.01684.

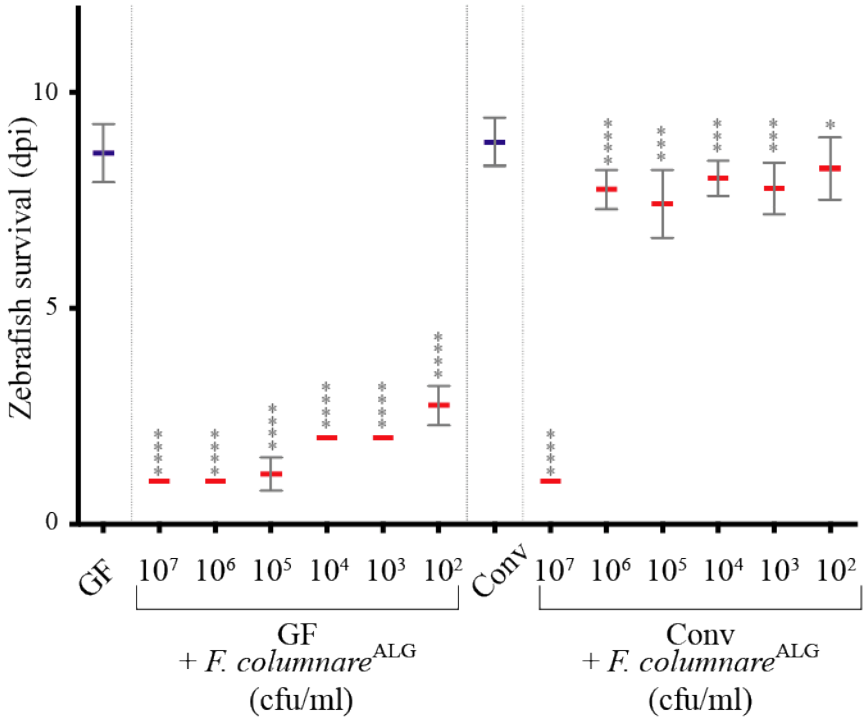
965 Pham, L. N., M. Kanther, I. Semova, and J. F. Rawls. 2008. "Methods for generating
966 and colonizing gnotobiotic zebrafish." *Nat Protoc* 3 (12):1862-75. doi:
967 10.1038/nprot.2008.186.
968 Qin, C., Z. Zhang, Y. Wang, S. Li, C. Ran, J. Hu, Y. Xie, W. Li, and Z. Zhou. 2017.
969 "EPSP of L. casei BL23 Protected against the Infection Caused by Aeromonas veronii
970 via Enhancement of Immune Response in Zebrafish." *Front Microbiol* 8:2406. doi:
971 10.3389/fmicb.2017.02406.
972 Quast, Christian, Elmar Pruesse, Pelin Yilmaz, Jan Gerken, Timmy Schweer, Pablo
973 Yarza, Jörg Peplies, and Frank Oliver Glöckner. 2012. "The SILVA ribosomal RNA
974 gene database project: improved data processing and web-based tools." *Nucleic Acids*
975 *Research* 41 (D1):D590-D596. doi: 10.1093/nar/gks1219.
976 Rawls, J. F., B. S. Samuel, and J. I. Gordon. 2004. "Gnotobiotic zebrafish reveal
977 evolutionarily conserved responses to the gut microbiota." *Proc Natl Acad Sci U S A*
978 101 (13):4596-601. doi: 10.1073/pnas.0400706101.
979 Rendueles, O., L. Ferrieres, M. Fretaud, E. Begaud, P. Herbomel, J. P. Levraud, and J.
980 M. Ghigo. 2012. "A new zebrafish model of oro-intestinal pathogen colonization
981 reveals a key role for adhesion in protection by probiotic bacteria." *PLoS Pathog* 8
982 (7):e1002815. doi: 10.1371/journal.ppat.1002815.
983 Robinson, C. J., B. J. Bohannan, and V. B. Young. 2010. "From structure to function:
984 the ecology of host-associated microbial communities." *Microbiol Mol Biol Rev* 74
985 (3):453-76. doi: 10.1128/MMBR.00014-10.
986 Roelofs, K. G., M. J. Coyne, R. R. Gentyala, M. Chatzidaki-Livanis, and L. E.
987 Comstock. 2016. "Bacteroidales Secreted Antimicrobial Proteins Target Surface
988 Molecules Necessary for Gut Colonization and Mediate Competition In Vivo." *MBio*
989 7 (4). doi: 10.1128/mBio.01055-16.
990 Rogers, G. B., L. R. Hoffman, M. P. Carroll, and K. D. Bruce. 2013. "Interpreting
991 infective microbiota: the importance of an ecological perspective." *Trends Microbiol*
992 21 (6):271-6. doi: 10.1016/j.tim.2013.03.004.
993 Rognes, T., T. Flouri, B. Nichols, C. Quince, and F. Mahe. 2016. "VSEARCH: a
994 versatile open source tool for metagenomics." *PeerJ* 4:e2584. doi:
995 10.7717/peerj.2584.
996 Rolig, A. S., R. Parthasarathy, A. R. Burns, B. J. Bohannan, and K. Guillemin. 2015.
997 "Individual Members of the Microbiota Disproportionately Modulate Host Innate
998 Immune Responses." *Cell Host Microbe* 18 (5):613-20. doi:
999 10.1016/j.chom.2015.10.009.
1000 Russell, A. B., A. G. Wexler, B. N. Harding, J. C. Whitney, A. J. Bohn, Y. A. Goo, B.
1001 Q. Tran, N. A. Barry, H. Zheng, S. B. Peterson, S. Chou, T. Gonen, D. R. Goodlett, A.
1002 L. Goodman, and J. D. Mougous. 2014. "A type VI secretion-related pathway in
1003 Bacteroidetes mediates interbacterial antagonism." *Cell Host Microbe* 16 (2):227-236.
1004 doi: 10.1016/j.chom.2014.07.007.
1005 Salonen, A., J. Nikkila, J. Jalanka-Tuovinen, O. Immonen, M. Rajilic-Stojanovic, R. A.
1006 Kekkonen, A. Palva, and W. M. de Vos. 2010. "Comparative analysis of fecal DNA
1007 extraction methods with phylogenetic microarray: effective recovery of bacterial and
1008 archaeal DNA using mechanical cell lysis." *J Microbiol Methods* 81 (2):127-34. doi:
1009 10.1016/j.mimet.2010.02.007.
1010 Shabgah, A. G., J. G. Navashenaq, O. G. Shabgah, H. Mohammadi, and A. Sahebkar.
1011 2017. "Interleukin-22 in human inflammatory diseases and viral infections."
1012 *Autoimmun Rev* 16 (12):1209-1218. doi: 10.1016/j.autrev.2017.10.004.

1013 Shade, A., H. Peter, S. D. Allison, D. L. Baho, M. Berga, H. Burgmann, D. H. Huber, S.
1014 Langenheder, J. T. Lennon, J. B. Martiny, K. L. Matulich, T. M. Schmidt, and J.
1015 Handelsman. 2012. "Fundamentals of microbial community resistance and resilience."
1016 *Front Microbiol* 3:417. doi: 10.3389/fmicb.2012.00417.
1017 Shafquat, A., R. Joice, S. L. Simmons, and C. Huttenhower. 2014. "Functional and
1018 phylogenetic assembly of microbial communities in the human microbiome." *Trends*
1019 *Microbiol* 22 (5):261-6. doi: 10.1016/j.tim.2014.01.011.
1020 Soto, E., M. J. Mauel, A. Karsi, and M. L. Lawrence. 2008. "Genetic and virulence
1021 characterization of Flavobacterium columnare from channel catfish (Ictalurus
1022 punctatus)." *J Appl Microbiol* 104 (5):1302-10. doi: 10.1111/j.1365-
1023 2672.2007.03632.x.
1024 Stecher, B., and W. D. Hardt. 2008. "The role of microbiota in infectious disease."
1025 *Trends Microbiol* 16 (3):107-14. doi: 10.1016/j.tim.2007.12.008.
1026 Stecher, B., and W. D. Hardt. 2011. "Mechanisms controlling pathogen colonization of
1027 the gut." *Curr Opin Microbiol* 14 (1):82-91. doi: 10.1016/j.mib.2010.10.003.
1028 Stephens, W. Z., A. R. Burns, K. Stagaman, S. Wong, J. F. Rawls, K. Guillemin, and B.
1029 J. Bohannan. 2016. "The composition of the zebrafish intestinal microbial community
1030 varies across development." *ISME J* 10 (3):644-54. doi: 10.1038/ismej.2015.140.
1031 van der Vaart, M., J. J. van Soest, H. P. Spaink, and A. H. Meijer. 2013. "Functional
1032 analysis of a zebrafish myd88 mutant identifies key transcriptional components of the
1033 innate immune system." *Dis Model Mech* 6 (3):841-54. doi: 10.1242/dmm.010843.
1034 van der Waaij, D., J.M. Berghuis-de Vries, and J.E.C. Lekkerkerk-van der Wees. 1972.
1035 "Colonization resistance of the digestive tract and the spread of bacteria to the
1036 lymphatic organs in mice." *The Journal of Hygiene* 70:335-342.
1037 Volant, Stevenn, Pierre Lechat, Perrine Woringer, Laurence Motreff, Christophe
1038 Malabat, Sean Kennedy, and Amine Ghozlane. 2019. "SHAMAN: a user-friendly
1039 website for metataxonomic analysis from raw reads to statistical analysis."
1040 *bioRxiv*:2019.12.18.880773. doi: 10.1101/2019.12.18.880773.
1041 Vollaard, E. J., and H. A. Clasener. 1994. "Colonization resistance." *Antimicrob Agents*
1042 *Chemother* 38 (3):409-14.
1043 Wang, Y., Z. Ren, L. Fu, and X. Su. 2016. "Two highly adhesive lactic acid bacteria
1044 strains are protective in zebrafish infected with Aeromonas hydrophila by evocation of
1045 gut mucosal immunity." *J Appl Microbiol* 120 (2):441-51. doi: 10.1111/jam.13002.
1046 Willing, B. P., S. L. Russell, and B. B. Finlay. 2011. "Shifting the balance: antibiotic
1047 effects on host-microbiota mutualism." *Nat Rev Microbiol* 9 (4):233-43. doi:
1048 10.1038/nrmicro2536.
1049 Yan, Q., C. J. van der Gast, and Y. Yu. 2012. "Bacterial community assembly and
1050 turnover within the intestines of developing zebrafish." *PLoS One* 7 (1):e30603. doi:
1051 10.1371/journal.pone.0030603.
1052 Yarza, P., P. Yilmaz, E. Pruesse, F. O. Glockner, W. Ludwig, K. H. Schleifer, W. B.
1053 Whitman, J. Euzeby, R. Amann, and R. Rossello-Mora. 2014. "Uniting the
1054 classification of cultured and uncultured bacteria and archaea using 16S rRNA gene
1055 sequences." *Nat Rev Microbiol* 12 (9):635-45. doi: 10.1038/nrmicro3330.
1056
1057

1058

1059 **FIGURES**

1060




1061

1062 **Figure 1. *Flavobacterium columnare* kills germ-free but not conventional zebrafish.**
1063 6 dpf (corresponding to 0 dpi) GF or Conv zebrafish larvae were exposed to different
1064 doses of *F. columnare*$^{ALG}$ by bath immersion and transferred after 3h into sterile water.
1065 Mean survival is represented by a thick horizontal bar with standard deviation. For each
1066 condition, n = 12 zebrafish larvae. Larvae mortality rate was monitored daily and
1067 surviving fish were euthanized at day 9 post infection. Statistics correspond to unpaired,
1068 non-parametric Mann-Whitney test comparing all conditions to non-infected GF (left) or
1069 Conv (right). ****: $p<0.0001$; ***: $p<0.001$; *: $p<0.05$, absence of *: non-significant.
1070 Blue mean bars correspond to non-exposed larvae and red mean bars correspond to larvae
1071 exposed to *F. columnare*.

1072

1073

1074

1075

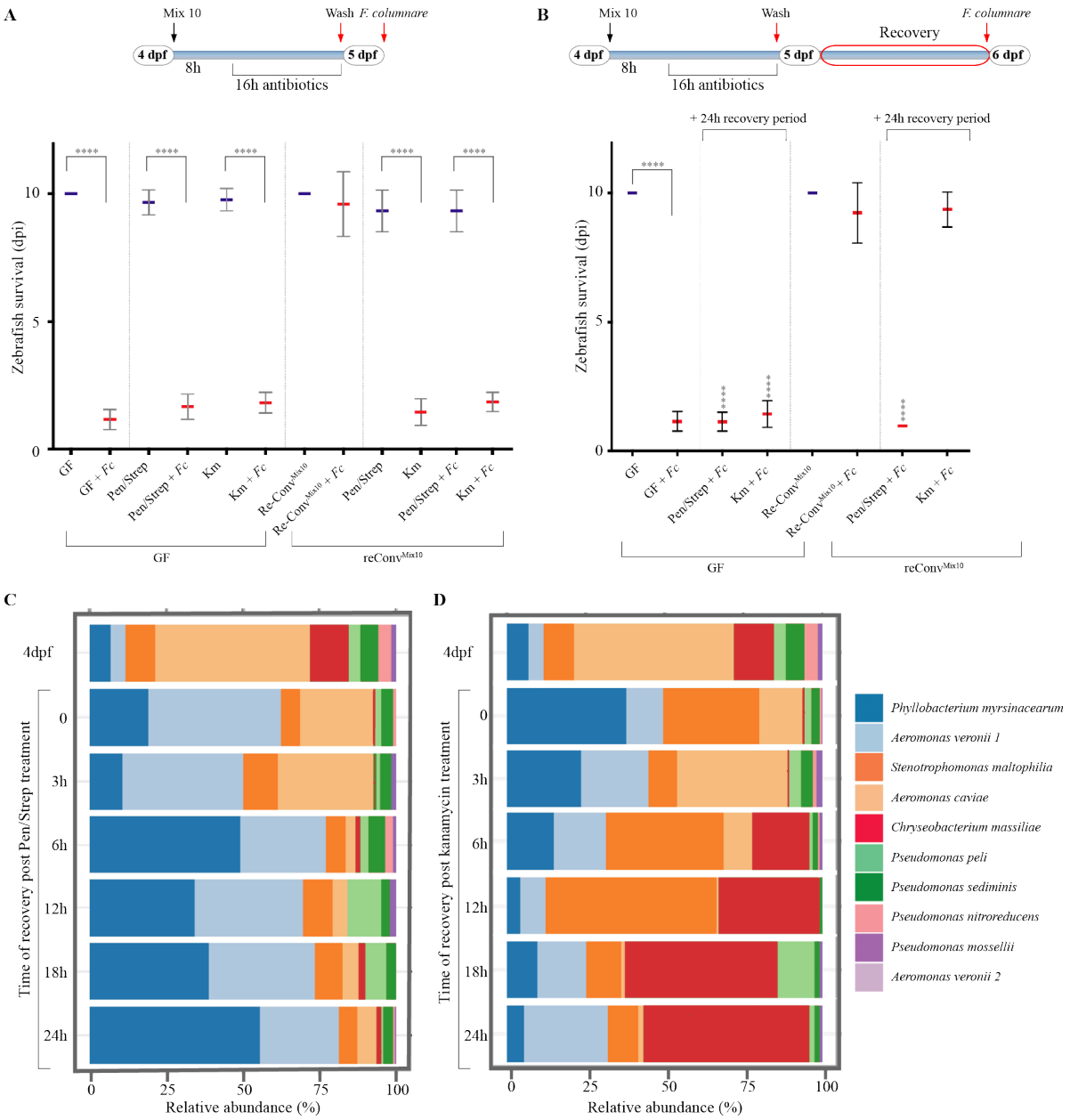



**Figure 2 Analysis of protection against *F. columnare* infection after antibiotic dysbiosis. A:** Response of zebrafish larvae to exposure to *F. columnare*$^{ALG}$ after antibiotic-induced dysbiosis with a diagram showing timing and treatments of the experiment. **B:** A 24h period after antibiotic 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.

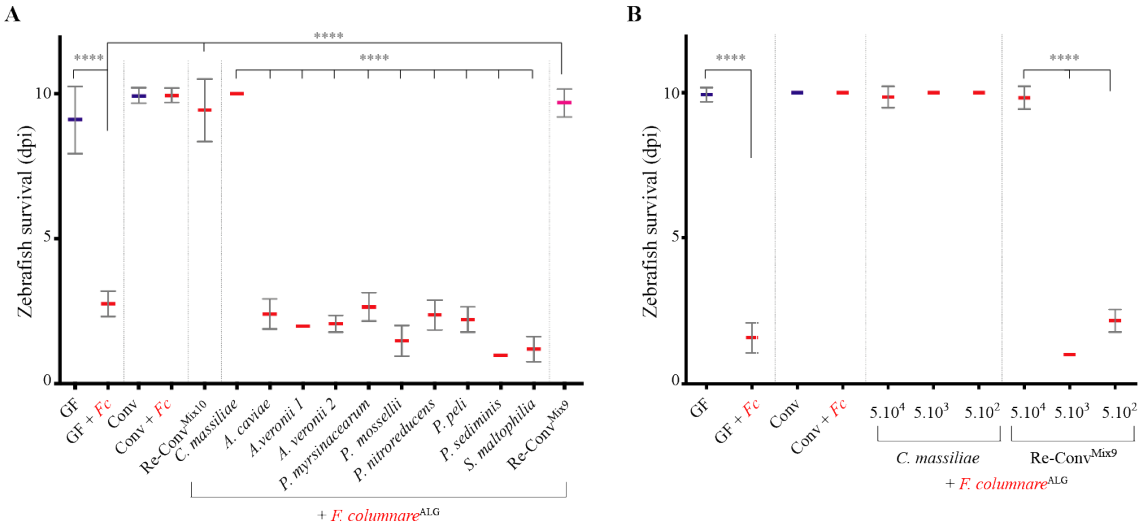



**Figure 3. Protection against *F. columnare* in zebrafish re-conventionalized with individual or mixed bacterial strains isolated from zebrafish. A**: Determination of the level of protection provided by each of the 10 bacterial strains composing the core protective zebrafish microbiota. Bacteria were 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.

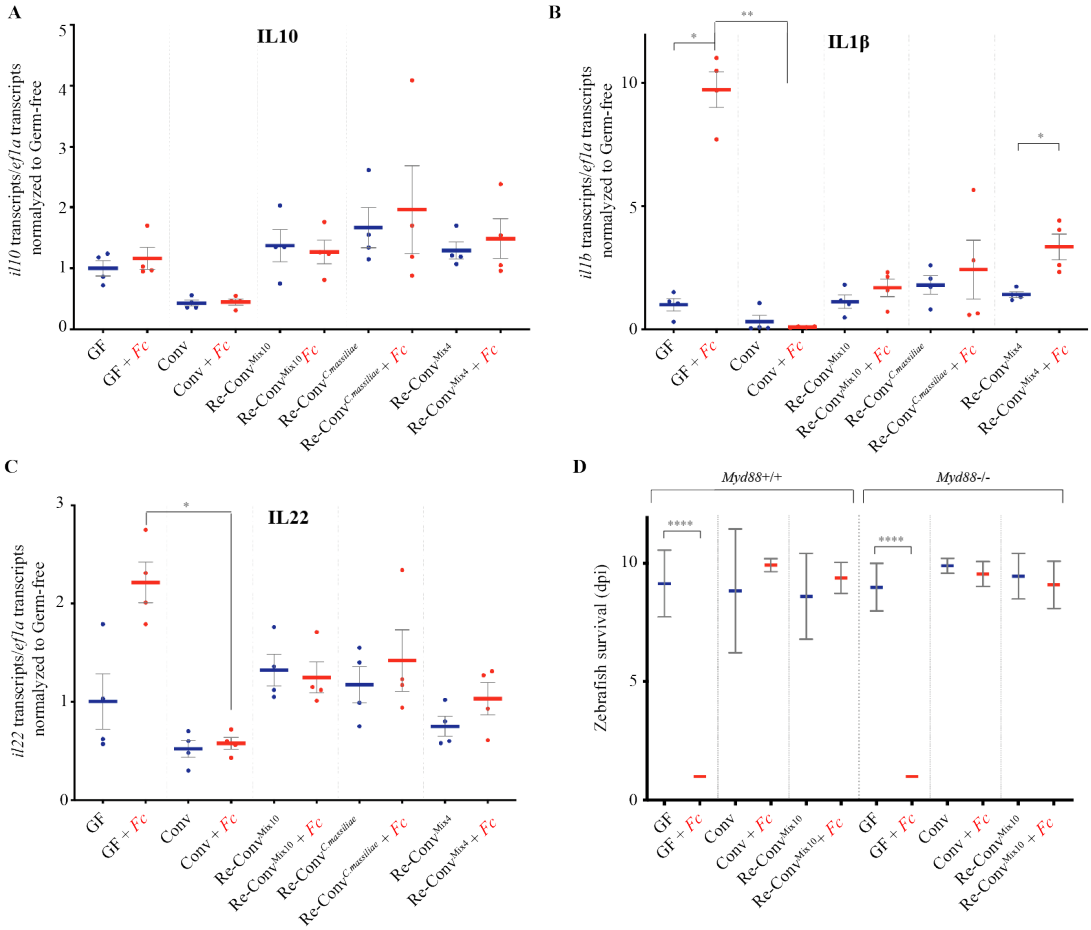



**Figure 4. Zebrafish immune response to *F.columnare* infection. A-C**: qRT-PCR analysis of host gene expression, 18 hours after exposure to *F. columnare,* in larvae re-conventionalized with indicated bacteria or bacterial mixes; each point corresponds to an individual larva. Expression of *il10* (**A**), *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.

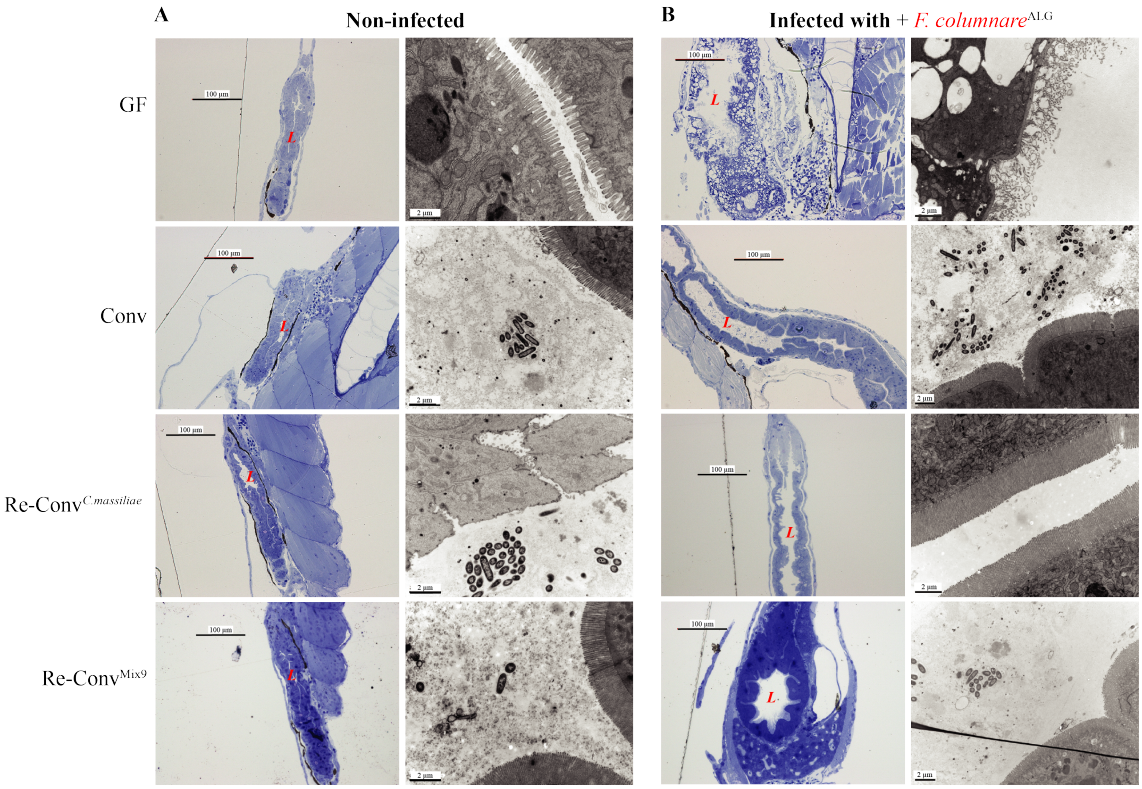



**Figure 5. Intestine of infected germ-free zebrafish displays severe disorganization.** Germ-free, conventional and re-conventionalized zebrafish larvae. Re-conventionalized zebrafish were inoculated at 4 dpf with Mix9 or *C. massiliae.* **A**: Representative picture of intestines of non-infected larvae. 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.

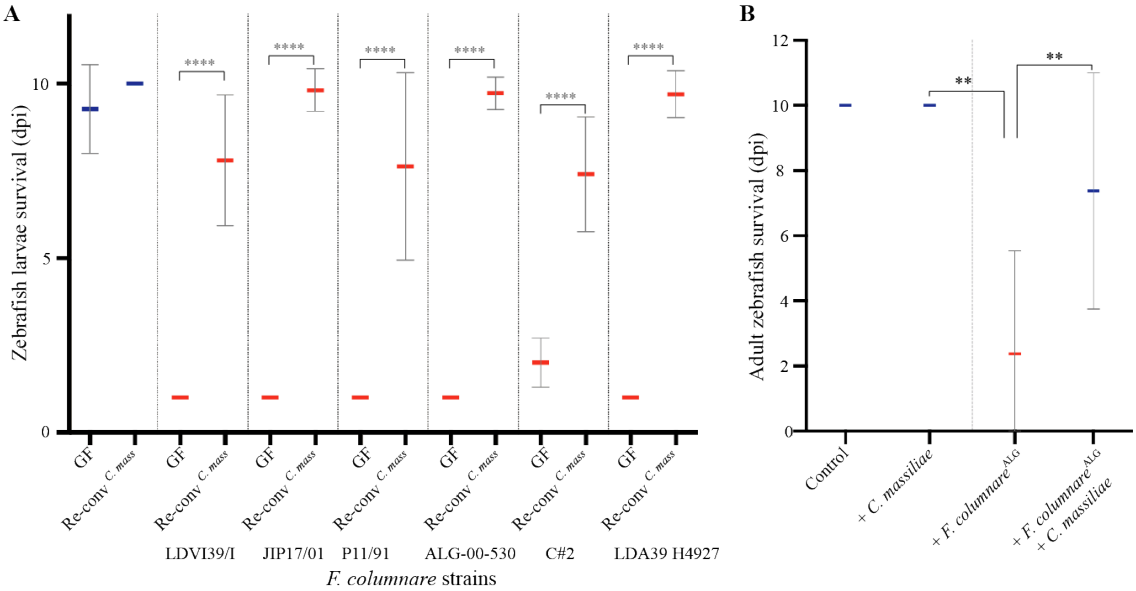



**Figure 6. Pre-exposure to *C. massiliae* protects larval and adult zebrafish against *F. columnare* infection**

**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.

1147 **TABLES**

1148

1149 **Table 1. The 10 strains composing the core of zebrafish larvae microbiota**

1150 Bacterial strains consistently detected at all time points (6 and 11 dpf) in all experiment
1151 runs and constituting the core of conventional zebrafish larval microbiota and their
1152 taxonomic affiliation.

1153

| **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* |

1154

1155 [a] Average Nucleotide Identity value
1156 [b] 16S rRNA gene sequence similarity
1157 [c] *recA* gene sequence similarity
1158 [d] *rplC* gene sequence similarity
1159 *Species ambiguously identified

1160