Regulation of Life Span by the Gut Microbiota in The Short-Lived African Turquoise Killifish

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ABSTRACT

Gut bacteria occupy the interface between the organism and the external environment, contributing to homeostasis and disease. Yet, the causal role of the gut microbiota during host aging is largely unexplored. Here, using the African turquoise killifish (*Nothobranchius furzeri*), a naturally short-lived vertebrate, we show that the gut microbiota plays a key role in modulating vertebrate life span. Recolonizing the gut of middle-age individuals with bacteria from young donors resulted in life span extension and delayed behavioral decline. This intervention prevented the decrease in microbial diversity associated with host aging and maintained a young-like gut bacterial community, characterized by overrepresentation of the key genera *Exiguobacterium, Planococcus, Propionigenium* and *Psychrobacter*. Our findings demonstrate that the natural microbial gut community of young individuals can causally induce long-lasting beneficial systemic effects that lead to life span extension in a vertebrate model.

27 **INTRODUCTION**

28

29 Life expectancy of different species in nature is regulated by a complex combination of
30 genetic and non-genetic factors. Genetic manipulations in model organisms have revealed key
31 conserved molecular pathways, including the insulin-IGF1 and the mTOR pathways, which
32 regulate aging and life span across several species, spanning from yeast to mammals (Kapahi
33 et al., 2010; Kenyon et al., 1993; Lapierre and Hansen, 2012). Environmental interventions
34 such as temperature and dietary manipulations have also been importantly associated with life
35 span modulation in several species. Among these, lower temperatures (Conti et al., 2006;
36 Miquel et al., 1976; Valenzano et al., 2006a; Van Voorhies and Ward, 1999) and reduced
37 nutrient intake (Fontana et al., 2010; Mair and Dillin, 2008) are key environmental factors that
38 have been associated with prolonged life span.

39 Complex microbial communities covering external surfaces live at the interface between
40 organisms and the external environment – from roots and leaves in plants, to skin, mucosal
41 surfaces and gut in animals. These microbial communities participate in a wide range of key
42 biological processes, including nutrient absorption (Semova et al., 2012), development
43 (Sommer and Backhed, 2013), metabolism (Nicholson et al., 2012), immune modulation
44 (Geva-Zatorsky et al., 2017), defence against pathogens (Kamada et al., 2013; Schuijt et al.,
45 2016) and disease (Sampson et al., 2016).

46 Individual gut microbiota (GM) composition changes dramatically in various diseases
47 (Baumgart and Carding, 2007; Garrett, 2015; Sokol et al., 2008) and during aging in flies,
48 mice and humans (Clark et al., 2015; Langille et al., 2014; O'Toole and Jeffery, 2015). Healthy
49 GM is typically characterized by large bacterial taxonomic diversity, whereas frailty is
50 associated with loss of diversity and expansion of more pathogenic bacterial species (Claesson
51 et al., 2012). Following antibiotic treatment, pathogenic bacterial species, such as *Clostridium*

52 *difficile* and *Enterococcus faecalis*, can restructure the human GM and cause severe chronic
53 conditions that pose a major threat for public health (Backhed et al., 2012; Cox and Blaser,
54 2015). Studies across different human age cohorts have shown that large changes in the
55 abundance of subdominant bacterial taxa in the gut are a hallmark of aging; moreover,
56 exceptionally long-lived individuals – including supercentenarians – are characterized by the
57 persistence of bacterial taxa associated with a more healthy status (Biagi et al., 2016). While
58 diversity-associated microbial taxa often decline during age, specific bacterial taxa, such as
59 Clostridiales, are associated with malnutrition and increased frailty (O'Toole and Jeffery,
60 2015). In flies, reducing GM dysbiosis by improving immune homeostasis promotes longer
61 life span (Guo et al., 2014; Li et al., 2016). Manipulating the GM towards a healthy state has
62 the therapeutic potential to improve health in specific diseases (Dodin and Katz, 2014; Kunde
63 et al., 2013). However, due to the lack of suitable short-lived vertebrate experimental models,
64 it is not known whether age-associated gut microbial community changes causally affect the
65 aging process and whether resetting a young GM in middle-age individuals can improve long-
66 term health and affect individual life span in normal aging individuals.
67 In this study, we develop the turquoise killifish (*Nothobranchius furzeri*), a naturally short-
68 lived vertebrate species with a life span of a few months in captivity (Valenzano et al., 2015),
69 as a new model organism to study aging in the host gut and microbiota. We show that
70 turquoise killifish (TK) have a complex GM (both in the wild and in captivity), similar in
71 taxonomic diversity to that of mammals. We also show that during aging the overall microbial
72 diversity in the TK gut decreases, with increased over-representation of pathogenic
73 Proteobacteria. By acutely recolonizing middle age individuals with GM from young donors,
74 we developed an intervention that enabled fish to live significantly longer, remain more active
75 at old age, and maintain a highly diverse GM. Transcriptome analysis additionally revealed
76 that gut aging is associated with increased inflammation and reduced proliferation. Here we

77 provide the first evidence that acute gut microbiota transfer in the context of normal aging can
78 significantly prolong life span in a vertebrate, becoming a novel candidate life span enhancing
79 intervention. Our study also promotes the turquoise killifish as a highly suitable vertebrate
80 model system to study the crosstalk between intestine and gut-microbiota during host aging.
81

82 **RESULTS**

83

84 **The TK is a short-lived vertebrate with a complex GM**

85 TK is a naturally short-lived vertebrate, whose genome sequence has become available
86 (Reichwald et al., 2015; Valenzano et al., 2015) and that is amenable to genetic manipulations
87 via transgenesis or genome editing (Harel et al., 2015; Valenzano et al., 2011). Remarkably,
88 this species is characterized by a broad spectrum of aging phenotypes, including cancer,
89 neurodegeneration, and behavioral decline (Harel and Brunet, 2015; Kim et al., 2016). TK are
90 adapted to living in conditions of intermittent availability of water and to surviving during
91 brief rainy seasons and long dry seasons (Cellerino et al., 2015). In captivity, it lives between
92 four to eight months, depending on the strain (Valenzano et al., 2015). However, nothing is
93 known about its associated commensal microbes and whether its gut microbial complexity and
94 taxonomic richness matches that of short-lived invertebrate model organisms, such as worms
95 and flies, or that of longer-lived vertebrate model organisms, such as mice and zebrafish. To
96 determine the TK's gut microbial composition, we sequenced the hyper-variable V3/V4
97 regions of the 16S rRNA gene amplicon from intestines of captive TK (N = 11, Materials and
98 Methods). We found that TK are characterized by a gut microbial taxonomic diversity similar
99 to other, longer-lived vertebrates, including zebrafish, mice and humans (Kostic et al., 2013;
100 Qin et al., 2010; Zac Stephens et al., 2016) (Figure 1A). This bacterial taxonomic diversity is
101 an order of magnitude higher than the gut microbial diversity present in invertebrate model
102 organisms such as worms (Cabreiro and Gems, 2013) and flies (Buchon et al., 2013) in the
103 laboratory (Figure 1A). Remarkably, the four most abundant bacterial phyla present in the
104 TK's gut, i.e. Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, are also the four
105 most abundant human gut bacterial divisions (Zoetendal et al., 2006) (Figure 1B and

106 Supplementary file 1), although in different proportions (Figure 1A). The unique combination

107 of a complex gut

108

FIGURE 1

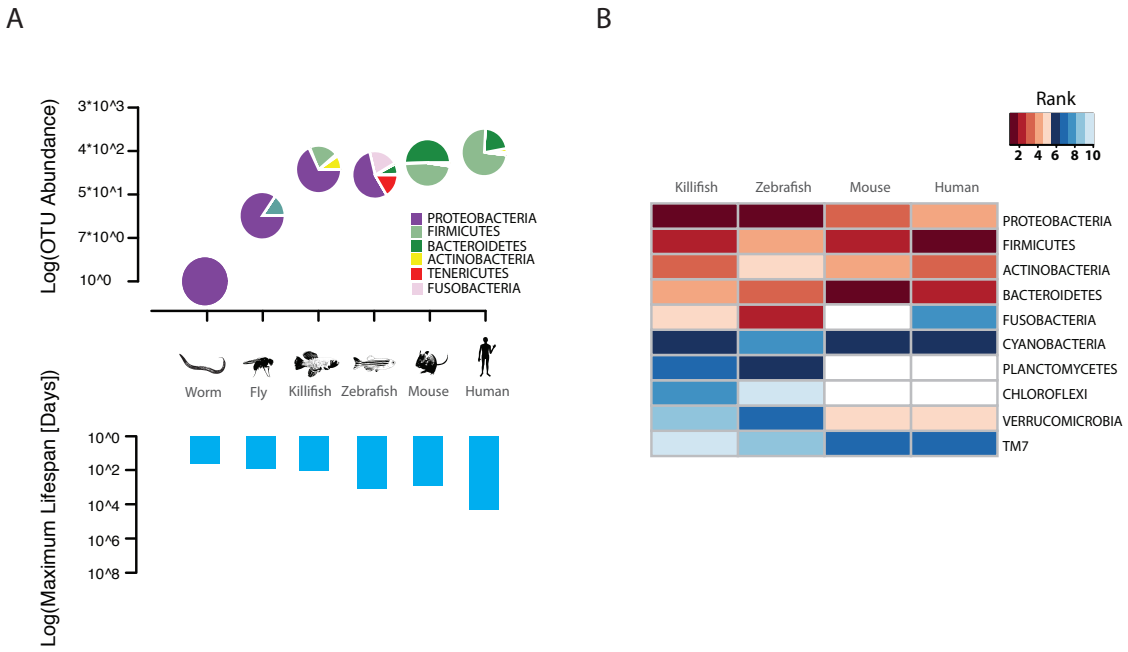


Figure 1. TK as a model to study aging of the GM

(A) Above: relative bacterial OTU composition (pie charts) and diversity (y-axis of upper plot) across different aging model organisms. Below: maximum life span (logarithm of days) in six model organisms, data from the AnAge longevity database. (B) Heatmap of ranked relative abundance of OTU composition at the phylum-level for different model organisms (Supplementary file 1). Bacterial phyla are ordered by their relative abundance in the TK. The ranked abundance is color-coded, with higher ranks (red) indicating greater relative abundance and lower ranks (blue) indicating lower relative abundance. White cells mark phyla that are not present in the respective model organism.

microbial composition, similar to that of other vertebrate aging model organisms, and its naturally short life span, combined with a wide spectrum of aging phenotypes, makes the turquoise killifish an ideal system to study the role of the gut microbiota during vertebrate aging.

Wild and captive TK populations share a core GM

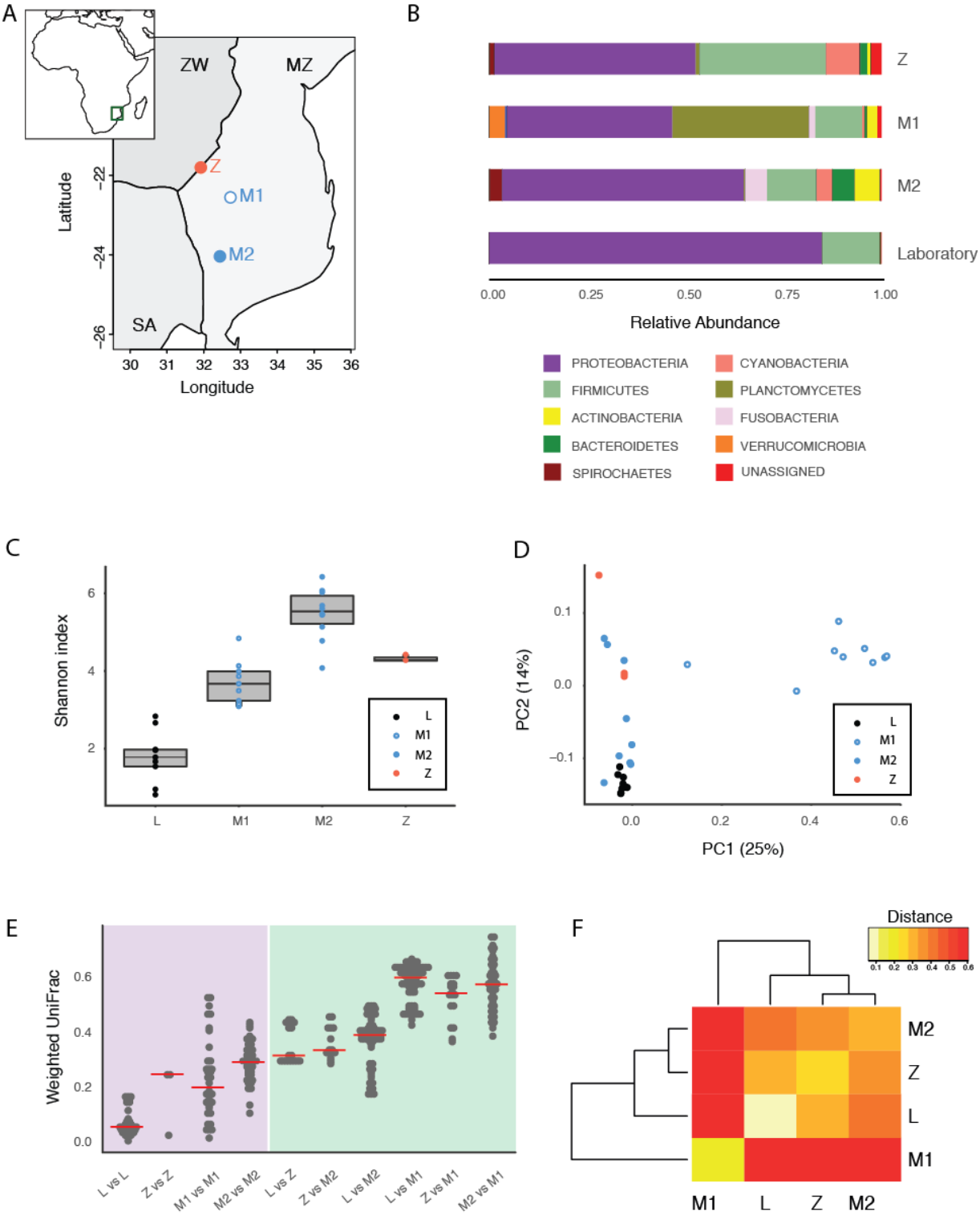
To assess whether the GM of captive TK was representative of the microbial communities associated with wild populations, we sequenced 16S rRNA gene amplicons from individual fish that we collected from different localities in the natural habitat of this species, ranging from the Gonarezhou National Park in Zimbabwe to the Gaza region in Mozambique (Figure 2A, Figure 2 – figure supplements 1B, Supplementary file 2A, Materials and Methods). Sequencing these wild populations confirmed that Proteobacteria was the dominant phylum also in most wild populations, similar to laboratory fish (Figure 2B, Figure 2 – figure supplements 1C). Individuals from all wild populations had a more diverse GM than laboratory fish (Figure 2C, Shannon alpha diversity, Dunn Kruskal-Wallis test, BH-adjusted p values < 0.05). While standard frequency-based diversity measures of observed bacterial taxonomic units (OTUs) (Simpson's and phylogenetic alpha diversity across the whole tree, OTU abundance, Figure 2 – figure supplements 1A) were higher in wild populations, Chao1 alpha diversity, which gives more weigh to rare bacterial OTUs, was higher in laboratory fish (Figure 2 – figure supplements 1A). These results possibly reflect the fact that laboratory fish are dominated by few, high-abundance OTUs, hence resulting in having more "rare" bacterial taxonomic units compared to wild populations. Differences in OTU abundance between laboratory-raised and wild fish might reflect ecological differences between the standardized laboratory conditions and the more heterogeneous wild environment, characterized by fluctuations in temperature, nutrients as well as other biotic and abiotic factors (Blažek et al.,

145 2017). To test whether differences in ecology across distinct wild fish populations influenced

146 bacterial diversity

147

FIGURE 2


A
ZW
MZ
Z
M1
M2
SA
Latitude
Longitude
B
Z
M1
M2
Laboratory
Relative Abundance
PROTEOBACTERIA
CYANOBACTERIA
FIRMICUTES
PLANCTOMYCETES
ACTINOBACTERIA
FUSOBACTERIA
BACTEROIDETES
VERRUCOMICROBIA
SPIROCHAETES
UNASSIGNED
C
Shannon index
L
M1
M2
Z
D
PC2 (14%)
PC1 (25%)
E
Weighted UniFrac
L vs L
Z vs Z
M1 vs M1
M2 vs M2
L vs Z
Z vs M2
L vs M2
L vs M1
Z vs M1
M2 vs M1
F
Distance
M2
Z
L
M1

151 **Figure 2. Core microbiota is conserved between wild and captive TK**

152 (A) Map location of wild populations collected in Zimbabwe (Z) and Mozambique (M1 and M2). ZW:
153 Zimbabwe; MZ: Mozambique; SA: South Africa. (B) Relative abundance of bacterial phyla in wild populations
154 and laboratory fish. (C) Shannon Index alpha diversity in laboratory and wild fish (L = laboratory, derived from
155 individuals originally collected in Zimbabwe). (D) PCoA of the Weighted UniFrac beta diversity distance for
156 wild and laboratory fish. Adonis test: L vs. all groups: p value < 0.001, M2 vs. M1: p value < 0.001, Z vs. M1
157 and M2: p value < 0.05. (E) Dotplot of the Weighted UniFrac distance values to visualize beta diversity within
158 (purple background) and between (green background) the different populations. Single dots represent
159 comparisons between individual fish. Red horizontal line indicates the median for each comparison. (F) Heatmap
160 of the Weighted UniFrac distance among wild populations and laboratory fish.

161

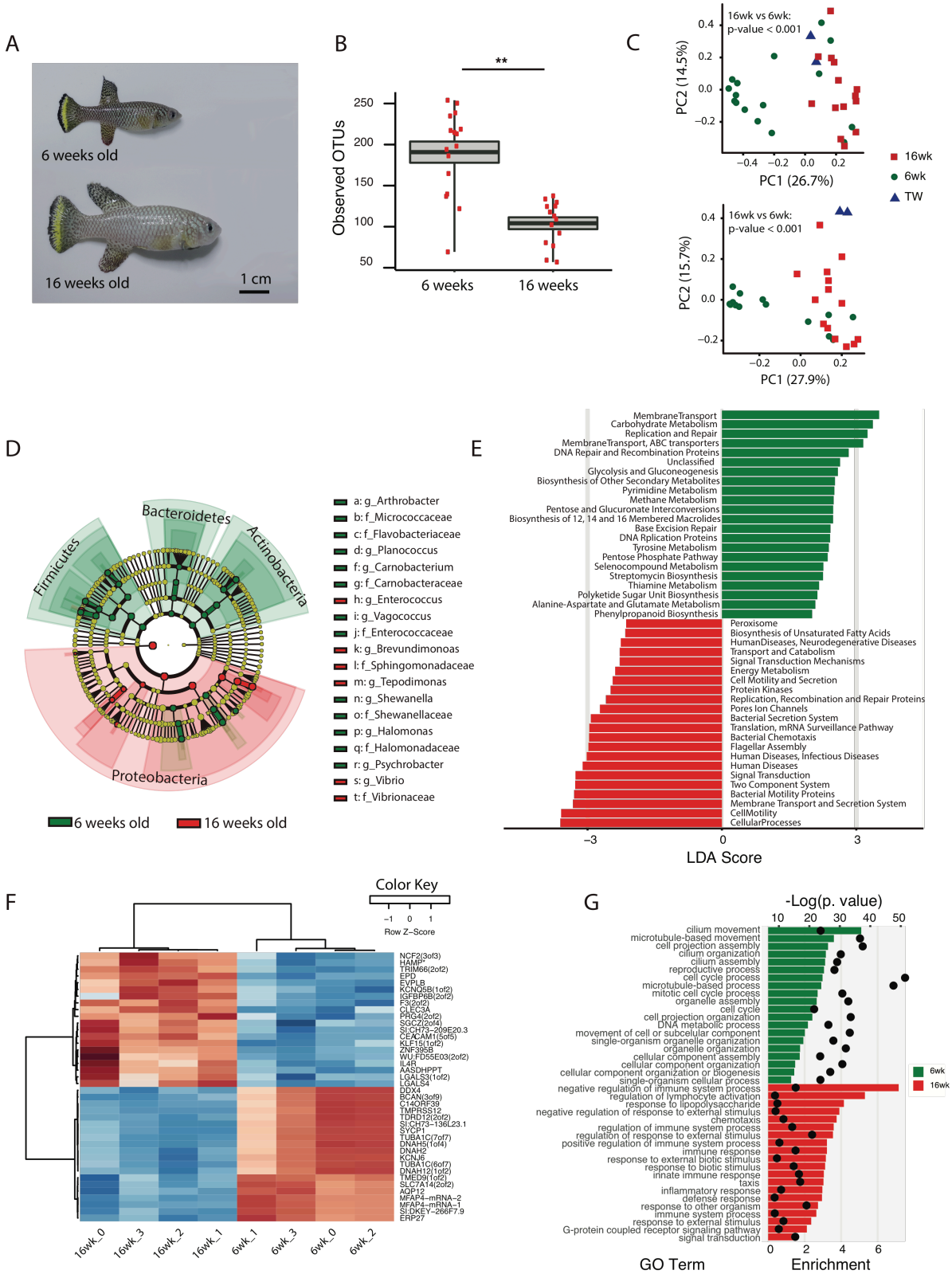
levels, we ran a regression model between diversity indexes and different recorded ecological factors, including altitude, pond size, maximum pond depth, vegetation, water conductivity, water temperature and water turbidity (Supplementary file 2B). None of the recorded factors was significantly correlated to alpha diversity levels (data not shown). This suggests that other parameters, such as food availability, fish genetics, presence of parasites or additional biotic or abiotic factors might be the key determinants of fish GM composition in the wild.

Although microbiota diversity in wild populations was higher than in laboratory-raised fish, the microbial composition of laboratory fish was not separated from the species-specific bacterial composition in wild fish, and was contained within the natural microbial variation of the species (Figure 2D). Importantly, one wild population (M1) had a divergent composition from all other populations, possibly due to over-representation of Planctomycetes (Figure 2B and D, Figure 2 – figure supplements 1C and E). Between-individuals diversity measures (beta diversity) showed that, while laboratory fish's bacterial diversity was lower than in the wild populations, between-group bacterial diversity was higher between population M1 and all the other populations, including laboratory fish (Figure 2E-F). These results indicate that population M1 had a more divergent microbial diversity of all the tested populations, and that the laboratory fish were not an outlier group (Figure 2D). Based on this, laboratory fish share a core microbiota with wild populations, despite the differences in culturing conditions between the laboratory and the wild, similar to what is seen in zebrafish (Roeselers et al., 2011). Thus, while wild turquoise killifish populations differ from one another in terms of microbial composition – possibly in association with ecological differences among different localities – laboratory fish recapitulate the core gut microbiota of wild populations, making them ideally suited to study how a complex microbiota influences host physiology.

186 **Aging in the TK is associated with loss of microbial diversity and decreased expression of**
187 **genetic markers of gut health**

188 An important question is what changes in the gut microbial population occur during aging in
189 vertebrates. To gain insight into the changes in GM composition occurring throughout aging in
190 the TK, we performed 16S rRNA gene amplicon surveys in the gastrointestinal tract of six-
191 week-old young-adult and 16-week-old individuals raised in captivity (Figure 3A, n = 16
192 young and 14 old fish). We found that bacterial taxonomic diversity (alpha diversity)
193 significantly decreased between 6-week-old and 16-week-old fish, while bacterial abundance
194 was not changed (Figure 3B, Figure 3 – figure supplements 1A and B). This indicates that,
195 similar to humans (Claesson et al., 2012), aging in the TK is characterized by a significant
196 reduction in gut bacterial richness. Bacterial composition was also significantly altered
197 between young and old fish guts (Figure 3C), including an age-dependent decrease in
198 Firmicutes (Kruskal-Wallis test, p value = 0.002) and Actinobacteria (Kruskal-Wallis test, p
199 value = 0.013). Young and old fish GM had significantly different community structures
200 (Figure 3C, UniFrac p value < 0.001; Bray-Curtis p value < 0.001, Adonis test, Figure 3 –
201 figure supplements 1C). While individual fish GM diversity was higher in young than in old
202 fish (Figure 3B), differences among old fish GM were more pronounced compared to young
203 fish (Unweighted UniFrac beta diversity, Bonferroni-corrected p value = 6.7E-06; Bray-Curtis
204 distance p value = 0.003; Figure 3 – figure supplements 1D and E). This indicates that
205 although aging in the TK was associated with decreased bacterial taxonomic diversity within
206 each fish's gut, distinct old fish guts had highly divergent bacterial communities. While young
207 fish guts were significantly enriched for Bacteroidetes, Firmicutes and Actinobacteria, old fish
208 were dominated by Proteobacteria (Figure 3D). We next investigated the predicted functional
209 metagenome biomarkers associated with young and old fish's guts using PICRUSt
210

FIGURE 3



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212

213

214 **Figure 3. Changes in GM and gut transcriptome between young and old fish**

215 (A) Representative 6-week-old (young) and 16-week-old (old) male TK. (B) Alpha diversity changes in observed
216 OTUs in young (6 weeks) and old (16 weeks) TK. N = 16 six-week-old fish and 14 16-week-old fish. The groups
217 are compared using the Mann Whitney U test; ** indicates a p value < 0.001. (C) Beta diversity microbiota
218 analysis separates samples based on age. Above: Bray-Curtis analysis, below: Unweighted UniFrac analysis. TW:
219 tank water control. Adonis test, p value < 0.001 in both comparisons between 6-week and 16-week-old fish. (D)
220 Cladogram representing microbial taxa enriched in young (green) versus old (red) individuals. (E) Predicted
221 metagenome function in young (green) and old (red) groups (LEfSe), representing functions with p value <
222 0.001. The x-axis indicates the Linear Discriminant Analysis score for all the significant metabolic functions. (F)
223 Expression heatmap for the twenty top differentially expressed genes (DEGs) between young and old fish (n = 4
224 for each group). Top 20 genes are highly expressed in old fish, the bottom 20 are highly expressed in young fish.
225 (G) Top 20 Gene Ontology (GO) terms of the DEGs between young and old fish. Enrichment values (bars) and
226 the negative natural logarithm of p values (black dots) are shown.

227 *HAMP is the best protein blast hit in *Danio rerio* of the TK gene NFURG05812010005.

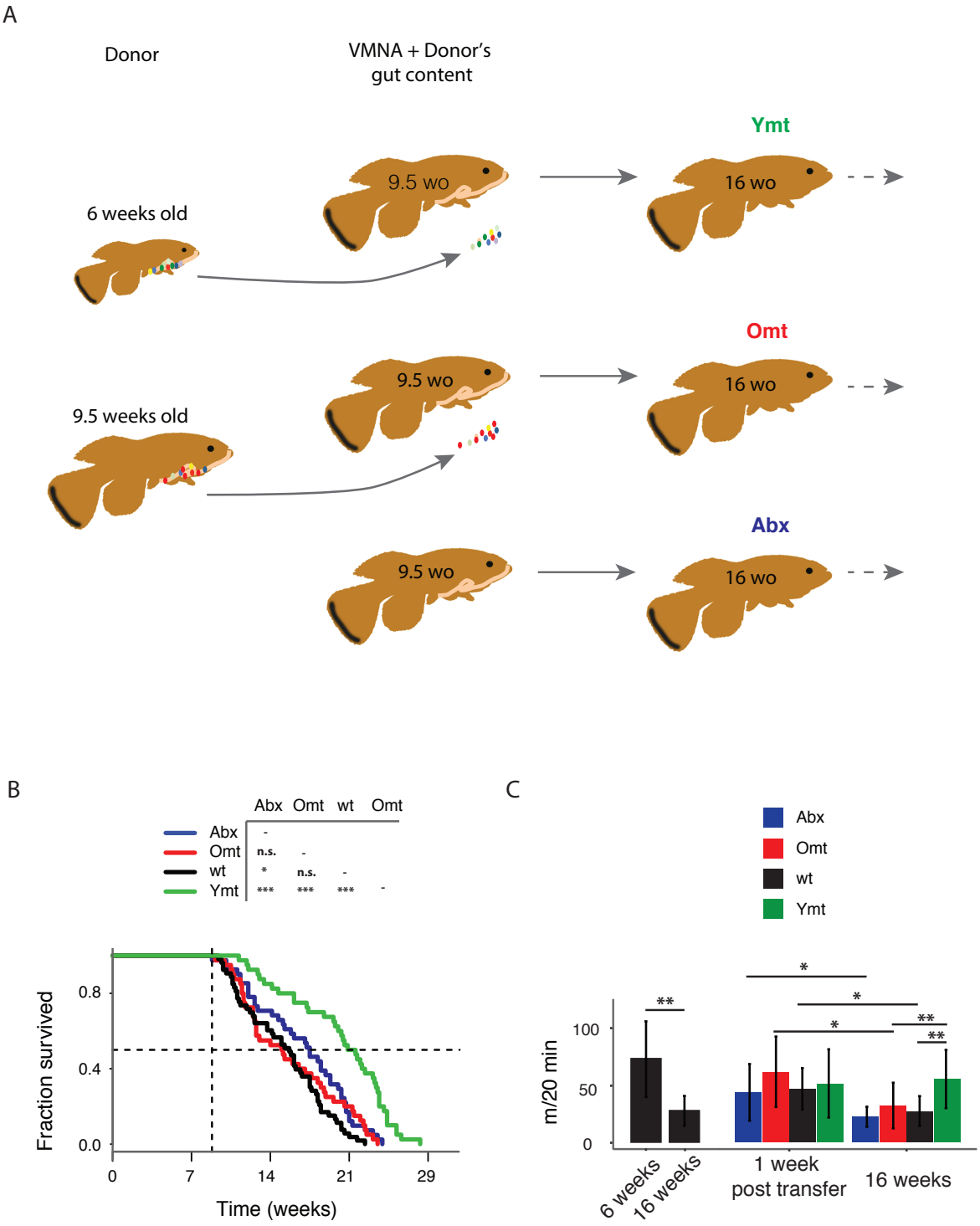
228

229 (Langille et al., 2013) and LEfSe (Segata et al., 2011). While young fish had GM strongly
230 associated with glycolysis and polysaccharide metabolism, old fish's GM was depleted of
231 bacteria associated with carbohydrate, nucleotide and amino acid metabolism, and was
232 enriched for bacteria associated with pathogenesis, transport and catabolism (Figure 3E). In
233 particular, bacterial motility and flagellar assembly was strongly increased in GM from old
234 fish. These terms are associated with increased virulence in bacteria (Josenhans and
235 Suerbaum, 2002), supporting that old fish had a higher prevalence of potentially pathogenic
236 bacteria.
237 To examine whether metagenomic biomarkers from bacterial taxonomic diversity (i.e. OTU)
238 data were consistent with host responses, we asked which were the transcriptional changes in
239 the gut associated with young and old status. To this end, we performed an RNA-Seq analysis
240 of whole gut in four 6-week old and four 16-week old fish (Figure 3F and G, Figure 3 – figure
241 supplements 2 and Materials and Methods). Young fish had a distinct expression signature of
242 active proliferation (Figure 3G, Figure 3 – figure supplements 2), consistent with the bacterial
243 metagenomic signature of replication. Old fish, on the other hand, had significant Gene
244 Ontology terms associated with immune and defense responses against pathogens as well as
245 inflammation, consistent with the bacterial metagenome signatures associated with host
246 disease and overall virulence. Together, these results strongly support that in old individuals
247 both changes in bacterial composition and gut transcriptome are consistent with a markedly
248 pathological gut environment, while young fish are characterized by a molecular signature of
249 healthy gut and a commensal bacterial community.
250
251 **Young GM transfer prolongs life span and delays age-dependent motor decline**
252 Interventions aimed at directly modifying the complex microbial composition in experimental
253 organisms and patients have been mostly focused on treating diseases such as *Clostridium*

254 *difficile* infections (Dodin and Katz, 2014; Lee et al., 2016), as well as obesity and type two
255 diabetes (Kootte et al., 2012; Turnbaugh et al., 2006). However, the impact of a young GM in
256 modulating aging and life span has not been explored to date in vertebrates (Clark et al.,
257 2015). To test whether resetting a young-like GM in middle age could impact aging and affect
258 life span, we treated middle-age fish (9.5 week-old) overnight with an antibiotic cocktail
259 (VMNA, i.e. vancomycin, metronidazole, neomycin, ampicillin) (Figure 4A, Figure 4 – figure
260 supplements 1A and Materials and Methods). The antibiotic treatment significantly reduced
261 gut microbial content compared to pre-treatment levels (Figure 4 – figure supplements 1B).
262 Antibiotic-treated fish were then exposed for 12 hours to the following conditions: six-week-
263 old donor fish gut content (Ymt), 9.5-week-old fish gut content (Omt) and sham (Abx) (Figure
264 4A and Figure 4 – figure supplements 1A). After antibiotic treatment and 12-hour acute gut
265 recolonization, fish were reintroduced in the water recirculation system in individual tanks and
266 were subjected to regular feeding (Materials and Methods). Their survival under the different
267 experimental conditions was then scored (Supplementary file 3A). Ymt fish underwent
268 dramatic life span prolongation compared to three control groups, which received: (i)
269 antibiotic-only (Abx) (21% life span increase in median life span, Logrank test p value =
270 5.89E-05), (ii) antibiotics and same-age (i.e. 9.5 weeks) gut content (Omt) (41% increase in
271 median life span, Logrank test p value = 5.08E-06), or (iii) no-treatment (wt) (37% increase in
272 median life span, Logrank test p value = 4.04E-09) (Figure 4B and Figure 4 – figure
273 supplements 1C-F). Noteworthy, acute antibiotic treatment alone was sufficient to increase
274 fish life span compared to the wt group (14% median life span increase, Logrank test p value =
275 0.0129) (Figure 4B). However, Omt fish did not live longer than the control, wt group (Figure
276 4B). Since Abx outlived Omt and wild-type fish, while Ymt fish outlived Abx fish, it is
277 plausible that middle-age GM composition might be primed to induce damage in the host and
278 that its removal is therefore beneficial. However, as the recolonization of middle-age

279 individuals with young fish gut content after antibiotic treatment prolongs life span even more,
280 this implies that young GM, *per se*, has beneficial effects on host physiology that are additive
281 to the effects of the antibiotic treatment.
282

FIGURE 4


A
Donor
VMNA + Donor's gut content
6 weeks old
9.5 wo
Ymt
16 wo
9.5 weeks old
9.5 wo
Omt
16 wo
9.5 wo
Abx
16 wo
B
Abx Omt wt Omt
Abx -
Omt n.s. -
wt * n.s. -
Ymt *** *** *** -
Fraction survived
0.8
0.4
0.0
0 7 14 21 29
Time (weeks)
C
Abx
Omt
wt
Ymt
m/20 min
100
50
0
**
*
*
*
**
**
6 weeks
16 weeks
1 week post transfer
16 weeks

283

284 **Figure 4. Transfering young GM to adult fish prolongs life span and delays motor**
285 **decline**

286 (A) Schematic representation of microbial transfer experiment (Materials and Methods). Experimental group
287 legend, Abx: fish receiving only antibiotic treatment at 9.5 weeks without direct recolonization. Omt: fish
288 receiving same-age GM transfer after antibiotic treatment at 9.5 weeks. Wt: wild-type, untreated fish. Ymt: fish
289 receiving 6-week-old fish GM transfer after antibiotic treatment at 9.5 weeks. VMNA: antibiotic cocktail of
290 vancomycin, metronidazole, neomycin and ampicillin. (B) Survival analysis. Statistical significance is calculated
291 by Logrank test. * indicates a p value < 0.05; *** indicates a p value < 0.001. (C) Exploratory behavior in
292 different treatments. Young and old wild-types are compared with a Kruskal-Wallis test (left), the remaining
293 groups are compared using a Dunn Kruskal-Wallis test for multiple comparisons, and the p values are adjusted
294 based on BH correction. Statistical significance: * indicates a p value < 0.05; ** indicates a p value < 0.01; ***
295 indicates a p value < 0.001.

296

297

298 We then asked whether treating young fish with old fish GM could also affect life span.
299 Compared to fish that received either the same-age GM or to untreated control fish, six-week-
300 old fish receiving 16-week-old fish's GM after antibiotic treatment did not have a different life
301 span (Figure 4 – figure supplements 1G). Additionally, unlike middle-aged fish treated with
302 antibiotics, young fish receiving antibiotic treatment did not live longer than untreated control
303 fish (Figure 4 – figure supplements 1G). These results suggest that the timing of GM transfer
304 is critical to inducing systemic effects and modulating life span.
305 It was shown in previous work that spontaneous exploratory behavior in TK decreases with
306 age (Genade et al., 2005; Valenzano et al., 2006b). We therefore asked whether treating
307 middle-age fish with young fish GM after antibiotic treatment could improve exploratory
308 behavior performance, considered as an integrated measure of individual health. Using an
309 automatic video-tracking system (Materials and Methods), we assayed spontaneous locomotor
310 activity. Young, six-week-old fish, were significantly more active than 16-week-old fish
311 (Supplementary file 3B and Figure 4C, Kruskal-Wallis chi-squared = 10.752, df = 1, p value =
312 0.00104). Remarkably, Ymt were more active at 16 weeks of life than Omt and wt fish at the
313 same age, resembling younger fish performance (Figure 4C, Dunn Kruskal-Wallis multiple
314 comparison test, BH-adjusted p value = 0.004). Additionally, all groups except Ymt
315 underwent a significant decrease in spontaneous locomotor activity from a week post transfer
316 to 16 weeks of life. This suggests that the transfer of young fish gut content had long-lasting
317 effects on a global measure of physiological health, influencing individual survival and
318 spontaneous exploratory behavior. Thus, depleting middle-age individuals from their resident
319 GM resulted beneficial when acutely recolonized by young-associated GM (Ymt), and in part
320 also when it was not followed by any acute recolonization (Abx). On the other hand, acutely
321 recolonizing the gut with same age GM after antibiotic treatment did not lead to differences

322 compared to the untreated control group. These results establish gut microbial recolonization
323 as a powerful life span enhancing intervention, which leads to significant effects also on
324 behavioral performance.
325
326 **Acute GM transfer affects microbial composition at old age**
327 To assess the extent to which one acute transfer reset the GM in recipient fish after VMNA
328 antibiotic treatment, we performed 16S rRNA gene amplicon surveys in fish that underwent
329 microbial transfer at 9.5 weeks of age. Untreated 6-week-old (6wk) and 16-week-old (16wk)
330 fish were also included in the analysis. One week post transfer there were no significant
331 differences in alpha diversity metrics among treatment groups (Figure 5 – figure supplements
332 1A and B). However, Ymt and Omt fish had already significantly different gut microbial
333 population diversity (UniFrac and Bray-Curtis distances, Figure 5 – figure supplements 1C
334 and D and Supplementary file 4). Seven weeks after the microbial transfer (16 weeks of age,
335 corresponding to the median life span for this species in captivity), Ymt fish had significantly
336 higher bacterial richness compared to wild-type, 16-week-old fish (Figure 5A, Dunn Kruskal-
337 Wallis test, BH-adjusted p value = 0.009). This shows that one acute transfer had long-lasting
338 effects on GM diversity. Bacterial OTU abundance at 16 weeks was higher in Ymt fish
339 compared to Omt fish (Figure 5A), but lower than 6-week old wild-type fish (6wk).
340 GM community structure in Ymt fish was also significantly altered compared to Omt and
341 16wk (Figure 5B and Figure 5 – figure supplements 1E); however, it did not statistically differ
342 from young wild-type fish, i.e. the 6wk group. Furthermore, based on hierarchical clustering,
343 Ymt fish clustered preferentially with 6wk fish (Figure 5C), showing that the GM-transfer
344 from young donors significantly reset a young-like GM.
345 Young fish (6wk), as well as fish treated with young GM (Ymt), were more enriched with
346 members of the Bacteroidetes and Firmicutes (Figure 5D) and with the genera

FIGURE 5

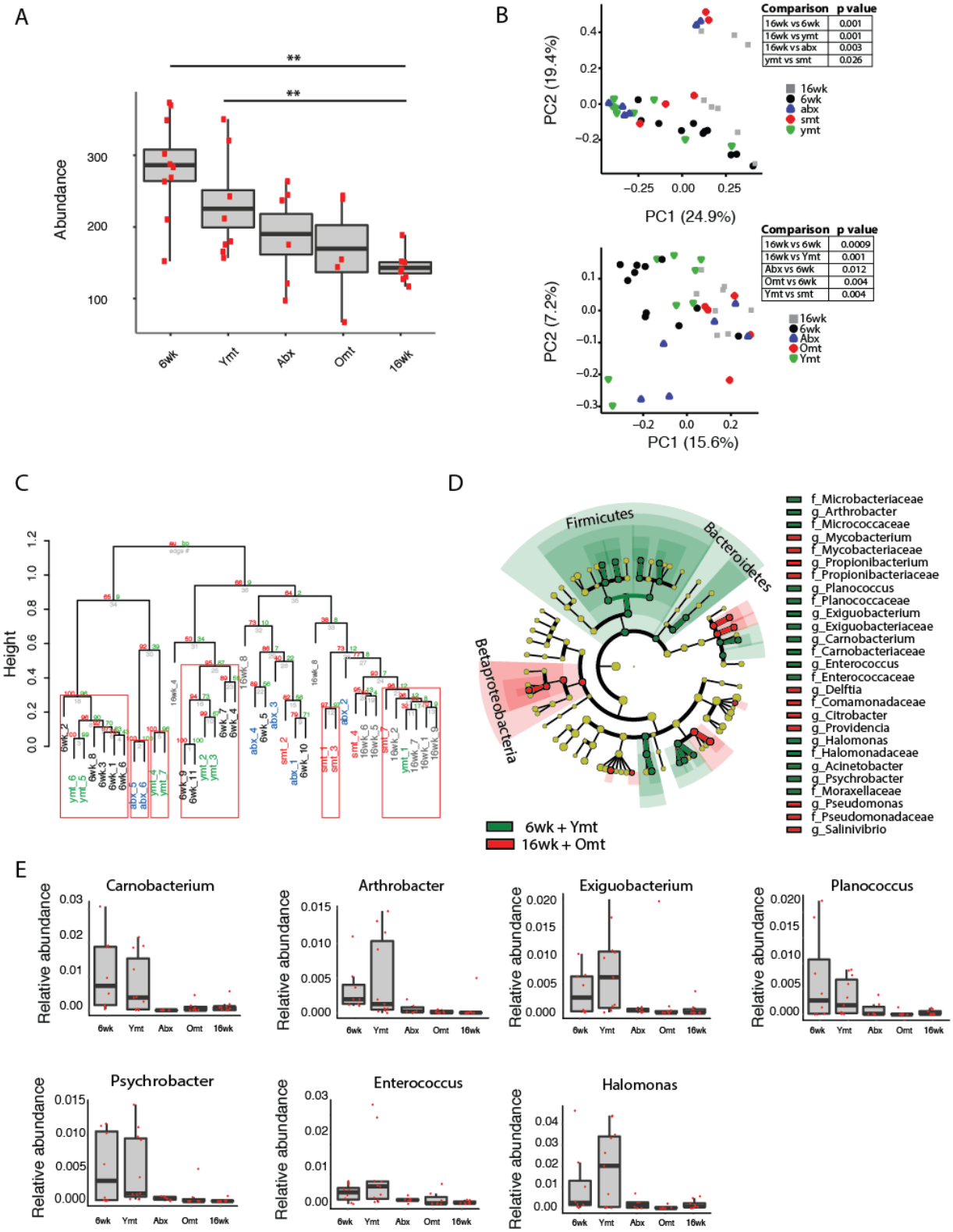


Figure 5. Microbiota transfer at 9.5 weeks influences microbial composition at 16 weeks

(A) Alpha diversity measured by number of observed OTUs in experimental groups at 16 weeks (16wk, Abx, Omt, Ymt) and young controls (6wk). Dunn Kruskal-Wallis test for multiple comparisons. The p values are

351 adjusted based on BH correction. Statistical significance: ** indicates a p value < 0.01. (B) Microbiota
352 community analysis using Bray-Curtis (above) and Unweighted UniFrac (below) separates samples based on
353 young vs. old GM treatment (significant p values are shown, Adonis test). (C) Hierarchical clustering on OTU
354 data. Significant clusters are highlighted by red rectangles. P values: au = approximately unbiased; bp: bootstrap
355 probability. (D) Cladogram of bacterial taxa enriched in young wild-type (6wk) and Ymt groups versus 16wk and
356 Omt. (E) Boxplots of selected bacterial genera relative abundance in experimental groups. Bacterial genera are
357 those represented in Figure 5D, filtered by r-square value larger than 0.8, based on the correlation between
358 bacterial genus abundance vs median life span in each individual group (Supplementary file 5).
359

360 *Carnobacterium*, *Arthrobacter*, *Exiguobacterium*, *Planococcus*, *Psychrobacter*, *Enterococcus*
361 and *Halomonas* (Figure 5E). Additionally, analyzing the correlation between bacterial genera
362 abundance and the group-specific median lifespan, we identified a set of bacterial genera
363 whose abundance is highly correlated with group-specific longevity (Supplementary file 5).
364 We then used predicted functional metagenome analysis from GM composition to compare
365 experimental fish groups receiving same-age GM transfer and no-treatment controls to fish
366 receiving young gut content. The latter group was enriched for increased saccharolytic
367 potential, DNA repair and recombination among other functions (Figure 5 – figure
368 supplements 1F), which are functional terms associated with a younger, healthy-like
369 physiological state. Together, these results show that gut microbial recolonization from young
370 killifish resulted in increased microbial diversity and a persistence of a young-like bacterial
371 community until old age, whose composition could be at least in part responsible for the life
372 span prolongation.
373
374 **Microbial transfer determines bacterium-to-bacterium connectivity at old age**
375 To investigate whether gut recolonization affected bacterium-to-bacterium association in
376 different experimental groups, we harnessed OTU co-occurrence to generate bacterial
377 connectivity networks (Agler et al., 2016; Biagi et al., 2016). To assess the bacterium-to-
378 bacterium connectivity in each experimental group, we built a network model for each
379 experimental group based on OTU co-occurrence. Significantly co-occurring genera within
380 each group composed a network, whose nodes were single bacterial genera. Bacterium-to-
381 bacterium connections (edges) were established based on r-square values (Materials and
382 Methods). Using this analysis, we found that Proteobacteria had higher connectivity in the
383 shorter-lived 16wk and Omt groups compared to 6wk, Ymt and Abx (Figure 6A). Overall,
384 6wk and Ymt fish had the largest networks and a higher number of highly connected nodes

FIGURE 6

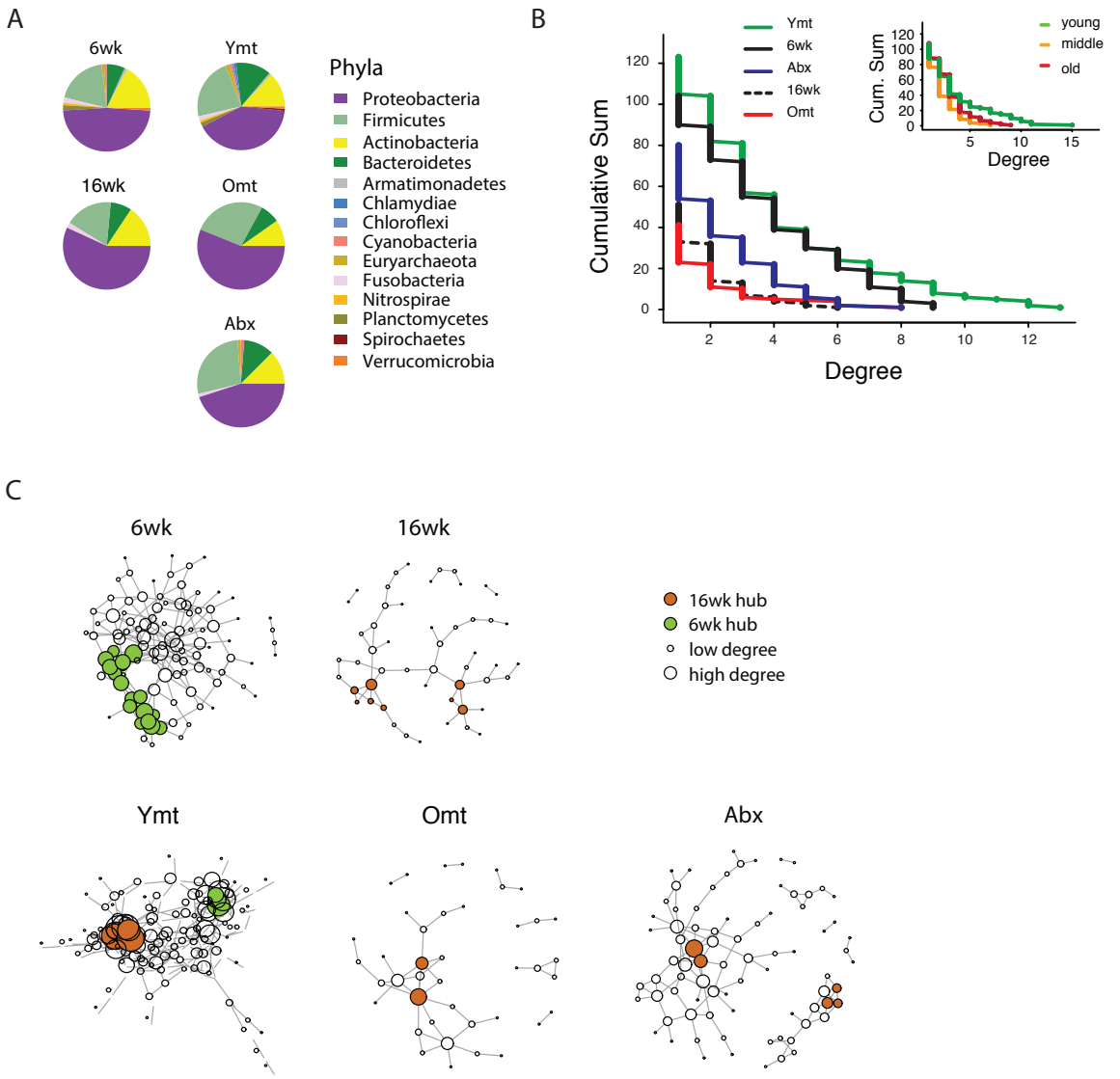


Figure 6. Bacterial co-occurrence connectivity

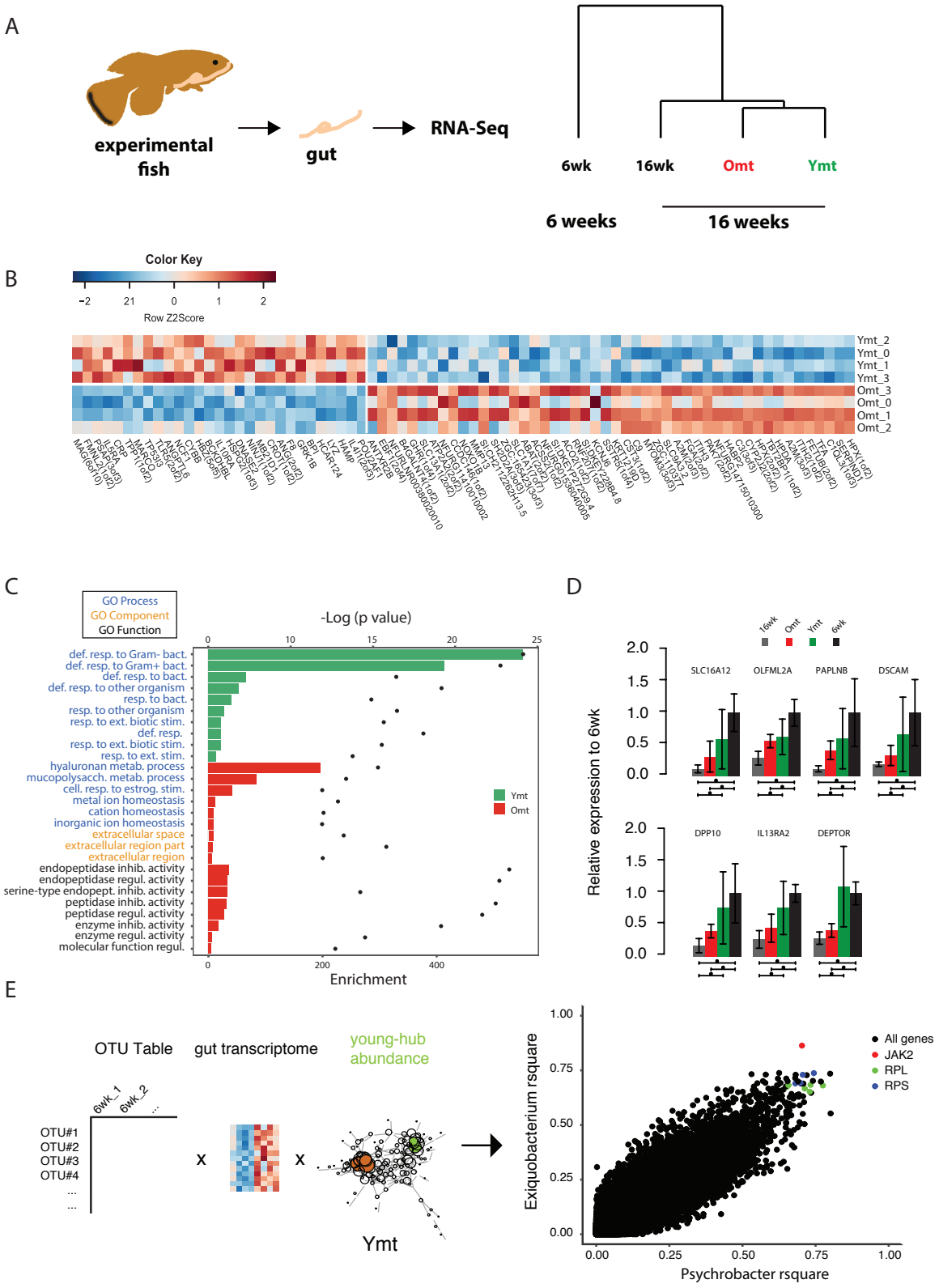
(A) Phyla composition of the bacterial co-occurrence networks in experimental groups at 16 weeks (16wk, Ymt, Omt Abx) and at 6 weeks (6wk) (Materials and Methods). (B) Cumulative sum of degree distribution in co-occurrence networks of all analyzed TK experimental groups (black: 6wk; dashed black line: 16wk; green: Ymt; red: Omt; blue: Abx) and a mouse cohort from (Langille et al., 2014) (Inlet, green: young mice; orange: middle-aged mice; red: old mice). X-axis shows the degree count and y-axis the cumulative sum of nodes with the corresponding degree. C) Visualization of co-occurrence networks in Fruchtermann-Reingold layout of 6wk, 16wk, Ymt, Omt, and Abx. Circle size increases with degree count and circle color corresponds to 6wk hubs (green) or 16wk hubs (orange).

395 (hub nodes), showing that a few genera of bacteria significantly co-occurred with a large set
396 of other bacterial genera (Figure 6B and C).
397 We then tested whether the relation between age of the host and bacterial connectivity
398 observed in TK guts was also conserved in mammals. We analyzed a published mouse 16S
399 amplicon survey from an aging cohort (Langille et al., 2014). Similarly to what we found in
400 the TK, young mice GM had more significantly connected nodes and a higher number of
401 bacterial hubs compared to middle age and old mice (Figure 6B, inset).
402 The identification of wild-type specific young and old-related hub bacterial genera enabled us
403 to study whether Ymt, Omt and Abx shared hub bacterial genera with either young or old fish
404 groups. Two hub bacterial clusters were identified in Ymt fish (Materials and Method).
405 Strikingly, one was composed of bacterial genera that were also hub-bacteria in young wild-
406 type fish (6wk) and included *Exiguobacterium*, *Planococcus*, *Propionigenium* and
407 *Psychrobacter* (Figure 6C, Ymt network, green nodes), while the other was composed of hub
408 bacteria from old wild-type fish (16wk) hub and included *Propionibacterium*, *Delftia*, and
409 *Citrobacter* (Figure 6C and Supplementary file 6). Remarkably, the bacterial hubs identified in
410 Omt and Abx overlapped exclusively with the old wild-type group (16wk) (Figure 6C, orange
411 nodes, and Supplementary file 6). These results support that bacterial network topology
412 reflects host age both in fish and mice, with younger biological age associated with larger
413 networks. Acute microbiota transfers significantly affect gut microbial population topology
414 and subsets of bacterial genera are identified as hub nodes in young-like and old-like microbial
415 communities. Therefore, not only bacterial composition, but also bacterium-bacterium co-
416 occurrence carries a key signature of host life span and network topology depends on a few
417 key bacterial hubs.
418

419 **Microbial transfer affects expression of host genes associated with defence to bacteria,**
420 **Tor pathway and extracellular matrix**

421 Because acute microbial transfer dramatically changed GM composition by resetting a young-
422 like microbial community in Ymt fish, we asked whether young GM transfer could also reset
423 the host transcriptome towards a young-like status. To this end, we performed host intestine
424 RNA-Sequencing in wild-type young (6wk) and old (16wk) fish, as well as in Omt and Ymt
425 fish (Figure 7A and B, Figure 7 – figure supplements 1 and Materials and Methods).
426 Interestingly, while young GM transfer reset a young-like GM environment in aged fish, host
427 gene transcripts separated based on fish age, with all the transcriptomes obtained from 16-
428 week old fish groups (16wk, Omt, Ymt) clustering together (Figure 7A). However, comparing
429 transcriptomes in Omt and Ymt revealed a distinct signature of defence response to bacteria in
430 Ymt and increased expression of genes associated with hyaluronic acid metabolism in Omt
431 (Figure 7B and C). Comparing the expression levels of all groups to the 6wk group, we
432 identified a set of genes whose expression in the gut was significantly changed comparing
433 16wk and Omt with 6wk; comparing 16wk and Ymt, but unchanged between Ymt and 6wk.
434 These were genes associated with the TOR-pathway (DEPTOR) and with cell adhesion and
435 extracellular matrix composition (DSCAM, OLFML2A, PAPLNB) (Figure 7D), suggesting a
436 potential difference in cell adhesion and gut permeability between 16wk and Omt from one
437 side, and Ymt and 6wk on the other side. Since we generated OTU abundance tables for
438 individual fish and we also sequenced intestinal transcripts for the same individuals, we then
439 generated an OTU-to-host trancripts correlation matrix (Data S1). Taking advantage of this
440 resource, we could identify the transcripts that were highly correlated with the hub genera that
441 are shared between Ymt and young fish (6wk) networks (Figure 6C, Figure 7E and Figure 7 –
442 figure supplements 2). Remarkably, these genes include Jak2, an important gene involved in
443 cellular proliferation and differentiation, and several genes encoding S and L- ribosomal

FIGURE 7



444

445

446

447 **Figure 7. Transcriptional changes in host intestine after acute GM transfer**

448 (A) Cluster analysis of gut RNA-Seq from intestines from six-week-old fish (6wk) and 16-week-old fish (16wk,
449 Omt (red) and Ymt (green)). (B) Expression heatmap for the 20 top differentially expressed genes (DEGs)
450 between Ymt and Omt fish (n = 4 for each group). The top 20 genes are highly expressed in Ymt fish, the bottom
451 20 are highly expressed in Omt fish. *HAMP is the best protein blast hit in *Danio rerio* of the TK gene
452 NFURG05812010005. (C) Gene Ontology (GO) analysis of the DEGs between Ymt and Omt fish. Enrichment
453 values (bars) and the negative natural logarithm of p values (black dots) are shown. (D) Relative gene expression
454 levels compared to six-week-old fish (6wk) of genes significantly differentially expressed between 16wk (gray),
455 Omt (red) and 6wk (black), but not between Ymt (green) and 6wk. These genes are also significantly
456 differentially expressed between 16wk and Ymt. (E) Based on the correlation matrix between OTU abundance
457 and transcript levels in individual fish (Data S1), we identified the gene transcripts whose expression correlates
458 more significantly with the hub bacteria shared between Ymt and 6wk fish (full analysis in Figure 7 – figure
459 supplements 2). Displayed are the top hits (chosen from an r-square value larger than 0.65 on both axes) shared
460 between *Psychrobacter* and *Exiguobacterium*, which have the highest OTU-transcript correlation trend based on
461 Figure 7 – figure supplements 2. RPLs include RPL7A, RPL8, RPL12, RPL37, RPL14, MRPL35; while RPS
462 include RPS3A, RPS7, RPS11, RPS24.

463

464 proteins (RPS and RPL genes) (Figure 7 – figure supplements 2), whose expression in other
465 species has been strongly associated with aging and longevity (Steffen et al., 2008). Thus,
466 while transfer of young GM to middle age fish maintained a young-like GM community
467 throughout old age, the gene-expression signature associated with host gut at 16 weeks of age
468 did not indicate overall rejuvenation. However, enhanced defense response against bacteria in
469 Ymt compared to Omt is compatible with an increased capacity to resist to the attack of
470 pathogenic gut bacteria, which could provide the basis for longer life span. Remarkably,
471 hyaluronic acid metabolism, altered between the experimental groups receiving young and old
472 gut contents, has been associated with increased inflammation, deregulated immune response
473 and risk for cancer (Cho et al., 2017; Tian et al., 2013), all of which could provide the basis for
474 life span modulation. Finally, we provide a dataset that allows to associating OTU levels in
475 individual fish to intestinal transcripts, enabling to investigate on how GM and host gene
476 expression are mutually regulated.
477

478 **DISCUSSION**

479

480 Key aims of research on aging are to understand the mechanisms behind the phenotypic
481 changes that occur during aging and to identify novel life span enhancing interventions. Using
482 the TK as a naturally short-lived vertebrate model system, we report the characterization of the
483 changes in GM composition occurring during aging and the discovery of a novel life span
484 enhancing intervention achieved by acutely transferring young GM to middle-age individuals
485 after antibiotic treatment. This intervention resulted in the maintenance of an overall healthier
486 physiological status, a highly diverse and young-like gut microbial community at late age and
487 in an enhanced transcriptional signature of defense responses to bacteria.

488

489 Our results show that TK are characterized by a complex GM community, more species-rich
490 than worms and flies and of the same order of magnitude of mammals, both in abundance and
491 composition. Indeed, the four most abundant bacterial phyla observed in the TK are also the
492 four most abundant phyla found in humans and mice. However, unlike mammals,
493 Proteobacteria is the most abundant gut bacterial phylum found in killifish, similarly to other
494 aquatic species, such as zebrafish (Roeselers et al., 2011). Since it is the shortest-lived
495 vertebrate to date reproduced in captivity, the TK can become an ideal model to dissect the
496 links between GM diversity and host aging.

497

498 Intriguingly, we show that while the gut bacterial diversity of captive-raised TK is lower than
499 that of wild-caught populations, still captive fish recapitulate the core microbial diversity of
500 wild TK. Laboratory-raised fish are not represented by distinct gut bacterial communities, but
501 are rather dominated by a few high-abundance bacterial taxa that are already present in wild
502 killifish populations. This supports the conclusion that fish raised in captivity have a GM

503 community that is representative for the species in nature. Additionally, we found that wild
504 populations of TK show large between-population gut bacterial diversity, with the emergence
505 of population-specific high-abundance taxa. This is plausibly caused by locality-specific
506 differences in ecological conditions, including climate, soil composition, parasites and
507 diversity of food sources (Nezhybová et al., 2017; Reichard et al., 2017).
508
509 Analyzing the changes in GM composition between young and old fish, we found that young
510 fish are characterized by a large taxonomic bacterial diversity. Old fish are less OTU-rich,
511 similar to what is observed in human cohorts from different age classes (Claesson et al., 2012).
512 Intriguingly, while individual bacterial diversity (alpha diversity) is higher in young
513 individuals, old fish are more dissimilar from one another, i.e. while each individual old fish
514 have a more homogeneous GM composition than young fish, any two old individuals have
515 more divergent bacterial communities compared to young individuals. This result raises the
516 possibility that the changes in composition and relative abundance in GM communities from
517 young to old individuals could be a function of i) initial individual GM composition, ii)
518 differences in individual immune system composition and function or iii) a combination of
519 initial individual GM composition and host immune function.
520 Aging in this experimental model was associated not only with reduced bacterial species
521 richness, but more specifically with loss of bacterial taxonomic units involved in carbohydrate,
522 nucleotide and amino acid metabolism, which in mice are associated with aging (Langille et
523 al., 2014). These same changes have been associated in humans with unhealthy aging
524 (Claesson et al., 2012; Rampelli et al., 2013), as well as with chronic conditions such as
525 obesity, type II diabetes and insulin resistance (Neis et al., 2015). The shift in microbial
526 composition between young and old fish was consistently characterized by a higher prevalence
527 of Proteobacteria in old individuals, while young individuals were significantly more enriched

528 in Firmicutes, Actinobacteria and Bacteroidetes. Additionally, functional metagenome
529 analysis showed that young fish had GM associated with carbohydrate metabolism, replication
530 and repair, as well as DNA repair, indicating the young GM's ability to protect itself against
531 assault and thus maintain homeostasis. On the other hand, old GM resulted more enriched in
532 pathogenic bacteria, associated with dysbiosis. Bacterial pathogenicity in the gut is associated
533 with the accumulation of mutations over time, induced by the failed capacity to repair them
534 (Leimbach et al., 2013). Bacterial communities present in old fish guts were indeed more
535 pathogenic and our functional metagenome analysis associated them with host disease.
536 Consistently, while young gut status was associated with high expression of genes involved in
537 cell cycle activity, likely associated with proliferation and differentiation, old gut status was
538 associated with host immune responses to pathogenic bacteria, reflecting the prevalence of
539 more pathogenic bacterial taxa.
540
541 Although GM transfers from young, healthy donors have found applications in the clinic to
542 treat acute gut infections such as those associated with *Clostridium difficile* (Lee et al., 2016)
543 and have been proposed to treat obesity, metabolic syndrome and even neurodegenerative
544 diseases (Marotz and Zarrinpar, 2016; Xu et al., 2015), the application of this methodology as
545 an anti-aging intervention has not been explored to date. Remarkably, despite single
546 associations of different bacterial diets have shown to significantly affect life span in
547 invertebrate model systems such as *Caenorhabditis elegans* (Zhao et al., 2013), a functional
548 test of the role of a complex GM community associated with young age as an intervention
549 aimed at modulating the recipient's life span has not yet been carried out to date. By acutely
550 exposing middle age individuals to young fish GM content – after antibiotic treatment – we
551 could prolong life span and retard the age-dependent decline in exploratory behavior.
552 Noteworthy, our results exclude that the effects of the interventions depend on repopulating

553 the intestine with any GM community or that antibiotic treatment alone was sufficient to
554 explain the full extent of life span increase achieved via transfer of young GM.
555 Additionally, life span was not affected in young fish exposed to GM from old, young, and
556 sham control fish after antibiotic treatment. These results are compatible with a scenario where
557 the age-associated decline of immune function might be responsible for the progressively
558 decreased capacity of the host to i) maintain the healthy portion of the GM community and ii)
559 counteract the proliferation of potentially pathogenic gut bacteria.
560 Fish treated with young GM after depletion of their own resident GM community not only
561 maintained a more diverse microbial community at old age compared to wild-type, age
562 matched control fish, but their microbial community remained more similar to that of young
563 fish. This raises the possibility that bacterial consortia associated with young fish can
564 contribute to increased life span and enhanced individual health status. Based on functional
565 metagenomic analysis, young fish and fish treated with young GM were enriched for bacteria
566 associated with carbohydrate metabolism and DNA repair, both importantly associated with
567 host metabolism, health and longevity.
568 Young fish, as well as fish treated with young GM, had a high number of bacterial taxa that
569 frequently co-occurred with one another, de facto contributing to a young-associated bacterial
570 network. On the other end, old wild-type controls (16wk), as well as old fish treated with same
571 age GM (Omt), had smaller bacterial networks, possibly associated with the higher inter-
572 individual variation in GM composition associated with these groups. Remarkably, applying
573 our analyses to a published mouse cohort (Langille et al., 2014), we extended this finding to
574 mammals, confirming that networks built on GM OTU abundance are associated with host's
575 chronological age. Our network analysis enabled us to identify a subset of highly frequent taxa
576 associated with a young-like status and with prolonged life span in fish treated with young
577 GM. These involved the genera *Exiguobacterium*, *Planococcus*, *Propionigenium* and

578 *Psychrobacter*, which are key bacterial genera responsible for structuring a healthy GM
579 community in TK. Interestingly, species belonging to each of these genera have been
580 associated with energy metabolism and potential health benefits. Specifically, species of
581 *Exiguobacterium* and *Propionigenium* are able to metabolize cellulose and ferment
582 carbohydrates to produce short chain fatty acids, which are known anti-inflammatory
583 mediators and can modulate the immune system. *Planococcus* species can hydrolyze gelatin to
584 produce essential amino acids for use by the host and certain *Psychrobacter* species are
585 capable of producing omega-fatty acids. Taken together, these key bacterial genera can
586 produce metabolites capable of maintaining immune system health and having anti-
587 inflammatory effects on the host, both of which have been associated with longevity.
588 While GM transfers significantly affected the GM composition of experimental fish, their
589 overall gut transcriptional profile showed that old fish clustered together regardless of the
590 treatment. This could be due to the lack of transcriptional programs associated with growth in
591 16-week-old fish. However, transcripts involved in defense against pathogens, extracellular
592 matrix components and the Tor pathway, are dramatically different among experimental
593 groups receiving young or same-age gut content, suggesting that these key aspects might
594 ultimately be fundamental modulators of organismal life span and health. Generating a
595 correlation matrix between bacterial abundance and gut transcripts, we could isolate host
596 transcripts whose expression was significantly correlated with specific OTUs. In particular,
597 bacterial genera associated with a healthier and longer life span, such as *Psychrobacter* and
598 *Exiguobacterium*, were strongly associated with genes importantly associated with aging
599 modulation.
600 Our results indicate that improving the ecological diversity of the GM in old individuals helps
601 to restore health and prolongs life span. Our approach could provide a key to slowing aging
602 and retarding the onset of age-associated diseases by specifically targeting the GM. Given its

603 large bacterial taxonomic diversity and the shortest life span for a vertebrate species raised in
604 captivity, the TK could become a key experimental species which will help to shed light on
605 the functional connection between GM dynamics and aging in vertebrates.

606 **MATERIALS AND METHODS**

607

608 **Wild fish samples**

609 The wild fish samples derived from Zimbabwe were collected during the expedition in
610 Gonarezhou National Park in 2015 (Permit No.: 23(1) (C) (II) 30/2015). Intestines were
611 collected at each location and preserved in pure ethanol. Sampling locations coordinates are
612 listed in Supplementary file 2.

613 **Fish husbandry and survival scoring**

614 Fish used for microbiota analysis and scored for survival were individually housed from week
615 4 post-hatching in single 2.8L tanks connected to a water recirculation system receiving 12h of
616 light and 12hr of dark every day. Water temperature was set to 28˚C and fish were fed blood
617 worm larvae and brine shrimp nauplii twice a day during the week and once a day during the
618 weekend. Dead fish were removed daily from the tanks, weighed and stored in 95% ethanol.

619

620 **DNA extraction, 16S rRNA gene amplification and sequencing**

621 All dissected intestinal samples were collected at the same time prior to morning feeding and
622 flash frozen in liquid nitrogen. For DNA isolation, frozen intestines were placed into
623 autoclaved 2ml screw caps tubes containing 1ml of lysis buffer (80mM EDTA, 200mM Tris
624 (pH 8.0) and 0.1M NaCl in PBS) and 0.4g of a mixture of 01.mm zirconia/silica and 1.4mm
625 stainless steel beads (Biospec Products). Samples were bead-beaten for 3 minutes at 30 Hz
626 (TissueLyzer II, Qiagen). Following the bead beating step, SDS (10% final concentration) and
627 RNase A (PureLink, Invitrogen) were added and samples were incubated for 30 minutes at
628 55°C. DNA was then isolated using phenol:chloroform:isoamyl alcohol (Invitrogen) as per
629 manufacturer's instructions with an additional chloroform step to remove excess phenol.

630 DNA was then used in one of two, two-step PCR methods designed to target the V3-V4 region
631 of the 16 rRNA gene (Klindworth et al., 2013). For the first method, initial primers consisted
632 of (5' to 3') a primer pad and linker (Caporaso et al., 2012) and the V3/V4 gene specific
633 forward or reverse primer sequences. The second step PCR used primers complementary for
634 the primer pad and linker followed by standard Illumina adaptors. The reverse primer for the
635 second step also contained a 12bp Golay barcode (Caporaso et al., 2012). For the second
636 method, initial primers consisted of (5' to 3') Illumina overhang adaptor sequences and the
637 V3/V4 gene specific primer sequences followed by a second step PCR using the Nextera XT
638 Index kit (Illumina). For both amplification methods cycling conditions were the same and the
639 first round of PCR was performed in triplicate with approximately equal amounts of DNA
640 template (250ng/reaction). PCR reactions were carried out with a two-minute denaturation
641 step at 98°C, followed by 25 cycles of 98°C for 30 seconds, 55°C for 30 seconds and 72°C for
642 30 seconds. Triplicate reactions were then pooled and cleaned using the Wizard SV Gel and
643 PCR Clean-up Kit (promega). The cycling conditions for the second step PCR were the same
644 as the first step, except annealing was performed at 60°C with only 8 cycles. Both PCR steps
645 used KAPA HiFi Hotstart ReadyMix (KAPA Biosystems) and 1µm of primers in 25µl total
646 volume. 2nd step PCR products were run on a 1.2% agarose gel and DNA products between
647 500-700 base pairs were excised and cleaned up as in step 1. PCR products were quantified by
648 Qubit (Life Technologies), diluted to 4nm and combined in equal volumes. The combined
649 amplicon libraries were then sequenced on the Illumina MiSeq, V3 reagents, 2x 300bp paired-
650 end reads.
651
652 **Antibiotic treatment and fish microbiota transfer**
653 Microbiota transfer experiments were based on those developed in zebrafish (Pham et al.,
654 2008). Recipient fish were removed from main water recirculating system and housed in 9L

655 tanks at a density of 10 fish per tank. Recipient fish (6wk or 9.5wk) were treated overnight
656 with a combination of Vancomycin (0.01g/L), Metronidazole (0.5g/L), Neomycin (0.5g/L)
657 and Ampicillin (0.5g/L) to diminish the resident bacterial community. Following antibiotic
658 treatment, recipient fish were washed twice for 10 minutes with autoclaved tank water.
659 Concurrently, whole intestines were isolated from donor fish and placed into 10 cm petri
660 dishes containing sterile PBS on ice. Intestines were then opened longitudinally, the intestinal
661 contents scraped out and then further cut into 0.5 cm pieces to facilitate the release of bacteria.
662 The collected intestinal contents were washed once in cold PBS and added to the fish tanks
663 containing autoclaved tank water and recipient fish at a ratio of 1 donor fish intestine/2
664 recipient fish. Fish were incubated overnight with the donor fish intestinal contents before
665 being returned to the main recirculating system and individually housed, where they were
666 regularly fed according to standard husbandry.
667
668 **Microbial community analysis**
669 Fastq files from paired end reads were joined, demultiplexed and subjected to quality filtering
670 with QIIME 1.8 (Q≥20) as previously described (Caporaso et al., 2012). For microbial
671 community analysis, QIIME was used to identify OTUs by open-reference picking using
672 UCLUST (97% similarity) and taxonomy was assigned with the Greengenes 13.8 database
673 (DeSantis et al., 2006; Edgar, 2010). A minimum OTU count of 5 was used to minimize
674 spurious OTUs. Representative OTU sequences were aligned with PYNAST (Caporaso et al.,
675 2010a) and FastTree 2 (Price et al., 2010) was used to build a phylogenetic tree. For diversity
676 analyses, OTU tables were rarefied to at least 5000 sequences, which allowed the majority of
677 samples to be kept. Qiime and R were used to calculate alpha and beta diversity metrics and
678 generate plots. To identify bacteria associated with specific groups, OTU tables were further
679 filtered for presence in at least 25% of samples with a collective abundance of greater than 100

680 reads. Significant changes in relative OTU abundance were identified with linear discriminant
681 analysis effect size (LEfSe) and visualized using GraPhlAn (Asnicar et al., 2015). For
682 metagenomics analysis, rarefied OTU tables were generated by closed reference picking and
683 the PICRUSt tool was used to normalize by 16S copy number and predict the metagenome
684 content of samples from 16S rRNA profiles. KEGG pathway functions were then categorized
685 at level 2 or 3 and LEfSe was used to identify significant changes among classes. The
686 accuracy of PICRUSt predictions was determined by nearest sequenced taxon index (NSTI).
687

688 **Bacterial diversity among model organisms**

689 To compare the overall bacterial diversity among different species, sequences were
690 downloaded from the European nucleotide archive (ENA), the NCBI Short Read Archive
691 (SRA) or MG-RAST and subjected to closed reference OTU picking. Taxa summary and
692 alpha diversity measures were computed using QIIME 1.8. Samples were chosen which used
693 Illumina sequencing of the V4 region of the 16 rRNA gene. Accession numbers. Human:
694 ERR561021UK, ERR560915UK, ERR560902UK, ERR560855UK, mgm4489670,
695 mgm4489628, mgm4489516, mgm4489454, mgm4538473, mgm4538468, mgm4538264,
696 mgm4538259, mgm4538238. Mouse: SRR1820108, SRR1820074, SRR1820073,
697 SRR1820072, SRR1820071, ERR706143, ERR706142, ERR706141. Zebrafish:
698 SRR1581750, SRR1581753, SRR1581759, SRR1581763, SRR1581766, SRR1581889,
699 SRR1581890, SRR1581891. Drosophila: SRR989472, SRR989473, SRR989474,
700 SRR989469, SRR989467, SRR952981.
701

702 **Bacterial load quantification**

703 To determine bacterial load, DNA was isolated from freshly collected faecal pellets as
704 described above. Real-time quantitative PCR was performed with DyNAmo Color Flash

705 SYBR green master mix (Thermo Scientific) and run with the BioRad CFX384 Real-Time
706 System. Samples were normalized to amount of input faecal material and graphed as 1/Ct
707 value. Primers targeting the 16s gene were described previously (Caporaso et al., 2010b). For:
708 5' TCCTACGGGAGGCAGCAGT 3' and Rev: 5' GGACTACCAGGGTATCTAATCCTGTT
709 3'.
710

711 **Quantitative analysis of spontaneous locomotor activity**

712 To measure total swimming distance, fish were placed in rectangular (16 x 90 cm) tanks filled
713 with 8 liters of water and allowed to acclimate for 30 minutes. The room and water
714 temperature and water quality were kept as similar to the fish's' normal environment as
715 possible. The fish were then filmed for 20 minutes with an overhead mounted camera and data
716 were analyzed using EthoVision XT11 (Noldus Information Technologies).
717

718 **Co-occurrence networks**

719 The OTU table was filtered by a minimum total count of 5509 per individual, based on the
720 rarefaction value (see Microbial community analysis). Subsequently, the table was divided
721 into subsets corresponding to the treatment groups. Normalization was achieved through
722 dividing the OTU sample count by the total sample count, scaled to 1000.
723 Co-occurrence networks were produced by applying the SparCC program (Friedman and Alm,
724 2012), a network inference tool specifically developed for analysis of correlations in
725 compositional data, such as 16S microbiome analyses. In order to avoid unreliable correlations
726 for very rare OTUs, the OTU tables were filtered for bacteria that were present in at least 25%
727 of samples of each group.
728 The SparCC pipeline was used to first calculate OTU-OTU correlations averaged over 20
729 iterations (Friedman and Alm, 2012). We then tested the significance of these correlations by

730 computing pseudo-p-values against 1000 bootstrap simulations, applying the same parameters.
731 All OTU-OTU correlations with a p-value lower than 0.05 were considered significant and
732 were included into network preparation. Nodes without any edges after filtering by significant
733 p-values were removed from the network. To analyze the network properties we used the
734 igraph package in R, version 1.0.1 (Csardi and Nepusz, 2006). For each group we generated an
735 undirected network, weighted by correlation magnitude. For biological interpretation we only
736 focused on positive correlations. To identify network size and composition, we calculated the
737 negative cumulative degree distribution and the percentage of genus present in the network
738 belonging to different phyla. The main clusters of each network were identified with two
739 different clustering algorithms of the igraph package (Csardi and Nepusz, 2006). The first
740 algorithm 'k-core' clusters based on the degree, with each member of the maximal subgraph
741 has at least a degree count of k. The second algorithm 'infomap community' searches for
742 community structures that minimize in the length of a random walker trajectory. We used the
743 overlap of the largest clusters within the two cluster algorithms to identify the main clusters in
744 each group. The members of clusters from young fish (wk6) and old fish (wk16) were used to
745 identify young-like or old-like clusters in the main clusters of the transfer groups (Ymt, Omt
746 and Abx).
747 In order to compare our findings to a different model organism, we used the data set from
748 Langille et al. (2014) (Langille et al., 2014). We scaled the relative frequencies from young,
749 middle and old mice to 1000 and excluded bacteria not present in at least 25% of samples.
750 Additionally, we excluded sample Y7.August14, because we included the same sample from
751 15th of August (Y7.August15). Identically to the previous analysis we calculated the negative
752 cumulative degree distribution.
753
754

755 **RNA extraction**

756 Intestines from four young (6 weeks old), four old (16 weeks old), four old received six-week-
757 old donor fish gut content (Ymt) and four old fish received 9.5-week-old fish gut content
758 (Omt) fish were dissected. Trizol (15596018, Thermo Fisher Scientific, USA) was used to
759 isolate total RNA, following manufacturer's instructions. Residual genomic DNA was
760 removed by DNaseI treatment (AM2222, Thermo Fisher Scientific, USA) and 1.5 µg of total
761 RNA was used as a template for reverse transcription (11754050, Thermo Fisher Scientific,
762 USA).

763

764 **RNA sequencing**

765 We sequenced four individuals of groups 6wk, 16wk, Ymt and Omt. Each sample sequenced
766 with 100bp paired end with 30 million reads. All samples were mapped to the African
767 Turquoise Killifish Genome (Valenzano et al., 2015) using STAR version 2.4.1c (Dobin et al.,
768 2013) with the default parameters, except for splice junction and chimeric junction parameters
769 set to 15 base-pairs, as well as allowing for only 5 mismatches over the whole fragment and
770 removing all reads that map to more than two locations.

771

772 **Differential gene expression**

773 The differential gene expression was performed using the Cufflinks suite (Trapnell et al.,
774 2010) version 2.2.1 and the edgeR package (Robinson et al., 2010) version 3.16.5. cuffquant
775 was used to generate feature abundances using the mapped reads and the annotation file from
776 (Valenzano et al., 2015) for each file independently. Subsequently cuffnorm was used to
777 obtain normalized feature counts of all genes of samples. The edgeR package was used to
778 identify differentially expressed genes between any of the groups resulting in six pairwise
779 comparisons. Additionally, we calculated the differentially expressed genes between the old

780 microbiome group (Omt and 16wk) and young microbiome group (Ymt and 6wk), as well for
781 the comparison of normal-lived group (Omt and 16wk) versus long-lived group (Ymt). For
782 these comparisons we excluded genes that were differentially expressed within pairwise
783 comparisons of each combined group. All edgeR runs were performed using TMM
784 normalization, followed by tagwise dispersion and an exact test (Robinson et al., 2010). We
785 considered only genes that had a counts-per-million value above 0.7 in at least 50% of
786 compared samples. The resulting p-values were multiple testing corrected with the Benjamini-
787 Hochberg method. Only genes with a FDR smaller or equal than 0.1 were considered as
788 differentially expressed. Gene enrichment analyses were performed using the GOrilla software
789 (Eden et al., 2009).
790
791 **Functional analysis of target genes**
792 To gain insights into the function of genes differing between young and old fish as well as
793 genes differing between fish of long or normal lifespan we used the GOrilla (Eden et al., 2009)
794 to perform enrichment tests. We used the zebrafish database for annotation. Therefore we used
795 the available reciprocal blast information (Valenzano et al., 2015). If top differentially
796 expressed genes were not found to have a zebrafish ortholog in this resource, we used blastp
797 (Altschul et al., 1990) to identify the corresponding zebrafish ortholog (Table S7). First all
798 genes were mapped to a GO-Term. The statistical enrichment test uses a hypergeometric
799 distribution to test for enrichment in the ratios of target genes associated with a GO-Term, and
800 the ratio of background genes associated with the same GO-Term to identify pathways or GO-
801 Terms that have a significantly larger amount of target genes than expected. Here, we used all
802 genes that were found to be expressed in the intestine of the experimental groups as
803 background for all analysis. The number of genes differentially expressed between long and
804 normal-lifespan fish was too low to reach significance for any GO-Term after adjustment for

multiple hypothesis testing (Bonferroni correction). The GOrilla results were visualized with the ggplot2 package in R.

Correlation between individual OTU and gut transcripts

Individual fish OTU tables and log2-transformed transcripts were used as a source to identify significant correlations using Pearson's correlation.

The python code used to perform this analysis was saved on github:

https://github.com/dvalenzano/uBiome_Network/blob/master/Notes_20-04-2016.ipynb

AUTHOR CONTRIBUTIONS

P.S. and D.R.V. conceived the study and planned all the experiments. P.S. performed all the experiments. P.S., D.W., M.L.P. and D.R.V. performed the statistical analyses. D.W. and F.M. analyzed the RNA-Seq data, D.W. performed the network analysis. M.L.P analyzed the wild fish GM data and helped with the network analysis. E.G. provided intellectual expertise and logistic support for the fielwork. D.R.V. and D.W. conducted the fieldwork. M.R. contributed with the wild specimens collection. D.R.V. designed, coordinated the study and wrote the manuscript.

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COMPETING INTEREST

There are no financial and non-financial competing interests for all the authors.

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