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.

INTRODUCTION

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

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

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

difficile and *Enterococcus faecalis*, can restructure the human GM and cause severe chronic conditions that pose a major threat for public health (Backhed et al., 2012; Cox and Blaser, 2015). Studies across different human age cohorts have shown that large changes in the abundance of subdominant bacterial taxa in the gut are a hallmark of aging; moreover, exceptionally long-lived individuals – including supercentenarians – are characterized by the persistence of bacterial taxa associated with a more healthy status (Biagi et al., 2016). While diversity-associated microbial taxa often decline during age, specific bacterial taxa, such as Clostridiales, are associated with malnutrition and increased frailty (O'Toole and Jeffery, 2015). In flies, reducing GM dysbiosis by improving immune homeostasis promotes longer life span (Guo et al., 2014; Li et al., 2016). Manipulating the GM towards a healthy state has the therapeutic potential to improve health in specific diseases (Dodin and Katz, 2014; Kunde et al., 2013). However, due to the lack of suitable short-lived vertebrate experimental models, it is not known whether age-associated gut microbial community changes causally affect the aging process and whether resetting a young GM in middle-age individuals can improve long-term health and affect individual life span in normal aging individuals.

In this study, we develop the turquoise killifish (*Nothobranchius furzeri*), a naturally short-lived vertebrate species with a life span of a few months in captivity (Valenzano et al., 2015), as a new model organism to study aging in the host gut and microbiota. We show that turquoise killifish (TK) have a complex GM (both in the wild and in captivity), similar in taxonomic diversity to that of mammals. We also show that during aging the overall microbial diversity in the TK gut decreases, with increased over-representation of pathogenic Proteobacteria. By acutely recolonizing middle age individuals with GM from young donors, we developed an intervention that enabled fish to live significantly longer, remain more active at old age, and maintain a highly diverse GM. Transcriptome analysis additionally revealed 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

RESULTS

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

TK is a naturally short-lived vertebrate, whose genome sequence has become available (Reichwald et al., 2015; Valenzano et al., 2015) and that is amenable to genetic manipulations via transgenesis or genome editing (Harel et al., 2015; Valenzano et al., 2011). Remarkably, this species is characterized by a broad spectrum of aging phenotypes, including cancer, neurodegeneration, and behavioral decline (Harel and Brunet, 2015; Kim et al., 2016). TK are adapted to living in conditions of intermittent availability of water and to surviving during brief rainy seasons and long dry seasons (Cellerino et al., 2015). In captivity, it lives between four to eight months, depending on the strain (Valenzano et al., 2015). However, nothing is known about its associated commensal microbes and whether its gut microbial complexity and taxonomic richness matches that of short-lived invertebrate model organisms, such as worms and flies, or that of longer-lived vertebrate model organisms, such as mice and zebrafish. To determine the TK's gut microbial composition, we sequenced the hyper-variable V3/V4 regions of the 16S rRNA gene amplicon from intestines of captive TK (N = 11, Materials and Methods). We found that TK are characterized by a gut microbial taxonomic diversity similar to other, longer-lived vertebrates, including zebrafish, mice and humans (Kostic et al., 2013; Qin et al., 2010; Zac Stephens et al., 2016) (Figure 1A). This bacterial taxonomic diversity is an order of magnitude higher than the gut microbial diversity present in invertebrate model organisms such as worms (Cabreiro and Gems, 2013) and flies (Buchon et al., 2013) in the laboratory (Figure 1A). Remarkably, the four most abundant bacterial phyla present in the TK's gut, i.e. Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, are also the four most abundant human gut bacterial divisions (Zoetendal et al., 2006) (Figure 1B and Table S1), although in different proportions (Figure 1A). The unique combination of a complex gut

FIGURE 1

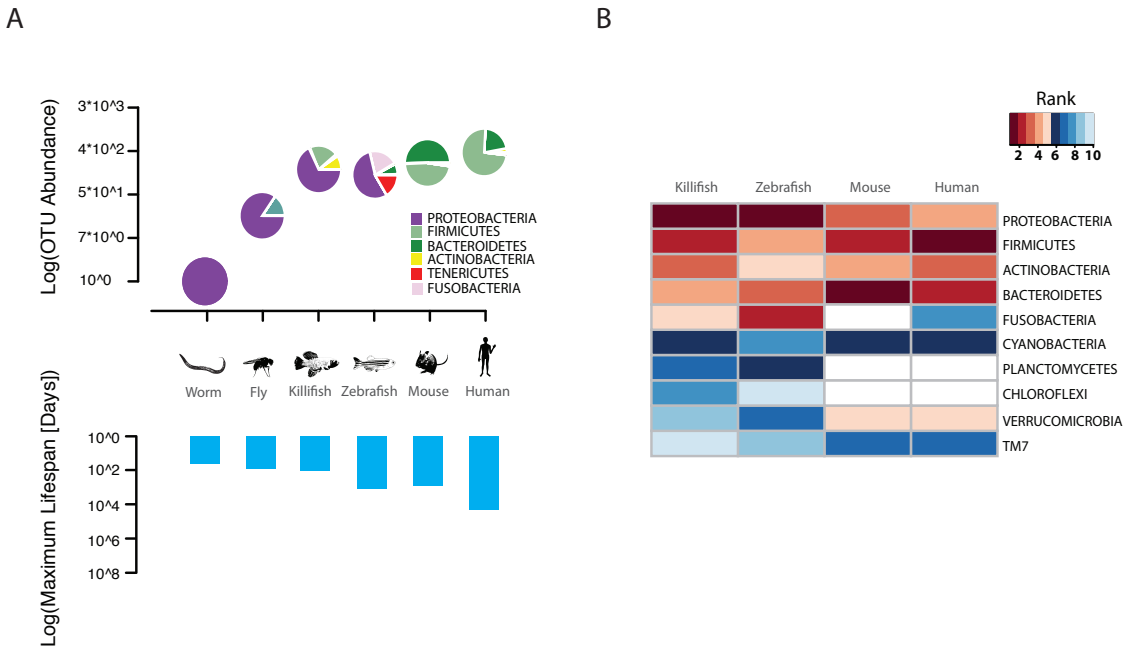
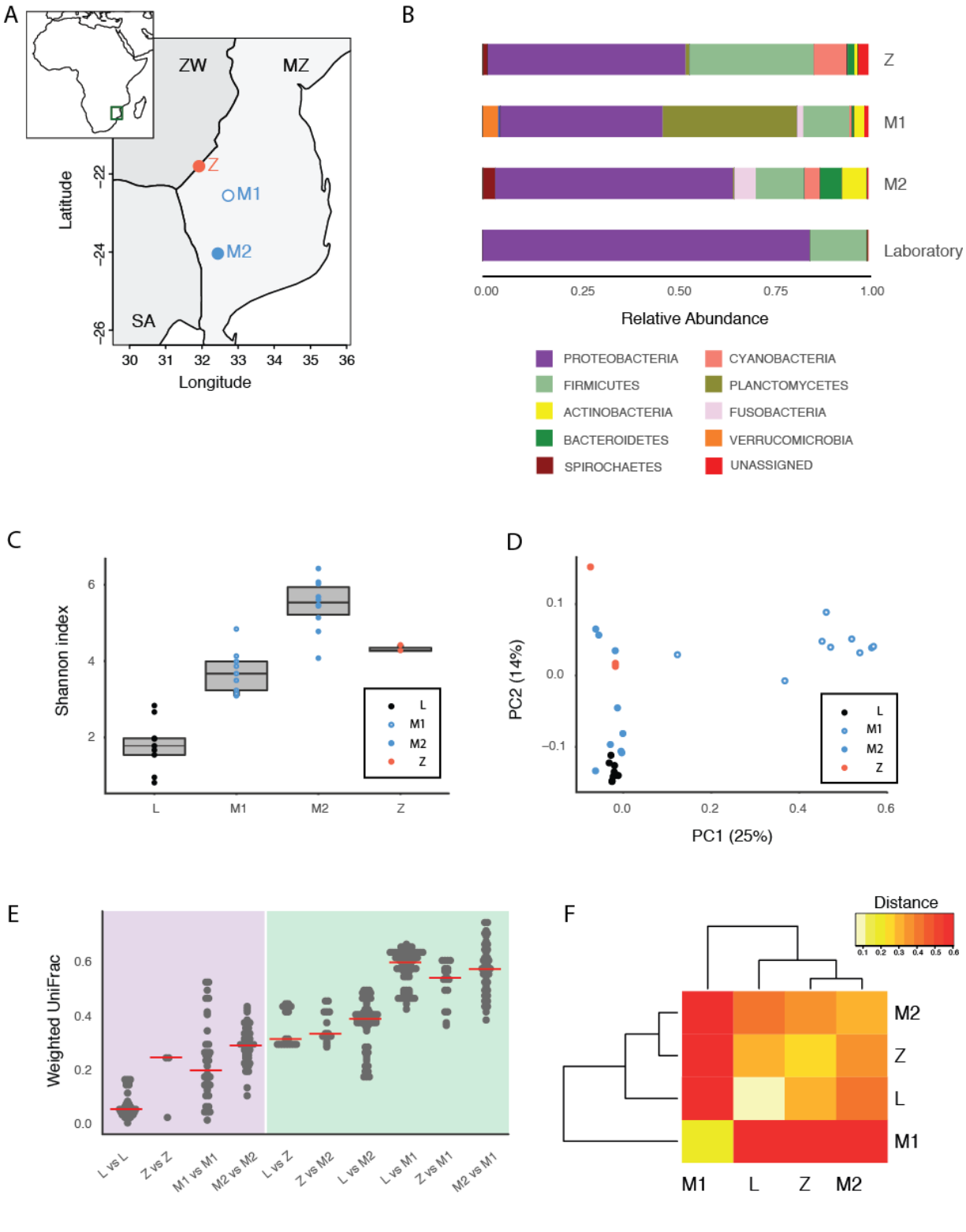


Figure 1. TK as a model to study aging of the GM

(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 (Table S1). 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.

118
119 microbial composition, similar to that of other vertebrate aging model organisms, and its
120 naturally short life span, combined with a wide spectrum of aging phenotypes, makes the
121 turquoise killifish an ideal system to study the role of the gut microbiota during vertebrate
122 aging.
123
124 **Wild and captive TK populations share a core GM**
125 To assess whether the GM of captive TK was representative of the microbial communities
126 associated with wild populations, we sequenced 16S rRNA gene amplicons from individual
127 fish that we collected from different localities in the natural habitat of this species, ranging
128 from the Gonarezhou National Park in Zimbabwe to the Gaza region in Mozambique (Figure
129 2A, Figure S1B, Table S2A, Materials and Methods). Sequencing these wild populations
130 confirmed that Proteobacteria was the dominant phylum also in most wild populations, similar
131 to laboratory fish (Figure 2B, Figure S1C). Individuals from all wild populations had a more
132 diverse GM than laboratory fish (Figure 2C, Shannon alpha diversity, Dunn Kruskal-Wallis
133 test, BH-adjusted p values < 0.05). While standard frequency-based diversity measures of
134 observed bacterial taxonomic units (OTUs) (Simpson's and phylogenetic alpha diversity
135 across the whole tree, OTU abundance, Figure S1A) were higher in wild populations, Chao1
136 alpha diversity, which gives more weigh to rare bacterial OTUs, was higher in laboratory fish
137 (Figure S1A). These results possibly reflect the fact that laboratory fish are dominated by few,
138 high-abundance OTUs, hence resulting in having more "rare" bacterial taxonomic units
139 compared to wild populations. Differences in OTU abundance between laboratory-raised and
140 wild fish might reflect ecological differences between the standardized laboratory conditions
141 and the more heterogeneous wild environment, characterized by fluctuations in temperature,
142 nutrients as well as other biotic and abiotic factors (Blažek et al., 2017). To test whether
143 differences in ecology across distinct wild fish populations influenced bacterial diversity

FIGURE 2



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

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

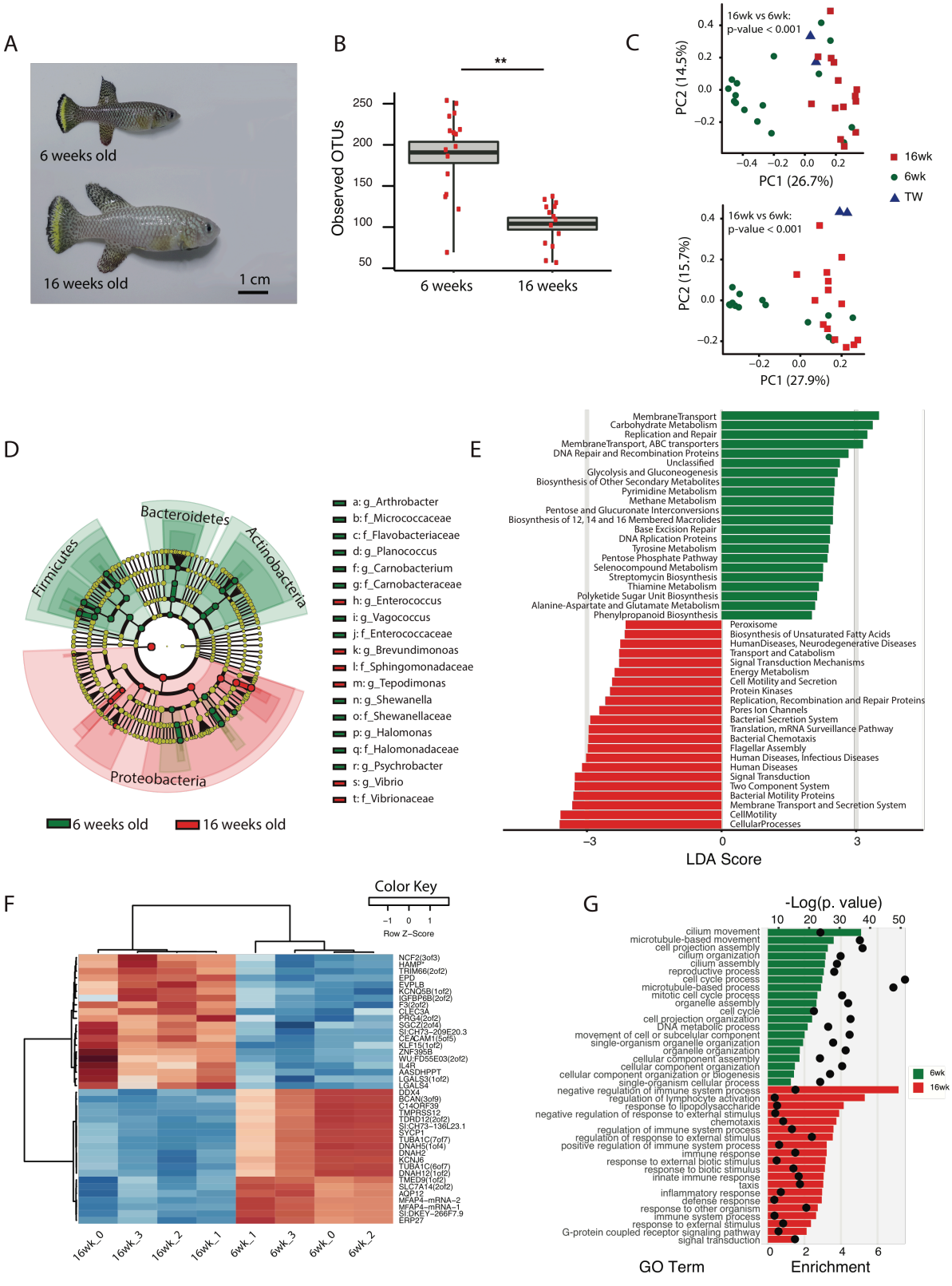
157

158 levels, we ran a regression model between diversity indexes and different recorded ecological
159 factors, including altitude, pond size, maximum pond depth, vegetation, water conductivity,
160 water temperature and water turbidity (Table S2B). None of the recorded factors was
161 significantly correlated to alpha diversity levels (data not shown). This suggests that other
162 parameters, such as food availability, fish genetics, presence of parasites or additional biotic or
163 abiotic factors might be the key determinants of fish GM composition in the wild.
164 Although microbiota diversity in wild populations was higher than in laboratory-raised fish,
165 the microbial composition of laboratory fish was not separated from the species-specific
166 bacterial composition in wild fish, and was contained within the natural microbial variation of
167 the species (Figure 2D). Importantly, one wild population (M1) had a divergent composition
168 from all other populations, possibly due to over-representation of Planctomycetes (Figure 2B
169 and D, Figure S1C and E). Between-individuals diversity measures (beta diversity) showed
170 that, while laboratory fish's bacterial diversity was lower than in the wild populations,
171 between-group bacterial diversity was higher between population M1 and all the other
172 populations, including laboratory fish (Figure 2E-F). These results indicate that population M1
173 had a more divergent microbial diversity of all the tested populations, and that the laboratory
174 fish were not an outlier group (Figure 2D). Based on this, laboratory fish share a core
175 microbiota with wild populations, despite the differences in culturing conditions between the
176 laboratory and the wild, similar to what is seen in zebrafish (Roeselers et al., 2011). Thus,
177 while wild turquoise killifish populations differ from one another in terms of microbial
178 composition – possibly in association with ecological differences among different localities –
179 laboratory fish recapitulate the core gut microbiota of wild populations, making them ideally
180 suited to study how a complex microbiota influences host physiology.
181

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

An important question is what changes in the gut microbial population occur during aging in vertebrates. To gain insight into the changes in GM composition occurring throughout aging in the TK, we performed 16S rRNA gene amplicon surveys in the gastrointestinal tract of six-week-old young-adult and 16-week-old individuals raised in captivity (Figure 3A, n = 16 young and 14 old fish). We found that bacterial taxonomic diversity (alpha diversity) significantly decreased between 6-week-old and 16-week-old fish, while bacterial abundance was not changed (Figure 3B, Figure S2A and B). This indicates that, similar to humans (Claesson et al., 2012), aging in the TK is characterized by a significant reduction in gut bacterial richness. Bacterial composition was also significantly altered between young and old fish guts (Figure 3C), including an age-dependent decrease in Firmicutes (Kruskal-Wallis test, p value = 0.002) and Actinobacteria (Kruskal-Wallis test, p value = 0.013). Young and old fish GM had significantly different community structures (Figure 3C, UniFrac p value < 0.001; Bray-Curtis p value < 0.001, Adonis test, Figure S2C). While individual fish GM diversity was higher in young than in old fish (Figure 3B), differences among old fish GM were more pronounced compared to young fish (Unweighted UniFrac beta diversity, Bonferroni-corrected p value = 6.7E-06; Bray-Curtis distance p value = 0.003; Figure S2D and E). This indicates that although aging in the TK was associated with decreased bacterial taxonomic diversity within each fish's gut, distinct old fish guts had highly divergent bacterial communities. While young fish guts were significantly enriched for Bacteroidetes, Firmicutes and Actinobacteria, old fish were dominated by Proteobacteria (Figure 3D). We next investigated the predicted functional metagenome biomarkers associated with young and old fish's guts using PICRUSt (Langille et al., 2013) and LEfSe (Segata et al., 2011). While young fish had GM strongly associated with glycolysis and polysaccharide metabolism, old fish's

FIGURE 3



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

212 (A) Representative 6-week-old (young) and 16-week-old (old) male TK. (B) Alpha diversity changes in observed
213 OTUs in young (6 weeks) and old (16 weeks) TK. N = 16 six-week-old fish and 14 16-week-old fish. The groups
214 are compared using the Mann Whitney U test; ** indicates a p value < 0.001. (C) Beta diversity microbiota
215 analysis separates samples based on age. Above: Bray-Curtis analysis, below: Unweighted UniFrac analysis. TW:
216 tank water control. Adonis test, p value < 0.001 in both comparisons between 6-week and 16-week-old fish. (D)
217 Cladogram representing microbial taxa enriched in young (green) versus old (red) individuals. (E) Predicted
218 metagenome function in young (green) and old (red) groups (LEfSe), representing functions with p value <
219 0.001. The x-axis indicates the Linear Discriminant Analysis score for all the significant metabolic functions. (F)
220 Expression heatmap for the twenty top differentially expressed genes (DEGs) between young and old fish (n = 4
221 for each group). Top 20 genes are highly expressed in old fish, the bottom 20 are highly expressed in young fish.
222 (G) Top 20 Gene Ontology (GO) terms of the DEGs between young and old fish. Enrichment values (bars) and
223 the negative natural logarithm of p values (black dots) are shown.
224 *HAMP is the best protein blast hit in *Danio rerio* of the TK gene NFURG05812010005.
225

GM was depleted of bacteria associated with carbohydrate, nucleotide and amino acid metabolism, and was enriched for bacteria associated with pathogenesis, transport and catabolism (Figure 3E). In particular, bacterial motility and flagellar assembly was strongly increased in GM from old fish. These terms are associated with increased virulence in bacteria (Josenhans and Suerbaum, 2002), supporting that old fish had a higher prevalence of potentially pathogenic bacteria.

To examine whether metagenomic biomarkers from bacterial taxonomic diversity (i.e. OTU) data were consistent with host responses, we asked which were the transcriptional changes in the gut associated with young and old status. To this end, we performed an RNA-Seq analysis of whole gut in four 6-week old and four 16-week old fish (Figure 3F and G, Figure S3 and Materials and Methods). Young fish had a distinct expression signature of active proliferation (Figure 3G, Figure S3), consistent with the bacterial metagenomic signature of replication. Old fish, on the other hand, had significant Gene Ontology terms associated with immune and defense responses against pathogens as well as inflammation, consistent with the bacterial metagenome signatures associated with host disease and overall virulence. Together, these results strongly support that in old individuals both changes in bacterial composition and gut transcriptome are consistent with a markedly pathological gut environment, while young fish are characterized by a molecular signature of healthy gut and a commensal bacterial community.

Young GM transfer prolongs life span and delays age-dependent motor decline

Interventions aimed at directly modifying the complex microbial composition in experimental organisms and patients have been mostly focused on treating diseases such as *Clostridium difficile* infections (Dodin and Katz, 2014; Lee et al., 2016), as well as obesity and type two diabetes (Kootte et al., 2012; Turnbaugh et al., 2006). However, the impact of a young GM in

modulating aging and life span has not been explored to date in vertebrates (Clark et al., 2015). To test whether resetting a young-like GM in middle age could impact aging and affect life span, we treated middle-age fish (9.5 week-old) overnight with an antibiotic cocktail (VMNA, i.e. vancomycin, metronidazole, neomycin, ampicillin) (Figure 4A, Figure S4A and Materials and Methods). The antibiotic treatment significantly reduced gut microbial content compared to pre-treatment levels (Figure S4B). Antibiotic-treated fish were then exposed for 12 hours to the following conditions: six-week-old donor fish gut content (Ymt), 9.5-week-old fish gut content (Omt) and sham (Abx) (Figure 4A and Figure S4A). After antibiotic treatment and 12-hour acute gut recolonization, fish were reintroduced in the water recirculation system in individual tanks and were subjected to regular feeding (Materials and Methods). Their survival under the different experimental conditions was then scored (Table S3A). Ymt fish underwent dramatic life span prolongation compared to three control groups, which received: (i) antibiotic-only (Abx) (21% life span increase in median life span, Logrank test p value = 5.89E-05), (ii) antibiotics and same-age (i.e. 9.5 weeks) gut content (Omt) (41% increase in median life span, Logrank test p value = 5.08E-06), or (iii) no-treatment (wt) (37% increase in median life span, Logrank test p value = 4.04E-09) (Figure 4B and Figure S4C-F). Noteworthy, acute antibiotic treatment alone was sufficient to increase fish life span compared to the wt group (14% median life span increase, Logrank test p value = 0.0129) (Figure 4B). However, Omt fish did not live longer than the control, wt group (Figure 4B). Since Abx outlived Omt and wild-type fish, while Ymt fish outlived Abx fish, it is plausible that middle-age GM composition might be primed to induce damage in the host and that its removal is therefore beneficial. However, as the recolonization of middle-age individuals with young fish gut content after antibiotic treatment prolongs life span even more, this implies that young GM, *per se*, has beneficial effects on host physiology that are additive to the effects of the antibiotic treatment.

FIGURE 4

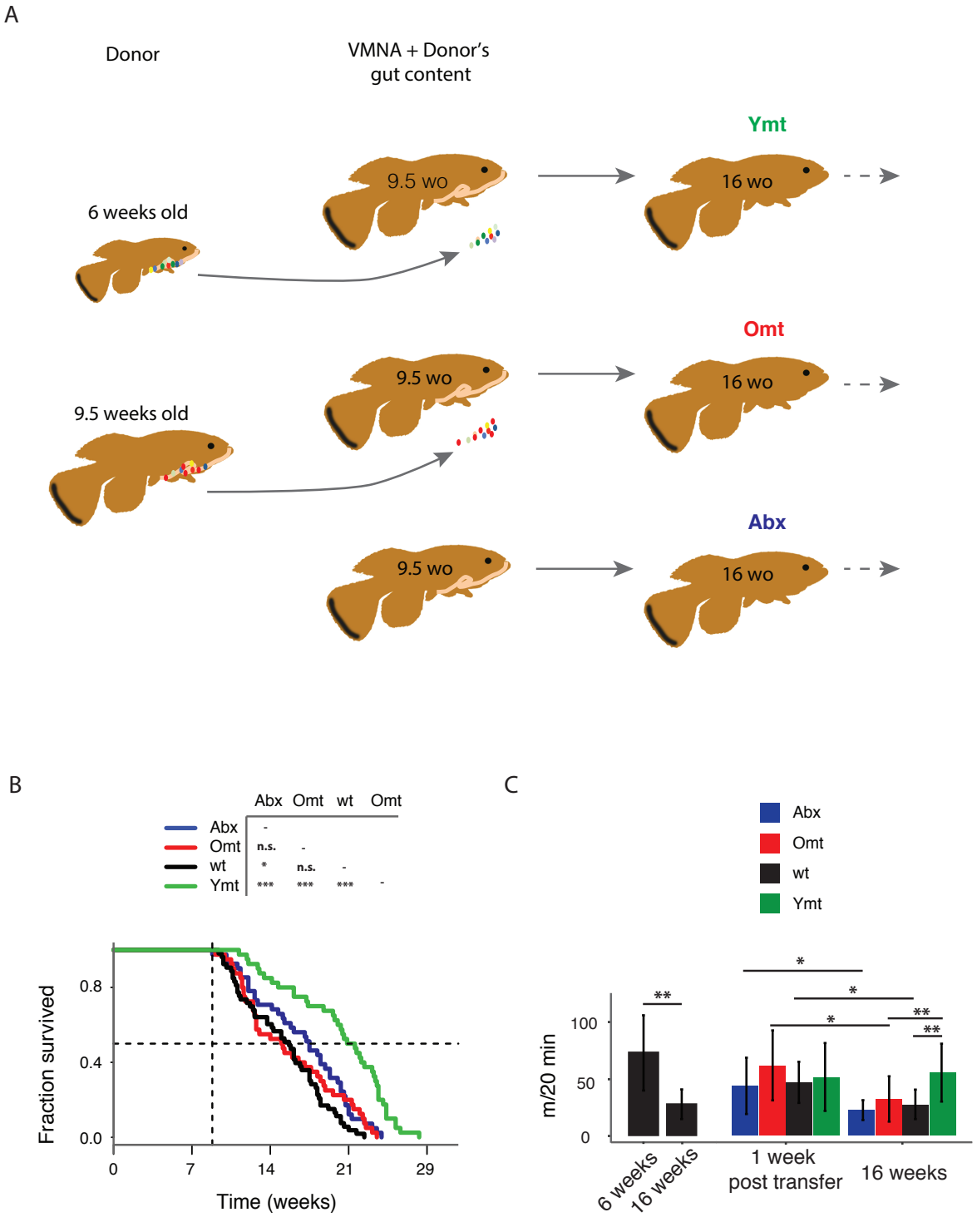


Figure 4. Transfering young GM to adult fish prolongs life span and delays motor decline

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

We then asked whether treating young fish with old fish GM could also affect life span. Compared to fish that received either the same-age GM or to untreated control fish, six-week-old fish receiving 16-week-old fish's GM after antibiotic treatment did not have a different life span (Figure S4G). Additionally, unlike middle-aged fish treated with antibiotics, young fish receiving antibiotic treatment did not live longer than untreated control fish (Figure S4G). These results suggest that the timing of GM transfer is critical to inducing systemic effects and modulating life span.

It was shown in previous work that spontaneous exploratory behavior in TK decreases with age (Genade et al., 2005; Valenzano et al., 2006b). We therefore asked whether treating middle-age fish with young fish GM after antibiotic treatment could improve exploratory behavior performance, considered as an integrated measure of individual health. Using an automatic video-tracking system (Materials and Methods), we assayed spontaneous locomotor activity. Young, six-week-old fish, were significantly more active than 16-week-old fish (Table S3B and Figure 4C, Kruskal-Wallis chi-squared = 10.752, df = 1, p value = 0.00104). Remarkably, Ymt were more active at 16 weeks of life than Omt and wt fish at the same age, resembling younger fish performance (Figure 4C, Dunn Kruskal-Wallis multiple comparison test, BH-adjusted p value = 0.004). Additionally, all groups except Ymt underwent a significant decrease in spontaneous locomotor activity from a week post transfer to 16 weeks of life. This suggests that the transfer of young fish gut content had long-lasting effects on a global measure of physiological health, influencing individual survival and spontaneous exploratory behavior. Thus, depleting middle-age individuals from their resident GM resulted beneficial when acutely recolonized by young-associated GM (Ymt), and in part also when it was not followed by any acute recolonization (Abx). On the other hand, acutely recolonizing the gut with same age GM after antibiotic treatment did not lead to differences compared to

315 the untreated control group. These results establish gut microbial recolonization as a powerful
316 life span enhancing intervention, which leads to significant effects also on behavioral
317 performance.
318
319 **Acute GM transfer affects microbial composition at old age**
320 To assess the extent to which one acute transfer reset the GM in recipient fish after VMNA
321 antibiotic treatment, we performed 16S rRNA gene amplicon surveys in fish that underwent
322 microbial transfer at 9.5 weeks of age. Untreated 6-week-old (6wk) and 16-week-old (16wk)
323 fish were also included in the analysis. One week post transfer there were no significant
324 differences in alpha diversity metrics among treatment groups (Figure S5A and B). However,
325 Ymt and Omt fish had already significantly different gut microbial population diversity
326 (UniFrac and Bray-Curtis distances, Figure S5C and D and Table S4). Seven weeks after the
327 microbial transfer (16 weeks of age, corresponding to the median life span for this species in
328 captivity), Ymt fish had significantly higher bacterial richness compared to wild-type, 16-
329 week-old fish (Figure 5A, Dunn Kruskal-Wallis test, BH-adjusted p value = 0.009). This
330 shows that one acute transfer had long-lasting effects on GM diversity. Bacterial OTU
331 abundance at 16 weeks was higher in Ymt fish compared to Omt fish (Figure 5A), but lower
332 than 6-week old wild-type fish (6wk).
333 GM community structure in Ymt fish was also significantly altered compared to Omt and
334 16wk (Figure 5B and Figure S5E); however, it did not statistically differ from young wild-
335 type fish, i.e. the 6wk group. Furthermore, based on hierarchical clustering, Ymt fish clustered
336 preferentially with 6wk fish (Figure 5C), showing that the GM-transfer from young donors
337 significantly reset a young-like GM.
338 Young fish (6wk), as well as fish treated with young GM (Ymt), were more enriched with
339 members of the Bacteroidetes and Firmicutes (Figure 5D) and with the genera

FIGURE 5

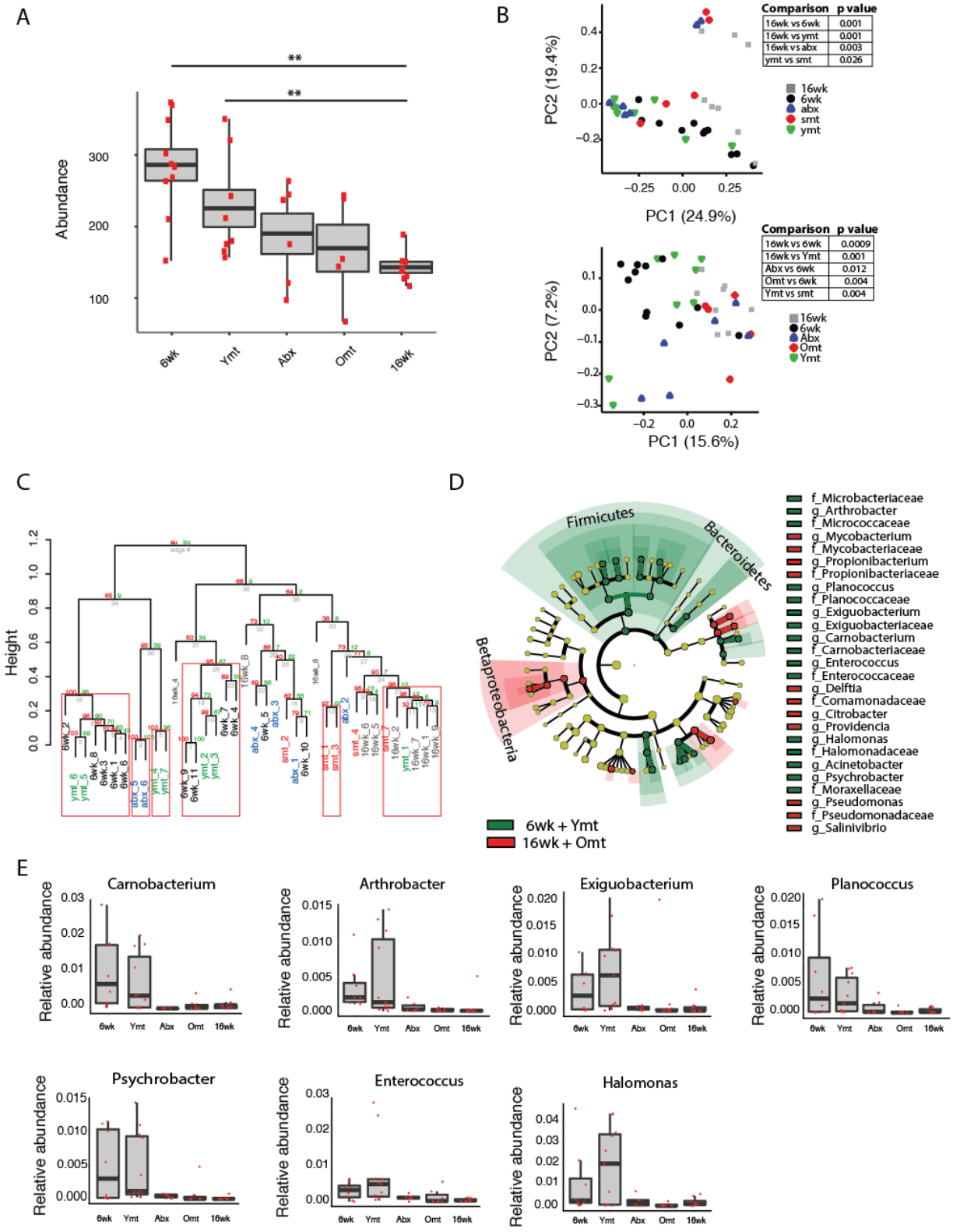


Figure 5. Microbiota transfer at 9.5 weeks influences microbial composition at 16 weeks

(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

adjusted based on BH correction. Statistical significance: ** indicates a p value < 0.01. (B) Microbiota community analysis using Bray-Curtis (above) and Unweighted UniFrac (below) separates samples based on young vs. old GM treatment (significant p values are shown, Adonis test). (C) Hierarchical clustering on OTU data. Significant clusters are highlighted by red rectangles. P values: au = approximately unbiased; bp: bootstrap probability. (D) Cladogram of bacterial taxa enriched in young wild-type (6wk) and Ymt groups versus 16wk and Omt. (E) Boxplots of selected bacterial genera relative abundance in experimental groups. Bacterial genera are those represented in Figure 5D, filtered by r-square value larger than 0.8, based on the correlation between bacterial genus abundance vs median life span in each individual group (Table S5).

353 *Carnobacterium*, *Arthrobacter*, *Exiguobacterium*, *Planococcus*, *Psychrobacter*, *Enterococcus*
354 and *Halomonas* (Figure 5E). Additionally, analyzing the correlation between bacterial genera
355 abundance and the group-specific median lifespan, we identified a set of bacterial genera
356 whose abundance is highly correlated with group-specific longevity (Table S5).
357 We then used predicted functional metagenome analysis from GM composition to compare
358 experimental fish groups receiving same-age GM transfer and no-treatment controls to fish
359 receiving young gut content. The latter group was enriched for increased saccharolytic
360 potential, DNA repair and recombination among other functions (Figure S5F), which are
361 functional terms associated with a younger, healthy-like physiological state. Together, these
362 results show that gut microbial recolonization from young killifish resulted in increased
363 microbial diversity and a persistence of a young-like bacterial community until old age, whose
364 composition could be at least in part responsible for the life span prolongation.
365
366 **Microbial transfer determines bacterium-to-bacterium connectivity at old age**
367 To investigate whether gut recolonization affected bacterium-to-bacterium association in
368 different experimental groups, we harnessed OTU co-occurrence to generate bacterial
369 connectivity networks (Agler et al., 2016; Biagi et al., 2016). To assess the bacterium-to-
370 bacterium connectivity in each experimental group, we built a network model for each
371 experimental group based on OTU co-occurrence. Significantly co-occurring genera within
372 each group composed a network, whose nodes were single bacterial genera. Bacterium-to-
373 bacterium connections (edges) were established based on r-square values (Materials and
374 Methods). Using this analysis, we found that Proteobacteria had higher connectivity in the
375 shorter-lived 16wk and Omt groups compared to 6wk, Ymt and Abx (Figure 6A). Overall,
376 6wk and Ymt fish had the largest networks and a higher number of highly connected nodes

FIGURE 6

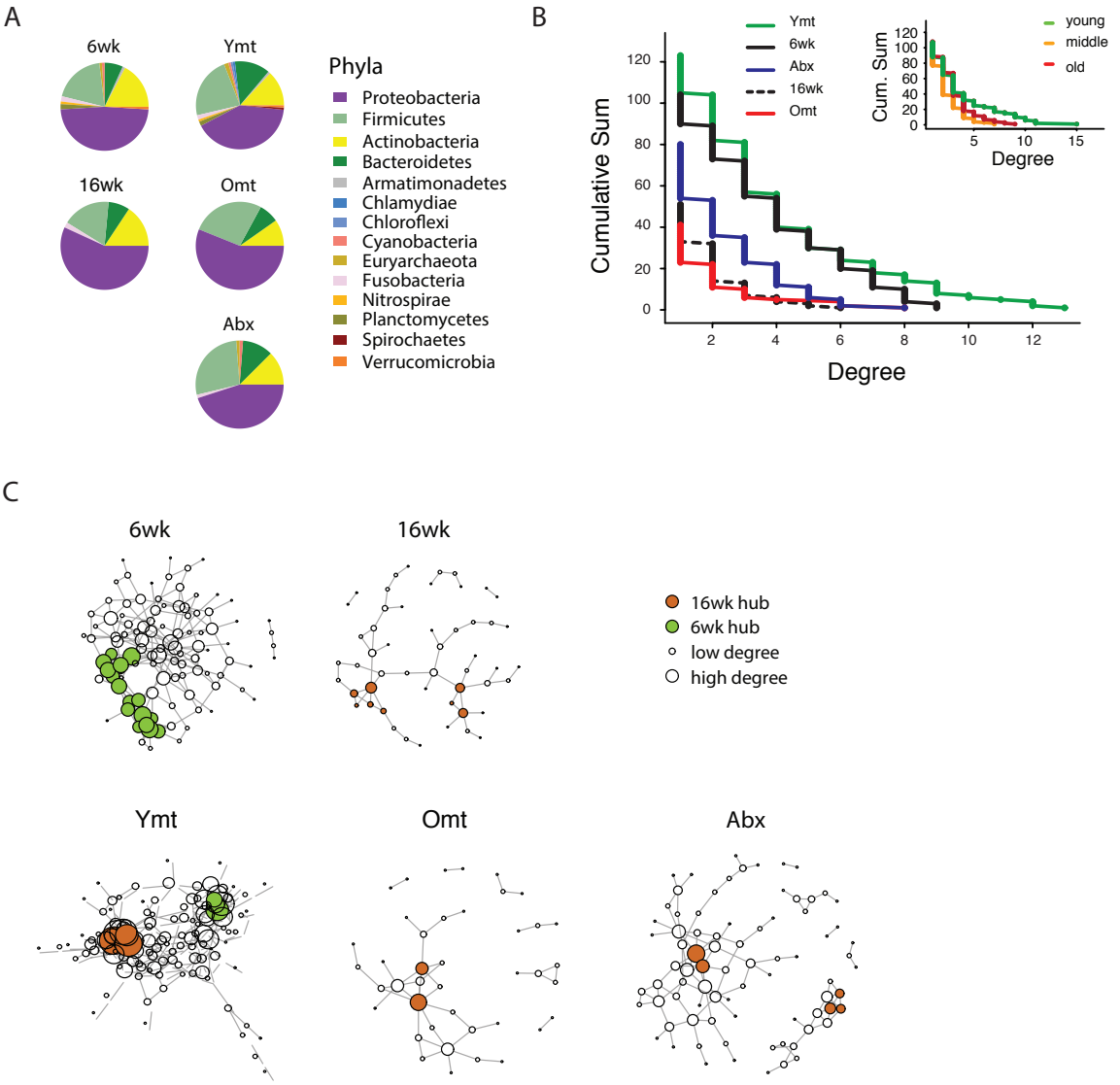


Figure 6. Bacterial co-occurrence connectivity

(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).

(hub nodes), showing that a few genera of bacteria significantly co-occurred with a large set of other bacterial genera (Figure 6B and C).

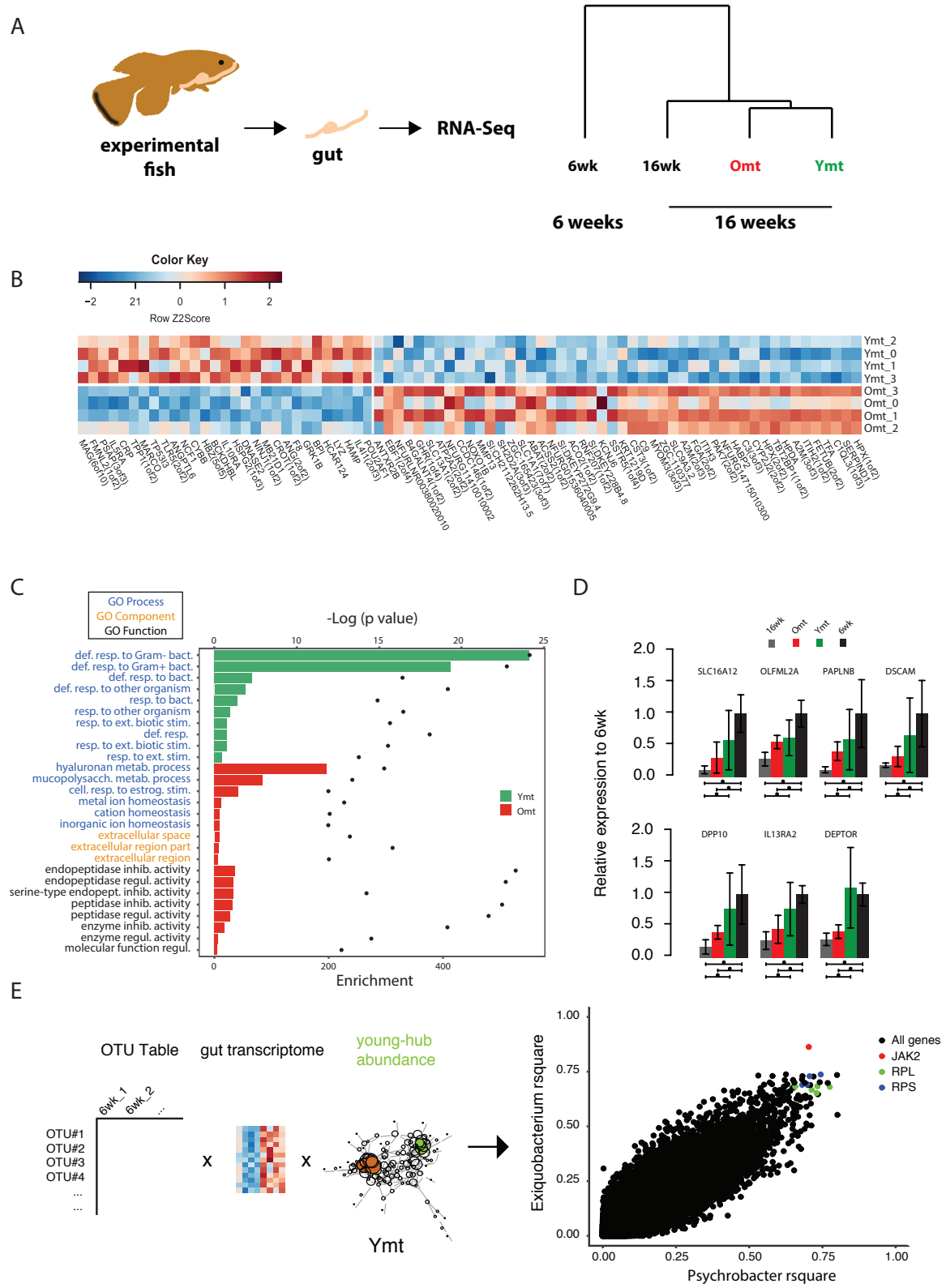
We then tested whether the relation between age of the host and bacterial connectivity observed in TK guts was also conserved in mammals. We analyzed a published mouse 16S amplicon survey from an aging cohort (Langille et al., 2014). Similarly to what we found in the TK, young mice GM had more significantly connected nodes and a higher number of bacterial hubs compared to middle age and old mice (Figure 6B, inset).

The identification of wild-type specific young and old-related hub bacterial genera enabled us to study whether Ymt, Omt and Abx shared hub bacterial genera with either young or old fish groups. Two hub bacterial clusters were identified in Ymt fish (Materials and Method). Strikingly, one was composed of bacterial genera that were also hub-bacteria in young wild-type fish (6wk) and included *Exiguobacterium*, *Planococcus*, *Propionigenium* and *Psychrobacter* (Figure 6C, Ymt network, green nodes), while the other was composed of hub bacteria from old wild-type fish (16wk) hub and included *Propionibacterium*, *Delftia*, and *Citrobacter* (Figure 6C and Table S6). Remarkably, the bacterial hubs identified in Omt and Abx overlapped exclusively with the old wild-type group (16wk) (Figure 6C, orange nodes, and Table S6). These results support that bacterial network topology reflects host age both in fish and mice, with younger biological age associated with larger networks. Acute microbiota transfers significantly affect gut microbial population topology and subsets of bacterial genera are identified as hub nodes in young-like and old-like microbial communities. Therefore, not only bacterial composition, but also bacterium-bacterium co-occurrence carries a key signature of host life span and network topology depends on a few key bacterial hubs.

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

412 Because acute microbial transfer dramatically changed GM composition by resetting a young-
413 like microbial community in Ymt fish, we asked whether young GM transfer could also reset
414 the host transcriptome towards a young-like status. To this end, we performed host intestine
415 RNA-Sequencing in wild-type young (6wk) and old (16wk) fish, as well as in Omt and Ymt
416 fish (Figure 7A and B, Figure S6 and Materials and Methods). Interestingly, while young GM
417 transfer reset a young-like GM environment in aged fish, host gene transcripts separated based
418 on fish age, with all the transcriptomes obtained from 16-week old fish groups (16wk, Omt,
419 Ymt) clustering together (Figure 7A). However, comparing transcriptomes in Omt and Ymt
420 revealed a distinct signature of defence response to bacteria in Ymt and increased expression
421 of genes associated with hyaluronic acid metabolism in Omt (Figure 7B and C). Comparing
422 the expression levels of all groups to the 6wk group, we identified a set of genes whose
423 expression in the gut was significantly changed comparing 16wk and Omt with 6wk;
424 comparing 16wk and Ymt, but unchanged between Ymt and 6wk. These were genes
425 associated with the TOR-pathway (DEPTOR) and with cell adhesion and extracellular matrix
426 composition (DSCAM, OLFML2A, PAPLNB) (Figure 7D), suggesting a potential difference
427 in cell adhesion and gut permeability between 16wk and Omt from one side, and Ymt and 6wk
428 on the other side. Since we generated OTU abundance tables for individual fish and we also
429 sequenced intestinal transcripts for the same individuals, we then generated an OTU-to-host
430 trancripts correlation matrix (Data S1). Taking advantage of this resource, we could identify
431 the transcripts that were highly correlated with the hub genera that are shared between Ymt
432 and young fish (6wk) networks (Figure 6C, Figure 7E and Figure S7). Remarkably, these
433 genes include Jak2, an important gene involved in cellular proliferation and differentiation,
434 and several genes encoding S and L- ribosomal proteins (RPS and RPL genes) (Figure S7),
435 whose expression in other species has been strongly associated with aging and longevity

FIGURE 7



436

437

438

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

(A) Cluster analysis of gut RNA-Seq from intestines from six-week-old fish (6wk) and 16-week-old fish (16wk, Omt (red) and Ymt (green)). (B) Expression heatmap for the 20 top differentially expressed genes (DEGs) between Ymt and Omt fish (n = 4 for each group). The top 20 genes are highly expressed in Ymt fish, the bottom 20 are highly expressed in Omt fish. *HAMP is the best protein blast hit in *Danio rerio* of the TK gene NFURG05812010005. (C) Gene Ontology (GO) analysis of the DEGs between Ymt and Omt fish. Enrichment values (bars) and the negative natural logarithm of p values (black dots) are shown. (D) Relative gene expression levels compared to six-week-old fish (6wk) of genes significantly differentially expressed between 16wk (gray), Omt (red) and 6wk (black), but not between Ymt (green) and 6wk. These genes are also significantly differentially expressed between 16wk and Ymt. (E) Based on the correlation matrix between OTU abundance and transcript levels in individual fish (Data S1), we identified the gene transcripts whose expression correlates more significantly with the hub bacteria shared between Ymt and 6wk fish (full analysis in Figure S7). Displayed are the top hits (chosen from an r-square value larger than 0.65 on both axes) shared between *Psychrobacter* and *Exiguobacterium*, which have the highest OTU-transcript correlation trend based on Figure S7. RPLs include RPL7A, RPL8, RPL12, RPL37, RPL14, MRPL35; while RPS include RPS3A, RPS7, RPS11, RPS24.

455 (Steffen et al., 2008). Thus, while transfer of young GM to middle age fish maintained a
456 young-like GM community throughout old age, the gene-expression signature associated with
457 host gut at 16 weeks of age did not indicate overall rejuvenation. However, enhanced defense
458 response against bacteria in Ymt compared to Omt is compatible with an increased capacity to
459 resist to the attack of pathogenic gut bacteria, which could provide the basis for longer life
460 span. Remarkably, hyaluronic acid metabolism, altered between the experimental groups
461 receiving young and old gut contents, has been associated with increased inflammation,
462 deregulated immune response and risk for cancer (Cho et al., 2017; Tian et al., 2013), all of
463 which could provide the basis for life span modulation. Finally, we provide a dataset that
464 allows to associating OTU levels in individual fish to intestinal transcripts, enabling to
465 investigate on how GM and host gene expression are mutually regulated.
466

DISCUSSION

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

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

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

492 community that is representative for the species in nature. Additionally, we found that wild
493 populations of TK show large between-population gut bacterial diversity, with the emergence
494 of population-specific high-abundance taxa. This is plausibly caused by locality-specific
495 differences in ecological conditions, including climate, soil composition, parasites and
496 diversity of food sources (Nezhybová et al., 2017; Reichard et al., 2017).
497
498 Analyzing the changes in GM composition between young and old fish, we found that young
499 fish are characterized by a large taxonomic bacterial diversity. Old fish are less OTU-rich,
500 similar to what is observed in human cohorts from different age classes (Claesson et al., 2012).
501 Intriguingly, while individual bacterial diversity (alpha diversity) is higher in young
502 individuals, old fish are more dissimilar from one another, i.e. while each individual old fish
503 have a more homogeneous GM composition than young fish, any two old individuals have
504 more divergent bacterial communities compared to young individuals. This result raises the
505 possibility that the changes in composition and relative abundance in GM communities from
506 young to old individuals could be a function of i) initial individual GM composition, ii)
507 differences in individual immune system composition and function or iii) a combination of
508 initial individual GM composition and host immune function.
509 Aging in this experimental model was associated not only with reduced bacterial species
510 richness, but more specifically with loss of bacterial taxonomic units involved in carbohydrate,
511 nucleotide and amino acid metabolism, which in mice are associated with aging (Langille et
512 al., 2014). These same changes have been associated in humans with unhealthy aging
513 (Claesson et al., 2012; Rampelli et al., 2013), as well as with chronic conditions such as
514 obesity, type II diabetes and insulin resistance (Neis et al., 2015). The shift in microbial
515 composition between young and old fish was consistently characterized by a higher prevalence
516 of Proteobacteria in old individuals, while young individuals were significantly more enriched

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

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

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

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

MATERIALS AND METHODS

Wild fish samples

The wild fish samples derived from Zimbabwe were collected during the expedition in Gonarezhou National Park in 2015 (Permit No.: 23(1) (C) (II) 30/2015). Intestines were collected at each location and preserved in pure ethanol. Sampling locations coordinates are listed in Table S2.

Fish husbandry and survival scoring

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

DNA extraction, 16S rRNA gene amplification and sequencing

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

DNA was then used in one of two, two-step PCR methods designed to target the V3-V4 region of the 16 rRNA gene (Segata et al., 2011). For the first method, initial primers consisted of (5' to 3') a primer pad and linker (Parks et al., 2014) and the V3/V4 gene specific forward or reverse primer sequences. The second step PCR used primers complementary for the primer pad and linker followed by standard Illumina adaptors. The reverse primer for the second step also contained a 12bp Golay barcode (Parks et al., 2014). For the second method, initial primers consisted of (5' to 3') Illumina overhand adaptor sequences and the V3/V4 gene specific primer sequences followed by a second step PCR using the Nextera XT Index kit (Illumina). For both amplification methods cycling conditions were the same and the first round of PCR was performed in triplicate with approximately equal amounts of DNA template (250ng/reaction). PCR reactions were carried out with a two minute denaturation step at 98°C, followed by 25 cycles of 98°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Triplicate reactions were then pooled and cleaned using the Wizard SV Gel and PCR Clean-up Kit (promega). The cycling conditions for the second step PCR were the same as the first step, except annealing was performed at 60°C with only 8 cycles. Both PCR steps used KAPA HiFi Hotstart ReadyMix (KAPA Biosystems) and 1um of primers in 25ul total volume. 2nd step PCR products were run on a 1.2% agarose gel and DNA products between 500-700 base pairs were excised and cleaned up as in step 1. PCR products were quantified by Qubit (Life Technologies), diluted to 4nm and combined in equal volumes. The combined amplicon libraries were then sequenced on the Illumina MiSeq, V3 reagents, 2x 300bp paired-end reads.

Antiobiotic treatment and fish microbiota transfer

Microbiota transfer experiments were based on those developed in zebrafish (Lopez-Otin et al., 2013). Recipient fish were removed from main water recirculating system and housed in 9L tanks at a density of 10 fish per tank. Recipient fish (6wk or 9.5wk) were treated overnight

644 with a combination of Vancomycin (0.01g/L), Metronidazole (0.5g/L), Neomycin (0.5g/L)
645 and Ampicillin (0.5g/L) to diminish the resident bacterial community. Following antibiotic
646 treatment, recipient fish were washed twice for 10 minutes with autoclaved tank water.
647 Concurrently, whole intestines were isolated from donor fish and placed into 10 cm petri
648 dishes containing sterile PBS on ice. Intestines were then opened longitudinally, the intestinal
649 contents scraped out and then further cut into 0.5 cm pieces to facilitate the release of bacteria.
650 The collected intestinal contents were washed once in cold PBS and added to the fish tanks
651 containing autoclaved tank water and recipient fish at a ratio of 1 donor fish intestine/2
652 recipient fish. Fish were incubated overnight with the donor fish intestinal contents before
653 being returned to the main recirculating system and individually housed, where they were
654 regularly fed according to standard husbandry.
655
656 **Microbial community analysis**
657 Fastq files from paired end reads were joined, demultiplexed and subjected to quality filtering
658 with QIIME 1.8 (Q≥20) as previously described (Rampelli et al., 2013). For microbial
659 community analysis, QIIME was used to identify OTUs by open-reference picking using
660 UCLUST (97% similarity) and taxonomy was assigned with the Greengenes 13.8 database
661 (Neis et al., 2015). A minimum OTU count of 5 was used to minimize spurious OTUs.
662 Representative OTU sequences were aligned with PYNAST (Klindworth et al., 2013) and
663 FastTree 2 (Caporaso et al., 2012) was used to build a phylogenetic tree. For diversity
664 analyses, OTU tables were rarefied to at least 5000 sequences, which allowed the majority of
665 samples to be kept. Qiime and R were used to calculate alpha and beta diversity metrics and
666 generate plots. To identify bacteria associated with specific groups, OTU tables were further
667 filtered for presence in at least 25% of samples with a collective abundance of greater than 100
668 reads. Significant changes in relative OTU abundance were identified with linear discriminant

669 analysis effect size (LEfSe) and visualized using GraPhlAn (Asnicar et al., 2015). For
670 metagenomics analysis, rarefied OTU tables were generated by closed reference picking and
671 the PICRUSt tool was used to normalize by 16S copy number and predict the metagenome
672 content of samples from 16S rRNA profiles. KEGG pathway functions were then categorized
673 at level 2 or 3 and LEfSe was used to identify significant changes among classes. The
674 accuracy of PICRUSt predictions was determined by nearest sequenced taxon index (NSTI,
675 Supplemental Table1).
676
677 **Bacterial diversity among model organisms**
678 To compare the overall bacterial diversity among different species, sequences were
679 downloaded from the European nucleotide archive (ENA), the NCBI Short Read Archive
680 (SRA) or MG-RAST and subjected to closed reference OTU picking. Taxa summary and
681 alpha diversity measures were computed using QIIME 1.8. Samples were chosen which used
682 Illumina sequencing of the V4 region of the 16 rRNA gene. Accession numbers. Human:
683 ERR561021UK, ERR560915UK, ERR560902UK, ERR560855UK, mgm4489670,
684 mgm4489628, mgm4489516, mgm4489454, mgm4538473, mgm4538468, mgm4538264,
685 mgm4538259, mgm4538238. Mouse: SRR1820108, SRR1820074, SRR1820073,
686 SRR1820072, SRR1820071, ERR706143, ERR706142, ERR706141. Zebrafish:
687 SRR1581750, SRR1581753, SRR1581759, SRR1581763, SRR1581766, SRR1581889,
688 SRR1581890, SRR1581891. Drosophila: SRR989472, SRR989473, SRR989474,
689 SRR989469, SRR989467, SRR952981.
690
691 **Bacterial load quantification**
692 To determine bacterial load, DNA was isolated from freshly collected faecal pellets as
693 described above. Real-time quantitative PCR was performed with DyNAmo Color Flash

694 SYBR green master mix (Thermo Scientific) and run with the BioRad CFX384 Real-Time
695 System. Samples were normalized to amount of input faecal material and graphed as 1/Ct
696 value. Primers targeting the 16s gene were described previously (Caporaso et al., 2010). For:
697 5' TCCTACGGGAGGCAGCAGT 3' and Rev: 5' GGACTACCAGGGTATCTAATCCTGTT
698 3'.
699
700 **Quantitative analysis of spontaneous locomotor activity**
701 To measure total swimming distance, fish were placed in rectangular (16 x 90 cm) tanks filled
702 with 8 liters of water and allowed to acclimate for 30 minutes. The room and water
703 temperature and water quality were kept as similar to the fish's' normal environment as
704 possible. The fish were then filmed for 20 minutes with an overhead mounted camera and data
705 were analyzed using EthoVision XT11 (Noldus Information Technologies).
706
707 **Co-occurrence networks**
708 The OTU table was filtered by a minimum total count of 5509 per individual, based on the
709 rarefaction value (see Microbial community analysis). Subsequently, the table was divided
710 into subsets corresponding to the treatment groups. Normalization was achieved through
711 dividing the OTU sample count (c_i) by the total sample count ($Sum(c_i)$), scaled to 1000.
712 Co-occurrence networks were produced by applying the SparCC program (Friedman and Alm,
713 2012), a network inference tool specifically developed for analysis of correlations in
714 compositional data, such as 16S microbiome analyses. In order to avoid unreliable correlations
715 for very rare OTUs, the OTU tables were filtered for bacteria that were present in at least 25%
716 of samples of each group.
717 The SparCC pipeline was used to first calculate OTU-OTU correlations averaged over 20
718 iterations (Friedman and Alm, 2012). We then tested the significance of these correlations by

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

742
743

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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754

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769

770

COMPETING INTEREST

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

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SUPPLEMENTAL FIGURES AND TABLES

FIGURE S1

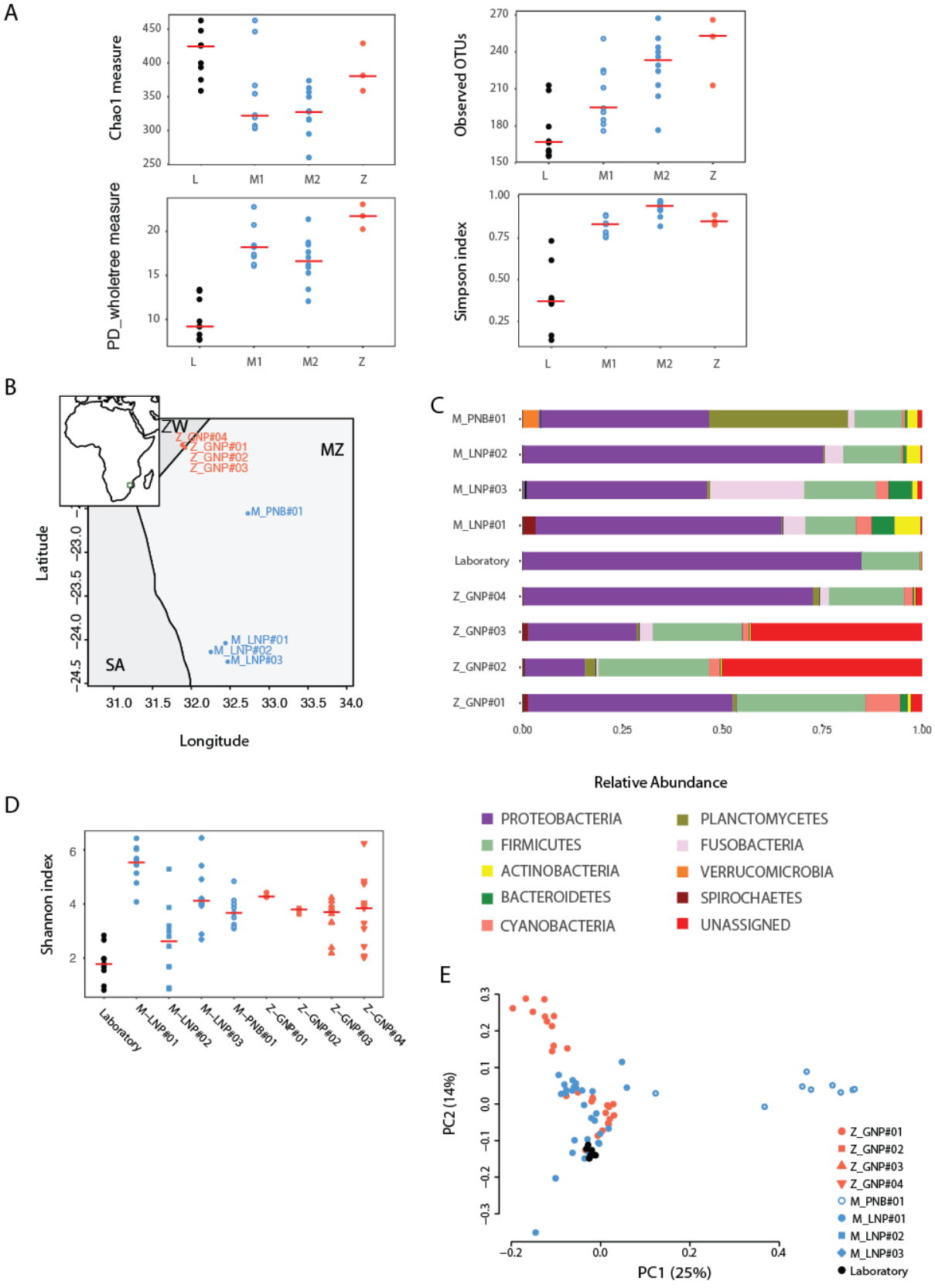

Figure S1. Related to Figure 2. Bacterial diversity measures in captive and wild turquoise killifish populations

(A) Chao1, Observed OTUs, Phylogenetic Distance and Simpson Index for laboratory ("L") and wild-caught populations (M1, M2, Z). (B) Map location of all the collected and sequenced populations. MZ: Mozambique; Z: Zimbabwe; SA: South Africa. (C) Phylum-level bacterial frequency in wild and laboratory populations. (D) Alpha diversity Shannon Index in laboratory and wild-caught populations. (E) PCoA of the Weighted UniFrac beta diversity between wild and laboratory fish.

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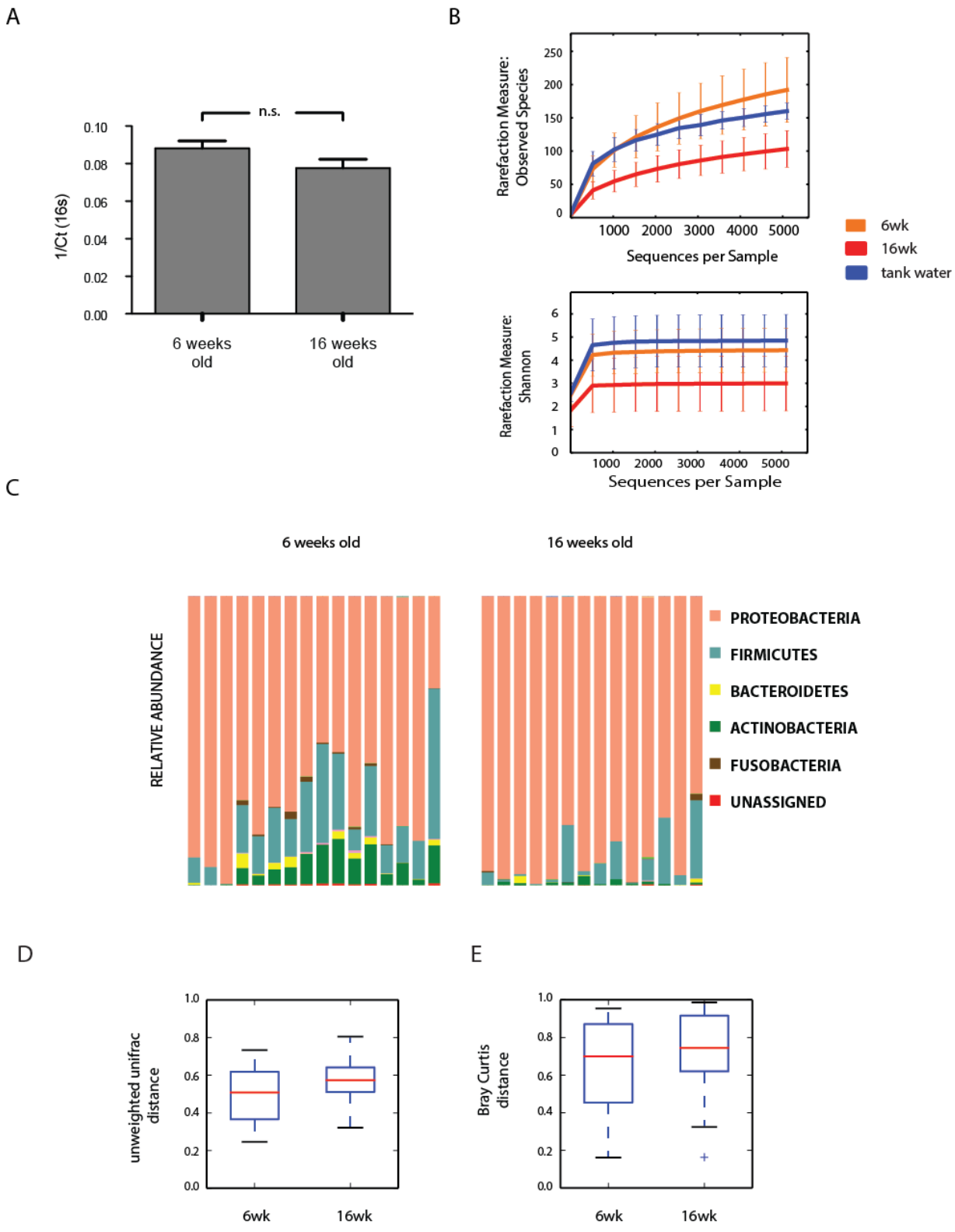
FIGURE S2



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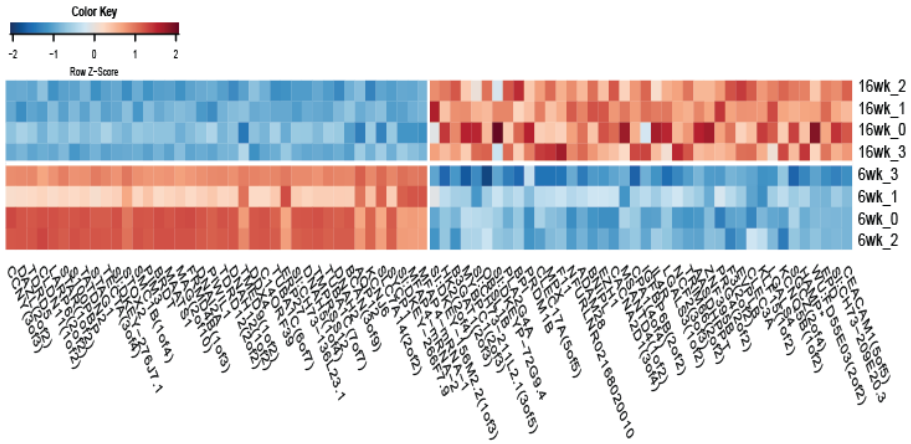
988 **Figure S2. Related to Figure 3. Aging in the GM: bacterial diversity**

989 A) Bacterial abundance in young and old fish measured from fecal pellets. (B) Rarefaction curves between
990 young, old fish, and two tank water control samples. (C) Relative abundance at the phylum level between young
991 and old subjects. (D-E) Beta diversity analysis between young and old fish OTUs. Unweighted UniFrac analysis,
992 non-parametric, Bonferroni-corrected p value = 0.001; Bray-Curtis beta diversity, non-parametric, Bonferroni-
993 corrected p value = 0.005.

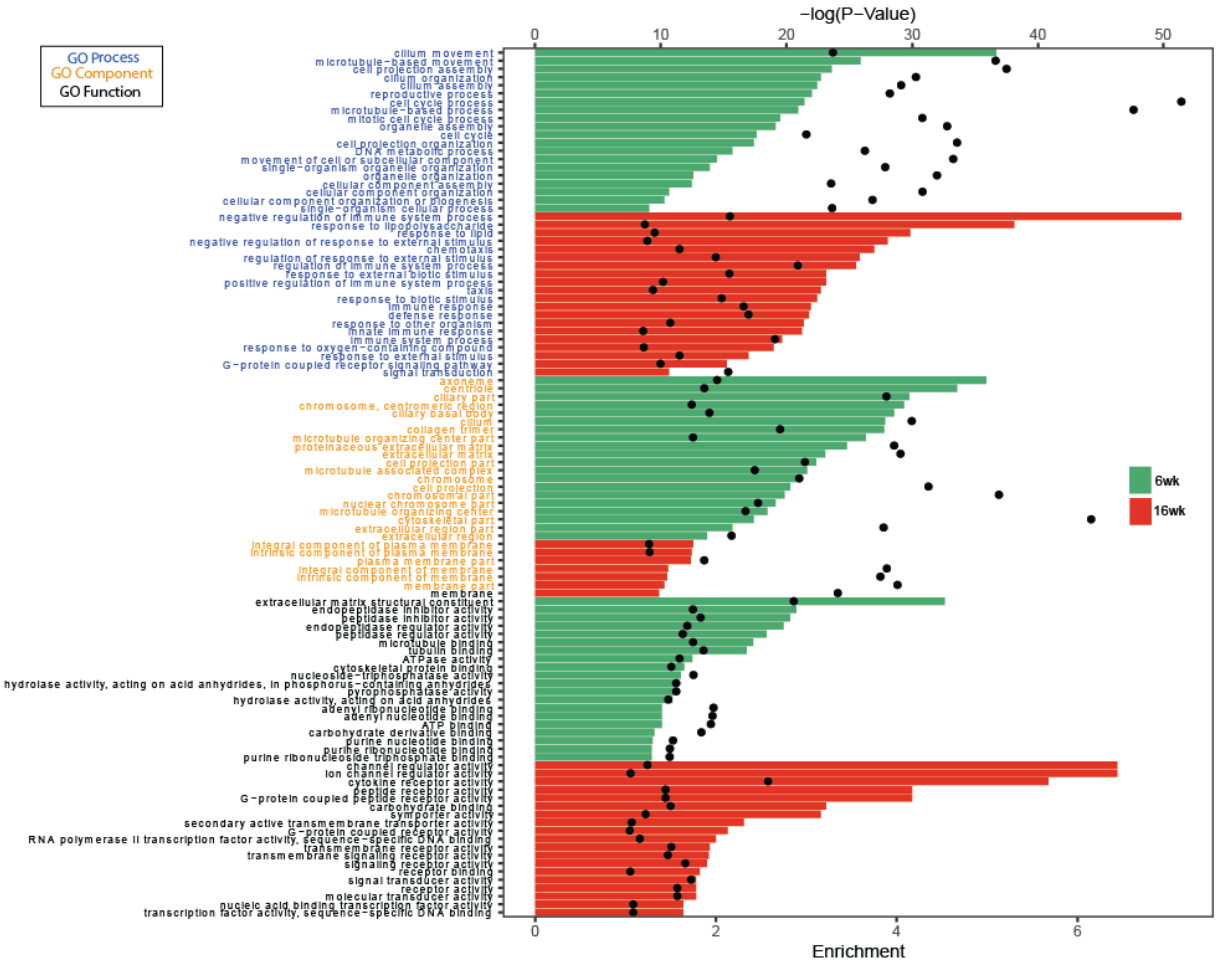
994

FIGURE S3

A



B



997 **Figure S3. Related to Figure 3. RNA-Seq analysis of young and old fish intestines**

998 (A) Expression heatmap for the top 80 differentially expressed genes (DEGs) between young and old fish (n = 4
999 for each group). Genes are shown in columns and individuals in rows. Highly expressed genes are colored red
1000 and low expressed genes are colored in blue.
1001 (B) Top Gene Ontology (GO) terms of the DEGs between young and old fish for GO categories process, function
1002 and component. The top twenty GO terms are listed based on significance and sorted by enrichment. Enrichment
1003 values (bars) and the negative natural logarithm of p values (black dots) are shown.
1004 *HAMP is the best protein blast hit in *Danio rerio* of the TK gene NFURG05812010005.
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FIGURE S4

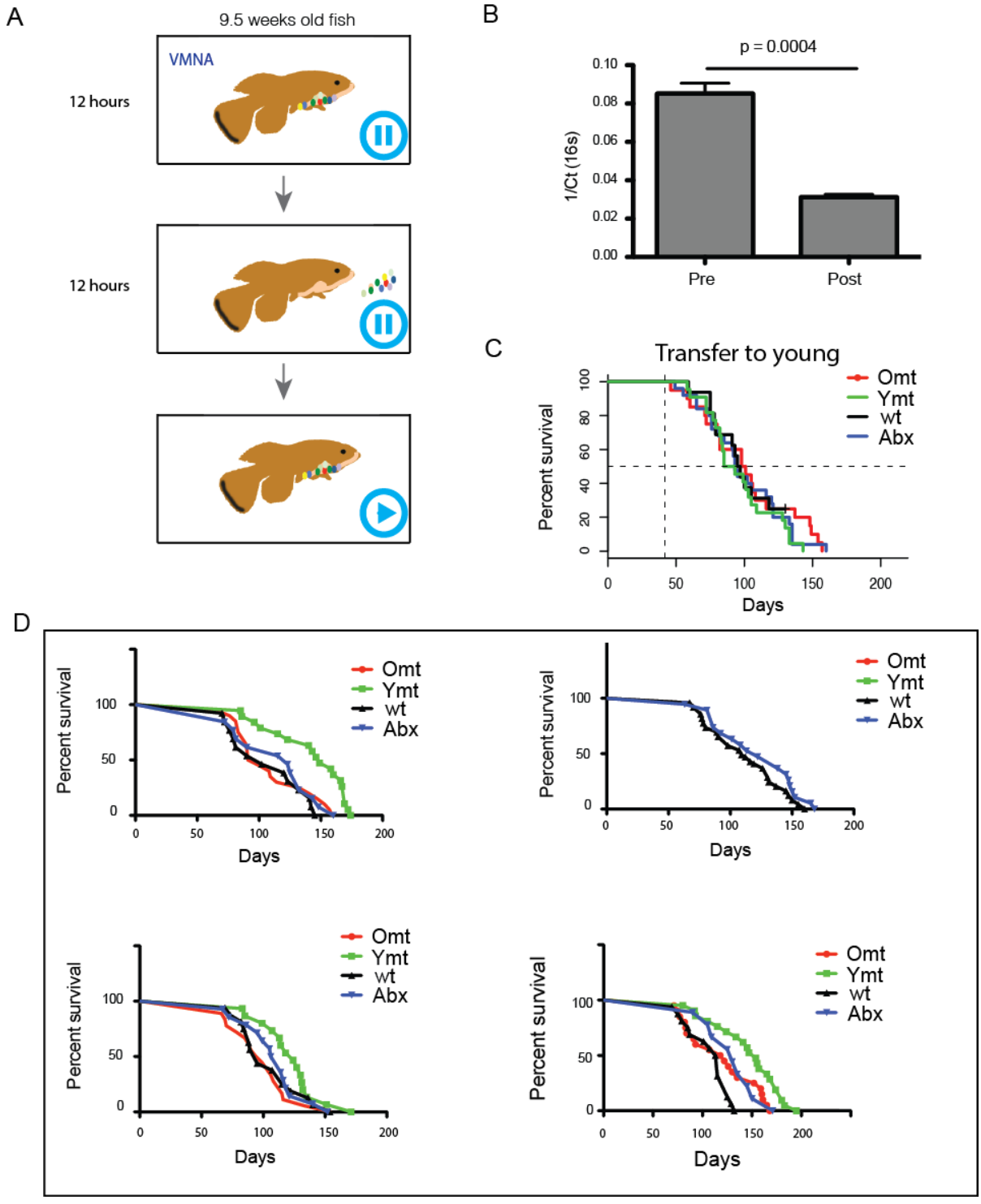

Figure S4. Related to Figure 4. Experimental design and consequences of gut microbiota transfer protocol

(A) Schematic of microbiota transfer protocol. During 12 hours of VMNA antibiotic cocktail administration fish are kept in tanks with no water filtration/recirculation. Next they are transferred to new tanks with autoclaved tank water where gut microbiota recolonization occurs for 12 hours without water filtration/circulation. After the second step, fish are transferred back to the water filtration/recirculation system and exposed to the regular fish room environment. (B) Bacterial load decreases after antibiotic treatment, measured as 1/Ct value after normalization to amount of input faecal material (N = 10 pre-treatment and 10 post-treatment; Materials and Methods). (C) Transfer to 6 week-old fish (data from Table S3A). Abx: fish receiving only antibiotic treatment at 6 weeks without direct recolonization. Omt: fish receiving 16-week-old GM transfer after antibiotic treatment at 6 weeks. Wt: wild-type, untreated fish. Ymt: fish receiving 6-week-old fish GM transfer after antibiotic treatment at 6 weeks. (D) Independent survival cohorts for the microbiota transfer experiment, with transfer towards 9.5-week-old fish (data from Table S3A). Abx: fish receiving only antibiotic treatment at 9.5 weeks without direct recolonization. Omt: fish receiving 9.5-week-old GM transfer after antibiotic treatment at 9.5 weeks. Wt: wild-type, untreated fish. Ymt: fish receiving 6-week-old fish GM transfer after antibiotic treatment at 9.5 weeks. VMNA: antibiotic cocktail of vancomycin, metronidazole, neomycin and ampicillin (Materials and Methods).

FIGURE S5

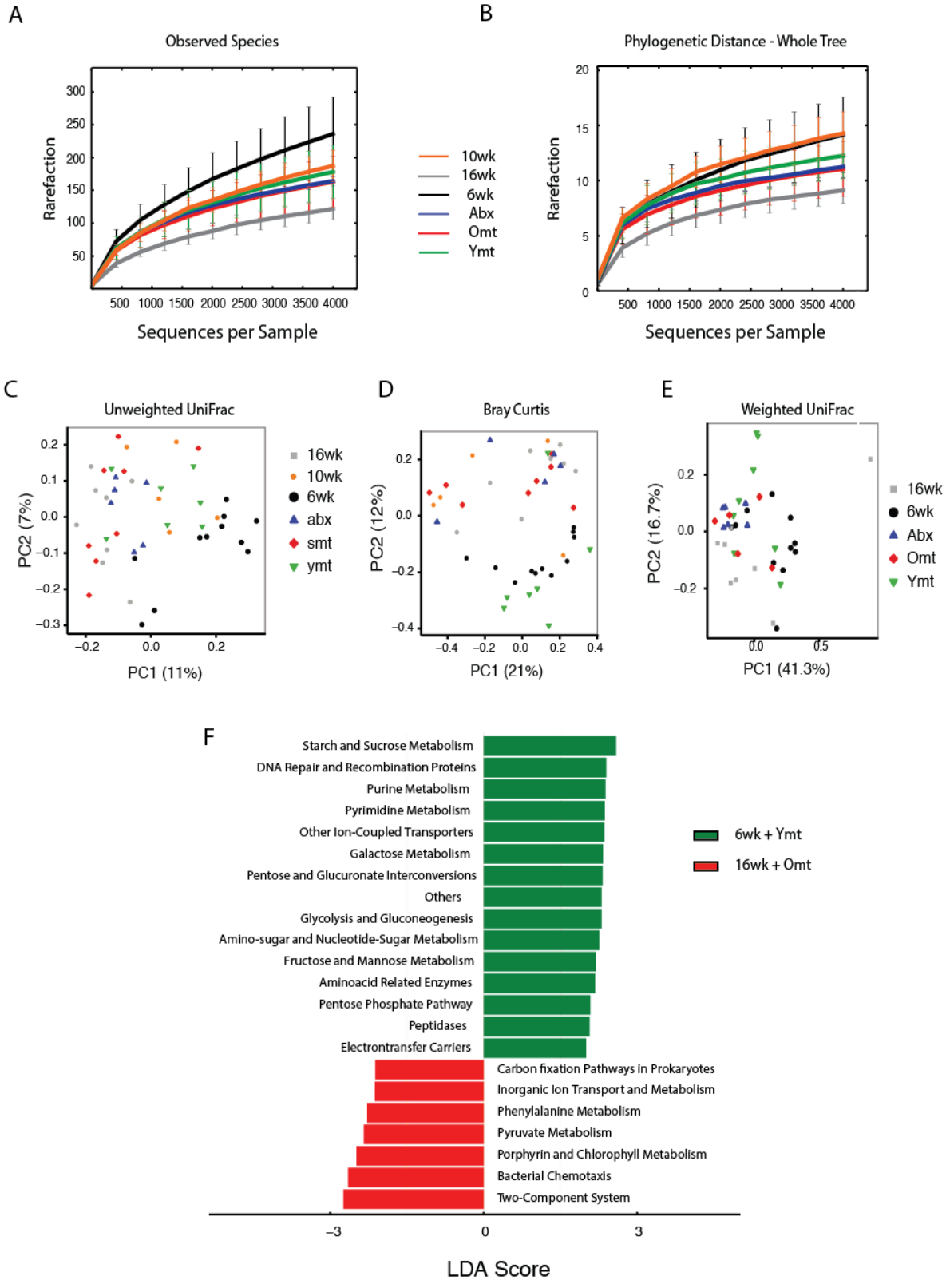

Figure S5. Related to Figure 5. Effects of gut microbiota transfer on OTU composition and metagenome

(A) Rarefaction curve for observed species alpha diversity at one week post-transfer shows no difference in transfer groups at one-week post transfer. (B) Rarefaction curve for phylogenetic distance (whole tree) alpha diversity at one week post-transfer shows no difference in transfer groups at one-week post transfer. (C) Unweighted UniFrac and Bray-Curtis (D) beta diversity distance at 1 week post transfer shows early segregation of young and Ymt away from old, Omt and Abx, while 10wk has an intermediate position. (E) Weighted UniFrac beta diversity at 16 weeks, i.e. 7 weeks post transfer. (F) Predicted metagenome function in 6wk and ymt (green) versus 16wk and smt (red) groups (LEfSe).

FIGURE S6

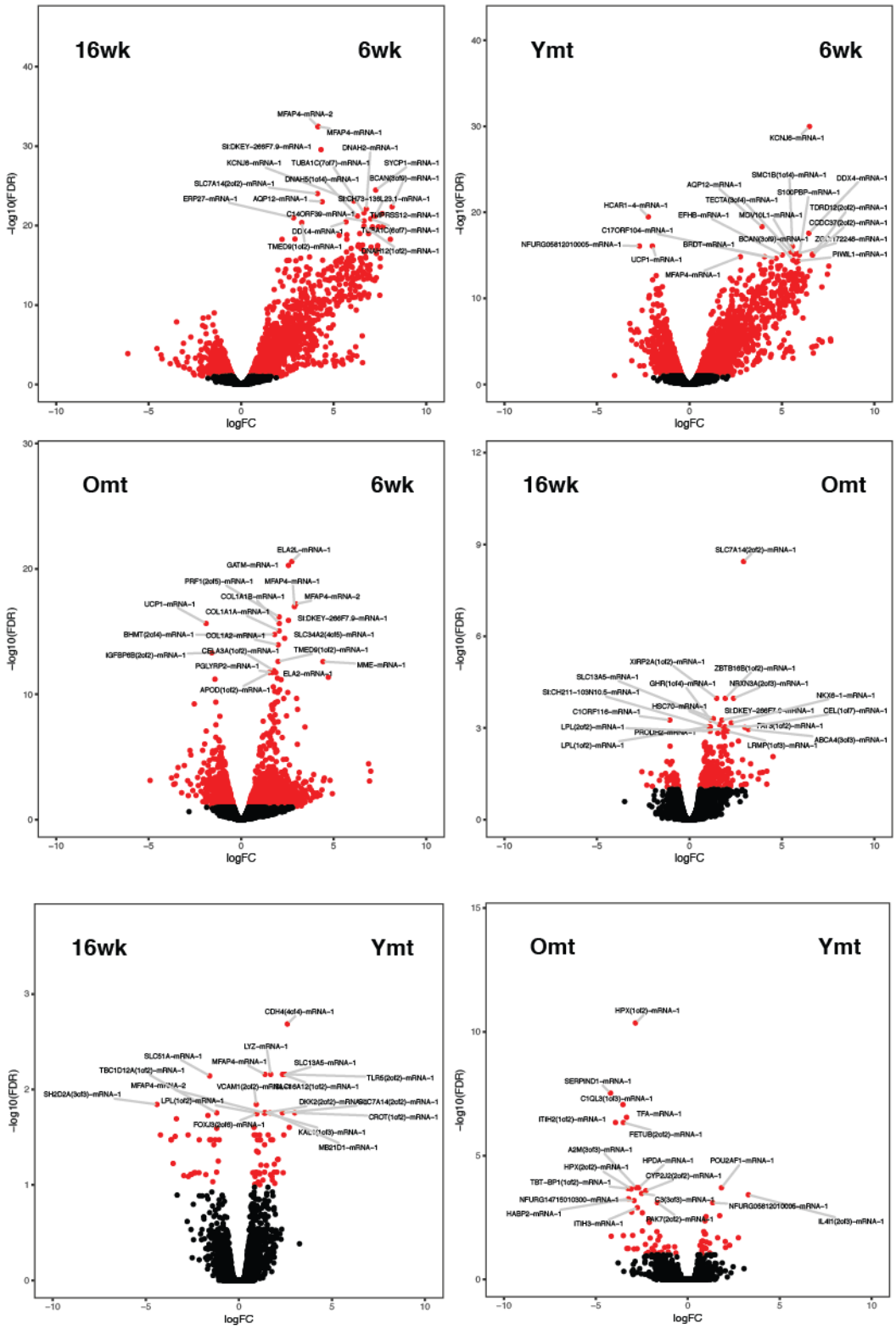

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Figure S6. Related to Figure 7. Volcano plots from the RNA-Seq data in the experimental groups

Differentially expressed genes between all the possible group to group comparisons. Genes belonging to the 0.999 quantile of the FDR (y) axis are shown.

FIGURE S7

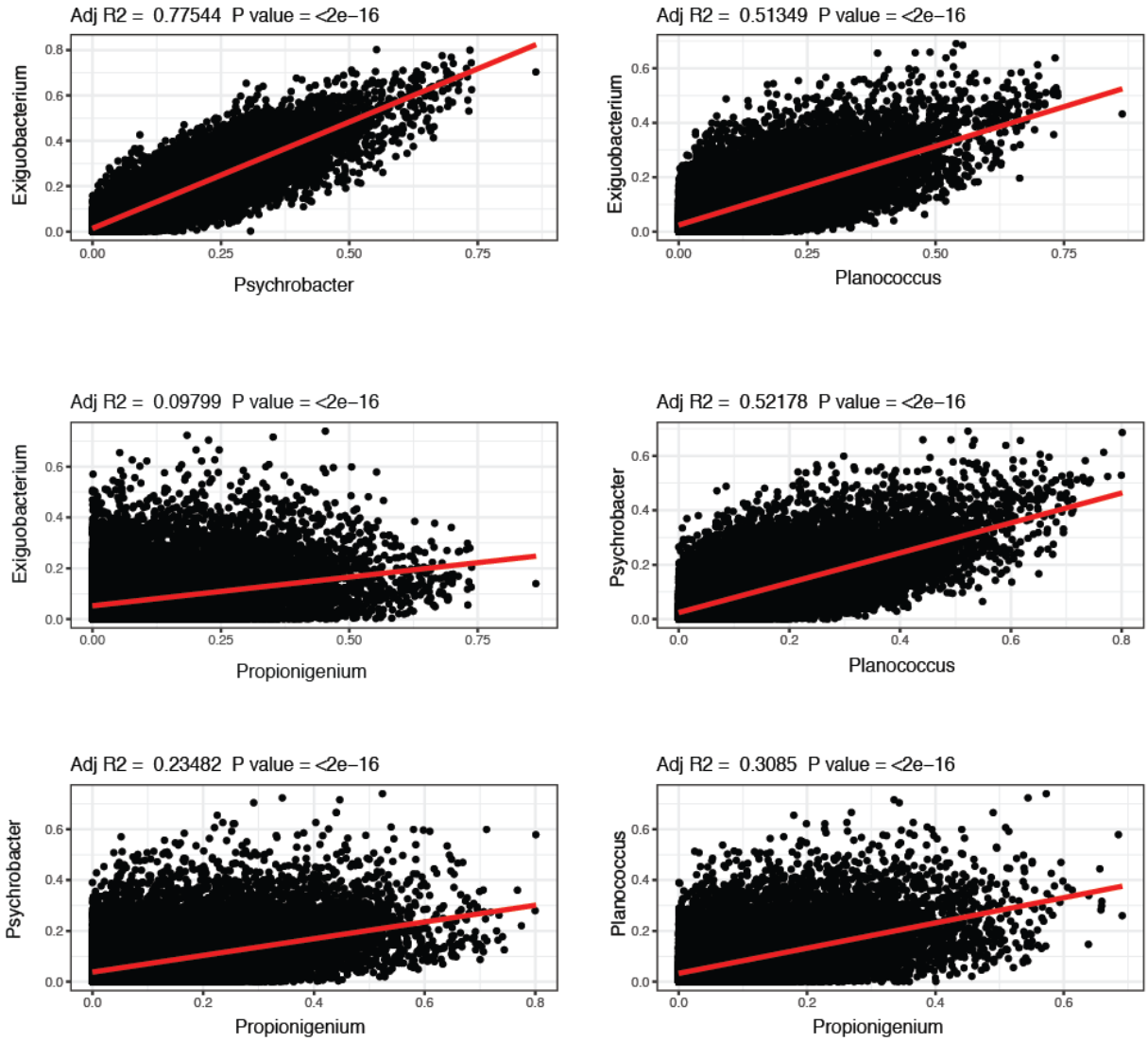

Figure S7. Related to Figure 7. OTU-transcript correlations between hub-genera.

Each plot represents on the x-axis the r-square values of the correlation between the bacterial genus indicated in the label and all gut transcripts (from Data S1), and on the y-axis the correlation coefficient (r-square) for the bacterial genus indicated on the label. Displayed are comparisons of hub bacteria in Ymt shared with 6wk.

1047 **Table S1A**

Ranked abundance of bacterial phyla shared with the turquoise killifish

	TK	Zebrafish	Mouse	Human
Proteobacteria	1	1	4	4
Firmicutes	2	5	2	1
Actinobacteria	3	6	5	3
Bacteroidetes	4	4	1	2
Fusobacteria	5	2	NA	10
Cyanobacteria	6	9	7	8
Planctomycetes	7	7	NA	NA
Chloroflexi	8	12	NA	NA
Verrucomicrobia	9	8	6	7
TM7	10	11	8	9
Acidobacteria	11	13	NA	11
SBR1093	12	NA	NA	NA
Euryarchaeota	13	NA	NA	6
Armatimonadetes	14	NA	NA	12
Spirochaetes	15	NA	NA	NA
TM6	15	NA	NA	NA
Nitrospirae	16	10	NA	NA
Tenericutes	16	3	3	5
[Thermi]	17	15	9	NA
Chlamydiae	18	14	NA	NA
WPS-2	19	NA	NA	NA

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Table S1B
Relative phylum abundance. A: Archaea; B: Bacteria

	Human	TK	Mouse	Zebrafish
Crenarchaeota (A)	0.0	0.0	0.0	1.33E-06
Euryarchaeota (A)	2.35E-03	6.06E-06	0.0	0.0
Acidobacteria (B)	2.33E-07	1.63E-05	0.0	1.86E-05
Actinobacteria (B)	2.59E-02	9.43E-02	1.59E-03	6.05E-03
Armatimonadetes (B)	1.17E-07	5.13E-06	0.0	0.0
Bacteroidetes (B)	2.13E-01	1.15E-02	5.00E-01	7.00E-02
Chlamydiae (B)	0.0	9.33E-07	0.0	9.30E-06
Chlorobi (B)	2.33E-07	0.0	0.0	1.33E-06
Chloroflexi (B)	0.0	2.42E-04	0.0	2.13E-05
Cyanobacteria (B)	3.82E-05	4.47E-03	2.74E-05	8.24E-05
Deferribacteres (B)	0.0	0.0	5.34E-03	0.0
Firmicutes (B)	7.40E-01	2.02E-01	4.71E-01	7.09E-03
Fusobacteria (B)	5.02E-06	8.60E-03	0.0	2.05E-01
Gemmatimonadetes (B)	1.17E-07	0.0	0.0	0.0
Lentisphaerae (B)	2.31E-05	0.0	0.0	0.0
Nitrospirae (B)	0.0	1.87E-06	0.0	6.51E-05
OD1 (B)	0.0	0.0	0.0	1.33E-06
Planctomycetes (B)	0.0	1.04E-03	0.0	2.34E-04
Proteobacteria (B)	9.07E-03	6.78E-01	4.75E-03	5.49E-01
SBR1093 (B)	0.0	9.33E-06	0.0	0.0
SR1 (B)	0.0	0.0	0.0	0.0
Spirochaetes (B)	0.0	4.20E-06	0.0	0.0
Synergistetes (B)	4.03E-05	0.0	0.0	0.0
TM6 (B)	0.0	4.20E-06	0.0	0.0
TM7 (B)	1.03E-05	3.36E-05	1.30E-05	2.92E-05
Tenericutes (B)	6.99E-03	1.87E-06	1.75E-02	1.62E-01
Verrucomicrobia (B)	2.06E-03	8.49E-05	2.88E-05	1.35E-04
WPS-2 (B)	0.0	4.66E-07	0.0	0.0
[Thermi] (B)	0.0	1.40E-06	7.20E-06	3.98E-06

Table S2A
Collection points of the wild fish populations

Abbreviation	Strain name	GPS coordinates
-	Z-GNP#00	S21˚ 48.8724’ E31˚ 55.9332’
Z	Z-GNP#01	S21˚ 48.198’ E31˚ 55.2306’
-	Z-GNP#02	S21˚ 48.1836’ E31˚ 55.2342’
-	Z-GNP#03	S21˚ 48.1512’ E31˚ 55.2756’
-	Z-GNP#04	S21˚ 46.4358’ E31˚ 52.9494’
M1	M-PNB#01	S22˚ 33.2778’ E32˚ 43.635’
M2	M-LNP#01	S24˚ 2.2848’ E32˚ 26.3592’
-	M-LNP#02	S24˚ 8.4156’ E32˚ 15.0816’
-	M-LNP#03	S24˚ 15.0756’ E32˚ 28.0428’

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Table S2B
Ecological factors associated with wild fish populations

Location	Altitude (m)	Size (m2)	Conductivity (microS per cm2)	Water temp. (C)	Turbidity (1 highest, 4 lowest)	Maximum pool depth (cm)	Littoral vegetation (coverage in %)
M_PNB#01	105.5	400	80	23	4	30	0
M_LNP#01	79.5	250	330	33.7	2	20	100
M_LNP#02	119.5	280	140	34	3	100	5
M_LNP#03	49.6	3500	35	29	2	50	100
Z_GNP#00	320.3	NA	NA	NA	NA	NA	NA
Z_GNP#01	324.6	NA	NA	NA	NA	NA	NA
Z_GNP#02	325.4	NA	NA	NA	NA	NA	NA
Z_GNP#03	326.3	NA	NA	NA	NA	NA	NA
Z_GNP#04	339.6	NA	NA	NA	NA	NA	NA

Table S3A

Transfer to 9.5 week-old fish					
Fish ID	**Days**	**Omt**	**Ymt**	**wt**	**Abx**
399	113			1	
400	115			1	
401	79			1	
402	115			1	
403	101			1	
404	85			1	
405	115			1	
550	52			1	
553	132			1	
558	75			1	
568	87			1	
571	125			1	
576	69			1	
579	125			1	
602	107			1	
562	129			1	
567	125			1	
703	81			1	
705	120			1	
707	141			1	
708	132			1	
709	145			1	
747	142			1	
2	70			1	
699	72			1	
696	90			1	
703	79			1	
746	102			1	
927	119			1	
909	67			1	
918	126			1	
929	112			1	
910	145			1	
911	147			1	
912	116			1	
931	160			1	
914	89			1	
932	150			1	
916	90			1	
924	130			1	

933	131		1	
925	155		1	
922	130		1	
923	137		1	
1	76		1	
917	80		1	
943	78		1	
906	77		1	
930	107		1	
926	76		1	
944	109		1	
904	98		1	
905	70		1	
939	98		1	
308	145			1
333	105			1
317	150			1
386	135			1
400	125			1
415	171			1
486	109			1
500	90			1
407	130			1
695	125			1
697	148			1
701	123			1
702	132			1
704	143			1
706	160			1
750	132			1
698	81			1
749	79			1
748	115			1
1278	72			1
1299	90			1
1100	72			1
379	93	1		
380	147	1		
381	115	1		
382	80	1		
383	157	1		
386	183	1		
387	168	1		

388	122	1	
389	168	1	
391	91	1	
392	75	1	
393	71	1	
394	130	1	
396	180		1
397	165		1
395	141		1
504	82	1	
505	118	1	
506	161	1	
507	107	1	
508	160	1	
511	77	1	
512	165	1	
513	84	1	
516	172		1
519	93	1	
520	123		1
521	135	1	
522	169		1
523	143		1
524	166		1
525	152	1	
518	105		1
517	83	1	
527	126	1	
641	140	1	
640	157	1	
625	146		1
626	153	1	
621	169		1
624	160		1
629	159	1	
634	147	1	
642	169		1
643	167		1
646	140		1
623	134	1	
670	91	1	
636	85		1
622	89	1	

633	114	1		
630	109	1		
637	100	1		
639	101		1	
638	115		1	
631	132	1		
644	76	1		
628	65	1		
648	149		1	
620	174		1	
626b	158		1	
348	180		1	
361	174		1	
320	174		1	
399	155		1	
401	145		1	
410	124		1	
417	134		1	
411	195		1	
466	154		1	
501	92		1	
1276	89	1		
1277	91	1		
1018	81	1		
1058	82	1		
1017	96		1	
1011	90	1		
1040	83	1		
1010	86		1	
1016	108	1		
1401	147			1
1405	138			1
1404	138			1
1421	148			1
1408	165			1
1428	168			1
1417	86			1
1420	86			1
1411	63			1
1424	86			1
1432	81			1
1410	113			1
1431	101			1

1427	92			1
1415	122			1
1423	108			1
14.02	150			1
1433	152			1
1426	145			1
1668	83		1	
1674	151		1	
1677	127		1	
1696	118		1	
1725	132		1	
1750	135		1	
1573	130		1	
1570	114		1	
1572	107		1	
1545	171		1	
1558	99		1	
1562	85		1	
1563	124		1	
1675	131		1	
1672	113		1	
1673	85	1		
1646	66	1		
1645	70	1		
1659	66	1		
1662	116	1		
1647	78	1		
1688	155	1		
1690	133	1		
1672	106	1		
1582	108	1		
1702	90	1		
1726	94	1		
1727	99	1		
1730	69	1		
1580	115	1		
1721	88	1		
1722	115	1		
1731	106	1		
1772	105			1
1729	99			1
1679	67			1
1703	152			1

1694	95				1
1680	86				1
1686	140				1
1708	114				1
1665	121				1
1547	116				1
1551	106				1
1728	72				1
1574	118				1
1580	114				1
1786	69			1	
1724	84			1	
1803	89			1	
1643	154			1	
1785	107			1	
1798	88			1	
1666	115			1	
1664	140			1	
1667	122			1	
1773	88			1	
1792	115			1	
1600	73			1	
1687	95			1	
1609	82			1	
1702	136			1	
1687	95			1	
1648	128			1	

Transfer to 6-week old fish

Fish ID	Days	Abx	wt	Old	6wk
1723	99				1
1640	101				1
1643	58				1
1716	72				1
1642	60				1
1769	82				1
1710	85				1
1720	84				1
1637	143				1
1717	83				1
1712	77				1
1715	79				1
1718	72				1

1638	130			1
1634	133			1
1635	133			1
1714	85			1
1773	93			1
1644	105			1
1641	103			1
1636	128			1
1639	109			1
1652	71	1		
1704	58	1		
1655	60	1		
1706	46	1		
1701	82	1		
1656	105	1		
1653	98	1		
1699	82	1		
1713	98	1		
1703	72	1		
1707	80	1		
1771	101	1		
1657	157	1		
1658	154	1		
1654	148	1		
1649	149	1		
1705	116	1		
1650	137	1		
1709	108	1		
1651	106	1		
1802	105		1	
1806	100		1	
1776	59		1	
1797	75		1	
1793	79		1	
1778	78		1	
1807	97		1	
1800	95		1	
1779	93		1	
1784	130		0	
1777	130		0	
1775	130		0	
1774	130		0	
1734	118		1	

1736	91	1	
1757	75	1	
1492	160		1
1533	133		1
1508	121		1
1495	120		1
1494	121		1
1487	116		1
1482	105		1
1490	102		1
1496	93		1
1547	95		1
1481	91		1
1480	92		1
1486	93		1
1479	76		1
1513	85		1
1491	74		1
1523	65		1
1485	49		1
1483	81		1
1489	55		1
1507	65		1
1503	135		1
1484	135		1
1493	135		1
1569	76		1

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Table S3B
Spontaneous Locomotor Activity (cm/20 min)

6wk				abx		Omt		ymt	
6wk	**-1wk**	**+1wk**	**16wk**	**+1wk**	**16wk**	**+1wk**	**16wk**	**+1wk**	**16wk**
4958	6709	3906	4220	3233	1501	7562	1681	9453	7321
6543	6130	3888	2674	1820	1500	5790	2886	7927	5219
1616	5089	3103	1397	5363	1889	4732	3182	3420	2638
7333	8300	4173	1417	7318	1786	4195	2794	6193	5345
3906	2254	3539	2934	2904	2105	7150	2219	3350	4346
13379	1263	5829	2804	1604	3235	12754	3390	5918	3222
8103	6187	1889	1437	2246	1680	1491	2340	3241	3371
10103	2643	6666	3334	7787	1732	6120	2504	3215	6643
7893	7777	7890	2282	6797	2389	4248	2483	10147	11626
8872	9121	5633	2719		4218	3925	2081	1847	5389
			2665			9656	2240	1662	7827
			1751				8987		3932
			2048				2485		10547
			6278				1445		3971
							2014		5912
							1159		6095
							5833		6042
							1729		1515
							3528		8767
							3837		6528
							8116		4341
							3190		4522
									1580

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Table S4

Beta diversity significance at one week post-transfer

Unweighted Unifrac	6wk	10wk	16wk	Ymt	Abx	Omt
6wk	-	0.014	0.001	0.004	0.001	0.002
10wk	0.014	-	0.002	0.016	0.045	0.059
16wk	0.001	0.002	-	0.003	0.05	0.242
Ymt	0.004	0.016	0.003	-	0.017	0.032
Abx	0.001	0.045	0.05	0.017	-	0.584
Omt	0.002	0.059	0.242	0.032	0.584	-

Bray-Curtis	6wk	10wk	16wk	Ymt	Abx	Omt
6wk	-	0.013	0.001	0.009	0.009	0.001
10wk	0.013	-	0.142	0.009	0.465	0.792
16wk	0.001	0.142	-	0.003	0.046	0.254
Ymt	0.009	0.009	0.003	-	0.007	0.007
Abx	0.009	0.465	0.046	0.007	-	0.387
Omt	0.001	0.792	0.254	0.007	0.387	-

Table S5
Significant genera from the regression between lifespan and genus abundance

Genus	P value	R-square	Adjusted R-square
Enterococcus	0.003706776	0.992600188	0.988900282
Plesiomonas	0.004338397	0.991342028	0.987013041
Aliivibrio	0.010432989	0.979242869	0.968864304
Leucobacter	0.012433899	0.975286804	0.962930206
Myroides	0.015826932	0.968596627	0.952894941
Jeotgalicoccus	0.018012821	0.96429882	0.94644823
Marinilactibacillus	0.022672523	0.955168996	0.932753495
Exiguobacterium	0.023563541	0.953428159	0.930142239
Leuconostoc	0.025705124	0.949250506	0.923875758
Psychrobacter	0.031568982	0.937858637	0.906787955
Paracoccus	0.035230192	0.930780783	0.896171174
Pseudoalteromonas	0.036045637	0.929208013	0.89381202
Arcobacter	0.043303847	0.915267529	0.872901293
Arthrobacter	0.04988702	0.902714674	0.854072011
Microbacterium	0.051662884	0.899343285	0.849014928
Chryseobacterium	0.057383533	0.888525803	0.832788705
Halomonas	0.060114797	0.883384194	0.825076291
Rhodobacter	0.06169409	0.880417981	0.820626972
Planococcus	0.062524949	0.878859472	0.818289208
Propionigenium	0.071110977	0.862834817	0.794252226
Brachybacterium	0.073041022	0.859252947	0.788879421
Dietzia	0.073041022	0.859252947	0.788879421
Microbispora	0.073041022	0.859252947	0.788879421
Fluviicola	0.073041022	0.859252947	0.788879421
Sphingobacterium	0.073041022	0.859252947	0.788879421
Brochothrix	0.073041022	0.859252947	0.788879421
Planomicrobium	0.073041022	0.859252947	0.788879421
Salinicoccus	0.073041022	0.859252947	0.788879421
Psychrilyobacter	0.073041022	0.859252947	0.788879421
Nitratireductor	0.073041022	0.859252947	0.788879421
Ruegeria	0.073041022	0.859252947	0.788879421
Chitinilyticum	0.073041022	0.859252947	0.788879421
Pseudidiomarina	0.073041022	0.859252947	0.788879421
Serratia	0.073041022	0.859252947	0.788879421
Proteiniclasticum	0.088315186	0.831169199	0.746753799
Carnobacterium	0.098165569	0.813305341	0.719958011
Flavobacterium	0.155189663	0.713704505	0.570556758
Acinetobacter	0.207527739	0.628012284	0.442018426
Citrobacter	0.211183494	0.62223148	0.433347221

Agrobacterium	0.236396917	0.583089668	0.374634502
Morganella	0.301990408	0.48721739	0.230826085
Lactococcus	0.349691759	0.422900808	0.134351212
Delftia	0.366005293	0.401949288	0.102923933
Vibrio	0.382646818	0.381124952	0.071687427
Mycobacterium	0.399926465	0.360088247	0.040132371
Pseudomonas	0.403033181	0.356369383	0.034554075
Propionibacterium	0.421686013	0.334447068	0.001670602
Leptothrix	0.427214853	0.328082824	-0.007875764
Salinivibrio	0.44411694	0.309005976	-0.036491036
Enhydrobacter	0.445172896	0.307833115	-0.038250327
Sphingomonas	0.473719587	0.276971074	-0.08454339
Photobacterium	0.517885801	0.232434101	-0.151348849
Vogesella	0.585140856	0.17210811	-0.241837836
Proteus	0.586163383	0.171260746	-0.243108881
Micrococcus	0.587043396	0.170533157	-0.244200265
Marinobacter	0.605497745	0.155632029	-0.266551957
Stenotrophomonas	0.610275233	0.151885394	-0.272171909
Granulicatella	0.623679614	0.141617033	-0.28757445
Bacillus	0.637296719	0.13155367	-0.302669495
Ochrobactrum	0.678456568	0.103390179	-0.344914732
Streptococcus	0.699081754	0.090551791	-0.364172313
Shewanella	0.704707995	0.087197368	-0.369203948
Klebsiella	0.715529319	0.080923568	-0.378614648
Staphylococcus	0.734480665	0.070500517	-0.394249224
Corynebacterium	0.761593239	0.056837784	-0.414743324
Vagococcus	0.922407082	0.006020661	-0.490969009
Aequorivita	0.926772679	0.00536224	-0.491956639
Brumimicrobium	0.937474235	0.003909471	-0.494135793
Candidatus Portiera	0.964841828	0.001236097	-0.498145854
Arenibacter	0.966292401	0.001136202	-0.498295697
Gelidibacter	0.966292401	0.001136202	-0.498295697
Winogradskyella	0.966292401	0.001136202	-0.498295697
Natronobacillus	0.966292401	0.001136202	-0.498295697
Clostridiisalibacter	0.966292401	0.001136202	-0.498295697
Peptoniphilus	0.966292401	0.001136202	-0.498295697
Limnobacter	0.966292401	0.001136202	-0.498295697
Neisseria	0.966292401	0.001136202	-0.498295697
Oceanimonas	0.966292401	0.001136202	-0.498295697
Alkalimonas	0.966292401	0.001136202	-0.498295697
Rheinheimera	0.966292401	0.001136202	-0.498295697
Providencia	0.998183343	3.30E-06	-0.49999505

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Table S6

Network hubs of OTU-based networks

Bacterial genus name	6wk	16wk	Ymt	Abx	Omt
Corynebacterium	YES	NO	NO	NO	NO
Dietzia	YES	NO	NO	NO	NO
Microbacterium	YES	NO	NO	NO	NO
Exiguobacterium	YES	NO	YES	NO	NO
Enterococcus	YES	NO	NO	NO	NO
Paracoccus	YES	NO	NO	NO	NO
Rhodobacter	YES	NO	NO	NO	NO
Ruegeria	YES	NO	NO	NO	NO
Photobacterium	YES	NO	NO	NO	NO
Arthrobacter	YES	NO	NO	NO	NO
Chryseobacterium	YES	NO	NO	NO	NO
Planococcus	YES	NO	YES	NO	NO
Carnobacterium	YES	NO	NO	NO	NO
Propionigenium	YES	NO	YES	NO	NO
Halomonas	YES	NO	NO	NO	NO
Psychrobacter	YES	NO	YES	NO	NO
Propionibacterium	NO	YES	YES	YES	NO
Delftia	NO	YES	YES	YES	NO
Vibrio	NO	YES	NO	NO	YES
Lactococcus	NO	YES	NO	YES	NO
Vogesella	NO	YES	NO	NO	YES
Citrobacter	NO	YES	YES	YES	NO
Morganella	NO	YES	NO	NO	NO
Acinetobacter	NO	YES	NO	YES	NO

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Table S7
Best hits from zebrafish pBlast on DEGs not annotated in the Valenzano et al. 2015 genome paper

Comparison	Group	Gene name	pBlast D.rerio best hit
16wk versus 6wk	6wk	MFAP4-mRNA-1	MFAP4
	6wk	MFAP4-mRNA-2	MFAP4
	6wk	C14ORF39-mRNA-1	-
	16wk	NFURG05812010005-mRNA-1	HAMP
16wk versus Ymt	Ymt	MFAP4-mRNA-1	MFAP4
	Ymt	MFAP4-mRNA-2	MFAP4
	16wk	NFURLNR02806021000-RNA-1	-
	16wk	NFURLNR02791010010-RNA-1	-
Omt versus Ymt	Ymt	POU2AF1-mRNA-1	POU2AF1
	Ymt	IL4I1(2of3)-mRNA-1	IL4I1
	Ymt	NFURG05812010005	HAMP
	Omt	TBT-BP1(1of2)-mRNA-1	-
	Omt	NFURG14715010300-mRNA-1	-
Omt + 16wk versus Ymt	Ymt	NFURG05812010005-mRNA-1	HAMP
	Ymt	IL4I1(2of3)-mRNA-1	IL4I1
	Ymt	IL5RA-mRNA-1	IL2RGA
	Ymt	POU2AF1-mRNA-1	POU2AF1
	Omt+16wk	DAZ2-mRNA-1	-
Omt+16wk vs. 6wk+Ymt	6wk+Ymt	MFAP4-mRNA-1	MFAP4
	6wk+Ymt	MFAP4-mRNA-2	MFAP4
	Omt+16wk	DAZ2-mRNA-1	-
Omt vs 6wk	Omt	TSPAN8(1of4)	TSPAN25,CD53
	Omt	NFURG05812010005-mRNA-1	HAMP
	6wk	MFAP4-mRNA-1	MFAP4
	6wk	MFAP4-mRNA-2	MFAP4
Ymt vs 6wk	Ymt	NFURG05812010005-mRNA-1	HAMP
	Ymt	F11-mRNA-1	-
	6wk	S100PBP-mRNA-1	KOP / ASKOPOS