Wild gut microbiome suppresses the opportunistic pathogen *Aeromonas* in medaka under domesticated rearing conditions

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Abstract

Background

The gut microbiome plays a crucial role in the metabolic health and pathogen resistance of various host animals. It is also well established that external environmental factors can influence the gut microbiome, leading to differences in its composition. However, the functional implications of these differences remain poorly understood. This study examined the gut microbiome of medaka (*Oryzias latipes* species complex) by comparing domesticated and wild populations, with the aim of gaining insights into the functional significance of their specific characteristics, particularly those of the wild-type microbiome.

Results

For the comparative analysis of the gut microbiome, 48 domesticated and 122 wild medaka were collected from multiple laboratories, pet stores and streams across Japan. The results showed that wild medaka exhibited significantly higher gut microbiome diversity, with a broader range of bacterial members. In contrast, the gut microbiome of domesticated medaka harbored lower microbial diversity and was consistently dominated by *Aeromonas*, a typical opportunistic pathogen in fish. Additionally, 88.6% of *Aeromonas* isolates from domesticated medaka exhibited haemolytic activity. Moreover, a domesticated rearing experiment with wild populations showed no proliferation or dominance of *Aeromonas* in their gut, as observed in domesticated medaka. A further rearing experiment revealed that pre-exposing antibiotic-treated medaka to sediments from their natural habitats prevented *Aeromonas* colonisation, even when reared under domesticated conditions.

Conclusions

These findings suggest that the habitat-derived wild gut microbiome can inhibit *Aeromonas* proliferation in domesticated fish, highlighting its potential to mitigate opportunistic diseases in aquaculture.

53 **Background**

54 Many animals host various microorganisms in their intestines, collectively known as the gut
55 microbiome [1]. These microbial communities play a crucial role in host physiology by
56 influencing metabolism, immune function and adaptation to environmental conditions [2-4]. For
57 example, woodrats (*Neotoma* spp.) rely on their gut microbiota to detoxify toxic desert plants,
58 enabling their survival in resource-limited environments [5]. Termites depend on their gut
59 microbiota to degrade indigestible lignin and cellulose in wood, allowing them to exploit highly
60 specialized ecological niches [6]. Similarly, the fruit fly (*Bactrocera dorsalis*) utilizes its gut
61 microbiota to recycle urea, a nitrogenous waste, for amino acid synthesis, supporting its
62 adaptation to nitrogen-poor habitats [7]. These examples highlight how gut microbiota perform
63 specialized functions linked to diet and habitat, profoundly influencing host physiology and
64 ecology.

65 Artificial rearing conditions, however, can induce substantial shifts in the gut
66 microbiome due to altered diets, antibiotic use, decreased exposure to environmental microbial
67 reservoirs and increased human interaction [8, 9]. Studies comparing animals in nature or reared
68 in artificial environments have documented changes such as reduced microbial diversity, shifts in
69 dominant taxa, and the loss of environmental microbial inputs [8]. However, the extent of
70 microbiome changes varies across species. Some exhibit marked reductions in microbial diversity,
71 leading to dysbiotic and imbalanced microbiota compared to their wild counterparts [10, 11],
72 whereas others retain a significant portion of their wild-type microbiota, demonstrating resilience
73 to artificial rearing conditions [12, 13]. Although some studies suggest that such alterations may
74 compromise immune function and increase disease susceptibility [14, 15], the functional
75 consequences of gut microbiome induced by artificial rearing conditions shifts remain unclear.

76 Fish gut microbiomes are similarly shaped by environmental factors such as diet, salinity,
77 and temperature [16, 17]. Wild fish generally harbor more diverse and complex gut microbiomes,
78 compared to domesticated fish which often exhibit reduced microbial diversity due to controlled

diets, artificial rearing environments and limited exposure to environmental microbes [18-21]. The fish gut microbiota plays critical roles in development of the digestive tract, nutrient absorption, immune regulation, and disease resistance [22-24], with specific bacterial taxa contributing to metabolic and protective functions [16, 25]. For example, genera such as *Bacillus*, *Lactobacillus* and *Clostridium* are commonly found in fish gut microbiomes and suggested to be involved in nutrient cycling and pathogen defense [16, 25, 26]. Given these functions of gut microbiota and the fact that they are shaped by environmental factors, it can be inferred that different functional microbiomes are formed depending on the rearing environment and natural habitats. While studies in mammals and insects suggest that wild-derived microbiomes confer enhanced pathogen resistance [14, 27], this hypothesis has not been thoroughly investigated in fish.

In this study, we focused on medaka (*Oryzias latipes* species complex [28, 29]), a widely used experimental model and ornamental freshwater fish with readily available wild populations across Japan [30]. Domesticated medaka are typically reared in plastic or glass tanks with stable water temperatures (25°C–28°C), dechlorinated tap water, and commercially formulated artificial feed designed for rapid growth [31]. These controlled conditions contrast sharply with wild medaka habitats, which are typically muddy puddles with fluctuating temperatures and diverse food sources, including zooplankton, phytoplankton and small invertebrates [32, 33]. We performed a comparative analysis of the gut microbiota in medaka from artificial environments and natural habitats, aiming to clarify the distinct characteristics of the gut microbiota in domesticated and wild medaka, as well as explore the effect of domesticated rearing condition on wild gut microbiota and its potential role in disease risk.

Methods

Sample collection

We collected 48 domesticated (D) medaka from three laboratories and three pet stores (**Fig. 1; Table 1**). The D medaka groups included inbred/conserved lines (D1–D4 groups) and the orange–red commercial strain (himedaka, D5–D8 groups), with each group reared separately in independent tanks (**Table 1**). Three other small fish species, the guppy (*Poecilia reticulata*), goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*), were obtained from the same pet store where the D8 group was purchased. All D medaka and other fish were maintained using standardised methods [31]: feeding on fishmeal-based artificial foods in dechlorinated tap water in glass or plastic tanks, with dechlorination achieved via aeration or chemical dechlorinators. Specifically, the D5 group, which is the generations of the D6 group, was maintained in our laboratory under domesticated conditions: fish were kept at 25°C in dechlorinated tap water in plastic tanks with aeration under long day conditions (14 h of light per day) and fed fishmeal-based artificial foods (TetraMin; Spectrum Brands Inc., Madison, WI, USA). Ammonium and nitrite concentrations in the rearing water were monitored daily using pack tests (Kyoritsu Chemical Lab., Tokyo, Japan); if they exceeded the safe standards for fish (ammonium, 0.2 mg/L; nitrite, 0.01 mg/L), one-third of the rearing water was replaced with fresh dechlorinated tap water. All D groups were used for the gut microbiome analysis, and each group consisted of six individuals, including three females and three males. The D8 group was also used for the isolation of *Aeromonas* strains and Rearing Experiment 2 as described below.

The W medaka groups (W1–W16), consisting of 122 individuals, were caught using nets in 16 streams across 10 prefectures in Japan from summer to fall between 2014 and 2019 (**Fig. 1; Table 1**). The collected individuals were transported alive to the laboratory within two days and were used for dissection to analyse the gut microbiome (W1–W16 groups) or for the subsequent rearing experiment (W10–W13 groups, Rearing experiment 1 as described below). Stream sediments (S6, S12, S14 and S15) were also collected from the sites where the W6, W12,

W14 and W15 medaka groups were captured. The mud from the stream was scooped up with a shovel, and water-saturated sediment from a depth of 0-5 cm was collected. The sediment was then transported to the laboratory and stored at 4°C until used for Rearing Experiment 2 as described below.

All fish in D and W groups analysed were adults over 2 cm in length. Comprehensive approval for the experimental use of fish was granted by the institutional animal care and use committee. Sampling and experiments followed the 'Guidelines for the Use of Fishes in Research' established by the Ichthyological Society of Japan in 2003 (http://www.fish-isj.jp/english/guidelines.html).

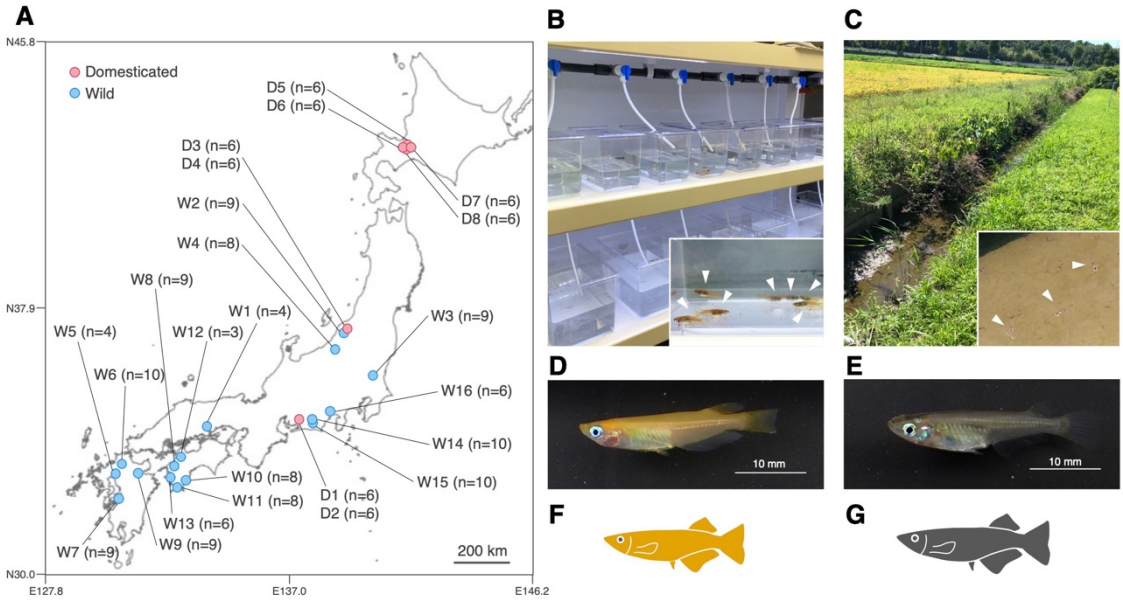


Fig. 1 Comparison of habitat and morphology between domesticated (D) and wild (W) groups of *Oryzias latipes* species complex in Japan. (A) Geographical locations of collection sites for D and W groups. Numbers in parentheses indicate the number of individuals used in the gut microbiome analysis, as shown in **Fig. 2**. Detailed information is summarized in **Table 1**. (B) Typical laboratory rearing conditions of the D groups. The inset photo shows medaka from the D groups (closed triangles). (C) Stream habitat of W groups. The inset photo showing wild medaka in a mud puddle (closed triangles). Photographs of the fish specimen in the D8 (D) and the W7 groups (E). Icons representing D medaka (F) and W medaka (G) used in subsequent figures. Map data sourced from the Geospatial Information Authority of Japan.

153 **Rearing Experiment 1: Domesticated rearing of wild medaka**

154 After the W10–W13 groups were transported alive from field sites to the laboratory within two
155 days, a subset of medaka from each group (n = 6 per group) was kept separately in a 10-L plastic
156 tank (15.3 × 27.8 × 16.5 cm) for 30 days under the same domesticated conditions as the D5 group,
157 as described above. Specifically, the tanks were maintained at 25°C with 8 L of dechlorinated tap
158 water under long day conditions and the individuals were fed 0.5 g of TetraMin flakes (Spectrum
159 Brands) per tank daily. Ammonium and nitrite concentrations in the rearing water were monitored
160 and managed as described above. After 30 days, the domestically reared wild medaka groups
161 (DW10–DW13) were dissected for gut microbiome analysis, as described below.

162

163 **Rearing Experiment 2: Domesticated rearing of medaka with transplanted environmental**
164 **microbes**

165 ***Step 1: Antibiotic treatment of medaka***

166 Two individuals from the D8 group were placed in pairs in a 250-mL sterile plastic flask with a
167 vented cap (ASONE, Osaka, Japan) containing 200 mL of sterile gnotobiotic zebrafish medium
168 (GZM) [34] with a 1% penicillin/streptomycin/amphotericin B mixture (FUJIFILM Wako Pure
169 Chemical Corporation) and 100 µL/mg erythromycin (Tokyo Chemical Industry Co., Tokyo,
170 Japan) for 24 h. After treatment, each medaka was rinsed three times in 200 mL of fresh sterile
171 GZM for 10 min to remove residual antibiotics. The treated medaka were then used in the next
172 step, i.e., for 'Step 2: Pre-rearing with stream sediments' as described below. Sterility was
173 confirmed using a culture-based method for germ-free zebrafish [34]. The guts were dissected,
174 homogenised in 100 µL of sterile GZM, diluted and spread on 1.5% agar plates containing Luria–
175 Bertani broth (Becton, Dickinson and Company, Sparks, MD, USA), Nutrient Broth (Becton,
176 Dickinson and Company) and R2A Broth (Nihon Pharmaceutical Co., Tokyo, Japan). After
177 incubation at 25°C for 3 days, colony-forming units (CFUs) were measured.

178 ***Step 2: Pre-rearing with stream sediments***

179 Initially, 200 g of each sediment (S6, S12, S14 and S15), consisting of water-saturated mud
180 collected from streams where wild medaka (W6, W12, W14 and W15 groups) were caught (**Table**
181 **1**), was added to 6 L of sterile dechlorinated tap water in separate 10-L plastic tanks (i.e., one tank
182 for each sediment type). After aerating the tanks at 25°C for one week, the antibiotic-treated
183 medaka, prepared as described above, were reared in these tanks for one week. Following this
184 period, the medaka gut, sediment, and rearing water were used for microbiome analyses. As a
185 control, the antibiotic-treated medaka were reared in 6 L of sterile dechlorinated tap water without
186 sediment for one week, with two separate tanks prepared for this condition.

187 ***Step 3: Domesticated rearing***

188 Medaka pre-reared with each sediment (DS6, DS12, DS14 and DS15 groups) or without sediment
189 (C1 and C2 groups) were rinsed in 200 mL of fresh sterile dechlorinated tap water three times for
190 10 min to remove residual sediment, and each group was then transferred to a dedicated 17-L
191 plastic tank (20.8 × 31.4 × 27.0 cm) with 10 L of rearing water routinely used for maintaining the
192 D5 group. The microbiome of this rearing water was also analysed. The medaka were reared for
193 three weeks under the same domesticated conditions as the D5 group, as described above, and
194 were then used for gut microbiome analysis.

195

196 **DNA extraction**

197 All fish were fasted for 1–3 days to remove transient gut contents and then anesthetised on ice.
198 After rinsing twice with ice-cold sterile distilled water, the entire gut was dissected using ethanol-
199 treated forceps and tweezers under a binocular microscope, transferred to a microcentrifuge tube
200 and homogenised with 180 µL of AL Buffer (Qiagen, Hilden, Germany) using a pestle. After 1 h
201 at 37°C with 20 µL of 20 mg/mL lysozyme (FUJIFILM Wako Pure Chemical Corporation, Osaka,
202 Japan), the homogenates were incubated overnight at 56°C with 20 µL of 20 mg/mL protease K
203 (Qiagen). The crude extract was purified using the QIAamp DNA Mini Kit (Qiagen). Water
204 samples (50–100 mL of rearing water) were first filtered through a 10 µm pore size membrane to

205 remove suspended debris, and then through a 0.22 μm pore size membrane to capture bacterial
206 cells. DNA extraction from the resulting membranes and sediment samples followed the same
207 method as for gut DNA extraction. All DNA samples were stored at −30°C until use.
208

209 **Diversity analysis of the microbiome**

210 The variable V4 region of the bacterial 16S rRNA gene was amplified using the universal primer
211 set 515F with Illumina P5 sequences and 806R with 12-base indexes and Illumina P7 sequences
212 [35], Q5 Hot Start High-Fidelity Master Mix (New England Biolabs, Beverly, MA, USA) and the
213 prepared DNA, as described previously [36]. Following 1.5% agarose gel electrophoresis using
214 Novel Juice dye (GeneDireX, Las Vegas City, NV, USA), appropriately sized PCR amplicons
215 were cut from the gels and purified using the Wizard SV Gel and PCR Clean-Up System (Promega,
216 Madison, WI, USA). Each purified DNA sample was quantified using a Qubit 2.0 Fluorometer
217 (Invitrogen, Carlsbad, CA, USA), diluted and mixed equally. Paired-end sequencing of the PCR
218 amplicon libraries was conducted with a PhiX control on a MiSeq or iSeq sequencing platform
219 (Illumina) using the MiSeq Reagent Kit v2 or iSeq 100 Reagent Kit (both Illumina), respectively,
220 following the manufacturer's instructions. Raw sequence preprocessing and quality filtering,
221 including paired-end sequence joining and removal of low-quality and chimeric sequences, were
222 performed as previously described [37]. Taxonomic assignment was performed using the RDP
223 classifier ver. 2.13 [38] with a 50% confidence threshold.
224

225 **Quantitative analysis of the microbiome**

226 Quantitative PCR (qPCR) was performed to amplify the V4 region of the bacterial 16S rRNA
227 gene using the universal primers 515F and 806R, along with a LightCycler 96 System (Roche
228 Diagnostics, Basel, Switzerland) and THUNDERBIRD SYBR qPCR Mix (Toyobo, Otsu, Shiga,
229 Japan), following the manufacturers' instructions. The thermal cycling conditions were as
230 follows: initial denaturation at 95°C for 30 s, followed by 45 cycles at 95°C for 10 s, 60°C for 30

231 s and 72°C for 30 s. The gene copy number of the bacterial 16S rRNA gene was calculated from
232 a standard curve based on a dilution series of *Aeromonas hydrophila* NBRC13286 PCR products.
233 Additionally, the abundance of *Aeromonas* was calculated by multiplying the bacterial abundance
234 through this qPCR by the proportion of *Aeromonas* in gut microbiome obtained from the diversity
235 analysis described above.
236
237 **Isolation and haemolysis assay of *Aeromonas* strains from the medaka gut**
238 To examine the pathogenic potential of *Aeromonas* in the D medaka gut, we isolated *Aeromonas*
239 strains and subjected them to a haemolysis assay on blood agar. Whole guts were dissected from
240 D8 samples and homogenised in 100 μL of GZM using a pestle. The homogenates were diluted
241 with GZM and spread on Trypticase soy agar containing 5% sheep blood (Becton, Dickinson and
242 Company). After incubation at 25°C for 3 days, halo formation around the colonies was monitored
243 for haemolysis activity. Colonies were picked and subjected to 16S rRNA gene sequencing, as
244 described previously [39]. Haemolytic *Aeromonas* strains were cultured overnight in Luria–
245 Bertani medium at 27°C and washed with GZM. After adjusting the concentration to OD_{600} =
246 0.01 using GZM, 10 μL of the bacterial suspension was dropped onto Trypticase soy agar with
247 5% sheep blood (Becton, Dickinson and Company) and cultured at 27°C for 3 days. Haemolysis
248 activity was evaluated based on the diameter of the halo formed around the drop. Halo size was
249 measured using Fiji [40]. *Aeromonas hydrophilia* NBRC 13286 was used as the control in the
250 haemolysis assay.
251
252 **Statistical analysis**
253 Beta and alpha diversity were analysed by normalising read counts to match the sample with the
254 minimum read counts using MicrobiomeAnalyst 2.0 [41]. Beta diversity at the bacterial genus
255 level among the gut microbiota was assessed using the Bray–Curtis dissimilarity index.
256 Nonmetric multidimensional scaling (NMDS) was used for data visualization. Differences in beta

257 diversity were statistically evaluated using PERMANOVA with sample type (D vs W, W vs DW,
258 D5 vs DW or C vs DS groups), using 999 permutations to assess statistical significance. Alpha
259 diversity was calculated using the Chao1 and Shannon indices. To identify specific bacterial
260 groups in medaka under each condition, linear discriminant effect size (LEfSe) analysis was
261 conducted using MicrobiomeAnalyst 2.0 [41]. For pairwise comparisons, the alpha indices and
262 *Aeromonas* proportions/abundance in the gut between each pair of groups, as well as CFUs before
263 and after antibiotic treatment, were analysed using the Mann–Whitney U-test (package
264 "exactRankTests" in R). For comparisons across multiple group, the haemolytic activity of
265 *Aeromonas* strains was analysed using the Mann–Whitney U-test with Bonferroni correction.
266 These statistical comparisons were conducted using R studio 4.2.2. [42].
267
268 **Availability of nucleotide sequence data**
269 Illumina sequencing data from all medaka gut, sediment and water samples were available in the
270 DDBJ/GenBank/EBI databases under accession PRJNA1102999. Additionally, Sanger
271 sequencing data from isolated *Aeromonas* strains were available in the same databases under
272 accessions PP474260, PP474261 and PP549924–PP549964.
273
274 **Results**
275 **Domesticated and wild medaka gut microbiome profiles**
276 The NMDS plot based on the Bray–Curtis distance revealed a clear distinction in gut microbiome
277 community structures between domesticated and wild populations (PERMANOVA: $F = 42.043$,
278 $r^2 = 0.200$, $p < 0.05$; **Fig. 2A**). Alpha diversity indices, Chao1 and Shanonn indices, showed that
279 the D groups had lower microbiome richness and evenness compared with the W groups (**Fig.**
280 **2B,C**), indicating greater diversity in wild populations. Taxonomic analysis at the phylum level
281 revealed *Proteobacteria* as the dominant group in both D and W groups (**Fig. 2D**), whereas
282 *Fusobacteria* were also abundant in half of the D groups (D5–D8) (**Fig. 2D**). Depending on the

collection site, the W groups exhibited a more diverse microbiome, including phyla such as *Planctomycetes*, *Verrucomicrobia*, *Firmicutes*, *Chloroflexi*, *Acidobacteria* and *Actinobacteria*, which were less prominent in the D groups (**Fig. 2D**).

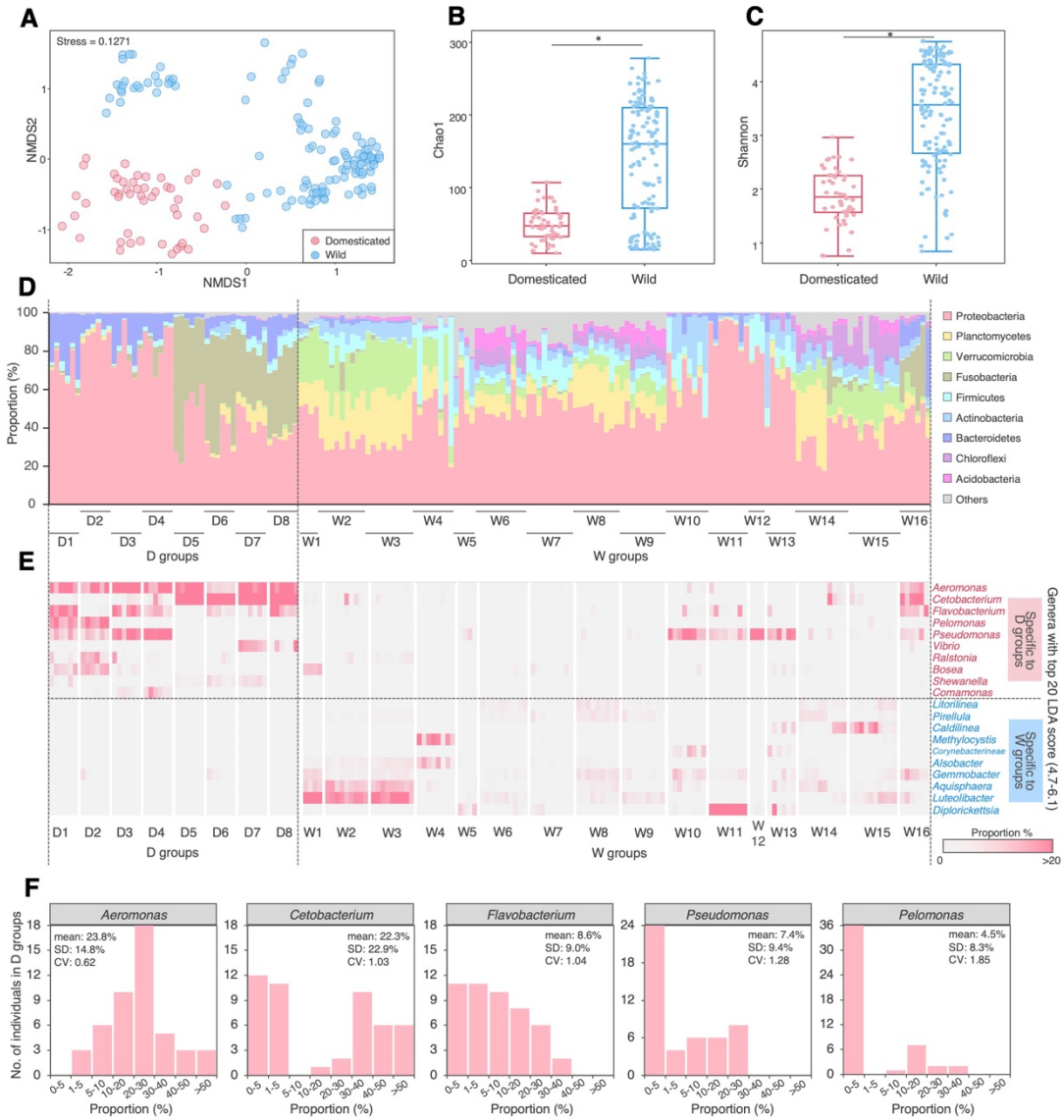


Fig. 2 Diversity and specificity of the gut microbiome of medaka between the D and W groups. (A) Nonmetric multidimensional scaling (NMDS) plot illustrating gut microbiome dissimilarity based on the Bray–Curtis distance. Microbial diversity comparison using Chao1 (B) and Shannon (C) indices. Asterisks indicate significant differences ($p < 0.05$, Mann–Whitney U-test). (D) Community structure of the gut microbiome according to bacterial phylum proportions. (E) Specific bacterial groups associated with the D or W groups, identified by LEfSe analysis, listing the top 10 genera with the highest LDA scores. (F) Histograms showing the top 5 groups in the D groups. SD, standard deviation; CV, coefficient of variation.

LEfSe analysis identified genera specific to each group. *Aeromonas*, *Cetobacterium* and

297 *Flavobacterium* were specific to the D groups, whereas *Diplorickettsia*, *Luteolibacter* and
298 *Aquishaera* were unique to W groups (**Fig. 2E; Supplementary Fig. S1**). The proportion of
299 *Cetobacterium* was high (>30%) in most D5–D8 groups (91.7%, 22/24 individuals), but lower
300 (<5%) in most D1–D4 groups (95.8%, 23/24 individuals) (**Fig. 2E,F**). In the W groups,
301 *Diplorickettsia* was predominantly found in the W11 group (33.7%–83.1%, 8 individuals) but
302 rarely in other W groups (0%–8.4%, 116 individuals) (**Fig. 2E**). *Aeromonas*, which had the highest
303 linear discriminant analysis (LDA) score and average proportion (**Supplementary Fig. S1A,B**),
304 was consistently detected in the D groups (**Fig. 2E**; **Supplementary Fig. S1B**), confirming it as
305 the most common genus in domesticated medaka. This is further supported by its lowest
306 coefficient of variation (CV) among the dominant genera in the D groups (**Fig. 2F**), indicating its
307 relatively stable abundance.
308

309 **Potentially pathogenic *Aeromonas* in the gut of domesticated fish**

310 *Aeromonas* was the dominant bacterial member in the gut of D groups (**Fig. 2E,F;**
311 **Supplementary Fig. S1B**), accounting for 4.2%–80.4% of the gut microbiome (mean ± SD =
312 23.8% ± 14.8%, n = 48; **Fig. 3A**). However, *Aeromonas* levels in the W groups were consistently
313 low (0%–8.6%; mean ± SD = 0.5% ± 1.2%, n = 116), except for the W16 group where they were
314 slightly higher (1.9%–15.8%; mean ± SD = 9.1% ± 4.5%, n = 6; **Fig. 3A**). Overall, *Aeromonas*
315 levels were significantly higher in the D groups compared with the W groups (**Fig. 3B**).
316 Quantitively, *Aeromonas* levels in the D groups ranged from 1.3×10^6 to 1.3×10^9
317 copies/individual (mean ± SD = $2.0 \pm 2.8 \times 10^8$ copies/individual, n = 48), whereas in the W
318 groups they were substantially lower (3.0×10^7 to 1.2×10^7; mean ± SD = $0.4 \pm 1.5 \times 10^6$
319 copies/individual, n = 116) (**Fig. 3C**). Both the proportion and quantity of *Aeromonas* were
320 significantly higher in the gut of D groups (**Fig. 3D**).

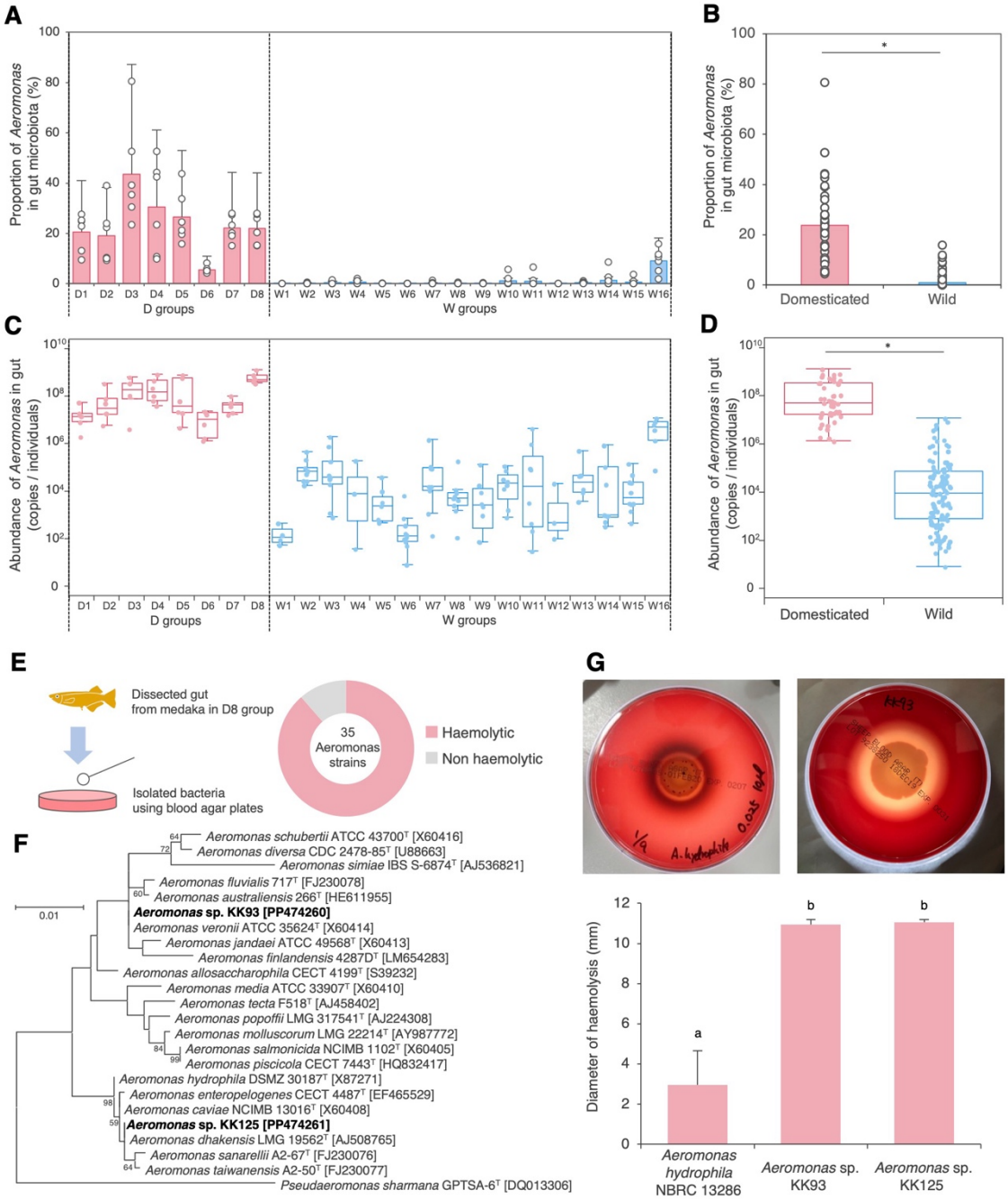


Fig. 3 Distribution and potential toxicity of *Aeromonas* in the medaka gut. (A) Proportion of *Aeromonas* in the gut microbiome of medaka samples in each group. (B) Average proportion of *Aeromonas* compared between the D and W groups. (C) Abundance of *Aeromonas* in the gut microbiome, estimated via amplicon sequencing and qPCR analyses. (D) Average abundance of *Aeromonas* compared between D and W medaka. The asterisks in (C) and (D) indicate statistically significant differences ($p < 0.05$, Mann–Whitney U-test). (E) Schematic overview of haemolytic bacterial strain isolation from the medaka gut in the D8 group, highlighting the composition of 35 *Aeromonas* strains. Isolates other than *Aeromonas* are summarised in **Supplementary table S1**. (F) Phylogenetic tree of *Aeromonas* strains isolated from the guts of domesticated medaka, based on 16S rRNA gene sequences. *Pseudaeromonas sharmana* GPTSA-6^T was used as the outgroup. Scale bar represents 0.01 substitutions per nucleotide position. (G) Haemolytic activity of *A. hydrophila* and medaka-derived *Aeromonas* strains, assessed via halo formation on blood agar (right photo: *A. hydrophila* NBRC 13286; left photo: *Aeromonas* sp. strain KK93). A bar graph displays the

mean with standard deviation from six replicates, with different letters indicating statistically significant differences ($p < 0.05$, Mann–Whitney U-test with Bonferroni correction).

Additionally, we analysed guppy (*Poecilia* sp.), goldfish (*Carassius* sp.) and zebrafish (*Danio rerio*) from the pet store as the D8 group. *Aeromonas* proportions in the gut microbiome were 5.0%–10.9% in guppy (mean ± SD = 7.5% ± 2.2%, n = 6), 10.4%–92.3% in goldfish (mean ± SD = 49.6% ± 20.6 %, n = 6) and 15.0%–27.9% in zebrafish (mean ± SD = 17.0% ± 5.8%, n = 6) (**Supplementary Fig. S2**).

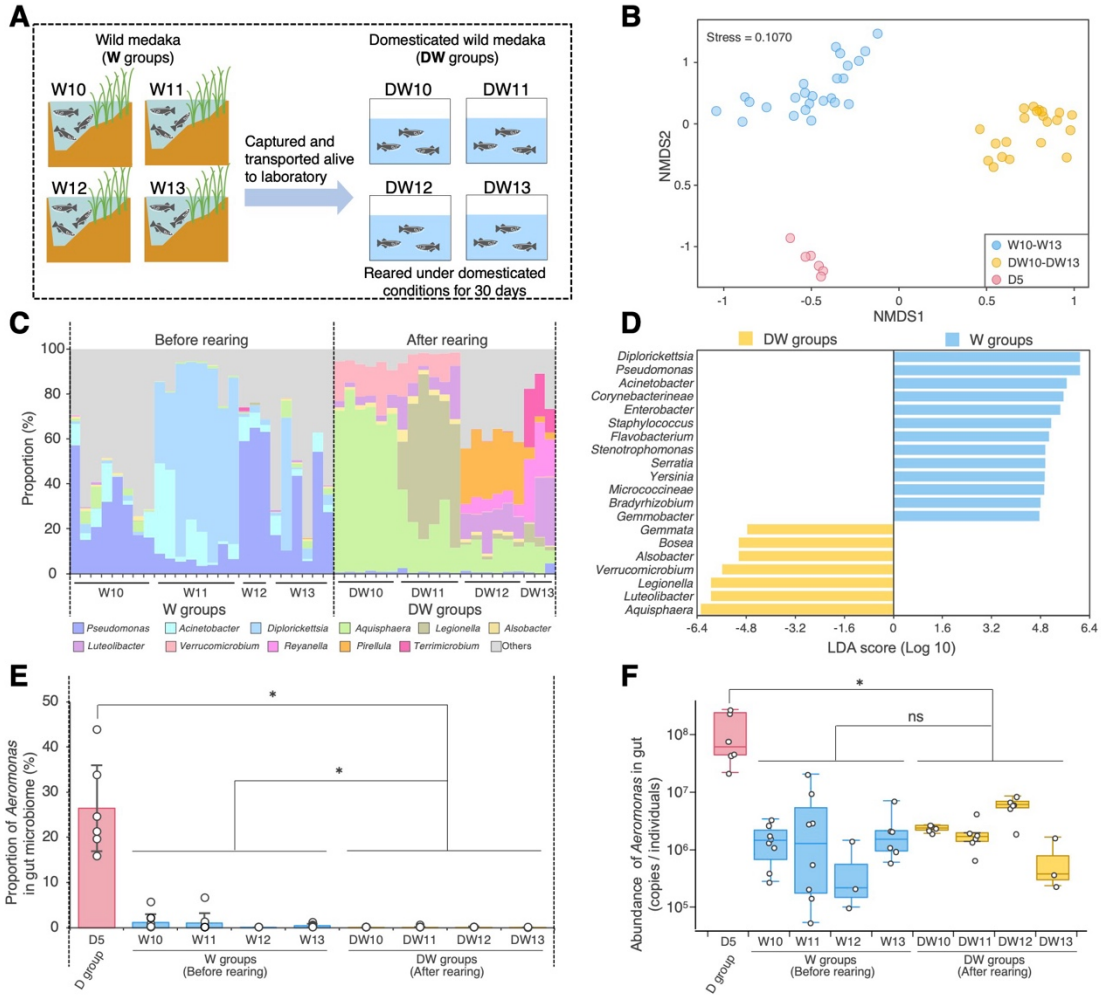
Specific *Aeromonas* species are opportunistic pathogens that cause diseases, such as haemorrhagic inflammation, ulcers and fin rot in fish [43]. Of the 35 *Aeromonas* strains isolated from the D5 group (**Fig. 3E; Supplementary Table S1**), 88.6% (31/35 strains) showed haemolytic activity, forming halos on the blood agar (**Fig. 3E**). Among 40 haemolytic isolates, 77.5% (31/40 strains) were *Aeromonas* (**Supplementary Table S1**). Two representative *Aeromonas* isolated strains, *A. veronii* KK93 and *A. dhakensis* KK125 (**Fig. 3F; Supplementary Table S1**), exhibited stronger haemolytic activity compared with *A. hydrophila* NBRC13286 isolated from the intestine of juvenile silver salmon (**Fig. 3G**).

Rearing Experiment 1: Gut microbiome of wild medaka reared under domesticated conditions

Rearing wild medaka in each W10–W13 group under the same domesticated conditions as D5 group (generating 'domesticated wild medaka', DW10–DW13 groups; **Fig. 4A**) resulted in significant shifts in their gut microbiome composition. An NMDS plot based on Bray–Curtis distances revealed that the gut microbiome of DW groups differed significantly from that of both relevant W and D5 groups (PERMANOVA: DW vs. W: $F = 31.01$, $r^2 = 0.41342$, $p < 0.05$; DW vs. D5: $F = 25.626$, $r^2 = 0.50618$, $p < 0.05$; **Fig. 4B**). The predominant gut bacteria also shifted markedly after domesticated rearing: although *Pseudomonas*, *Acinetobacter* and *Diplorickettsia* were dominant in the W10–W13 groups, these genera were nearly absent in the DW10–DW13

361 groups, replaced by *Aquisphaera*, *Luteolibacter* and *Legionella* among others (**Fig. 4C**). The

362 specificity of these genera to W or DW groups was supported through LEfSe analysis (**Fig. 4D**).



363
364 **Fig. 4** Gut microbiome of wild medaka reared under domesticated conditions. (A) Schematic overview of
365 Rearing Experiment 1: the experimental design for preparing domestically reared wild (DW) medaka. The
366 W10–W13 groups, collected from four different locations (Table 1), were placed in separate tanks and
367 reared under domesticated conditions for 30 days. (B) NMDS plot based on the Bray–Curtis distance,
368 illustrating dissimilarity in the community structure of the gut microbiome. (C) Community structure of the
369 gut microbiome at the bacterial genus level before (W10–W13) and after (DW10–DW13) rearing under
370 domesticated conditions. (D) Specific bacterial groups in W or DW medaka identified via LEfSe analysis.
371 Genera with an LDA score >4.0 are shown. (E) Proportion of *Aeromonas* in the gut microbiome of each
372 medaka sample. (F) *Aeromonas* abundance in the gut microbiome of each medaka sample, estimated
373 through amplicon sequencing and qPCR analyses. The asterisks in (E) and (F) indicate statistically
374 significant differences ($p < 0.05$, Mann–Whitney U-test). Data for D5 and W10–W13 groups are the same
375 as in **Fig. 2**.
376

377

378

379 Notably, although the DW groups were reared under the same domesticated conditions
380 as the D5 group, the proportion of *Aeromonas* in their gut of the DW groups (mean ± SD = 0.1%
381 ± 0.1%, n = 21) remained significantly lower than in the D5 group (mean ± SD = 26.5% ± 9.5%,
382 n = 6) and was even lower than in the corresponding W groups (mean ± SD = 0.8% ± 1.6%, n =
383 25) (**Fig. 4E**). While the quantity of *Aeromonas* in the DW groups (mean ± SD = $1.4 \pm 1.9 \times 10^5$,
384 n = 21) did not differ from that in the corresponding W groups (mean ± SD = $2.6 \pm 8.6 \times 10^5$, n =
385 25), it remained lower than in the D5 group (mean ± SD = $2.4 \pm 3.1 \times 10^8$, n = 6) (**Fig. 4F**).
386
387 **Rearing Experiment 2: Effect of sediment pretreatment on the gut microbiome of medaka**
388 **reared under domesticated conditions**
389 Based on the results of Rearing Experiment 1, we conducted an additional experiment to examine
390 the influence of environmental microbes on the dominance of *Aeromonas* in the gut by pre-
391 exposing medaka to these microbes before domesticated rearing (**Fig. 5A**).
392 Prior to rearing with field-derived environmental microbes, medaka in the D8 group
393 underwent antibiotic treatment to remove their existing gut microbiota (**Step 1 in Fig. 5A**). This
394 reduced CFUs on three types of agar media to less than 1/10,000 of the initial CFUs
395 (**Supplementary Fig S3**), with no adverse effects on medaka health. The antibiotic-treated
396 medaka were then pre-reared for one week with each sediment (S6, S12, S14 and S15) from the
397 streams where wild medaka (W6, W12, W14 and W15) were captured (**Step 2 in Fig. 5A**). After
398 this period, genera such as *Aquisphaera*, *Zavarzinella*, *Pirellula* and *Reyranella*, originating from
399 the sediments, colonised the gut as well as rearing water (**Supplementary Tables S2–S5**).

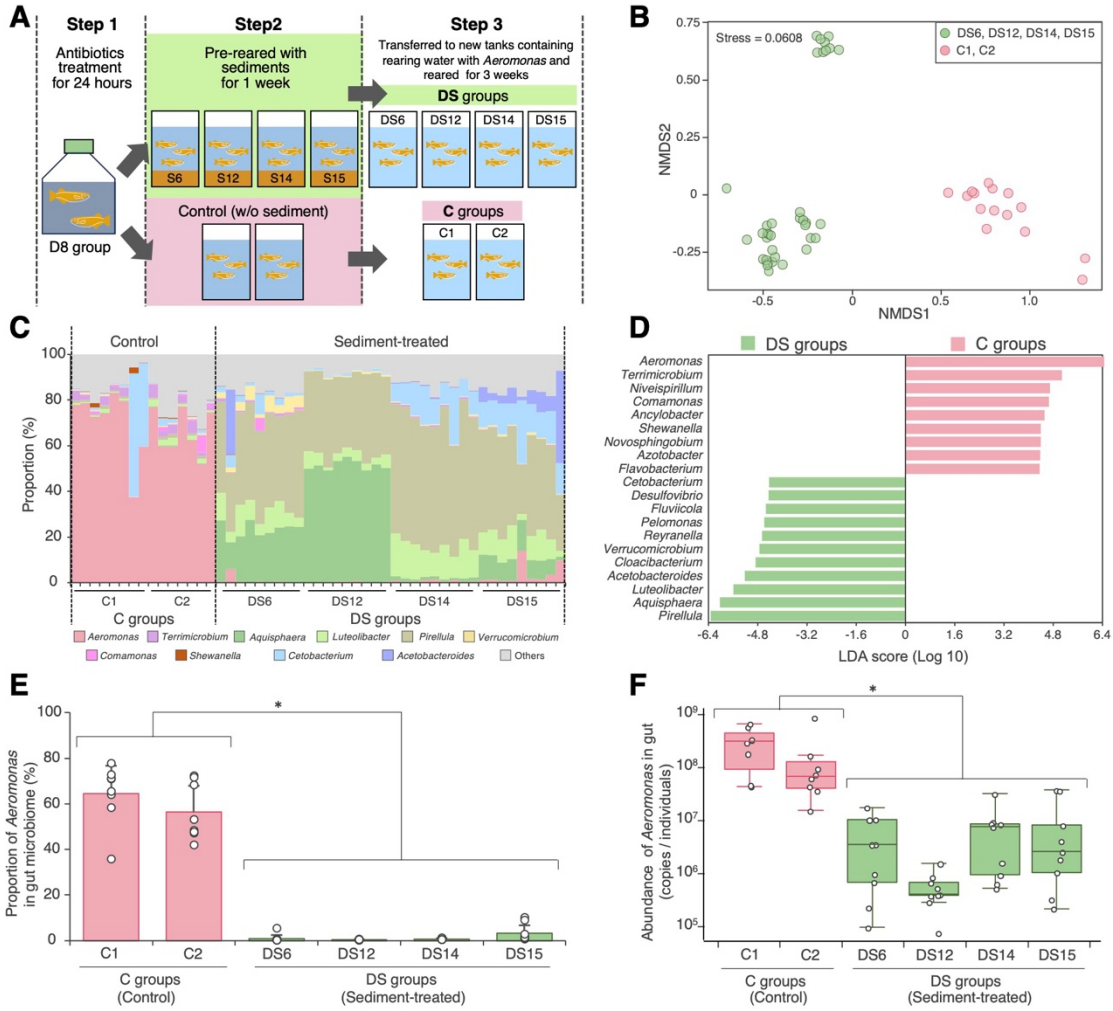


Fig. 5 Effect of sediment pretreatment on the gut microbiome of medaka reared under domesticated conditions. (A) Schematic overview of Rearing Experiment 2: the experimental design for DS groups (DS6, DS12, D14 and D15), i.e., domesticated medaka (D8 group) pre-reared with sediment from W6, W12, W14 and W15 medaka habitats. (B) NMDS plot illustrating gut microbiome dissimilarity based on the Bray–Curtis distance. (C) Community structure of the gut microbiome at the bacterial genus level with (DS groups) or without (C groups) sediment pretreatment. (D) Specific bacterial groups in the DS or C groups identified via LEfSe analysis. Genera with an LDA score >4.0 are shown. (E) Proportion of *Aeromonas* in the gut microbiome of each medaka sample. (F) *Aeromonas* abundance in the gut microbiome of each medaka sample, estimated through amplicon sequencing and qPCR analyses. The asterisks in (E) and (F) indicate statistically significant differences ($p < 0.05$, Mann–Whitney U-test).

These sediment-treated medaka (DS6, DS12, DS14 and DS15 groups) were transferred into new tanks filled with rearing water containing *Aeromonas* (**Supplementary Table S6**) and reared for an additional three weeks under domesticated conditions (**Step 3 in Fig. 5A**). After rearing, the gut microbiome in the DS groups structure differed significantly from that of control medaka (C1 and C2 groups) reared without sediment exposure (PERMANOVA: DS vs. C:

417 $F = 100.71$, $r^2 = 0.6727$, $p < 0.05$; **Fig. 5B**). The duplicate control groups exhibited gut
418 microbiomes dominated by *Aeromonas* (C1: mean ± SD = 64.5% ± 12.2%, n = 8; C2: mean ± SD
419 = 57.7% ± 11.8%, n = 7), similar levels to the D groups (**Fig. 3A,B**), with *Aeromonas* being the
420 most dominant genus in almost all control samples (14/15 individuals; **Fig. 5C**). In contrast, the
421 gut microbiomes of the DS groups were dominated by *Pirellula*, *Aquisphaera* and/or
422 *Luteolibacter* (**Fig. 5C**). The specificity of these predominant genera in C or DS groups was
423 confirmed statistically via LEfSe analysis (**Fig. 5D**). The proportion of *Aeromonas* in the gut of
424 DS medaka (mean ± SD = 1.3% ± 2.2%, n = 36) was significantly lower than that in the C groups
425 (mean ± SD = 61.3% ± 12.5%, n = 15) (**Fig. 5E**). These levels were comparable to those found in
426 the W groups (**Fig. 3A,B**). Additionally, the quantity (copies/individual) of *Aeromonas* in the DS
427 medaka gut (mean ± SD = $6.1 \pm 9.9 \times 10^6$, n = 36) was two orders of magnitude lower than that
428 in the controls (mean ± SD = $2.6 \pm 2.6 \times 10^8$, n = 15) (**Fig. 5F**).
429

430 **Discussion**

431 This study reports the difference in gut microbiome diversity between domesticated and wild
432 medaka (**Fig. 1**). Wild medaka had a significantly higher microbial diversity than domesticated
433 medaka (**Fig. 2B,C**), with a broader range of bacterial phyla, including *Planctomycetes*,
434 *Verrucomicrobia*, *Firmicutes* and *Acidobacteria*, which were largely absent in domesticated
435 medaka (**Fig. 2D**). In contrast, domesticated medaka exhibited reduced diversity and a higher
436 abundance of Proteobacteria and Fusobacteria, along with consistent *Aeromonas* dominance (**Fig.
437 2E,F; Supplementary Fig. S1A,B**). This trend aligns with previous studies showing higher gut
438 microbiome diversity in wild versus domesticated animals [44-47], with the pattern likely due to
439 more varied diets and exposure to diverse environmental microbes.

440 The dominance of *Aeromonas* in the gut of domesticated medaka has also been reported
441 in previous studies [48, 49]. This trend is commonly observed in other domesticated freshwater
442 fish species, including zebrafish, guppy, rainbow trout and various cyprinid species [26, 44, 50-

443 54] (**Supplementary Fig. S2**). However, in wild fish, such as guppy, stickleback, gizzard shad
444 and cyprinid species, *Aeromonas* is less dominant [44, 51, 55-57]. These findings suggest that the
445 reduced dominance of *Aeromonas* in wild populations, compared to their domesticated
446 counterparts, may be relatively common across freshwater fish species.

447 Several factors may explain *Aeromonas* proliferation in the guts of domesticated fish.
448 First, *Aeromonas* is a mesophilic bacterium, with the rearing temperature for medaka (24°C–
449 28°C) providing optimal conditions for its growth [58]. Additionally, the provision of nutrient-
450 rich artificial feed supports its growth, with *Aeromonas* known to thrive under nutrient-abundant
451 conditions [58]. Furthermore, overcrowded conditions in domesticated rearing environments may
452 weaken immune defences, making it easier for opportunistic pathogens, including *Aeromonas*, to
453 proliferate. Although these factors likely encourage *Aeromonas* dominance in domesticated fish,
454 with this phenomenon observed in various fish species, the present study revealed that wild
455 medaka, even when reared under domesticated conditions, did not exhibit *Aeromonas*
456 proliferation or dominance in the gut (Rearing Experiment 1, **Fig. 4**). This suggests that the wild
457 gut microbiome may exert stronger selective pressure on *Aeromonas* relative to domesticated
458 rearing conditions. Longer-term studies could clarify how sustained *Aeromonas* suppression by
459 the wild gut microbiome occurs under domesticated conditions.

460 A notable finding is that introducing environmental microbes into medaka successfully
461 suppressed *Aeromonas* dominance under domesticated rearing conditions (Rearing Experiment 2,
462 **Fig. 5**). In the D8 groups, which typically exhibit *Aeromonas* dominance under these conditions
463 (**Figs. 2E and 3A**), pretreatment with sediment from wild medaka habitats prevented *Aeromonas*
464 from becoming dominant (**Fig. 5**). This suggests that the established gut microbiota has a stronger
465 influence on *Aeromonas* suppression than either the medaka's genetic background or rearing
466 environment. This suppression can be explained by the priority effect, a phenomenon in which
467 established microbial communities prevent the invasion of new microbes [59]. This effect has
468 been widely observed in the guts of various animals and in plant rhizospheres [60]. In the present

469 study, the microbes introduced from the sediment may have outcompeted *Aeromonas* for
470 resources, occupied key ecological niches, or modified the gut environment in a way that hindered
471 *Aeromonas* colonisation. Therefore, this study emphasises the critical role of the environmental
472 microbiome in preventing *Aeromonas* overgrowth, highlighting the importance of the priority
473 effect.

474 *Pirellula*, *Aquisphaera* and *Luteolibacter* were consistently associated with wild
475 medaka, domesticated wild-reared medaka and sediment-exposed medaka (**Figs. 2, 4 and 5**),
476 suggesting that they may inhibit *Aeromonas* through priority effects and/or direct antagonistic
477 interactions. *Pirellula* and *Aquisphaera* belong to the phylum Planctomycetes, commonly found
478 in aquatic and terrestrial environments [61, 62], and are frequently detected in fish gut
479 microbiomes in natural or semi-natural settings [63, 64]. *Luteolibacter*, a Verrucomicrobia
480 member, is found in diverse environments, including seawater, soil and activated sludge [65-67],
481 as well as the guts of annelids, including the medicinal leech [68]. Given their association with
482 lower *Aeromonas* levels, these bacteria may use antibiotics or injectors (such as those from the
483 Type VI secretion system) to attack *Aeromonas*, or they may form robust communities, such as
484 biofilms, that prevent its colonisation. However, the ecological roles of these bacteria are not well
485 understood; therefore, future research should isolate strains from medaka guts and test their
486 antagonism against *Aeromonas* through coculture experiments *in vitro* and i*n vivo*.

487 The consistent dominance of *Aeromonas* in domesticated medaka is an important
488 finding that should also be highlighted from the perspective of applied science. Although
489 *Aeromonas* can aid digestion and immune activation [69], it is more commonly recognised as an
490 opportunistic pathogen in fish [70, 71]. Environmental stressors, such as water quality or
491 temperature changes, can trigger *Aeromonas*-related infections [72], with a high abundance of
492 *Aeromonas* in the gut increasing infection risk. In this study, 88.6% of *Aeromonas* isolates from
493 domesticated medaka exhibited haemolytic activity (**Fig. 3E**), suggesting a potential health risk
494 for these fish. In aquaculture, efforts are being made to reduce the prevalence of *Aeromonas* in

495 fish and their environments given the pathogen's role in disease outbreaks [70, 71, 73]. Current
496 *Aeromonas* control methods rely on antibiotics, but this bacterium is highly prone to developing
497 antibiotic resistance, and fish farms are major sources of resistant *Aeromonas* strains [74-78].
498 Therefore, there is an urgent need for alternative control strategies.

499 This study introduces a novel concept as a promising approach for aquaculture:
500 temporarily rearing fish in sediment to introduce 'wild-type' gut microbiota. The 'dirt is good'
501 hypothesis [79] challenges traditional aquaculture practices that emphasise sterile environments.
502 Although maintaining hygiene remains important, this study shows that introducing beneficial
503 environmental microbes can prevent the overgrowth of opportunistic pathogens, such as
504 *Aeromonas*, which tend to proliferate under domesticated conditions, in the guts of domesticated
505 fish. Previous studies have shown that exposure to diverse microbial communities enhances host
506 immunity in medaka and other species [49, 80, 81], supporting the notion that controlled microbial
507 exposure may be a key strategy for disease prevention. This approach could reduce the
508 dependence on antibiotics and chemical treatments in aquaculture, offering more sustainable
509 disease management practices.

510 In conclusion, this study provides compelling evidence that 'wild-type' gut microbiota
511 can suppress the opportunistic pathogen *Aeromonas* in medaka under domesticated conditions.
512 These findings highlight the crucial role of microbial diversity in maintaining gut health and
513 suggest that introducing environmental microbes could offer a sustainable strategy for disease
514 prevention in aquaculture. Furthermore, by shedding light on the relationship between animals
515 and the environmental microbes in their habitats, this research underscores the significance of
516 understanding these interactions for improving animal health and welfare. While we observed
517 marked differences in microbiome composition, the underlying mechanisms, especially those
518 related to *Aeromonas* suppression in 'wild-type' gut microbiota, remain unclear. Further research
519 is needed to identify specific bacterial genera in wild gut microbiomes that inhibit pathogen
520 growth, as well as to assess host responses, including immunity and stress, through survival tests,

histological analyses, and immune response evaluations.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

K.K. and H.I. designed the study with insights from M.K. and Y.K., and H.I. supervised the project. K.K., K.Kb., Y.S., Y.K. and H.I. collected fish and sediment samples. K.K., K.Kb., Y.S., T.H. and H.I. conducted laboratory experiments. K.K. and H.I. analysed and interpreted the data. K.K. and H.I. wrote the paper with substantial input from all authors. All authors reviewed the manuscript.

547 **Figure legends**

548 **Fig. 1** Comparison of habitat and morphology between domesticated (D) and wild (W) groups of
549 *Oryzias latipes* species complex in Japan. (A) Geographical locations of collection sites for D and
550 W groups. Numbers in parentheses indicate the number of individuals used in the gut microbiome
551 analysis, as shown in **Fig. 2**. Detailed information is summarized in **Table 1**. (B) Typical
552 laboratory rearing conditions of the D groups. The inset photo shows medaka from the D groups
553 (closed triangles). (C) Stream habitat of W groups. The inset photo showing wild medaka in a
554 mud puddle (closed triangles). Photographs of the fish specimen in the D8 (D) and the W7 groups
555 (E). Icons representing D medaka (F) and W medaka (G) used in subsequent figures. Map data
556 sourced from the Geospatial Information Authority of Japan.

557

558 **Fig. 2** Diversity and specificity of the gut microbiome of medaka between the D and W groups.
559 (A) Nonmetric multidimensional scaling (NMDS) plot illustrating gut microbiome dissimilarity
560 based on the Bray–Curtis distance. Microbial diversity comparison using Chao1 (B) and Shannon
561 (C) indices. Asterisks indicate significant differences ($p < 0.05$, Mann–Whitney U-test). (D)
562 Community structure of the gut microbiome according to bacterial phylum proportions. (E)
563 Specific bacterial groups associated with the D or W groups, identified by LEfSe analysis, listing
564 the top 10 genera with the highest LDA scores. (F) Histograms showing the top 5 groups in the D
565 groups. SD, standard deviation; CV, coefficient of variation.

566

567 **Fig. 3** Distribution and potential toxicity of *Aeromonas* in the medaka gut. (A) Proportion of
568 *Aeromonas* in the gut microbiome of medaka samples in each group. (B) Average proportion of
569 *Aeromonas* compared between the D and W groups. (C) Abundance of *Aeromonas* in the gut
570 microbiome, estimated via amplicon sequencing and qPCR analyses. (D) Average abundance of
571 *Aeromonas* compared between D and W medaka. The asterisks in (C) and (D) indicate statistically
572 significant differences ($p < 0.05$, Mann–Whitney U-test). (E) Schematic overview of haemolytic

573 bacterial strain isolation from the medaka gut in the D8 group, highlighting the composition of
574 35 *Aeromonas* strains. Isolates other than *Aeromonas* are summarised in **Supplementary table**
575 **S1**. (F) Phylogenetic tree of *Aeromonas* strains isolated from the guts of domesticated medaka,
576 based on 16S rRNA gene sequences. *Pseudaeromonas sharmana* GPTSA-6^T was used as the
577 outgroup. Scale bar represents 0.01 substitutions per nucleotide position. (G) Haemolytic activity
578 of *A. hydrophila* and medaka-derived *Aeromonas* strains, assessed via halo formation on blood
579 agar (right photo: *A. hydrophila* NBRC 13286; left photo: *Aeromonas* sp. strain KK93). A bar
580 graph displays the mean with standard deviation from six replicates, with different letters
581 indicating statistically significant differences ($p < 0.05$, Mann–Whitney U-test with Bonferroni
582 correction).
583
584 **Fig. 4** Gut microbiome of wild medaka reared under domesticated conditions. (A) Schematic
585 overview of Rearing Experiment 1: the experimental design for preparing domestically reared
586 wild (DW) medaka. The W10–W13 groups, collected from four different locations (Table 1), were
587 placed in separate tanks and reared under domesticated conditions for 30 days. (B) NMDS plot
588 based on the Bray–Curtis distance, illustrating dissimilarity in the community structure of the gut
589 microbiome. (C) Community structure of the gut microbiome at the bacterial genus level before
590 (W10–W13) and after (DW10–DW13) rearing under domesticated conditions. (D) Specific
591 bacterial groups in W or DW medaka identified via LEfSe analysis. Genera with an LDA score
592 >4.0 are shown. (E) Proportion of *Aeromonas* in the gut microbiome of each medaka sample. (F)
593 *Aeromonas* abundance in the gut microbiome of each medaka sample, estimated through
594 amplicon sequencing and qPCR analyses. The asterisks in (E) and (F) indicate statistically
595 significant differences ($p < 0.05$, Mann–Whitney U-test). Data for D5 and W10–W13 groups are
596 the same as in **Fig. 2**.
597
598 **Fig. 5** Effect of sediment pretreatment on the gut microbiome of medaka reared under

599 domesticated conditions. (A) Schematic overview of Rearing Experiment 2: the experimental
600 design for DS groups (DS6, DS12, D14 and D15), i.e., domesticated medaka (D8 group) pre-
601 reared with sediment from W6, W12, W14 and W15 medaka habitats. (B) NMDS plot illustrating
602 gut microbiome dissimilarity based on the Bray–Curtis distance. (C) Community structure of the
603 gut microbiome at the bacterial genus level with (DS groups) or without (C groups) sediment
604 pretreatment. (D) Specific bacterial groups in the DS or C groups identified via LEfSe analysis.
605 Genera with an LDA score >4.0 are shown. (E) Proportion of *Aeromonas* in the gut microbiome
606 of each medaka sample. (F) *Aeromonas* abundance in the gut microbiome of each medaka sample,
607 estimated through amplicon sequencing and qPCR analyses. The asterisks in (E) and (F) indicate
608 statistically significant differences ($p < 0.05$, Mann–Whitney U-test).

Table 1. Medaka examined in this study.

Sample ID	No. of individuals[a]	Condition[b]	Purchase and collection site[c]	Collection date	Note[d]
Domesticated populations					
D1	6	Conventional lab aquaculture (indoor)	NIBB, Okazaki, Aichi pref.	4 Jul, 2013	Inbred strains HNI-II, originated from northern Japan population
D2	6	Conventional lab aquaculture (indoor)	NIBB, Okazaki, Aichi pref.	4 Jul, 2013	Inbred strains HdrR-II1, originated from southern Japan population
D3	6	Conventional lab aquaculture (outdoor)	Niigata University, Niigata, Niigata pref.	4 Jul, 2013	Inbred strains HSOK, originated from the east Korean population
D4	6	Conventional lab aquaculture (outdoor)	Niigata University, Niigata, Niigata pref.	4 Jul, 2013	Conserved strains WS267, originated from the China-west Korean population
D5	6	Conventional lab aquaculture (indoor)	AIST Hokkaido, Sapporo, Hokkaido pref.	28 Nov, 2014	F1 generations originated from commercial strains D6
D6	6	Aquaculture (indoor)	A pet store 1, Sapporo, Hokkaido pref.	11 Jan, 2014	Commercial strains, himedaka
D7	6	Aquaculture (indoor)	A pet store 2, Sapporo, Hokkaido pref.	8 Dec, 2019	Commercial strains, himedaka
D8	6	Aquaculture (indoor)	A pet store 3, Sapporo, Hokkaido pref.	19 May, 2020	Commercial strains, himedaka
Wild populations					
W1	4	-	Setouchi, Okayama pref.	12 Jul, 2014	Southern Japan population
W2	9	-	Niigata, Niigata pref.	17 Jul, 2014	Northern Japan population
W3	9	-	Mito, Ibaraki pref.	19 Jul, 2014	Southern Japan population
W4	8	-	Myoko, Niigata pref.	10 Oct, 2015	Northern Japan population
W5	4	-	Kanzaki, Saga pref.	29 Oct, 2015	Southern Japan population
W6	10	-	Onga-gun, Fukuoka pref.	30 Oct, 2015	Southern Japan population
W7	9	-	Tamana, Kumamoto pref.	30 Oct, 2015	Southern Japan population

W8	9	-	Ozu, Ehime pref.	1 Nov, 2015	Southern Japan population
W9	9	-	Kusu-gun, Oita pref.	2 Nov, 2015	Southern Japan population
W10	8	-	Shimanto, Kochi pref.	20 Nov, 2017	Southern Japan population
W11	8	-	Sukumo, Kochi pref.	20 Nov, 2017	Southern Japan population
W12	3	-	Matsuyama, Ehime pref.	20 Nov, 2017	Southern Japan population
W13	6	-	Uwajima, Ehime pref.	20 Nov, 2017	Southern Japan population
W14	10	-	Site 1, Hamamatsu, Shizuoka pref.	6 Sep, 2019	Southern Japan population
W15	10	-	Site 2, Hamamatsu, Shizuoka pref.	6 Sep, 2019	Southern Japan population
W16	6	-	Iwata, Shizuoka, pref.	10 Oct, 2019	Southern Japan population

[a] Numbers indicate the number of individuals used for the gut microbiome analysis shown in Figs. 2 and 3.

[b] All of domesticated fish were reared in dechlorinated tap water and fed with artificial food. D1, D2 and D5-D8 groups were kept indoors at a water temperature of around 25°C. D3 and D4 groups were maintained at variable water temperature because they reared in outdoor tanks.

[c] NIBB, National Institute for Basic Biology; AIST, National Institute of Advanced Industrial Science and Technology.

[d] A category of wild medaka populations was referred to Takehana et al., 2003 [23].

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