Transplantation of high fat fed mouse microbiota into zebrafish larvae identifies MyD88-dependent acceleration of hyperlipidaemia by Gram positive cell wall components

Pradeep Manuneedhi Cholan[1], Simone Morris[1], Kaiming Luo[1], Jinbiao Chen[2], Jade A Boland[2], Geoff W McCaughan[2,3], Warwick J Britton[1,4], Stefan H Oehlers[1,5] *

[1] Tuberculosis Research Program at the Centenary Institute, The University of Sydney, Camperdown, NSW 2050, Australia

[2] Liver Injury and Cancer Program at the Centenary Institute, The University of Sydney, Camperdown, NSW 2050, Australia

[3] AW Morrow Gastroenterology and Liver Centre, Royal Prince Alfred Hospital, Camperdown, NSW 2050, Australia

[4] Department of Clinical Immunology, Royal Prince Alfred Hospital, Camperdown, NSW 2050, Australia

[5] The University of Sydney, Discipline of Infectious Diseases & Immunology and Marie Bashir Institute, Camperdown, NSW 2050, Australia

Corresponding author: Dr Stefan Oehlers s.oehlers@centenary.org.au

ORCID: 0000-0003-0260-672X Twitter: @oehlerslab

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Abstract

Gut dysbiosis is an important modifier of pathologies including cardiovascular disease but our understanding of the role of individual microbes is limited. Here, we have used transplantation of

mouse microbiota into microbiota-deficient zebrafish larvae to study the interaction between members of a mammalian high fat diet-associated gut microbiota with a lipid rich diet challenge in a tractable model species. We find zebrafish larvae are more susceptible to hyperlipidaemia when exposed to the mouse high fat-diet-associated microbiota and that this effect can be driven by two individual bacterial species fractionated from the mouse high fat-diet-associated microbiota. We find *Stenotrophomonas maltophilia* increases the hyperlipidaemic potential of chicken egg yolk to zebrafish larvae independent of direct interaction between *S. maltophilia* and the zebrafish host. Colonisation by live, or exposure to heat-killed, *Enterococcus faecalis* accelerates hyperlipidaemia via host Myd88 signalling. The hyperlipidaemic effect is replicated by exposure to the Gram positive Toll-like receptor agonists peptidoglycan and lipoteichoic acid in a MyD88-dependent manner. In this work, we demonstrate the applicability of zebrafish as a tractable host for the identification of gut microbes that can induce conditional host phenotypes via microbiota transplantation and subsequent challenge with a high fat diet.

Introduction

The metagenome encoded by the gut microbiome is an essential component of the animal digestive system [1, 2]. Microbes can affect digestion directly through the breakdown of indigestible material such as fibre to short chain fatty acids and indirectly by stimulating the differentiation of the intestinal epithelium.

The microbiome is shaped by host and environment selective pressures [3, 4]. Lipid-rich Western diets that cause obesity in susceptible individuals are associated with gut dysbiosis which can drive an unwanted increase in nutrient absorption and epithelial leakiness [5]. Transplantation of human lean and obese microbiota into germ-free mice transmits these phenotypes across host mammalian species [6]. While the germ-free mouse has been invaluable for the study of host-

microbe interactions through gnotobiotic and transplantation studies, there is a need for faster and more accessible models that serve as functional screening tools for disease-associated microbiota.

Zebrafish embryos require microbial colonisation during development for full physiological digestive function and are a simple platform for studying host-microbiota-environment interactions [7, 8]. Zebrafish embryos develop *ex utero* within a chorion that can be surface sterilised and are tolerant of antibiotics in their media making them a technically simple model for raising germ-free experimental subjects. Probiotic transfer has been shown to increase resistance to pathogen colonisation [9]. The zebrafish gut microbiome is sensitive to challenge with a HFD, feeding larval zebrafish a diet supplemented with 10% fat for 25 days altered the distribution of bacterial phyla in the gut which correlated with increased expression of host inflammatory genes compared to larvae fed the control diet [3].

Microbiota transplantations have been previously performed from mice and humans to zebrafish demonstrating the feasibility of using the zebrafish as an in vivo screening tool for studying the effects of defined microbiota on host physiology [10]. Here we use this technically simple model to rapidly identify specific members of the mouse HFD-associated microbiota that accelerate a diet-induced hyperlipidaemic phenotype in zebrafish embryos and the mechanisms by which these pathobionts interact with the zebrafish host.

Results

<u>Transplantation of microbiota from HFD fed mice accelerates hyperlipidaemia in zebrafish embryos</u>

75 Microbiome-depleted (MD) zebrafish embryos were exposed to mouse faecal microbiota
76 preparations generated from mice fed a conventional chow diet or HFD from 3-5 dpf (days post
77 fertilization) and challenged with chicken egg yolk feeding from 5-7 dpf (Figure 1A).
78
79 We first investigated if colonisation with the control or HFD-fed mouse microbiota affected
80 zebrafish intestinal physiology at 5 dpf before the onset of exogenous feeding. The absorptive
81 activity of zebrafish midgut lysosome-rich intestinal epithelial cells can be visualised by neutral red
82 staining, previous studies have demonstrated that this phenotype requires microbial colonisation
83 and is impeded by inflammation [11, 12]. We used neutral red staining visually to examine the
84 absorptive function of colonised embryos and observed higher staining in 5 dpf larvae colonised
85 with the HFD-fed microbiota compared to chow diet colonised controls (Figure 1B and 1C).
86
87 We next collected colonised zebrafish larvae and stained with Oil Red O to visualise neutral lipids
88 but did not observe any differences in lipid staining. To determine if colonisation with the HFD-fed
89 mouse microbiota affected the zebrafish response to challenge with a chicken egg yolk challenge
90 [13], we challenged 5 dpf zebrafish with chicken egg yolk and stained with Oil Red O to visualise
91 neutral lipids (Figure 1D). Zebrafish larvae colonised with the faecal microbiota from HFD fed mice
92 had more vascular Oil Red O staining after 2 day of chicken egg yolk feeding challenge compared
93 to zebrafish larvae colonised with the faecal microbiota from chow diet fed mice (Figure 1E).
94
95 These data demonstrate the responsiveness of MD zebrafish larvae to the transferable effects of
96 dysbiotic mammalian gut microbiome-associated microbiota when challenged with a complex
97 environmental stimulus in the form of lipid-rich feeding.
98
99 <u>Identification of individual microbes with pathobiont activity</u>

100 To isolate individual species that would be amenable to *in vitro* handling and growth, faecal
101 homogenate supernatants were plated on LB agar and grown at 28°C to select for species that
102 would be easily handled and most likely to colonise zebrafish embryos (Figure 2A). The best
103 growing isolate in LB broth culture from two chow diet and HFD fed faecal preparations were
104 selected and sequenced. We identified *Enterococcus faecalis* strain YN771 (*E.f* on figures) and
105 *Stenotrophomonas maltophilia* strain CD103 (*S.m*) from HFD-fed mouse faecal lysate, and
106 *Escherichia* species PYCC8248 (*E.s*) and *Escherichia coli* strain Y15-3 (*E.c*) from chow diet-fed
107 mouse faecal lysate.
108
109 We colonised MD zebrafish larvae with individual isolates to determine if monoassociation could
110 replicate the effects of bulk microbiota transfer. To determine if exposure of larvae to
111 preparations of *E. faecalis, S. maltophilia,* or either of the *E. coli* strains resulted in gut colonisation
112 we dissected guts from rinsed 5 dpf larvae after 2 days of monoassociation exposure and
113 recovered bacteria onto LB agar. All tested strains yielded recoverable colonisation levels of
114 approximately 100 CFU per larval gut. Gnotobiotic zebrafish larvae colonised with *E. faecalis* or *S.*
115 *maltophilia* had increased Oil Red O staining compared to larvae colonised with either of the
116 *Escherichia* strains after chicken egg yolk challenge (Figure 2B).
117
118 Interestingly, colonisation with *E. faecalis* or *S. maltophilia* did not increase the absorptive activity
119 of the midgut intestinal epithelium compared to larvae colonised with either of the *Escherichia*
120 strains suggesting the absorptive phenotype seen in bulk microbiota transplant is the product of
121 multiple microorganisms or metabolites present in the complex mouse faecal microbiota
122 preparations (Figure 2C).
123

124 We next sought to confirm our observations using a second strain of each bacterial species, *E.*
125 *faecalis* UNSW 054400 type strain (*E.f* UNSW) and *S. maltophilia* yy01 (*S.m* yy01) isolated from
126 another mouse in the same facility. Colonisation of MD zebrafish embryos with either strain
127 replicated the hyperlipidaemic phenotype seen with our original isolates (Figure 2D). Interestingly,
128 the type UNSW 054400 strain increased hyperlipidaemia beyond that seen with our YN771 isolate
129 strain, while conversely the *S. maltophilia* yy01 strain was not as potent as our original CD103
130 strain, demonstrating strain-specific variability in our zebrafish embryo system.
131
132 *Stenotrophomonas maltophilia* can accelerate hyperlipidaemia by digesting food external to the
133 host
134 To identify the mechanisms by which *E. faecalis* and *S. maltophilia* were accelerating HFD-induced
135 hyperlipidaemia in zebrafish larvae, we sought to investigate if the bacteria needed to be alive
136 and/or in contact with the host.
137
138 We adapted our gnotobiotic methodology to expose MD embryos to heat killed bacterial
139 preparations prior to chicken egg yolk feeding (Figure 3A). Exposure to heat-killed *E. faecalis*
140 replicated the live *E. faecalis* hyperlipidaemic phenotype when compared MD embryos that had
141 been exposed to either heat killed *S. maltophilia* or either of the *Escherichia* strains (Figure 3B).
142 Conversely, heat-killed *S. maltophilia* did not induce hyperlipidaemia in concert with chicken egg
143 yolk challenge.
144
145 We hypothesised *S. maltophilia* might interact with the chicken egg yolk independently of the host
146 colonisation. To test this hypothesis, we incubated chicken egg yolk with each of our 4 bacterial
147 isolates in conditions representative of the zebrafish embryo media and then sterilised the "pre-
148 digested" chicken egg yolk by autoclaving prior to feeding to MD zebrafish embryos (Figure 3C).

149 Compared to either untreated chicken egg yolk, autoclaved chicken egg yolk, or chicken egg yolk
150 incubated with the other three bacterial isolates, the chicken egg yolk that had been incubated
151 with *S. maltophilia* increased hyperlipidaemia in zebrafish embryos (Figure 3D). A weaker, but
152 statistically significant, effect was also seen with chicken egg yolk that had been incubated with
153 *Escherichia coli* strain Y15-3 (*E.c*).
154
155 We next repeated this experiment in conventionally raised embryos and found the increased
156 hyperlipidaemic potential of *S. maltophilia* "pre-digested" chicken egg yolk was replicated in
157 larvae with a conventional microbiome (Figure 3E).
158
159 To examine the substrate specificity of *S. maltophilia*, we incubated commercially available fish
160 embryo food with *Escherichia coli* strain Y15-3 or *S. maltophilia* and observed an increase in body
161 size of only in embryos fed the commercial feed that had been "pre-digested" with *S. maltophilia*
162 compared to untreated commercial feed (Figure 3F).
163
164 Visual inspection of *S. maltophilia* "pre-digested" chicken egg yolk suspensions suggested *S.*
165 *maltophilia* had broken apart the chicken egg yolk resulting in smaller particles that could be more
166 easily ingested and altered the biochemical properties of the chicken egg yolk as the solution
167 contained much finer particles than for other treatments (Figure 3G). An intermediate phenotype
168 was seen in chicken egg yolk that had been "pre-digested" by *E. coli* strain Y15-3. CFU recovery
169 assays demonstrated higher growth of *S. maltophilia* than *E. coli* strain Y15-3 suggesting the better
170 growth of *S. maltophilia* may convert chicken egg yolk into components that could be digested by
171 zebrafish embryos (Figure 3H). We performed nutritional panel and free fatty acid analyses of
172 chicken egg yolk that had been "pre-digested" by *S. maltophilia* or *E. coli* strain Y15-3 as an
173 additional control (Table 1). These analyses revealed only a modest increase in energy content by

174 *E coli* strain Y15-3 and *S. maltophilia*, and an increase in total fat content made up of
175 monounsaturated and saturated fatty acids in *S. maltophilia*-incubated samples that was not seen
176 in control or *E. coli* strain Y15-3-incubated samples.
177
178 These data illustrate a colonisation-independent mechanism by which *S. maltophilia* may enhance
179 lipid uptake in zebrafish larvae by modifying food in the aqueous environment external to the
180 host.
181
182 *Enterococcus faecalis* accelerates hyperlipidaemia via host MyD88-mediated signalling
183 Our analyses had shown *E. faecalis* did not need to be alive but did need to be pre-associated with
184 the hatching zebrafish larvae to accelerate hyperlipidaemia suggesting colonisation of the gut or
185 other mucosal surfaces and subsequent recognition by the host may been necessary to accelerate
186 hyperlipidaemia. Host innate immune signalling via the Myd88 adaptor protein is essential for the
187 zebrafish intestinal epithelium to respond to microbial colonisation [14].
188
189 We performed knockdown of host *myd88* expression using multiple CRISPR-Cas9 gRNAs (Figure
190 4A). Host *myd88* expression was necessary for transducing the *E. faecalis*-induced hyperlipidaemic
191 signal as *myd88* crispants had significantly less vascular Oil Red O staining than scrambled
192 gRNA/Cas9-injected control embryos after colonisation with *E. faecalis* and chicken egg yolk
193 challenge (Figure 4B).
194
195 Gram positive cell wall components accelerate hyperlipidaemia in zebrafish embryos
196 *E. faecalis* is a Gram-positive bacterium. To determine if *E. faecalis*-driven hyperlipidaemia was
197 due to a conserved Gram-positive cell wall component, we initially compared the hyperlipidaemic
198 potential of heat-killed *E. faecalis* to heat killed *Staphylococcus xylosus* (*S.x*), another Gram-

199 positive bacterium obtained from the faecal microbiota of a mouse from the same facility. We
200 found exposure of zebrafish larvae to heat-killed *S. xylosus* replicated the hyperlipidaemic effect of
201 heat-killed *E. faecalis* after chicken egg yolk challenge (Figure 5A).
202
203 Next we directly soaked MD larvae in a sublethal dose of 25 µg/mL purified lipoteichoic acid (LTA)
204 or peptidoglycan (PGL), which are major components of the Gram-positive cell wall, prior to
205 challenge with chicken egg yolk feeding [15]. Either one of these purified ligands were able to
206 accelerate hyperlipidaemia in MD larvae (Figure 5B).
207
208 To test the requirement for intact *E. faecalis* PGL to accelerate hyperlipidaemia, we exposed MD
209 larvae to lysozyme-digested heat-killed *E. faecalis* prior to chicken egg yolk feeding. Lysozyme
210 digestion ablated the ability of heat-killed *E. faecalis* to accelerate hyperlipidaemia (Figure 5C).
211
212 To determine if host innate immune signalling via the Myd88 adaptor protein transduced the LTA
213 or PGL-induced signal that accelerates hyperlipidaemia, we again knocked down *myd88* using a
214 pooled CRISPR-Cas9 gRNA approach and exposed crispants to LTA or PGL. Depletion of host *myd88*
215 ablated the sensitivity of embryos to LTA and PGL-induced accelerated hyperlipidaemia (Figure
216 5D).
217
218 Together, these experiments demonstrate larval zebrafish lipid metabolism is sensitive to the
219 presence of Gram-positive bacterial cell wall components via Myd88-mediated host signalling
220 pathways.
221
222 **Discussion**

223 Our study demonstrates the utility of the gnotobiotic zebrafish platform to screen donor
224 microbiota samples for transplantable biological activities in combination with an exogenous
225 environmental factor. The addition of an exogenous trigger to the experimental system is an
226 important permutation that allows the identification of microbes whose interactions with the host
227 only become apparent in combination with an environmental challenge such as diet.
228
229 We applied the gnotobiotic zebrafish platform to explore the diet-hyperlipidaemia axis as there is
230 a growing interest in microbiome studies within the cardiovascular disease field [16]. The
231 gnotobiotic zebrafish platform represents a disruptive technology that could be adapted to
232 identify pathobiont species from human cardiovascular disease patients and as a "first pass"
233 platform in mechanistic studies with appropriate zebrafish models of cardiovascular pathology.
234
235 Our investigation of *E. faecalis*-accelerated hyperlipidaemia uncovered a surprising role of Gram-
236 positive cell wall component-triggered Myd88 signalling in zebrafish embryo lipid metabolism. The
237 presence of this response in zebrafish embryos has broad implications for the use of zebrafish
238 embryos to study transplanted mammalian microbiota as it may not be representative of the
239 mammalian response to colonisation with Gram positive organisms. Most zebrafish embryo mono-
240 association studies have been carried out with Gram negative organisms including *E. coli, A.*
241 *veronii*, *V. cholerae*, and *P. aeruginosa* [17-19].
242
243 The comparison of heat killed *E. faecalis* to heat killed *S. xylosus* demonstrated an increased ability
244 of heat killed *E. faecalis* to accelerate diet-induced hyperlipidaemia. This suggests that there is
245 variability in hyperlipidaemia accelerating potential amongst Gram positive organisms in zebrafish
246 embryos. The basis of this difference could be further explored using model Gram positive
247 organisms and Gram-positive organisms that are natural commensals in the zebrafish gut. A recent

248 paper by Griffin *et al.* has demonstrated a role for *E. faecalis* SagA enzyme in producing immuno-
249 stimulatory muropeptides [20], as we have previously demonstrated a conserved function of the
250 zebrafish NOD orthologs it is possible that SagA-mediated production of muropeptides from
251 bacterial peptidoglycan contributes to our hyperlipidaemia-accelerating phenotype.
252
253 *Caenorhabditis elegans* fed with *S. maltophilia* accumulate neutral lipids within intracellular lipid
254 droplets driven by a bacterially-encoded mechanism that is independent of the innate immune
255 response [21]. Our finding that two distinct mouse-associated *S. maltophilia* strains were able to
256 increase the hyperlipidaemic potential of chicken egg yolk suggests the digestive ability of *S.*
257 *maltophilia* is potentially conserved between strains and potentially between host species.
258 Previous studies have identified *S. maltophilia* strains within zebrafish gut microbiomes [9], these
259 zebrafish-associated strains could be analysed to determine if increased lipid uptake is a
260 consequence of natural host-*S. maltophilia* pairs and for comparative studies to identify the food
261 modifying mechanisms employed by our *S. maltophilia* CD103 strain isolate.
262
263 We also found an embryo growth-enhancing effect of pre-digesting commercially available
264 zebrafish embryo feed with *S. maltophilia* which suggests *S. maltophilia* could have commercial
265 applications as an aquaculture feed additive. The use of *S. maltophilia* as a feed additive is
266 potentially risky as this organism is associated with opportunistic infections in humans and is
267 resistant to a wide range of antibiotics so care should be taken to avoid cross over into the food
268 chain [22, 23].
269
270 As a proof-of-principle study, our work demonstrates the feasibility of studying the interaction of
271 bacterial species transplanted from a mammalian host with an environmental factor to result in a
272 conditional phenotype in the tractable zebrafish model system. Further work is required to

273 examine effects of the two pathobiont species identified in our study on mammalian models of
274 hyperlipidaemia and to correlate their colonisation with the progression of cardiovascular disease
275 phenotypes in mammalian models and human samples.
276
277 **Methods**
278 Zebrafish handling
279 Adult zebrafish were housed at the Centenary Institute (Sydney Local Health District Animal
280 Welfare Committee Approval 17-036). Zebrafish embryos were obtained by natural spawning and
281 conventionally raised embryos were maintained in E3 media at 28°C.
282
283 Generation of microbiome-depleted (MD) zebrafish embryos
284 Microbiome-depleted (MD) zebrafish were created and maintained as previously described [24].
285 Briefly, freshly laid embryos were rinsed with 0.003% v/v bleach in sterile E3 and rinsed 3 times
286 with sterile E3. Bleached embryos were raised in sterile E3 supplemented with 50 µg/mL ampicillin
287 (Sigma), 5 µg/mL kanamycin (Sigma) and 250 ng/mL amphotericin B (Sigma) in sterile tissue
288 culture flasks. Dead embryos and chorions were aseptically removed at one and three dpf
289 respectively.
290
291 Conventionalised zebrafish were used as a control, where 3 dpf MD zebrafish were inoculated
292 with system water from the aquarium at Centenary Institute.
293
294 Generation of mouse faecal microbiota specimens
295 Mice were housed at the Centenary Institute (Sydney Local Health District Animal Welfare
296 Committee Approval 2018/016). C57BL/6J mice were housed in a pathogen-free and temperature-
297 controlled environment, with 12 hours of light and 12 hours of darkness, and free access to food

298 and water. Mice were provided with a High Fat Diet (HFD) or chow from 6 to 30 weeks of age. The
299 HFD was prepared in-house based on rodent diet no. D12451 (Research Diets New Brunswick,
300 USA) and its calories were supplied as: fat 45%, protein 20%, and carbohydrate 35% [25]. The
301 chow diet was commercially produced by Speciality Feeds as "Irradiated Rat and Mouse Diet" and
302 its calories were supplied as: fat 12%, protein 23%, and carbohydrate 65%.
303
304 Faecal pellets were collected from mice that were housed in different cages. Individual faecal
305 pellets were collected into sterile 1.7 mL microcentrifuge tubes and homogenised in 1 mL of sterile
306 E3 by pipetting. Homogenised specimens were centrifuged at 500 G for 2 minutes to sediment
307 fibrous material and the supernatant was collected. Supernatants were supplemented with
308 glycerol to a final concentration of 25% v/v, aliquoted, and frozen at -80°C for experimental use.
309
310 Colonisation of zebrafish with mouse faecal microbiota
311 MD zebrafish were colonised at 3 dpf by transfer into sterile E3 and addition of 200 µL thawed
312 faecal homogenate supernatant. At 5 dpf, embryos were rinsed with E3 and placed on a chicken
313 egg yolk diet.
314
315 Neutral red staining and morphology measurements
316 Neutral red staining was performed as previously described [26]. Briefly, 2.5 µg/mL neutral red
317 was added to the media of 4 dpf embryos and incubated overnight. Embryos were rinsed with
318 fresh E3 to remove unbound neutral red and live imaged on a Leica M205FA microscope with a
319 consistent zoom between specimens in a single experiment. The area of neutral red-stained
320 midgut was measured in pixels using ImageJ. Body size was measured in pixels using ImageJ.
321
322 Zebrafish high fat diet challenge with chicken egg yolk

323 High fat diet challenge with chicken egg yolk was performed as previously described [13]. Briefly, 5
324 dpf zebrafish embryos were placed in an E3 solution containing 0.05% w/v emulsified hard boiled
325 chicken egg yolk in glass beakers. Beakers were housed in a 28°C incubator with a 14:10 hour
326 light:dark cycle. The emulsified hard boiled chicken egg yolk solution was changed daily.
327
328 Oil Red O staining assay
329 Oil Red O staining and analysis was performed as previously described [13, 27, 28]. Briefly, 7 dpf
330 embryos were fixed overnight at 4°C in 4% paraformaldehyde, rinsed with PBS, and rinsed
331 stepwise through to propylene glycol. Embryos were stained with filtered 0.5% (w/v) Oil Red O
332 dissolved in propylene glycol overnight at room temperature. Unbound dye was removed by
333 washing with propylene glycol and embryos were rinsed stepwise through to PBS for imaging.
334
335 Embryos were imaged on a Leica M205FA microscope. Experimental batches of colour images
336 were analysed in ImageJ by adjusting the colour threshold function to eliminate non-red signal,
337 this output was then converted to a binary mask and the tail region posterior to the swim bladder
338 was selected to measure the area of particles.
339
340 Isolation, identification, and handling of bacterial isolates from mouse faecal microbiota samples
341 Faecal homogenate supernatants were plated onto LB Agar (Amyl Media) and incubated at 28°C
342 for two days. Individual isolates were grown in broth culture in Luria Broth (Miller's LB Broth Base,
343 Thermofisher) at 28°C overnight with 200 RPM shaking.
344
345 For identification, bacteria were harvested from broth culture and subjected to PCR using
346 universal 16S primers (5′-3′) Fw Rv. PCR products were sequenced by Sanger Sequencing (AGRF)

347 and NCBI records were searched by BLAST to identify the closest matching bacterial strain by
348 sequence identity.
349
350 For gnotobiotic experiments, bacteria were harvested from overnight broth cultures and
351 resuspended in sterile E3 zebrafish embryo media at a concentration of OD600 0.2. MD zebrafish
352 in autoclaved E3 were then inoculated with the bacterial suspension at a ratio of 1:200.
353
354 For heat killed bacterial inoculations, bacteria were resuspended in E3 at a concentration of
355 OD600 0.2 and heat-killed in a 95°C heat block for 30 minutes. Heat killed bacterial solutions were
356 then added at 1:200 ratio to 3 dpf MD zebrafish.
357
358 For purified ligand exposure, 3 dpf MD zebrafish were soaked in 25 μg/mL lipoteichoic acid
359 (Sigma) or 25 μg/mL peptidoglycan (Sigma).
360
361 For lysozyme digestion of heat-killed *E. faecalis*, a suspension of heat-killed bacteria was incubated
362 with 50 μg/mL lysozyme (Sigma) at 37°C for 6 h before being used to inoculate 3 dpf MD zebrafish
363 at a ratio of 1:200.
364
365 Digestion of chicken egg yolk
366 20 g hard-boiled chicken egg yolk was mixed with 40 mL E3 and emulsified using a Branson Digital
367 Sonifier sonicator. Emulsified egg yolk was inoculated with bacterial isolates in E3 (described
368 above) at a 1:200 ratio. Egg yolk mixture was then placed in a shaker at 28 °C at 200 rpm and for
369 48 h. Samples were then autoclaved prior to feeding experiments.
370
371 Analysis of chicken egg yolk composition

372 “Pre-digested” samples were autoclaved and mailed to Australian Laboratory Services (VIC,
373 Australia) for commercial grade nutritional analyses.
374
375 Gene knockdown with CRISPR-Cas9
376 gRNA templates for *myd88* (5`- 3`): Target 1
377 TAATACGACTCACTATAGGCAGTTTCCGAAAGAAACTGTTTTAGAGCTAGAAATAGC, Target 2
378 TAATACGACTCACTATAGGAAAAGGTCTTGACGGACTGTTTTAGAGCTAGAAATAGC, Target 3
379 TAATACGACTCACTATAGGAACTGTTTGATCATCTCGGTTTTAGAGCTAGAAATAGC, Target 4
380 TAATACGACTCACTATAGGTTTTTTCGATAAGCTCACGTTTTAGAGCTAGAAATAGC. gRNA was
381 synthesized as previously described [29].
382
383 A 1:1 solution of gRNA and 500 µg/mL of Cas9 nuclease V3 (Integrated DNA Technology, Sigma, or
384 or Sydney Analytical) was prepared with phenol red dye (Sigma, P0290). Freshly laid eggs were
385 collected from breeding tanks and the solution was injected in the yolk sac of the egg before the
386 emergence of the first cell with a FemtoJet 4i (Eppendorf).
387
388 Knockdown efficacy was monitored by RT-qPCR as previously described [30]. *myd88*-specific
389 primers (5`- 3`): Fw ACAGGGACTGACACCTGAGA, Rv GACGACAGGGATTAGCCGTT.
390
391 To derive MD crispant embryos, injected embryos were placed in E3 containing ampicillin,
392 kanamycin and amphotericin B as described previously. Bleaching injected embryos caused high
393 mortality rates.
394
395 Statistics

396 All statistical analyses (t-tests and ANOVA where appropriate) were performed using GraphPad
397 Prism 8. Outliers were removed using ROUT, with Q = 1%. All data shown are representative of at
398 least 2 biological replicates.
399
400 **Data availability**
401 Source data are provided with this paper.
402 Raw image and analysis data is archived for 10 years by The Centenary Institute and available on
403 request from the corresponding author (Sydney, Australia).
404
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420

421 **References**

422 1. Sekirov, I., et al., *Gut microbiota in health and disease.* Physiol Rev, 2010. **90**(3): p. 859-904.
423 2. Backhed, F., et al., *Mechanisms underlying the resistance to diet-induced obesity in germ-*
424 *free mice.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 979-84.
425 3. Arias-Jayo, N., et al., *High-Fat Diet Consumption Induces Microbiota Dysbiosis and Intestinal*
426 *Inflammation in Zebrafish.* Microb Ecol, 2018. **76**(4): p. 1089-1101.
427 4. Rawls, J.F., et al., *Reciprocal gut microbiota transplants from zebrafish and mice to germ-*
428 *free recipients reveal host habitat selection.* Cell, 2006. **127**(2): p. 423-33.
429 5. Murphy, E.A., K.T. Velazquez, and K.M. Herbert, *Influence of high-fat diet on gut*
430 *microbiota: a driving force for chronic disease risk.* Curr Opin Clin Nutr Metab Care, 2015.
431 **18**(5): p. 515-20.
432 6. Ridaura, V.K., et al., *Gut microbiota from twins discordant for obesity modulate metabolism*
433 *in mice.* Science, 2013. **341**(6150): p. 1241214.
434 7. Lopez Nadal, A., et al., *Feed, Microbiota, and Gut Immunity: Using the Zebrafish Model to*
435 *Understand Fish Health.* Front Immunol, 2020. **11**: p. 114.
436 8. Brinkmann, B.W., et al., *Colonizing microbiota protect zebrafish larvae against silver*
437 *nanoparticle toxicity.* Nanotoxicology, 2020. **14**(6): p. 725-739.
438 9. Stressmann, F.A., et al., *Mining zebrafish microbiota reveals key community-level resistance*
439 *against fish pathogen infection.* ISME J, 2021. **15**(3): p. 702-719.
440 10. Valenzuela, M.J., et al., *Evaluating the Capacity of Human Gut Microorganisms to Colonize*
441 *the Zebrafish Larvae (Danio rerio).* Front Microbiol, 2018. **9**: p. 1032.
442 11. Bates, J.M., et al., *Distinct signals from the microbiota promote different aspects of*
443 *zebrafish gut differentiation.* Dev Biol, 2006. **297**(2): p. 374-86.
444 12. Oehlers, S.H., et al., *A chemical enterocolitis model in zebrafish larvae that is dependent on*
445 *microbiota and responsive to pharmacological agents.* Dev Dyn, 2011. **240**(1): p. 288-98.
446 13. Morris, S., et al., *Glucose inhibits haemostasis and accelerates diet-induced hyperlipidaemia*
447 *in zebrafish larvae.* Sci Rep, 2021.
448 14. Cheesman, S.E., et al., *Epithelial cell proliferation in the developing zebrafish intestine is*
449 *regulated by the Wnt pathway and microbial signaling via Myd88.* Proc Natl Acad Sci U S A,
450 2011. **108 Suppl 1**: p. 4570-7.
451 15. Novoa, B., et al., *LPS response and tolerance in the zebrafish (Danio rerio).* Fish Shellfish
452 Immunol, 2009. **26**(2): p. 326-31.
453 16. Kaye, D.M., et al., *Deficiency of Prebiotic Fiber and Insufficient Signaling Through Gut*
454 *Metabolite-Sensing Receptors Leads to Cardiovascular Disease.* Circulation, 2020. **141**(17):
455 p. 1393-1403.
456 17. Tan, F., et al., *The Responses of Germ-Free Zebrafish (Danio rerio) to Varying Bacterial*
457 *Concentrations, Colonization Time Points, and Exposure Duration.* Front Microbiol, 2019.
458 **10**: p. 2156.
459 18. Weitekamp, C.A., et al., *Monoassociation with bacterial isolates reveals the role of*
460 *colonization, community complexity and abundance on locomotor behavior in larval*
461 *zebrafish.* Anim Microbiome, 2021. **3**(1): p. 12.
462 19. Robinson, C.D., et al., *Experimental bacterial adaptation to the zebrafish gut reveals a*
463 *primary role for immigration.* PLoS Biol, 2018. **16**(12): p. e2006893.
464 20. Griffin, M.E., et al., *Enterococcus peptidoglycan remodeling promotes checkpoint inhibitor*
465 *cancer immunotherapy.* Science, 2021. **373**(6558): p. 1040-1046.
466 21. Xie, K., et al., *Dietary S. maltophilia promotes fat storage by enhancing*
467 *lipogenesis and ER-LD contacts in C. elegans.* bioRxiv, 2020: p.
468 2020.04.29.067793.

469 22. Brooke, J.S., *Stenotrophomonas maltophilia: an emerging global opportunistic pathogen.*
470 Clin Microbiol Rev, 2012. **25**(1): p. 2-41.
471 23. Pathmanathan, A. and G.W. Waterer, *Significance of positive Stenotrophomonas*
472 *maltophilia culture in acute respiratory tract infection.* Eur Respir J, 2005. **25**(5): p. 911-4.
473 24. Melancon, E., et al., *Best practices for germ-free derivation and gnotobiotic zebrafish*
474 *husbandry.* Methods Cell Biol, 2017. **138**: p. 61-100.
475 25. Henderson, J.M., et al., *Multiple liver insults synergize to accelerate experimental*
476 *hepatocellular carcinoma.* Sci Rep, 2018. **8**(1): p. 10283.
477 26. Oehlers, S.H., et al., *Chemically induced intestinal damage models in zebrafish larvae.*
478 Zebrafish, 2013. **10**(2): p. 184-93.
479 27. Fang, L., et al., *In vivo visualization and attenuation of oxidized lipid accumulation in*
480 *hypercholesterolemic zebrafish.* J Clin Invest, 2011.
481 28. Johansen, M.D., et al., *Mycobacterium marinum infection drives foam cell differentiation in*
482 *zebrafish infection models.* Dev Comp Immunol, 2018. **88**: p. 169-172.
483 29. Wu, R.S., et al., *A Rapid Method for Directed Gene Knockout for Screening in G0 Zebrafish.*
484 Dev Cell, 2018. **46**(1): p. 112-125 e4.
485 30. Cholan, P.M., et al., *Conserved anti-inflammatory effects and sensing of butyrate in*
486 *zebrafish.* Gut Microbes, 2020. **12**(1): p. 1-11.
487

488 **Figure Legends**

489 Figure 1: Transplantation of microbiota from HFD-fed mice accelerates hyperlipidaemia in
490 zebrafish embryos.

491 (a) Schematic describing method of faecal microbiome inoculation in MD zebrafish and chicken
492 egg yolk challenge diet. (b) Representative images of neutral red staining in mid-gut of 5 dpf
493 zebrafish embryos. Red brackets indicate mid-gut region used for quantification. (c) Quantification
494 of neutral red staining area in mid-gut of 5 dpf zebrafish embryos. (d) Representative images of Oil
495 Red O staining of 7 dpf chow-fed and HFD-fed mouse faecal microbiome-inoculated zebrafish
496 embryos. Red brackets indicate tail region posterior to the swim bladder used for quantification
497 (e) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
498 bladder in MD zebrafish inoculated with mouse faecal microbiota and challenged with chicken egg
499 yolk diet from 5-7 dpf. Scale bar represents 500 μm. Results are expressed as mean ± SD.

500

501 Figure 2: Identification of individual microbes with pathobiont activity.

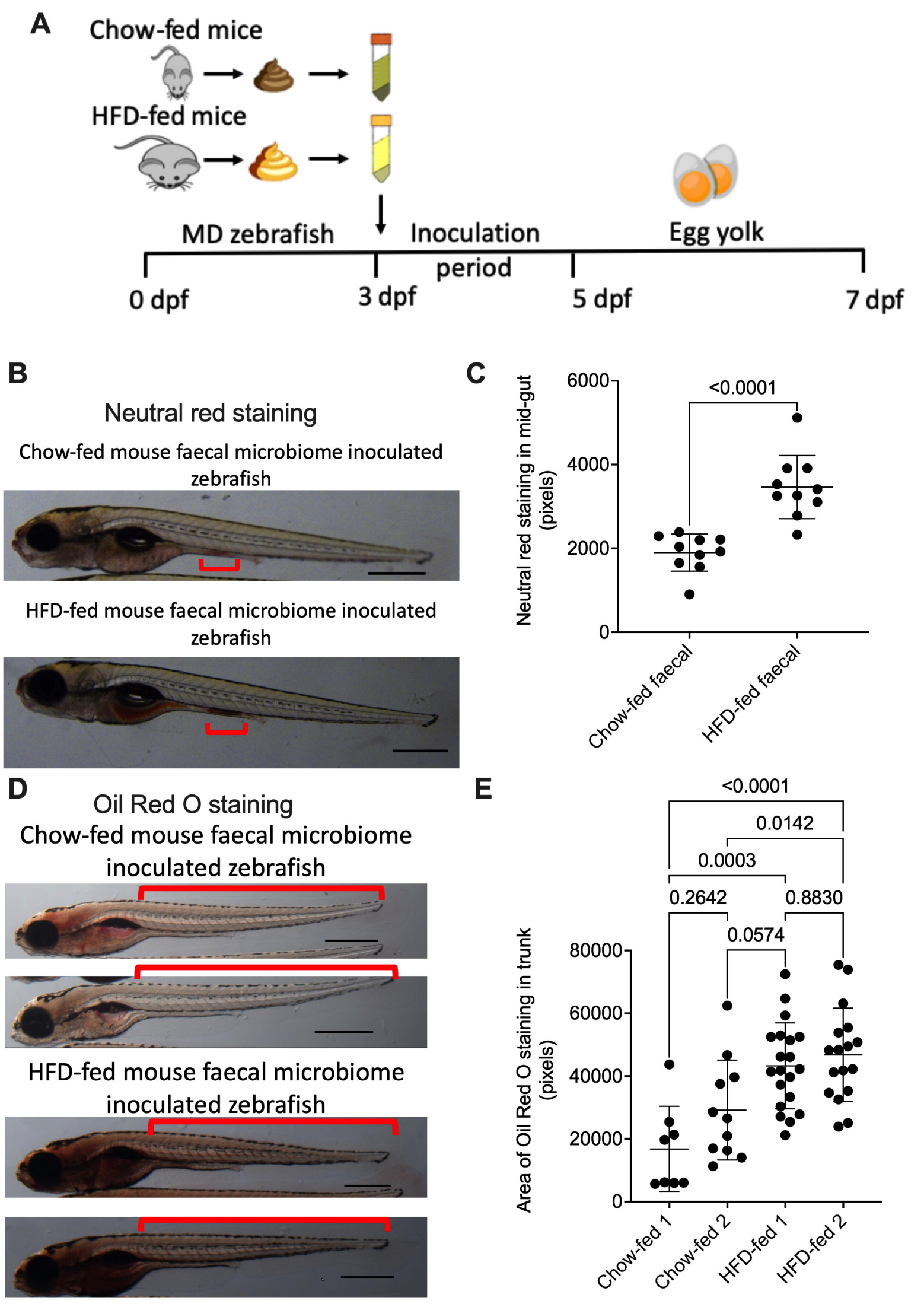
502 (a) Schematic describing workflow to isolate *Escherichia species* PYCC8248 (*E.s*), *Escherichia coli*
503 strain Y15 (*E.c*), and *Enterococcus faecalis* strain YN771 (*E.f*) and inoculate into MD zebrafish with
504 subsequent chicken egg yolk diet challenge. (b) Quantification of trunk vascular Oil Red O staining
505 from the tail region posterior to the swim bladder in MD zebrafish inoculated with bacterial
506 isolates *E.s*, *E.c*, and *E.f* and challenged with a chicken egg yolk diet from 5-6 dpf. (c) Area of
507 neutral red stained in the mid-gut of 5 dpf gnotobiotic zebrafish embryos mono-associated with
508 bacterial isolates *E.s*, *E.c*, and *E.f*. (d) Quantification of trunk vascular Oil Red O staining from the
509 tail region posterior to the swim bladder in MD zebrafish inoculated with bacterial isolates *E.f* and
510 *E. faecalis* UNSW 054400 type strain (*E.f* UNSW) then challenged with a chicken egg yolk diet from
511 5-6 dpf. Results are expressed as mean ± SD.
512
513 Figure 3: Stenotrophomonas maltophilia accelerates hyperlipidaemia by digesting food.
514 (a) Schematic describing creation of "pre-digested" chicken egg yolk by incubation with bacterial
515 isolates *E.*s, *E.c*, *S.m*, and *E.f*, autoclaving, for feeding to 5-7 dpf zebrafish embryos. (b)
516 Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
517 bladder in MD zebrafish fed "pre-digested" egg yolk with bacterial isolates *E.*s, *E.c*, *S.m*, and *E.f*
518 from 5-7 dpf. (c) Quantification of trunk vascular Oil Red O staining from the tail region posterior
519 to the swim bladder in conventionally raised zebrafish fed "pre-digested" egg yolk with bacterial
520 isolate *S.m* from 5-7 dpf. (d) Quantification of trunk vascular Oil Red O staining from the tail region
521 posterior to the swim bladder in conventionally raised zebrafish fed "pre-digested" fish embryo
522 food with bacterial isolates *E.c* and *S.m* from 5-7 dpf. (e) Representative images of "pre-digested"
523 egg yolk with bacterial isolates *E.c* and *S.m* red brackets indicate fraction of the water column
524 containing large particulates after autoclaving. (f) CFU recovery from chicken egg yolk "pre-
525 digestion" reactions. Results are expressed as mean ± SD.
526

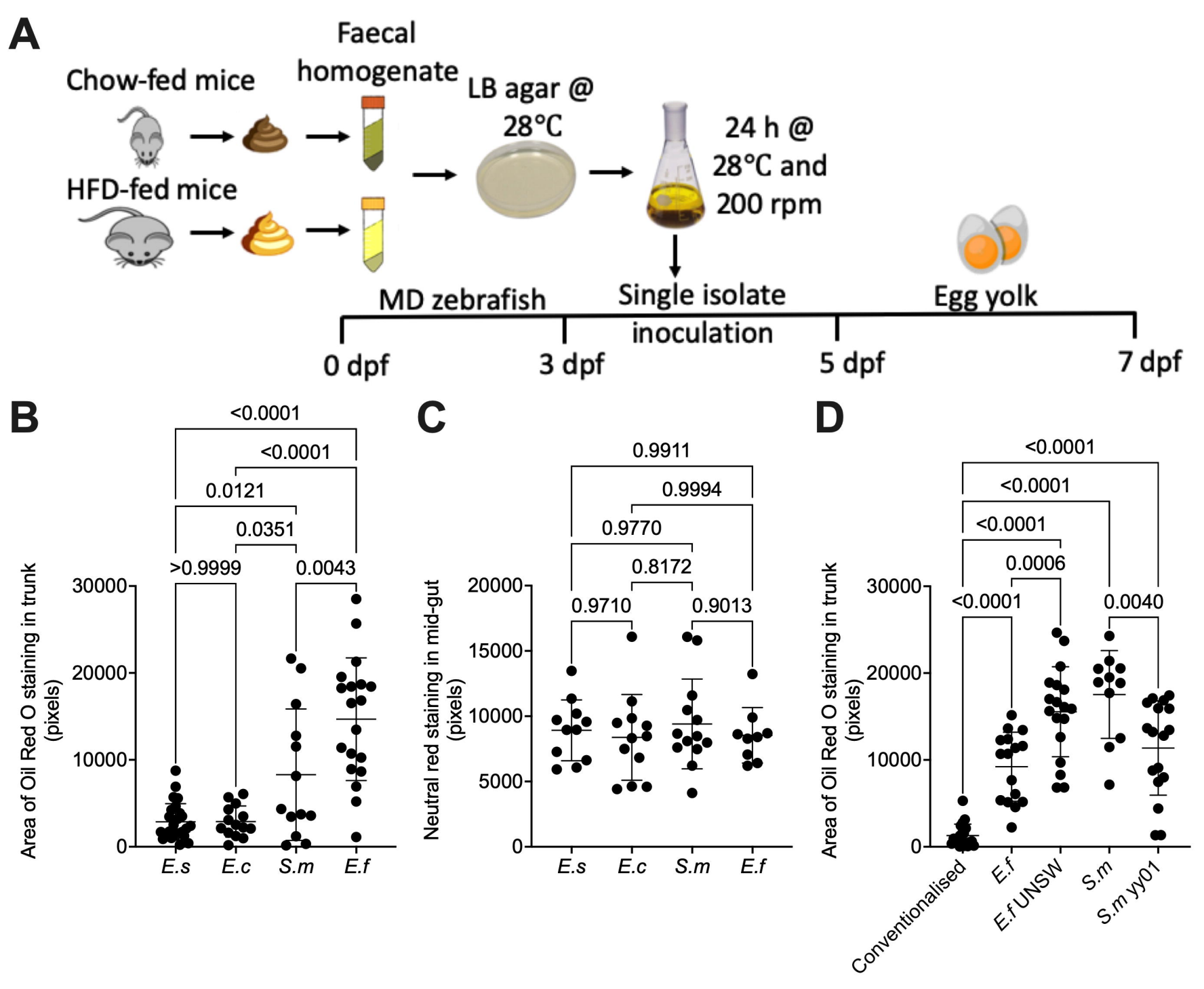
527 Figure 4: *Enterococcus faecalis* (*E.f*) accelerates hyperlipidaemia via host MyD88-mediated
528 signalling.
529 (a) Schematic describing inoculation of MD zebrafish embryos with heat-killed bacterial isolates
530 *E.s*, *E.c*, and *E.f* from 3-5 dpf, followed by chicken egg yolk diet challenge from 5-7 dpf. (b)
531 Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
532 bladder in MD zebrafish inoculated with heat killed bacterial isolates *E.s*, *E.c*, and *E.f* and
533 challenged with a chicken egg yolk diet. (c) Quantification of *myd88* expression in zebrafish
534 embryos injected with *myd88*-targeting CRISPR-Cas9 complexes at 5 dpf. Each dot represents a
535 biological replicate of at least 10 embryos. (d) Quantification of trunk vascular Oil Red O staining
536 from the tail region posterior to the swim bladder in control scrambled and *myd88* crispant
537 embryos exposed to *E.f* and challenged with a chicken egg yolk diet. Results are expressed as
538 mean ± SD.
539
540 Figure 5: Gram-positive cell wall components accelerate hyperlipidaemia in zebrafish embryos.
541 (a) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
542 bladder in MD zebrafish inoculated with heat killed bacterial isolates *E.f* and Staphylococcus
543 xylosus (*S.x*) from 3-5 dpf and challenged with a chicken egg yolk diet from 5-7 dpf. (b)
544 Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
545 bladder in MD zebrafish pre-incubated with LTA or peptidoglycan from 3-5 dpf before co-
546 challenge with chicken egg yolk diet from 5-7 dpf. (c) Quantification of trunk vascular Oil Red O
547 staining from the tail region posterior to the swim bladder in MD zebrafish pre-incubated with
548 lysozyme-treated heat killed *E.f* from 3-5 dpf and challenged with a chicken egg yolk diet from 5-7
549 dpf. (d) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the
550 swim bladder in control scrambled and *myd88* crispant embryos pre-incubated with LTA or

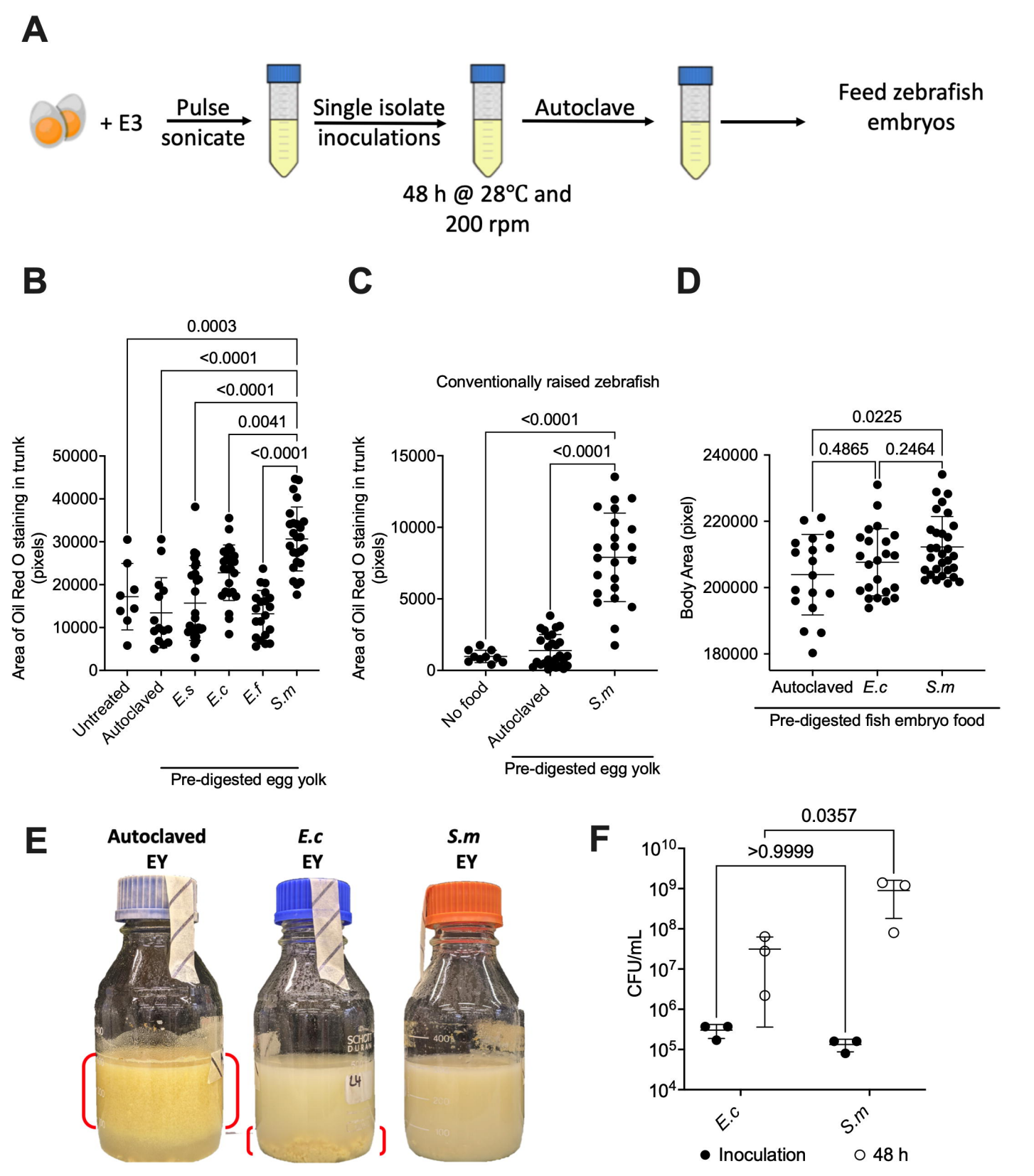
551 peptidoglycan from 3-5 dpf before co-challenge with chicken egg yolk diet from 5-7 dpf. Results

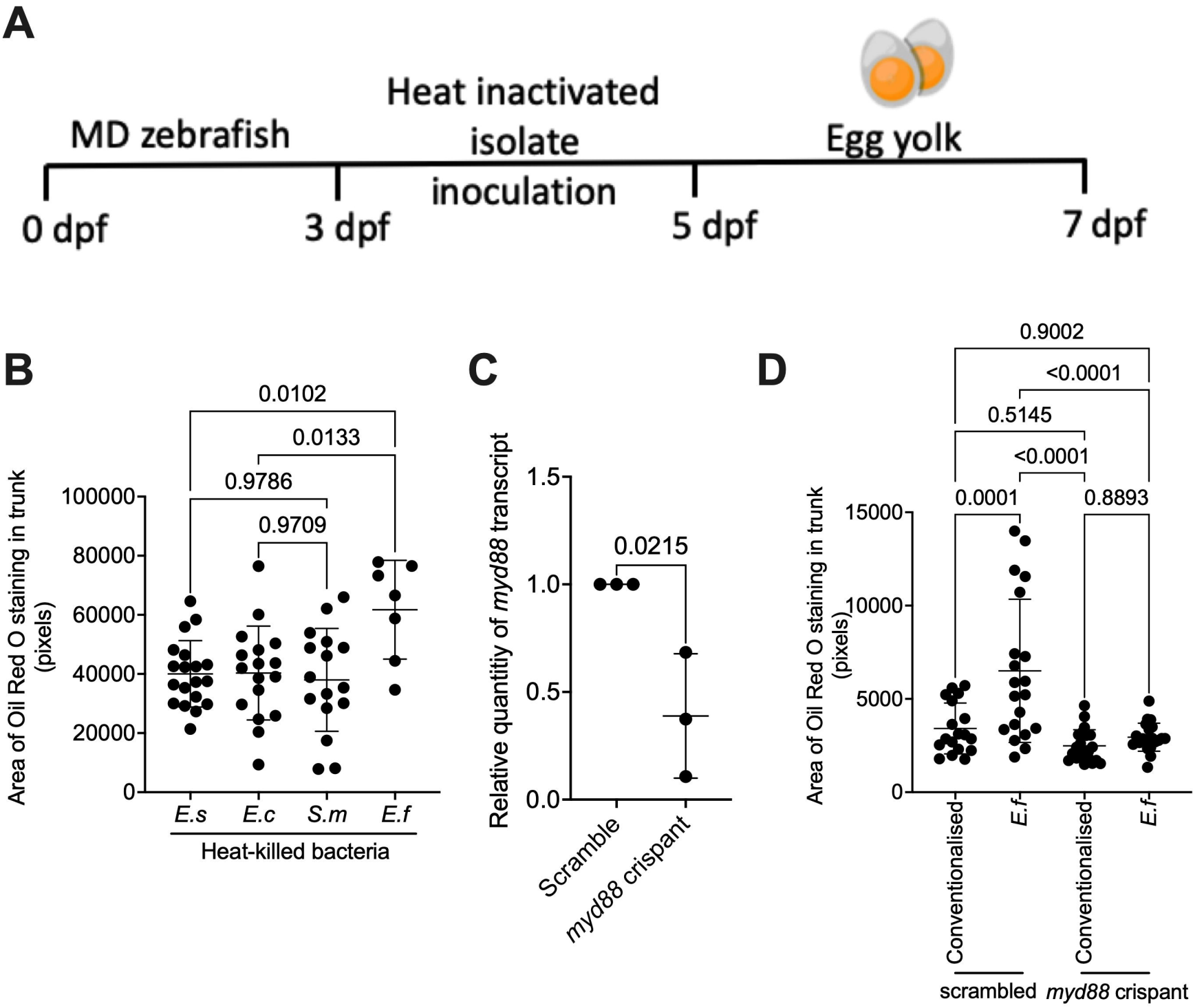
552 are expressed as mean ± SD.

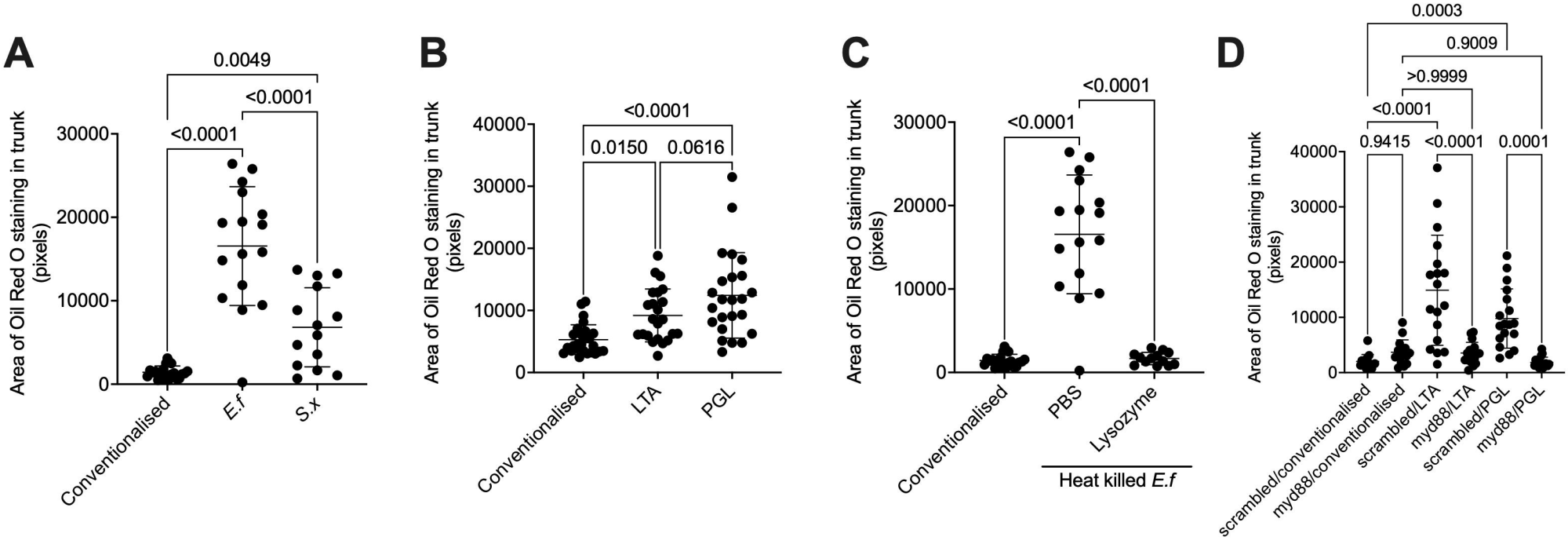
553


A
Chow-fed mice
HFD-fed mice
MD zebrafish
Inoculation period
Egg yolk
0 dpf
3 dpf
5 dpf
7 dpf
B
Neutral red staining
Chow-fed mouse faecal microbiome inoculated zebrafish
HFD-fed mouse faecal microbiome inoculated zebrafish
C
Neutral red staining in mid-gut (pixels)
6000
4000
2000
0
<0.0001
Chow-fed faecal
HFD-fed faecal
D
Oil Red O staining
Chow-fed mouse faecal microbiome inoculated zebrafish
HFD-fed mouse faecal microbiome inoculated zebrafish
E
Area of Oil Red O staining in trunk (pixels)
80000
60000
40000
20000
0
<0.0001
0.0142
0.0003
0.2642
0.8830
0.0574
Chow-fed 1
Chow-fed 2
HFD-fed 1
HFD-fed 2


A
Chow-fed mice
HFD-fed mice
Faecal homogenate
LB agar @ 28°C
24 h @ 28°C and 200 rpm
Single isolate inoculation
MD zebrafish
Egg yolk
0 dpf
3 dpf
5 dpf
7 dpf
B
Area of Oil Red O staining in trunk (pixels)
<0.0001
<0.0001
0.0121
0.0351
>0.9999
0.0043
E.s
E.c
S.m
E.f
C
Neutral red staining in mid-gut (pixels)
0.9911
0.9994
0.9770
0.8172
0.9710
0.9013
E.s
E.c
S.m
E.f
D
Area of Oil Red O staining in trunk (pixels)
<0.0001
<0.0001
<0.0001
0.0006
<0.0001
0.0040
Conventionalised
E.f
E.f UNSW
S.m
S.m yy01


A
+ E3
Pulse sonicate
Single isolate inoculations
Autoclave
Feed zebrafish embryos
48 h @ 28°C and 200 rpm
B
Area of Oil Red O staining in trunk (pixels)
0.0003
<0.0001
<0.0001
0.0041
<0.0001
Untreated
Autoclaved
E.s
E.c
E.f
S.m
Pre-digested egg yolk
C
Conventionally raised zebrafish
Area of Oil Red O staining in trunk (pixels)
<0.0001
<0.0001
No food
Autoclaved
S.m
Pre-digested egg yolk
D
Body Area (pixel)
0.0225
0.4865
0.2464
Autoclaved
E.c
S.m
Pre-digested fish embryo food
E
Autoclaved EY
E.c EY
S.m EY
F
CFU/mL
0.0357
>0.9999
E.c
S.m
Inoculation
48 h


A
MD zebrafish
Heat inactivated isolate
Egg yolk
inoculation
0 dpf
3 dpf
5 dpf
7 dpf
B
0.0102
0.0133
0.9786
0.9709
Area of Oil Red O staining in trunk (pixels)
100000
80000
60000
40000
20000
0
E.s
E.c
S.m
E.f
Heat-killed bacteria
C
0.0215
Relative quantitiy of myd88 transcript
1.5
1.0
0.5
0.0
Scramble
myd88 crispant
D
0.9002
<0.0001
0.5145
<0.0001
0.0001
0.8893
Area of Oil Red O staining in trunk (pixels)
15000
10000
5000
0
Conventionalised
E.f
Conventionalised
E.f
scrambled
myd88 crispant


A
0.0049
<0.0001
<0.0001
Area of Oil Red O staining in trunk (pixels)
30000
20000
10000
0
Conventionalised
E.f
S.x
B
<0.0001
0.0150
0.0616
Area of Oil Red O staining in trunk (pixels)
40000
30000
20000
10000
0
Conventionalised
LTA
PGL
C
<0.0001
<0.0001
Area of Oil Red O staining in trunk (pixels)
30000
20000
10000
0
Conventionalised
PBS
Lysozyme
Heat killed E.f
D
0.0003
0.9009
>0.9999
<0.0001
0.9415
<0.0001
0.0001
Area of Oil Red O staining in trunk (pixels)
40000
30000
20000
10000
0
scrambled/conventionalised
myd88/conventionalised
scrambled/LTA
myd88/LTA
scrambled/PGL
myd88/PGL