Transplantation of high fat fed mouse microbiota into zebrafish embryos identifies pathobiont species

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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 embryos 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 embryos are more susceptible to hyperlipidaemia when colonised with mouse high fat-diet-associated microbiota and that this effect can be driven by individual bacterial species. Colonisation or exposure to *Enterococcus faecalis* activates host Myd88 signalling, an effect that is phenocopied by the Gram positive Toll-like receptor agonists peptidoglycan and lipoteichoic acid, to drive hyperlipidaemia. In contrast, we find *Stenotrophomonas maltophilia* increases the hyperlipidaemic potential of chicken egg yolk independent of gut colonisation. In this work, we demonstrate the applicability of zebrafish as a tractable host for the identification of gut pathobionts 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 can act as function screening tools for disease-associate 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 vivo* 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 HFD challenge [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

Transplantation of microbiota from HFD fed mice accelerates hyperlipidaemia in zebrafish embryos

Microbiome-depleted (MD) zebrafish embryos were exposed to mouse faecal microbiota preparations generated from mice fed a conventional chow diet or HFD from 3-5 dpf (days post fertilization) and challenged with chicken egg yolk feeding from 5-7 dpf (Figure 1A).

We first investigated if colonisation with the control or HFD-associated mouse microbiota affected zebrafish embryo intestinal physiology at 5 dpf before the onset of exogenous feeding. The absorptive activity of zebrafish midgut lysosome-rich intestinal epithelial cells requires microbial colonisation and is impeded by inflammation [11,12]. We used neutral red staining visually to examine the absorptive function of colonised embryos and observed higher staining in 5 dpf embryos colonised with the HFD fed microbiota compared to chow diet colonised controls (Figure 1B and 1C).

We next collected colonised zebrafish embryos one and two days after exposure to the chicken egg yolk challenge and stained with Oil Red O to visualise neutral lipids (Figure 1D). Zebrafish embryos colonised with the faecal microbiota from HFD fed mice had more vascular Oil Red O staining after 2 day of compared to zebrafish embryos colonised with the faecal microbiota from chow diet fed mice (Figure 1E)

These data demonstrate the responsiveness of MD zebrafish embryos to the transferable effects of dysbiotic mammalian gut microbiome-associated microbiota when challenged with a complex environmental stimulus in the form of lipid-rich feeding.

Identification of individual microbes with pathobiont activity

To isolate individual species that would be amenable to *in vitro* handling and growth, faecal homogenate supernatants were plated on LB agar and grown at 28°C to select for species that would be easily handled and most likely to colonise zebrafish embryos (Figure 2A). The best growing isolate in LB broth culture from two chow diet and HFD fed faecal preparations were selected and sequenced. We identified *Enterococcus faecalis* strain YN771 (*E.f*) and *Stenotrophomonas maltophilia* strain CD103 (*S.m*) from HFD-fed mouse faecal lysate, and

Escherichia species PYCC8248 (*E.s*) and *Escherichia coli* strain Y15-3 (*E.c*) from chow diet-fed mouse faecal lysate.

We colonised MD zebrafish embryos with individual isolates to determine if specific pathobionts from HFD-fed mouse microbiomes could phenocopy the effects of bulk microbiota transfer. Gnotobiotic zebrafish embryos colonised with *E. faecalis* or *S. maltophilia* had increased Oil Red O staining compared to embryos colonised with either of the *Escherichia* strains (Figure 2B).

Interestingly, colonisation with *E. faecalis* or *S. maltophilia* did not increase the absorptive activity of the midgut intestinal epithelium compared to embryos colonised with either of the *Escherichia* strains suggesting the absorptive phenotype seen in bulk microbiota transplant is the product of multiple microorganisms (Figure 2C).

We next sought to confirm our observations using a second strain of each bacterial species, *E. faecalis* UNSW 054400 type strain (*E.f* UNSW) and *S. maltophilia* yy01 (*S.m* yy01) isolated from another mouse in the same facility. Colonisation of MD zebrafish embryos with either strain phenocopied the hyperlipidaemic phenotype seen with our original isolates (Figure 2D). Some variability was observed between strains of the same species suggesting strain-specific effects in our zebrafish embryo system.

Together this data demonstrates gnotobiotic zebrafish embryos can be used to identify individual pathobionts from the microbiomes of diet-challenged mice.

Enterococcus faecalis accelerates hyperlipidaemia by activating host immune signalling pathways

123 To investigate the mechanisms by which *E. faecalis* and *S. maltophilia* accelerate diet-induced
124 hyperlipidaemia in zebrafish embryos, we first asked if these bacteria needed to be alive to exert
125 their pathobionic effect. We adapted our gnotobiotic methodology to expose MD embryos to heat
126 killed bacterial preparations prior to chicken egg yolk feeding (Figure 3A).
127
128 Exposure to heat-killed *E. faecalis* phenocopied the live *E. faecalis* hyperlipidaemic phenotype
129 when compared MD embryos that had been exposed to either heat killed *S. maltophilia* or either
130 of the *Escherichia* strains (Figure 3B). Conversely, heat-killed *S. maltophilia* did not induce
131 hyperlipidaemia in concert with chicken egg yolk challenge.
132
133 Host innate immune signalling via the Myd88 adaptor protein is essential for the zebrafish
134 intestinal epithelium to respond to microbial colonisation [13]. We performed knockdown of host
135 *myd88* expression using multiple CRISPR-Cas9 gRNAs (Figure 3C). Host *myd88* expression was
136 necessary for transducing the *E. faecalis*-induced hyperlipidaemic signal as *myd88* crispants had
137 significantly less Oil Red O staining than scrambled gRNA/Cas9-injected control embryos after
138 colonisation with *E. faecalis* and chicken egg yolk challenge (Figure 3D).
139
140 Gram positive cell wall components accelerate hyperlipidaemia in zebrafish embryos
141 *E. faecalis* is a Gram-positive bacterium. To determine if *E. faecalis*-driven hyperlipidaemia was
142 due to a conserved Gram-positive cell wall component, we initially compared the hyperlipidaemic
143 potential of heat-killed *E. faecalis* to heat killed *Staphylococcus xylosus* (*S.x*), another Gram-
144 positive bacterium obtained from the faecal microbiota of a mouse from the same facility. We
145 found exposure to heat-killed *S. xylosus* phenocopied the hyperlipidaemic effect of heat-killed *E.*
146 *faecalis* after chicken egg yolk challenge (Figure 4A).
147

Next we directly soaked MD embryos in a sublethal dose of 25 µg/mL purified lipoteichoic acid (LTA) or peptidoglycan (PGL), which are major components of the Gram-positive cell wall, prior to challenge with chicken egg yolk feeding [14]. Either one of these purified ligands were able to accelerate hyperlipidaemia in MD embryos (Figure 4B).

To test the requirement for intact *E. faecalis* PGL to accelerate hyperlipidaemia, we exposed MD embryos to lysozyme-digested heat-killed *E. faecalis* prior to chicken egg yolk feeding. Lysozyme digestion ablated the ability of heat-killed *E. faecalis* to accelerate hyperlipidaemia (Figure 4C).

To determine if host innate immune signalling via the Myd88 adaptor protein transduced the LTA or PGL-induced signal that accelerates hyperlipidaemia, we again knocked down *myd88* using a pooled CRISPR-Cas9 gRNA approach and exposed crispant embryos to LTA or PGL. Depletion of host *myd88* ablated the sensitivity of embryos to LTA and PGL-induced accelerated hyperlipidaemia (Figure 4D).

Together, these experiments demonstrate zebrafish embryo lipid metabolism is sensitive to the presence of Gram-positive bacterial cell wall components via Myd88-mediated host signalling pathways.

Stenotrophomonas maltophilia accelerates hyperlipidaemia by digesting food

Since we had previously shown *S. maltophilia* needed to be alive to accelerate hyperlipidaemia, we hypothesised *S. maltophilia* might interact with the chicken egg yolk independently of the host colonisation. To test this hypothesis, we incubated chicken egg yolk with each of our 4 bacterial isolates in conditions representative of the zebrafish embryo media and then sterilised the "pre-digested" chicken egg yolk by autoclaving prior to feeding to MD zebrafish embryos (Figure 5A).

Compared to either untreated chicken egg yolk, autoclaved chicken egg yolk, or chicken egg yolk incubated with the other three bacterial isolates, the chicken egg yolk that had been incubated with *S. maltophilia* increased hyperlipidaemia in zebrafish embryos (Figure 5B).

We next repeated this experiment in conventionally raised embryos and found the increased hyperlipidaemic potential of *S. maltophilia* "pre-digested" chicken egg yolk was independent of colonisation by commensal microbes (Figure 5C). To examine the substrate specificity of *S. maltophilia*, we incubated commercially available fish embryo food with *S. maltophilia* and observed an increase in body size of embryos fed the "pre-digested" commercial feed compared to untreated commercial feed (Figure 5D).

Visual inspection of *S. maltophilia* "pre-digested" chicken egg yolk suspensions suggested *S. maltophilia* had broken apart the chicken egg yolk resulting in smaller particles that could be more easily ingested and altered the biochemical properties of the chicken egg yolk as the solution contained much finer particles than for other treatments (Figure 5E). An intermediate phenotype was seen in chicken egg yolk that had been "pre-digested" by *E. coli* strain Y15-3. CFU recovery assays demonstrated higher growth of *S. maltophilia* than *E. coli* strain Y15-3 suggesting the better growth of *S. maltophilia* may convert chicken egg yolk into components that could be digested by zebrafish embryos (Figure 5F). We performed nutritional panel and free fatty acid analyses of chicken egg yolk that had been "pre-digested" by *S. maltophilia* or *E. coli* strain Y15-3 as an additional control. Fatty acid analysis revealed an increase in total fat content, made up of monounsaturated and saturated fatty acids, in *S. maltophilia*-incubated samples that was not seen in control or *E. coli* strain Y15-3-incubated samples (Table 1).

These data illustrate an intestinal colonisation-independent mechanism by which *S. maltophilia* may enhance lipid uptake in zebrafish embryos by increasing the lipid content and decreasing the physical size of food.

Discussion

Our study demonstrates the utility of the gnotobiotic zebrafish platform to screen donor microbiota samples for transplantable biological activities in combination with an exogenous environmental factor. The addition of an exogenous trigger to the experimental system is an important permutation that allows the identification of pathobionts whose disease-associated interactions with the host only become apparent in combination with an environmental challenge such as diet.

We applied the gnotobiotic zebrafish platform to the diet-hyperlipidaemia axis as there is a growing interest in microbiome studies within the cardiovascular disease field [15]. The gnotobiotic zebrafish platform represents a disruptive technology that could be used to identify additional pathobiont species from human cardiovascular disease patients and as a "first pass" platform in mechanistic studies to link these species to cardiovascular pathology in mammalian systems.

Our investigation of *E. faecalis*-accelerated hyperlipidaemia uncovered a surprising role of Gram-positive cell wall component-triggered Myd88 signalling in zebrafish embryo lipid metabolism. The presence of this response in zebrafish embryos has broad implications for the use of zebrafish embryos to study transplanted mammalian microbiota as it may not be representative of the mammalian response to colonisation with Gram positive organisms. Most zebrafish embryo mono-association studies have been carried out with Gram negative organisms including *E. coli, A. veronii, V. cholerae,* and *P. aeruginosa* [16-18].

The comparison of heat killed *E. faecalis* to heat killed *S. xylosus* demonstrated an increased ability of heat killed *E. faecalis* to accelerate diet-induced hyperlipidaemia. This suggests that there is variability in hyperlipidaemia accelerating potential amongst Gram positive organisms in zebrafish embryos. The basis of this difference could be further explored using model Gram positive organisms and Gram-positive organisms that are natural commensals in the zebrafish gut.

Caenorhabditis elegans fed with *S. maltophilia* accumulate neutral lipids within intracellular lipid droplets driven by a bacterially-encoded mechanism that is independent of the innate immune response [19]. Our finding that two distinct mouse-associated *S. maltophilia* strains were able to increase the hyperlipidaemic potential of chicken egg yolk suggests the digestive ability of *S. maltophilia* is potentially conserved between strains and potentially between host species. Previous studies have identified *S. maltophilia* strains within zebrafish gut microbiomes [9], these zebrafish-associated strains could be analysed to determine if increased lipid uptake is a consequence of natural host-*S. maltophilia* pairs.

We also found an embryo growth-enhancing effect of pre-digesting commercially available zebrafish embryo feed with *S. maltophilia* which suggests *S. maltophilia* could have commercial applications as an aquaculture feed additive. The use of *S. maltophilia* as a feed additive is potentially risky as this organism is associated with opportunistic infections in humans and is resistant to a wide range of antibiotics [20,21].

As a proof-of-principle study, our work demonstrates the feasibility of studying the interaction of transplanted pathobiont species, from a mammalian host, with an environmental factor to result in a conditional pathological phenotype in the tractable zebrafish model system. Further work is

248 required to examine effects of the two pathobiont species identified in our study on mammalian
249 models of cardiovascular disease and to correlate their colonisation with the progression of
250 cardiovascular disease phenotypes in mammalian models and human samples.
251
252 **Methods**
253 Zebrafish handling
254 Adult zebrafish were housed at the Centenary Institute (Sydney Local Health District Animal
255 Welfare Committee Approval 17-036). Zebrafish embryos were obtained by natural spawning and
256 conventionally raised embryos were maintained in E3 media at 28°C.
257
258 Generation of microbiome-depleted (MD) zebrafish embryos
259 Microbiome-depleted (MD) zebrafish were created and maintained as previously described [22].
260 Briefly, freshly laid embryos were rinsed with 0.003% v/v bleach in sterile E3 and rinsed 3 times
261 with sterile E3. Bleached embryos were raised in sterile E3 supplemented with 50 µg/mL ampicillin
262 (Sigma), 5 µg/mL kanamycin (Sigma) and 250 ng/mL amphotericin B (Sigma) in sterile tissue
263 culture flasks. Dead embryos and chorions were aseptically removed at one and three dpf
264 respectively.
265
266 Conventionalised zebrafish were used as a control, where 3 dpf MD zebrafish were inoculated
267 with system water from the aquarium at Centenary Institute.
268
269 Generation of mouse faecal microbiota specimens
270 Mice were housed at the Centenary Institute (Sydney Local Health District Animal Welfare
271 Committee Approval 2018/016). C57BL/6J mice were housed in a pathogen-free and temperature-
272 controlled environment, with 12 hours of light and 12 hours of darkness, and free access to food

and water. Mice were provided with a High Fat Diet (HFD) or chow from 6 to 30 weeks of age. The HFD was prepared in-house based on rodent diet no. D12451 (Research Diets New Brunswick, USA) and its calories were supplied as: fat 45%, protein 20%, and carbohydrate 35% [23]. The chow diet was commercially produced by Speciality Feeds as "Irradiated Rat and Mouse Diet" and its calories were supplied as: fat 12%, protein 23%, and carbohydrate 65%.

Faecal pellets were collected from mice that were housed in different cages. Individual faecal pellets were collected into sterile 1.7 mL microcentrifuge tubes and homogenised in 1 mL of sterile E3 by pipetting. Homogenised specimens were centrifuged at 500 G for 2 minutes to sediment fibrous material and the supernatant was collected. Supernatants were supplemented with glycerol to a final concentration of 25% v/v, aliquoted, and frozen at -80°C for experimental use.

Colonisation of zebrafish with mouse faecal microbiota

MD zebrafish were colonised at 3 dpf by transfer into sterile E3 and addition of 200 µL thawed faecal homogenate supernatant. At 5 dpf, embryos were rinsed with E3 and placed on a chicken egg yolk diet.

Neutral red staining and morphology measurements

Neutral red staining was performed as previously described [24]. Briefly, 2.5 µg/mL neutral red was added to the media of 4 dpf embryos and incubated overnight. Embryos were rinsed with fresh E3 to remove unbound neutral red and live imaged on a Leica M205FA microscope with a consistent zoom between specimens in a single experiment. The area of neutral red-stained midgut was measured in pixels using ImageJ. Body size was measured in pixels using ImageJ.

Zebrafish high fat diet challenge with chicken egg yolk

High fat diet challenge with chicken egg yolk was performed as previously described [25]. Briefly, 5 dpf zebrafish embryos were placed in an E3 solution containing 0.05% w/v emulsified hard boiled chicken egg yolk in glass beakers. Beakers were housed in a 28°C incubator with a 14:10 hour light:dark cycle. The emulsified hard boiled chicken egg yolk solution was changed daily.

Oil Red O staining assay

Oil Red O staining and analysis was performed as previously described [25-27]. Briefly, 7 dpf embryos were fixed overnight at 4°C in 4% paraformaldehyde, rinsed with PBS, and rinsed stepwise through to propylene glycol. Embryos were stained with filtered 0.5% (w/v) Oil Red O dissolved in propylene glycol overnight at room temperature. Unbound dye was removed by washing with propylene glycol and embryos were rinsed stepwise through to PBS for imaging.

Embryos were imaged on a Leica M205FA microscope. Experimental batches of colour images were analysed in ImageJ by adjusting the colour threshold function to eliminate non-red signal, this output was then converted to a binary mask and the tail region posterior to the swim bladder was selected to measure the area of particles.

Isolation, identification, and handling of bacterial isolates from mouse faecal microbiota samples

Faecal homogenate supernatants were plated onto LB Agar (Amyl Media) and incubated at 28°C for two days. Individual isolates were grown in broth culture in Luria Broth (Miller's LB Broth Base, Thermofisher) at 28°C overnight with 200 RPM shaking.

For identification, bacteria were harvested from broth culture and subjected to PCR using universal 16S primers (5-3) Fw Rv. PCR products were sequenced by Sanger Sequencing (AGRF)

and NCBI records were searched by BLAST to identify the closest matching bacterial strain by sequence identity.

For gnotobiotic experiments, bacteria were harvested from overnight broth cultures and resuspended in sterile E3 zebrafish embryo media at a concentration of OD600 0.2. MD zebrafish in autoclaved E3 were then inoculated with the bacterial suspension at a ratio of 1:200.

For heat killed bacterial inoculations, bacteria were resuspended in E3 at a concentration of OD600 0.2 and heat-killed in a 95°C heat block for 30 minutes. Heat killed bacterial solutions were then added at 1:200 ratio to 3 dpf MD zebrafish.

For purified ligand exposure, 3 dpf MD zebrafish were soaked in 25 µg/mL lipoteichoic acid (Sigma) or 25 µg/mL peptidoglycan (Sigma).

For lysozyme digestion of heat-killed *E. faecalis*, a suspension of heat-killed bacteria was incubated with 50 µg/mL lysozyme (Sigma) at 37°C for 6 h before being used to inoculate 3 dpf MD zebrafish at a ratio of 1:200.

Gene knockdown with CRISPR-Cas9

gRNA templates for *myd88* (5`- 3`): Target 1 TAATACGACTCACTATAGGCAGTTTCCGAAAGAAACTGTTTTAGAGCTAGAAATAGC, Target 2 TAATACGACTCACTATAGGAAAAGGTCTTGACGGACTGTTTTAGAGCTAGAAATAGC, Target 3 TAATACGACTCACTATAGGAACTGTTTGATCATCTCGGTTTTAGAGCTAGAAATAGC, Target 4 TAATACGACTCACTATAGGTTTTTTCGATAAGCTCACGTTTTAGAGCTAGAAATAGC. gRNA was synthesized as previously described [28].

A 1:1 solution of gRNA and 500 µg/mL of Cas9 nuclease V3 (Integrated DNA Technology) was prepared with phenol red dye (Sigma, P0290). Freshly laid eggs were collected from breeding tanks and the solution was injected in the yolk sac of the egg before the emergence of the first cell with a FemtoJet 4i (Eppendorf).

Knockdown efficacy was monitored by RT-qPCR as previously described [29]. *myd88*-specific primers (5`- 3`): Fw ACAGGGACTGACACCTGAGA, Rv GACGACAGGGATTAGCCGTT.

To derive MD crispant embryos, injected embryos were placed in E3 containing ampicillin, kanamycin and amphotericin B as described previously. Bleaching injected embryos caused high mortality rates.

Digestion of chicken egg yolk

20 g hard-boiled chicken egg yolk was mixed with 40 mL E3 and emulsified using a Branson Digital Sonifier sonicator. Emulsified egg yolk was inoculated with bacterial isolates in E3 (described above) at a 1:200 ratio. Egg yolk mixture was then placed in a shaker at 28 °C at 200 rpm and for 48 h. Samples were then autoclaved prior to feeding experiments.

Analysis of chicken egg yolk composition

“Pre-digested” samples were autoclaved and mailed to Australian Laboratory Services (VIC, Australia) for commercial grade nutritional analyses.

Statistics

371 All statistical analyses (t-tests and ANOVA where appropriate) were performed using GraphPad
372 Prism 8. Outliers were removed using ROUT, with Q = 1%. All data shown are representative of at
373 least 2 biological replicates.
374
375 **Data availability**
376 Source data are provided with this paper.
377 Raw image and analysis data is archived for 10 years by The Centenary Institute and available on
378 request from the corresponding author (Sydney, Australia).
379
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394
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Figure Legends

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

(a) Schematic describing method of faecal microbiome inoculation in MD zebrafish and chicken egg yolk challenge diet. (b) Representative images of neutral red staining in mid-gut of 5 dpf zebrafish embryos. Red brackets indicate mid-gut region used for quantification. (c) Quantification of neutral red staining area in mid-gut of 5 dpf zebrafish embryos. (d) Representative images of Oil

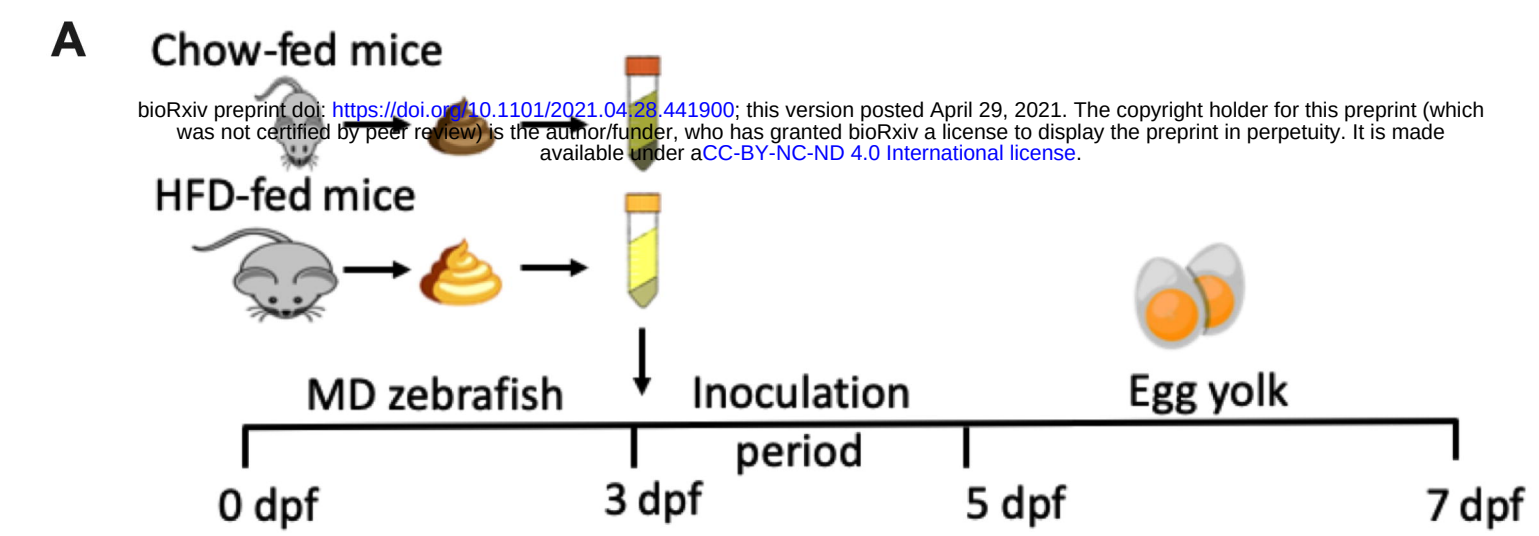
485 Red O staining of 7 dpf chow-fed and HFD-fed mouse faecal microbiome-inoculated zebrafish
486 embryos. Red brackets indicate tail region posterior to the swim bladder used for quantification
487 (e) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
488 bladder in MD zebrafish inoculated with mouse faecal microbiota and challenged with chicken egg
489 yolk diet from 5-7 dpf. Scale bar represents 500 μm. Results are expressed as mean ± SD.
490
491 Figure 2: Identification of individual microbes with pathobiont activity.
492 (a) Schematic describing workflow to isolate *Escherichia species* PYCC8248 (*E.s*), *Escherichia coli*
493 strain Y15 (*E.c*), *Stenotrophomonas maltophilia* strain CD103 (*S.m*) and *Enterococcus faecalis* strain
494 YN771 (*E.f*) and inoculate into MD zebrafish with subsequent chicken egg yolk diet challenge. (b)
495 Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
496 bladder in MD zebrafish inoculated with bacterial isolates *E.s*, *E.c*, *S.m*, and *E.f* and challenged with
497 a chicken egg yolk diet from 5-6 dpf. (c) Area of neutral red stained in the mid-gut of 5 dpf
498 gnotobiotic zebrafish embryos mono-associated with bacterial isolates *E.s*, *E.c*, *S.m* and *E.f*. (d)
499 Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
500 bladder in MD zebrafish inoculated with bacterial isolates *E.f*, *E. faecalis* UNSW 054400 type strain
501 (*E.f* UNSW), *S.m* and *S. maltophilia* yy01 (*S.m* yy01) and challenged with a chicken egg yolk diet
502 from 5-6 dpf. Results are expressed as mean ± SD.
503
504 Figure 3: *Enterococcus faecalis* (*E.f*) accelerates hyperlipidaemia by activating host immune
505 signalling pathways.
506 (a) Schematic describing inoculation of MD zebrafish embryos with heat-killed bacterial isolates
507 *E.s*, *E.c*, *S.m* and *E.f* from 3-5 dpf, followed by chicken egg yolk diet challenge from 5-7 dpf. (b)
508 Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim
509 bladder in MD zebrafish inoculated with heat killed bacterial isolates *E.s*, *E.c*, *S.m*, and *E.f* and

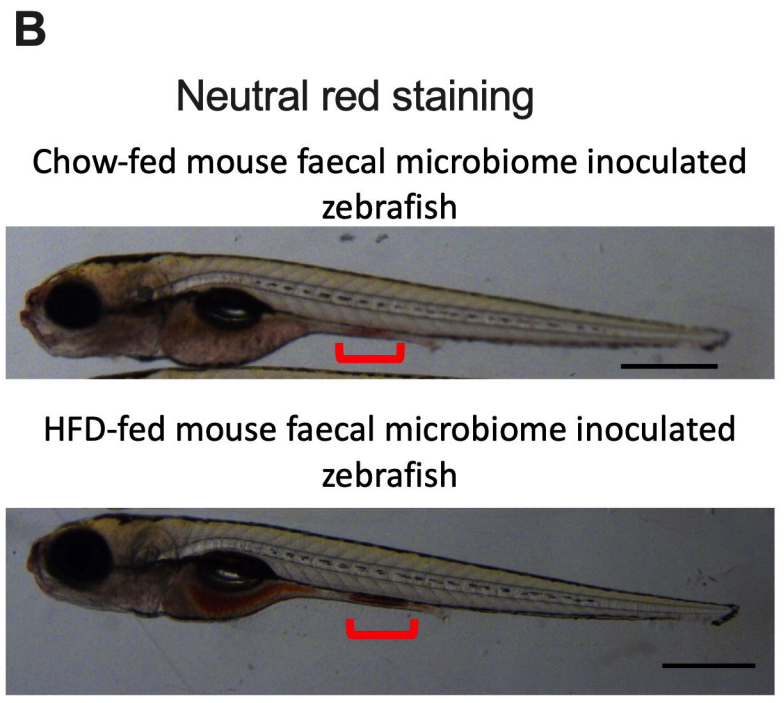
challenged with a chicken egg yolk diet. (c) Quantification of *myd88* expression in zebrafish embryos injected with *myd88*-targeting CRISPR-Cas9 complexes at 5 dpf. Each dot represents a biological replicate of at least 10 embryos. (d) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim bladder in control scrambled and *myd88* crispant embryos exposed to *E.f* and challenged with a chicken egg yolk diet. Results are expressed as mean ± SD.

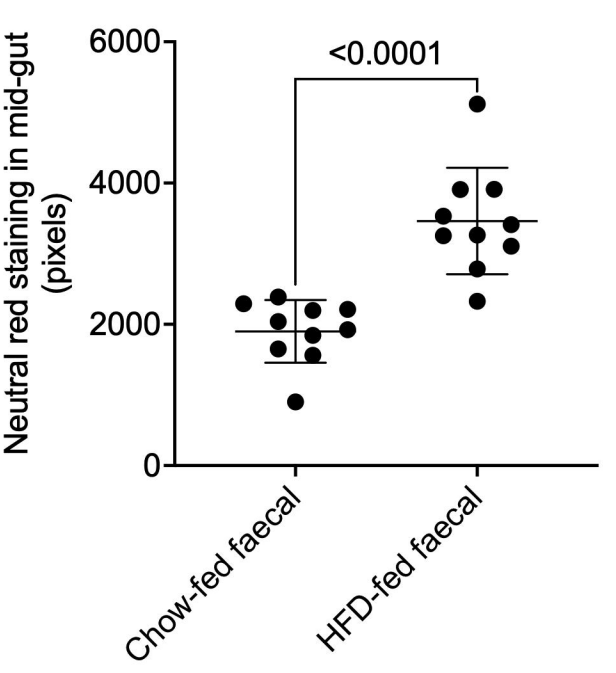
Figure 4: Gram-positive cell wall components accelerate hyperlipidaemia in zebrafish embryos. (a) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim bladder in MD zebrafish inoculated with heat killed bacterial isolates *E.f* and Staphylococcus xylosus (*S.x*) from 3-5 dpf and challenged with a chicken egg yolk diet from 5-7 dpf. (b) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim bladder in MD zebrafish pre-incubated with LTA or peptidoglycan from 3-5 dpf before co-challenge with chicken egg yolk diet from 5-7 dpf. (c) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim bladder in MD zebrafish pre-incubated with lysozyme-treated heat killed *E.f* from 3-5 dpf and challenged with a chicken egg yolk diet from 5-7 dpf. (d) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim bladder in control scrambled and *myd88* crispant embryos pre-incubated with LTA or peptidoglycan from 3-5 dpf before co-challenge with chicken egg yolk diet from 5-7 dpf. Results are expressed as mean ± SD.

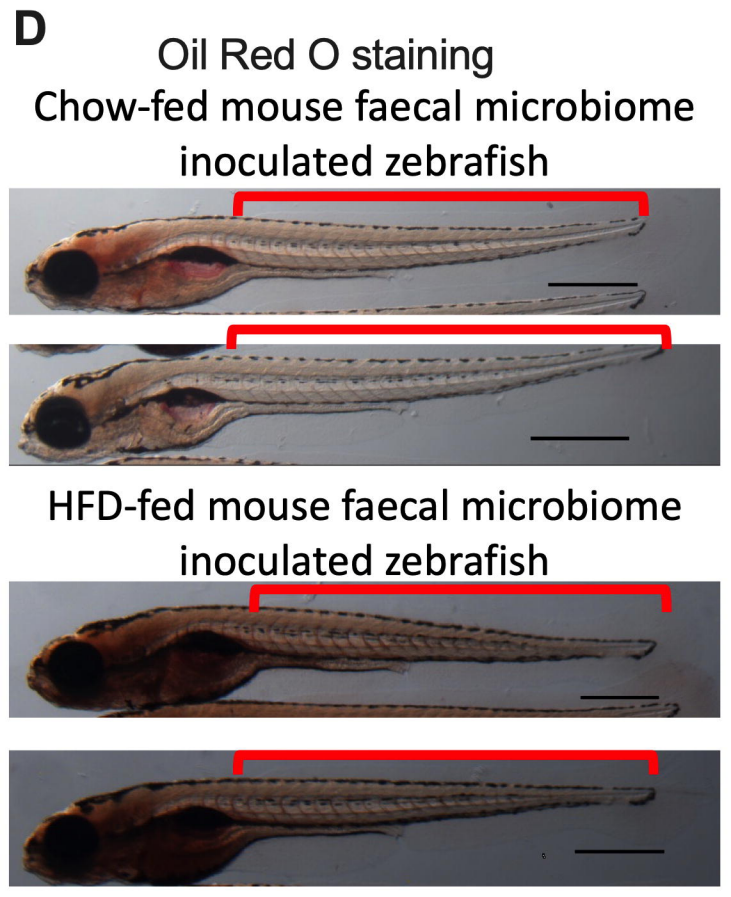
Figure 5: Stenotrophomonas maltophilia accelerates hyperlipidaemia by digesting food. (a) Schematic describing creation of "pre-digested" chicken egg yolk by incubation with bacterial isolates *E.s*, *E.c*, *S.m*, and *E.f*, autoclaving, for feeding to 5-7 dpf zebrafish embryos. (b) Quantification of trunk vascular Oil Red O staining from the tail region posterior to the swim

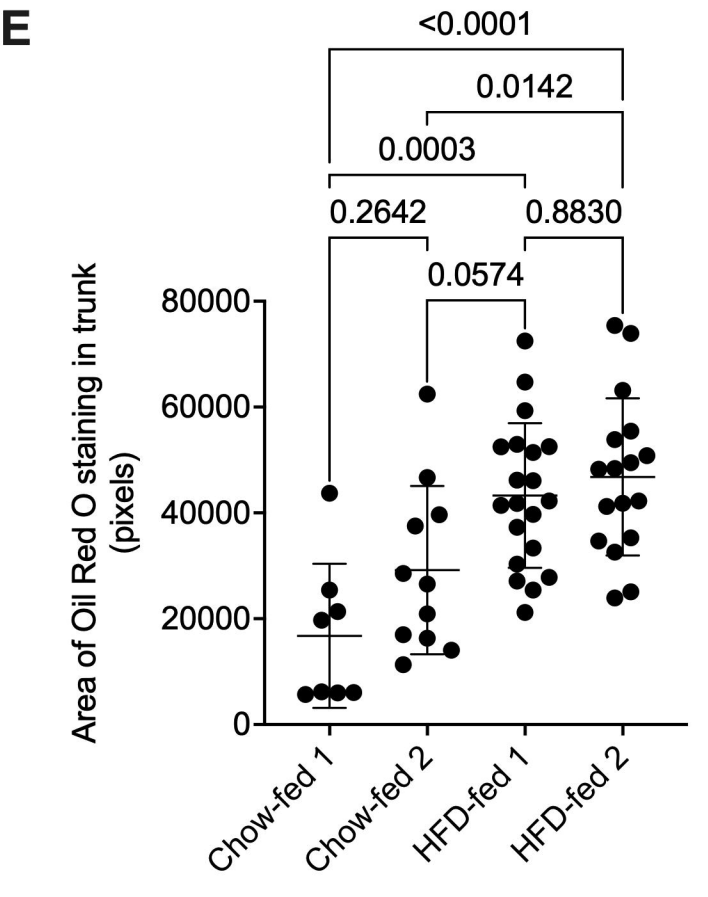
535 bladder in MD zebrafish fed "pre-digested" egg yolk with bacterial isolates *E.s*, *E.c*, *S.m*, and *E.f*
536 from 5-7 dpf. (c) Quantification of trunk vascular Oil Red O staining from the tail region posterior
537 to the swim bladder in conventionally raised zebrafish fed "pre-digested" egg yolk with bacterial
538 isolate *S.m* from 5-7 dpf. (d) Quantification of trunk vascular Oil Red O staining from the tail region
539 posterior to the swim bladder in conventionally raised zebrafish fed "pre-digested" fish embryo
540 food with bacterial isolates *E.c* and *S.m* from 5-7 dpf. (e) Representative images of "pre-digested"
541 egg yolk with bacterial isolates *E.c* and *S.m* red brackets indicate fraction of the water column
542 containing large particulates after autoclaving. (f) CFU recovery from chicken egg yolk "pre-
543 digestion" reactions. Results are expressed as mean ± SD.

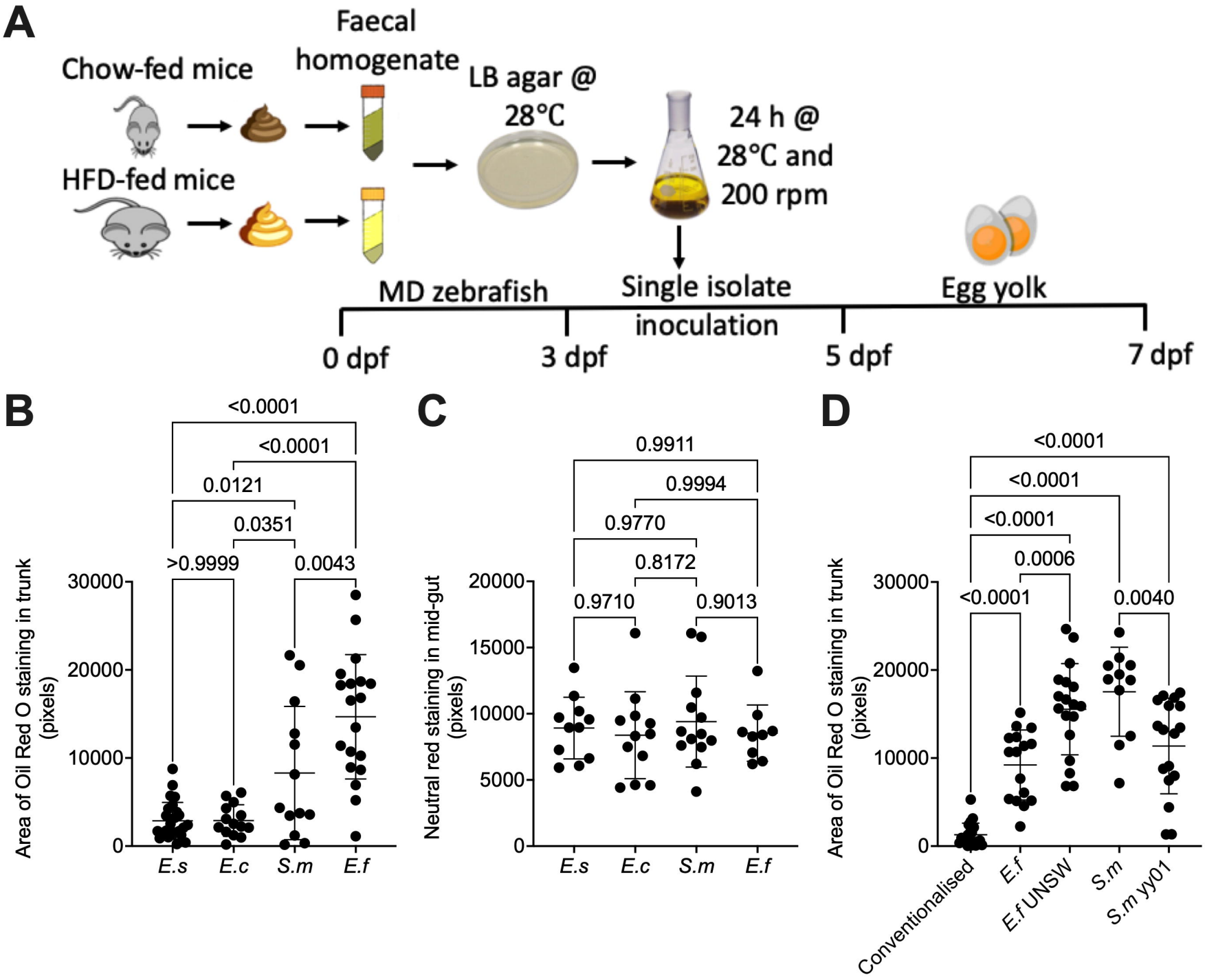


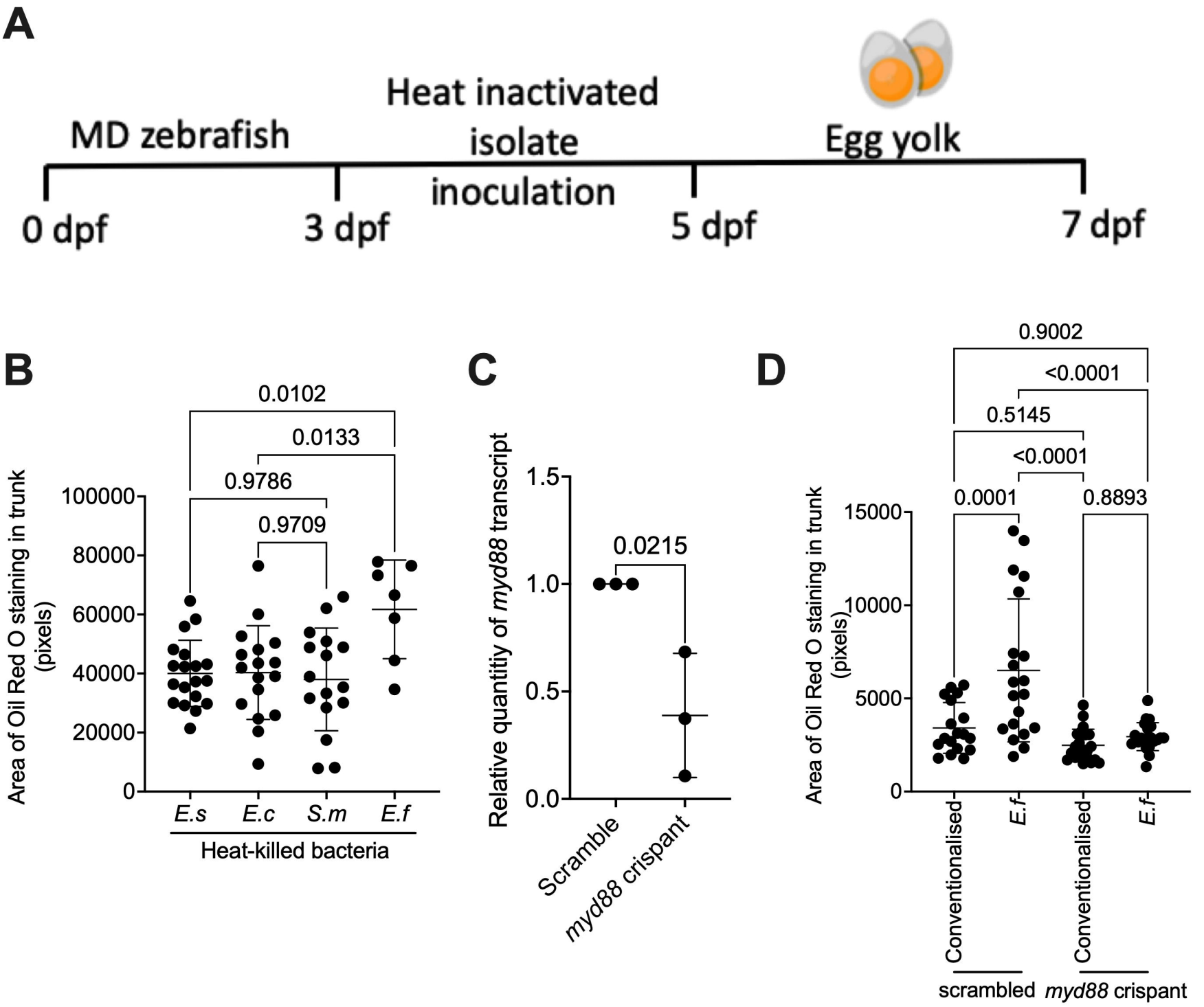


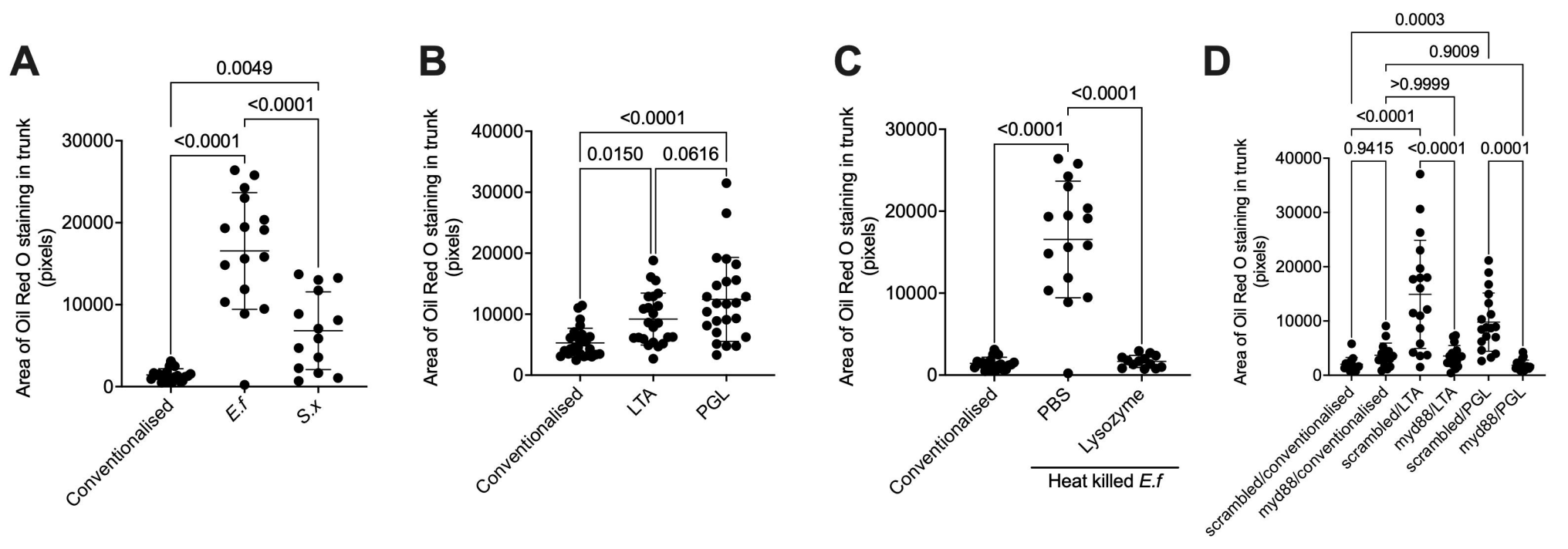


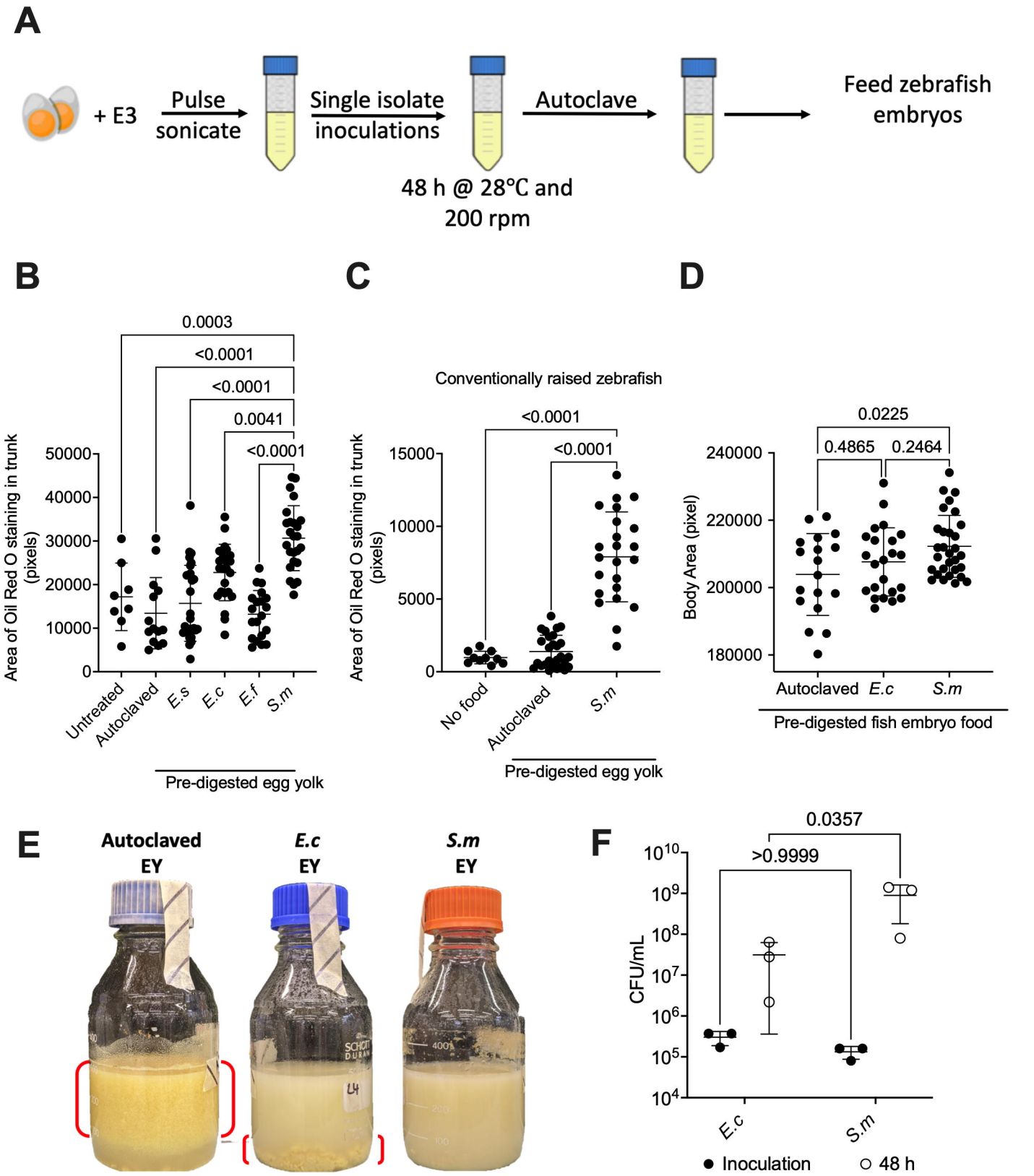





A
Chow-fed mice
HFD-fed mice
Faecal homogenate
LB agar @ 28°C
24 h @ 28°C and 200 rpm
MD zebrafish
Single isolate inoculation
Egg yolk
0 dpf
3 dpf
5 dpf
7 dpf
B
Area of Oil Red O staining in trunk (pixels)
<0.0001
<0.0001
0.0121
0.0351
>0.9999
0.0043
E.s
E.c
S.m
E.f
C
Neutral red staining in mid-gut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


A
+ E3
Pulse sonicate
Single isolate inoculations
Autoclave
Feed zebrafish embryos
48 h @ 28°C and 200 rpm
B
0.0003
<0.0001
<0.0001
0.0041
<0.0001
Area of Oil Red O staining in trunk (pixels)
50000
40000
30000
20000
10000
0
Untreated
Autoclaved
E.s
E.c
E.f
S.m
Pre-digested egg yolk
C
Conventionally raised zebrafish
<0.0001
<0.0001
Area of Oil Red O staining in trunk (pixels)
15000
10000
5000
0
No food
Autoclaved
S.m
Pre-digested egg yolk
D
0.0225
0.4865
0.2464
Body Area (pixel)
240000
220000
200000
180000
Autoclaved
E.c
S.m
Pre-digested fish embryo food
E
Autoclaved EY
E.c EY
S.m EY
F
0.0357
>0.9999
CFU/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
E.c
S.m
Inoculation
48 h