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Variation in diet concentration and bacterial inoculum size in larval habitats shapes the performance of the Asian tiger mosquito, *Aedes albopictus*

Vincent Raquin[1,2], Edwige Martin[1], Guillaume Minard[1] and Claire Valiente Moro[1*]

Abstract

Background Ecological niches present unique environmental and biological trademarks such as abiotic conditions, nutrient availability, and trophic interactions that may impact the ecology of living organisms. Female mosquitoes deposit their eggs in aquatic niches with fluctuating diet sources and microbial communities. However, how niche's diet and microbial composition impact mosquito performance (i.e., traits that maximize mosquito fitness) are not well understood. In this study, we focused on the Asian tiger mosquito, *Aedes albopictus*, one of the most invasive species in the world and a competent vector for human pathogens. To remove any external microbes, *Ae. albopictus* eggs were surface-sterilized then hatching larvae were exposed to a gradient of bacterial inoculum (i.e., initial microbial load) and diet concentrations while their impact on mosquito performance traits during juvenile development was measured.

Results Our results showed that *Ae. albopictus* larvae develop faster and give larger adults when exposed to microbiota in rearing water. However, mosquito performance, up to the adult stage, depends on both bacterial inoculum size and diet concentration in the aquatic habitat. Upon low inoculum size, larvae survived better if the diet was in sufficient amounts whereas a higher inoculum size was associated with optimal larvae survival only in the presence of the lower amount of diet. Inoculum size, and to a lesser extent diet concentration, shaped bacterial community structure and composition of larval-rearing water allowing the identification of bacterial taxa for which their abundance in larvae-rearing water correlated with niche parameters and/or larval traits.

Conclusions Our work demonstrates that both diet concentration and bacterial inoculum size impact mosquito performance possibly by shaping bacterial community structure in the larval habitat, which accounts for a large part of the juvenile's microbiota. Host-microbe interactions influence several mosquito life-history traits, and our work reveals that niche parameters such as inoculum size and diet concentration could have numerous implications on the microbiota assembly and host evolutionary trajectory. This underlies that host-microbe-environment interactions are an important yet overlooked factor of mosquito adaptation to its local environment, with potential future implications for vector control and vector ecology.

Keywords Mosquito ecology, Symbiosis, Microbiota, Diet, *Aedes albopictus*, Vector, Complex interactions

*Correspondence:
Claire Valiente Moro
claire.valiente-moro@univ-lyon1.fr
Full list of author information is available at the end of the article

Background

Across various models ranging from mammals to insects, host-microbiota interactions have been shown to influence important fitness and development traits linked to host performance [1–3]. Microbial symbionts can impact their host in several ways, for instance by serving as a nutrient source, detoxifying diet, participating in nutrient digestion/intake, or supplementing diet with essential molecules as in nutritional symbiosis [2, 4]. Converging evidence shows that diet is a driving force towards host-associated microbial communities, for instance, by selecting diet-adapted microbial communities [5, 6]. In the fruit fly *Drosophila melanogaster*, bacteria establish a diet-dependent nutritional symbiosis to provide their host with essential nutrients when raised on a scarce diet [7–9]. All stages in the life cycle of mosquitoes are dependent upon several environmental factors for their survival and development. Upon egg hatching, larvae and pupae are cloistered in their aquatic habitat until the adult stage. These natural breeding sites contain variable resource inputs that larvae can directly consume while such resources stimulate microbial growth, triggering the decomposition of organic matter and the release of nutrients that can be uptake by larvae [10–12]. Both larval diet quantity and quality affect a range of traits of mosquitoes from juvenile growth, development, and survival to adult immunity and vector competence [12–23]. The mosquito microbiota is mainly acquired from the environment, particularly from the water where eggs are laid, and larvae develop. A part of this microbiota is thus transferred from female to offspring during egg laying or egg smearing, allowing microorganism dispersal in the water and further acquisition by its offspring or conspecific larvae already present in the breeding site. This acquisition of microbes with each generation helps mosquitoes adapt to their specific ecological niche and can influence various aspects of their biology [24–27]. Notably, environmental microorganisms have strong implications on mosquito development, with previous studies demonstrating that axenic individuals (those lacking gut microbiota) experience larval developmental delays and reduced adult size compared to conventional counterparts [28, 29]. Recently, studies on the mosquito *Aedes aegypti* suggested that more than each factor independently, mosquito-microbe-diet interactions could be of primary importance for mosquito performance [30]. On the one hand, diet abundance impacts mosquito microbiota size and composition [31]. On the other hand, larvae-associated microbial communities mediate larval growth by provisioning essential dietary factors that are limiting in the niche [28–30]. Altogether, this underlines that *Ae. aegypti* larval development partly results from complex interactions between the microbiota and the quality or quantity of diet with carry-over effects on adult traits [32].

Among mosquitoes, *Ae. albopictus* is an important vector of human pathogens [33, 34] and a worldwide invasive species [35]. *Ae. albopictus* larval niches vary in terms of physiochemical composition (pH, temperature, oxygen level, detritus input, nutrients ratio) [36–38] and *Ae. albopictus* larvae harbor a bacterial microbiota that varies according to the niche [39]. Bacteria influence many host traits in *Ae. albopictus* from oviposition site selection [40] to sugar feeding [41] or larval development [42]. *Ae. albopictus* juvenile development also depends on diet concentration as larvae present a developmental delay below a given diet concentration that microbiota, including the native intracellular bacterium *Wolbachia* cannot counterbalance [13]. Together, it suggests that both microbiota and diet impact *Ae. albopictus* performance. But to the best of our knowledge, no study has yet investigated the concomitant impact of diet and microbiota concentration on *Ae. albopictus* development and its consequences regarding vector ecological persistence in the environment.

In this study, we specifically assessed if and how the diet concentration and the size of the bacterial inoculum in the larval niche influence *Ae. albopictus* mosquito performance. To that end, eggs were surface-sterilized (to get rid of native microbiota with the exception of intracellular bacteria such as *Wolbachia*) and then re-associated to a batch of bacteria derived from larvae-rearing water or sterile water using a procedure that allowed larval development up to the adult stage [43]. Selected performance-related life history traits were measured in re-associated or control larvae along a gradient of diet concentrations. Then, we questioned if the impact of diet concentration and bacterial inoculum size on *Ae. albopictus* performance could correlate with changes in the composition and the structure of the niche bacterial microbiota. Water microbial community composition and relative abundance were determined across diet and inoculum gradients using 16S rRNA gene amplicon sequencing. Altogether, our study demonstrates that diet concentration and bacterial inoculum size drive the ecological performance of *Ae. albopictus* larvae with carry-over effects on adult mosquitoes, and this effect could be originating from the modeling of larvae aquatic habitat bacterial community structure by inoculum size and, to a lesser extent, diet concentration.

Material and methods

Mosquito colony maintenance

F_9 and F_{10} from an *Ae. albopictus* colony named AealbVB (referred hereafter as VB) established from field mosquitoes collected in 2017 in Villeurbanne and Pierre-Bénite

(France) were used. Larvae were maintained at 26℃ with dechlorinated water and Tetramin fish food. Adults were raised at 28℃, 80% relative humidity, 16:8 light:dark photoperiod in mass rearing. Egg papers were stored at 28℃ for up to 2 months.

Diet plug preparation

Sterile agar diet plugs of various concentrations were prepared. Commercial tropical fish flakes (Tetra) were used as a complex diet for mosquito larvae. The product guaranteed a minimum of 46% crude protein, 11% crude fat, vitamin D3 1990 IU/kg, a maximum of 3% crude fiber, and 6% moisture as well as trace elements such as manganese (manganese (II) sulfate, monohydrate) 96 mg/kg, zinc (zinc sulfate monohydrate) 57 mg/kg, and iron (iron(II) sulfate, monohydrate) 37 mg/kg. The flakes were finely ground and mixed with sterile water at final concentrations of 20%, 12%, 10%, 8%, 5%, 2%, 1%, 0.5%, and 0.1% (w:v). Agar was added at a final concentration of 1.6% (w:v). Diet suspensions were autoclaved (120℃, 20 min), poured into 90-mm Petri dishes (20 mL per dish), and stored at 4℃ for up to 3 days. Die-cut of 0.6 g agar food plugs using a 15-mL sterile tube (Falcon) allowing an accurate control of diet quantity.

Microbial inoculum production

Ae. albopictus VB eggs (no surface sterilization) were allowed to hatch for 2 h at − 20 ATM in a vacuum chamber. Then, 3 plastic trays (Gilac, 24 × 32 × 9 cm) were prepared with 200 first instar larvae per tray in 1.5 L of dechlorinated water. In each tray, 0.1 g/tray of ground fish flakes (Tetramin) supplemented with yeast extract (Biover) (3:1, w:w) were added every second day. This food mixture favors a synchronized larval growth with only 4th instar larvae after 7 days at 26℃. At day 7 post-hatching, 50 mL of rearing water (without larvae) from each of the 3 independent trays were pooled to form the microbial inoculum.

Larvae microbiota manipulation

Previous studies showed that mosquito larvae deprived of surface microorganisms only develop in the dark [29], this being confirmed in our experimental set-up (Fig. S1B). VB eggs were first surface-sterilized and allowed to hatch in sterile conditions (Fig. S1A). First-instar larvae were placed in 6-well plates (3 larvae per well) with an autoclaved 0.6 g diet plug at the selected concentration (see above). The number of larvae per well was set at three to limit food competition between larvae and provide good rearing conditions in our experimental design (considering the size of the food plug and the duration of the experiment) while keeping enough individuals to support conclusions. Each well contained 5 mL of microbial inoculum (as described above) diluted from 10^{-4} to 10^{-8} in sterile water to obtain larvae re-associated to a microbiota (re-associated) or 5 mL of sterile water to obtain control larvae unexposed to environmental microorganisms (control). Plates were incubated at 28℃ for up to 22 days in complete darkness [28]. The well is the biological replicate unit and at least 6 wells per condition were prepared for each independent experiment. For each experiment, PBS solution (in which surface-sterilized larvae hatched) as well as food plugs were inoculated on modified lysogeny broth and brain heart infusion agar plates for 7 days at 30℃ to test for the presence of cultivable microorganisms. All wells of control larvae were observed daily and discarded in the presence of turbid water, while some random wells were also examined in each plate at × 1000 magnification to assess potential microbial contamination. To control for the impact of the egg surface sterilization procedure on larval performance, larvae derived from non-surface sterilized eggs (i.e., conventional, without control of microbial inoculum load) were monitored under similar rearing conditions (28℃, dark) across a gradient of 1, 2, 5, 10, and 12% food concentrations (Fig. S8).

Larvae survival and development time measurement

The presence of pupae was recorded daily at fixed hours. Larvae-to-pupae survival represents the percentage of larvae that reached the pupal stage. Development time represents the time (in days) needed to reach 50% of the total number of pupae (Day_{50}). On the day of emergence, pupae were individually transferred together with ~ 300 µL of rearing water in a sterile 2-mL microcentrifuge tube and allowed to emerge at 28℃. Upon emergence, the sex of adults was recorded and tubes were stored at − 80℃ until use.

Wing length measurement

Wing length is a proxy for *Ae. albopictus* adult performance [44]. Adults stored at − 80℃ were thawed, and both wings were dissected under a Leica M80 stereomicroscope. Wings were included in Eurapal (Roth) on a 10-well epoxy-coated glass slide (Labelians). Slides were photographed at × 20 magnification with a Leica MC170 HD camera. Wing length was measured between the intersection of the second and third vein and the intersection of the seventh vein with the wing border using ImageJ v.2.1.0/1.53c [45].

DNA extraction from larvae rearing water and 16S rRNA gene quantification

After 5 days at 28℃, 500 µL of rearing water was collected in each well under sterile conditions and stored at − 20℃ prior to DNA extraction. Samples were centrifuged for 20

min, 4℃, at 17,000 g, and the total DNA was extracted from the pellet with the DNeasy Blood and Tissue kit following manufacturer recommendations (Qiagen). DNA concentration was estimated by Qubit dsDNA HS kit (Thermo Fisher Scientific), and samples were stored at − 20℃. A blank control was performed by using only DNA lysis buffer to control for external DNA microbial contaminations during the DNA extraction procedure.

The 16S rRNA load was measured by quantitative PCR using the Itaq SYBR green supermix kit (Bio-Rad), 784F (5'-AGGATTAGATACCCTGGTA-3'), and 1061R (5'-CRRCACGAGCTGAC') primers and 5 ng of template DNA isolated from water samples. The 16 µL reaction comprised 0.48 µL of each primer at 10 µM, 8 µL of master mix, and 5.04 µL of PCR-grade water. After a single denaturation step at 95℃ for 3 min, a two-step amplification was performed including 10 s at 95℃ followed by 30 s at 60℃, for 40 cycles on a Bio-Rad CFX96 machine. The number of 16S rRNA copies per µL was calculated using tenfold serial dilutions (from 10^8 to 10 copies/µL) of a bacterial (*Acinetobacter* sp.) purified PCR amplicon. Sterile water was used as the template for qPCR-negative controls.

High-throughput sequencing and bioinformatic analysis

For 16S and 18S rRNA amplicon sequencing, two-step Nextera PCR libraries were created following the manufacturer's protocol (Illumina). Prokaryotic and eukaryotic barcodes were amplified in a first-step PCR of a ~ 280 bp fragment of the 16S rRNA gene and a ~ 430 bp fragment of the 18S rRNA gene, respectively [45, 46]. Primers for the 16S rRNA gene are V5-784F (5′-AGGATT AGATACCCTGGTA-3′) and V6-1061R (5′-CRRCAC GAGCTGACGAC-3′), while for the 18S rRNA gene, the primers Euk82F (5′-GAAACTGCGAATGGCTC-3′) and Euk516R (5′-ACCAGACTTGCCCTCC-3′) were used. PCR amplifications were carried out in duplicates on a Bio-Rad C1000 thermal cycler (Bio-Rad, Irvine, CA) in a 25-µL reaction. PCR reactions were performed using the 5× BioAmp master mix (Biofidal) containing a 2-µL sample DNA template, 5 µL of Mix HotStarTaq 5×, 1× of GC rich Enhancer, 0.2 mg.mL^{-1} of bovine serum albumin (New England Biolabs), and 0.2 µM of each primer. Amplifications were conducted for 10 min at 96℃ followed by 35 cycles at 96℃ for 20 s, 54℃ for 1 min (16S rRNA gene) or 30 s (18S rRNA gene), 72℃ for 30 s, and a final extension at 72℃ for 10 min. Duplicate PCR products were pooled, and 5 µL of the pooled products was separated by electrophoresis on a 1.5% agarose gel supplemented with 2.5 µL of clear sight DNA stain for 17 min at 100 V. All the 18S rRNA gene PCR were negative. For the 16S rRNA gene, all PCR were positive at the expected size. A total of 188 libraries from 16S rRNA gene amplicons were constructed, including controls. Sequencing was performed on Illumina MiSeq (2 × 300 bp, paired-end) at Biofidal. In total, 20,764,855 reads were obtained. The paired-end reads were demultiplexed, and the Illumina adaptor was trimmed. Sequence quality control and analysis were carried out using the FROGS pipeline [47] as previously described [48]. Briefly, FROGS clustering was performed using Swarm [49], based on a local clustering threshold level and an aggregation distance of 3 to identify operational taxonomic units (OTUs). FROGS OTU filters (OTUs whose proportion of sequences was below the threshold of 5×10^{-5} were removed) and FROGS chimeras were removed (i.e., sequences formed from two or more biological sequences joined together were removed). OTUs were mapped to the SILVA database 138.1 [50] and assigned to specific taxa using the naïve Bayesian classifier [51] at a 80% minimum bootstrap [52–54]. A normalization of 11,780 sequences per sample was performed to enable further comparisons. A total of 184 operational taxonomic units (OTUs) were identified in the whole normalized dataset. OTUs with a relative abundance less than 10 times greater than that observed in the negative control were removed [39]. All FastQ files were deposited in the EMBL European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the project accession number PRJEB57586.

Statistical analysis

Analyses and graphical representations were made with R software (http://www.r-project.org/). Larvae- and pupae-to-adult survival (binary response variable) were analyzed by generalized linear mixed-effect models (GLMM) [55]. GLMM was performed with a binomial distribution and a probit link function. The development time (Day_{50}) and wing length (in mm) were analyzed using linear mixed models (LMM). For each of those models, variations in the response variables (either survival, development time, or wing length) were explained by the microbial status and diet concentration (categorical) as well as their interaction (i.e., fixed effects) and were corrected for the experiment and/or batch of inoculum (i.e., random effects). The GLMM and LMM models were conducted with the *lme4* package version 1.1–25 [56]. The inference of fixed effects on variations of the response variables was tested with a Wald χ^2 test or a type II ANOVA for binomial and continuous data, respectively. Post hoc comparisons after GLMM/LMM were performed with *emmeans* package version 1.5.2–1 [57] to estimate pairwise differences between diet concentrations and microbial status using Tukey-HSD tests including *p*-value corrections for multiple comparisons. Adonis-ANOVA and non-metric multidimensional scaling ordination were performed with the *ade4* and *vegan*

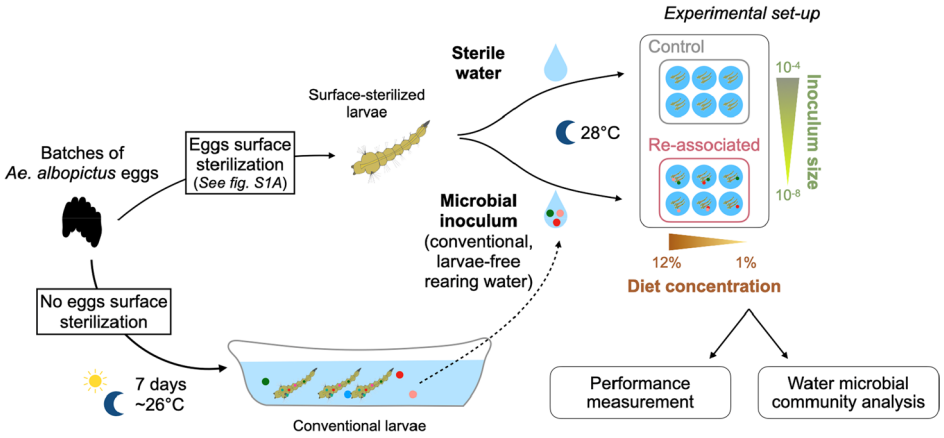

Fig. 1 Experimental design to study the impact of diet and microbiota on mosquito performance upon juvenile exposure. Microbial inoculum was prepared using larvae-free, a 7-day-old water of conventionally reared larvae. This inoculum was inoculated to larvae hatched from surface-sterilized eggs (re-associated), and larvae hatched from surface-sterilized eggs and incubated in sterile water were used as controls (control). Re-associated and control larvae were kept in 6-well plates ($n = 3$ larvae/well) and exposed concomitantly to a gradient of diet and microbial inoculum, then scored for several performance traits (survival, development time, adult wing length, and sex ratio). The whole bacterial community from re-associated and control larvae was analyzed on day 5 using 16S rRNA metabarcoding

packages [52, 53]. Nonlinear Spearman correlations were performed with the *Hmisc* package [54]. Other R packages were used for data organization and representation such as *plyr* version 1.8.6 [58] and *ggplot2* version 3.3.2 [59].

Results

Bacterial community in larvae-rearing water impacts juvenile development in a diet-dependent manner

Experiments to address the impact of microbiota and diet on mosquito traits were conducted on larvae hatched from surface-sterilized eggs and further reseeded with conventional microbiota (re-associated) or sterile water (control), to avoid bias due to native microbiota, upon a gradient of diet and/or microbial inoculum (Fig. 1).

First, *Ae. albopictus* larvae were exposed to sterile water (control) or re-associated to a constant microbial inoculum size (10^{-6} dilution of conventional larvae rearing water) upon a range of four diet concentrations (2, 5, 10, and 12%). Individuals were monitored for larval and adult traits according to the design detailed in Fig. S2A. Larvae-to-pupae survival depended on the interaction between microbial status and diet concentration (Wald χ^2, Chisq = 162.8, Df = 3, $P_{\text{diet x microbial status}} < 2.2 \times 10^{-16}$) (Fig. 2A). Overall survival was ~ 2 times higher in control larvae (63% ± 29) compared to re-associated siblings (34.5% ± 28) although significant variations were observed according to diet concentration with maximal survival reached at 10% diet concentration (78% ± 9.8) in control larvae but 2% diet concentration (57.7% ± 35) in re-associated larvae. In control larvae, survival from 5 to 12% diet concentration (74.4% ± 23, 78% ± 9.8, and 73.5% ± 29 for 5, 10, and 12% diet, respectively) was similar but ~ 3 times lower than at 2% (21.4% ± 9.4) ($P < 0.0001$, Tukey-HSD post hoc comparisons) while an opposite trend was observed in re-associated larvae, with a ~ 3 times higher survival at 2 and 5% diet concentration (57.7% ± 34.9, 49.1% ± 18.7) compared to 10 and 12% (21.3% ± 16, 10% ± 11) ($P < 0.0001$, Tukey-HSD post hoc comparisons) (Fig. 2A). The pupae-to-adult survival was not impacted by diet concentration nor microbial status, remaining above 83% regardless of the condition (Fig. S3).

The time (in days) needed to reach 50% of the final number of pupae (Day_{50}) was measured as a proxy of larvae development time. As observed for survival, juvenile development time depended on the interaction between microbial status and diet concentration (Wald χ^2, Chisq = 123.6, Df = 3, $P_{\text{diet x microbial status}} < 2.2e^{-16}$) (Fig. 2B). Overall, re-associated larvae pupariated ~ 6 days earlier (7.3 days ± 2.5) than the control (13.1 days ± 2.6). The Day_{50} of re-associated larvae at 2, 5, and 10% diet concentrations (7.11 days ± 2.2, 7.18 days ± 1.8, 7.28 days ± 2.7) was similar but ~ 1 day shorter than at 12% (8.6 days ± 4.8) although Day_{50} of 10 and 12% were not significantly different ($P_{\text{Day50 2\%v 12\%}} = 0.004$, $P_{\text{Day50 5\%v 12\%}} = 0.005$, and $P_{\text{Day50 10\%v 12\%}} = 0.055$, respectively, Tukey-HSD post hoc comparison). The Day_{50} of control larvae at 2% diet concentration (17.3 days ± 1.5) was ~ 4 days higher than at 5% (13.6 days ± 2.3) ($P < 0.0001$, Tukey-HSD post hoc comparison), which in turn was ~ 1.5 days higher than at 10 and 12% (12 days ± 1.5, 11.9 days ± 2.2) ($P < 0.0001$, Tukey-HSD post hoc comparison) (Fig. 2B).

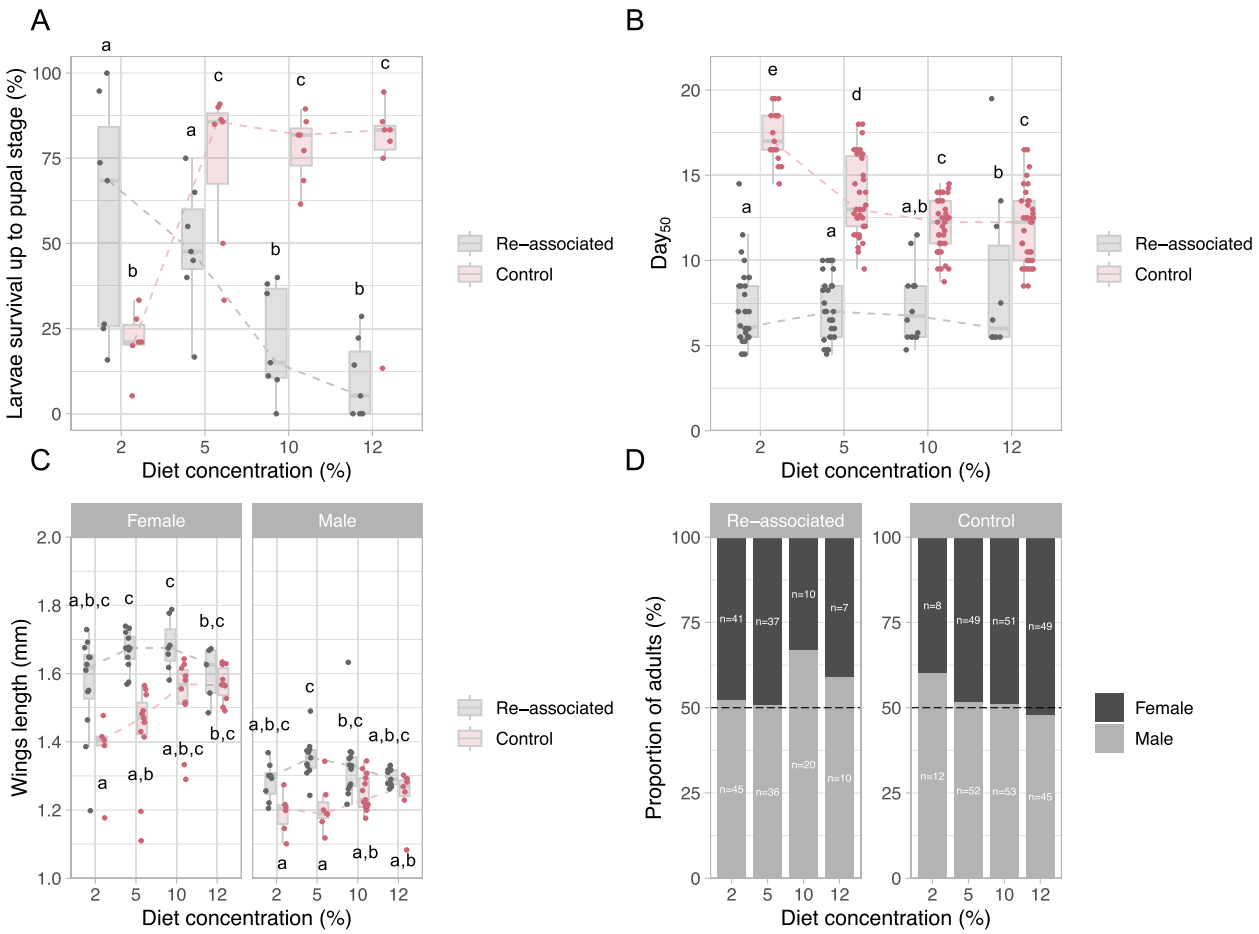

Fig. 2 Performance of *Ae. albopictus* mosquitoes according to microbial status and diet concentration. **A** Larvae-to-pupae survival for re-associated (gray) and control (red) larvae expressed as the proportion (in %) of mosquito larvae that reached the pupal stage as a function of diet concentration (in %). Each dot represents the mean survival for a 6-well plate (3 larvae per well, i.e., 18 larvae per plate) and 3 experiments were conducted (from 1 to 3 plates per experiment). Different letters indicate statistically significant viability following Tukey-HSD post hoc pairwise comparisons. **B** The larval median development time into pupae (Day_{50}) for re-associated and control individuals was represented as a function of diet concentration (in %). Each dot represents the Day_{50} for a single well (3 larvae per well) from a 6-well plate, and 3 experiments were conducted (from 1 to 3 plates per experiment). Different letters indicate statistically significant viability following Tukey-HSD post hoc pairwise comparisons. **C** Adult wings length. Each dot represents the mean length (in mm) of both wings together (± standard deviation). From 11 to 23 individuals (males and females together) were analyzed for each combination of diet and microbial status, representing between 2 and 11 individuals per experiment (3 experiments). **D** Proportion of female and male adult mosquitoes. The number of individuals (*n*) for each condition was reported. For panels (**A** to **C**), median values are connected by a dashed line to facilitate trend visualization

Adult wing length varied according to the interaction between microbial status and diet concentration (Wald χ^2, Chisq = 11.5, Df = 3, $P_{\text{diet x microbial status}} = 0.009$) (Fig. 2C). As expected, females wing length was higher compared to males, so each sex was separated for the rest of the analysis (Fig. 2C). In males, no significant differences in wing length were found according to diet concentration within re-associated or within control adults (Fig. 2C). In females, wing length was similar regardless of the diet concentration in re-associated but, in control adults, individuals reared with 12% diet concentrations had 0.02-mm larger wings than those reared in 2% diet concentrations ($P = 0.04$, Tukey-HSD post hoc comparison). However, significant differences in wing length were reported between re-associated and control within each sex. In males, re-associated adults at 5% showed ~ 0.1-mm larger wings compared to any control individuals while re-associated male wings length at 10% was ~ 0.1-mm larger than control males at 2% and 5% ($P = 0.018$ and $P = 0.046$, respectively, Tukey-HSD post hoc comparison). In females, the wing length of re-associated individuals at 5%, 10%, and 12% was ~ 0.3-mm larger than control at 2% ($P = 0.001$, 0.004, and 0.046 respectively, Tukey-HSD post hoc comparison) notably (Fig. 2C).

The proportion of adults from each sex was not different when microbial status and diet concentration varied (Wald χ^2, Chisq = 0.64, Df = 3, $P_{diet} = 0.88$; Chisq = 0.43, Df = 1, $P_{microbial\ status} = 0.51$; Chisq = 3.87, Df = 3, $P_{diet\ x\ microbial\ status} = 0.25$) (Fig. 2D).

Inoculum size and diet concentration in larval rearing water are strong predictors of bacterial community structure and performance of juvenile *Ae. albopictus*

The larval performance pattern was consistent between re-associated larvae and siblings derived from non-surface sterilized eggs across a diet gradient, although re-associated larvae exhibited a 10–30% decrease in survival. This reduction may be attributed to the impact of the egg surface sterilization procedure and/or the microbial inoculum load (Fig. S8). Therefore, microbiota promote mosquito performance in a diet-dependent manner (Fig. 2), but how the breeding site microbial community is shaped by inoculum size and diet concentration remained unclear. Therefore, re-associated larvae were exposed to a gradient of inoculum size upon three diet concentrations to monitor bacterial community structure and juvenile development (see design in Fig. S2B). In our experimental conditions, rearing water of conventional larvae contained no detectable microeukaryotes using 18S rRNA gene PCR amplification (not shown). On the day of exposure, the amount of 16S rRNA copies measured in the inoculum was on average 1.5×10^5 copies/µL (Fig. S4A). This inoculum was composed of 11 major (> 5% relative abundance) bacterial operational taxonomic units (OTUs) belonging to the genera *Brevundimonas*, *Cnuella*, *Delftia*, *Edaphobaculum*, *Limnobacter*, *Peredibacter*, *Piscinibacter*, *Pseudomonas*, and *Sphingobacterium* as well as the families *Flavobacteriaceae* and *Sphingobacteriaceae* (Fig. S4B). This inoculum was serially diluted (10^{-4}, 10^{-6}, and 10^{-8} dilution) in sterile water and used as an initial load for re-exposed larvae. The rearing water of control larvae was also tested at a single, latter time point (day 5 in the presence/absence of larvae and/or diet) and showed ~ 10,000 times less 16S rRNA copies. In total, DNA from 5 OTUs (> 5% relative abundance) was found in the water of control larvae that correspond to the genera *Aeromonas*, *Bacteroides*, *Dysgonomonas*, *Pseudomonas*, and *Stenotrophomonas* (Fig. S4A and S4C). No cultivable bacteria were isolated from control larvae water, although residual 16S rRNA sequences (~ 1000 copies) were detected. These OTUs are distinct from those retrieved from those found in re-associated siblings. It is likely that this DNA became detectable following metabarcoding 16S rRNA PCR rounds, as it was amplified from the only DNA template available. However, our results support that larval performance can reliably be attributed to the experimental conditions (diet and inoculum manipulation) rather than the presence of a residual active bacterial community. Together, these data reinforce the importance of including both qPCR and metabarcoding analyses to ensure the rigor and transparency of the results while acknowledging the difficulty of eliminating microbial DNA elimination with current methods.

The range of inoculum size tested started at 10^{-4} dilution as a lower dilution (10^{-3}) was associated with extensive larval mortality during a pilot experiment (Fig. S5). For this experiment, the bacterial inoculum was produced simultaneously from three independent batches (B1, B2, and B3), and the batch effect was controlled in subsequent statistical analyses (Fig. S2B). The bacterial load in water of re-associated larvae varied with the inoculum size and diet concentration with no detectable interactions (Wald χ^2, Chisq = 17.3, Df = 2, $P_{diet} = 0.00017$; Chisq = 145.3, Df = 2, $P_{inoculum} < 2.2e-16$; Chisq = 0. 8, Df = 4, $P_{diet\ x\ inoculum} = 0.93$). Post hoc comparisons showed that bacterial load was similar (10^6–10^7 16S rRNA copies/µL) upon high and medium inoculum size (10^{-4} and 10^{-6} dilution, respectively) but ~ 10 times higher (10^5–10^6 16S rRNA copies/µL) compares to low inoculum size (10^{-8} dilution) regardless of diet concentration (Fig. 3A).

Overall, larvae survival pattern suggests that high diet concentration and high inoculum size, or low diet concentration and low inoculum size could be detrimental to larvae survival although this trait depends on the interaction between inoculum size and diet concentration (Wald χ^2, Chisq = 34.17, Df = 4, $P_{diet\ x\ inoculum} = 6.86e^{-7}$) (Fig. 3B). At medium inoculum size (10^{-6}), re-associated larvae displayed similar survival regardless of diet concentration (63.6% ± 25, 49.7% ± 15, and 37% ± 8 at 1, 5, and 12% diet concentration, respectively) (Fig. 3B). At high and low inoculum size (10^{-4} and 10^{-8}), larvae survival for 12% diet concentration was different by ~ twofold compare to 1% although in an opposite direction according to the inoculum dilution (12.9% ± 6 v 44% ± 19 for 12 and 1% at 1×10^{-4}, $P = 0.01$ and 71.2% ± 25 v 36.6% ± 14.6 for 12 and 1% at 1×10^{-8}, $P = 0.008$, Tukey-HSD post hoc test) (Fig. 3B).

The Day_{50} of re-associated larvae was similar within each inoculum size although differences were observed between inoculums, with inoculum size and diet concentration having a significant impact on Day_{50} without any detectable interaction (Wald χ^2, Chisq = 7.99, Df = 2, $P_{diet} = 0.018$, Chisq = 64.4, Df = 2, $P_{inoculum} = 1 \times 10e^{-14}$, Chisq = 7.4, Df = 4, $P_{diet\ x\ inoculum} = 0.11$). The mean Day_{50} increased by 3 days at the low inoculum size (10^{-8}, 9.49 days ± 2.5) compared to medium (10^{-6}, 6.49 days ± 0.8) regardless of the diet concentration. In addition, the shortest Day_{50} was measured at 5% diet concentration

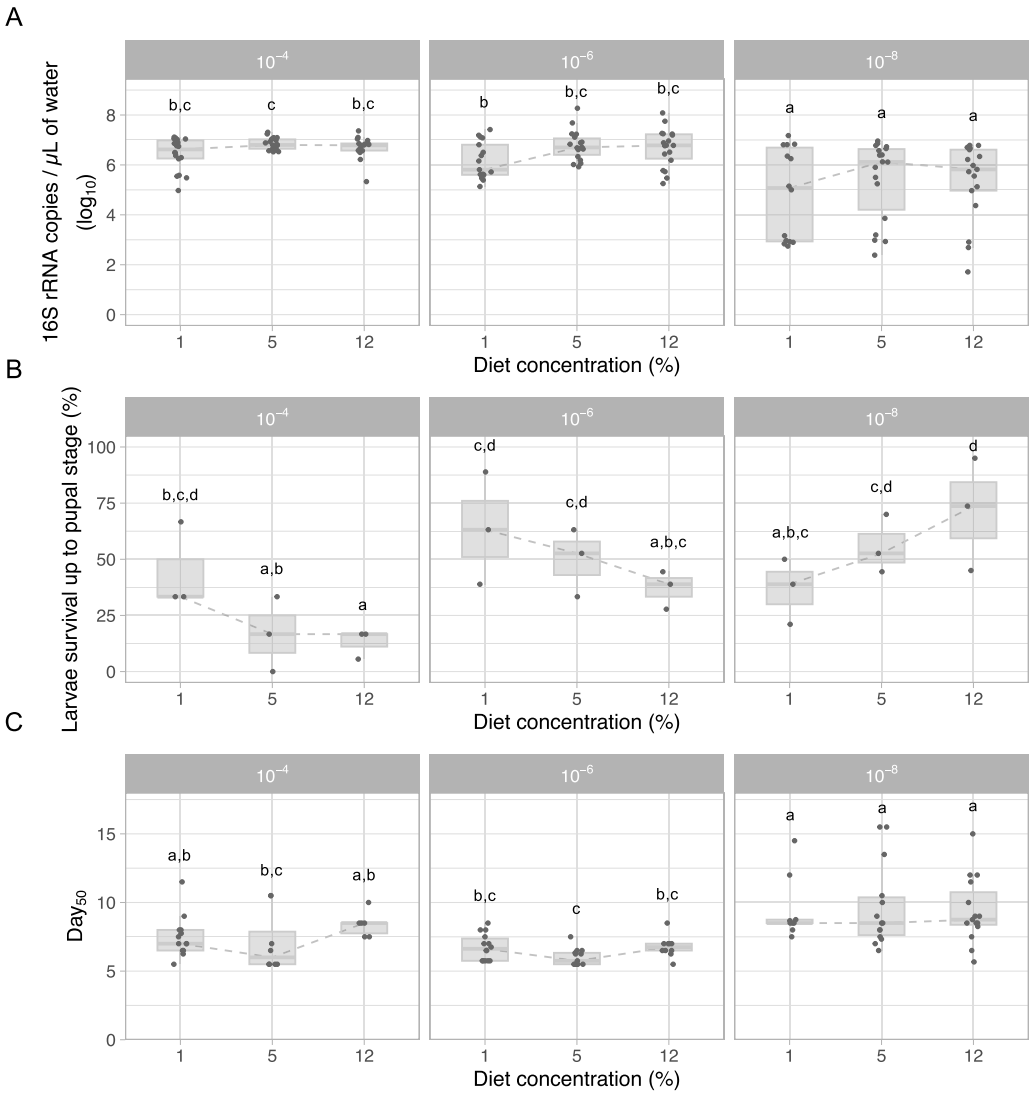

Fig. 3 Juvenile performance of *Ae. albopictus* re-associated larvae upon concomitant variations of bacterial inoculum sizeand diet concentration. **A** Quantification of bacterial 16S rRNA copies per µL of larvae rearing water estimated by qPCR. Each dot corresponds to a single well, with 18 wells tested per combination of diet and inoculum concentration (6 wells per batch of inoculum). **B** Re-associated larvae survival expressed as the proportion (in %) of larvae that reached pupal stage as a function of diet concentration and inoculum size (gray labels). Each dot corresponds to the mean survival per plate (6 wells of 3 larvae per plate) for each batch of inoculum. **C** Larval median development time to expressed in days. Each dot represents the mean Day_{50} (± standard deviation). Different letters indicate statistically significant viability following Tukey-HSD post hoc pairwise comparisons. Each dot corresponds to a single well, with 6 to 15 wells tested per combination of diet and inoculum concentration. The missing wells compared to panel A correspond to wells where no larvae reached the pupal stage (100% mortality)

for medium inoculum size (10^{-6}, 6 days ± 0.6), which was similar to 5% at high inoculum size (10^{-4}, 7.06 days ± 2.2) but at least 1.4 days lower than 1 and 12% at high inoculum size (10^{-4}, 7.5 days ± 1.5 and 8.41 ± 0.9) and at least 2.6 days lower of than low inoculum size (10^{-8} dilution) irrespective of the diet concentration (9.43 days ± 2.28, 9.66 days ± 3, and 9.37 days ± 2.4 at 1, 5, and 12% diet concentration, respectively) (Fig. 3B).

High inoculum size correlates with a stable and homogenous bacterial community in larval habitat, with a smaller impact of diet concentration on community structure

Eleven bacterial OTUs (> 5% relative abundance) dominated the microbial inoculum used to generate re-associated larvae (Fig. S4B). Six out of these 11 OTUs (*Brevundimonas*_OTU_20, *Delftia*_OTU_2, *Pseudomonas*_OTU_18, and *Sphingobacterium*_OTU_3 genera, *Flavobacteriaceae*_OTU_5, and *Sphingobacteriaceae*_OTU_16

families) were still detectable at day 5 in the water of re-associated larvae while 25 other OTUs (*Aeromonas*_OTU_22, *Brevundimonas*_OTU_12, *Brevundimonas*_OTU_37, *Pseudomonas*_OTU_21, *Pseudomonas*_OTU_33, *Rhizobium*_OTU_8, *Bacteroides*_OTU_9, *Blastomonas*_OTU_26, *Bosea*_OTU_31, *Cedecea*_OTU_6, *Chryseobacterium*_OTU_1, *Clostridium*_OTU_24, *Devosia*_OTU_65, *Dysgonomonas*_OTU_17, *Luteolibacter*_OTU_103, *Massilia*_OTU_48, *Microbacterium*_OTU_13, *Microbacterium*_OTU_32, *Microbacterium*_OTU_4, *Shinella*_OTU_19, *Sphingomonas*_OTU_15, *Sphingomonas*_OTU_41, and *Stenotorophomonas*_OTU_7 genera, *Acetobacteraceae*_OTU_34 family and *Enterobacterales*_OTU_14 *order*) were detected only at day 5 but not in the bacterial input (Figs. S4B and S6). A total of 13 OTUs presented the highest abundance in the water of larvae exposed to a high and medium bacterial inoculum, sometimes beyond 10^{10} per mL of water (number of 16S rRNA reads normalized by 16S rRNA qPCR copies) such as *Delftia*_OTU_2, *Rhizobium*_OTU_8, *Sphingobacterium*_OTU_3, *Chryseobacterium*_OTU_1, and *Cedecea*_OTU_6. Interestingly, when inoculum size is low (10^8), these OTUs were less present for the benefit of other OTUs notably *Dysgonomonas*_OTU_17 or *Bacteroides*_OTU_9 (Fig. 4A). This suggests a strong structuration of bacterial microbiota according to the initial inoculum size. When assessing the effect of diet concentration (1, 5, or 12%), inoculum size (10^{-4}, 10^{-6}, or 10^{-8}), and a batch of inoculum (B1, B2, B3) on beta diversity, the triple interaction was significant (PERMANOVA, $P_{\text{inoculum x diet x batch}}=0.006$, $R^2=0.047$) although the strongest impact on bacterial community structure was observed with inoculum size (PERMANOVA, $P_{\text{inoculum}}=0.001$, $R^2=0.16$) (Fig. 4B, C). With low inoculum size, bacterial community in rearing water exhibited relative stability whereas a lower inoculum size triggers a shift towards a more variable community.

To decipher the impact of specific OTUs on mosquito larvae, correlation analyses between OTUs abundance (number of reads normalized by 16S rRNA qPCR) in larval water and performance-related traits (median Day_{50}, larvae-to-pupae survival, inoculum size, and diet concentration) were performed. The abundance of sixty OTUs was significantly correlated (Spearman correlation, $P<0.05$) with one of the four above traits with a correlation coefficient below −0.4 or above 0.4 (Fig. 5). Out of these 60 OTUs, 57 (95%) had their abundance correlated to inoculum size, while 16 (26.6%) were correlated to larval viability, 5 (8.3%) to diet concentration, and 1 (1.6%) to Day_{50} with both positive and negative correlations (Fig. 5). Interestingly, some of the most abundant OTUs in water (e.g., *Delftia*_OTU_2, *Chryseobacterium*_OTU_1, *Cedecea*_OTU_6, *Sphingobacterium*_OTU_3, *Pseudomonas*_OTU_18) showed abundances that were negatively correlated with both inoculum dilution and larval viability (Figs. 5 and Fig. S7). *Delftia*_OTU_2 showed an additional positive correlation with the diet concentration.

Discussion

Over the past decade, the host-diet-microbiota interaction has emerged as a main driver of health, from flies to humans, offering promising health benefits, particularly through personalized nutrition [60, 61]. While this tripartite interaction has been extensively studied in a few model organisms like *Drosophila melanogaster*, it has been largely overlooked in most species. This underscores the need to reconsider the current host-diet-microbiota interaction framework in the light of non-model biological systems, particularly arthropod vectors like mosquitoes, where such an approach could enhance vector control strategies and reduce pathogen transmission. Previous studies showed that mosquito larval habitats are complex niches with specific physical, chemical, and biological characteristics, including variable nutrient quantity/quality and flexible microbial communities [24, 37, 62]. Diet-microbiota interaction in the aquatic niche influences mosquito performance, as shown by differences in the development of *Ae. aegypti* larvae with a simplified bacterial community when exposed to two diet regimens or by bacteria-mediated larval provisioning of limiting dietary factors [30, 63]. However, how diet and microbiota influence each other in the niche, how these two factors (notably their quantity) interact to drive mosquito performance, and what are the underlying mechanisms remain poorly characterized in most mosquito species. To the best of our knowledge, our study is the first to address the impact of concomitant quantitative variations in diet and microbiome on juvenile performance in mosquitoes using gnotobiotic specimens. To that aim, we used an experimental approach to test the impact of a range of concentrations of complex diet and bacterial inoculum on performance-related mosquito traits in *Ae. albopictus*. Our results showed that diet and bacterial communities in larvae rearing water are intricately linked to impact *Ae. albopictus* larval performance with carry-over effects on adult mosquitoes. This correlates with an impact of initial bacterial load, and to a lesser extent diet concentration, on the abundance and the structure of bacterial communities in larvae rearing water. Therefore, larvae performance patterns could take three forms according to diet and inoculum size. First, a low inoculum size triggers a stochastic bacterial community assembly associated with an overall lower bacterial biomass, a higher larvae survival as diet concentration increases together, and a longer development time but in a diet-independent manner. Second, a high inoculum size

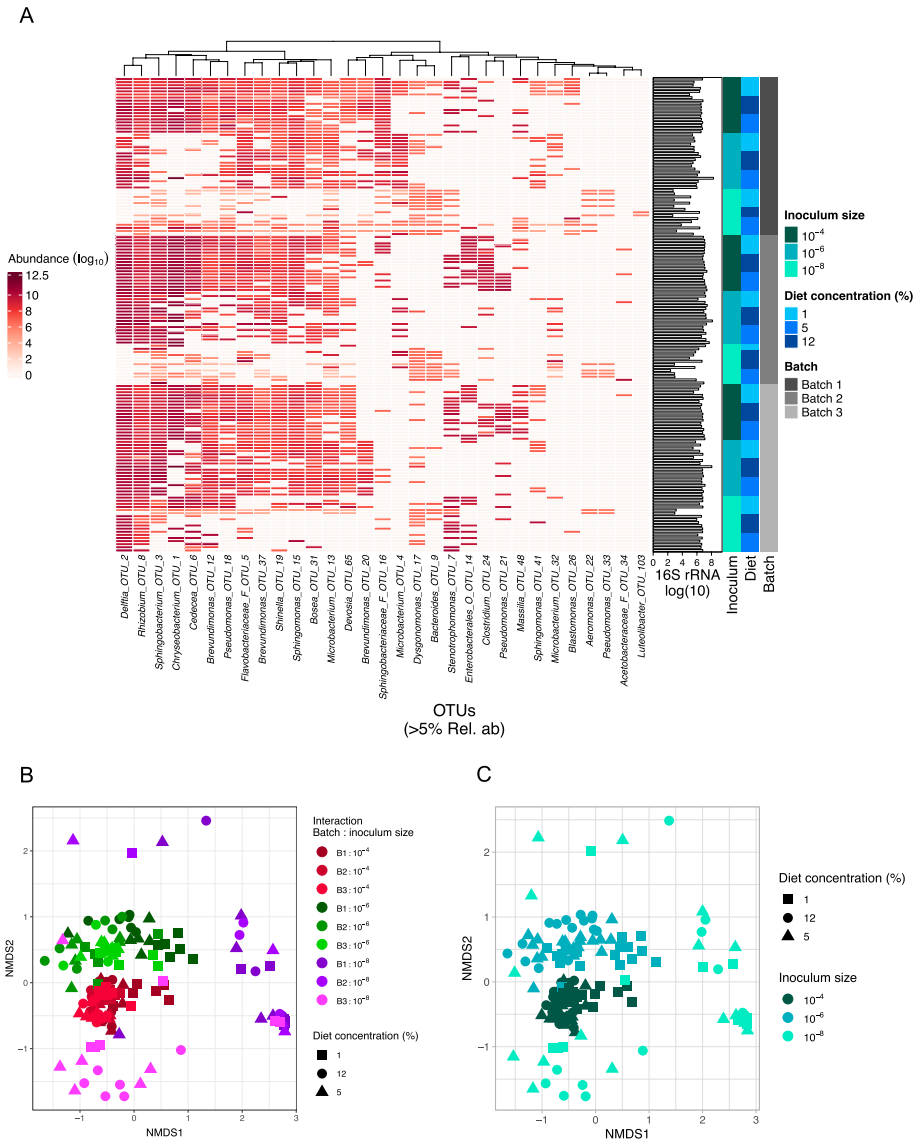

Fig. 4 Bacterial community structuration in larval rearing water upon a range of bacterial inoculum size and diet concentration. **A** Heatmap of the abundance (normalized by 16S rRNA qPCR and $\log_{10}$-transformed) of the 31 main OTUs (> 5% relative abundance in at least one sample) detected in larval water according to the inoculum size, the diet concentration, and the batch of inoculum. Each line represents a sample, each row an OTU. Barplot shows the number of 16S rRNA copies (in $\log_{10}$) for each sample for the three batches of microbial inoculum (B1, B2, B3). **B**, **C** Non-metric multidimensional scaling (NMDS) analyses of the Bray–Curtis dissimilarity distances with a highlight on (**B**) the diet concentration and inoculum size or (**C**) the interaction between a batch of inoculum and inoculum size

triggers a deterministic bacterial community assembly associated with a higher biomass, and a short development time (regardless of diet concentration) although a decrease in larvae survival was observed as diet concentration increased. Third, larvae exposed to an intermediate inoculum size show similar development time but a higher survival compared to high inoculum size, despite a similar 16S rRNA load at day 5 post-inoculation regardless of diet concentration.

Interestingly, we show that this tripartite interaction, although similar in some respects, exhibits strong differences between mosquitoes and flies. Bacteria buffer diet scarcity to maintain *Ae. albopictus* larvae development time around 7 days, as shown in *Drosophila* [64]. Bacteria buffer diet scarcity to maintain the development time of *Ae.albopictus* larvae at around 7 days, as observed in *Drosophila* [64]. While both *Drosophila* and mosquito germ-free larvae exhibit delayed development compared to conventional larvae, this delay is abolished under rich

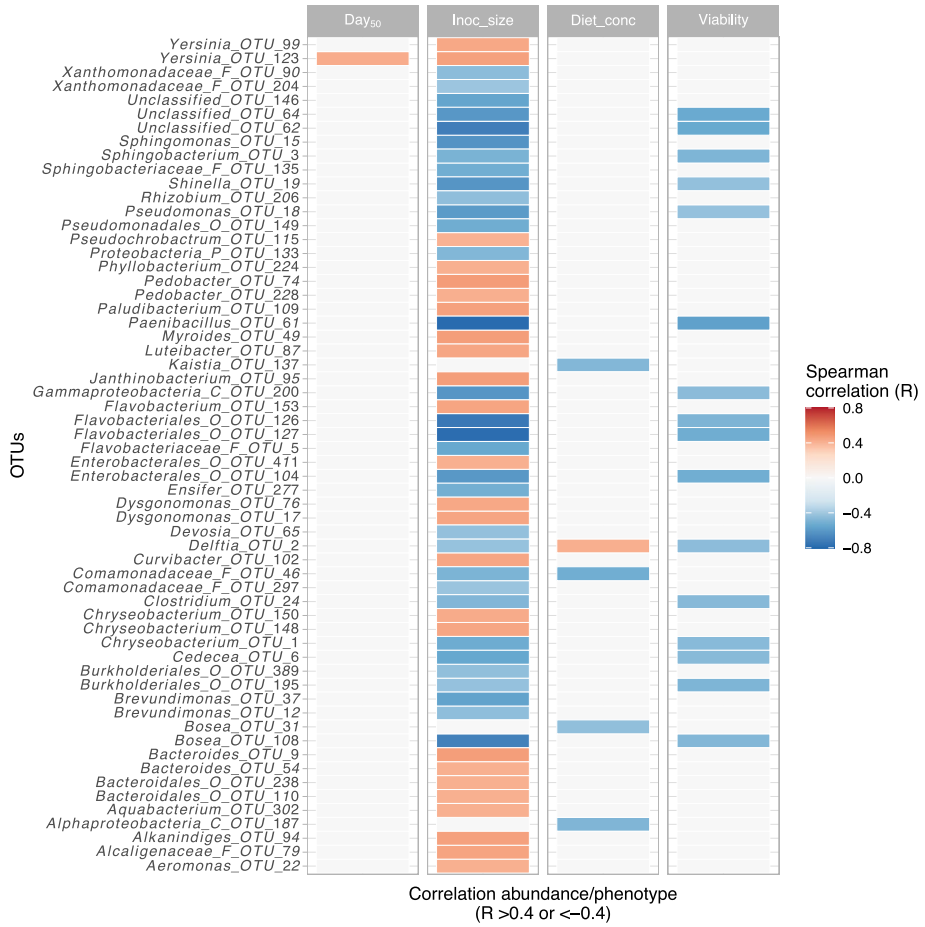

Fig. 5 Correlation between OTU abundance in rearing water and larval traits. Heatmap representing Spearman correlation coefficient between phenotype of interest (larvae-to-pupae development time—Day_{50}, inoculum size—Inoc_size, diet concentration—Diet_conc and larvae-to-pupae viability—viability) as a function of OTU abundance (normalized by 16S rRNA qPCR). Only OTUs with a significant Spearman correlation (*P*-value < 0.05) and a coefficient (*R*) < 0.4 or > 0.4 are represented. When genus level information was not available, the family (*F*), order (*O*), or class (C) level are indicated

diet conditions in flies [7] but persists for over 5 days in mosquito control larvae compared to their re-associated counterparts, regardless of diet conditions. This suggests that bacteria are not solely passive sources of food for mosquito larvae but actively contribute to *Ae.albopictus* development. This could occur through bacteria-mediated diet processing and/or metabolite production, possibly involving insect endocrine function and/or enhancing insect cell and tissue differentiation, as demonstrated in *Ae.aegypti* [23, 63, 65]. This underlines that the relationship between diet, microbiota, and performance is not fully understood and emphasizes the need for studies of non-model species to disentangle such a complex tripartite interaction. Additionally, it suggests that mosquito performance is strongly influenced by rearing water bacteria, making them major targets for vector control strategies. Several bacterial OTUs (25/36) were below the detection threshold in the inoculum at day 0 but became detectable in the water after 5 days. This suggests that their growth may have been promoted by diet and/or inoculum concentration during larval development. Notably, we showed that microbial inoculum concentration has a major founder effect on larvae microbiota structure. It has not been well explored in most model organisms so far despite identifying mechanisms at play during microbial community assembly is a major question in biology. In aquatic environments, previous studies showed that both deterministic (through specific environmental factors), stochastic, or ecologically neutral processes impact microbial community assembly [66–68]. Depicting the influence of assembly processes on the stochastic-deterministic spectrum would be of primary importance in predicting how diversity changes at different spatial and temporal scales. We could hypothesize that a low bacterial inoculum triggers bottlenecks that result in a more variable composition of the bacterial

community whereas a higher inoculum converges to a more deterministic bacterial community structure. Diet concentration also influenced bacterial community structure, although to a lesser degree compared to inoculum size, but did not impact the bacterial load in larval water as measured by qPCR on 16 s rRNA gene thereby only partially supporting recent results in *Ae. aegypti* [31]. A holistic diet for *Ae. albopictus*, as developed in *Ae. aegypti* [69], could help to disentangle the role of diet concentration and inoculum size on mosquito bacterial community assembly while controlling for diet composition, together with cultivable bacterial load and mosquito tissue-specific microbiota analysis. In addition, the establishment and assemblage of microbial communities in mosquitoes does not only rely on inoculum and diet concentration but also on mosquito genotype, diet availability, biotic, and abiotic factors of the niche as well as their interactions [23].

Ecological performance is a multifaceted phenotype driven by a set of traits that are not equally impacted by microbiota and diet. Our work suggests a trade-off between diet concentration and bacterial inoculum size with consequences for larval survival that could be driven by specific bacterial taxa. Indeed, at a medium–high bacterial load, an increase in diet concentration can be detrimental to larval survival. As the overall bacterial community load remains stable (as estimated by 16S rRNA qPCR), this effect is likely explained by the dynamics of specific bacterial isolates in the breeding site. Several bacterial OTUs (25/36) were below the detection threshold in the inoculum at day 0 but became detectable in the water after 5 days. This suggests that their growth may have been promoted by diet and inoculum concentration during larval development. The abundance of some of these OTUs significantly correlated with niche conditions (diet concentration and inoculum size) or larval performance traits (Day_{50} and larvae survival). For instance, the increase in *Yersinia* abundance was associated with a slower *Ae. albopictus* larvae development. Bacteria from the *Yersinia* genus are considered commensal in *Ae. albopictus* microbiota, but their impact on mosquito performance has poorly been described until now [70, 71]. They widely express genes encoding for *Enhancin*, a protein that increases *Ae. aegypti* permissiveness to dengue virus (DENV) when expressed by *Serratia* [72]. It would be interesting to evaluate if exposure of *Ae. albopictus* larvae to *Yersinia* can trigger developmental delay with carry-over effects on adult vector competence. The increase in abundance of *Delftia*_OTU_2, *Chryseobacterium*_OTU_1, *Cedecea*_OTU_6, *Sphingobacterium*_OTU_3, and *Pseudomonas*_OTU_18 correlated with an increase in diet concentration and a decrease in larval viability. These genera are frequently identified in the microbiota of field-caught *Ae. albopictus* although their occurrence varies according to the mosquito's geographical origin or life stage [73–75]. A strain of *Sphingobacterium multivorum* was shown to elicit egg-laying in *Ae. albopictus*, while an isolate of *Pseudomonas protegens* has larvicidal activity upon exposure to high dose (1×10^8 CFU/mL) in the water [40, 76]. *Chryseobacterium* was found to be an important component of the gut microbiota of female *Ae. albopictus* before and after blood feeding, as well as in eggs, suggesting that it can be transmitted among generations [73]. Targeting *Chryseobacterium* with an antimicrobiota vaccine in *Ae. albopictus* decreased *Chryseobacterium* relative abundance, altered the bacterial community structure, and impaired fecundity and egg-hatching rate in vaccinated mosquitoes [77]. While these OTUs emerge as potential keystone species of *Ae. albopictus* microbiome with a potential for vector control, it is worth noting that interbacterial or even interkingdom interactions can shape the microbiome of their host, with an impact on its performance [78, 79].

From an evolutionary perspective, mosquitoes could maximize juvenile performance by selecting a niche with a low bacterial density but a high diet concentration although larvae developmental time could be delayed increasing the risks of predation notably, or a niche with a higher bacterial density but nutrient-poor to maximize both juvenile development time and survival beyond a certain diet concentration. Implementing behavior assays to monitor how mosquitoes evaluate water suitability for egg laying as a function of microbial inoculum and diet concentration could be of interest to studying evolutionary adaptations and manipulating mosquito behavior towards vector control [62, 80].

Conclusions

This work expands the host-diet-microbiota interaction concept and demonstrates its uniqueness in non-model, medically relevant organisms like mosquitoes. By addressing the effects of varying food availability and microbial abundance on mosquito performance, our findings provide valuable insights into the influence of this tripartite interaction on mosquito larvae ecology and evolution. These results pave the way for future research in vector ecology and control. Nutrient and microbial levels in larval aquatic habitats are variable in natural ecosystems and such results demonstrate how the habitat may impact developmental responses of mosquitoes and regulate natural populations. Recognition and selection of breeding sites by gravid females is a key step in mosquito life cycles. Further studies are needed to evaluate how these variations impact ovipositing females and shape the niche construction and partitioning initiated

by the gravid female. More broadly, those complex interactions raise questions about the existence of species-specific effects as well as the genetic and environmental basis of mosquito traits and resource allocation on the mosquito vectorial capacity.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-025-02067-0.

Supplementary Material 1. Figure S1. Development of *Ae. albopictus* juveniles issued from conventional and surface-sterilized eggs. (**A**) Procedure for eggs surface sterilization adapted from [29]. *Ae. albopictus* eggs between 2 weeks and 2 months old were used. Careful visual inspection of egg papers under a stereomicroscope was conducted prior to each experiment to avoid unfertilized eggs. Under a safety cabinet, eggs were rinsed twice by dipping the paper in sterile water to discard debris. Egg papers were then soaked in a petri dish containing 70% ethanol solution (in sterile water) for 5 min then transferred for 5 min in a 50-mL falcon tube containing 30 mL of sodium hypochlorite (3% active chlorite) supplemented with 4 mg/mL ampicillin. Contact with sodium hypochlorite detached the eggs from the paper, that was removed using sterile forceps. Within 5 min, eggs quickly settled at the bottom of the tube allowing the complete removal of sodium hypochlorite without the need of centrifugation. Eggs were resuspended in 25 ml of 70% ethanol and incubated for 5 min. We ensured that all eggs remained immersed in ethanol and gently agitated the tube to allow a complete contact of the eggs with ethanol solution. Eggs were rinsed three times in sterile water (Gibco) for 5 min then 30 mL of sterile 1X PBS was added. The tube was closed with a 0.2 µm filtered cap and transferred in a vacuum chamber outside the cabinet for 40 min at −20 ATM to allow hatching. The sterile first instar larvae were transferred under the safety cabinet in a sterile petri dish and immediately transferred in 6-well plates using a micropipette according to the experimental design. (**B**) Conventionally-raised *Ae. albopictus* larvae (6-well-plate image, left side) after 7 days under 16 h:8 h light:dark cycle (up) or complete darkness (bottom). Control larvae (6-well-plate image, right side) after 11 days under 16 h:8 h light:dark cycle (up) or complete darkness (bottom). Three different diet concentrations were used (1, 5 and 12.5% w:v) as indicated. White arrowheads indicate > 2nd instar larvae/pupae as a sign of larval development. (**C**) Inoculation of water from conventional or control larvae on non-selective bacterial agar. Rearing water of conventional on the day of hatching (1 µL, day 0) or after 7 days (50 µL of a 10^{-6} dilution) (left panel). Rearing water (100 µL) and sterile diet plug (plug was removed to facilitate visualization of potential contaminants underneath) on the day of hatching (day 0) and rearing water (100 µL) after 10 days (Right panel: Left, central and right image, respectively). All pictures show Luria-Bethani modified (LBm) agar except the right image of right panel (Day 10, control water) that was spread on both Lysogeny Broth modified and Brain Heart Infusion agar plates. Incubation of agar plates were performed for the indicated number of days at 28–30℃. bacteria were detected after plating water from control larvae.

Supplementary Material 2. Figure S2. Experimental designs used in this study. (**A**) Design corresponding to Fig. 2. Three independent experiments (Exp. #1, 2 and 3) were performed on different days but following the same protocol. Re-associated and control larvae were prepared concomitantly but incubated in separate 6-well plates, with re-associated larvae being exposed to a single dilution of water from conventionally-reared larvae used as inoculum (1×10^{-6}). Larvae were exposed to 4 diet concentrations (12, 10, 5 and 2%) using a single agar plug at the selected concentration deposited in each well, then scored for performance. Exp. #1 was conducted with 1 plate per condition (8 plates total) while Exp. #2 and 3 had 3 plates per condition (24 plates per experiment). (**B**) Design corresponding to Figs. 3 to 5. Within a single experiment, 3 batch of eggs (laid at different days) were hatched to produce 3 batch of inoculum (B1, B2 and B3). Re-associated larvae exposed to B1, B2 or B3 at three dilutions (1×10^{-4}, 1×10^{-6} or 1×10^{-8}) for 3 diet concentrations (1, 5 and 12%) were incubated in 6-well plates (1 plate per condition) and scored for juvenile development. At day 5, the bacterial load and OTUs composition in the water was tested using 16S qPCR and amplicon sequencing respectively. A plate of control larvae (not shown) was also prepared to test bacterial load and OTUs composition in the water after 5 days.

Supplementary Material 3. Figure S3. *Ae. albopictus* pupae-to-adult survival according to larval diet concentration and microbial status. Pupal survival expressed as the proportion (in %) of pupae that reached adult stage according to microbial status (re-associated or control) and diet concentration (2, 5, 10 and 12%). Each dot represents the mean survival for a 6-well plate (3 larvae per well, *i.e.* 18 larvae per plate) and 3 experiments were conducted (from 1 to 3 plate per experiment). Missing dots compare to Fig. 2A correspond to conditions were no pupae were obtained. No significant differences in pupal viability was detected according to diet concentration, microbial status, or their interaction (Wald χ^2, Chisq = 5.33, Df = 3, $P_{diet} = 0.15$; Chisq = 0.79, Df = 1, $P_{microbial\ status} = 0.37$; Chisq = 2.26, Df = 3, $P_{diet\ x\ microbial\ status} = 0.51$).

Supplementary Material 4. Figure S4. Load, taxonomic identity, and relative abundance of bacterial OTUs in water. (**A**) Bacterial load ($\log_{10}$ 16S rRNA copies per µL) determined by qPCR at Day 0 in the water inoculum used to generate re-associated larvae in Fig. 3 to 5 (corresponding to panel B) or per µL of water from control larvae at Day 5 (corresponding to panel C). (**B**) Relative abundance of bacterial operational taxonomic units (OTUs) at genus or family (F) level in the inoculum at Day 0 used in Fig. 3 to 5. Two samples (S1, S2) per batch of inoculum (Batch 1, 2 and 3) were sequenced except for Batch 3 (only one sample). (**C**) Relative abundance of bacterial OTUs at genus level in control water (5-days-old water from control larvae). The five control water samples originated from wells with sterile diet but no larvae (S1, S2), with larvae and diet (S4, S5) or without larvae nor diet (S3). OTUs representing less than 5% in relative abundance were grouped (red, < 5% Rel. ab.)

Supplementary Material 5. Figure S5. Development of *Ae. albopictus* larvae according to diet concentration upon exposure to low inoculum dilution. Larvae-to-pupae survival for re-associated larvae exposed 1×10^{-3} dilution of microbial inoculum expressed as the proportion (in %) of larvae that reached pupal stage as a function of diet concentration (in %). Each dot represents the mean survival (± standard deviation). The panel corresponds to one pilot experiment with one 6-well plate per condition (3 larvae per well).

Supplementary Material 6. Figure S6. OTUs relative abundance upon bacterial inoculum size and diet concentration gradients. Relative abundances (in %) of bacterial OTUs at the genus level were represented for each combination of inoculum and diet concentration, for the three batches of microbial inoculum (B1, B2, B3). OTUs representing less than 5% in relative abundance were grouped (red, < 5% Rel. ab.)

Supplementary Material 7. Figure S7. Correlation between the abundance of selected OTUs and larval traits. OTUs abundance (normalized by 16S rRNA qPCR, $\log_{10}$ transformed) as a function of the larval trait. Linear correlation (+ in red,—in blue) is shown. Relevant experimental condition (diet concentration and inoculum size) is highlighted by color codes. Each dot represents a unique sample, while samples in which the OTUs were not detected were discarded. As the inoculum size is a major driver of bacterial community structure, dots were coloured accordingly.

Supplementary Material 8. Figure S8. (**A**) Experimental design. (**B**) Larvae-to-pupae survival expressed as the proportion (in %) of mosquito larvae that reached pupal stage as a function of diet concentration (in %). Each dot represents the mean survival for a 6-well plate (3 larvae per well, *i.e.* 18 larvae per plate) with 4 (2 and 5% diet concentration) to 7 plates (1%, 10 and 12% diet concentration) per condition. Of note, no larvae survived at the 12% diet concentration. Different letters indicate statistically significant viability following Tukey-HSD *post-hoc* pairwise comparisons. (**C**) Median development time into pupae (Day_{50}) for surviving re-associated and control individuals was represented as a function of diet concentration (in %). Each dot represents the Day_{50} for a single well (3 larvae per well) from a 6-well plate. Different letters indicate statistically significant viability following Tukey-HSD *post-hoc* pairwise comparisons. (**D**) Adult wings length. Each dot represents the mean length (in mm) of

both wings together (± standard deviation). 10 females and 10 males were randomly picked and analysed for each diet concentration.

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Authors' contributions
All authors contributed intellectually to and agreed to this submission. VR, GM, and CVM designed the experiments. VR and EM conducted the experiments and collected the data. VR, GM, and CVM analyzed the data. VR wrote the initial draft of the manuscript, while GM and CVM provided substantial feedback. The authors read and approved the final manuscript.

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Data availability
All FastQ files were deposited in the EMBL European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the project accession number PRJEB57586.

Declarations

Ethics approval and consent to participate
Female mosquitoes were blood-fed on mice in accordance with the Institutional Animal Care and Use Committee from Lyon1 University (Apafis #31807–2021052715018315).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Universite Claude Bernard Lyon 1, CNRS, INRAE, VetAgro Sup, UMR Ecologie Microbienne, Villeurbanne F-69622, France. [2]Present Address: Universite Claude Bernard Lyon1, INRAE, EPHE, PSL Research University, IVPC Umr754, Lyon F69007, France.

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