Identification of culturable fungi and bacteria in mosquito saliva and impact on arbovirus infection *in vitro*

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Abstract

Mosquito saliva was shown to play a key role in arbovirus transmission and pathogenesis. In this study, we explored the presence of microbiota in mosquito saliva and their potential effect on mosquito-borne virus infection *in vitro*. Culturable fungal and bacterial colonies were isolated and identified from saliva harvested from *Aedes aegypti* (lab strain) and *Culex pipiens* (field-collected mosquitoes). For the first time, the fungal species *Penicillium crustosum* was identified in mosquitoes. Culturable bacteria detected in mosquito saliva included *Serratia marcescens, Serratia nematodiphila*, *Enterobacter* spp. and *Klebsiella* spp., which were previously identified as mosquito or insect endosymbionts in the midgut or other organs. Oral treatment of adult mosquitoes with antibiotics or an antifungal drug resulted in a significant reduction of bacteria or fungi in saliva. Saliva depleted from fungi or bacteria significantly decreased Semliki Forest virus infection in human skin fibroblasts compared to non-treated saliva when the virus was preincubated with saliva or when virus and saliva were added to the cells at the same time. These results suggest that the salivary microbiota may play an essential role in mosquito-borne viral replication in human skin cells. Nevertheless, the potential effect of salivary microbiota warrants further investigation *in vivo*.

Introduction

Mosquitoes of the *Culicidae* family are considered the deadliest animals on the planet[1], due to the ability of female mosquitoes to transmit pathogens such as arboviruses during blood feeding. Arboviruses (i.e. arthropod-borne viruses) are an emerging threat to the health of humans and animals worldwide[2]. Epidemics with arboviruses have increased in recent decades as a consequence of the development of global transportation systems, deforestation, urbanization, and an increasing human population density. The main mosquito vectors of medically important arboviruses, such as chikungunya virus (CHIKV), Zika virus (ZIKV), dengue virus (DENV), Yellow fever virus (YFV) and West Nile virus (WNV), are *Aedes aegypti* and *Culex pipiens*[1]. Symptoms caused by arboviruses can vary from asymptomatic or mild febrile illness to severe disease, such as chronic arthralgia and arthritis (CHIKV), congenital microcephaly and Guillain-Barré syndrome (ZIKV), dengue hemorrhagic fever and shock syndrome (DENV) and viral encephalitis (WNV), which can potentially be fatal[3–5].

Several decades of global efforts towards the development of arbovirus vaccines have resulted in few successful candidates. Since the 1930s to date, the live-attenuated 17D vaccine (YFV-17D) against YFV has been the most successful arbovirus vaccine. However, despite the high vaccination coverage rates in some countries, YFV outbreaks continue to be reported[6]. Other recently licensed vaccines are the live-attenuated tetravalent dengue vaccine Dengvaxia (CYD-TDV) and QDENGA (TAK-003). However, their efficacy varies between the four DENV serotypes because of the risk of antibody-dependent enhancement, leading to the limited use of Dengvaxia to date. Dengvaxia (CYD-TDV) is only licensed for use in individuals with a confirmed prior DENV infection[7,8], and QDENGA (TAK-003) was approved to be used in individuals older than 4 years regardless of previous dengue exposure in the EU and Brazil[9]. Furthermore, there are currently no approved antiviral drugs available for arbovirus infections. Therefore, the primary strategy to control arbovirus outbreaks in high-risk countries relies on mosquito vector control aiming at reducing contact with potentially infected mosquitoes[10]. Due to the long-term usage of insecticides of the same chemical classes, including organophosphates and synthetic pyrethroids, insecticide-resistant mosquitoes are widely spread and are considered a major threat to vector control by the World Health Organization[11]. Other known limitations of using insecticides are ineffective timing of application, low efficacy, low community acceptance, and high economic costs[12]. Therefore, new strategies for vector control are needed to reduce the global burden caused by arboviruses.

When feeding on an infected host, the mosquito ingests the virus simultaneously with the blood into the midgut. In order to be efficiently transmitted to another vertebrate host, the virus must replicate in the midgut and then disseminate to secondary organs including the salivary glands. The virus needs to cross the salivary gland infection barrier to infect and replicate in salivary glands. Finally, the virus should be released into the mosquito saliva to be deposited into the skin of a new host during a subsequent blood feeding[13]. Virus transmission by mosquitoes can be influenced by extrinsic factors (temperature, humidity and nutrition), intrinsic factors (innate immune responses and microbiota) and genetic factors[14].

Both the midgut and the salivary glands have been characterized as significant barriers for systemic arbovirus infection in the mosquito and transmission in the saliva. Both organs are also known to be colonized by a dynamic microbial community[15–18]. Previous research has found that the interaction of microbial components with viruses can either negatively or positively affect arbovirus transmission. For example, *Serratia odorifera*, a bacterium inhabiting the midgut of *Aedes aegypti*, has been shown to enhance mosquito susceptibility to DENV and CHIKV by suppressing the mosquito immune response[19,20]. Furthermore, *Serratia marcescens* was identified as a midgut commensal bacterium with the ability to promote mosquito permissiveness to arboviruses by secreting SmEnhancin, a protein to digest the mosquito midgut membrane-bound mucins[21]. In addition to bacteria, a *Talaromyces* fungus was found in the midgut of field-captured *Ae. aegypti*, rendering the mosquito more permissive to DENV infection by down-regulating digestive enzyme genes and their activity in the midgut[22].

While biting a host, female mosquitoes probe into the host skin in search for a blood vessel and meanwhile inject saliva into the epidermis and dermis of the vertebrate host. Mosquito saliva contains vasodilatory, anti-hemostatic, angiogenic and anti-inflammatory molecules that ensure a continuous blood flow to the mosquito during feeding[23,24]. Additionally, mosquito saliva contains molecules that could alert the immune system of the host which potentially may block viral transmission. Therefore, mosquito saliva components are being studied as promising candidates for vaccine development[25]. Mosquito saliva has recently also been shown to contain bacteria[26]. Theoretically, these bacteria present in the saliva are also deposited into the host skin[27], but their potential role in virus transmission and infection during this process is unclear. With regards to the impact of mosquito microbiota on vector competence, most research so far has focused on the midgut and the salivary glands, while

the microbiota present in the saliva of mosquitoes remain largely unexplored. Here we describe that both fungi and bacteria can be cultured from saliva of *Aedes aegypti* from a laboratory colony and from field-collected *Culex pipiens* mosquitoes. The effect of these microbes on arbovirus replication in the vertebrate host skin was investigated in a human skin fibroblast cell line.

Methods

Ethics statement

Permits for mosquito field collections in Leuven were obtained from KU Leuven and the City Green Management (CGM) of Leuven. Mosquitoes of the *Aedes aegypti* lab strain were maintained by blood feeding on mice and were performed with the approval and under the guidelines of the Ethical Committee of the University of Leuven (license P053/2019).

Mosquito strains

Aedes aegypti Paea lab strain (provided by Pasteur Institute Paris via the EU-funded Infravec consortium) was reared and maintained under controlled conditions (28 ± 1°C, relative humidity of 80%, light: dark cycle of 16 h: 8 h). Field *Culex pipiens* mosquitoes were collected in Leuven (N 50°52'41, E 4°41'21, Belgium) during the summer of 2021. Adult mosquitoes were trapped with BG-Sentinel traps (BioGents GmbH, Germany) with the help of BG-lure (BioGents GmbH, Germany). After sorting mosquitoes based on morphology, *Culex pipiens* were maintained under conditions of 25 ± 1°C, relative humidity of 50%, and light: dark cycle of 14 h: 10 h. Adult mosquitoes were housed in Bugdorm-1 insect cages (30 x 30 x 30 cm, Bugdorm, Taiwan, China) in the insectary of the Rega Institute and fed with 10% sterile sugar solution.

Species identification of field mosquitoes by multiplex RT-qPCR

Species identification was performed as described earlier[28]. In brief, wings and legs of individual female mosquitoes were homogenized by bead disruption (2.8 mm Precellys, Bertin Instruments). Subsequently, the supernatant was hydrolysed at 95°C for 10 min. Multiplex RT-qPCR was performed using a forward primer (5'-GCGGCCAAATATTGAGACTT-‘3) and reverse primer (5'-CGTCCTCAAACATCCAGACA-‘3) targeting the gene locus for acetylcholinesterase 2 to identify the *Culex pipiens* complex[29]. Additionally, subspecies-specific probes were used by targeting the CQ11 microsatellite locus to distinguish between *Culex pipiens pipiens* (5'-GCTTCGGTGAAGGTTTGTGT-'3)[29] and *Culex pipiens molestus* (5'-TGAACCCTCCAGTAAGGTATCAACTAC-‘3)[30]. Reactions were prepared in a final volume of 25 µL containing 3 µL of template DNA, 6.25 µL of 2x TaqMan™ PCR Master mix, 0.38 µL of each primer at 10 µM, 1 µL of each probe at 5 µM, 0.06 µL Reverse Transcriptase and 12.94 µL of nuclease-free water. The thermal profile consisted of an initial step of 15 min at 95°C, followed by 40 amplification cycles of 1 min at 94°C, 1 min at 48°C and 1 min at 72°C.

Forced salivation and midgut/salivary gland dissection

Mosquitoes were starved 24h prior to salivation. Female *Aedes aegypti* or *Culex pipiens* mosquitoes were anaesthetized by placing them under cold conditions (4-8°C) for 5 min, or by CO_2 released by dry ice for 2 min, respectively. While sedated, legs and wings were removed to immobilize the mosquito during salivation. Saliva for culturing was harvested by gently placing the proboscis of a living female into a 20 µL pipette tip (uTIP, Biotix) containing 20 µL of a 1:1 solution of 10% sterile sugar solution and 1X PBS. Saliva for the *in vitro* infection assays was harvested by gently placing the proboscis of a living female into the pipette tip containing only 1 µL of silicon oil as described previously[26]. Blank tips containing only the salivation solution were placed aside on the bench as the environment control during each salivation. After 1h of forced salivation, saliva was harvested and stored at -80°C either individually or pooled for further studies.

The surface of the mosquito body was disinfected individually by 75% ethanol and rinsed twice with 1X PBS buffer before dissection. Salivary glands were harvested before the midgut to avoid potential contamination as the midgut harbours the highest bacterial load. The salivary glands and midguts were pooled (8 to 12 females) in homogenization tubes (2.8 mm Precellys, Bertin Instruments) containing 150-200 µL of 1X PBS and then stored at -80°C for further analysis.

Artificial blood feeding, antibiotic and antifungal treatment

To study the effects of a blood meal on salivary microbiota, 5 to 7-day-old *Aedes aegypti* female mosquitoes were orally fed for 30 min using an artificial membrane feeding system (Hemotek, UK). The bloodmeal contained fresh rabbit erythrocytes washed with PBS and plasma was replaced by the same volume of sterile FBS. Fully engorged females were cold-anesthetized and separated to be maintained up to two weeks until forced salivation, under the same controlled conditions mentioned above. In parallel, the blood meal was cultured on Luria-Bertani (LB) plates to determine the absence of bacteria in the blood meal.

Adult mosquitoes were treated with an antibiotic cocktail with doses ranging from 10 units/mL penicillin and 10 µg/mL streptomycin/gentamycin (Penicillin-Streptomycin 100X, Gentamycin, Gibco™) up to 200 units/mL penicillin and 200 µg/mL streptomycin/gentamycin into the 10% sugar solution for 6 days. For the antifungal treatment, 25 µg/mL of Fungin™ (InvivoGen) was added into the 10% sugar solution for 10 days. The sugar solution containing antibiotic and/or antifungal compounds was refreshed every day throughout the treatment.

Bacteria and fungi culturing on agar plates

Mosquito-derived samples (saliva, salivary glands and midguts) were cultured on LB broth, Brain Heart Infusion (BHI) broth or blood agar plates at 28°C to compare microbial growth on different media. Approximately 50 µL of saliva was cultured per agar plate. For the salivary gland samples, 50 µL of supernatant post-homogenization was used. For the midgut, the supernatant was diluted 10 to 100 times in sterile water post-homogenization before culturing 50 µL of supernatant on the agar plates. As a negative control, salivation solution was cultured in parallel. Plates were checked after 24, 48 and 120 h of incubation for bacteria, and up to one month for fungi. Colony-forming units (CFU) were calculated to determine the bacterial and fungal loads.

Bacterial identification by 16S Sanger sequencing

Individual colonies were selected from the agar plates and resuspended in 30 µL of sterile water. The bacterial suspension was heat-inactivated at 95°C for 15 min. When necessary, the DNA concentration was diluted to 100 ng/µL. PCR amplification of 467 bp in the 16S ribosomal RNA gene was performed with the 16S forward universal primer (5' TCC TAC GGG AGG CAG CAG T '3) and 16S reverse universal primer (5' GGA CTA CCA GGG TAT CTA ATC CTG TT '3) as described previously by Romoli et al[31]. The PCR product was confirmed by gel electrophoresis (1% agarose gel). PCR samples were purified by the Wizard SV Gel and PCR Clean system following manufacturers' instructions. After purification, samples were sent to Macrogen Europe for Sanger sequencing. The DNA sequences obtained were trimmed using TrimAl (v. 1.3) and searched for homology to those available at the GenBank-EMBL database using the Basic Local Alignment Search Tool (BLAST) program (NCBI).

Fungal identification by ITS3-ITS4 and CRU-specific Sanger sequencing

A portion of the fungal colony was collected into a homogenization tube with 500 µL of PBS. Homogenization was performed at 2 cycles of 6800 rpm for 10 sec with a pause of 20 sec between each cycle. After homogenization, 200 µL of the supernatant was used for DNA extraction, which was performed using the QIAamp DNA Kit 250 (51306, QIAGEN) following manufacturers' instructions. The universal forward primer targeting the ITS3 region (5' GCA TCG ATG AAG AAC GCA GC '3) and universal reverse primer targeting the ITS4 region (5' TCC TCC GCT TAT TGA TAT GC '3) were used for ITS PCR, amplifying a 350 bp PCR product as described previously[32]. For species-specific detection of *Penicillium crustosum*, the CRUF

forward primer (5' TCC CAC CCG TGT TTA TTT TA '3) and CRUR reverse primer (5' TCC CTT TCA ACA ATT TCA CG '3) were used, amplifying a 892 bp PCR product[33]. PCR products were confirmed by gel electrophoresis (1% agarose gel). PCR samples were purified by the Wizard SV Gel and PCR Clean system (Promega) following manufacturers' instructions. The protocol for Sanger sequencing and analysis is identical as described above for the bacterial identification.

DNA extraction from midgut, salivary glands and saliva samples

DNA extractions were strictly performed under a laminar flow cabinet. Blank Eppendorf tubes were added per experiment as a negative environmental control. DNA extractions were performed using the QIAamp DNA Kit 250 (51306, QIAGEN) following an adapted version of the manufacturers' protocol including an additional step of adding 0.5 µL of Ready-Lyse™ Lysozyme Solution (Lucigen). Next, 100 µL ATL buffer and 20 µL proteinase K were added to each sample, followed by an incubation step at 56°C for 5 min. After pulse-vortexing for 15 sec, samples were incubated at 70°C for 10 min and 230 µL of ethanol was added. The following steps followed the original protocol according to manufacturers' instructions. Samples were eluted in 50 µL AE buffer and were stored at -20°C until further analysis.

Quantification of bacterial and fungal loads by RT-qPCR

The bacterial and fungal loads in extracted DNA samples were quantified by RT-qPCR using the iTaq Universal SYBR Green One-Step kit (Bio-Rad). Each reaction was prepared in a final volume of 20 µL containing 5 µL of template DNA, 10 µL of 2x one-step SYBR Green reaction mix, 0.4 µL of each primer at 10 µM and 4.2 µL of nuclease-free water. The same primers were used as for Sanger sequencing (described previously). The thermal profile consisted of an initial step of 10 min at 95°C, followed by 40 amplification cycles of 15 sec at 95°C and 1 min at 50°C for bacteria, while a 45°C annealing temperature was used for fungal quantification. The genome copies/mosquito for each sample where quantified based on the use of a gBlocks™ Gene Fragment (IDT) as a standard dilution for both the 16S and ITS qPCR. Limit of detection (LOD) was determined as the lowest bacterial/fungal load able to be detected by the RT-qPCR assay in 95% of experiments.

Phylogenetic analysis

Sanger sequencing was performed by Macrogen Europe. The consensus sequence of fungal or bacterial colonies together with reference sequences from GenBank were aligned with MEGA-

X. The resulting alignment was trimmed by using TrimAl (v.1.3). A maximum likelihood (ML) tree was constructed using MEGA-X with the substitution model being Kamura 2-parameter model (K2) and Gamma distributed (G). 500 bootstrap replicates were used. Finally, trees were visualized using FigTree v1.4.4.

Virus strains and cells

The SFV Vietnam strain (Genbank EU350586.1) belongs to the collection of the Rega Institute for Medical Research (Belgium). Virus stocks were propagated in *Aedes albopictus*-derived C6/36 cells (ATCC CRL-1660) at 28°C and stored at -80°C. Viral titers were determined by endpoint titration and plaque assay on Vero cells. African green monkey kidney epithelial cells (Vero cells, ATCC CCL-81) were maintained in minimal essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% L-glutamine (L-glu, Gibco), 1% sodium bicarbonate ($NaHCO_2$, Gibco), and 1% non-essential amino acids (NEAA, Gibco). Human skin fibroblasts (ATCC CRL-2522) were grown in MEM supplemented with 10% FBS, 1% L-glu, 1% $NaHCO_2$, 1% NEAA and 1% sodium pyruvate (Gibco). Cell cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 95%-99% relative humidity. Assays were performed using similar medium but supplemented with 2% FBS (2% assay medium).

Effect of mosquito saliva microbiota on Semliki Forest virus infection of skin fibroblasts

Human skin fibroblast cells were pre-seeded (12 000 cells/well, in 2% assay medium) in 96 well-plates 24h before the infection. Saliva from non-treated *Aedes aegypti*, AF saliva from antifungal-treated females, AB saliva from antibiotic-treated females, and filtered saliva from non-treated *Aedes aegypti* using a 0.2µm filter were used. Three scenarios were evaluated: (1) cells were directly inoculated with saliva and virus (MOI:0.001) for 1h; (2) virus was pre-incubated with saliva for 1h at 28°C before being added to the cells for 1h (MOI:0.001); (3) cells were preincubated with saliva for 1h followed by virus infection (MOI: 0.001) for 1h. After virus infection for 1h, the virus inoculum was removed, cells were washed 3X with PBS and subsequently, fresh 2% assay medium was added. The plates were maintained at 37°C with 5% of CO_2 until 24h post infection (hpi), when supernatant was harvested and stored at -80°C for further analysis. The levels of infectious virus in the supernatant were determined by end-point titrations on Vero cells. Cells were pre-seeded in 96 well-plates (25 000 cells/well) in 2% assay medium and incubated overnight (37°C, 5% CO_2). The next day, 10-fold serial dilutions of the samples were prepared in triplicate in the plates and incubated for 3 days (37°C, 5% CO_2). At day 3 pi, cells were scored microscopically for virus-induced cytopathogenic effect

(CPE). The tissue culture infectious dose$_{50}$/ml ($TCID_{50}$/ml) was calculated using the Reed and Muench method[34]. Limit of quantification (LOQ) was determined as the lowest $TCID_{50}$ value that could be quantified by the end-point titration assay.

Data availability

The *Penicillium crustosum* sequence that was identified in this study has been deposited in GenBank under the accession number OL436123. Additionally, bacterial sequences retrieved in this study have been deposited in GenBank under the accession numbers: *Asaia* sp. ON527783; *Chryseobacterium* sp. ON527789; *Enterobacter* sp. ON527791; *Microbacterium* sp. ON527869; *Klebsiella* sp. ON527877; *Serratia nematodiphila* ON527870; *Serratia marcescens* ON527875. The other sequences used for the analysis were available from GenBank.

Results

1. Identification of fungi in mosquito saliva and organs

Mosquito saliva pooled from 8 to 12 *Aedes aegypti* mosquitoes was cultured on LB agar plates for at least 5 days at 28°C. Fungi appeared as grey-blue powdery or fuzzy colonies indicated with black arrows (Fig 1A), which were distinguishable from bacterial colonies. The fungi colony-forming units (CFU) per mosquito were recorded after culturing for at least 120h. No fungal growth was observed in the negative control containing only blank salivation solution. Next, saliva collected from single *Aedes aegypti* (lab strain) and *Culex pipiens* (field) mosquitoes were cultured in similar conditions. 40% of *Aedes aegypti* (12/30 females) and 33% of *Culex pipiens* (7/21 females) had culturable fungi in their saliva (Fig 1B). ITS qPCR was performed to verify the presence of fungal DNA in midguts, salivary glands, and saliva samples of *Aedes aegypti*. The 17S mosquito housekeeping gene was used as an internal control. The highest fungal load was detected in midgut samples, with 4.5×10^6 median ITS copies/mosquito (Fig 1C). Salivary glands contained a fungal load of 3.0×10^3 median copies/mosquito, which was higher than the median fungal load in saliva (83 copies/mosquito). The latter was not significantly different from the fungal load in the negative control.

To identify the fungal colonies cultured from *Culex pipiens* or *Aedes aegypti* saliva on agar plates, DNA was extracted from individual colonies and subjected to an universal ITS3-4 fungal identification PCR[32] targeting a 350 bp-long segment. Sanger sequencing results showed a 99% identity similarity by BLAST (NCBI) for both *Penicillium expansum* and *Penicillium crustosum*. Therefore, a specific PCR for *Penicillium crustosum* was performed to distinguish which of these two species was present in the saliva[33]. From all samples tested, an 892 bp-long segment could be amplified by the *P. crustosum* specific PCR (Fig S1), demonstrating that the fungal colonies were *Penicillium crustosum* rather than *Penicillium expansum*.

Phylogenetic analysis was performed to compare the ITS3-4 segments of *Penicillium crustosum* detected in the mosquito saliva from this study to the *Penicillium crustosum* and *Penicillium expansum* species previously reported in GenBank (Fig 1D). The bootstrap values indicate how many times (out of 500) the sequences were clustered when repeating the tree construction on a re-sampled set of the data. In 89% of the trees, the *Penicillium* clade, including the species identified in the mosquito saliva, was related to the previously described mosquito inhabitant *Penicillium chrysogenum*[35]. In 97% of the trees, the *Penicillium* clade was

related to a clade containing *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus*, which were previously reported in mosquitoes[36].

2. Identification of bacteria in mosquito saliva

Culturing of mosquito salivary bacteria was performed on LB agar plates, as no significant difference was detected when comparing the bacterial load and diversity of mosquito saliva from LB agar plates with BHI and blood agar plates (Fig S2, Table S1 and S2). Bacteria were characterized by morphological features (Fig 2A, bacteria indicated with black arrows). The bacterial CFU/mosquito saliva was defined at 48h post-culture at 28°C for both *Ae. Aegypti* and field-collected *Culex pipiens* (Fig 2B). No colonies grew in the environmental control containing only the salivation solution. Furthermore, no colonies were observed on the plates with salivary gland samples from the same mosquitoes (Fig S3). Using 16S qPCR on *Aedes aegypti* samples, the highest bacterial load was found in the midgut (10^4 mean copies/mosquito), followed by saliva (170 mean copies/mosquito) and salivary glands (38 mean copies/mosquito) which were both very close to the quantification limit and not significantly different from the NC samples (Fig 2C).

Individual bacterial colonies were picked from the plates based on their characteristics (shape, color and size) and were identified by 16S Sanger sequencing (Fig 2D). *Klebsiella* spp., *Enterobacter* spp., *Asaia* spp., *Serratia marcescens*, *Serratia nematodiphila, Microbacterium* spp., and *Chryseobacterium* sp. were identified in the saliva of *Aedes aegypti*, whereas only *Serratia marcescens* and *Serratia nematodiphila* were found in the saliva of field-collected *Culex pipiens* mosquitoes. Next, phylogenetic analysis was performed to study the relationship between the sequence of the bacterial species detected in the mosquito saliva from this study and other species previously described in mosquitoes (Fig 2E). In 98% of the trees, *Serratia* sp. sequences of this study clustered with *Serratia* sp. (FJ372764) previously found in mosquitoes. Additionally, the clade containing *Enterobacter* spp. and *Klebsiella* spp. was related to the previously described mosquito inhabitants (OK035565, OL441036) in 87% and 89% of the trees, respectively. In 74% of the trees, the *Asaia* spp. detected in saliva were closely related to the already detected *Asaia* sp. (FJ372761). Finally, the *Chryseobacterium* sp. and *Microbacterium* sp. found in 100% of the trees related to the species previously reported in mosquitoes (KY002136 and OM033360).

3. Microbiota composition in mosquitoes in different life stages

To determine whether life stage affects the mosquito microbiota in the *Ae. aegypti* Paea lab colony, culturable bacterial and fungal genomes were determined in larvae, pupae and adults (Fig 3A – Fig S4). During the whole larval stage, the median CFU increased through the four larval instar stages form 10^4 CFU/larva (L1/L2) to 10^5 CFU/larva (L3/L4). Subsequently, a drop in bacterial load and diversity was observed during the metamorphosis of male and female pupae into newly emerged male and female mosquitoes, although non-significant (Kruskal-Wallis test; ns, $p>0.05$). Overall, female mosquitoes tended to have a higher median CFU/mosquito compared to male mosquitoes in each life stage, with a statistically significant difference in median bacterial colonies of 6.5×10^4 CFU/mosquito between 2-weeks old males and females (Kruskal-Wallis test; $p<0.01$). At the end of their life, 4-weeks old females also had a significantly higher median CFU/mosquito of 1.8×10^5 compared to 4-weeks old males (1.5×10^3 CFU/mosquito) (Kruskal-Wallis test; $p=0.04$). In contrast, no statistically significant differences in fungal genomes were observed between different life stages, although a similar trend was observed during metamorphosis (drop in fungal genome copies during metamorphosis from pupae to newly emerged adult mosquitoes, Fig S4).

The culturable bacteria identified by Sanger sequencing in *Aedes aegypti* at different life stages belonged to three phyla, including *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*. Bacteria from five different genera were identified, including *Acinetobacter, Chryseobacterium, Delftia, Enterobacter* and *Microbacterium* (Fig 3B). For the pupae, males were dominated by the *Microbacterium* genus with 73%, while females were mainly colonized with bacteria belonging to the *Chryseobacterium* genus with 36%. After emergence of pupae into adult mosquitoes, a diminishment of the *Microbacterium* genera was found in both males and females, which became completely absent in 2-weeks old mosquitoes. For newly emerged males and 2-weeks old females, the dominant genera were *Chryseobacterium*, for 75% and 69% respectively, while in contrast, for newly emerged females and 2-weeks old males, the highest percentage of bacterial colonies was identified as *Enterobacter*, with 100% and 72% respectively. These results suggest that the bacterial composition reshaped during different life stages for both male and female mosquitoes.

Correlation analysis were performed for male and female mosquitoes to investigate the relationship between bacterial and fungal loads for the adult life stages. A significant negative correlation was observed between bacterial and fungal load in males (Pearson Correlation;

R2=0.3281; p<0.05) (Fig 3C). This was in contrast to the significant positive correlation determined between bacterial and fungal load in females (Pearson Correlation; R2=0.1395; p<0.05) (Fig 3D), suggesting that an increased bacterial load was related to an increased fungal load, and vice versa.

4. Effect of blood meal on the microbiota in mosquito saliva

To assess whether blood feeding could influence the salivary microbiota, culturable fungi and bacteria were determined in the saliva of *Aedes aegypti* following a blood meal. No fungal or bacterial colonies were observed after one week of culture of the blood itself, indicating there was no input fungi or bacteria from the bloodmeal. No significant differences were observed in fungal loads between the saliva of non-fed and fed mosquitoes or between the saliva of fed mosquitoes at one week and two weeks post-feeding (Fig 3E). On the other hand, bacterial loads were significantly higher at one-week post-bloodmeal (Mann-Whitney test p=0.002) compared to two weeks post-feeding, which was not observed between the unfed groups (Fig 3F). This suggests that the bloodmeal might affect the bacterial load but not the fungal load in the saliva of the mosquito, and that this effect could be time-dependent.

5. Antifungal and antibiotic treatment of mosquitoes reduced the microbiota in saliva

Female *Aedes aegypti* mosquitoes of 5-7 days old were provided with 10% sterile sugar solution supplemented with 25 µg/mL Fungin for 10 days (Fig 4A). Antifungal treatment of the adult mosquito resulted in the complete absence of fungi in the saliva, whereas bacteria were not affected by the treatment. In addition, females were provided with 10% sterile sugar solution supplemented with 20 units/mL of penicillin and 20 µg/mL streptomycin for 6 days, as described previously[21]. Oral antibiotic treatment resulted in a significant reduction of bacterial load in the saliva (Mann-Whitney test, p=0.028), but not in a complete inhibition (Fig 4B). These data suggest that the culturable bacteria and fungi detected in mosquito saliva have their origin in the mosquito body.

Bacterial colonies of the saliva that resisted the antibiotic treatment were found to be susceptible to gentamycin (a drug effective against both gram-positive and gram-negative bacteria) (data not shown). Therefore, gentamycin was added to the antibiotic cocktail and different doses of penicillin, streptomycin and gentamycin were evaluated to optimize the treatment. Compared to untreated mosquitoes which solely received a 10% sterile sugar solution, significant lower CFU was observed in the bodies of mosquitoes receiving antibiotic doses of 100 units/mL of penicillin and 100 µg/mL streptomycin/gentamycin or higher (Fig 4C).

Mosquito mortality was not significantly affected when compared with untreated control groups (Fig 4D). Therefore, a dose of 100 units/mL of penicillin and 100 µg/mL streptomycin/gentamycin was selected for further studies. Interestingly, the bacterial species which were found to be naturally resistant to the antibiotic cocktail were *C. cucumeris, M. maritypicum*, *M. oxydans* and *S. maltophilia*, representing 17%, 69%, 12% and 2.7% of all colonies, respectively (Fig 4E). 81% of these bacteria were gram-positive bacteria and only 19% were gram-negative bacteria.

6. Effect of saliva microbiota on the replication of Semliki Forest virus *in vitro*

To investigate the potential effects of fungi and bacteria in mosquito saliva on arbovirus replication *in vitro*, saliva was collected from non-treated female *Ae. aegypti* (NT), antifungal-treated females (AF), or antibiotic-treated females (AB) (Fig 5A). Additionally, filtered saliva, from which fungi and bacteria were removed physically using a 0.2 µm filter, was tested. Interestingly, the direct inoculation of human skin fibroblasts with SFV and AB saliva or AF saliva significantly reduced SFV replication compared to the NT saliva (Kruskal-Wallis test; $p<0.01$) (Fig 5B). This inhibition was not observed when skin fibroblasts were incubated with filtered saliva (Fig 5B). Similarly, pre-incubation of saliva and SFV for 1h prior to inoculation on human skin fibroblasts, significantly lowered SFV replication for AB saliva or AF saliva compared to NT saliva (Kruskal-Wallis test; $p<0.01$). Again, there was no difference between NT saliva and filtered saliva (Fig 5C). Finally, saliva was pre-incubated with skin fibroblast cells for 1h, after which the cells were infected with SFV. No significant difference was observed between the NT saliva, AF saliva or AB saliva conditions; however, SFV replication was significantly reduced when cells were pre-incubated with filtered saliva (Kruskal-Wallis test; $p<0.01$) compared with NT saliva (Fig 5D).

Discussion

Mosquito saliva has recently been shown to play an important role in arbovirus transmission[26]. The saliva is a complex mixture of salivary and microbial components, of which the latter has been rarely studied so far. Herein, we aimed to explore the presence of microbiota in mosquito saliva and to assess their effect on virus infection. For the first time, the fungus *Penicillium crustosum* was isolated and identified from the saliva of both *Aedes aegypti* lab strain and *Culex pipiens* field-collected mosquitoes (Fig 1). *Penicillium crustosum* is a common blue mold, morphologically characterized by typically gray-blue-green conidia. This species has been frequently detected in fruits (e.g. peaches, apples, pears), wheat, peanuts and other nuts, propolis[37] and food waste[38]. It is a food–borne ubiquitous fungal species which produces mainly roquefortine C and penitrems[39]. Interestingly, penitrems have demonstrated insecticidal activity against the large milkweed bug (*Oncopeltus fasciatus*) and the Mediterranean fruit fly (*Ceratitis capitate*)[40]. It is unknown whether similar insecticidal effects are exerted on mosquitoes. Symbiotic relationships between fungi and their hosts are well established in three models, including *mutualism,* (e.g. fungus *Mortierella elongata* and algae *Nannochloropsis oceanica*)[41], *commensalism* (e.g. *Saccharomyces cerevisiae* or *Candida albicans* in the human digestive tract)[42], or *parasitism* (e.g. *Beauveria bassiana* in arthropods)[43]. In Iran, *Penicillium* spp. were found to be the most abundant entomopathogenic fungi isolated and identified from the natural larval habitats of *Anopheles* mosquitoes[36]. It would therefore be interesting to investigate whether *Penicillium crustosum* and its mycotoxins have an effect on the life history traits and vector competence of mosquitoes.

In contrast, most salivary bacteria identified in this study were previously reported in either the midgut or other mosquito organs (Fig 2). Identified gram-negative genera were *Asaia*, *Enterobacter*, *Klebsiella*, *Chryseobacterium* and *Serratia*. *Asaia*, belonging to the *Acetobacteraceae* family, has been previously shown to colonize the salivary glands and midgut of mosquito species such as *Aedes aegypti, Aedes albopictus, Anopheles stephensi* and *Anopheles gambiae*[15,44]. Interestingly, *Asaia* has been considered as a potential vector of anti-plasmodial factors through a paratransgenic approach[45]. The bacterial genera *Enterobacter* and *Klebsiella*, belonging to the family *Enterobacteriaceae,* are both frequently found in the gut of adult *Aedes* spp. and *Culex pipiens*[16,18]. Furthermore, *Serratia* of the *Yersiniaceae* family was identified in both the salivary glands and the midgut of *Aedes* and *Anopheles* mosquitoes[15],

and in the midgut of *Culex* mosquitoes[16]. Interestingly, *Serratia marcescens* was found to have the ability to render mosquitoes more permissive to arbovirus infection[21]. *Chryseobacterium,* a member of the *Weeksellaceae* family, was detected in all life stages of *Aedes aegypti*, which is in line with our observations that this species was found in pupae and saliva samples of adult females. *Microbacterium,* belonging to the family *Microbacteriaceae,* was reported to be abundantly present in the larval stage of *Aedes aegypti*[46]. In this study, these gram-positive bacteria (e.g. *M. maritypicum*, *M. oxydans*) were mainly identified in the pupal stage, but also in *Aedes aegypti* adults, suggesting that some core bacteria such as *Chryseobacterium* and *Microbacterium* might be conserved in *Aedes aegypti* colonies although maintained in different labs[47].

It has been shown previously that the diversity of bacterial communities varies during mosquito development, more significantly during the metamorphosis from pupae into adults. Moreover, fungal colonization may compete with other microbiota at different developmental stages of the mosquito. For example, it was found earlier that the larval gut fungus *Zancudomyces culisetae* affected the structure of bacterial communities and relative abundances of certain genera in both larval and adult stages of *Aedes aegypti* mosquitoes[48]. Furthermore, the presence of gut fungi was shown to collaborate with other gut microbiota to increase the mortality of mosquitoes[49]. In this study, the co-existence of fungi and bacteria in female and male adults correlated in two opposite directions. As shown before, the core microbiota in females and males may be different because of different physical needs. Females need the assistance of several bacterial species to digest the bloodmeal and guarantee the production of eggs of good quality[50] which is not necessary for males. Therefore, the fungi - bacteria interactions within males and females could be different. In addition, it was reported previously that blood feeding can plan an important role in shaping the mosquito gut microbial composition and diversity, since it may favor proliferation of particular bacterial taxa over others. One week after blood feeding, the bacterial diversity in the midgut and body was shown to return to the same state as before the bloodmeal[51], which is in line with the reduction observed in saliva in this study. However, there were no significant changes found for fungi. We hypothesize that the majority of energy offered by the bloodmeal is mainly used for the maturation of eggs and partially for the proliferation of bacteria, while the proliferation cycle of fungi takes much longer and it could not similarly benefit from the bloodmeal.

The presence of fungi in saliva was eliminated by treating adult mosquitoes with the antifungal drug Fungin via the sugar meal. In contrast, an antibiotic cocktail containing 20 units/mL of penicillin and 20 µg/mL streptomycin[21] was not able to totally deplete the saliva bacteria from the *Aedes aegypti* lab strain. Therefore, the antibiotic cocktail was adapted to contain 100 units/mL of penicillin and 100 µg/mL of streptomycin / gentamycin for further studies. Naturally resistant bacterial species were observed and these results were in line with the previous study demonstrating that antibiotic-resistant bacteria can exist in mosquitoes[47].

As the knowledge on microbiota-arbovirus interactions increases, there is a growing interest to exploit the microbiota for vector control and arbovirus outbreak containment. Microbial taxa that negatively impact mosquito physiology or display an antiviral effect could be employed in order to reduce arbovirus transmission in the field[52]. Therefore, salivary microbiota and their potential contributors to the crucial steps in mosquito-borne virus infections are worth to be investigated in more detail. During a mosquito bite, the salivary microbiota are injected simultaneously into the skin together with the arbovirus. Therefore, we investigated whether salivary microbes could interact with viral particles in the saliva during the biting or when delivered in the host environment. In human skin fibroblasts, mosquito saliva of *Aedes aegypti* did not significantly decrease infectious virus titers of SFV compared to the virus control without saliva (Fig 5). This is in contrast to earlier work showing that mosquito saliva slightly reduced SFV replication in skin fibroblasts[26]. The authors hypothesized that the presence of saliva microbiota activated immune responses in skin cells, resulting in increased viral resistance. On the other hand, another study demonstrated that salivary gland extracts enhanced dengue virus replication in human keratinocytes[53]. The difference between this result and the results of our study might be explained by the use of salivary gland extracts instead of saliva itself, and/or the use of another arbovirus and cell type. Interestingly, the removal of fungi or bacteria from saliva by chemical treatment resulted in a significant reduction of virus replication compared to saliva of non-treated mosquitoes, when virus was preincubated with saliva or when virus and saliva were added to cells simultaneously (Fig 5B-C). When cells were pre-treated with saliva, no significant reduction of virus infection was observed for saliva without bacteria or fungi when compared to normal saliva (Fig 5D). In contrast, a previous study reported that SFV replication was similar to that of the virus control, when skin fibroblasts were pre-incubated with saliva without bacteria, whereas saliva with bacteria slightly reduced SFV replication[26]. It is currently not clear why different results were

obtained. Firstly, it could due to the different antibiotic cocktails used to clear bacteria from the mosquitoes. Furthermore, the core microbiota present in saliva may differ between different strains used (*Aedes aegypti* Liverpool strain vs Pacific Paea strain). Importantly, it has been equally shown that the virus-enhanced infection phenotype by mosquito saliva observed in mouse models could not be recapitulated *in vitro* or *ex vivo* [26]. Therefore, it will be important to perform *in vivo* studies to elucidate the potential effect of salivary bacteria and fungi on virus infection and pathogenesis.

In conclusion, mosquito salivary bacteria and fungi were observed by culturing saliva of lab colony and field-collected mosquitoes of two different species. Oral treatment of adult mosquitoes with antibiotics or an antifungal drug significantly reduced microbiota in the saliva, suggesting that the salivary microbiota have an origin in the mosquito body. Furthermore, the depletion of fungi and bacteria from saliva may alter resistance to viral infection *in vitro*. However, the effects of salivary microbiota warrant further investigation *in vivo*.

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Competing interests

The authors declare no competing interests.

Figures

Figure 1. Presence of fungi in the saliva of *Aedes aegypti* and *Culex pipiens* mosquitoes. (A) Mosquito saliva of *Aedes aegypti* (lab strain) was harvested by forced salivation and cultured on LB agar plates for 120 h at 28°C. Fungal colonies are indicated by black arrows. (B) Mosquito saliva from single *Aedes aegypti* (lab strain) and *Culex pipiens* (field) mosquitoes was cultured on LB agar plates. The number of CFU was quantified after culturing for 120 h at 28°C. (C) Genome copies of fungi in different organs of *Aedes aegypti* were quantified by ITS qPCR. Negative control (NC) consisted of the blank solution used as environment control for forced salivation. Each point represents an independent pool containing samples harvested from 8-12 mosquitoes. lines show the median value of the samples tested. LOQ presents the limit of quantification of the qPCR. SG= salivary glands. (D) ML tree was constructed using MEGA-X with the substitution model Kamura 2-parameter model (K2) and Gamma distributed (G) (500 bootstrap replicates), by using the CRU sequence of the fungal colony identified in mosquito saliva (OL436123) and other fungal species from GenBank which were previously detected in mosquito body and organs.

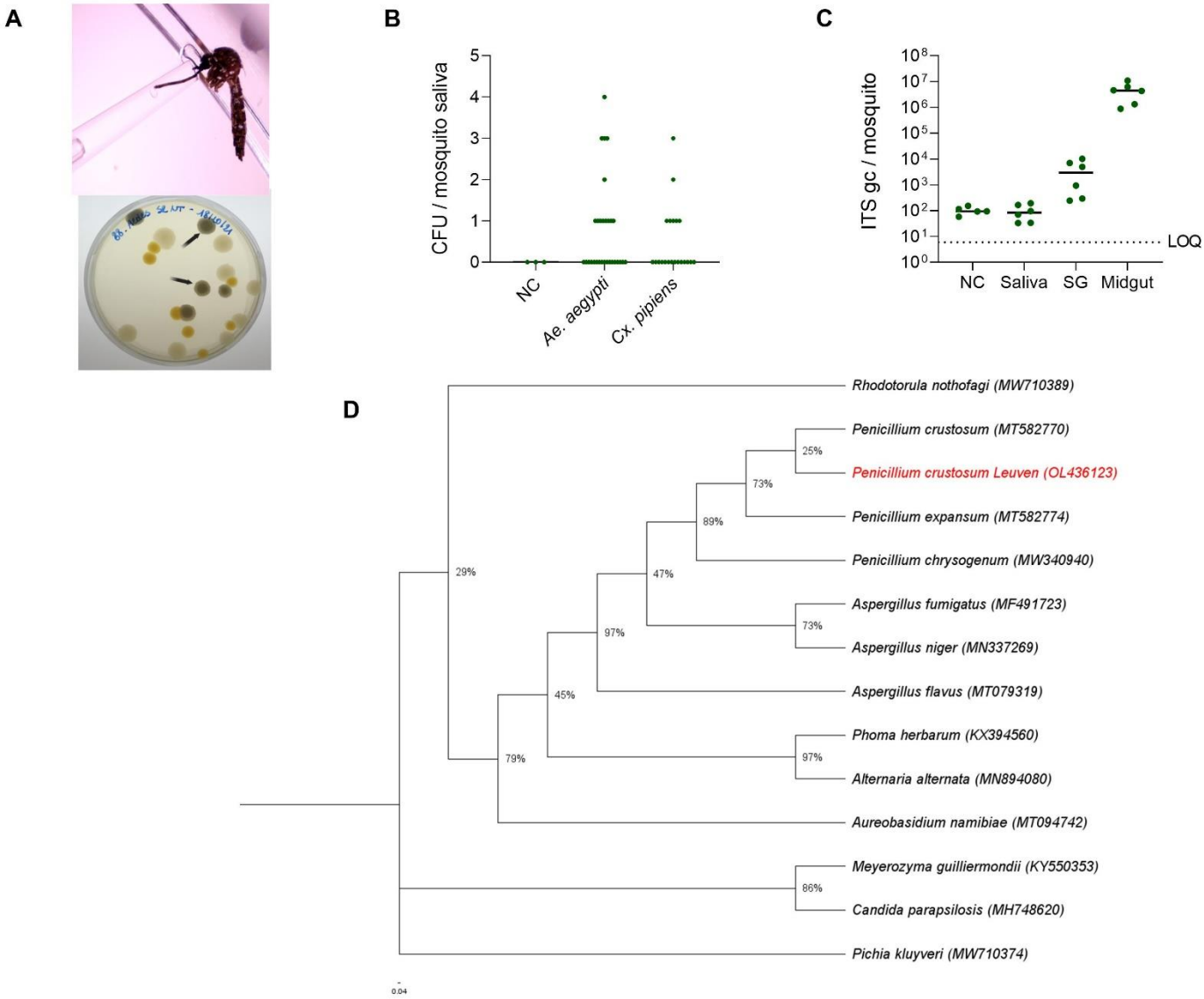

A
B
C
D
CFU / mosquito saliva
NC
Ae. aegypti
Cx. pipiens
ITS gc / mosquito
LOQ
NC
Saliva
SG
Midgut
Rhodotorula nothofagi (MW710389)
Penicillium crustosum (MT582770)
Penicillium crustosum Leuven (OL436123)
Penicillium expansum (MT582774)
Penicillium chrysogenum (MW340940)
Aspergillus fumigatus (MF491723)
Aspergillus niger (MN337269)
Aspergillus flavus (MT079319)
Phoma herbarum (KX394560)
Alternaria alternata (MN894080)
Aureobasidium namibiae (MT094742)
Meyerozyma guilliermondii (KY550353)
Candida parapsilosis (MH748620)
Pichia kluyveri (MW710374)
25%
73%
89%
29%
47%
73%
97%
45%
97%
79%
86%
0.04

Figure 2. Presence of bacteria in the saliva of *Aedes aegypti* and *Culex pipiens* mosquitoes. (A) Mosquito saliva of *Aedes aegypti* (lab strain) was harvested by forced salivation and cultured on LB agar plates. Bacterial colonies are indicated by black arrows. (B) Mosquito saliva pools from both *Aedes aegypti* (lab strain) and *Culex pipiens* (field collection) were cultured on LB agar plates at 28°C. The CFU/saliva pool was quantified after culturing for 48 h. Each dot represents one pool of 8-12 saliva samples. (C) Genome copies of the 16S bacterial gene in different organs of *Aedes aegypti* were quantified by 16S-qPCR. NC as environmental control consisted of the solution used for forced salivation. Each point represents an independent pool containing samples harvested from 8-12 mosquitoes. The dotted line represents the LOQ (limit of quantification) of the qPCR. SG= salivary glands. (D) ML tree was constructed using MEGA-X with the substitution model being Kamura 2-parameter model (K2) and Gamma distributed (G) (500 bootstrap replicates), by using the Sanger sequences of bacterial colonies identified in mosquito saliva and other species from GenBank which were previously detected in mosquitoes.

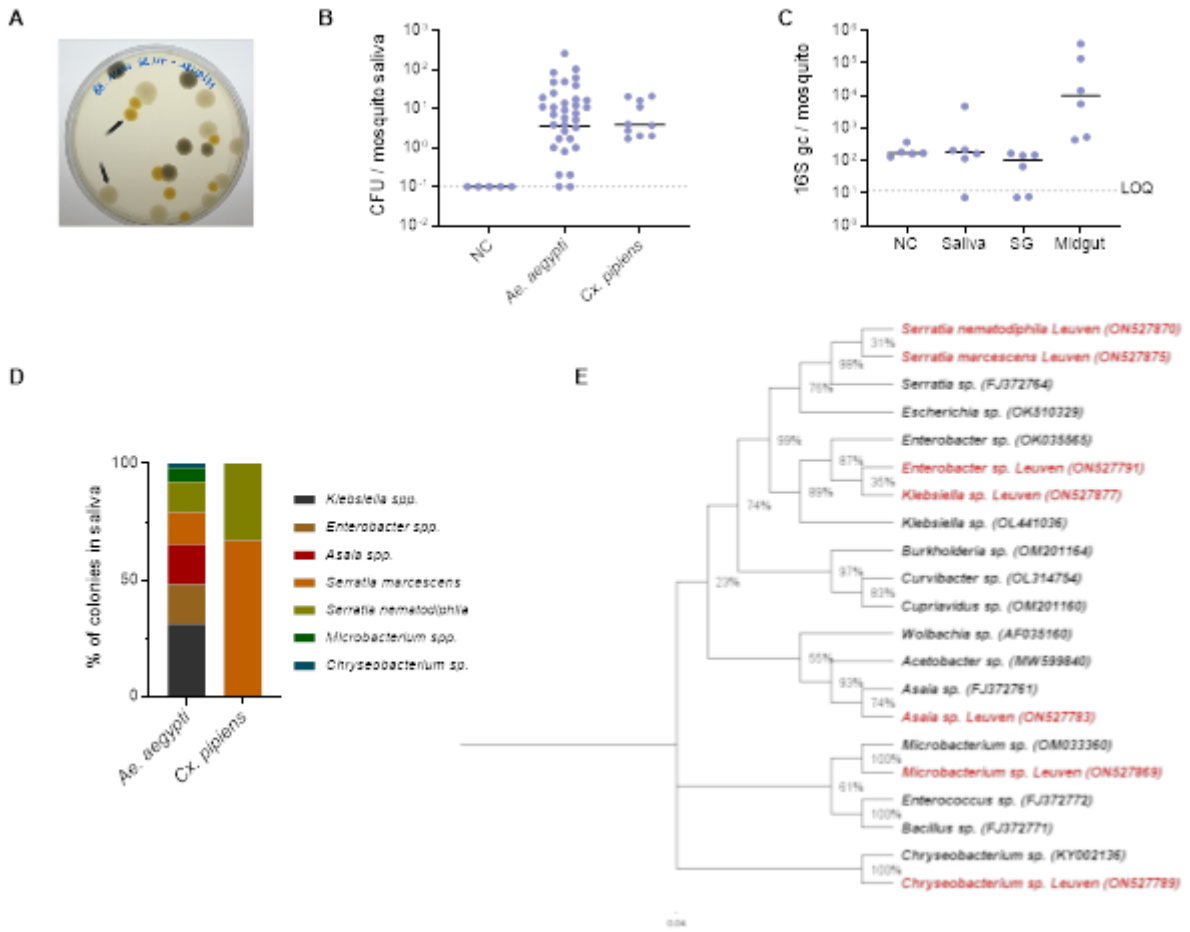


Figure 3. The effects of age, sex and blood meal on the microbiota in mosquitoes. (A) CFU for bacteria of individual *Aedes aegypti* mosquitoes at different life stages. The lines present the median values. D0= newly emerged adults. (B) Based on the 16S Sanger sequencing, number of colonies (%) per genus was calculated among pupae males; newly emerged males; 2-week-old males; pupae females; newly emerged females; and 2-week-old females. (C-D) 16S and ITS3-4 RT-qPCR was performed on extracted DNA of individual adult *Aedes aegypti* mosquitoes: (C) Correlation between bacterial and fungal load in males. (D) Correlation between bacterial and fungal load in females. (E-F) Bacterial and fungal CFU of saliva from one-week-old female *Aedes aegypti* adults fed on rabbit blood. Saliva was harvested after one week and two weeks post-blood meal. The CFU/mosquito saliva was calculated for each condition after culturing on LB agar plates at 28°C for 120 h. (E) fungal load and (F) bacteria load were compared between fed and unfed groups. Significant differences were demonstrated by the Mann-Whitney test (two groups) or Kruskal-Wallis test (⩾ two groups) (*, p<0.05; **, p<0.01).

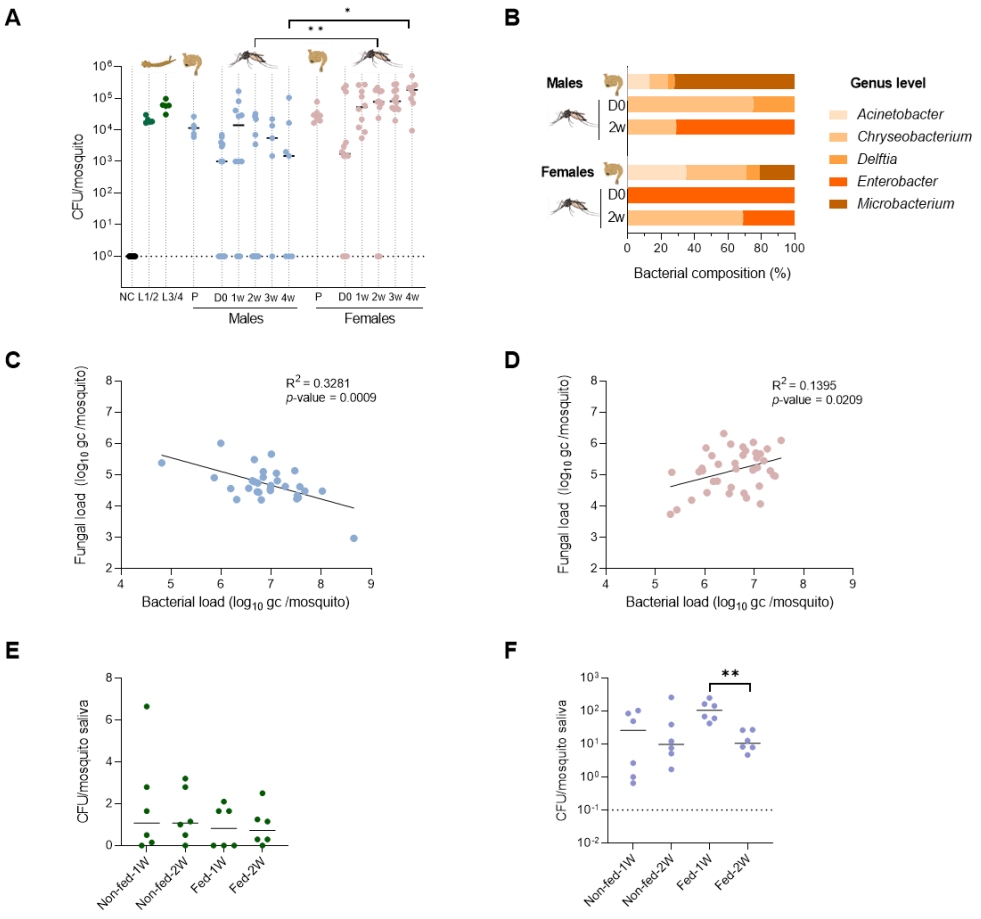


Figure 4. Antifungal and antibiotic treatment reduced the fungal and bacterial load in mosquito saliva. (A) Fungal load in saliva from antifungal-treated mosquitoes (AF; 25 µg/mL of Fungin in 10% sucrose for 10 days) and non-treated saliva (NT), quantified by determining CFU/mosquito. (B) Bacterial load in saliva from antibiotics-treated mosquitoes (AB; 20 units/mL of penicillin and 20 µg/mL streptomycin in 10% sucrose for 6 days) and non-treated saliva (NT), quantified by determining CFU/mosquito. Each dot represents an independent pool of saliva from 8-12 mosquitoes. (C) Female *Aedes aegypti* adults (5-7 days old) were divided between three treatment groups: untreated control group (NT: only 10% sucrose), antifungal-treated (AF: 25 µg/mL of Fungin in 10% sucrose for 10 days) and antibiotic-treated (AB: serial dilution up to 200 units/mL of penicillin and 200 µg/mL streptomycin + gentamycin in 10% sucrose for 6 days). Bacterial loads in the individual body were quantified by determining CFU/mosquito. The dotted line represents the limit of detection (LOQ). (D) Survival rate of mosquitoes recorded for each treatment group. (F) Identification of naturally resistant bacterial species after high-dose antibiotic treatment (⩾100 units/mL of penicillin and ⩾100 µg/mL streptomycin/ gentamycin) by Sanger sequencing. Saliva was cultured on LB agar plates at 28°C for 120 h. The CFU/mosquito was calculated for each condition. Significant differences were demonstrated by the Mann-Whitney test (two groups) or Kruskal-Wallis test (⩾ two groups) (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

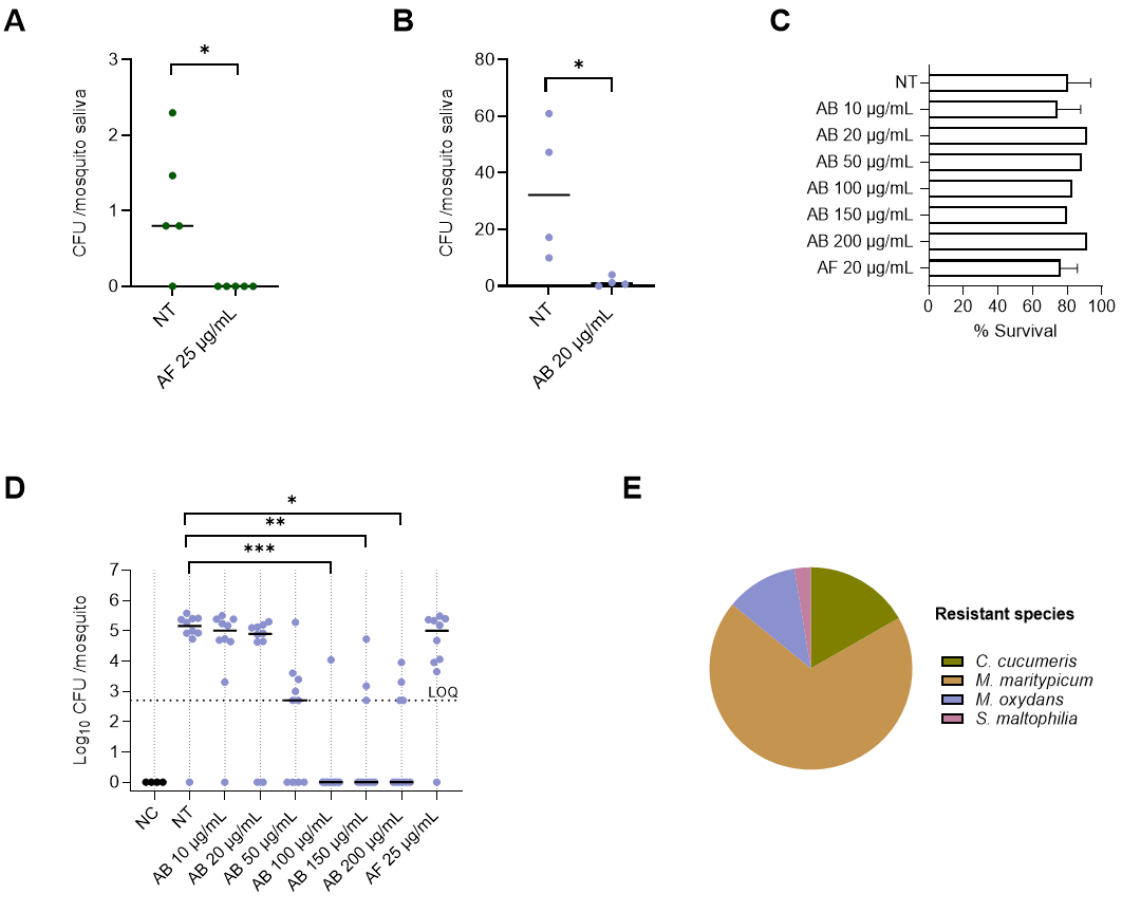
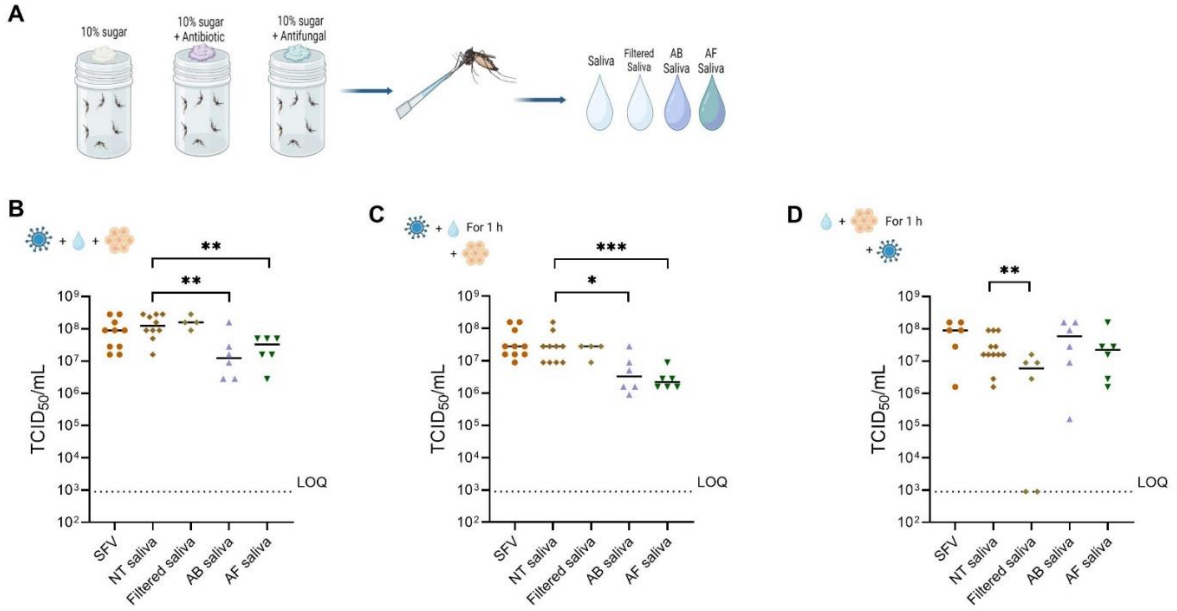

A
CFU /mosquito saliva
NT
AF 25 µg/mL
B
CFU /mosquito saliva
NT
AB 20 µg/mL
C
NT
AB 10 µg/mL
AB 20 µg/mL
AB 50 µg/mL
AB 100 µg/mL
AB 150 µg/mL
AB 200 µg/mL
AF 20 µg/mL
% Survival
D
Log10 CFU /mosquito
LOQ
NC
NT
AB 10 µg/mL
AB 20 µg/mL
AB 50 µg/mL
AB 100 µg/mL
AB 150 µg/mL
AB 200 µg/mL
AF 25 µg/mL
E
Resistant species
C. cucumeris
M. maritypicum
M. oxydans
S. maltophilia

Figure 5. Effect of mosquito saliva on Semliki Forest virus infectivity in skin fibroblast cells. (A) Female *Aedes aegypti* adults (5-7 days) were provided with 10% sterile sugar solution (NT), 10% sugar containing 25 µg/mL Fungin (AF), or 10% sugar containing 100 units/mL of penicillin and 100 µg/mL streptomycin/gentamycin (AB). Filtered saliva was obtained from the untreated saliva using a 0.2 µm filter. (B-D) SFV infectious titers following exposure to mosquito saliva. (B) Human skin fibroblast cells were directly inoculated with saliva (saliva from 5 females/well) and Semliki Forest virus (MOI:0.001), (C) Semliki Forest virus (MOI:0.001) was pre-incubated with saliva for 1 h before being added to human skin fibroblasts, (D) Skin fibroblast cells were preincubated with saliva 1 h before infection with Semliki Forest virus (MOI: 0.001). The supernatant was harvested at 24 h post-infection and the viral yield was determined by end-point titrations on Vero cells. $TCID_{50}$/ml was calculated using the Reed and Muench method. Kruskal-Wallis test was used for statistical analysis (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). The dotted line indicates the limit of quantification (LOQ).



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