Native *Wolbachia* from *Aedes albopictus* Blocks Chikungunya Virus Infection *In Cellulo*

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**Abstract**

*Wolbachia*, a widespread endosymbiont of terrestrial arthropods, can protect its host against viral and parasitic infections, a phenotype called "pathogen blocking". However, in some cases *Wolbachia* may have no effect or even enhance pathogen infection, depending on the host-*Wolbachia*-pathogen combination. The tiger mosquito *Aedes albopictus* is naturally infected by two strains of *Wolbachia*, *wAlbA* and *wAlbB*, and is a competent vector for different arboviruses such as dengue virus (DENV) and chikungunya virus (CHIKV). Interestingly, it was shown in some cases that *Ae.* *albopictus* native *Wolbachia* strains are able to inhibit DENV transmission by limiting viral replication in salivary glands, but no such impact was measured on CHIKV replication *in vivo*. To better understand the *Wolbachia*/CHIKV/*Ae. albopictus* interaction, we generated a cellular model using *Ae.* *albopictus* derived C6/36 cells that we infected with the *wAlbB* strain. Our results indicate that CHIKV infection is negatively impacted at both RNA replication and virus assembly/secretion steps in presence of *wAlbB*. Using FISH, we observed CHIKV and *wAlbB* in the same mosquito cells, indicating that the virus is still able to enter the cell in the presence of the bacterium. Further work is needed to decipher molecular pathways involved in *Wolbachia*-CHIKV interaction at the cellular level, but this cellular model can be a useful tool to study the mechanism behind virus blocking phenotype induced by *Wolbachia*. More broadly, this put into question the ecological role of *Wolbachia* symbiont in *Ae. albopictus*, but also the ability of the CHIKV to counteract *Wolbachia*’s antiviral potential *in vivo*.

**Introduction**

Human infectious diseases caused by vector-borne pathogens have an increasing incidence worldwide, accounting for 17% of the estimated burden of infectious diseases as referred by World’s Health Organization [1]. Notably, arthropod-borne viruses (arboviruses) are emerging or re-emerging viruses transmitted to vertebrate hosts by the bite of infected arthropod vectors, mainly mosquitoes. Among them, Chikungunya is a mosquito-borne viral infection caused by
an alphavirus from the Togaviridae family. Chikungunya virus (CHIKV) is transmitted to humans by Aedes (Stegomyia) spp mosquitoes, primarily Aedes aegypti. Since 2004, CHIKV started a global spread with severe outbreaks in the Indian Ocean region, the Indian subcontinent and Central Africa, all associated with a single amino-acid change in the virus E1 glycoprotein that allowed an enhanced transmission by a secondary mosquito species, Aedes albopictus [2–4]. Autochthonous transmissions in Europe were also reported from Italy, with 217 confirmed cases in 2007 [5] and from France with two confirmed cases in 2010 [6]. Consecutively to a major chikungunya outbreak started in the French Antilles in 2013, autochthonous cases were reported in the United States [7] and more recently in France [8], bringing the threat of multiple outbreaks caused by virus-carrying travellers; in both temperate areas, Ae. albopictus was the vector responsible for CHIKV transmission.

The species Ae. albopictus, also known as tiger mosquito, is native from Southern and Eastern Asia but recently spread worldwide [9]. The rapid extension of Ae. albopictus combined with its ecological plasticity and vector competence for diverse arboviruses make the tiger mosquito a significant threat for public health [10]. In absence of effective vaccines or prophylaxis against most of arboviruses included CHIKV, current efforts are mainly based on controlling vector populations with insecticides. However, the development of mosquito resistance, as well as environmental contamination and side effects on non-target organisms has called chemical-based control methods into question [11]. Consequently, alternative and innovative vector control strategies emerged, and one of the most promising is based on the use of symbiotic bacteria [12]. In this framework, the endosymbiont Wolbachia has been the most studied candidate including arboviruses and parasites transmission control [13–15].

Wolbachia is an obligate intracellular bacterium that infects around 40% of arthropods [16], and manipulates their reproduction to facilitate its own spread among populations [17]. When the wMel strain of Wolbachia, originated from Drosophila, was transfected into Ae. aegypti embryos, mosquitoes presented limited vector competence for a large panel of pathogens including dengue virus (DENV) [18,19], CHIKV [19], yellow fever (YFV) [20], West-Nile virus (WNV) [21] and Plasmodium parasite [14,19]. However, it appears that Wolbachia-transinfected mosquitoes are markedly associated with a viral inhibition phenotype compared to naturally infected populations, which most of time exhibit no inhibition or even an enhancing of the infection [22]. In the field, Ae. aegypti lacks this association with Wolbachia while Ae. albopictus mosquitoes naturally carry two strains, namely wAlbA and wAlbB [23,24]. The native Wolbachia from Ae. albopictus was associated with a decrease of DENV transmission in mosquitoes from La Réunion island [25]. However, this phenotype was shown to be dependent on the mosquito population considered as no inhibition was observed in population from Houston, Texas [18]. Intriguingly, no significant impact of Wolbachia was observed on CHIKV transmission in Ae. albopictus population from La Réunion [26]. This suggests that the Wolbachia inhibition phenotype also depends on the viral strain considered. Together, these observations clearly indicate that the tripartite interaction between Wolbachia, arboviruses and their mosquito host is complex and varies according to the nature of the interacting partners.

The molecular and cellular mechanisms of Wolbachia-mediated inhibition of arboviruses are poorly known, but current hypotheses suggest a competition for host cell resources, supported by the bacterial density-dependent interference and the intra-host competition for amino acids and cholesterol [27,28]. Insect immune pathways activated upon Wolbachia infection have been also suggested to mediate the blocking phenotype, like autophagy [29], oxidative stress [30] or miRNA pathway [31]. It appears that Wolbachia-mediated activation of the Toll and Imd immune pathways was unlikely to trigger antiviral interference, as suggested by a recent study in Drosophila [32]. In addition, as being an obligate intracellular bacterium, studies on Wolbachia are difficult using standard techniques. Interestingly, Wolbachia-infected cell
lines were used as a tool to study the mechanisms involved in *Wolbachia*-pathogen interaction [29,30,33–35]. To facilitate the understanding of the *Wolbachia/CHIKV/Ae. albopictus* interaction, we built a cellular model by culturing the *wAlbB* strain *in vitro* into the *Ae. albopictus* CHIKV-permissive cell line C6/36. Using this simplified *in vitro* model, we measured the viral dynamic in the presence or absence of *Wolbachia*, and tried to decipher at which step of the viral cycle *Wolbachia* interferes with CHIKV infection. More broadly, this work provides a suitable tool to study *Wolbachia*-arbovirus interaction at the cellular level.

**Material and Methods**

**Establishment of *Wolbachia*-infected mosquito cell line**

The C6/36 cells, derived from *Ae. albopictus* larvae and originally non-infected by *Wolbachia*, were used for culturing wAlbB strain. This bacterial strain originated from naturally infected Aa23 cells isolated from *Ae. albopictus* eggs [36]. Both cell types were cultured at 28°C in growth medium consisting of equal volumes of Mitsubishi/Maramorosh (Bioconcept, Switzerland) and Schneider’s insect medium (Sigma, France) supplemented with 10% (v/v) of heat-inactivated foetal bovine serum (PAA, USA) and penicillin/streptomycin (50 U/50 μg/mL; Gibco, Invitrogen, France). Briefly, three 25 cm² flasks of confluent Aa23 cells were scraped, pelleted for 10 min at 300×g and crushed by vortexing 10 min with 5-mm diameter sterile borosilicate beads (Biospec, OK, USA). Cell lysates were centrifuged for 5 min at 300×g, and supernatants were filtered through a 5-μM syringe filter (Millipore) to eliminate cellular debris. Fresh filtrate (500 μL) containing bacteria was inoculated onto 80% confluent monolayer of C6/36 cells, in shell vial tube (Sterilin, UK). After centrifugation 5 min at 2000×g, cells were incubated overnight at 28°C then the overspill bearing cells was transferred into a 25 cm² flask with fresh culture medium and incubation period extended to reach 80% confluence. After this first round of infection, cells were harvested, resuspended in 500 μL of fresh medium and used for a second infection procedure. The *Wolbachia* infection in cells was characterized using electron microscopy, Fluorescent *In Situ* Hybridization (FISH) and quantitative PCR (qPCR). For each assay, we used as control tetracycline-treated cells (TET) to remove bacteria without modifying the host cell genetic background. This was achieved by adding 10 μg/mL of tetracycline hydrochloride (Sigma, France) in culture media of *Wolbachia*-infected (wAlbB) cells for 5 passages, and then cells were maintained in culture without tetracycline until use. The original C6/36 uninfected (CTRL), TET and wAlbB infected cells were continuously passaged in 25 cm² flasks by scraping and seeding a new flask with 1:5 of the cell suspension in 5 mL of fresh medium, every 4 days.

**Electron microscopy**

The presence of *Wolbachia* in C6/36 cells was observed using electron microscopy at the Centre Technologique des Microstructures, University Lyon I (http://microscopies.univ-lyon1.fr/index.htm). Briefly, cells were washed in PBS twice and fixed in a 2% glutaraldehyde solution containing cacodylate buffer at pH 6.5, then postfixed in 1% osmium tetroxide in cacodylate buffer. Samples were then dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections of 60 nm were performed using an UC7 ultramicrotome (Leica). After a contrast with uranyl acetate and lead citrate, the sections were observed using a Philips CM 120 Transmission Electron Microscope.

**Virus**

The CHIKV 06.21 strain derived from newborn serum sample with neonatal encephalopathy, was collected in La Reunion Island in 2005 [37]. This isolate was highly passaged in C6/36.
Viral stocks were produced on C6/36 cells in 25-cm² flasks, at Multiplicity Of Infection (MOI) of 0.01. After 3 days at 28°C, supernatants from infected cells were recovered and virus titration was performed using plaque assay on Vero E6 (green monkey kidney) cells [38]. To measure the impact of tetracycline treatment on viral dynamics, CHIKV RNA titer was compared between CTRL and TET cells using quantitative RT-PCR (RT-qPCR), at two different MOI of 0.1 and 3. To that end, cells were transferred into 12-well plates at 1×10⁶ cells per well and allowed to attach for 24 h, at 28°C. Infection with CHIKV 06.21 was performed in 2% FBS medium, using virus-free medium as control. After 1 h, 1.5 mL of fresh media with 10% FBS was added. Cells and supernatants were harvested at 2, 4, 6, 8, 10, 24, 48, 72, 96 and 168 hours post-infection. Residual cells were removed from supernatant by centrifugation for 3 min at full-speed and samples were stored at -80°C until titration. Adherent cells were rinsed twice in PBS and scrapped, pelleted by centrifugation and kept at -80°C prior to RNA isolation. Experiment was conducted with two independent replicates. To assess the role of Wolbachia during CHIKV infection, we compared virus titer between TET and wAlbB bearing cells. The day prior infection, cells from three to six independent flasks were transferred in 12-well plates at 1×10⁶ cells per well while another fraction was inoculated in shell vial tubes at 5×10⁵ cells per tube for FISH staining. CHIKV 06.21 infection was performed as mentioned above, at MOI of 0.1 and 3, with cells and supernatant harvested at 1, 3, 5 and 7 days post-infection. Samples were stored at -80°C until use.

**DNA and RNA isolation**

Genomic DNA isolation was performed using DNaseasy blood and tissues kit (Qiagen, France) following manufacturer’s recommendations. After lysis in 180 μL of ATL buffer, samples were incubated for 2 h at 37°C with lysozyme (Euromedex, France) at a final concentration of 2 mg/mL. Residual co-extracted RNA was eliminated by adding 100 mg/mL of RNase A, for 2 min at room temperature. The isolated DNA was eluted in 30 μL of DNase-free water. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, France) as recommended by supplier. Cell pellets were crushed in 350 μL RLT lysis buffer using RNase-free piston pellet (Kontes, USA), and RNA was eluted in 37 μL of RNase-free water. RNA solution was treated with DNase using the Ambion TURBO-DNA free kit (Ambion, USA) in 50 μL final volume following the manufacturer’s instructions. DNA and RNA were quantified using a UV-mc2 spectrophotometer and diluted to 5 ng/μL, then frozen at -20°C (DNA) or -80°C (RNA) until use.

**Quantitative Wolbachia PCR analysis**

The relative density of Wolbachia per cell was monitored by qPCR using Wolbachia Surface Protein (wsp) gene for the bacterium and actin gene for the host cell. Standard curves were drawn on 10-fold serial dilutions from 1×10⁸ to 1×10¹ copies/μL of the DNA plasmid pQuantAlb16S containing wsp and actin gene fragments [23]. Each 20 μL reaction contained 10 ng (2 μL) of template DNA, 10 μL Fast-SYBR-Green Master Mix (Roche, Suisse), 200 mM (wsp) and 300 mM (actin) of primers (Table 1). Amplification was performed on LC480 LightCycler (Roche, France) and consisted of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 65°C, and a final elongation at 72°C for 30 s. All PCR reactions were done in triplicate and DNA from C6/36_TET was used as negative control.

**CHIKV RT-qPCR analysis**

The CHIKV RNA copy number was quantified by RT-qPCR targeting the envelope E2 gene. Viral RNA copies were assessed using a standard curve of 10-fold serial dilution of a synthetic CHIKV RNA transcript [26]. One-step RT-qPCR was performed using EXPRESS One-Step
SYBR GreenER Kit (Invitrogen, France) in a volume of 20 μL containing 10 ng (2 μL) of RNA template, 10 μL EXPRESS SYBR GreenER SuperMix Universal, 200 nM of sense Chik/E2/9018/+ and anti-sense Chik/E2/9235/− primers (Table 1) and 0.5 μL EXPRESS Superscript Mix. Amplification was performed on a LC480 LightCycler (Roche, France) and consisted of 15 min at 50°C followed by 95°C for 2 min, then 40 cycles of 95°C for 15 s and 63°C for 1 min. All PCR reactions were performed in triplicate and RNA from CHIKV-uninfected C6/36 cells was used as negative control.

**Fluorescent focus assay (FFA)**

Virus infectious titer was quantified using an indirect immunofluorescent detection of infectious foci on C6/36 monolayer [39]. Cells were seeded in 96-well plates at a density of 3×10^6 cells/well and incubated for 36 h at 28°C to produce confluent monolayers. Ten-fold serial dilutions of sample supernatants were inoculated in a final volume of 50 μL/well. After 1 h incubation at 28°C to allow viral adsorption, with gently rocking every 15 min to spread viral inoculum, an overlay consisting of 5% FBS, 1.6% of carboxymethyl cellulose (CMC, VWR) was added in a final volume of 200 μL per well. Plates were incubated 3 days at 28°C then 150 μL of freshly prepared 4% formaldehyde solution in PBS was added without removing the overlay. Cell monolayers were fixed for 10 min in freshly prepared 4% formaldehyde in PBS. Hybridization was conducted overnight at 37°C in 1 mL of hybridization buffer [formamide 50%, SSC (saline-sodium citrate) 5X, 200 mg dextran sulfate per mL and 250 μg poly(A) per mL, 250 μg salmon sperm DNA per mL, 250 μg tRNA per mL, DTT 250 μg poly(A) per mL].

**Fluorescent In Situ Hybridization**

After two washes in PBS, cells were fixed on the coverslip for 10 min in freshly prepared 4% formaldehyde in PBS. Hybridization was conducted overnight at 37°C in 1 mL of hybridization buffer [formamide 50%, SSC (saline-sodium citrate) 5X, 200 mg dextran sulfate per mL and 250 μg poly(A) per mL, 250 μg salmon sperm DNA per mL, 250 μg tRNA per mL, DTT 250 μg poly(A) per mL]
(1,4-dithiothreitol) 0.1 mg/L, Denhartdt’s solution 0.5X containing 200 ng of Wolbachia probes W2 and Wol3 labelled in their 5’-end with Rhodamine Red-X and CHIKV probe labelled in 5’-end with Alexa488 fluor (Table 1). After hybridization, samples were washed twice in 1X SSC-10 mmol/L DTT and then twice in 0.5X SSC-10 mmol/L DTT at 55°C for 15 min each. Cells were then rinsed in PBS, mounted on a glass slide with 3 μL of DAPI (4’,6-diamidino-2-phenylindole, dihydrochloride) solution (1 μg/mL of dye) in glycerol/PBS (1:1). Samples were viewed under a fluorescence microscope (AXIO Imager.Z1; Zeiss, France). To estimate the proportion of cells infected by Wolbachia, five different microscope fields were analyzed with at least 50 cells per field [40].

Statistics
The continuous response variables (viral and bacterial titers) were log10-transformed. They were analysed using a multifactorial linear model, with a normal error distribution and an identity link function that included the effect of the time and MOI as ordinal variables, treatment as discrete variable and their interactions. All the statistical analysis was performed using R environment (version 3.1.0).

Results
Characterization of wAlbB infection in mosquito cells
Previous studies mentioned that the wAlbB strain could be maintained in C6/36 [41,42]. Despite this, wAlbB dynamics of infection in C6/36 remains unknown. The wAlbB cells were purified from Aa23 cells, as they were already adapted to cell line culture. The C6/36 cells tend to grow in adhesive cell clusters, forming patchy monolayers independently of Wolbachia infection (S1 Fig). Two attempts were necessary to obtain Wolbachia infected cells, designated C6/36_wAlbB, with a wsp signal in PCR persisting in cells after several passages (not shown). Electron microscopy of C6/36_wAlbB cells (P.30) revealed the presence of Wolbachia as round-shaped particles of varying size inside the cytoplasm, surrounded by a host cell membrane were the bacteria seem to divide (Fig 1). As expected, no Wolbachia was seen outside a cell, while some bacteria could be released after the lysis of their host cell. In C6/36_TET cells, i.e. cells cured from Wolbachia by tetracycline treatment, no difference in cell aspect was noted compared to Wolbachia-infected cells, despite the absence of Wolbachia infection. The C6/36_wAlbB cells were maintained in continuous culture for 40 passages, corresponding to approximately 5 months. Quantitative PCR analysis showed that the density of Wolbachia was highly dynamic according to the passages (Fig 2), with the lowest density of 0.9 wsp/actin ratio at P.7 to 67.6 wsp/actin ratio at P.17 for the highest. After P.17, Wolbachia’s density decreased to remain around 10 wsp/actin ratio from P.36 to P.40. The C6/36_TET cells were negative for Wolbachia infection in qPCR. The FISH also confirmed the absence of Wolbachia in C6/36_TET cells whereas the bacteria were detected in C6/36_wAlbB cytoplasm (Fig 3A), even if the infection did not reach 100% of the cultured cells (Fig 3B). Along with the density of bacteria measured in qPCR, the Wolbachia fluorescent signal decreased from P.15 to P.37 and that goes together with a significantly lower proportion of Wolbachia-infected cells from 92.4% to 45.3% at P.15 and P.37, respectively (P < 2.2e^16) (Fig 3B).

Reduced CHIKV infection by wAlbB in vitro
As no viral inhibition was measured for CHIKV 06.21 in orally infected Ae. albopictus mosquitoes [26], we tested the interaction of wAlbB and CHIKV 06.21 in C6/36. First, we assessed that CHIKV replication was not affected by anti-Wolbachia tetracycline treatment, as viral
Fig 1. Electron microscopy of Wolbachia in Aedes albopictus C6/36 cells. Low-magnification transmission electron micrograph of C6/36_TET cells with no bacterial signal in host cell cytoplasm (A) whereas Wolbachia (white arrowhead) are seen throughout the cytoplasm of C6/36_wAlbB cells (B). Wolbachia presumably is undergoing the process of cell division (C). High-magnification micrograph of Wolbachia in cytoplasm of the host cell showing a membranous structure surrounding the bacterium (black arrowhead) (D).

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Fig 2. Dynamics of wAlbB infection in C6/36 cells. Ratio of Wolbachia wsp copies per host actin copies during continuous cell culture, measured by qPCR on total genomic DNA (error bars represent the standard deviation of the mean of 3 independent samples).

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RNA titer was not significantly different between C6/36_TET and C6/36_CTRL cells at MOIs of 0.1 ($P = 0.45$) and 3 ($P = 0.68$) (Fig 4). The viral RNA titer increased from 2 h to 72 h post-infection (pi), with a short eclipse phase between 8 h and 10 h pi, then decreased until 96 h to reach a plateau until day 7 pi. The viral replication was dramatically reduced in C6/36_wAlbB compared to C6/36_TET cells as measured by RT-qPCR after infection at MOI 0.1 (Fig 5A). The RNA titer significantly decreased in C6/36_wAlbB cells by at least ten-fold across all time-points. Interestingly, *Wolbachia*-mediated inhibition depended on the time of infection (*Wolbachia*/C3 time interaction, $P < 2E^{-16}$). It seems that *Wolbachia* antiviral effect was lower as time increased. Although viral RNA titer decreased, inhibition was not complete with at least 4.81 log$_{10}$ CHIKV RNA copies per ng total RNA in C6/36_wAlbB cells at day 1 pi, where *Wolbachia* antiviral effect seemed to be the strongest. CHIKV inhibition by wAlbB was also measured at the RNA infectious particles level using FFA assay on cell supernatants (Fig 5B). A major decrease of viral infectious titer was detected in C6/36_wAlbB compared to C6/36_TET cells, depending on the time post-infection (*Wolbachia* time interaction, $P = 0.00177$). As for viral RNA, this suggests that *Wolbachia*-mediated inhibition of viral infectious particles production decreases with the time of infection, even if the time effect is lower than for viral RNA decrease. The wAlbB density was monitored in both CHIKV infected (CHIKV+) and uninfected (CHIKV-) cells using qPCR (Fig 6). The bacterial load did not vary according to viral infection ($P = 0.228$) but time had a significant effect ($P < 2E^{-16}$). The *Wolbachia* titer increased with time, ranging from 13.3 to 25.7 wsp/actin ratio at day 1 and 7 pi, respectively.

**CHIKV infection of wAlbB-colonized cells**

The FISH technique was shown to be an efficient method to detect viruses in mosquito cells [43]. This is the first time such a technique was used to detect CHIKV. The oligonucleotide-probes designed can also detect other alphaviruses, namely Sindbis virus and Ross River virus (not shown). The results showed that CHIKV could be labelled in the cytoplasm of infected cells whereas no CHIKV signal was detected in uninfected cells (Fig 7). Moreover, viral RNA was also detected in cells previously infected with *Wolbachia*, indicating that at least in some cells the virus is able to penetrate in spite of the presence of the bacterium. However, the co-localization of both *Wolbachia* and CHIKV was not detected in many cells, and the use of FISH
technique did not allowed us to tell if the presence of both micro-organisms in the same host cell was correlated with the load of either bacterium or virus.

Discussion

* Ae. albopictus* is naturally infected by *Wolbachia* and remains an important vector of CHIKV [44,45] and in a lesser extent of DENV [46,47]. Intriguingly, the pattern of *Wolbachia*-arbovirus interaction in *Ae. albopictus* remains unclear. Previous studies showed that transinfection of *Ae. albopictus* with the wMel strain of *Wolbachia* is likely to induce DENV and CHIKV inhibition [48,49]. However, *Ae. albopictus* is naturally co-infected with *Wolbachia* wAlbA and wAlbB strains but no blocking phenotype was measured against DENV and CHIKV in populations from Houston [18,50] and La Réunion [26], respectively. Conversely, a decrease of DENV titer was observed in the saliva of symbiotic females in the *Ae. albopictus* population from La Réunion [25]. This suggests that *Wolbachia*’s potential to interact with viral replication in its native mosquito host depends on the combination of bacterial strain, vector and virus factors thus making the study of this multipartite interaction very complex. Therefore, simplified models are needed to explore *Wolbachia*-pathogen interaction in mosquito. As *Wolbachia* is an obligate intracellular bacterium, insect cell lines have been widely used for culturing the bacterium with special emphasis on *Ae. albopictus* derived cells [29,36,41,42,51–54]. Moreover,
mosquito cell lines are generally permissive to arbovirus infection, providing a useful tool to study Wolbachia-arbovirus interaction [21,35,50]. In adult mosquito, during the Extrinsic Incubation Period (EIP), the virus infects essentially somatic tissues including midgut and salivary glands [55] which are both infected by Wolbachia in Ae. albopictus [23]. C6/36 cells, which originated from uninfected somatic tissue appear to be an appropriate model, in complement to Aa23 cells, to study Wolbachia-arbovirus interaction in an Ae. albopictus background.

Previous studies suggested that DENV inhibition seems to depend on Wolbachia density [50,56]. We showed that in C6/36, wAlbB density is highly dynamic but remains low compared to Aa23 with a maximum at 72.5 wsp/actin copies against 1,888.3 wsp/actin, respectively [50]. However, we observed a significant CHIKV interference in C6/36_wAlbB at a relative

**Fig 5. Effect of Wolbachia on CHIKV replication and infectiosity.** Kinetic at MOI 0.1 of CHIKV RNA titer measured by RT-qPCR on total cellular RNA (A) and CHIKV infectious titer in supernatant measured by FFA (B) in presence of Wolbachia (wAlbB) or in cells cured from the bacteria by tetracycline treatment (TET). Error bars represent the standard deviation of the mean of 3 independent samples.

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Fig 6. Dynamics of wAlbB in C6/36 during CHIKV infection. Ratio of Wolbachia wsp copies per host actin copies during CHIKV infection at MOI 0.1, measured by qPCR on genomic DNA. Error bars represent the standard deviation of the mean of 3 independent samples.
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Fig 7. Detection and localization of wAlbB and CHIKV in cellulo by FISH. Detection of Wolbachia 16S rDNA (red) and CHIKV Env RNA (green) using oligonucleotide probes labelled with Rhodamine and Alexa488, respectively. Wolbachia signal is detected in C6/36_wAlbB but not in tetracycline treated cells (C6/36_TET). CHIKV signal is detected only in CHIKV infected modality, in the absence or in the presence of Wolbachia where it co-localize with the bacteria in the cytoplasm of C6/36_wAlbB cells. Nuclei of host cells are shown in blue after DAPI labelling (bars = 10 μm).
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Wolbachia density of 13.7 to 25.6 wsp/actin, although inhibition was not complete. These results suggest that Wolbachia-mediated antiviral activity can occur in vitro even at low bacterial density. Interestingly, Lu and colleagues extrapolated from their observations in Aa23_wAlbB cells that a relative density of wAlbB of 0.3, 5.3, and 12.3 wsp/actin in midgut, salivary gland and fat body of Ae. albopictus, respectively was too low to interfere with DENV infection in vivo. The lower abundance of wAlbB in Ae. albopictus organs compared to C6/36 cells [23] is in line with this observation, and with the absence of CHIKV inhibition measured in vivo in Ae. albopictus. Conversely, the viral load did not seem to counteract with virus blocking by Wolbachia as demonstrated in C6/36_wMelPop-CLA cells infected with DENV [35]. Using the Ae. aegypti cell line Aag-2 to culture wMelPop-CLA, it was recently shown that Wolbachia-induced antiviral activity occurred as soon as the RNA replication step for DENV, but only at the step of virion assembly/secretion for WNV [21]. These results emphasize the importance of measuring both RNA and infectious particles to assess Wolbachia-antiviral activity, and suggest that distinct antiviral cellular mechanisms are involved during Wolbachia-virus interaction. In our model, CHIKV replication is inhibited by wAlbB in C6/36 cells, in a time-dependent manner with the lowest viral RNA load measured at 24 h pi. We also observed a decrease of infectious particles titer in supernatant as early as 24 h pi, indicating that viral blocking could occur at both stages of the viral cycle. This also suggests that CHIKV blocking by wAlbB could occur at the early stage of viral infection. Considering this, FISH was used to label both Wolbachia and CHIKV during co-infection of C6/36 cells. The FISH experiment showed that Wolbachia and CHIKV could be localized in the same host cell, indicating that wAlbB did not seem to inhibit CHIKV infection by preventing viral entry, at least in some cells. This hypothesis is reinforced by in vivo confocal microscopy where Wolbachia was co-localized with DENV in salivary glands of Ae. albopictus [25]. This is in contrast with observations made in Ae. Aegypti where detection by FISH supported a cellular exclusion of DENV by the wMel strain of Wolbachia [19]. However, even if Wolbachia-virus co-infected cells or tissues are detected in Ae. albopictus both in vitro and in vivo, their magnitude cannot exclude that viruses preferentially infect Wolbachia-free compartment.

The cellular pathways involved during virus blocking by Wolbachia are poorly known and indirect effects were mentioned to explain Wolbachia-mediated antiviral phenotype. The mechanisms of antiviral response in insects relies on different innate immune pathways, the main one being the small interfering RNA (siRNA) pathway [57]. It was recently shown that C6/36 lacks a functional siRNA mechanism [58], suggesting that siRNA pathway is not involved in wAlbB-mediated CHIKV interference. Wolbachia was shown to manipulate another RNA interference pathway, the micro-RNA (miRNA) pathway, to facilitate its own spread in the mosquito, and this mechanism could be involved in DENV interference [59,60]. It has been proposed that Wolbachia-induced antiviral phenotype relies through the activation of mosquito innate immune system, including Imd and Toll pathways [19]. However, a recent study using Drosophila mutant’s deficient for Toll and Imd genes conclude that neither is required for the bacteria to inhibit DENV [32]. In the meantime, it has been suggested that Wolbachia and the virus could engage a direct competition for host cell resources, as underlined by the importance of host cholesterol levels for Drosophila C virus blocking in D. melanogaster [27]. We demonstrated in previous work that wMel manipulates iron metabolism in Ae. albopictus RML-12 cells through bacterioferritin expression [61], another potential explanation for its antiviral activity as iron load is involved in the modulation of innate immunity [62]. Further unexplored hypothesis is autophagy, a mechanism that has been shown recently to regulate Wolbachia density across different arthropod hosts including mosquito cells [29]. The autophagy pathway is required by CHIKV to replicate [63], and this cellular function could be involved in Wolbachia antiviral interference.
Overall, insect cell lines may represent a promising tool to facilitate the understanding of Wolbachia-pathogen interaction notably through electron microscopic observations of cell structural changes, and transcriptomic or proteomic studies which could allow to identify host infection regulatory pathways influenced by Wolbachia [34,64–66]. The potential direct activity of Wolbachia derived compounds against pathogens remains unknown but need further exploration, especially in the light of recent results suggesting the direct anti-DENV activity of a Chromobacterium sp (Csp_P) isolated from Ae. aegypti midgut [67]. Our results showed a significant antiviral effect of wAlbB against CHIKV in cellulo that was not measured in vivo at the mosquito organ level, even if CHIKV RNA load was constraint in a smaller range in symbiotic females organs [26]. This emphasizes the need to better understand Wolbachia symbiosis in its native host Ae. albopictus, and its impact on vector competence. Mosquito vector competence for arboviruses depends on multiple factors such as mosquito genotype, virus genotype and their interaction [68] but also temperature [69,70] or mosquito microbiota [71]. Recent studies showed that pathogen blocking by Wolbachia was influenced by temperature [72] and that bacteria from the genus Asaia can inhibit vertical transmission of Wolbachia in An. gambiae [73]. Together, these results underline the importance of exploring Wolbachia-pathogen interaction, especially in a context where Wolbachia-infected mosquitoes represent a promising strategy to control vector-borne diseases.

Supporting Information

S1 Fig. C6/36_wAlbB cells in transmission-light microscopy. Pictures in light microscopy of C6/36 cells infected by Wolbachia (C6/36_wAlbB) or tetracycline-treated (C6/36_TET) during their growth in F25 cm² flasks, between two passages (bars = 20 μm). (TIF)

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Author Contributions

Conceived and designed the experiments: VR CVM PM. Performed the experiments: VR CVM YS FHT. Analyzed the data: VR CVM PM. Contributed reagents/materials/analysis tools: PP PM. Wrote the paper: VR CVM PM.

References

1. WHO | About vector-borne diseases. In: WHO [Internet]. Available: http://www.who.int/campaigns/world-health-day/2014/vector-borne-diseases/en/
2. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 2007; 3: e201. doi:10.1371/journal.ppat.0030201 PMID: 18069894
3. Tsetsarkin KA, Weaver SC. Sequential adaptive mutations enhance efficient vector switching by Chikungunya virus and its epidemic emergence. PLoS Pathog. 2011; 7: e1002412. doi:10.1371/journal.ppat.1002412 PMID: 22174678
4. De Lamballerie X, Leroy E, Charrel RN, Tsetsarkin K, Higgs S, Gould EA. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? Virol J. 2008; 5: 33. doi: 10.1186/1743-422X-5-33 PMID: 18304328

5. Angelini R, Finarelli AC, Angelini P, Po C, Petropoulakos K, Silvi G, et al. Chikungunya in north-eastern Italy: a summing up of the outbreak. Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull. 2007; 12: E071122.2.

6. La Ruche G, Souarès Y, Armengaud A, Peloux-Petiot F, Delaunay P, Després P, et al. First two autochthonous dengue virus infections in metropolitan France, September 2010. Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull. 2010; 15: 19676.

7. Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. Lancet. 2014; 383: 514. doi: 10.1016/S0140-6736(14)60185-9 PMID: 24506907

8. WHO | Chikungunya — France. In: WHO [Internet]. Available: http://www.who.int/csr/don/23-october-2014-chikungunya/en/

9. Bonizzoni M, Gasperi G, Chen X, James AA. The invasive mosquito species Aedes albopictus: current knowledge and future perspectives. Trends Parasitol. 2013; 29: 460–468. doi: 10.1016/j.pt.2013.07.003 PMID: 23916878

10. Benedict MQ, Levine RS, Hawley WA, Lounibos LP. Spread of the tiger: global risk of invasion by the mosquito Aedes albopictus. Vector Borne Zoonotic Dis Larchmt N. 2014; 7: 76–85. doi: 10.1089/vbz.2006.0562 PMID: 17417960

11. David J-P, Coissac E, Melodelima C, Poupardin R, Riaz MA, Chandor-Proust A, et al. Transcriptome sequencing technology. BMC Genomics. 2010; 11: 216. doi: 10.1186/1471-2164-11-216 PMID: 20356352

12. Christodoulou M. Biological vector control of mosquito-borne diseases. Lancet Infect Dis. 2011; 11: 84–85. PMID: 21351390

13. Slatko BE, Luck AJ, Dobson SL, Foster JM. Wolbachia endosymbionts and human disease control. Mol Biochem Parasitol. 2014; 195: 88–95. doi: 10.1016/j.molbiopara.2014.07.004 PMID: 25046729

14. Bian G, Yoshi D, Dong Y, Lu P, Zhou G, Pan X, et al. The endosymbiotic bacterium Wolbachia invades Anopheles stephensi Populations and Induces Refractoriness to Plasmodium Infection. Science. 2013; 340: 748–751. doi: 10.1126/science.1236192 PMID: 23661760

15. Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, van den Hurk A, et al. Limited dengue virus replication in field-collected Aedes aegypti mosquitoes infected with Wolbachia. PLoS Negl Trop Dis. 2014; 8: e2688. doi: 10.1371/journal.pntd.0002688 PMID: 24587459

16. Zug R, Hammerstein P. Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS One. 2012; 7: e38544. doi: 10.1371/journal.pone.0038544 PMID: 22685581

17. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol. 2006; 8: 741–751. doi: 10.1038/nrmicro1969 PMID: 18794912

18. Bian G, Xu Y, Lu P, Xie Y, Xi Z. The endosymbiotic bacterium Wolbachia induces resistance to dengue virus in Aedes aegypti. PLoS Pathog. 2010; 6: e1000833. doi: 10.1371/journal.ppat.1000833 PMID: 20368968

19. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. Cell. 2009; 139: 1268–1278. doi: 10.1016/j.cell.2009.11.042 PMID: 20064373

20. Van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, et al. Impact of Wolbachia on infection with chikungunya and yellow fever viruses in the mosquito vector Aedes aegypti. PLoS Negl Trop Dis. 2012; 6: e1892. doi: 10.1371/journal.pntd.0001892 PMID: 23133693

21. Hussain M, Lu G, Torres S, Edmonds JH, Kay BH, Khromykh AA, et al. Effect of Wolbachia on replication of West Nile virus in a mosquito cell line and adult mosquitoes. J Virol. 2013; 87: 851–858. doi: 10.1128/JVI.01837-12 PMID: 23115298

22. Zélé F, Nicot A, Berthomieu A, Weill M, Duron O, Rivero A. Wolbachia increases susceptibility to Plasmodium infection in a natural system. Proc Biol Sci. 2014; 281: 20132387. doi: 10.1098/rspb.2013.2837 PMID: 24500167

23. Zouache K, Voronin D, Tran-Van V, Mousson L, Failloux A-B, Mavingui P. Persistent Wolbachia and cultivable bacteria infection in the reproductive and somatic tissues of the mosquito vector Aedes albopictus. PloS One. 2009; 4: e6388. doi: 10.1371/journal.pone.0006388 PMID: 19633721

24. Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LHR, Ravelonandro P, et al. Bacterial diversity of field-caught mosquitoes, Aedes albopictus and Aedes aegypti, from different geographic
regions of Madagascar. FEMS Microbiol Ecol. 2011; 75: 377–389. doi: 10.1111/j.1574-6941.2010.01012.x PMID: 21175696

25. Mousson L, Zouache K, Arias-Goeta C, Raquin V, Mavingui P, Failloux A-B. The Native Wolbachia Symbions Limit Transmission of Dengue Virus in Aedes albopictus. PLoS Negl Trop Dis. 2012; 6: e1898. doi: 10.1371/journal.pntd.0001989 PMID: 23301109

26. Mousson L, Martin E, Zouache K, Madec Y, Mavingui P. Wolbachia modulates Chikungunya replication in Aedes albopictus. Mol Ecol. 2010; 19: 1953–1964. doi: 10.1111/j.1365-294X.2010.04606.x PMID: 20345866

27. Caragata EP, Rancès E, Hedges LM, Goffton AW, Johnson KN, O’Neill SL, et al. Dietary cholesterol modulates pathogen blocking by Wolbachia. PLoS Pathog. 2013; 9: e1003459. doi: 10.1371/journal.ppat.1003459 PMID: 23825950

28. Caragata EP, Rancès E, O’Neill SL, McGraw EA. Competition for amino acids between Wolbachia and the mosquito host, Aedes aegypti. Microb Ecol. 2014; 67: 205–218. doi: 10.1007/s00248-013-0339-4 PMID: 24337107

29. Voronin D, Cook DAN, Steven A, Taylor MJ. Autophagy regulates Wolbachia populations across diverse symbiotic associations. Proc Natl Acad Sci U S A. 2012; 109: E1638–1646. doi: 10.1073/pnas.1203519109 PMID: 22645363

30. Brennan LJ, Keddie BA, Braig HR, Harris HL. The endosymbiont Wolbachia pipientis induces the expression of host antioxidant proteins in an Aedes albopictus cell line. PloS One. 2008; 3: e2083. doi: 10.1371/journal.pone.0002083 PMID: 18461124

31. Hussain M, Frentiu FD, Moreira LA, O’Neill SL, Asgari S. Wolbachia uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector Aedes aegypti. Proc Natl Acad Sci U S A. 2011; 108: 9250–9255. doi: 10.1073/pnas.1105469108 PMID: 21576469

32. Rancès E, Ye YH, Woolfit M, McGraw EA, O’Neill SL. The relative importance of innate immune priming in Wolbachia-mediated dengue interference. PLoS Pathog. 2012; 8: e1002548. doi: 10.1371/journal.ppat.1002548 PMID: 22383881

33. Hussain M, O’Neill SL, Asgari S. Wolbachia interferes with the intracellular distribution of Argonaute 1 in the dengue vector Aedes aegypti by manipulating the host microRNAs. RNA Biol. 2013; 10: 1868–1875. doi: 10.4161/ma.27392 PMID: 24351659

34. Hughes GL, Ren X, Ramirez JL, Sakamoto JM, Bailey JA, Jedlicka AE, et al. Wolbachia infections in Anopheles gambiae cells: transcriptomic characterization of a novel host-symbiont interaction. PLoS Pathog. 2011; 7: e1001296. doi: 10.1371/journal.ppat.1001296 PMID: 21379333

35. Frentiu FD, Robinson J, Young PR, McGraw EA, O’Neill SL. Wolbachia-mediated resistance to dengue virus infection and death at the cellular level. PloS One. 2010; 5: e13398. doi: 10.1371/journal.pone.0013398 PMID: 20756219

36. O’Neill SL, Pettigrew MM, Sinkins SP, Braig HR, Andreadis TG, Tesh RB. In vitro cultivation of Wolbachia pipientis in an Aedes albopictus cell line. Insect Mol Biol. 1997; 6: 33–39. PMID: 9013253

37. Schuffenecker I, Itenam I, Michault A, Murri S, Frangeul L, Vaney M-C, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med. 2006; 3: e263. doi: 10.1371/journal.pmed.0030263 PMID: 16700631

38. Dubrulle M, Mousson L, Moutailleur S, Vazeille M, Failloux A-B. Chikungunya virus and Aedes mosquitoes: saliva is infectious as soon as two days after oral infection. PloS One. 2009; 4: e5895. doi: 10.1371/journal.pone.0005895 PMID: 19521520

39. Payne AF, Binduga-Gajewska I, Kaufman EB, Kramer LD. Quantitation of flaviviruses by fluorescent focus assay. J Virol Methods. 2006; 134: 183–189. doi: 10.1016/j.jviromet.2006.01.003 PMID: 16510196

40. Venard CM-P, Crain PR, Dobson SL. SYTO11 staining vs FISH staining: a comparison of two methods to stain Wolbachia pipientis in cell cultures. Lett Appl Microbiol. 2011; 52: 168–176. doi: 10.1111/j.1472-765X.2010.02986.x PMID: 21214605

41. Fenollar F, La Scala B, Inokuma H, Dumler JS, Taylor MJ, Raoult D. Culture and phenotypic characterization of a Wolbachia pipientis isolate. J Clin Microbiol. 2003; 41: 5434–5441. PMID: 14662922

42. Voronin D, Tran-Van V, Potter P, Mavingui P. Transinfection and growth discrepancy of Drosophila Wolbachia strain wMel in cell lines of the mosquito Aedes albopictus. J Appl Microbiol. 2010; 108: 2133–2141. doi: 10.1111/j.1365-2672.2009.04621.x PMID: 19951376

43. Raquin V, Wannagat M, Zouache K, Legras-Lachuer C, Moro CV, Mavingui P. Detection of dengue group viruses by fluorescence in situ hybridization. Parasit Vectors. 2012; 5: 243. doi: 10.1186/1756-3305-5-243 PMID: 23110979

44. Bonilauri P, Bellini R, Calzolari M, Angelini R, Venturi L, Fallacara F, et al. Chikungunya virus in Aedes albopictus, Italy. Emerg Infect Dis. 2008; 14: 852–854. doi: 10.3201/eid1405.071144 PMID: 18439383
56. Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, Guerbois M, et al. Multi-peaked adaptive landscape for chikungunya virus evolution predicts continued fitness optimization in Aedes albopictus mosquitoes. Nat Commun. 2014; 5: 4084. doi: 10.1038/ncomms5084 PMID: 24933611

57. Delattre H, Paupy C, Dehecq JS, Thiria J, Failloux AB, Fontenille D. [Aedes albopictus, vector of chikungunya and dengue viruses in Reunion Island: biology and control]. Parasite Paris Fr. 2008; 15: 3–13.

58. Ponlawat A, Scott JG, Harrington LC. Insecticide susceptibility of Aedes aegypti and Aedes albopictus across Thailand. J Med Entomol. 2005; 42: 821–825. PMID: 16363166

59. Blagrove MSC, Arias-Goeta C, Giannou C, Failloux AB, Fontenille D. [Aedes albopictus, vector of chikungunya virus]. J Comp Pathol. 2005; 133: 1–8.

60. Zhang G, Hussain M, Asgari S. Regulation of arginine methyltransferase 3 by a microRNA in Aedes albopictus cells. PLoS Negl Trop Dis. 2013; 7: e2152. doi: 10.1371/journal.pntd.0002152 PMID: 23556030

61. Blagrove MSC, Arias-Goeta C, Failloux AB, Sinks SP. Wolbachia strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in Aedes albopictus. Proc Natl Acad Sci U S A. 2012; 109: 255–260. doi: 10.1073/pnas.1112021108 PMID: 22123944

62. Fallon AM, Wittuhhn BA. Proteosome activity in a naïve mosquito cell line infected with Wolbachia piipientis wAlbB. In Vitro Cell Dev Biol Anim. 2009; 45: 460–466. doi: 10.1007/s11626-009-9193-6 PMID: 19296184

63. Fallon AM, Baldrige GD, Higgins LA, Wittuhhn BA. Wolbachia from the planthopper Laodelphax striatellus establishes a robust, persistent, streptomycin-resistant infection in clonal mosquito cells. In Vitro Cell Dev Biol Anim. 2013; 49: 66–73. doi: 10.1007/s11626-012-9571-3 PMID: 23273164

64. Khoo CCH, Venard CMP, Fu Y, Mercer DR, Dobson SL. Infection, growth and maintenance of Wolbachia piipientis in clonal and non-clonal Aedes albopictus cell cultures. Bull Entomol Res. 2013; 103: 251–260. doi: 10.1017/S0007485312000648 PMID: 23113940

65. Salazar MI, Richardson JH, Sánchez-Vargas I, Olson KE, Beaty BJ. Dengue virus type 2: replication and tropisms in orally infected Aedes aegypti mosquitoes. BMC Microbiol. 2007; 7: 9. doi: 10.1186/1471-2180-7-9 PMID: 17263893

66. Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, O’Neill SL, Johnson KN. Antiviral Protection and the Importance of Wolbachia in Drosophila simulans. Appl Environ Microbiol. 2012; 78: 6922–6929. doi: 10.1128/AEM.01727-12 PMID: 22843518

67. Kingsolver MB, Huang Z, Hardy RW. Insect antiviral innate immunity: pathways, effectors, and connections. J Mol Biol. 2013; 425: 4921–4936. doi: 10.1016/j.jmb.2013.10.006 PMID: 24120681

68. Brackney DE, Scott JC, Sagawa F, Woodward JE, Miller NA, Schilkey FD, et al. C6/36 Aedes albopictus cells have a dysfunctional antiviral RNA interference response. PLoS Negl Trop Dis. 2010; 4: e856.: doi: 10.1371/journal.pntd.0000856 PMID: 21049065

69. Zhang G, Hussain M, O’Neill SL, Asgari S. Wolbachia uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in Aedes aegypti. Proc Natl Acad Sci U S A. 2013; 110: 10276–10281. doi: 10.1073/pnas.1303603110 PMID: 23739960

70. Zhang G, Hussain M, Asgari S. Regulation of arginine methyltransferase 3 by a Wolbachia-induced microRNA in Aedes aegypti and its effect on Wolbachia and dengue virus replication. Insect Biochem Mol Biol. 2014; 53: 81–88. doi: 10.1016/j.ibmb.2014.08.003 PMID: 25158106

71. Kremer N, Voronin D, Charif D, Mavingui P, Mollereau B, Vavre F. Wolbachia interferes with ferritin expression and iron metabolism in insects. PLoS Pathog. 2009; 5: e1000630. doi: 10.1371/journal.ppat.1000630 PMID: 19851452

72. Schaible UE, Kaufmann SHE. Iron and microbial infection. Nat Rev Microbiol. 2004; 2: 946–953. doi: 10.1038/nrmicro1046 PMID: 15550940

73. Krejčich-Trotot P, Gay B, Li-Pat-Yuen G, Hoarau J-I, Jaffar-Bandjee M-C, Briant L, et al. Chikungunya triggers an autophagic process which promotes viral replication. Virol J. 2011; 8: 432. doi: 10.1186/1743-422X-8-432 PMID: 21902836

74. Baldridge GD, Baldridge AS, Wittuhhn BA, Higgins L, Markowski TW, Fallon AM. Proteomic profiling of a robust Wolbachia infection in an Aedes albopictus mosquito cell line. Mol Microbiol. 2014; 94: 537–556. doi: 10.1111/mmi.12768 PMID: 25155417

75. Darby AC, Christina Gill A, Armstrong SD, Hartley CS, Xia D, Wastling JM, et al. Integrated transcriptomic and proteomic analysis of the global response of Wolbachia to doxycycline-induced stress. ISME J. 2014; 8: 925–937. doi: 10.1038/ismej.2013.192 PMID: 24152719
66. Kambris Z, Blagborough AM, Pinto SB, Blagrove MSC, Godfray HCJ, Sinden RE, et al. Wolbachia stimulates immune gene expression and inhibits Plasmodium development in Anopheles gambiae. PLoS Pathog. 2010; 6: e1001143. doi: 10.1371/journal.ppat.1001143 PMID: 20949079

67. Ramirez JL, Short SM, Bahia AC, Saraiva RG, Dong Y, Kang S, et al. Chromobacterium Csp_P Reduces Malaria and Dengue Infection in Vector Mosquitoes and Has Entomopathogenic and In Vitro Anti-pathogen Activities. PLoS Pathog. 2014; 10: e1004398. doi: 10.1371/journal.ppat.1004398 PMID: 25340821

68. Fansiri T, Fontaine A, Diancourt L, Caro V, Thaisomboonsuk B, Richardson JH, et al. Genetic mapping of specific interactions between Aedes aegypti mosquitoes and dengue viruses. PLoS Genet. 2013; 9: e1003621. doi: 10.1371/journal.pgen.1003621 PMID: 23935524

69. Richards SL, Anderson SL, Lord CC, Tabachnick WJ. Effects of virus dose and extrinsic incubation temperature on vector competence of Culex nigripalpus (Diptera: Culicidae) for St. Louis encephalitis virus. J Med Entomol. 2012; 49: 1502–1506. PMID: 23270182

70. Carrington LB, Seifert SN, Armijos MV, Lambrechts L, Scott TW. Reduction of Aedes aegypti vector competence for dengue virus under large temperature fluctuations. Am J Trop Med Hyg. 2013; 88: 689–697. doi: 10.4269/ajtmh.12-0488 PMID: 23438766

71. Jupatanakul N, Sim S, Dimopoulos G. The Insect Microbiome Modulates Vector Competence for Arboviruses. Viruses. 2014; 6: 4294–4313. doi: 10.3390/v6114294 PMID: 25393895

72. Murdock CC, Blanford S, Hughes GL, Rasgon JL, Thomas MB. Temperature alters Plasmodium blocking by Wolbachia. Sci Rep. 2014; 4: 3932. doi: 10.1038/srep03932 PMID: 24488176

73. Hughes GL, Dodson BL, Johnson RM, Murdock CC, Tsujimoto H, Suzuki Y, et al. Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. Proc Natl Acad Sci U S A. 2014; 111: 12498–12503. doi: 10.1073/pnas.1408888111 PMID: 25114252