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RESEARCH ARTICLE

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 underlines that despite *Wolbachia* antiviral potential other complex interactions occur *in vivo* to determine mosquito vector competence in *Ae. albopictus*.

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,

1), part of the 2012-2013 BiodivERsA call for research proposals.

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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*-transfected 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 Mitsuhashi/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 scrapped, 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 coverslip 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 scrapping 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 samples. 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 *DNeasy 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.

Table 1. List of primers and probes used in this study.

Primers	Sequence (5'-3')	Reference
183F	AAGGAACCGAAGTTCATG	[77]
QBrev2	AGTTGTGAGTAAAGTCCC	[77]
actAlb-dir	GCAAACGTGGTATCCTGAC	[77]
actAlb-rev	GTCAGGAGAACTGGGTGCT	[77]
Chik/E2/9018/+	CACCGCCGCAACTACCG	[78]
Chik/E2/9235/-	GATTGGTGACCGCGGCA	[78]
Oligonucleotide probes		
W2	Rhodamine-CTTCTGTGAGTACCGTCATTATC	[79]
Wol3	Rhodamine-TCCTCTATCCTCTTTCAATC	[80]
Chiknsp2	Alexa488-CAAGTCAGCTATTATCAAGAACCCTAGTTAC	this study
ChikE2	Alexa488-GAGATAATTCTGTATTATTATGAGCTGTAC	this study

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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 μ L containing 10 ng (2 μ L) of RNA template, 10 μ L EXPRESS SYBR GreenER SuperMix Universal, 200 nM of sense Chik/E2/9018/+ and anti-sense Chik/E2/9235/- primers (Table 1) and 0.5 μ L 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 μ L/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 μ L per well. Plates were incubated 3 days at 28°C then 150 μ L of freshly prepared 4% formaldehyde solution in PBS was added without removing the overlay. Cell monolayers were fixed for 20 min at RT, washed three times in PBS, then incubate for 30 min at RT in PBS-0.1% Triton X-100. Plates were stained for 1 h at 37°C with a 1:1000 dilution of hyper-ascetic immune fluid specific to CHIKV 06.21 in PBS-0.1% Bovine Serum Albumin (BSA, Sigma, France). After 3 washes in PBS, cells were incubated for 1 h at 37°C with an anti-mouse Alexa488-conjugated antibody (Molecular probes, Invitrogen, France) diluted at 1:200 in PBS-0.1% BSA followed by three washes in PBS and a final wash in distilled water. Cell monolayers were observed using an EVOS inverted fluorescence microscope (Life Technologies, France) with a FITC-filter, under 10X objective. The total number of fluorescent foci was counted from 5 to 50 at the appropriate dilution, and virus titer was calculated as fluorescent focus unit per mL. The titer represents a mean of two independent samples.

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 (1,4-dithiothreitol) 0.1 mg/L, Denhardt'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.ZI; 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 where 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).

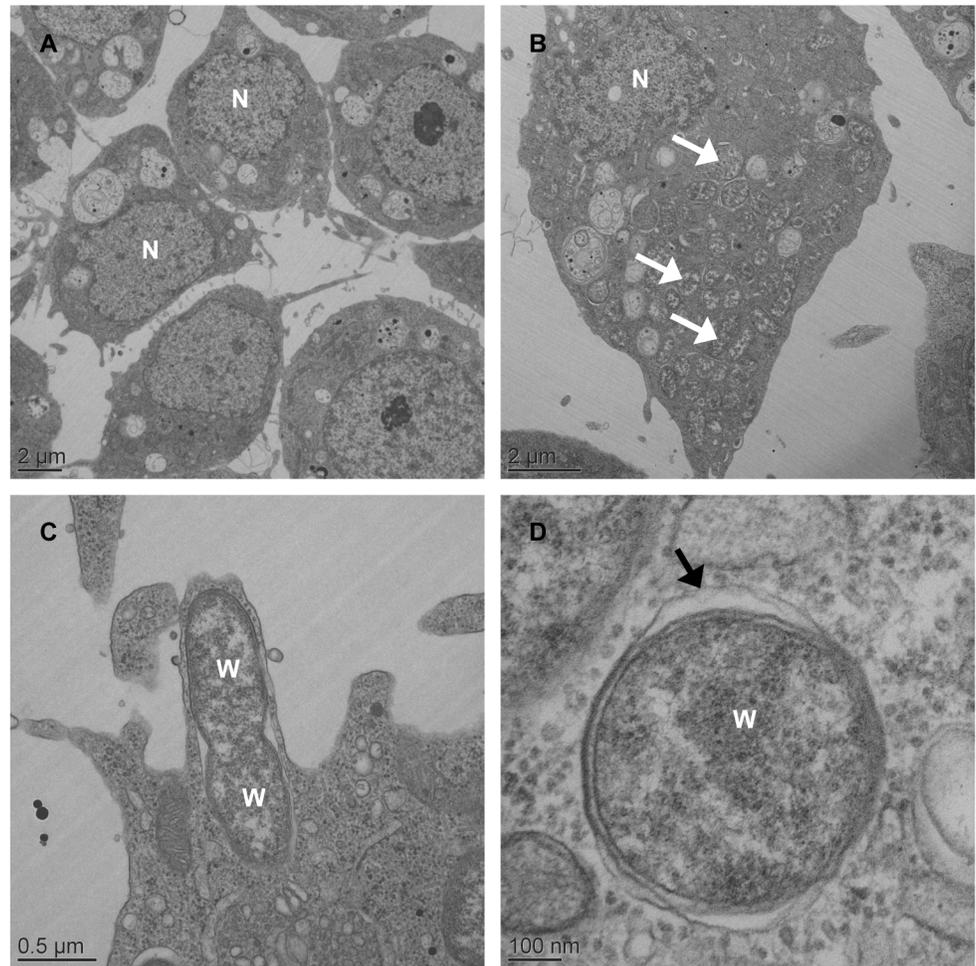


Fig 1. Electron microscopy of *Wolbachia* in *Aedes albopictus* C6/36 cells. Low-magnification transmission electron micrograph of C6/36_TET cells with no bacterial signal in host cell cytoplasm (A) whereas *Wolbachia* (white arrowhead) are seen throughout the cytoplasm of C6/36_wAlbB cells (B). *Wolbachia* 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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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 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**time interaction, $P < 2E-16$), suggesting that presence of *Wolbachia* could delay virus replication as previously mentioned [43]. Although viral RNA titer decreased, inhibition was

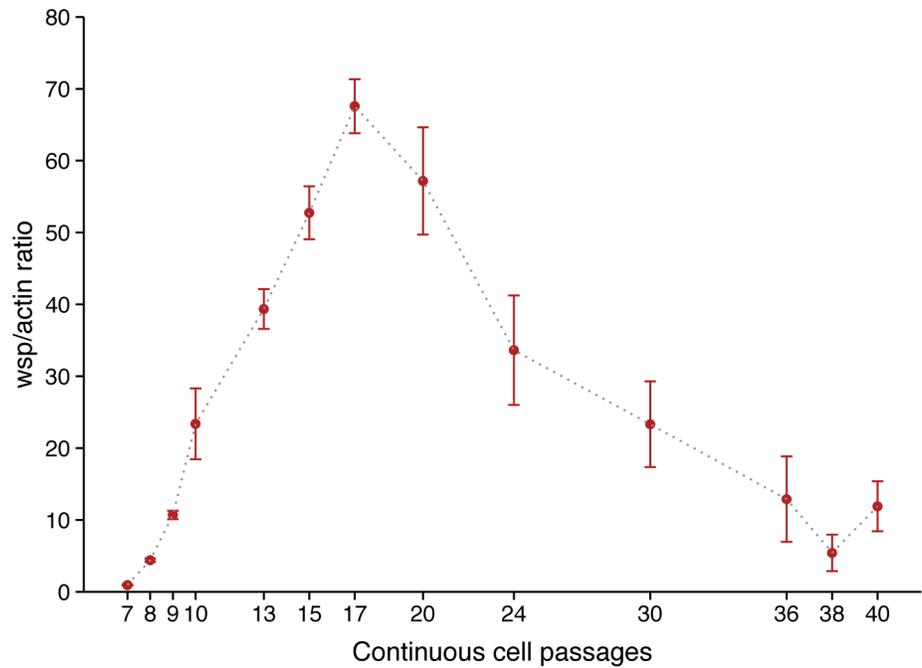


Fig 2. Dynamics of wAlbB infection in C6/36 cells. Ratio of *Wolbachia* *wsp* copies per host *actin* copies during continuous cell culture, measured by qPCR on total genomic DNA (error bars represent the standard deviation of the mean of three independent samples).

doi:10.1371/journal.pone.0125066.g002

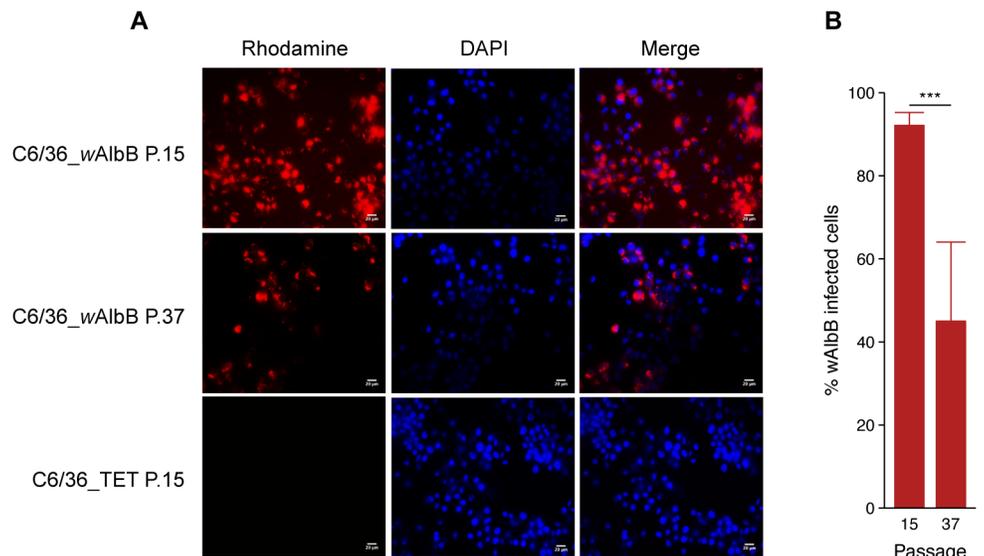


Fig 3. Proportion of *Wolbachia*-infected cells detected by Fluorescence *in situ* Hybridization. Rhodamine-labelled oligonucleotide probe designed on *Wolbachia* 16S rRNA gene (red) detected the bacteria in the cytoplasm of the host cell at passages P.15 and P.37 (A). Nuclei of the host cells are shown in blue after DAPI labelling (bars = 20µm). Percentage of cells with a *Wolbachia*-positive signal in FISH at P.15 and P.37 (B) (Error bars represent the standard deviation of the mean of 50 independent microscope fields from three independent samples).

doi:10.1371/journal.pone.0125066.g003

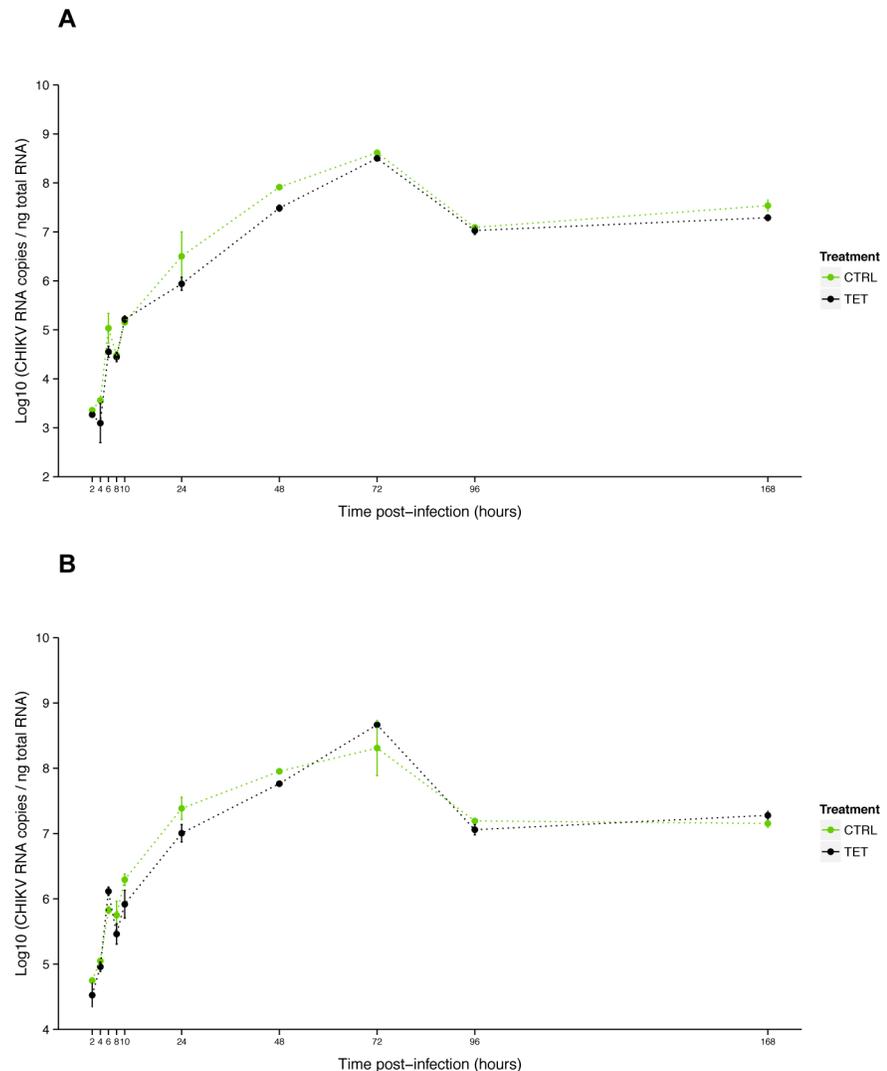


Fig 4. Effect of tetracycline treatment on CHIKV growth in C6/36. Kinetics of CHIKV RNA titer at MOI 0.1 (A) and 3 (B) measured by RT-qPCR on total cellular RNA isolated from C6/36 cells (non infected by *Wolbachia*) treated with tetracycline (TET) or not (CTRL). Error bars represent the standard deviation of the mean of two independent samples.

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not complete with at least 4.81 log₁₀ 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.

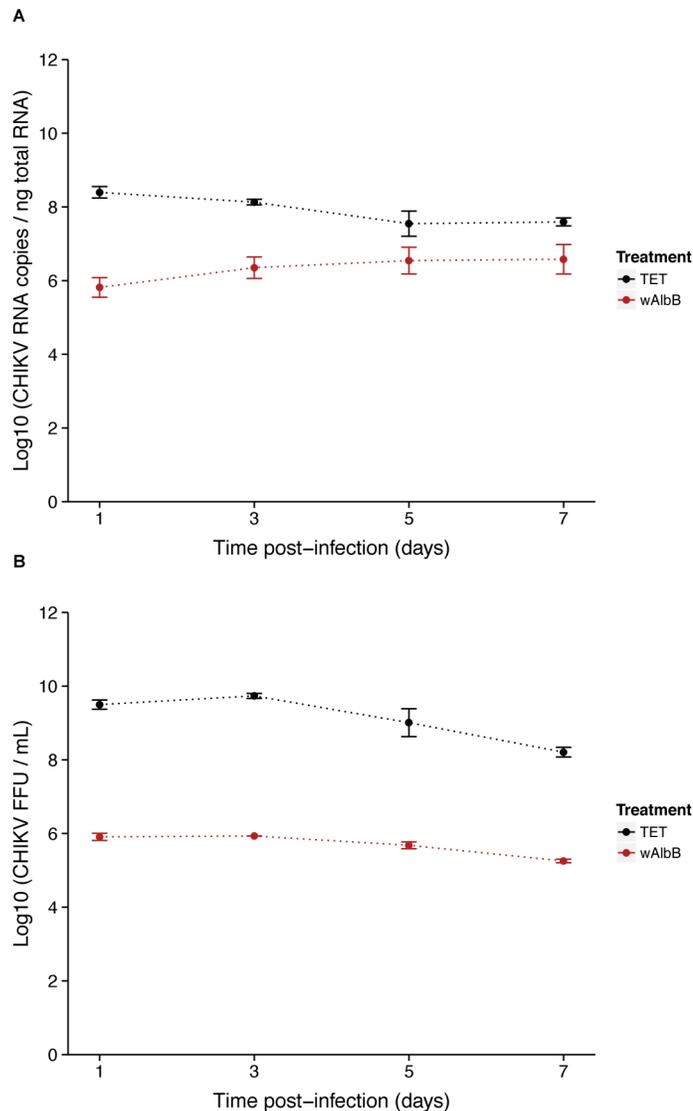


Fig 5. Effect of *Wolbachia* on CHIKV replication and infectivity. Kinetics at MOI 0.1 of CHIKV RNA titer measured by RT-qPCR on total cellular RNA (A) and CHIKV infectious titer in supernatant measured by FFA (B) in presence of *Wolbachia* (wAlbB) or in cells cured from the bacteria by tetracycline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

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CHIKV infection of wAlbB-colonized cells

The FISH technique was shown to be an efficient method to detect viruses in mosquito cells [44]. 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

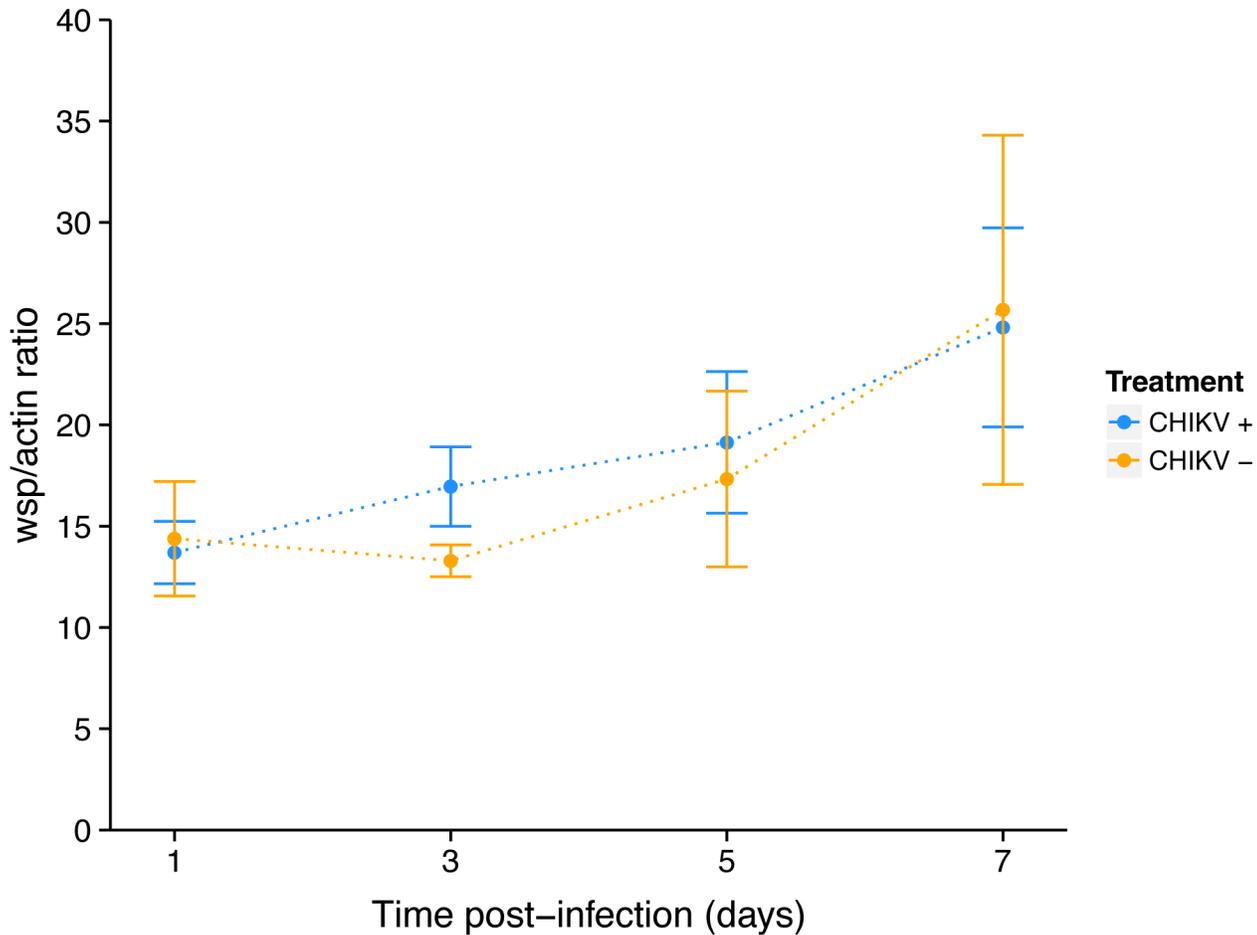


Fig 6. Dynamics of wAlbB in C6/36 during CHIKV infection. Ratio of *Wolbachia* wsp copies per host actin copies during CHIKV infection at MOI 0.1, measured by qPCR on genomic DNA. Error bars represent the standard deviation of the mean of three independent samples.

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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 [45,46] and in a lesser extent of DENV [47,48]. 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 [49,50]. 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 [18,51] 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

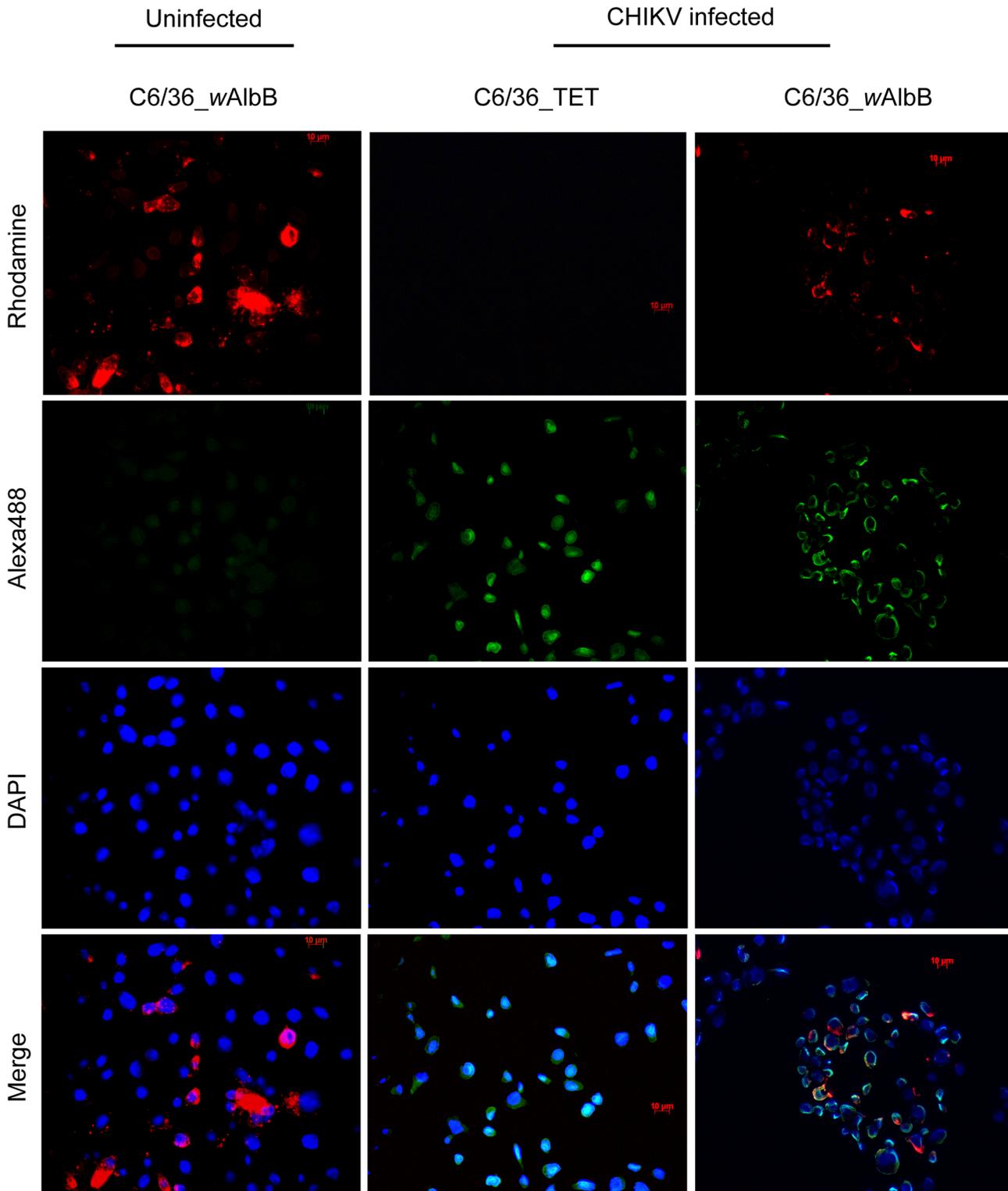


Fig 7. Detection and localization of wAlbB and CHIKV in cellulo by FISH. Detection of *Wolbachia* 16S rRNA gene (red) and CHIKV *Env* RNA (green) using oligonucleotide probes labelled with Rhodamine and Alexa488, respectively. *Wolbachia* signal is detected in C6/36_wAlbB but not in tetracycline treated 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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bacterium with special emphasis on *Ae. albopictus* derived cells [29,36,41,42,52–55]. It is worth noting that previous studies showed a high discrepancy in *Wolbachia* density in cell culture [42,55,56], possibly due to cells passaging method that could result in reduction or loss of bacterial infection [55]. The initial burst of infection followed by a rapid decrease of *wAlbB* density in C6/36 could be interpreted as an adaptation of the bacterium to cell culture as previous work noticed a shift in *Wolbachia* phenotype in its natural host after long-term passage in mosquito cell line [52]. It would be worth to study the mechanisms underpinning *Wolbachia* establishment in mosquito cell line and explore possible correlation with antiviral activity. Moreover, mosquito cell lines are generally permissive to arbovirus infection, providing a useful tool to study *Wolbachia*-arbovirus interaction at a finer scale [21,35,51]. In adult mosquito, during the Extrinsic Incubation Period (EIP), the virus infects essentially somatic tissues including midgut and salivary glands [57] 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 [51,58]. 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 [51]. However, we observed a significant CHIKV interference in C6/36_ *wAlbB* at a relative *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* [51]. 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 *Ae. albopictus* salivary glands [25] as well in *Ae. aegypti* tissues, 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 [59]. It was recently shown that C6/36 lacks a functional siRNA mechanism [60], 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 [61,62]. 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 [63], another potential explanation for its antiviral activity as iron load is involved in the modulation of innate immunity [64]. 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 [65], 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* [34,66–68]. 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 *A. aegypti* midgut [69]. 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 [70]. Mosquito vector competence for arboviruses depends on multiple factors such as mosquito genotype, virus genotype and their interaction [71] but also temperature [72,73] or mosquito microbiota [74]. Recent studies showed that pathogen blocking by *Wolbachia* was influenced by temperature [75] and that bacteria from the genus *Asaia* can inhibit vertical transmission of *Wolbachia* in *An. gambiae* [76]. 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.

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