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Selective Elimination of *Wolbachia* from the Leafhopper *Yamatotettix flavovittatus* Matsumura

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Abstract

Wolbachia infections affect the reproductive system and various biological traits of the host insect. There is a high frequency of *Wolbachia* infection in the leafhopper *Yamatotettix flavovittatus* Matsumura. To investigate the potential roles of *Wolbachia* in the host, it is important to generate a non-*Wolbachia*-infected line. The efficacy of antibiotics in eliminating *Wolbachia* from *Y. flavovittatus* remains unknown. This leafhopper harbors the mutualistic bacterium *Candidatus* Sulcia muelleri, which has an important function in the biological traits. The presence of *Ca.* S. muelleri raises a major concern regarding the use of antibiotics. We selectively eliminated *Wolbachia*, considering the influence of antibiotics on leafhopper survival and *Ca.* S. muelleri prevalence. The effect of artificial diets containing different doses of tetracycline and rifampicin on survival was optimized; high dose (0.5 mg/ml) of antibiotics induces a high mortality. A concentration of 0.2 mg/ml was chosen for the subsequent experiments. Antibiotic treatments significantly reduced the *Wolbachia* infection, and the *Wolbachia* density in the treated leafhoppers sharply declined. *Wolbachia* recurred in tetracycline-treated offspring, regardless of antibiotic exposure. However, *Wolbachia* is unable to be transmitted and restored in rifampicin-treated offspring. The dose and treatment duration had no significant effect on the infection and density of *Ca.* S. muelleri in the antibiotic-treated offspring. In conclusion, *Wolbachia* in *Y. flavovittatus* was stably eliminated using rifampicin, and the *Wolbachia*-free line was generated at least two generations after treatment. This report provides additional experimental procedures for removing *Wolbachia* from insects, particularly in host species with the coexistence of *Ca.* S. muelleri.

Introduction

Numerous insects harbor intracellular bacteria, which are commonly categorized as obligate primary symbionts (P-symbionts) and facultative or secondary symbionts (S-symbionts). P-symbionts are mutualistic and are essential for host survival and development. In contrast, the S-symbionts interact in broader ways, ranging from mutualism to parasitism. They are not critical for host survival but play an important biological role [1, 2]. Among the S-symbionts, the members of the genus *Wolbachia*, which infect 20–70% of all insect species [3]. *Wolbachia* plays various roles in their hosts, including inducing abnormalities in the reproductive system and affecting biological traits [4–7]. Induced phenotypes, such as cytoplasmic incompatibility, reduction of pathogen transmission in insect vectors, and induction of deleterious effects on host fitness, could be used for developing novel control strategies against insect pests [8–10]. Therefore, investigating the roles of *Wolbachia* in insect hosts could expand the current knowledge on insect–bacteria interactions and allow us to exploit *Wolbachia* as a potential control agent.

To explore the role of *Wolbachia* within the host, the target traits of the *Wolbachia*-infected and non-infected insect lines with identical genotypes or genetic backgrounds should be evaluated [11, 12]. *Wolbachia* infections can be eliminated in *vivo* using antibiotics, and this method was used to establish a non-*Wolbachia*-infected insect lineage. Tetracycline and rifampicin are widely used, and *Wolbachia* has been successfully eliminated in various insect hosts, such as

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fruit flies, beetles, mosquitoes, wasps, whiteflies, and planthoppers [13–18]. However, the efficiency of antibiotics in eliminating *Wolbachia* varies and is highly dependent on insect species, type and doses of antibiotics, and treatment duration [19]. In addition, a major concern regarding the use of antibiotics is the coexistence of other bacterial symbionts within the individual host species. The antibiotics could affect the other bacteria in the host insects and, thus, could have direct effects on the biology of host insects [20].

The leafhopper *Yamatotettix flavovittatus* Matsumura (Hemiptera: Cicadellidae) is an important insect pest of sugarcane in Southeast Asia because it is a phytoplasma transmitter that causes white leaf disease [21–23]. *Wolbachia* is abundant in populations of *Y. flavovittatus*, and the influence of *Wolbachia* infection on some leafhopper traits was investigated [24]. Previous reports used different lineages with different genotypes originating from different geographical locations. Therefore, the traits may be partially influenced by the differences in the genetic backgrounds of the leafhoppers. In addition, important questions on the induced phenotypes, such as whether *Wolbachia* infections are related to pathogen transmission, remain unanswered. Therefore, it is important to obtain a non-*Wolbachia*-infected lineage and minimize the differences in the genetic background of the hosts. The selectivity and efficacy of antibiotics in *Wolbachia* elimination for establishing a non-infected lineage are needed in the leafhopper *Y. flavovittatus*.

Y. flavovittatus typically harbors two types of the P-symbionts: *Candidatus* Sulcia muelleri (Bacteroidetes) and *Candidatus* Yamatotia cicadellidicola (Gammaproteobacteria) [25]. The presence of P-symbionts in the hosts raises a major concern regarding the use of antibiotics. In particular, the bacterium *Ca.* S. muelleri is well known for providing essential nutrients and is necessary for host survival and development [26, 27]. Therefore, we aimed to determine the efficacy of antibiotics (tetracycline and rifampicin) for the removal of *Wolbachia* infection from *Y. flavovittatus*. In addition, the effect of antibiotics on the infection and density of the P-symbiont was evaluated. The co-existing bacterium *Ca.* S. muelleri was targeted as it plays a crucial role in influencing the life history traits of the leafhopper.

Material and Methods

Leafhopper Collection and Rearing

Adult *Y. flavovittatus* were collected by setting light traps in sugarcane plantations located in the Udon Thani Province of Thailand. The natural population of this lineage has a high prevalence of *Wolbachia* [24]. Some of the specimens were immersed in absolute ethanol and stored at − 20 °C until DNA extraction. In addition, some of the adults were kept in plastic cages and transferred to the laboratory. For mass rearing, the leafhoppers were maintained in sugarcane plant cages (10 males and 10 females per cage, total 10 cages), where they were allowed to mate and females laid their eggs. After the new generation emerged, leafhoppers from this stock were used for studying the effect of antibiotics solutions. The presence of *Wolbachia* and *Sulcia* was evaluated in both the natural populations and new generation that emerged in the laboratory to confirm infection status prior to the experiments.

Effect of Antibiotics on Survival

Artificial feeding through a parafilm membrane was used as described earlier [28], for delivering antibiotics to the adult *Y. flavovittatus* leafhoppers. A plastic tube (5 cm diameter and 10 cm height) that was open at both ends was used as the feeding chamber. The top end was covered with a layer of stretched parafilm; the artificial diet (0.2 ml) was dropped on the outer surface, and a layer of parafilm was used to wrap the solution. A fresh sugarcane leaf was placed on the upper layer to attract the leafhoppers to feed on the diet solution. The bottom end was covered with two layers of parafilm with a small hole to release the leafhoppers into this chamber.

The control feeding solution contained 5% sucrose (w/v) in 5 mM phosphate buffer (pH 7.0). The antibiotic treatments contained the same solution with the addition of a series of different concentrations of tetracycline or rifampicin (0.1, 0.2, and 0.5 mg/ml). The feeding time was 96 h for all the concentrations. Newly emerged adult *Y. flavovittatus* leafhoppers were introduced into each feeding chamber through the lower open end. The experiment used 10 leafhoppers (5 males and 5 females) per chamber, with a total of 6 chambers (replications) for each treatment. After feeding, as scheduled, leafhoppers were transferred to the sugarcane plant cages for maintenance. The effect of artificial feeding on the survival of the leafhoppers was determined based on the survival at 10-day intervals until 30 days. Suitable concentrations of the artificial solutions were selected for further experiments.

Wolbachia Elimination Using Antibiotic Treatments and Specimen Sampling

A suitable concentration of antibiotics was selected based on its effect on leafhopper survival; then a dose of 0.2 mg/ml was chosen for subsequent experiments. The newly emerged adult *Y. flavovittatus* leafhoppers were set up for artificial feeding through a parafilm membrane as described above. Sixty males and females were used for each feeding treatment, which included 0.2 mg/ml of tetracycline, 0.2 mg/ml rifampicin, and the control without antibiotics. The

populations that were directly exposed to the solutions were designated as G1. After feeding, they were maintained in sugarcane plant cages and allowed to mate, and the females laid their eggs normally. During this period, random specimens were sampled at 10, 20, and 30 days (about 10–15 of male and female leafhoppers for each age stage).

Fresh nymphal instars that emerged from G1 parents were immediately separated and reared on sugarcane plants throughout the developmental stages. These populations were designated as G2, and they were reared similarly to obtain the G3 generation. The adults of G2 and G3 generations were sampled at 10, 20, and 30 days old, similar to the G1 generation (at least 10 individual leafhoppers for males and females). The collected leafhoppers were kept in absolute ethanol and stored at − 20 °C until DNA extraction.

DNA Extraction

Insect DNA was extracted using the phenol–chloroform method [29] with minor modifications for leafhoppers, as described previously [24]. DNA quantity was measured using a Nanodrop spectrophotometer (NanoDrop Lite; Thermo Scientific). The concentration of genomic DNA in the specimens was adjusted to 50 ng/µl and stored at − 20 °C until analysis.

Diagnostic PCR

The leafhoppers were tested for the presence of *Wolbachia* using PCR with specific primers that amplify the 610-bp *Wolbachia* surface protein-encoding gene (*wsp*). The forward primer was 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and the reverse primer was 961R (5′-AAAAATTAAACGCTACTCCA-3′) [30]. The PCR conditions used were as described previously [24]. The prevalence of *Ca.* S. muelleri was detected using the specific primers of 16S rRNA gene: forward primer 10CFBFF (5-AGAGTTTGATCATGGCTCAGGATG-3) and the reverse primer 1515R (5-GTACGGCTACCTTGTTACGACTTAG-3) [31]. The PCR conditions used were as described previously [25]. In brief, reactions were performed in 25 µl final volume comprising the following components: 2 µl DNA template, 1 × reaction buffer, 2.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM dNTPs, and 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were visualized on 1% agarose gels, and the DNA bands were stained using SYBR Safe DNA Gel Stain (Invitrogen).

Construction of qPCR Standard Curves

The *wsp* gene of *Wolbachia* and the 16S rDNA gene of *Ca.* S. muelleri were amplified using the respective primer sets. The *wsp*-specific primers were the forward *w*Yfla-F (5′-GGTGTTGGTGCAGCGTATGT-3′) and the reverse *w*Yfla-R (5′-TCCGCCATCATCTTTAGCTGT-3′), which were used to amplify a 198-bp fragment of *wsp* [24]. For *Sulcia*, specific primers were designed based on the 16S rDNA gene of *Ca.* S. muelleri from *Y. flavovittatus* (accession number MH678721). The Primer-BLAST at NCBI was used to design the forward SulYfla-F (5′-CGTTCCCCCACATTGGTACT-3′) and the reverse SulYfla-F (5′-CGACTGCTGGCACAGAGTTA-3′) primers which were used to amplify a 225-bp fragment. The fragments were amplified using PCR as described above (except for an annealing and extension of 30 s each). The amplicons were cloned into a pCR™4-TOPO® TA vector, and the recombinant plasmids were transformed into TOP10 competent cells (TOPO-TA cloning kit; Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Plasmid DNA was purified using the Purelink Quick Plasmid Miniprep Kit (Life Technologies). The concentration of the plasmids was determined using a Nanodrop spectrophotometer, and the copy numbers of *wsp* or 16S rRNA gene fragments were calculated using an established equation [32]. A standard curve was generated using the plasmids containing the target sequence, at five serial dilutions (10^7−10^3 copies).

Quantitative Real-Time PCR (qPCR)

Leafhoppers from previous experiment that were PCR-positive for *Wolbachia* were used to quantify the *wsp* gene. Four to five individual male and female leafhoppers at 10, 20, and 30 days old were selected from each treatment. The leafhoppers that were negative in the PCR reaction or the treatment that had insufficient specimens (less than four individual leafhoppers) was excluded from the qPCR analysis. For analysis of *Ca.* S. muelleri density, the leafhoppers that were PCR-positive were sufficient for quantification. The 16S rRNA gene of *Ca.* S. muelleri was quantified in five individual males and females per age stage for each treatment.

qPCR was performed using the Applied Biosystems StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA). Absolute quantification was conducted as described previously [33], with minor modifications. In brief, reactions were performed in a final volume of 20 µl consisting of 1 µl (final 50 ng) template DNA, 0.5 µl (0.5 M) of each primer, and 10 µl of SYBR Green Master Mix (Applied Biosystems). The cycling conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s, 55 °C for 30 s, and 60 °C for 30 s. The samples and

serial dilutions of the standards were distributed in duplicate wells. The reaction mixtures without DNA were used as negative controls for all amplifications. The copy numbers of *wsp* and 16S rDNA genes were quantified by comparing the Ct values (cycle threshold) against that in the serial dilutions of standards.

Bacterial 16S rRNA Gene Sequences in Tested Leafhoppers

To confirm the presence of the bacteria in the tested leafhoppers, diversity was screened by amplifying, cloning, and sequencing bacterial 16S rRNA genes using the universal primers 27F and 1513R [34]. This analysis used the DNA template from two individual specimens at 10 days old (one male and one female), which were selected from each treatment of first and second generation (total 12 individual specimens). The fragments of 16S rRNA genes were amplified by PCR as described above. PCR products were visualized on a 1% agarose gel; the positive samples were cloned, and plasmid DNA was purified as described above. Five recombinant plasmid clones were randomly selected from each leafhopper DNA template for sequencing, which was carried out by Bio Basic Inc. (Singapore). All sequences were compared with the sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database, using the basic local alignment search tool (BLAST).

Statistical Analysis

The survival percentage of the leafhoppers and the detection rate of bacteria were calculated. The statistical significance of the survival rate (alive = 1, death = 0) and the detection rate of bacteria (positive = 1, negative = 0) were tested. The distribution of *wsp* and 16S rRNA gene copies was evaluated using the Kolmogorov–Smirnov test. Data were normally distributed; therefore, data transformation before analysis was not performed. Statistically significant differences were determined using one-way analysis of variance (ANOVA), and the comparisons of the means were performed using Tukey's HSD test at a significance level of 0.05. All statistical analyses were performed using the IBM SPSS Statistics 20.

Sequence Accession Numbers

The 55 consensus sequences of the 16S rRNA bacterial genes from *Y. flavovittatus* were deposited into the GenBank database under the accession numbers OM489160-OM489214 Table S1.

Results

Optimization of Antibiotics Treatment

The status of bacterial infection in the natural population and the new generation that emerged in the laboratory was confirmed, and > 90% and > 95% of the individuals tested positive for *Wolbachia* and *Ca.* S. muelleri, respectively (data not shown).

To establish an experimental procedure for eliminating *Wolbachia* infection from *Y. flavovittatus*, we evaluated the effects of artificial diets containing varying doses of antibiotics on the survival of the leafhoppers. High doses of antibiotics, both tetracycline and rifampicin, affected the viability of the treated leafhoppers. The survival rates in the 0.5 mg/ml antibiotic treatments were significantly lower than those in the treatments with lower concentrations (0.1 and 0.2 mg/ml) and in the control populations ($P < 0.001$). However, there were no significant differences in the survival rates among the 0.1 mg/ml, 0.2 mg/ml, and the control treatments, in which approximately 68.33–90.00% of the insects survived (Table 1). Therefore, a concentration of 0.2 mg/ml of antibiotics was selected for the subsequent experiments.

Effect of Antibiotics on *Wolbachia* and *Ca.* S. muelleri Infection

The results of *wsp* gene detection in the first-generation (G1) leafhoppers (directly fed antibiotic-containing solution) and their offspring at the second and third generations (G2 and G3) are summarized in Table 2. In the control group of G1 leafhoppers, 90.00–100% of the insects were positive for *Wolbachia*. The *Wolbachia* infection in the antibiotics-fed leafhoppers decreased significantly; however, the infection rates were dependent on the type of antibiotics and the leafhopper's age ($P < 0.001$). In the tetracycline treatment, the *Wolbachia* infection rates were 25.29%, 52.00%, and 90.00% in the leafhoppers at 10, 20, and 30 days old, respectively. However, they were 57.69%, 69.57%, and 60.00%, respectively, at 10, 20, and 30 days in the rifampicin treatment (Table 2).

Similar trends were observed in G2, in which the *Wolbachia* infection rates in the antibiotic-treated offspring were significantly lower than that in the control populations ($P < 0.001$). In the tetracycline-treated offspring, infection rates were 20.00%, 45.00%, and 85.71% at 10, 20, and 30 days, respectively. The *Wolbachia* infections in the leafhoppers from the rifampicin-treated offspring were extremely low at 15.00%, 0%, and 20% at 10, 20, and 30 days, respectively (Table 2). There was a decreasing

Table 1 Effect of feeding artificial diet with different doses of antibiotics on the survival of *Y. flavovittatus* leafhoppers

Treatments	Concentration (mg/ml)	Total samples	Survival rates (%, mean ± SE)		
			10 days[1,2]	10 days[1,2]	10 days[1,2]
Control	No antibiotic	60	90.00 ± 8.94[a]	88.33 ± 7.53[a]	81.67 ± 4.08[a]
Tetracycline	0.1	60	76.67 ± 15.06[a]	73.33 ± 13.66[a]	68.33 ± 14.72[a]
	0.2	60	80.00 ± 12.65[a]	80.00 ± 12.65[a]	76.67 ± 12.11[a]
	0.5	60	36.67 ± 11.55[b]	33.33 ± 15.26[b]	30 ± 10.00[b]
F			6.12	6.41	8.22
P			< 0.001	< 0.001	< 0.001
Rifampicin	0.1	60	80.00 ± 8.94[a]	73.33 ± 12.11[a]	68.33 ± 14.72[a]
	0.2	60	86.67 ± 10.33[a]	81.67 ± 9.83[a]	73.33 ± 8.16[a]
	0.5	60	46.67 ± 5.77[b]	43.33 ± 5.77[b]	43.33 ± 5.77[b]
F			6.68	6.79	6.37
P			< 0.001	< 0.001	< 0.001

[1]Leafhopper's age

[2]Values in a column are compared between the treatments with each antibiotic and the control

[a,b]Indicate the values are statistical significant difference (Tukey HSD test)

trend in the *Wolbachia* infections in the G3 leafhoppers that emerged from the antibiotic-treated insects. Infection rates were significantly lower in all the antibiotic treatments compared to those in the control populations ($P < 0.001$). In the tetracycline-treated offspring, infection rates were 15%, 40%, and 50% at 10, 20, and 30 days, respectively; however, in the rifampicin-treated offspring, no *Wolbachia* was detected at 10 and 20 days, whereas 10% infection occurred at 30 days (Table 2).

Ca. S. muelleri prevalence in the individual leafhoppers was also determined. PCR revealed no significant differences in the levels of infection in the leafhoppers. Consistent results were found through the three generations; *Ca.* S. muelleri infection was at high frequencies, ranging from 90 to 100% of the individuals tested (Table 3).

Effect of Antibiotics on *Wolbachia* and *Ca.* S. muelleri Density

PCR analysis of the leafhoppers from the control population (without antibiotic treatment) showed clear visible bands on gel electrophoresis; this was an indication of *Wolbachia* infection. In addition, the invisible or faint bands on the samples from the antibiotic treatments were also considered as *Wolbachia* infection. Subsequently, the samples from the antibiotic-treated leafhoppers (four or five leafhoppers for each sex and age) were evaluated for *Wolbachia* density using qPCR. The treatments that were negative in the PCR reactions and the groups that had insufficient leafhoppers were excluded from the qPCR analysis. The copy number of *wsp* in the three generations is summarized in Fig. 1a–c.

Wolbachia density in the antibiotic-treated G1 leafhoppers was significantly lower than those in the control leafhoppers ($P < 0.001$) (Fig. 1a). The average number of *wsp* in the control was 5.80×10^4 copies and 6.92×10^4 copies in male and female leafhoppers, respectively. However, the titers in the antibiotic-treated insects decreased by approximately tenfold. The average copy number of *wsp* was 0.53×10^4 and 0.56×10^4 copies for males and females, respectively, in the tetracycline treatment; it was 0.93×10^4 and 0.52×10^4 copies for the males and females, respectively, in the rifampicin treatment.

Similar trends were found in the G2 leafhoppers that were not treated with antibiotics. The *Wolbachia* titers in the antibiotic-treated insects were significantly lower than those in the control group ($P < 0.001$) (Fig. 1b). The average number of *wsp* in the control was 5.59×10^4 copies (males) and 6.79×10^4 copies (females). In the tetracycline-treated offspring, average copy number of *wsp* was 2.36×10^4 copies (males) and 3.22×10^4 copies (females). However, *Wolbachia* could not be recovered in the leafhoppers from the rifampicin-treated offspring (> 90% negative for PCR test); therefore, the offspring was excluded from the qPCR analysis.

A similar trend was observed in the G3 leafhoppers; a significant difference in the *Wolbachia* titers among the treatment groups was observed ($P < 0.001$). *Wolbachia* titers were reestablished in the offspring of the tetracycline-treated offspring. The number of copies of *wsp* was 3.26×10^4 copies (males) and 5.36×10^4 copies (females). However, there was no recovery of *Wolbachia* in the leafhoppers from the rifampicin-treated offspring (almost 100% negative for PCR test); therefore, the samples were excluded from the qPCR analysis (Fig. 1c).

Ca. S. muelleri density was determined by quantifying the 16S rRNA gene in the G1, G2, and G3 generations, and

Table 2 Detection of *Wolbachia* in *Y. flavovittatus* leafhoppers

Generations/treatment[1]		*Wolbachia* infections								
		10 days			20 days			30 days		
		No. tested[2]	No. positive	Infection (%)[3]	No. tested	No. positive	Infection (%)	No. tested	No. positive	Infection (%)
First generation (G1)										
Control (without antibiotic)		15 M, 15F	15 M, 15F	100[a]	9 M, 12F	9 M, 11F	91.67[a]	12 M, 12F	10 M, 12F	91.67[a]
Tetracycline solution		14 M, 13F	2 M, 5F	25.29[c]	12 M, 13F	5 M, 8F	52.00[b]	10 M, 10F	8 M, 10F	90.00[a]
Rifampicin solution		13 M, 13F	9 M, 6F	57.69[b]	12 M, 11F	7 M, 9F	69.57[b]	10 M, 10F	7 M, 5F	60.00[b]
	F			27.37			7.897			8.491
	P			< 0.001			< 0.001			< 0.001
Second generation (G2)										
Offspring-control		10F, 11F	10F, 10F	95.23[a]	14F, 14F	14F, 14F	100[a]	11 M, 11F	11 M, 10F	95.45[a]
Offspring- tetracycline		12 M, 13F	1 M, 4F	20.00[b]	10 M, 10F	4 M, 5F	45.00[b]	10 M, 11F	7 M, 11F	85.71[a]
Offspring-rifampicin		10 M, 10F	0 M, 3F	15.00[b]	11 M, 10F	0 M, 0F	0.00[c]	10 M, 10F	3 M, 2F	20.00[b]
	F			46.68			81.39			31.01
	P			< 0.001			< 0.001			< 0.001
Third generation (G3)										
Offspring-control		10 M, 10F	10 M, 10F	100[a]	10 M, 10F	9 M, 9F	90[a]	10 M, 10F	10 M, 10F	100[a]
Offspring- tetracycline		10 M, 10F	0 M, 3F	15.00[b]	10 M, 10F	4 M, 4F	40[b]	10 M, 10F	6 M, 4F	50.00[b]
Offspring-rifampicin		10 M, 10F	0 M, 0F	0.00[b]	10 M, 10F	0 M, 0F	0[c]	10 M, 10F	1 M, 0F	10.00[c]
	F			130.02			192.11			57.00
	P			< 0.001			< 0.001			< 0.001

[1]First generation (G1) was directly fed antibiotics-containing artificial diet (0.2 mg/ml/96 h.). The offspring in the second (G2) and third (G3) generations are not treated with antibiotics

[2]Leafhopper sex, *M* male, *F* female

[3]Values in a column are compared between the treatments with each antibiotic and the control

[a,b,c]Indicate the values are statistical significant difference (Tukey HSD test)

Table 3 Detection of *Ca.* S. muelleri in *Y. flavovittatus* leafhoppers

Generations/treatment[1]		*Sulcia* infections								
		10 days			20 days			30 days		
		No. tested[2]	No. positive	Infection (%)[3]	No. tested	No. positive	Infection (%)	No. tested	No. positive	Infection (%)
First generation (G1)										
Control (without antibiotic)		15 M, 15F	14 M, 15F	96.67	9 M, 12F	8 M, 11F	90.48	12 M, 12F	10 M, 12F	91.67
Tetracycline solution		14 M, 13F	12 M, 13F	92.59	12 M, 13F	11 M, 13F	96.00	10 M, 10F	9 M, 10F	95.00
Rifampicin solution		13 M, 13F	12 M, 12F	92.31	12 M, 11F	12 M, 10F	95.65	10 M, 10F	8 M, 9F	90.00
	F			0.29NS			0.37NS			0.75NS
	P			0.747			0.69			0.473
Second generation (G2)										
Offspring-control		10F, 11F	10F, 11F	100	14F, 14F	12F, 14F	92.86	11 M, 11F	10 M, 10F	90.91
Offspring-tetracycline		12 M, 13F	11 M, 12F	92.00	10 M, 10F	8 M, 10F	90.00	10 M, 11F	10 M, 10F	95.24
Offspring-rifampicin		10 M, 10F	10 M, 9F	95.00	11 M, 10F	10 M, 9F	90.48	10 M, 10F	8 M, 10F	90.00
	F			0.83NS			0.07NS			0.21NS
	P			0.44			0.932			0.808
Third generation (G3)										
Offspring-control		10 M, 10F	10 M, 10F	100	10 M, 10F	10 M, 10F	100	10 M, 10F	9 M, 10F	95.00
Offspring-tetracycline		10 M, 10F	9 M, 10F	95.00	10 M, 10F	10 M, 10F	100	10 M, 10F	9 M, 9F	90.00
Offspring-rifampicin		10 M, 10F	10 M, 10F	100	10 M, 10F	8 M, 10F	90.00	10 M, 10F	9 M, 10F	95.00
	F			1.00NS			2.11NS			0.25NS
	P			0.374			0.13			0.774

[1]First generation (G1) was directly fed antibiotics-containing artificial diet (0.2 mg/ml/96 h.). The offspring in the second (G2) and third (G3) generations are not treated with antibiotics

[2]Leafhopper sex, *M* male, *F* female

[3]*NS* no significant difference at the 5% level (Tukey HSD test)

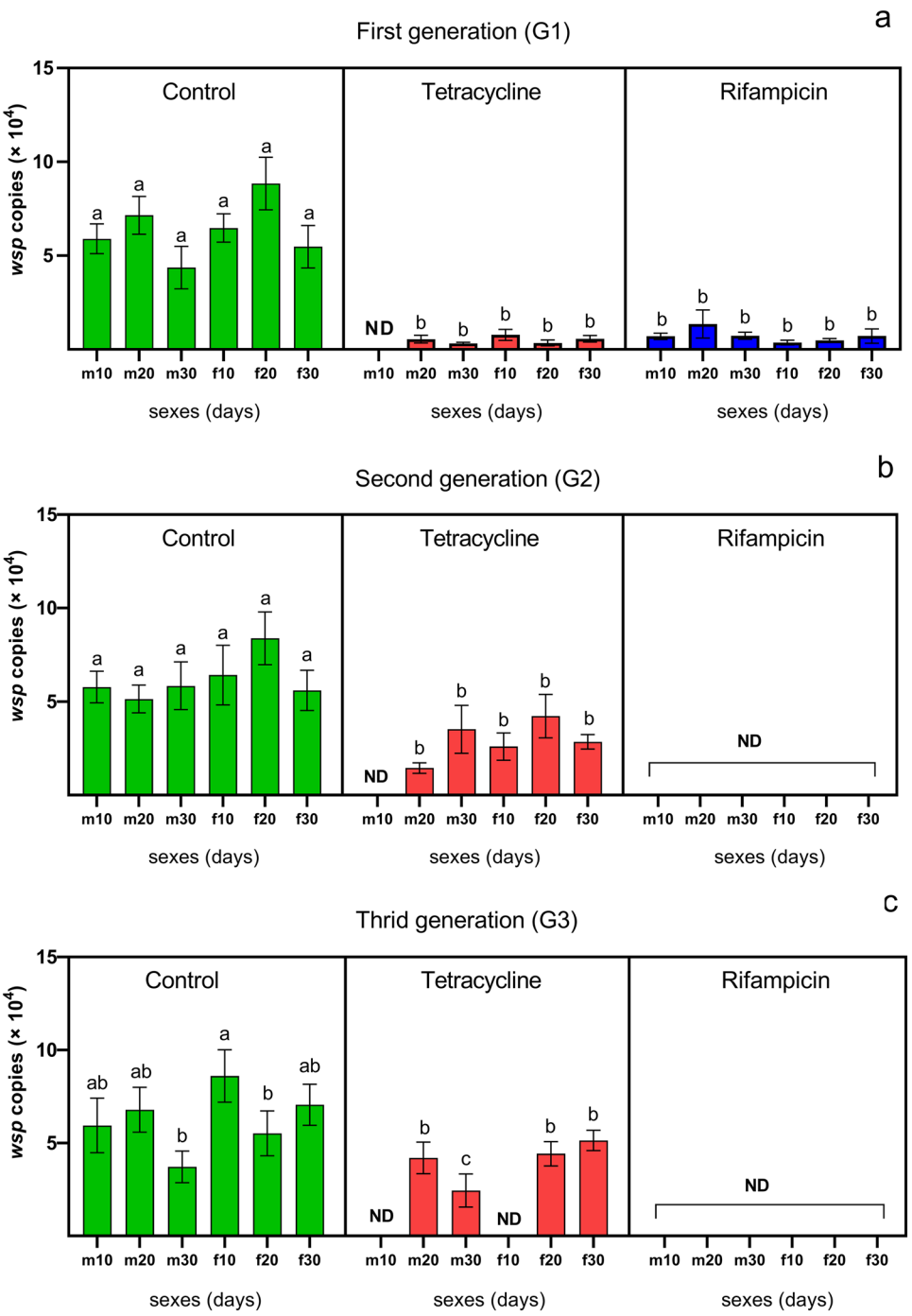

Fig. 1 *Wolbachia* density in *Y. flavovittatus* leafhoppers: **a** first-generation (G1) directly fed antibiotics-containing artificial diet (0.2 mg/ml/96 h), **b** second (G2) and **c** third (G3) generations are not treated with antibiotics. *m* male, *f* female; 10, 20, and 30 days old. Values represent the mean (± SE) of *wsp* gene copies per 50 ng of host genomic DNA. Different letters indicate significant difference determined using Tukey's HSD test (G1; $F = 8.12$, $P < 0.001$, G2; $F = 28.49$, $P < 0.001$, (G3; $F = 67.92$, $P < 0.001$). *ND* no data, due to insufficient specimen availability (less than four leafhoppers)

the results are summarized in Fig. 2a–c. Tetracycline treatment induced an increase in the *Ca.* S. muelleri density in the G1 females, unlike the effects on the *Wolbachia* density. The average copy numbers of 16S rDNA were 10.76×10^5, 9.46×10^5, and 12.37×10^5 copies in the female leafhoppers at 10, 20, and 30 days, respectively, which were significantly higher than those in the control populations ($P < 0.001$) (Fig. 2a). In the rifampicin-treated offspring, the average copy number of 16S rDNA was 1.98×10^5 and 3.21×10^5 copies for the male and female leafhoppers, respectively. This was lower (but not significantly) than that in the leafhoppers from the control populations, which showed 3.24×10^5 and 5.75×10^5 copies for male and female leafhoppers, respectively (Fig. 2a).

There were differences in the *Ca.* S. muelleri density among the treatments in the G2 and G3 leafhoppers (G2; $P = 0.02$, G3; $P < 0.001$). The variations in the density may be influenced by the age and sex of the leafhoppers, rather than by the antibiotics. In the G2 population, the highest density was found in the females of the

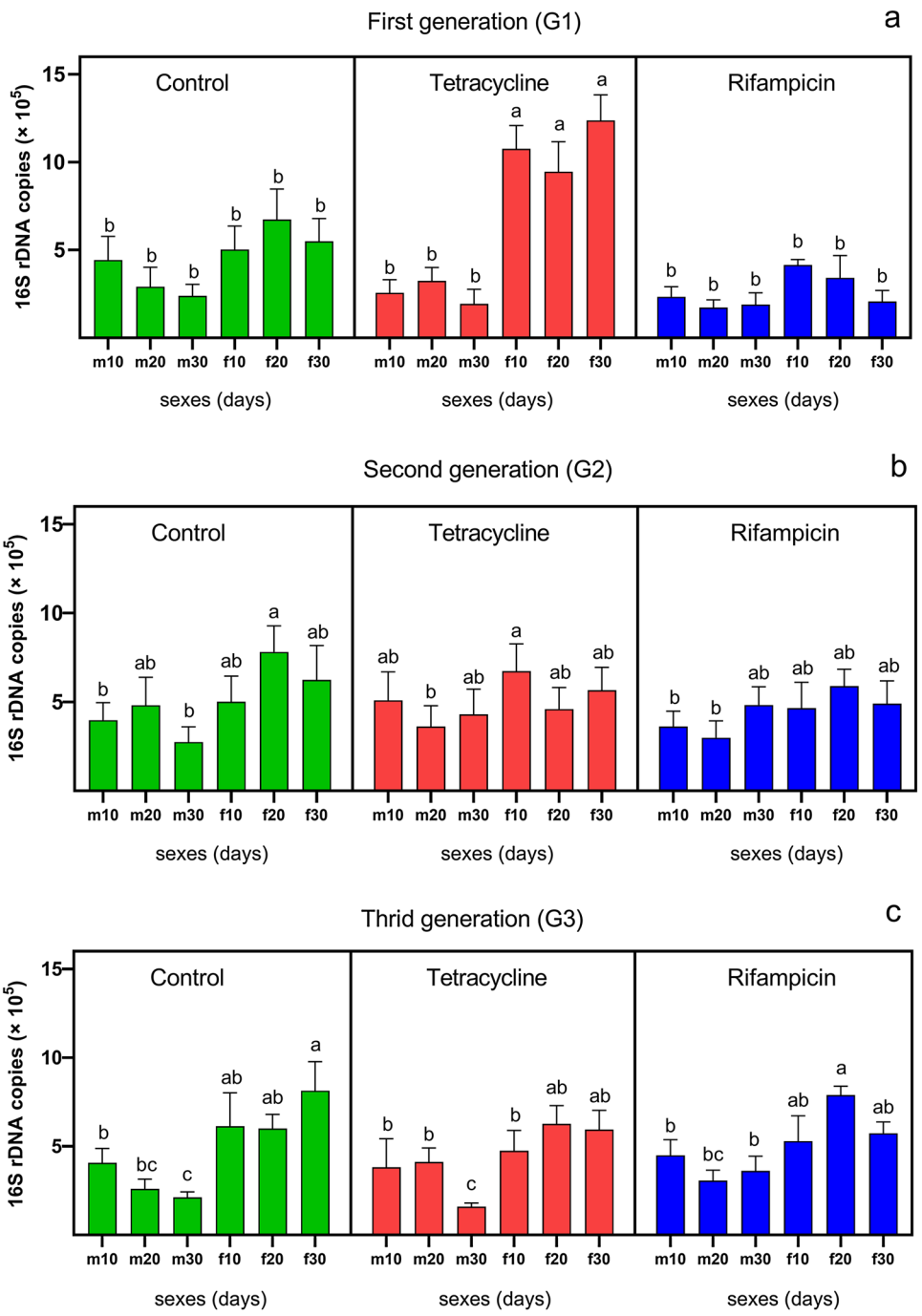

Fig. 2 *Ca.* S. muelleri density in *Y. flavovittatus* leafhoppers: **a** first-generation (G1) directly fed antibiotics-containing artificial diet (0.2 mg/ml/96 h), **b** second (G2) and **c** third (G3) generations are not treated with antibiotics. *m* male; *f* female; 10, 20, and 30 days old. Values represent the mean (±SE) of 16S rDNA gene copies per 50 ng of host genomic DNA. Different letters indicate significant difference determined using Tukey's HSD test (G1; $F=8.87$, $P<0.001$, G2; ($F=2.49$, $P=0.02$, G3; $F=4.48$, $P<0.001$)

control populations (20 days old) and tetracycline treatments (10 days old). However, the *Ca.* S. muelleri density was low in the male leafhoppers from the control and both antibiotic treatments (Fig. 2b). In the G3 population, a high density was found in the females from control populations (30 days old) and rifampicin treatments (20 days old), whereas a low density was found in the male leafhoppers from the control populations and tetracycline treatments (30 days old) (Fig. 2c).

16S rRNA Gene Sequencing

The types of bacteria infecting *Y. flavovittatus* were confirmed in representative specimens that were selected from

each treatment of G1 and G2 leafhoppers (10 days old). The 55 sequencing clones were obtained, and the remaining six clones had low-quality reads and omitted from the analysis. BLAST search results showed that two types of bacterial symbionts were found, including 39 clones identical to *Ca.* S. muelleri and 16 clones identical to *Ca.* Y. cicadellidicola. These were detected in most of the tested leafhoppers both in the antibiotic-treated and untreated groups (Table S1). However, *Wolbachia* was not detected during the 16S rRNA gene sequencing. This may be due to a low number of clones being randomly selected from DNA templates of individual leafhoppers.

Discussion

To identify the potential role of *Wolbachia* in their host insects, it is important to obtain a non-*Wolbachia*-infected lineage using antibiotics. However, the coexistence of other bacterial symbionts in the individual host species makes this challenging. In particular, the P-symbionts provide the hosts with essential nutrients and play a crucial function in determining the biological traits. Therefore, suitable evaluation of the types, concentrations, and period of exposure to antibiotic treatment is required to establish a *Wolbachia*-free line [19, 35]. To the best of our knowledge, this is the first report on an experimental procedure for eliminating *Wolbachia* infections from *Y. flavovittatus*. The results provide a practical method for establishing a non-*Wolbachia*-infected lineage. The subsequent generations following the rifampicin treatment (0.2 mg/ml, 96 h) are suitable for exploring the effect of *Wolbachia,* with minimal differences in the genetic background and confounding factors such as P-symbionts that may influence the interpretations of the *Wolbachia*–host interactions.

Our results indicate that high concentrations of tetracycline and rifampicin (0.5 mg/ml) immediately caused a high mortality. This could be attributed to the direct effect of the antibiotics on the insect host. In *Drosophila*, tetracycline treatment decreases ATP production and increases mtDNA density [20]. In the beetle, *Tribolium confusum* (Jacquelin du Val), rearing the insects on a diet containing tetracycline (5.0 and 10 mg/g) or rifampicin (1.0 mg/g) for one generation, caused a high mortality [36]. However, tetracycline treatment (100 µg/ml, 48 h) was not suitable for the whitefly *Bemisia tabaci* (Gennadius) because the antibiotics may act as an antifeedant in the insect [37]. In this study, the *Y. flavovittatus* leafhoppers survived artificial feeding with a concentration of 0.1 and 0.2 mg/ml of antibiotics. Therefore, a concentration of 0.2 mg/ml was used for evaluating *Wolbachia* elimination and the effect on the prevalence of *Ca.* S. muelleri.

The efficacy of the elimination of *Wolbachia* in the first generation of *Y. flavovittatus* was similar for tetracycline and rifampicin treatments. In the leafhoppers that were directly treated with antibiotics, *Wolbachia* infections were reduced, but not completely removed. There were residual amounts of *Wolbachia*, but the titers in both antibiotic treatments were significantly lower than that in the corresponding control populations. Similarly, *Wolbachia* could not be completely eliminated from *B. tabaci*, using 50 µg/ml tetracycline (48 h) [37], and from the wasp *Encarsia Formosa* Gahan, using 10–50 mg/ml tetracycline for one generation [38]. This could be attributed to the insufficient concentration and period of antibiotic exposure, leading to a reduction, but not total elimination of *Wolbachia*. The duration of antibiotic exposure required to completely remove *Wolbachia* from the hosts could be a few days or an entire lifetime [19]. *Wolbachia* was completely removed from the spider mite *Tetranychus piercei* McGregor by administering tetracycline (1 mg/ml) for four generations [39], and from the springtail *Folsomia candida* Willem by continuous exposure to 2.7% rifampicin over two generations and several weeks [40]. Longer periods of exposure have higher efficacy; however, the appropriate exposure period depends on the insect species because some insects are too weak to withstand and persist in artificial feeding systems with antibiotic exposure for a long time.

To allow colonies to recover from the potential direct effects of antibiotics, the treated specimens are maintained for a number of generations prior to use for studying *Wolbachia*–host interactions. It is necessary to investigate stable elimination; therefore, the infection rates and titers of *Wolbachia* in *Y. flavovittatus* leafhoppers were continuously investigated in the immediate two generations. In the G2 and G3 leafhoppers, the variation in infection levels and titers of *Wolbachia* were highly dependent on the type of antibiotics. *Wolbachia* was likely transmitted and restored in the tetracycline-treated offspring. Recovery was reported in the filarial nematode *Brugia pahangi*, in which using rifampicin treatment significantly reduced *Wolbachia* titers; however, after 8 months, the titers rebounded to normal levels. During this period, *Wolbachia* was observed within the ovaries, which allows the bacteria to persist and repopulate in ovarian tissues, then transmitted to following generations [41]. This may be an explanation for the restoration of *Wolbachia* in the tetracycline-treated offspring from this study. In the *Y. flavovittatus* leafhoppers, *Wolbachia* localized in the egg and was concentrated in the bacteriomes of the nymph and adult, thereby vertically transmitted in *Y. flavovittatus* [24]. In the present study, *Wolbachia* density was reduced but not completely to zero following antibiotic treatment. Although localization was not tested, we believe that vertical transmission through the egg is the origin of *Wolbachia* restoration and transmission to the following generations.

However, different antibiotic types and doses had differential efficacy for the elimination of bacteria in host insects. For example, in the flour beetle *T. confusum,* complete removal of *Wolbachia* required 3.0 to 10 mg/g of tetracycline, whereas it required 0.1 to 0.5 mg/g of rifampicin[36]. Similarly, *Wolbachia* was removed from springtail *F. candida* through the use of rifampicin treatment, but not with tetracycline when applied at the same concentration [40]. These could be attributed to the different mechanisms or modes of action of antibiotics. Tetracycline inhibits protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome [19, 42]. Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells, therefore preventing transcription of messenger RNA and the subsequent translation to protein [19, 43]. This difference in mechanism could be the reason for rifampicin's efficacy in stable elimination and the interference with the recurrence of *Wolbachia* infection in *Y. flavovittatus* leafhoppers. This treatment resulted in low levels of *Wolbachia* infection and density in G1. Moreover, there was a great reduction in the *Wolbachia* infection rates in their offspring (G2 and G3), even though they were not exposed to this antibiotic. However, there was negligible transmission of *Wolbachia* to the offspring of rifampicin-treated insects.

The differential efficacy or suitability of selective antibiotics depends on the host insect species, *Wolbachia* strains, and the interaction between these factors [28, 44], which contribute to the varying levels of resistance and recovery. In contrast with our study, tetracycline treatments in the planthopper *Laodelphax striatellus* (Fallen) were effective in removing *Wolbachia*; it was entirely cured and was not restored until 10 generations post-treatment [45]. However, the treatment period was much longer than that in our experiment at five generations of the entire nymphal stage of the planthopper *L. striatellus*.

Considering the effect of antibiotics on the coexistent bacterium symbiont, we suggest that after antibiotic treatments, it has no effect on the prevalence on the *Ca.* S. muelleri which was confirmed by PCR detection. However, tetracycline-treated leafhoppers, in particular the females, had twice the *Ca.* S. muelleri density. This irregularity has not been reported previously, we hypothesized two possible reasons to explain this. First, tetracycline might hinder the growth of other microorganisms that are antagonistic to *Ca.* S. muelleri*,* therefore imparting a proliferation advantage without any regulated mechanisms. Second, under the stress conditions from tetracycline treatment, there might be an overproduction of the *Ca.* S. muelleri strain in the *Y. flavovittatus* leafhoppers. Further investigations are required to elucidate the actual mechanism. In the subsequent generations following the antibiotics treatment, there was a restoration of *Ca.* S. muelleri density to normal levels. We believe that this is related to the coevolution and function of this bacterium, which are discussed below.

In addition, the 16S rRNA gene sequencing approach was used to confirm the presence of *Ca.* Y. cicadellidicola, which is one of the common bacteria in this leafhopper. The result obtained from this study was consistent with previous reports [25], in which *Ca.* Y. cicadellidicola was observed in the leafhoppers sampled from both the antibiotics-treated and untreated groups. Because the exact function of *Ca.* Y. cicadellidicola in the host is currently unknown, the infection levels and density of this bacterium were not tested in the present study. For increased accuracy, further studies on the effect of antibiotics on infection and density of *Ca.* Y. cicadellidicola are needed if the exact function is clarified in the future.

The suitable antibiotics for *Wolbachia* elimination that have no effect on the coexistence of P-symbionts was reported. For example, *Wolbachia* was completely inactivated from the whitefly, *B. tabaci* (MED) using rifampicin, without affecting the P-symbiont, *Portiera aleyrodidarum* [46]. Similarly, antibiotics eliminate *Wolbachia* and *Arsenophonus*, with an efficacy of 50–80%, but without significant impact on the P-symbiont *P. aleyrodidarum* in the whitefly *B. tabaci* [28]. Different types of intracellular bacteria in insects could lead to differential responses to antibiotic treatment [47]. However, the differences between *Wolbachia* and *Ca.* S. muelleri in their resistance to antibiotics could be attributed to the varied aspects of coevolution with the host [12]. *Ca.* S.muelleri is a mutualistic obligate symbiont that is closely associated and has a long-term evolutionary history with host insects [26]. In addition, *Ca.* S. muelleri provides the majority of essential amino acids, and it is required for the growth and development of host insects [48]. Therefore, even under stress, *Ca.* S. muelleri can persist in an appropriate range or it is protected by the hosts. Facultative symbionts, such as *Wolbachia*, have a more recent origin in the hosts [49], resulting in *Wolbachia* being eliminated more easily than *Ca.* S. muelleri during antibiotic treatments. However, there are exceptions, depending on the host species and their associated microorganisms. Disruption of P-symbionts using antibiotics is possible with the proper types, concentrations, and treatment durations. For instance, the density of the P-symbionts, *Ca.* S. muelleri, and *Candidatus* Nasuia deltocephalinicola significantly decreases following tetracycline treatment during entire nymphal instars in the leafhopper *Nephotettix cincticeps* (Uhler) [50]. However, if dose and time of antibiotic exposure increase, these may have an effect on *Ca.* S. muelleri and other common bacteria in the *Y. flavovittatus* leafhoppers.

This study has a few limitations: (1) the method of administering antibiotics to the leafhoppers using a parafilm membrane could result in the uptake of insufficient nutrients

during the feeding on artificial diets and, therefore, affect biological traits. It is essential to develop other methods of antibiotic delivery such as using plant cultures in antibiotic solutions. (2) The stable elimination of *Wolbachia* was investigated only in two generations following the exposure to the antibiotics; accurate results could be achieved by increasing the number of generations following treatment.

Conclusions

Treating *Y. flavovittatus* leafhoppers with 0.2 mg/ml of rifampicin for 96 h could reduce *Wolbachia* infection and density in the treated generation. *Wolbachia* could not be transmitted and restored in two subsequent generations, even though they had no direct exposure to the antibiotics. Rifampicin had no significant effect on the infection density of the co-existing mutualistic bacterium *Ca.* S. muelleri. Stable elimination resulted in a non-*Wolbachia*-infected line, which was obtained at least two generations after treatment. The non-infected line could be used to explore the potential role of individual *Wolbachia* in *Y. flavovittatus*, such as inducing cytoplasmic incompatibility, pathogen transmission capability, and other biological traits. However, the effects of antibiotic treatments on other microorganisms, in particular, those impacting biological traits could act as confounding factors that may influence the explanations of *Wolbachia*-host interaction. We suggest that for increased reliability, the *Wolbachia*-induced phenotypes should be further investigated alongside other analysis, i.e., basic mechanisms underlying, gene expression analysis, as well as whole genome sequencing of *Wolbachia*.

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Code Availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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