Colonization of the tsetse fly midgut with commensal *Kosakonia cowanii* Zambiae inhibits trypanosome infection establishment

Brian L. Weiss*1*, Michele A. Maltz*2*, Aurélien Vigneron1, Yineng Wu1, Katharine S. Walter1, Michelle B. O’Neill1, Jingwen Wang1, Serap Aksoy*1*

1 Yale School of Public Health, Department of Epidemiology of Microbial Diseases, New Haven, Connecticut, United States of America, 2 Southern Connecticut State University, New Haven, Connecticut, United States of America

☯ These authors contributed equally to this work.
* brian.weiss@yale.edu (BLW); serap.aksoy@yale.edu (SA)

Abstract

Tsetse flies (*Glossina* spp.) vector pathogenic trypanosomes (*Trypanosoma* spp.) in sub-Saharan Africa. These parasites cause human and animal African trypanosomiases, which are debilitating diseases that inflict an enormous socio-economic burden on inhabitants of endemic regions. Current disease control strategies rely primarily on treating infected animals and reducing tsetse population densities. However, relevant programs are costly, labor intensive and difficult to sustain. As such, novel strategies aimed at reducing tsetse vector competence require development. Herein we investigated whether *Kosakonia cowanii* Zambiae (*Kco_Z*), which confers *Anopheles gambiae* with resistance to *Plasmodium*, is able to colonize tsetse and induce a trypanosome refractory phenotype in the fly. *Kco_Z* established stable infections in tsetse’s gut and exhibited no adverse effect on the fly’s survival. Flies with established *Kco_Z* infections in their gut were significantly more refractory to infection with two distinct trypanosome species (*T. congoense*, 6% infection; *T. brucei*, 32% infection) than were age-matched flies that did not house the exogenous bacterium (*T. congoense*, 36% infected; *T. brucei*, 70% infected). Additionally, 52% of *Kco_Z* colonized tsetse survived infection with entomopathogenic *Serratia marcescens*, compared with only 9% of their wild-type counterparts. These parasite and pathogen refractory phenotypes result from the fact that *Kco_Z* acidifies tsetse’s midgut environment, which inhibits trypanosome and *Serratia* growth and thus infection establishment. Finally, we determined that *Kco_Z* infection does not impact the fecundity of male or female tsetse, nor the ability of male flies to compete with their wild-type counterparts for mates. We propose that *Kco_Z* could be used as one component of an integrated strategy aimed at reducing the ability of tsetse to transmit pathogenic trypanosomes.

Author summary

Tsetse flies transmit pathogenic African trypanosomes, which are the causative agents of socio-economically devastating human and animal African trypanosomiases. These
diseases are currently controlled in large part by reducing the population size of tsetse vectors through the use of insecticides, traps and sterile insect technique. However, logistic and monetary hurdles often preclude the prolonged application of procedures necessary to maintain these control programs. Thus, novel strategies, including those aimed at sustainably reducing the ability of tsetse to transmit trypanosomes, are presently under development. Herein we stably colonize tsetse flies with a bacterium (Kosakonia cowanii Zambiae, Kco_Z) that acidifies their midgut, thus rendering the environment inhospitable to infection with two distinct, epidemiologically important trypanosome strains as well as an entomopathogenic bacteria. In addition to inducing a trypanosome refractory phenotype, colonization of tsetse with Kco_Z exerts only a modest fitness cost on the fly. Taken together, these findings suggest that Kco_Z could be applied to enhance the effectiveness of currently employed tsetse control programs.

Introduction

Insects transmit numerous vertebrate pathogens that cause devastating disease throughout tropical and subtropical regions around the globe. The lack of effective and affordable vaccines, coupled with insect and pathogen resistance to pesticides and drug treatments, respectively, severely limits disease control. Many vertebrate pathogens are acquired by insect vectors via the ingestion of an infectious blood meal. The disease causing agents must then establish an infection in the insect’s gut prior to being transmitted to a new vertebrate host during a subsequent bite. In most cases pathogens are eliminated from the insect vector prior to transmission to a new vertebrate host. This outcome reflects the presence of dynamic active and passive immune barriers that function locally in the insect gut and systemically in the hemocoel [1–3].

Although few insect vectors support transmissible infections with vertebrate pathogens, all house symbiotic microorganisms in their gut that influence numerous aspects of their host’s physiological homeostasis. Symbiotic associations between arthropod disease vectors and enteric bacteria have been particularly well-studied in an effort to determine how these microbes influence their host’s ability to transmit disease [4–8]. Tsetse flies, which are the prominent vectors of pathogenic African trypanosomes, house a taxonomically diverse enteric microbiota that includes endosymbiotic Wigglesworthia and Sodalis [9] as well as an assemblage of bacteria obtained from the fly’s environment [10–12]. Both Wigglesworthia and Sodalis are maternally transmitted to developing intrauterine larvae during tsetse’s unique mode of viviparous reproduction [9,13]. Wigglesworthia influences trypanosome infection establishment in tsetse by regulating the production of trypanocidal PGRP-LB [14,15]. Additionally, tsetse that undergo larval development in the absence of this bacterium fail to synthesize a gut-associated peritrophic matrix during adulthood [16]. This structure is an important mediator of tsetse’s vector competence because it serves as a physical barrier that ingested parasites must traverse in order to successfully colonize the fly’s gut [17] and subsequently the salivary glands for transmission in saliva [18]. Sodalis’ impact on tsetse vector competency is less known, although studies suggest that a positive correlation exists between the prevalence and density of this bacterium and trypanosome infection prevalence [19–23]. Like mosquitoes, tsetse’s gut also harbors a diverse population of bacteria obtained from the fly’s environment [10–12]. However, the effect of these bacteria on tsetse vector competency is poorly understood.

Mosquitoes, including Anopheles gambiae and Aedes aegypti, also house bacteria in their gut, and these microbes play a significant role in the ability of their host to transmit vertebrate pathogens. Boissiere et al. [24] discovered a positive correlation between the density of enteric
Enterobacteriaceae and *Plasmodium* infection prevalence in field-captured *An. gambiae*. These midgut microbes, as well as the enteric microbiota found in *Ae. aegypti*, indirectly regulate their host’s vector competency by modulating basal expression of genes that encode anti-*Plasmodium* and anti-dengue effector proteins [5,25,26]. Other members of the mosquito enteric microbiota exert direct effects on their host’s vector competency. Specifically, a *Chromobacterium* isolated from *Ae. aegypti* secretes factors that exhibit anti-*Plasmodium* and anti-Dengue activity [27]. Also, laboratory reared *A. gambiae* present an abnormal *Plasmodium* refractory phenotype when their guts are colonized with *Kosakonia cowanii* Zambiae, (*Kco_Z*; previously designated *Enterobacter* sp Zambiae, *Esp_Z* [28]) that had been isolated from field-captured mosquitoes. *Kco_Z* was determined to produce reactive oxygen intermediates (ROIs) that exhibit direct anti-*Plasmodium* properties [29,30].

In this study we investigated whether *Kco_Z* isolated from *A. gambiae* is able to successfully colonize tsetse’s gut and induce parasite and pathogen refractory phenotypes in the fly. We found that this bacterium can reside stably in tsetse’s midgut without imparting a detrimental fitness cost on the fly. *Kco_Z* colonized tsetse present an acidified midgut environment that is inhospitable to both African trypanosomes and entomopathogenic *Serratia marcescens*. We discuss the potential utility of *Kco_Z* as a novel component of currently used area wide integrated pest management strategies aimed at controlling tsetse populations and thus transmission of African trypanosomes.

**Results**

**Bacterial infection outcomes in tsetse’s midgut, and subsequent fly survival**

We investigated the ability of *Kco_Z* and *Sodalis* (as a control) to colonize the gut of both wild-type (hereafter referred to as ‘*Gmm^WT*’) and symbiont-free tsetse (aposymbiotic, hereafter referred to as ‘*Gmm^Apo*’). *Gmm^WT* flies were used to interrogate the interaction between *Kco_Z* and the natural tsetse microbiota, while the use of *Gmm^Apo* individuals allowed us to correlate the presence of distinct, experimentally introduced bacterial taxa with specific fly phenotypes. We challenged all flies *per os* with 1x10^3 CFU of either *Kco_Z* or *Sodalis* in their first blood meal and then monitored bacterial proliferation over a 28 day period. By 7 days post-inoculation, midgut bacterial load in *Gmm^WT* flies that housed *Kco_Z* (*Gmm^WT/Kco_Z*) and *Sodalis* (*Gmm^WT/Sgm*) was 1.9x10^6 ± 6.4x10^5 CFU and 4.5x10^5 ± 6.4x10^5 CFU, respectively, and *Gmm^Apo/Kco_Z* (9.3x10^5 ± 5.3x10^5 CFU) and *Gmm^Apo/Sgm* (1.4x10^6 ± 3.9x10^5 CFU) flies harbored a similar bacterial load at the same time point post-inoculation (Fig 1A). The midgut load of *Kco_Z* and *Sodalis* did not change significantly in any of the fly groups over the following 21 days (Fig 1A), thus suggesting that the bacteria had achieved stable-state infections within their fly hosts by one week post-acquisition.

We next examined whether midgut infections with *Kco_Z* or *Sodalis* impacted tsetse survival. We found that 76% of *Gmm^WT/Kco_Z* individuals, and 84% of *Gmm^WT/Sgm* individuals, survived for 28 days following bacterial inoculation. Similarly, 84% and 80% of *Gmm^Apo/Kco_Z* and *Gmm^Apo/Sgm* flies, respectively, survived the duration of the experiment (Fig 1B). Percent survival was not significantly different between any of these groups, indicating that *Kco_Z* and *Sodalis* both exhibit commensal phenotypes in wild-type and aposymbiotic tsetse.

*Kco_Z* is resistant to Peptidoglycan Recognition Protein-LB (PGRP-LB)

The midgut of adult tsetse expresses *peptidoglycan recognition protein LB* (*pgrp-lb*), which encodes a pattern recognition receptor that exhibits potent antimicrobial activity [14,15]. Thus, in order to colonize tsetse’s midgut, a microorganism must be resistant to this molecule. We investigated whether innate resistance to PGRP-LB represents one mechanism that allows
Kco_Z to colonize tsetse’s gut. We found that 108% (±16) of Kco_Z cells were able to survive 1 h in the presence of recPGRP-LB, while only 2.3% (±1.0) of E. coli cells survived for the same time period. Additionally, 154% (±14) of Sodalis cells survived following a 12 h incubation with recPGRP-LB (Fig 2). These findings suggest that like native Sodalis, Kco_Z is resistant to the antimicrobial properties of PGRP-LB and is able to survive in the presence of this protein.
as indicated by a slight increase in bacterial load compared to the initial inoculate). This phenotype may facilitate this bacterium’s ability to successfully colonize tsetse’s gut.

**Kco\_Z colonized aposymbiotic tsetse present a trypanosome refractory phenotype**

*Kco\_Z* successfully colonizes the gut of *Gmm\(^{Apo}\)* flies, resides in the niche for at least 28 days, and has no impact on fly survival during that time period. Thus, we next evaluated whether colonization with this bacterium impacts trypanosome infection establishment in tsetse’s midgut. We began by challenging mature *Gmm\(^{Apo}\)* flies because they are highly susceptible to trypanosome infection (~50%) while their age-matched *Gmm\(^{WT}\)* counterparts are refractory (~3%) [16]. Distinct groups of eight day old *Gmm\(^{Apo/Sgm}\)* and *Gmm\(^{Apo/Kco\_Z}\)* flies, which housed similar numbers of their respective exogenous bacteria (S1A Fig), were administered a meal supplemented with 1x10\(^6\) blood stream form (BSF) trypanosomes per ml of blood. Thereafter all flies were maintained on regular blood for two weeks, at which point their midguts were dissected and microscopically examined for the presence of parasites. An age-matched control cohort consisted of similarly challenged *Gmm\(^{Apo}\)* flies. We found that infection prevalence in the *Gmm\(^{Apo/Sgm}\)* group (57%) was similar to that of *Gmm\(^{Apo}\)* controls (52%), while infection prevalence in *Gmm\(^{Apo/Kco\_Z}\)* individuals was significantly lower (19%) (Fig 3A). These data indicate that the presence of *Kco\_Z* in tsetse’s gut interferes with the ability of
trypanosomes to establish an infection in this niche. This parasite resistant phenotype is similar to that which occurs in the gut of Kco_Z colonized mosquitoes following exposure to malaria parasites [29,30].

African trypanosomes are not susceptible to Kco_Z generated reactive oxygen intermediates

In A. gambiae, Kco_Z produces reactive oxygen intermediates (ROIs) that are directly toxic to Plasmodium [29,30]. ROIs have also been implicated as mediators of trypanosome infection outcomes in tsetse. Specifically, tsetse are rendered susceptible to trypanosome infection when the initial infectious blood meal is supplemented with the antioxidants vitamin C or cysteine [18,31]. These antioxidants detoxify ROIs that otherwise induce programmed cell death processes in trypanosomes [32]. In light of this information, we investigated the correlation between Kco_Z generated ROIs and the trypanosome refractory phenotypes we observed in adult Gmm_Apo/Kco_Z individuals. As an indicator of bacterial ROI production, we quantified H$_2$O$_2$ concentrations in supernatants from mid-log phase Kco_Z and Sodalis cultures. Kco_Z and Sodalis supernatants contained 127nM ($\pm$15) and 142nM ($\pm$13) of H$_2$O$_2$, respectively (Fig 3B).

We next tested whether ROIs produced by Kco_Z inhibit the ability of trypanosome to infect Gmm_Apo/Kco_Z flies. Individual groups of eight day old Gmm_Apo and Gmm_Apo/Kco_Z individuals were offered a blood meal containing infectious trypanosomes together with the antioxidant vitamin C. All trypanosome challenged flies were subsequently maintained on vitamin C supplemented blood for 14 days. Under these conditions, 74% of Gmm_Apo/vitC flies were infected with trypanosomes, while only 11% of their Gmm_Apo/Kco_Z+vitC counterparts housed parasite infections (Fig 3C). These results suggest that ROIs produced by Kco_Z that reside
stably in tsetse’s gut are not the sole determinants of the fly’s susceptibility to infection with trypanosomes.

**Kco_Z produces acid that is toxic to trypanosomes**

We observed that Gmm\textsuperscript{Apo/Kco\_Z} flies are significantly more refractory to infection with trypanosomes than are Gmm\textsuperscript{Apo/Sgm} individuals, despite the fact that Sodalis and Kco\_Z produce similar amounts of H\textsubscript{2}O\textsubscript{2}. This outcome implies that Kco\_Z modulates trypanosome infection outcomes in tsetse via a mechanism other than ROI production. Members of the genus Kosakonia [33–35], as well as several enteric commensals including Enterobacter spp. [36–38], produce organic acids, and these products can inhibit pathogen growth by creating an acidic environment [39,40]. Because many trypanosomatids, including members of the genera Trypanosoma and Leishmania, are highly sensitive to environmental pH [41,42], we investigated whether Kco\_Z creates an acidic environment that prohibits T. brucei growth in vitro. Specifically, we heat killed (HK) Kco\_Z (1x10\textsuperscript{6} log-phase in 500 μl of LB media) and added the solution to trypanosome cultures maintained in vitro. This medium includes phenol red, which is a pH-sensitive dye that when in solution turns from red-pink to yellow as the quantity of acid in the environment increases. Addition of this HK Kco\_Z extract immediately turned the Beck’s media yellow, and the pH measured at 5.8 (± 0.39). This value was significantly lower than trypanosome cultures that were supplemented with 500 μl of 1x10\textsuperscript{6} log-phase HK trypanosomes (pH 7.3 ± 0.28), HK Sodalis (pH 7.4 ± 0.39) or LB (Kco\_Z growth media; pH 7.1 ± 0.28) or MM media (Sodalis growth media; pH 7.2 ± 0.29) alone (Fig 4A). We subsequently monitored trypanosome growth in cultures that received the above-mentioned supplements. We observed that trypanosomes failed to replicate in Beck’s medium that contained HK Kco\_Z extracts, while trypanosomes multiplied in all of the other culture conditions (Fig 4B).

Heat-killed Kco\_Z extracts create an acidic environment when added to trypanosome cultures, and trypanosomes fail to replicate in this environment. These findings do not rule out the possibility that trypanosomes are capable of surviving Kco\_Z-induced acidic conditions, and instead, some other unknown component of the medium [e.g., a bacterium-derived trypanocidal molecule(s)] exhibits toxic properties. To address this possibility, we monitored trypanosome growth in Beck’s medium, the pH of which was artificially decreased to 5.8 (the same as that achieved by adding HK Kco\_Z extracts) via the addition of exogenous acid. Under these conditions trypanosomes failed to replicate (Fig 4C). Furthermore, when we buffered Beck’s medium containing HK Kco\_Z extracts back up to pH 7.2, trypanosomes replicated normally (Fig 4D). Taken together, these data indicate that Kco\_Z produces an acidic environment that is toxic to trypanosomes, thus impeding their growth in vitro.

**Kco\_Z acidifies tsetse’s gut**

We observed that trypanosomes are unable to multiply when cultured in medium supplemented with acidic Kco\_Z extracts. Thus, we next investigated whether Kco\_Z produces acid in vivo in tsetse’s gut. To do so we colonized teneral, aposymbiotic flies with either Kco\_Z or Sodalis, and 5 days later fed them a meal containing 2.5% sucrose and 0.04% phenol red solubilized in water. Twenty-four hours later, midguts from a sample of flies (n = 8 per group) were excised and plated on solid medium containing phenol red. Gmm\textsuperscript{Apo/Kco\_Z} and Gmm\textsuperscript{Apo/Sgm} flies housed similar densities of the introduced bacteria (S1B Fig), and their respective mediums changed color to reflect corresponding pH shifts (S1C Fig). The remaining flies were dissected to expose their midgut in situ, and the color of the gut contents was visualized microscopically. We observed that the gut contents of Gmm\textsuperscript{Apo/Kco\_Z} flies were yellow in color.
Thus indicating that the environment had become acidified. Conversely, the gut contents of \( Gmm^{\text{Apo/Sgm}} \) individuals were red, which is similar to the more alkaline environment present in the gut of \( Gmm^{\text{WT}} \) tsetse (Fig 5).

Finally, we investigated whether \( Kco_Z \) also produces acid in the gut \( Gmm^{\text{WT}} \) tsetse by inoculating teneral individuals with \( 1 \times 10^3 \) CFU of the bacterium (these flies were designated \( Gmm^{\text{WT}}/Kco_Z \)). Five days later a cohort of \( Gmm^{\text{WT}}/Kco_Z \) females (these flies housed 1.27 \( \times \) 10^6 ±

Fig 4. \( Kco_Z \) produces a low pH environment that is toxic to trypanosomes. (A) Early log phase trypanosomes (\( T. b. \) brucei YTAT 1.1), cultured in 10ml of Beck’s medium containing the pH sensitive dye phenol red, exposed to 1ml of heat treated LB media (\( Kco_Z \) culture medium), 1ml of heat treated BHI media (Sodalis culture medium), heat killed (HK) \( Kco_Z \) (5x10^6 cells) in 1ml of LB media and HK Sodalis (5x10^6 cells) in 1ml of BHI media. Controls are trypanosomes alone (tryps). All heated treatments and controls were allowed to cool to room temperature prior to adding them to the trypanosome cultures. Two hours post-treatment, culture pH was measured. HK \( Kco_Z \) significantly reduced the pH of the trypanosome culture (\( p<0.001 \)). The experiment was repeated using 6 distinct clonal trypanosome populations (the image represents one of the six replicates). (B) Density of trypanosomes in culture 2h, 5h and 24h after addition of the treatments described in (A) above. At the 24h time point, all trypanosomes exposed to HK \( Kco_Z \) extracts were dead while those from the other groups were replicating similarly to controls. (C) Density of trypanosomes cultured in normal (pH 7.2) and artificially produced (via the addition of 0.1N HCl) acidic (pH 5.8) environments. Artificial acidic conditions kill all trypanosomes with 24 h. (D) The density of cultured trypanosomes exposed to HK \( Kco_Z \) extracts buffered to pH 7.2 (via the addition of 0.1N NaOH). The buffering treatment rescues parasite growth. In (A), (B) and (C), statistical significance was determined using a one-way ANOVA followed by Tukey’s HSD post-hoc analysis in (A), and a two-way ANOVA followed by Tukey’s HSD post-hoc analysis in (B) and (C). Different letters represent statistical significance between treatments and controls. In (B), (C) and (D), experiments were performed using 5 or 6 distinct clonal trypanosome populations.

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(Fig 5), thus indicating that the environment had become acidified. Conversely, the gut contents of \( Gmm^{\text{Apo/Sgm}} \) individuals were red, which is similar to the more alkaline environment present in the gut of \( Gmm^{\text{WT}} \) tsetse (Fig 5).
8.6×10^4 Kco_Z at this time point; S1D Fig), as well as age matched Gmm^WT \textsuperscript{WT} controls, were fed a sugar meal containing phenol red (as described above) to observe gut pH. Similar to our results noted in Gmm^Apo/Kco_Z flies, we observed that the gut of Gmm^WT/Kco_Z individuals was yellow, thus indicative of an acidified environment. Conversely, the gut environment of Gmm^WT flies was red and thus comparatively alkaline (Fig 5). Thus, the presence of indigenous symbionts does not impede the ability of Kco_Z to acidify the gut of wild-type flies.

Gmm^WT/Kco_Z are highly refractory to infection with trypanosomes and entomopathogenic bacteria

We hypothesized that exogenous microorganisms would be unable to successfully infect Gmm^WT/Kco_Z due to their acidified midgut environment. To test this hypothesis we first co-inoculated teneral Gmm^WT males with Kco_Z and T. congolense parasites. Two weeks post-challenge we observed no significant difference in the percentage of Gmm^WT/Kco_Z (15%) and control Gmm^WT (23%) flies that harbored trypanosome infections in their midguts (Fig 6A). We next inoculated teneral Gmm^WT males with Kco_Z and then three days later (5 day old adults) challenged Gmm^WT/Kco_Z individuals with either T. congolense or T. brucei parasites (both of these parasite species are naturally transmitted by G. m. morsitans; [43,44]). Under these conditions we observed that Gmm^WT/Kco_Z males were significantly more refractory to infection with both parasite species (T. congolense, 6%; T. brucei, 32%) than were their age-matched Gmm^WT counterparts (T. congolense, 36%; T. brucei, 70%; Fig 6B). Thus, tsetse must house an established Kco_Z infection in its gut at the time of trypanosome challenge in order to present a refractory phenotype. Finally, we found that trypanosome infected Gmm^WT/Kco_Z house similar densities of Kco_Z as do age-matched individuals that eliminated their trypanosome infection (S1E Fig), again indicating that exogenous Kco_Z appears to be resistant to tsetse’s trypanocidal immune response. Additionally, the presence of tsetse’s indigenous,
Enteric microbiota does not interfere with Kco_Z mediated obstruction of trypanosome infection establishment.

Finally, we investigated whether Kco_Z also protects tsetse against infection with an entomopathogenic bacteria. To do so tender Gmm^WT males were fed 1x10^3 CFU of Kco_Z, and then three days later, the same dose of Serratia marcescens strain db11, which is highly virulent to wild-type tsetse [17,18,45]. Five day old Gmm^WT males infected with the same dose of S. marcescens were used as controls. Fly survival following Serratia inoculation was monitored over a 14 day period in both fly groups. We observed that 51% and 9% of Gmm^WT/Kco_Z and Gmm^WT individuals, respectively, survived their infection with S. marcescens (Fig 6C).

Taken together, our results detailed above indicate that wild-type tsetse present a parasite and entomopathogen refractory phenotype when they house an established Kco_Z infection in their gut. This phenotype like occurs because the acidified nature of the gut environment is inhospitable to trypanosomes. To address whether decreased midgut pH adversely impacts tsetse fitness, we quantified several fitness parameters in Gmm^WT/Kco_Z flies (a sample of these individuals housed 1.39x10^6 ± 1.1x10^5 Kco_Z at the time they were used for experimentation; S1F Fig).

We began by measuring midgut weight, which reflects overall digestive health. We observed no significant difference in midgut weight between 8 day old Gmm^WT/Kco_Z and Gmm^WT males (3.8 ± 1.1 mg and 3.2 ± 1.2 mg, respectively) and females (12.8 ± 1.8 mg and 13.0 ± 1.8 mg, respectively) 24 hrs after acquiring their last blood meal (Fig 7A). We next measured fecundity parameters in female and male Gmm^WT/Kco_Z and Gmm^WT flies to determine if stable infection with this bacterium would alter their reproductive capacity. We began by...
measuring gonotrophic cycle (GC) duration of Gmm\textsuperscript{WT/Kco\_Z} and Gmm\textsuperscript{WT} females. The length of the 1\textsuperscript{st} GC was not significantly different between Gmm\textsuperscript{WT/Kco\_Z} (24.0 ± 1.2 days) and Gmm\textsuperscript{WT} females (24.0 ± 0.9 days) (Fig 7B). However, the 2\textsuperscript{nd} and 3\textsuperscript{rd} GCs of Gmm\textsuperscript{WT/Kco\_Z} females (13.0 ± 1.1 and 14.0 ± 1.1 days, respectively) were significantly longer than those of
their age-matched WT counterparts (11.7 ± 1.0 and 11.5 ± 1.1 days, respectively) (Fig 7B). We also determined that pupal weight from all three GCs was similar between both fly groups (GC1, Gmm<sup>WT/Kco_Z</sup> = 23.1 ± 1.5 mg, Gmm<sup>WT</sup> = 22.9 ± 1.4 mg; GC2, Gmm<sup>WT/Kco_Z</sup> = 23.4 ± 1.7 mg, Gmm<sup>WT</sup> = 24.2 ± 1.8 mg; GC3, Gmm<sup>WT/Kco_Z</sup> = 24.7 ± 1.7 mg, Gmm<sup>WT</sup> = 24.4 ± 1.6 mg) (Fig 7C). Thus, Kco_Z infection impacts the reproductive physiology of female tsetse by increasing GC duration and hence the number of offspring infected individuals are able to produce over the course of their lifespan. However, despite this effect, infection with this bacterium does not impact pupal weight.

We also investigated the effect of Kco_Z infection on the reproductive fitness of male tsetse by comparing the mating competitiveness of Gmm<sup>WT/Kco_Z</sup> and Gmm<sup>WT</sup> individuals. To do so we set up 80 individual cages, each of which contained one sexually mature virgin female. We subsequently placed one sexually mature Gmm<sup>WT/Kco_Z</sup> and Gmm<sup>WT</sup> male in each cage and monitored the arena to determine which of the two males successfully mated with the female. We observed that 47.5% of matings occurred between Gmm<sup>WT/Kco_Z</sup> males and females (neither male mated with the female in two of the cages) (Fig 7D), thus indicating that Kco_Z infection does not significantly alter male mating competitiveness ($\chi^2 = 0.1025641$, df = 1, $p = 0.748774$). Next we compared the number of sperm present in three and 14 day old Gmm<sup>WT/Kco_Z</sup> and Gmm<sup>WT</sup> males by quantifying transcript abundance of sperm-specific dynein intermediate chain (sdic). The Drosophila homologue of this gene is transcribed exclusively in sperm cells [46] and is used to quantify sperm abundance [47]. We observed no significant difference in sdic transcript abundance between three day old or 14 day old Gmm<sup>WT/Kco_Z</sup> and Gmm<sup>WT</sup> males (Fig 7E).

Finally, we examined whether the presence of Kco_Z impacts the load of endosymbiotic Wigglesworthia and Sodalis. These measurements, which were taken at 14 days post-inoculation with Kco_Z, are important because tsetse’s microbiota impact many aspects of their host’s fitness, including fecundity and immune system development and function [48,49]. We observed that infection with Kco_Z did not significantly alter the load of tsetse’s midgut (bacteriome) population of obligate Wigglesworthia in Gmm<sup>WT/Kco_Z</sup> males or females (Fig 7F). Conversely, midguts from Gmm<sup>WT/Kco_Z</sup> males and females housed significantly fewer Sodalis than did midguts from their Gmm<sup>WT</sup> counterparts (Fig 7F). Taken together, these data indicate that Kco_Z significantly impacts specific fitness parameters in both female and male flies.

**Discussion**

Morbidity and mortality caused by vector-borne diseases currently inflicts a devastating socioeconomic burden on a significant percentage of the global population. To reduce this burden, novel disease control strategies that inhibit pathogen maturation within arthropod disease vectors require development. The enteric microbiota is being increasingly studied for use in this context, and one such novel strategy could employ the use of ‘probiotic’ bacteria, the presence of which would alter the physiology of the vector’s gut to make the environment inhospitable to pathogens. Herein we use the tsetse fly model system to highlight how an exogenous bacterium can be employed in this capacity to impede infection establishment of two pathogens in this insect disease vector. Specifically, we determined that Kco_Z, which is a bacterium found naturally in the gut of some An. gambiae populations, and directly kills Plasmodium by producing anti-parasitic ROIs [29,30], can stably colonize tsetse’s gut for at least 28 days. When the bacterium is present in this niche, tsetse are significantly more refractory to infection with parasitic African trypanosomes and entomopathogenic S. marcescens than are flies that house only their indigenous microbiota. Kco_Z creates this inimical environment by acidifying tsetse’s gut such that trypanosomes and S. marcescens, which are sensitive to these conditions,
are no longer able to successfully infect the fly. While infection with Kco_Z exerts only a negligible effect on tsetse’s reproductive fitness, the bacterium’s presence does reduce the load of endosymbiotic Sodalis. Cumulatively, our findings suggest that Kco_Z could be used in a natural setting to artificially reduce disease transmission by this arthropod vector.

Herein we demonstrate that Kco_Z is able to stably colonize tsetse's gut, which is an outcome that likely results at least in part from the bacterium’s resistance to antimicrobial PGRP-LB. This protein is constitutively produced in the fly’s midgut and directly kills trypanosomes and E. coli K12 (Fig 2 in this study, and [16]). Kco_Z resistance to tsetse PGRP-LB may reflect one of many bacterial adaptations that result from residing within the immunologically hostile environment of the insect midgut. While the specific physiological mechanism(s) that Kco_Z uses to facilitate its colonization of tsetse's midgut are currently unknown, the bacterium survives for prolonged periods within the gut of An. gambiae in part by increasing expression of genes that encode a type III secretion system apparatus protein as well as glutathione S-transferase and oxidoreductase [30]. Type III secretion system proteins can facilitate bacterial penetration into host cells [50] and be involved in subversion of host immunity [51], while the latter two proteins are antioxidant pathway components that mediate redox homeostasis in oxidatively stressful environments such as the insect midgut [52,53]. Kco_Z may employ similar mechanisms to survive in tsetse’s immunologically hostile gut. Sodalis is also resistant to tsetse antimicrobial immune response [9,54], which may be the result of structural adaptations present in exposed bacterial surface coat molecules, including lipopolysaccharide [55] and outer membrane protein A [56]. Furthermore, Sodalis enters into host cells through the use of a type III secretion system [57], which may further protect the bacterium from tsetse’s immunologically hostile midgut environment. Likewise, similar mechanisms may facilitate Kco_Z survival in this niche. Additionally, Kco_Z, like Sodalis [58], may reside extracellularly in the endoperitrophic space of tsetse’s midgut. In this position, the fly’s peritrophic matrix barrier would physically separate the bacteria from immunocompetent epithelial cells, thus reducing their exposure to harmful antimicrobial responses.

Microbes can alter their environment such that it either favors or hinders its own prosperity as well as the prosperity of other resident organisms [59]. Depending on specific physiological circumstances, these effects can reflect the consumption of resources and/or the production of beneficial or harmful metabolic byproducts [60,61]. Tsetse’s sole energy source, vertebrate blood, is rich in glucose. Many bacterial taxa, including members of the Enterobacteriaceae (in which Kosakonia and Enterobacter spp. cluster), ferment this sugar, thus producing hydrogen that acidifies their environment (38,61]. The acidic environment present in tsetse’s gut when Kco_Z resides stably in the tissue likely results at least in part from the bacterium’s utilization of blood glucose as an energy source. The pH in the gut of insect vectors, including tsetse flies [62], sand flies [63] and mosquitoes [64] is normally alkaline, and the parasites they transmit, as well as other enteric microbes, are adapted to survive in this environment. Correspondingly, our results indicate that Kco_Z induced conditions in tsetse’s gut detrimentally impact not only trypanosomes but also other enteric microbes including entomopathogenic S. marcescens and symbiotic Sodalis. Kco_Z mediated suppression of S. marcescens, or any other pathogen, would have obvious benefits to the fly. However, dysbiosis of tsetse’s facultative and commensal enteric microbiota could impact the fly’s overall fitness and/or vector competency. For example, reducing Sodalis density significantly decreases tsetse longevity [20]. This may prove beneficial because flies with a reduced life span could perish before trypanosomes are able to complete their 20–30 extrinsic incubation period [65]. A reduction in Sodalis density could be further beneficial because tsetse that house relatively low densities of the bacterium are less likely to be infected with trypanosomes than are individuals that house more of the symbiont [19–23]. Thus, the trypanosome refractory phenotype presented by Kco_Z colonized tsetse
may result in part from, or be enhanced by, the fact that they contain fewer Sodalis. Finally, the midgut of wild tsetse is colonized by a transient population of environmentally acquired bacteria [9]. The contribution of these bacteria to tsetse’s physiology has not been characterized, and as such, interference with this microbial population could further alter the fly’s physiological homeostasis. To the contrary, the environmentally acquired microbiota could out-compete Kco_Z that reside in tsetse’s gut, or could prevent the bacterium from acidifying the environment. Future studies are required to elucidate microbe-microbe interactions in the gut Kco_Z colonized flies after their release into the field.

Reducing the incidence of African trypanosomiases has to date been achieved largely by controlling the size of tsetse populations. This process is currently accomplished by employing area wide integrated pest management (AW-IPM) strategies that make use of insecticides, traps and sterile insect technique (SIT) [66,67]. SIT involves sequentially releasing a large number of sterilized males (achieved by irradiating pupae or adults) into the target environment. These males reproductively outcompete wild males for female mates, and the population size drops significantly, or the fly is completely eradicated [68]. The efficacy of SIT as a means of controlling tsetse populations is well exemplified on Unguja Island (the large island of the Zanzibar archipelago), where the technique was used to eradicate G. austeni, the main vector of trypanosomes that cause animal African trypanosomiasis in that locale [69]. One shortcoming of this procedure is that releasing large numbers of sterile males significantly increases the population of potential disease vectors in that environment (male tsetse also feed exclusively on vertebrate blood). One way to overcome this obstacle is to release sterilized males that present enhanced refractoriness to parasite infection. This outcome is currently achieved by feeding the sterilized males twice with the drug isometamidium chloride prior to their release [70]. However, treated flies are not 100% resistant to infection [70], and the risk exists that the parasite will eventually develop resistance to the drug. Our data presented herein indicate that inoculating sterilized males with Kco_Z prior to their release would serve as an alternative, or supplemental, means of making the flies resistant to infection. Specifically, Gmm WT/Kco_Z males (and females) are significantly more refractory to infection with trypanosomes than are their wild-type counterparts. This finding implies that sterilized, Gmm WT/Kco_Z individuals would be relatively poor vectors of disease-causing trypanosomes and thus safer to release than sterilized males that do not house this bacterium. Furthermore, our preliminary analyses suggest that Kco_Z infection does not compromise the mating competitiveness nor sperm abundance of male tsetse, thus suggesting that Gmm WT/Kco_Z individuals would be as successful as their wild counterparts at locating females and engaging in viable matings. Finally, male tsetse could be irradiated as pupae or teneral adults [71], prior to colonization with Kco_Z, thus eliminating the possibility that this treatment could detrimentally impact the bacterium’s fitness and thus its effect on fly vector competency. These characteristics provide preliminary evidence that releasing sterilized, Kco_Z infected male tsetse as part of an AW-IPM program would significantly reduce the capacity of these flies to transmit disease.

In conclusion, data presented in this study indicates that Kco_Z could effectively complement currently used AW-IPM programs aimed at reducing or eliminating tsetse populations by inhibiting trypanosome infection establishment in the fly’s gut. However, the complex relationship between tsetse, its indigenous (endosymbionts) and exogenous (trypanosomes and environmentally acquired microorganisms) microbiota, and Kco_Z must be studied in more detail before the bacterium is used in this capacity. Of particular importance are studies aimed at determining whether Kco_Z presents trypanocidal activity in other epidemiologically important tsetse species (e.g., G. fuscipes). Furthermore, field-based studies would shed light on how the ecology of tsetse’s natural environment influences the overall efficacy of the system.
**Materials and methods**

**Ethical consideration**

This work was carried out in strict accordance with the recommendations in the Office of Laboratory Animal Welfare at the National Institutes of Health and the Yale University Institutional Animal Care and Use Committee. The experimental protocol was reviewed and approved by the Yale University Institutional Animal Care and Use Committee (Protocol 2011–07266).

**Tsetse, bacteria and trypanosomes**

Tsetse flies (*Glossina morsitans morsitans*) were maintained in Yale University’s insectary at 24˚C with 55% relative humidity. Flies received defibrinated (via mechanical agitation for 20 minutes) bovine blood (Quad Five, Ryegate, MT, USA) through an artificial membrane feeding system every 48 h [72]. Aposymbiotic tsetse (*Gmm*~Apo~) were generated and maintained as described previously [73]. Throughout the manuscript, flies referred to as ‘teneral’ were unfed adults recently eclosed (< 24h) from their pupal case. All tsetse lines used in this study are described in S1 Table.

*Sodalis* were isolated from tsetse pupae as described previously [74], and subsequently maintained in liquid brain heart infusion (BHI) media (Becton Dickinson). When necessary, *Sodalis* were plated on either Brain Heart Infusion agar supplemented with 10% defibrinated bovine blood (BHIB) or Mitsubishi-Maramorosch (MM)-agar plates. *Kosakonia cowanii* Zambiae [previously designated *Enterobacter* sp Zambiae (*Esp_Z*); GenBank accession number CP022690.1; 28], isolated from the gut of the mosquito, *Anopheles gambiae* [25]), and *Serratia marcescens* (strain db11; isolated from a moribund *Drosophila* sp.) [75] were grown in liquid LB media or on LB-agar plates at 30˚C.

*In vivo* *Trypanosoma congolense* and *T. brucei brucei* (YTAT 1.1) were expanded in rats and harvested from infected blood at peak parasitemia. Rat blood containing blood stream form (BSF) parasites was aliquoted and cryopreserved (in the liquid nitrogen vapor phase, approximately -150˚C) for subsequent tsetse challenge experiments.

**Recombinant PGRP-LB antibacterial assays**

Antibacterial activity of recombinant (rec) PGRP-LB was determined as described previously [15], with minor modification. Specifically, recPGRP-LB was added (10 μg/ml of media) to early log-phase (OD = 0.2–0.4) cultures of *Kco_Z*, *Sodalis* and *E. coli*. Controls consisted of bacterial cultures exposed to bovine serum albumin. Using a plate-based quantification assay [58], *E. coli* and *Kco_Z* load was subsequently measured 1 hr. later, while *Sodalis* load was measured 24 hr. later. Results are presented as % of initial inoculum, which was determined by dividing the number of bacterial CFU present after treatment with recPGRP-LB by the number of CFU present prior to inoculation.

**Microbial infection assays**

*Per os* bacterial challenge of wild-type (*Gmm*~WT~) and *Gmm*~Apo~ flies was performed by feeding teneral adults a heat inactivated (HI; 56˚C for 1 hr) blood meal (to inactivate the vertebrate complement system) inoculated with 5x10^4 colony forming units (CFU) of each bacterial strain per ml of blood. Because tsetse flies consume approximately 20 μl of blood per feeding, each fly is inoculated with 1x10^5 bacterial cells. *Gmm*~Apo~ flies colonized with either *Sodalis* or *Kco_Z* are designated *Gmm*~Apo/Sgm~ and *Gmm*~Apo/Kco_Z~, respectively, and *Gmm*~WT~ flies colonized with *Kco_Z* are designated *Gmm*~WT/Kco_Z~. For all experiments that employed tsetse flies...
inoculated with either *Sodalis* or *Kco_Z*, bacterial midgut load was determined by homogenizing microscopically dissected gut tissue in 0.85% NaCl and serially diluting and plating the samples on LB-agar (*E. coli*, *Kco_Z* and *Serratia*) or BHIB or MM (*Sodalis*) plates supplemented with antibiotics [58]. CFU per plate were counted manually, and counts are presented in the corresponding Results subsections.

For trypanosome infections, all flies received infectious blood meals containing $1 \times 10^6$/mL BSF *T. congolense* or *T. b. brucei* parasites. *Gmm*<sup>Apo</sup>, *Gmm*<sup>Apo/Gsm</sup> and *Gmm*<sup>Apo/Kco_Z</sup> were challenged as eight day old adults (3<sup>rd</sup> blood meal), while *Gmm*<sup>WT</sup> and *Gmm*<sup>WT/Kco_Z</sup> flies were challenged as five day old adults (2<sup>nd</sup> blood meals). For *Kco_Z*/trypanosome co-infection experiments, distinct groups of mature *Gmm*<sup>WT</sup> individuals were inoculated with $1 \times 10^6$/mL BSF *T. congolense* parasites and $5 \times 10^4$ CFU/ml of *Kco_Z*. Two weeks post-trypanosome challenge, all flies were dissected and their midguts microscopically examined to determine parasite infection status.

**Detection and inhibition of tsetse reactive oxygen intermediates**

*Kco_Z* and *Sodalis* cultures were grown to mid-log phase (OD = 0.25), and cell-free supernatants were generated via centrifugation. Hydrogen peroxide ($H_2O_2$) concentrations in bacterial culture supernatants were determined using an Amplex Red Hydrogen Peroxide/Peroxidase assay kit according to the manufacturer’s (Invitrogen) protocol. In brief, supernatants were incubated for 30 min. with the assay reagent, and resulting fluorescence units were quantified using a Bio-Tek plate reader.

Antioxidants were used to inhibit tsetse ROI activity *in vivo*. The assay used was similar to those described previously [18,29,30]. In brief, treated flies were offered a blood meal inoculated with trypanosomes [$1 \times 10^6$/mL BSF *T. b. brucei* (YTAT 1.1)] and supplemented with vitamin C (10mM) or cysteine (10μM). All subsequent meals also contained antioxidant supplements.

**Determination of bacterial acid production *in vitro***

*Sodalis* and *Kco_Z* were grown in their respective liquid media to an O.D. of 1.0. Subsequently, $5 \times 10^6$ cells (this value represents the approximate maximum load to which these bacteria grow in tsetse’s gut; see Fig 1A) were diluted to a volume of 1 ml (again in respective liquid media) and heat-killed (80°C for 1.5 hr). Conditioned media containing dead cells was added to early log growth phase *T. b. brucei* YTAT 1.1 grown in a Beck’s medium (GE Hyclone), which contains phenol red. When in solution this compound serves as a pH-sensitive colorimetric indicator that changes from pink-red to yellow as environmental pH drops. Other treatment groups were inoculated with 1 ml of heated, clean LB (*Kco_Z* growth medium) or clean BHI (*Sodalis* growth medium), while the control group consisted of trypanosomes alone. Two hours after exposing *T. b. brucei* to treatment conditions, cultures were assayed to determine pH using a Mettler Toledo pH meter. The pH of trypanosome containing Beck’s medium was experimentally reduced (to pH 5.8) via the addition of 0.1N HCl, while HK *Kco_Z* extracts were buffered to pH 7.2 via the addition of 0.1N NaOH. Trypanosome load in all treatment and control groups was determined at 2, 5 and 24 hour time points by counting live parasites using a Brite-Line hemocytometer.

**Determination of bacterial acid production *in vivo***

Microbial regulation of pH in tsetse’s midgut was determined by feeding teneral *Gmm*<sup>Apo</sup> flies a HI blood meal inoculated with either *Sodalis* or *Kco_Z* ($5 \times 10^6$ CFU/ml of blood). Additionally, teneral *Gmm*<sup>WT</sup> flies received the same quantity of *Kco_Z*. Five days post-bacterial
challenge, colonized individuals were administered a meal composed of sucrose (2.5%) and phenol red (0.04%) solubilized in water. Twenty-four hours later, the color of the solution contained in the midgut was determined by incising the fly abdomen and observing the intact gut using a dissecting microscope (Zeiss Discovery) equipped with a digital camera (Zeiss Axio-Cam MRc 5). Remaining flies were dissected and their guts were harvested, homogenized in 0.85% NaCl, serially diluted and plated onto MM-agar plates (prepared as described in [56]) supplemented with phenol red (0.025 g/L) and sucrose (a 2.5% sucrose solution was spread onto plates immediately prior to applying tsetse gut extracts). CFU per plate was counted manually, and the growth medium was monitored to observe pH-induced changes in color.

Fitness assays

For all fitness assays, GmmWT teneral females and males were infected with Kco_Z during their first blood meal. To determine midgut weight, midguts were dissected from 9 day old GmmWT/Kco_Z and GmmWT females and males (24 h after consuming their last blood meal) and weighed using a Mettler Toledo (AL104) balance. The effect of Kco_Z infection on female fecundity was measured by quantifying the length of three gonotrophic cycles (GC) and by weighing pupal offspring. To measure GC length, GmmWT/Kco_Z and GmmWT females were mated as 5 day old adults and thereafter maintained in individual cages. All females were monitored daily to determine when they deposited larvae, and all deposited larvae were weighed.

The effect of Kco_Z infection on male reproductive fitness was measured by quantifying the mating competitiveness and sperm abundance of individuals that housed the bacterium versus those that did not. Mating competitiveness assays were performed in individual tubular cages (height, 6 cm; diameter 12.7 cm), each of which housed one 5 day old virgin female (fed twice). Subsequently, one age-matched GmmWT/Kco_Z and GmmWT male (also fed twice) was added to each cage. These males were distinguished from one another by removing the proximal tarsus of the right foreleg from one of the individuals. The arena was observed until one of the males had successfully mounted the female, at which point the cage was submerged in ice and the free male identified. To eliminate any bias associated with removal of the foreleg tarsus, the experiment was repeated twice (n = 40 cages per experiment), each time with either GmmWT/Kco_Z or GmmWT males receiving the distinguishing procedure. Sperm abundance was measured by RT-qPCR quantification of sperm-specific dynein intermediate chain (sdic) expression in the reproductive tracts of three and 14 day old (fed twice) virgin GmmWT/Kco_Z and GmmWT males. Absolute sdic transcript abundance was determined by comparing experimental sample cycle threshold (Ct) values to those derived from an sdic internal standard curve.

Sodalis fliC and Wigglesworthia thiC gene specific primers were used to quantify the absolute abundance of these bacteria. This was performed by comparing Sodalis fliC and Wigglesworthia thiC cycle threshold (Ct) values in GmmWT/Kco_Z and GmmWT females and males to those derived from symbiont gene-specific internal standard curves. Because Wigglesworthia and Sodalis can be polyploid [76,77], we normalized symbiont genome copy number to constitutively expressed tsetse gapdh copy number. All RT-qPCR primers are listed in S2 Table. All RT-qPCR assays were carried out in duplicate, and replicates were averaged for each sample. Negative controls were included in all amplification reactions.

Statistical analyses

For trypanosome infection experiments, statistical analyses were carried out using the R software for macOS (version 3.3.2) or GraphPad Prism (v.6). A generalized linear model (GLM) was generated using binomial distribution with a logit transformation of the data. The binary
infection status (infected or recovered) was analyzed as a function of the bacterium used to colonize the insects (or its absence). For experiments requiring a pairwise comparison, we performed a Wald test on the individual regression parameter (nature of the bacterium used to colonize) to test its statistical difference. For experiments requiring multiple comparisons, multiple pairwise tests were generated using Tukey contrasts on the generalized linear model (GLM) using glht function of "multcomp" package in R. For the mating assay, under the null hypothesis "Kco_Z does not change the attractiveness of the male", the probability that a female will choose either of the males is $p = 0.5$. We tested the validity of the null hypothesis by using a Chi-square goodness of fit test. Details of the statistical tests described above are indicated in S1 Dataset. All statistical tests used, and statistical significance between treatments, and treatments and controls, are indicated on the figures or in their corresponding legends. All samples sizes are provided in corresponding figure legends or are indicated graphically as points on dot plots. Biological replication implies distinct groups of flies were collected on different days, and where applicable, treated with distinct treatments (e.g., different bacterial cultures or individual aliquots of trypanosomes).

Supporting information

S1 Fig. Load [colony forming units (CFU) per fly gut] of exogenous Kco_Z and Sodalis in the gut of experimental flies, and Kco_Z acid production in vitro. Kco_Z and Sodalis CFU/gut in 8 day old Gmm_Apo/Kco_Z (Kco_Z) and Gmm_Apo/Sgm (Sodalis) flies prior to (A) challenge with $1 \times 10^6$ blood stream form (BSF) trypanosomes per ml of blood and (B) measuring gut pH in vivo. (C) Guts from Gmm_Apo/Kco_Z (Kco_Z) and Gmm_Apo/Sgm (Sodalis) flies homogenized and plated onto MM-agar plates supplemented with phenol red (0.025 g/L) and sucrose (a 2.5% sucrose solution was spread onto plates immediately prior to applying gut extracts). Plate color reflects bacteria induced changes in pH relative to the empty control. (D) Kco_Z density in the gut of Gmm_WT/Kco_Z (Kco_Z) flies prior to measuring gut pH in vivo. (E) Kco_Z density in the gut of trypanosome infected (Kco_Z TI) and trypanosome refractory (Kco_Z TR) Gmm_WT/Kco_Z flies. Measurements were taken at the time infection status was determined (14 days post-challenge). (F) Kco_Z density in the gut of a random sample of Gmm_WT/Kco_Z (Kco_Z) flies used to determine the bacterium’s impact of tsetse fitness parameters. In panels (A), (B) and (D-F) bacterial load (CFU/gut) was determined via a plating assay described in the Materials and Methods (subsection Microbial infection assays) and in reference [58]. (TIF)

S1 Table. Tsetse cohorts used in this study, and the status of their enteric microbiota. (XLSX)

S2 Table. RT-qPCR primers used in this study. (XLSX)

S1 Dataset. Statistical tests, and their results, used in this study. (XLSX)

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**Author Contributions**

**Conceptualization:** Brian L. Weiss, Michele A. Maltz, Aurélien Vigneron, Serap Aksoy.

**Data curation:** Brian L. Weiss, Michele A. Maltz, Aurélien Vigneron, Michelle B. O’Neill.

**Formal analysis:** Brian L. Weiss, Michele A. Maltz, Aurélien Vigneron, Katharine S. Walter, Serap Aksoy.

**Funding acquisition:** Brian L. Weiss, Serap Aksoy.

**Investigation:** Brian L. Weiss, Michele A. Maltz, Aurélien Vigneron, Yineng Wu, Katharine S. Walter, Michelle B. O’Neill, Jingwen Wang, Serap Aksoy.

**Methodology:** Brian L. Weiss, Michele A. Maltz, Aurélien Vigneron, Jingwen Wang, Serap Aksoy.

**Project administration:** Brian L. Weiss, Michele A. Maltz, Serap Aksoy.

**Resources:** Brian L. Weiss, Serap Aksoy.

**Software:** Brian L. Weiss, Aurélien Vigneron.

**Supervision:** Brian L. Weiss, Serap Aksoy.

**Validation:** Brian L. Weiss.

**Visualization:** Brian L. Weiss, Michele A. Maltz, Aurélien Vigneron.

**Writing – original draft:** Brian L. Weiss, Michele A. Maltz, Aurélien Vigneron, Serap Aksoy.

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