# Colonization of the tsetse fly midgut with commensal *Enterobacter* inhibits trypanosome infection establishment

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## 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 an *Enterobacter* bacterium (*Esp_Z*), which confers *Anopheles gambiae* with resistance to *Plasmodium*, is able to colonize tsetse and induce a trypanosome refractory phenotype in the fly. *Esp_Z* established stable infections in tsetse's gut, and exhibited no adverse effect on the survival of individuals from either group. Flies with established *Esp_Z* infections in their gut were significantly more refractory to infection with two distinct trypanosome species (*T. congolense*, 6% infection; *T. brucei*, 32% infection) than were age-matched flies that did not house the exogenous bacterium (*T. congolense*, 36% infected; *T. brucei*, 70% infected). Additionally, 52% of *Esp_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 *Esp_Z* acidifies tsetse's midgut environment, which inhibits trypanosome and *Serratia* growth and thus infection establishment. Finally, we determined that *Esp_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 *Esp_Z* could be used as one component

40 of an integrated strategy aimed at reducing the ability of tsetse to transmit pathogenic

41 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 (*Enterobacter* sp. *Z*, *Esp_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 *Esp_Z* exerts only a modest fitness cost on the fly. Taken together, these findings suggest that *Esp_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 agent 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 (Baxter et al., 2017; Saraiva et al., 2016; Aksoy et al., 2013).

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 (Weiss and Aksoy, 2011; Cirimotich et al., 2011; Narasimhan and Fikrig, 2015; Song et al., 2018; Dey et al., 2018). Tsetse flies, which are the prominent vectors of pathogenic African trypanosomes, house a taxonomically diverse enteric microbiota that includes endosymbiotic *Wigglesworthia* and *Sodalis* (Wang et al., 2013) as well as an assemblage of bacteria obtained from the fly's environment (Aksoy et al., 2014; Geiger et al., 2011; Lindh and Lehane, 2011). Both *Wigglesworthia* and *Sodalis* are maternally

transmitted to developing intrauterine larvae during tsetse's unique mode of viviparous reproduction (Wang et al. 2013; Benoit et al., 2015). *Wigglesworthia* influences trypanosome infection establishment in tsetse by regulating the production of trypanocidal PGRP-LB (Wang et al., 2009; Wang et al., 2012). Additionally, tsetse that undergo larval development in the absence of this bacterium fail to synthesize a gut-associated peritrophic matrix during adulthood (Weiss et al., 2013) 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 (Weiss et al., 2014) and subsequently the salivary glands for transmission in saliva (Vigneron et al., 2018). *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 (Welburn et al., 1993; Dale and Welburn, 2001; Farikou et al., 2010; Soumana et al., 2013; Griffith et al., 2018). Like mosquitoes, tsetse's gut also harbors a diverse population of bacteria obtained from the fly's environment (Geiger et al., 2011; Lindh and Lehane, 2011; Aksoy et al., 2014). 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. (2012) 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

104 competency by modulating basal expression of genes that encode anti-*Plasmodium* and
105 anti-dengue effector proteins (Cirimotich et al., 2011; Dennison et al., 2014; Bahia et al.,
106 2014). Other members of the mosquito enteric microbiota exert direct effects on their
107 host's vector competency. Specifically, a *Chromobacterium* isolated from *Ae. aegypti*
108 secretes factors that exhibit anti-*Plasmodium* and anti-Dengue activity (Ramirez et al.,
109 2014). Also, laboratory reared *A. gambiae* present an abnormal *Plasmodium* refractory
110 phenotype when their guts are colonized with a strain of *Enterobacter* (*Esp_Z*) that had
111 been previously isolated from field-captured mosquitoes. *Esp_Z* was determined to
112 produce reactive oxygen intermediates (ROIs) that exhibit direct anti-*Plasmodium*
113 properties (Cirimotich et al., 2011; Dennison et al., 2016).
114 In this study we investigated whether *Esp_Z* isolated from *A. gambiae* is able to
115 successfully colonize tsetse's gut and induce parasite and pathogen refractory
116 phenotypes in the fly. We found that this bacterium can reside stably in tsetse's midgut
117 without imparting a detrimental fitness cost on the fly. *Esp_Z* colonized tsetse present
118 an acidified midgut environment that is inhospitable to both African trypanosomes and
119 entomopathogenic *Serratia marcescens*. We discuss the potential utility of *Esp_Z* as a
120 novel component of currently used area wide integrated pest management strategies
121 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 *Esp_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 *Esp_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 *Esp_Z* or *Sodalis* in their first blood meal and then monitored bacterial proliferation over an 28 day period. By 7 days post-inoculation, midgut bacterial density in *Gmm*$^{WT}$ that housed *Esp_Z* (*Gmm*$^{WT/Esp\_Z}$) and *Sodalis* (*Gmm*$^{WT/Sgm}$) was $1.9x10^7 \pm 6.4x10^6$ CFU and $4.5x10^5 \pm 6.4x10^6$ CFU, respectively, and *Gmm*$^{Apo/Esp\_Z}$ ($9.3x10^6 \pm 5.3x10^5$ CFU) and *Gmm*$^{Apo/Sgm}$ ($1.4x10^6 \pm 3.9x10^5$ CFU) flies harbored a similar bacterial density at the same time point post-inoculation (Fig. 1A). The midgut density of *Esp_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 *Esp_Z* or *Sodalis* impacted tsetse survival. We found that 76% of *Gmm*$^{WT/Esp\_Z}$ and 84% of *Gmm*$^{WT/Sgm}$ survived for 28 days following bacterial inoculation. Similarly, 84% and 80% of *Gmm*$^{Apo/Esp\_Z}$ and *Gmm*$^{Apo/Sgm}$, respectively, survived the duration of the experiment (Fig. 1B). Percent survival was not significantly different between any of these groups, indicating that

144 *Esp_Z* and *Sodalis* both exhibit commensal phenotypes in wild-type and aposymbiotic
145 tsetse.
146
147 *Enterobacter is resistant to Peptidoglycan Recognition Protein-LB (PGRP-LB)*
148 The midgut of adult tsetse expresses *peptidoglycan recognition protein LB* (*pgrp-lb*),
149 which encodes a pattern recognition receptor that exhibits potent antimicrobial activity
150 (Wang et al., 2009; Wang et al., 2012). Thus, in order to colonize tsetse's midgut, a
151 microorganism must be resistant to this molecule. We investigated whether innate
152 resistance to PGRP-LB represents one mechanism that allows *Esp_Z* to colonize
153 tsetse's gut. We found that 108% (±16) of *Esp_Z* cells were able to survive 1 h in the
154 presence of recPGRP-LB, while only 2.3% (±1.0) *E. coli* cells survived for the same time
155 period. Additionally, 154% (±14) of *Sodalis* cells survived following a 12 h incubation
156 with recPGRP-LB (Fig. 2). These findings suggest that like native *Sodalis*, *Esp_Z* is
157 resistant to the antimicrobial properties of PGRP-LB and is able to replicate in the
158 presence of this protein (as indicated by an increase in bacterial density compared to
159 the initial inoculate). This phenotype may facilitate this bacterium's ability to successfully
160 colonize tsetse's gut.
161
162 *Esp_Z colonized aposymbiotic tsetse present a trypanosome refractory phenotype*
163 *Esp_Z* successfully colonizes the gut of *Gmm*$^{Apo}$ flies, resides in the niche for at least 28
164 days, and has no impact on fly survival during that time period. Thus, we next evaluated
165 whether colonization with this bacterium impacts trypanosome infection establishment in
166 tsetse's midgut. We began by challenging mature *Gmm*$^{Apo}$ because they are highly

susceptible to trypanosome infection (~50%) while their age-matched *Gmm*$^{WT}$ counterparts are refractory (~3%) (Weiss et al., 2013). Distinct groups of eight day old *Gmm*$^{Apo/Sgm}$ and *Gmm*$^{Apo/Esp\_Z}$, which housed similar numbers of their respective exogenous bacteria (S1 Fig. A), 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*$^{Apo/Esp\_Z}$ individuals was significantly lower (19%) (Fig. 3A). These data indicate that the presence of *Esp_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 *Esp_Z* colonized mosquitoes following exposure to malaria parasites (Cirimotich et al., 2011; Dennison et al., 2016).

*African trypanosomes are not susceptible to Esp_Z generated reactive oxygen intermediates*

In *A. gambiae*, *Esp_Z* produces reactive oxygen intermediates (ROIs) that are directly toxic to *Plasmodium* (Cirimotich et al., 2011; Dennison et al., 2016). 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 (MacLeod et al.,

2007; Vigneron et al., 2018). These antioxidants detoxify ROIs that otherwise induce programmed cell death processes in trypanosomes (Ridgely et al., 1999). In light of this information, we investigated the correlation between *Esp_Z* generated ROIs and the trypanosome refractory phenotypes we observed in adult *Gmm*$^{Apo/Esp\_Z}$. As an indicator of bacterial ROI production, we quantified $H_2O_2$ concentrations in supernatants from mid-log phase *Esp_Z* and *Sodalis* cultures. *Esp_Z* and *Sodalis* supernatants contained 127nM (±15) and 142nM (±13) of $H_2O_2$, respectively (Fig. 3B).

We next tested whether ROIs produced by *Esp_Z* inhibit the ability of trypanosome to infect *Gmm*$^{Apo/Esp\_Z}$. Individual groups of eight day old *Gmm*$^{Apo}$ and *Gmm*$^{Apo/Esp\_Z}$ 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*$^{Apo+vitC}$ were infected with trypanosomes, while only 11% of their *Gmm*$^{Apo/Esp\_Z+vitC}$ counterparts housed parasite infections (Fig. 3C). These results suggest that ROIs produced by *Esp_Z* that reside stably in tsetse's gut are not the sole determinants of the fly's susceptibility to infection with trypanosomes.

*Enterobacter produces acid that is toxic to trypanosomes*

We observed that *Gmm*$^{Apo/Esp\_Z}$ are significantly more refractory to infection with trypanosomes than are *Gmm*$^{Apo/Sgm}$ individuals, despite the fact that *Sodalis* and *Esp_Z* produce similar amounts of $H_2O_2$. This outcome implies that *Esp_Z* modulates trypanosome infection outcomes in tsetse via a mechanism other than ROI production. Several enteric commensals, including *Enterobacter* spp. (Podlesny et al., 2017;

Fischer et al., 2017; Goldford et al., 2018), produce organic acids, and these products can inhibit pathogen growth by creating an acidic environment (Buffie et al., 2013; Neal-McKinney et al., 2012). Because many trypanosomatids, including members of the genera *Trypanosoma* and *Leishmania*, are highly sensitive to environmental pH (Nolan et al., 2000; Zilberstein and Shapira, 1994), we investigated whether *Esp_Z* creates an acidic environment that prohibits *T. brucei* growth *in vitro* and *in vivo*. Specifically, we added heat killed (HK) *Esp_Z* ($1x10^6$ 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 *Esp_Z* extract immediately turned the Beck's media yellow, and the pH measured at 5.8 ($\pm$ 0.39). This value was significantly lower than trypanosome cultures that were supplemented with 500 µl of $1x10^6$ log-phase HK trypanosomes (pH 7.3 $\pm$ 0.28), HK *Sodalis* (pH 7.4 $\pm$ 0.39) or LB (*Esp_Z* growth media; pH 7.1 $\pm$ 0.28) or MM media (*Sodalis* growth media; pH 7.2$\pm$ 0.29) alone (Figure 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 *Esp_Z* extracts, while trypanosomes multiplied in all of the other culture conditions (Figure 4B).

Heat-killed *Esp_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 *Esp_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 *Esp_Z* extracts) via the addition of exogenous acid. Under these conditions trypanosomes failed to replicate (Figure 4C). Furthermore, when we buffered Beck's medium containing HK *Esp_Z* extracts back up to pH 7.2, trypanosomes replicated normally (Figure 4D). Taken together, these data indicate that *Esp_Z* produces an acidic environment that is toxic to trypanosomes, thus impeding their growth *in vitro*.

*Esp_Z acidifies tsetse's gut*

We observed that trypanosomes are unable to multiply when cultured in medium supplemented with acidic *Esp_Z* extracts. Thus, we next investigated whether *Esp_Z* produces acid *in vivo* in tsetse's gut. To do so we colonized teneral, aposymbiotic flies with either *Esp_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*$^{\text{Apo}/Esp\_Z}$ and *Gmm*$^{\text{Apo}/Sgm}$ housed similar densities of the introduced bacteria (S1 Fig. B), and their respective mediums changed color to reflect corresponding pH shifts (S1 Fig. C). 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*$^{\text{Apo}/Esp\_Z}$ were yellow in color (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).

Finally, we investigated whether *Esp_Z* also produces acid in the gut *Gmm*$^{WT}$ by inoculating teneral individuals with 1x10$^{3}$ CFU of the bacterium (these flies were designated *Gmm*$^{WT/Esp\_Z}$). Five days later a cohort of *Gmm*$^{WT/Esp\_Z}$ females (these flies housed 1.27x10$^{6}$ ± 8.6x10$^{4}$ *Esp_Z* at this time point; S1 Fig. D), as well as age matched *Gmm*$^{WT}$ controls, were fed a sugar meal containing phenol red (as described above) to observe gut pH. Similar to our results observed in *Gmm*$^{Apo/Esp\_Z}$, we observed that the gut of *Gmm*$^{WT/Esp\_Z}$ individuals was yellow, thus indicative of an acidified environment. Conversely, the gut environment of *Gmm*$^{WT}$ was red and thus comparatively alkaline (Fig. 5). Thus, the presence of indigenous symbionts does not impede the ability of *Esp_Z* to acidify the gut of wild-type flies.

*Gmm*$^{WT/Esp\_Z}$ *are highly refractory to infection with trypanosomes and entomopathogenic bacteria*

We hypothesized that exogenous microorganisms would be unable to successfully infect *Gmm*$^{WT/Esp\_Z}$ due to their acidified midgut environment. To test this hypothesis we first co-inoculated teneral *Gmm*$^{WT}$ males with *Esp_Z* and *T. congolense* parasites. Two weeks post-challenge we observed no significant difference in the percentage of *Gmm*$^{WT/Esp\_Z}$ (15%) and control *Gmm*$^{WT}$ (23%) that harbored trypanosome infections in their midguts (Fig. 6A). We next inoculated teneral *Gmm*$^{WT}$ males with *Esp_Z* and then three days later (5 day old adults) challenged *Gmm*$^{WT/Esp\_Z}$ individuals with either *T. congolense* or *T. brucei* parasites (both of these parasite species are naturally transmitted by *G. m. morsitans*; Harley and Wilson, 1968; Moloo *et al*., 1992). Under these conditions we observed that *Gmm*$^{WT/Esp\_Z}$ 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 *Esp_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*$^{WT/Esp\_Z}$ house similar densities of *Esp_Z* as do age-matched individuals that eliminated their trypanosome infection (S1 Fig. E), again indicating that exogenous *Esp_Z* appears to be resistant to tsetse's trypanocidal immune response. Additionally, the presence of tsetse's indigenous, enteric microbiota does not interfere with *Esp_Z* mediated obstruction of trypanosome infection establishment.

Finally, we investigated whether *Esp_Z* also protects tsetse against infection with an entomopathogenic bacteria. To do so teneral *Gmm*$^{WT}$ males were fed 1x10$^3$ CFU of *Esp_Z*, and then three days later, the same dose of *Serratia marcescens* strain db11, which is highly virulent to wild-type tsetse (Weiss et al., 2014; Aksoy et al., 2016; Vigneron et al., 2018). Five day old *Gmm*$^{WT}$ 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*$^{WT/Esp\_Z}$ 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 *Esp_Z* infection in their gut. This phenotype like occurs because the acidified nature of the gut environment is inhospitable to the development of exogenous microbes.

305

306 *Esp_Z infection exerts a minimal fitness cost on tsetse*

307 *Esp_Z* produces acid in tsetse's gut such that the environment becomes inhospitable to
308 trypanosomes. To address whether decreased midgut pH adversely impacts tsetse
309 fitness, we quantified several fitness parameters in $Gmm^{WT/Esp\_Z}$ (a sample of these
310 individuals housed $1.39x10^6 \pm 1.1x10^5$ *Esp_Z* at the time they were used for
311 experimentation; S1 Fig. F).

312 We began by measuring midgut weight, which reflects over all digestive health.
313 We observed no significant difference in midgut weight between 8 day old $Gmm^{WT/Esp\_Z}$
314 and $Gmm^{WT}$ males (3.8 ± 1.1 mg and 3.2 ± 1.2 mg, respectively) and females (12.8 ±
315 1.8 mg and 13.0 ± 1.8 mg, respectively) 24 hrs post last blood meal acquisition (Fig.
316 7A). We next measured fecundity parameters in female and male $Gmm^{WT/Esp\_Z}$ and
317 $Gmm^{WT}$ to determine if stable infection with this bacterium would alter their reproductive
318 capacity. We began by measuring gonotrophic cycle (GC) duration of $Gmm^{WT/Esp\_Z}$ and
319 $Gmm^{WT}$ females. The length of the 1st GC was not significantly different between
320 $Gmm^{WT/Esp\_Z}$ (24.0 ± 1.2 days) and $Gmm^{WT}$ females (24.0 ± 0.9 days) (Fig. 7B).
321 However, the 2nd and 3rd GCs of $Gmm^{WT/Esp\_Z}$ females (13.0 ± 1.1 and 14.0 ± 1.1 days,
322 respectively) were significantly longer than those of their age-matched WT counterparts
323 (14.0 ± 1.0 and 11.5 ± 1.1 days, respectively) (Fig. 7B). We also determined that pupal
324 weight from all three GCs was similar between both fly groups (GC1, $Gmm^{WT/Esp\_Z}$ =
325 23.1 ± 1.5 mg, $Gmm^{WT}$ = 22.9 ± 1.4 mg; GC2, $Gmm^{WT/Esp\_Z}$ = 23.4 ± 1.7 mg, $Gmm^{WT}$ =
326 24.2 ± 1.8 mg; GC3, $Gmm^{WT/Esp\_Z}$ = 24.7 ± 1.7 mg, $Gmm^{WT}$ = 24.4 ± 1.6 mg)(Fig. 7C).
327 Thus, *Esp_Z* infection impacts the reproductive physiology of female tsetse by

328 increasing GC duration and hence the number of offspring infected individuals are able
329 to produce over the course of their lifespan. However, despite this effect, infection with
330 this bacterium does not impact pupal weight.

331 We also investigated the effect of *Esp_Z* infection on the reproductive fitness of
332 male tsetse by comparing the mating competitiveness of *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$
333 individuals. To do so we set up 80 individual cages, each of which contained one
334 sexually mature virgin female. We subsequently placed one sexually mature
335 *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ male in each cage and monitored the arena to determine
336 which of the two males successfully mated with the female. We observed that 47.5% of
337 matings occurred between *Gmm*$^{WT/Esp\_Z}$ males and females (neither male mated with
338 the female in two of the cages) (Fig. 7D), thus indicating that *Esp_Z* infection does not
339 significantly alter male mating competitiveness. Next we compared the number of sperm
340 present in three and 14 day old *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ males by quantifying
341 transcript abundance of *sperm-specific dynein intermediate chain* (*sdic*). The *Drosophila*
342 homologue of this gene is transcribed exclusively in sperm cells (Nurminsky et al.,
343 1998) and is used to quantify sperm abundance (Yeh et al., 2012). We observed no
344 significant difference in *sdic* transcript abundance between three day old or 14 day old
345 *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ males (Fig. 7E).

346 Finally, we examined whether the presence of *Esp_Z* impacts the density of
347 endosymbiotic *Wigglesworthia* and *Sodalis*. These measurements, which were taken at
348 14 days post-inoculation with *Esp_Z*, are important because tsetse's microbiota impact
349 many aspects of their host's fitness, including fecundity and immune system
350 development and function (Michalkova et al., 2014; Vigneron and Weiss, 2017). We

observed that infection with *Esp_Z* did not significantly alter the density of tsetse's midgut (bacteriome) population of obligate *Wigglesworthia* in $Gmm^{WT/Esp\_Z}$ males or females (Fig. 7F). Conversely, midguts from $Gmm^{WT/Esp\_Z}$ males and females housed significantly fewer *Sodalis* than did midguts from their $Gmm^{WT}$ counterparts (Fig. 7F). Taken together, these data indicate that *Esp_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 *Esp_Z*, which is a bacterium found naturally in the gut of some *An. gambiae* populations, and directly kills *Plasmodium* by producing anti-parasitic ROIs (Cirimotich et al., 2011), 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. *Esp_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 *Esp_Z* exerts only a negligible effect on tsetse's reproductive fitness, the bacterium's presence does reduce the density of endosymbiotic *Sodalis*. Cumulatively, our findings suggest that *Esp_Z* could be used in a natural setting to artificially reduce disease transmission by this arthropod vector.

379 Herein we demonstrate that *Esp_Z* is able to stably colonize tsetse's gut, which
380 is an outcome that likely results at least in part from the bacterium's resistance to
381 antimicrobial PGRP-LB. This protein is constitutively produced in the fly's midgut and
382 directly kills trypanosomes (Wang et al., 2012) and *E. coli* K12 (Fig. 2 in this study, and
383 Wang et al., 2012). *Esp_Z* resistance to tsetse PGRP-LB may reflect one of many
384 bacterial adaptations that result from residing within the immunologically hostile
385 environment of the insect midgut. While the specific physiological mechanism(s) that
386 *Esp_Z* uses to facilitate its colonization of tsetse's midgut are currently unknown, the
387 bacterium survives for prolonged periods within the gut of *An. gambiae* in part by
388 increasing expression of genes that encode a type III secretion system apparatus
389 protein as well as glutathione S-transferase and oxidoreductase (Dennision et al.,
390 2016). Type III secretion system proteins can facilitate bacterial penetrance into host
391 cells (Dale and Moran, 2006) and be involved in subversion of host immunity (Raymond
392 et al., 2013), while the latter two proteins are antioxidant pathway components that
393 mediate redox homeostasis in oxidatively stressful environments such as the insect
394 midgut (Ketterman et al., 2011; Pedrini et al., 2015). *Esp_Z* may employ similar
395 mechanisms to survive in tsetse's immunologically hostile gut. *Sodalis* is also resistant
396 to tsetse antimicrobial immune response (Hu and Aksoy, 2005; Wang et al., 2013),
397 which may be the result of structural adaptations present in exposed bacterial surface
398 coat molecules, including lipopolysaccharide (Toh et al., 2006) and outer membrane
399 protein A (Weiss et al., 2008). Furthermore, *Sodalis* enters into host cells through the
400 use of a type III secretion system (Dale et al., 2001), which may further protect the
401 bacterium from tsetse's immunologically hostile midgut environment. Likewise, similar

mechanisms may facilitate *Esp_Z* survival in this niche. Additionally, *Esp_Z*, like *Sodalis* (Maltz et al., 2012), 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 (Ratzke et al., 2018a). Depending on specific physiological circumstances, these effects can reflect the consumption of resources and/or the production of beneficial or harmful metabolic byproducts (Celiker and Gore, 2013; Ratzke et al., 2018b). Tsetse's sole energy source, vertebrate blood, is rich in glucose. Many bacterial taxa, including *Enterobacter* spp., ferment this sugar, thus producing hydrogen that acidifies their environment (Ratzke et al., 2018b, Goldford et al., 2018). The acidic environment present in tsetse's gut when *Esp_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 (Liniger et al., 2003), sand flies (Rosenzweig et al., 2007) and mosquitoes (del Pilar Corena et al., 2005) 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 *Esp_Z* induced conditions in tsetse's gut detrimentally impact not only trypanosomes but also other enteric microbes including entomopathogenic *S. marcescens* and symbiotic *Sodalis*. *Esp_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

425 impact the fly's overall fitness and/or vector competency. For example, reducing *Sodalis*
426 density significantly decreases tsetse longevity (Dale and Welburn, 2001). This may
427 prove beneficial because flies with a reduced life span could perish before
428 trypanosomes are able to complete their 20-30 extrinsic incubation period (Aksoy et al.,
429 2001). A reduction in *Sodalis* density could be further beneficial because tsetse that
430 house relatively low densities of the bacterium are less likely to be infected with
431 trypanosomes than are individuals that house more of the symbiont (Welburn et al.,
432 1993; Dale and Welburn, 2001; Farikou et al., 2010; Soumana et al., 2013; Griffith et al.,
433 2018). Thus, the trypanosome refractory phenotype presented by *Esp_Z* colonized
434 tsetse may result in part from, or be enhanced by, the fact that they contain fewer
435 *Sodalis*. Finally, the midgut of wild tsetse is colonized by a transient population of
436 environmentally acquired bacteria (Wang et al., 2013). The contribution of these
437 bacteria to tsetse's physiology has not been characterized, and as such, interference
438 with this microbial population could further alter the fly's physiological homeostasis. To
439 the contrary, the environmentally acquired microbiota could out-compete *Esp_Z* that
440 reside in tsetse's gut, or could prevent the bacterium from acidifying the environment.
441 Future studies are required to elucidate microbe-microbe interactions in the gut *Esp_Z*
442 colonized flies after their release into the field.

443 Reducing the incidence of African trypanosomiases has to date been achieved
444 largely by controlling the size of tsetse populations. This process is currently
445 accomplished by employing area wide integrated pest management (AW-IPM)
446 strategies that make use of insecticides, traps and sterile insect technique (SIT)
447 (Vreyson et al., 2013; Percoma et al., 2018). SIT involves sequentially releasing a large

448 number of sterilized males (achieved by irradiating pupae) into the target environment.
449 These males reproductively outcompete wild males for female mates, and the
450 population size drops significantly, or the fly is completely eradicated (McGraw and
451 O'Neill, 2013). The efficacy of SIT as a means of controlling tsetse populations is well
452 exemplified on Unguja Island (the large island of the Zanzibar archipelago), where the
453 technique was used to eradicate *G. austeni*, the main vector of trypanosomes that
454 cause animal African trypanosomiasis in that locale (Vreysen et al., 2000). One
455 shortcoming of this procedure is that releasing large numbers of sterile males
456 significantly increases the population of potential disease vectors in that environment
457 (male tsetse also feed exclusively on vertebrate blood). One way to overcome this
458 obstacle is to release sterilized males that present enhanced refractoriness to parasite
459 infection. This outcome is currently achieved by feeding the sterilized males twice with
460 the drug isometamidium chloride prior to their release (Bouyer, 2008). However, treated
461 flies are not 100% resistant to infection (Bouyer, 2008), and the risk exists that the
462 parasite will eventually develop resistance to the drug. Our data presented herein
463 indicate that inoculating sterilized males with *Esp_Z* prior to their release would serve as
464 an alternative, or supplemental, means of making the flies resistant to infection.
465 Specifically, $Gmm^{WT/Esp\_Z}$ males (and females) are significantly more refractory to
466 infection with trypanosomes than are their wild-type counterparts. This finding implies
467 that sterilized, $Gmm^{WT/Esp\_Z}$ individuals would be relatively poor vectors of disease-
468 causing trypanosomes and thus safer to release than sterilized males that do not house
469 this bacterium. Furthermore, our preliminary analyses suggest that *Esp_Z* infection
470 does not compromise the mating competitiveness nor sperm abundance of male tsetse,

thus suggesting that *Gmm*$^{WT/Esp\_Z}$ 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 (de Beer et al., 2017), prior to colonization with *Esp_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, *Esp_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 *Esp_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 *Esp_Z* must be studied in more detail before the bacterium is used in this capacity. Of particular importance are studies aimed at determining whether *Esp_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 bovine blood through an artificial membrane feeding system every 48 h. Aposymbiotic tsetse (*Gmm*$^{Apo}$) were generated and maintained as described previously (Weiss et al., 2012). 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 (Dale and Maudlin, 1999), and subsequently maintained in liquid brain heart infusion (BHI) media. When necessary, *Sodalis* were plated on either Brain Heart Infusion agar supplemented with 10% defibrinated bovine blood (BHIB) or Mitsuhashi-Maramorosch (MM)-agar plates. *Enterobacter* sp Z (*Esp_Z;* isolated from the gut of the mosquito, *Anopheles gambiae*) (Cirimotich et al., 2011) and *Serratia marcescens* (strain db11; isolated from a

511 moribund *Drosophila* sp.) (Nehme et al., 2007) were grown in liquid LB media or on LB-
512 agar plates at 30°C.

513 *In vivo Trypanosoma congolense* and *T. brucei brucei* (YTAT 1.1) were
514 expanded in rats, and harvested from infected blood at peak parasitemia. Rat blood
515 containing blood stream form (BSF) parasites was aliquoted and cryopreserved for
516 subsequent tsetse challenge experiments.

517

518 *Recombinant PGRP-LB antibacterial assays*

519 Antibacterial activity of recombinant (rec) PGRP-LB was determined as described
520 previously (Wang et al., 2012), with minor modification. Specifically, recPGRP-LB was
521 added (10 µg/ml of media) to early log-phase (OD = 0.2-0.4) cultures of *Esp_Z*, *Sodalis*
522 and *E. coli*. Controls consisted of bacterial cultures exposed to bovine serum albumin.
523 Using a plate-based quantification assay (Maltz et al., 2012), *E. coli* and *Esp_Z* density
524 was subsequently measured 1 hr. later, while *Sodalis* density was measured 24 hr.
525 later. Results are presented as % of initial inoculum, which was determined by dividing
526 the number of bacterial CFU present after treatment with recPGRP-LB by the number of
527 CFU present prior to inoculation.

528

529 *Microbial infection assays*

530 *Per os* bacterial challenge of wild-type (*Gmm*$^{WT}$) and *Gmm*$^{Apo}$ flies was performed by
531 feeding teneral adults a heat inactivated (HI; 56°C for 1 hr) blood meal inoculated with
532 5x10$^4$ colony forming units (CFU) of each bacterial strain per ml of blood. Because
533 tsetse flies consume approximately 20 µl of blood per feeding, each fly is inoculated

534 with 1x10$^3$ bacterial cells. *Gmm*$^{Apo}$ flies colonized with either *Sodalis* or *Esp_Z* are
535 designated *Gmm*$^{Apo/Sgm}$ and *Gmm*$^{Apo/Esp\_Z}$, respectively, and *Gmm*$^{WT}$ flies colonized with
536 *Esp_Z* are designated *Gmm*$^{WT/Esp\_Z}$. For all experiments that employed tsetse flies
537 inoculated with either *Sodalis* or *Esp_Z*, bacterial midgut density was determined by
538 homogenizing microscopically dissected gut tissue in 0.85% NaCl and serially diluting
539 and plating the samples on LB-agar (*E. coli*, *Esp_Z* and *Serratia*) or BHIB or MM
540 (*Sodalis*) plates supplemented with antibiotics. CFU per plate were counted manually,
541 and counts are presented in the corresponding Results subsections.
542 For trypanosome infections, all flies received infectious blood meals containing
543 1x10$^6$/mL BSF *T. congolense* or *T. b. brucei* parasites. *Gmm*$^{Apo}$, *Gmm*$^{Apo/Sgm}$ and
544 *Gmm*$^{Apo/Esp\_Z}$ were challenged as eight day old adults (3$^{rd}$ blood meal), while *Gmm*$^{WT}$
545 and *Gmm*$^{WT/Esp\_Z}$ flies were challenged as five day old adults (2$^{nd}$ blood meals). For
546 *Esp_Z*/trypanosome co-infection experiments, distinct groups of mature *Gmm*$^{WT}$
547 individuals were inoculated with 1x10$^6$/mL BSF *T. congolense* parasites and 5x10$^4$
548 CFU/ml of *Esp_Z*. Two weeks post-trypanosome challenge, all flies were dissected and
549 their midguts microscopically examined to determine parasite infection status.
550
551 *Detection and inhibition of tsetse reactive oxygen intermediates*
552 *Esp_Z* and *Sodalis* cultures were grown to mid-log phase (OD = 0.25), and cell-free
553 supernatants were generated via centrifugation. Hydrogen peroxide ($H_2O_2$)
554 concentrations in bacterial culture supernatants were determined using an Amplex Red
555 Hydrogen Peroxide/Peroxidase assay kit according to the manufacturer's (Invitrogen)
556 protocol. In brief, supernatants were incubated for 30 min. with the assay reagent, and

557 resulting fluorescence units were quantified using a Bio-Tek plate reader.

558 Antioxidants were used to inhibit tsetse ROI activity *in vivo*. The assay used was
559 similar to those described previously (MacLeod et al., 2007; Cirimotich et al., 2011;
560 Vigneron et al., 2018). In brief, treated flies were offered a blood meal inoculated with
561 trypanosomes [1x$10^6$/mL BSF *T. b. brucei* (YTAT 1.1)] and supplemented with vitamin C
562 (10mM) or cysteine (10μM). All subsequent meals also contained antioxidant
563 supplements.

564

565 *Determination of bacterial acid production in vitro*

566 *Sodalis* and *Esp_Z* were grown in their respective liquid media to an O.D. of 1.0.
567 Subsequently, 5x$10^6$ cells (this value represents the approximate maximum density to
568 which these bacteria grow in tsetse's gut; see Fig. 1A) were diluted to a volume of 1 ml
569 (again in respective liquid media) and heat-killed (80°C for 1.5 hr). Conditioned media
570 containing dead cells was added to early log growth phase *T. b. brucei* YTAT 1.1 grown
571 in a Beck's medium (GE Hyclone), which contains phenol red. When in solution this
572 compound serves as a pH-sensitive colorimetric indicator that changes from pink-red to
573 yellow as environmental pH drops. Other treatment groups were inoculated with 1 ml of
574 heated, clean LB (*Esp_Z* growth medium) or clean BHI (*Sodalis* growth medium), while
575 the control group consisted of trypanosomes alone. Two hours after exposing *T. b.*
576 *brucei* to treatment conditions, cultures were assayed to determine pH using a Mettler
577 Toledo pH meter. The pH of trypanosome containing Beck's medium was
578 experimentally reduced (to pH 5.8) via the addition of 0.1N HCl, while HK *Esp_Z*
579 extracts were buffered to pH 7.2 via the addition of 0.1N NaOH. Trypanosome density in

580 all treatment and control groups was determined at 2, 5 and 24 hour time points by
581 counting live parasites using a Brite-Line hemocytometer.
582
583 *Determination of bacterial acid production in vivo*
584 Microbial regulation of pH in tsetse's midgut was determined by feeding teneral *Gmm*$^{Apo}$
585 flies a HI blood meal inoculated with either *Sodalis* or *Esp_Z* (5x10$^4$ CFU/ml of blood).
586 Additionally, teneral *Gmm*$^{WT}$ flies received the same quantity of *Esp_Z*. Five days post-
587 bacterial challenge, colonized individuals were administered a meal composed of
588 sucrose (2.5%) and phenol red (0.04%) solubilized in water. Twenty-four hours later, the
589 color of the solution contained in the midgut was determined by incising the fly abdomen
590 and observing the intact gut using a dissecting microscope (Zeiss Discovery) equipped
591 with a digital camera (Zeiss AxioCam MRc 5). Remaining flies were dissected and their
592 guts were harvested, homogenized in 0.85% NaCl, serially diluted and plated onto MM-
593 agar plates (prepared as described in Weiss et al., 2008) supplemented with phenol red
594 (0.025 g/L) and sucrose (a 2.5% sucrose solution was spread onto plates immediately
595 prior to applying tsetse gut extracts). CFU per plate was counted manually, and the
596 growth medium was monitored to observe pH-induced changes in color.
597
598 *Fitness assays*
599 For all fitness assays, *Gmm*$^{WT}$ teneral females and males were infected with *Esp_Z*
600 during their first blood meal. To determine midgut weight, midguts were dissected from
601 9 day old *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ females and males (24 h after consuming their last
602 blood meal) and weighed using a Mettler Toledo (AL104) balance. The effect of *Esp_Z*

603 infection on female fecundity was measured by quantifying the length of three
604 gonotrophic cycles (GC) and by weighing pupal offspring. To measure GC length,
605 *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ females were mated as 5 day old adults and thereafter
606 maintained in individual cages. All females were monitored daily to determine when they
607 deposited larvae, and all deposited larvae were weighed.
608 The effect of *Esp_Z* infection on male reproductive fitness was measured by
609 quantifying the mating competitiveness and sperm abundance of individuals that
610 housed the bacterium versus those that did not. Mating competitiveness assays were
611 performed in individual cages, each of which housed one 5 day old virgin female (fed
612 twice). Subsequently, one age-matched *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ male (also fed twice)
613 was added to each cage. These males were distinguished from one another by
614 removing the proximal tarsus of the right foreleg from one of the individuals. The arena
615 was observed until one of the males had successfully mounted the female, at which
616 point the cage was submerged in ice and the free male identified. To eliminate any bias
617 associated with removal of the foreleg tarsus, the experiment was repeated twice (*n*=40
618 cages per experiment), each time with either *Gmm*$^{WT/Esp\_Z}$ or *Gmm*$^{WT}$ males receiving
619 the distinguishing procedure. Sperm abundance was measured by RT-qPCR
620 quantification of *sperm-specific dynein intermediate chain* (*sdic*) expression in the
621 reproductive tracts of three and 14 day old (fed twice) virgin *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$
622 males. Absolute *sdic* transcript abundance was determined by comparing experimental
623 sample cycle threshold ($C_t$) values to those derived from an *sdic* internal standard
624 curve.

625 *Sodalis fliC* and *Wigglesworthia thiC* gene specific primers were used to quantify
626 the absolute abundance of these bacteria. This was performed by comparing *Sodalis*
627 *fliC* and *Wigglesworthia thiC* cycle threshold ($C_t$) values in $Gmm^{WT/Esp\_Z}$ and $Gmm^{WT}$
628 females and males to those derived from symbiont gene-specific internal standard
629 curves. Because *Wigglesworthia* and *Sodalis* can be polyploid (Rio et al., 2006; Weiss
630 et al., 2006), we normalized symbiont genome copy number to constitutively expressed
631 tsetse *gapdh* copy number. All RT-qPCR primers are listed in S2 Table. All RT-qPCR
632 assays were carried out in duplicate, and replicates were averaged for each sample.
633 Negative controls were included in all amplification reactions.
634
635 *Statistical analyses*
636 For trypanosome infection experiments, statistical analyses were carried out using the R
637 software for macOS (version 3.3.2) or GraphPad Prism(v.6). A generalized linear model
638 (GLM) was generated using binomial distribution with a logit transformation of the data.
639 The binary infection status (infected or recovered) was analyzed as a function of the
640 bacterium used to colonized the insects (or its absence). For experiments requiring a
641 pairwise comparison, we performed a Wald test on the individual regression parameter
642 (nature of the bacterium used to colonize) to test its statistical difference. For
643 experiments requiring multiple comparisons, multiple pairwise tests were generated
644 using Tukey contrasts on the generalized linear model (GLM) using glht() function of
645 "multcomp" package in R. Details of the statistical tests described above are indicated
646 in S1 Dataset. All statistical tests used, and statistical significance between treatments,
647 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.

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957 **Figure legends**

958 Figure 1. Bacterial colonization of tsetse's midgut, and the effect on fly survival. Distinct
959 groups of newly emerged adult wild-type (*Gmm*$^{WT}$) and aposymbiotic (*Gmm*$^{Apo}$) females
960 were colonized with 1x10$^3$ CFU of either *Enterobacter* (*Esp_Z*) or *Sodalis* (*Sgm*), and
961 then bacterial density and fly survival was measured. (A) Average number (±SEM) of
962 bacterial CFUs per tsetse gut per group per time point. $n \geq 5$ individuals per group per
963 timepoint. (B) Kaplan-Meier plot depicting survival of *Gmm*$^{WT}$ and *Gmm*$^{Apo}$ females
964 colonized with either *Esp_Z* or *Sgm*. Infection experiments were performed in triplicate,
965 using 25 flies per replicate. No significant difference in survival was observed between
966 any of the fly groups ($p$=0.88; log-rank test).

967

968 Figure 2. *Esp_Z* is resistant to normally bactericidal Peptidoglycan Recognition Protein-
969 LB. Survival of cultured *Esp_Z*, *Sodalis* and *E. coli* following exposure (1 hr for *Esp_Z*
970 and *E. coli*, and 24 hr for *Sodalis*) to recombinant (rec) PGRP-LB (10 µg/ml). Results
971 are presented as % of initial inoculum, which was determined by dividing the number of
972 bacterial CFU present after treatment with recPGRP-LB by the number of CFU present
973 prior to inoculation. Statistical significance was determined using a one-way ANOVA
974 followed by Tukey's HSD post-hoc analysis

975

976 Figure 3. The trypanosome refractory phenotype exhibited by *Gmm*$^{Apo/Esp\_Z}$ flies is not
977 directly caused by reactive oxygen intermediates. (A) Percentage of *Gmm*$^{Apo}$,
978 *Gmm*$^{Apo/Sgm}$ and *Gmm*$^{Apo/Esp\_Z}$ flies harboring midgut infections with bloodstream form
979 (BSF) YTAT 1.1 trypanosomes. Statistical analysis was performed using a GLM

980 followed by multiple comparisons and Tukey contrasts, and different letters represents
981 statistical significance between treatments and controls. (B) Mid-log phase cultures of
982 *Esp_Z* and *Sodalis* synthesize similar quantities of $H_2O_2$ ($p$=0.6; paired t-test).
983 Measurements were taken from 7 distinct clonal populations of each bacterium. (C)
984 Percentage of *Gmm*$^{Apo}$ and *Gmm*$^{Apo/Esp\_Z}$ flies infected with BSF YTAT 1.1
985 trypanosomes after being fed blood meals containing antioxidant vitamin C over the
986 course of the 14 day experiment. Despite exposure of both tsetse groups to the ROI-
987 suppressing vitamin, *Gmm*$^{Apo/Esp\_Z}$ flies were still significantly more refractory to
988 trypanosome infection than were *Gmm*$^{Apo}$ individuals ($p$=0.002; GLM Wald test). In (A),
989 (B) and (C) different letters represents statistical significance between treatments and
990 treatments and controls.
991
992 Figure 4. *Esp_Z* produces a low pH environment that is toxic to trypanosomes. (A) Early
993 log phase trypanosomes (*T. b. brucei* YTAT 1.1), cultured in 10ml of Beck's medium
994 containing the pH sensitive dye phenol red, exposed to 1ml of heat treated LB media
995 (*Esp_Z* culture medium), 1ml of heat treated BHI media (*Sodalis* culture medium), heat
996 killed (HK) *Esp_Z* ($5x10^6$ cells) in 1ml of LB media and HK *Sodalis* ($5x10^6$ cells) in 1ml
997 of BHI media. Controls are trypanosomes alone (tryps). All heated treatments and
998 controls were allowed to cool to room temperature prior to adding them to the
999 trypanosome cultures. Two hours post-treatment, culture pH was measured. HK *Esp_Z*
1000 significantly reduced the pH of the trypanosome culture ($p$<0.001). The experiment was
1001 repeated using 6 distinct clonal trypanosome populations (the image represents one of
1002 the six replicates). (B) Density of trypanosomes in culture 2h, 5h and 24h after addition

1003 of the treatments described in (A) above. At the 24h time point, all trypanosomes
1004 exposed to HK *Esp_Z* extracts were dead while those from the other groups were
1005 replicating similarly to controls. (C) Density of trypanosomes cultured in normal (pH 7.2)
1006 and artificially produced (via the addition of 0.1N HCl) acidic (pH 5.8) environments.
1007 Artificial acidic conditions kill all trypanosomes with 24 h. (D) The density of cultured
1008 trypanosomes exposed to HK *Esp_Z* extracts buffered to pH 7.2 (via the addition of
1009 0.1N NaOH). The buffering treatment rescues parasite growth. In (A), (B) and (C),
1010 statistical significance was determined using a one-way ANOVA followed by Tukey's
1011 HSD post-hoc analysis in (A), and a two-way ANOVA followed by Tukey's HSD post-
1012 hoc analysis in (B) and (C). Different letters represents statistical significance between
1013 treatments and controls. In (B), (C) and (D), experiments were performed using 5 or 6
1014 distinct clonal trypanosome populations.
1015
1016 Figure 5. *Esp_Z* acidifies tsetse's gut. Midgut pH of $Gmm^{Apo/Esp\_Z}$, $Gmm^{Apo/Sgm}$,
1017 $Gmm^{WT/Esp\_Z}$ and $Gmm^{WT}$ flies. Distinct groups of teneral $Gmm^{Apo}$ flies were inoculated
1018 *per os* with either *Esp_Z* or *Sodalis*, while $Gmm^{WT}$ individuals received *Esp_Z* (all flies
1019 received $5x10^4$ CFU of bacteria per ml of blood) or no bacteria. Five days post-
1020 inoculation, all individuals were offered a meal containing sucrose (2.5%) and phenol
1021 red (0.04%) dissolved in water. Twenty-four hours later, fly abdomens were excised and
1022 the color of the solution found within the midgut was observed. Each image represents
1023 one of five flies monitored for each treatment.
1024

Figure 6. *Gmm*$^{WT/Esp\_Z}$ flies are significantly more refractory to infection with parasitic trypanosomes and entomopathogenic *S. marcescens*. (A) Percentage of *Gmm*$^{WT}$ and *Gmm*$^{WT/Esp\_Z}$ flies infected with *T. congolense* 14 days after they were co-inoculated with *Esp_Z* and parasites in their first (teneral) blood meal. (B) Percentage of *Gmm*$^{WT}$ and *Gmm*$^{WT/Esp\_Z}$ flies infected with *T. congolense* (left graph) and *T. brucei* (right graph). For these experiments *Gmm*$^{WT/Esp\_Z}$ flies housed the exogenous bacteria for fives prior to challenge with trypanosomes. In (A) and (B) Statistical analysis was performed using a GLM followed by multiple comparisons and Tukey contrasts, and different letters represents statistical significance between treatments and controls. (C) Kaplan-Meier plot depicting survival of *Gmm*$^{WT}$ and *Gmm*$^{WT/Esp\_Z}$ flies following challenge with *S. marcescens*. Infection experiments were performed in triplicate, using 25 flies per replicate. *Gmm*$^{WT/Esp\_Z}$ flies were also significantly more refractory to *S. marcescens* infection than were their *Gmm*$^{WT}$ counterparts ($p$=0.001; log-rank test).

Figure 7. *Esp_Z* infection impacts specific tsetse fitness parameters. (A) Midgut weight, as an indicator of blood meal digestion proficiency, in six day old *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ males and females (guts were weighed 24 h after the flies had consumed their last blood meal). Each point on the graph represents one individual, and statistical significance was determined via multiple t-tests. (B). Gonotrophic cycle (GC) length of *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ females. Age-matched, pregnant females from each group ($n$=35 per group) were housed in individuals cages and monitored daily to observe frequency of pupal deposition. Statistical significance was determined via log-rank test. (C) Weight of pupae from *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ females over three GCs. Each point

on the graph represents one individual, and statistical significance was determined via multiple t-tests. (D) Mating competitiveness of *Gmm*$^{WT/Esp\_Z}$ compared to *Gmm*$^{WT}$ males. Matings were setup in individual cages (*n*=80). Each cage housed a virgin female, to which one virgin *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ male was added. Males and females were age-matched, and each had fed twice prior to exposure. Statistical significance was determined via Chi-squared test. (E) Sperm abundance in the reproductive tracts of three and 14 day old (fed twice) virgin *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ males. Sperm quantity is a reflection of *sperm-specific dynein intermediate chain* (*sdic*) transcript abundance. Absolute *sdic* transcript abundance was determined by comparing experimental sample cycle threshold ($C_t$) values to those derived from an *sdic* internal standard curve. Each point on the graph represents one individual, and statistical significance was determined via multiple t-tests. (F) Quantitation of *Wigglesworthia* and *Sodalis* in *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ male and female tsetse. Abundance of symbiont specific gene transcripts (*Wigglesworthia*, *thiC*; *Sodalis*, *fliC*) was used as a proxy to quantify bacterial load. This was performed by comparing *thiC* and *fliC* cycle threshold ($C_t$) values in *Gmm*$^{WT/Esp\_Z}$ and *Gmm*$^{WT}$ flies to those derived from symbiont gene-specific internal standard curves. *Wigglesworthia* and *Sodalis* can be polyploid (Rio et al., 2006; Weiss et al., 2006), and as such, we normalized symbiont genome copy number to constitutively expressed tsetse *gapdh* copy number. Each point on the graph represents one individual, and statistical significance was determined via multiple t-tests.

## Supporting information

**S1 Fig. Density [colony forming units (CFU) per fly gut] of exogenous *Esp_Z* and *Sodalis* in the gut of experimental flies, and *Esp_Z* acid production *in vitro*.** *Esp_Z* and *Sodalis* CFU/gut in *Gmm*$^{Apo/Sgm}$ and *Gmm*$^{Apo/Esp\_Z}$ flies prior to (A) challenge with trypanosomes and (B) measuring gut pH *in vivo*. (C) Guts form *Gmm*$^{Apo/Sgm}$ and *Gmm*$^{Apo/Esp\_Z}$ flies homogenized and plates 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) *Esp_Z* density in the gut of *Gmm*$^{WT/Esp\_Z}$ flies prior to measuring gut pH *in vivo*. (E) *Esp_Z* density in the gut of trypanosome infected (TI) and trypanosome refractory (TR) *Gmm*$^{WT/Esp\_Z}$ flies. Measurements were taken at the time infection status was determined (14 days post-challenge). (F) *Esp_Z* density in the gut of a random sample of *Gmm*$^{WT/Esp\_Z}$ flies used to determine the bacterium's impact of tsetse fitness parameters.

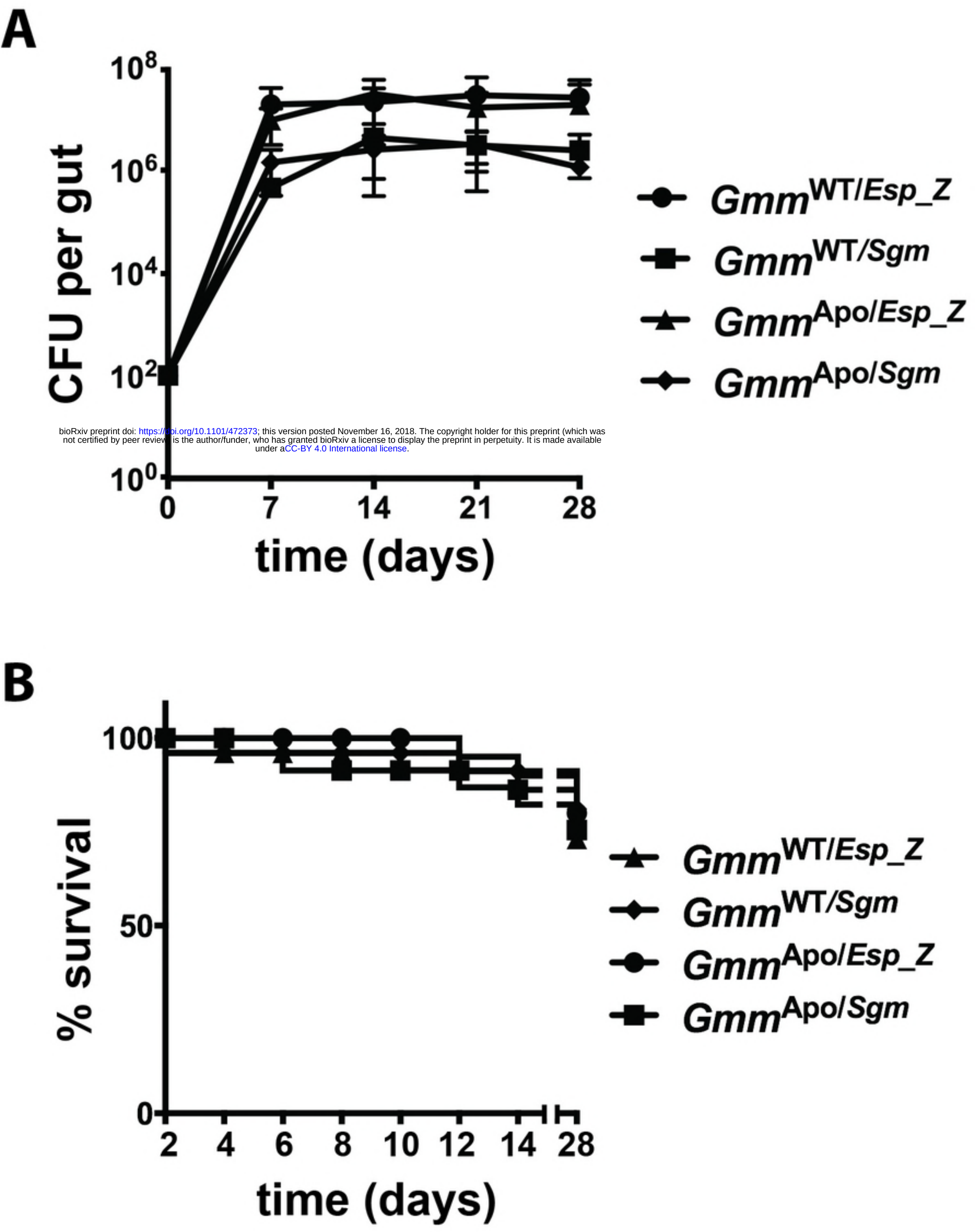



Figure1

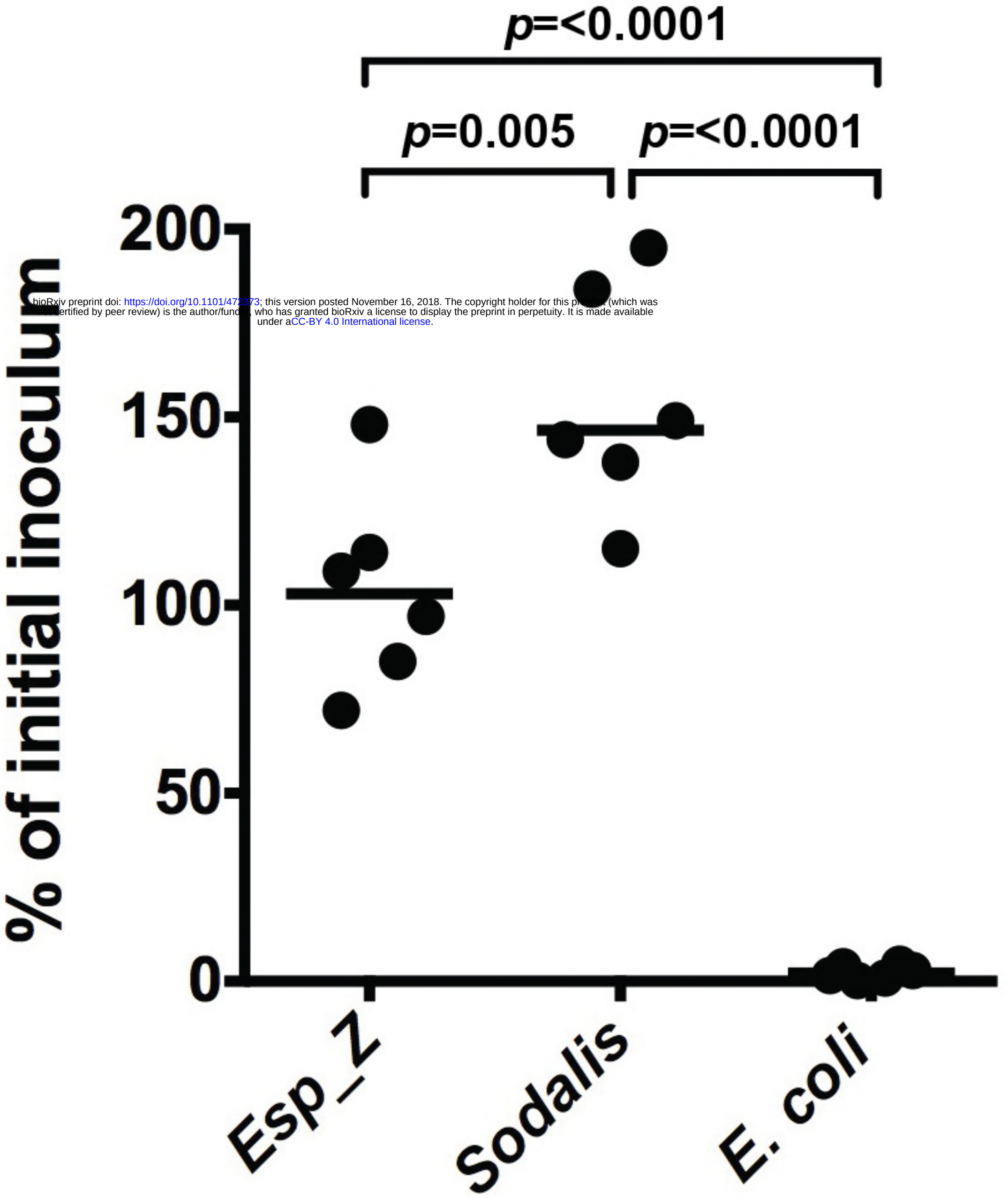



Figure2

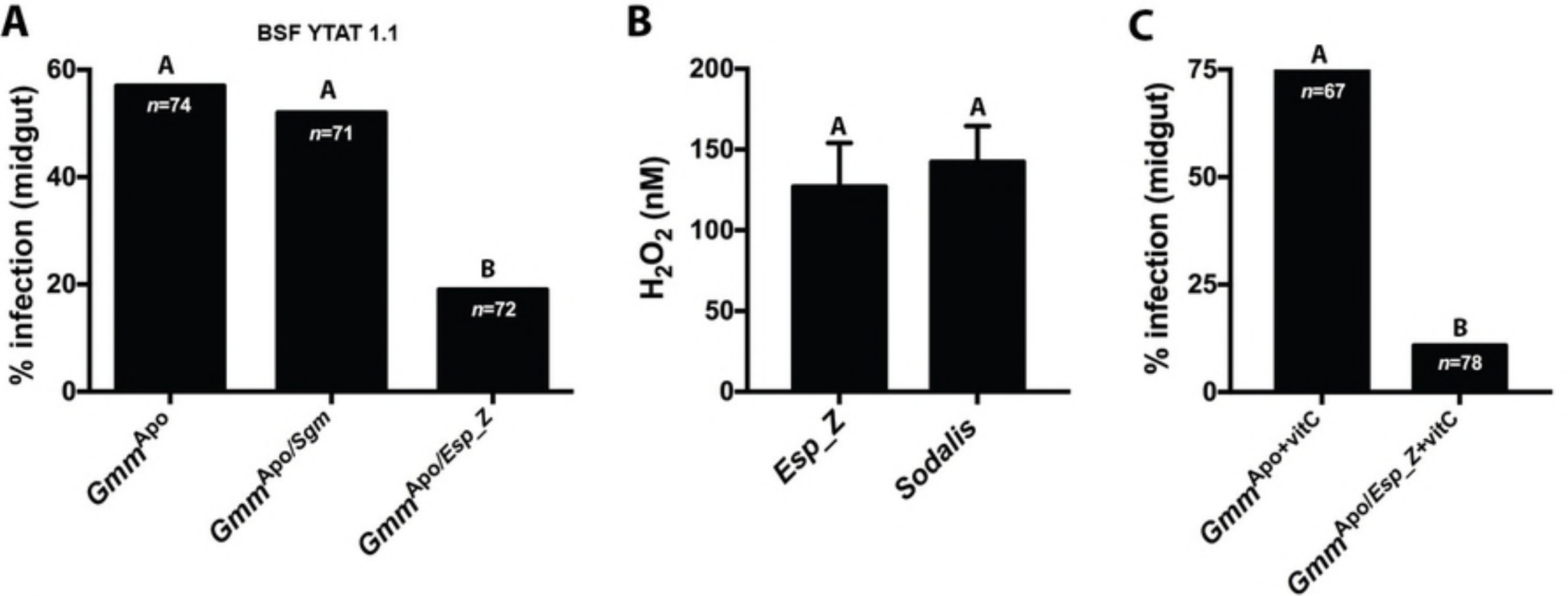
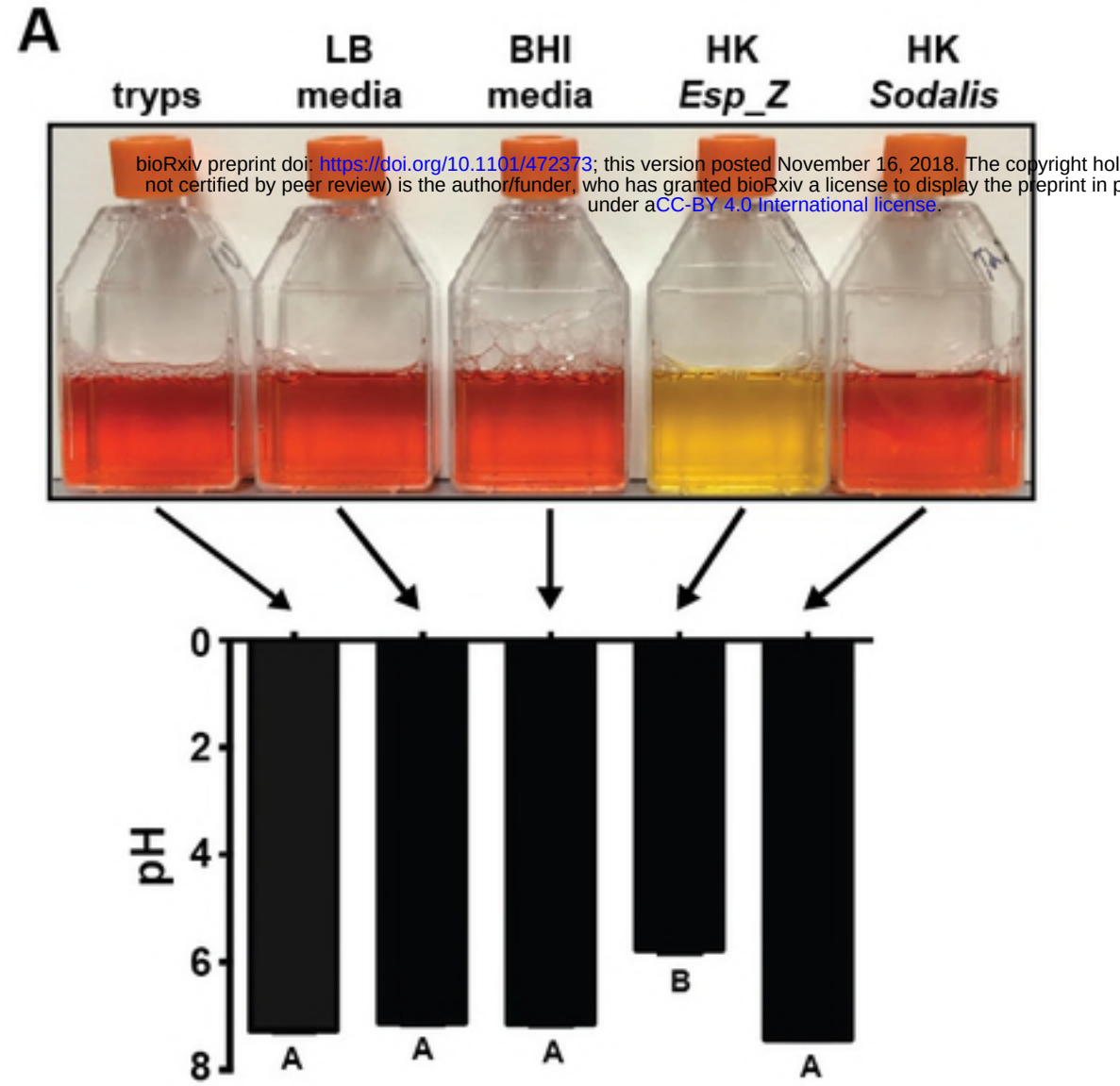
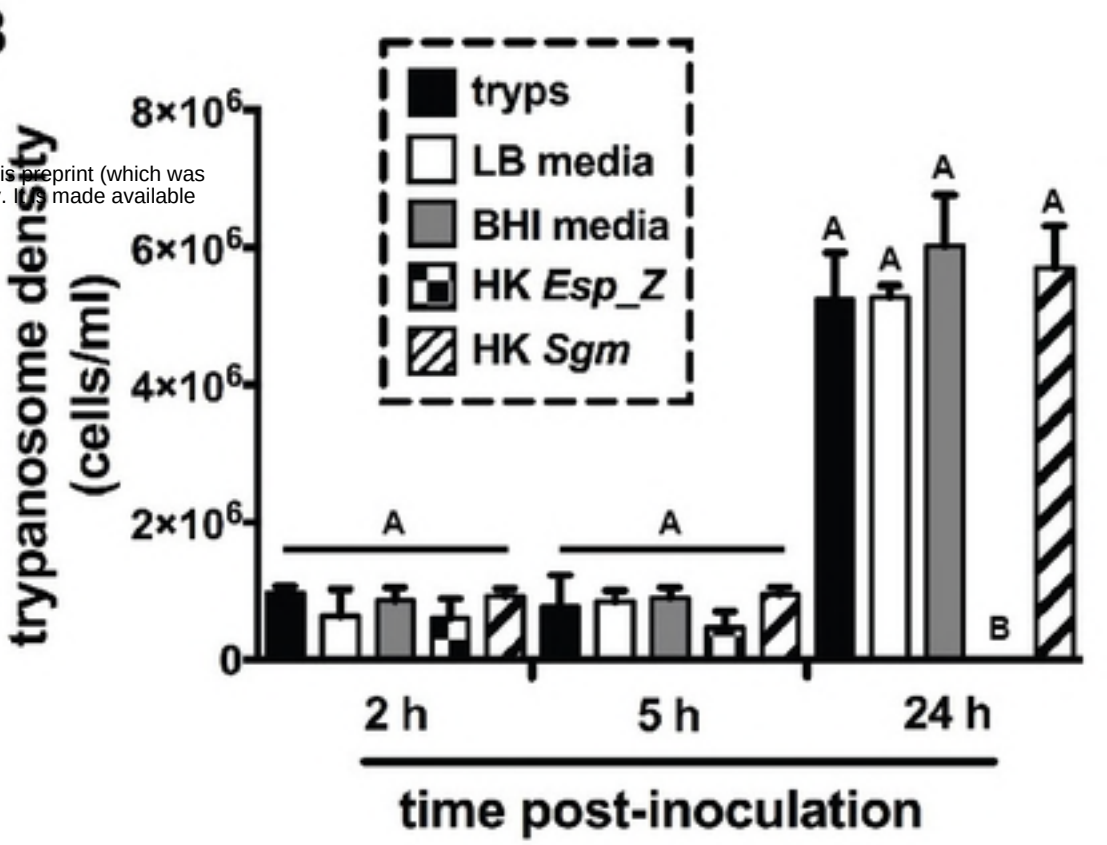
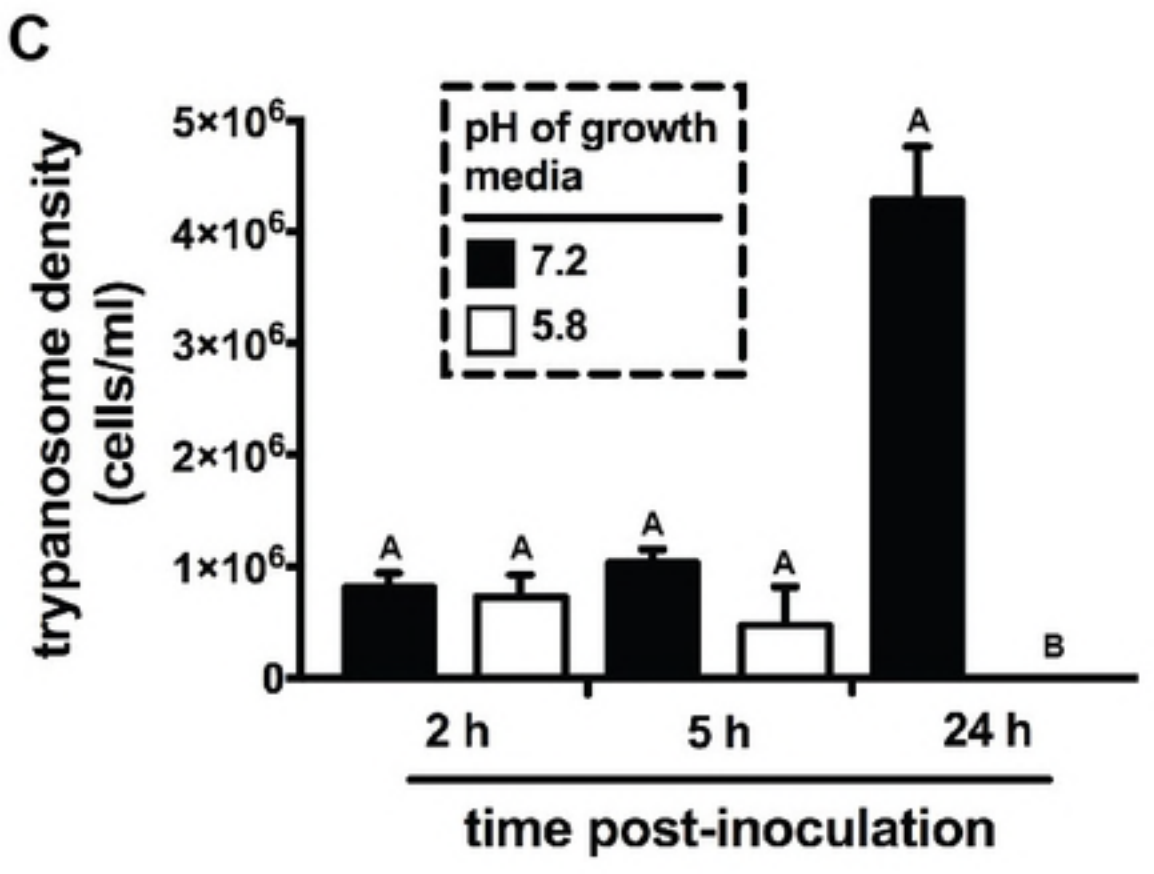

A
BSF YTAT 1.1
% infection (midgut)
60
40
20
0
A
n=74
A
n=71
B
n=72
GmmApo
GmmApo/Sgm
GmmApo/Esp_Z
B
H2O2 (nM)
200
150
100
50
0
A
A
Esp_Z
Sodalis
C
% infection (midgut)
75
50
25
0
A
n=67
B
n=78
GmmApo+vitC
GmmApo/Esp_Z+vitC

Figure3



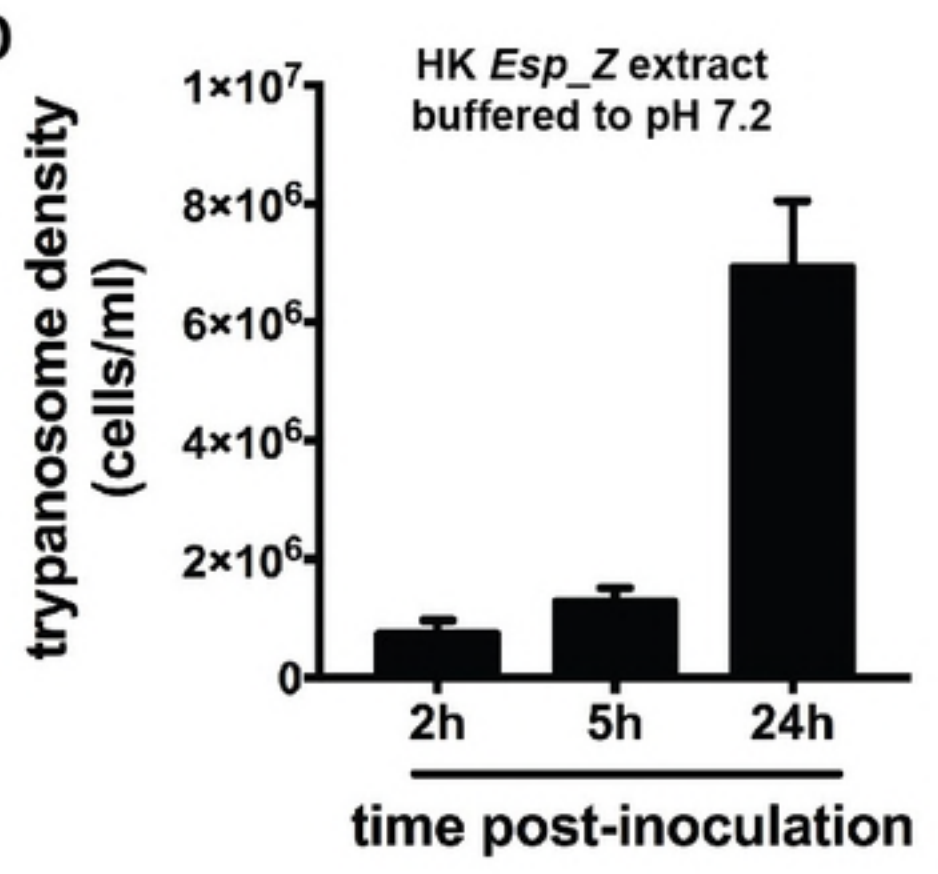




Figure4

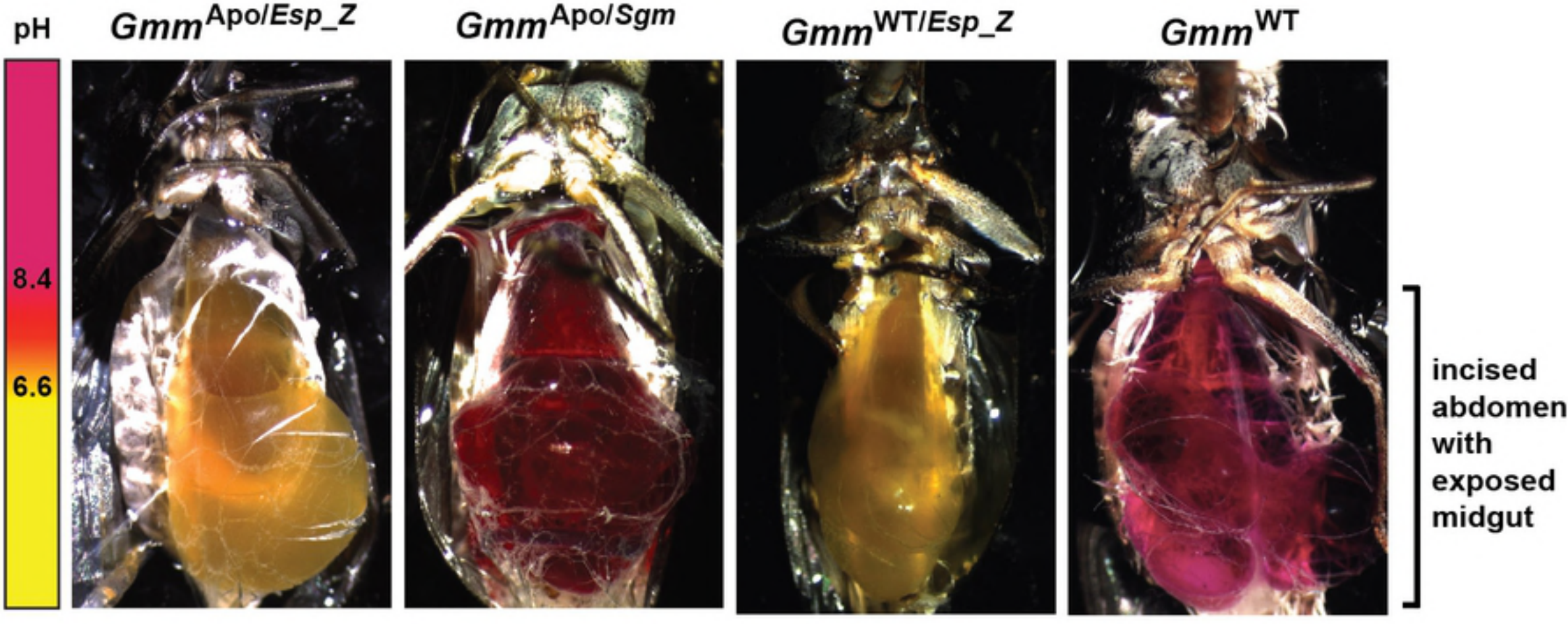

pH
Gmm Apo/Esp_Z
Gmm Apo/Sgm
Gmm WT/Esp_Z
Gmm WT
8.4
6.6
incised abdomen with exposed midgut

Figure5

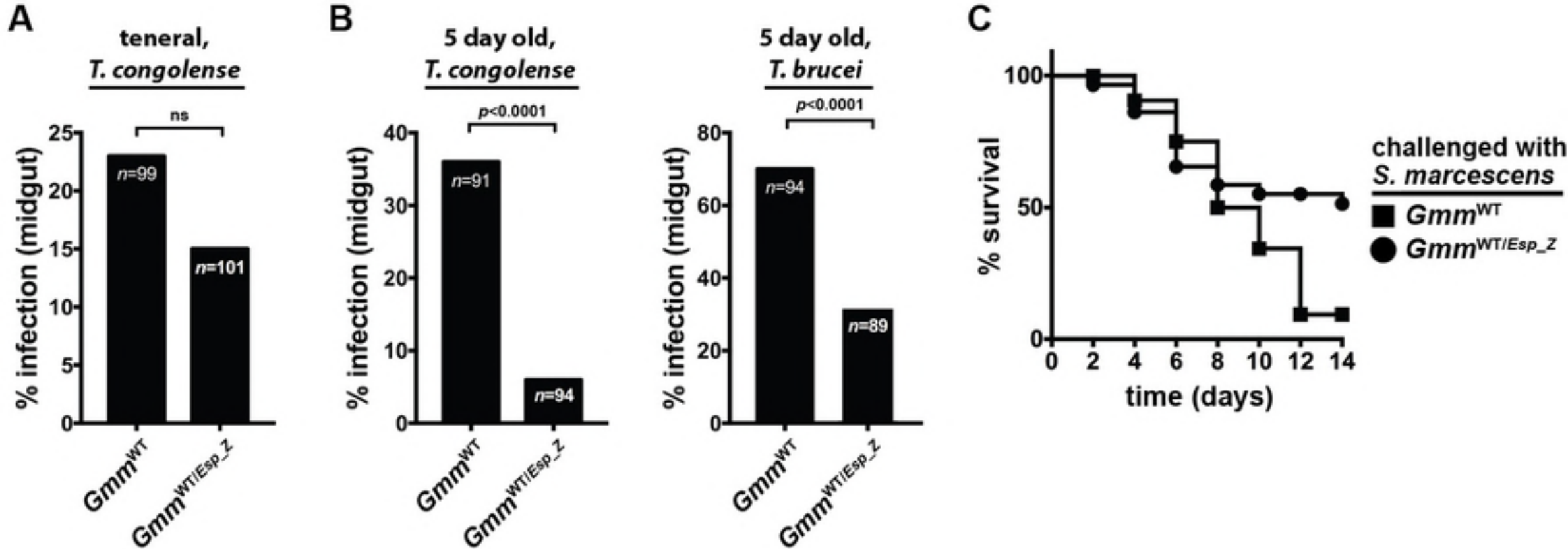

A
teneral,
T. congolense
ns
% infection (midgut)
n=99
n=101
Gmm^WT
Gmm^WT/Esp_Z
B
5 day old,
T. congolense
p<0.0001
% infection (midgut)
n=91
n=94
Gmm^WT
Gmm^WT/Esp_Z
5 day old,
T. brucei
p<0.0001
% infection (midgut)
n=94
n=89
Gmm^WT
Gmm^WT/Esp_Z
C
% survival
time (days)
challenged with
S. marcescens
Gmm^WT
Gmm^WT/Esp_Z

Figure6

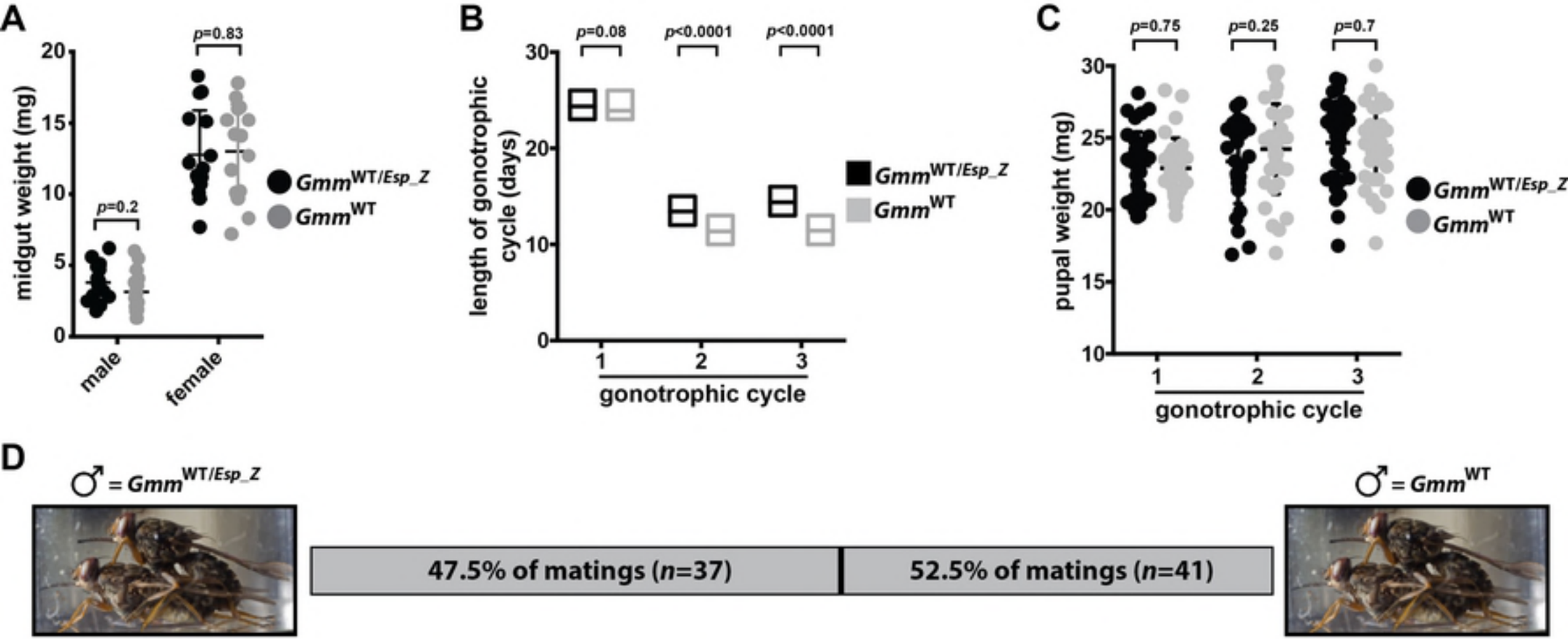

A
p=0.83
p=0.2
midgut weight (mg)
male
female
Gmm WT/Esp_Z
Gmm WT
B
p=0.08
p<0.0001
p<0.0001
length of gonotrophic cycle (days)
gonotrophic cycle
C
p=0.75
p=0.25
p=0.7
pupal weight (mg)
gonotrophic cycle
D
♂ = Gmm WT/Esp_Z
47.5% of matings (n=37)
52.5% of matings (n=41)
♂ = Gmm WT
E
p=0.06
p=0.98
total sdic transcript abundance
days post-emergence
F
ns
p<0.0001
symbiont genomes/fly
male
female
WT
WT/Esp_Z
Gmm cohort
Wigglesworthia (thiC)
Sodalis (fliC)

Figure7