

Gut dysbiosis allows foodborne *salmonella* colonization in edible crickets: a probiotic strategy for enhanced food safety

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Abstract

Aims: Edible insects, including crickets, represent a promising protein source, yet concerns over foodborne pathogens limit consumer acceptance. This study investigated whether gut microbiota modulates colonization by *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE) in the two-spotted cricket (*Gryllus bimaculatus*).

Methods and Results: Under standard conditions, SE was undetectable in crickets despite prolonged exposure; however, antibiotic-induced dysbiosis enabled stable SE colonization. Long-read 16S rRNA sequencing revealed significant microbiota shifts, notably a reduction in *Lactococcus garvieae*. *In vitro* assays showed strong inhibitory effects of *L. garvieae* against SE, and supplementation of dysbiotic crickets with *L. garvieae* reduced SE colonization by ~1000-fold.

Conclusions: The native cricket gut microbiota, especially *L. garvieae*, plays a protective role against SE colonization. Enhancing beneficial gut bacteria could mitigate pathogen risks and promote edible insects as a sustainable protein.

Impact Statement

Control of pathogens is essential for ensuring the safety of insect-based food products, especially as edible insects gain popularity as sustainable protein sources. This research contributes to food safety by revealing microbiome-based pathogen control mechanisms and guiding probiotic strategies to enhance product safety and consumer confidence.

Keywords: food safety; edible crickets; *Salmonella*; *Lactococcus*; probiotics; microbiome

Introduction

The increasing global population and the need for environmentally sustainable protein sources have spurred significant interest in edible insects, with crickets (*Gryllus bimaculatus*) emerging as a particularly promising option (FAO 2013). Their high protein content, favorable amino acid profile, and low environmental impact align well with efforts to meet future food demands while reducing agricultural footprints (Raheem et al. 2019a,b, Rumpold and Schlüter 2013, Musungu et al. 2023). Additionally, global production of premium-quality edible insects currently reaches about 10 000 t year⁻¹ and is projected to rise 50-fold within the next decade, underscoring the rapidly growing demand for insect-based foods (Aleknavičius et al. 2022). Despite these advantages, concerns regarding food safety remain an obstacle to wider consumer acceptance of cricket-based products. Potential hazards include microbial pathogens (e.g. *Salmonella* spp., *Listeria*), insect-specific viruses, parasitic pathogens, and chemical contaminants like heavy metals or pesticide residues (Grabowski and Klein 2017, Kooh

et al. 2019, Garofalo et al. 2019, Baiano 2020, Traynor et al. 2024).

Recent surveys of farmed crickets offer encouraging data: common foodborne bacteria such as *Salmonella*, *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium perfringens* were largely undetected in final products under hygienic rearing practices (Garofalo et al. 2017). Additionally, the presence of foodborne viruses in edible insects was investigated, and hepatitis A and E virus and norovirus genogroup II were not detected. Hence, the food safety risks related to these viruses can be stated to be very low (Vandeweyer et al. 2020). Nonetheless, contamination events remain possible; if insects, including crickets are exposed to pathogen-laden feed or unhygienic conditions, they may carry pathogens through their life stages (Pava-Ripoll et al. 2015, Walia et al. 2019).

A key component in understanding such risks involves the insect's gut microbiota. Like other animals, crickets harbor a diverse microbial community on their surface and within their digestive tract (Garofalo et al. 2017). Fluorescence *in situ* hybridization (FISH) studies demonstrated that "The

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Bacteroides-Prevotella probe, of the eight eubacterial probes tested, stained the largest percentage of cells in all crickets” (Santo Domingo et al. 1998), while high-throughput sequencing of Jamaican and house crickets revealed that “*Parabacteroides* was the most dominant taxon at genus level (36.17% for JC and 31.97% for HC)” (Aleksavičius et al. 2022). These Bacteroidetes genera form part of a conserved core that supports host health; indeed, “Representatives of these genera can resist intestinal inflammation, suppress the growth of pathogens, and accelerate the establishment of intestinal microbial balance.” Beneficial low-abundance taxa such as *Lactococcus*, *Akkermansia*, and *Coproccoccus* are likewise noted for “their ability to regulate the gut floramicrobiota and improve host health” (Aleksavičius et al. 2022). However, the same *in-situ* probe work showed that “hybridizations with group-specific probes indicated that dietary changes altered the structure of the hindgut community, although it did not change its predominantly eubacterial nature” (Santo Domingo et al. 1998). Such community shifts are thought to erode colonization resistance, providing a mechanistic link between microbiota balance and susceptibility to foodborne pathogens.

This resident microbiota can shape susceptibility to pathogens by outcompeting harmful microbes or stimulating the host’s innate immune responses (Steele et al. 2021, Wang et al. 2023). Although *Salmonella* has not been detected among the typical gut microbiota of farmed crickets, evidence from other insects suggests that disruptions to the microbiome or exposure to contaminated environments can alter pathogen resistance (Khan and Rolff 2024).

Given that *Salmonella enterica* serovar Enteritidis (SE) is a leading cause of foodborne gastroenteritis worldwide (Silva et al. 2014), its potential interactions with crickets warrant closer examination. In particular, whether a healthy cricket gut microbiota can inhibit *Salmonella* colonization remains an open question. Addressing this gap has both practical and scientific implications: from guiding good farming practices (e.g. hygienic rearing, possible probiotic supplementation) to deepening our understanding of host-microbe symbioses in insects. Therefore, the present study aims to evaluate how the native gut microbiome of *G. bimaculatus* influences *Salmonella* colonization under different microbiota states—thereby providing insights into microbiota-mediated defense mechanisms and informing strategies to further ensure the safety of edible cricket products.

Materials and methods

Crickets

Adult two-spotted crickets (*G. bimaculatus*) used in this study were obtained from an established laboratory colony [originally maintained for leg regeneration research (Bando et al. 2022)]. All crickets were reared under standard conditions (28°C, 12 h light:12 h dark photoperiod) as described by Mito and Noji (2008). The insects were fed *ad libitum* with a powdered feed (MF; Oriental Yeast Co., Ltd., Tokyo, Japan). Water was provided continuously using a 15 ml tube filled with water and fitted with a paper towel wick, following standard cricket husbandry practices.

Experimental groups and infection overview

Five experimental groups were defined for the present study. Crickets that received standard MF diet for 12 days without

Salmonella exposure served as an uninfected control and are referred to as MF-Ctrl. A second control cohort, 4Mix-Ctrl, was fed MF diet containing the four-antibiotic cocktail (4Mix, see Antibiotic Administration) for the same 12-day period to account for any effects of antibiotic treatment alone. The MF-Ctrl and 4Mix-Ctrl groups consisted of 12 crickets each. To evaluate infection in animals with an intact microbiota, the MF-SE^{high} group was maintained on MF diet for 9 days and then challenged with a high-dose (see Infection of Crickets with SE) *Salmonella*-infused paste for the subsequent 7 days. The 4Mix-SE^{high} group underwent 8 days of 4Mix feeding followed by a 24 h antibiotic-free washout and was subsequently exposed to the same high-dose *Salmonella* paste for 7 days. An additional infection cohort, 4Mix-SE^{low}, followed the identical antibiotic and wash-out schedule but was challenged with a low-dose (see Infection of Crickets with SE) *Salmonella* paste during the final 7-day phase. MF-SE^{high}, 4Mix-SE^{high}, and 4Mix-SE^{low} groups each consisted of 14 crickets. In all *Salmonella*-exposed groups, the infected paste was provided *ad libitum* and renewed daily. Intestinal tracts were sampled at 2, 4, and 7 days postinfection. A schematic timeline of treatments and sampling points is presented in Figure 1a, and operative details are described in the section “Infection of Crickets with SE.”

Bacterial strain and culture conditions

Salmonella Enteritidis ATCC 31194 (SE) was used for all infections. A frozen glycerol stock (−80°C) was thawed, streaked onto Luria–Bertani (LB) agar, and incubated overnight at 37°C. A single colony from this plate was inoculated into 100 ml of LB broth in a 300 ml Erlenmeyer flask and cultured at 37°C with shaking (200 rpm) until the culture reached an optical density at 600 nm (OD₆₀₀) of ~1.5. The culture was then centrifuged at 4°C, 12 000 × g for 10 min, and the resulting bacterial pellet was used for oral inoculation.

Infection of crickets with SE

For the oral infection test, SE culture broth incubated overnight was adjusted to ~5 × 10⁹ (high) or 5 × 10⁸ (low) CFU ml^{−1} based on OD₆₀₀, and preparations were made for inoculation into the feed. The feed paste was prepared by mixing 1 g of wheat flour and 2 g of sterilized cricket feed (MF). Two milliliters of the prepared SE suspension were thoroughly mixed into this paste to create SE-containing feed (hereinafter referred to as “SE paste”). Experimental crickets were allowed to freely consume the SE paste for 24 h, after which the remaining paste was replaced daily with fresh SE paste, continuing for a total of seven days. Preliminary survival rate tests showed that the number of viable SE in the paste decreased by approximately one order of magnitude (~5 × 10⁸ CFU ml^{−1}) after 24 h at 28°C. To ensure consistent infection levels throughout the experiment, freshly prepared SE paste was provided for free access, replaced every 24 h, and continued for a total of 7 days.

Evaluation of feeding amount using fluorescent tracers

To assess the quantity of feed consumed by crickets, we employed uranine (fluorescein) as a fluorescent tracer, which is considered relatively safe based on previous toxicity evaluations (Alford et al. 2009). Four types of feed were prepared: standard feed (MF), SE-paste, antibiotic-containing and *L.*

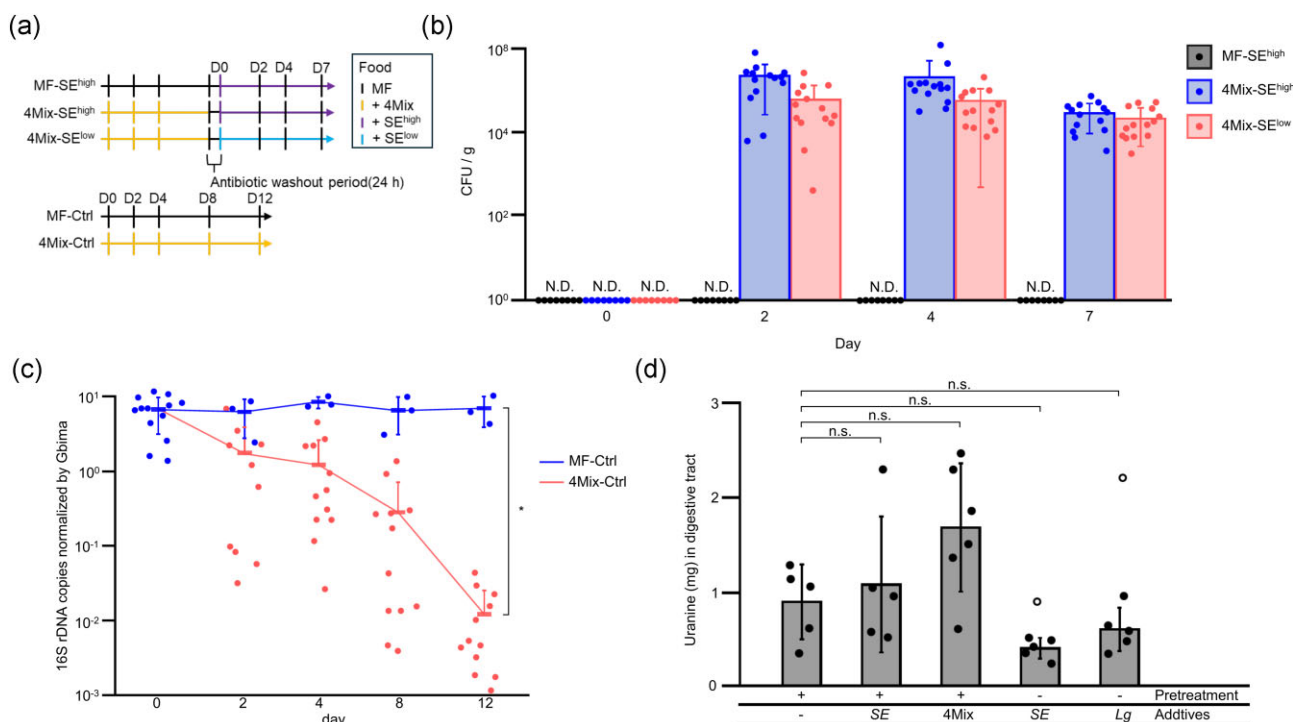


Figure 1. *Salmonella Enteritidis* (SE) colonization in crickets' gut. (a) Schema of the experimental workflow of the infection assay. (b) SE colonization levels in crickets in pretreatment with or without antibiotics. Bars represent the mean $\pm$ SD. High and low condition indicated 5×10^9 and 5×10^8 CFU of SE in 1 g of feed respectively. N.D. indicates not detected, meaning that the bacterial count was below the detection limit (<20 CFU g⁻¹), as determined by culture-based methods. (c) The relative abundance of gut bacterial DNA was assessed by quantifying 16S rDNA copies using quantitative polymerase chain reaction (qPCR), normalized to *G. bimaculatus* (Gbima) DNA. Bars represent the mean $\pm$ SD, with individual data points shown. All post-treatment values for either group were significantly different using Two-way ANOVA ($*P < 0.05$) when compared to control values. (d) Comparison of food intake using a fluorescent dye (uranine). Food intake was assessed by measuring the amount of uranine retained in the homogenized digestive tract. Data were shown in mean $\pm$ SD. Pretreatment conditions were categorized as either antibiotic-treated (+) or untreated (-). Additives included *Salmonella Enteritidis* (SE), a four-antibiotic mix (4Mix), and *Lactococcus garvieae* (Lg). Statistical comparisons were performed using Welch's *t*-tests with Bonferroni correction (n.s., not significant).

garvieae-containing feed. Each feed was supplemented with uranine at 50 mg ml⁻¹, and crickets were allowed to feed *ad libitum* for 24 h. After feeding, the entire intestinal tract was dissected from each cricket under a stereomicroscope, homogenized with PBS, centrifuged and fluorescence intensity of supernatant was measured using the FlexStation[®]3 Multi-Detection Reader (Applied Biosystems, Foster City, CA, USA) to quantify the amount of feed ingested.

Antibiotic administration

To deplete the gut microbiota, we adapted a protocol originally developed for *Drosophila melanogaster* (Téfit and Leulier 2017), scaling the antibiotic concentrations to account for the larger body mass of crickets. Ampicillin (500 μ g ml⁻¹), erythromycin (100 μ g ml⁻¹), kanamycin (500 μ g ml⁻¹), and tetracycline (100 μ g ml⁻¹) were combined in feed paste and thoroughly mixed to produce what is herein referred to as "4Mix." Crickets were allowed continuous access to this 4Mix feed for 12 days, with fresh 4Mix provided every 24 h.

Inhibition of SE establishment by ingestion of *L. garvieae*

To examine whether supplementing crickets with *L. garvieae* could inhibit gut colonization by SE, crickets were first fed 4Mix for 12 days to reduce native microbiota. *Lactococcus garvieae* was cultured on MRS agar overnight (conditions as

above) and then suspended in sterile water at $\sim 1 \times 10^8$ CFU mL⁻¹ (adjust as necessary based on pilot tests). A total volume of 2 ml of this suspension was mixed thoroughly with 2 g of MF feed to create an *L. garvieae*-enriched pellet. The insects which were recovered from antibiotics by normal feed for 24 h were allowed to feed on the *L. garvieae ad libitum* for 24 h. Following this period, crickets received SE-paste feed for an additional 24 h. The number of SE cells present in the gut was then enumerated by plating serial dilutions of gut homogenates onto MLCB agar, as described above.

PCR-based detection of SE

Aseptically dissected entire digestive tracts—comprising the foregut, midgut, and hindgut—were collected from eight to twelve final (eighth-instar) nymphs of *G. bimaculatus* during each sampling period (September 2022, November 2022, and February 2023). The full sets of organs from each period were pooled and homogenized to generate one composite gut sample per period, and total DNA was extracted from each composite using the DNeasy PowerSoil Pro Kit (QIAGEN Inc., USA) for subsequent polymerase chain reaction (PCR) analyses. The presence of SE DNA was tested by PCR using SE-specific primers IE1 L (5'-AGTGCCATACTTTTAATGAC-3') and IE1R (5'-ACTATGTGCGATACGGTGGG-3') (Wang and Yeh 2002). PCR reactions were prepared with GoTaq Master Mix (Promega) and performed under the following conditions: initial denaturation at 95°C for 3 min; 35 cycles of

95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; followed by a final extension at 72°C for 5 min. As an internal control for cricket DNA, a fragment of the cricket mitochondrial cytochrome oxidase c subunit I (COI) gene was amplified using primer pair Gbima-COI-F (5'-TCCACCTCTATCAACAGGAATTG-3') and Gbima-COI-R (5'-AATTTTCYTCAATTCTAGGAGCT-3') (Garino et al. 2021). PCR conditions were the same as above, except that the annealing temperature was set to 50°C. Amplification products were examined by electrophoresis on a 1.5% agarose gel to confirm the expected band sizes.

Culture-based detection of SE

To isolate live SE from the gut, each dissected digestive tract was placed in a sterile microtube with 1 ml of phosphate-buffered saline (PBS) and two sterile stainless-steel beads (3.175 mm diameter). Tissues were homogenized using a Beads Crusher μ T12 (TAITEC) at 2600 rpm for 1 min. The homogenate was then serially diluted 10-fold (up to 10⁻⁶) in physiological saline. Aliquots of each dilution were spread onto Mannitol Lysine Crystal Violet Brilliant Green (MLCB) agar plates and incubated aerobically at 37°C for 24 h (Brandt et al. 2004). After incubation, black colonies indicative of H₂S-producing *Salmonella* were counted on plates with countable colony numbers. This plating assay was conducted at 2, 4, and 7 days post-SE administration to monitor the persistence of the bacteria in the cricket gut.

Evaluation of gut bacterial load by real-time PCR

On days 2, 4, 8, and 12 of antibiotic treatment (4Mix feeding), crickets were dissected, and their intestinal tracts were collected for DNA extraction (using, for example, the DNeasy PowerSoil Pro Kit or an equivalent method). Total bacterial load was quantified by real-time PCR using universal eubacterial 16S rRNA primers, 16S-F (5'-ACTCCTACGGGAGGAGCAGCT-3') and 16S-R (5'-ATTACCGCGGCTGCTGGC-3') (Bergström et al. 2012). Reactions were carried out with KOD SYBRTM qPCR Mix (Toyobo, Japan), and serial dilutions of a known bacterial DNA standard (Zymo Research) were included for absolute quantification. No-template controls (NTCs) were also included to confirm the absence of contamination. The cricket mitochondrial COI gene (Gbima-COI-F, Gbima-COI-R) served as an internal reference for normalization.

Gut microbiota analysis

For taxonomic profiling of the gut microbiota, total DNA was extracted from the dissected intestinal tracts. PCR amplification of bacterial 16S rRNA genes was performed using primers from the 16S Barcoding Kit 1–24 (Oxford Nanopore Technologies), following the manufacturer's protocol with KAPA2G Robust HotStart ReadyMix (KAPA Biosystems). Amplified products were purified with magnetic beads (KAPA Pure Beads, KAPA Biosystems) and used for library preparation (16S Barcoding Kit 1–24 manual). Sequencing was carried out on a MinION device (Oxford Nanopore Technologies) equipped with an R9.4.1 flow cell. Basecalling was performed using Guppy (Oxford Nanopore Technologies), and reads were quality-trimmed with NanoFilt (Lee et al. 2021). Taxonomic classification was then conducted using Emu (Curry et al. 2022) or an equivalent pipeline. Poison-Tweedie model-based differentially expressed genes (DEGs)

analysis against count data of each bacteria species was performed using R package “tweeDEseq.” Calculated false discovery rate (FDR) and difference ratio between two groups were plotted using R package “ggplot2.”

Cricket's gut bacterial culture

To isolate lactic acid bacteria (LAB) from the cricket gut, intestines of healthy, colony-reared crickets were dissected and homogenized in sterile PBS. Aliquots of the homogenate were streaked onto de Man–Rogosa–Sharpe (MRS) agar and Tryptic Soy Broth (TSB) agar (1.5% agar) for general bacteria and incubated at 27°C for 24 h. Colony morphology was noted, and single colonies were subcultured until pure isolates were obtained. Species-level identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; MALDI Biotyper, Beckman Coulter).

Dual culture overlay assay

A dual culture overlay assay was conducted with minor modifications from a previously described method (Mami et al. 2012) to assess potential antagonism of test strains including LAB against SE. Briefly, 3 μ l of each bacterial isolate was spotted onto LB agar plates and incubated at 27°C for 24 h. Then, a top layer of LB soft agar (0.75% agar) was poured over the surface. After solidification, 1 ml of a fresh suspension of SE (prepared in LB broth) was added onto the surface of the soft agar, and the plate was gently tilted to spread the suspension evenly. Excess liquid was carefully removed using a pipette to ensure uniform coverage. Plates were incubated overnight at 27°C, and zones of inhibition (clearing) around spot of test strains were recorded as evidence of antagonistic activity.

Results

Impact of microbiota status on SE colonization of the cricket gut

qPCR and culture detected no SE (<10³ CFU), indicating the lab-reared crickets were SE-free. We evaluated whether SE could colonize the cricket digestive tract by screening gut samples after the insects consumed an SE-supplemented diet (see Methods). Throughout the study, SE levels in all gut samples remained below the culture-based limit of detection under standard rearing conditions (Figure. 1b).

To determine whether the gut microbiota suppresses SE colonization, we first induced dysbiosis by antibiotic treatment against cricket gut microbiome. After 12 days of treatment, bacterial 16S rRNA levels were reduced to 1/1 000 of baseline levels (Figure. 1c). Under these dysbiotic conditions, SE reached 10⁷ CFU g⁻¹ by day 2 and remained stable for 7 days, in both high-dose (5 $\times$ 10⁹ CFU g⁻¹ feed) and low-dose (5 $\times$ 10⁸ CFU g⁻¹ feed) exposure groups, with no evident dose effect within this range (Figure. 1b).

These results suggest that the gut microbiota plays a critical role in preventing SE colonization in crickets. Food intake did not differ between antibiotic-treated and control crickets (Figure. 1d), ruling out altered feeding as a confounder.

Gut microbiota analysis under normal and dysbiotic conditions

To identify the gut bacteria contributing to the suppression of SE colonization, we performed full-length 16S rRNA gene sequencing on a long-read platform. In control crickets, obligate-anaerobe families were predominant: *Lachnospiraceae*, *Ruminococcaceae*, and *Tannerellaceae* together formed the core community (Figure. 2a). In addition, *Morganellaceae* constituted a notable fraction of the control microbiota, further characterizing the baseline community structure. 16S rRNA profiling revealed a pronounced community shift in antibiotic-treated crickets, including a marked enrichment of *Enterobacteriaceae* (Figure. 2a). After treatment, *Lachnospiraceae*, *Ruminococcaceae*, and *Tannerellaceae* each fell below 1%, whereas *Enterobacteriaceae* rose to ~60% and other γ -Proteobacteria expanded strongly (Figure. 2a). Hierarchical clustering at the family level split all samples clearly by treatment, indicating the extent of the compositional shift. Consistent with these taxonomic changes, α -diversity reduced significantly: the antibiotic group showed a mean Shannon index of 2.49 ± 0.83 , whereas the control group averaged 3.46 ± 0.35 (Welch's *t*-test, $P = 0.013$), indicating a loss of within-sample diversity. Taken together—loss of core obligate anaerobes, overgrowth of *Enterobacteriaceae*, clear clustering by treatment, and reduced Shannon diversity—the antibiotic-treated community represents a canonical antibiotic-associated dysbiotic state.

Species-level differential abundance testing using a Poisson–Tweedie count model with BH–FDR 5% identified significant reductions (>30-fold) in four taxa—*L. garvieae*, *P. vulgaris*, *Parabacteroides chongii*, and *E. faecalis* (Figure. 2B). Based on these results, we hypothesize that these four species may inhibit SE growth.

In vitro and *in vivo* suppression of SE growth by resident *Lactococci* in crickets

Based on the comparative microbiome analysis, we cultured candidate taxa and successfully isolated *Candida tropicalis*, *Enterococcus raffinosus*, *P. vulgaris*, and *L. garvieae* (Table. 1). Each isolate was then tested for its ability to inhibit SE. Spotted *L. garvieae* and *E. raffinosus* strains produced inhibition zones against lawn growth of SE, with *L. garvieae* showing the effective zones of inhibition, whereas the other bacterial species isolates showed no inhibition at all (Figure 3a). While *E. raffinosus* also displayed inhibitory activity, the zones were smaller in diameter. These findings suggest that certain LAB from the cricket gut may exert antimicrobial activity against SE *in vitro*.

In vitro assays showed that *L. garvieae* inhibits the growth of SE; therefore, we tested whether live *L. garvieae* could similarly suppress SE colonization *in vivo*. Feeding live *L. garvieae* strain to sntibiotic-induced dysbiotic crickets reduced SE loads from 10^7 to 10^4 CFU g⁻¹ relative to the vehicle control (Figure. 3b). Feed-intake did not differ between *L. garvieae*-treated and control groups (Figure. 1d), indicating that the suppression was not attributable to altered food consumption. These results suggest that *L. garvieae* may contribute to the suppression of SE colonization within the digestive tracts of crickets.

Discussion

Our findings indicate that the cricket digestive tract, under normal rearing conditions, does not naturally retain SE. This point is pivotal because *Salmonella* spp. are designated “control pathogens” in international food-safety guidance for edible insects (EFSA 2015, EFSA 2021, EFSA 2024). Even when SE was introduced via feed, no detectable colonization occurred unless the resident gut bacterial community was disrupted by antibiotics. These results support the notion that an intact gut microbiota is crucial for suppressing pathogenic bacteria in insects, as previously noted in other host–microbe models (Ryu et al. 2010, Weiss et al. 2011). This resistance may involve both gut commensal bacteria (Steele et al. 2021) and insect's immune factors, such as antimicrobial peptides (Vizioli et al. 2001), which together create an inhospitable environment for SE colonization.

Although SE was our focus, similar microbiota-mediated exclusion has been reported for other edible-insect contaminants, including *L. monocytogenes*, *B. cereus*, and *Staphylococcus aureus* (Wynants et al. 2019, EFSA 2021). This suggests that the mechanism we uncovered may extend to a broader spectrum of control pathogens. This resistance may involve both gut commensal bacteria (Steele et al. 2021) and immune factors such as antimicrobial peptides (Vizioli et al. 2001), which together create an inhospitable environment for SE colonization.

The antibiotic-induced dysbiosis drastically lowered overall bacterial abundance, creating an invasive environment for SE colonization at high levels over an extended period. A microbiome screen for SE-suppressing factors identified the lactic-acid bacterium *L. garvieae* as a leading candidate. The *L. garvieae* strain isolated from cricket intestines inhibited SE growth across an intervening agar layer, indicating that it secretes diffusible antimicrobial agents such as a bacteriocin-like peptide. Although we could not directly show that *in situ* antimicrobial agents from *L. garvieae* suppress SE in the cricket gut, administering live *L. garvieae* strain to SE-susceptible crickets partially restored resistance, lowering the SE burden but not eliminating it. The incomplete clearance suggests that additional factors—such as other gut microbes or host immune defenses—may also be required for full protection. Nevertheless, incorporating *L. garvieae* as a feed additive could be a promising strategy to prevent intestinal colonization by pathogens such as SE. Similar probiotic-like strategies have been used in livestock and other invertebrate models to protect against enteric pathogens (Vonaesch et al. 2018). For the edible-insect industry, supplementing feed or post-harvest treatments with *Lactococcus* spp. may help lower *Salmonella* risk and warrants evaluation as an additional hurdle within hazard analysis critical control point (HACCP) programs, pending further validation.

We disrupted the cricket gut microbiota with an antibiotic cocktail, choosing drug types and estimating dosage by scaling *Drosophila* protocols to cricket body mass. This approach has several limitations. First, antibiotics can alter host physiology; second, resistant bacteria may persist—as evidenced by the survival of the notoriously drug-tolerant *Enterobacteriaceae*. By contrast, *Lactococcus* is generally susceptible to tetracycline, ampicillin, erythromycin, and kanamycin (the four drugs in our cocktail), which likely explains why it was among the most strongly depleted taxa in our screen. These differential susceptibilities complicate causal inference. While

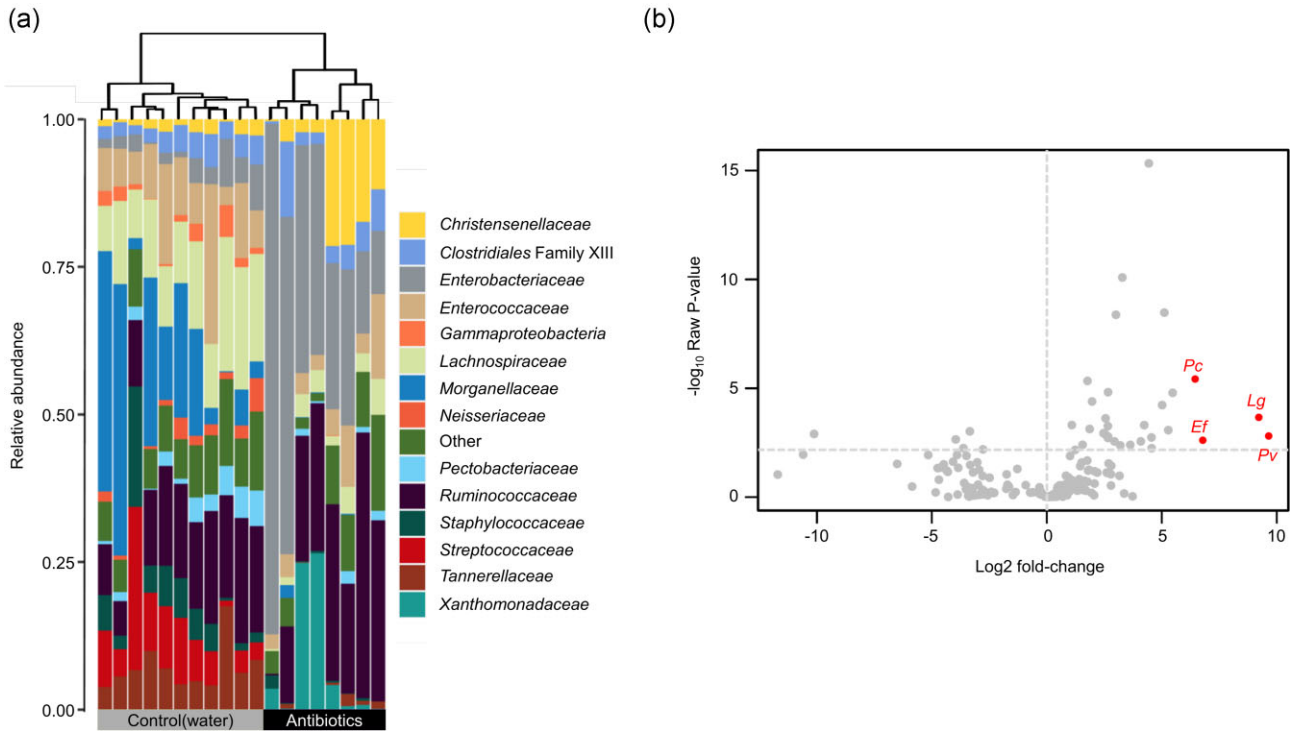


Figure 2. The impact of antibiotics mixture on cricket gut bacterial community. (a) Relative abundance of gut microbiota at the family level in crickets under control and antibiotic-treated conditions. The composition of gut microbiota in crickets was analyzed at the family level based on 16S rRNA sequencing. The control group (gray) and antibiotics group (black) indicated with or without 4Mix feed. Hierarchical clustering was performed to illustrate the similarity between samples based on microbiota composition. (b) Volcano plot of species-level bacterial changes in response to antibiotic treatment, analyzed using the Poisson–Tweedie distribution model. Log₂ fold-change indicated the ratio of bacterial abundance of control group to antibiotic-treated groups. The dashed horizontal line represents the FDR threshold of 5%. The four most significantly different species are highlighted in red on the plot: Lg, *L. garvieae*; Ef, *Enterococcus faecalis*; Pv, *Proteus vulgaris*; and Pc, *Proteus chongii*.

Table 1. Screening of SE-inhibiting bacteria.

Medium	Apecies	Number of isolate
MRS	<i>Lactococcus garvieae</i>	28
	<i>Enterococcus raffinosus</i>	14
	<i>Proteus vulgaris</i>	6
	<i>Candida tropicalis</i>	2
TSB	<i>Morganella morganii</i>	18
	<i>Klebsiella variicola</i>	3
	<i>Klebsiella oxytoca</i>	3

Using the spread plate technique, 50 isolates from MRS agar and 24 isolates from TSB agar were obtained. The isolated bacteria were identified using a mass spectrometer.

germ-free (axenic) insects would provide the most rigorous test, establishing such conditions was beyond the scope of the present study.

Our results align with prior studies indicating that insect microbiota can confer “colonization resistance,” similar to what is observed in vertebrate systems (Lee et al. 2018, Park et al. 2021). For instance, black soldier fly (*Hermetia illucens*) larvae have been reported to significantly reduce *Salmonella* and *S. aureus* in contaminated substrates and lose this capability when reared germ-free (Bruno et al. 2019). In this context, our observation that cricket gut-derived *L. garvieae* inhibits SE further confirms that indigenous insect symbionts can protect their hosts. Moreover, LAB are frequently detected in other farmed insect species such as mealworms and wax-moth larvae, where their antagonistic activity against *Listeria*

and *Enterococcus* has been documented (Stoops et al. 2017). Thus, LAB-based defense appears to be a recurring theme across edible-insect taxa. The presence of LAB in various insects (e.g. honeybees, bumblebees) and their known antagonistic effects on pathogens (Kwong and Moran 2016) also align with our findings, suggesting that LAB-based defense may be widespread among insects. However, our study adds new insight by specifically identifying *L. garvieae* as a key inhibitory species, potentially via production of bacteriocins or other antimicrobial factors. Future work should (i) determine whether *L. garvieae* supplementation lowers the burden of additional control pathogens (e.g. *L. monocytogenes*, *B. cereus*) under industry-mimicking conditions, (ii) elucidate the molecular basis of its antagonism, and (iii) assess its persistence during downstream processing such as blanching or drying. Addressing these points will clarify how microbiota-based interventions can be integrated into comprehensive safety protocols for edible insects.

In conclusion, we demonstrate that an intact gut microbiota of *G. bimaculatus* confers strong colonization resistance against SE, that antibiotic-induced dysbiosis abolishes this protection, and that the lactic-acid bacterium *L. garvieae*—depleted during dysbiosis yet abundant in healthy crickets—can partly restore resistance when supplied as a live feed supplement. These findings highlight resident LAB as key antagonists of food-borne pathogens in edible insects and suggest probiotic fortification as a practical, microbiome-friendly hurdle to enhance HACCP-based safety programs for the emerging insect-protein industry.

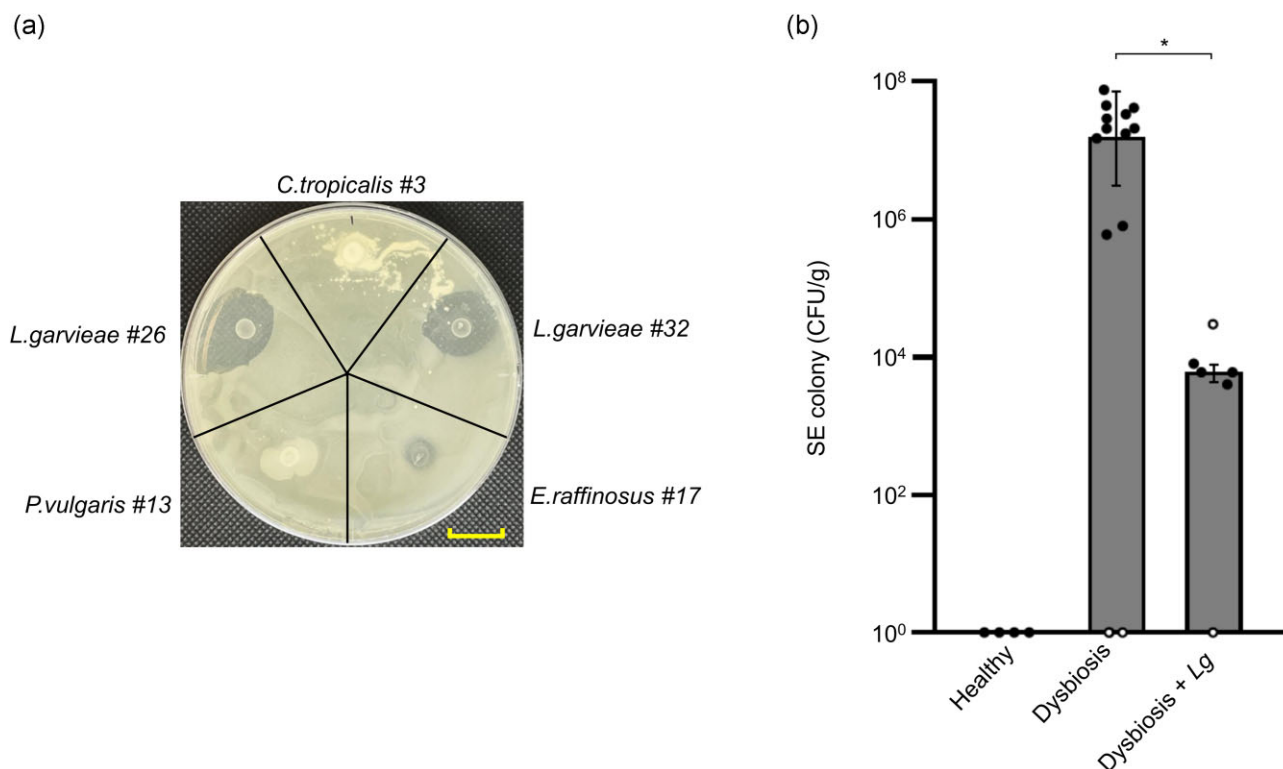


Figure 3. SE inhibitory effect of *L. garvieae*. (a) Image of the Dual Culture-Overlay Assay. The scale bar represents 20 mm. (b) *In vivo* inhibition of SE colonization by *L. garvieae* in crickets. SE colonization levels in crickets were compared across three conditions: healthy and dysbiosis condition were prepared by normal feed and antibiotics-containing feed before SE-infected. *Lg* indicated 24 h-post antibiotics crickets were supplemented with *L. garvieae*-containing feed, before SE-infected. Bars represent the mean $\pm$ SD. Open circles indicated outlier values. Welch's *t*-test was performed for statistical test (* $P < 0.05$).

Author contributions

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Data availability

The data underlying this article are available from the corresponding author on reasonable request.

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