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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

N-acyl-homoserine lactone produced by *Rahnella inusitata* isolated from the gut of *Galleria mellonella* influences *Salmonella* phenotypes

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Abstract

The most studied mechanism of quorum sensing in Gram-negative bacteria is mediated by autoinducer 1 (AI-1), namely, acyl-homoserine lactone (AHL). This system allows communication among different bacterial species and regulates the expression of virulence genes in many pathogens. Although AHL-producing bacteria have been detected in the intestines of humans and other animals, no report was found about AHL-producing bacteria in the insect gut and the possible effects of these autoinducers on enteropathogenic bacteria. Therefore, this study aimed to identify AHL-producing bacteria in the gut of larvae of *Galleria mellonella* and to evaluate the influence of this quorum sensing signal on the regulation of adhesion and motility phenotypes in the intestinal pathogen *Salmonella.* Sequencing of the 16S rRNA gene, 16S rRNA gene-based phylogenetic analyses, and phenotypic characterization of gut isolates was performed. The profile of AHLs produced by the isolates was determined using thin-layer chromatography (TLC) and revealed with the biosensor strain *Chromobacterium violaceum* CV026. Sequencing, phylogenetic analyses and phenotypic characterization of gut isolates showed that the three AHL-producing strains belong to the species *Rahnella inusitata*, named GM34, GM56, and GM60. The TLC showed that *R. inusitata* produces a six-carbon AHL. In the presence of cell-free extract of *R. inusitata* containing AHL and under anaerobic conditions*, Salmonella enterica* increased the adhesion to stainless steel coupons and presented swarming motility. Extracts from the culture medium of *R. inusitata* isolates containing AHL increased the adhesion on stainless steel coupons and swarming motility of *Salmonella enterica* serovar Enteritidis PT4 under anaerobic conditions. The results suggest the possibility of communication between members of the *G. mellonella* intestinal microbiota with pathogens such as *Salmonella*.

Keywords Cell communication · Intestinal microbiota · Autoinducer · Biofilm · Motility

Introduction

The cell–cell communication system, called quorum sensing (QS), allows bacteria to collectively regulate the expression of different genes in response to changes in cell density. This mechanism involves producing, releasing, and detecting signaling molecules known as autoinducers (AIs) [1]. Some Gram-negative bacteria can produce several AIs, including *N*-acyl-homoserine lactones (AHLs). In general, two proteins belonging to the LuxI-LuxR families are required for AHL-mediated QS. The LuxI-type proteins are responsible for the synthesis of AHL, whereas LuxR-type proteins are transcriptional regulators that bind to AHLs and regulate the expression of targeted genes [2, 3]. Some bacteria of the Enterobacteriaceae family, such as *Salmonella* and *Escherichia coli*, do not produce AHLs but can detect these molecules produced by other bacteria [4, 5]. The AHLs contain a homoserine lactone ring and an acyl side chain with 4 to 19 carbon atoms and can have modifications in their structure, with the presence of hydroxyl or oxhydryl groups. These variations are essential for distinguishing the types of AHLs and their biological role [6, 7]. Phenotypes such as bioluminescence, motility, biofilm formation, and virulence are controlled by AHL-mediated QS [8–10].

The AHLs can be used for intra- and inter-species communication, contributing to a coordinated organization of

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the microbial community [3]. The gut is a complex environment, with several Gram-positive and Gram-negative species [11]. However, the presence of bacteria that produce AHL and the role of these molecules in the communication QS and among the resident microbiota are currently not fully understood. In a previous study, AHL-producing bacteria were isolated from cattle rumen and swine intestines [12], and AHLs have also been detected in human feces [13]. The evidence of signaling molecules in the gastrointestinal tract leads to a speculation regarding the involvement of the QS in the regulation of intestinal microbiota. In the intestine of invertebrates, the presence of AHL-producing bacteria has already been confirmed [14], but no study has investigated AHL-producing bacteria from the intestine of *Galleria mellonella* larvae.

Galleria mellonella is a promising insect species to study microbial infections and toxicity. The *G. mellonella* larvae have a large size, facilitating direct infection in the hemolymph or oral infection. They can be reared at 37 °C, the optimum temperature for many human pathogens [15–17]. The species has an innate immune response, with characteristics similar to those of the mammalian immune system [18, 19]. In addition, *G. mellonella* can be used as an oral infection model for human bacterial pathogens [20]. The invertebrate gut microbiota is dominated mainly by Gram-positive bacteria, with a low concentration of Gram-negative bacteria [21]. As is supposed to happen in other living animals, it would not be surprising if members of the intestinal microbiota of larvae could communicate with each other to coordinate maintenance processes from the commensal population to resistance to intestinal pathogens. This study aimed to detect AHL-producing bacteria from the *G. mellonella* gut and verify these molecules' influence on the adhesion and motility of *Salmonella* Enteritidis PT4.

Material and methods

Bacterial strains

Salmonella enterica subspecies *enterica* serovar Enteritidis phage type 4 (*Salmonella* PT4) 578 (GenBank: 16S ribosomal RNA gene, MF066708.1), isolated from chicken meat (FIOCRUZ, Rio de Janeiro, Brazil) and the biosensors of AHL *Chromobacterium violaceum* CV026, and *E. coli* pSB403 were used in this study. *Salmonella* PT4 was grown in tryptone soy broth (TSB; Sigma, India), *C. violaceum* CV026 and *E. coli* pSB403 were grown in Luria–Bertani broth (LB; Sigma, USA) supplemented with kanamycin (20 µg/mL) and tetracycline (20 µg/mL), respectively.

Larval culture

G. mellonella larvae (250–300 mg weight) were donated by the Laboratory of Molecular Genetics of Microorganisms at the Universidade Federal de Viçosa (UFV, Viçosa, Brazil). Larvae were reared at 28 °C in the dark and fed on an artificial diet [22]. Before the intestines were extracted, the larvae were kept in Petri dishes at 28 °C for a 24 h fasting period.

Detection of AHL-producing bacteria in the larvae intestine

Gut dissection

Gut dissection was performed as described by Ignasiak and Maxwell [23], with modifications. Larvae were anesthetized on ice and surface-disinfected with 70% ethanol for 45 s then washed with sterile distilled water in a biological safety cabinet. The insect was stabilized with needles on a previously disinfected surgical table, and an incision was made in the abdomen to expose the digestive tract using a sterile razor. The digestive tract ends were cut using a sterile dissecting scissor, and the entire gut was carefully removed. The guts were placed inside 2 mL tubes on ice. Five guts were collected per tube.

Isolation of bacteria

An aliquot of 500 µL cold peptone saline solution (0.1%) was added to the tube containing the dissected guts. Subsequently, the guts were macerated with a pestle and sonicated with an ultrasonic processor (Vibra Cell, USA) equipped with a 6-mm stainless steel probe at continuous mode (130 W, 20 kHz) for 30 s, which was repeated twice [24]. Serial dilutions were performed in phosphate-buffered saline (PBS; 10 mM, pH 7.2), and 200 µL were plated on LB and MacConkey agar (Merck, Germany). The plates were incubated for 24–48 h at 30 °C under aerobic and anaerobic conditions using anaerobic jars with Anaerobac (Probac, Brazil). As we focused on the Gram-negative AHL producers, only colonies grown on MacConkey agar were streaked again on the same agar and on Plate Count Agar (PCA) to obtain pure cultures. The plates were incubated for 24–48 h at 30 °C.

Detection of AHL-producing bacteria

Colonies were selected according to morphological differences. The isolates were grown in 2 mL of LB broth at 28 °C for 24 h and screened for AHL production using *C.violaceum* CV026 and *E. coli* pSB403 as biosensors. The

tested strains were streaked in perpendicular to *C. violaceum* CV026 on LB agar plates and the presence of the pigment violacein indicated exogenous AHLs [25]. *C. violaceum* CV026 is sensitive to unsubstituted chains varying in size from C4 to C8 [25]. *E. coli* pSB403 exhibits the highest sensitivity towards 3-oxo-C6-HSL although several other AHL molecules can be detected [26]. The test was performed as described by Martins et al. [26], with modifications. A volume of 800 µL of *E. coli* pSB403, grown in LB broth supplemented with tetracycline (20 µg/mL) for 12 h at 37 °C, was incubated with 200 µL of the isolate's supernatant obtained by centrifugation at 10,000 *g* for 10 min. After incubation at 37 °C for 12–24 h, the bioluminescence was visualized in a darkroom.

AHL identification

The AHL-producing isolates were grown in 200 mL of LB broth for 36 h at 28 °C, with an agitation of 150 rpm. Bacteria were removed by centrifugation at 6000 *g* for 20 min, and the supernatants were extracted three times with equal volumes of ethyl acetate acidified with 0.5% of formic acid [25]. Subsequently, the extracts were evaporated to dryness, and the residues were dissolved in 800 µL of HPLC-grade acetonitrile (Merck, Germany). The extracts containing AHL (ExCA) were stored at − 20 °C.

Volumes of 2–10 µL of commercial AHLs (Fluka, Germany) or ExCA dissolved in acetonitrile were applied onto C18 reversed-phase TLC plates (aluminum sheets 20 × 20 cm; RP-18 F254 S, Merck, Germany) and chromatographed using a methanol/water (60:40, v/v) solvent system. The solvent was evaporated at room temperature for 1 h, and the plate was overlaid with a thin film of agar with the biosensor strain *C. violaceum* CV026 and was incubated at 28 °C for 24–48 h [25, 27]. The migration pattern was used to compare the profiles of the AHLs produced by the isolates with the commercial AHLs [*N*-butanoyl-homoserine lactone (C4-HSL), *N*-hexanoyl-homoserine lactone (C6-HSL), *N*-octanoyl-homoserine lactone (C8-HSL), and *N*-oxooctanoyl-homoserine lactone (Oxo-C8-HSL)].

Sequencing of AHL-producing bacteria and phylogenetic analysis

The DNA of isolates producing AHLs was extracted with the Wizard® Genomic DNA Purification Kit (Promega, USA), following the procedures described by the manufacturer. The isolates were identified by sequencing the 16S rRNA gene, using the primers 10f (GAGTTTGATCCTGGCTCAG) and 1100r (AGGGTTGCGCTCGTTG) in a SeqStudio™ Genetic Analyzer (Thermo Fisher Scientific, USA). The NCBI BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the sequence of the 16S gene of the isolates with the Prokaryotic 16S ribosomal RNA database. The phylogenetic tree was constructed using sequences deposited in the Ribosomal Database Project (RDP). Multiple alignments were performed using the CLUSTAL_X software, applying a neighbor-joining method and the Jukes-Cantor model. Bootstrap analysis was performed based on 1000 replicates [28].

Phenotypic characterization of isolates

The phenotypic characteristics of each AHL-producing isolate were evaluated using routine biochemical tests suggested by Brenner et al. [29] by using standardized procedures. Carbon source utilization tests were performed with cultures previously grown in LB broth for 24 h at 30 °C and then inoculated separately in media containing different carbohydrate sources at 1% (glucose, arabinose, glucose, fucose, lactose, maltose, mannose, rhamnose, sucrose, trehalose, xylose). The cultures were incubated for 24–48 h at 30 °C, and the growth ability was observed. Other biochemical assays were also performed, such as Voges-Proskauer and methyl red tests, indole production, citrate utilization, and catalase and hydrogen sulfide production [29].

AHL quantification

The concentration of AHLs in the extract was estimated according to Blosser and Gray [30], with modifications. *C. violaceum* CV026 was cultivated for 24 h at 28 °C, and 500 µL of the culture was inoculated in 4.5 mL of LB broth supplemented with kanamycin (20 µg/mL) or 10 µL ExCA. The tubes were incubated for 24 h at 28 °C with shaking at 200 rpm. After incubation, 1 mL of each *C. violaceum* CV026 culture was centrifuged at 10,000 *g* for 10 min, the supernatant was discarded, and 200 µL of sodium dodecyl sulfate (SDS, 10%) was added. The mixture was vortexed vigorously for 45 s and incubated for 5 min at room temperature. Then, 900 µL of butanol were added, and the mixture was vortexed for 5 min to solubilize the violacein and centrifuged at 10,000 *g* for 5 min to remove cell debris. A volume of 200 µL of the violacein-containing supernatants was added to 96-well microplates, and absorbance was measured with a microplate reader (Thermo Fisher Scientific, Finland) at a wavelength of 585 nm.

To estimate the concentration of AHL in each extract, a regression analysis was performed using C6-HSL in different concentrations (0.5, 1, 5, 10, 15, 20, 30, 40, and 50 nM) as standard. Violacein was extracted as described above. A linear equation was generated ($y = 81.084 \times OD585 - 8.4278$), where y is the AHL concentration in nM and OD585 is the absorbance value at 585 nm. The curve obtained showed an R^2 of 0.958. These values were used to calculate the volume

of ExCA to be used in the *Salmonella* PT4 adhesion and motility assays.

Effects of ExCA on *Salmonella* PT4 phenotypes

Salmonella cell preparation

Salmonella PT4 was cultivated in TSB under O_2-free conditions, as previously described by Almeida et al. [31]. Cells in the stationary phase were transferred to 10 mL of anaerobic TSB and incubated for 4 h at 37 °C to reach the exponential phase. After incubation, cells were harvested by centrifugation, and the pellet was washed twice in phosphate-buffered saline (PBS; 10 mM, pH 7.2). The inoculum was standardized to 0.1 optical density at 600 nm (OD600nm), using a spectrophotometer. Then, 10 mL of anaerobic TSB (pH 7.0) was inoculated with 0.1 mL and supplemented with 50, 150, and 250 nM of ExCA or commercial C6-HSL. Controls without ExCA or C6-HSL were also prepared. The bottles were incubated for 7 h at 37 °C.

Quantification of adhered and planktonic cells of *Salmonella* PT4

Salmonella PT4 cells grown for 7 h as described above were standardized to an OD600 nm of 0.1, and 100 µL was inoculated in anaerobic TSB supplemented with 50, 150, and 250 nM of ExCA or C6-HSL containing stainless steel AISI 304 (#4) (SS) coupons with dimensions of 1 × 1 cm, previously sanitized as described by Oliveira et al. [32]. The SS coupons were placed vertically in bottles to allow biofilm formation on both sides. To quantify the sessile cells, the coupons were removed from the anaerobic bottles after 40 h of incubation at 37 °C and washed with PBS to remove the planktonic cells. Each coupon was placed in a tube containing 3 mL of PBS and sonicated with an ultrasonic processor (130 W, 20 kHz) for 1 min to remove attached cells. Subsequently, the suspension was homogenized, diluted, and plated by the drop plate method [33] on PCA, and after incubation at 37 °C for 8–24 h, the colonies were counted. For planktonic cell quantification, 1 mL of the supernatant was collected; tenfold serial dilutions were generated and plated on PCA.

Microscopy assay

After 40 h of incubation, the coupons were removed from the anaerobic bottles, washed with PBS, and stained with a Live/Dead® BacLight™ (Invitrogen, USA) kit for 20 min in the dark. Then, the coupons were washed, and biofilm was visualized using an epifluorescence microscope (Thermo Fisher Scientific, EVOS M5000 Imaging System, USA).

Determination of swarming motility

The TSB added with 0.7 (w/v) of agar was used for swarming motility assay according to Rashid and Kornberg [34], with modifications. A volume of 15 mL of the medium at 50 °C was supplemented with a final concentration of 250 nM of one of the three ExCA or C6-HSL and poured into Petri dishes. The plates were dried for 3 h at room temperature before use. Swarming motility was analyzed by pipetting 5 µL of *Salmonella* PT4 grown for 7 h, as described above, in the center of the Petri dish containing TSB agar. After the aliquot had dried, plates were incubated at 37 °C for 24 h in anaerobic jars with Anaerobac (Probac, Brazil).

Statistical analysis

The analysis of variance (ANOVA) and Turkey's test were used for multiple comparisons between *Salmonella* grown in the presence and absence (control) of the isolates extracts containing AHLs or C6-HSL. The GraphPad Prism 5.00 software was used and the *p*-value adopted was 0.05, 0.01, and 0.001.

Results and discussion

Identification of AHL-producing isolates

The atmosphere used in the incubation of the plates, whether aerobiosis or anaerobiosis, influenced the percentage of colony-forming units per larva (CFU/larva) (Fig. 1). Aerobic incubation resulted in a higher percentage of CFU/larva in LB and MacConkey agar than anaerobiosis. The Gram-negative cultivable bacterial population, represented by colonies formed on MacConkey agar, was less than half of the total population grown on LB agar, both under aerobic or anaerobic conditions (Fig. 1). These results are in agreement with Allonsius et al. [21] and Ignasiak and Maxwell [23]. They reported that Gram-negative bacteria are relatively rare in the *G. mellonella* gut and that Gram-positive bacteria, such as *Enterococcus* and *Lactobacillus*, predominate in this environment. In previous studies, Gram-negative bacteria such as *Enterobacter, Pseudomonas, Acinetobacter*, *Aeromonas,* and *Shewanella* were detected as part of the gut microbiota of *G. mellonella* [35, 36].

A total of 138 isolates were analyzed for AHL production using *C. violaceum* CV026 and *E. coli* pSB403 as biosensors, and only three were identified as AHL-producers, one from the anaerobic MacConkey (isolate 34) and two from the aerobic MacConkey (isolates 56 and 60). As shown in Fig. 2A, the isolates induced violacein production when cross-streaked with the biosensor strain *C. violaceum* CV026. The three isolates also induced bioluminescence

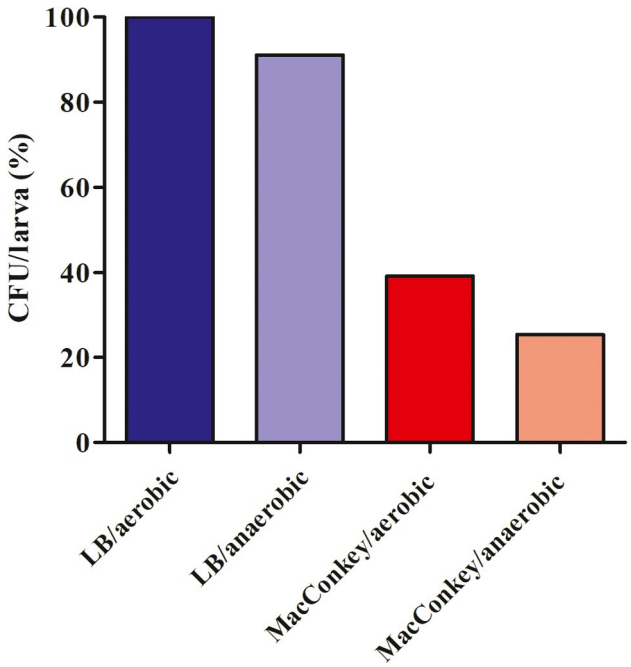

Fig. 1 Standard count of bacteria from the gut of *G. mellonella*. Results are expressed as CFU per larva (CFU/larva) by the ratio of the number of total CFU and the number of larvae used (five larvae). The CFU/larva from LB in aerobiosis (LB/aerobic) was considered 100%

in *E. coli* pSB403 (data not shown). *C. violaceum* CV026 responds to the AHLs with an acyl chain length of C4 to C8, *E. coli* pSB403 responds to AHLs with carbon side chains of C6 or C8 [25, 37]. In Fig. 2B, the profile of AHLs present in the extracts of the isolates, revealed by TLC, is shown. Spots with violet pigments indicate the production of violacein by *C. violaceum* CV026, induced by the presence of AHLs. The isolates produced a signal molecule with migration equal to the C6-HSL standard, indicating that the three isolates produced the same signal molecule. These results suggest that GM34, GM56, and GM60 isolates produce an AHL with six carbons. We do not rule out the possibility that the isolates produce AHLs with longer side chains and with different modifications that were not detected by the used biosensors. Therefore, additional analyses must be carried out to determine the spectrum of AHLs produced by the intestinal microbiota of larvae.

The sequencing data indicated a strong homology between the AHL-producers with the 16S rRNA gene of *Rahnella inusitata* strain DSM 30,078 (Accession number: NR_146846.1). The percent identity of GM34, GM56, and GM 60 were 100%, 100%, and 99.84%, respectively. During the cultivation of the isolates, we observed that the GM34 isolate grows more slowly than the GM56 and GM60 isolates. We also observed that the GM60 isolate induces violacein production in CV 026 faster than GM56 and GM34. However, we cannot state that they are different biotypes as more in-depth analyses were not yet been carried out. The sequence with 16 S did not show any difference between them. In addition, the phylogenetic tree (Fig. 3) showed that AHL-producing strains form a tight subcluster with *R. inusitata* (previously *Rahnella* genome sp. 3, type strain DSM 30078 T) [38]. Also, some phenotypic characteristics of isolates obtained in this study were compared with the data of Lee et al. [28], Brenner et al. [29], and Brady et al. [38]. The biochemical tests performed were similar between isolates and *R. inusitata* (Supplementary Table S1). Therefore, the isolates were named *R. inusitata* GM34, *R. inusitata* GM56, and *R. inusitata* GM60. The genus *Rahnella* belongs to the family Enterobacteriaceae and comprises the six recognized species *R. aquatilis*, *R. variigena*, *R. inusitata*, *R. bruchi*, *R. woolbedingensis*, and *R. victoriana* [38]. These bacteria are distributed in different environments such as soil, food, plant rhizosphere, water, and human clinical samples [38–40]. *Rahnella* spp. is a Gram-negative bacterium and

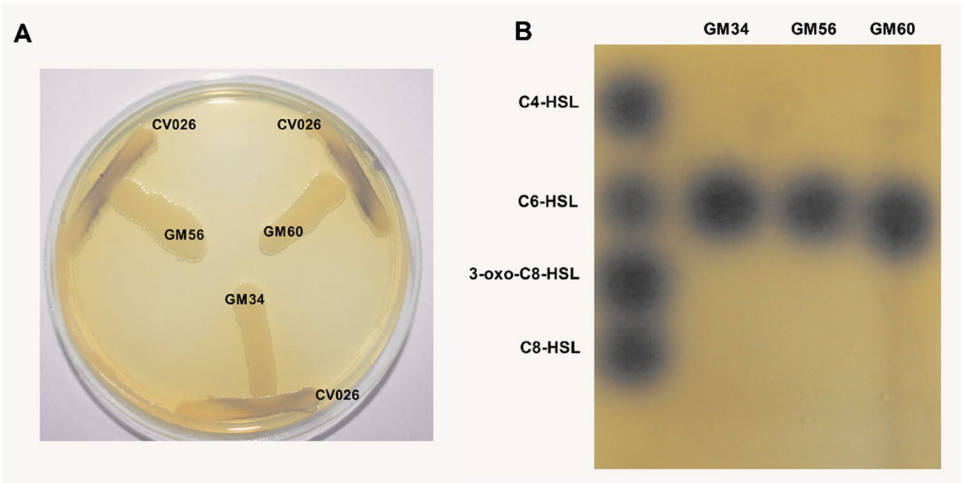

Fig. 2 AHL screening of *R. inusitata* (GM34, GM56, and GM60) isolated from the gut of *G. mellonella* revealed with *C. violaceum* CV026. **A** cross-streaking of strains with *C. violaceum* CV026. **B** AHL profile present in the extracts of the *Rahnella* isolates by thin-layer chromatography (TLC) with *C. violaceum* CV026. The first column is the migration profile of AHL standards

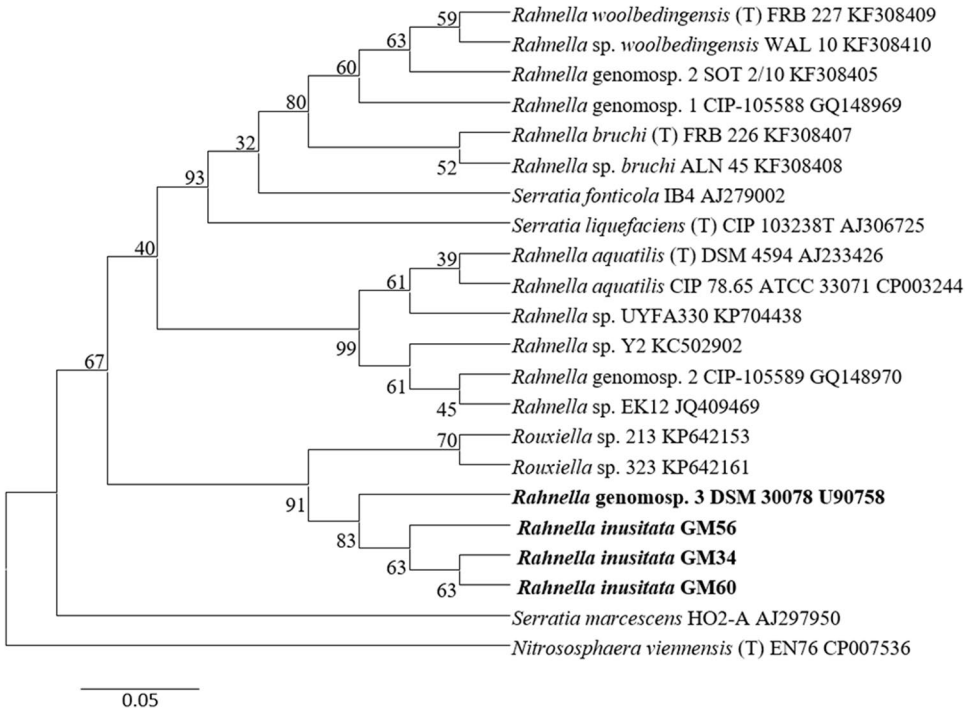

Fig. 3 Neighbor-joining phylogenetic tree of *R. inusitata* (GM34, GM56, and GM60) isolated from the gut of *G. mellonella*. Alignments were performed with the 16S rRNA gene sequences of *R. inusitata* isolates and sequences deposited in the RDP database. *Nitrososphaera viennensis* was employed as outgroup, and 1000 replicates are shown at branch nodes. *Rahnella* genomo sp. 3 DSM 30078 ^{T} was identified as *R. inusitata* by Brady et al. [38]

has been isolated from the gut of invertebrates [1, 38, 41], but to our knowledge, no study has detected *Rahnella* in the *G. mellonella* gut. Moreover, diet is a factor that can alter the microbiota, resulting in the modification and predominance of particular species in the intestinal environment [23, 42].

The cell-to-cell communication system of the genus *Rahnella* is little known, but the AHL production by this genus has already been described in the literature. Different strains of *R. aquatilis* from the tomato rhizosphere produce 3-oxo-C6-HSL and 3-oxo-C8-HSL [43], and *R. aquatilis* isolated from cabbage produces C8-HSL, C10-HSL, and 3-oxo-C14-HSL [44]. The confirmation that *R. inusitata* isolated from the intestine of *G. mellonella* produces C6-HSL, contributes to clarifying the mechanism of QS in this genus, and reinforces the evidence that species of the genus *Rahnella* use this AI-1 for cellular communication. In the gut of animals, QS possibly participates in maintaining the commensal microbiota and the homeostasis in this environment by controlling the predominance of certain species [45]. However, this QS function in the gut is not well characterized, and the role of AHLs in the *G. mellonella* microbiota is a field to be explored.

Effect of ExCA of *Rahnella* isolates on *Salmonella* adhesion and motility

The AHL concentrations in the extracts of the isolates GM34, GM56, and GM60 were 5.9, 7.2, and 6.3 µM, respectively. The ExCA were named as follows: extract of isolate GM34 (ExCA-GM34), extract of isolate GM56 (ExCA-GM56), and extract of isolate GM60 (ExCA-GM60). The results of *Salmonella* PT4 adhesion in the presence and absence of ExCA or C6-HSL (50, 150, and 250 nM) are shown in Fig. 4. It is evident that after 40 h of incubation, the presence of 250 nM AHL significantly influenced the number of sessile cells of *Salmonella* PT4 in SS coupons under anaerobic conditions (Fig. 4A-D) without interfering with planktonic cell numbers (Fig. 4E-H). Both ExCA-GM56 and C6-HSL increased the count of adhered *Salmonella* PT4 cells in SS coupons, also in 150 nM (Fig. 4B and D). Thus, the results show that *Salmonella* adherence is affected by AHL present in extracts of *R. inusitata* isolates, which reinforces the evidence about communication mechanisms between different species.

Although *Salmonella* does not produce AHL, this pathogen synthesizes the protein SdiA, homologous to LuxR, and can sense the AHL produced by other Gram-negative bacteria. The molecular docking results among the modeled SdiA protein from *Salmonella* and AHLs show that AHLs with a shorter chain, such as C6-HSL, bind to SdiA, although they presented less affinity than those with 12 and 10 carbons [46]. Salmonella's more significant biofilm formation in the presence of exogenous AHL under anaerobiosis without influencing the growth has been described before [46, 47].

The results of epifluorescence microscope analyses were consistent with the quantitative analysis of adhered cells on the SS coupon, with the highest population of adhered *Salmonella* cells cultured in the presence of the ExCA of *R. inusitata* and C6-HSL (Fig. 5). As shown in Fig. 5A, few *Salmonella* PT4-adhered cells were observed in the control

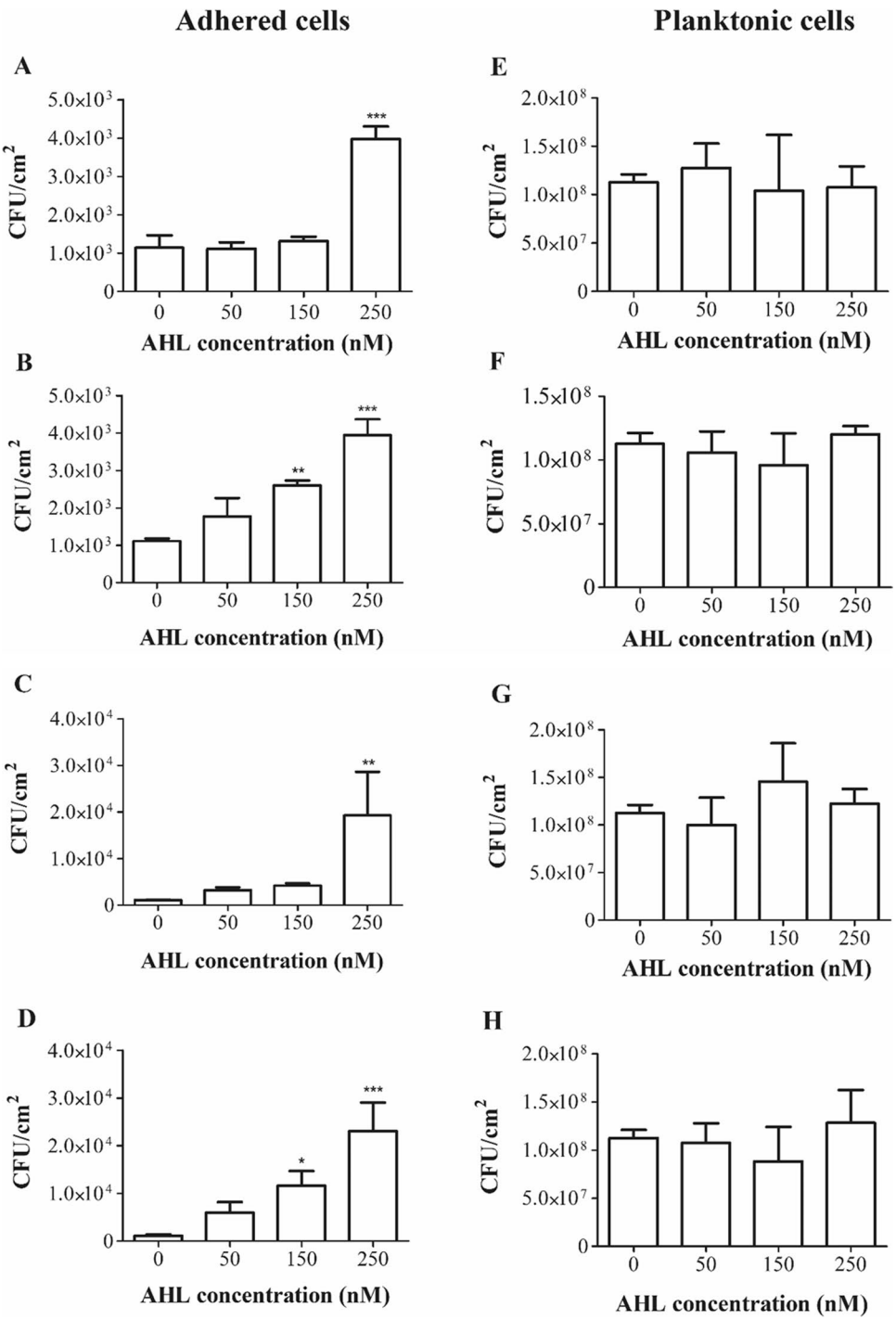

Fig. 4 CFU/cm2 of sessile and planktonic *Salmonella* cells on SS coupons in the presence of ExCA of *R. inusitata* and commercial C6-HSL after 40 h of incubation at 37 °C under anaerobic conditions. **A** and **E** in the presence of ExCA-GM34; **B** and **F** in the presence of ExCA-GM56; **C** and **G** in the presence of ExCA-GM60; (D and H) in the presence of C6-HSL. Error bars indicate the standard error ($n = 3$), and values that are significantly different to the control (0 nM) by Tukey's test are indicated; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

treatment, i.e., without ExCA or C6-HSL, whereas 250 nM of ExCA or C6-HSL increased the population of adhered cells (Fig. 5B-E). Observing small cell aggregations on the SS surface when TSB broth was added to ExCA suggests the initial biofilm formation process. The results also indicate that *R. inusitata* ExCA can influence *Salmonella* adherence, a phenotype essential for *Salmonella* during the infectious process. The addition of 50 nM of C6-HSL significantly stimulated the adhesion of *Salmonella* on the surface of polystyrene [47], which indicates that C6-HSL can control phenotypes in this pathogen. *Salmonella* needs to adhere to the intestinal epithelial surface in the gut lumen to begin the host invasion process [48]. However, since the surface material influences adhesion and biofilm formation, extrapolation of results from abiotic to biotic surfaces should be performed with caution. In general, the changes caused by AHL in *Salmonella* behavior suggest that this pathogen perceives the presence of a competitive microbiota and uses

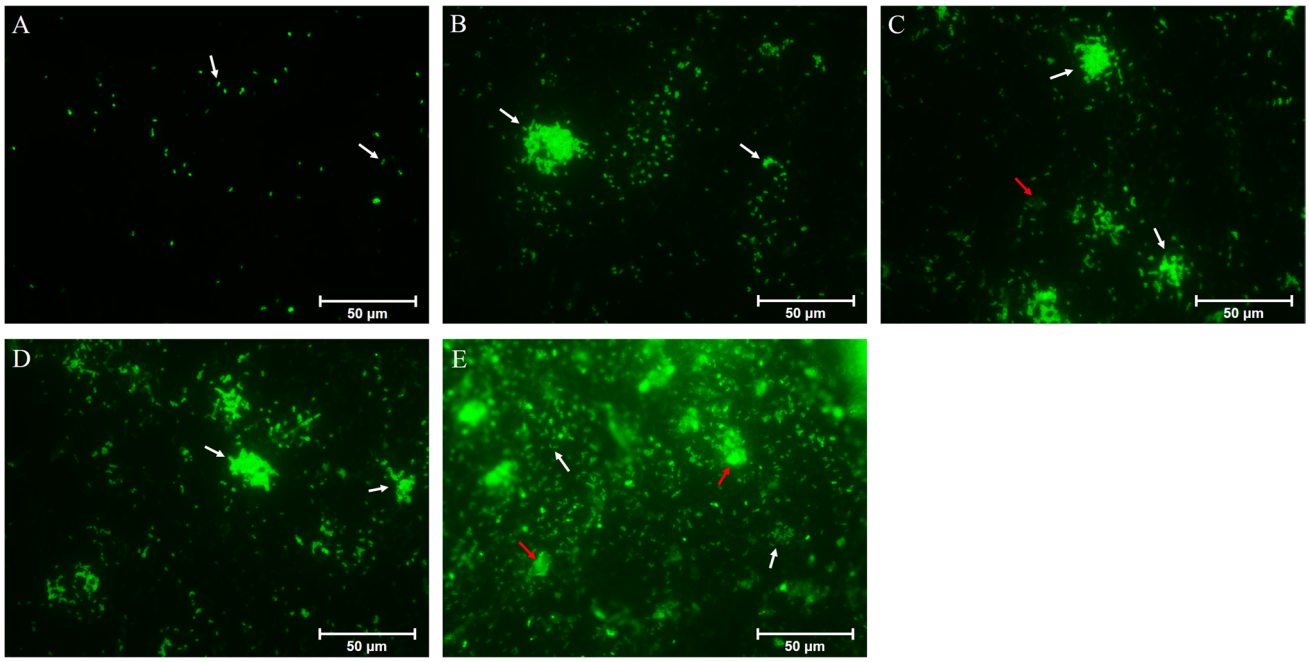

Fig. 5 Epifluorescence microscopy micrographs of *Salmonella* adhesion on SS coupons in TSB broth and in the presence of ExCA *R. inusitata* and commercial C6-HSL after 40 h of anaerobic incubation at 37 °C. **A** control; **B** in the presence of ExCA-GM34; **C** in the presence of ExCA-GM56; **D** in the presence of ExCA-GM60; **E** in the presence of C6-HSL. The final concentration of AHL was 250 nM. White arrows indicate adhered cells and red arrows indicate artifacts. Magnification 40×

this information to develop mechanisms to become more competitive, guaranteeing its persistence in the environment in addition to regulating the production of host colonization factors.

The motility of *Salmonella* PT4 was affected by ExCA-GM60 and C6-HSL (Fig. 6). In the presence of AHL, strong swarming motility was observed when compared to the control group. The absence of swarming motility in *Salmonella* Enteritidis PT4 578 under anaerobic incubation has been described by Almeida et al. [49], and the addition of 50 nM of 12-carbon AHL (*N*-dodecanoyl-homoserine lactone: C12-HSL) did not change this behavior. The fact that we used a different AHL and in concentrations five times higher (250 nM) may explain the divergence of the results. *Salmonella* uses flagella to swarm over semi-solid surfaces [50], and genes involved in virulence, lipopolysaccharide (LPS) biosynthesis, and iron metabolism in *Salmonella* typhimurium are upregulated during swarming [51]. In addition, swarmer differentiation in this serovar is related to antibiotic and other antimicrobial agent resistance, including

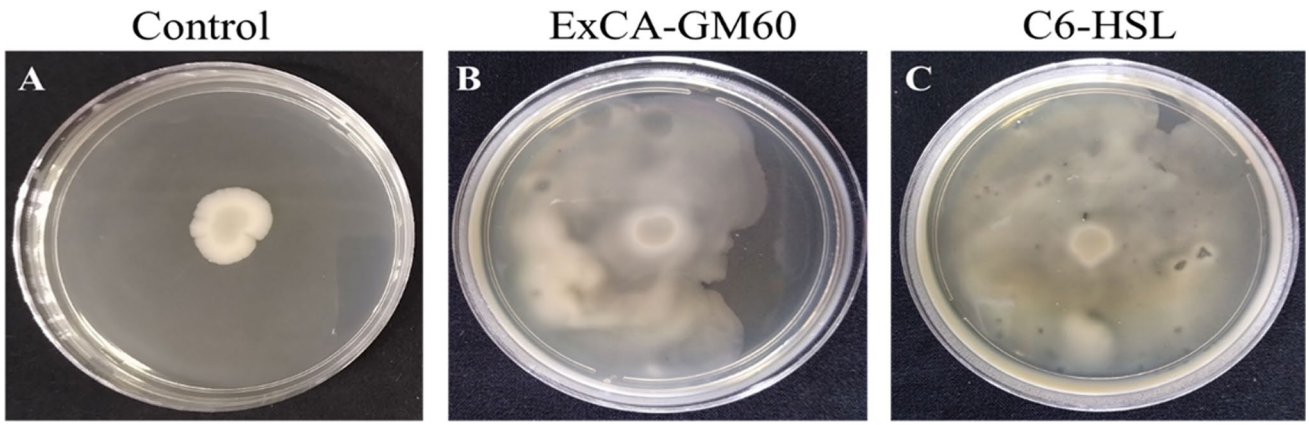

Fig. 6 Swarming motility assays of *Salmonella* PT4 in the presence of extract of isolate GM60 (ExCA-GM60) *R. inusitata* and commercial C6-HSL at a final concentration of 250 nM after 24 h of anaerobic incubation at 37 °C

cationic peptides [52]. Interestingly, C12-HSL increased the resistance of *Salmonella* Enteritidis PT4 to nisin, a cationic peptide [53]. In *E. coli*, *Pseudomonas aeruginosa*, *Burkholderia dolosa,* and *Yersinia enterocolitica,* QS is required during swarming motility [54].

Conclusion

Our findings show that isolates of *R. inusitata* from the *G. mellonella* gut are producers of AHLs and that the extracts containing AHLs from these isolates increased the adhesion and motility of *Salmonella* PT4 under anaerobic conditions. These results strongly suggest that enteric pathogens, such as *Salmonella* can detect signaling molecules in the gastrointestinal tract of animals and activate multiple virulence factors that can contribute to an infection. However, in-depth investigations need to be carried out to understand better this "eavesdropping" between different bacterial species and its role in pathogenicity.

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Author contribution Leonardo Luiz de Freitas: conceptualization, methodology, investigation, writing — original draft preparation. Deisy Guimarães Carneiro: investigation. Gabriel Silva Oliveira: investigation. Maria Cristina Dantas Vanetti: conceptualization, supervision, project administration, writing—reviewing and editing.

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Availability of data and material GenBank accession numbers The GenBank accession numbers for 16S rRNA sequences generated in this study are as follows: MW900260 (isolated GM34), MW900261 (isolated GM56), and MW900262 (isolated GM60).

Declarations

Ethics approval Not required.

Consent to participate Not applicable.

Consent for publication The authors Leonardo Luiz de Freitas, Deisy Guimarães Carneiro, Gabriel Silva Oliveira, and Maria Cristina Dantas Vanetti are in accordance with the submission of this manuscript to the Brazilian Journal of Microbiology.

Competing interests The authors declare no competing interests.

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