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# Detection of Quorum Sensing Signal Molecules, Particularly N-Acyl Homoserine Lactones, 2-Alky-4-Quinolones, and Diketopiperazines, in Gram-Negative Bacteria Isolated From Insect Vector of Leishmaniasis

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Gram-negative bacteria are known to use a quorum sensing system to facilitate and stimulate cell to cell communication, mediated via regulation of specific genes. This system is further involved in the modulation of cell density and metabolic and physiological processes that putatively either affect the survival of insect vectors or the establishment of pathogens transmitted by them. The process of quorum sensing generally involves N-acyl homoserine lactones and 2-alkyl-4-quinolones signaling molecules. The present study aimed to detect and identify quorum sensing signaling molecules of AHLs and AHQs type that are secreted by intestinal bacteria, and link their production to their extracellular milieu and intracellular content. Isolates for assessment were obtained from the intestinal tract of *Pintomyia evansi* (*Leishmania* insect vector). AHLs and AHQs molecules were detected using chromatography (TLC) assays, with the aid of specific and sensitive biosensors. For identity confirmation, ultra-high-performance liquid chromatography coupled with mass spectrometry was used. TLC assays detected quorum sensing molecules (QSM) in the supernatant of the bacterial isolates and intracellular content. Interestingly, *Pseudomonas otitidis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Pantoea ananatis* isolates showed a migration pattern similar to the synthetic molecule 3-oxo-C6-HSL (OHHL), which was used as a control. *Enterobacter cancerogenus* secreted C6-HSL, a related molecules to N-hexanoyl homoserine lactone (HHL), while *Acinetobacter gyllenbergii* exhibited a migration pattern similar to 2-heptyl-4-quinolone (HHQ) molecules. In comparison to this, 3-oxo-C12-HSL (OdDHL) type molecules were produced by *Lysobacter soli*, *Pseudomonas putida*, *A. gyllenbergii*, *Acinetobacter calcoaceticus*, and *Pseudomonas aeruginosa*, while *Enterobacter cloacae* produced molecules similar to 2-heptyl-3-hydroxy-4-quinolone (PQS). For *Pseudomonas putida*, *Enterobacter aerogenes*, *P. ananatis*, and *Pseudomonas otitidis* extracts, peak chromatograms with distinct

retention times and areas, consistent with the molecules described in case of TLC, were obtained using HPLC. Importantly, *P. ananatis* produced a greater variety of high QSM concentration, and thus served as a reference for confirmation and identification by UHPLC-MRM-MS/MS. The molecules that were identified included N-hexanoyl-L-homoserine lactone [HHL, C<sub>10</sub>H<sub>18</sub>NO<sub>3</sub>, (M + H)], N-(3-oxohexanoyl)-L-homoserine lactone [OHHL, C<sub>10</sub>H<sub>16</sub>NO<sub>4</sub>, (M + H)], N-(3-oxododecanoyl)-L-homoserine lactone [OdDHL, C<sub>16</sub>H<sub>28</sub>NO<sub>4</sub>, (M + H)], and 2-heptyl-3-hydroxy-4(1H)-quinolone [PQS, C<sub>16</sub>H<sub>22</sub>NO<sub>2</sub>, (M + H)]. Besides this, the detection of diketopiperazines, namely L-Pro-L-Tyr and ΔAla-L-Val cyclopeptides was reported for *P. ananatis*. These molecules might be potentially associated with the regulation of QSM system, and might represent another small molecule-mediated bacterial sensing system. This study presents the first report regarding the detection and identification of QSM and diketopiperazines in the gut sand fly bacteria. The possible effect of QSM on the establishment of *Leishmania* must be explored to determine its role in the modulation of intestinal microbiome and the life cycle of *Pi. evansi*.

**Keywords:** AHLs, AHQs, diketopiperazines, gram-negative bacteria, sandflies

## INTRODUCTION

Quorum Sensing (QS) signaling molecules constitute a complex environmental system that is regulated according to the density dynamics of the bacterial population. It mainly involves N-acyl homoserine lactones (AHLs) and/or alkyl quinolones (AHQ) signaling pathways (1, 2). These signals are generally involved in various gene regulatory mechanisms, and are responsible for modifying the behavior, which is mediated by chemical communication (3, 4). Many gram-negative bacteria are known to regulate different physiological processes through AHL signal, and their biological activity depends on the stereochemistry mediated by homologous proteins related to the LuxI/R system (4, 5). However, AHL type auto inducers are not the only type of molecules that are dependent on the cell density (6, 7).

In this context, pathogen–bacteria–host interactions, found in insect vectors involved tropical diseases, might depend on a wide variety of molecular signals that allow the regulation of activities in a coordinated way, thereby affecting the population abundance of three entities and possibly the vector competition (8). In *Aedes aegypti aegypti* (Linnaeus), QS generated by bacteria has been shown to have implications in the decision of mosquitoes, particularly regarding the detection, location, and host preferences (8). Additionally, it has been previously reported that quorum sensing molecules (QSM) are considered as “Integral components of global gene regulatory networks”, and these molecules allow bacteria to exhibit physiological responses as per the host environment, such as biofilm formation, virulence, motility, bioluminescence, symbiosis, conjugation, and production of antibiotics and some secondary metabolites (8, 9).

Bacterial intercellular communication (QS) also plays an important role in bacterial cell density and microbiota modulation. However, the gut microbiota of insect vectors is a dynamic and complex system. Regardless of the nature of the

association, success of symbiosis majorly depends upon a complex interplay between bacterial symbionts and their insect hosts throughout the course of insect development and reproduction. In fact, many symbiotic bacteria preferentially colonize specialized insect tissues or cells, wherein they can achieve extremely high infection densities; however, QSMs produced by one species or through bacterial consortia can play a decisive role in their establishment. It has been previously reported that cell culture supernatant of *P. aeruginosa* is capable of mediating cell death of *E. coli* via a QS mediated process (10).

One of the fundamental aspects of bacterial communication, involving AHLs and AHQ molecules, is their ability to move through the membrane and quickly reach a balance between the intracellular and extracellular compartments at high concentrations (11). This further facilitates the detection of these molecules using qualitative and quantitative bioassays. It also enables the use of complete cells that are capable of detecting a wide variety of signals, bioreporters, and robust and sensitive tools such as HPLC and LC-MS/MS (2, 11–13). It is necessary to specify that biological activity of AHLs molecules is dependent on a wide variety of parameters, including relatively acidic pH that is associated with closed lactone ring, high rate of biosynthesis, presence of an N-acyl chain comprising of at least four carbons in length, and high density of AHLs in the extracellular medium (11, 14).

In case of certain species of medical importance, such as *Lutzomyia longipalpis*, under normal conditions (without blood in the gut), the gut of adults are characterized by the presence of normal pH of 6, while more alkaline pH is observed after blood intake (pH of 8). However, this pH keeps on dropping throughout the process of digestion and returns to pH 6 at the end (15). In case of larvae, the physiological conditions are completely different. Fazito et al. (16) reported a pH gradient,

varying from pH > 9 in the anterior midgut to pH of 6.5–7.0 in the posterior midgut (16).

These findings might suggest that communities present in the gut of phlebotomines act as a strong biological study model for the detection and isolation of physiologically active AHLs molecules. Previous studies of the lepidopteran larvae gut have shown that long chain molecules, like N-acyl carbonates (3-oxo-C12-HSL), are more resistant to basic pH ( $\geq 7.4$ ), owing to the hydrophobic nature of the carbon chain and the presence of less electrophilic lactone ring. This makes it less susceptible to attack by hydroxide ( $-\text{OH}$ ) ions and more resistant to higher pH (17). These studies further justify the search of QSMs associated with the intestines of vector insects, such as phlebotomines due to the compartment pH. During blood digestion, QSM are in a more stable environment (alkaline condition). It is known that during the blood digestion, the bacterial population increase (18). This is because the insect produces less hydrogen peroxide and hypochlorite, molecules produced by the insect's immune system to control the bacterial population in the gut. The QSMs of sand flies putatively might play an important role in the modulation of the development of parasites (*Leishmania*) in the intestine and/or mechanisms associated with the insect defense systems or the processing of nutrients acquired during its life cycle.

Currently, no studies are available that demonstrated the interaction between *Leishmania* and QSM. However, studies conducted in other biological models (merely parasites), reported cross species QS that can operate in trypanosomes. For example, the involvement of QS signals in proteolytic processing of host proteins, which resulted in the generation of short oligopeptides that are important for parasitic differentiation, as reported in case of *Trypanosoma brucei* (19). Additionally, it was reported that QS signal could be used to communicate between mixed infections of different African trypanosome species (19, 20).

Studies exploring QSMs in bacterial communities present in environmentally complex surroundings, such as insects gut, are often complicated. This is mainly attributed to the occurrence of a variety of intrinsic and extrinsic cellular factors that modulate QS kinetics (21) and signal production, detection, and response. QSMs studies conducted in arthropods are mainly restricted to *Sodalis praecaptivus* (22), *Aedes aegypti aegypti*, *Spodoptera* spp., and tsetse flies (17, 20, 22).

Currently, only midgut bacterial genera and endosymbionts, such as *Pintomyia evansi* and *Lutzomyia longipalpis* (vectors of visceral leishmaniasis), are known to be present in natural populations of phlebotomine sand flies of Colombia (23–26). Recently, this species was selected to reinforce, deepen, and understand the dynamics of the gut microbiota in the presence of *Leishmania infantum* infection (27). Dominant intestinal bacteria, including *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Bacillus*, *Ochrobactrum*, *Pantoea*, *Ralstonia*, and *Methylobacterium*, and some endosymbionts, such as *Cardinium* and *Wolbachia*, have been previously reported in *Pi. evansi*. This study highlighted the enzymatic, anti leishmanicidal, and antimicrobial bioactivities of these QS molecules (28). However, details of QSMs detection in

*leishmania* vectors and its importance in the physiology of intestinal bacterial microbiota are not been fully established.

Importantly, QS molecules are not only involved in inter-microbial communication, but these molecules can possibly assist cross-talk of bacteria directly or indirectly with their host, and thus can modulate pathogen transmission.

QS molecules produced by gram-negative bacteria predominantly include AHLs and AHQ molecules, while gram-positive bacteria mainly use peptides (autoinducer peptides, AIP, or quorum sensing peptides). The present study aimed to detect and identify QSMs in gram-negative bacteria isolated from the gut of *Pi. evansi*. The findings of the study would assist the generation of new and complementary information, which would be useful in understanding the tripartite interactions that occurs between host–parasite–microorganism.

## MATERIALS AND METHODS

### Ethics Statement

The sandfly collection was performed following the parameters of Colombian decree N° 1376. This study was performed under the permission granted by Ministry of Environment and Sustainable Development (Permission contract N. 121 22/01/2016 otrosí #23). No specific permits were required for this study.

### Isolation of Bacteria and Culture Conditions

Bacterial strains used in this study were isolated from the guts of larvae and adults (females) of *Pi. evansi*, and these were identified by 16S rDNA gene sequencing (23). In particular, sandflies were collected from two locations of the Department of Sucre (Caribbean coast of Colombia). Further, the guts were dissected under a stereoscope using sterile 1X PBS buffer and stiletos on a glass, under aseptic conditions. Cultivable and aerobic midgut bacteria were isolated. The methods and culture media used in the present study have been previously described in other studies conducted on intestinal microbiota of sand flies. Following this, the gut suspension was streaked on Luria-Bertani (LB) agar (Merck, Bogota, Colombia) and MacConkey Agar (Merck, Bogota, Colombia) plates using serial dilution, and incubated for 48 h at room temperature. Next, the isolates were selected, identified macroscopically and microscopically by Gram's staining, and molecular identity was established. All isolates were cryopreserved in 20% glycerol at  $-80^{\circ}\text{C}$ . For further experiments, only gram-negative bacteria were selected.

All the isolated 12 strains and biosensors (pSB401, pSB1142, and PqsA-Lux) were grown in LB medium at  $37^{\circ}\text{C}$  overnight and sub-cultured in LB agar. A single colony was inoculated and incubated overnight at  $37^{\circ}\text{C}$  at 200 rpm until the cell culture reached an optical density of 1.0 ( $\text{OD}_{600}$ ). Next, 0.25 mL of each culture was transferred to a 250 mL Erlenmeyer flask containing 25 mL of LB broth, and incubated overnight at  $37^{\circ}\text{C}$  with continuous stirring at 200 rpm (1, 2).

## Extraction of AHL and AHQ Molecules From Cells and Supernatant

AHL and AHQ extraction was performed following the method previously described by Fletcher and co-workers (1). Briefly, 10 ml of each stationary phase culture was separated by centrifugation at  $10000 \times g$  for 10 mins at  $4^{\circ}\text{C}$ . AHL and AHQ molecules associated with the cells or cell-free supernatant were extracted, as previously described (1, 2).

Supernatants were filtered through sterile  $0.22 \mu\text{m}$  filters (Minisart, NML, Sartorius, EE. UU.), and transferred to a 50 mL falcon tube. Following this, 10 ml of acidified ethyl acetate (0.01% acetic acid) was added to filtered supernatant, and the sample was vortexed for 30 sec to mix two phases (organic phase/aqueous phase) (1). The mixture was then transferred to a separating funnel, previously washed with acetone, and it was left undisturbed until two phases were separated. Next, the organic phase was transferred into another 50 mL falcon tube (1). The extraction process was repeated twice or more until a total volume of 30 mL was obtained. The cell pellet was resuspended in 10 mL of LB broth and centrifuged at  $10,000 \times g$  for 10 min, and the resulting supernatant was discarded. This process was developed three times to completely remove QSMs from the supernatants. Further, 10 mL of methanol (100% v/v) was added to final cell pellet for re-suspension, and the resulting sample was centrifuged at  $10000 \times g$  for 10 mins at  $4^{\circ}\text{C}$  (1, 2).

The extracts obtained from the cells and supernatants were separately transferred to 50 mL round bottom flasks, and subjected to rotary evaporation until liquid phase was completely dried. Each extract was washed with methanol and transferred to 2 mL vials (1). The resulting extracts were dried using a rotary vacuum concentrator (Concentrator 5301, Eppendorf) and stored at  $-20^{\circ}\text{C}$  until used for further analysis.

## Synthetic Molecules AHL and AHQ

AHL and AHQ synthetic molecules (HHL, OHHL, OdDHL, PQS, and HHQ) that were used as standards and the biosensing strains were received as a kind gift from the Bioscience School of Nottingham University, UK. These samples were conserved as previously described (1). All experiments were carried out in a cell-free bioassay. **Table 1** describes the names and some characteristics of AHLs molecules and biosensors used in the present study.

The identification of large number of QS systems has been made possible mainly by the use of biosensors that are capable of detecting the presence of AHLs. These mechanisms are primarily based on the coupling between a transcriptional regulator and an inducible promoter (Protein/Promoter) that responds to different environmental, nutritional, or cellular communication conditions. This interaction between the target molecule and transcriptional regulator either activates or represses a reporter gene, resulting in a measurable signal change. In gram-negative bacteria, a typical AHL-QS system is mediated by two proteins belonging to the LuxI/LuxR family, which are involved in the bioluminescence production and cell density. Importantly, this AHL-protein complex binds specifically to the box-lux (promoter) to promote the expression of QS genes.

In the present study, all experiments were developed from cell-free supernatants because AHLs that were extracted from cell culture supernatants in their exponential phase significantly increased the sensitivity of biosensors to detect AHL and AHQ molecules.

## Detection of AHL Molecules Using Thin Layer Chromatography

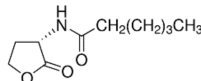
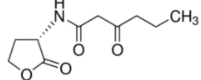
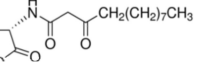
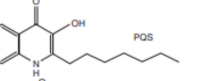
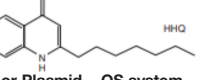
First, crude extracts were re-suspended in  $100 \mu\text{L}$  of methanol, and synthetic molecules were brought to a final concentration of 10 mM, 1.0 mM, 0.1 mM, and  $1.0 \mu\text{M}$ . Following this,  $5 \mu\text{L}$  of each sample and  $2 \mu\text{L}$  of each synthetic molecule were loaded onto TLC plate ( $20 \times 20 \text{ cm}$ , 60F254 normal phase silica TLC plates, Merck) (29, 30). Chromatographic separation was performed using a solvent phase of dichloromethane-methanol solution (95:5, v/v). After the solvent front reached the required level, plate was removed, dried, and overlaid with 1 mL of *E. coli* (pSB401 and pSB1142) and *P. aeruginosa* (PqsA-Lux) biosensor strains using semi-solid LB agar (100 mL). The plates were further incubated at  $37^{\circ}\text{C}$  for 18 h. Bioluminescence signals were detected on TLC plate overlays using an X-ray film for each specific biosensor, at three-time intervals (1, 3, and 10 mins) (29, 30). For each sample, the migration value,  $R_f$ , was calculated as  $R_f = X/Y$ , wherein “X” represented the distance from the origin to the pattern of the detected molecule and “Y” represented the distance from the origin to the end of the silica gel plate (29, 30). The resulting  $R_f$  values for the samples were compared with AHL standards. Samples (cell and supernatants extract) and standards (synthetic molecules) were processed three times to achieve adequate reproducibility.

## High-Performance Liquid Chromatography Analysis

For HPLC analysis,  $600 \mu\text{L}$  of methanol was added to  $200 \mu\text{L}$  of each sample. Each sample was subsequently filtered using  $0.22 \mu\text{m}$  nylon filter, and the filtered samples were deposited in mass-type vials. The reagents used for HPLC analysis included acetonitrile (Gradient type, HPLC grade), methanol (HPLC grade, MERCK<sup>®</sup>), and water-type I (Milli-Q, Millipore, MERCK<sup>®</sup>). For HPLC analysis, a Chromaster Hitachi<sup>®</sup> HPLC equipped with a DAD detector at a wavelength of 210 nm was used. A Synergi<sup>™</sup> 4  $\mu\text{m}$  Hydro-RP 80  $\text{\AA}$  LC Column ( $150 \times 4.6 \text{ mm}$ ), with a KJ0-4282 precolumn with C-18 cartridges both brands Phenomenex was also used (13, 31). The mobile phase comprised of 100% Milli Q water in pump A and 100% acetonitrile in pump B. Both the solvents were maintained at room temperature. The samples were injected in volumes of  $10 \mu\text{L}$  each, and separated using reverse phase chromatography method as described below (13, 31).

Briefly, HPLC separation was carried out at a flow rate of 2 mL/min. The total run time for HPLC separation was 42 mins, which involved initial isocratic conditions of 5% of B and 95% of A for first 2 mins, which changed to 5% to 20% of B in the next minute. This was followed by a gradient change from 20% to 95% of B in 30 minutes, which was maintained for next 3 mins. Subsequently, it returned to the initial condition in 5 mins, which was maintained for additional 2 mins (13, 31).

**TABLE 1** | List of synthetic Quorum Sensing molecules and specific biosensors used in detection bioassays on TLC and UHPLC-MS.

Chemical name QSM	Common name	Chemical structure	Formula (M+H)	Mono isotopic mass	Relevant biological significance	References
N-Hexanoyl-L-homoserine lactone	HHL		C <sub>10</sub> H <sub>18</sub> NO <sub>3</sub>	200.1281	Biofilm formation Adaptation	Winson et al. (26)
N-(3-Oxohexanoyl)-L-homoserine lactone	OHHL		C <sub>10</sub> H <sub>16</sub> NO <sub>4</sub>	214.1073	"Swarming" mobility Iron transport	Winson et al. (26)
N-(3-Oxododecanoyl)-L-homoserine lactone	OdC		C <sub>16</sub> H <sub>28</sub> NO <sub>4</sub>	298.2012	Biofilm formation Virulence Membrane protein degradation Apoptosis	Winson et al. (26)
2-Heptyl-3-hydroxy-4(1H)-quinolone	PQS		C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub>	260.1650	Inhibition of fungi and Gram-positive Immune response modulation Virulence factors Bacterial lysis	Winson et al. (26), Diggle et al. (29)
2-Heptyl-4(1H)-quinolone	HHQ		C <sub>16</sub> H <sub>22</sub> NO	244.1695		Winson et al. (26), Diggle et al. (29)
<b>AHL Biosensor</b>	<b>Strain or Plasmid</b>	<b>QS system</b>	<b>AHL Detected</b>	<b>Reporter</b>	<b>Relevant feature</b>	<b>References</b>
pSB401	<i>E. coli</i> JM109	<i>LuX R/PluxI</i>	C4- a C12-AHL, 3-oxo-C4 a 3-oxoC14-AHL	LuxCDABE	Contains a luxRl::luxCDABE fusion in a pACYC184 plasmid	Winson et al. (26).
pSB1142	<i>E. coli</i> JM109	<i>LasR/LasI</i>	C12 AHL	LuxCDABE	Long-chain reporter carrying promoters R and <i>P. aeruginosa</i> fused to <i>luxCDABE</i> cassette from <i>Photobacterium luminescens</i> , which respond to long AHLs (C10-C14) chains.	Winson et al. (26); How et al. (27)
PqsA-Lux	<i>P. aeruginosa</i>	<i>LysR-type</i>	2-heptyl-4-quinolone, 2-Heptyl-3-hydroxy-4(1H)-quinolone	pqsABCDE	Dual biosensor for detection of 2-alkyl-4-quinolone signal molecules.	Coleman et al. (28); Diggle et al. (29)

AHL, N-Acylhomoserine lactones (AHL). The molecular formulas are protonated (M+H) as well as the respective monoisotopic mass.

## Mass Spectrometry (UHPLC-MRM-MS/MS) Analysis

Mass spectrometry analysis was performed using two buffers, 100% Milli Q water with 0.1% formic acid in pump A and 100% acetonitrile with 0.1% formic acid in pump B. Both the solvents were maintained at 40°C. The chromatographic separation was performed with UHPLC using an initial isocratic flow with 5% of B for 3 mins, which changed from 5% to 20% of B in next 1 min. Subsequently, the gradient increased from 20% to 95% of B in 18 mins, and this condition of B was maintained for next 3 mins. Finally, it returned to the initial condition in next 3 mins. The total run time of separation was 32 mins (1, 32).

To confirm the exact masses of the standards, the samples were injected in volumes of 1  $\mu$ L each, and separated by reverse-phase chromatography using Thermo<sup>®</sup> Ultimate 3000, with a Synergy 2.1  $\mu$ m Hydro-RP 80<sup>a</sup> column (3 x 50 mm) Phenomenex<sup>®</sup>, which was coupled to a Bruker<sup>®</sup> Impact II ESI-QTOF in positive ion scan mode, with auto MS/MS in a range of 50–1200 m/z. The spectrometer conditions that were used for MS included Dry Heater: 200°C, Nebulizer: 29 psi, Dry Gas: 8 L/min, Capillary: 4500 V, Charging Voltage: 2000 V, and Fragment: 700Vp. Spectra in MS/MS mode were collected for MS (full scan) range of 100–1200 m/z. Auto MS/MS was performed in a range of 50–1200 m/z, which was collected at a rate of 5 spectra, with a maximum of 3 selected and fragmented precursors, using a collision energy of 5eV. To search the masses of the exact standard and possible adducts with sodium and methanol, the spectra were processed using Bruker<sup>®</sup> Data Analysis software (1–32) and *in silico* fragmenter MetFrag (33).

Once the masses of the exact standard were confirmed, an MRM mode test was carried out based on monoisotopic mass of ions (M + H) 200.1281, 214.1073, 298.1073, 244.1695, and 260.1650, which corresponded to the molecules HHL, OHHL, OdDHL, HHQ, and PQS. MRM mode test was performed using a Reprosil-Pur Basic C18 column (brand DR Maisch) and same equipment, under same conditions (31). Samples (cell and supernatants extract) and standards (synthetic molecules) were processed three times to achieve adequate reproducibility.

## RESULTS

### Detection of AHL and AHQ Molecules Using Specific Bioreporters

All the isolates, except *O. anthropi*, generated QS molecules that were associated with the cell fraction or supernatant. These molecules showed bioluminescence patterns that were related to different type of organic AHL molecules, which were detected by *pSB401-pSB1142* of *E. coli* and *PqsA-Lux* of *P. aeruginosa* bioreporters on TLC plates (**Supplementary Material 1** and **Table 2**). Greater production of QS was evident in the supernatant as compared to the cell fraction. QS is known to involve the production of extracellular signaling molecules, which are spread to the external environment *via* accumulation according to growth and cell density. But it is noteworthy that some bacteria, such as *E. hormaechei*, generate QS only in the cell fraction.

This might be associated with autoinducers that were bound by specific receptors residing in the inner membrane or cytoplasm.

*P. otitidis*, *E. cloacae*, and *P. ananatis* presented a migration pattern similar to 3-oxo-C6-HSL (Rf = 0.63), whereas *E. cancerogenus* secreted molecules related to HHL (Rf = 0.58). In comparison to these, AHQ molecules were visualized in the pellet and supernatant of *A. gyllenbergii* on biosensor *PqsA-Lux* and had a migration pattern similar to HHQ (Rf = 0.457), with closely related Rf values (**Table 2**). *L. soli*, *P. putida*, *P. aeruginosa*, *A. gyllenbergii*, *P. otitidis*, *A. calcoaceticus*, and *E. cloacae* isolates were also found to secrete QS molecules related to the supernatant or cell pellet, with migration similar to the positive control OdDHL, 3-oxo-C12-HSL (Rf = 0.024–0.575–0.914) (**Supplementary Material 1** and **Table 1**). Some of the detected QS did not correlate with Rf values expressed by the synthetic molecules that were used as a control, which indicated that these are molecules of a similar nature, but require further analysis by LC/MS-MS for accurate identification.

On TLC, the residues of molecules that were found to be different from the controls migrated with Rf values < 0.1 (for example *E. hormaechei*, *E. cancerogenus*, and *P. putida*), as observed in *pSB401*. This could represent 3-oxo-C8-HSL or C8-HSL. In comparison to this, the uncharacterized signals in *PqsA-Lux* exhibited Rf value < 0.5 (*E. cancerogenus*, *P. otitidis*, *A. gyllenbergii*, and *P. aeruginosa*), which might belong to PQS-type molecules; however, additional analysis is required to confirm the same.

### QSM Detection by High-Performance Liquid Chromatography

As expected, singular/single peak chromatograms were obtained for each sample of synthetic AHL and AHQ molecules, which were used as standards and controls. Retention times and peak heights (**Supplementary Materials 2A, B**) were estimated for these molecules, which were later used as a library to compare the presence of QSM in the bacterial extracts that were assessed.

Interestingly, the chromatographic separation yielded defined and distinctive peaks and retention times for supernatants and cell fraction of *P. putida*, *E. aerogenes*, *P. otitidis*, and *P. ananatis*, when compared with previously characterized synthetic QS patterns (**Table 3**). Among these QSM, OHHL signal was detected repeatedly in the chromatograms obtained from HPLC, with retention times ranging from 5.037–5.063 mins and peak areas varied from 255.370–5052.365 mAU\*s. The supernatants of *E. aerogenes* and *P. ananatis* were found to be unique, as their respective chromatograms made it feasible to confirm the possible presence of QSM type HHL, HHQ, and PQS, respectively (**Table 3**).

### QSM Identification With Mass Spectrometry (UHPLC-MRM-MS/MS)

As shown in **Figure 1**, mass spectra and fragmentation patterns were obtained for each AHL and AHQ synthetic molecules, which were used as standards (33). It should be pointed out that UHPLC-MRM-MS/MS analysis focused mainly on *P. ananatis* for certain reasons. During HPLC analysis, the concentration of AHLs and AHQs were recorded to be comparatively lower in

case of other bacterial extracts. Consequently, during the first-run of UHPLC-MRM-MS/MS, adequate ionization was not achieved to obtain characteristic mass spectra. Additionally, little degradation was observed in the extracts of the supernatants obtained from the rest of the bacterial isolates. Thus, the bacterium *P. ananatis* presented the greatest richness of QSM, as reported by TLC, HPLC, and UHPLC-MRM-MS/MS analyses. As a result, *P. ananatis* was considered as the biological reference sample for the validation and identification of QSM.

A chromatogram of total ions (TIC) was obtained for the supernatant extract of *P. ananatis* (Figure 2A), and subsequently, protonated ions were identified in the mass spectra belonging to HHL (C<sub>10</sub>H<sub>18</sub>NO<sub>3</sub>) molecules and their lactone ring (Figure 2B), OHHL (C<sub>10</sub>H<sub>16</sub>NO<sub>4</sub>), OdDHL (C<sub>16</sub>H<sub>28</sub>NO<sub>4</sub>), and PQS (C<sub>16</sub>H<sub>22</sub>NO<sub>2</sub>) molecule (Figures 2C-E). The masses of HHL and OHHL molecules were fragmented (Figure 2). Molecules having nature different from AHLs and AHQs were also identified in *P. ananatis* supernatant. These molecules corresponded to diketopiperazines (L-Pro-L-Tyr and ΔAla-L-Val cyclopeptides), as revealed by mass spectra (Figure 3).

## DISCUSSION

Regulation of gene expression in bacteria is an important adaptive trait that influences the dynamics of the intestinal microbiomes of insects, their physiology and survival, and

establishment of other microorganisms (34). One of these regulatory modes is related to the QS system (35), wherein bacteria synthesize an autoinductive signal pheromone that increases according to the bacterial density and interacts with response regulators in a concentration dependent manner (threshold). This in turn facilitates changes in the host gene expression (suppression or expression of immune response genes) or increases the production of secondary metabolites from inter-specific interactions (34).

It has been previously shown that QS molecules could participate in the interactions between insect vectors and vertebrates, preferably (*Aedes aegypti aegypti*) increasing their attraction (8) or other insects (tsetse flies) that could attenuate the virulence of their pathogens. Such a process has been reported in bacterium *Sodalis glossinidius* (22). The microbiota and signaling molecules in invertebrates are also known to exert

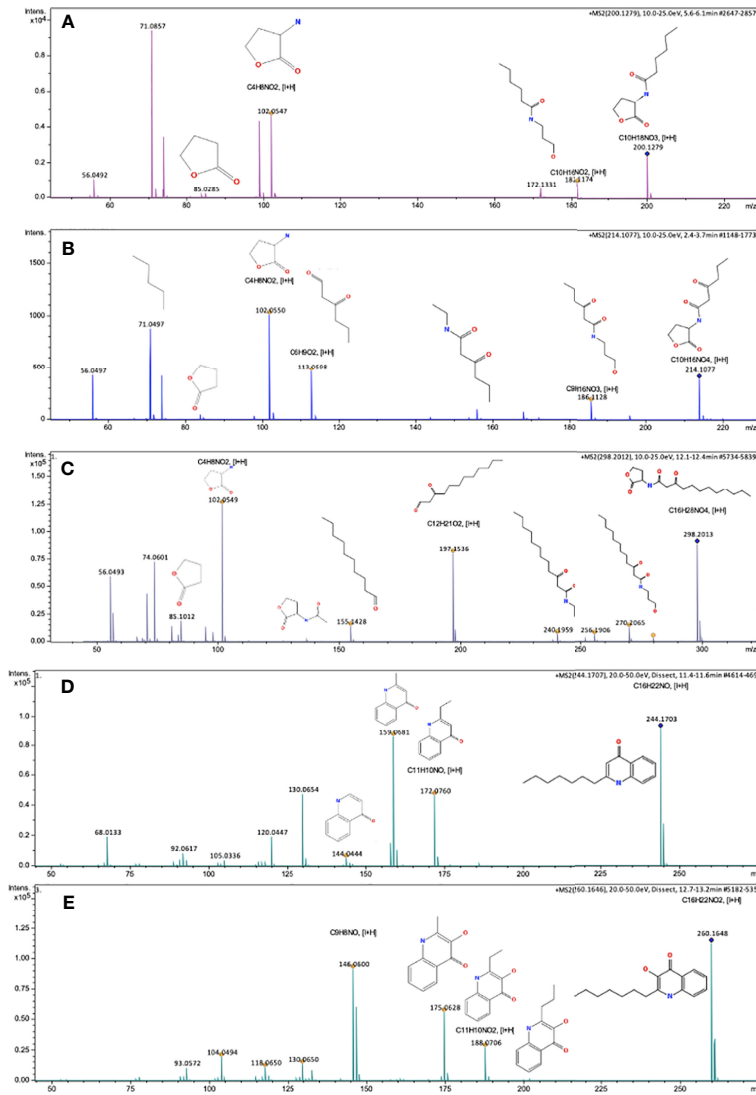
**TABLE 3 |** HPLC analysis of QSM in Gram-negative bacteria supernatants.

Gram-negative strain	QSM type	Retention time (Min)	Area (mAU)
<i>P. putida</i>	OHHL	5.037-5.040	501.427-803.781
<i>E. aerogenes</i>	HHL	7.063	1081.052
	HHQ	15.748	868.829
	OHHL	5.063	5052.365
<i>P. otitidis</i>	OHHL	5.037	255.370
<i>P. ananatis</i>	PQS	16.686	156.174

**TABLE 2 |** Detection of Quorum Sensing molecules (QSM) AHLs and AHQs with pSB401, pSB1142, and PqsA-Lux biosensors as well as migration values (Rf) of each molecule on TLC plates.

Gram-negative strains	Specific Biosensors					
	pSB401		pSB1142		PqsA-Lux	
	Cell (Value Rf)	Sup (Value Rf)	Cell (Value Rf)	Sup (Value Rf)	Cell (Value Rf)	Sup (Value Rf)
<i>E. cancerogenus</i>	0,03	0,04	-	0,77	-	0,29
<i>P. putida</i>	0,04	0,03	1,12	0,99	-	-
<i>E. aerogenes</i>	0,53 - 0,03	0,05	-	-	-	-
<i>A. calcoaceticus</i>	-	-	-	0,97	-	-
<i>P. otitidis</i>	-	0,69 -0,09	-	0,67	-	0,26
<i>E. cloacae</i>	-	0,72	0,55	0,89	-	-
<i>O. anthropi</i>	-	-	-	-	-	-
<i>P. ananatis</i>	-	0,97-0,71 -0,09	-	-	-	-
<i>A. gyllenbergii</i>	-	-	-	0,63	0,40	0,44
<i>E. hormaechei</i>	0,05	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	0,62	0,61	-	0,23
<i>L. soli</i>	-	-	-	0,98	-	-
<b>Synthetic QSM</b>						
HHQ					10mM* (0,45)	
PQS					10mM* (0,68)	
OdDHL				1.0mM* (0,02 - 0,57 - 0,91)		
OHHL	1.0μM* (0,63)					
HHL	0,1mM* (0,58)					

Absence (-) of QSM in Gram-negative strains; Cell, QSM related to cell pellets; Sup, QSM related to the supernatant. pSB401 and pSB1142: Biosensor of *E. coli*; PqsA-Lux, Biosensor of *P. aeruginosa*; \*Final concentration of synthetic QS molecules used as positive control; mM: final concentration in millimolar, μM: final concentration in micromolar.



**FIGURE 1** | Mass spectra consistent with AHLs and AHQs standard molecules obtained through UHPLC-MRM-MS. **(A)** Mass spectra of HHL molecule [M+H]= 200.1279, Fragments [I+H]= 182.1174, 102.0547, 85.0285, 71.0857, 56.0492 **(B)** Mass spectra of OHHL molecule [M+H]= 214.1077, Fragments [I+H]= 186.1128, 113.0598, 102.0550, 71.0497, 56.0497, **(C)** Mass spectra of OdDHL molecule, [M+H]= 298.2013, Fragments [I+H]= 270.2065, 256.1906, 240.1959, 197.1536, 155.1428, 102.0549, 85.1012, 74.0601, 56.0493, **(D)** Mass spectra of HHQ molecule [M+H]= 244.1703, Fragments [I+H]= 172.0760, 159.0681, 130.0654, 120.0447, 68.0133, **(E)** Mass spectra of PQS molecule [M+H]= 260.1648, Fragments [I+H]= 188.0706, 175.0628, 146.0600, 130.0650, 104.0494, 93.0572. Impact II ESI-QTOF de Bruker® equipment. Reprosil-Pur Basic C18 column, DR Maisch Brand (characterized of standards **A–C**). Synergy Hydro-RP 80<sup>Å</sup> column, Phenomenex® Brand (characterized of standards **D, E**).

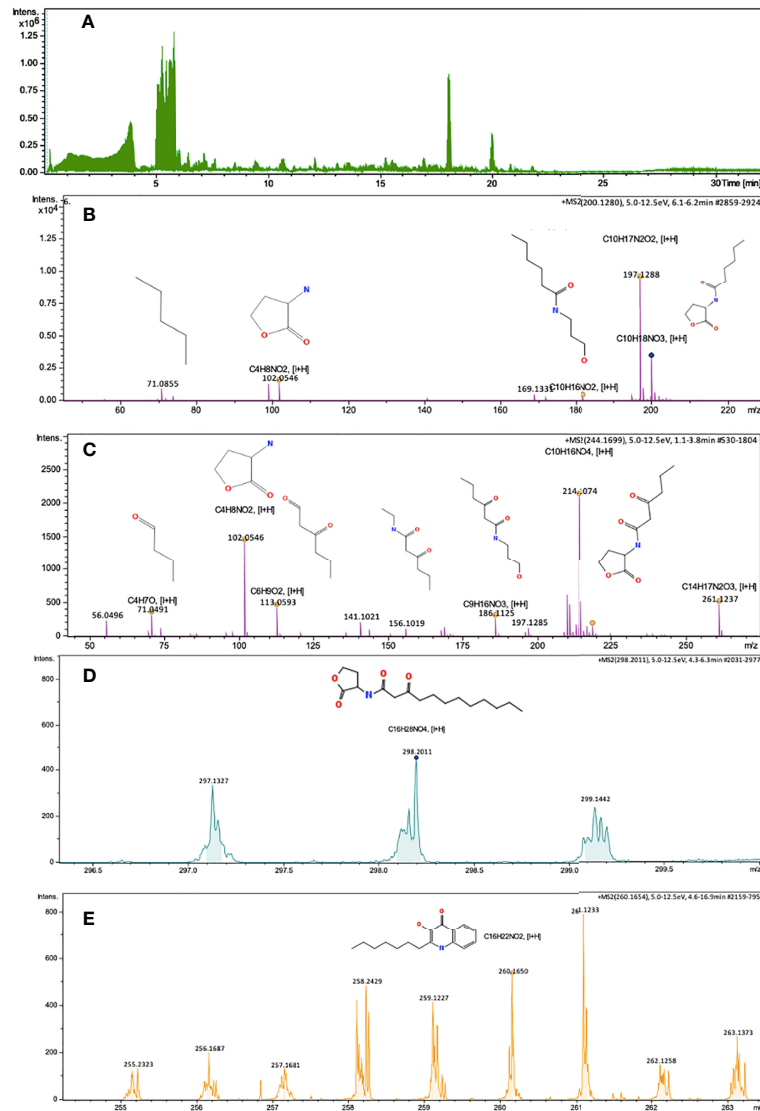
strong effects on fitness, nutrient supply, maturation of the immune system, development, and resistance to colonization against pathogens (36).

All these findings motivated both the detection and identification of QS molecules (N-acyl homoserine lactones and 2-alkyl-4-quinolones) in gram-negative bacteria isolated from the intestinal tract of *Pi. evansi*, a blood-sucking insect that interacts with viruses (arbovirus), parasites (*Leishmania*), and bacteria. QS molecules described in the present study varied in terms of the length of carbon chain and associated functional

groups. Thus, the present study is the first report associated with this vector.

TLC enabled significant detection of QS molecules as these molecules were found to be present in eleven of the twelve isolates, in both supernatant and cell extracts. In particular, a greater number of molecules were detected mainly in the supernatant obtained from *Enterobacter-Pantoea* and *Pseudomonas-Acinetobacter* genera, with bioreporter *pSB401* and *pSB1142*, respectively. In TLC test, migration patterns showed high similarity to synthetic molecules OHHL and





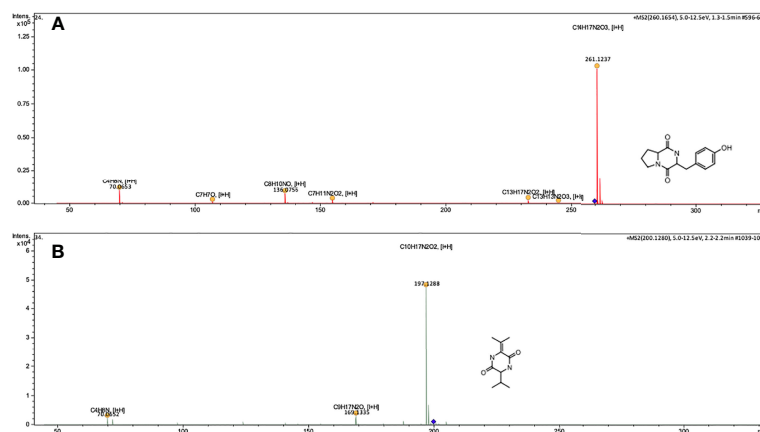
**FIGURE 2** | Chromatogram of total ions and mass spectra consistent with AHLs and AHQs molecules, obtained from the supernatant extract of *P. ananatis* (Isolate 140), using UHPLC-MRM-MS/MS. **(A)** Total ions chromatogram, **(B)** Mass spectra of HHL molecule and HHL lactone ring, C<sub>10</sub>H<sub>17</sub>NO<sub>2</sub> [M+H]<sup>+</sup>=200.1280, Fragments [I+H]<sup>+</sup>= 182.1174, 101.0546, 71.0855, **(C)** Mass spectra of OHHL molecule, C<sub>10</sub>H<sub>16</sub>NO<sub>4</sub>, [M+H]<sup>+</sup>= 214.1074, Fragments [I+H]<sup>+</sup>= 186.1125, 113.0593, 102.0546, 71.0491, 56.0496, **(D)** Mass spectra of OdDHL molecule, C<sub>16</sub>H<sub>28</sub>NO<sub>4</sub>, [M+H]<sup>+</sup>= 298.2011 **(E)** Mass spectra of PQS molecule, C<sub>16</sub>H<sub>22</sub>NO<sub>2</sub>, [M+H]<sup>+</sup>= 260.1650. Impact II ESI-QTOF Bruker® equipment, Reprosil-Pur Basic C18 column, DR Maisch brand.

HHL. In addition to this, TLS test consistently detected HHQ molecule and signals with migration patterns close to the migration value (*R<sub>f</sub>*) of PQS, using *PqsA-Lux* reporter.

AHQ biosensor provided a simple, rapid, and sensitive means to detect QS molecules, without requiring the use of any sophisticated instrumentation. In many cases, it has been observed that the detection, identification, quantification, and migration patterns of AHL and PQS are usually inaccurate, and the sensitivity is generally lower than the expected (10). It has been previously reported that TLC could possibly assist in obtaining masked results (molecules of same *R<sub>f</sub>* but different identity), which is attributed to polar nature of AHL.

In particular, it could detect the presence of diketopiperazines or non-AHL type components that interfere or activate biosensors response (37, 38). However, it is also necessary to indicate that a lower concentration of control compounds would allow better visualization of the spots. This is a potential limitation of the present study.

Thus, significant care should be taken while interpreting the data obtained using AHLs biosensors, owing to aforementioned limitations. In the view of the above, the use of more than one bioreporter and standard synthetic QS molecules would assist in achieving a better profile from environmental isolates (10), as developed/observed in this study. At the same time, TLC could



**FIGURE 3** | Mass spectra consistent with Diketopiperazines-DCPs type molecules, obtained from the supernatant extract of *P. ananatis* (Isolate 140), using UHPLC-MRM-MS/MS. **(A)** Mass spectra of the molecule Cyclo (L-Pro-L-Tyr), C14H16N2O3, [M+H]=261.1234, **(B)** Mass spectra of molecule Cyclo (ΔAla-L-Val), C10H16N2O2, [M+H]= 197.1284. Impact II ESI-QTOF Bruker® equipment, Reprosil-Pur Basic C18 column, DR Maisch brand.

also help to observe the development of organic reactions, ensure the purity of the evaluated products, and determine the different types of AHL and PQS molecules (39).

As reported in most of the studies, the detection of QS signal molecules in the supernatant was higher as compared to the cells for most of the gram-negative bacteria (*E. cancerogenus*, *P. putida*, *E. aerogenes*, *A. calcoaceticus*, *P. otitidis*, *E. cloacae*, *P. ananatis*, and *P. aeruginosa*) isolated from *Pi. evansi*. This might be attributed to the ability of bacteria to modulate their cellular communication using diffusible signal molecules in the extracellular content. The concentrations of these molecules are strictly related to the density of microorganisms or surrounding cells (40). This process of massive generation (threshold value) of signal molecules in the supernatant by gram-negative bacteria is important because it facilitates the increase in the activity of QS-regulated genes, stimulates the differential production of AHLs-AHQs, and induces biofilm formation. In addition to this, it can even improve the secretion of extracellular polymeric substances (41), which in the case of insects vector could play a decisive role in either favoring or inhibiting the establishment and development of parasites or viruses.

A greater number of QS signal molecules were also detected in the intracellular content using *pSB401* and *pSB1142* biosensors, which were mainly associated with *Enterobacter* and *Pseudomonas* genera. In general, QS signal molecules are synthesized and released into the environment. Although further studies are necessary, the findings of the present study might indicate the presence of these molecules at the level of bacterial cell envelope. In fact, their presence might be potentially associated with membrane vesicles to later disseminate these molecules to the extracellular content, as demonstrated in *Pseudomonas* and *Vibrio harveyi* (42–44). It has been previously reported that PQS type molecules of *Pseudomonas* are required for membrane vesicles formation (45). In addition to this, it has been described that some QS signal molecules are hydrophobic and probably cannot diffuse (43). The occurrence

of this type of molecules in gram-negative bacteria is highly relevant, which might directly influence cell signaling and intra-specific and inter-specific gene regulation (46, 47).

Definitive chemical analysis was done using UHPLC-MRM-MS/MS, which is useful for separation and fragmentation of AHLs and AHQs by structural analysis. This method then contemplated mass spectrometry (MS/MS) of the same extracts, and other coupled approaches, such as the mode MRM (Multiple Reaction Monitoring), were further used to generate the identity of greater precision. The supernatant extracts from *P. putida*, *E. aerogenes*, *P. otitidis*, and *P. ananatis* showed the highest concentration of QS signal molecules, particularly OHHL, HHL, HHQ, and PQS, as indicated by the areas and retention times obtained by HPLC, which were similar to those previously established in the chromatograms of synthetic controls. These HPLC findings confirmed the detection of QS signal molecules in the isolates obtained from *Pi. evansi*. Although the results of UHPLC-MRM-MS/MS confirmed the presence of these molecules only in *P. putida*, *E. aerogenes*, *P. otitidis*, and *P. ananatis*, the results obtained for QS signal molecules detected in the rest of the bacterial strains, using TLC, should be considered important for the sensitivity of the biosensors and their specificity, for which an adequate reproducibility of each evaluated extract was obtained.

It is possible that before conducting UHPLC-MRM-MS/MS analysis, QS signal molecules were inhibited or degraded in some of the extracts, possibly by the action of antimicrobial components naturally produced by the bacteria, which are endowed with the potential to exert bacteriostatic or bactericidal effects to reduce cell density. Importantly, cell density is known to be the main stimulus for QS signal molecules production. In addition to this, the interference in the production of these molecules could be due to the presence of diketopiperazines (48). Besides these, two functional groups of AHLs-degrading enzymes have been reported, which include AHL-lactonase that catalyzes the opening of the lactone ring and AHL-acylase that hydrolyzes the amide bond (49).

The results for TLC and HPLC analyses for *P. ananatis* encouraged its selection for in-depth analysis of UHPLC-MRM-MS/MS. In particular, it presented the greatest variety of QS signal molecules, as assessed by TLC. In fact, *P. ananatis* exhibited the best concentration of QSM, as estimated by HPLC. Besides this, it is known to have endosymbiotic association with blood-sucking insects like *Pi. evansi*, and exhibits wide biotechnological potential. Interestingly, *P. ananatis* supernatant sample and UHPLC-MRM-MS/MS analysis allowed consolidated identification of these molecules, which included HHL, OHHL, OdDHL, and PQS. Previous studies provided evidences for the relationship between these molecules and expression of genes involved in iron transport, metabolism, and oxidative stress in insects (31), detoxification, and induction of the synthesis of a surfactant necessary for “Swarming” bacterial displacement (50, 51). In addition to this, these molecules influence the production of extracellular enzymes (proteases) and increase the virulence of bacteria (52, 53), which might be important for the survival of *Pi. evansi* and establishment of pathogens that develop in the digestive tract.

Specifically, OHHL molecule is known to play an important role in the pathogenesis and regulation of genes in insects of agricultural importance, which allow better adaptation to different ecological niches (53). OHHL is freely diffusible, and it modulates the expressions of genes involved in the transport and storage of metal ions (iron metabolism). At the same time, it is very effective in activating bioluminescence at low concentrations and acts as a determinant of autoinduction mechanisms of genes that condition the production of signals for the increase of bacterial populations in growth media (54). Few studies reported that OHHL increased the expression of genes involved in the production of iron siderophores and bacterioferritin, which have been shown to play a role in decrease the oxidative stress (54). Pontes and co-workers (2008) reported that OHHL increased the expression of a large number of genes in *Sodalis glossinidius* that are known to be involved in the cellular response to oxidative stress (34). This oxidative stress response is likely mandated under conditions of dense intracellular symbiont infection, when intense metabolic activity is expected to generate a heavy oxidative burden (34). Many bacteria are known to undergo an “adaptive response” when challenged with a sublethal dose of ROS. Thus, it appears that symbionts and host cells work together to modulate their gene expression profiles and metabolic activities to minimize the deleterious effects of oxidative stress encountered during their symbiotic interactions.

It is important to highlight the frequent detection of OdDHL molecule in most of the gram-negative bacteria. This molecule has been previously shown to be involved in modulating the activation of genes that are involved in the interaction of proteins of the host immune system with the membrane proteins of bacteria. Additionally, this molecule plays a decisive role in parasite–insect–microbiota relationship. However, this hypothesis still needs to be validated in *Pi. evansi*. HHL molecule is relevant to stimulate the production of natural pigments, such as violacein in *Chromobacterium violaceum*,

which has different biological properties against fungi, bacteria and viruses (55).

One of the most relevant findings is perhaps the identification of PQS, mainly due to the physiology and homeostasis participation of gram-negative bacteria with *Pi. evansi*. Recent data suggested that this molecule might also be involved in mediating the acquisition of iron, cytotoxicity, and biogenesis of external vesicles of membranes. Additionally, it might exert modulating activities on the host’s immune response (56). Furthermore, PQS not only affects cells by altering the transcriptional profiles of genes, but it can also bind directly to hundreds of proteins present in the host surroundings, thus PQS can directly interact with several key virulence pathways (56, 57). Many of these compounds (PQS) have been previously isolated for their antibiotic, antifungal, and anti-algal activity against human, plant, and animal pathogens (58). Several previous studies have also explored the antimalarial potential of alkylquinolones (59–61). In particular, the compounds were isolated from different strains of *P. aeruginosa*, which inhibited the growth *P. falciparum* (58). These studies suggested that PQS is a good candidate for designing biological control applications associated with diseases, such as leishmaniasis.

The present study also highlighted the discovery of a different family of signaling compounds, diketopiperazines (DKPs), which were initially discovered in cell-free supernatants for genus *Pseudomonas*. DKPs are naturally occurring cyclic dipeptides that have been isolated from yeasts, lichens, fungi, bacteria, and marine sponges. Molecular characterization by UHPLC-MRM-MS/MS facilitated the identification of DKPs cyclo(L-Pro-L-Tyr) and cyclo( $\Delta$ Ala-L-Val) in the extract of free supernatant obtained from *P. ananatis*. These DKPs could activate biosensors on TLC assays, underscoring and suggesting the importance of chemical characterization of molecules identified in the bioassay (62, 63). DKPs naturally show a wide range of antimicrobial, antiviral, antitumor, and immunosuppressive activities (55, 63). In a previous study, 64 reported insecticidal activity of DPKs in Diptera, specifically in *Ceratitis capitata* (64).

*P. ananatis* has been widely explored, especially for being an opportunistic pathogen and causing disease symptoms in a wide range of crops and forest species of global economic importance. However, few recent studies reported leishmanicidal activity against *Le. infantum* and *Le. mexicana* (24, 26). Some studies reported the presence of *P. agglomerans* and *P. ananatis* in sandflies (*Phlebotomus kandelakii*, *Phlebotomus papatasi*, *Pi. evansi*, and *Lu. longipalpis*) and mosquitoes (*Aedes albopictus* and *Anopheles stephensi*), suggesting a possible effect on the *Leishmania* development stages. The relationships of *P. ananatis* with other microorganisms and with the host have been previously studied using various *in vitro* and *in silico* approaches, which showed that they perform a variety of functions, including transportation, metabolism, and defense, and are also involved in the generation of specific associated molecules. Altogether, these QSM findings might suggest that these molecules could impact cell density and molecular signaling in various processes, such as biofilm formation,

interaction with the host, or production of antibiotics and secondary metabolites in the gut of *Pi. evansi*.

In conclusion, the present study reported the identification of QSM molecules, including HHL, OHHL, OdDHL, and PQS. Interestingly, the study also identified diketopiperazines, cyclo(L-Pro-L-Tyr) and cyclo( $\Delta$ Ala-L-Val), in *P. ananatis*. The characterization of these molecules by bioassays (TLC), use of behavioral biosensors to respond to the stimulation of specific auto inductors, and the implementation of HPLC-MRM allowed the detection (65), characterization, and identification of QSM from gram-negative bacteria isolated from insects. It is necessary to deepen the understanding regarding the detected QSM on the establishment of *Leishmania* in *Pi. evansi*, and explore its biotechnological potential to stimulate the production of natural compounds of great pharmaceutical, entomopathogenic, and even industrial interest.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

RV-G, CXMH, GC-R, SR: Designed the study and contributed to writing the manuscript. RV-G, GB-M, and JH-C: Performed the experiments, analyzed the data, and contributed to writing the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ftd.2021.760228/full#supplementary-material>

**Supplementary Figure 1** | AHL and AHQ molecules were detected by bioluminescence signals in the supernatant of Gram-negative strains isolated from the intestinal tract of *Pintomyia evansi* using Thin Layer Chromatography-TLC methodology. **(A)** Bioluminescence signals with biosensor *pSB401* Line 1: OHHL standard; Line 2: HHL standard; Line 3: CFSs of *P. otitidis*; Line 4: CFS of *E. cloacae*; Line 5: CFSs of *P. ananatis*. **(B)** Bioluminescence signals with biosensor *pSB401* Line 1: OHHL standard; Line 2: HHL standard; Line 6: CFS of *E. cancerogenus*; Line 7: *P. putida*; Line 8: *E. aerogenes*. **(C)** Bioluminescence signals with biosensor *pSB1142* Line 9: OdDHL Standard; Line 10: CFS of *Lysobacter soli*; Line 11: CFS of *P. putida*; Line 12: CFS of *A. calcoaceticus*. **(D)** Bioluminescence signals with biosensor *PqsA-Lux*. PQS Standard; HHQ Standard; Line 18: CFS of *E. cancerogenus*; Line 19: CFSs of *P. otitidis*; Line 20: CFS of *A. gyllenbergii*; Line 21: CFS of *P. aeruginosa*.

**Supplementary Figure 2** | HPLC Chromatogram of synthetic QSM **(A)** with the mixture of synthetic QSM used as controls, **(B)** retention times and estimated heights from individual chromatography.

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