LEONARDO LUIZ DE FREITAS

PHENOTYPES REGULATED BY *N*-ACYL HOMOSERINE LACTONES IN *Salmonella enterica*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientadora: Maria Cristina Dantas Vanetti

Coorientadores: Denise Mara Soares Bazzolli
Gustavo Ferreira Martins
Leandro Licursi de Oliveira

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Assentimento:

Leonardo Luiz de Freitas
Autor

Maria Cristina Dantas Vanetti
Orientadora

Dedico
À minha família e amigos.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.

(Madre Teresa de Calcutá)

RESUMO

FREITAS, Leonardo Luiz de, D.Sc., Universidade Federal de Viçosa, abril de 2021. **Fenótipos regulados por N-acil homoserina lactonas em *Salmonella enterica*.** Orientadora: Maria Cristina Dantas Vanetti. Coorientadores: Denise Mara Soares Bazzolli, Gustavo Ferreira Martins e Leandro Licursi de Oliveira.

Quorum sensing (QS) é um sistema de comunicação bacteriano mediado por moléculas químicas sinalizadoras que permitem a uma comunidade microbiana regular um conjunto de genes de forma coordenada. Diferentes mecanismos de comunicação célula-célula têm sido descritos em bactérias que infectam humanos, incluindo *Salmonella*. Nesse patógeno, o QS mediado pelo autoindutor 1 (AI-1), também conhecido como acil homoserina lactonas (AHLs), é incompleto em razão da ausência de proteínas (LuxI ou homóloga) responsáveis pela síntese dessas moléculas. Entretanto, *Salmonella* possui a proteína SdiA, homóloga a proteína LuxR, que permite reconhecer e responder às AHLs sintetizadas por outras bactérias ou adicionadas ao meio de cultura. A elucidação dos mecanismos de virulência de *Salmonella* regulados por AHLs poderá contribuir para o desenvolvimento de estratégias de controle desse patógeno. Assim, os objetivos do presente estudo foram investigar a função da molécula sinalizadora de quórum, *N*-dodecanoil homoserina lactona (C12-HSL), na regulação de genes de virulência e de reposta geral ao estresse, na resistência à bacteriocina nisina e ao estresse ácido, bem como avaliar a influência de C12-HSL na virulência de *Salmonella* Enteritidis PT4, usando larvas de *Galleria mellonella*. Nesse estudo, também foi avaliado o efeito de extratos do meio de cultura de *Rahnella inusitata* contendo AHLs na adesão e motilidade de *Salmonella*. Células de *Salmonella* cultivadas em anaerobiose a 37 °C na presença de 50 nM de C12-HSL aumentou a expressão dos genes *phoP*, *phoQ*, *pmrA*, e *pmrB* que estão diretamente envolvidos com a resistência a estresses, como também aumentou a resistência a nisina e ao estresse ácido. Alterações em ácidos graxos da membrana celular e na carga da superfície celular podem ter contribuído para a sobrevivência do patógeno em condições de estresses e na redução do extravazamento de NADPH e íons potássio. O cultivo na presença de C12-HSL também aumentou a expressão dos genes *rpoS*, *arcA*, *arcB*, e *invA* e a virulência de *Salmonella* inoculada na hemolinfa de *G. mellonella*, resultando em maior mortalidade das larvas, além de aumentar a sobrevivência de *Salmonella* na hemolinfa e dentro dos hemócitos. *G. mellonella* inoculada com *Salmonella* cultivada na presença de C12-HSL aumentou a produção de pigmento, óxido nítrico, enzimas antioxidantes, caspases-3 e LC3 nesse modelo de infecção. A

presença de extratos de *R. inusitata* contendo AHLs aumentou a adesão de *Salmonella* em cupons de aço inoxidável e a motilidade. As evidências demonstradas de que maior resistência a estresses e virulência em *Salmonella* Enteritidis PT4 são regulados por AHLs reforçam a necessidade de compreender profundamente a função dessas moléculas sinalizadoras neste patógeno, considerando sua importância como agente de doenças de origem alimentar e responsável por milhares de mortes anualmente.

Palavras-chave: *Salmonella*. Quorum sensing. N-acil homoserina lactonas. Virulência. Resistência a estresses.

ABSTRACT

FREITAS, Leonardo Luiz de, D.Sc., Universidade Federal de Viçosa, April, 2021. **Phenotypes regulated by *N*-acyl homoserine lactones in *Salmonella enterica*.** Adviser: Maria Cristina Dantas Vanetti. Co-Advisers: Denise Mara Soares Bazzolli, Gustavo Ferreira Martins and Leandro Licursi de Oliveira.

Quorum sensing (QS) is a bacterial communication system mediated by signaling molecules that allow a microbial community to regulate a set of genes in a coordinated way. Different mechanisms of cell-cell communication have been described in bacteria that infect humans, including *Salmonella*. In this pathogen, the QS mediated by autoinducer 1 (AI-1), also known as acyl homoserine lactones (AHLs), is incomplete due to the absence of proteins (LuxI or homologous) responsible for the synthesis of these molecules. However, *Salmonella* has the SdiA protein, homologous to the LuxR protein, which allows it to recognize and respond to AHLs synthesized by other bacteria or added to the culture medium. The elucidation of *Salmonella* virulence mechanisms regulated by AHLs may contribute to the development of strategies to control this pathogen. Thus, the aims of the present study were to investigate the function of the quorum signaling molecule, *N*-dodecanoyl homoserine lactone (C12-HSL), in regulating virulence genes and general stress response, resistance to nisin bacteriocin and acid stress, as well as to evaluate the influence of C12-HSL on the virulence of *Salmonella* Enteritidis PT4, using *Galleria mellonella* larvae. In this study, the effect of extracts from the culture medium of *Rahnella inusitata* containing AHLs on the adhesion and motility of *Salmonella* was also evaluated. *Salmonella* cells cultured in anaerobiosis at 37 °C in the presence of 50 nM C12-HSL increased the expression of the *phoP*, *phoQ*, *pmrA*, and *pmrB* genes that are directly involved with stress resistance, as well as increased nisin resistance and to acid stress. Changes in cell membrane fatty acids and cell surface charge may have contributed to the survival of the pathogen under stress conditions and in the reduction of leakage of NADPH and potassium ions. Cultivation in the presence of C12-HSL also increased the expression of the *rpoS*, *arcA*, *arcB*, and *invA* genes and the virulence of *Salmonella* inoculated in the *G. mellonella* hemolymph, resulting in higher larval mortality, in addition to increasing *Salmonella* survival in the hemolymph and within the hemocytes. *G. mellonella* inoculated with *Salmonella* grown in the presence of C12-HSL increased the production of pigment, nitric oxide, antioxidant enzymes, caspases-3 and LC3 in this model of infection. The presence of *R. inusitata* extracts containing AHLs increased the adhesion of *Salmonella* in

stainless steel coupons and motility. The demonstrated evidence that greater resistance to stress and virulence in *Salmonella* Enteritidis PT4 are regulated by AHLs reinforce the need to deeply understand the function of these signaling molecules in this pathogen, considering its importance as an agent of foodborne diseases and responsible for thousands of deaths annually.

Keywords: *Salmonella*. Quorum sensing. *N*-acyl homoserine lactones. Virulence. Stress resistance.

SUMÁRIO

GENERAL INTRODUCTION

Quorum sensing (QS) is a bacterial molecular communication system that allows the regulation of gene expression in response to changes in population density, contributing to a coordinated organization of the microbial community. During growth, the bacteria synthesize signaling molecules called autoinducers (AIs). These Als are released into the surrounding medium and when it reaches a high concentration in the external environment, they bind to specific receptors within the cell and simultaneously, regulate the differential genes expression. The capacity to behave collectively as a group has obvious advantages, as the ability to migrate to a more suitable environment or to a better nutrient supply and to adopt new modes of growth, such as biofilm formation, entry in dormancy state, as spores which may afford protection from deleterious environments.

Bacterial language is chemical in nature, and a large number of bacteria use this cell-to-cell communication to organize their collective behavior in diverse environments. In pathogens, the AIs are used for communication during the colonization process to overcome the barriers found in the host. Currently, a wide variety of AIs is described in the literature, but there are four well-defined classes: (i) autoinducer-1 (AI-1), named *N*-acyl homoserine lactones (AHLs), used for communication by Gram-negative bacteria; (ii) autoinducer-2 (AI-2), which are a group of molecules produced and used by both Gram-positive and Gram-negative bacteria; (iii); autoinducer-3 (AI-3), used for inter-kington communication and (iv) autoinducing peptides (AIPs), used for communication by Gram-positive bacteria. Therefore, different signaling molecules are produced by bacteria for communicate, adapt and survival in stressful niches.

The Al-1 are fatty acid derivatives produced by an AHL synthase from the substrates *S*-adenosyl-L-methionine (SAM) and an acylated acyl carrier protein (acyl-ACP). Two proteins belonging to the LuxI-LuxR families are required for the AHL-mediated QS. The LuxI or

homologous proteins are responsible for the synthesis of AHLs, while LuxR or homologous proteins are transcriptional regulators that bind to AHLs and regulates the expression of targeted genes. However, some phenotypes are also regulated in pathogens that do not produce AHL, such as *Escherichia coli* O157: H7 and *Salmonella*. These pathogens do not have an AHL synthase but can detect AHLs in the environment because they have a protein called SdiA, a LuxR homolog. In *Salmonella,* some phenotypes are identified to be regulated by exogenous AHLs. For example, the expression of *rck* (resistant to complement killing) and *srgE* genes of *Salmonella* Typhimurium and invasion in epithelial cell is increased by exogenous AHLs. Also, the presence of *N*-dodecanoyl-homoserine lactone (C12-HSL) increased the expression of genes related to the formation of biofilm and virulence as well as altered the protein profile in *Salmonella* Enteritidis. The addition of C12-HSL in the culture medium affected important metabolic processes for *Salmonella* Enteritidis and promoted a greater abundance of proteins involved with acid and oxidative stress. Molecular docking analysis showed that C12-HSL has the highest binding affinity with the Sdia protein of *Salmonella* when compared to other smaller chain AHLs. Intriguingly, twelve-carbon AHLs have been found in human feces, suggesting that *Salmonella* can detect these signaling molecules in the human intestine and activate numerous virulence factors to achieve a successful infection. However, more studies need to be carried out to elucidate this hypothesis.

Thus, understanding the resistance and pathogenicity phenotypes regulated by the QS is crucial for the development of a control strategy for *Salmonella* and other pathogens. Considering the importance of *Salmonella* as a frequent foodborne pathogen that causes gastrointestinal diseases, and complications that can lead to death, reducing its virulence is of interest and of high impact in public health. Estimates show that in 2017 more than 500,000 cases of *Salmonella* infection occurred, with a prevalence of serovars Typhimurium and Enteritidis, and 77,500 deaths. Thus, this study aimed to evaluate the influence of C12-HSL

and extracts containing AHLs on resistance and virulence phenotypes of *Salmonella* Enteritidis PT4.

CHAPTER 1

Nisin and acid resistance in *Salmonella* is enhanced by *N*-dodecanoyl-homoserine lactone

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Nisin and acid resistance in *Salmonella* is enhanced by *N*-dodecanoyl-homoserine lactone

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ABSTRACT

Salmonella is a foodborne pathogen that can develop resistance to different stresses, which is essential for successful infection of the host. Some genes directly related to acid resistance are also involved in cationic peptide resistance in Gram-negative bacteria and could be under the control of quorum sensing (QS) mediated by autoinducer 1, known as acyl-homoserine lactone. Here, we investigated the influence of autoinducer 1, *N*-dodecanoyl-homoserine lactone (C12-HSL) on the resistance of *Salmonella enterica* subspecies *enterica* serovar Enteritidis to nisin and acid stress. *Salmonella* cells growing in anaerobic tryptic soy agar (TSB) at a pH of 7.0 for 7 h were submitted to acid stress at a pH of 4.5 in the presence and absence of nisin and were either supplemented or not with C12-HSL. Viable cell counts, gene expression, membrane charge alterations, fatty acid composition, and intracellular content leakage were observed. The autoinducer C12-HSL increased nisin resistance and survival at a pH of 4.5 in *Salmonella*. Also, C12-HSL increased the expression of the genes, *phoP*, *phoQ*, *pmrA,* and *pmrB*, which are involved with antimicrobial and acid resistance. The positive charge on the cell surface and concentration of cyclopropane fatty acid of the cellular membrane were increased in the presence of C12-HSL under acidic conditions, whereas membrane fluidity decreased. The loss of K^+ and NADPH, promoted by nisin, was reduced in the presence of C12-HSL at a pH of 4.5. Taken together, these findings suggest that quorum sensing plays an important role in enhanced nisin and acid resistance in *Salmonella.*

1. Introduction

Salmonella is a food and waterborne pathogen responsible for outbreaks worldwide. *Salmonella* can survive during passage through the stomach and persist inside the acidic vacuolar environment of defense cells and endure the presence of cationic antimicrobial peptides [1,2]. Resistance to antimicrobial peptides and acidic conditions is directly associated with the ability of these bacteria to express a set of genes to survive in these environments [2,3]. Transcriptome analysis revealed that differentially expressed genes induced by exposure to acid stress can contribute to antimicrobial peptide resistance, as well as affect the virulence of *Salmonella* [4]. The cell to cell communication system, called quorum sensing (QS), can regulate the production of different proteins involved with antimicrobial and acid stress resistance in *Salmonella* [5,6]. QS is a density-dependent mechanism controlled via autoinducer (AI) molecules that are used for cell signaling and gene regulation [7]. In Gram-negative bacteria, the most studied mechanism of QS is mediated by the AI-1, namely acyl-homoserine lactone (AHL). This system relies on signal molecules, AHLs, an AHL synthase (LuxI), and a transcriptional regulator (LuxR) [8]. In *Salmonella*, this mechanism is incomplete because it is not able to synthesize its own AHL, but contains a homolog of LuxR, known as SdiA. Thus, *Salmonella* can regulate gene expression by detecting AHLs produced by other Gram-negative bacteria [9].

The *Salmonella* SdiA protein regulates the *rck* operon, which is associated with resistance to the complement system and adhesion and invasion of epithelial cells [10]. Moreover, there is evidence that SdiA when linked to *N*-dodecanoyl-DL-homoserine lactone (C12-HSL) decreases the abundance of outer membrane proteins (OMPs), such as OmpA, OmpC, and OmpD, while the abundance of Adi increases [5]. Adi is an important protein related to the survival of *Salmonella* in acidic conditions [11]. The abundance of proteins, such as TolC and PhoP, also increases when *Salmonella* is cultivated in the presence of C12-HSL under anaerobic conditions [6]. In addition to their relationship with resistance to an acidic environment, these proteins also participate in antimicrobial resistance [12–15]. Genes related to biofilm formation are up-regulated in the presence of C12-HSL [16], which is another strategy used by bacteria to resist antimicrobial agents [17].

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Resistance of pathogens to antibiotics has become a major health concern. The US Center for Disease Control and Prevention (CDC) has estimated that over two million people are infected with antibiotic-resistant bacteria and at least 35,000 people die annually [18]. Therefore, there is great interest in the search for non-antibiotic antimicrobial agents [19]. Bacteriocins are widely studied antimicrobial agents and have a promising future in the control of bacterial infections [20]. Nisin is a cationic bacteriocin used as an additive in the food industry of over 50 countries and displays a wide spectrum of activity against several food and waterborne bacteria. This bacteriocin uses lipid II as a docking molecule to facilitate the formation of pores in the cell membrane, resulting in the dissipation of membrane potential, a loss of electrolytes and other metabolites and, ultimately, cell death. Additionally, this bacteriocin inhibits cell-wall biosynthesis, by binding to lipid II, an essential cell-wall precursor [21]. Gram-negative bacteria are slightly more resistant to nisin due to the outer membrane, which acts as a protective barrier for the cell [22,23]. In *Salmonella*, this bacteriocin is more effective when associated with other techniques, such as a low pH [24,25]. Nevertheless, the role of QS in antimicrobial peptide and acid resistance remains unknown in *Salmonella*. Therefore, this study aimed to evaluate the influence of C12-HSL on the resistance of *Salmonella* to nisin and acid stress.

2. Material and methods

2.1. Bacterial strain

Stock culture of *Salmonella enterica* subspecies *enterica* serovar Enteritidis phage type 4 (PT4) 578 (GenBank: 16S ribosomal RNA gene - MF066708.1), isolated from chicken meat (FIOCRUZ, Rio de Janeiro, Brazil) in Tryptic Soy Broth (TSB; Sigma-Aldrich, India) supplemented with 20% (v/v) sterilized glycerol was maintained at −20 °C.

2.2. Preparation of nisin

The stock solution of nisin (2.5%, Nisaplin®, Danisco Brazil) was prepared in phosphate-buffered saline (PBS, 10 mM, pH 2.0). The final stock concentration was 1500 μM and was stored in the refrigerator (4 °C) until use.

2.3. Preparation of inoculum and cultivation with C12-HSL

Salmonella Enteritidis PT4 578 was cultivated in anaerobiosis [6]. The TSB was prepared under O_2-free conditions by bubbling the medium with CO_2 (anoxic gas) to expel all O_2. The medium was dispensed in bottles, sealed with butyl rubber stoppers and sterilized by autoclaving (anaerobic TSB). The inoculation was done with syringes, which pierced the lids, but there is no entry of O_2. Briefly, *Salmonella* was incubated in 10 mL of anaerobic TSB for 4 h at 37 °C to reach the exponential phase. Next, the cells were harvested by centrifugation, and the pellet was washed and resuspended with anaerobic PBS (10 mM, pH 7.2). The inoculum was standardized to 0.1 optical density at 600 nm (OD_{600nm}) using a spectrophotometer (Thermo Fisher Scientific, Finland). Then, 10 mL of anaerobic TSB (pH 7.0) was inoculated with 1 mL (10^7 CFU/mL) and supplemented with C12-HSL (PubChem CID: 11565426; Fluka, Switzerland) at a final concentration of 50 nM. A control without C12-HSL was also prepared. The confirmation of bacterial load was done by plating on Plate Count Agar (PCA, Himedia, India). The plates were incubated aerobically at 37 °C for 24 h. The bottles were incubated for 7 h at 37 °C and aliquots were collected for analysis of gene expression and the cytochrome *c* binding assay.

2.4. Real-time quantitative PCR (RT-qPCR)

Total RNA of cells grown in the presence or absence of C12-HSL for 7 h was extracted with Trizol® Reagent (Invitrogen, USA) following the procedures described by the manufacturer. Complementary DNA (cDNA) synthesis was performed using the ImProm-II Reverse Transcription kit with a Random Hexamer Primer (Promega, USA). Specific primers for *phoP*, *phoQ*, *pmrA,* and *pmrB* genes were designed using the program GenScript (Table 1). The expression of endogenous genes 16S and *gyrA* were used to normalize the data. RT-qPCR was performed with 20 ng of cDNA and Sybr Green I Master Mix (Promega, USA) in 96-well plates using a Bio-Rad C1000 Thermal Cycler. The relative standard curve was used to calculate the relative quantity (Rq) of each sample for each gene as previously described [26].

Table 1
Primers used in this study.

Primer	Oligonucleotide sequences (5′–3′)
*phoP*F	TCACTGCCGGTTCTGGTGTT
*phoP*R	GATCACCTGGGAGGCCAGAC
*phoQ*F	CACGCTGTTGAGCGAAGACC
*phoQ*R	ACGAACCAGCTCCACACCAT
*pmrA*F	ACCGAAGGCTATGCGTGTGA
*pmrA*R	TCGGGCAGCCCTAAATCCAG
*pmrB*F	TAGCGTCTCCCAGCTTCTGC
*pmrB*R	CGCGTTTCCAGCATGGTGTT
*gyrA*F	ATCGCCGAGCTGGTGAAAGA
*gyrA*R	ACCTGTAGCTGGGTCTGGGA
*16S*F	GCATTCGAAACTGGCAGGCT
*16S*R	CCTTCGCCACCGGTATTCCT

2.5. Determination of nisin and acid resistance

Cells cultured in the presence of C12-HSL were standardized to an OD_{600nm} of 0.1 and were used to inoculate anaerobic TSB with a pH of 4.5 acidified with 5 M HCl, (Vetec, Brazil) supplemented with 50 nM C12-HSL and 50 μM nisin (C12-HSL + nisin).

The control included cells growing in the absence of C12-HSL but exposed to 50 μM nisin at a pH of 4.5. To determine acid resistance, cells growing in the presence of C12-HSL were used to inoculate anaerobic TSB with a pH of 4.5 containing 50 nM C12-HSL. Cells growing in media without the signal molecule were used as controls and were used to inoculate anaerobic TBS (pH 4.5) without C12-HSL. The bottles were incubated at 37 °C and the number of viable cells was assessed at 0, 1, 2, 4, 6, and 7 h by the drop plate method [27] on PCA. The plates were incubated aerobically at 37 °C for 8–24 h. At 2 and 7 h, aliquots (10 mL each) were collected for analysis of cytochrome *c* binding, fatty acids, and leakage of intracellular content. All experiments were repeated three times.

2.6. Cytochrome c binding assay

Cytochrome *c* binding was performed as previously described [28] with modifications. After sample collection, cells were centrifuged at 2500 *g* for 10 min. The cells were washed twice in morpholinepropanesulfonic acid (MOPS) buffer (20 mM, pH 7.0), concentrated to a final OD_{578} of 7.0 and incubated with 0.5 mg/mL cytochrome *c* (95% purity; Sigma Chemicals, USA) for 15 min. Cell suspensions were centrifuged at 10,000 *g* for 5 min and the cytochrome *c* remaining in the supernatant was determined at a wavelength of 530 nm.

2.7. Fatty acid profile analysis

Aliquots of *Salmonella* cultures were collected and centrifuged at 5000 *g* at 4 °C for 15 min (Sorvall, USA). The pellet was resuspended in 1 mL of sterilized distilled water and centrifuged again. The pellet was freeze-dried and transferred to glass tubes free of fatty acids and then, the fatty acids were saponified, methylated, extracted, and identified by the procedure from the Sherlock® Analysis Manual (version 6.2; MIDI, USA).

2.8. Analysis of intracellular content leakage

Samples collected for analysis of K^+ and NADPH leakage were centrifuged at 2500 *g* at 4 °C for 10 min. The supernatant was filtered on membranes with 0.22 μm pores (Millipore®, Merck, Germany) and the filtrate was used to analyze. The extracellular K^+ concentration was determined in a flame photometer (Corning, UK) and the concentrations of NADPH were estimated with a spectrophotometer [29].

2.9. Statistic analyses

The Student's t-test was used to evaluate statistical differences between *Salmonella* grown in the presence and absence of C12-HSL. Also, analysis of variance (ANOVA) and Tukey's test were used for multiple comparisons. All statistical analyses were performed using GraphPad Prism 5.00 software and the significance level adopted was 5% for all statistical tests.

3. Results

3.1. RT-qPCR

Initially, RT-qPCR analysis was performed to evaluate the expression of *phoP*, *phoQ*, *pmrA,* and *pmrB* in *Salmonella* cells growing in the presence of C12-HSL for 7 h before nisin and acid treatments. The results showed that C12-HSL significantly increased the expression of all genes evaluated (Fig. 1). Increased expression of *phoP* and *phoQ* indicates that QS can prepare the cells for possible acid stress. Furthermore, *phoP*, *phoQ*, *pmrA,* and *pmrB* genes are directly related to antimicrobial peptide resistance.

3.2. C12-HSL increases resistance to nisin and survival to acid stress

To evaluate nisin resistance, *Salmonella* growing with C12-HSL was used to inoculate anaerobic TSB (pH 4.5) plus C12-HSL and nisin (C12-HSL + nisin). Cells growing in the presence of C12-HSL and exposed to nisin at a pH of 4.5 exhibited significantly higher bacteriocin resistance ($P < 0.05$) than those that were cultivated in the absence of C12-HSL (Fig. 2A). The presence of C12-HSL in treatments resulted in 1.2 $\log_{10}$ CFU/mL more bacteria ($P < 0.05$) than the control treatment after 7 h of incubation, without the AI-1 (Fig. 2A). Similarly, *Salmonella* cells cultivated in the presence of C12-HSL for 7 h and subjected to acidic growth condition in O_2-free TSB supplemented with C12-HSL grew more ($P < 0.05$) than the control cells at a pH of 4.5 without C12-HSL (Fig. 2B). The QS molecule, C12-HSL, induces slight resistance to a low pH (4.5) and the maximum population reached 8.1 $\log_{10}$ CFU/mL at the end of the 7 h incubation period. In contrast, cells growing in the absence of C12-HSL achieved a population of 7.4 $\log_{10}$ CFU/mL after the same period of incubation. These results suggest that the QS can affect the survival of *Salmonella* in the presence of the antimicrobial peptide nisin under acid conditions and directly contributes to cationic peptide resistance.

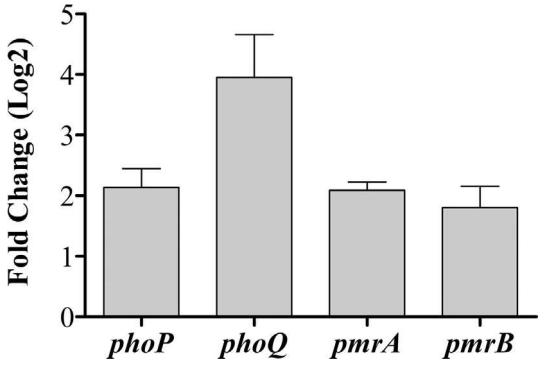

Fig. 1. Gene expression of *Salmonella* growing in the presence of C12-HSL for 7 h at a pH of 7.0 before nisin and acid treatments. Expression values were obtained by log2 of the ratio between cells treated with C12-HSL and the control (without C12-HSL). The expression of significantly regulated genes ($P < 0.05$) is shown. Error bars indicate standard error (n = 3).

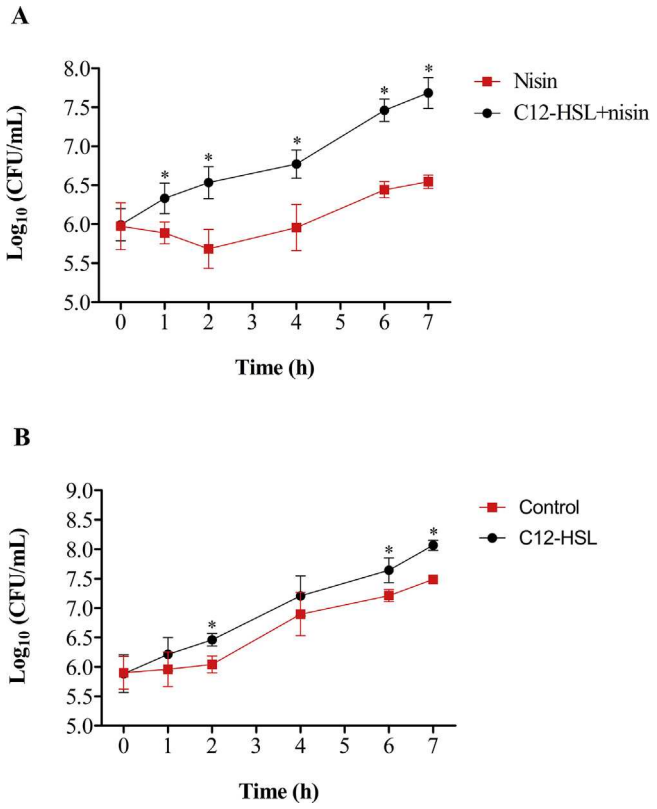

Fig. 2. Survival of *Salmonella* grown at a pH of 4.5 in the presence or absence of C12-HSL and exposed to nisin. Cells were grown in the presence of C12-HSL for 7 h at a pH of 7.0 and then used to inoculate the media (pH 4.5) with (A) nisin alone or C12-HSL + nisin; (B) without C12-HSL (control) or C12-HSL. The asterisk indicates statistically significant differences between treatments at the same sample collection time by the Student's t-test ($P < 0.05$). Error bars indicate standard error (n = 3).

3.3. C12-HSL alters cell membrane charge

To estimate the relative bacterial cell surface charge of *Salmonella,* a cytochrome *c* binding assay was used. The supernatant of the cells cultivated in the presence of C12-HSL for 7 h before nisin and acid treatments presented a higher concentration of cytochrome *c* ($P < 0.05$) in comparison to the control treatment, without C12-HSL (Fig. 3A). This same pattern was observed in the 2 h incubation samples in the supernatant of cells treated with C12-HSL under acid stress conditions (Fig. 3B). However, no statistical difference was observed at 7 h under acidic conditions. These results show that the presence of AI-1 promotes an increase in positive charge density on the cell surface, which might contribute to nisin resistance.

3.4. C12-HSL alters the fatty acid profile

Fatty acids composition analysis was performed to determine if C12-HSL causes membrane changes, which can contribute to nisin and acid stress resistance. The presence of C12-HSL alters the fatty acid composition of *Salmonella* cultivated for 2 and 7 h at a pH of 4.5 (Table 2). The results showed that C12-HSL causes major changes in cyclopropane fatty acids (CFA). At 2 h of treatment, the proportion of fatty acids,

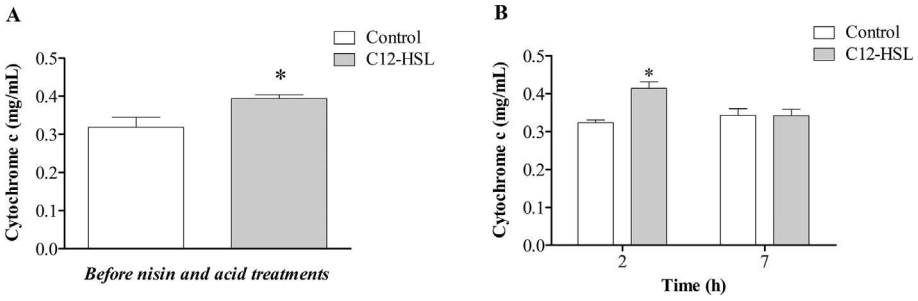

Fig. 3. Cytochrome *c* assay for an estimate of the relative surface charge of the cell membrane. (A) *Salmonella* was growing in the presence of C12-HSL for 7 h at a pH of 7.0 before nisin and acid treatments; (B) then used to inoculate the media (pH 4.5) without C12-HSL (control) or supplemented with C12-HSL. The graph represents the concentration of cytochrome *c* in the supernatant. The asterisk indicates statistically significant differences between treatments at the same sample collection time by the Student's t-test ($P < 0.05$). Error bars indicate standard error (n = 3).

C17:0 and C19:0 cyclo, in cells treated with C12-HSL were significantly higher when compared to cells growing without C12-HSL ($P < 0.05$). Moreover, C12-HSL assists in maintaining a higher 17:0 cyclo fatty acid concentration after 7 h of incubation. Interestingly, a smaller ratio of unsaturated/saturated fatty acids (UFA/SFA) was observed in cells treated with C12-HSL after 2 h at a pH of 4.5 (Fig. 4). Thus, these modifications could help the cell survive in acid stress environments and increase nisin resistance.

3.5. C12-HSL reduces K^+ and NADPH leakage

The main antimicrobial mechanism of nisin in bacterial death is membrane pore formation and intracellular content leakage. Thus, we evaluated K^+ and NADPH leakage in cells submitted to different treatments at a pH of 4.5. The K^+ and NADPH leakage of cells cultivated in the presence or absence of C12-HSL without nisin did not differ statistically at either analyzed time (Fig. 5). Interestingly, the extracellular K^+ and NADPH concentrations were lower in cells cultivated with C12-HSL and exposed to nisin (C12-HSL + nisin) when compared to the treatment with nisin alone (Fig. 5A and B). At 7 h of cultivation, a lower concentration ($P < 0.05$) of K^+ and NADPH was observed in the treatment with the QS molecule in comparison to the control treatment (only nisin). *Salmonella* cells growing in the presence of C12-HSL leaked less of their intracellular content into the environment, suggesting that C12-HSL causes changes in the cell membrane and prevents pore formation by nisin.

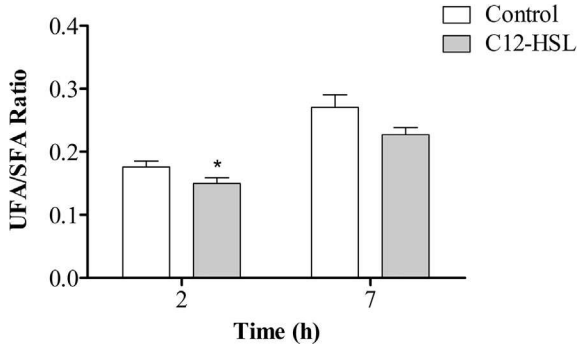

Fig. 4. UFA/SFA ratio of *Salmonella* growing in the presence or absence of C12-HSL at a pH of 4.5. (UFA) unsaturated fatty acids; (SFA) saturated fatty acids. The asterisk indicates statistically significant differences between treatments at the same sample collection time by the Student's t-test ($P < 0.05$). Error bars indicate standard error (n = 3).

4. Discussion

Nisin is an antimicrobial peptide used in the food industry and is a strong candidate for combating bacterial pathogens [20]. However, Gram-negative bacteria can present resistance to cationic antimicrobial peptides through the outer membrane, which acts as a permeability barrier, preventing the entry of antimicrobials, as well as through the regulation of different genes that result in membrane modifications [2,30]. In *Salmonella*, the role of QS in antimicrobial peptide resistance

Table 2
Fatty acids composition of *Salmonella* cultivated in the presence or absence of C12-HSL in pH 4.5.

Fatty acids	Time (h)			
	2		7	
	Control	**C12-HSL**	**Control**	**C12-HSL**
Saturated fatty acid				
C12:0	4.26 ± 0.18	4.37 ± 0.24	4.04 ± 0.20	4.23 ± 0.41
C14:0	8.93 ± 0.19	9.57 ± 0.41	10.03 ± 0.30	11.33 ± 0.99
C16:0	36.84 ± 0.32	35.33 ± 0.70	39.24 ± 0.18	39.01 ± 2.52
C18:0	0.54 ± 0.02	0.56 ± 0.03	0.76 ± 0.03	0.74 ± 0.05
C19:0	0.18 ± 0.05	0.19 ± 0.01	0.74 ± 0.18	0.94 ± 0.14
Monounsaturated				
C16:1	1.63 ± 0.11	1.83 ± 0.16	1.20 ± 0.06	1.22 ± 0.04
C17:1	0.51 ± 0.17	0.39 ± 0.07	0.63 ± 0.09[a]	0.83 ± 0.07[b]
C18:1 ω7c 11-methyl	0.61 ± 0.01	0.55 ± 0.06	0.86 ± 0.12	1.06 ± 0.14
C18:1	5.00 ± 0.13[a]	3.72 ± 0.23[b]	10.57 ± 0.08[a]	8.24 ± 0.04[b]
Polyunsaturated Hydroxy				
C20:2	0.41 ± 0.01	0.39 ± 0.04	0.71 ± 0.13	1.07 ± 0.29
C18:1 2OH	0.61 ± 0.08	0.65 ± 0.03	0.84 ± 0.10	1.01 ± 0.07
Cyclopropane				
C17:0 cyclo	19.42 ± 0.14[a]	21.57 ± 0.06[b]	13.33 ± 0.15[a]	14.98 ± 0.19[b]
C19:0 cyclo	15.65 ± 0.68[a]	18.62 ± 0.14[b]	12.13 ± 0.73	12.93 ± 1.68
Total minor fatty acids	5.39 ± 0.46[a]	2.26 ± 0.12[b]	4.93 ± 0.89[a]	2.41 ± 0.51[b]

Different letters indicate a significant difference between treatments within the same sample collection time, by the Student's t-test ($P < 0.05$). Where a letter is not shown, no statistical difference between treatments was observed.

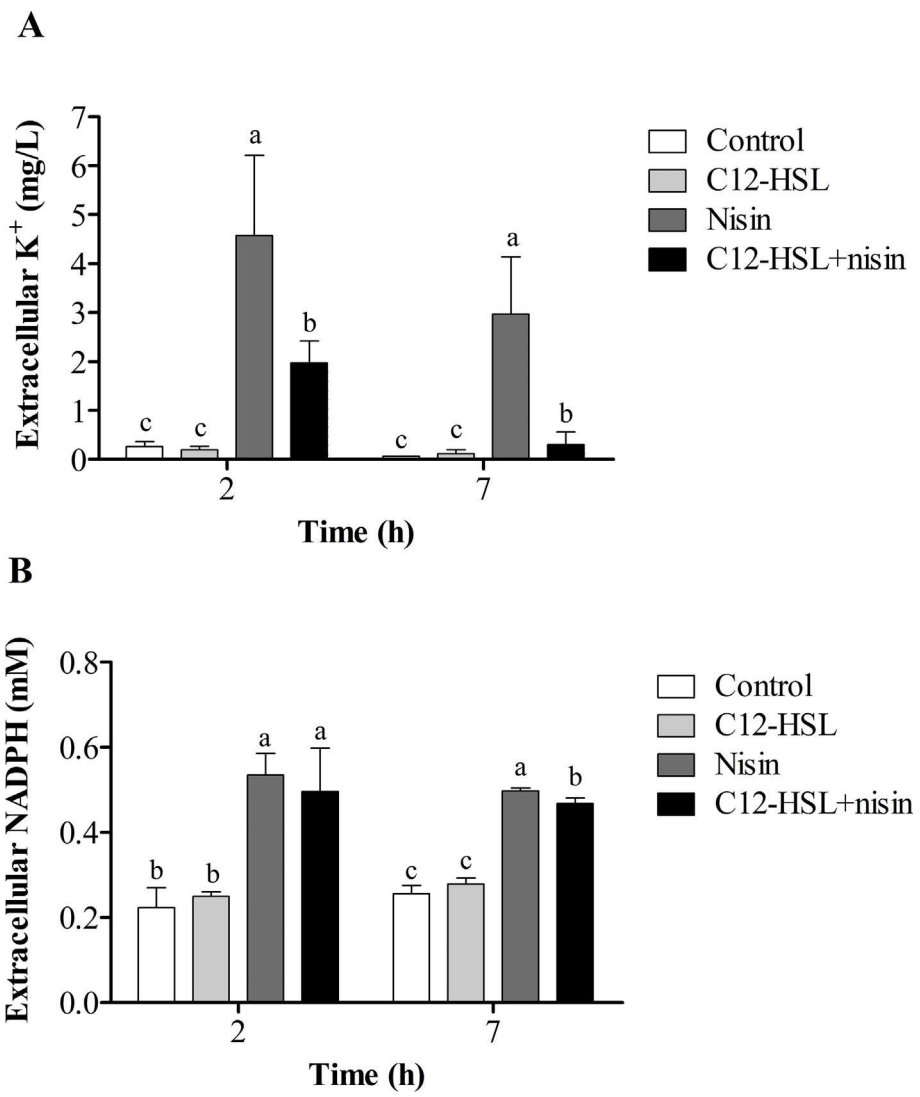

Fig. 5. Effect of C12-HSL on the release of intracellular content by *Salmonella* exposed to nisin at a pH of 4.5. Cells were grown in the presence of C12-HSL for 7 h at a pH of 7.0 and then used to inoculate media (pH 4.5) without C12-HSL (control), with C12-HSL, nisin alone, or C12-HSL + nisin. Extracellular (A) K^+ and (B) NADPH leakage of cells. Different letters indicate significant differences between treatments at the same sample collection time by Tukey's test ($P < 0.05$). Error bars indicate standard error (n = 3).

has not yet been described. In this study, *phoP*, *phoQ*, *pmrA,* and *pmrB* gene expression was up-regulated when *Salmonella* was cultured with C12-HSL. PhoP is a member of the two-component regulatory system PhoP/PhoQ, which is involved in the control of acid resistance genes [31,32]. The PhoPQ system indirectly activates the PmrAB system by the ability of the PhoP protein to activate transcription of the *pmrD* gene [33]. Furthermore, the PhoPQ and PmrAB systems are involved in cationic antimicrobial peptide and polymyxin B resistance in Gram-negative bacteria [34]. Here, we report that C12-HSL enhanced *Salmonella* survival in the presence of 50 μM nisin at a pH of 4.5. Cells growing in the presence of C12-HSL have an increased positive charge density on the cell surface. In *Salmonella*, PhoP induces activation of PmrA, which contributes to the production of lipid A with modifications, such as the addition of 4-amino-4-deoxy-L-arabinose (Ara4N) and phosphoethanolamine (pEtN) to phosphate groups. These modifications can increase the positive charge of the bacterial cell surface, affecting electrostatic interactions with cationic peptides and favoring bacterial resistance [35].

Many studies have focused on *Salmonella* resistance to acid stress conditions, however, until now, no study correlating the role of AHL-mediated QS to this adaptation has been identified. Thus, the influence of QS on *Salmonella* resistance to acid stress is unknown. C12-HSL increased the survival of *Salmonella* at a low pH in anaerobic conditions and is the first evidence of this behavior. Some studies have shown that C12-HSL can increase the abundance of different acid stress-related proteins. For instance, the Adi and TolC proteins were more abundant when *Salmonella* was cultivated in the presence of C12-HSL in anaerobic conditions [5,6]. The Adi protein is involved in resistance to acid stress by the amino acid decarboxylation system, which consumes protons increasing intracellular pH [11]. TolC is a protein channel located on the bacterial outer membrane that is associated with acid tolerance and drug resistance [12,14].

Another important observation of our study was that C12-HSL promotes alterations in the fatty acid composition of *Salmonella,* increasing the proportion of CFA. These fatty acids are synthesized by transmethylation of *cis* monounsaturated fatty acids at the end of the exponential phase and onset of the stationary phase of growth [36]. In *Escherichia coli*, the increase in CFA levels in the cell membrane helps to reduce the impact of acid stress by decreasing H+ permeability and H + extrusion, an enhanced ability of bacterial membranes that assists in the maintenance of intracellular pH [37]. In addition, CFA synthesis and a decrease of the UFA/SFA ratio are directly related to the

reduction of membrane fluidity to prevent the penetration of undesirable compounds [36,38].

Nisin acts on the bacterial cell causing pore formation in the membrane and consequently leakage of intracellular content [21]. Metabolic alterations caused by growing *Salmonella* in the presence of C12-HSL make it more resistant to this antimicrobial peptide, probably by altering the binding capacity of nisin to the cell membrane. As a result, little intracellular content is leaked, preventing cell death. Although different compounds have been analyzed to evaluate the effect of bacteriocins on cells [25,39], the loss of K^+ and NADPH is a reliable measure of pore formation in the cellular membrane. All alterations, including the increase in the membrane's positive charge, modifications of fatty acid composition, and reductions in the permeability of the outer membrane, can alter the sensitivity of bacteria to cationic peptides, such as nisin [40]. In conclusion, the results support the hypothesis that C12-HSL regulates different phenotypes in *Salmonella*, increasing nisin resistance, and acid stress survival. However, a more in-depth investigation needs to be performed to better elucidate the mechanisms involved with both resistances, as well as the role of C12-HSL in antibiotic resistance.

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CRediT authorship contribution statement

Leonardo Luiz de Freitas: Conceptualization, Methodology, Investigation, Writing - original draft. **Clarissa Isabela Aparecida dos Santos:** Investigation. **Deisy Guimarães Carneiro:** Methodology, Investigation. **Maria Cristina Dantas Vanetti:** Conceptualization, Supervision, Project administration, Writing - review & editing.

Declaration of competing interest

The authors have no conflict of interest to declare.

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CHAPTER 2

The virulence of *Salmonella* Enteritidis in *Galleria mellonella* is improved by *N*-dodecanoyl-homoserine lactone

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The virulence of *Salmonella* Enteritidis in *Galleria mellonella* is improved by *N*-dodecanoyl-homoserine lactone

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ABSTRACT

Salmonella is a food and waterborne pathogen responsible for outbreaks worldwide, and it can survive during passage through the stomach and inside host phagocytic cells. Virulence genes are required for infection and survival in macrophages, and some are under the regulation of the quorum sensing (QS) system. This study investigated the influence of the autoinducer 1 (AI-1), *N*-dodecanoyl-homoserine lactone (C12-HSL), on the virulence of *Salmonella* PT4 using *Galleria mellonella* as an infection model. *Salmonella* PT4 was grown in the presence and absence of C12-HSL under anaerobic conditions for 7 h, and the expression of *rpoS*, *arcA*, *arcB*, and *invA* genes was evaluated. After the inoculation of *G. mellonella* with the median lethal dose (LD_{50}) of *Salmonella* PT4, the survival of bacteria inside the larvae and their health status (health index scoring) were monitored, as well as the pigment, nitric oxide (NO), superoxide dismutase (SOD), and catalase (CAT) production. Also, the hemocyte viability, the induction of caspase-3, and microtubule-associated light chain 3 (LC3) protein in hemocytes were evaluated. *Salmonella* PT4 growing in the presence of C12-HSL showed increased *rpoS*, *arcA*, *arcB*, and *invA* expression and promoted higher larvae mortality and worse state of health after 24 h of infection. The C12-HSL also increased the persistence of *Salmonella* PT4 in the hemolymph and in the hemocytes. The highest pigmentation, NO production, and antioxidant enzymes were verified in the larva hemolymph infected with *Salmonella* PT4 grown with C12-HSL. Hemocytes from larvae infected with *Salmonella* PT4 grown with C12-HSL showed lower viability and higher production of caspase-3 and LC3. Taken together, these findings suggest that C12-HSL could be involved in the virulence of *Salmonella* PT4.

1. Introduction

Salmonella enterica is a foodborne pathogen of extreme concern to public health officials and the food industry. Non-typhoid *Salmonella* (NTS) infections generally cause gastrointestinal problems, but they can evolve into more serious conditions through the invasion of bacteria into other tissues, causing bacteremia and other local infections [1,2]. Worldwide, it was estimated that there were 535,000 cases of invasive NTS in 2017, with 77,500 deaths [3]. The *S. enterica* subspecies *enterica* is responsible for most cases of infections, with a prevalence of NTS serovars Typhimurium and Enteritidis [4].

The *Salmonella* pathogenesis is related to its ability to survive and replicate under adverse conditions [5–7]. This pathogen may survive during stomach passage and reach the gut lumen, where the process of adhesion and invasion in the intestinal epithelium will begin [8]. After crossing the intestinal barrier, *Salmonella* may be internalized by phagocytes such as macrophages. Within the defense cells, the pathogen can survive and replicate inside a compartment called the *Salmonella*-containing vacuole (SCV) by activating several virulence factors [9,10]. Finally, macrophages will undergo apoptosis, allowing *Salmonella* to escape the cell, and reinvade epithelial cells or other phagocytic cells of the immune system [10]. In the *Salmonella* genome, there are approximately 4,500 genes, and more than 100 genes, grouped into *Salmonella* pathogenicity islands (SPIs), have been associated with virulence [11–13]. Some evidence has indicated that *Salmonella* can regulate virulence factors by the mechanism of cell-cell communication, known

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as quorum sensing (QS) [14–16].

The QS system allows intraspecies and interspecies communication, wherein bacteria secrete signal sensing molecules known as autoinducers (AIs). Thus, bacteria can collectively regulate differential gene expression when AI concentrations reach a threshold [17,18]. In Gram-negative bacteria, the most studied mechanism of QS is mediated by the AI-1, acyl-homoserine lactone (AHL), synthesized by a synthase called LuxI and detected by a transcriptional regulator called LuxR or by homologous proteins [19,20]. In *Salmonella*, this system is incomplete because this bacterium does not have the gene that encodes LuxI; therefore, it is not able to synthesize AHL. However, *Salmonella* contains a transcriptional regulator homologous to LuxR, known as SdiA. Thus, this pathogen can regulate gene expression by detecting AHLs produced by other Gram-negative bacteria [21,22].

In *Salmonella*, the Rck protein, an outer membrane protein encoded by the *rck* (resistant to complement killing) operon, is directly involved with the resistance to the bactericidal action of the complement system, as well as the adhesion and invasion of host cells [23]. The expression of *rck* and *srgE* genes in *Salmonella* Typhimurium is upregulated in the presence of exogenous AHLs [24,25]. Increased expression of *hilA*, *invA*, and *invF* genes, associated with virulence and *glgC*, *fliF*, *lpfA*, and *fimF* genes, which are related to biofilm formation, was verified when *Salmonella* Enteritidis PT4 was grown in the presence of the AHL, *N*-dodecanoyl homoserine lactone (C12-HSL), under anaerobic conditions [16]. In addition, C12-HSL increased the abundance of Adi proteins, which are important in the resistance to acid stress [26]. The lack of the Adi system impairs the ability of *Salmonella* to replicate inside macrophages [27]. Almeida et al. [28] also showed that the abundance of different proteins of *Salmonella* Enteritidis PT4, such as Fdx, FldA, GrxA, GrxC, YdhD, NfsB, SodC1, and YfgD, related to oxidation-reduction processes, and SspB, PhoP, and TolC, involved with virulence, increased in the presence of C12-HSL under anaerobic conditions. Interestingly, some of these proteins are important for *Salmonella* during invasion into epithelial cells and survival within SCV in macrophages, suggesting that cells growing in the presence of C12-HSL can be best prepared for the infection process [29–31]. Although different genes are under the regulation of AHLs, some global regulators important for survival under adverse conditions and for the virulence of *Salmonella*, such as *rpoS* and *arcAB*, have not yet been linked to AHLs.

There is no evidence that *Salmonella* may detect the AHL produced by the intestinal microbiota and use this information to regulate the production of host colonization factors. However, the SdiA of *Salmonella* Typhimurium was activated during transit through the gastrointestinal tract of turtles colonized with *Aeromonas hydrophila* [32] and of mice colonized with *Yersinia enterocolitica* [33], which may suggest the occurrence of gene regulation of this pathogen by AI-1 in the intestine. Both *A. hydrophila* and *Y. enterocolitica* are AHL producers [32,33]. The detection of 14 different AHLs from human feces [34] also suggests that these autoinducing molecules may have some regulatory effect on the metabolism and virulence of Gram-negative intestinal pathogens in humans.

The study of host-pathogen interactions is an ever-emerging and evolving field, and the exploration of alternative infection models, such as the use of the larvae of the greater wax moth, *Galleria mellonella*, is of interest. The innate immune response of *G. mellonella* shares a high similarity with the mammalian system [35]. *G. mellonella* larvae contain phagocytic immune cells, called hemocytes, that are responsible for the cellular immune response, including phagocytosis, encapsulation, and nodulation, while the humoral immune response comprises opsonization, melanization, and secretion of antimicrobial peptides (AMPs) [36,37]. Some studies have successfully used *G. mellonella* to analyze the virulence of *Salmonella* strains [38–40] but none with the approach of QS regulation. Therefore, this study aimed to evaluate the influence of the C12-HSL signal molecule on the virulence of *Salmonella* Enteritidis PT4 using *G. mellonella* as an infection model.

2. Materials and methods

2.1. Bacterial strain and larval culture

Salmonella enterica subspecies *enterica* serovar Enteritidis phage type 4578 (*Salmonella* PT4) (GenBank: 16S ribosomal RNA gene, MF066708.1) isolated from chicken meat (FIOCRUZ, Rio de Janeiro, Brazil) was used in this study. The strain was stored at −20 °C in tryptone soy broth (TSB; Sigma, India) supplemented with 20% (v/v) glycerol. *G. mellonella* larvae were reared at 28 °C in the dark and fed an artificial diet containing a mixture of 27.3 g of wheat bran, 13.6 g of wheat germ, 13.6 g of milk powder, 8.2 g of brewer's yeast, 23.1 g of honey, and 14.2 g of glycerol per 100 g of food. Before all experiments, last instar larvae were selected (250–300 mg weight) and acclimatized to 37 °C for 1 h.

2.2. In vitro analysis

2.2.1. Effect of C12-HSL on Salmonella PT4 growth and gene expression

The inoculum standardization was performed as described by Almeida et al. [28], with minor modifications. *Salmonella* PT4 was grown twice in 10 mL of O_2-free TSB (anaerobic TSB) at 37 °C for 24 h. Then, 1 mL of culture was inoculated into 10 mL of anaerobic TSB and incubated at 37 °C for 4 h to reach the exponential phase. After incubation, cells were harvested by centrifugation at 10,000 *g* at 4 °C for 5 min, washed twice with phosphate-buffered saline (PBS; 10 mM, pH 7.2), and standardized to an optical density at 600 nm (OD_{600}) of 0.10 (approximately 10^7 CFU/mL) using a spectrophotometer (Thermo Fisher Scientific, Finland). The influence of C12-HSL on *Salmonella* PT4 growth was evaluated by inoculating 1 mL of the standardized inoculum in 10 mL of anaerobic TSB supplemented with 50 nM of *N*-dodecanoyl-DL-homoserine lactone (C12-HSL; PubChem CID:11565426; Fluka, Switzerland) [28]. The cultures were incubated at 37 °C, and aliquots were collected to assess bacterial growth by the drop plate method [41] on plate count agar (PCA; Himedia, India). The control experiment was done using *Salmonella* PT4 cells grown under the same conditions in broth without C12-HSL.

The total RNA of *Salmonella* PT4 grown for 7 h in the presence and absence of C12-HSL was extracted with Trizol® Reagent (Invitrogen, USA) following the procedures described by the manufacturer. The concentration and purity of the RNA samples were measured with a NanoDrop Spectrophotometer (Thermo Scientific, USA), and the integrity was evaluated in agarose gel. A total of 1 μg was treated with RNase-Free DNase. Complementary DNA (cDNA) synthesis was performed using 1 μg of treated RNA and the ImProm-II Reverse Transcription kit with Random Hexamer Primer (Promega, USA) according to the procedures described by the manufacturer. Specific primers for *rpoS*, *arcA*, *arcB*, and *invA* genes were designed using the program GenScript (Table 1). Self-annealing loops and dimer formation were evaluated using the Oligo Explorer tool, and the primer specificity was verified by electronic polymerase chain reaction (e-PCR) using the *Salmonella*

Table 1
Primers used in this study.

Primer	Oligonucleotide sequences (5′–3′)
*rpoS*F	GGCGTTGCTGGACCTGATTG
*rpoS*R	GGTTCATGATCGCCCGTTCG
*arcA*F	TGAAGCCGCATGACCGTACT
*arcA*R	AGCGATAACCTTCGCCGTGA
*arcB*F	GATCTCACCGCCGAACAGGA
*arcB*R	TGAACCTTACGCCGCTCCAT
*invA*F	ACAGTGCTCGTTTACGACC
*invA*R	ACTGGTACTGATCGATAAT
*gyrA*F	ATCGCCGAGCTGGTGAAAGA
*gyrA*R	ACCTGTAGCTGGGTCTGGGA
*16S*F	GCATTCGAAACTGGCAGGCT
*16S*R	CCTTCGCCACCGGTATTCCT

Enteritidis P125109 genome as a template. The expressions of the endogenous genes *gyrA* and 16S were used to normalize the data. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with 100 ng of cDNA and SYBR Green I Master Mix (Promega, USA) in the 96-well plates in a Bio-Rad C1000 Thermal Cycler under custom thermal cycling conditions (denaturation step at 95 °C for 2 min, followed by 40 cycles of a denaturation step at 95 °C for 15 s and an annealing/elongation step at 60 °C for 1 min). The relative standard curve was used to calculate the relative quantity (Rq) values of each sample for each gene, as previously described [42,43]. Expression values were obtained by the $\log_2$ of the ratio between the cells treated with C12-HSL and the control (without C12-HSL).

2.3. In vivo analysis

2.3.1. Determination of median lethal dose (LD_{50})

Cells of *Salmonella* PT4, grown anaerobically in anaerobic TSB at 37 °C for 24 h, were washed twice in PBS by centrifugation at 10,000 *g* at 4 °C for 5 min. The cell pellet was resuspended in PBS, and suspensions with different cell concentrations were prepared. Groups of 10 larvae were inoculated with bacterial loads ranging from 10^2 to 10^5 colony-forming units per larva (CFU/larva) by injection of 10 μL of bacterial suspensions into the last right pro-leg with an ultra-fine insulin syringe (BD, USA). The confirmation of bacterial load was done by the drop plate method [41] on PCA, and after incubation at 37 °C for 8–24 h the colonies were counted. Uninoculated larvae or those injected with PBS were used as controls. Post-injection, the larvae were placed in Petri dishes and incubated at 37 °C in the dark. Larvae that did not move when touched with a pipette tip were considered dead after 24 h of infection. The experiments were performed three times. In addition, health index scoring was calculated according to the criteria (activity, cocoon formation, melanization, and survival) established by Loh et al. [44], which assesses the larvae health status during infection (Supplementary Table S1).

2.3.2. Salmonella PT4 infection in G. mellonella

Salmonella PT4 cells growing in 10 mL of anaerobic TSB containing 50 nM C12-HSL were incubated at 37 °C for 7 h [28]. Cells were harvested by centrifugation at 10,000 *g* at 4 °C for 5 min, washed twice in PBS to remove all extracellular C12-HSL from the medium, and standardized to an OD_{600} of 0.10. Then, 10-fold serial dilutions were performed to obtain approximately 1×10^3 CFU per volume of injection (LD_{50}), and 10 μL of this bacterial suspension was injected into the last right pro-leg *G. mellonella*, as described above. The determination of bacterial load in the injection volume was done by the drop plate method [41] on PCA. The larvae were placed in Petri dishes and incubated at 37 °C in the dark. Larval mortality was recorded after 24 h of infection. The tests were performed in three biological replicates (30 larvae for replicated), totaling 90 larvae per group. The health index scoring was also calculated [44]. Larvae injected with *Salmonella* PT4 untreated with C12-HSL, as well as uninoculated larvae and PBS-injected larvae, were included in every experiment as controls.

2.3.3. Hemolymph collection

Hemolymph was collected as previously described by Pereira et al. [45]. After 2, 4, 12, and 24 h of injection, larvae infected with *Salmonella* PT4 grown in the presence or absence of C12-HSL were anesthetized on ice and surface disinfected with 70% ethanol followed by washing with sterile distilled water. The hemolymph was also extracted from uninoculated larvae or PBS-injected larvae and used as controls. The larvae were turned in the ventral position, and an incision was made in the last right pro-leg with a sterile needle. Hemolymph was collected from ten larvae and pooled (10 μL of each larva, totaling 100 μL). The volume of 10 μL of each larva was collected using a micropipette.

2.3.4. The proliferation of Salmonella PT4 within the larvae

For extracellular bacterial counting, 100 μL of the hemolymph extracted as described above was serially diluted in PBS, and the CFU per larva was quantified on PCA by incubation at 37 °C for 24 h [45]. Results were expressed as CFU per larva by the ratio of the number of total CFU and the number of larvae used (10 larvae). For intracellular bacterial counting, the gentamicin protection assay was used, with modifications [46]. A volume of 100 μL of the hemolymph was collected and diluted in 900 μL of insect physiologic saline (IPS; 150 mM sodium chloride, 5 mM potassium chloride, 100 mM TRIS hydrochloride [pH 6.9], 10 mM ethylenediaminetetraacetic acid, and 30 mM sodium citrate). Hemocytes were harvested by centrifugation at 500 *g* at 4 °C for 10 min. The supernatant was discarded, and 500 μL of IPS with 100 mg/L of gentamicin was added. After 1 h of incubation, the hemocytes were centrifuged at 500 *g* at 4 °C for 10 min, and the death of all extracellular bacteria was confirmed by plating the supernatant on PCA at 37 °C for 24 h. Then, the hemocytes were washed three times with IPS and lysed with 0.5% (vol/vol) Triton X-100 for 20 min, and the number of CFU was determined on PCA at 37 °C for 24 h. Before the lyse stage, the hemocytes were counted in a hemocytometer using a light microscope (Olympus CX40, Japan). The results are presented as the number of CFU per hemocyte. No CFU was recovered from uninoculated larvae or PBS-injected larvae.

2.3.5. Hemolymph pigmentation and nitric oxide (NO) quantification

Larvae were bled, and hemolymph was collected as previously described, centrifuged at 500 *g* at 4 °C for 5 min and used for quantification of the pigmentation (melanization). The supernatant was diluted (1:3) in cold IPS and added to 96-well plates, and the optical density was read at 405 nm [47]. The Griess test was used to measure nitric oxide (NO) concentrations. For the assay, 100 μL of the hemolymph was diluted in 900 μL of the IPS, and the solution was strongly homogenized and centrifuged at 500 *g* at 4 °C for 5 min. Next, 100 μL of the supernatant was placed in a 96-well microplate. An equal volume of Griess reagent was added, and the solution was incubated in the dark for 15 min. The absorbance was measured on a microplate reader with a wavelength of 570 nm. A standard curve was prepared using hemolymph of uninfected larvae with $NaNO_2$.

2.3.6. Hemolymph antioxidant enzyme activity

To assess the antioxidant activity, the hemolymph was collected as described above, centrifuged at 500 *g* at 4 °C for 5 min, and filtered on membranes with 0.22 μm pores (Merck, Germany). The superoxide dismutase (SOD) activity was determined according to the method described by Zheng et al. [48], and the catalase (CAT) activity was determined according to Hadwan and Abed [49]. The data were normalized by the protein concentration found in the hemolymph, measured according to Bradford [50], using bovine serum albumin as a standard.

2.3.7. Hemocyte viability, caspase-3, and LC3 analysis

Flow cytometry measurements were performed on a BD FACSVerse cytometer (BD Bioscience, USA) [51]. The hemolymph (100 μL) was collected as described above. The hemocytes were recovered by centrifugation at 500 *g* at 4 °C for 10 min, washed twice, and resuspended in 300 μL of PBS. The propidium iodide (PI) was added according to the manufacturer (Invitrogen, USA), and viability was calculated as the percentage of PI-negative hemocytes.

The hemocytes were also prepared for caspase-3 and LC3 analysis by fixing in Zamboni's fixative (2% paraformaldehyde containing 15% picric acid in 0.1 M sodium phosphate buffer) for 20 min at room temperature. After fixation, the samples were centrifuged at 1,500 *g* for 10 min and washed three times with PBS (0.1 M, pH 7.2). The hemocytes were incubated for 1 h with 1% PBS–Triton-X100 and then incubated at 4 °C with the primary antibodies for 24 h, separately. The primary antibodies used were anti-caspase-3 (1:500; Sigma-Aldrich, USA) and anti-

LC3 A/B (1:100; Cell Signaling Technology, USA). Next, the hemocytes were washed in PBS three times and incubated with a secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, USA) at 4 °C for 24 h. Finally, the hemocytes were washed in PBS three times and examined by flow cytometry. For the negative control, hemocytes were treated as mentioned above, but the treatment with the primary antibody was replaced by PBS incubation.

2.4. Statistical analyses

The Student's *t*-test was used for comparison between two treatments and analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. All statistical analyses were performed using GraphPad Prism 5.00 software, and the significance level adopted was 5% for all statistical tests.

3. Results

3.1. RT-qPCR

The C12-HSL did not interfere with *Salmonella* PT4 growth under anaerobic conditions in TSB (Fig. S1). The RT-qPCR analysis was performed to evaluate the expression of *rpoS*, *arcA*, *arcB*, and *invA* in *Salmonella* PT4 cells growing in the presence of C12-HSL before infecting the larva, and the results indicated increased gene expression (Fig. 1). The *rpoS* gene, involved in the general response to stress, was upregulated 1.9-fold in the presence of C12-HSL, while the *arcA* and *arcB* genes increased 2.4- and 1.8-fold, respectively, in the presence of the QS signal. Also, the upregulation of the gene involved in cellular invasion (*invA*) was detected.

3.2. LD_{50}

The inoculation of 10^2–10^5 CFU/larva of *Salmonella* PT4 into the hemolymph of *G. mellonella* larvae indicated an LD_{50} of 10^3 CFU/larva (Fig. 2A). Dose of *Salmonella* PT4 of 10^5 CFU/larva killed all larvae with 24 h of infection. In addition, the health index scoring was used to assess larva health status during the infection period. Healthy larva received a score of 10 points, while very sick larvae received a very low score (less than 5). The results showed that 10^3 CFU/larva caused an intermediate infectious process, with a health score of approximately 5 during the infection period (Fig. 2B). Therefore, the dose of approximately 1×10^3 CFU/larva was chosen for subsequent studies to assess *Salmonella* PT4 virulence.

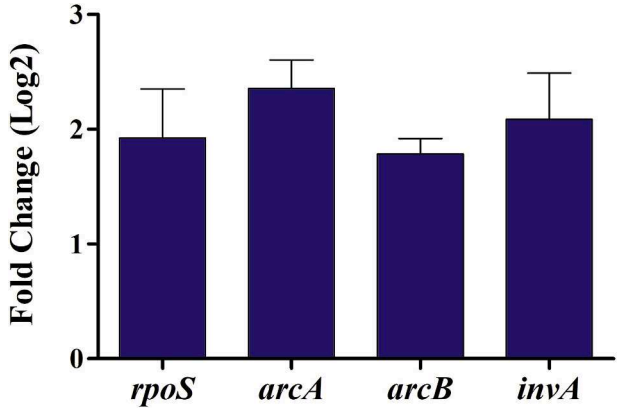

Fig. 1. Gene expression of *Salmonella* grown in the presence of *N*-dodecanoyl-homoserine lactone (C12-HSL) for 7 h before inoculation in *G. mellonella*. Expression values were obtained by $\log_2$ of the ratio between cells treated with C12-HSL and the control (without C12-HSL). The expression of significantly regulated genes ($p < 0.05$) is shown. Error bars represent the standard deviation of the mean ($n = 3$).

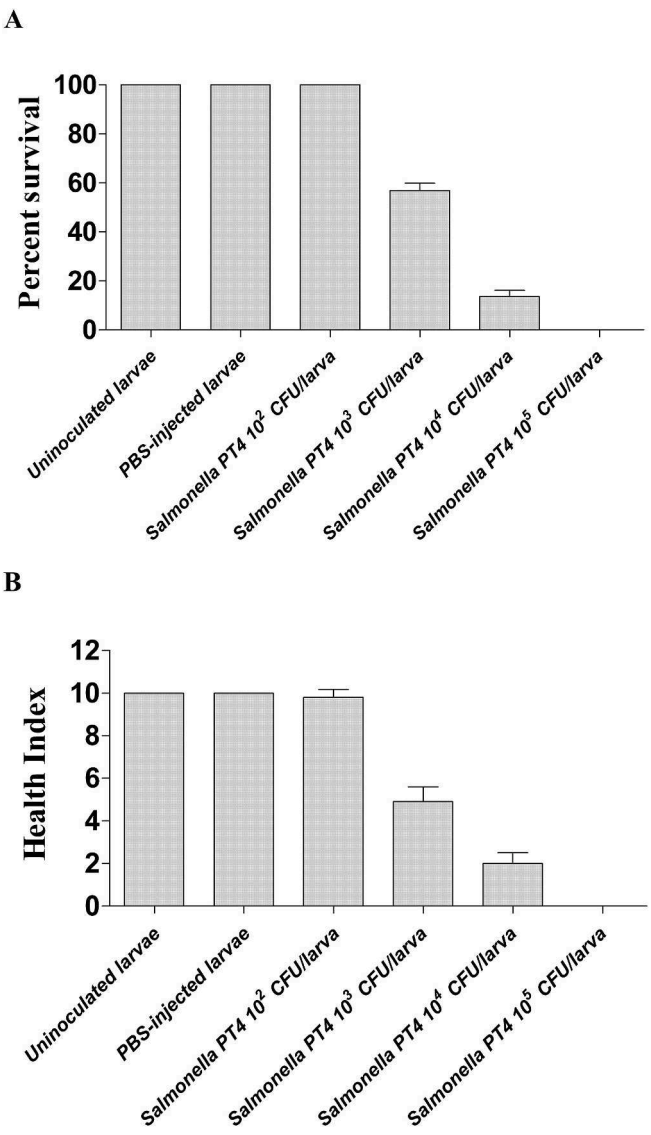

Fig. 2. Survival and health index of *G. mellonella* larvae after 24 h of infection with *Salmonella* PT4. (A) Percentual survival of *G. mellonella* inoculated with *Salmonella* PT4 at different concentrations. (B) Mean health index scores of *G. mellonella* 24 h post-infection with different concentrations of bacteria. Error bars represent the standard deviation of the mean.

3.2.1. Effect of C12-HSL on Salmonella PT4 virulence in vivo

The results showed that the survival of *G. mellonella* larvae was reduced when the pathogen grew in the presence of C12-HSL (Fig. 3A). In the first 24 h after inoculation, 35% survival of the infected larvae with *Salmonella* PT4 grown with C12-HSL (*Salmonella* PT4 + C12-HSL) was registered, while 52% survival occurred in the control group, i.e., inoculated with *Salmonella* PT4 cultivated in the absence of the AI. In addition, the health status (health index) and visual appearance showed a difference ($p < 0.05$) between larvae infected with *Salmonella* PT4 that was grown with C12-HSL and *Salmonella* PT4 that was not grown with C12-HSL, at 24 h post-infection (Fig. 3B and C). Considering that the number of *Salmonella* PT4 cells treated with C12-HSL and inoculated into the larvae and the number of *Salmonella* PT4 cells not treated with C12-HSL and inoculated into the larvae was equal during the infection procedure ($p > 0.05$) (Fig. 3D), it can be suggested that C12-HSL enhances *Salmonella* PT4 virulence. During the experiments, 100% of the uninoculated or PBS-injected larvae remained alive (Fig. 3A) and

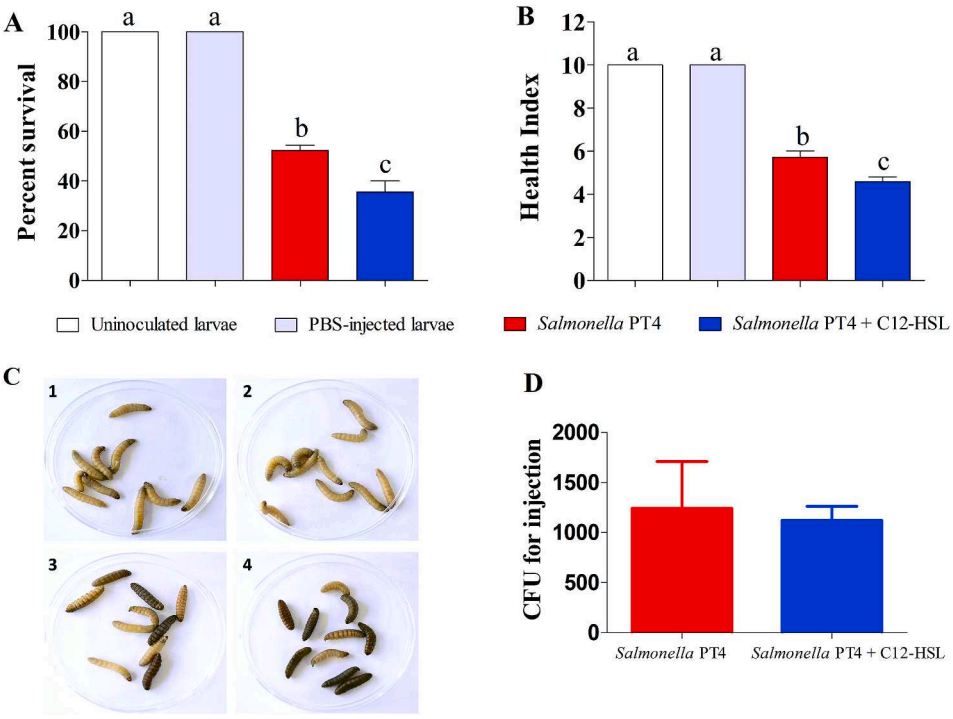

Fig. 3. Modulation of the virulence of *Salmonella* PT4 by C12-HSL. (A) Survival of *G. mellonella* infected with *Salmonella* PT4 grown in the presence or absence of C12-HSL 24 h post-infection. (B) Mean health index scores of *G. mellonella* 24 h post-infection. (C) Live and dead larvae from different treatments 24 h post-infection: 1, uninoculated larvae; 2, PBS-injected larvae; 3, larvae inoculated with *Salmonella* PT4 grown in the absence of C12-HSL; 4, larvae inoculated with *Salmonella* PT4 grown in the presence of C12-HSL. (D) Colony-forming units of *Salmonella* PT4 cultured in the presence or absence of C12-HSL immediately before infection. *Salmonella* PT4: *Salmonella* PT4 grown in the absence of C12-HSL; *Salmonella* PT4 + C12-HSL: *Salmonella* PT4 grown in the presence of C12-HSL. For each group in the same time, means that do not share the same letter are significantly different and means that share the same letter are not significantly different by Tukey's test ($p < 0.05$).

healthy (Fig. 3B and C).

3.2.2. C12-HSL influences Salmonella PT4 survival within insects

C12-HSL increased the persistence of *Salmonella* PT4 in the hemolymph and within the hemocytes (Fig. 4). Although the number of bacteria recovered from the hemolymph did not differ between the treatments in the first 12 h of infection, at 24 h post-infection it was observed that the number of CFU/larva was higher when *Salmonella* PT4 was cultivated with C12-HSL ($p < 0.05$) (Fig. 4A). At 2 h post-infection, the bacteria/hemocyte ratio was significantly higher when *Salmonella* PT4 cells were cultivated in the presence of C12-HSL compared to the control (Fig. 4B). These observations indicate that this AHL can increase the invasion at the beginning of the infection process and contributes directly to the survival of *Salmonella* PT4 within the hemolymph of *G. mellonella*.

3.2.3. Increased pigmentation and NO production in G. mellonella by Salmonella PT4

The defense response of *G. mellonella* larvae infected with *Salmonella* PT4 was shown by an increase in dark pigmentation for up to 4 h of infection in the hemolymph of larvae infected with *Salmonella* PT4 whether treated with C12-HSL or not (Fig. 5A). However, at 12 h and 24 h post-infection, the highest pigmentation was quantified in the hemolymph infected with *Salmonella* PT4 grown with C12-HSL (Fig. 5A). Furthermore, the production of NO is a response to control infectious pathogens, and the results showed that at 12 h post-infection the NO concentration was higher in the hemolymph of larvae infected with *Salmonella* PT4 cultured with C12-HSL (Fig. 5B). It was also observed that at 12 h and 24 h, uninfected or PBS-infected larvae increased the production of NO, possibly due to the stress caused by the temperature of 37 °C.

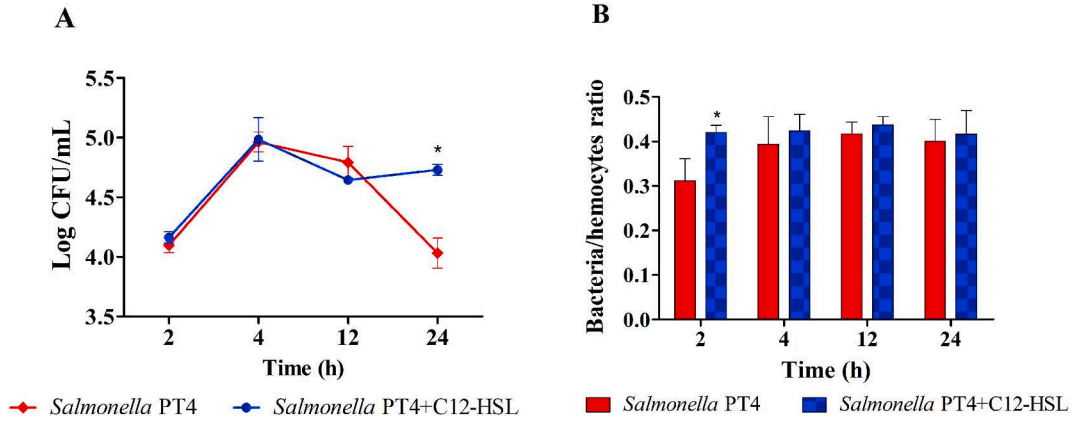

Fig. 4. *Salmonella* PT4 displays differential abilities to persist in *G. mellonella* when grown with C12-HSL. (A) Count of viable *Salmonella* PT4 cells in the hemolymph from live larvae of *G. mellonella* post-infection. (B) Ratio between the number of CFU of *Salmonella* PT4 and the hemocytes of *G. mellonella* post-infection. *Salmonella* PT4: *Salmonella* PT4 grown in the absence of C12-HSL; *Salmonella* PT4 + C12-HSL: *Salmonella* PT4 grown in the presence of C12-HSL. The asterisk indicates statistically significant differences between treatments at the same sample collection time determined by the Student's *t*-test ($p < 0.05$). Error bars represent the standard deviation of the mean ($n = 3$).

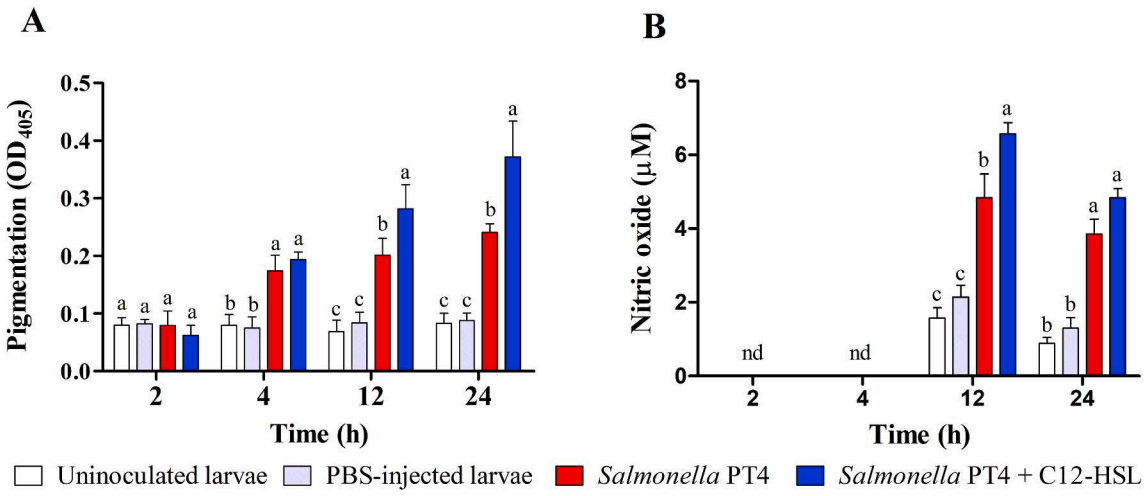

Fig. 5. Pigmentation and nitric oxide (NO) production of live larvae after infection with *Salmonella* PT4 grown in the presence or absence of C12-HSL. (A) Measurement of the pigmentation of the hemolymph by the spectrophotometric method (405 nm). (B) NO quantification by the Griess test (nd: not detected). *Salmonella* PT4: *Salmonella* PT4 grown in the absence of C12-HSL; *Salmonella* PT4 + C12-HSL: *Salmonella* PT4 grown in the presence of C12-HSL. For each group in the same time, means that do not share the same letter are significantly different and means that share the same letter are not significantly different by Tukey's test ($p < 0.05$). Error bars represent the standard deviation of the mean (n = 5).

3.2.4. Salmonella PT4 grown with C12-HSL influences the antioxidant response of G. mellonella

The antioxidant activity in the hemolymph was measured, and the results are shown in Fig. 6. Larvae inoculated with *Salmonella* PT4 presented an increase in SOD activity, and the difference between larvae infected with *Salmonella* PT4, whether grown with C12-HSL or not, was only detected at 2 h of infection (Fig. 6A). In contrast, the CAT activity was higher in larvae infected with *Salmonella* PT4 treated with C12-HSL at 24 h (Fig. 6B).

3.2.5. Salmonella PT4 cultured with C12-HSL influences the immune response of G. mellonella

The viability of hemocytes, analyzed by flow cytometry using PI, did not differ statistically between uninoculated larvae and PBS-injected larvae at either analyzed time (Fig. 7). However, the hemocytes from the larvae inoculated with *Salmonella* PT4 grown with C12-HSL presented a lower viability percentage compared with *Salmonella* PT4 not treated with C12-HSL at 12 h and 24 h post-infection (Fig. 7).

The presence of caspase-3 was monitored in the hemocytes from larvae infected with *Salmonella* PT4 grown in the presence or absence of C12-HSL. It was observed that caspase-3 activity increased only for the treatment with C12-HSL after 2 h of infection (Fig. 8A), which indicates that the cells were in the process of cell death. To determine the autophagy process, the LC3 antibody was used. The results indicated that hemocytes from larvae infected with *Salmonella* PT4 cultured with C12-HSL showed 55% and 68% LC3-positive staining 2 h and 24 h post-infection, respectively. In contrast, hemocytes from larvae inoculated with *Salmonella* PT4 and not treated with C12-HSL showed a lower percentage, with 39% and 59% LC3-positive staining after 2 h and 24 h of infection, respectively (Fig. 8B). Furthermore, hemocytes from uninoculated or PBS-infected larvae showed caspase-3 and LC3 activity, possibly because of the incubation temperature of 37 °C. Together, the data suggest that C12-HSL increases the virulence of *Salmonella* PT4 once this pathogen promotes a reduction in hemocyte viability after 24 h of infection and increases apoptosis and autophagy activity in hemocytes.

4. Discussion

Salmonella coordinates the expression of different virulence genes to ensure successful infection, and some of these genes can be regulated by AHL-mediated QS [14–16,52]. In the present study, *Salmonella* PT4 was

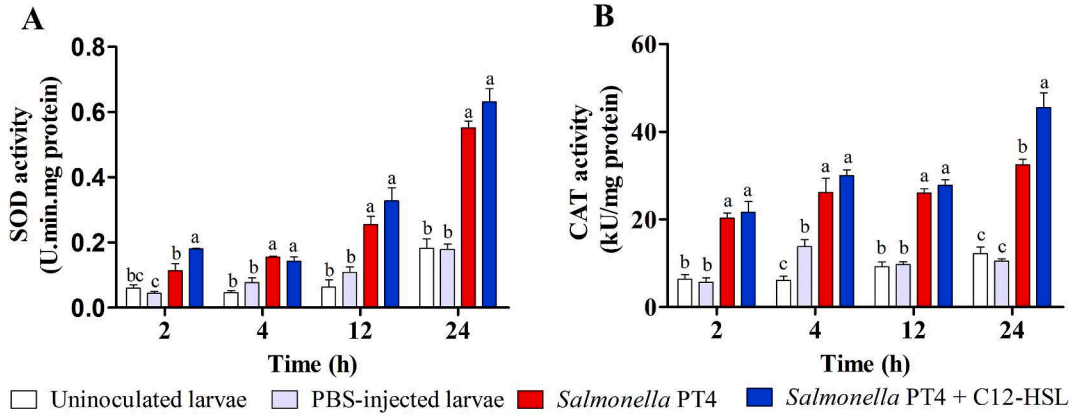

Fig. 6. Antioxidant enzyme activity of the *G. mellonella* hemolymph after infection with *Salmonella* PT4. (A) Superoxide dismutase (SOD) activity. (B) Catalase (CAT) activity. *Salmonella* PT4: *Salmonella* PT4 grown in the absence of C12-HSL; *Salmonella* PT4 + C12-HSL: *Salmonella* PT4 grown in the presence of C12-HSL. For each group in the same time, means that do not share the same letter are significantly different and means that share the same letter are not significantly different by Tukey's test ($p < 0.05$). Error bars represent the standard deviation of the mean (n = 3).

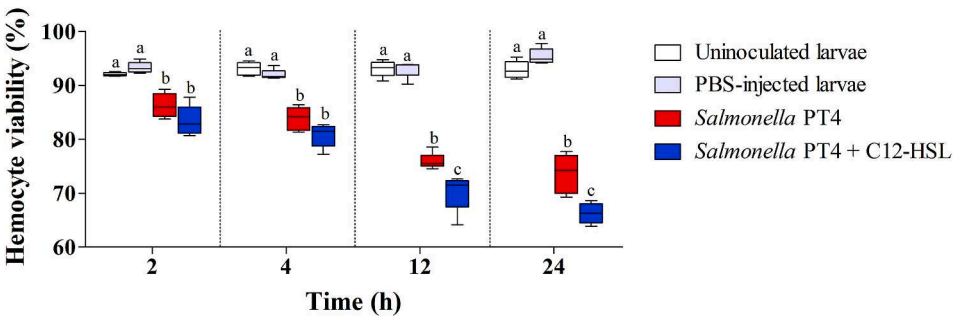

Fig. 7. Hemocyte viability after infection with *Salmonella* PT4. Hemocytes were stained with propidium iodide (PI) and analyzed by flow cytometry. *Salmonella* PT4: *Salmonella* PT4 grown in the absence of C12-HSL; *Salmonella* PT4 + C12-HSL: *Salmonella* PT4 grown in the presence of C12-HSL. For each group in the same time, means that do not share the same letter are significantly different and means that share the same letter are not significantly different by Tukey's test ($p < 0.05$). Error bars represent the standard deviation of the mean ($n = 5$).

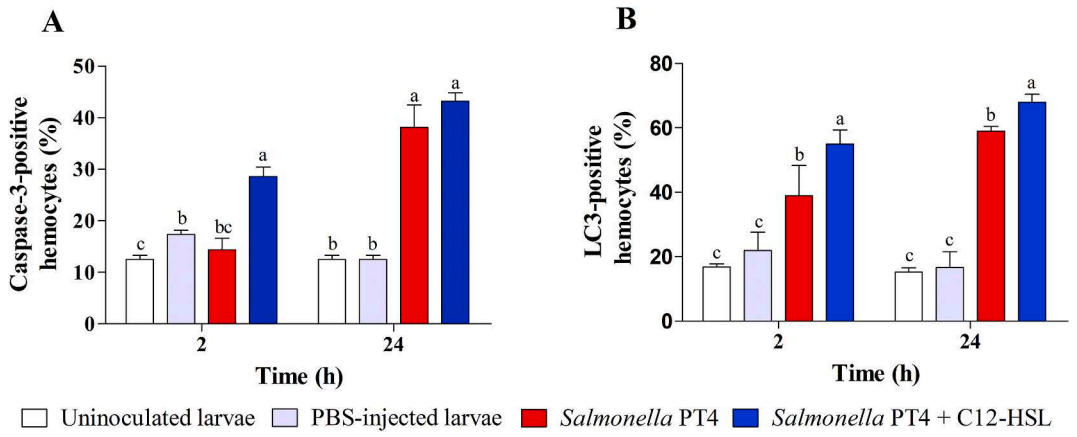

Fig. 8. Caspase-3-positive and LC3-positive hemocytes from live larvae after infection with *Salmonella* PT4 grown in the presence or absence of C12-HSL. (A) Percentage of caspase-3-positive hemocytes. (B) Percentage of LC3-positive hemocytes. *Salmonella* PT4: *Salmonella* PT4 grown in the absence of C12-HSL; *Salmonella* PT4 + C12-HSL: *Salmonella* PT4 grown in the presence of C12-HSL. For each group in the same time, means that do not share the same letter are significantly different and means that share the same letter are not significantly different by Tukey's test ($p < 0.05$). Error bars represent the standard deviation of the mean ($n = 5$).

grown with 50 nM of C12-HSL under anaerobic conditions. The concentration of 50 nM of C12-HSL increased expression of virulence and biofilm genes, biofilm formation and metabolism optimization in *Salmonella* PT4 without changing growth [16,43,53]. According to Michael et al. [24], depending on the type of AHL, concentration up to 1 nM de AHL could be detected by the SdiA of *Salmonella* Typhimurium. The increased expression of the *rpoS* gene in *Salmonella* PT4 cells cultured with C12-HSL is an indicator that QS signals also increased the resistance of the pathogen to the adversities found in the host cells. The *rpoS* gene is extremely important for *Salmonella* survival under several stress conditions, including nutrient deprivation, acid stress, and oxidative stresses [54,55]. In addition, the RpoS protein is a global regulator directly related to the activation of virulence genes required for intracellular growth and infection in mice [56–58]. RpoS is also essential for the colonization of lymphoid organs, for adherence to murine Peyer's patches, and for resistance from NO produced by macrophages [59–61].

The increased expression of *arcA* and *arcB* observed in the presence of C12-HSL could also contribute to enhancing the virulence of *Salmonella* PT4. To achieve successful infection, *Salmonella* activates the *arc* two-component system for adaptation under low oxygen tension conditions, located in the gastrointestinal tract and within host cells [62–64]. Thus, this two-component system participates in bacterial adaptation to changing oxygen levels and plays an important role in intracellular survival in macrophages [64,65]. As observed by Campos-Galvão et al. [16], the invasion gene, *invA*, was also upregulated in the presence of C12-HSL. The InvA is an export apparatus component of the Type III secretion system (T3SS), which is essential during the invasion process in host epithelial cells [66,67]. This genic expression pattern suggests that C12-HSL can enhance the response to stresses encountered during infectious processing and can contribute to increased virulence of *Salmonella* PT4.

The results obtained using *G. mellonella* larvae as an infection model are relevant and may be considered to better understand the effect of QS in the virulence of *Salmonella*. *G. mellonella* is a promising model organism in the study of infectious agents [68–71]. The larvae can be reared at 37 °C, which is the optimal physiological temperature for most pathogens that infect humans [36,72]. This insect has been used as an infection model to differentiate the virulence of different strains of the same species, such as *Streptococcus pneumoniae* [73], extraintestinal pathogenic *Escherichia coli* [74], *Actinobacillus pleuropneumoniae* [45], *Shigella flexneri* [75], and *Legionella pneumophila* [76]. In research with *Salmonella*, *G. mellonella* was used to differentiate the virulence of different isolates [40], to demonstrate the role of endoribonucleases E and III [77], and the LPS structure and PhoQ activity [78]. Therefore, in this study, we utilized this host-pathogen model to explore the effect of QS on some virulence factors. First, the infectious dose was determined, and, although differences in the virulence and host range displayed by the various *Salmonella* subspecies and serovars were described, the LD_{50} of *Salmonella* Enteritidis PT4 in *G. mellonella* (around 10^3 CFU/larva) was similar to that determined for others *Salmonella* serovars, such as *Salmonella* Typhimurium [78] or *Salmonella* Gallinarum and Kentucky (10^2 CFU/larva) [38]. Additional metric methods used to increase the sensitivity of the infection test rather than just assessing larvae survival, such as the health index scoring system, corroborate the evaluation of the health status of the larva during the infectious process [44]. This parameter is also used to measure more subtle differences in virulence of other bacterial pathogens in *G. mellonella* [44,79,80].

The QS signal molecules have been associated with virulence gene expression, and the 17% increase in mortality and low health index (24 h post-infection) of *G. mellonella* inoculated with *Salmonella* PT4 cells

grown in the presence of C12-HSL reinforce this evidence. Our finding shows that *Salmonella* grown with C12-HSL causes higher mortality in the larvae when compared to *Salmonella* not grown with C12-HSL. The effect of exogenous AHLs on *Salmonella* virulence is little studied, especially using C12-HSL. *Salmonella* Typhimurium cultivated with *N*-hexanoyl-DL-homoserine lactone (C6-AHL) or *N*-octanoyl-DL-homoserine lactone (C8-AHL) increase the invasion in epithelial cell and the expression of *rck* and *srgE*, which are important genes for *Salmonella* virulence [14]. In another study, the adhesion in HeLa cells by *Salmonella* Typhi ST_8 is increased when this pathogen was cultivated with C8-AHL [15]. Thus, the results of gene expression could help to explain the lower survival of the larvae and greater survival of *Salmonella* PT4 in the hemolymph and within the hemocytes, suggesting that C12-HSL modulates different virulence genes that contribute to the persistence of bacteria in larvae. This increased persistence of *Salmonella* PT4 within the insect reinforces the effect of QS in enhancing the pathogen virulence.

In *G. mellonella*, the production of melanin is activated during the infectious process to kill the invading pathogen [35,81]. The increase in the black pigmentation of the hemolymph is attributed to the greater production of melanin [82]. At 12 h and 24 h post-infection, the hemolymph pigmentation of larvae infected with *Salmonella* PT4 cultured with C12-HSL was greater. This indicates that *Salmonella* PT4 cultivated with C12-HSL can alter the larval humoral response, suggesting differential activation of the enzyme phenoloxidase (PO), which is essential for activating melanin production [37]. Moreover, the hemocytes of *G. mellonella* can produce NO to increase the effectiveness of the immune response [83]. Here, we also observed a differential modulation in response to NO production because *Salmonella* PT4 cultured with C12-HSL induced the highest NO production 12 h after infection, but no difference was observed at 24 h post-infection. One possible explanation for these results is that the inactivation of a pathogen which can be accompanied by melanization (the oxygen-consuming process) results in the formation of the melanotic capsules, where the nitrite reduction activity of hemocytes may increase, causing a local burst of NO [83,84]. However, *Salmonella* treated with C12-HSL is more resistant to NO and the hypoxic environment, which induces the larva to produce a greater amount of NO. We report that C12-HSL increased the expression of the *rpoS* gene, which is directly involved with resistance to NO. In 24 h post-infection, the regulation of resistance genes by C12-HSL in *Salmonella* PT4 may have been normalized when compared to the control. Another critical factor is that NO production and PO activity can be activated independently of each other, although they are activated by the same stimulus [83]. To our knowledge, until now, none study has assessed the influence of QS on *Salmonella* resistance to NO, especially in the *G. mellonella* infection model.

Hemocytes of *G. mellonella* can engulf pathogenic microorganisms and produce reactive oxygen species (ROS) to kill the pathogen [85,86]. During infection, the concentration of ROS increases inside the cells, generating oxidative stress, which can cause various damages to the host cell. On the other hand, many antioxidant enzymes, such as SOD and CAT, are produced by hemocytes and efficiently convert ROS to less reactive substances [85,87]. *Salmonella* PT4 grown in the presence of C12-HSL promotes greater production of SOD (2 h post-infection) and CAT (24 h post-infection) in *G. mellonella*. C12-HSL may anticipate a resistant response in *Salmonella* to oxidative stress, promoting greater resistance to the immune response and, as a result, hemocytes produce more ROS and, hence, antioxidant enzymes. The results show that 2 h post-infection, the bacteria/hemocyte ratio was higher when the pathogen was treated with C12-HSL. Possibly, during the invasion process at the beginning of the infection *Salmonella* PT4 cultivated with C12-HSL triggers a differentiated response for ROS production. In addition, the higher production of CAT in 24 h after infection may be associated with more remarkable persistence of the bacteria treated with C12-HSL in the hemolymph. Proteomics analysis performed by Almeida et al. [28] showed that C12-HSL changes the protein profile in *Salmonella* PT4 at different incubation times. The increase in the abundance of proteins related to the oxidation-reduction process, and levels of free cellular thiol in *Salmonella* grown in the presence of C12-HSL is more pronounced in the early incubation times. These observations suggest that C12-HSL anticipates the response to oxidative stress, which helps *Salmonella* PT4 to survive during the infection process. It is probable that C12-HSL increases the expression of genes involved with resistance to oxidative stress, contributing to greater survival of bacteria within the hemolymph and hemocytes of *G. mellonella*. Thus, these changes in the physiology of *Salmonella* PT4 by C12-HSL can alter the production of ROS in *G. mellonella* and, consequently, in a disbalance in the synthesis of antioxidant enzymes. However, it is not clear how *Salmonella* PT4 grown with an exogenous AI-1 can alter the production of SOD and CAT at different times.

The larval hemocyte viability decreased over time, and when *Salmonella* PT4 treated with C12-HSL was present, lower viability percentages were verified. The increase in the production of ROS may potentiate the activation of the cell death process of the hemocytes [88,89]. This indicates that *Salmonella* PT4 cultured with C12-HSL can reduce the population of circulating hemocytes and contribute to the increase in virulence. Furthermore, the increased caspase-3 activity after 2 h of infection with cells treated with C12-HSL was also indicative of enhanced virulence. Caspase-3 is an apoptosis marker that plays a key role in programmed cell death. The high expression of caspase-3 by bacterial pathogens contributes to the activation of apoptosis, allowing the pathogen to escape [90,91]. Also, C12-HSL increased *Salmonella* PT4 virulence and induced more significant expression of LC3, an autophagy marker, in the circulating hemocytes of *G. mellonella*. This indicates that the expression of LC3 is related to the virulence of the bacteria [51]. In conclusion, the findings of this study suggest that the presence of C12-HSL can alter the phenotypes in *Salmonella* PT4 and increase its virulence. Thus, the presence of AHLs in some environments such as human gut can anticipate the coordinated expression of virulence factors, increasing the success of infection. Moreover, *G. mellonella* was confirmed to be a useful infection model for analyzing *Salmonella* virulence. However, in-depth investigations need to be carried out to better understand the role of QS in modulating *Salmonella* virulence.

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Ethical approval

Not required.

CRediT authorship contribution statement

Leonardo Luiz de Freitas: Conceptualization, Methodology, Investigation, Writing - original draft. **Fernanda Pereira da Silva:** Investigation. **Kenner Morais Fernandes:** Investigation. **Deisy Guimarães Carneiro:** Investigation. **Leandro Licursi de Oliveira:** Investigation. **Gustavo Ferreira Martins:** Methodology. **Maria Cristina Dantas Vanetti:** Conceptualization, Supervision, Project administration, Writing - review & editing.

Declaration of competing interest

None to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2021.104730.

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Supplementary material

Table S1.
Criteria used for determination of the health index score in *G. mellonella* infected with *Salmonella* PT4 (Loh et al. [1], modified).

Category	Description	Score
Activity	No activity	0
	Minimal activity on stimulation	1
	Active when stimulated	2
	Active without stimulation	3
Cocoon formation	No cocoon	0
	Partial cocoon	0.5
	Full cocoon	1
Melanization	Complete melanization (black)	0
	Melanization (many black spots)	1
	Partial melanization (few black spots)	2
	No melanization	4
Survival	Dead	0
	Alive	2

[1] J.M. Loh, N. Adenwalla, S. Wiles, T. Proft, *Galleria mellonella* larvae as an infection model for group A streptococcus, Virulence. 4 (2013) 419–428. https://doi.org/10.4161/viru.24930

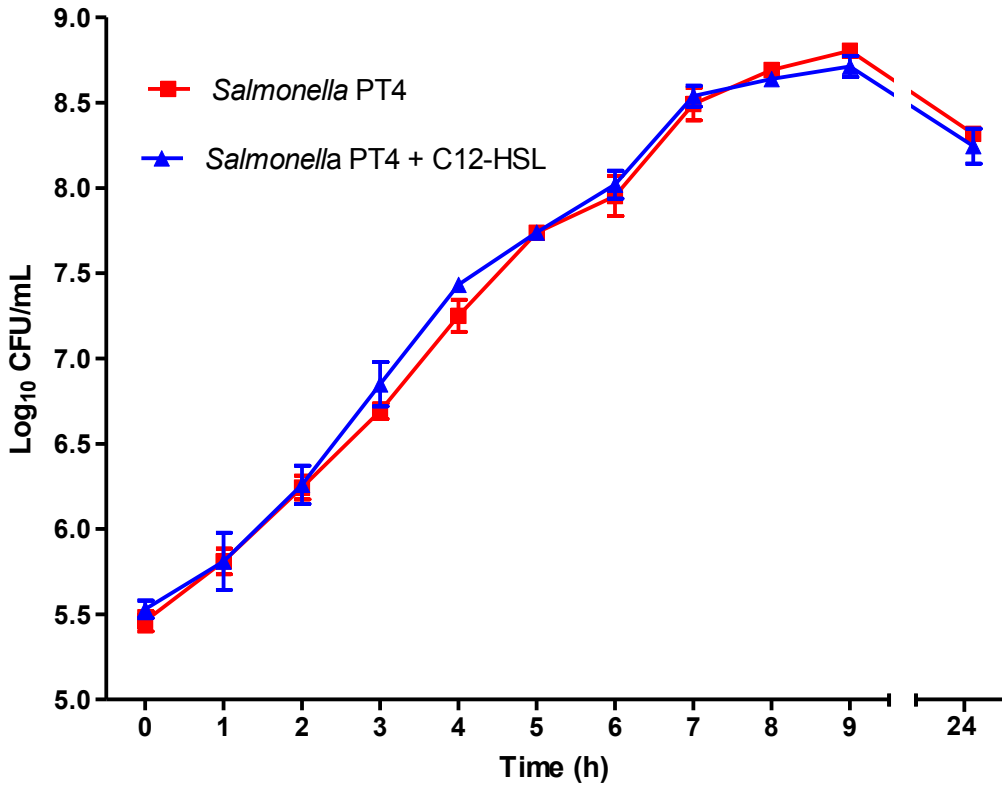

Figure S1. *Salmonella* PT4 growth in anaerobic tryptone soy broth (TSB) in the presence and absence of C12-HSL. *Salmonella* PT4: *Salmonella* PT4 grown in the absence of C12-HSL; *Salmonella* PT4 + C12-HSL: *Salmonella* PT4 grown in the presence of C12-HSL.

CHAPTER 3

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

Article formatted according to Brazilian Journal of Microbiology

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 the 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 the intestinal microbiota. Therefore, the aim of this study was to identify AHL-producing bacteria in the gut of larvae of *Galleria mellonella* and to evaluate the influence of these QS molecules 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 were 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*; they were named GM34, GM56, and GM60. The TLC showed that *R. inusitata* produces a six-carbon AHL. Extracts from the culture medium of *R. inusitata* isolates containing AHL were obtained; they increased the adhesion on stainless-steel coupons and swarming and twitching motilities of *Salmonella enterica* serovar Enteritidis PT4 under anaerobic conditions. The results suggest the possibility of communication between members of the *G. mellonella* microbiota with pathogens such as *Salmonella*.

1. 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) (Fuqua et al. 1994). 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 (Reading and Sperandio 2006; Papenfort and Bassler 2016). Some bacteria of the Enterobacteriaceae family, such as *Salmonella* and *Escherichia coli*, do not produce their own AHLs but are able to detect these molecules produced by other bacteria (Ahmer 2004; Kendall and Sperandio 2014). 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 important for distinguishing the types of AHLs and for their biological role (Liu et al. 2017; Saurav et al. 2020). Phenotypes such as bioluminescence, motility, biofilm formation, and virulence are controlled by AHL-mediated QS (Zhu et al. 2019; Cellini et al. 2020; Hayek et al. 2020).

The AHLs can be used for intra- and interspecies communication, contributing to a coordinated organization of the microbial community (Papenfort and Bassler 2016). The gut is a complex environment, with several Gram-positive and Gram-negative species (Lagier et al. 2012). 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 pig intestine (Yang et al. 2018), and AHLs have also been detected in human feces (Landman et al. 2018).

Evidence of these signaling molecules in the gastrointestinal tract leads to speculation regarding the involvement of these signal molecules in the regulation of intestinal microbiota. In the intestine of invertebrates, the presence of AHL-producing bacteria has already been confirmed (Borlee et al. 2008), 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 37ºC, which is the optimum temperature for many human pathogens (Fuchs et al. 2010; Tsai et al. 2016; Mukherjee et al. 2020). The species has an innate immune response, with characteristics similar to those of the mammalian immune system (Kavanagh and Reeves 2004; Wojda 2017). The invertebrate gut microbiota is largely dominated by Gram-positive bacteria, with a low concentration of Gram-negative bacteria (Allonsius et al. 2019). In addition, *G. mellonella* can be used as an oral infection model for human bacterial pathogens (Fedhila et al. 2010). Thus, the detection of signaling molecules in the intestine by Gram-negative pathogens could occur, and its effect on the infection course was unrevealed. Here, we detected AHL-producing bacteria from the *G. mellonella* gut and verified the influence of these molecules on the adhesion and motility of *Salmonella* Enteritidis PT4.

2. Material and methods

2.1. 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), *Chromobacterium violaceum* CV026, and *E. coli* pSB403 were used in this study. *Salmonella* PT4 was grown in tryptone soy broth (TSB; Sigma, India),

and the biosensors *C. violaceum* CV026 and *E. coli* pSB403 were grown in Luria-Bertani broth supplemented with kanamycin (20 μg/mL) and tetracycline (20 μg/mL), respectively (LB; Sigma, USA).

2.2. Larval culture

We used *G. mellonella* larvae, 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 (Freitas et al. 2021). Before the intestines were extracted, the larvae (250–300 mg weight) were kept in Petri dishes at 28°C for a 24-h fasting period.

2.3. Detection of AHL-producing bacteria in the larvae intestine

2.3.1. Gut dissection

Gut dissection was performed as described by Ignasiak and Maxwell (2018), with modifications. Larvae were anesthetized on ice and surface-disinfected with 70% ethanol for 45 s, followed by washing 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.

2.3.2. 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 (Berasategui et al. 2017). Serial dilutions were performed in phosphate-buffered saline (PBS; 10 mM, pH 7.2), and 200 µL were plated on LB agar 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.

2.3.3. 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 on LB agar plates with the biosensor strains *C. violaceum* CV026 (McClean et al. 1997) and *E. coli* pSB403, which was performed as described by Martins et al. (2014), 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 37ºC, 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.

2.3.4. 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 r.p.m. Bacteria were removed by centrifugation at 6,000 *g* for 20 min, and the supernatants were extracted three times with equal volumes of ethyl acetate acidified with 0.5% of formic acid (Pinto et al. 2007). 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 -20ºC.

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 (Shaw et al. 1997; Pinto et al. 2007). The retention factor 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)].

2.3.5. 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 1,000 replicates (Lee et al. 2020).

2.3.6. Phenotypic characterization of isolates

We characterized the phenotypic characteristics of each AHL-producing isolate. For this, the isolates were grown in LB broth for 24 h at 30ºC and then inoculated separately in media containing different carbohydrate sources at 1% (glucose, arabinose, glucose, fucose, lactose, maltose, mannose, rhamnose, sucrose, trehalose, xylose). The tubes were incubated for 24-48 h at 30ºC. Other biochemical assays were also performed, such as Voges-Proskauer, indole production, methyl red, citrate use, and catalase and hydrogen sulfide production assays.

2.4. AHL quantification

The concentration of AHLs in the extract was estimated according to Blosser and Gray (2000), with modifications. For this, *C. violaceum* CV026 was cultivated for 24 h at 28ºC, and 500 µL of the culture were inoculated in 4.5 mL of LB broth supplemented with kanamycin (20 µg/mL) and 10 µL ExCA. The tubes were incubated for 24 h at 28°C with shaking at 200 r.p.m. After incubation, 1 mL of each *C. violaceum* CV026 was centrifuged at 10,000 *g* for 10 min, the supernatant was discarded, and 200 µL of sodium dodecyl sulfate (SDS, 10%) were 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 ($y = 81.084 \times OD_{585} - 8.4278$), where y is the AHL concentration in nM and OD_{585} 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.

2.5. Effects of ExCA on *Salmonella* PT4 phenotypes

2.5.1. *Salmonella* cell preparation

Salmonella PT4 was cultivated in TSB under O_2-free conditions, as previously described by Almeida et al. (2018). 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 (OD_{600nm}), using a spectrophotometer. Then, 10 mL of anaerobic TSB (pH 7.0) were 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 37ºC.

2.5.2. Quantification of adhered and planktonic cells of *Salmonella* PT4

Salmonella PT4 cells grown for 7 h as described above were standardized to an OD_{600nm} of 0.1, and 100 μL were 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. (2019). 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 37ºC 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 (Morton 2001) 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; 10-fold serial dilutions were generated and plated on PCA.

2.5.3. 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).

2.5.4. Determination of swarming and twitching motilities

The TSB added with 0.7 or 1% (w/v) of agar was used for swarming and twitching motility assays, respectively, according to Rashid and Kornberg (2000), with modifications. A volume of 15 mL of the medium at 50ºC 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. Twitching motility was observed using an inoculum of 10 µL. After the aliquot had dried, plates were incubated at 37°C for 24 h in anaerobic jars with Anaerobac (Probac, Brazil).

2.6. Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were used for multiple comparisons. All statistical analyses were performed using the GraphPad Prism 5.00 software.

3. Results and discussion

3.1. Identification of AHL-producing isolates

The microbiota from the gut of *G. mellonella* was cultured in an aerobic and an anaerobic atmosphere on LB medium to count Gram-positive and Gram-negative bacteria and on MacConkey agar, with the objective to determine the Gram-negative microbiota. As shown in Figure 1, the colony-forming units per larva (CFU/larva) differed depending on the culture conditions. Aerobic incubation resulted in higher numbers of CFU/larva both in LB agar and MacConkey agar than in 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. (2019) and Ignasiak and Maxwell (2018), who 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* (Chung et al. 2018, Cassone et al. 2020).

A total of 138 isolates were analyzed for AHL production using *C. violaceum* CV026 and *E. coli* pSB403 as bioreporters, and only three were identified as AHL-producers, one from the anaerobic MacConkey (isolated 34) and two from the aerobic MacConkey (isolated 56 and 60). As shown in Figure 2A, the isolates induced violacein production when cross-streaked with the biosensor strain *C. violaceum* CV026. The three isolates also induced bioluminescence 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 (McClean et al. 1997; Winson et al. 1998). In Figure 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 produce the same signal molecule. These results suggest that GM34, GM56, and GM60 isolates produce an AHL with six carbons.

The sequencing data indicated a strong homology between the AHL-producers with the 16S rRNA gene of *Rahnella inusitata* (GM 34: 100%, GM56: 100%, and GM 60: 99.84%). In addition, the phylogenetic tree (Fig. 3) showed that AHL-producing strains form a tight subcluster with *R. inusitata* (previously *Rahnella* genomosp. 3, type strain DSM 30078) (Brady et al. 2014). Also, some phenotypic characteristics of isolates obtained in this study were compared with data from previously published studies (Brady et al. 2014; Lee et al. 2020). The results of 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* (Brady et al. 2014). These bacteria are distributed in different environments such as soil, food, plant rhizosphere, water, and human clinical samples (Brady et al. 2014; Godziszewska et al. 2017; Lee et al. 2019). *Rahnella* spp. is a Gram-negative bacterium and has been isolated from the gut of invertebrates (Brady et al. 2014; Wang and Zhang 2015; Gonçalves et al. 2019), 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 (Ignasiak and Maxwell 2018; Lou et al. 2020).

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 (Steidle

et al. 2001), and *R. aquatilis* isolated from cabbage produces C8-HSL, C10-HSL, and 3-oxo-C14-HSL (Myszka et al. 2015). The confirmation that *R. inusitata* isolated from the intestine of *G. mellonella* produces C6-HSL contributes to clarify 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 (Wu and Luo 2021). 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.

3.2. 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 Figure 4. It is evident that after 40 h of incubation, the presence of 250 nM AHL significatively 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 4D). 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 synthetizes the protein SdiA, homologous to LuxR, and can sense the AHL produced by other Gram-negative bacteria. The results of molecular docking 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 (Almeida et al. 2016). The greater biofilm formation by *Salmonella* in the presence of exogenous AHL under anaerobiosis has been described before, without influencing the growth (Campos-Galvão et al. 2015; Almeida et al. 2017).

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 Figure 5A, few *Salmonella* PT4-adhered cells were observed in the control treatment, i.e., without ExCA or C6-HSL, whereas the presence of 250 nM of ExCA or C6-HSL increased the population of adhered cells (Fig. 5B-E). The observation of 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 (Campos-Galvão et al. 2015), which indicates that C6-HSL can control phenotypes in this pathogen. In the gut lumen, *Salmonella* needs to adhere to the intestinal epithelial surface to begin the host invasion process (Dougan et al. 2011). 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 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 and twitching motilities were observed when compared

to the control group. The absence of swarming and twitching motilities in *Salmonella* Enteritidis PT4 578 under anaerobic incubation has been described by Almeida et al. (2017), 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 (Kim and Surette 2005), and genes involved in virulence, lipopolysaccharide (LPS) biosynthesis, and iron metabolism in *Salmonella* Typhimurium are upregulated during swarming (Wang 2004). In addition, swarmer differentiation in this serovar is related to antibiotic and other antimicrobial agent resistance, including cationic peptides (Kim et al. 2003). Interestingly, C12-HSL increased the resistance of *Salmonella* Enteritidis PT4 to nisin, a cationic peptide (Freitas et al. 2020). In some microorganisms, such as *E. coli*, *Pseudomonas aeruginosa*, *Burkholderia dolosa,* and *Yersinia enterocolitica,* QS is required during swarming motility (Jose and Singh 2020). Twitching motility is a surface movement that allows bacteria to explore an area through the extension and retraction of type IV pili (Park et al. 2015). This type of motility contributes to adhesion to the surface, biofilm formation, and virulence in some bacterial species (Merz et al. 2000; Mattick 2002; Weller-Stuart et al. 2017). However, in *Salmonella*, this mechanism is poorly understood, especially regarding the participation of QS in the regulation of type IV pili synthesis.

4. 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 infection and spread. However, in-depth investigations need to be carried out to better understand this "eavesdropping" between different bacterial species and its role in pathogenicity.

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).

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Declarations

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CRediT author statement

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.

Figures

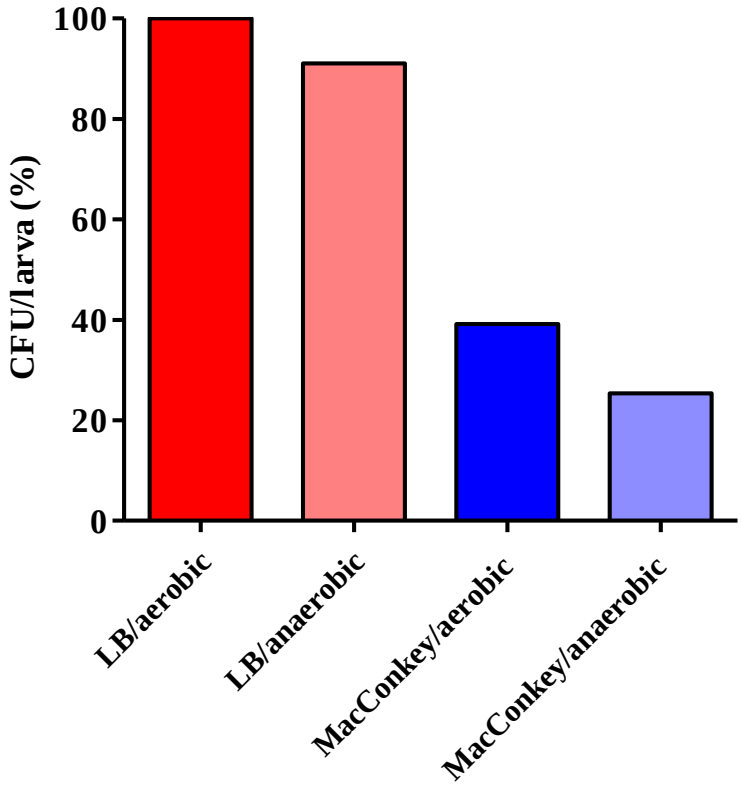

Figure 1: Standard count of bacteria from the gut of *G. mellonella*. Results are expressed as CFU per larva (CFU/larva) by the ratio of the number of total CFU and the number of larvae used (five larvae). The CFU/larva from LB in aerobiosis (LB/aerobic) was considered 100%.

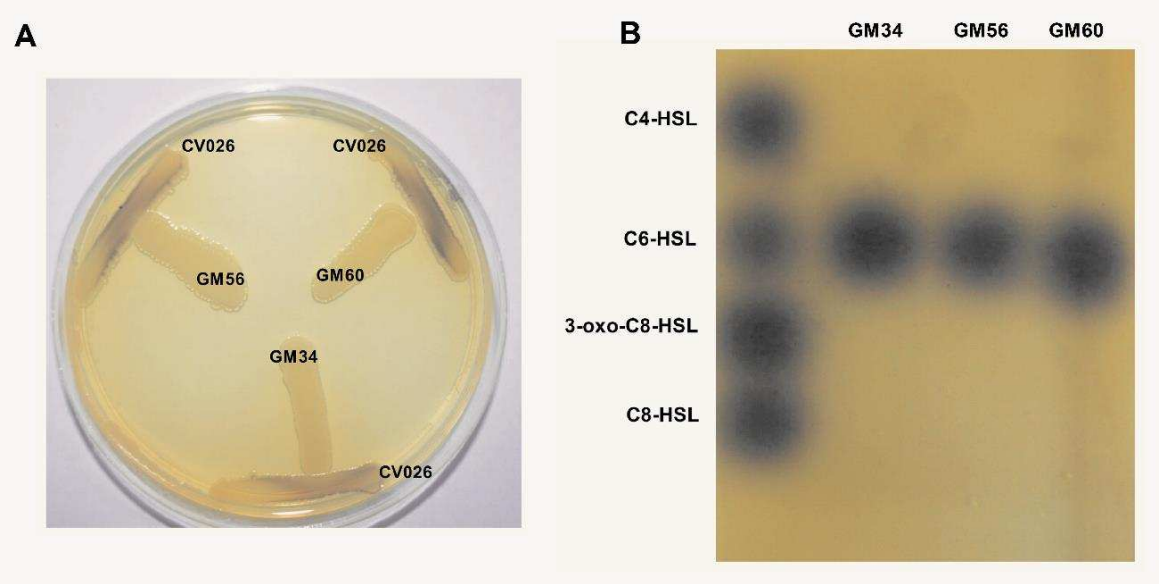

Figure 2: AHL screening of *R. inusitata* (GM34, GM56, and GM60) isolated from the gut of *G. mellonella* revealed with *C. violaceum* CV026. A: Cross-streaking of strains with *C. violaceum* CV026. B: AHL profile present in the extracts of the *Rahnella* isolates by thin-layer chromatography (TLC) with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

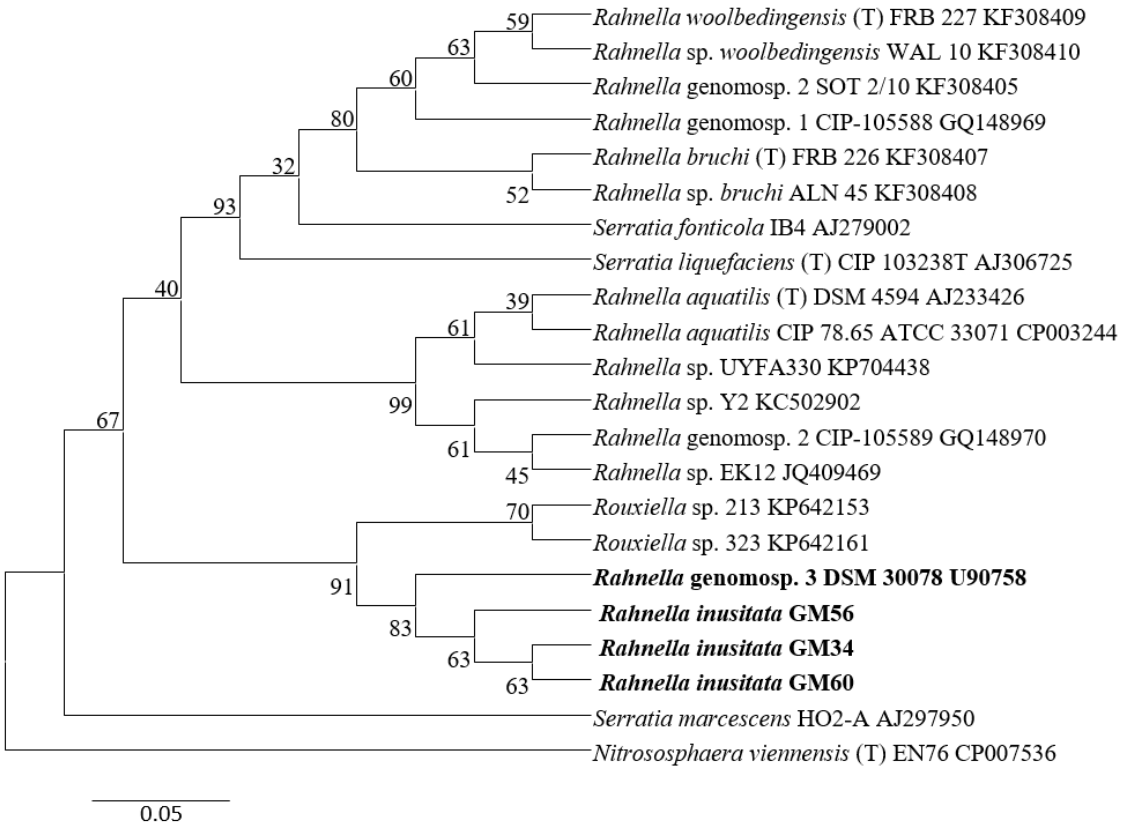

Figure 3: Neighbor-joining phylogenetic tree of *R. inusitata* (GM34, GM56 and GM60) isolated from the gut of *G. mellonella*. Alignments were performed with the 16S rRNA gene sequences of *R. inusitata* isolates and sequences deposited in the RDP database. *Nitrososphaera viennensis* was employed as outgroup, and 1,000 replicates are shown at branch nodes. *Rahnella* genomosp. 3 DSM 30078 was identified as *R. inusitata* by Brady et al. (2014).

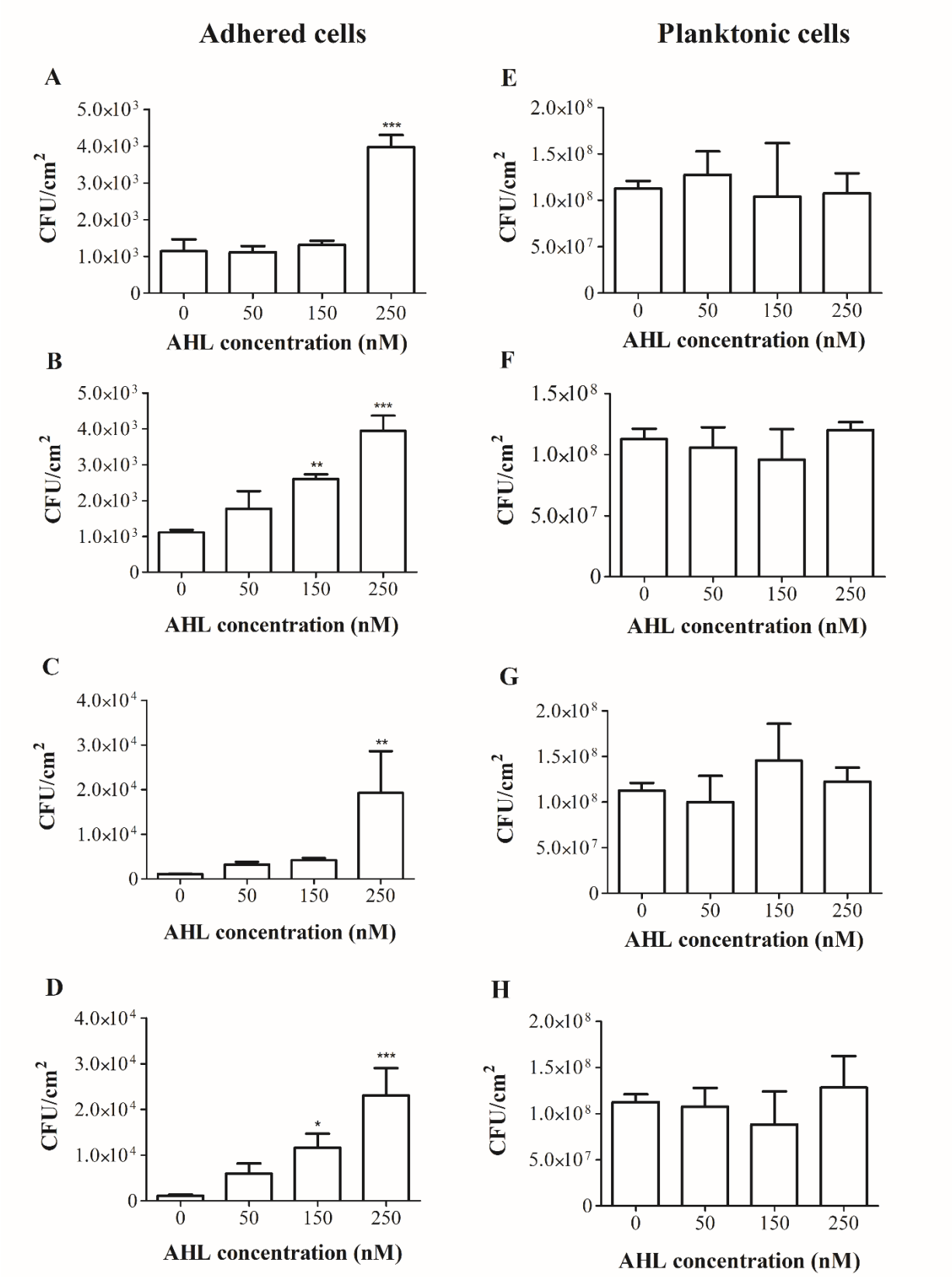

Figure 4: CFU/cm^2 of sessile and planktonic *Salmonella* cells on SS coupons in the presence of ExCA of *R. inusitata* and commercial C6-HSL after 40 h of incubation at 37°C under

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.

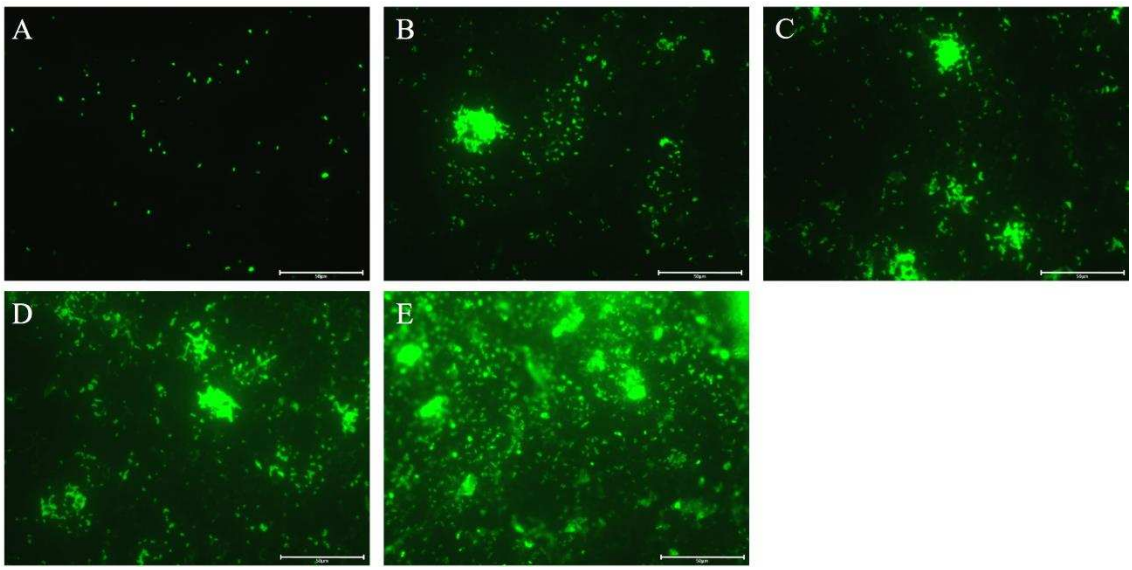

Figure 5: Epifluorescence microscopy micrographs of *Salmonella* adhesion on SS coupons in TSB broth and in the presence of ExCA *R. inusitata* and commercial C6-HSL after 40 h of anaerobic incubation at 37ºC. (A) control; (B) in the presence of ExCA-GM34; (C) in the presence of ExCA-GM56; (D) in the presence of ExCA-GM60; (E) in the presence of C6-HSL. The final concentration of AHL was 250 nM.

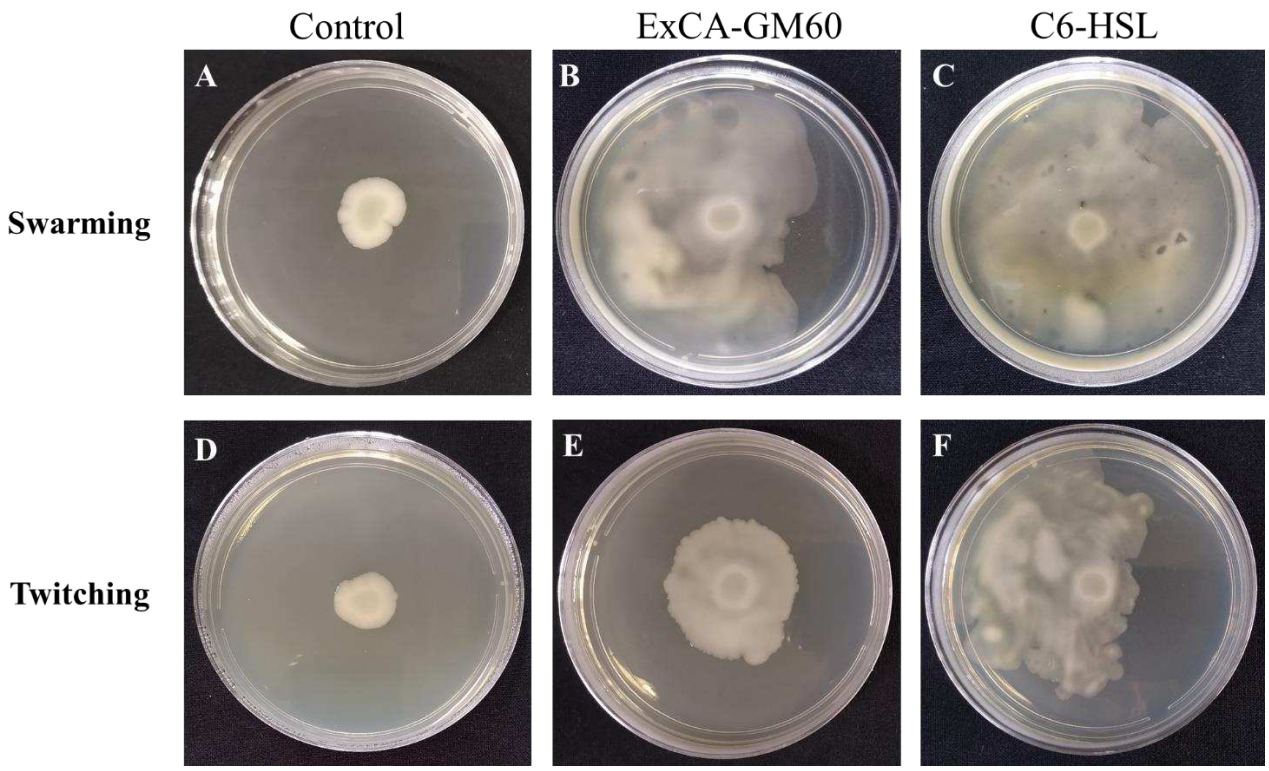

Figure 6: Motility assays of *Salmonella* PT4 in the presence of extract of isolate GM60 (ExCA-GM60) *R. inusitata* and commercial C6-HSL at a final concentration of 250 nM after 24 h of anaerobic incubation at 37ºC. (A-C) Swarming motility; (D-F) twitching motility.

Supplementary Material

Table S1: Phenotypic characteristics of *R. inusitata* isolates from the gut of *G. mellonella*.

Characteristic	GM34	GM56	GM60
Voges-Proskauer	+	+	+
Indole production	-	-	-
Methyl red	-	-	-
Citrate use (Simmons)	-	-	-
H_2S on Triple-sugar iron agar	-	-	-
Catalase	+	+	+
Use of			
D-Arabinose	+	+	+
D-Glucose	+	+	+
D-Fucose	+	+	+
D –Lactose	+	+	+
D- maltose	+	+	+
D-mannose	+	+	+
L-rhamnose	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
Xylose	+	+	+

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CONCLUSION AND PERSPECTIVES

Stressful environments are found in the food industry, in the passage through the gastrointestinal tract and within phagocytic cells and some human pathogens such as *Salmonella* have developed strategies to survive in those environments whose QS is critical to success this adaption. The results of the present study showed that the QS mediated by AHLs regulates multiple phenotypes in *Salmonella* Enteritidis PT4 including resistance to nisin and acidic stress, virulence, adhesion and motility. The deepening of the knowledge of the involvement of the QS in the resistance and virulence of *Salmonella* is of interest because strategies to inhibit cell communication can be used in order to reduce the intensity of these phenotypes in this pathogen, contributing to reduce the number of individuals infected by this foodborne pathogen annually.