DEISY GUIMARÃES CARNEIRO

WHOLE GENOME SEQUENCING ANALYSIS AND BIOFILM FORMATION IN *Salmonella* Enteritidis PT4 578

Thesis submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Maria Cristina Dantas Vanetti

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Assent:

Deisy Guimarães Carneiro
Author

Maria Cristina Dantas Vanetti
Adviser

To my family and
to everyone who cheers for me.

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ABSTRACT

CARNEIRO, Deisy Guimarães, D.Sc., Universidade Federal de Viçosa, December, 2022. **Whole genome sequencing analysis and biofilm formation in *Salmonella* Enteritidis PT4 578**. Adviser: Maria Cristina Dantas Vanetti.

Salmonella is an important and versatile foodborne pathogen responsible for numerous infections and deaths worldwide. The difficulty of controlling and eradicating this pathogen is related to its ability to form complex aggregates of cells, called biofilms. In a biofilm, cells are surrounded by a matrix of self-produced extracellular polymeric substances that protect against various types of stress, such as desiccation, sanitization, and host defense. The *Salmonella* biofilm matrix is composed mainly of curli fimbriae and cellulose. A highly complex genetic network regulates the synthesis of these components. The main regulators associated with biofilm formation are the CsgD protein and the bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), which activate the biosynthesis of most polymers. Quorum sensing, a cellular communication mechanism, is also described as an important regulator of biofilm formation in *Salmonella*. The objective of the present work was to sequence the genome of *Salmonella enterica* Enteritidis PT4 578, analyze the main characteristics related to virulence and biofilm formation, and compare the genome and morphotype formation, biofilm and motility phenotypes with four related serotypes. Furthermore, evaluate the influence of temperature, atmosphere, and quorum sensing on biofilm formation by this serotype. Genome analyzes showed that *Salmonella* Enteritidis PT4 578 has 165 virulence genes, representing 3.66% of the coding sequences. Twelve *Salmonella* pathogenicity islands (SPI) were identified, some extremely conserved, such as SPI1 and SPI2, that encode the apparatus of intracellular invasion and colonization,. A total of thirteen gene clusters related to fimbriae biosynthesis, including the *csg* operon, were also annotated. Under aerobic and anaerobic conditions, *Salmonella* Enteritidis PT4 578 can form a biofilm on stainless steel coupons at 28 °C and only under aerobic conditions at 37 °C. However, adding of *N*-hexanoyl homoserine lactone (C12-HSL) to the anaerobic culture medium reverses the biofilm formation phenotype at 37 °C. In this condition, C12-HSL increased the expression of the *adrA* gene, a diguanylate cyclase related to the synthesis of c-di-GMP and the *luxS* gene, which is part of another quorum sensing mechanism that uses autoinducer-2 (AI-2), present in *Salmonella*. Although it did not influence biofilm formation at 28 ºC, C12-

HSL also increased *luxS* expression. The data presented here reinforce the role of conditions of atmosphere and temperature, as well as quorum sensing in *Salmonella* biofilm formation. Finally, it reports a possible connection between this pathogen's two main quorum sensing mechanisms. Understanding the factors that influence *Salmonella* biofilm formation can contribute to developing control and eradication strategies.

Keywords: Genome analyses. Biofilm. Quorum sensing.

RESUMO

CARNEIRO, Deisy Guimarães, D.Sc., Universidade Federal de Viçosa, dezembro de 2022. **Análise de sequenciamento do genoma completo e formação de biofilme em *Salmonella* Enteritidis PT4 578**. Orientadora: Maria Cristina Dantas Vanetti.

Salmonella é um importante e versátil patógeno de origem alimentar, responsável por numerosas infecções e mortes no mundo todo. A dificuldade de controle e erradicação da transmissão está relacionada com a sua capacidade de formar agregados complexos de células, chamados de biofilme. Em um biofilme, as células são envoltas por uma matriz de substâncias poliméricas extracelulares autoproduzidas que confere proteção a diversos tipos de estresse, como dessecação, sanitização e defesa do hospedeiro. A matriz do biofilme de *Salmonella* é constituída principalmente por fímbrias curli e celulose. A síntese desses componentes é regulada por uma rede gênica altamente complexa. Os principais reguladores associados à formação de biofilme são a proteína CsgD e o monofosfato de guanosina bis-(3'-5')-cíclico dimérico (c-di-GMP), que ativam a biossíntese da maioria dos polímeros. O *quorum sensing*, um mecanismo de comunicação celular, também é descrito como um importante regulador da formação de biofilme em *Salmonella*. Deste modo, o objetivo do presente trabalho foi sequenciar o genoma de *Salmonella enterica* Enteritidis PT4 578 e analisar as principais características relacionadas à virulência e a formação de biofilme, bem como comparar o genoma e os fenótipos de formação de morfotipo, biofilme e motilidade com quatro sorotipos relacionados. Além disso, foi avaliada a influência da temperatura, atmosfera e do *quorum sensing* na formação de biofilme por esse sorotipo. As análises do genoma demonstraram que *Salmonella* Enteritidis PT4 578 possui 165 genes de virulência, o que representa 3,66% das sequências codificadoras. Doze ilhas de patogenicidade de *Salmonella* (SPI), algumas extremamente conservadas, como SPI1 e SPI2 que codificam o aparato de invasão e colonização intracelular foram identificadas. Um total de treze clusters de genes relacionados à biossíntese de fímbrias, incluindo o operon *csg*, também foram encontrados. Em condições aeróbias e anaeróbias, *Salmonella* Enteritidis PT4 578 é capaz de formar biofilme em cupons de aço inoxidável a 28 ºC enquanto a 37 ºC, o biofilme foi formado apenas em condições aeróbias. Entretanto, a adição de *N*-hexanoil homoserina lactona (C12-HSL) ao meio de cultura anaeróbio resultou na formação de biofilme a 37 °C. Nessa condição, C12-HSL aumentou a expressão do gene *adrA*, uma diguanilato ciclase

relacionada com a síntese de c-di-GMP e do gene *luxS*, que faz parte do mecanismo de *quorum sensing* que utiliza o autoindutor-2 (AI-2), presente em *Salmonella*. Embora não tenha influenciado a formação de biofilme a 28 ºC, a C12-HSL também aumentou a expressão de *luxS*. Os dados apresentados reforçam a atuação das condições de atmosfera e temperatura, bem como do sistema *quorum sensing* na formação de biofilme em *Salmonella*. Por fim, relata uma possível conexão entre os dois principais mecanismos de *quorum sensing* neste patógeno. A compreensão dos fatores que influenciam a formação de biofilme em *Salmonella* pode contribuir para a elaboração de estratégias para seu controle e erradicação.

Palavras-chave: Análises do genoma. Biofilme. Quorum sensing.

SUMMARY

GENERAL INTRODUCTION

Salmonella enterica serotype Enteritidis is a generalist pathogen infecting a wide variety of animals and has a low rate of human invasiveness, usually causing self-limiting gastroenteritis. In addition, this serotype has a remarkable ability to adapt to niches and hosts and has been frequently explored in research for understanding behaviors such as virulence, biofilm formation, and quorum sensing.

The pathogenicity of *Salmonella* is related to the presence of genes that encode virulence factors. Most of these genes are grouped in *Salmonella* Pathogenicity Islands (SPIs) clusters. Sequence analyzes of different *Salmonella* genomes demonstrated that these genes have distinct properties from the rest of the genome, such as GC content, indicating that they were acquired from other species by horizontal gene transfer. The acquisition of SPIs was a defining event in *Salmonella* evolution and is thought to have occurred after common ancestor divergence with *Escherichia coli*.

The ability of *Salmonella* to adhere to surfaces and form complex communities, called biofilms, contributes to its persistence and colonization of different niches, inside and outside the host. While the bacteria reside within a biofilm, they are protected from various challen ges such as environmental stress, antimicrobial agents, host defense mechanisms, and antibiotics, so they can be challenging to eradicate. Moreover, the detachment of cells from the biofilm is a frequent source of cross-contamination in the food industry and infections. The protection offered by the biofilm is due to the difference in the physiological state of the cells and the polymeric matrix, called extracellular polymeric substances (EPS). The main components of the biofilm matrix produced by *Salmonella* are curli fimbriae, the major proteinaceous component, and cellulose.

A highly complex genetic network regulates the synthesis of biofilm structural components. The central regulators associated with biofilm formation are the CsgD protein and the bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) messenger molecule, which activate the biosynthesis of most polymers. Numerous environmental factors affect the expression of curli and cellulose fimbriae in many *Salmonella* isolates. Environmental conditions are transduced into intracellular signals via outer membrane proteins, two-component signal transduction systems, regulatory proteins, and enzymes associated with c-

di-GMP production. A large 521 bp intergenic region is located upstream of the *csgD* promoter allowing a range of regulatory interactions.

Quorum sensing is a cellular communication system that allows the regulation of gene expression synchronously in response to changes in population density. This mechanism has already been described as a regulator of *Salmonella* biofilm formation in some specific conditions. For example,the quorum sensing system mediated by autoinducer 1, the acyl homoserine lactones (AHLs), promotes biofilm formation and the expression of some genes related to motility in *Salmonella* cells cultivated anaerobically at 37 ºC. However, the mechanisms behind this regulation remain unknown.

Thus, understanding the different factors that regulate biofilm formation in *Salmonella* is crucial for developing efficient strategies for its control. Therefore, this study aims to understand how different environmental factors and quorum sensing influence biofilm formation by *Salmonella* Enteritidis PT4 578. Furthermore, through genome sequencing and genome annotation, we seek to understand the intrinsic characteristics of this strain.

CHAPTER 1

An overview of factors that influence biofilm formation by *Salmonella enterica*

Abstract

The survival in the host and the persistence of *Salmonella enterica* in the environment are improved by its enormous adaptive capacity and ability to form biofilms in different conditions and surfaces. Biofilms are formed by communities of sessile cells surrounded by a tridimensional matrix of extracellular polymeric substances (EPS) that can be fixed to the substrate or not. In *S. enterica*, this matrix is mainly comprises of curli fimbriae, cellulose, and colanic acid. A biofilm matrix offers tremendous protection against antimicrobials, desiccation, sanitizers, and host defense. For this reason, eradicating *S. enterica* from the food production chain is challenging. In addition, biofilms are highly dynamic, and some conditions favor the detachment of cells or even cell blocks. In this way, pathogens can overcome adverse conditions due to the protection provided by biofilms and detach themselves when conditions are more favorable for planktonic growth to restart a new cycle of contamination. Considering that *S. enterica* biofilms represent a problem for health and the food processing industry, this review brings an approach to the factors described in the current literature that affect the formation of biofilms in *S. enterica*.

Keywords: foodborne pathogen, biofilm regulation, c-di-GMP, quorum sensing.

1 Introduction

During most of the history of microbiology, microorganisms were characterized as unicellular and freely suspended life forms due to observations of growth in laboratory culture medium. Today, the most current conception is that biofilm is the way of growth in nature due to the numerous advantages that this lifestyle offers (Steenackers et al., 2012). Biofilms are a fantastic lifestyle in which microorganisms switch from free-floating (planktonic) to sessile and collective lifestyles, with a high level of organization. Enclosed in a biofilm, cells have different metabolism and gene expression than planktonic cells (White et al., 2010; Giaouris et al., 2013).

In the biofilm, microbial cells form aggregates and are incorporated into a three-dimensional, self-produced matrix of extracellular polymeric substances (EPS) and may or may not attach to a surface (Flemming et al., 2016; Tursi and Tükel, 2018). Matrix components vary by microorganism, surface, substrate, temperature, oxygen tension, presence of nutrients, inhibitors, enzymes, and other microorganisms, as well as other factors (Steenackers et al., 2012). The EPS matrix also increases resistance to stresses such as host immune defense or antimicrobial substances (Hall-Stoodley et al., 2004; Cadena et al., 2019).

Biofilm may be constituted by single species (monospecies biofilm), by two or more species (multispecies biofilm), or even by microorganisms of different taxonomic levels, such as fungi and bacteria (polyspecies biofilm) (Guzman-Soto et al., 2021). Although the biological diversity in biofilms is well recognized, the number of cells needed to compose it remains debatable (Flemming and Wuertz, 2019)

Biofilms develop through five stages, which can be reversible or irreversible: (a) reversible bacterial adhesion to the surface, (b) irreversible adhesion to the surface, (c) microcolony development, (d) maturation of the biofilm architecture, (e) dispersion. (Gupta et al., 2016; Guzmán-Soto et al., 2021). It is noteworthy that the dispersion of cells or parts of biofilms can occur at any stage of biofilm formation, and due to this dynamic nature, biofilms are frequent sources of cross-contamination, also causing an enormous economic impact (Winder et al., 2008; Galié et al., 2018; Merino et al., 2019).

Biofilms formed in the environment are not just a persistence strategy for facultative pathogens outside the host but a relevant reservoir for triggering new infections (Schulze et al., 2021). For example, an investigation involving feces and food related to outbreaks in

Brazil found several *Salmonella* serotypes circulating for decades, mainly within the poultry production chain (de Melo et al., 2021). Therefore, biofilm formation is an essential aspect of many, if not all, chronic diseases (Vestby et al., 2020). Bacteria belonging to the *Salmonella enterica* are mostly pathogenic and responsible for countless deaths and infections worldwide (Balasubramanian et al., 2019; Stanaway et al., 2019). Adhesion and biofilm formation are essential to ensure persistence outside the host and contribute to the development of infection within the host (Adcox et al., 2016). This review will address various factors that influence biofilm formation in *S. enterica*.

2 *Salmonella enterica*, a persistent problem

Salmonella belongs to the Enterobacteriaceae family and comprises Gram-negative, neutrophilic, facultatively anaerobic, non-sporulating bacilli. It was isolated for the first time in 1885 by Theobald Smith and Daniel Salmon, who called it "The bacterium of swine plague". In 1900, Joseph Leon Lignières named this pathogen *Salmonella* as a tribute to Daniel Salmon (Eng et al., 2015). The taxonomic classification of the genus is very complex and controversial. Based on differences in the *16s RNA* sequence, it divides into just two species, *Salmonella enterica* and *Salmonella bongori* (Popoff et al., 2003). *S. enterica* is generally subdivided into seven subspecies based on genomic relationships and biochemical properties. However, genotyping analyzes indicated the probable existence of at least three new genetically distinct subspecies (Alikhan et al., 2018). Phenotypically, through the use of specific antibodies to identify the three major antigens, somatic (O), capsular (K), and flagellar (H), *Salmonella* is further divided into more than 2,659 serotypes (Issenhuth-Jeanjean et al., 2014). Serotyping is the most used method for epidemiological screening (Diep et al., 2019). Serotypes that cause disease in humans and warm-blooded animals mainly belong to the *S. enterica* subspecies enterica (I). These serotypes are often clinically divided into typhoidal and non-typhoidal *Salmonella* (NTS) (Andino and Hanning, 2015). Although *S. bongori* has been reported to cause human disease, this species is more closely related to cold-blooded animals (Fookes et al., 2011)

Salmonella has been a public health concern for over a century, contributing to the economic burden of food contamination in developed and developing countries associated with the costs of treating and preventing infections (Majowicz et al., 2010). Chickens, pigs, and cattle are the main reservoirs, and animal foods play a significant role in human NTS (Stevens and Kingsley, 2021). Therefore, human infections are most associated with

consuming contaminated food, especially animal origin. An estimated 535,000 NTS diseases and 77,500 deaths due to this disease occurred in 2017 (Marchello et al., 2022). Among many factors, the difficulty of controlling and eradicating *Salmonella* from the food production chain is due to its ability to adhere to and form biofilms (Merino et al., 2019).

3 Main structural components of *Salmonella* biofilms

The matrix of *Salmonella* biofilm is composed mainly of proteins and exopolysaccharides. The protein fraction consists of curli fimbriae and the BapA protein, while the exopolysaccharides fraction consists of cellulose and colanic acid. Other components, such as fatty acids and extracellular DNA, also comprise the *Salmonella* biofilm matrix (Steenackers et al., 2012). A deep understanding of EPS would help control biofilms for purposes of safety and biotechnology, including a combination of bacterial genetics, systems biology, and surfaces (Tan et al., 2014).

The fimbriae are one of the most important factors in the specific adhesion of *Salmonella* to the intestinal epithelium during the infectious process and for biofilm formation (Humphries, 2001). Fimbriae are divided into different types based on the way of assembly, where each type has its structural subunit and biogenesis genes encoded by a cluster of fimbrial genes (Yue et al., 2012). Thirty-eight unique clusters of fimbrial genes have been identified in 111 sequenced *Salmonella* genomes from 34 serotypes (Rehman et al., 2019). It has already been demonstrated that, in *S. enterica* serotype Typhimurium, type 1 fimbria (*fim*), plasmid-encoded fimbria (*pfe*), aggregative fimbriae or curli fimbriae (*csg*), long polar fimbriae (*lpf*), and bovine colonization factor (*bcf*) are related to the formation biofilm on epithelial cells (Ledeboer and Jones, 2005; Ledeboer et al., 2006). The curli fimbriae encoded by the bidirectional *csgBAC-csgDEFG* (curli-specific gene) operons are fimbriae with a *β*-sheet conformation known as amyloid (Van Gerven et al., 2018; Tursi and Tükel, 2018). Due to their morphology, they are more resistant than common fimbriae and are the main protein components of the biofilm matrix (Barnhart and Chapman, 2006; Erskine et al., 2018).

The biofilm-associated (BapA) protein, a large cell surface protein, also makes up the protein part of the biofilm matrix. Deletion of the *bapA* gene in *S. enterica* serotype Enteritidis causes loss of ability to form biofilms. However, this deficiency in biofilm formation by the *bapA* mutant strain is compensated for by the overproduction of curli fimbriae (Latasa et al., 2005).

The main component of the exopolysaccharides fraction is cellulose, a *β*-1-4-D–glucose polymer encoded by the *bcsABZC-bcsEFG* (bacterial cellulose synthesis) operons (Solano et al., 2002). Cellulose provides cohesion and structural integrity to mature biofilms (Jonas et al., 2007), and biofilm strength depends on it (Kim et al., 2022).

Colanic acid is another exopolysaccharide component of the *Salmonella* biofilm, a negatively charged polysaccharide polymer composed of repeating units of glucose, fructose, and glucuronic acid (Tursi and Tükel, 2018).

4 Regulators of biofilm formation in *Salmonella*

A highly complex genetic network regulates the synthesis of biofilm structural components (Fig. 1). The central regulators associated with biofilm formation are the protein CsgD and the messenger molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), which activate the biosynthesis of most biofilm polymers (Steenackers et al., 2012).

The transcriptional regulator CsgD acts to synchronize several processes that determine the production of the cell matrix (Gerstel and Römling, 2003). This protein consists of an *N*-terminal receptor domain with a conserved aspartate (D59) as a putative target site for phosphorylation and a *C*-terminal helix-turn-helix DNA binding motif similar to LuxR (Romling et al., 2000). In its active, non-phosphorylated form, the CsgD protein activates the expression of divergently transcribed biogenesis of *csgDEFG* and *csgBAC* operons (Zakikhany et al., 2010). The *csgDEFG* operon encodes regulatory proteins that regulate, export, and assemble the curli fimbriae. In contrast, the *csgBAC* operon encodes proteins that make up the curli fimbriae (Bamhart and Chapman, 2006).

CsgD positively regulates cellulose biosynthesis through the *adrA* gene encoding a diguanylate cyclase. The AdrA protein promotes cellulose biosynthesis through interaction at the post-transcriptional level with the products of the two divergent operons *bcsABZC* and *bcsEFG* or through the production of c-di-GMP, which acts as an activator of the biosynthesis of this polymer (Römling, 2002).

The expression of the *bapA* gene and the *bapBCD* operon that encode a type I protein secretion system is coordinated with the expression of the genes that encode curli fimbriae and cellulose through the action of CsgD (Latasa et al., 2005). Additionally, O-antigen

capsule production via the *yihU-yshA* and *yihVW* operons is co-regulated with the expression of curli fimbriae and cellulose via CsgD (Gibson et al., 2006).

The c-di-GMP is recognized as a bacterial second messenger and a key regulator of biofilm formation (Jenal et al., 2017). It is produced through two molecules of guanosine triphosphate (GTP) by diguanylate cyclases (such as AdrA), proteins characterized by the domain of amino acids GGDEF, which are essential for its activity (García et al., 2004; Paul, 2004). The broad range of mechanisms by which c-di-GMP affects biofilm formation is due to its ability to allosterically bind to effector components, which can be proteins or RNAs, and alter their structure and, or function (Hengge, 2009). At increased levels, this messenger suppresses motility and promotes the synthesis of matrix compounds (Kader et al., 2006). Furthermore, the c-di-GMP signaling mediates the transition between virulence properties and biofilm formation in the host (Ahmad et al., 2011). Cellulose is an antivirulence factor, and within macrophages, the MgtC protein prevents cellulose synthesis by regulating c-di-GMP levels during acute infection. However, the expression of cellulose within macrophages is a resource used by *Salmonella* to slowly exploit host resources, prolonging the infection and increasing the chance of transmission to new hosts (Pontes et al., 2015).

As CsgD can be seen as the control point of biofilm formation, regulating the expression of the main constituents, multiple environmental factors such as temperature, oxygen tension, and nutrient availability act as inducing or repressor signals of its expression (Fig. 1) (Gerstel and Römling, 2003). Environmental conditions are transduced into intracellular signals via outer membrane proteins, two-component signal transduction systems, regulatory proteins, and enzymes associated with c-di-GMP production (Steenackers et al., 2012). A large 521 bp intergenic region is located upstream of the *csgD* promoter and allows numerous regulatory interactions (Gerstel et al., 2006). Several regulators that modulate *csgD* promoter transcription have already been reported, such as OmpR, PhoP, Crl, RpoS, MlrA, CpxR, H-NS, and IHF. In *Escherichia coli*, CpxR, H-NS, IHF, OmpR, and RstA bind directly, and it is believed that there is cooperation in regulation between the positive factors (OmpR and IHF; and RstA and IHF) and also between the negative factors (CpxR and H-NS). Complex and redundant regulation allows fine-tuning of the regulatory network and the generation of fast and well-controlled responses to changes in environmental conditions (Ogasawara et al., 2010; Steenackers et al., 2012).

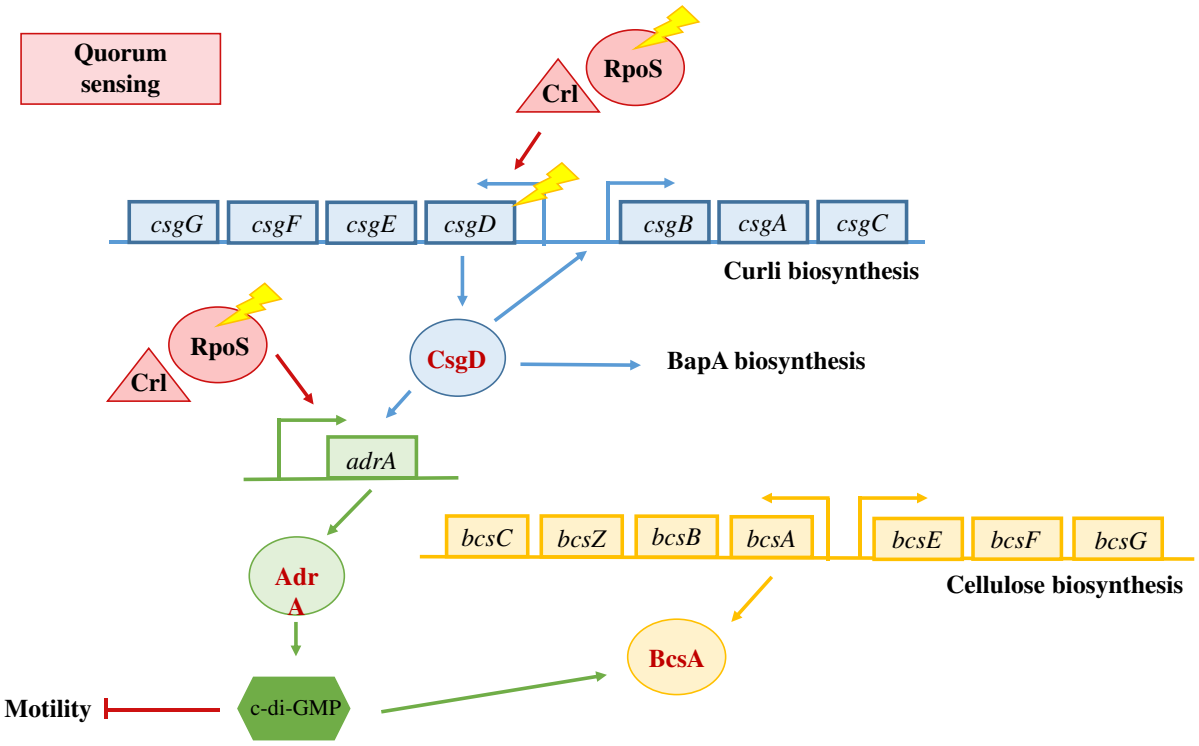

Fig. 2. Major components of the complex regulatory network that governs *Salmonella* biofilm formation. Arrows and flat-headed arrows represent an activation and a suppression effect, respectively. Blue, green and orange rectangles represent the genomic organization of the genes that encode the main structural components of the biofilm. Circles and triangles represent major regulatory proteins. Lightning symbols represent the entry and integration of different environmental signals through these general regulators into the regulatory system Adapted (Steenackers et al., 2012).

5 Main factors that influence the formation of *Salmonella* biofilms

5.1 Serotype

As discussed earlier, the genus *Salmonella* has a high diversity, and within the two species, there is an enormous variety of serotypes. There is extensive variation between strains of *S. enterica* regarding the ability to form a biofilm and how the different environmental parameters affect this ability (Lianou and Koutsoumanis, 2012). It is believed that the amount of biofilm produced under specific environmental stimuli may represent complex selection pressures to which certain bacterial strains were exposed (Lianou and Koutsoumanis, 2012). For example, it has been shown that there is a correlation between the ability to form biofilm and the capacity of strains to colonize and replicate within the intestines of various host species (MacKenzie et al., 2017). The biofilm formation is related to

generalist strains, whereas most host-adapted strains cannot form biofilms (MacKenzie et al., 2017). Genetic comparisons between serotypes demonstrated that fimbrial operons, especially the *csg* operon, are conserved in the genus *Salmonella* (Suez et al., 2013; Ksibi et al., 2022). The comparison between gene sequences also demonstrated that the loss or reduction of the ability to form biofilm in many serotypes is related to mutations in the *csgD* promoter region or gene that regulates flagellar gene expression and motility, such as *sirA* (Ramachandran et al., 2016; MacKenzie et al., 2019).

According to Agarwal et al. (2011), the intrinsic characteristics of the strains, including the presence of fimbria, flagellum, and other components, may be more relevant for biofilm formation than environmental conditions. Although this issue is debated, the available results suggest an intense interaction between genetic components and environmental factors.

5.2 Motility

Bacteria have evolved different strategies for moving on different surfaces. Through chemotaxis, cells can monitor changes in environmental signals and adapt their motility machinery to seek nutrients, evade toxic compounds, and colonize new niches (Wadhams and Armitage, 2004; Colin et al., 2021). Flagella are the main and best-understood motility organs (Jarrell and McBride, 2008). *Salmonella* exhibits two types of flagella-mediated motility: swimming and swarming. Swimming is an individual bacterial movement driven by rotating flagella, and swarming is a multicellular movement involving the generation of viscous colonies that expand across the surface. Swimming can be visualized in the laboratory using a medium with a 0.1 to 0.3% agar while swarming with a concentration of 0.4–0.7% (Kearns, 2010). Even in rich medium, supplementation with a source of sugar is necessary for *Salmonella* Typhimurium swarming movement (Harshey and Matsuyama, 1994).

Flagella play an essential role in the initial adhesion and dispersal stages (Verstraeten et al., 2008). *Salmonella* mutants *flgE* (flagellar hook) and *fliC* (flagellin protein) formed less biofilm than wild type in the early stages (Wang et al., 2020). However, at other stages of biofilm development, the expression of flagella is inhibited. It is known that the intracellular second messenger c-di-GMP plays a key role in this transition between mobile and sessile lifestyles, increasing the synthesis of cellulose and fimbriae and decreasing the synthesis and functioning of flagella (Hengge, 2009). This coordinated regulation occurs through two proteins, BcsA and YcgR, which have a c-di-GMP binding domain. When binding to c-di-

GMP, BcsA synthesizes cellulose, and YcgR inhibits motility through its interaction with flagellar motor proteins (Zorraquino et al., 2013).

5.3 Surface

Bacteria can move across a surface using chemotaxis through motility organs such as flagella, or they can be moved by physical forces such as Brownian diffusion, gravitational sedimentation, and hydrodynamic forces (Kimkes and Heinemann, 2020). The first step in adhesion involves the attraction of cells to the surface, followed by adsorption and attachment (Katsikogianni and Missirlis, 2004). Once on the surface, fixation is determined by physicochemical interactions such as Van der Waals forces, acid-base, and electrostatic interactions. At this stage, synthesizing bacterial appendages, such as the fimbriae and pili, reduces repulsion and increases adhesion (Guzmán-Soto et al., 2021).

Environmental conditioning, properties, and material surface characteristics also influence bacterial adhesion. Conditioning is related to the accumulation of molecules at the solid-liquid interface that will alter the physicochemical properties of the surface (Palmer et al., 2007). Material surface characteristics that affect adhesion include chemical composition, surface charge, and hydrophobicity (Katsikogianni and Missirlis, 2004). In addition, the presence of nanoscale asperities reduces the physical-chemical potential barrier a bacterial cell encounters when it approaches the surface (Ammar et al., 2015).

There are reports of *Salmonella* adhesion to abiotic (plastic, rubber, glass, stainless steel) and biotic (plants, animal epithelial cells, gallstones) surfaces (Steenackers et al., 2012). In industry, the main materials on the surface in contact with food are stainless steel, glass, plastic, Teflon, and rubber (Chia et al., 2009; Fink et al., 2017). The nature of these surfaces plays a crucial role in biofilm formation in food processing industries (Merino et al., 2019). Most laboratory biofilm studies primarily use polystyrene, stainless steel, and glass to simulate biofilm formation in these environments (Steenackers et al., 2012). Some reports of *Salmonella* biofilm formation on these surfaces have shown that the ability to adhere to hydrophobic surfaces, such as polystyrene, is more significant compared to hydrophilic ones, such as stainless steel and glass (Agarwal et al., 2011; Steenackers et al., 2012; Borges et al., 2018).

5.4 Oxygen tension

Salmonella has a cyclic life cycle, in which colonization of the host, an anaerobic environment, is alternated with periods of survival outside the host, an aerobic environment (Winfield and Groisman, 2003). In an aerobic environment, adhesion and biofilm formation play a clear role in resistance, persistence, and transmission between hosts (Fabrega and Vila, 2013). Numerous studies demonstrate the ability of different serotypes of *Salmonella* to form biofilm under aerobic conditions (Stepanovic et al., 2004; Lamas et al., 2016a; Borges et al., 2018).

The effect of anaerobiosis in biofilm formation in *Salmonella* still needs to be fully elucidated. Gerstel and Romling (2001) demonstrated that the response of the *csg* promoter to oxygen tension depends on the composition of the medium. At the temperature of 28 °C, at low oxygen tensions, the expression is negatively regulated in a minimal medium and positively regulated in a rich medium. However, Lamas et al. (2016b) demonstrated that the expression of the *adrA*, *csgD*, *luxS*, and *sdiA* genes is downregulated in microaerobiosis and anaerobiosis in tryptone soy broth (TSB), decreasing biofilm formation at 37 °C. In addition to the host, anaerobic environments can be found in the food industry. Controlled atmospheric storage is used to preserve food (Deuchande et al., 2016). Using modified atmosphere packaging by eliminating oxygen or replacing it with other gases is a strategy to inhibit microbial growth and promote oxidative stability (Arvanitoyannis and Stratakos, 2012). Furthermore, an oxygen gradient is present in biofilms. Due to diffusion limitations, bacteria in the biofilm's innermost layers find themselves in a particular environment with poor oxygen and nutrients (Stepanović et al., 2003).

5.5 Temperature

Generally, the temperatures evaluated for biofilm formation in *Salmonella* are important for the life cycle in food processing and infection. Temperatures from 20 to 30 °C were described as optimal for expressing the main components of the extracellular matrix of the *Salmonella* biofilm (Steenackers et al., 2012). Stepanović et al. (2003) observed increased biofilm production at 30 °C compared to incubation at 37 °C. Piras et al. (2015) reported a higher biofilm production at 22 °C than at 35 °C. Biofilm formation at temperatures below 30 °C was related to the thermoregulated Crl protein, which was higher at temperatures 30 ºC than 37 ºC in *Salmonella* (Bougdour et al., 2004; Robbe-Saule et al., 2006). The Crl protein stimulates the sigma factor σ^S (RpoS) activity, increasing the *csg* operon's transcription rate (Pratt and Silhavy, 1998; Bougdour et al., 2004).

High temperatures reduce curli fimbriae biosynthesis, but some environmental signals, such as iron depletion, can revert this inhibition (Gerstel and Römling, 2003). Mutations in the *csgD* promoter region can also restore *csgD* transcription and curli fimbriae production at higher temperatures (Romling et al., 1998). These mutations render transcription from the *csgD* promoter completely independent of RpoS (Romling et al., 1998). Temperatures below 30 ºC are recognized as optimal for forming standardized aggregative colonies in agar containing Congo Red, known as the red, dry, and rough (rdar) morphotype (Römling, 2005). This morphotype is an extensively studied phenotype of biofilm formation in *Salmonella* and is related to the expression of curli fimbriae and cellulose (Römling et al., 2003; White and Surette, 2006). Although reduced, the ability of *Salmonella* to form biofilms has been reported at temperatures below 20 °C, including refrigeration temperatures on different food industry surfaces (Borges et al., 2018; Webber et al., 2019).

5.6 Quorum sensing

Quorum sensing is a widely studied cell-cell communication mechanism between microorganisms. It consists of a cell-density-dependent chemical signaling system through which bacteria manage to act collectively and increase resistance to stressful situations. Quorum sensing is based on synthesizing small molecules called autoinducers (AI) that are secreted into the environment and, when internalized, they bind to receptor molecules that regulate the expression of target genes (Keller and Surette, 2006; Hense and Schuster, 2015). Quorum sensing has been implicated in biofilm formation and virulence in several bacteria (Mukherjee and Bassler, 2019). In *Salmonella*, three quorum sensing systems, mediated by acyl homoserine lactones (AHLs or AI-1), AI-2, and AI-3, have already been described (Walters and Sperandio, 2006).

Acyl homoserine lactones (AHLs) are signaling molecules of the quorum sensing mechanism in Gram-negative bacteria (Lazdunski et al., 2004). *Salmonella* does not synthesize AHLs but encodes a protein known as SdiA, which has an amino acid sequence similar to that of LuxR-type transcriptional activators and enables the detection of AHLs produced by other bacterial species, leading to the regulation of gene expression (Ahmer, 2003; Janssens et al., 2007). The first correlation between SdiA and biofilm formation was in *Salmonella* Typhimurium. In this serotype, it was demonstrated that SdiA regulates the *rck* operon that affects the expression and function of the *pef* operon, which encodes plasmid-coded fimbriae whose function is adhesion to crypt epithelial cells, induction of inflammatory

response and biofilm formation (Smith and Ahmer, 2003). It has already been shown that SdiA is activated during the intestinal transit of *Salmonella* Typhimurium in mice. A *Salmonella* Typhimurium mutant strain, *sdiA*$^{-}$, was rapidly overcome by AHL-producing mutant *Salmonella* Typhimurium strains in the intestinal transit of mice (Dyszel et al., 2010). The intestinal environment has an abundant bacterial community, mostly Gram-negative bacteria, which can produce AHLs (Lahiri et al., 2010; Arumugam et al., 2011; Sankar et al., 2015) that *Salmonella* can detect.

As previously mentioned, anaerobiosis is a food preservation strategy used in the food industry. Therefore, under these conditions, Salmonella could respond to AI-1 production by natural food microbiota and increase biofilm formation (Sholpan et al., 2021). Campos-Galvão et al. (2015) demonstrated that, in TSB and under anaerobic conditions at 37 ºC, *Salmonella* Enteritidis could form a biofilm on polystyrene microplates after 36 h, in the presence of *N*-dodecanoyl homoserine lactone (C12-HSL). Under the same conditions, there was an increase in the expression of the genes *glgC*, *fimF*, and *fliF*, involved in forming biofilms after 7 h of cultivation (Campos-Galvão et al., 2015). Biofilm formation was also evaluated in the presence of other AHLs, with carbon chains ranging from six to 10 carbons; however, in the presence of only C12-HSL, *Salmonella* Enteritidis formed a more compact and mature biofilm. Molecular docking analyses performed by Almeida et al. (2016) demonstrated that C12-HSL has a higher binding affinity to the SdiA protein of *Salmonella* Enteritidis when compared to AHLs with smaller carbon chains. Almeida et al. (2017) demonstrated C12-HSL influences biofilm maturation in anaerobiosis but did not influence the initial adhesion process of cells in *Salmonella* Enteritidis. It should be clear that the results obtained regarding the effect of AHL on *Salmonella* vary with serotype and culture conditions. For example, different behavior was observed in *Salmonella* Typhimurium, cultivated aerobically at 20 °C. Under these conditions, the AHLs produced by *Hafnia alvei* did not influence the formation of biofilms on two abiotic substrates, stainless steel coupon, and polystyrene microplate and the result was confirmed using 3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) (Blana et al., 2017).

The AI-2-mediated quorum sensing, derived from 4,5-dihydroxy-2,3-pentanedione (DPD), is considered a universal microbial language, as it is present in Gram-negative and Gram-positive bacteria (Geethanjali et al., 2019). AI-2 is produced from *S*–adenosyl methionine (SAM) by the LuxS protein (Pereira et al., 2013). In *Salmonella*, AI-2 is recognized by the periplasmic protein LrsB and is internalized by the ABC transporter Lsr,

composed of LsrA and LsrC. Once internalized, AI-2 is phosphorylated by LsrK and interacts with the transcriptional repressor LsrR to alleviate repression of the *lsr* operon (*lsrACDBFGE*). The *lsrACDB* genes encode proteins responsible for AI-2 internalization, while *lsrFG* encodes proteins for degrading AI-2 (Taga and Bassler, 2003; Pereira et al., 2013). Deletion of *luxS* in *Salmonella* Typhimurium resulted in reduced swimming and swarming motility, reduced biofilm formation, and downregulation of genes related to motility, chemotaxis, biofilm, and pathogenicity (Jesudhasan et al., 2010).

In addition to the above, there are relevant data on the connection between quorum sensing and the second messenger c-di-GMP (Condinho et al., 2022; Prentice et al., 2022). However, in *Salmonella*, this issue needs to be explored.

7 Conclusion and perspectives

Biofilms are the predominant lifestyle of bacteria due to the numerous advantages. The protective characteristics of the matrix make eradication difficult, making biofilm a problem for the food industry and public health. In the enteric pathogen *Salmonella*, biofilm formation is an extremely complex and highly regulated process involving genetic and environmental factors. Understanding the global mechanisms that affect biofilm formation in one of the main foodborne pathogens allows the control of relevant bacterial processes, mainly for the development of anti-biofilm strategies. This review highlights current knowledge about components, regulation, and the main factors affecting biofilm formation in *Salmonella*. Furthermore, the role of quorum sensing was also discussed to draw attention to this avenue of research exploring its impact on biofilm formation, mainly under anaerobic conditions.

8 References

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CHAPTER 2

Genome sequencing and analysis of *Salmonella enterica* subsp. enterica serotype Enteritidis PT4 578 and phenotypes

Abstract

Salmonella enterica serotype Enteritidis is a generalist serotype that adapts to different hosts and transmission niches. It has great epidemiological relevance, being one of the most prevalent serotypes distributed in several countries. *Salmonella* Enteritidis causes self-limited gastroenteritis in humans, which can progress to systemic infection in immunocompromised individuals. Poultry products are considered significant reservoirs of many *Salmonella* serotypes, and *Salmonella* Enteritidis infections are often related to the consumption of chicken meat and eggs. This work presents the whole-genome sequence of *Salmonella* Enteritidis PT4 strain 578. A total of 165 genes (3.66%) of the 4,506 coding sequences (CDS) predicted in the genome are virulence factors associated with cell invasion, intestinal colonization, and intracellular survival. Twelve *Salmonella* pathogenicity islands (SPIs) were detected, and the SPI-1 and SPI-2 genes encode type III secretion systems (T3SS) were highly conserved. Six prophage-related sequences were found. Two regions of intact prophages correspond to *Salmon_118970_sal3* and *Gifsy-2*. Two CRISPR systems were identified. Comparative genome analysis with three other serotypes of *Salmonella* demonstrates that most unshared genes are related to metabolism, membrane and hypothetical proteins. Finally, the phenotypic characterization of *Salmonella* Enteritidis PT4 578 and the other three serotypes regarding the expression of the red, dry, and rough (rdar) morphotype and biofilm formation showed differences among them. Overall, the genomic characterization and phenotypic properties expand knowledge of the mechanisms of pathogenicity in *Salmonella* Enteritidis PT4 578.

Keywords: Foodborne pathogen, serotype Enteritidis, whole genome analysis, virulence determinants, biofilm.

1 Introduction

Salmonella is one of the main pathogens responsible for millions of foodborne infections worldwide and has great relevance for public health systems and food production (Eng et al., 2015; Wessels et al., 2021; WHO, 2022). The genus *Salmonella* is a complex group divided into only two species, *Salmonella bongori* and *Salmonella enterica*, based on differences between *16S rRNA* sequences (Popoff et al., 2003). The species *S. enterica* can be further subdivided into seven subspecies, based on genomic genotyping, and more than 2,659 serotypes based on serotyping (Issenhuth-Jeanjean et al., 2014; Brown et al., 2021). Most pathogenic serotypes responsible for causing infections in humans and animals belong to *S. enterica* subsp. *enterica* and are clinically divided into typhoidal and non-typhoidal serotypes (NTS) (Andino and Hanning, 2015).

S. enterica serotype Enteritidis and *S. enterica* serotype Typhimurium are the NTS with the most significant epidemiological relevance globally (Luvsansharav et al., 2020; Food and Authority, 2021). Although data on prevalent serotypes in Brazil are scarce, *Salmonella* Enteritidis has been the leading serotype isolated from gastroenteritis cases for decades (Campioni et al., 2018; Morasi et al., 2022). Most strains belonged to the globally disseminated phage type 4 (PT4) (Nunes et al., 2003; Monte et al., 2020). *Salmonella* Enteritidis is an example of a successful *Salmonella* serotype, with a remarkable ability to adapt to niches and hosts as long as specialization opportunities present themselves (Feasey et al., 2016). This serotype has been frequently explored in research for understanding behaviors concerning quorum sensing, biofilm, and virulence mechanisms (Almeida et al., 2018; Byun et al., 2021; Luiz de Freitas et al., 2021; Kim et al., 2022).

The increasing number of available genomic sequences of *S. enterica* allowed for defining the “core” genome, which is conserved and shared by most of its members, and the “accessory” genomic elements, which are present in some, but absent in other *Salmonella* strains (Medini et al., 2005; Jacobsen et al., 2011). Accessory genomic elements are unnecessary for survival; however, they are a source of diversity, favor adaptability to specific colonization niches, and confer unique virulence attributes (Land et al., 2015). Furthermore, comparative analyzes of closely related genomes revealed that these genes were acquired mainly by several horizontal transfer events. Thus, recombination between genomes is believed to be a significant driver of diversity within the *Salmonella* genus (Didelot et al., 2011; Ilyas et al., 2017).

In *Salmonella*, many of these horizontally acquired genes linked to infection are found on the chromosome as units of one or a few virulence genes or large cassettes composed of a series of genes and operons called *Salmonella* Pathogenicity Islands (SPIs) (Marcus et al., 2000; Ilyas et al., 2017). The virulence factors encoded by SPIs, such as type III secretion systems 1 and 2 (T3SS1 and T3SS2, respectively), are responsible for the invasion of the epithelium and induction of the infectious process in the host (Ibarra and Steele-Mortimer, 2009; Fattinger et al., 2021). The pan-genome of *Salmonella* includes 24 different SPIs, and although not all have been experimentally validated, most of them are present in both species and all subspecies (Lerminiaux et al., 2020). In addition to SPIs, the major components of the accessory genome of *Salmonella* are prophage sequences, transposable elements, and plasmids (Jacobsen et al., 2011). These genes have detectable properties that differentiate them from the rest of the host genome, such as having a different genomic signature and containing mobility genes so they can be integrated into the genome (Che et al., 2014).

Thus, the objectives of this work were to perform the sequencing and annotation of the genome of *S. enterica* serotype Enteritidis PT4 578 to expand the understanding of structural features of the core genome and accessory genomic elements. Moreover, perform several analyzes to identify SPIs, virulence factors, resistance genes, prophage, plasmid region, mobile elements, restriction-modification site (RM), and the CRISPR-Cas system, as well as comparative genomic analyses with three other closely related serotypes. In addition, selected phenotypic characteristics of these strains, such as the ability to form the red, dry, and rough (rdar) morphotype, motility, and biofilm will also be evaluated. These results help explain the pathogenic characteristics of *Salmonella* Enteritidis PT4 578 and the phenotypic divergences observed between closely related serotypes.

2 Materials and methods

2.1 Bacterial strain

Salmonella Enteritidis PT4 578 isolated from chicken breasts (GenBank: 16S ribosomal RNA gene MF066708.1) and obtained from the bacterial culture collection of the Oswaldo Cruz Foundation (FIOCRUZ; Rio de Janeiro, Brazil) was used in this study. This strain was chosen because it is a PT of clinical importance in Brazil. In addition, it is a model for the study of several subjects, such as virulence, quorum sensing, and biofilm formation (Almeida et al., 2018; Campioni et al., 2018; Byun et al., 2021; Luiz de Freitas et al., 2021; Kim et al., 2022). The culture was maintained in Luria Bertani broth (LB; Himedia, India) (tryptone 1%, yeast extract 0.5%, and NaCl 0.5%) with 20% (v/v) sterile glycerol at -20 °C.

The other strains, *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium ATCC 13311, and *Salmonella* Typhimurium ATCC 14028, were chosen because they are directly related to the Enteritidis PT4 serotype. The strains maintenance conditions were the same as those described above.

2.2 Genome sequencing, assembly, and annotation

The total DNA of *Salmonella* Enteritidis PT4 578 was obtained from an overnight culture in 5 mL Tryptic Soy Broth (TSB; Sigma, United States) at 37 °C using Wizard® Genomic DNA Purification Kit (Promega, USA) following the instructions provided by the manufacturer. The quality of DNA was assessed using a NanoDrop (Thermo Fisher Scientific, Delaware, USA) and agarose gel. The double-stranded DNA (dsDNA) was quantified using a Qubit 3.0 fluorimeter (Life Technologies, Paisley, UK). According to the manufacturer's instructions, library preparation was performed with the Rapid Sequencing Kit (SQK-RAD004, Oxford Nanopore Technologies, Oxford, UK). The library was sequenced on a MinION sequencing device equipped with a flow cell (FLO-MIN106 R9.4.1, Oxford Nanopore Technologies, Oxford, UK) for approximately 2 h. The sequencing was managed using MinKNOW software v4.3.4. Real-time base-calling was performed using Guppy v5.0.11 in the high-accuracy mode (parameters: -c dna_r9.4.1_450bps_hac.cfg). Adaptor sequences were trimmed with Porechop v0.2.4 (https://github.com/rrwick/Porechop), and the quality of the filtered data was assessed using PycoQC v2.5.2 (https://github.com/tleonardi/pycoQC). Genomes were *de novo* assembled by Flye v.2.9.0 (Kolmogorov et al., 2019) (parameters: --iterations 5 --plasmids --genome-size 4.8m –nano-raw). The assembled genomes were corrected using Racon v1.4.3 (Vaser et al., 2017) and Medaka v1.3.3 (Oxford Nanopore Technologies, Oxford, UK). Genome completeness was assessed using BUSCO v5.0.0 (parameters: --lineage_dataset enterobacterales_odb10). Finally, the assembled genome in FASTA format was annotated using the Prokka tool (Seemann, 2014). Standard annotation files, including GFF and GBK, were generated by running Prokka. The general characteristics of the *Salmonella* Enteritidis PT4 578 genome were compared with the reference genomes of *Salmonella* Enteritidis P125109 and *Salmonella* Typhimurium LT2.

2.3 Identification of pathogenicity islands (SPIs), virulence factors, and resistance genes

The SPIs were identified using the SPIFinder with a 95% of identity threshold and 60% of minimum length (Roer et al., 2016). The virulence factor database (VFDB) was used

to identify virulence factors in the genome (Chen, 2004). The identification of acquired resistance genes was performed through ResFinder 4.1 (Bortolaia et al., 2020) and the resistance gene identifier from the CARD database.

2.4 Phage insertion, prediction of plasmid regions, integrative and conjugative elements

The insertion of prophages into the genome was evaluated using the Phage Search Tool (PHAST) (Zhou et al., 2011). Scores above 90 were known as an intact prophage, scores 70–90 as questionable prophage, and regions scoring below 70 as incomplete prophage (Zhou et al., 2011). The VFDB was used to identify virulence factors in the prophage regions (Chen, 2004). PlasmidFinder was used to analyze plasmid regions in the genome (Carattoli et al., 2014). The ICEberg 2.0 was used for the identification of integrative and conjugative elements (ICEs) (Liu et al., 2019).

2.5 Determination of restriction-modification sites (RM) and CRISPR regions

The Restriction-ModificationFinder-1.1 was used to identify the RM sites (Roer et al., 2016). CRISPR regions were analyzed using CRISPRCasFinder (Couvin et al., 2018). Subsequently, the file generated by CRISPRCasFinder (result.json) was loaded in CRISPRTarget to identify the spacers (Biswas et al., 2013).

2.6 Comparative genome analysis

Comparative analyzes were performed between the *Salmonella* Enteritidis PT4 578 genome and three other closely related serotypes available from GenBank, *Salmonella* Enteritidis ATCC 13076 (NC_ LSHA01) and two strains of another NTS, *Salmonella* Typhimurium ATCC 13311 (NC_ ASM74305v1) and *Salmonella* Typhimurium ATCC 14028 (NC_ ASM199711v1). OrthoVenn was used to perform the analysis of the cluster of orthologs groups of proteins (COGs) with default parameters (*E*-value: 1e-10 and inflation value: 1.5) (Xu et al., 2019). In addition, the Roary pipeline 3.11.2 was used to predict the pan-genome of the sequenced *Salmonella* Enteritidis PT4 578 (Page et al., 2015) and whole-genome comparison with the three serotypes previously mentioned.

2.7 Phenotypic analysis

2.7.1 Swimming and swarming motilities

The ability of the four strains to move by flagella was examined. The four strains were grown for 20 h at 37 °C in a TSB medium. Swimming motility was performed as described

previously by Sperandio et al. (2002). The cultures were standardized to 107 CFU mL^{-1}, and an aliquot of 3 µL was plated onto a 0.3% tryptone agar plate (1% tryptone and 0.25% NaCl). For the swarming motility, a volume of 3 µL was inoculated onto a 0.5% LB agar plate supplemented with 0.4% glucose (Harshey and Matsuyama, 1994; Kearns, 2010). The plates were incubated at 28 ºC for 10 h for swimming motility and 40 h for swarming motility. The halos from the center to the edge of the swimming and swarming motilities were evaluated by measuring their diameter.

2.7.2 Colony morphology

The colony morphology was evaluated. A volume of 5 µL of the standardized inoculum in subitem 2.7.2 was spotted into the LB agar plate, supplemented with 40 $mg.L^{-1}$ of Congo red (Sigma-Aldrich, United States) and 20 $mg.L^{-1}$ of Coomassie brilliant blue (Sigma-Aldrich, United States) (Römling et al., 2003a). The colony morphology was judged visually after 72 h incubation under 28 ºC as red, dry, and rough (rdar; expression of curli fimbriae and cellulose), pink, dry, and rough (pdar; expression of cellulose), brown, dry, and rough (bdar; expression of fimbriae) and white and smooth (saw; no expression of matrix components). Pictures of the colonies were taken using a Canon EOS 450D camera.

2.7.3 Biofilm formation

Biofilm formation was also quantified in the four strains. The measurement was performed in 96-well polystyrene microplates according to the protocol described by Stepanovic et al. (2004), with modifications. Briefly, the polystyrene microplates were filled with 230 µL of TSB and 20 µL of the standardized inoculum in subitem 2.7.2 and then incubated for 40 h. After incubation, the total cells' optical density (OD) was determined at 600 nm. The culture supernatant was discarded, and the microplate was washed three times with 300 µ L of distilled water. The surface-attached cells were then fixed by adding 250 µL of methanol for 15 min. The methanol was discarded, and the microplate was air-dried for 20 min and stained with 250 µL of 0.1% (w/v) crystal violet for 30 min. Then, the violet crystal was removed, and the cells were washed three times with water. Next, the microplates were air-dried for 15 min at 40 °C, and the dye bound to the adherent bacterial cells was resolubilized in 250 µL of 33% glacial acetic acid. The OD of attached cells was determined at 590 nm. The OD was used to classify the isolates. The cutoff OD (ODc) was defined as three standard deviations above the mean OD of the negative controls. Thus, isolates were classified as a non-biofilm producer ($OD \leq ODc$), a weak biofilm producer $ODc < OD \leq$

(2xODc), a moderate biofilm producer (2xODc) < OD ≤ (4xODc), or a strong biofilm producer (4xODc) < OD.

2.7.4 Statistical analysis

GraphPad Prism software was used to analyze the statistical significance of the differences observed in the motilities and biofilm formation between the strains through analysis of variance (ANOVA) and Tukey's test. *P* <0.05 were defined as statistically significant.

3 Results

3.1 Structure and general information of *Salmonella* Enteritidis PT4 578 genome

Salmonella Enteritidis PT4 578 presents a chromosome of 4,685,705 bp with 4,615 putative genes, 4,506 coding DNA sequence (CDS), and 52.2% GC content. The general characteristics of this genome are represented in Fig. 1 and summarized in Table 1.

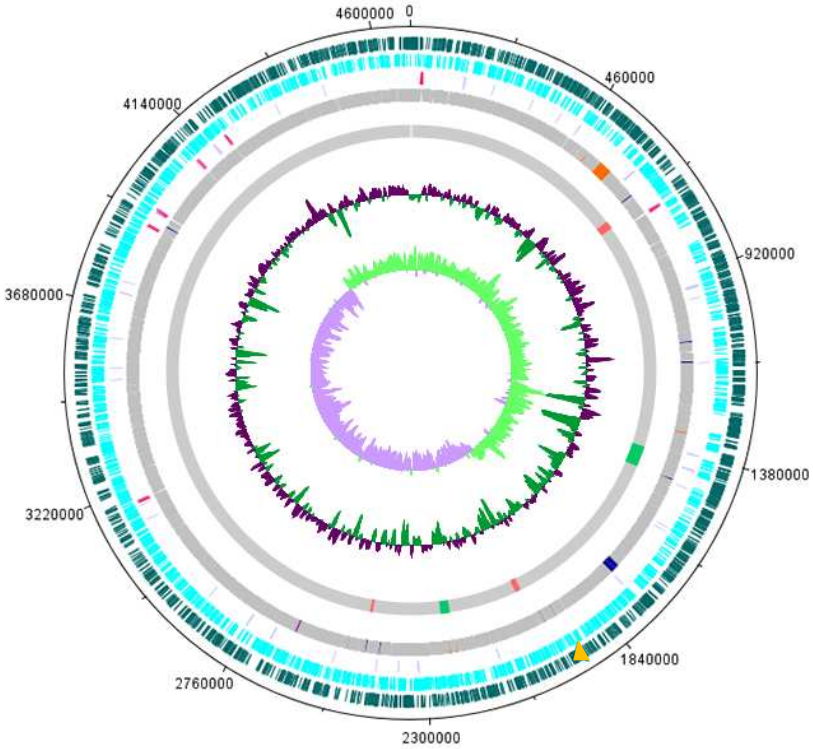

Fig. 1. Circular map of *Salmonella* Enteritidis PT4 578 chromosome. Forward and reverse CDS represented in dark green and light blue respectively. In the yellow resistance gene was marked. More internally, light purple represents tRNA and pink represents rRNA. In orange, the virulence factors of SPI-1 and in blue SP-2. In red regions of incomplete prophage and in green regions of intact prophage. The two inner tracks are G+C content and GC skew. The circular map was generated in DNAPlotter (Carver et al., 2009).

Table 1. General genome characteristics of *Salmonella* Enteritidis PT4 578 compared to *Salmonella* Enteritidis P125109 and *Salmonella* Typhimurium LT2.

Features	***Salmonella* Enteritidis PT4 578**	***Salmonella* Enteritidis P125109**	***Salmonella* Typhimurium LT2**
Size (bp)	4,685,705	4,685,848	4,857,432
G+C (%)	52.20	52.17	53.0
CDS	4,506	4,318	4,489
rRNA	7	7	22
tRNA	84	84	85
Reference	This work	(Thomson et al., 2008)	(McClellan et al., 2001)

3.2 Pathogenicity islands (SPIs), virulence factors, and resistance genes

SPIFinder reveal 12 SPIs in the genome of *Salmonella* Enteritidis PT4 578, namely SPI-1, SPI-2, SP-3, SP-4, SP-5, SP-9, SP-10, SP-12, SPI-13, SP-14, centisome 63 pathogenicity island (C63PI), and centisome 54 pathogenicity island (CS54). A total of 165 genes (3.66%) of the 4,506 CDS annotated in the genome of *Salmonella* Enteritidis PT4 578 were predicted as virulence factors in the VFDB (Table S2). A total of 82 virulence factors encode the required proteins for the invasion of cells and secretion. Of these, 37 were carried in *Salmonella* Enteritidis PT4 578 SPI-1 (Fig. 2A) and 31 in SPI-2 (Fig. 2B). VFDB results revealed three virulence factors encoding by SPI-5 and SPI-12, both related to T3SS effector. SPI-14 carries one virulence factor, a type IV secretion system effector. *Salmonella* Enteritidis PT4 578 carries the C63PI that encodes four virulence factors related to iron uptake, namely *sitABCD*. Meanwhile, three virulence factors related to intestinal colonization and persistent determinants, *ratB*, *shdA*, and *sinH*, are encoded by CS54. The SP-3, SP-4, SP-9, SP-10, and SPI-13 did not carry any identified or known virulence factors. In addition to the SPI-encoded secretion apparatus, the *Salmonella* Enteritidis PT4 578 repertoire of virulence factors also include fimbrial and non-fimbrial adherence determinants, macrophage inducible genes, magnesium uptake, stress, and anaerobic adaptation (Table S2). Thirteen clusters of fimbriae *csg*, *bcf*, *fim*, *lpf*, *saf*, *sef*, *stb*, *std*, *ste*, *stf*, *sth*, *sti,* and *peg* were identified in *Salmonella* Enteritidis PT4 578 (Table S2). About the *peg* operon, only *pegA* and *pegD* genes were found.

RESFinder and CARD showed that *Salmonella* Enteritidis PT4 578 contains the cryptic resistance gene, *aac(6')-Iy*, related to resistance to the aminoglycoside antibiotic class (Fig. 1).

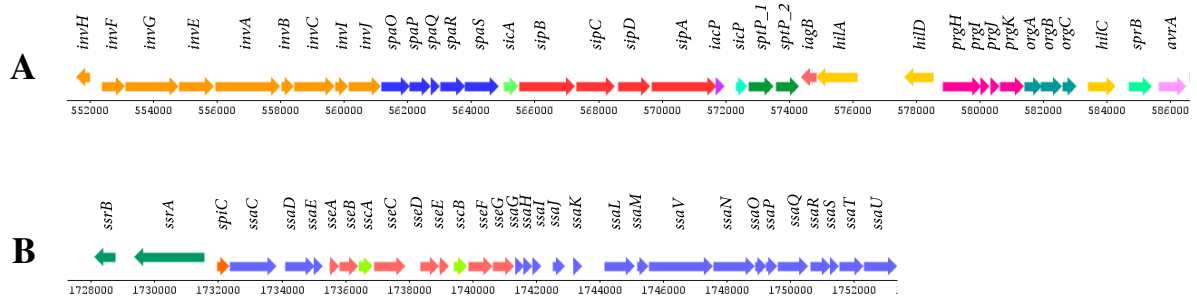

Fig. 2. Genetic organization of virulence factors in SPI-1 (A) and SPI-2 (B). The linear map was generated in DNAPlotter (Carver et al., 2009).

3.3 Mobile elements

The PHAST program revealed the presence of six prophage regions (Fig. 1). Two regions of intact prophages (region 2 and region 5), and four incomplete regions. Region 2 contains 62,6 kb, 47.77% of GC content, and 63 ORFs, of which nine encode hypothetical proteins and 54 encode proteins with correspondence in the phage protein database. The phage with the highest number of proteins, like those in this region, was *Salmon_118970_sal3* (NC_031940), with 52 matches. Region 5 contains 31,1 kb, 47.22% of GC content, and 40 ORFs, 10 encode hypothetical proteins and 30 encode proteins with correspondence in the phage protein database. The phage with the highest number of proteins, like those in this region, was *Gifsy-2* (NC_ 010393), with 16 matches. BLAST analysis of the prophage sequences identified against the VFDB revealed the presence of phage-encoded virulence factors, such as T3SS effector, *sseK*2 and *nleB*1 in region 2, superoxide dismutase precursor, *sodCI* in region 5 and T3SS effector, *sseI*/*srfH*, *sspH2* and *sspH1* in region 6. However, conjugative plasmids were not identified in *Salmonella* Enteritidis PT4 578 genome. In addition, PlasmidFinder did not identify any plasmid regions in the genome.

A region of 13379 bp was predicted as ICE in the genome of *Salmonella* Enteritidis PT4 578. This region encodes thirteen proteins, one integrase and one relaxase.

3.4 Identification restriction-modification (RM) sites and CRISPR loci

In *Salmonella* Enteritidis PT4 578 genome, three RM sites were detected by Restriction-ModificationFinder, comprising type I, II, and III. The type I, *Sty*SBLI, enzymes consist of a hetero-oligomeric protein complex encoded by three closely linked genes, S.Sen1427II (specificity subunit), M.StySBLI (MT), and StySBLI (RE). The recognition CGANNNNNNTRCC motif, in which the residues underlined are the substrates for cleavage. Type II methyltransferase M.Sen158Dcm and M.Sen641III recognizes CCWGG and ATGCAT motifs, respectively. In type III, the SenAZII and M.Sen1427I recognize CAGAG and CAGAG motifs, respectively. However, the cleavage site of SenAZII is unknown.

Through CRISPRCasFinder it was discovered that *Salmonella* Enteritidis PT4 578 contains two CRISPR systems at the evidence of level 4, CRISPR 1 and CRISPR 2 (Fig. 3). Both contain 29 bp, with nine and eight unique spacers, respectively. A total of three out of seventeen were recognized as protospacers (Table S1), homologs to plasmid sequences and phage sequences. The Cas cassette, composed of *cas3*, *cse1*, *cse2*, *cas7*, *cas5*, *cas6*, *cas1*, and *cas2*, was found between CRISPR1 and CRISPR2 locus, starting at 510618 bp and ending at 519071 bp (Fig. 3).

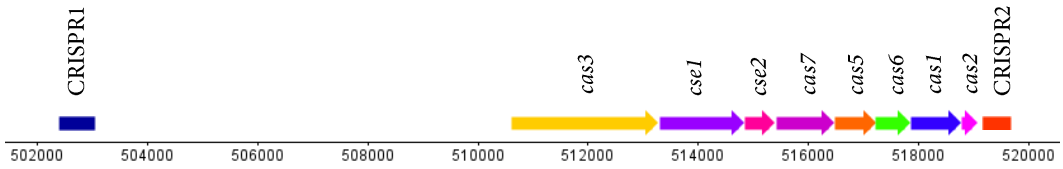

Fig. 3. Genetic organization of CRISPR-Cas system in *Salmonella* Enteritidis PT4 578. The linear map was generated in DNAPlotter (Carver et al., 2009).

3.5 Comparison of the genome of *Salmonella* Enteritidis PT4 578 with that of other serotypes

The comparative analysis of *Salmonella* Enteritidis PT4 578 was performed with three other closely related genomes, one of the same serotype, *Salmonella* Enteritidis ATCC 13076, and two of a different NTS serotype, *Salmonella* Typhimurium ATCC 13311 and *Salmonella* Typhimurium ATCC 14028. These four genomes share a core of 3,878 orthologous groups of proteins (COGs) (Fig. 4A). In total, *Salmonella* Enteritidis PT4 578 showed 4,214 COGs (Fig 4A). Of these, only one cluster, which contains two plasma membrane-related proteins, is specific to *Salmonella* Enteritidis PT4 578 (Fig. 4A). The number of clusters shared between strains of the same serotype was greater than between strains of different serotypes (Fig. 4B).

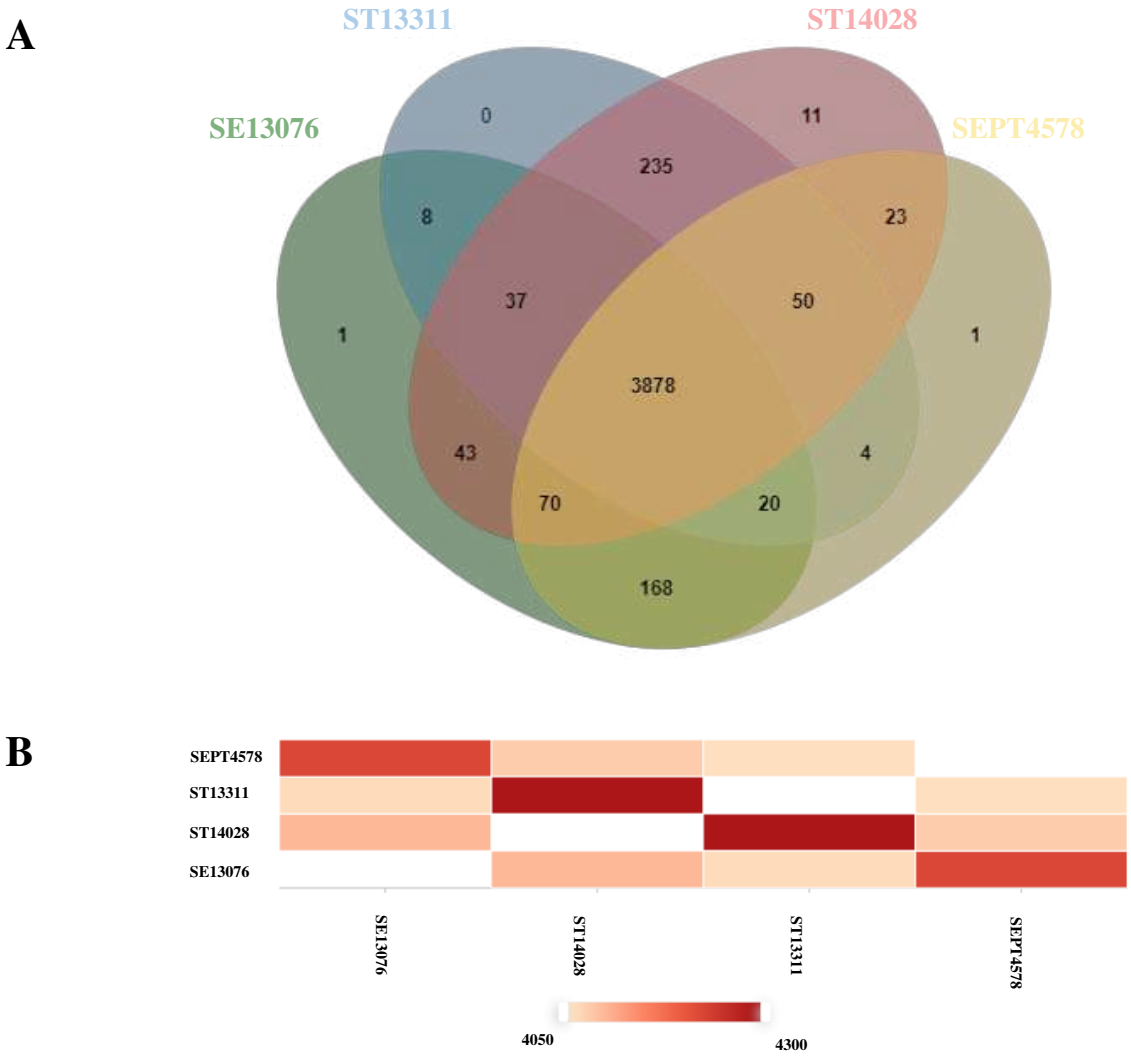

Fig. 4. Venn diagram of single protein clusters of *Salmonella* Enteritidis PT4 578 and shared with *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium ATCC 14028, and *Salmonella* Typhimurium ATCC 13311 (A). Similarity matrix for pairwise genome comparisons, where the heatmap shows the ortholog cluster between any pair of genomes (B).

The pan-genome of these four genomes was estimated at 5,317 genes. Of these, 250 genes were found exclusively in *Salmonella* Enteritidis PT4 578, mainly related to hypothetical proteins, membrane, and metabolism proteins.

3.6 Phenotypes

The four strains were phenotypically analyzed for motility, colony morphology, and biofilm formation. All strains showed swimming and swarming motilities at 28 ºC after 10 h, and 40 h, respectively (Fig. 5A). After 10 h, *Salmonella* Typhimurium ATCC 14028 showed significantly higher swimming motility than the other evaluated strains (Fig. 5A). The colony

morphology at 28 ºC was highly variable. *Salmonella* Enteritidis PT4 578 did not show a rdar morphotype in Congo red agar,, forming white and smooth colonies (saw). *Salmonella* Typhimurium ATCC 14028 was the serotype that showed the typical rdar morphotype. *Salmonella* Typhimurium ATCC 13311 and *Salmonella* Enteritidis ATCC 13076 showed a rdar morphotype with less roughness than *Salmonella* Typhimurium 14028 (Fig. 5B). The four strains were grown on polystyrene microplates to assess biofilm formation at 28 ºC. The OD 590 nm value was used to classify the strains, according to Stepanovic et al. (2004). *Salmonella* Enteritidis PT4 578 was classified as a non-producing biofilm in the condition evaluated. *Salmonella* Typhimurium ATCC 14028 demonstrated strong biofilm formation ability, while *Salmonella* Typhimurium ATCC 13311 and *Salmonella* Enteritidis ATCC 13076 showed a weak biofilm formation (Fig. 5C).

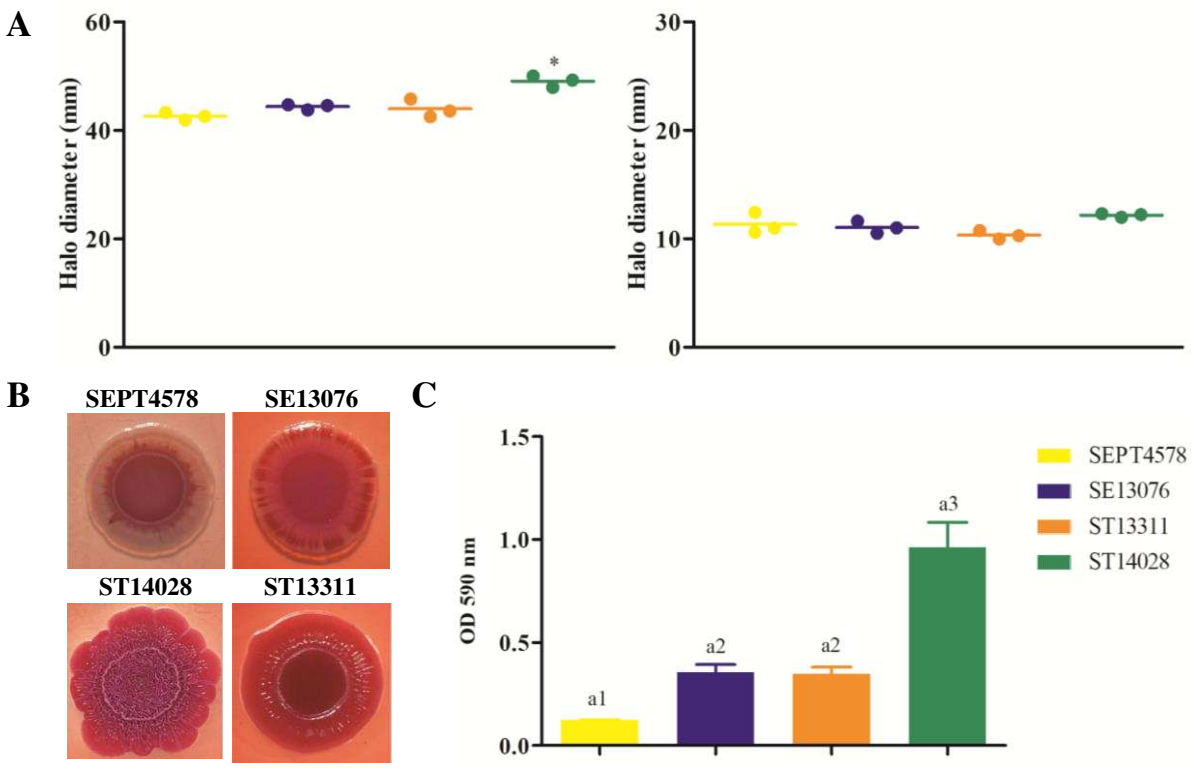

Fig. 5. Swimming and swarming motilities of *Salmonella* Enteritidis PT4 578 (SEPT4578), *Salmonella* Enteritidis ATCC 13076 (SE13076), *Salmonella* Typhimurium ATCC 13311 (ST13311), and *Salmonella* Typhimurium ATCC 14028 (ST14028) at 28 ºC for 10 and 40 h, respectively (A). Colony morphology of SEPT4578, SE13076, ST13311, and ST14028 in Congo red agar (B). Adhered cells of SEPT4578, SE13076, ST13311, and ST14028 on polystyrene microplates cultivated at 28 ºC for 40 h (C). Error bars indicate a standard deviation, * and the different numbers mean significant difference ($p < 0.05$).

4 Discussion

This study analyzed the main features of the *Salmonella* Enteritidis PT4 578 genome, a serotype and a phage type of significant clinical relevance and used in several studies of virulence, quorum sensing, and biofilm (Campos-Galvão et al., 2015; Carneiro et al., 2020; Byun et al., 2021; Luiz de Freitas et al., 2021; Kim et al., 2022). Furthermore, *Salmonella* Enteritidis PT4 is predominant in outbreaks in the western states of the United States and is the most frequently isolated from chicken in Europe and South America (Kipper et al., 2022). Table 1 shows the general characteristics of this genome and two reference genomes, *Salmonella* Enteritidis P125109 (AM933172.1) and *Salmonella* Typhimurium LT2 (NC_003197.2). Comparatively, the *Salmonella* Enteritidis PT4 578 genome has 143 bp less than *Salmonella* Enteritidis P125109 and 171 bp less than *Salmonella* Typhimurium LT2. The genomes analyzed are according to Lyu et al. (2021), who analyzed genomes of 314 *Salmonella* isolates from retail meat and demonstrated that the average lengths ranged from 4.50 to 5.15 Mbp, with an overall average length of 4.78 Mbp. The GC content of the strains is similar. *Salmonella* Enteritidis PT4 578 has more CDS than the reference strains. Minor differences observed in these genomes demonstrate great conservation in the functional capacity.

The SPIs have been described as the "molecular toolbox" for *Salmonella* pathogenesis (Gal-Mor and Finlay, 2006). Of the 24 SPIs identified, 12 were predicted in the genome of *Salmonella* Enteritidis PT4 578. It is known that SPI-1 and SPI-2 encode T3SS-1 and T3SS-2, which play a crucial role in the invasion, survival, and replication of the host cells (Lou et al., 2019). The structure of SPI-1 and SPI-2 was completely conserved, indicating that these systems are functional in *Salmonella* Enteritidis PT4 578 (Fig. 2A). Some effectors translocated were variably distributed, including *sopABDE sspH2*, *sseK1*, *sseK2*, and *srfJ*, encoded outside of SPIs and phage-encoded. In *Salmonella* Enteritidis P125109, 14 SPIs have been described, including SPI-6, SPI-17, SPI-18, and SPI-19 (Thomson et al., 2008) that were not identified in *Salmonella* Enteritidis PT4 578. Suez et al. (2013) reported the universal presence of SPIs 1–5, 9, 13, and 14, the absence of SPIs 7, 8 and 15, and the variable or mosaic presence of SPIs 6, 10–12 and 16–19 in the genome of all non-typhoidal *Salmonella* isolates. Moreover, an analysis of 45 strains of *Salmonella* Enteritidis demonstrated that six SPIs, including SPI-1, SPI-2, SPI-3, SPI-9, SPI-13, and CS54, are conserved in all isolates while SPI-11 and SPI-12 were absent (Ksibi et al., 2022).

Salmonella Enteritidis PT4 578 also has a diverse repertoire of fimbriae (Table S2). The thirteen fimbrial operons (*csg*, *bcf*, *fim*, *lpf*, *saf*, *sef*, *stb*, *std*, *ste*, *stf*, *sth*, *sti* and *peg*) are

shared with the reference genome *Salmonella* Enteritidis P125019. However, the *pegB* and *pegC* genes of the *peg* operon have not been identified in *Salmonella* Enteritidis PT4 578. This operon is considered to influence the caecal colonization of chickens by *Salmonella* Enteritidis (Vaid et al., 2021).

Furthermore, the *mig-14* gene (macrophage-inducible gene-14) was detected in *Salmonella* Enteritidis PT4 578. Acquired as a mobile genetic element, this gene encodes a periplasmic protein that inhibits the entry of antimicrobial peptides into the *Salmonella* cytoplasm, making the macrophage a good niche for pathogen replication and survival (Valdivia et al., 2000; Brodsky et al., 2004). In addition,this gene has been shown to play an important role in the long-term survival of *Salmonella* Typhimurium in some tissues of infected mice (Brodsky et al., 2002).

The presence of mobile genetic elements is crucial for the pathogenesis of *Salmonella*. Prophage sequences are vehicles for horizontal gene exchange between different bacterial species and are responsible for much of the diversity between strains of the same species (Brüssow et al., 2004; Wahl et al., 2019). No accident, several SPIs are close to phage-encoding genes or phage-binding sites (Ilyas et al., 2017).

Salmonella Enteritidis PT4 578 has six prophage regions in its genome, two of which are intact (Fig. 1). The intact regions correspond to *Salmon_118970_sal3* and *Gifsy-2*. These are common regions for the serotype Enteritidis (Ksibi et al., 2022). *Gifsy-2* is a lambda-related prophage that may play an important role in resistance to oxidative stress in *Salmonella* Enteritidis PT4 578 since it it harboredsuperoxide dismutase *sodCI*. The region *Salmon_118970_sal3* assigned to may play a relevant role in invasion as it has been shown to contain genes encoding type III effector proteins, *sseK2,* and *nleB1*. Three other genes that also encode type III effector proteins (SseI/SrfH (*Gifsy-2*), SspH2, SspH1(*Gifsy-3*)) are contained in the incomplete region 6. This demonstrates that these three genes have also been acquired by lysogenic conversion, and these phages have subsequently been disrupted (Ehrbar and Hardt, 2005).

Plasmids are also part of the accessory genome of bacteria, contributing to antimicrobial resistance and virulence factors, such as *rck* encoded by pSLT in *Salmonella* Typhimurium (Hiley et al., 2019). However, no plasmid was detected in *Salmonella* Enteritidis PT4 578. Conjugative plasmids and ICEs are mobile genetic elements that carry genes that encode the machinery necessary for conjugation (Johnson and Grossman, 2015). A region predicted as ICE was found integrated into the genome of *Salmonella* Enteritidis PT4 578. The presence of the gene *aac(6')-Iy*, does not confer resistance, as they are weakly

expressed or not expressed at all (Magnet et al., 1999). Aminoglycoside resistance in *Salmonella* is usually secondary to increased gene expression following regulatory mutations (Magnet et al., 1999).

The RM system is important component of prokaryotic defense mechanisms against the uptake of foreign DNA (Vasu and Nagaraja, 2013). There are four categories of RM systems: type I, II, III and IV (Roer et al., 2016). Except for type IV, the other three types were detected in *Salmonella* Enteritidis PT4 578. The CRISPR-Cas system is another important defense mechanism against foreign DNA and, associated with the RM system, can increase phage resistance (Dupuis et al., 2013). Two CRISPR loci with eight and nine spacers each, 11 of which were protospacers identified (Table 2) in plasmids and phages, were detected in *Salmonella* Enteritidis PT4 578. This result demonstrates that these systems efficiently generated immunity against exogenous DNA from bacteriophages and plasmids.

Flagella are the main motility structures of bacteria. Although associated with the planktonic lifestyle, flagella also appear necessary for the early stages of biofilm formation (Wang et al., 2020). In the evaluated conditions, all serotypes analyzed presented the two types of flagellar motility, swimming and swarming.

The rdar is a widely used manifestation of *Salmonella* multicellular behavior to evaluate the expression of the adhesive extracellular matrix components cellulose and curli fimbriae (Romling et al., 1998; Steenackers et al., 2012). Variations in the expression of cellulose and curli fimbriae result in the development of different morphotypes. *Salmonella* Enteritidis PT4 578 presented the saw morphotype, indicating no expression of these components at 28 °C. On the other hand, *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteritidis ATCC 13076, and *Salmonella* Typhimurium ATCC 13311 presented the rdar morphotype, indicating the expression of fimbriae and cellulose. More than 90% of *Salmonella* Typhimurium and *Salmonella* Enteritidis strains expressed the rdar morphotype at 28 °C (Römling et al., 2003b). The divergent *csg* operon is necessary for the curli fimbriae biosynthesis and includes genes for fimbrial protein subunits (*csgBA*), transcriptional regulation (*csgD*), and curli assembly machinery (*csgC* and *csgEFG*) (Liu et al., 2014; Van Gerven et al., 2018). Cellulose is encoded by the *bcsABZC-bcsEFG* operons, which are under the regulation of *adrA* and *csgD* (Solano et al., 2002). A manual analysis of the genome confirmed that the *csg* and *bsc* operons and *adrA* genes were conserved in *Salmonella* Enteritidis PT4 578 and in the other three serotypes analyzed. This indicates that *Salmonella* Enteritidis PT4 578 has all the genetic requirements to form the rdar morphotype. However,

for some reason, it has lost this ability. Some strains may lose the ability to express the rdar morphotype due to regulatory mutations that affect *csgD* expression (White and Surette, 2006). The loss of the rdar morphotype may also be related to mutations in the *rpoS* locus (Davidson et al., 2008).

Salmonella strains presenting the rdar morphotype have a great capacity to produce biofilm on abiotic surfaces since the curli fimbriae and cellulose are the main components of the matrix of biofilm (Paz-Méndez et al., 2017). The biofilm formation was consistent with the morphotype results (Fig. 5B). Under the evaluated conditions, *Salmonella* Enteritidis PT4 578, which expressed the saw morphotype, did not form biofilm. *Salmonella* Typhimurium ATCC 14028 formed more biofilm than *Salmonella* Typhimurium ATCC 13311, *Salmonella* Enteritidis ATCC 13076, and a rougher morphotype. However, despite not expressing the rdar morphotype and being classified as non-biofilm forming, some studies have shown that *Salmonella* Enteritidis PT4 578 is capable of forming a compact and mature biofilm in the presence of acyl-homoserine lactones, a quorum sensing signal molecule (Campos-Galvão et al., 2015; Almeida et al., 2017). This finding reflected the plastic nature of genomes and demonstrated that specific genes might cease to be essential under certain conditions, become redundant, or even be lost throughout evolution.

5 Conclusion

The results of this study expand the understanding of the diversity and pathogenicity of *Salmonella* Enteritidis PT4 578. An increasing number of available genomes allows expanding the specie's pan-genome and specifying the core genome. Although many virulence genes demonstrate the pathogenic potential of the *Salmonella* Enteritidis PT4 578 strain, many genes may lose functionality or become redundant, resulting in different phenotypes. Likewise, horizontal gene transfer can increase virulence, resistance, or host range. The expansion of knowledge about the accessory genome of *Salmonella* is essential contributing to the development of detection and control strategies for this pathogen.

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Supplementary Table

Table S1. Identified protospacers in the CRISPR cluster of *Salmonella* Enteritidis PT4 578.

CRISPR	Spacer nº	Prophage/plasmid	Accession number
1	6	Plasmid pYRKMM821_2 of *Yersinia ruckeri* KMM821	NZ_CP071804.1
		Plasmid p17Y0153.1 of *Yersinia ruckeri* 17Y0153	NZ_CP084651.1
		Plasmid p17Y0189.1 of *Yersinia ruckeri* 17Y0189	NZ_CP084640.1
		Plasmid p16Y0180.1 of *Yersinia ruckeri* 16Y0180	NZ_CP084653.1
		Plasmid p17Y0155.1of *Yersinia ruckeri* 17Y0155	NZ_CP084649.1
		Plasmid pYR4 of *Yersinia ruckeri* NHV_3758	NZ_CP032236.1
2	1	*Escherichia* phage vB_EcoM-783R5	ON470627
		Escherichia phage vB_EcoM-705R4	ON470624
		Escherichia phage vB_EcoM-720R5	ON470604
		Escherichia phage vB_EcoM-569R10	ON470596
	6	Plasmid of *Salmonella* Senftenberg NCTC10384	NZ_LN868944.1

Table S2. Virulence factors present in the *Salmonella* Enteritidis PT4 genome.

Virulence factor class	Virulence factors	Related genes	*Salmonella* Enteritidis PT4 578
Capsule	Vi antigen	*tviA*	-
		tviB	-
		tviC	-
		tviD	-
		tviE	-
		vexA	-
		vexB	-

		vexC	-
		vexD	-
		vexE	-
Fimbrial adherence determinants	Agf/Csg	*csgA*	PT4578_01467
		csgB	PT4578_01466
		csgC	PT4578_01468
		csgD	PT4578_01465
		csgE	PT4578_01464
		csgF	PT4578_01463
		csgG	PT4578_01462
	Bcf	*bcfA*	PT4578_03450
		bcfB	PT4578_03449
		bcfC	PT4578_03448
		bcfD	PT4578_03447
		bcfE	PT4578_03446
		bcfF	PT4578_03445
		bcfG	PT4578_03444
	Fim	*fimA*	PT4578_02927
		fimC	PT4578_02925
		fimD	PT4578_02924
		fimF	PT4578_02922
		fimH	PT4578_02923
		fimI	PT4578_02926
		fimW	PT4578_02919
		fimY	PT4578_02920
		fimZ	PT4578_02921
	Lpf	*lpfA*	PT4578_04416
		lpfB	PT4578_04417
		lpfC	PT4578_04419; PT4578_04420
		lpfD	PT4578_04421
		lpfE	PT4578_04422
	Pef	*pefA*	-
		pefB	-
		pefC	-
		pefD	-
	Peg	*pegA*	PT4578_01203
		pegB	-
		pegC	-
		pegD	PT4578_01209
	Saf	*safA*	PT4578_03179
		safB	PT4578_03178
		safC	PT4578_03177
		safD	PT4578_03175
	Sef	*sefA*	PT4578_03596
		sefB	PT4578_03595
		sefC	PT4578_03593
		sefD	PT4578_03592
	Sta	*staA*	-
		staB	-
		staC	-
		staD	-
		staE	-
		staF	-
		staG	-

Stb	*stbA*	PT4578_03134
	stbB	PT4578_03136
	stbC	PT4578_03137
	stbD	PT4578_03138
	stbE	PT4578_03139
Stc	*stcA*	-
	stcB	-
	stcC	-
	stcD	-
Std	*stdA*	PT4578_00430
	stdB	PT4578_00431
	stdC	PT4578_00432
Ste	*steA*	PT4578_00512
	steB	PT4578_00511
	steC	PT4578_00510
	steD	PT4578_00509
	steE	PT4578_00508
	steF	PT4578_00507
Stf	*stfA*	PT4578_03267
	stfC	PT4578_03266
	stfD	PT4578_03265
	stfE	PT4578_03264
	stfF	PT4578_03263
	stfG	PT4578_03262
Stg	*stgA*	-
	stgB	-
	stgC	-
	stgD	-
Sth	*sthA*	PT4578_03476
	sthB	PT4578_03477
	sthC	PT4578_03478
	sthD	PT4578_03479
	sthE	PT4578_03480
Sti	*stiA*	PT4578_03286
	stiB	PT4578_03287
	stiC	PT4578_03288
	stiH	PT4578_03289
Stj	*Undetermined*	-
	Undetermined	-
	Undetermined	-
	stjB	-
	stjC	-
Stk	*stkA*	-
	stkB	-
	stkC	-
	stkD	-
	stkE	-
	stkF	-
	stkG	-
Tcf	*tcfA*	-
	tcfB	-

		tcfC	-
		tcfD	-
Macrophage inducible genes	Mig-14	*mig-14*	PT4578_00691
	Mig-5	*mig-5*	-
Magnesium uptake	Mg2+ transport	*mgtB*	PT4578_04284
		mgtC	PT4578_04283
Nonfimbrial adherence determinants	MisL	*misL*	PT4578_04290
	RatB	*ratB*	PT4578_00830
	ShdA	*shdA*	PT4578_00832; PT4578_00833
	SinH	*sinH*	PT4578_00827
Regulation	PhoPQ	*phoP*	PT4578_01559
		phoQ	PT4578_01558
Secretion system	TTSS (SPI-1 encode)	*hilA*	PT4578_00590
		hilC	PT4578_00600
		hilD	PT4578_00592
		iacP	PT4578_00584
		iagB	PT4578_00589
		invA	PT4578_00569
		invB	PT4578_00570
		invC	PT4578_00571
		invE	PT4578_00568
		invF	PT4578_00566
		invG	PT4578_00567
		invH	PT4578_00565
		invI	PT4578_00572
		invJ	PT4578_00573
		orgA	PT4578_00597
		orgB	PT4578_00598
		orgC	PT4578_00599
		prgH	PT4578_00593
		prgI	PT4578_00594
		prgJ	PT4578_00595
		prgK	PT4578_00596
		sicA	PT4578_00579
		sicP	PT4578_00586
		sipD	PT4578_00582
		spaO	PT4578_00574
		spaP	PT4578_00575
		spaQ	PT4578_00576
		spaR	PT4578_00577
		spaS	PT4578_00578
		sprB	PT4578_00601
	TTSS (SPI-2 encode)	*ssaC*	PT4578_01730
		ssaD	PT4578_01731
		ssaE	PT4578_01732
		ssaG	PT4578_01743
		ssaH	PT4578_01744
		ssaI	PT4578_01745
		ssaJ	PT4578_01747
		ssaK	PT4578_01749
		ssaL	PT4578_01750
		ssaM	PT4578_01751
		ssaN	PT4578_01753
		ssaO	PT4578_01754

		ssaP	PT4578_01755
		ssaQ	PT4578_01756
		ssaR	PT4578_01757
		ssaS	PT4578_01758
		ssaT	PT4578_01759
		ssaU	PT4578_01760
		ssaV	PT4578_01752
		sscA	PT4578_01735
		sscB	PT4578_01740
		sseA	PT4578_01733
		sseB	PT4578_01734
		sseC	PT4578_01736
		sseD	PT4578_01738
		sseE	PT4578_01739
		ssrA	PT4578_01728
		ssrB	PT4578_01725
	TTSS effectors translocated via both systems	*slrP*	PT4578_02679; PT4578_02680; PT4578_02681
		sspH1	-
	TTSS-1 translocated effectors	*avrA*	PT4578_00602
		sipA	PT4578_00583
		sipB	PT4578_00580
		sipC	PT4578_00581
		sopA	PT4578_01298
		sopB/sigD	PT4578_02466
		sopD	PT4578_00522
		sopE2	PT4578_02232
		sopE	PT4578_02257
		sptP	PT4578_00587; PT4578_00588
	TTSS-2 translocated effectors	*gogB*	-
		pipB2	PT4578_00693
		pipB	PT4578_02468
		sifA	-
		sifB	PT4578_01945
		sopD2	PT4578_02543
		spiC/ssaB	PT4578_01729
		spvC	-
		spvD	-
		sseF	PT4578_01741
		sseG	PT4578_01742
		sseI/srfH	PT4578_02504
		sseJ	PT4578_01978
		sseK1	PT4578_03895
		sseK2	PT4578_01452
		sseL	PT4578_01073
		sspH2	PT4578_01122
Serum resistance	Rck	*rck*	-
Stress adaptation	SodCI	*sodCI*	PT4578_02264
Toxin	SpvB	*spvB*	-
	Typhoid toxin	*cdtB*	-
		pltA	-
		pltB	-

Anaerobic respiration	Fused nitrate reductase(Mycobacterium)	*narX*	PT4578_02141

CHAPTER 3

The quorum sensing molecule C12-HSL promotes biofilm formation and increases *adrA* expression in *Salmonella* Enteritidis under anaerobic conditions

Abstract

The genus *Salmonella* represents the most common cause of foodborne diseases. One of the difficulties in eradicating this pathogen is related to its ability to adhere to the surface and form biofilm. Biofilms afford protection from various environmental challenges, such as antimicrobial agents, dehydration, antibiotics, and host immune defense mechanisms. Acyl-homoserine lactones (AHLs) are quorum sensing signaling molecules that mediate cell-to-cell communication in Gram-negative bacteria and positively regulate biofilm formation in *Salmonella* cultivated anaerobic at 37 ºC on a polystyrene surface. In this work, the biofilm formation by *Salmonella* Enteritidis PT4 578 on stainless steel surface at two temperatures, 28 and 37 ºC, and two atmospheres, aerobiosis, and anaerobiosis, were evaluated. Moreover, the influence of the *N*-dodecanoyl-DL-homoserine lactone (C12-HSL) in the biofilm formation and expression of genes related to the synthesis of structural components, regulation, and quorum sensing was evaluated in anaerobiosis at two temperatures, 28 and 37 ºC on a stainless-steel surface. C12-HSL enhances biofilm formation on stainless steel at 37 °C under anaerobic conditions. At this temperature, the quorum sensing molecule also increases the expression of the *adrA* gene, a diguanylate cyclase involved in cellulose biosynthesis, through synthesizing the bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) messenger and the *luxS* response regulator. Although it does not influence biofilm formation or the expression of related genes, C12-HSL also increases the expression of *luxS* at 28 ºC. These results bring a new perspective regarding regulating biofilm formation in *Salmonella* under anaerobic conditions.

Keywords: foodborne pathogen, temperature, quorum sensing, cellulose, c-di-GMP.

1 Introduction

Bacteria can form dense, three-dimensional structured communities embedded in a self-produced matrix of extracellular polymeric substances (EPS) that may adhere to a surface, known as biofilms (Flemming et al., 2016). Biofilm cells have a different physiology than actively growing planktonic cells (Joo and Otto, 2012; Giaouris et al., 2013). These characteristics associated with a matrix make the biofilm inherently tolerant to antibiotics, disinfectants, desiccation, and increased tolerance to host immune defense mechanisms (Del Pozo, 2018; Cadena et al., 2019). However, the persistence and difficulty of eradicating biofilms cause enormous concerns for the food industry, making it a frequent source of the contamination of foodstuffs associated with outbreaks of foodborne illness (Galié et al., 2018; Merino et al., 2019). Additionally, there is a correlation between biofilm formation and the ability of strains to colonize and replicate within the intestines of multiple host species, including humans (MacKenzie et al., 2017).

The biofilm formed by the foodborne enterobacterial pathogen *Salmonella* is particularly relevant to world public health. Salmonellosis, the *Salmonella* food poisoning, significantly impacts the global economic burden through product contamination, and costs associated with disease treatment, surveillance, and prevention (Majowicz et al., 2010; Havelaar et al., 2015). Usually, infections caused by *Salmonella* present in two clinical forms: typhoid fever and nontyphoidal diseases. Nontyphoidal infections result in gastroenteritis, and *Salmonella* Typhimurium and *Salmonella* Enteritidis are frequently involved (López et al., 2012; Jajere, 2019).

The biofilm matrix comprises exopolysaccharides, proteins, lipids, extracellular nucleic acids (eDNA and eRNA), and other biomolecules (Karygianni et al., 2020). In *Salmonella*, curli fimbriae and cellulose are the main structural components (Romling et al., 1998; Jonas et al., 2007). Curli are an amyloid-like proteinaceous substance that promotes surface colonization and cell-cell interactions (Tursi and Tükel, 2018). They are encoded by the *csgBAC* (structural proteins) and *csgDEFG* genes (accessory proteins required for curli assembly) (Barnhart and Chapman, 2006). Cellulose, a β-1-4-D-glucose polymer, provides cohesion and structural integrity to mature biofilms (Solano et al., 2002). This polymer is also related to effectively interacting with the surrounding environment (Maruzani et al., 2019). Its synthesis requires genes encoded in the *bcsABZC* and *bcsEFG* operons (Solano et al., 2002). The CsgD protein is the control point for biofilm formation, directly regulating the expression

of *csg* operons and indirectly involved in cellulose production by activating transcription of *adrA* (Gerstel and Römling, 2003; Zakikhany et al., 2010; Liu et al., 2014). The AdrA protein promotes cellulose biosynthesis through the interaction at the post-transcriptional level with the products of the *bcs* operon or through the production of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), which acts as an activator of the biosynthesis of this polymer (Römling, 2002). A large intergenic region is located upstream of the *csgD* promoter enabling a range of regulatory interactions (Gerstel et al., 2006). Thus, different environmental signals and surfaces can influence the transcription of the *csgD* through several global transcriptional regulators, such as RpoS, OmpR, H-NS, IHF, and Crl (Gerstel and Römling, 2003; Ogasawara et al., 2010). As several biofilm components have been shown to be conserved pathogen-associated molecular patterns recognized by the immune system (Tursi and Tükel, 2018), it is interesting to understand the mechanisms by which biofilm components are synthesized and recognized by the innate immune system.

Quorum sensing is another factor related to regulating biofilm formation in *Salmonella*. This is a chemical system of communication among cells based on producing signal molecules called autoinducers (AI) that diffuse into the environment. After being internalized, they induce a response in a coordinated manner by regulating gene expression (Fuqua et al., 1994). Acyl homoserine lactones (AHLs) are a type of AI recognized by SdiA, a transcriptional activator protein (Fuqua and Greenberg, 2002; Papenfort and Bassler, 2016). *Salmonella* does not produce these autoinducers but senses AHLs produced by other bacteria (Michael et al., 2001; Smith and Ahmer, 2003).

In *Salmonella* Enteritidis cultivated in anaerobiosis at 37 ºC, the AHL named *N*-dodecanoyl-DL-homoserine lactone (C12-HSL) induces the formation of a compact biofilm on the polystyrene surface and positively regulates the expression of biofilm genes, such as *lpfA*, *fimF*, *fliF*, and *glgC* after 7 h of culture in planktonic cells (Campos-Galvão et al., 2015). However, it does not influence the initial adhesion processes (Almeida et al., 2017). In *Salmonella* Typhimurium, cultivated in aerobiosis at 20 °C, the AHLs produced by *Hafnia alvei* did not influence the formation of biofilms on stainless steel coupon and polystyrene microplate, and the result was confirmed with the use of 3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) (Blana et al., 2017).

Although there is a positive correlation between AHL-mediated quorum sensing and biofilm formation in anaerobiosis, it is necessary to understand how these molecules regulate

this complex phenomenon. Thus, this study aimed to evaluate how C12-HSL affects the expression of the main biofilm components and regulators in *Salmonella* Enteritidis PT4 578 under anaerobic conditions by comparing two temperatures, 28 and 37 ºC. For this, biofilm formation and the expression of significant regulators of biofilm formation, global regulators of anaerobiosis, and quorum sensing regulators genes were evaluated.

2 Materials and methods

2.1 Bacterial strain, growth media, and chemicals

S. enterica serotype Enteritidis PT4 578, isolated from chicken breasts (GenBank: 16S ribosomal RNA gene MF066708.1) and obtained from the bacterial culture collection of the Oswaldo Cruz Foundation (FIOCRUZ; Rio de Janeiro, Brazil), was used in this study. The culture was maintained in Luria Bertani (LB) broth (tryptone 1%, yeast extract 0.5%, and NaCl 0.4%) with 20% (v/v) sterile glycerol at -20 °C. Cells were cultured in aerobic or anaerobic Tryptic Soy Broth (TSB; Sigma-Aldrich, St. Louis, United States). The anaerobic TSB was prepared with CO_2 under O_2-free conditions and dispensed in anaerobic bottles sealed with a butyl rubber stopper. All culture medium was autoclaved at 121 ºC,120 psi,15 min. C12-HSL (PubChem CID:11565426; Fluka, St. Gallen, Switzerland) was used as a quorum sensing signal molecule (treatment). The C12-HSL was suspended in acetonitrile (Merck, Darmstadt, Germany) at a concentration of 10 $mmol.L^{-1}$ and further diluted to a working solution of 100 $\mu mol.L^{-1}$ in acetonitrile. The C12-HSL was added to the culture medium to a final concentration of 50 $nmol.L^{-1}$. The choice of C12-HSL and concentration was based on previous studies (Campos-Galvão et al., 2015; Almeida et al., 2016; Carneiro et al., 2020). Acetonitrile was used as a control. All experiments were performed in laboratories with biosafety level 2.

2.2 Inoculum standardization

The inoculum was prepared as established by Almeida et al. (2017). Before each experiment, cells were cultured twice in 10 mL of aerobic or anaerobic TSB and incubated at 37 °C. Then an aliquot was transferred to 10 mL of aerobic or anaerobic TSB and incubated for 4 h at 37 °C. Finally, a volume of 2 mL was centrifuged, washed, and resuspended with sterile 0.1% (w/v) peptone saline. The optical density at 600 nm (OD600nm) of the cells was standardized at 0.1 corresponding to approximately 10^7 $CFU.mL^{-1}$.

2.3 Biofilm formation on stainless steel coupons

Stainless steel coupons with dimensions of 1 x 1 cm were the abiotic substrates used for biofilm development, as described previously by Oliveira et al. (2019), with some modifications. Briefly, the coupons were sanitized and fixed with cotton thread at the bottom of bottles and covered by adding 10 mL of aerobic or anaerobic TSB and subsequently autoclaved. The biofilm was conducted in two temperatures, 28 and 37 ºC. These temperatures were chosen because 28 °C is the optimum temperature for expressing extracellular matrix components of the biofilm, and 37 °C is the optimum temperature for *Salmonella* growth and human body temperature (Gerstel and Römling, 2003; Steenackers et al., 2012). Two different atmospheres were also evaluated aerobiosis and anaerobiosis. The inoculum was prepared as described in item 2.2, with the following observation: for the aerobiosis experiments, the inoculum was prepared in aerobic TSB, and for the anaerobiosis experiments, the inoculum was prepared in anaerobic TSB. The volume of 1 mL of the standardized inoculum was inoculated in 10 mL of aerobic or anaerobic TSB with stainless steel coupons and incubated at 28 and 37 ºC for 40 h, but for anaerobic conditions, it was incubated in incubation jars (Probac, São Paulo, Brazil) with Anaerobac™ atmospheric generator (Probac, São Paulo, Brazil).

2.3.1 Quantification

The coupons were washed with 4 mL of sterile 0.1% (w/v) peptone saline to remove the planktonic cells for sessile cell quantification,. Then, each coupon was transferred to a tube containing 3 mL of sterile 0.1% (w/v) peptone saline and was subjected to ultrasound (Sonics & Materials Inc., United States) for 30 s at a frequency of 20 kHz to remove firmly attached cells. Decimal dilutions were prepared, plated into Plate Count Agar (PCA) (Himedia, Mumbai, India), and incubated at 37 ºC for 40 h. Subsequently, colonies were enumerated.

2.3.2 Microscopy analysis

Biofilm formation by *Salmonella* Enteritidis PT4 578 on stainless steel surface was also observed using microscopy. The same experimental design described in item 2.3.1 for quantifying sessile cells was used for this. After 40 h incubation, the coupons were collected, washed with 4 mL of sterile 0.1% (w/v) peptone saline, and air-dried at room temperature. After, they were stained with Live/Dead™ BacLight Bacterial Viability Kit (Invitrogen, Oregon, United States). Cells with a compromised membrane that are considered dead or

dying will stain red, whereas cells with an intact membrane will stain green. After 20 min, coupons were washed with sterile 0.1% (w/v) peptone saline to remove excess dye and observed using an epifluorescence microscope EVOS M5000 (Invitrogen, United States).

2.4 Evaluation of the influence of C12-HSL on biofilm formation under anaerobic conditions on stainless steel coupons

The influence of the quorum sensing molecule C12-HSL was evaluated under anaerobic conditions at 28 and 37 ºC. First, the bottles containing 10 mL of anaerobic TSB were supplemented with 5.5 µL of C12-HSL at a final concentration of 50 $nmol.L^{-1}$ (treatment) or with the same volume of acetonitrile (control). Then, 1 mL of the standardized inoculum (10^7 $CFU.mL^{-1}$) was added to each bottle and incubated using incubation jars with Anaerobac™ atmospheric generator under the two temperatures. After 20 h incubation, the culture medium was replaced by a new anaerobic TSB medium supplemented with 5.5 µL of C12-HSL or 5.5 µL of acetonitrile and 1 mL of a new standardized inoculum. After 40 h incubation, the coupons were collected, and the number of adhered cells was quantified as described in item 2.3.1. Biofilm formation was also observed by microscopy, as described in item 2.3.2.

2.5 Expression of genes related to biofilm and quorum sensing

The expression of four biofilm-related genes and two quorum sensing-related genes (Table 1) was evaluated in biofilm cells after 40 h. The biofilm was formed in a 6-well polystyrene microplate. The culture of *Salmonella* Enteritidis PT4 578 was obtained as described in item 2.2. The microplate wells were filled with 10 mL of anaerobic TSB, 5.5 µL of C12-HSL at a final concentration of 50 $nmol.L^{-1}$ or with the same volume of acetonitrile, and 1 mL of the standardized inoculum. After 20 h incubation, the culture medium was replaced by a new culture medium supplemented with 50 $nmol.L^{-1}$ C12-HSL or acetonitrile and 1 mL of a new standardized inoculum. After 40 h of cultivation, the wells were washed two times with 5 mL of sterile 0.1% (w/v) peptone saline to remove planktonic cells. Adhered cells were scraped and resuspended in the same solution for RNA extraction and enumeration of sessile cells. The total RNA was extracted with Trizol™ Reagent (Invitrogen™, Thermo Fisher Scientific, United States) according to the supplier's instructions. RNA concentration and quality were analyzed using NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, United States). Complementary DNA (cDNA) synthesis was performed using 1 µg treated RNA and the ImProm-II Reverse Transcription kit with Random Hexamer Primer (Promega,

United States). Specific primers (Table S1) were designed in the program GenScript, and the quality of these primers was evaluated using the Oligo Explorer tool and electronic PCR with the *Salmonella* Typhimurium LT2 (GenBank: NC_ 003197.2) genome as a template. The expressions of endogenous gene *gyrA* were used to normalize the data. Real time-qPCR was performed with Sybr Green I Master Mix (Promega, United States) in the 96-well microplates using the StepOnePlusTM Real-Time PCR System (Applied Biosystems, United States). Conditions used for the amplification were 95 °C for 10 min, 40 denaturing cycles at 95 °C for 30 s, followed by annealing and extension at 60 °C for 1 min. After the 40 cycles, the temperature was gradually increased, by 1 °C increments, to 95 °C, resulting in the generation of a melting curve (Carneiro et al., 2020). The efficiency of amplification was determined by running a standard curve for each primer with serial dilutions DNA and calculated with the formula $E=(10^{(1/\text{-slope})} - 1)$ x 100. The relative standard curve was used to calculate each sample's relative quantity (Rq) values for each gene (Mendes et al., 2013).

2.6 Statistical analysis

All experiments were carried out in three biological replicates. Data were analyzed by the GraphPad Prism 5.0 program (GraphPad Inc.) using Tukey's test. A $p < 0.05$ was considered statistically significant. The results were expressed as mean values and their standard deviations. Graphics were built in the same software.

3 Results

The influence of temperature and atmosphere on biofilm formation by *Salmonella* Enteritidis PT4 578 was evaluated on stainless steel coupons immersed in aerobic and anaerobic TSB after 40 h. The atmosphere and temperature did not influence the sessile cell number at 28 °C. However, at 37 °C, the cell number in the biofilm was significantly reduced under anaerobic conditions (Fig. 1).

The images obtained in epifluorescence microscopy corroborated the sessile cell count results. In stainless steel, there was no difference in the biofilm of *Salmonella* Enteritidis PT4 578 cultivated at 28 ºC in aerobiosis and anaerobiosis (Fig. 2A and 2B). However, at 37 ºC, there were more clusters of sessile cells in aerobic than in anaerobic conditions (Fig. 2C and 2D). Furthermore, using the Live/Dead® kit dyes, it is possible to observe the predominance of live cells, characterized by their green color. These results reinforce that environmental conditions, such as atmosphere and temperature, ultimately dictate biofilm formation properties.

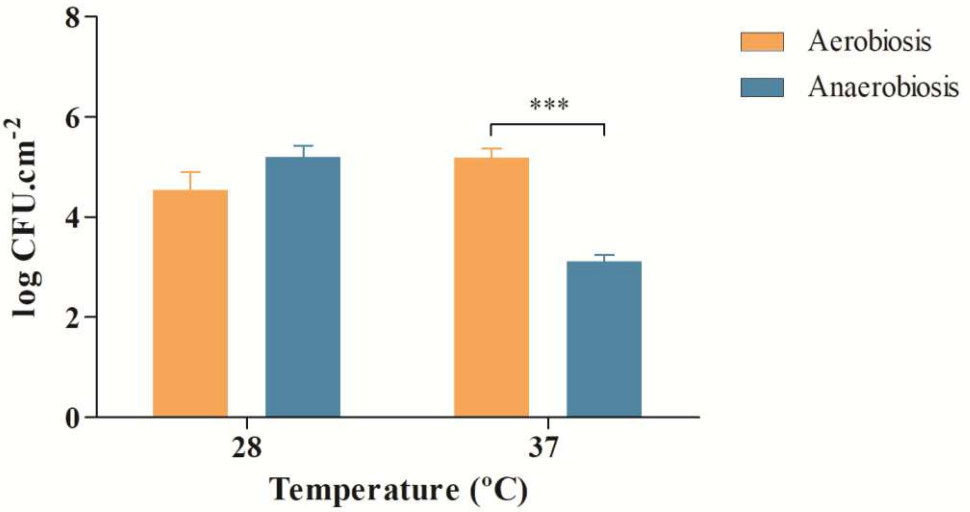

Fig. 1. Number of *Salmonella* Enteritidis PT4 578 sessile cells in stainless steel coupons, expressed in log CFU.cm^{-2}, after 40 h of cultivation in TSB in two temperatures, 28 and 37 ºC and atmosphere conditions of aerobiosis and anaerobiosis. Error bars indicate the standard deviation and values that are significantly different by Tukey's test are indicated: *p < 0.05, **p < 0.005 and ***p < 0.001.

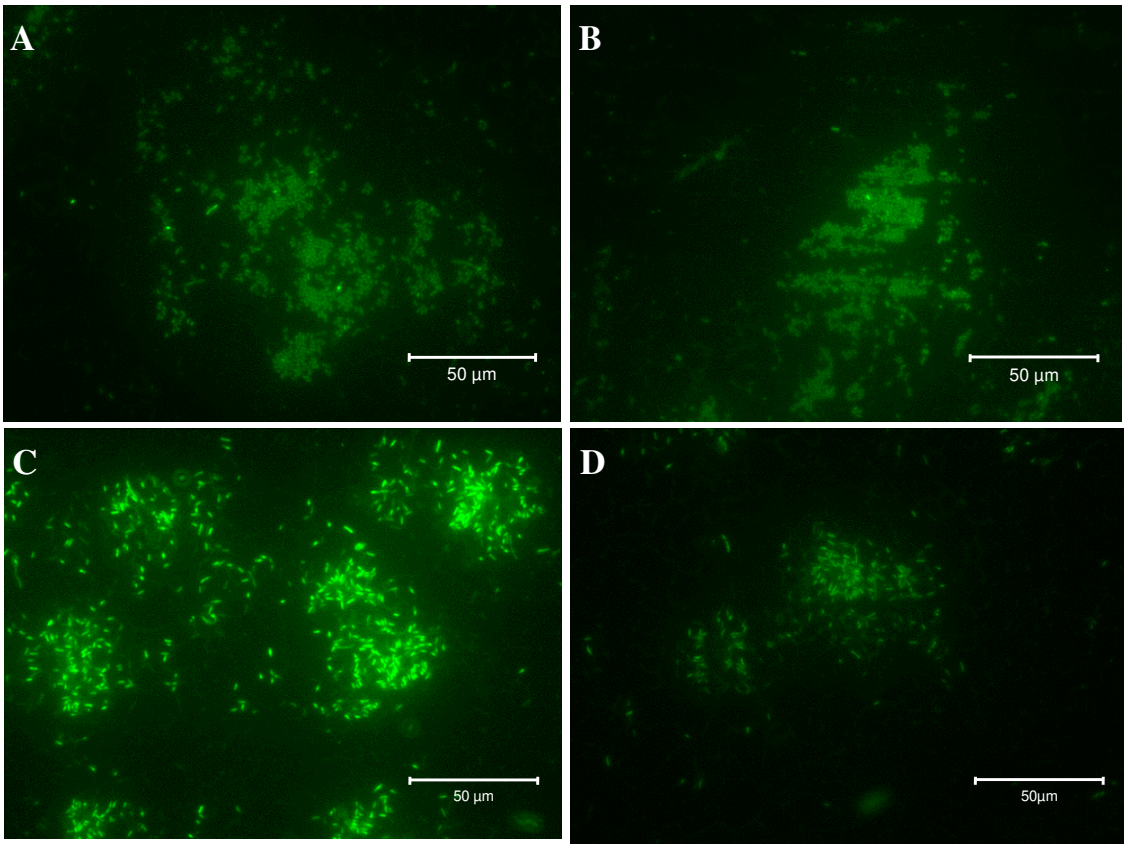

Fig. 2. Biofilm formed by *Salmonella* Enteritidis PT4 578 on stainless steel coupons immersed in aerobic (A) and anaerobic TSB (B) at 28 ºC and in aerobic (C) and anaerobic (D) TSB at 37 ºC for 40 h.

As there is still a need to clarify the effect of AHLs on *Salmonella* biofilm formation under different experimental conditions, biofilm formation by *Salmonella* Enteritidis PT4 578 in the presence of 50 nmol.L^{-1} of C12-HSL was evaluated at 28 and 37 ºC on stainless steel coupons, under anaerobic conditions. The anaerobic atmosphere was chosen to follow the experiments considering the previous results indicating that in this condition, there is a reduction in biofilm formation at 37 °C. Again, after incubation at 28 ºC for 40 h, no significant difference was observed in the cells number in biofilm formed in the absence (control) or in the presence of C12-HSL (treatment) (Fig. 3). However, at 37 ºC the stimulatory effect of C12-AHL on biofilm formation by *Salmonella* Enteritidis PT4 578 was noted, due to the more significant number of sessile cells (Fig. 3).

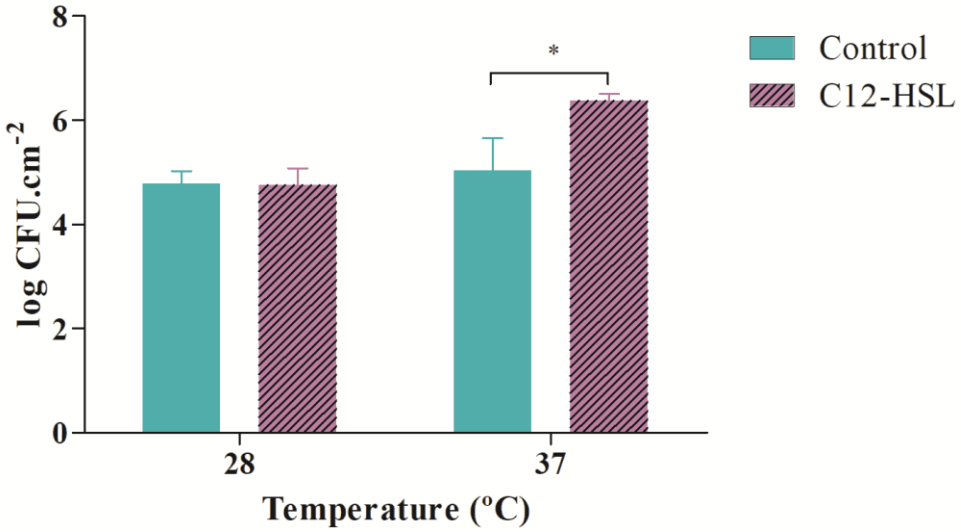

Fig. 3. Number of *Salmonella* Enteritidis PT4 578 sessile cells on stainless steel coupons, expressed in log UFC.cm^{-2}, after 40 h of cultivation in TSB supplemented with C12-HSL (treatment) or acetonitrile (control) in two temperatures 28 and 37 ºC in anaerobiosis. Error bars indicate the standard deviation and values that are significantly different from the control by Tukey's test are indicated, * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.001$.

Epifluorescence microscopy allows visualization of groups of viable sessile cells (Fig 4) in all experimental conditions. After 40 h, no difference between the control and treatment was observed in coupons incubated at 28 °C, corroborating the quantitative results (Fig. 4A and 4B). At 37 ºC, it is possible to visualize small groups of sessile cells without forming layers and compaction (Fig. 4C). In the presence of C12-HSL, large groups of cells were observed, forming extensive and compact structures (Fig. 4D).

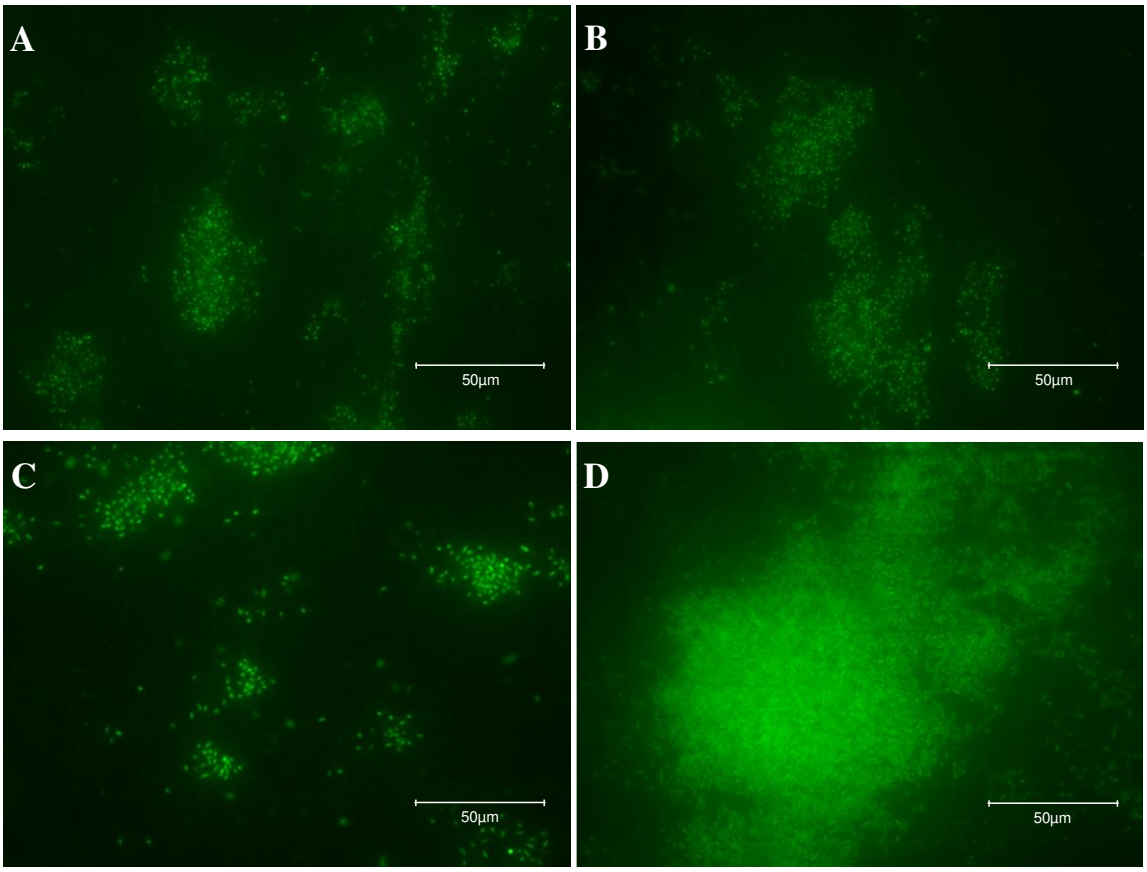

Fig. 4. Biofilm formation by *Salmonella* Enteritidis PT4 578 on stainless steel coupons immersed for 40 h at 28 ºC in anaerobic TSB supplemented with acetonitrile (control) (A) and 50 nmol.L^{-1} of C12-HSL (treatment) (B) and at 37 ºC supplemented with acetonitrile (control) (C) and 50 nmol.L^{-1} of C12-HSL (treatment) (D).

The number of sessile cells reached around 10^6 and 10^7 CFU per well of polystyrene microplates in the control and treatment with C12-HSL at 28 °C, respectively. At 37 °C, the sessile population reached 10^7 and 10^8 CFU per well in control and treatment, respectively.

The RNA extracted from cells grown in polystyrene microplates with anaerobic TSB incubated at 28 and 37 ºC was used for the real time-qPCR. The expression levels of *csgD*, *adrA*, and *csrA* were not significantly affected by the presence of C12-HSL in biofilm cells cultured at 28 ºC (Fig. 5A). This result supports previous observations indicating that this AI-1 does not alter biofilm formation at 28 °C. However, gene expression was substantially reduced when biofilm formation was conducted at 37°C. An exception was observed for the *crl* expression, which practically did not vary with temperature (Fig. 5A and 5B). Furthermore, the presence of C12-HSL did not revert the reduced expression of *csgD* and *csrA* in sessile cells cultured at 37 °C, but C12-HSL significantly increased *adrA* expression (Fig. 5B).

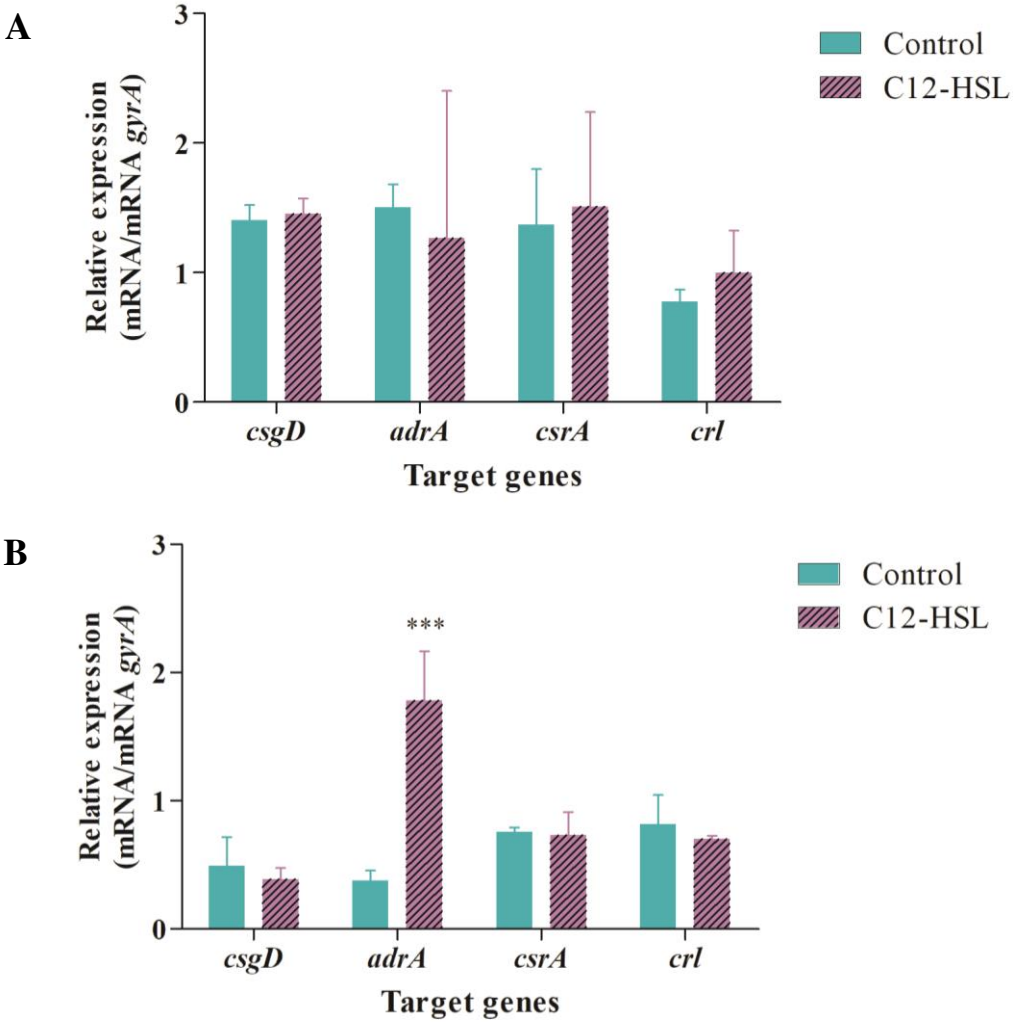

Fig. 5. The relative gene expression of biofilm-related genes *csgD*, *adrA*, *csrA* and *crl*, from *Salmonella* Enteritidis PT4 578 anaerobically cultivated in TSB at 28 ºC (A) and 37 ºC (B) with the addition of acetonitrile (blue bar) or 50 nmol L^{-1} of C12-HSL (red bar). Error bars indicate the standard deviation and values that are significantly different by Tukey's test are indicated: *$p < 0.05$, **$p < 0.005$ and ***$p < 0.001$.

As observed in the biofilm-related genes, the expression levels of the *sdiA* and *luxS* genes were higher in the biofilm cells cultivated at 28 ºC (Fig. 6A and 6B). Although *sdiA* expression did not vary significantly with C12-HSL at both evaluated temperatures, *luxS* was significantly more expressed at 28 and 37 ºC in the presence of this AI-1 (Fig.6A and 6B). These results indicate an intercommunication between quorum sensing systems that needs to be better understood and explored.

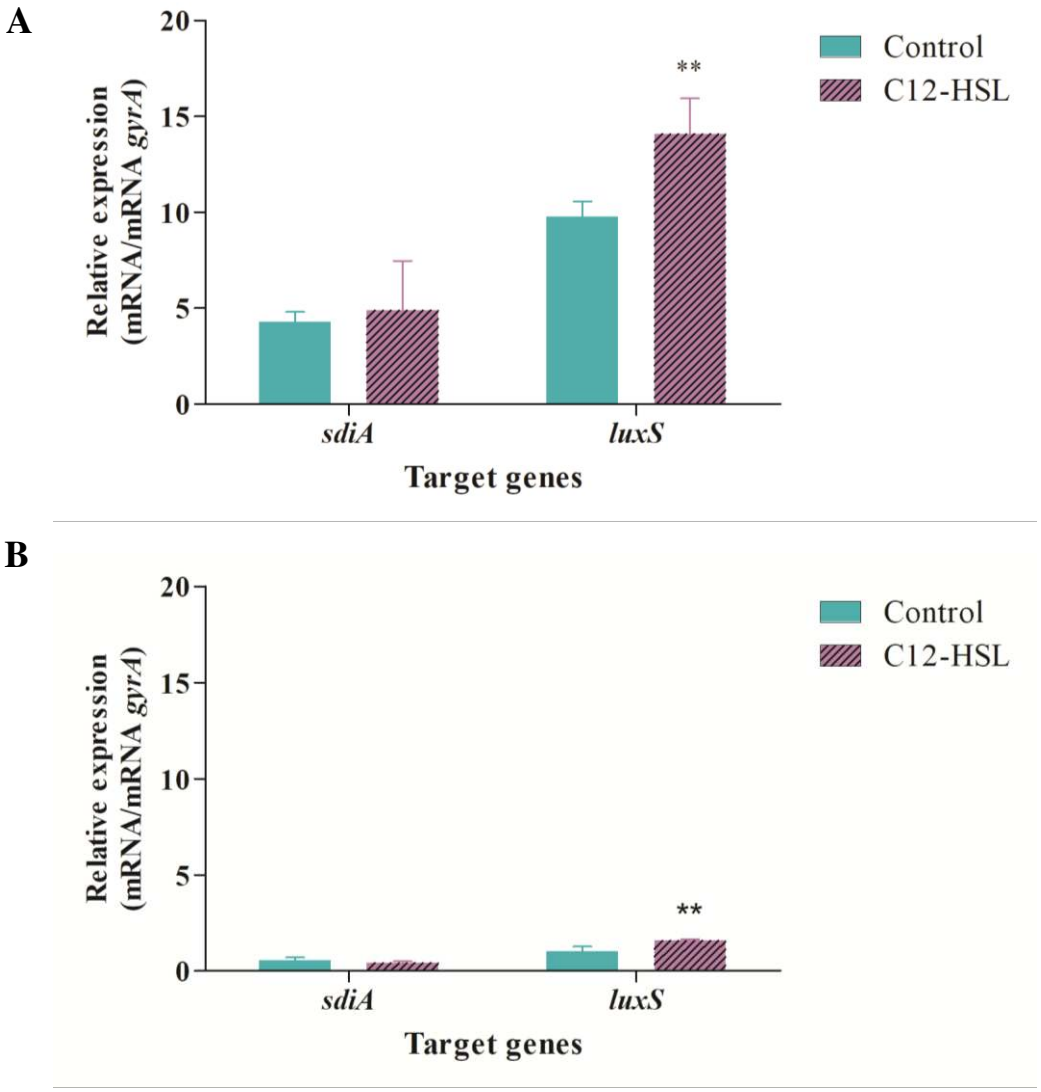

Fig. 6. The relative gene expression of quorum sensing genes *sdiA* and *luxS*, from *Salmonella* Enteritidis PT4 578 anaerobically cultivated in TSB at 28 ºC (A) and 37 ºC (B) with the addition of acetonitrile (blue bar) or 50 nmol L^{-1} of C12-HSL (red bar). Error bars indicate the standard deviation and values that are significantly different by Tukey's test are indicated: *$p < 0.05$, **$p < 0.005$ and ***$p < 0.001$.

4 Discussion

Multiple factors strongly influenced by different environmental signals are determinants for biofilm formation by *Salmonella*. *Salmonella* Enteritidis PT4 578 forms a biofilm at 28 °C and 37 °C under aerobic and anaerobic conditions, confirming that this characteristic of the genus is maintained in this serotype. Numerous studies confirm the

biofilm formation by *Salmonella* serotypes at different temperatures in aerobic conditions. For example, Borges et al. (2018) evaluated 243 *Salmonella enterica* belonging to 11 serotypes isolated from either poultry or food involved in salmonellosis outbreaks in southern Brazil. These authors demonstrated, in aerobic conditions, that moderate and strong producer isolates were more common when the incubations were at 37 and 28 °C, respectively.

The significant reduction of biofilm formed by *Salmonella* Enteritidis PT4 578 at 37 ºC in the anaerobic condition is in agreement with other studies that demonstrated that high temperatures and low oxygen tensions are factors that reduce or even inhibit biofilm formation in many *Salmonella* isolates (Gerstel and Romling, 2001; Joo and Otto, 2012; Lamas et al., 2016). However, the absence of oxygen does not influence the number of sessile cells of *Salmonella* Enteritidis PT4 578 at 28 ºC; this could be related to EPS. The temperature of 28 ºC is described as optimal for synthesizing matrix constituents, such as fimbria curli and cellulose (Steenackers et al., 2012). Tursi and Tükel (2018) reinforced that curli expression is triggered when enteric bacteria are grown under stressful environmental conditions that favor biofilm formation over planktonic cell growth. Stress factors include temperature, osmolarity, and oxygen or nutrient availability.

It is possible to hypothesize that the more remarkable synthesis of EPS constituents favored by the temperature of 28 °C has superimposed the effect of C12-HSL added to the medium. However, in anaerobiosis, at 37 ºC, a condition with a lower stimulus for EPS production, the positive effect of C12-HSL was notable. The results obtained reinforce the observations of previous studies that demonstrated the ability of the *Salmonella* Enteritidis PT4 578 to form a mature biofilm on polystyrene when grown in anaerobic TSB at 37 ºC in the presence of the quorum sensing molecule C12-HSL (Campos-Galvão et al., 2015; Almeida et al., 2017). Furthermore, it demonstrates that the influence of C12-HSL on biofilm formation may be temperature and atmosphere-dependent but not influenced by the surface of stainless steel or polystyrene.

The quantitative and qualitative results indicating that C12-HSL did not interfere with biofilm formation at 28 °C in anaerobiosis could be explained with gene expression analysis. No influence of the AI-1 molecule upon gene *csgD*, *adrA*, *csrA*, and *crl* was observed after 40 h incubation at 28 °C in anaerobiosis. The lower formation of biofilm by *Salmonella* Enteritidis PT4 578 at 37 ºC in anaerobiosis could also be influenced by the lower expression of genes evaluated and related to curli and cellulose synthesis, as well as quorum sensing.

However, growth in the presence of C12-HSL altered the expression of only *adrA* after 40 h incubation at 37° C in anaerobiosis.

CsgD is key to regulating the transition from motile to sessile behavior (Tursi and Tükel, 2018), and an essential protein in the regulatory network of the biofilm in *Salmonella*, related to cellulose biosynthesis through direct binding and subsequent transcriptional stimulation of *adrA* (Steenackers et al., 2012). The *adrA* gene encodes a membrane-bound GGDEF domain protein with diguanylate cyclase activity, involved in the production of c-di-GMP (Römling, 2002). Therefore, C12-HSL stimulates *adrA* expression in a condition that reduces the expression and other related genes. Elevated levels of c-di-GMP due to overexpression of *adrA* may be sufficient to overcome temperature-based *csgBAC* repression (Kader et al., 2006; Joo and Otto, 2012). Thus, a possibile quorum sensing mechanism to stimulate biofilm formation at 37 ºC in anaerobiosis may be the increase in c-di-GMP through increased diguanylate cyclase *adrA* expression (Fig. 6). The c-di-GMP is recognized as a bacterial second messenger and controls several physiological functions, such as the mobile-sessile transition of numerous bacteria (Römling et al., 2005; Jenal et al., 2017; Lamprokostopoulou and Römling, 2022). Quorum sensing controls biofilm formation through c-di-GMP levels, regulating the transcription of fourteen genes that encode a group of proteins that synthesize and degrade this molecule in *Vibrio cholerae* (Waters et al., 2008). Quorum sensing also monitors c-di-GMP levels in *Burkholderia cenocepacia* (Deng et al., 2012) and *Pseudomonas aeruginosa* (Kim et al., 2018).

The c-di-GMP is produced through two molecules of guanosine triphosphate (GTP). Interestingly, an analysis of the metabolome of planktonic *Salmonella* Enteritidis PT4 578 grown at 37 °C under anaerobic conditions demonstrated that C12-HSL strongly influenced purine metabolism (Carneiro et al., 2020). Although the physiology of planktonic and sessile cells differes (Joo and Otto, 2012; Giaouris et al., 2013), increased purine metabolism in the presence of C12-HSL may indicate increased c-di-GMP pools. At increased levels, this messenger suppresses motility and promotes the synthesis of biofilm matrix compounds (Kader et al., 2006).

In addition to mediating the transition between motility and biofilm, c-di-GMP mediates the transition between virulence and biofilm formation in the host. Cellulose is considered an antivirulence factor. However, it is believed that *Salmonella* has used its

expression within macrophages as a strategy to prolong the infection and increase the chance of infection for new hosts (Pontes et al., 2015).

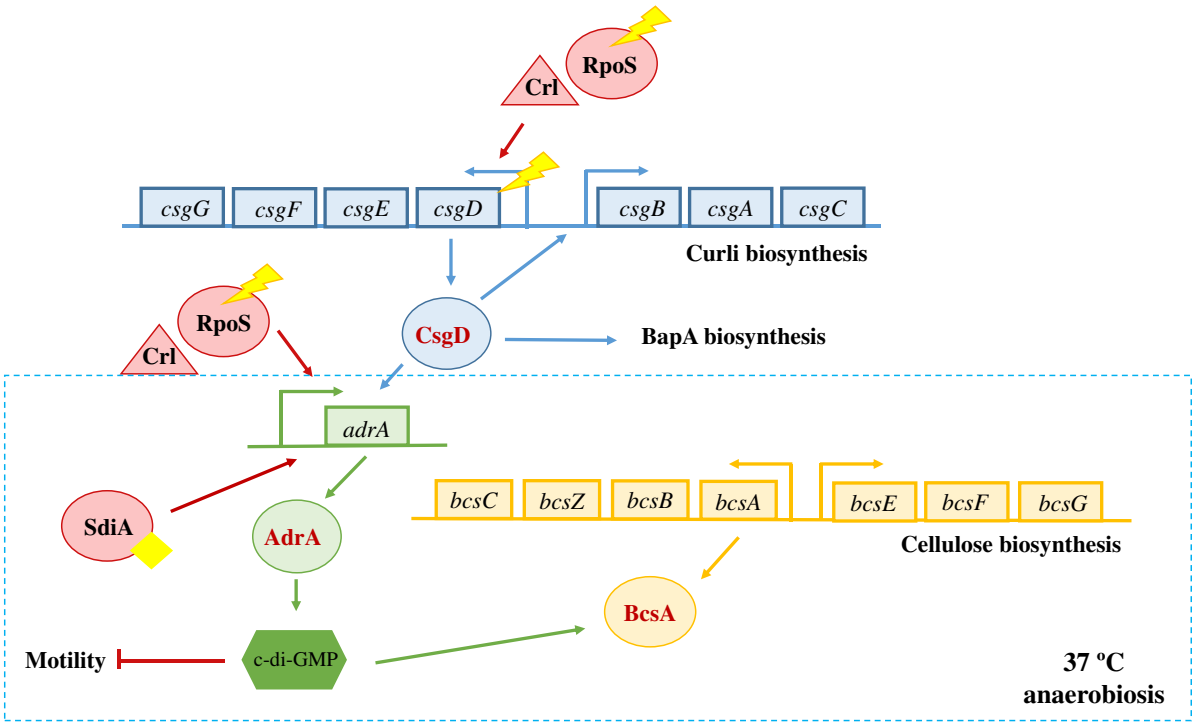

Fig. 6. The hypothetical influence of C12-HSL on anaerobic biofilm formation at 37 ºC in *Salmonella* Enteritidis PT4 578. Arrows and flat-headed arrows represent an activation and a suppression effect, respectively. Blue, green and orange rectangles represent the genomic organization of the genes that encode the main structural components of the biofilm. Circles and triangles represent major regulatory proteins. Lightning symbols represent the entry and integration of different environmental signals through these general regulators into the regulatory system. Adapted (Steenackers et al., 2012).

This study also evaluated the expression of some crucial regulators for biofilm formation. The CsrA, a key negative regulator of biofilm formation, acts by stimulating motility, promoting FlhDC expression, an important regulator of flagellum biosynthesis, and directly and indirectly regulates the expression of at least eight genes involved in this network that encode GGDEF domain proteins (Steenackers et al., 2012). C12-HSL did not significantly influence *csrA* expression at the two tested temperatures. Another evaluated gene was *crl* which interacts directly with RpoS promoting the binding of the σ^S holoenzyme to the *csgBA* promoter. The bidirectional *csgBAC* operon is involved in the amyloid curli production at 28 ºC not 37 ºC. Therefore, it is a thermoregulated protein (Robbe-Saule et al., 2006).

However, C12-HSL did not influence the expression of this protein at any of the evaluated temperatures.

The expression of quorum sensing related gene *sdiA* and *luxS* by sessile cells of *Salmonella* Enteritidis PT4 578 reduced when the temperature incubation increased from 28 °C to 37 °C. The *sdiA* was not influenced by C12-HSL at any of the temperatures. Carneiro et al. (2020) observed that C12-HSL also does not influence *sdiA* expression in *Salmonella* Enteritidis PT4 planktonic cells at 37 °C incubation in anaerobiosis. However, C12-HSL increased *luxS* expression at 28 and 37 °C. LuxS is responsible for the synthesis of autoinducer-2 (AI-2). A study with encapsulated colonies have shown that spatial distribution has a crucial impact on bacterial quorum sensing (Gao et al., 2016). Large cell clusters exhibit stronger quorum sensing. Although C12-HSL does not significantly influence the number of sessile cells, through the images, it is possible to see that this molecule stimulates the formation of large and dense aggregates of cells. Increased *luxS* expression in the presence of C12-HSL could result in increased AI-2 production and, consequently, stimulate these aggregates, or the aggregation of *Salmonella* cells can be a stimulus for communication via AI-2. Therefore, more studies are needed to clarify how AI-1 and AI-2 act together in forming biofilm by *Salmonella*.

Continued understanding of *Salmonella* fitness, diversification, virulence, and survivability will be essential to our ability to manage, treat, and prevent its contamination of humans and human-associated upstream niches (Brown et al., 2021).

5 Conclusion

External factors such as temperature and oxygen tension strongly influence biofilm formation in *Salmonella*. The results demonstrate that C12-HSL can overcome temperature regulation and low oxygen tension, inducing biofilm formation in stainless steel coupons. Furthermore, analysis of biofilm gene expression demonstrated that *adrA* expression is significantly increased by this quorum sensing signal molecule. Therefore, it is likely that the mechanism of biofilm formation activated by C12-HSL is through the increase of the second messenger, c-di-GMP. Through the integration between quorum sensing, *Salmonella* can finely control essential phenotypes, such as virulence and biofilm, and combine information from the surrounding bacterial population with environmental signals. Furthermore, C12-HSL has been shown to increase *luxS* expression in biofilm cells, indicating a connection between communication mechanisms. Although additional studies are needed, this work reinforces the

role of quorum sensing in *Salmonella* biofilm formation and provides new information on the mechanism of action. From this perspective, in addition to quorum sensing, c-di-GMP-related genes can be an exciting target to combat the biofilm of this highly clinically relevant pathogen.

6 References

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Supplementary Table

Table S1. Primers used for RT-qPCR assays.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Reference
gyrA	ATCGCCGAGCTGGTGAAAGA	ACCTGTAGCTGGGTCTGGGA	(Luiz de Freitas et al., 2020)
csgD	AGGGTATTCTGCGTGGCGAA	TAATGCGGACTCGGTGCTGT	This study
adrA	GAAGCTCGTCGCTGGAAGTC	TTCCGCTTAATTTAATGGCCG	This study
crl	TCGATTGCCTGGCAGTTTGC	TCGCTCCACCACTTCGGTTT	This study
sdiA	ACGCGCAATGTTGTTACGCT	TTCCAGCCGCTGTGTCTGAT	(Carneiro et al., 2020)
luxS	CGATGGCGGATGTGCTGAAA	GCAATGTCCTGCGCTTCACT	This study

CONCLUSION AND PERSPECTIVES

Salmonella Enteritidis has a vast arsenal of virulence factors related to host invasion and colonization in its genome. In addition, it has all the genes related to biofilm formation. Although this serotype is not a good biofilm producer under conditions considered optimal for biofilm formation in the genus, that are temperatures below 30 ºC and aerobic conditions, *Salmonella* Enteritidis can form biofilm at 37 ºC under anaerobic conditions in the presence of C12-HSL on a stainless steel, a surface widely used in the food industry. The mechanism involved in biofilm formation in this condition may be increased synthesis of the bacterial second messenger, c-di-GMP, through increased *adrA* expression. The messenger c-di-GMP is related to the regulation of important phenotypes, such as virulence, motility, and biofilm formation. Therefore, the connection of this messenger with quorum sensing could be an interesting strategy for the fine control of important phenotypes. Future research needs to be done to confirm this association in other serotypes and environmental conditions. Furthermore, it may be interesting to evaluate the expression of other diguanylate cyclases. These results may direct future strategies to eradicate the *Salmonella* biofilm.