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Anti-quorum sensing effects of licochalcone A and epigallocatechin-3-gallate against *Salmonella* Typhimurium isolates from poultry sources

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Abstract

Quorum sensing (QS) is a cell density-dependent mechanism used by many pathogenic bacteria for regulating virulence gene expression. Inhibition or interruption of QS by medicinal plant remedies has been suggested as a new strategy for fighting against antibiotic-resistant bacteria. This study aimed to assess the impact of sub-inhibitory concentrations of licochalcone A (LAA) and epigallocatechin-3-gallate (EGCG) as natural plant products on the QS-associated genes (*sdiA* and *luxS*) expression. The PCR test was used to confirm the presence of *sdiA* and *luxS* genes in 23 *S*. Typhimurium isolates from poultry. The quantitative real-time PCR assay was used to analyze the expression of *sdiA* and *luxS* in *S*. Typhimurium isolates in response to the treatment with sub-inhibitory concentrations of LAA and EGCG at 45-min time point. All *S*. Typhimurium isolates showed the presence of *sdiA* and *luxS* genes (100%). As result, the expression of QS-related genes was significantly reduced in *S.* Typhimurium isolates following treatment with LAA and EGCG. In conclusion, LAA and EGCG showed anti-QS activity with down-regulation of both *sdiA* and *luxS* genes in *S.* Typhimurium, suggesting potential therapeutic use of them against salmonellosis. However, it must be pointed out that the safety and efficiency of these compounds need more thorough research.

Introduction

The Gram-negative bacilli, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a widely distributed food-borne pathogen and one of the primary enteric pathogen infecting both humans and animals.[1] This pathogen is also one of the most frequent serotypes of *Salmonella* associated with salmonellosis worldwide[2] and is transmitted to humans primarily through the consumption of raw or uncooked eggs, vegetables, fruits and poultry.[3,4]

Many Gram-negative bacteria including *S.* Typhimurium employ a mechanism of intercellular communication known as quorum sensing (QS) to coordinate their gene expression in response to its population size. In *S.* Typhimurium, virulence determinants such as those encoded within the so-called *Salmonella* pathogenicity islands (SPIs) and the *Salmonella* plasmid virulence, adhesions, flagella, and biofilm-related proteins are under the control of QS pathways.[1] *Salmonella* bacteria utilize three main types of QS systems including acyl-homoserine lactone (AHL), autoinducer-2 (AI-2), and AI-3 signalings.[5] *S.* Typhimurium encodes a transcription factor of the LuxR family, named SdiA which detects and responds to AHLs produced by other species of bacteria.[6] To date, SdiA is known to regulate seven genes located in virulence plasmid and in the chromosome of *Salmonella* which including *pefI/srgC* operon, *srgE* and *sirA*.[7] The *luxS* gene is also directly involved in the AI-2 synthesis and induces the expression of the virulence genes of SPI-1, which are required for the efficient invasion of intestinal epithelial cells and are therefore crucial in the pathogenesis of *Salmonella* infections in several animal species.[8]

Antibiotics are commonly used therapeutically and prophylactically to treat *S.* Typhimurium infections in humans and animals.[9] However, increased occurrences of antimicrobial-resistant *S.* Typhimurium have been reported from different food animals around the world.[10] The emergence of multidrug-resistant strains of *S.* Typhimurium, particularly *S*. Typhimurium definitive phage type 104, is

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also a particular concern for animal husbandry and in human medicine.[10] In recent years, therefore, there has been a growing interest in the discovery of novel anti-microbial agents from natural sources to combat bacterial resistance. Anti-virulence drugs are a new type of therapeutic agent aiming at virulence factors rather than killing the pathogen, thus providing less selective presser for the evolution of resistance. One promising example of this therapeutic concept targets bacterial QS, which is a key regulatory system in the pathogenesis of various bacterial infections.[11] More recently, plant-derived natural products and phytochemicals have been emphasized as quorum sensing inhibitors (QSI).[12] Licochalcone A (LAA), a major phenolic constituent of the licorice species (*Glycyrrhiza inflate*), and epigallocatechin-3-gallate (EGCG), the most abundant catechin extracted from green tea (*Camellia sinensis*), have been reported to possess remarkable antibacterial activity against various microorganisms.[13,14] Although LAA and EGCG have been proven to have anti-QS activity against a range of bacteria, there is no report on the anti-QS activity of these two compounds against *S*. Typhimurium. Therefore, the purpose of this study was to investigate the effects of phytochemicals LAA and EGCG on two regulatory genes (*luxS* and *sdiA*) of food-borne pathogen *S.* Typhimurium.

Materials and Methods

Bacterial strains and reagents. *S.* Typhimurium RITCC1730 was obtained from Razi Institute Culture Collection Center (Razi Vaccine and Serum Research Institute, Karaj, Iran). A total of 23 clinical isolates of *S.* Typhimurium from poultry flocks were also included in this study (kindly provided by Professor Taghi Zahraei-Salehi, Faculty of Veterinary Medicine, Tehran University, Tehran, Iran). These isolates had previously been identified by biochemical and molecular tests to be representative of *S.* Typhimurium.[15] According to our previous study, 14 out of 23 isolates (60.78%) were resistant to one or more antibiotics and LAA and EGCG inhibited their growth at a minimum inhibitory concentration (MIC) of 62.50~1000 and 1.56~400 μg mL^{-1}, respectively.[16] The LAA and EGCG were purchased from Sigma-Aldrich (Taufkirchen, Germany) and a stock solution was made in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The final concentration of DMSO for dissolving compounds was 10.00% (v/v). The LAA and EGCG stock solution concentrations were 2.00 mg mL^{-1} and 0.80 mg mL^{-1}, respectively.

Detection of *16S rRNA*, *sdiA*, and *luxS* genes by PCR. We used the PCR test to confirm the presence of *16SrRNA*, *sdiA*, and *luxS* genes in the studied isolates. The PCR amplification was carried out in a 25.00 μL reaction mixture containing 2.00 μL of the DNA as the template, 12.50 μL of 2X PCR master mix (3.00 mM $MgCl_2$, 0.04 U μL^{-1} Taq polymerase, reaction buffer, 0.40 mM of each dNTPs), 1.00 μL (0.40 mM) from the forward and reverse primers (SinaClon, Tehran, Iran), (Table 1). The PCR amplification was conducted in a thermal cycler (CP2-003; Corbett, Sydney, Australia). The cycling program consisted of denaturation at 94.00 ˚C for 5 min, followed by 35 cycles of 94.00 ˚C for 1 min, 60.00 ˚C for 1 min, 72.00 ˚C for 1 min. A final extension was performed at 72.00 ˚C for 10 min. Both positive and negative control reactions were included in each PCR amplification experiment. For negative controls, template DNA was replaced with sterile water. The *S*. Typhimurium RITCC1730 was used as a positive control. PCR products were resolved by electrophoresis in 2.00% (w/v) agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, USA). Visualizations were undertaken using a UV trans-illuminator (BTS-20, Tokyo, Japan) and the 100 bp plus DNA ladder (Thermo Scientific, Karlsruhe, Germany) was used as a molecular size marker.

Growth curves. For each of the LAA and EGCG, *S.* Typhimurium RITCC1730 was grown to an optical density of 0.10 at 600 nm in Mueller-Hinton Broth (MHB; Merck, Darmstadt, Germany) and distributed as 100 mL volumes into six 500 mL Erlenmeyer flasks (Azmatajhiz, Karaj, Iran). The LAA and EGCG (dissolved in 10.00% DMSO) were added to five of the cultures to obtain final concentrations of 1/4 MIC, 1/2 MIC, 1 MIC, 2 MIC, and 4 MIC, respectively. The control culture included the addition of 10.00% DMSO alone. The cultures were incubated further and cell growth was monitored spectrophotometrically in optical density at 600 nm. For this, 3.00 mL samples of each culture were collected immediately at 15 min intervals after the addition of LAA and EGCG (t_0). Also, the total number of viable bacteria was estimated by plating dilutions of the culture on Muller Hinton Agar without antibiotic and counting the numbers of CFU after 24 hr at 37. 00 ˚C.

Treatment with LAA and EGCG. To obtain RNA for investigating the effects of EGCG and LAA on the expression of *sdiA* and *luxS* genes, *S.* Typhimurium RITCC1730 and each of the clinical isolates of *S.* Typhimurium was grown overnight at 37.00 ˚C in 10.00 mL of MHB. Two 250 mL Erlenmeyer flasks, each of which contained 100 mL of MHB, were inoculated with an overnight culture to an initial OD600 of 0.10. Subsequently, the stock solution of LAA or EGCG prepared in 10.00% DMSO was added to one culture (experimental culture), giving a final concentration of 1/2 MIC. Another culture supplemented with vehicle only (DMSO 10.00% v/v) was used as a control culture. All bacterial suspensions (both experimental and control suspensions) were further incubated for 45 min at 37.00 ˚C and then RNA isolation was performed at this time. Three independent bacterial cultures for each LAA and EGCG treatment or control condition were prepared as biological replicates for RNA isolation on different days.

Table 1. Primers used in PCR.

Primer	Sequence (5'→3')	Amplicon	Reference
16s rRNA	F: AGGCCTTCGGGTTGTAAAGT R: GTTAGCCGGTGCTTCTTCTG	97 bp	40
sdiA	F: AATATCGCTTCGTACCAC R: GTAGGTAAACGAGGAGCAG	274 bp	21
luxS	F: ATGCCATTATTAGATAGCTT R: GAGATGGTCGCGCATAAAGCCAGC	204 bp	32

Total RNA isolation. Four hundred microliter of the bacterial suspensions were removed and combined with 800 µL of RNA Protect Bacteria Reagent (Qiagen, Valencia, USA) to minimize RNA degradation immediately before harvesting for RNA isolation. Then, cells were collected by centrifugation and kept at – 80.00 ˚C. The extraction of total RNA was carried out from both treated and non-treated bacteria using the Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's instructions. All RNA samples were treated with RNase-free DNase I (TaKaRa, Kusatsu, Japan) to remove contaminating DNA. The RNAs quality and quantity were monitored by agarose gel electrophoresis as well as measuring the absorbance at 260 and 280 nm using a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific, Wilmington, USA).

Real-time quantitative PCR assay. The RT-qPCR analysis was performed using the One-Step SYBR Prime-Script RT-PCR Kit (Perfect Real Time; TaKaRa). Each reaction mixture (20.00 µL) was prepared as follows: 10.00 µL of 2X One-Step SYBR RT-PCR Buffer III (Takara), 0.40 µL of TaKaRa Ex Taq HS (5.00 U μL^{-1}), 0.40 µL of PrimeScript RT enzyme Mix II, 0.40 µL of each primer (4.00 mM), 0.40 µL of ROX reference dye I, 2.00 µL template RNA and 6.00 µL of RNase-free dH_2O. Amplification and detection were performed with StepOne Real-Time PCR System (Applied Biosystems, Waltham, USA). Cycling condition included 42.00 ˚C for 5 min, 95.00 ˚C for 10 sec and then 40 cycles of 95.00 ˚C for 5 sec and 60.00 ˚C for 34 sec. To verify the identity of the amplified product, post-amplification melting curve analysis was conducted as follows: 95.00 ˚C for 15 sec followed by stepwise elevation of the temperature from 60.00 ˚C to 95.00 ˚C by 0.30 ˚C at a rate of 0.30 ˚C per sec with continuous fluorescence collection. To confirm that that no contamination exists; RNA template-free and a primer-free negative control were included in each run. All real-time PCR reactions were performed in triplicates and normalized against *16S rRNA* housekeeping gene expression. To minimize data variation in separate runs, paired treated and non-treated samples from the same isolate were examined on the same runs. The expression of the target genes *sdiA* and *luxS* was determined as relative to the expression of the endogenous control gene *16S rRNA* using the comparative cycle threshold (ΔΔCT) method of RT-PCR. Fold changes in *sdiA* and *luxS* expressions between treated isolates and matched non-treated isolates were also determined by the $2^{-\Delta\Delta CT}$ method.[17]

Statistical analysis. All experiments were performed in triplicate and repeated three times and the data are expressed as the mean ± SD. The statistical calculations were performed using GraphPad Prism Software (version 6.0; GraphPad Software Inc., San Diego, USA). A Student's *t*-test was used to analyze the data. A *p*-value of < 0.05 was considered to be statistically significant.

Results

Detection of *16S rRNA*, *sdiA*, and *luxS* genes by PCR. The primer pairs targeted against *16S rRNA*, *sdiA,* and *luxS* genes were able to amplify 97, 204, and 274 bp products, respectively, from all *S.* Typhimurium, tested isolates (Fig. 1).

Growth of *S.* Typhimurium in the presence of sub-inhibitory concentrations of LAA and EGCG. After 15 min of LAA and EGCG treatment, no obvious difference was observed in the OD600 value among all cultures. A steady increase in optical density occurred after 30 min. With 125, 250 and 500 µg mL^{-1} of LAA treatment, the optical density increased at a slower rate than it did with the lower concentrations. Somewhat similar results were recorded for EGCG treatment. After 300 min, the OD value of the *S.* Typhimurium treated with 31.25, 62.50, 125, 250 and 500 µg mL^{-1} of LAA was approximately 97.22%, 93.05%, 72.22%, 44.44% and 31.94% of control culture, respectively. The corresponding values of the *S.* Typhimurium treated with 25, 12.50, 6.25, 3.125, 1.562 µg mL^{-1} of EGCG were approximately 95.83%, 88.88%, 66.66%, 43.05% and 30.55% of control culture, respectively.

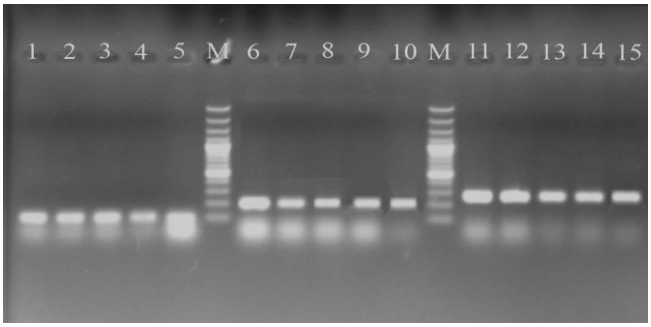

Fig. 1. Agarose gel electrophoresis of PCR product of *16S rRNA*, *luxS,* and *sdiA* genes for DNA extracted from analyzed *S.* Typhimurium isolates. Lane M: GeneRuler™ 100 bp plus DNA ladder; Lanes 1-5: Amplified 16S rRNA gene (97 bp) in some representative isolates; Lanes 6-10: Amplified *luxS* gene (204 bp) in some representative isolates; Lanes 11-15: Amplified *luxS* gene (274 bp) in some representative isolates.

According to the results, LAA and EGCG concentrations of 1 MIC, 2 MIC, and 4 MIC strongly inhibited the growth of *S.* Typhimurium RITCC1730. Therefore, to study the effects of a low LAA and EGCG concentrations on the transcription of *S.* Typhimurium, we reduced the inhibitory LAA and EGCG concentrations to 1/2 MIC (62.50 and 3.125 µg mL^{-1}, respectively) according to the growth curve. We chose the 45-min time point for LAA and EGCG treatment in the experiment based on the results obtained from the growth curve and our preliminary experiments. In preliminary experiments, cultures were harvested for RNA preparation following 15, 45, and 120 min of treatment. Results demonstrated that the 45-min time point was appropriate and produced the most meaningful results (data not shown). The growth curve of *S.* Typhimurium RITCC1730 in the presence of a sub-inhibitory concentration of LAA is shown in Figure 2.

Influence of sub-inhibitory concentration of LAA and EGCG on the expression levels of *sdiA* and *luxS* genes. Quantitative RT-PCR analysis indicated that relative expression of *sdiA* and *luxS* genes in 23 treated *S.* Typhimurium strains with LAA was significantly down-regulated in comparison with non-treated ones of the same strain ($p < 0.0001$ and $p = 0.0012$, respectively; Figs. 3A and 3B). The results also demonstrated significant down-regulation of *sdiA* and *luxS* in EGCG-treated strains in comparison with non-treated isolates ($p < 0.0001$ and $p = 0.0012$, respectively; Figs. 3C and 3D).

Comparison of LAA and EGCG effects on the relative expressions of *sdiA* and *luxS* genes. The impact of sub-inhibitory concentrations of LAA and EGCG on the relative expression of the *sdiA* and *luxS* genes of 23 *S.* Typhimurium strains indicated that LAA was significantly more effective than EGCG in reducing *sdiA* gene expression ($p = 0.0026$; Fig. 3E). However, EGCG was significantly more effective than LAA in decreasing the expression of the *luxS* gene ($p = 0.0004$; Fig. 3F).

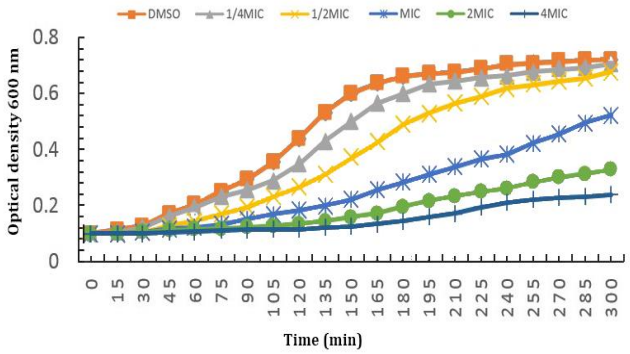

Fig. 2. Growth curve for *S.* Typhimurium RICC1730 in the presence or absence of LAA. —■—: *S.* Typhimurium plus 10.00% DMSO; —▲—: *S.* Typhimurium plus 31.25 µg mL^{-1} LAA; —×—: *S.* Typhimurium plus 62.50 µg mL^{-1} LAA; —✱—: *S.* Typhimurium plus 125 µg mL^{-1} LAA; —●—: *S.* Typhimurium plus 250 µg mL^{-1} LAA; —+—: *S.* Typhimurium plus 500 µg mL^{-1} LAA.

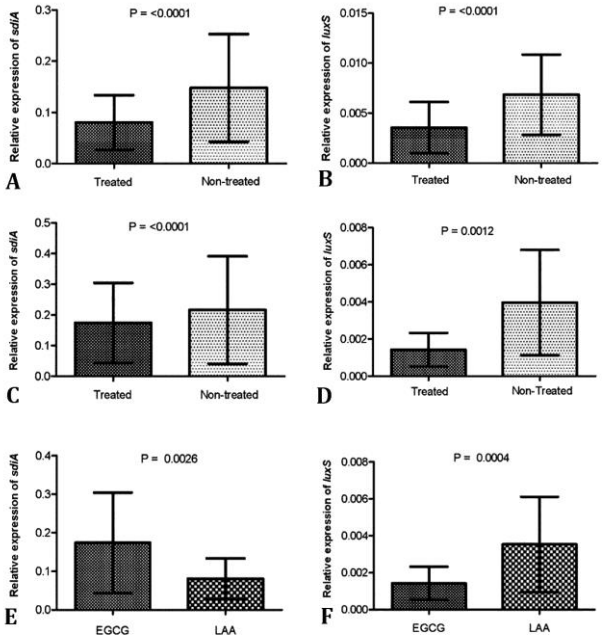

Fig. 3. Relative expression of **A)** *sdiA* and **B)** *luxS* in response to LAA. Relative expression of **C)** *sdiA* and **D)** *luxS* in response to EGCG. Comparison of LAA and EGCG effects on the expression levels of **E)** *sdiA* and **F)** *luxS* genes.

Discussion

Quorum-sensing systems are major regulators of *Salmonella* intestinal survival, colonization, and virulence.[18] The SdiA is a QS receptor in *S.* Typhimurium exclusively detecting the AHLs signals of other bacterial species and LuxS catalyzes the synthesis of the QS signaling molecule AI-2.[19,20] Furthermore, molecular assays targeting various *Salmonella* genes such as *16S rRNA, sdiA* and *luxS* have been used reliably for identifying *Salmonella* spp.[21-23] Consistent with other studies, *sdiA* and *luxS* genes were detected in all *S.* Typhimurium clinical isolates as well as *S.* Typhimurium RITCC1730.[21,24,25]

The use of QSIs for treatment may provide new tools in future veterinary medicine.[26] Plants are lacking a sophisticated immunity system to fight bacterial infections, therefore, instead of relying on cellular and biochemical defense systems, plant cells may be able to produce anti-QS compounds that can be used to defeat QS pathogens.[27] Some studies have shown that components derived from plants serve functionally as anti-QS and can be used for the development of novel anti-infective measures.[12] In this study, the expression levels of *sdiA* and *luxS* in LAA/EGCG-treated and non-LAA/EGCG-treated *S.* Typhimurium isolates were assessed by Quantitative Real-Time PCR. According to the results, the *sdiA* gene was found to be significantly down-regulated in LAA and EGCG treated isolates compared to the matched non-treated ones. Since a broad spectrum of virulence genes in *S.*

Typhimurium including *pefI/srgC* operon, *srgE* and *sirA* genes is under the control of SdiA QS system,[7] thus reductions in *sdiA* gene expression can affect the expression of these QS-controlled virulence factors which in turn will decrease the flagella formation (motility), fimbria formation, bacterial invasion, biofilm production, virulence-associated type III secretion systems and the phenotypes derived from genes located on the pathogenic islands 1 and 4.[18,28-31]

Another way to interrupt QS is to inhibit the synthase enzymes producing AIs. The LuxS is the key enzyme directly involving in AI-2 molecules production, so its inhibition would decrease the amount of AI-2. In the present study, LAA and EGCG also reduced the expression of the *luxS* gene in treated isolates compared to non-treated ones. Down-regulation of the *luxS* gene may affect the QS system and some other genes involved in virulence in treated isolates such as genes expressed from SPI-1. Choi *et al*. have reported that LuxS-mediated QS is required for normal expression of a subset of genes within SPI-1 and contributes to virulence of *S*. Typhimurium because deletion of the *luxS* gene decreased the transcription of SPI-1 genes and impaired invasion of *Salmonella*.[32] In another study, expression of SPI-1 and flagella genes was also reduced by over-expression of the LsrR regulator from a plasmid but was relieved by exogenous AI-2, which binds to and inactivates LsrR.[24] Therefore, down-regulation of the *luxS* gene in LAA- or EGCG-treated isolates can reduce the synthesis of AI-2 internalization structure as well as the expression of genes located on SPI-1, which in turn might lead to a decrease in bacterial invasion. In one mutational study, it has been shown that the LuxS-generated AI-2 signaling molecule plays a major role in *S.* Typhimurium biofilm formation[23], hence the formation of biofilm in this organism could be disrupted by reducing the *luxS* gene expression. The study by Jesudhasan *et al.* has also indicated that both *luxS* and AI-2 play a vital role in the expression of different genes of *S*. Typhimurium including those involved in motility, biofilm formation, virulence, translation, transcription, and other key cellular functions.[33]

Although there is no documented scientific report on the anti-QS potential of LAA and EGCG against *S*. Typhimurium, the results of this study showed that both LAA and EGCG have anti-QS effects by decreasing the expression of *luxS* and *sdiA* genes. These results, however, are consistent with the findings of Shen *et al.* and Qiu *et al*. showing that LAA can down-regulate the expression of the *agrA* gene which is one of the major genes of the QS system in *Staphylococcus aureus*.[34,35] Also, green tea polyphenols (particularly EGCG) have been shown to have a certain degree of anti-QS potential against *Shewanella baltica*[36] as well as an anti-microbial effect against the food-borne pathogen *Campylobacter jejuni* by disruption of QS.[37] Recently, several reports have demonstrated that EGCG could inhibit the expression of QS–regulated virulence genes in diverse bacterial pathogens such as *Porphyromonas gingivalis*,[38] *Enterococcus faecalis*,[39] and *Escherichia coli* O157:H7.[40]

The results of this study also revealed that LAA was more effective than EGCG in reducing *sdiA* gene expression in *S*. Typhimurium isolates, while EGCG was more effective than LAA in decreasing the expression of the *luxS* gene. These results indicate that the anti-QS activity of LAA and EGCG was different. This can be attributed to the difference of their antimicrobial mechanisms which have not yet been elucidated.

In conclusion, the results of this study revealed that sub-MICs of LAA and EGCG can inhibit the expression of QS-associated genes of *S.* Typhimurium. This may open doors for anti-QS based prophylactic/therapeutic strategies against salmonellosis. However, further studies are needed to assess their safety and efficiency through experimental tests.

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Conflict of interest

The authors do not have any particular conflicts of interest to declare.

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