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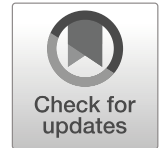

Antibiofilm activity of coenzyme Q0 against *Salmonella* Typhimurium and its effect on adhesion–invasion and survival–replication

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Abstract

Salmonella Typhimurium, a common Gram-negative foodborne pathogen, threatens public health and hinders the development of the food industry. In this study, we evaluated the antibiofilm activity of coenzyme Q0 (CoQ0) against *S.* Typhimurium. Besides, the inhibition of the *S.* Typhimurium's adhesion to and invasion of Caco-2 cells and its survival and replication in RAW 264.7 cells by CoQ0 were also explored. The minimum inhibitory concentrations and minimal bactericidal concentrations of CoQ0 against *Salmonella* were both 100–400 μg/mL. *Salmonella* Typhimurium biofilm formation was effectively inhibited by subinhibitory concentrations (SICs) of CoQ0. The CoQ0-affected biofilm morphology was observed with light microscopy and field-emission scanning electron microscopy. CoQ0 at SICs reduced the swimming motility and quorum sensing of *S.* Typhimurium and repressed the transcription of critical virulence-related genes. CoQ0 at SICs also clearly reduced the adhesion of *S.* Typhimurium to and its invasion of Caco-2 cells and reduced its survival and replication within RAW 264.7 macrophage cells. These findings suggest that CoQ0 has strong antibiofilm activity and can be used as an anti-infectious agent against *Salmonella.*

Keywords *Salmonella* Typhimurium · Coenzyme Q0 · Antibiofilm · Anti-infectious · RAW264.7 · Subinhibitory concentration

Introduction

Salmonella is a ubiquitous, Gram-negative, flagellated bacillus belonging to the family *Enterobacteriaceae* (Mathur et al. 2012). Among its more than 2600 serovars, defined according to its surface antigens, *Salmonella enterica* serovar Typhimurium is one of the commonest causes of human infection (Fàbrega and Vila 2013). The ingestion of food products contaminated with *S.* Typhimurium, such as poultry, beef, pork, eggs, milk, seafood, and fresh produce, can cause many diseases, including gastroenteritis, with symptoms such as diarrhea, vomiting, cramps, fever, and headache (Gal-Mor 2019). It is estimated that food contaminated with *Salmonella* species is responsible for 94 million cases of gastroenteritis annually, which result in 155,000 deaths globally every year (Ros-Chumillas et al. 2017). In total, 94,530 cases of *Salmonella* infection were confirmed by the European Union in 2016, with an incidence of 20.4 cases per 100,000 (European Food Safety Authority (EFSA) 2017).

Biofilm formation always renders foodborne pathogens more persistent and resistant to antimicrobial stress, limited nutrient supply, and inappropriate pH or temperature, increasing their threat to public health and posing a huge challenge for food industries worldwide (Fàbrega and Vila 2013). *Salmonella* can form biofilms on various surfaces, including poultry skin, stainless steel, concrete, tile, glass, granite, rubber, and polyvinyl surfaces (Cappitelli et al. 2014; Dhowlaghar et al. 2018). Stepanović et al. (2004) isolated 122 *Salmonella* spp. from humans, animals, and food and confirmed that all of the strains had the capacity to form a

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biofilm. The formation of biofilms by pathogens is involved in over 80% of microbial infections (Bjarnsholt et al. 2018). The ability to form a biofilm is also thought to be a major virulence factor in *Salmonella* and is closely related to quorum sensing (QS) and motility (Chakroun et al. 2018). The chemical signaling molecules of the QS system, called "autoinducers" (AIs), are reported to activate the signal transduction cascade that regulates biofilm formation (Ng and Bassler 2009). The initial attachment of pathogens to the surfaces of biotic or abiotic objects depends on their motility, and these adherent cells constitute the basis of the biofilm (Srey et al. 2013).

Pathogenic *Salmonella* strains are quite different from non-pathogenic *Salmonella* strains because they express virulence genes, which are usually organized into regions known as "*Salmonella* pathogenicity islands" (SPIs) (Kaur and Jain 2012). SPI-1 encodes a needle-like structure, termed "type III secretion system 1" (T3SS-1). For *S.* Typhimurium, T3SS-1 is directly related to the biofilm formation, the bacterium's ability to invade the host intestinal epithelial cells, and the initiation of inflammation (Roche et al. 2018). "Type III secretion system 2" (T3SS-2), encoded by SPI-2, contributes to the survival of *Salmonella* in macrophages, the spread of the bacterium, and systemic infections (Zhao et al. 2016).

Fluoroquinolones, trimethoprim-sulfamethoxazole, ampicillin, and expanded-spectrum cephalosporins are used to efficiently treat *Salmonella* (Fàbrega and Vila 2013). However, antibiotic resistance has disseminated rapidly for many reasons, including the overuse or misuse of antibiotics and some complex ancient antibiotic resistance mechanisms bacteria (Bao et al. 2018). Consequently, it is essential to find a substitute for antibiotics to control bacterial contamination. Natural plant products have the potential to inhibit bacterial virulence rather than the viability of the pathogen, resulting in less-severe infections and more repaid immune responses in their hosts (Silva et al. 2016).

Coenzyme Q is a member of the ubiquinone compound family, consists of a benzoquinone ring conjugated to an isoprenoid chain. The number of isoprenoid units determines the name of the coenzyme (Q0-Q10) (Fan et al. 2018). Coenzyme Q0 (CoQ0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone) is a natural compound containing no isoprenoid unit extracted from the filtrates of submerged cultures of *Antrodia cinnamomea* (Chung et al. 2014). Zhao et al. (2014) and Fan et al. (2018) demonstrated that CoQ0 has antimicrobial activity against *Staphylococcus aureus* and *Listeria monocytogenes*, respectively. Yang et al. (2016) proved that CoQ0 inhibited LPS-induced inflammation and the redox imbalance in RAW 264.7 cells and mice. Chung et al. (2014) reported that CoQ0 induced reactive oxygen species generation and apoptosis in A549 human lung cancer cells. Moreover, CoQ0 has been demonstrated to have anti-metastatic (Yang et al. 2019) and anti-angiogenic (Yang et al. 2015) activities.

Although the antimicrobial characteristics of CoQ0 have been extensively investigated, little research has been directed towards its antibiofilm and anti-infection activities against *Salmonella*. In this study, the effects of CoQ0 on the biofilm formation, QS, and motility of *S.* Typhimurium were evaluated. The effects of CoQ0 treatment on the capacities of *S.* Typhimurium to adhere to and invade Caco-2 cells and to survive and replicate in RAW 264.7 cells were also investigated. Finally, the mechanism by which CoQ0 regulates the transcription levels of critical virulence-related genes of *S.* Typhimurium was examined.

Materials and methods

Reagents

Coenzyme Q0 (HPLC > 99%, CAS 605-94-7) was purchased from J&K Scientific Co., Ltd (Beijing, China). Before each assay, CoQ0 was dissolved in dimethyl sulfoxide (DMSO) and vortexed for 30 s at room temperature. The final concentration of DMSO in all of the sample solutions (treatment and control samples) was 0.1% (v/v), which has no apparent effect on the growth of *S.* Typhimurium.

Bacterial strains and culture conditions

Salmonella Typhimurium SL1344 (DSM 24522) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), *S.* Typhimurium ATCC 14028, and *Chromobacterium violaceum* ATCC 12472 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *Salmonella* Typhimurium CMCC 50115 was obtained from the National Center for Medical Culture Collections (CMCC, Beijing, China). Nine other *Salmonella* strains were from our laboratory strain collection and were originally isolated from various raw chicken samples in China. All of the *Salmonella* isolates were used in minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays. *Salmonella* Typhimurium SL1344 was used for further experiments because it is commonly used in *Salmonella* virulence studies and its phenotypic and genotypic characteristics are well-documented (Li et al. 2014). Because *S.* Typhimurium ATCC 14028 displayed stronger biofilm production, it was used to perform the assays related to biofilm. *Chromobacterium violaceum* ATCC 12472 was used as the QS indicator strain to evaluate the effect of CoQ0 on the QS system (Ma et al. 2018).

Before each assay, Luria–Bertani (LB) agar was streak-inoculated with the *Salmonella* strains, which were cultured at 37 °C for 12 h to activate the bacteria. To obtain fresh overnight cultures, 30 mL of LB broth was inoculated with a

single colony and incubated at 37 °C for 12 h with shaking at 130 rpm. The culture was then diluted in LB broth to an optical density at a wavelength of 600 nm ($OD_{600\ nm}$) of 0.5 (approximately 10^9 CFU/mL).

Determination of MICs and MBCs

The MICs of CoQ0 against the test *Salmonella* strains were determined with the broth dilution method based on the Clinical and Laboratory Standards Institute guidelines (CLSI 2009). Each well of a 96-well plate was inoculated with 200 μL of diluted bacterial strain culture, with a final bacterial concentration of approximately 5×10^5 CFU/mL. CoQ0 solution was added to each well to final concentrations of 800, 400, 200, 100, 50, 25, or 0 μg/mL. LB broth containing 0.1% DMSO was used as the negative control. The samples were then incubated at 37 °C for 24 h. The MIC of CoQ0 was the lowest concentration of CoQ0 that resulted in no visible *Salmonella* growth. To determine the MBCs, 100 μL of bacterial solution from each well was plated on an LB agar plate and cultured for 24 h. The MBC of CoQ0 was defined as the minimum concentration of CoQ0 that killed 99.9% of the test bacteria.

Determination of the subinhibitory concentrations (SICs)

The SICs of CoQ0 against *S.* Typhimurium SL1344 and *S.* Typhimurium ATCC 14028 were determined with the Bioscreen C Automated Microbiology Growth Curve Analysis System (Labsystems, Helsinki, Finland). The bacterial suspension was diluted to 5×10^5 CFU/mL with LB broth and 125 μL of the diluted culture was added to the individual cells of 100-well plates. Equal volumes of LB broth containing different concentrations of CoQ0 were added to the individual wells to achieve final CoQ0 concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, or 0 μg/mL. LB broth containing 0.1% DMSO was used as the negative control. The samples were further cultured at 37 °C for 24 h, and the $OD_{600\ nm}$ was monitored automatically every 1 h. Concentrations of CoQ0 that showed no inhibitory effect on bacterial growth were deemed to be SICs.

Motility assay

A motility assay was performed on medium containing 20 mL of LB broth and 0.3% (v/v) agar, as described by Li et al. (2014) with some modifications. CoQ0 was added to the medium at a final concentration of 25, 12.5. 6.25, 3.125, or 0 μg/mL, and the plates were left to dry for 1 h. The center of this semisolid medium surface was inoculated with 5 μL of overnight culture ($OD_{600\ nm} = 0.5$) and the samples were incubated upright at 37 °C for 7 h. Images of the motile bacteria were collected with the Gel Imaging System (Bio-Rad, USA), and the ImageJ software was used to measure the diameters of the areas of motility.

Specific biofilm formation (SBF) inhibition assay

To evaluate the effect of CoQ0 on the SBF of *S.* Typhimurium SL1344 and ATCC 14028, the crystal violet staining method was used, with some modifications (Shi et al. 2017). Tryptic soy broth (TSB, 30 mL) was inoculated with a single colony of *S.* Typhimurium and incubated at 37 °C overnight. CoQ0 was added to the overnight bacterial suspension ($OD_{600\ nm}$ = 1) to a concentration of 25, 12.5, 6.25, or 0 μg/mL, and then 200 μL of the incubated mixture was added to the wells of a 96-well plate. TSB containing 0.1% DMSO was used as the negative control. The samples were incubated at 37 °C for 24, 48, or 72 h. After incubation, the absorbance of each culture was measured at 630 nm and the wells were washed with distilled water. The plates were air-dried for 30 min, and 200 μL of 0.1% (w/v) crystal violet was added to each well for 20 min at room temperature to stain the biofilm. The uncombined dye was then removed by washing the wells three times with 300 μL of distilled water. After the wells were dried, the bound dye was solubilized in 200 μL of 33% (v/v) glacial acetic acid and the plates were shaken for 20 min at 25 °C. The ODs were read at 570 nm with a microtiter spectrophotometer. SBF was determined by calibrating the $OD_{570\ nm}$ with the $OD_{630\ nm}$.

Light microscope analysis

To visualize the effect of CoQ0 on biofilm formation by *S.* Typhimurium ATCC 14028, the method of Bai et al. (2019) was used. Briefly, glass slides of the same size were placed in each well of a 24-well plate, and were covered with *S.* Typhimurium ATCC 14028 bacterial suspension ($OD_{600\ nm}$ = 1) treated with 25, 12.5, 6.25, or 0 μg/mL CoQ0. After incubation at 37 °C for 72 h, the fluid in each well was removed and the plate was washed twice with phosphate-buffered saline (PBS). The glass slides were removed and stained with 0.4% crystal violet solution for 20 min. They were then washed three times with 300 μL of distilled water to remove any excess stain and air-dried. A light microscope (BX53, Olympus, Tokyo, Japan) was used to observe the stained biofilms.

Field-emission scanning electron microscopy (FESEM)

Biofilms were formed on glass slides as described in "Light microscope analysis" section. After the removal of planktonic cells, PBS containing 2.5% glutaraldehyde was added to fix the biofilm for 12 h at 4 °C. The glass slides were then washed

twice with PBS and dehydrated with a series of ethanol (50%, 70%, 80%, 90%, and 100%) to fix the cells. The glass slides were dried at room temperature and coated with gold, and the samples were examined under a field-emission scanning electron microscope (S-4800, Hitachi, Tokyo, Japan).

Quantitative QS inhibition assay

The QS indicator strain *C. violaceum* ATCC 12472 was used to determine the effect of CoQ0 on the QS-inhibitory activity of CoQ0 by quantifying its violacein production (Ma et al. 2018). First, the effects of SICs of CoQ0 on the growth of *C. violaceum* ATCC 12472 were determined using the method described in "Determination of the subinhibitory concentrations" Section. The three highest concentrations of CoQ0 that had no effect on the growth of *C. violaceum* ATCC 12472 were selected as the SICs for subsequent experiments.

An overnight culture of *C. violaceum* ATCC 12472, diluted to $OD_{600\ nm} = 0.2$, was added to LB broth containing CoQ0 at a concentration of 12.5, 6.25, 3.125, or 0 μg/mL. The samples were cultured at 30 °C for 24 h with shaking at 130 rpm. Violacein was extracted and quantified according to the method described by Choo et al. (2006) with some modifications. Briefly, 3 mL of culture was centrifuged (5000×*g*, 5 min, 4 °C) to precipitate the insoluble violacein. The liquid supernatant was discarded and 1 mL of DMSO was added to the centrifuge tube. After shaking, the mixture was centrifuged (10,000×*g*, 10 min, 4 °C) to remove the cells. The violacein solution (200 μL) was added to 96-well microtiter plates and the $OD_{585\ nm}$ was read.

Adhesion and invasion of Caco-2 cells

In this study, the human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection and cultured according to the method described by Shi et al. (2017). The effects of CoQ0 on bacterial adhesion and invasion were determined according to the method described by Fan et al. (2018) with some modifications. Briefly, Caco-2 cells were seeded in a 24-well plate, grown in DMEM with 10% FBS (10^5 cells per well) at 37 °C and 5% CO_2 for 18 h, and then rinsed twice with PBS. *S.* Typhimurium SL1344 bacterial strain culture was added to LB broth containing different concentrations (25, 12.5, 6.25, or 0 μg/mL) of CoQ0 and cultured at 37 °C for 12 h with shaking at 130 rpm before Caco-2 cells adhesion and invasion assays. The bacterial suspensions were centrifuged and resuspended in PBS to remove any residual CoQ0. After then, the samples were diluted 1000 times with DMEM and added to each well at a multiplicity of infection (MOI) of 10. The samples were incubated at 37 °C in a humidified 5% CO_2 incubator for 1 h.

For the adhesion assay, the infected monolayers of cells were washed twice with PBS and lysed with 1 mL of 0.1% Triton X-100. The number of *S.* Typhimurium SL1344 cells was counted after they were serially diluted, plated onto LB agar, and incubated for 24 h at 37 °C. For the invasion assay, the Caco-2 monolayers were washed once with PBS and incubated for 1 h with 1 mL of DMEM containing gentamicin (100 μg/mL) to kill the extracellular bacteria. The monolayers were then washed three times and lysed with 0.1% Triton X-100 at 4 °C. The number of bacteria was counted by colony plating, as described for the adhesion assay. The adhesion and invasion rates of *S.* Typhimurium in the control groups were taken to be 100%, and those for the treatment groups were calculated as a percentage of the control value.

Survival and intracellular replication in macrophages

In this study, the murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection. The intracellular survival and replication of *S.* Typhimurium SL1344 in RAW 264.7 were examined according to the method described by Ryan et al. (2018) with minor modifications. Briefly, RAW 264.7 cells were seeded in a 24-well plate and cultured in DMEM with 10% FBS (10^5 cells per well) at 37 °C and 5% CO_2 for 12 h. *S.* Typhimurium SL1344 bacterial suspension was pre-treated with or without CoQ0 (12.5, 6.25, 3.125, or 0 μg/mL) before adding into each well containing RAW 264.7 cells. After incubation overnight, the bacterial suspension was washed twice with PBS to remove any extra CoQ0, and diluted to a concentration of $OD_{600\ nm} = 0.5$. After that, the samples were diluted 1000 times with DMEM, yielding a final bacterial concentration of approximately 10^6 CFU/mL. The bacterial suspension (1 mL, MOI = 10) was added to each well containing RAW 264.7 cells. After subsequent incubation for 45 min, the cells were washed once with PBS and incubated for 30 min with 1 mL of DMEM containing gentamicin (100 μg/mL) to kill any extracellular bacteria.

For the survival assay, the macrophages were lysed by the addition of 0.1% Triton X-100 for 20 min at 4 °C and then serially diluted and plated on LB agar. To estimate intracellular replication, 1 mL of DMEM containing 10 μg/mL gentamicin was added to each well. After incubation for 24 or 72 h, the cells were lysed and the appropriate dilution was plated on LB agar plates, as described in the survival assay. The number of bacteria was counted after incubation for 24 h.

RNA extraction and reverse transcription–quantitative PCR (RT–qPCR)

To determine the effect of CoQ0 on the transcription of motility-, biofilm formation–, invasion-, and virulence-related genes (Table 1), *S.* Typhimurium SL1344 was grown in LB broth containing different concentrations (25, 12.5, or 0 μg/

Table 1 Differentially expressed virulence-related genes in *S.* Typhimurium SL1344 with or without CoQ0

Gene	Primer sequence (5′–3′)[c]	Relative gene expression at:		Reference
		25 µg/mL	12.5 µg/mL	
16s rRNA	F, AGGCCTTCGGGTTGTAAAGT R, GTTAGCCGGTGCTTCTTCTG	1	1	Lamas et al. (2018)
arcZ	F, ACTGCGCCTTTGACATCATC R, CGAATACTGCGCCAACACCA	− 2.56 ± 0.16b	− 2.44 ± 0.40b	Lamas et al. (2018)
sroC	F, GGGACTCCTGTCCTCTCGAT R, CAGCGCTACCCTCGAAGATT	− 1.76 ± 0.10b	− 1.61 ± 0.29b	Lamas et al. (2018)
csrB	F, CAAAGTGGAAAGCGCAGGAT R, TGACCTTACGGCCTGTTCAT	− 2.02 ± 0.55b	− 1.33 ± 0.12	Lamas et al. (2018)
fimD	F, CGCGGCGAAAGTTATTTCAA R, CCACGGACGCGGTATCC	− 13.15 ± 0.36b	− 6.74 ± 0.55b	Li et al. (2014)
fljB	F, TGGATGTATCGGGTCTTGATG R, CACCAGTAAAGCCACCAATAG	− 5.20 ± 0.18b	− 3.48 ± 0.50b	Li et al. (2014)
flhD	F, CTCCTTGCACAGCGTTTGAT R, TCTCCGCCAGTTTGACCAT	− 8.13 ± 0.24b	− 8.83 ± 0.39b	Wu et al. (2016)
adrA	F, GAAGCTCGTCGCTGGAAGTC R, TTCCGCTTAATTTAATGGCCG	− 9.89 ± 0.34b	− 5.71 ± 0.28b	Lamas et al. (2018)
csgD	F, TCCTGGTCTTCAGTAGCGTAA R, TATGATGGAAGCGGATAAGAA	− 9.46 ± 0.23b	− 6.32 ± 0.25b	Lamas et al. (2018)
hilA	F, AATGGTCACAGGCTGAGGTG R, ACATCGTCGCGACTTGTGAA	− 2.76 ± 0.21b	− 1.50 ± 0.18	Salaheen et al. (2016)
invA	F, CGCGCTTGATGAGCTTTACC R, CTCGTAATTCGCCGCCATTG	− 6.24 ± 0.31b	− 4.96 ± 0.27b	Salaheen et al. (2016)
invH	F, CCCTTCCTCCGTGAGCAAA R, TGGCCAGTTGCTCTTTCTGA	− 3.88 ± 0.18b	− 1.54 ± 0.12a	Li et al. (2014)
orf245	F, CAGGGTAATATCGATGTGGACTACA R, GCGGTATGTGGAAAACGAGTTT	− 5.83 ± 0.29b	− 5.59 ± 0.21b	Li et al. (2014)
pipB	F, GCTCCTGTTAATGATTTCGCTAAAG R, GCTCAGACTTAACTGACACCAAACTAA	− 6.73 ± 0.05b	− 7.93 ± 0.22b	Li et al. (2014)
sdiA	F, TTACATTGGGATGACGTGCT R, AACTGCTACGGGAGAACGAT	− 3.09 ± 0.13b	− 1.86 ± 0.22b	Li et al. (2014)
srgE	F, GCGCAGGTTGGTATTACTTG R, GGCAGATTGTTCATGATTGC	− 1.60 ± 0.17a	− 1.33 ± 0.16	Li et al. (2014)
sodC	F, CACATGGATCATGAGCGCTTT R, CTGCGCCGCGTCTGA	− 4.54 ± 0.28b	− 2.28 ± 0.15b	Li et al. (2014)

[a] $P < 0.05$

[b] $P < 0.01$

[c] F, forward; R, reverse

mL) of CoQ0 at 37 °C for 8 h. Total RNA was extracted with the Tiangen RNAprep Pure Cell/Bacteria Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The RNA quality and concentration were determined with a nucleic acid and protein spectrophotometer (Nano-200, Aosheng Instrument Co., Ltd, Hangzhou, China). The Takara PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan) was used to reverse transcribe the RNA into cDNA, according to the manufacturer's instructions. The cDNA samples were stored at − 20 °C until analysis. The primer sequences used for RT–qPCR are listed in Table 1.

The qPCR reactions (25 µL) with SYBR® Premix Ex Taq™ II (Takara) were performed using the IQ™5 system (Bio-Rad) with the following cycling conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, and a dissociation step at 95 °C for 15 s and 60 °C for 30 s. The relative gene transcription in the samples was analyzed using the $2^{-\Delta\Delta Ct}$ method, relative to the reference gene 16s rRNA.

Statistical analysis

All statistical analyses were performed with SPSS 23.0 (IBM, New York, NY, USA). The data are presented as means ± standard deviations (SD) and the differences between the means were tested with the independent-sample Student's *t* test. $P < 0.05$ and $P < 0.01$ were considered statistically significant and extremely significant, respectively. All experiments were measured independently three times.

Results

MICs and MBCs

The MICs and MBCs of CoQ0 against *Salmonella* spp. are listed in Table 2. The MICs of CoQ0 against *Salmonella* ranged from 100 to 400 μg/mL. The MBC values were equal to the MIC values.

SICs

As can be seen in Fig. 1a and b, the growth conditions of *S.* Typhimurium SL1344 were similar to those for *S.* Typhimurium ATCC 14028. When the concentrations of CoQ0 ranged from 50 to 100 μg/mL, the lag phase of *S.* Typhimurium was lengthened. When the concentration of CoQ0 was reduced to 25 μg/mL or below, there was no apparent effect on the growth of *S.* Typhimurium. Therefore, concentrations of 25, 12.5, 6.25, and 3.125 μg/mL CoQ0 were selected as SICs to study the effects of CoQ0 on the virulence of *S.* Typhimurium.

Motility

The motility of *S.* Typhimurium SL1344 was reduced by CoQ0 (Fig. 2a–f). The area of motility of untreated *S.* Typhimurium SL1344 was 16.75 ± 0.06 cm^2, whereas those of the strains treated with 25, 12.5, 6.25, or 3.125 μg/mL, CoQ0 were 7.27 ± 0.04 cm^2, 9.57 ± 0.08 cm^2, 11.93 ± 0.05 cm^2, or 12.02 ± 0.06 cm^2, respectively (about 43.37%, 57.14%, 71.24%, or 71.75% of the control motility area, respectively).

Inhibition of biofilm formation by CoQ0

As shown in Fig. 3a and b, the formation of biofilm by *S.* Typhimurium SL1344 and ATCC 14028 grown at 37 °C for 24, 48, or 72 h was effectively and concentration-dependently inhibited by CoQ0 at SICs. For *S.* Typhimurium ATCC 14028, the biofilm biomass treated with 6.25, 12.5, or 25 μg/mL CoQ0 was reduced by 21.93%, 39.53%, or 48.70% of the control level, respectively, after 24 h. At 25 μg/mL, CoQ0 inhibited the biofilm formation of *S.* Typhimurium SL1344 after 24, 48, or 72 h by 55.39–60.77% compared with that of the control.

Light microscopic and FESEM observations

The *S.* Typhimurium ATCC 14028 biofilm was observed at a × 400 magnification by light microscopy (Fig. 4a–d). The biofilms of CoQ0-treated *S.* Typhimurium were markedly reduced on the glass slides. The biofilm biomass was also dose-dependently reduced by CoQ0 treatment.

As shown in Fig. 4e–l, FESEM observations at × 1500 and × 4000 magnification demonstrated that the formation of *S.* Typhimurium ATCC 14028 biofilm on the surfaces of glass slides was inhibited by CoQ0 at SICs, consistent with the crystal violet staining results. After incubation for 72 h, untreated *S.* Typhimurium showed a dense biofilm layer, and most cells had gathered into large clusters and multilayer structures (Fig. 4e). In contrast to the control, as the concentration of CoQ0 increased, *Salmonella* displayed severe damage to the typical biofilm structure and lower biofilm cell densities (Fig. 4b–d).

Anti-QS activity of CoQ0

Figure 5a shows that the concentrations of CoQ0 used in this study (25, 12.5, and 6.25 μg/mL) had no apparent inhibitory effect on the growth of *C. violaceum*. As seen in Fig. 5b, the production of violacein decreased to about 89.4%, 87.0%, or 68.8% of the control after treatment with 3.125, 6.25, or 12.5 μg/mL CoQ0, respectively.

Adhesion to and invasion of Caco-2 cells

The effects of CoQ0 on the adhesion of *S.* Typhimurium to Caco-2 cells are shown in Fig. 6a. Compared with the control,

Table 2 MICs and MBCs of CoQ0 for different *Salmonella* strains

Strain	Serovar	Source	MIC (μg/mL)	MBC (μg/mL)
SL1344	Typhimurium		200	200
ATCC 14028	Typhimurium		200	200
CMCC 50115	Typhimurium		200	200
ATCC 13076	Enteritidis		100	100
S8XC004c	Shubra	Whole chicken	400	400
44-1	Indiana	Chicken liver	400	400
76D	Indiana	Whole chicken	200	200
1087R	Ball	Whole chicken	200	200
59-1	Infantis	Chicken breast	400	400
60505-10cTT	Thompson	Whole chicken	200	200

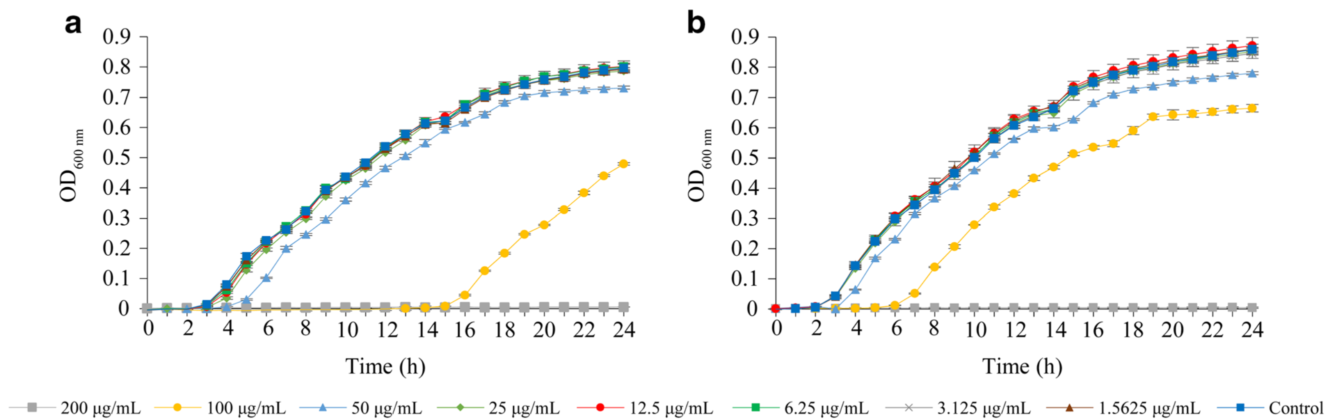


Fig. 1 Growth of *S.* Typhimurium SL1344 (**a**) and *S.* Typhimurium ATCC 14028 (**b**) in LB with various concentrations of CoQ0. Error bars represent the standard deviations of three replicate experiments

6.25, 12.5, and 25 μg/mL CoQ0 inhibited the adhesion of *S.* Typhimurium to 59.1%, 50.9%, and 52.3% of the control value, respectively. The invasion of Caco-2 cells by *S.* Typhimurium was also inhibited by CoQ0, decreasing to 84.3%, 69.8%, and 40.6% of the control value, respectively, at the SICs (Fig. 6b).

Survival and replication within macrophage cells

As shown in Fig. 7, only 25 μg/mL CoQ0 significantly inhibited the intracellular survival of *S.* Typhimurium in RAW264.7 cells ($P < 0.01$), and the quantity of intracellular bacteria decreased by 42.9% of the control level. However, the intracellular replication of *S.* Typhimurium in RAW264.7 cells was significantly reduced by all of the SICs of CoQ0 in a dose-dependent manner relative to the control after 72 h ($P < 0.01$). *Salmonella* Typhimurium replication in RAW264.7 cells decreased by 80.7–87.1% in the presence of 6.25, 12.5, or 25 μg/mL CoQ0.

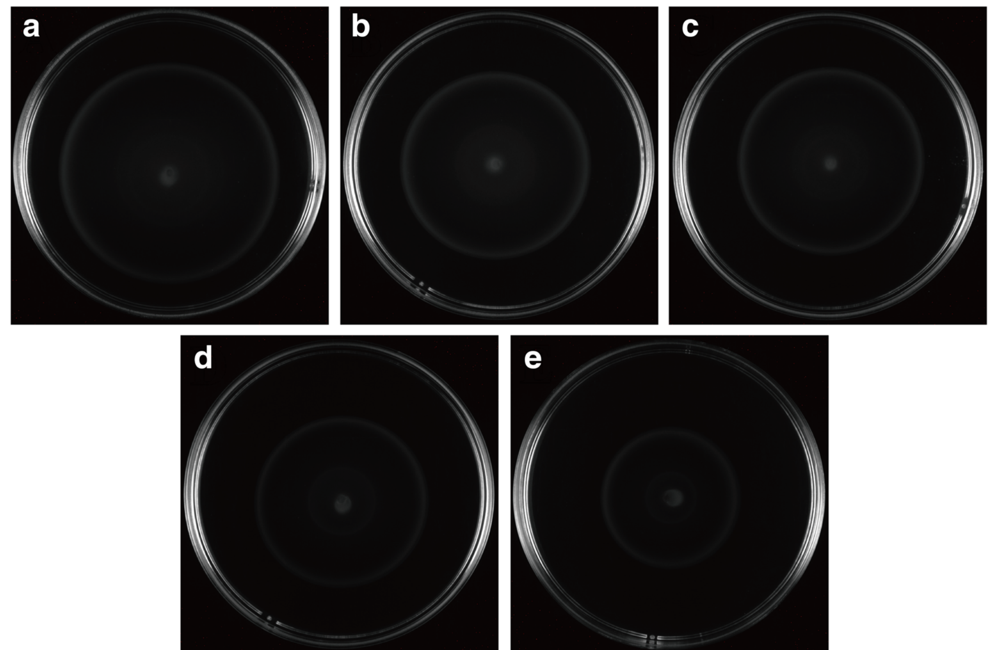
RT–qPCR analysis of virulence-related genes

CoQ0 at SICs downregulated the transcription of 13 genes in *S.* Typhimurium SL1344 that are associated with its virulence (Table 1). CoQ0 downregulated the transcription of genes *fljB* and *flhD* (critical for flagellar regulation); *fimD* (motility); *arcZ*, *sroC*, *csrB*, *csgD*, and *adrA* (biofilm formation); *hilA*, *invA*, and *invH* (adherence and invasion); *pipB* and *orf245* (T3SS); *sdiA* and *srgE* (QS); and *sodC* (survival in macrophages) to various degrees.

Discussion

Motility allows *Salmonella* to form biofilm, to approach the host cell, and to initiate adhesion and invasion processes, so that it can successfully infect mammalian cells (Fàbrega and Vila 2013). In this study, the swimming motility of *Salmonella* was significantly inhibited by CoQ0 at SICs ($P < 0.05$) (Fig. 2). Similarly, a previous study reported that the swimming motility of *Escherichia coli* O157:H7 was dose-dependently blocked by grape seed extract (Zhu et al. 2015). Li et al. (2014) reported that the areas of motility were reduced to 23.17% and 39.01% of the control area by 15.625 and 31.25 μg/mL punicalagin, respectively, while the flagellum-associated genes were downregulated. The flagellum is a component of *Salmonella* that is essential for its motility, and also contributes to its chemotaxis, adherence to and invasion of host cells, colonization, and even subsequent innate immune signaling (Horstmann et al. 2017). The *fljB* gene encodes the flagellin protein, which forms the filament structure of the flagellar apparatus and is considered a primary proinflammatory determinant of *Salmonella* (Zeng et al. 2003). The *flhD* gene encodes the flagellar switch protein FlhD4C2, which is the flagellar operon master regulator and the transcriptional activator of all of the flagellar genes. The *fimD* gene partly encodes the type 1 fimbriae, which reportedly play roles in adherence, invasion, biofilm formation, and the immune response (Li et al. 2017). Our results show that all three genes, *fljB*, *flhD*, and *fimD*, were repressed at the transcriptional level by CoQ0 at different SICs. Therefore, it can be inferred that CoQ0 restricts the motility of *Salmonella* by mediating the function of the flagellum, which may influence other features of infection, such as adherence, invasion, and biofilm formation.

Salmonella can form biofilms on various surfaces, including stainless steel, aluminum, plastic, and glass (Merino et al. 2019). The great damage caused by *Salmonella* to the food processing industry is mainly attributable to the persistence and resistance of mature biofilms (Shi and Zhu 2009). Our results show that CoQ0 at SICs exerts a significant inhibitory effect on the biofilm formation of *S.* Typhimurium on polystyrene and glass surfaces (Figs. 3 and 4). These results are similar to those of a study of *S. aureus*, in which its biofilm formation was effectively inhibited by 0.3125 mg/mL shikimic acid, as determined by confocal laser scanning microscopy,



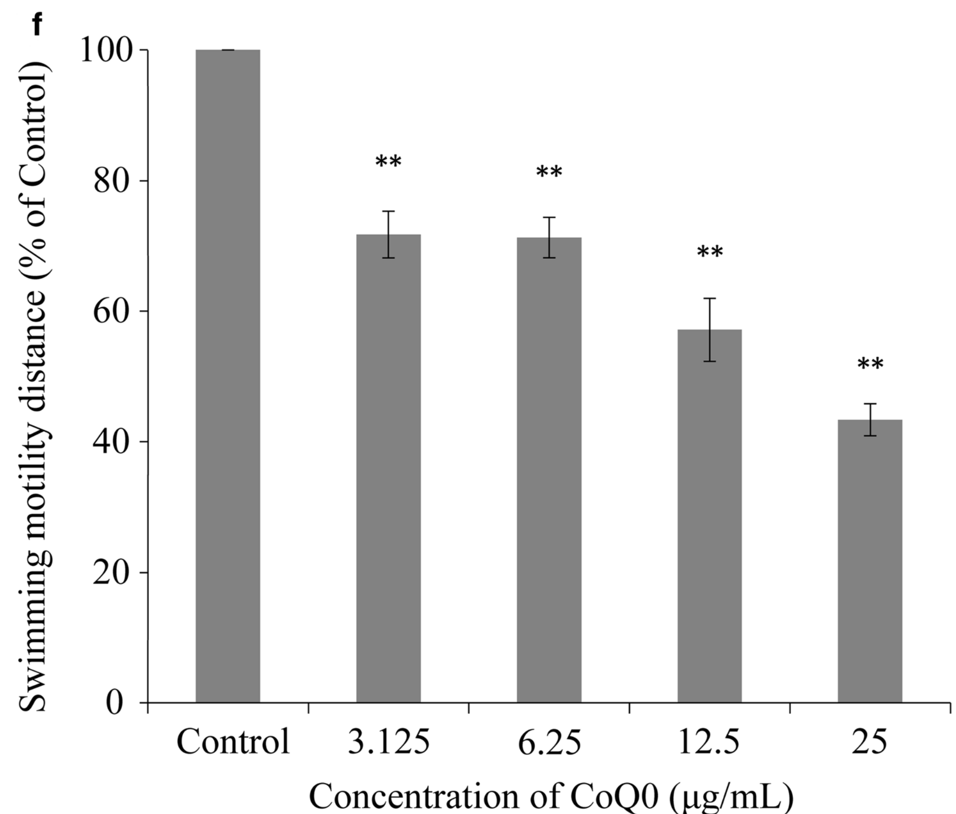


Fig. 2 Swimming motility of *S.* Typhimurium on soft agar plates containing different concentrations of CoQ0. **a** Untreated cells; **b**, **c**, **d**, **e** cells treated with 3.125, 6.25, 12.5, or 25 mg/mL CoQ0, respectively. **f** Quantification of *S.* Typhimurium SL1344 swimming motility in the presence of CoQ0. Relative swimming motility area of the strain was measured after treatment with CoQ0. Values are normalized to the 100% motility area measured in the absence of CoQ0. Bars indicate means ± the standard deviation. **P* < 0.05, ***P* < 0.01

light microscopy, and scanning electron microscopy, which were used to analyze the biofilm biomass, the viability of the biofilm cells, and the biofilm architecture in different strains (Bai et al. 2019). Similarly, Shi et al. (2017) reported that thymoquinone prevented biofilm formation by *Cronobacter sakazakii* by inhibiting the production of cellulose and the flagellum. Cellulose is a crucial component of the *Salmonella* biofilm (Steenackers et al. 2012) and is encoded by *csgD* and *adrA*. Some *Salmonella* small RNAs (sRNAs) have been shown to play a role in biofilm formation (Ryan et al. 2017). In the present study, CoQ0 at SICs down-regulated the transcription of various genes, including *csgD*

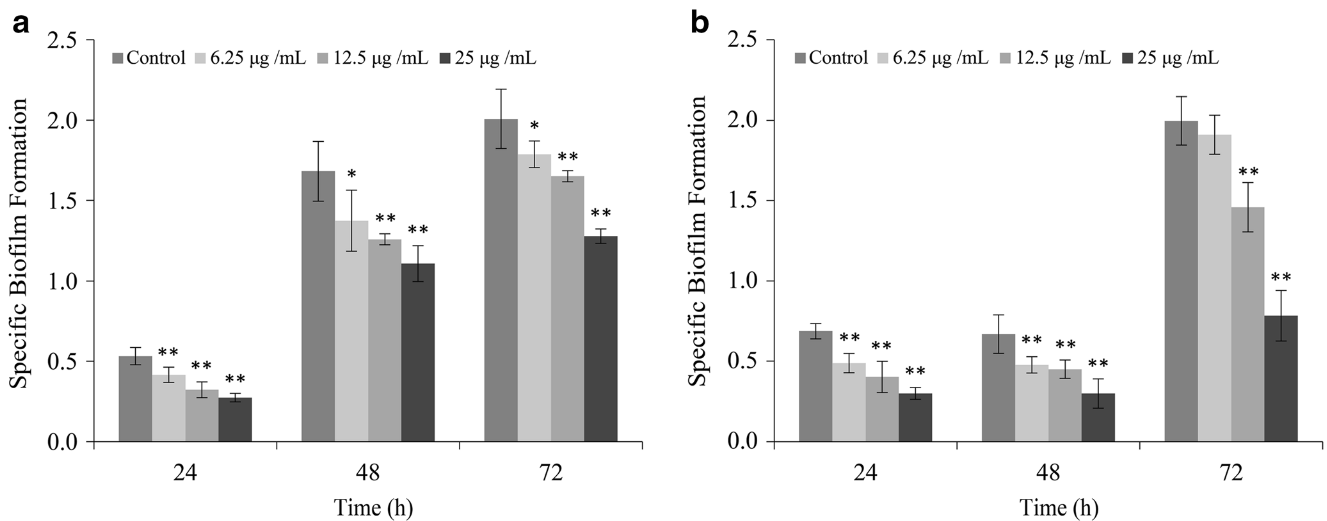


Fig. 3 Inhibition of *S.* Typhimurium ATCC 14028 (**a**) and *S.* Typhimurium SL1344 (**b**) biofilm formation by CoQ0 at different concentrations. Bars show the means ± the standard deviations. $^*P < 0.05$, $^{**}P < 0.01$

and *adrA*, and some sRNAs, including *arcZ*, *sroC*, and *csrB*, which trigger biofilm formation by regulating biofilm-associated genes, such as *csgD* and the flagellar genes (Fuentes et al. 2015). Therefore, CoQ0 may control the biofilm formation of *S.* Typhimurium by mediating cellulose and the function of related sRNAs.

QS is a chemical cell-to-cell communication system relying on the small signaling molecules called AIs. By the production, release, and detection of AIs, *Salmonella*'s biofilm formation, motility, and production of virulence factors could be regulated (Defoirdt et al. 2013). N-Acyl homoserine lactone (AHL), a type of membrane-permeable small chemical

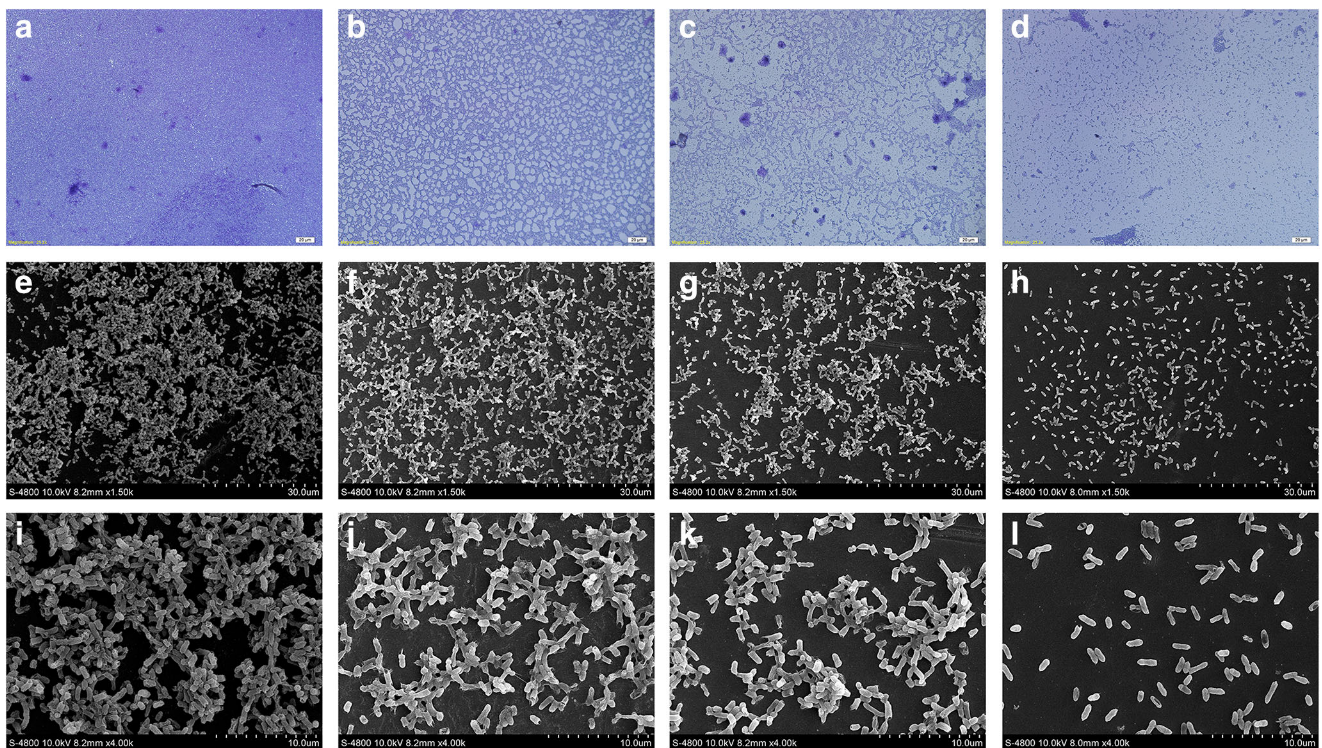


Fig. 4 Light microscopic images of *S.* Typhimurium ATCC 14028 biofilm in the presence of CoQ0 at concentrations of 0 (**a**), 3.125 (**b**), 6.25 (**c**), or 12.5 mg/mL (**d**). Scanning electron microscopic images at a × 1500 magnification of *S.* Typhimurium ATCC 14028 biofilm after treatment with CoQ0 at 0 (**e**), 3.125 (**f**), 6.25 (**g**), or 12.5 mg/mL (**h**). Scanning electron microscopic images at × 4000 magnification of *S.* Typhimurium ATCC 14028 biofilm after treatment with CoQ0 at 0 (**g**), 3.125 (**h**), 6.25 (**i**), or 12.5 mg/mL (**l**)

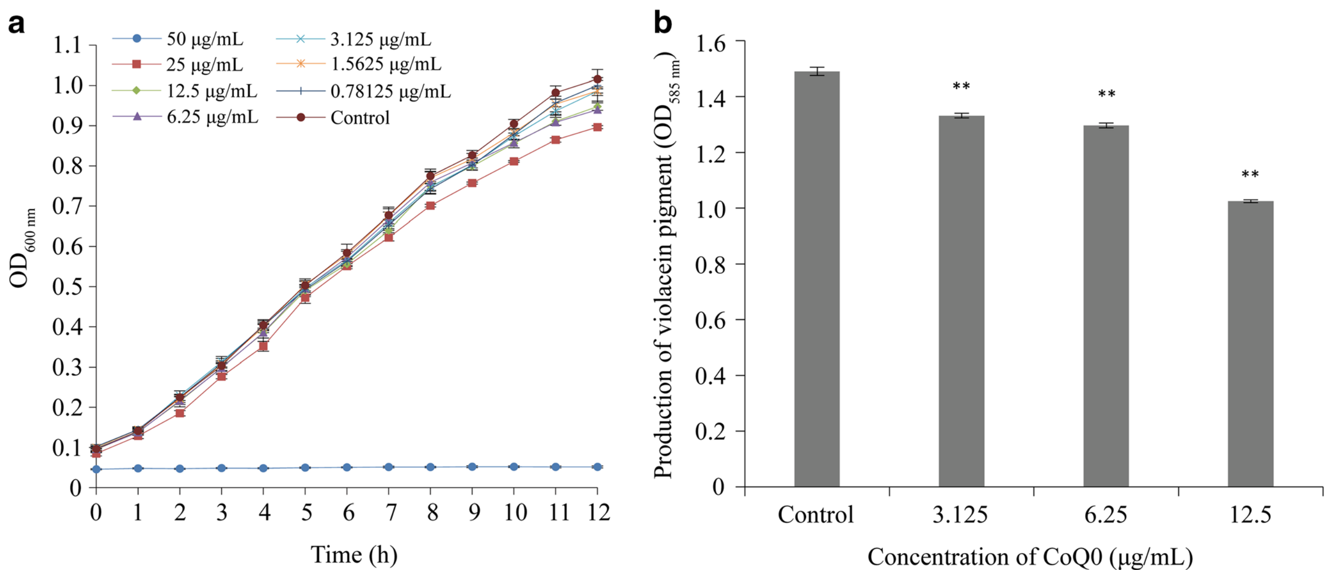


Fig. 5 **a** Growth of *C. violaceum* ATCC 12472 in LB with various concentrations of CoQ0. Error bars represent the standard deviations of three replicate experiments. **b** Inhibition of violacein production by *C. violaceum* ATCC 12472 at different concentrations of CoQ0. Bars represent the means ± standard deviations ($n = 3$). $*P < 0.05$, $**P < 0.01$

compound, is classified as one of these signaling molecules and combines with cytoplasmic receptors to play a regulatory role (Thoendel et al. 2011). In this study, by measuring the concentration of violacein pigment in *C. violaceum*, we showed that CoQ0 markedly interferes with the production of the QS signaling molecule AHL (Fig. 5). Similarly, Borges et al. (2014) showed that six phenolic products extracted from natural sources reduced AHL-regulated violacein pigment to disturb the QS system. Vinothkannan et al. (2018) reported that fructose furoic acid ester curbed the QS system of *E. coli* by downregulating *sdiA* expression and that it is a potential QS inhibitor. The gene *sidA* encodes an AHL receptor, SdiA (a LuxR homolog), that activates *srgE* expression and helps *S.* Typhimurium sense and respond to the AHLs produced by other bacteria (Dyszel et al. 2010). Our RT–qPCR assay demonstrated that CoQ0 significantly downregulated the transcription of *sdiA* and *srgE* in *Salmonella*. We assume that CoQ0 reduced the *Salmonella* QS system through inhibiting the synthesis of AHL receptor protein. The intracellular physiological state affected by CoQ0 will be investigated in further studies. The pathway or receptor protein by which external CoQ0 enter into the cell will be also studied.

The adhesion to and invasion of host epithelial cells by *Salmonella* depends on functions encoded by SPI-1. *Salmonella* Typhimurium SPI-1 T3SS shifts effector proteins into host cells across the plasma membrane so that the

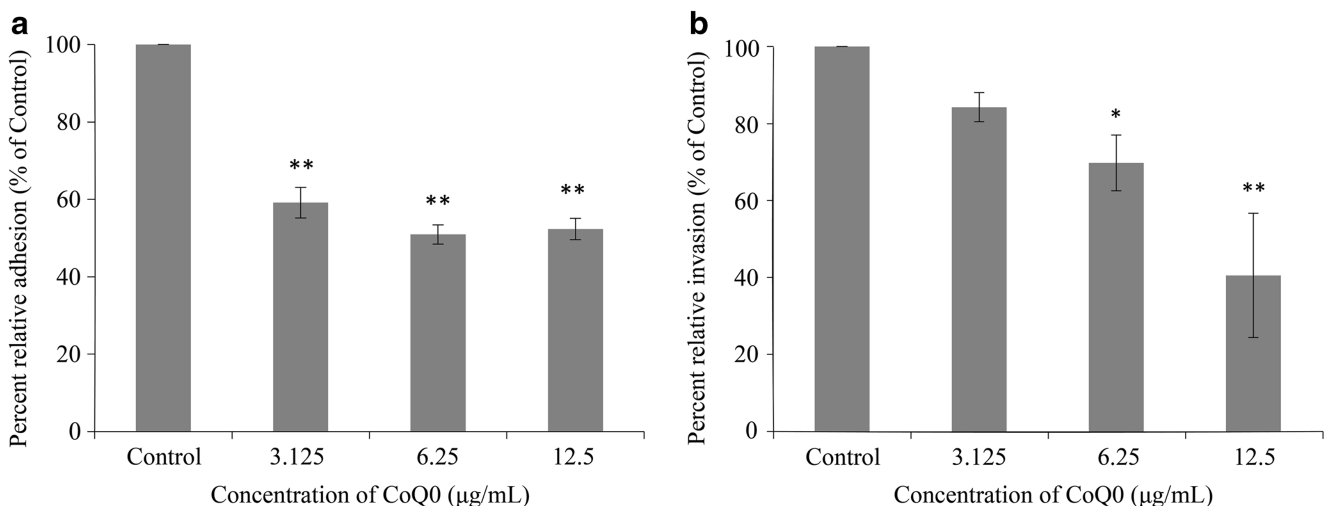


Fig. 6 Effects of CoQ0 on adhesion to (**a**) and invasion of (**b**) Caco-2 cells by *S.* Typhimurium SL1344. Bars represent the means ± standard deviations ($n = 3$). $*P < 0.05$, $**P < 0.01$ compared with the control

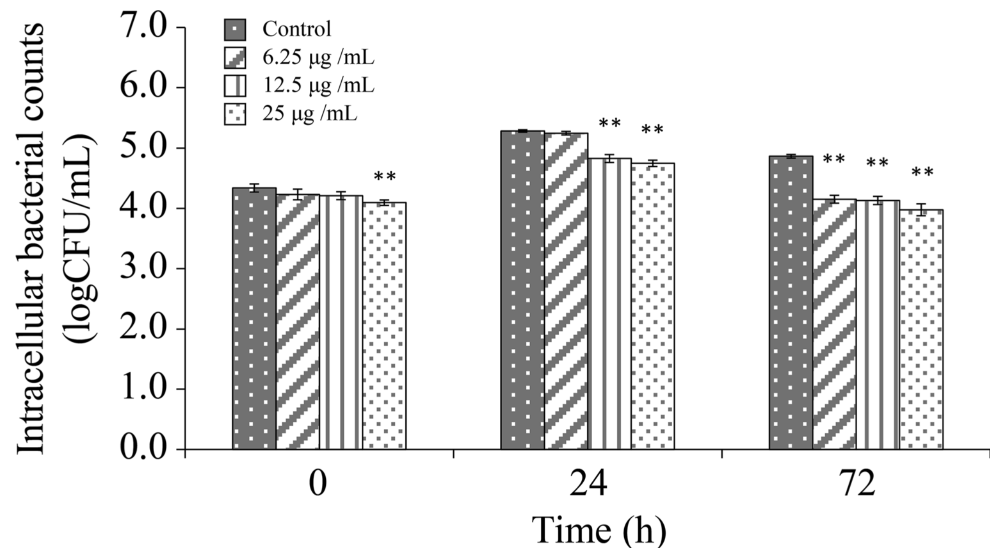


Fig. 7 Effect of CoQ0 on intracellular survival and replication of *S.* Typhimurium SL1344 within macrophages. Bars represent the means ± standard deviations ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$

bacterium can invade the cells and induce an intestinal inflammatory response (LaRock et al. 2015). Baxter and Jones (2015) reported that *hilA*, the master transcriptional activator of SPI-1, regulates at least 10 activators and eight repressors, and that cell invasion requires the upregulation of *hilA* transcription. The *invA* and *invH* genes encoded by *S.* Typhimurium SPI-1 are responsible for stimulating the inflammatory responses in cultured epithelial cells and causing inflammation in the gut (Pati et al. 2013). In the present study, the abilities of *Salmonella* to adhere to and invade Caco-2 cells were reduced by CoQ0 at SICs, and CoQ0 significantly downregulated *hilA*, *invA*, and *invH* transcription ($P < 0.05$) (Fig. 6). In a previous study, Wu et al. (2018) reported that 20 μg/mL baicalin inhibited the invasion of Caco-2 cells by *S.* Typhimurium and downregulated its transcription of the *sopB*, *sopE*, and *sopE2* genes, which are associated with SPI-1. Likewise, 30 μg/mL methyl gallate inhibited 43.75% of *S.* Typhimurium adhesion to and invasion of Caco-2 cells and downregulated the transcription of the *cheY*, *ompD*, *sipB*, *lexA*, and *ompF* genes, which are essential for its invasion and adhesion. Our results demonstrate that CoQ0 has potential utility as an antibiotic substitute to reduce *Salmonella* adhesion to and invasion of enterocytes and to ultimately alleviate inflammation in the gut.

During its infection of the gastrointestinal tract, *S.* Typhimurium targets both epithelial cells and macrophages (Hautefort et al. 2008). After entering the small intestine, *Salmonella* invades and penetrates the intestinal epithelium, which results in the systemic spread of the bacteria, which are taken up by macrophages (Jiang et al. 2017). Birhanu et al. (2018) have shown that the intracellular survival of *S.* Typhimurium in RAW 264.7 cells was effectively reduced by 30 μg/mL methyl gallate. In the present study, CoQ0 reduced the ability of *S.* Typhimurium to survive and reproduce intracellularly (Fig. 7). Our results indicate that CoQ0 reduces the ability of *S.* Typhimurium to utilize macrophages to evade the immune response and invade bodily organs. Cu, Zn superoxide dismutase has been shown to be expressed in several bacteria, including *Salmonella*, and confers protection against extracellular reactive oxygen species (Pacello et al. 2008). A deficiency in Cu, Zn superoxide dismutase, which is encoded by the *sodC* gene, reduces *Salmonella* survival in macrophages and attenuates its virulence in mice (DeGroote et al. 1997). In the present study, CoQ0 significantly downregulated the transcription of *sodC* (Table 1). Therefore, we hypothesize that CoQ0 inhibits the survival and replication of *S.* Typhimurium within macrophages by inhibiting Cu, Zn superoxide dismutase expression, ultimately attenuating *Salmonella* infection.

In conclusion, SICs of CoQ0 effectively prevent the formation of *Salmonella* biofilm by reducing the biofilm biomass, collapsing the biofilm architecture, and downregulating the expression of biofilm-associated genes and sRNAs. SICs of CoQ0 also inhibit the swimming motility and QS of *S.* Typhimurium, its adhesion to and invasion of Caco-2 cells, and its intracellular survival in RAW 264.7 macrophage cells, and repress its transcription of critical virulence-associated genes. Our results demonstrate that CoQ0 has potential utility as an antibiofilm and anti-infectious agent against *Salmonella*. However, further studies are required to confirm the anti-infection effects of CoQ0 in vivo before its practical application.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical statements This paper is our original work. It has not been submitted elsewhere, and it is not under consideration in any other Journal. This article does not contain any studies with human participants or animals performed by any of the authors. All the authors have seen the manuscript and approved its submission to Applied Microbiology and Biotechnology.

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