

Inhibition of *Cronobacter Sakazakii* Biofilm Formation and Expression of Virulence Factors by Coenzyme Q₀

Ning Guan

Northwest Agriculture and Forestry University

Yiqi Shi

Northwest Agriculture and Forestry University

Haoyu Tong

Northwest Agriculture and Forestry University

Yanpeng Yang

Northwest Agriculture and Forestry University

Jiahui Li

Northwest Agriculture and Forestry University

Du Guo

Northwest Agriculture and Forestry University

Yutang Wang

Northwest Agriculture and Forestry University

Xiaodong Xia

Northwest Agriculture and Forestry University

Chao Shi (✉ meilixinong@nwsuaf.edu.cn)

Northwest Agriculture and Forestry University

Research article

Keywords: *Cronobacter sakazakii*, Coenzyme Q₀, Antibiofilm, Adhesion, Invasion, Survival and replication

Posted Date: August 18th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-58153/v1>

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Abstract

Objectives—Here, we investigated the inhibitory effects of coenzyme Q₀ (CoQ₀) on biofilm formation and the expression of virulence genes by *Cronobacter sakazakii*.

Results—We found that the minimum inhibitory concentration of CoQ₀ against *C. sakazakii* strains ATCC29544 and ATCC29004 was 100 µg/mL, while growth curve assays showed that sub-inhibitory concentrations (SICs) of CoQ₀ for both strains were 6.4, 3.2, 1.6 and 0.8 µg/mL. Assays exploring the inhibition of specific biofilm formation showed that SICs of CoQ₀ inhibited biofilm formation by *C. sakazakii* in a dose-dependent manner, which was confirmed by scanning electron microscopy and confocal laser scanning microscopy analyses. CoQ₀ inhibited the swimming and swarming motility of *C. sakazakii* and reduced its ability to adhere to and invade HT-29 cells. In addition, CoQ₀ impeded the ability of *C. sakazakii* to survive and replicate within RAW 264.7 cells. Finally, real time polymerase chain reaction analysis confirmed that nine *C. sakazakii* genes associated with biofilm formation and virulence were down-regulated in response to CoQ₀ treatment.

Conclusion—Overall, our findings suggest that CoQ₀ is a promising antibiofilm agent and provide new insights for the prevention and control of infections caused by *C. sakazakii*.

Introduction

Cronobacter sakazakii (formerly *Enterobacter sakazakii*) is a motile, Gram-negative, rod-shaped opportunistic pathogen belonging to the family *Enterobacteriaceae* [1]. It is widely dispersed in the environment (e.g., domestic environments, manufacturing plants) and is commonly isolated from clinical specimens (e.g., cerebrospinal fluid, blood, sputum), food (e.g., cheese, meat, vegetables), and animals (e.g., rats, flies) [2–4]. Infants under the age of one are particularly susceptible to developing severe *C. sakazakii* infections, especially newborns and babies with low birth weight or compromised immunity [5]. Concerningly, several cases of neonatal *C. sakazakii* infection has been attributed to consumption of powdered infant formula [6]. The most common outcomes of foodborne *C. sakazakii* infection in infants are necrotizing enterocolitis, bacteremia, and meningitis [7, 8], often leading to neurological sequelae and even death [9]). Recently, the Centers for Disease and Control Prevention estimated that *C. sakazakii* infection case fatality rates are as high as 40% [10].

Biofilms are complex microbial communities that adhere to biotic or abiotic surfaces. Within the biofilm, microbes are firmly embedded in an extracellular matrix composed of polysaccharides, proteins, and DNA [11]. This matrix acts as a physical barrier against chemical agents and provides a protective ecological niche, enhancing the survival of biofilm-associated microorganisms [12]. Given the public health importance of *C. sakazakii*, its ability to form a biofilm has been the subject of much research. *C. sakazakii* forms a biofilm on various surfaces, including glass, stainless steel, silicon, latex, and polycarbonate, all of which are used in the processing and preparation of milk powder [13]. Biofilm-associated *C. sakazakii* show increased resistance to antibiotics, detergents, and other environmental

stresses [14]. As such, compared with planktonic cells, biofilm-based *C. sakazakii* infections are significantly more difficult to resolve [15]. In an industrial setting, biofilm formation by *C. sakazakii* increases the risk of contamination of foodstuffs, which has significant health and financial repercussions [16].

Virulence factors are gene-mediated molecules produced by microorganisms that enhance their ability to invade a host, cause disease, or evade host defenses [17]. Functions such as motility are also considered virulence factors. *C. sakazakii* produces a variety of virulence factors, including proteins involved in motility, host cell adhesion and invasion [18], and replication and survival within macrophages [19]. Motility is essential for the virulence of *C. sakazakii*, allowing it to pass through the intestine and colonize more favorable host environments such as mucous membranes, the gastric and intestinal epithelia, and endothelial tissues [20–22]. Outer membrane proteins OmpA and OmpX were shown to play an important role in *C. sakazakii* adhesion to and invasion of human intestinal epithelial Caco-2 and HT-29 cells [23]. Further study confirmed that OmpA contributes significantly to the pathogenicity of *C. sakazakii* as an essential factor in the invasion of various epithelial and endothelial cells of human and animal origin [24], while OmpX plays a crucial role in the basolateral invasion of host cells [25]. The ability of *C. sakazakii* to survive and replicate inside immune cells such as macrophages is critical for establishing infection and is the first step in the development of severe illnesses such as sepsis and meningitis [26].

Over the past decade, the use of plant-derived compounds as alternative antimicrobials has gained significant attention as a result of increasing concerns over the safety of synthetic antimicrobial agents and the emergence of antibiotic-resistant bacteria [27]. Coenzyme Q₀ (CoQ₀; 2,3-dimethoxy-5-methyl-1,4-benzoquinone; C₉H₁₀O₄) is a benzoquinone compound extracted from the fungus *Antrodia camphorata* [28]. It has demonstrated anti-tumor, anti-inflammatory, and anti-angiogenic properties [29], as well as antibacterial activity against *Staphylococcus aureus* and *Listeria monocytogenes* [30, 31]. In addition, CoQ₀ successfully inhibited biofilm formation by *Salmonella enterica* serovar Typhimurium [32].

Although multiple reports show that CoQ₀ has inhibitory effects against a variety of microorganisms, few studies have examined the effects of CoQ₀ on *C. sakazakii* biofilm formation or the expression of virulence factors. In the current study, we first determined the minimum inhibitory concentration (MIC) and sub-inhibitory concentration (SIC) of CoQ₀ against several *C. sakazakii* strains. Crystal violet staining, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM) were then used to study the effects of SICs of CoQ₀ on *C. sakazakii* biofilm formation. We then assessed the effects of CoQ₀ on various *C. sakazakii* virulence traits, including motility, adhesion to and invasion of HT-29 cells, and survival and replication within macrophages. Finally, the expression of genes associated with biofilm formation and other virulence factors following CoQ₀ treatment was examined using real time polymerase chain reaction (RT-PCR) analyses.

Methods

Chemicals and reagents

CoQ₀ (HPLC \geq 99%, CAS 605-94-7) was obtained from J&K Scientific Co., Ltd (Beijing, China) and dissolved in dimethyl sulfoxide (DMSO) for use in all experiments. The final concentration of DMSO in all sample solutions was 0.1% (v/v), which has no apparent effect on the growth of *C. sakazakii*. All other chemicals were of analytical grade and were unaltered.

Bacterial strains and culture conditions

C. sakazakii strains ATCC 29004 and ATCC 29544 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Strain ATCC 29004, which is a relatively strong biofilm producer, was used in the biofilm assay. Prior to each assay, bacteria were inoculated onto tryptic soy agar (TSA) medium and incubated at 37°C for 12 h. To obtain fresh overnight cultures, a single colony was inoculated into 30 mL of tryptic soy broth (TSB) medium and incubated with shaking at 130 rpm for 12 h at 37°C. Following incubation, cultures were centrifuged (4°C, 8000 \times g, 5 min), washed three times with sterile phosphate-buffered saline (PBS), and diluted in TSB medium to an optical density at 600 nm (OD₆₀₀) of 0.5 (approximately 4 \times 10⁸ colony-forming units (CFU)/mL).

MICs and SICs determinations

The MICs and SICs of CoQ₀ against *C. sakazakii* ATCC 29004 and ATCC 29544 were determined as described previously [33], with some modifications. Briefly, overnight bacterial culture was diluted 400 \times in TSB medium (approximately 1 \times 10⁶ CFU/mL) before 125 μ L of the diluted culture were added to individual wells of a 96-well plate. Equal volumes of CoQ₀ solution were gently added to each well to achieve final CoQ₀ concentrations of 0 (control), 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, and 51.2 μ g/mL. TSB medium containing 0.1% DMSO was used as the negative control. Plates were incubated at 37°C for 24 h, and cell growth was monitored at 600 nm at 1-h intervals using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The MIC of CoQ₀ was defined as the lowest concentration at which there was no visible growth of *C. sakazakii*. SICs of CoQ₀ were defined as concentrations at which no significant inhibition of *C. sakazakii* growth was observed.

Inhibition of specific biofilm formation (SBF) assay

The inhibition of SBF assay was carried out using a crystal violet staining method as described previously [34], with minor modifications. Briefly, an overnight culture of *C. sakazakii* ATCC 29004 was diluted in TSB medium to an OD₆₀₀ of 1.0. CoQ₀ was added to culture aliquots to obtain final concentrations of 0 (control), 1.6, 3.2, and 6.4 μ g/mL. Aliquots (200 μ L) of the mixtures were then pipetted into individual wells of a 96-well plate. Uninoculated TSB containing 0.1% DMSO was used as the negative control. Plates were incubated statically at 37°C or 25°C for 24, 72, or 120 h and the absorbance of the mixtures was monitored using a microplate reader (Model 680; Bio-Rad) at 630 nm. Following incubation, bacterial cultures were aspirated and the plates were washed with 300 μ L of

distilled water before being air-dried for 30 min. The wells were stained with 250 μL of 1% (w/v) crystal violet (Tianjin Kermel Chemical Regent Co., Ltd, Tianjin, China) for 20 min and any excess stain was removed using sterile distilled water. After drying for 30 min, 250 μL of 33% (v/v) glacial acetic acid was added to each well and the plates were shaken at ambient temperature for 20 min. The biofilm biomass in each well was quantified by measuring the OD at 570 nm. The SBF was determined from the ratio of the OD₅₇₀ and OD₆₃₀ values.

SEM observation

Confirm the effects of CoQ₀ on biofilm formation, the *C. sakazakii* ATCC 29004 biofilms were further analyzed by SEM as described previously [31], with minor modifications. Briefly, *C. sakazakii* ATCC 29004 cultures (OD₆₀₀ = 1.0) mixed with CoQ₀ solution (6.4, 3.2, 1.6, or 0 $\mu\text{g}/\text{mL}$) were added to individual wells of a 24-well plate containing sterile glass slides of the same diameter as the bottoms of the wells. Culture without CoQ₀ was used as a control. Following incubation at 25°C for 48 h, culture supernatants were removed, and the samples were fixed with 2 mL of 2.5% (v/v) glutaraldehyde overnight at 4°C. The glass slides were then removed and washed with sterile PBS, followed by treatment with 1% (v/v) osmic acid at 4°C for 5 h. Subsequently, the glass slides were dehydrated using a graded ethanol series (30%, 50%, 60%, 70%, 80%, 90%, and 100%). After being dried and coated with gold, the slides were examined under a field-emission scanning electron microscope (S-4800; Hitachi, Tokyo, Japan) at 4000 \times and 1500 \times magnification.

CLSM observation

To examine the effects of CoQ₀ on the viability of biofilm-associated *C. sakazakii* ATCC 29004 cells, biofilms were next strained using a LIVE/DEAD *BacLight* Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA), consisting of SYTO 9 and propidium iodide (PI) dyes. Biofilms were cultured on stainless steel coupons in a 24-well plate in the presence or absence of CoQ₀, as described in section 2.5. Following removal of the supernatant, the plate was washed twice with sterile water. Stainless-steel coupons were then stained with SYTO 9 and PI in the dark for 8 min as per the manufacturer's instructions. After being washed with sterile water, the stained biofilms on the stainless-steel coupons were examined using a confocal laser scanning microscope (A1; Nikon, Tokyo, Japan).

Motility assay

Swimming and swarming motility assays were conducted as described previously [35], with minor modifications. Bacterial swimming motility assays were conducted using 20 mL of LB broth containing 0.3% (w/v) agar, while swarming motility assays were conducted in 20 mL of LB broth supplemented with 0.5% (w/v) agar and 0.5 (w/v) glucose. CoQ₀ was added to the warm media (45°C) to achieve final concentrations of 0 (control), 1.6, 3.2, and 6.4 $\mu\text{g}/\text{mL}$, and the resulting mixtures poured into Petri dishes. The resulting agar plates were dried at ambient temperature for 1 h. Aliquots (5 μL) of bacterial cultures (OD₆₀₀ = 0.5) were then inoculated onto the center of each plate and incubated upright at 37°C for 7 h.

Images of the resulting bacterial halos and swarm areas were obtained using a Gel Imaging System (Bio-Rad).

Cell culture

HT-29 human colon carcinoma cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco, Grand Island, NY, USA). RAW 264.7 murine macrophage cells (ATCC) were cultured in DMEM (Gibco). Culture media were prepared as described by Shi et al. [33]. Cell lines were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Adhesion and invasion assay

The effects of CoQ₀ on bacterial adhesion and invasion were examined as described previously [36], with some modifications. For both assays, HT-29 cells in DMEM/F-12 were inoculated into 24-well plates (10⁵ cells/well) and incubated overnight before being washed twice with sterile PBS. Aliquots of *C. sakazakii* ATCC 29544 culture (OD₆₀₀ = 0.5) were mixed with CoQ₀ solution (6.4, 3.2, 1.6, 0.8, or 0 µg/mL final concentration) and cultured at 37°C for 6 h. Bacterial cultures were then washed with sterile PBS and the cell pellets resuspended in TSB medium to an OD₆₀₀ of 0.5 (approximately 4 × 10⁸ CFU/mL). Cultures were diluted 40× in DMEM/F-12 and inoculated onto the HT-29 cell monolayers at a multiplicity of infection (MOI) of 10. Following centrifugation (600 × *g*, 5 min), the plates were incubated in a humidified, 5% CO₂ incubator at 37°C for 2 h.

For adhesion assays, the incubated plates were rinsed three times with sterile PBS and the cells lysed by the addition of 1 mL of 0.1% (v/v) Triton X-100 (Amresco, Solon, OH, USA) followed by incubation at 4°C for 20 min. The lysed cells were then serially diluted in sterile PBS and plated on TSA for colony counting. For invasion assays, the incubated plates were washed once with sterile PBS and 1 mL of DMEM/F-12 containing gentamicin (100 µg/mL; Amresco) was added to each well. The plates were then incubated for a further 45 min to kill the extracellular bacteria. Following incubation, the cells were washed three times before being lysed by the addition of 1 mL of 0.1% (v/v) Triton X-100 followed by incubation at 4°C for 20 min. The lysed bacteria were then serially diluted and plated on TSA for colony counting. Results were expressed as the percentage of colonies on the treatment plates relative to those on the control plates.

Intracellular survival and replication assay

The effects of CoQ₀ on the intracellular survival and replication of *C. sakazakii* ATCC 29544 in RAW 264.7 cells were examined as described previously [34], with some modifications. RAW 264.7 cells cultured in DMEM were seeded into 24-well plates (10⁵ cells/well) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 16 h. Aliquots of bacterial suspension (OD₆₀₀ = 0.5) were mixed with CoQ₀ at a final concentration of 0 (control), 0.8, 1.6, 3.2, or 6.4 µg/mL and incubated at 37°C for 6 h. Following incubation, the suspensions were washed once with sterile PBS and the resulting cell pellets resuspended in TSB to an OD₆₀₀ of 0.5 (approximately 4 × 10⁸ CFU/mL). The cell suspensions were then diluted in

DMEM to a density of 1×10^6 CFU/mL and inoculated onto the RAW 264.7 cell monolayers at a MOI = 10. Following incubation at 37°C in the presence of 5% CO₂ for 2 h, the plates were rinsed once with sterile PBS, and 1 mL of DMEM containing gentamicin (100 µg/mL) was added to each well. The plates were then incubated for a further 45 min.

For the intracellular survival assays, the incubated plates were rinsed with sterile PBS and the cells lysed by the addition of 1 mL of 0.1% (v/v) Triton X-100 followed by incubation at 4°C for 20 min. The lysed cells were serially diluted before being plated on TSA plates. The results are expressed as the number of viable *C. sakazakii* cells (CFU/mL) before and after CoQ₀ treatment. Infected cells that were not treated with CoQ₀ were used as the control. For the intracellular replication assay, the incubated plates were again rinsed with sterile PBS and 1 mL of DMEM containing gentamicin (10 µg/mL) was added to each well. Following incubation at 37°C in the presence of 5% CO₂ for either 24 h or 48 h, the cells were lysed, serially diluted, and plated on TSA plates, as described in the intracellular survival assay.

Isolation of RNA and RT-PCR assays

To assess the effects of CoQ₀ on the expression of virulence genes (Table 1), *C. sakazakii* ATCC 29004 was cultured in the presence (1.6 or 3.2 µg/mL) or absence (control) of CoQ₀ at 37°C for 8 h. Total RNA was then extracted from each culture using an RNA Prep Pure Cell/Bacteria Kit (Tiangen, Beijing, China) as per the manufacturer's instructions. The quality and concentration of the extracted RNA were determined using a nucleic acid and protein spectrophotometer (Nano-200; Aosheng Instrument Co., Ltd, Hangzhou, China). RNA was then reverse-transcribed into cDNA using a Takara PrimeScript RT Reagent Kit (Takara, Dalian, China) as per the manufacturer's instructions. RT-PCR assays were carried out in 25-µL reaction volumes with SYBR Green reagents (Takara) using an IQ 5 Multicolor Real-Time PCR Detection System (Bio-Rad). The reaction parameters included an initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, with a dissociation step of 95°C for 15 s and 60°C for 30 s. Specific primers corresponding to each of the target genes (Table 1) were based on those from previous reports [24, 37]. The 16S rRNA gene was used as an internal control for normalization of gene expression. The $2^{-\Delta\Delta C_t}$ method was used to compare the expression of genes from different samples [38]. The means and standard deviations were calculated from triplicate experiments.

Table 1 Primers used in this study.

Primers	Gene amplified	Primer sequences (5'–3')
16S rRNA	Control-RT- F	CCAGGGCTACACACGTGCTA
	Control- RT-R	TCTCGCGAGGTCGCTTCT
<i>bcsA</i>	<i>bcsA</i> -RT- F	CACGATGGTGGCGTTGTTC
	<i>bcsA</i> - RT-R	CCTTTGGCGGGTGACGTTAA
<i>fliD</i>	<i>fliD</i> -RT- F	AAAACCGCAACATGGAATTCA
	<i>fliD</i> - RT-R	CCGCAAACGCGGTATTG
<i>flgJ</i>	<i>flgJ</i> -RT- F	GACGGCGGGCAAAGG
	<i>flgJ</i> - RT-R	GCCGCCCATCTGTTTGAC
<i>motA</i>	<i>motA</i> -RT- F	GGTGTGGGTGCGTTTATCGT
	<i>motA</i> - RT-R	GCCTTCAGCGTGCCTTTG
<i>motB</i>	<i>motB</i> -RT- F	ACGGCTCGTGGAAAATCG
	<i>motB</i> - RT-R	CCAGGAAGAAGGCCATCATG
<i>ompX</i>	<i>ompX</i> -RT- F	GTCTTTCAGCACTGGCTTGTGT
	<i>ompX</i> - RT-R	GGTGCCAGCAACAGCAGAA
<i>uvrY</i>	<i>uvrY</i> -RT-F	GCGAGGACGCCATCAAAT
	<i>uvrY</i> -RT-R	ATCCATCAGCACCACATCCA
<i>lpxB</i>	<i>lpxB</i> -RT-F	GCACGACACTTTCCGTAAACTG
	<i>lpxB</i> -RT-R	CGCCTGTTTCATCGGCATT
<i>bcsG</i>	<i>bcsG</i> -RT-F	ACGACTGGCTAACAGCTTTTAC
	<i>bcsG</i> -RT-R	GCCGGGAAGGTTGTCTGA

Statistical analyses

All experiments were carried out independently three times. The data were presented as means \pm standard deviations (SD) and analyzed using SPSS 23.0 software (IBM, New York, NY, USA). A Student's *t*-test was used to analyze differences between means. Differences were considered statistically significant at $P < 0.05$ and extremely significant at $P < 0.01$.

Results

MICs

CoQ₀ showed strong antibacterial activity against *C. sakazakii* ATCC 29004 and ATCC 29544, with an observed MIC of 100 µg/mL for both strains (data not shown).

SICs and growth curve analyses

The effects of CoQ₀ on the growth of *C. sakazakii* strains ATCC 29004 and ATCC 29544 are shown in Fig. 1. At a CoQ₀ concentration of 51.2 µg/mL, the lag phase of both *C. sakazakii* cultures was longer than that of the control culture grown in the absence of CoQ₀. However, the growth curves of *C. sakazakii* ATCC 29004 and ATCC 29544 treated with 6.4, 3.2, 1.6, or 0.8 µg/mL CoQ₀ were not significantly different from that of the untreated control ($P>0.05$). Therefore, these concentrations were used as SICs in subsequent biofilm formation, virulence, and RT-PCR assays.

Effects of CoQ₀ on *C. sakazakii* biofilm formation

The effects of CoQ₀ on the ability of *C. sakazakii* ATCC 29004 to form biofilms following incubation at 25°C or 37°C for 24, 72, or 120 h are shown in Fig. 2. The total biofilm biomass was significantly ($P<0.05$) decreased compared with the control following treatment with CoQ₀ at 6.4 µg/mL and incubation at 25°C (Fig. 2A). At 37°C, biofilm biomass was significantly ($P<0.05$) decreased compared with the control at a CoQ₀ concentration of 1.6 µg/mL, with further decreases in biomass observed with increasing CoQ₀ concentration (Fig. 2B).

SEM observation

SEM images of CoQ₀-treated *C. sakazakii* biofilms at 4000× and 1500× magnification are shown in Fig. 3. In the absence of CoQ₀, the biofilms exhibited a typical three-dimensional morphology with thick aggregates. In comparison, the biofilms became monolayers and cell clusters became looser with increasing CoQ₀ concentrations, indicative of significant disruption.

CLSM observation

As shown in Fig. 4A, the untreated biofilm appeared almost completely green, indicative of live cells, with few bacteria exhibiting PI fluorescence. However, increasing levels of red fluorescence (PI) were observed with increasing concentrations of CoQ₀, suggesting a breakdown in membrane integrity. The highest level of red fluorescence intensity was observed at a CoQ₀ concentration of 6.4 µg/mL (Fig. 4d).

Motility

As shown in Fig. 5A, CoQ₀ treatment resulted in a decrease in the swimming motility of *C. sakazakii* compared with the untreated control, with the observed decreases in motility occurring in a dose-dependent manner. Similarly, the swarming motility of *C. sakazakii* decreased compared with that of the control in response to increasing CoQ₀ concentration (1.6–6.4 µg/mL) (Fig. 5B).

Adhesion to and invasion of HT-29 cells

We next examined the effects of CoQ₀ on the ability of *C. sakazakii* ATCC 29544 to adhere to and invade HT-29 cells. As shown in Fig. 6A, CoQ₀ significantly ($P<0.01$) impeded the ability of *C. sakazakii* ATCC 29544 to adhere to host cells, with the decreases in adherence compared with the control occurring in a dose-dependent manner. At 0.8, 1.6, 3.2, and 6.4 µg/mL, CoQ₀ treatment decreased the adherence of *C. sakazakii* ATCC 29544 by 36.8%, 39.3%, 40.2%, and 56.2%, respectively, compared with the control. Further, CoQ₀ at concentrations ≥ 1.6 µg/mL significantly ($P<0.05$) inhibited the ability of *C. sakazakii* ATCC 29544 to invade host cells (Fig. 6B). Specifically, 5.3%, 21.2%, 34.8%, and 42.8% decreases in the percent relative invasion of host cells were observed at CoQ₀ concentrations of 0.8, 1.6, 3.2, and 6.4 µg/mL, respectively.

Intracellular survival and replication in RAW 264.7 cells

The effects of CoQ₀ on the intracellular survival and replication of *C. sakazakii* in RAW 264.7 cells are shown in Fig. 7. At concentrations of 3.2 and 6.4 µg/mL, CoQ₀ significantly ($P<0.05$) decreased the intracellular survival of *C. sakazakii* ATCC 29544 compared with the control at 48 h post-inoculation. However, at CoQ₀ concentrations of 0.8 µg/mL and 1.6 µg/mL, the intracellular survival and replication of *C. sakazakii* ATCC 29544 did not significantly differ from the control at 24 h and 48 h post-inoculation.

RT-PCR analyses

RT-PCR analyses showed that following treatment with CoQ₀ at 3.2 µg/mL, the expression of *uvrY* (adherence and invasion) in *C. sakazakii* cells was significantly ($P<0.05$) down-regulated compared with the untreated control (Fig. 8). Similarly, the mRNA levels of *flgJ* and *fliD* (flagellar assembly), *motA* and *motB* (flagellar motor proteins), *bcsG* (cell biosynthesis and biofilm formation), *bcsA* (cellulose synthase operon), and *ompX* and *lpxB* (LPS biosynthesis) were significantly ($P<0.01$) lower than those in the control. However, no differences in the levels of transcription of *flgJ*, *uvrY*, and *motB* compared with the control were observed at CoQ₀ concentrations ≤ 1.6 µg/mL.

Discussion

C. sakazakii, with its array of virulence factors, poses a significant threat to the health of infants and young children because of its ability to form biofilms on surfaces in facilities that process milk and dairy products [39]. With the global increase in antibiotic resistance and safety concerns surrounding the use of chlorine-based disinfectants, the antibacterial properties of plant-derived compounds are increasingly being investigated [40, 41]. CoQ₀, derived from *A. cinnamomea*, has attracted attention for its potential as a natural food preservative and as a therapeutic antibiotic [28]. Based on growth curve analysis, we identified appropriate SICs of CoQ₀ that had no effect on growth for use in subsequent assays. These experiments showed that at SICs, CoQ₀ has significant anti-biofilm and antibacterial activities that cannot

be attributed to inhibition of bacterial growth, indicating that low concentrations of CoQ₀ affect bacterial virulence factors instead.

Biofilm formation contributes to the survival of *C. sakazakii* in sub-optimal environments, is involved in evading and circumventing the host immune system, and provides protection against antibiotics and disinfectants [42]. Therefore, identifying potential anti-biofilm strategies has been a major focus for controlling the growth of *C. sakazakii* [12]. In the current study, the effects of CoQ₀ on the formation of *C. sakazakii* biofilms was first evaluated via crystal violet staining. The results showed that at SICs, CoQ₀ significantly reduced *C. sakazakii* biofilm biomass at 25°C (normal room temperature) and biofilm formation at 37°C (the optimal temperature for *C. sakazakii* growth) (Fig. 2). In addition, CoQ₀ significantly reduced the expression of genes related to biofilm formation, including *bcsA* and *bcsG* (Fig. 8). *bcsA* encodes the catalytic subunit of cellulose synthase, while *bcsG* encodes a conserved hypothetical protein involved in cellulose biosynthesis. Cellulose is an important component of the extracellular matrix of *C. sakazakii* biofilms and is essential for biofilm formation [43]. Previous studies have shown that cell-free supernatants derived from goat milk-origin lactobacilli cultures can prevent the formation of *C. sakazakii* biofilms [44], while pomegranate and rosemary extracts were shown to have a synergistic effect in combination with traditional antibiotics against biofilm formation by *Pseudomonas aeruginosa* [45]. Shi et al. (2018) reported that thymequinone significantly inhibited the biofilm formation of *C. sakazakii* by reducing the production of cellulose [33]. Therefore, our results suggest that CoQ₀ reduces the transcription of genes required for cellulose biosynthesis, ultimately inhibiting *C. sakazakii* biofilm formation.

With increasing CoQ₀ concentration, *C. sakazakii* biofilms contained fewer cells overall and a higher percentage of damaged and dead cells compared with the control (Fig. 3). These SEM- and CLSM-based findings supported the results of quantitative crystal violet staining analysis, confirming that CoQ₀ inhibits *C. sakazakii* biofilm formation. Similarly, Yang et al. (2019) used SEM analysis to show that shikimic acid (0.3125 mg/mL) effectively inhibits the formation of *Salmonella* Typhimurium biofilms, while CLSM observations by Nair et al. (2018) demonstrated that selenium damages *Escherichia coli* O157:H7 biofilms and decreases live cell counts [32, 46].

Bacterial invasion of host cells requires motility, which is an important factor in the early stages of infection. Swimming motility allows bacteria to move through liquid media, while swarming and clustering are observed in broth/semi-solid media and on solid surfaces/agar, respectively [14]. Our findings demonstrated that both the swimming and swarming motilities of *C. sakazakii* were decreased in a concentration-dependent manner compared with the control as a result of CoQ₀ treatment (Fig. 5). Consistent with this observation, motility-associated genes *motA*, *motB*, *flgJ*, and *fliD* were significantly down-regulated compared with the control following CoQ₀ treatment (Fig. 8). Other researchers have shown that the swimming and swarming motility of *Listeria monocytogenes* can be significantly reduced compared with morin-treated controls following treatment with a *Bifidobacterium*-derived bacteriocin [36] while the inhibition of flagellum biosynthesis and function as a result of citral treatment helped reduce

the motility of *C. sakazakii* [18]. Flagella are the main structures responsible for bacterial motility and are essential for maximum virulence [47]. *fliD* and *flgJ* encode the flagellum capping protein and a muramidase involved in flagella rod assembly, respectively. Hu et al. (2015) reported that *motA* and *motB* form a bicistronic operon encoding two proteins that form the flagellum matrix [48]. Therefore, we predict that SICs of CoQ₀ inhibit *C. sakazakii* flagellum biosynthesis, reducing its mobility and capacity to invade host cells.

The ability of *C. sakazakii* to adhere to and invade host cells, including various epithelial cell lines and brain endothelial cells, is an important virulence factor in the establishment of infection [49]. In *C. sakazakii*, outer membrane protein OmpX also contributes to host cell invasion [50]. In the current study, we found that CoQ₀ significantly inhibited the adhesion and invasion of *C. sakazakii* in HT-29 cells, likely by downregulating the transcription of *ompX* and *uvrY*, two genes that are positively correlated with bacterial adhesion/invasion of epithelial cells (Fig. 8). Similarly, CoQ₀ treatment downregulated the expression of adhesion/invasion-associated genes in *L. monocytogenes*, resulting in a significant decrease in the adherence to and invasion of Caco-2 cells [31]. *Pediococcus acidilactici* K10, isolated from kimchi, was also shown to reduce the adherence of *S. Typhimurium* KCTC 1925 and *E. coli* O157:H7 ATCC 35150 to HT-29 cells [51]. In light of these findings, it is likely that CoQ₀ plays a significant role in reducing *C. sakazakii* adhesion to and invasion of enterocytes by regulating associated gene expression.

Clinical *C. sakazakii* isolates have been observed to continuously reproduce in macrophages and microglia [52]. Similar studies have reported that *C. sakazakii* tolerates the intracellular environment of macrophages, hiding inside these cells to evade the immune response and, ultimately, invade other organs [49, 53]. In the present study, SICs of CoQ₀ prominently inhibited the survival and replication of *C. sakazakii* inside macrophages (Fig. 7), supporting findings by Yang et al. (2019) who reported that the ability of *S. Typhimurium* to survive and reproduce intracellularly was impacted by CoQ₀ treatment [32]. Based on these findings, we speculate that CoQ₀ can reduce the ability of *C. sakazakii* to overcome host barriers and evade the immune response.

In summary, the current study confirms that CoQ₀ is an ideal antibiofilm and anti-virulence agent. We demonstrated that SICs of CoQ₀ effectively inhibited the initial formation of biofilms at 25°C and 37°C, prevented the adherence of the biofilm to glass slides, and caused significant collapse of the biofilm structure. In addition, CoQ₀ inhibited the swimming and swarming motilities of *C. sakazakii* cells and prevented their adhesion to and invasion of HT-29 cells. As well as decreasing the intracellular survival and replication of *C. sakazakii* in RAW 264.7 macrophages, SICs of CoQ₀ down-regulated the transcription of virulence-associated genes. Together, these activities significantly impacted the virulence of *C. sakazakii*. Our findings suggest that CoQ₀ is a promising broad-spectrum anti-virulence therapeutic agent that could be used to control *C. sakazakii* biofilm pollution and provide new avenues for the prevention and control of infections.

Declarations

Acknowledgements

We appreciate the support of State Key Laboratory of Crop Stress Biology in Arid Areas and College of Veterinary Medicine in Northwest Agriculture and Forestry University for providing us with scanning electron microscopy and confocal laser scanning microscopy.

Authors' contributions

CS, NG, and YqS conceived and designed the experiments. NG, YqS, HyT, YpY and JhL performed the experiments. NG, YqS, DG and YtW analyzed the data. CS and XdX contributed reagents, materials, and analysis tools. NG, and YqS wrote the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (31801659), the Shaanxi Key Research and Development Program (2019NY-118), the General Financial Grant from the China Postdoctoral Science Foundation (No. 2017M623256), and the class General Financial Grant from the Shaanxi Province Postdoctoral Science Foundation (2018BSHEDZZ150).

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no conflicts of interest.

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Figures

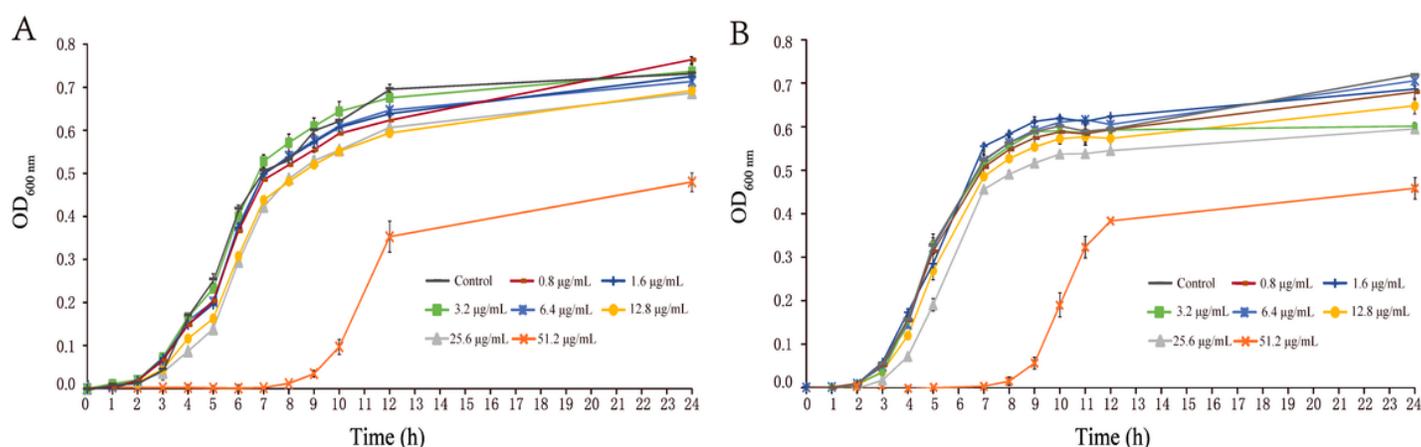


Figure 1

Growth curves of *C. sakazakii* ATCC 29004 (A) and *C. sakazakii* ATCC 29544 (B) cultured in TSB medium supplemented with CoQ0 at the indicated concentrations. Each value represents the average of three

independent experiments. Control, *C. sakazakii* cultured in the absence of CoQ0.

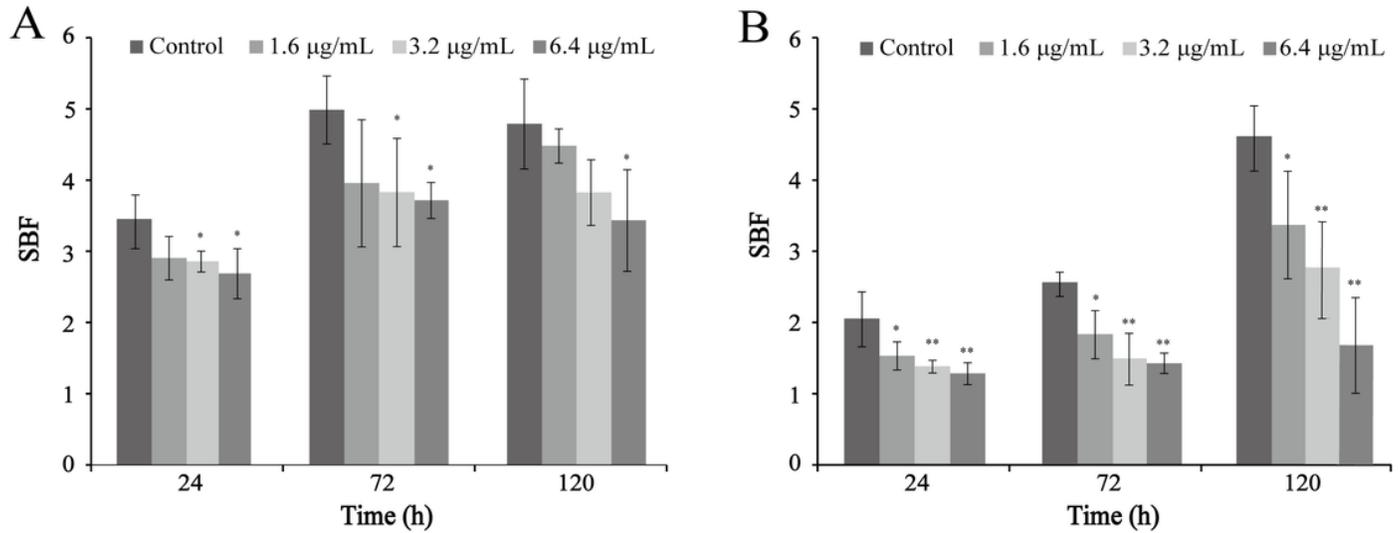


Figure 2

Effects of CoQ0 on *C. sakazakii* ATCC 29004 biofilm formation at 25°C (A) and 37°C (B). Values are the mean \pm SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ versus the control.

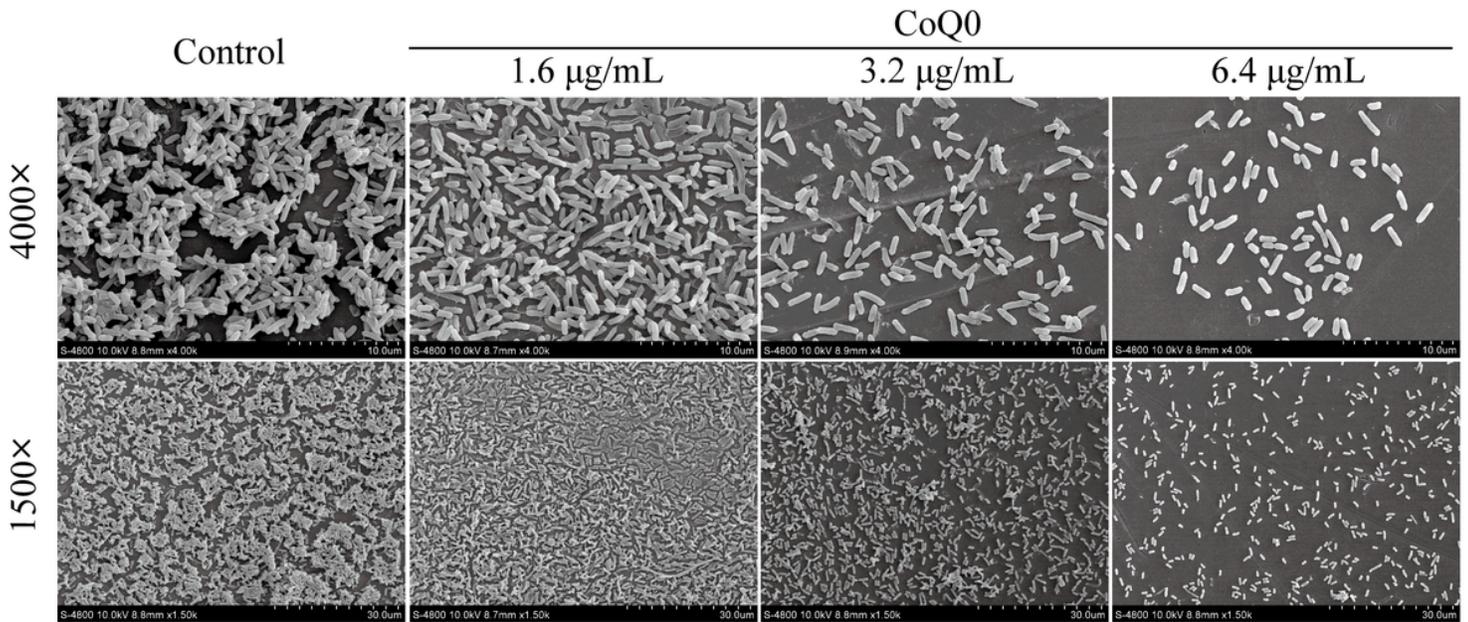


Figure 3

SEM micrographs of *C. sakazakii* ATCC 29004 biofilms formed on glass surfaces in the presence and absence of SICs of CoQ0. Magnification, 4000 \times and 1500 \times .

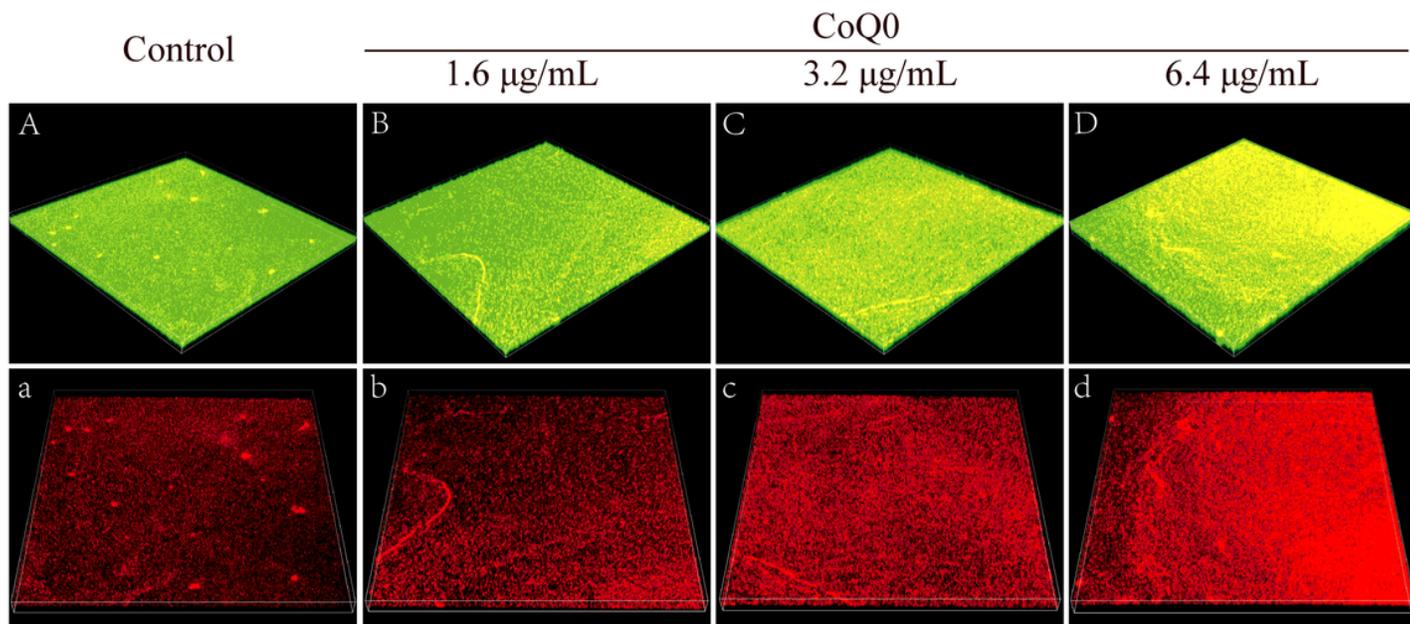


Figure 4

CLSM images of *C. sakazakii* ATCC 29004 biofilms following treatment with CoQ0 at 0 (A, a), 1.6 (B, b), 3.2 (C, c), or 6.4 $\mu\text{g/mL}$ (D, d).

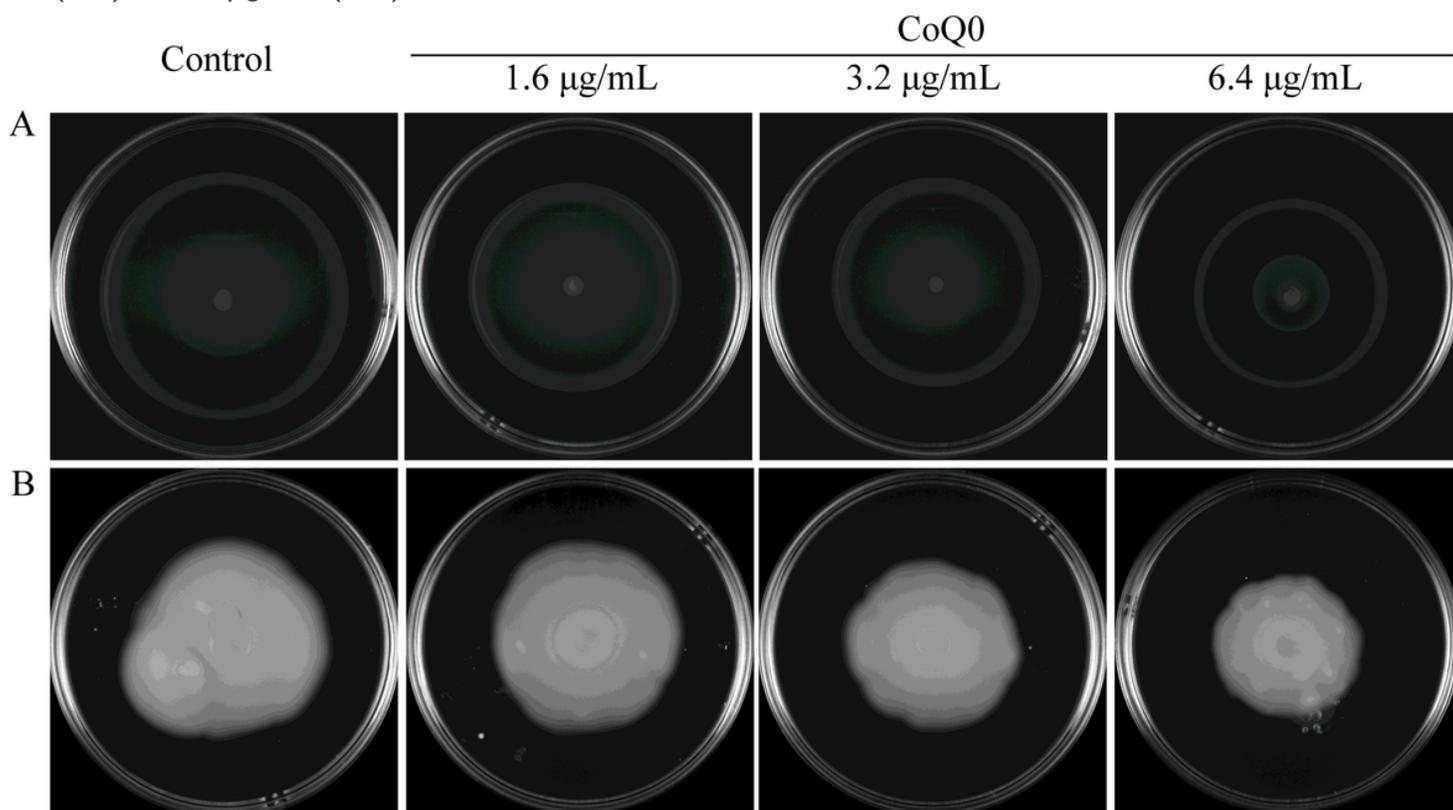


Figure 5

Effects of SICs of CoQ0 on the swimming (A) and swarming (B) motility of *C. sakazakii* ATCC 29544.

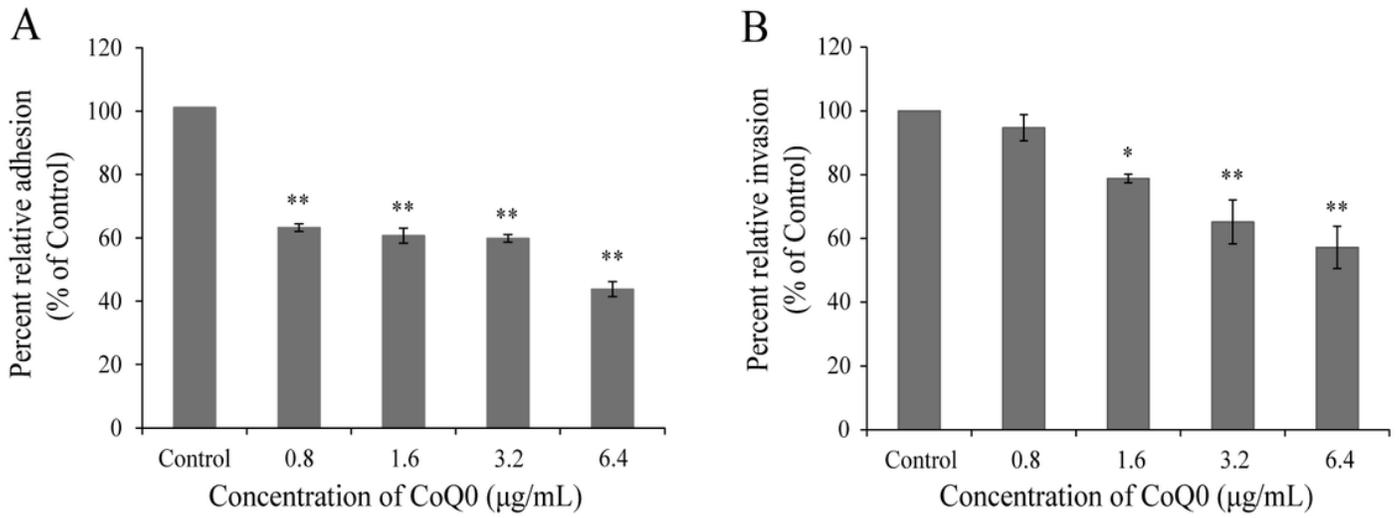


Figure 6

Effects of CoQ0 on the adhesion to (A) and invasion of (B) HT-29 cells by *C. sakazakii* ATCC 29544. Values are the mean \pm SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ versus the control.

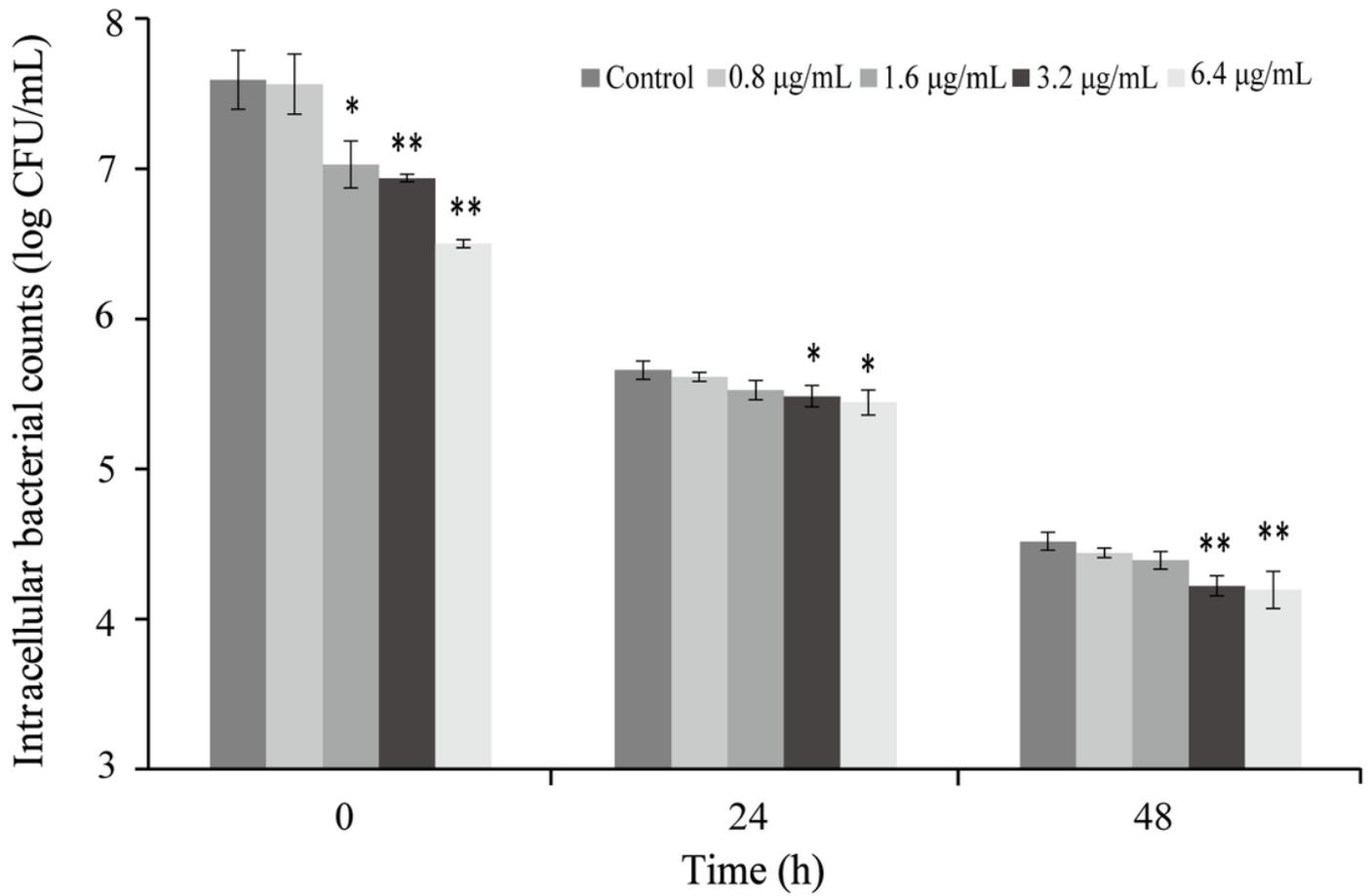


Figure 7

Inhibitory effects of CoQ0 the intracellular survival and replication of *C. sakazakii* ATCC 29544 in RAW 264.7 cells. Values are the mean \pm SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ versus the control.

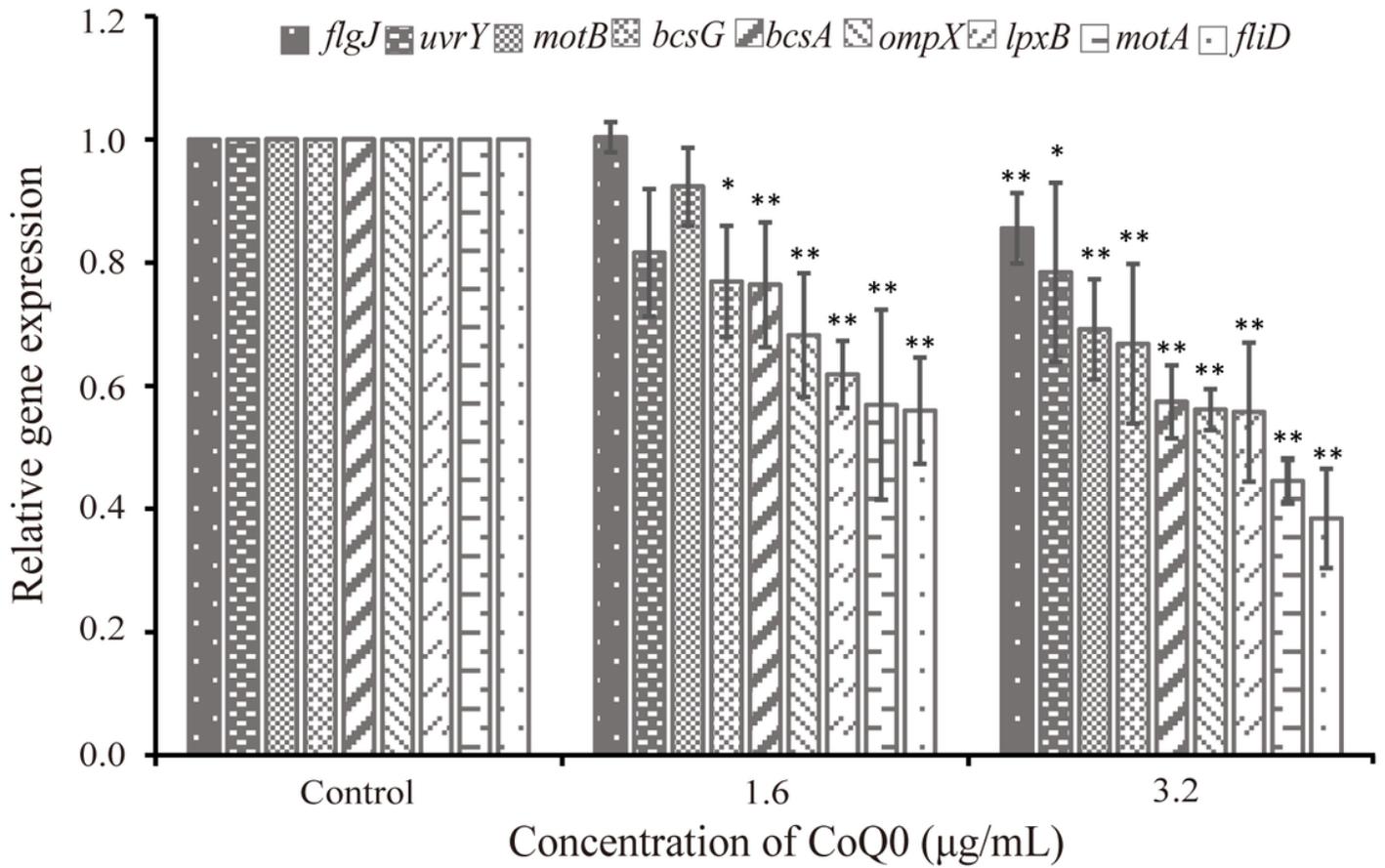


Figure 8

Effects of CoQ0 on the relative transcription of virulence-associated genes in *C. sakazakii* ATCC 29004. *, P<0.05; **, P<0.01 versus the control.