

Article

# Antimicrobial Activity and Antibiofilm Potential of Coenzyme Q<sub>0</sub> against *Salmonella* Typhimurium

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**Abstract:** Coenzyme Q<sub>0</sub> (CoQ<sub>0</sub>) has anti-inflammatory and anti-tumor effects; however, the antimicrobial and antibiofilm activities of CoQ<sub>0</sub> against *Salmonella enterica* serovar Typhimurium are unknown. Thus, we investigated the bacteriostatic and antibiofilm activities, along with the underlying mechanism, of CoQ<sub>0</sub> against *S. Typhimurium*. The minimum inhibitory concentration (MIC) of CoQ<sub>0</sub> against *S. enterica* serovars Typhimurium was 0.1–0.2 mg/mL (549–1098 μM), and CoQ<sub>0</sub> at MIC and 2MIC decreased viable *S. Typhimurium* counts below detectable limits within 6 and 4 h, respectively. CoQ<sub>0</sub> at 20MIC (4 mg/mL) reduced *S. Typhimurium* on raw chicken by 1.5 log CFU/cm<sup>3</sup> within 6 h. CoQ<sub>0</sub> effectively disrupted cell membrane integrity and induced morphological changes in the cell, resulting in hyperpolarization, decreased intracellular ATP concentrations, and cellular constituents leakage. Biofilm-associated *S. Typhimurium* cells were killed by CoQ<sub>0</sub> treatment. These findings suggest that CoQ<sub>0</sub> could be applied as a natural antibacterial substance for use against *S. Typhimurium* by the food industry.

**Keywords:** coenzyme Q<sub>0</sub>; *Salmonella* Typhimurium; cell membrane; biofilm; raw chicken



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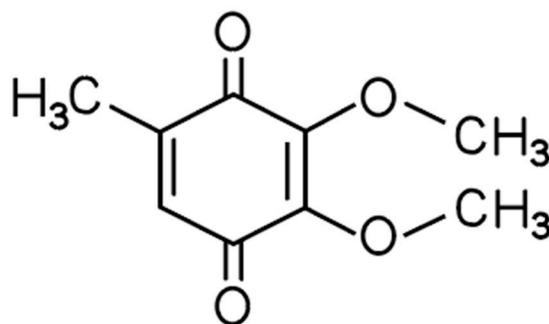
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## 1. Introduction

The genus *Salmonella* comprises several species and numerous sub-species and serovars, all of which are foodborne opportunistic bacterial pathogens that can be transmitted between humans and animals [1]. These Gram-negative, rod-shaped, flagellated bacteria are widely distributed and are common contaminants of foods, especially meat, egg, and dairy products, which are some of the main sources of foodborne illness [2]. *Salmonella* infection can cause gastroenteritis, typhoid fever, sepsis, and other syndromes, the combined effects of which have the greatest impact on *Salmonella* mortality rates [3]. Worldwide, an estimated 94 million disease cases and 155,000 deaths have been attributed to *Salmonella* infection in the last decade [4].

Biofilms are aggregates of bacterial cells with a certain spatial structure. Within these aggregates, bacteria proliferate and secrete an extracellular matrix that helps to anchor the cells to each other and, often, to a surface [5]. *Salmonella* readily form biofilms on multiple surface types found in the food production chain, including plastics, glass, stainless steel, wood, and rubber [6]. When a biofilm reaches the stage of irreversible adhesion, it cannot be removed by gentle cleaning [7]. In addition, compared with planktonic cells, biofilm-associated *Salmonella* demonstrate increased pathogenicity, including enhanced adaptability and resistance to various antibiotics, physical and chemical disinfectants, environmental stress, and the human immune response [8].

Preventing *Salmonella* contamination of food products, corrosion of food production facilities, and human infections is of significance to public health worldwide [9]. Various chemical preservatives are commonly used in food processing, production, and storage to kill *Salmonella* and other pathogenic microorganisms in food; however, the extensive use of chemical preservatives has led to increased resistance among pathogenic microorganisms [10]. Further, traditional methods of removing *Salmonella* biofilms have limitations: disinfectants used in the food industry have significant scavenging effects on biofilm but can produce carcinogenic by-products [11]. In recent years, there has been increased interest in plant-based antimicrobials, which are widely distributed in nature and have a variety of physiological activities. More importantly, these compounds can be used as highly effective broad-spectrum bacteriostatic agents in place of chemical preservatives to control foodborne pathogens [12]. *Antrodia camphorata* is a wild fungus with significant utility in the production of plant-based antimicrobials. It contains high levels of coenzyme Q<sub>0</sub> (CoQ<sub>0</sub>; 2,3-dimethoxy-5methyl-1,4-benzoquinone; molecular weight, 182.1733, Figure 1), an anthraquinone substance with anti-cancer and anti-inflammatory activities [13–16]. CoQ<sub>0</sub> has also been shown to inhibit and kill important human pathogens such as *Cronobacter sakazakii*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, and *Staphylococcus aureus*. However, there is little information on the effects of CoQ<sub>0</sub> on *Salmonella* or its ability to remove biofilms [17–19].



**Figure 1.** The chemical structure of CoQ<sub>0</sub>.

In this study, the effects of CoQ<sub>0</sub> on *S. Typhimurium* were investigated to discern the mechanism of CoQ<sub>0</sub> inhibition/killing of planktonic bacteria, and to assess its ability to eliminate *S. Typhimurium* biofilms. Based on the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CoQ<sub>0</sub> against *S. Typhimurium*, its effects on bacterial survival, intracellular ATP concentration, cell membrane potential, intracellular compound leakage, cell membrane permeability, and cell morphology were investigated. We also assessed the ability of CoQ<sub>0</sub> to eliminate *S. Typhimurium* biofilms.

## 2. Material and Methods

### 2.1. Reagents

Coenzyme Q<sub>0</sub> (HPLC  $\geq$  99%, CAS 605-94-7) was purchased from J&K Scientific Co. (Beijing, China) and was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in all sample solutions was 1% (*v/v*), which has no apparent effect on the growth of *S. Typhimurium*. Luria–Bertani (LB) and buffered peptone water (BPW) were purchased from Land Bridge Technology Co. (Beijing, China). All other chemicals and reagents were of analytical grade.

### 2.2. Bacterial Strains and Growth Conditions

*Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, and *S. Typhimurium* AS1.1174 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Five other *S. Typhimurium* strains (Table 1) were isolated from raw chicken and beef by our experimental team. All seven *S. Typhimurium* strains

were used in MIC and MBC assays, while *S. Typhimurium* ATCC 14028 was used in all subsequent experiments.

**Table 1.** The MICs and MBCs of CoQ<sub>0</sub> against *S. Typhimurium*.

Strain	Serovar	MIC (mg/mL)	MIC (μM)	MBC (mg/mL)	MBC (μM)
ATCC 14028	Typhimurium	0.2	1098	0.2	1098
AS1.1174	Typhimurium	0.2	1098	0.2	1098
22	Typhimurium	0.2	1098	0.2	1098
63	Typhimurium	0.1	549	0.1	549
64	Typhimurium	0.2	1098	0.2	1098
80	Typhimurium	0.2	1098	0.2	1098
98	Typhimurium	0.2	1098	0.2	1098

*S. Typhimurium* strains were stored at  $-80\text{ }^{\circ}\text{C}$  before being cultured at  $37\text{ }^{\circ}\text{C}$  for 18 h on LB agar. To obtain fresh overnight cultures, stored isolates were inoculated into LB broth medium in 50-mL conical flasks and incubated at  $37\text{ }^{\circ}\text{C}$  for 12 h with shaking at 130 rpm. The cultures were then centrifuged ( $8000\times g$ , 5 min,  $4\text{ }^{\circ}\text{C}$ ), washed twice with sterile phosphate-buffered saline (PBS; pH 7.2), and resuspended in LB broth to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.5, which was equivalent to a cell concentration of approximately  $10^9$  colony-forming units (CFU/mL).

### 2.3. Determination of MIC and MBC Values

The MIC of CoQ<sub>0</sub> against the different *S. Typhimurium* strains was determined using the broth microdilution method described by Guo et al. [17], with some modifications. Briefly, bacteria were diluted to a concentration of  $5 \times 10^5$  CFU/mL in LB broth. Equivalent volumes (100 μL) of prepared bacterial suspension and CoQ<sub>0</sub> solution were added to the wells of 96-well microtiter plates to achieve final CoQ<sub>0</sub> concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, and 0 (control) mg/mL. LB broth containing 1% (v/v) DMSO was used as the negative control. The  $\text{OD}_{600}$  of each well was measured using a microtiter plate reader (Model 680; Bio-Rad, Saint Louis, MO, USA), and then plates were incubated at  $37\text{ }^{\circ}\text{C}$  for 24 h.  $\text{OD}_{600}$  measurements were repeated at the end of the incubation period. The MIC of CoQ<sub>0</sub> was defined as the lowest concentration that resulted in a change in  $\text{OD}_{600}$  (final  $\text{OD}_{600}$  value minus initial  $\text{OD}_{600}$  value) of  $\leq 0.05$ . For MBC determinations, 100 μL of bacterial suspension from each well was plated onto an LB agar plate. Following incubation for 48 h at  $37\text{ }^{\circ}\text{C}$ , the MBC was defined as the lowest antimicrobial concentration that did not allow any bacterial growth.

### 2.4. Inactivation of *S. Typhimurium* by CoQ<sub>0</sub> in LB

The bactericidal activity of CoQ<sub>0</sub> against *S. Typhimurium* ATCC 14028 was tested using the plate count method described by Kang and Song [20], with slight modification. *S. Typhimurium* was first diluted to a concentration of  $1 \times 10^8$  CFU/mL. Equal volumes (10 mL) of LB broth and CoQ<sub>0</sub> were mixed in 15-mL test tubes to achieve final CoQ<sub>0</sub> concentrations of 0, 0.1 (1/2MIC), 0.2 (MIC), and 0.4 mg/mL (2MIC). A 100-μL aliquot of *S. Typhimurium* suspension was then added to each test tube and cultured at  $37\text{ }^{\circ}\text{C}$ . Samples were collected at 0, 1, 2, 4, 6, 9, and 12 h post-inoculation, serially diluted in PBS (pH 7.2), plated on LB agar, and incubated at  $37\text{ }^{\circ}\text{C}$  for 18 h. Following incubation, the numbers of *S. Typhimurium* colonies were counted.

### 2.5. Antimicrobial Effect of CoQ<sub>0</sub> on Inoculated *S. Typhimurium* on Raw Chicken

The experimental method of Roh et al. [21] was used to test the antibacterial effect of CoQ<sub>0</sub> on *S. Typhimurium* on raw chicken, with slight modification. Boneless, skinless chicken breasts were purchased by online shopping (Jindong, China). The chicken breasts were first cut into uniform pieces ( $1.5 \times 1.5 \times 1$  cm) and placed in a sterile sealed bag for

irradiation. After 2 s of 4 kGy irradiation, the background bacteria on raw chicken were completely removed, then raw chicken was stored in the refrigerator at  $-20\text{ }^{\circ}\text{C}$  until use. *S. Typhimurium* was diluted to a concentration of  $1 \times 10^9$  CFU/mL, then the bacteria solution was inoculated on the surface of chicken breast meat (20  $\mu\text{L}$  per piece), and dried for 30 min. BPW broth (10 mL), DMSO (1%), and CoQ<sub>0</sub> were mixed in aseptic sealed bags to achieve final CoQ<sub>0</sub> concentrations of 0 (Control), 1 (5MIC), 2 (10MIC), and 4 mg/mL (20MIC). The chicken samples were added to each sterile sealed bag and cultured for 30 min, and then the chicken samples were taken out and stored in the refrigerator at  $12\text{ }^{\circ}\text{C}$ . At 0, 3, and 6 h, the chicken samples were taken out and shaken in a homogenizer in 0.1% BPW for 3 min, collection fluid (1 mL) serially diluted in PBS (pH 7.2), plated on LB agar, and incubated at  $37\text{ }^{\circ}\text{C}$  for 12 h. Following incubation, the numbers of *S. Typhimurium* colonies were counted.

## 2.6. Determination of the Antibacterial Mechanism of CoQ<sub>0</sub> against Planktonic *S. Typhimurium* Cells

### 2.6.1. Membrane Potential

The membrane potential of *S. Typhimurium* ATCC 14028 cells following CoQ<sub>0</sub> treatment was determined as described by Shi et al. [22]. Briefly, *S. Typhimurium* diluted in PBS ( $\text{OD}_{600} = 0.5$ ) was seeded into black 96-well microtiter plates, and the same volume of PBS was also seeded into black 96-well microtiter plates as background. Following incubation, 1  $\mu\text{M}$  bis-(1,3-dibutyl barbituric acid) trimethine oxonol (DiBAC<sub>4</sub>(3); Molecular Probes, Eugene, OR, USA) was used as a membrane potential-sensitive fluorescent probe and added to the *S. Typhimurium* suspensions and PBS for 30 min at  $37\text{ }^{\circ}\text{C}$  in the dark. CoQ<sub>0</sub> was then added to each well to achieve final concentrations equivalent to 0 (control), MIC, and 2MIC.

Following a further incubation for 5 min on the bench, fluorescence was measured using a fluorescence microplate reader (Spectra Max M2; Molecular Devices, San Jose, CA, USA) at excitation and emission wavelengths of 492 and 515 nm, respectively. Membrane potential was illustrated in terms of relative fluorescence units (RFUs;  $\text{RFU} = (\text{FU}_{\text{treatment}} - \text{FU}_{\text{background}}) - (\text{FU}_{\text{control}} - \text{FU}_{\text{background}})$ ), with three independent replicates included in each experiment.

### 2.6.2. Intracellular ATP

Intracellular ATP concentrations were determined as described by Fei et al. [23], with some modification. Briefly, *S. Typhimurium* ATCC 14028 resuspended in PBS to  $\sim 10^9$  CFU/mL was mixed with CoQ<sub>0</sub> at concentrations equivalent to 0 (control), MIC, and 2MIC, and incubated for 30 min in  $37\text{ }^{\circ}\text{C}$ . Following incubation, all samples were ultrasonicated on ice. Following ultrasonication, *S. Typhimurium* suspensions were heated at  $100\text{ }^{\circ}\text{C}$  for 180 s and then centrifuged for 5 min ( $5000 \times g$ ,  $4\text{ }^{\circ}\text{C}$ ). The resulting supernatants were collected and stored on ice for measurement of intracellular ATP concentration.

ATP concentrations were measured using an ATP assay kit as per the manufacturer's instructions (Beyotime Bioengineering Institute, Shanghai, China). Briefly, 100  $\mu\text{L}$  aliquots of the extracted ATP-containing supernatants and the ATP standard solutions were added to white 96-well microtiter plates. An equal volume of ATP detection reagent was then added to each well. The luminescence of each well was determined using a multimode microplate reader (Spectra Max M2; Molecular Devices). The intracellular ATP concentrations of the samples were calculated from a standard curve generated from the luminescence values of the ATP standard solutions. Three independent replicates of each experiment were performed.

### 2.6.3. Release of Cellular Constituents

The release of cellular constituents from *S. Typhimurium* ATCC 14028 was measured as described by Li et al. [24]. *S. Typhimurium* resuspended in PBS to an  $\text{OD}_{600}$  of 0.5 (30 mL) was added to three 50-mL centrifuge tubes, and 30-mL PBS was added to the other

three 50-mL centrifuge tubes as background. CoQ<sub>0</sub> was added to each tube to achieve final concentrations equivalent to 0 (control), MIC, and 2MIC. Following vortex oscillation, the samples were incubated at 37 °C for 240 min. Each sample was then centrifuged (5000× *g*, 4 °C, 15 min) and filtered through a 0.22-μm filter membrane. The absorbance measured at 260 nm using a microplate reader (Spectra Max M2; Molecular Devices) indicated the concentration of the constituents released from the cells (Absorbance = Abs<sub>treatment</sub> − Abs<sub>background</sub>).

#### 2.6.4. Membrane Integrity

The effect of CoQ<sub>0</sub> on the integrity of *S. Typhimurium* cell membranes was assessed using two nucleic acid dyes, SYTO 9 (green) and propidium iodide (PI, red), as described previously [25], with minor modification. Briefly, *S. Typhimurium* ATCC 14028 cells were washed three times in 0.85% (*w/v*) NaCl solution before being resuspended in 0.85% (*w/v*) NaCl solution to a concentration of ~1 × 10<sup>9</sup> CFU/mL. CoQ<sub>0</sub> was added to the bacterial cell suspensions at concentrations equivalent to 0, MIC, or 2MIC and incubated at 37 °C for 30 min. Following incubation, the suspensions were centrifuged at 10,000× *g* for 2 min and the resulting bacterial pellets resuspended in 1 mL of 0.85% (*w/v*) NaCl solution. The resuspended cells were then mixed with 3 μL of fluorescent marker solution, consisting of 1.5 μL of SYTO 9 and 1.5 μL of PI, and incubated at room temperature for 5 min in the dark. Finally, one drop of each sample was placed onto a glass slide, covered with a coverslip, and observed by confocal laser scanning microscopy (CLSM; A1; Nikon, Tokyo, Japan) at 800× magnification.

#### 2.6.5. Bacterial Morphology

The morphology of *S. Typhimurium* ATCC 14028 was observed using field emission scanning electron microscopy (FESEM), as described by Shi et al. [22]. Briefly, *S. Typhimurium* ATCC 14028 cells (~1 × 10<sup>9</sup> CFU/mL) were treated with CoQ<sub>0</sub> at concentrations equivalent to 0, MIC, 2MIC, and 4MIC and incubated at 37 °C for 2 or 4 h. Cells were then harvested by centrifugation (5000× *g*, 5 min, 4 °C), rinsed twice with PBS, and resuspended in 2.5% (*v/v*) glutaraldehyde at 4 °C for initial fixation. The cells were then washed with PBS and sterile water before being dehydrated gradually in a graded water-ethanol series (30, 50, 70, 80, 90, and 100% ethanol) for 10 min. Finally, the samples were dried, gold sputtered, and observed using a field-emission scanning electron microscope (S-4800; Hitachi, Tokyo, Japan) at 20,000× magnification. In the 20,000× magnification field of view, areas with less background impurities and low-density cells were searched, and then *S. Typhimurium* ATCC 14028 cells with typical characteristics under the treatment of the corresponding CoQ<sub>0</sub> concentration were identified and selected to make SEM images.

#### 2.7. CoQ<sub>0</sub> Inactivation of *S. Typhimurium* Biofilms on Stainless Steel Surfaces

The ability of CoQ<sub>0</sub> to inactivate biofilm-associated *S. Typhimurium* ATCC 14028 on stainless steel surfaces was examined as described by Zheng et al. [26], with slight modification. *S. Typhimurium* ATCC 14028 suspension was centrifuged (4000× *g*, 15 min, 4 °C) and resuspended in PBS to achieve a concentration of 7.0 log CFU/mL. To encourage biofilm formation, 30-mL aliquots of *S. Typhimurium* ATCC 14028 were added to separate sterile 50-mL test tubes containing a sterile stainless steel sheet (5 cm × 2 cm) and incubated at 25 °C for 72 h.

Following biofilm formation, the sheets were removed from the test tubes using sterile forceps, soaked in 400 mL of sterile distilled water at 25 °C for 15 s, and then rinsed in 400 mL of sterile distilled water with gentle agitation for 5 s to remove weakly adherent cells. The stainless steel sheets were then transferred to sterile tubes containing 30 mL of PBS with/without CoQ<sub>0</sub> (0, 5MIC, 8MIC, or 10MIC) and incubated at 25 °C for 0, 30, 60, 90, 120, or 180 min. At each sampling point, stainless steel sheets were rinsed in 400 mL of sterile distilled water with agitation for 15 s and then in 400 mL of sterile distilled water for 5 s, before being placed in sterile 50-mL centrifuge tubes containing 3 g of glass beads

(G8772, 425–600  $\mu\text{m}$ ; Sigma-Aldrich, St. Louis, MO, USA) and 30 mL of sterile PBS, and then vortexed for 5 min. Finally, the bacterial suspensions were plated onto LB plates and incubated at 37 °C for 24 h.

### 2.8. Statistical Analysis

The whole experiment of each assay was repeated three times independently. SPSS statistical analysis software v. 19.0 (SPSS Inc., Chicago, IL, USA) was used to process the data, and the results were expressed as mean  $\pm$  SD. Differences were analyzed by one-way analysis of variance, and significance was determined using Tukey's test and least significant difference analysis. Significance was indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

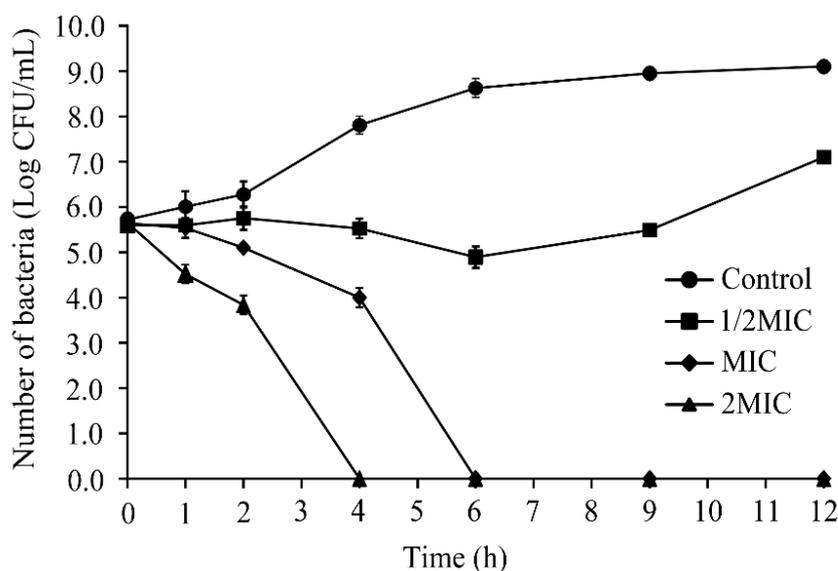
## 3. Results

### 3.1. MICs and MBCs

As shown in Table 1, the MIC of CoQ<sub>0</sub> against *S. Typhimurium* ATCC 14028, *S. Typhimurium* AS1.1174, and food isolates 22, 64, 80, and 98 was 0.2 mg/mL (1098  $\mu\text{M}$ ), and the MIC of CoQ<sub>0</sub> against food isolate 63 was 0.1 mg/mL (549  $\mu\text{M}$ ). The MBC values of all tested strains in this study were identical to the MIC values. *S. Typhimurium* ATCC 14028 was selected as a model organism for use in all subsequent experiments.

### 3.2. Inactivation of *S. Typhimurium* by CoQ<sub>0</sub> in LB

Based on the results of MIC assays, various concentrations of CoQ<sub>0</sub> were used to investigate its inhibitory effects on *S. Typhimurium* ATCC 14028. As shown in Figure 2, total viable cell counts were initially the same across all treatment groups (~5.8 log CFU/mL). However, cell counts of *S. Typhimurium* ATCC 14028 exposed to CoQ<sub>0</sub> at 0 and 1/2MIC were 9.1 and 6.9 log CFU/mL at 12 h post-inoculation, respectively, while those of cultures exposed to CoQ<sub>0</sub> at MIC and 2MIC were below detectable levels at 6 and 4 h post-treatment.

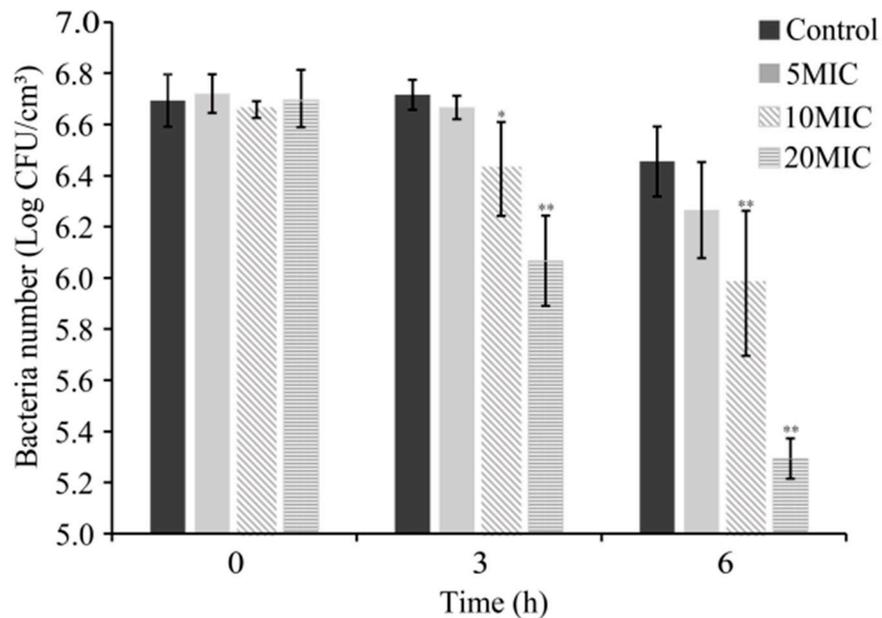


**Figure 2.** Inactivation of *S. Typhimurium* by CoQ<sub>0</sub> in LB. (■) Control, (●) 1/2MIC, (▲) MIC, (◆) 2MIC. Control represents no CoQ<sub>0</sub> added.

### 3.3. Antimicrobial Activity of CoQ<sub>0</sub> on Raw Chicken

The antimicrobial activity of CoQ<sub>0</sub> on *S. Typhimurium* on raw chicken is shown in Figure 3. The initial number of viable *S. Typhimurium* cells on raw chicken was similar among all treatment groups (~6.7 log CFU/cm<sup>3</sup>). The bacterial count of the control group declined from 6.7  $\pm$  0.1 to 6.5  $\pm$  0.1 log CFU/cm<sup>3</sup> within 6 h, while the bacterial count of 5MIC and 10MIC groups decreased by 0.4 and 0.7 log CFU/cm<sup>3</sup>, respectively.

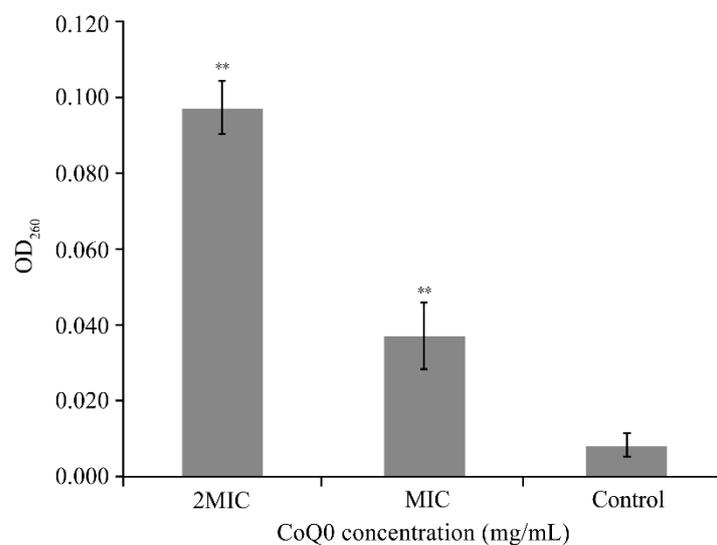
Simultaneously, the bacterial count of the 20MIC group decreased significantly ( $p < 0.01$ ) to  $5.3 \pm 0.1$  log CFU/cm<sup>3</sup> within 6 h.



**Figure 3.** Antimicrobial effect of CoQ<sub>0</sub> on inoculated *S. Typhimurium* on raw chicken. Bars represent the standard deviation ( $n = 3$ ). Control represents no CoQ<sub>0</sub> added. \*  $p < 0.5$  compared with the control, \*\*  $p < 0.01$  compared with the control.

### 3.4. Release of Cell Constituents

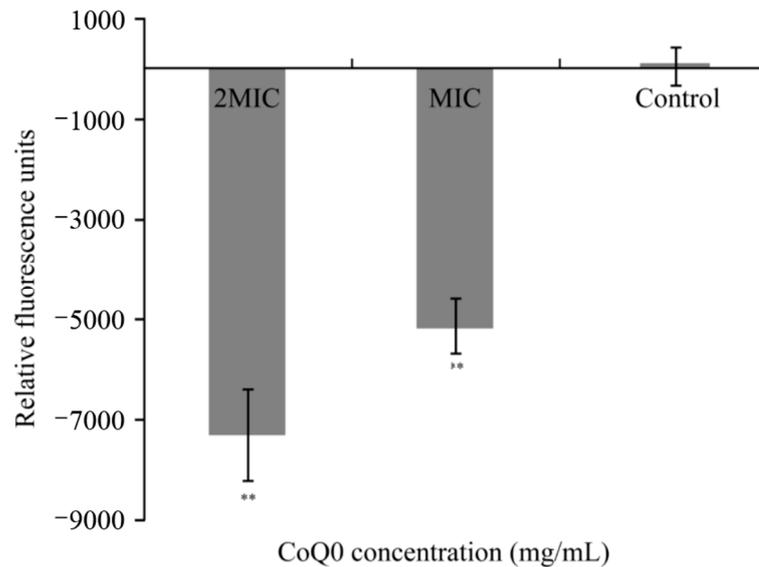
Changes in optical density, indicative of the release of intracellular constituents from *S. Typhimurium* ATCC 14028 cells following treatment with CoQ<sub>0</sub> for 4 h, are shown in Figure 4. There was a significant increase ( $p < 0.01$ ) in the release of cell constituents following treatment with CoQ<sub>0</sub> at concentrations equivalent to the MIC ( $OD_{260} = 0.04 \pm 0.02$ ) and 2MIC ( $OD_{260} = 0.1 \pm 0.02$ ) compared with the control ( $OD_{260} = 0.008 \pm 0.003$ ).



**Figure 4.** Effect of CoQ<sub>0</sub> treatment on the release of constituents from *S. Typhimurium* ATCC 14028 cells. Values represent the means of triplicate measurements. Bars represent the standard deviation ( $n = 3$ ). Control represents no CoQ<sub>0</sub> added. \*\*  $p < 0.01$  compared with the control.

### 3.5. Membrane Potential

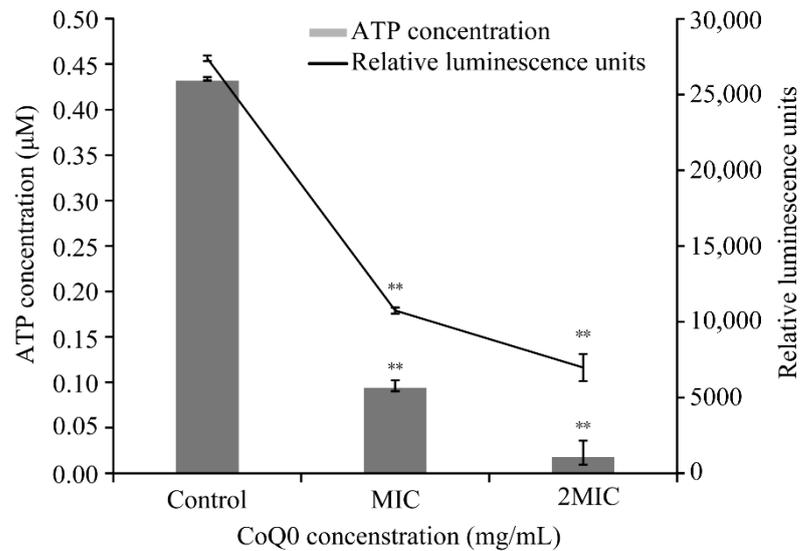
The effects of CoQ<sub>0</sub> on the membrane potential of *S. Typhimurium* ATCC 14028 are shown in Figure 5. Hyperpolarization (indicated by negative relative fluorescence values) was observed in *S. Typhimurium* ATCC 14028 samples treated with CoQ<sub>0</sub> at concentrations equivalent to the MIC and 2MIC, with the degree of hyperpolarization increasing with increases in CoQ<sub>0</sub> concentration.



**Figure 5.** Effects of CoQ<sub>0</sub> treatment on the membrane potential of *S. Typhimurium* ATCC 14028. Values represent the means of triplicate measurements. Bars represent the standard deviation ( $n = 3$ ). Control represents no CoQ<sub>0</sub> added. \*\*  $p < 0.01$  compared with the control.

### 3.6. Intracellular ATP

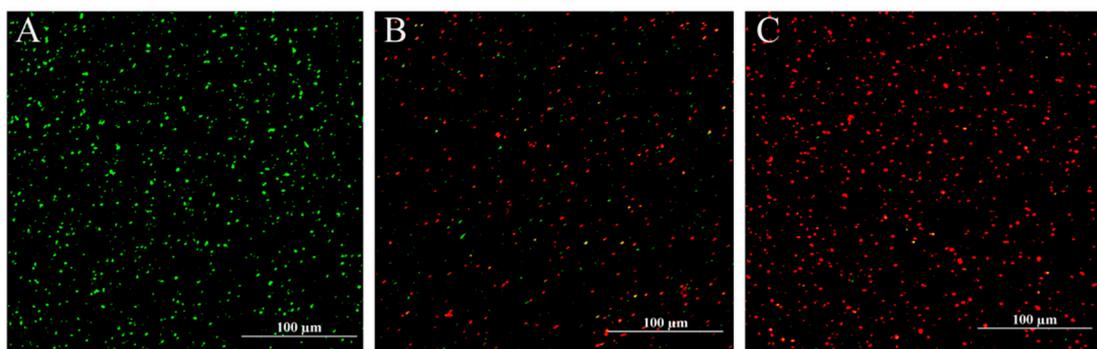
Relative luminescence units and ATP concentration were used to establish a standard curve ( $y = 9249x + 6094.7$ ,  $R^2 = 0.9998$ , the standard curve is not shown) to measure ATP concentration by fluorescence units. As shown in Figure 6, the *S. Typhimurium* ATCC 14028 intracellular ATP concentration was significantly decreased ( $p < 0.01$ ) following CoQ<sub>0</sub> treatment (MIC,  $0.094 \pm 0.01 \mu\text{mol/L}$ ; 2MIC,  $0.02 \pm 0.004 \mu\text{mol/L}$ ) for 30 min compared with the control ( $0.43 \pm 0.02 \mu\text{mol/L}$ ).



**Figure 6.** Effects of CoQ<sub>0</sub> treatment on intracellular ATP concentration of *S. Typhimurium* ATCC 14028. Values represent the means of triplicate measurements. Bars represent the standard deviation ( $n = 3$ ). Control represents no CoQ<sub>0</sub> added. \*\*  $p < 0.01$  compared with the control.

### 3.7. Membrane Integrity

SYTO 9, which is a green fluorescent nucleic acid stain that can label all bacteria with intact membranes and damaged membranes, and PI, which is a red fluorescent nucleic acid stain that only penetrates bacteria with damaged membranes, stain bacteria with intact cell membranes with green fluorescence, while bacteria with damaged membranes are stained with red fluorescence to evaluate the injuries of CoQ<sub>0</sub> to the cell membrane of *S. Typhimurium* ATCC 14028 in CLSM (Figure 7). As shown in Figure 7A, only green fluorescence was observed for control cell cultures. In comparison, increases in the proportion of red versus green fluorescence were observed in cultures treated with increasing concentrations of CoQ<sub>0</sub> (MIC or 2MIC) (Figure 7B,C).

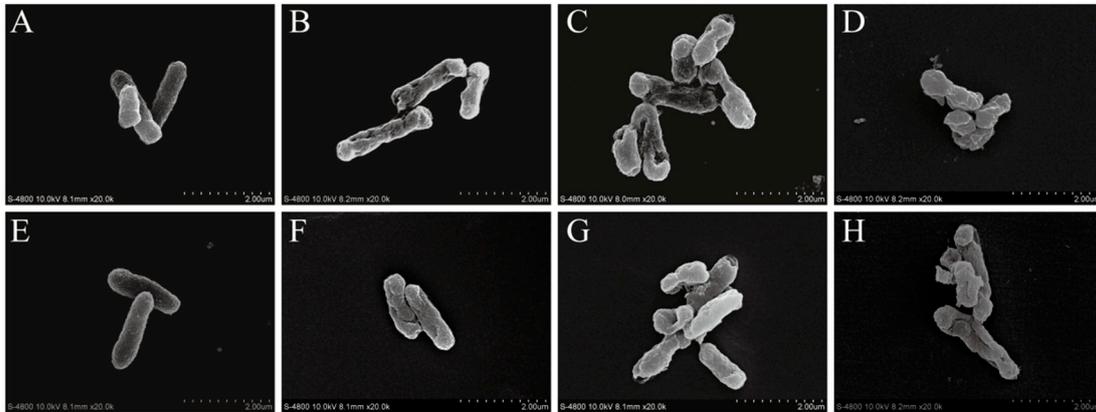


**Figure 7.** CLSM-based observation of changes in the membrane integrity of untreated *S. Typhimurium* ATCC 14028 cells (A) versus those treated with CoQ<sub>0</sub> at MIC (B) and 2MIC (C), as demonstrated by the proportions of green versus red fluorescence.

### 3.8. FESEM-Based Observation of Cell Morphology

SEM images of CoQ<sub>0</sub>-treated and untreated *S. Typhimurium* ATCC 14028 cells are shown in Figure 8. Untreated *S. Typhimurium* ATCC 14028 cells were rod-shaped with a smooth surface (Figure 8A,E), while *S. Typhimurium* cells treated with CoQ<sub>0</sub> at the MIC appeared wrinkled with a rough surface and notable depressions (Figure 8B,F). When cultures were treated with CoQ<sub>0</sub> at 2MIC, cell size was dramatically reduced and normal

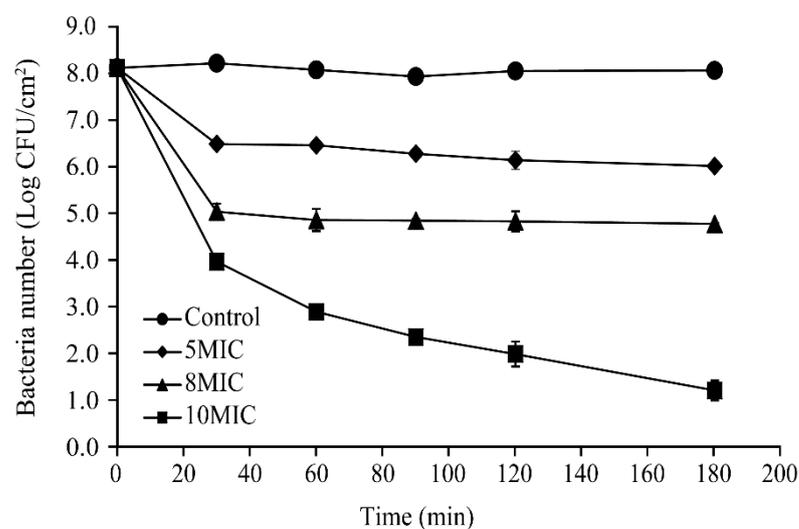
cell morphology was absent (Figure 8C,G). Treatment with CoQ<sub>0</sub> at 4MIC resulted in membrane rupture (Figure 8D,H).



**Figure 8.** FESEM-based observations (20,000 $\times$  magnification) of untreated *S. Typhimurium* ATCC 14028 at 2 (A) and 4 h (E), and of *S. Typhimurium* ATCC 14028 treated with 0.2 mg/mL CoQ<sub>0</sub> for 2 (B) and 4 h (F); treated with 0.4 mg/mL CoQ<sub>0</sub> for 2 (C) and 4 h (G); and treated with 0.8 mg/mL CoQ<sub>0</sub> for 2 (D) and 4 h (H).

### 3.9. Inactivation of *S. Typhimurium* Biofilms on Stainless Steel Surfaces by CoQ<sub>0</sub> Treatment

Changes in the number of viable biofilm-associated cells following treatment with CoQ<sub>0</sub> at 25 °C are shown in Figure 9. At the beginning of treatment, the total number of viable *S. Typhimurium* cells in biofilms on stainless steel surfaces was similar among all treatment groups (~8.1 log CFU/cm<sup>2</sup>). After 3 h, the cell population in untreated biofilms remained at about 8.2 log CFU/cm<sup>2</sup>. However, the number of viable cells in the 5MIC and 8MIC CoQ<sub>0</sub>-treated biofilms decreased significantly to ~6.5 and 5.1 log CFU/cm<sup>2</sup>, respectively, between the 0 and 30 min timepoints before stabilizing. In addition, the number of viable cells in biofilms exposed to 10MIC CoQ<sub>0</sub> decreased rapidly in the first 30 min, dropping by approximately 4.0 log CFU/cm<sup>2</sup> within this period, and then continued to decline between 30 and 180 min post-treatment.



**Figure 9.** Inactivation of biofilm-associated *S. Typhimurium* ATCC 14028 cells on stainless steel surfaces following CoQ<sub>0</sub> treatment. (●) Control, (◆) 5MIC, (▲) 8MIC, (■) 10MIC. Control represents no CoQ<sub>0</sub> added.

#### 4. Discussion

In this study, an MIC for CoQ<sub>0</sub> of 549–1098 µM was observed against *S. Typhimurium*. Previous studies have reported similar inhibitory effects of natural substances against *S. Typhimurium*. For example, Sun, Joy, and Huang reported MICs of carvacrol and thymol against *S. Typhimurium* in the range of 3329–6657 µM, while the MIC of citral against *S. Typhimurium* was  $\geq 13,138$  µM [27]. In addition, Lin, Liao, and Cui [28] determined an MIC of thyme essential oil against *S. Typhimurium* of 1664 µM, and Buket et al. [10] determined MICs of conventional preservatives, including sodium nitrite, methyl paraben, propyl paraben, potassium sorbate, and sodium benzoate, against *Salmonella* in the range of 110–3624 µM. Therefore, based on our current results, CoQ<sub>0</sub> appears to have stronger antimicrobial activity against *S. Typhimurium* compared with carvacrol, thymol, thyme essential oil, and citral; besides, the antimicrobial activity of CoQ<sub>0</sub> is comparable to conventional preservatives.

The inactivation experiment of CoQ<sub>0</sub> on *S. Typhimurium* in LB showed that CoQ<sub>0</sub> at concentrations equivalent to the MIC or higher involved significantly decreasing the viable numbers of *S. Typhimurium* cells in LB broth, and confirmed the bactericidal action was irreversible (Figure 1). In comparison, Kang et al. [29] showed that *Shigella flexneri* cells at an initial concentration of 6.4 log CFU/mL decreased to about 1.0 log CFU/mL within 6 h following treatment with gallic acid (GA) at MBC, while incubation with GA at the MIC only resulted in a 1.3-log decline in viable cell counts after 12 h. Prakash et al. [30] found that nanoemulsions of linalool at the MIC reduced *S. Typhimurium* cell counts to below the limits of detection within 2 h; however, after 8 h, the *S. Typhimurium* culture showed renewed growth, suggesting that the effects of linalool nanoemulsions at MIC are reversible. Therefore, in comparison with these other natural compounds, CoQ<sub>0</sub> shows good inhibitory and germicidal activity against *S. Typhimurium*. Further, the damage caused by CoQ<sub>0</sub> at concentrations greater than or equal to the MIC is irreversible in *S. Typhimurium*.

The inhibition experiment of CoQ<sub>0</sub> on *S. Typhimurium* on raw chicken showed that CoQ<sub>0</sub> (0.5%) significantly decreased ( $p < 0.01$ ) the viable numbers of *S. Typhimurium* on raw chicken (Figure 2). However, comparing the antibacterial effects of CoQ<sub>0</sub> in LB broth and on raw chicken (Figures 1 and 2), we found that the antibacterial effect weakened on raw chicken. We speculate that the difference is caused by the temperature change when CoQ<sub>0</sub> acts (in LB: 37 °C, on raw chicken: 12 °C); besides, the protein and texture of raw chicken reduce the effective contact concentration of *S. Typhimurium* and CoQ<sub>0</sub>. Jang and Rhee reported that the antibacterial effect of caprylic acid on *Cronobacter* spp. (*Enterobacter sakazakii*) is enhanced by the increase in temperature [31], and Guo et al. [18] showed that the antibacterial activity of CoQ<sub>0</sub> on *Cronobacter sakazakii* decreases as the temperature decreases from 55 to 25 °C. In addition, Jeyakumar et al. [32] found that microalgal fatty acid esters with 1/2MIC concentration can reduce *Listeria monocytogenes* in tryptic soy broth by about 12 log CFU/mL within 9 h, but *L. monocytogenes* on raw chicken only reduced by about 2 log CFU/mL within 9 h. Similarly, Zheng et al. [26] found that CoQ<sub>0</sub> with a concentration of 100MIC reduced the *Vibrio parahaemolyticus* in fresh shrimp from 6.3 to 3.0 log CFU/g within 6 h. Thereby, methods such as synergy with other antibacterial agents, combined with ultrasound, mild heating, or other treatment should be investigated to further enhance the antibacterial effect of CoQ<sub>0</sub> on *S. Typhimurium* in food.

Our study showed that CoQ<sub>0</sub> treatment resulted in a significant increase ( $p < 0.01$ ) in the amount of extracellular cell constituents in *S. Typhimurium* cultures. Similarly, Sun et al. [33] examined the effect of anthocyanins on the membrane permeability of *S. Enteritidis* and found that following anthocyanin treatment, cell constituent leakage from *S. Enteritidis* cells increased by 54.1%. Further, Campos et al. [34] showed that cell constituent leakage from *S. Enteritidis* and *S. Typhimurium* cells increased by 51.0 and 50.0%, respectively, following treatment with essential oil extracted from *Mitracarpus frigidus*. Therefore, we speculate that CoQ<sub>0</sub> causes changes in the permeability of the

*S. Typhimurium* cell membrane, resulting in intracellular cell constituent leakage and inhibiting growth.

The difference in potential between the inner and outer cell membrane is called the membrane potential and, in the resting state, membrane potential supports membrane integrity and nutrient transport [23]. In the current study, CoQ<sub>0</sub> was shown to significantly hyperpolarize the membrane of *S. Typhimurium*, as shown by the negative membrane potential. We have previously shown that citral can also hyperpolarize the membrane of *Cronobacter sakazakii* [22], while membrane hyperpolarization was implicated as the antimicrobial mechanism of CoQ<sub>0</sub> against *S. aureus* [19]. Similarly, polyphenols extracted from olive oil can cause cell membrane depolarization in *Cronobacter sakazakii* and *Bacillus cereus* [23,35]. Guo et al. [17] suggested that hyperpolarization or depolarization of cell membranes is caused by changes in K<sup>+</sup> concentration as a result of increased membrane permeability. Therefore, we speculate that CoQ<sub>0</sub> alters the permeability of the *S. Typhimurium* cell membrane, resulting in changes in cell membrane potential.

ATP is vitally important to many different physiological processes in bacteria [36]. Our results show a significant reduction ( $p < 0.01$ ) in intracellular ATP concentration in *S. Typhimurium* cells treated with CoQ<sub>0</sub>. Lin, Wang, and Cui found that thyme essential oil reduced the intracellular ATP concentration of *S. Typhimurium* by 52.6% [28], and later demonstrated that the intracellular ATP concentration of *Escherichia coli* O157:H7 was reduced by 49.5% following treatment with *Litsea cubeba* essential oil [37]. The decrease in intracellular ATP concentration of *S. Typhimurium* likely results from the rapid hydrolysis of ATP or changes in cell membrane permeability.

To verify the above hypothesis, we next explored the effects of CoQ<sub>0</sub> on *S. Typhimurium* membrane integrity. CLSM-based analyses showed that CoQ<sub>0</sub> treatment significantly reduced the integrity of *S. Typhimurium* cell membranes (Figure 6). Consistent with this observation, Fan et al. [19] and Guo et al. [17] used CLSM to show that CoQ<sub>0</sub> at concentrations ranging from the MIC to 4MIC caused obvious damage to the cell membranes of *S. aureus* and *Cronobacter sakazakii*. FESEM analysis also showed morphological alterations to the cell membrane following CoQ<sub>0</sub> treatment (Figure 7). Similarly, *Mitracarpus frigidus* and anthocyanins cause obvious morphological changes in the bacterial cell surface, resulting in shrinkage, corrugation, and other damage to the membranes of *S. enterica* and *S. enteritidis* cells [33,35]. Based on these observations, we propose that the morphological changes that occur in *S. Typhimurium* as a result of CoQ<sub>0</sub> treatment are caused by membrane damage and permeabilization, which ultimately result in the loss of cell contents.

*S. Typhimurium* biofilms attached to various surfaces are surrounded by a matrix mainly composed of polysaccharide substances. These biofilms can cause cross-infection during food production [9]. Our study determined that CoQ<sub>0</sub> at concentrations ranging from 5MIC to 10MIC caused a significant decline in the number of viable cells in *S. Typhimurium* biofilms on stainless steel surfaces. Thus, our results suggest that CoQ<sub>0</sub> has the potential to control *S. Typhimurium* biofilms in the food production environment. However, because *S. Typhimurium* cells contained within a biofilm are surrounded by the matrix, it is difficult to eliminate them completely. In recent studies, Kang et al. [29] reported that a high concentration of cinnamaldehyde (18,916 μM) can make *Vibrio parahaemolyticus* in shrimp decrease to the minimum level of detection; Mohan and Purohit [38] demonstrated that pyruvic and succinic acid, and oregano essential oil had obvious antibacterial effects on planktonic *S. Typhimurium*, while high concentrations (52,000–254,000 μM) of these plant-derived antimicrobials were required to kill biofilm-associated *S. Typhimurium*. In addition, we found that conventional disinfectants have a far greater activity on the inactivation of *Salmonella* biofilms than natural substances. Byun et al. [39] found that on stainless steel surfaces, 1343 μM sodium hypochlorite and 1483 μM chlorine dioxide, respectively, reduced the number of *Salmonella* cells in the biofilm by about 4 log CFU/cm<sup>2</sup> and dropped below the detection limit within 1 min. Therefore, under the premise of ensuring the good antimicrobial effect of CoQ<sub>0</sub>, new antibacterial technologies such as light-emitting diodes at

specific wavelengths, ultrasound, and cold plasma methods could be used in combination with plant-based antimicrobials to further reduce the concentration of CoQ<sub>0</sub> and effectively control food-borne pathogens and biofilms in future studies. In summary, CoQ<sub>0</sub> demonstrates good bacteriostatic and bactericidal activity against *S. Typhimurium* in LB broth and raw chicken, and effectively destroyed cell membrane integrity and induced morphological changes in the cell. CoQ<sub>0</sub> damages and permeabilizes the *S. Typhimurium* cell membrane, resulting in membrane hyperpolarization, decreased intracellular ATP concentration, and intracellular compound leakage. CoQ<sub>0</sub> also kills biofilm-associated *S. Typhimurium* cells. Therefore, CoQ<sub>0</sub> has significant potential for use as a new natural antibacterial agent for the control of *S. Typhimurium* in the food production chain, and the pharmacokinetics and toxicity of CoQ<sub>0</sub> will be explored before it is used as a bacteriostatic agent.

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