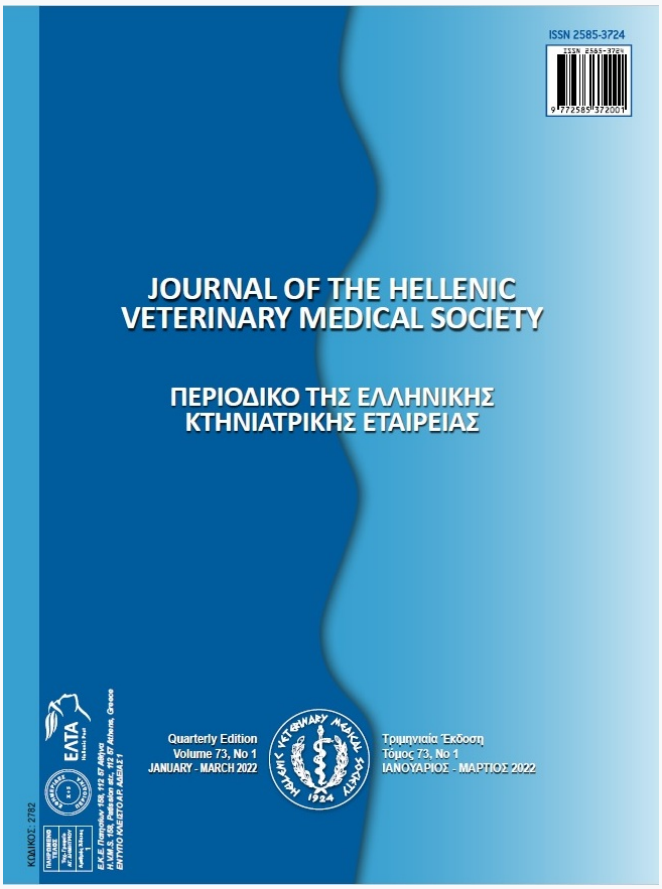
Περιοδικό της Ελληνικής Κτηνιατρικής Εταιρείας

Τόμ. 73, Αρ. 1 (2022)



Antibacterial activity of cinnamon oil against multidrug-resistant Salmonellaserotypes and anti-quorum sensing potential

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doi: 10.12681/jhvms.25514

Βιβλιογραφική αναφορά:

Kiymaci, M. E., Kaskatepe, B., & Soyer, Y. (2023). Antibacterial activity of cinnamon oil against multidrug-resistant Salmonellaserotypes and anti-quorum sensing potential. *Περιοδικό της Ελληνικής Κτηνιατρικής Εταιρείας*, *73*(1), 3707–3714. https://doi.org/10.12681/jhvms.25514 (Original work published 29 Απρίλιος 2022)

J HELLENIC VET MED SOC 2022, 73 (1): 3707-3714
ΠΕΚΕ 2022, 73 (1): 3707-3714

Research article
Ερευνητικό άρθρο

Antibacterial activity of cinnamon oil against multidrug-resistant *Salmonella* serotypes and anti-quorum sensing potential

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ABSTRACT: The antimicrobial resistance of *Salmonella* and other foodborne pathogenic bacteria, witnessed in recent years, has become a significant health concern. Bacteria use chemical signals to communicate each other and regulate their behavior including virulence. Due to increasing antibiotic resistance, new drug development strategies are being investigated and the use of active ingredients of various medicinal and aromatic plants as alternatives to antibiotics is tested. This study aimed to determine the anti-quorum sensing (QS) activity of cinnamon oil (CO) on *Chromobacterium violaceum* and to evaluate antimicrobial activity of CO against multidrug-resistant (MDR) *Salmonella enterica* serotypes. Anti-QS activity was tested using biosensor strain and antibacterial activity was determined by a microdilution method according to EUCAST standards. CO was found effective on QS system of *Chromobacterium violaceum*. Nineteen foodborne pathogens isolated from different poultry/cow sourced foods and serotyped as *Salmonella enterica* subsp. *Enterica* serotype Infantis (15), Kentucky (1), Newport (1), Telaviv (1), and Typhimurium (1). *Salmonella* Infantis strains were found resistant to three or more antibiotic classes (with resistance to at least one antibiotic from each class) and categorized as MDR. The results concluded that CO has strong antibacterial activity against all *Salmonella enterica* serotypes with MIC between 0.125 μg/ml and 1.0 μg/ml. This research demonstrates that CO is a potential candidate for developing new antimicrobial agents, antiseptic solutions or natural food preservatives against MDR *Salmonella* serotypes while is also a potential anti-QS agent.

***Keywords*:** antibacterial activity; anti-quorum sensing activity; cinnamon oil; multidrug-resistant; *Salmonella enterica*

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Date of initial submission: 09-12-2020
Date of revised submission: 09-01-2021
Date of acceptance: 12-02-2021

INTRODUCTION

Quorum sensing (QS) is a communication system, includes the secretion of extracellular chemical signals that regulates the gene expression of bacterial virulence like biofilm formation, movement, pigment and enzyme production, depending on bacterial population density (Papenfort and Bassler 2016). Bacterial infections have become an alarming health problem, especially due to the increasing number of multiple antimicrobial drug-resistant bacteria and raise concerns about success in therapy due to resistance. And QS is one of the most important barriers to overcome in the fight against bacteria, as a mechanism responsible for the generation of virulence factors that play an important role in the pathogenicity and resistance (Zhao et al. 2020). Due to the increased antibiotic resistance, researchers are in search of new drug development strategies and also looking for different solutions as alternative treatment methods like the usability of active ingredients of various medicinal and aromatic plants (essential oils, herbs, spices), bacteriophages, probiotic microorganisms and their metabolites alongside synthesizing new chemicals (Kiymaci et al. 2018; Waters and Smyth 2015). Aromatic and medicinal plants are natural sources of various industries like pharmaceutical, food, chemical, cosmetics, and cinnamon, a tropical spice, is one of the most investigated sources of these plants (Akthar 2014; Arumugam et al. 2016; Swamy 2012; Swamy and Sinniah 2015). Cinnamon (extracts, essential oils) has some primary and secondary metabolites that show antibacterial activity including compounds like cinnamic acid, trans-cinnamaldehyde, cinnamate, etc. against some different pathogens (Kaskatepe et al. 2016; Vasconcelos et al. 2018).

Diarrheal illnesses are the most common foodborne diseases that affect millions of people worldwide. A World Health Organization (WHO) report claims these to be the leading cause of death in children under five years of age. According to the WHO report 2018 (WHO 2018), *Salmonella* is one of the four key global causative agents of gastroenteritis. *Salmonella* infection or salmonellosis, caused by consumption of foods contaminated with non-typhoidal *Salmonella enterica* (NTS) serotypes, is one of the major foodborne diseases worldwide and considered an important public health problem. The infection is self-limiting in the majority of cases, with no requirement for antibiotics. However, antibiotic medication is recommended to control infection in severe cases. Quinolones possess a broad range of antimicrobial activity against enteric pathogens but are not recommended for use in children. Instead, third generation cephalosporins are prescribed for treating critical infections in children. However, recent studies have reported increased resistance to antibiotics, particularly those used to treat infections by multi-drug resistant (MDR) NTS strains. These strains are associated with high mortality and morbidity and are therefore considered a major public health concern (Medalla et al. 2017).

In light of the recent surge in the development of MDR strains of enteric pathogens, past years have seen the use of natural products, particularly essential oils (EOs) with potent antimicrobial activity, for treating these infections. For instance, cinnamon oil (CO) is one such product that is used as a spice, condiment, and flavoring agent. Moreover, it has been a component of traditional medicine to treat anaphrodisia, vaginitis, shortness of breath, eye inflammation, rheumatism, neuralgia, leucorrhea, wounds, and sore teeth. Additionally, it has antioxidant and hypoglycemic properties and has been used against various pathogenic microorganisms. There is no literature investigating the efficacy of CO against *Salmonella* Infantis, *Salmonella* Kentucky, and *Salmonella* Telaviv.

In the present study, our first aim was to investigate the anti-QS activity of CO on *Chromobacterium violaceum (C. violaceum).* In Gram-negatives, the QS mechanism occurs via acyl-homoserine lactone (AHL) signal molecules. While *C. violaceum* (wild type) is a strain capable of producing short-chain AHLs and purple colored violacein pigment; mutant *C. violaceum* CV026 is a biosensor strain that does not produce AHL and pigments but can produce violacein pigment by recognizing short-chain AHL molecules (AHL molecules with chain length varying from C4 to C8) (McClean et al. 1997). With the addition of N-hexanoyl-L-homoserine lactone signal molecule to the medium, the mutant CV026 strain produces violacein pigment due to the QS mechanism.

Additionally, our aim was to determine the antibacterial activity of CO against *Salmonella enterica* subsp. *enterica* serotypes including MDR Infantis, Kentucky, Newport, Telaviv, and Typhimurium, isolated as foodborne pathogens from different poultry-cow sourced foods. To the best of our knowledge this is the first study that examines the inhibitory activity of CO against MDR *Salmonella* Infantis isolates.

MATERIALS AND METHODS

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) analysis of cinnamon oil

CO was purchased from a herbalist (Ministry of Food, Agriculture and Livestock of Turkey registration number TR-34-K-000495) and in our previous study the composition of compounds was analyzed by GC and GC-MS on an Agilent 6890N Network GC system and GC/MS analysis was performed on Agilent 5973 Network Mass Selective Detector integrated with the GC system, using an HP Innowax Capillary column with dimensions 60.0 m × 0.25 mm × 0.25 mm and helium as carrier gas (1.2 ml/min). The oven temperature was set to 60° for 10 min after injection, then increased to 220° with 4°/min heating ramp for 10 min and increased to 240° with 1°/min heating ramp without hold. Both injector and detector (FID) temperatures were 250°; split ratio was adjusted to 50:1. Injection volume was 2.0 µl. MS conditions were as follows: ionization energy, 70 eV; ion source temperature, 280°; interface temperature, 250°; mass range, 34-450 atomic mass units and the cinnamaldehyde content of CO was determined to be equal to 99.54% (Kaskatepe et al. 2016).

Anti-quorum sensing activity of cinnamon oil

A modified method was used, the fresh culture of *C. violaceum* CV026 at 30°C for 18 hours was taken and adjusted to Mc Farland 0.5 density (10^8cfu/ml). A hundred microliters *C. violaceum* CV026, and 50 µl N-hexanoyl-L-homoserine lactone was added to 10 ml soft Luria Bertani agar (0.9%) medium and poured into Petri plates after vortexing. CO (15 µl) was dropped on agar plates and incubated 30°C for 48 hours. Tests were carried out in duplicate (Erdonmez et al. 2018).

Bacteria and their characteristics (serotypes and antibacterial phenotypic and genotypic resistance profiles)

S. enterica strains (n =56), isolated from chicken meat, and cow ground meat and offal, were obtained from the Department of Food Engineering of the Middle East Technical University (METU, TURKEY) and in their previous study molecular confirmation of these strains was conducted by determining *invA* gene (*F:5'-GAACCCTCAGTTTTTCAACGTTTC-3', R:5'-TAGCCGTAACAACCAATACAAATG-3'*) by PCR (Kim et al. 2007) and serotyping of strains was performed using the White-Kauffmann-Le Minor scheme (Grimont and Weil 2007) at the laboratory of Public Health Agency of Turkey in Ankara. Antimicrobial resistance of fifty-six *S. enterica* isolates have been phenotypically determined by agar disk diffusion (CLSI 2016) test using eighteen antibiotics, including amikacin (AK) 30 µg, ampicillin (AMP) 10 µg, amoxicillin-clavulanic acid (AMC) 20/10 µg, cefoxitin (FOX) 30 µg, cephalothin (KF) 30 µg, ceftiofur (EFT) 30 µg, ceftriaxone (CRO) 30 µg, chloramphenicol (C) 10 µg, ciprofloxacin (CIP) 5 µg, ertapenem (ETP) 10 µg, gentamicin (GN) 10 µg, imipenem (IPM) 10 µg, kanamycin (K) 30 µg, nalidixic acid (N) 30 µg, streptomycin (S) 10 µg, sulfisoxazole (SF) 10 µg, sulfamethoxazole-trimethoprim (SXT) 10 µg, and tetracycline (T) 30 µg discs and also the antimicrobial gene resistance profiles of these strains have been investigated by screening 21 antimicrobial resistance genes shown in Table 1, encoding resistance against Class A beta-lactamase, ceftiofur, ceftriaxone, beta-lactamases, chloramphenicol, streptomycin, gentamicin, kanamycin, trimethoprim, sulfisoxazole, and tetracycline (Acar et al. 2017, Durul et al. 2015). The isolates that show resistance to three or more antibiotic classes (with resistance to at least one antibiotic from each class) were considered to be MDR.

Table 1. The antimicrobial resistance genes that were screened in the isolates (Soyer et al. 2013)

Antimicrobial gene	Antimicrobial
*bla*TEM-1	Class A beta-lactamase
*bla*PS13E-1	Class A beta-lactamase
*bla*CMY-2	Ceftiofur, Ceftriaxone
ampC	Beta-lactamases
cat1	Chloramphenicol
cat2	Chloramphenicol
Flo	Chloramphenicol
cmlA	Chloramphenicol
aadA1	Streptomycin
aadA2	Streptomycin
strA	Streptomycin
strB	Streptomycin
aacC2	Gentamicin, Kanamycin
aphA1-Iab	Kanamycin
dhfrI	Trimethoprim
dhfrXII	Trimethoprim
sulI	Sulfisoxazole
sulII	Sulfisoxazole
tetA	Tetracycline
tetB	Tetracycline
tetG	Tetracycline

Antibacterial activity of cinnamon oil

The minimal inhibitory concentration (MIC) of

CO was determined using the broth microdilution test, based on the recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (EUCAST 2021). For this purpose, 100 µl cation adjusted Mueller-Hinton Broth (MHB) was added to 96-well micro-titer plates. Then, 100 µl CO was added into the first well and then was two-fold serially diluted. From a fresh culture of the bacterium, a suspension was prepared in phosphate-buffered saline; its turbidity was adjusted to an optical density equivalent to McFarland 0.5 using a nephelometer. The suspension was diluted to a ratio of 1:100 in MHB to obtain a final density of 5×10^5 cfu/ml. Finally, 100 µl of this bacterial suspension was added to each well. Micro-titer plates were incubated at 37 °C for 16 to 20 h. After incubation period, the MIC value was determined as the lowest CO concentration that inhibited bacterial growth. All tests were performed in duplicate.

RESULTS

In this study, anti-QS activity of the CO was evaluated as an inhibition of violacein pigment although signal molecule and CV026 strain together in the environment. As a result, it was determined that CO has inhibitory activity on N-hexanoyl-L-homoserine lactone molecule, in other words decreasing production of violacein pigment depending on the quorum sensing bacterial communication system.

For the antibacterial activity part of the study, nineteen of fifty-six *Salmonella enterica* isolates from chicken meat, and cow ground meat and offal, representing serotypes Infantis (15), Kentucky (1), Newport (1), Telaviv (1), and Typhimurium (1), were determined as resistant or MDR. All Infantis isolates were obtained from chicken meat. All foodborne *Salmonella* isolates in our study were resistant to at least one antibiotic. *Salmonella* Infantis strains were found resistant to three or more antibiotic classes (with resistance to at least one antibiotic from each class) and categorized as MDR. All *Salmonella* Infantis isolates (15) were resistant to N, and eight of these were resistant to K, S, SF, T, and N antibiotics. The results of the antibiotic resistance of these isolates are shown in Table 2.

Cinnamaldehyde (99.54%) was determined as the major component of the tested CO by GC and GC-MS. The antibacterial activity of CO was studied using the broth microdilution test, and the results were expressed as MIC value, as shown in Table 3. The lowest MIC value was determined as 0.125 (µg/ml) against some *Salmonella* Infantis and Kentucky serotypes.

Table 2. Antibiotic resistance of *Salmonella* isolates (Acar et al., 2017)

*METU ID	Isolates	The list of resistant antibiotics	The number of resistant antibiotics
MET S1-056	*S. enterica* Infantis	K, S, T, AMP, KF, SF, SXT, C, N	9
MET S1-050	*S. enterica* Infantis	K, S, T, AMP, SF, N	6
MET S1-674	*S. enterica* Infantis	K, S, T, SF, N	5
MET S1-669	*S. enterica* Infantis	S, AMP, KF, N	4
MET S1-788	*S. enterica* Infantis	SF, SXT, C, S, CIP, N, T	7
MET S1-785	*S. enterica* Infantis	SF, SXT, C, S, N, T	6
MET S1-750	*S. enterica* Infantis	SF, SXT, K, N, T	5
MET S1-782	*S. enterica* Infantis	SF, SXT, K, S, N, T	6
MET S1-759	*S. enterica* Infantis	SF, SXT, N, T	4
MET S1-777	*S. enterica* Infantis	SF, SXT, S, CIP, N, T	6
MET S1-792	*S. enterica* Infantis	SF, SXT, S, N, T	5
MET S1-668	*S. enterica* Infantis	S, SF, N	3
MET S1-492	*S. enterica* Infantis	S, T, N	3
MET S1-606	*S. enterica* Infantis	S, T, SF, N	4
MET S1-673	*S. enterica* Infantis	S, T, SF, N	4
MET S1-313	*S. enterica* Kentucky	T, N	2
MET S1-670	*S. enterica* Newport	SF	1
MET S1-063	*S. enterica* Telaviv	N	1
MET S1-625	*S. enterica* Typhimurium	S	1

*Middle East Technical University, Food Engineering Department Laboratory ID
AMP: Ampicillin, KF: Cephalothin, C: Chloramphenicol, CIP: Ciprofloxacin, N: Nalidixic acid, S: Streptomycin, SF: Sulfisoxazole, SXT: Sulfamethoxazole-trimethoprim, T: Tetracycline.

Table 3. Antibacterial activity of cinnamon oil

*METU ID	Isolates	MIC (µg/ml)
MET S1-056	*S. enterica* Infantis	0.125
MET S1-050	*S. enterica* Infantis	0.125
MET S1-674	*S. enterica* Infantis	0.50
MET S1-669	*S. enterica* Infantis	0.50
MET S1-788	*S. enterica* Infantis	1
MET S1-785	*S. enterica* Infantis	0.25
MET S1-750	*S. enterica* Infantis	0.25
MET S1-782	*S. enterica* Infantis	0.25
MET S1-759	*S. enterica* Infantis	0.50
MET S1-777	*S. enterica* Infantis	0.50
MET S1-792	*S. enterica* Infantis	1
MET S1-668	*S. enterica* Infantis	0.125
MET S1-492	*S. enterica* Infantis	0.125
MET S1-606	*S. enterica* Infantis	0.125
MET S1-673	*S. enterica* Infantis	0.50
MET S1-313	*S. enterica* Kentucky	0.125
MET S1-670	*S. enterica* Newport	0.50
MET S1-063	*S. enterica* Telaviv	0.50
MET S1-625	*S. enterica* Typhimurium	0.25

*Middle East Technical University, Food Engineering Department Laboratory ID

DISCUSSION

In the studies, it was reported that if the bacterial communication system is disrupted for any reason, the bacteria are not able to act in a coordinated manner and cannot create a successful infection process (Rasko and Sperandio 2010). Today, the methods preferred to combat bacterial infections are classical methods such as killing bacteria or stopping reproduction. However, the continuous use of these methods (such as protein synthesis inhibition, prevention of DNA replication, and effect on cell wall synthesis) results in the formation of resistant bacterial populations and ineffective treatment methods as well as economic losses (Aminov 2010). In the present study, we aimed to examine the inhibitory effect of CO against bacterial communication mechanism as a violacein inhibition like an initial level alternative treatment strategy for the attenuation of bacteria and their virulence and found CO effective.There are a limited number of studies on this subject. Similar to our study, Domínguez-Borbor et al. (2020) found that CO has QS inhibitory activity as anti-virulence therapy for vibriosis control, Alibi et al. (2020) specified the anti QS activity of *Cinnamomum* essential oil. Kavyani et al. (2019) underlined that cinnamon has inhibitory activity on the QS gene expression against *Pseudomonas aeruginosa* PAO1. Kalia et al. (2015) found that CO caused a decrease in biofilm-related DNA content and exopolysaccharide production of *Pseudomonas aeruginosa* in addition to inhibiting QS.

Salmonella genus is one of the primary causative agents of human and animal foodborne illnesses. Apart from being a public health concern, it adds to the economic costs in both developing and developed countries (Majowicz et al. 2010). *Salmonella* spp. commonly reside in poultry, beef, eggs, and some other fruits and vegetables. The symptoms of salmonellosis include diarrhea, fever, and abdominal cramps that develop between 12 and 72 h after infection in most people. Diarrhea is regarded as a major symptomatic factor as it can turn into a complication in infants, older people, and individuals with a compromised immune system. According to the CDC data, active food-related *Salmonella* outbreaks observed in 2018 were associated with *Salmonella* Enteritidis, Infantis, and Newport. The CDC report 2018 considered *Salmonella* serovars, particularly Typhimurium, to be the most frequently isolated agents from foods.

Recent years have witnessed a surge in both the number and variety of foodborne diseases. In particular, the increasing severity of salmonellosis, manifested as septicemia, is attributed to the development of MDR *Salmonella* strains that mainly affect children than adults (Ranjbar et al. 2012). The increasing use and misuse of antibiotics in human and veterinary

medicine has introduced antimicrobial resistance among *Salmonella* isolates in recent years (Foley and Lynne 2008). Its spread to the blood-stream and other body sites in hospitalized patients may turn fatal unless treated with antibiotics. Therefore, MDR *Salmonella* serotypes are regarded as an important health concern while treating *Salmonella* infections (CDC 2018).

To overcome the problem of multidrug resistance, recent studies have adopted treatment approaches based on antibiotic-free procedures to fight bacterial infections, for example, the use of EOs with antimicrobial properties. These natural products have widespread applications in the food and have been tested against different microorganisms as inhibitory agents in the form of food preservatives (Anwar et al. 2009; Burt 2004). Spices and their EOs, such as cinnamon, in addition to their use as a flavoring agent, have been the components of traditional medicine. These have been used for treating anaphrodisia, vaginitis, shortness of breath, eye inflammation, leucorrhea, rheumatism, neuralgia, wounds, and sore tooth. Moreover, some studies have reported cinnamon to possess hypoglycemic properties that are beneficial in lowering cholesterol levels (Bandara et al. 2012). It also has wound healing properties and acts as an anti-inflammatory compound (Gunawardena et al. 2014; Haddi et al. 2017; Tung et al. 2008). The major component of cinnamon EO that is responsible for its antimicrobial activity is cinnamaldehyde. This is compatible with our results that indicate high cinnamaldehyde content.

However, to our knowledge, no data related to anti-inhibitory effects of CO on MDR *Salmonella* serotypes exist. Therefore, the present study evaluated the inhibitory activity of CO against MDR *Salmonella* Infantis and some other serotypes (Kentucky, Newport, Typhimurium, and Telaviv). We believe this study to be the first of its kind, and therefore the results cannot be compared with other studies. *Salmonella* Infantis is known as the most frequent host-unspecific serovar to be associated with infections and outbreaks. It is also the fourth most common cause of human salmonellosis in Europe, with 1, 846 cases reported by the EU/EEA countries in 2014 (European Food Safety Authority [EFSA] and European Centre for Disease Prevention) (Almeida et al. 2013; ECDC 2016; McEwen and Fedorka-Cray 2002). The CO tested in the present study exhibited a high antibacterial activity between 0.125 μg/ml and 1.0 μg/ml against these isolates. Although some studies exist in the literature reporting significant antibacterial activity against *Salmonella enterica,* only few of these have specified the serotypes. For example, Yostawonkula et al. (2021) exhibited antibacterial activity of nano/microstructured hybrid composite particles containing CO against *Salmonella enterica*, Bagheri (2020) evaluated an antibacterial activity of CO against *Salmonella enterica* strains, Li et al. (2019) tested antimicrobial properties of hydroxypropyl methylcellulose-cinnamon essential oil emulsions and found effective against *Salmonella* Typhi, Paudel (2019) determined antimicrobial activity of cinnamon oil nanoemulsions on *Salmonella sp*., Chuesiang (2019) found effective cinnamon oil in oil-in-water nanoemulsions against *Salmonella* Typhimurium. Chen et al. (2013), in their study, determined the effect of CO on *Salmonella* Typhimurium proliferation; they reported a 1.3-log reduction (1.3 log) in *Salmonella* Typhimurium count in ground pork after treatment with CO. The minimum concentration required to achieve more than 1-log reduction in *Salmonella* population was 0.8% CO. On the same lines, Brnawi et al. (2018) tested the effects of cinnamon leaf and bark oil on *Salmonella* Typhimurium in strawberry shakes and observed a significant difference ($p< 0.05$) in log reduction of bacterial growth. Upadhyaya et al. (2015) indicated trans-cinnamaldehyde to reduce *Salmonella* Enteritidis colonization on eggshell and in yolk, whereas Todd et al. (2013) found a considerable decrease in bacterial population after CO treatment of infected romaine, iceberg, and spinach with *Salmonella* Newport at high concentrations and long treatment durations. Piovezan et al. (2014) found CO to effectively inhibit *Salmonella* Saintpaul growth at a concentration of 312 μg/ml.

Cinnamon has been found to have a strong antibacterial activity, consistent with other studies. In fact, in the present study, it was determined that multi-drug resistant isolates are sensitive to cinnamon oil and can also be effective as virulence inhibitors. However, to determine the usability of cinnamon in terms of public health and its ability to be added to existing treatment plans, in vivo studies and additional toxicity studies are required, and this situation appears as factors that limit the present study.

CONCLUSION

The results of the present study demonstrate that CO has strong antimicrobial activity against all tested foodborne *Salmonella enterica* isolates and anti-quorum sensing potential. Considering its inhibitory activity, especially against MDR *Salmonella* Infantis isolates, we believe CO could act as a natural antimi-

crobial ingredient in foods, thereby protecting from foodborne pathogens. Additionally, it is a potential candidate for developing new antimicrobial agents against MDR *Salmonella* isolates and also as a quorum sensing inhibitory agent. *Salmonella enterica* serotypes produce autoinducer 2 (AI-2) via the lux S synthase gene to coordinate virulence lux S gene expression with the QS mechanism, depending on the population density. Based on this, it is aimed to study the effectiveness of CO against AL-2 synthesis and QS gene expression of *Salmonella* serotypes in future studies.

CONFLICT OF INTEREST

The authors have no declaration of competing interests.

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