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Original Research Article

Effect of Carvacrol on *Salmonella* Saintpaul Biofilms on Stainless Steel Surface

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Abstract

Purpose: *To evaluate the effect of carvacrol against Salmonella Saintpaul biofilms on stainless steel surface.*

Methods: *The effects of carvacrol on planktonic cells were evaluated by determining the minimum inhibitory concentration and minimal bactericidal concentration. The action of carvacrol on Salmonella Saintpaul biofilms on stainless steel surface was evaluated on established biofilm and on biofilm formation by counting the number of bacterial cells that adhered to the surface using scanning electron microscopy.*

Results: *The antimicrobial activity of carvacrol in planktonic cells of Salmonella Saintpaul was observed. The highest inhibitory effect of carvacrol was observed on biofilm formation at different sub-inhibitory concentrations.*

Conclusion: *Carvacrol reduced the number of bacterial cells that adhered to stainless steel surface, making it a potential compound for Salmonella Saintpaul control.*

Keywords: *Salmonella spp, Biofilm, Planktonic cells, Carvacrol, Stainless steel*

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INTRODUCTION

Salmonella infection constitutes a major public health problem in many countries and millions of cases of salmonellosis are reported worldwide [1]. The ability of *Salmonella* spp. to adhere and form biofilm is an important factor that contributes to its resistance and persistence in different environments, especially in the food processing industry. The surfaces of stainless steel equipment and utensils are known to be major sites of bacterial adhesion and biofilm formation and can consequently lead to food deterioration or foodborne disease transmission [2].

Several strategies for controlling bacterial adhesion to surfaces have been proposed, including the use of natural compounds. Many studies have demonstrated the action of essential oils in the adhesion of bacteria on different surfaces [2-4].

Carvacrol is an important component of the essential oils of oregano and thyme and is considered a broad-spectrum antimicrobial compound [5]. Although the antimicrobial effect of carvacrol is well documented, few studies have evaluated its effects on bacterial biofilms [6-8]. The effects of carvacrol on *Salmonella* spp.

biofilms were evaluated by Knowles and Roller [9] and by Knowles *et al* [10] on *Salmonella typhimurium* NCTC 74 biofilms on stainless steel.

In this context, the objective of the present study was to evaluate the effect of carvacrol on biofilms of *Salmonella* Saintpaul on stainless steel surface.

EXPERIMENTAL

Microorganism

S. Saintpaul used in this research was isolated from raw material used for animal feed and was stored at the Laboratory of Food Microbiology, Department of Biomedicine and Clinical Analysis, State University of Maringá.

Effect of carvacrol on planktonic cells

The minimum inhibitory concentration (MIC) of carvacrol was determined using the broth microdilution method according to M7-A8 of the Clinical Laboratory Standards Institute [11]. Carvacrol (98 % purity) was obtained from Sigma (Steinheim, Germany). The compound was diluted in ethanol and tested at final concentrations that ranged from 19 to 5000 µg ml^{-1}. The MIC was defined as the lowest concentration of carvacrol that inhibited bacterial growth. Two bacterial growth controls that consisted of Mueller Hinton Broth (MHB; Difco, Le Pont de Claix, France) and 0.5 % ethanol (v/v) and one control that consisted of carvacrol in MHB were included. Ampicillin was used as a standard drug at concentrations of 0.5 to 128 µg ml^{-1} against *E. coli* ATCC 25922. Each test was performed in duplicate and repeated three times.

Biofilm formation on steel

Stainless steel surfaces

AISI 304 stainless steel coupons (1 × 8 × 9 mm) were washed with 100 % acetone by immersion, dried, and cleaned with 70 % alcohol (v/v). After hygienization, they were washed with distilled water, dried for 2 h at 60 °C and autoclaved at 121 ºC for 15 min.

Inoculum preparation

To biofilm formation on stainless steel, overnight cultures *S.* Saintpaul at 35 ºC were diluted 1:100 in Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) to obtain approximately 10^7 CFU ml^{-1} (colony-forming units per milliliter), confirmed by counting the CFU on Hektoen agar.

Biofilm formation

The stainless steel coupons were individually placed in sterile microtubes in triplicate that contained 1500 µl of the bacterial inoculum and incubated at 35 ºC for 24 h. After incubation, the contents were aspirated and replaced by 1500 µl TSB, and the microtubes were re-incubated at 35 ºC for 24 h. TSB was used as the negative control, and the positive controls were *P. aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603.

Effect of carvacrol on biofilm

Established biofilms

After biofilm formation on stainless steel for 48 h, the coupons with attached bacteria were washed with 0.85 % sterile saline solution and exposed to carvacrol at concentrations of 39, 78, and 117 µg ml^{-1}, corresponding to 25 %, 50 %, and 75 % of the MIC, respectively, for 1 h at room temperature. The control, consisting of TSB and bacterial inoculum, were included in the experiment. The tests were performed in triplicate and repeated twice.

Biofilm formation

Carvacrol (39, 78 and 117 µg ml^{-1}) was added to the microtubes that contained the coupons and bacterial inoculum. After 24 h, the contents of the microtubes were replaced by 1500 µl TSB with the same concentrations of carvacrol followed by incubation at 35 °C for 24 h. A control of biofilm formation with the same inoculum without carvacrol was included in the assay. The tests were performed in triplicate with two independent repetitions.

Counting of adhered bacterial cells

After 48 h of incubation, the microtube contents were aspirated, and the control coupons without carvacrol and coupons exposed to different concentrations of carvacrol (39, 78, and 117 µg ml^{-1}) were washed with 0.85 % sterile saline solution, placed in the 0.85 % sterile saline solution, and subjected to an ultrasonic bath at 25 Hz for 5 min (Ultra Cleaner 750A, Unique). Serial dilutions were performed in sterile saline solution, plated on MHA (Difco, Le Pont de Claix, France), and incubated at 35 °C for 24 h. The results were expressed in log CFU cm^{-2}.

Scanning electron microscopy

Biofilm formation on stainless steel was analyzed using scanning electron microscopy (SEM). The

coupons were washed with 0.85 % sterile saline solution, fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, and kept for 48 h under refrigeration. The coupons were washed twice in 0.1 M sodium cacodylate buffer and dehydrated in a graded series of ethanol solution (50 %, 70 %, 80 %, and 90 %) and twice in 100 % ethanol. The coupons were subjected to critical-point-dried in CO_2, coated with gold, and examined by scanning electron microscopy (Shimadzu SS-550).

Statistical analysis

The statistical analysis was performed using Statistica 8.0 and 2.14.0 software. We used descriptive measures (mean and standard deviation) followed by t-tests to compare differences between the control group and carvacrol-treated groups. The level of statistical significance was set at $p < 0.05$.

RESULTS

Effect of carvacrol on planktonic cells

Carvacrol was able to inhibit *S.* Saintpaul growth, with a MIC of 156 µg ml^{-1}.

Biofilm formation on stainless steel surface

The number of *S.* Saintpaul cells on stainless steel was approximately 8 log CFU cm^{-2} (Table 1). The bacterial adhesion to stainless steel was uniform (Figure 1).

Effect of carvacrol on established biofilm

The effects of carvacrol on preformed *S.* Saintpaul biofilm on stainless steel are shown in Table 1. The greatest reduction of bacterial counts was observed with 117 µg ml^{-1} carvacrol (75 % of MIC), in which we observed a decrease of approximately 3 log cycles in bacterial counts. However, this reduction was not statistically significant ($p > 0.05$) at any of the subinhibitory concentrations tested (25 %, 50 %, and 75 % of MIC) when comparing the control group with the carvacrol-treated groups.

Effect of carvacrol on biofilm formation

A reduction of the number of *S.* Saintpaul cells on stainless steel after carvacrol treatment is shown in Table 1. A statistically significant reduction ($p < 0.05$) was observed on biofilms treated with carvacrol at concentrations of 39, 78, and 117 µg ml^{-1} (25 %, 50 %, and 75 % of MIC, respectively). SEM (Figure 1) revealed the effects of carvacrol on *S.* Saintpaul biofilm formation on stainless steel. The biofilm was reduced and almost absent at 75 % of the MIC (117 µg ml^{-1}).

DISCUSSION

In the present study, we evaluated the effects of carvacrol on *S.* Saintpaul biofilms. This compound inhibited biofilm formation on stainless steel.

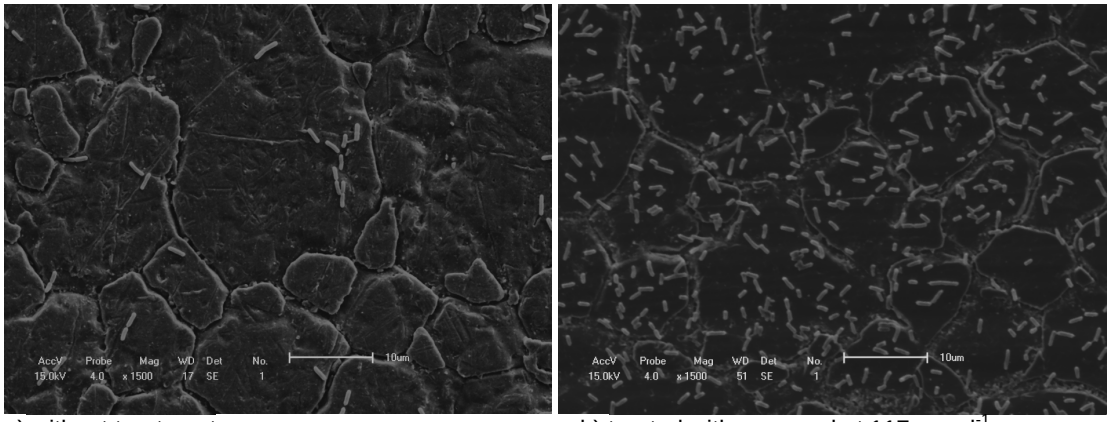
Salmonella spp. biofilms on food contact surfaces are widely spread in food processing environments [2]. The use of carvacrol as an antimicrobial agent is an efficient alternative for *Salmonella* spp. control, however, only few studies have reported the effects of carvacrol on the adhesion of *Salmonella* spp. to stainless steel surfaces [9,10].

The number of *S.* Saintpaul cells on stainless steel after 48 h was 7.61 and 8.77 log CFU cm^{-2}.

Table 1: Effect of carvacrol on preformed *Salmonella* Saintpaul biofilm and *Salmonella* Saintpaul biofilm formation on stainless steel surface

Biofilm	Concentration of carvacrol (µg ml^{-1})*	log CFU cm^{-2}	DP	*p* value **
Preformed	0	7.61	0.900	Reference
	117	4.76	4.179	0.3126
	78	6.49	1.732	0.3762
	39	8.25	0.366	0.3182
Formation	0	8.77	0.113	Reference
	117	5.18	0.233	0.00001***
	78	5.35	0.078	0.00001***
	39	7.00	0.620	0.0082***

Tests were performed in triplicate in two different experiments. **117 µg ml^{-1} (75 % MIC), 78 µg ml^{-1} (50 % MIC), and 39 µg ml^{-1} (25 % MIC);* ** Unpa*ired t-test using a reference group without treatment;* *** *Difference between mean p < 0.05*



a) without treatment b) treated with carvacrol at 117 µg ml^{-1}

Figure 1: Scanning electron microscopy images of *Salmonella* Saintpaul biofilm on stainless steel surface

These findings are consistent with studies that evaluated *Salmonella* spp. biofilm formation on steel, with counts that ranged from 5.26 to 8.01 CFU cm^{-2} after 8-120 h of incubation [12,13]. Kim and Wei [14] showed that the initial adhesion of *Salmonella* spp. to steel occurred after 12 h of incubation, with microcolonies formation within 24 h and mature biofilm formed 48 h later. According to Wirtanen *et al* [4], to consider biofilms, counts above 10^3 CFU cm^{-2} are necessary. Therefore, the results in the present study indicated the formation of biofilms on stainless steel. Our results (Figure 1) showed that bacterial adhesion was uniform on the stainless steel, in which the bacteria were individualized without the presence of aggregated bacteria or an extracellular matrix. Kim and Wei [14] found the formation of microcolonies on the steel surface using SEM when *Salmonella* spp. was grown on turkey and beef broths, but when grown on lettuce broth, adhesion was similar to the present study. In fact, according to Steenackers *et al* [2], the extracellular matrix components of *Salmonella* biofilms vary considerably with environmental conditions, which may explain this result.

In the established biofilm, carvacrol had few effects, the largest of which occurred after treatment with 117 µg ml^{-1} (75 % MIC), but this reduction was not statistically significant compared with the biofilm control (Table 1). Moreover, the biofilm formation results showed that the treatment with the three concentrations of carvacrol resulted in a significant reduction of the number of cells that adhered compared with controls (Table 1).

These results may be attributable to the characteristics of biofilm formation that occur in two phases. During the initial phase, adhesion is reversible, and the bacteria are easily removed by the application of minimal force. In a subsequent phase, the removal of irreversibly adhered cells is difficult in mature biofilm, requiring the application of strong mechanical forces or chemical interruption of the adherence by applying enzymes, detergents, surfactants, disinfectants, or heat [15].

Similar results were observed by Knowles and Roller [9], in which the lowest concentration of carvacrol (0.5 mmol l^{-1}) had no effect on biofilms formed by *S. typhimurium*, but the highest concentration (2.0 mmol l^{-1}) resulted in a 2.6 log CFU/surface reduction, indicating the concentration-response effects of carvacrol.

According to Knowles *et al* [10], during the initial phase of biofilm development (24 h), the number of viable *S. typhimurium* was reduced by 3 log CFU/biofilm when treated with 1 mmol carvacrol per hour. However, a rapid recovery of viable cell numbers was observed that exceeded and eventually equaled the cell numbers on the biofilm without treatment. In mature biofilm, after 12 days of formation, carvacrol (1 mmol l-1/h) also caused a 3 log CFU/biofilm reduction after the initial exposure and an average 1 log reduction during subsequent pulses.

Nostro *et al* [6] also observed significant differences in *S. aureus* and *S. epidermidis* biofilm formation when treated with different concentrations of carvacrol. The concentration of 78 µg ml^{-1} produced a greater reduction in the number of bacterial cells that adhered to the microplate. The authors used SEM and found that the amount of biofilm was reduced in the presence of 39 µg ml^{-1} carvacrol and was almost absent at 78 µg ml^{-1}.

Interesting findings were also reported by Perez-Conesa *et al* [7] who assessed the effects of encapsulated carvacrol on the inactivation of *L. monocytogenes* and *E. coli* O157:H7 biofilms on

stainless steel. The morphological evaluation of biofilm revealed an increasing number of dead cells when the biofilms were treated with sufficiently high concentrations of carvacrol and a reduction of bacterial counts.

CONCLUSION

The present study demonstrated that carvacrol reduced the number of bacterial cells that adhered to stainless steel surface, showing that this compound may be an alternative for *Salmonella* spp. control. The action of carvacrol together with other natural compounds increases its effectiveness against bacterial biofilm and may even replace the use of chemicals to disinfect surfaces during food production.

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REFERENCES

1. *World Health Organization. Drug-resistant Salmonella. [cited 2011 Nov 21]. Available from:http://www.who.int/mediacentre/factsheets/fs139 /en/.*
2. *Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SCJ. Salmonella biofilms: an overview on occurrence, structure, regulation and eradication. J Food Research Int 2012; 45: 502-531.*
3. *Budzyñska A, Wiêckowska-Szakiel M, Sadowska B, Kalemba D, Rózalska B. Antibiofilm activity of selected plant essential oils and their major components. Polish J Microbiol 2011; 60: 35-41.*
4. *Wirtanen G, H Husmark U, Mattila-Sandholm T. Microbial evaluation of the biotransfer potencial from surfaces with Bacillus biofilms after rinsing and cleaning procedures in closed food processing systems. J Food Prot 1996; 59: 727-733.*
5. *Burt S. Essential oils: their antibacterial properties and potential application in foods: a review. Int J Food Microbiol 2004; 94: 223-253.*
6. *Nostro A, Sudano Roccaro A, Bisignano G, Marino A, Cannatelli MA, Pizzimenti FC, Cioni PL, Procopio F, Blanco AR. Effects of oregano, carvacrol and thymol on Staphylococcus aureus and Staphylococcus epidermidis biofilms. J Med Microbiol 2007; 56: 519-523.*
7. *Pérez-Conesa D, Cao J, Chen L, McLandsborough L, Weiss J. Inactivation of Listeria monocytogenes and Escherichia coli O157:H7 biofilms by micelle encapsulated eugenol and carvacrol. J Food Prot 2010; 74: 55-62.*
8. *Iannitelli A, Grande R, Di Stefano A, Di Giulio M, Sozio P, Bessa LJ, Laserra S, Paolini C, Protasi F, Cellini L. Potential antibacterial activity of carvacrol-loaded poly (DL-lactide-co-glycolide) (PLGA) nanoparticles against microbial biofilm. Int J Mol Sci 2011; 12: 5039-5051.*
9. *Knowles J, Roller S. Efficacy of chitosan, carvacrol, and a hydrogen peroxide-based biocide against foodborne microorganisms in suspension and adhered to stainless steel. J Food Prot 2001; 64: 1542-1548.*
10. *Knowles JR, Roller S, Murray DB, Naidu AS. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by Staphylococcus aureus and Salmonella enterica serovar Typhmurium. Appl Environ Microbiol 2055; 71:797-803.*
11. *Clinical Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, approved standard M07-A8. 2009; 29: 1-88.*
12. *Jun W, Kim MS, Cho BK, Millner PD, Chao K, Chan DE. Microbial biofilm detection on food contact surfaces by macro-scale fluorescence imaging. J Food Engineering 2010; 99: 314-322.*
13. *Speranza B, Corbo MR, Sinigaglia M. Effects of nutritional and environmental conditions on Salmonella sp. biofilm formation. J Food Sci 2011; 76: M12-M16.*
14. *Kim SH, Wei CI. Biofilm formation by multidrug-resistant Salmonella enterica serotype Typhimurium phage type DT 104 and other pathogens. J Food Prot 2007; 70: 22- 29.*
15. *Chmielewski RAN, Frank J. Biofilm formation and control in food processing facilities. Int J Food Sci Technol 2003; 2: 22-32.*