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Cinnamon essential oil reduces adhesion of food pathogens to polystyrene

[1]Ferreira, L. R., [1]Rosário, D. K. A., [1]Silva, P. I., [1]Carneiro, J. C. S., [2]Pimentel Filho, N. J. and [1*]Bernardes, P. C.

[1]Food Engineering Department, Center of Agriculture Science and Engineering, Espírito Santo Federal University, Alegre, Espírito Santo 29500-000, Brazil
[2]Center for Natural Sciences, Federal University of São Carlos, Buri, São Paulo 18290-000, Brazil

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Abstract

Substances with antimicrobial properties able to prevent microbial adhesion to the surfaces are of interest to the industry and consumers. Microorganisms attached to surfaces can form mature biofilms that are a risk for public health. In the present work, the antimicrobial activity of the essential oils of rosemary, cinnamon, peppermint, ginger, orange and Tahiti lemon was evaluated in vitro against the pathogenic bacteria *Staphylococcus aureus* (SA), *Escherichia coli* (EC) and *Salmonella enterica* (SE). In addition, the inhibition of adhesion of these foodborne pathogens by cinnamon essential oil was also tested. The cinnamon essential oil showed the lowest MIC values of 6.25%, 3.12% and 3.12% (v/v) for SA, EC and SE, respectively, when compared with the others evaluated oils. Subinhibitory concentration of cinnamon oil such as 0.78% was able to inhibit the adhesion of all foodborne pathogens to polystyrene surface. The result emphasises the potential application of essential oils as an alternative natural compound that can help to prevent the bacterial adhesion on surfaces, extending the shelf life and improving the sensory characteristics of foods.

Introduction

Diseases caused by the consumption of food contaminated by pathogenic microorganisms are a public health concern (Bajpai *et al.*, 2015). Contamination by these microorganisms can occur through handlers with poor hygiene practices, contaminated water and raw materials, and cross contamination (Brugnera *et al.*, 2011). A wide diversity of pathogenic and spoilage microorganisms is able to adhere and form biofilms, and failures in hygienic procedure favour adhesion process and biofilm establishment in food processing surfaces (Andrade, 2008).

Biofilms are microbial cells immobilised in a matrix of extracellular polymers that act as an independent ecosystem (Muazu *et al.*, 2015). The adhesion of microorganisms and biofilm formation capacity depend on parameters such as pH, temperature, growth phase of cells, growth medium, contact time, and type and properties of the surface material (Dimakopoulou-Papazoglou *et al.*, 2016). Therefore, certain conditions throughout the food chain could enable biofilm formation on biotic and abiotic surfaces (Bridier *et al.*, 2015). Extracellular polymeric substances, which are part of the mature biofilm, act as a barrier and provide protection to the cells, allowing microorganisms better resistance to stress conditions such as treatment with sanitisers and other antimicrobial agents (Pimentel-Filho *et al.*, 2014). Biofilms can also cause corrosion in equipment, and by releasing pathogenic or spoilage cells, act as constant contamination points throughout the food chain (Boari *et al.*, 2009). They are difficult to remove and according to Muazu *et al.* (2015), approximately 80% of human infections can be associated with biofilms. Worldwide data have highlighted *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* spp. as the major food pathogens involved in food poisoning outbreaks and infections, and they are able to form biofilms.

Staphylococcal food poisoning is a common foodborne disease in several countries. *S. aureus*, a Gram-positive opportunist bacterium, adapts easily in a wide variety of environments. However, it is poorly competitive, and only presents a greater risk of

*Corresponding author.
Email: patricia.bernardes@ufes.br

contamination in food where the normal microbiota is already inhibited or destroyed. One of the biggest causes of toxin formation by *S. aureus* in food is related to temperature abuse during storage (Rode *et al.*, 2007).

E. coli, a Gram-negative bacterium, has high versatility, ranging from a harmless gut commensal to an intra- or extra-intestinal pathogen. Apart from gastrointestinal problems, *E. coli* may produce enterotoxins responsible for urinary tract infections. It is the predominant species between facultative anaerobic bacteria of the intestinal tract, and develops more easily in multiple species biofilms (Beloin *et al.*, 2008).

Salmonella is a Gram-negative pathogenic bacterium spread throughout the world, and considered one of the most common causes for foodborne diseases. It is often detected in a variety of animal products that, when consumed under-cooked, can lead to the development of acute gastroenteritis characterised by vomiting and diarrhoea (Wang *et al.*, 2016).

Currently, it is usually accepted that the foodborne pathogens grow predominantly as biofilm on solid surfaces, both in their natural habitats and human surrounding. Biofilms of foodborne pathogens are widely present in a variety of food processing sites and many foodborne outbreaks have been linked to them (Wang *et al.*, 2016).

With growing demands for foods with fewer additives, due to their possible toxicity, and consumers demanding for healthier foods with less preservatives, the search for natural products with antimicrobial properties is also growing (Aquino *et al.*, 2010). Many spices for flavouring foods and their essential oils could serve as alternatives to reduce the growth of microorganisms and the consequent deterioration of foods, preventing also adhesion and biofilm formation (Silva *et al.*, 2010).

Essential oils are secondary metabolites of plants and complex mixtures of biologically active volatile substances (Bajpai *et al.*, 2015). In plants, they play an important role in the development and growth, as well as response to stress and pathogen attack. These antimicrobials can be used as natural preservatives (Siddiqua *et al.*, 2015). Some studies have demonstrated the inhibitory effects of essential oils such as those from thyme, oregano and cinnamon on microbial adhesion and biofilm formation (Nuryastuti *et al.* 2009; Soni *et al.*, 2013; Szczepanski and Lipski, 2014).

Therefore, the present work aimed to evaluate in vitro antimicrobial activity of essential oils against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enterica*, and evaluate the subinhibitory concentrations of cinnamon essential oil on the adhesion of these pathogenic bacteria to polystyrene.

Materials and methods

Essential oils

Essential oils extracted from rosemary (*Rosmarinus officinalis*), cinnamon (*Cinamommum cassia*), ginger (*Zingiber officinale*), peppermint (*Mentha piperita*), sweet orange (*Citrus sinensis*) and Tahiti lemon (*Citrus aurantifolia*) were kindly provided by Phytoterápica Company, São Paulo, Brazil. According to the manufacturer, the main components of essential oils are: 1,8 cineol 48%, camphor 12%, beta-pinene 8% (rosemary); cinnamic aldehyde 81%, coumarin, benzaldehyde, cinnamic alcohol and styrene 3% (cinnamon); zingiberene 33% (ginger); i-menthol 33%, menthone 30%, eucalyptol 6%, menthol acetate 4% (peppermint); L-menthol 33%, menthone 30%, eucalyptol 6%, menthol acetate 4% (sweet orange); and D-limonene 55%, P-cymene 8%, terpinene and A-pinene 5%, myrcene 1.5%, neral 0.5% (Tahiti lemon).

Microorganisms

Gram-positive bacterium *Staphylococcus aureus* ATCC 6538 (SA) and Gram-negative bacteria *Escherichia coli* ATCC 11229 (EC) and *Salmonella enterica* subsp. *enterica* (SE) were used. SE was isolated from vegetables commercialised in the city of Alegre, Espírito Santo, Brazil, and identified by the sequencing of the 16S rRNA gene in a previous work (data not published).

In all tests, the suspensions of microorganisms were standardised. The suspensions of microorganisms were activated twice in Brain Heart Infusion (BHI) broth (Himedia, India), and incubated at 35°C for 18–24 h. To obtain isolated colonies, streaking was done in Petri dishes containing plate count agar (PCA, Himedia, India), and incubated at 35°C for 18–24 h. Isolated colonies were selected to prepare the bacterial inoculum in saline solution at 0.85% (w/v). The optical density of bacterial inocula was adjusted spectrophotometrically at 600 nm (Thermo Scientific Multiskan Go, USA) to give approximately 1.0×10^8 cfu.mL^{-1} ($Abs_{600nm} = 0.1$). For the evaluation of growth and adhesion on polystyrene plates, cultures were used after being activated twice in BHI broth and incubated at 37°C for 18 h.

Disc diffusion assay

Disc diffusion assays were carried out following the methodology of Clinical And Laboratory

Standards Institute (CLSI, 2003) with modifications. Aliquot of 0.1 mL bacterial suspension (Abs_{600nm} = 0.1) was spread onto Müller-Hinton agar plate. Discs (6 mm) were placed on the surface of the agar impregnated with 5 µL of each pure essential oil separately. Inoculated plates were incubated at 37°C for 24 h. At the end of incubation, the diameter of inhibition zones was measured with a ruler and recorded in mm. Ampicillin (10 µg/disc) and tetracycline (30 µg/disc) (Bio-Rad, France) were used as positive controls, and sterilised distilled water as negative control.

Microdilution assay

In order to determine the Minimal Inhibitory Concentration (MIC), 200 µL nutrient broth was supplemented with increasing concentrations of the essential oils. A two-fold dilution factor was used, ranging from 0.78 to 50% v/v, and essential oils were transferred to 96-wells microtiter plates. Microtiter plates were inoculated with 5×10^5 cfu. mL^{-1} of exponentially growing cells of EC, SA, and SE previously propagated in nutrient broth and confirmed by plate counts. Tween 80 at 0.1% (v/v) was employed to increase the solubility of essential oils. Wells of plates treated with essential oils without microorganisms and wells without essential oils were used as control. The minimal concentration that prevented growth after 24 h incubation at 37°C was designated as MIC as measured by absorbance at 500 nm (Pimentel-Filho *et al.*, 2014).

Adhesion testing on polystyrene surface

Based on the results from disc diffusion assay and MIC for the tested oils, cinnamon essential oil was selected to evaluate its effect on the bacterial adhesion. Subinhibitory concentrations of 0.78% and 1.56% (v/v) were tested. Assays were carried out using the same experimental design as previously described for MIC experiments. Following 48 h incubation, the optical density of total cells (planktonic and adhered cells) was determined at 600 nm. Afterwards, the culture supernatant was discarded, and the surface-attached cells were stained with 200 µL 0.1% (w/v) crystal violet for 30 min. Subsequently, the crystal violet was removed, and the plate was washed three times with water. Following air-drying for 15 min at 40°C, the attached cells were determined at 590 nm with the microtiter plate reader (Thermo Scientific Multiskan Go, USA) by addition of 200 µL 95% (v/v) ethanol (Pimentel-Filho *et al.*, 2014). Data were expressed as the ratio between the absorbance of violet crystal extract (adhered cells) and the optical density of total cells (Viana *et al.*, 2009). This ratio was used to unlink growth and adhesion. Ratio below 1.0 indicates that adhesion is smaller than growth. Wells inoculated with microorganisms without cinnamon oil served as control.

Statistical analysis

The experiment was conducted under completely randomised design (CRD) with three repetitions in duplicate. Data from the agar diffusion test were analysed with the aid of the system software for Statistical Analyses (SAEG, 2007), and were subjected to analysis of variance (ANOVA) and Tukey's test at 5% of probability. Means with $p < 0.05$ were considered significantly different.

Table 1. Antimicrobial activity of essential oils (EOs) by disc diffusion assay.

Essential oil	Diameter of inhibitory zone (mm)		
	Bacteria		
	Staphylococcus aureus	*Escherichia coli*	*Salmonella enterica*
Cinnamon	18.2 ± 1.8^{aA}	19.7 ± 1.8^{aA}	15.5 ± 2.8^{aA}
Pepper mint	12.4 ± 2.3^{bA}	5.8 ± 0.3^{cB}	NA
Rosemary	9.6 ± 2.5^{bcA}	7.9 ± 1.8^{cA}	9.0 ± 1.2^{bA}
Sweet orange	7.8 ± 0.6^{bcA}	8.7 ± 1.4^{cA}	7.9 ± 0.7^{bA}
Tahiti lemon	6.8 ± 0.4^{c}	NA	NA
Ginger	6.0 ± 2.2^{c}	NA	NA
Ampicillin	$20.8 \pm 2,5^{aA}$	$16.0 \pm 1,8^{bB}$	NT
Tetracycline	$23.0 \pm 1,6^{aA}$	$20.0 \pm 1,3^{aB}$	NT

Data are means of three repetitions (n = 3) ± standard error. Means followed by similar lowercase in a column, and similar uppercase in a row do not differ by Tukey's test ($p > 0.05$). NA: no activity. NT: not tested.

Results and discussion

Disc diffusion assay

Disc diffusion assay was performed to evaluate the potential inhibitory effect of the essential oils against foodborne pathogens at 37ºC for 24 h. There was a significant difference ($p < 0.05$) in the size of the inhibition halos of the different essential oils studied for all three tested microorganisms. The essential oil of cinnamon significantly differed from other essential oils for all target microorganisms, with the largest halos of inhibition (Table 1).

Of the six essential oils studied, three (cinnamon, rosemary, sweet orange) did not significantly differ ($p > 0.05$) between Gram-negative and Gram-positive bacteria thereby showing a good spectrum of action (Table 1). While the essential oil of peppermint had no inhibitory effect on SE, the essential oils of

Table 2. Minimum inhibitory concentration (%) of essential oils (EOs) using broth microdilution assay.

Bacteria	Minimum inhibitory concentration (%)					
	Rosemary	Cinnamon	Ginger	Pepper Mint	Sweet Orange	Tahiti Lemon
Staphylococcus aureus	25.00	6.25	> 50.00*	6.25	25.00	25.00
Escherichia coli	12.50	3.12	> 50.00*	6.25	12.50	50.00
Salmonella enterica	25.00	3.12	50.00	6.25	12.50	50.00

*no activity at the maximum concentration (50.00% v/v) tested.

Tahiti lemon and ginger showed inhibitory effect only against SA. All essential oils tested had some inhibitory effect on SA. The largest inhibition was observed by cinnamon essential oil, followed by peppermint, rosemary and sweet orange. The latter two did not significantly differ from the essential oils of Tahiti lemon and ginger. In general, Gram-negative bacteria (SE, EC) were less sensitive to the action of essential oils when compared with Gram-positive bacterium (SA). This could probably be due to the difficulty of the compounds to act on the complex structure of the cell wall of Gram-negative. This result corroborates the work of Kotzekidou *et al.* (2008) wherein among the species tested, the most sensitive to essential oils were the Gram-positive bacteria *S. aureus*, *Listeria monocytogenes* and *Bacillus cereus*. According to Nazzaro *et al.* (2013), generally, Gram-negative bacteria are more resistant to the essential oils than Gram-positive bacteria. The cell wall structure of Gram-negative bacteria is more complex and consists of an outer membrane (consisting of a bilayer of phospholipids and lipopolysaccharides) and a thin layer of peptidoglycan firmly connected by lipoproteins. The outer membrane contains abundant amounts of porins, which serve as hydrophilic transmembrane channels, allowing the passage of small hydrophilic solutes. The hydrophobic character of essential oils would be the reason why Gram-negative bacteria are relatively more resistant when compared with Gram-positive. The outer membrane is therefore enough, but not totally impervious to hydrophobic molecules. As the cell wall of Gram-positive bacteria consists of peptidoglycan bound to other molecules such as proteins and teichoic acid, this structure enables hydrophobic molecules such as essential oils to act on the cell wall and cytoplasm as they penetrate more easily.

According to Lucena *et al.* (2015), the hydrophobic nature of certain components of natural products such as essential oils, inhibit the growth of microorganisms by means of several mechanisms. Growth inhibition can occur due to alterations in the permeability of the cell membrane, causing the suspension of vital cellular activity, or by the direct action in the respiratory chain and production of energy due to the interactions of the components of the essential oils with lipid bilayer of the cell membrane.

Trajano *et al.* (2009) analysed antimicrobial activity of 11 essential oils, including cinnamon, mint, black pepper, rosemary and ginger, against 10 strains of Gram-positive and Gram-negative bacteria. They reported that among the oils tested, cinnamon showed greater inhibitory action.

Minimal inhibitory concentration of essential oils

Cinnamon essential oil showed the lowest MIC values of 6.25%, 3.12% and 3.12% (v/v) for SA, EC and SE, respectively (Table 2). The lower inhibitory action on these pathogens was observed on the essential oil of ginger whose MIC was not possible to be determined for SA and EC, and only inhibited the growth of SE at the maximum concentration tested (50% v/v).

SE was the only one whose MIC was detected for all essential oils. However, in the agar diffusion test, in which all essential oils were evaluated at a concentration of 100% (v/v), SE growth was not inhibited by essential oils of peppermint, Tahiti lemon and ginger. In the agar diffusion test, it is necessary to have diffusion of the compound through agar in order to reveal an inhibitory effect. The diffusion of antimicrobial agent through agar depends on its chemical composition and interaction with the components of culture medium. On the other hand, in the test for determining the MIC, the contact between essential oil and the microorganism was enhanced as they were in liquid medium. This may explain the absence of inhibition of some oils in agar diffusion test, so it is important that more than one antimicrobial test be performed.

The greatest growth inhibition of EC was observed when it was exposed to cinnamon essential oil. The MIC value for Tahiti essential oil was 50% (v/v) and greater than 50% (v/v) for ginger essential oil. In agar diffusion assay, Tahiti lemon and ginger

essential oils had no effect against EC which confirms the low effect of such oils on this bacterium.

For SA, cinnamon and peppermint essential oils showed the lowest MIC values (6.25% v/v). In the disc diffusion test, cinnamon essential oil showed the greatest inhibition halo, 18.2 mm, significantly differing from others ($p < 0.05$).

According to Burt (2004), natural compounds such as essential oils can act on bacterial cells by disintegration of the cell membrane by destabilising the proton force, electron flow, active transport and coagulation of cell contents. However, considering that essential oils have many different groups of chemical compounds, the antibacterial activity may be related to its composition and the mechanism of action cannot be assigned to a specific mechanism. Besides, there may be other targets in the cell, not only the cytoplasmic membrane. The direction for proper use of essential oil may be closely related to its composition. High concentrations can denature proteins and low concentrations can interfere with the activity of enzymes involved in the energy production of the cell (Tiwari *et al.*, 2009).

Therefore, MIC evaluation is necessary because natural products are constantly subjected to changes by several factors, e.g., the secondary metabolism of plants which originate the essential oils, which if altered, will also alter its composition. Some of these factors are related to planting and growth of the plants such as soil, climate, altitude, brightness and growing region or factors involving processing and oil storage after extraction (Grossman, 2005).

Silva *et al.* (2010) analysed the effect of essential oils on *E. coli* and *Salmonella* spp. isolated from humans and ATCC cultures. Cinnamon essential oil showed the lowest MIC values for the studied microorganisms thereby confirming its high inhibitory activity. The active compounds present in the essential oil of cinnamon such as eugenol and cinnamaldehyde are responsible for causing damage to the structure of bacterial cell wall and has the capacity to interfere with the synthesis of certain bacterial enzymes (Matan *et al.*, 2006).

Hoffman *et al.* (1999) compared the antimicrobial activity of essential oils of cinnamon, cloves, ginger and mint in concentrations of 10%, 1% and 0.1% against 21 microorganisms (seven yeasts and 14 bacteria, including *S. aureus* and *Salmonella*) and showed that cinnamon and mint essential oils caused greater inhibition. The essential oil of ginger was not effective as an antimicrobial agent, corroborating the results obtained in the present work.

Nabavi *et al.* (2015) presented several studies with results indicating that cinnamon essential oil significantly reduced the bacterial growth rate of samples artificially contaminated as compared to the control (uncontaminated). In many studies with foodborne pathogens in food matrices (e.g. meat and cheese), positive effect on microbial growth inhibition was observed. The inhibitory activity was attributed by several authors to their bioactive components, especially cinnamaldehyde, suggesting that essential oil of cinnamon could be used as natural alternative for food preservative for reducing or inhibiting the growth of spoilage and pathogenic bacteria, and thus extending the food self-life.

Inhibition of adhesion on polystyrene surface by cinnamon essential oil

Subinhibitory dosages (0.78% and 1.56% v/v) of cinnamon essential oil were tested for their capacity to inhibit adhesion of the three foodborne pathogens; EC, SA, and SE on polystyrene 96-well microtiter plates after 48 h incubation by using crystal violet method. Adhesion was estimated by calculating the ratio between absorbance of violet crystal extract and optical density of total cells. The presence of essential oil reduced adhesion of all bacteria (Figure 1). The highest concentration evaluated was able to completely inhibit bacterial adhesion. Even though bacterial adhesion was reduced, EC was the most adherent and resistant strain to the lowest concentration of the oil. Although EC and SE are Gram-negative, they have different virulence factors and appendages that certainly interfere with the effect of the essential oil causing differences in their sensitivity profile.

Microorganisms attached to surfaces can form biofilm which are a risk for public health. Biofilms are a challenge for food industries where the need for surfaces free from contamination is essential. Biofilm-associated cells are protected by an extracellular matrix consisting mainly of exopolysaccharides, proteins, DNA and lipids (Flemming and Wingender, 2010), and its effective removal requires in most of the times substantial mechanical treatment (Simões *et al.*, 2010). There is a large and increasing interest among the industries in substances able to prevent biofilms formation. The results obtained in the present work revealed that presence of subinhibitory dosages of cinnamon oil inhibited the biofilm formation on polystyrene surface by all three foodborne pathogens. Normally, the application of essential oils against pathogens and food spoilers in preformed biofilms require high concentrations (Kavanaugh and Ribbeck, 2012). However, many studies have reported the potential activity of cinnamon oil on microbial biofilms. Low

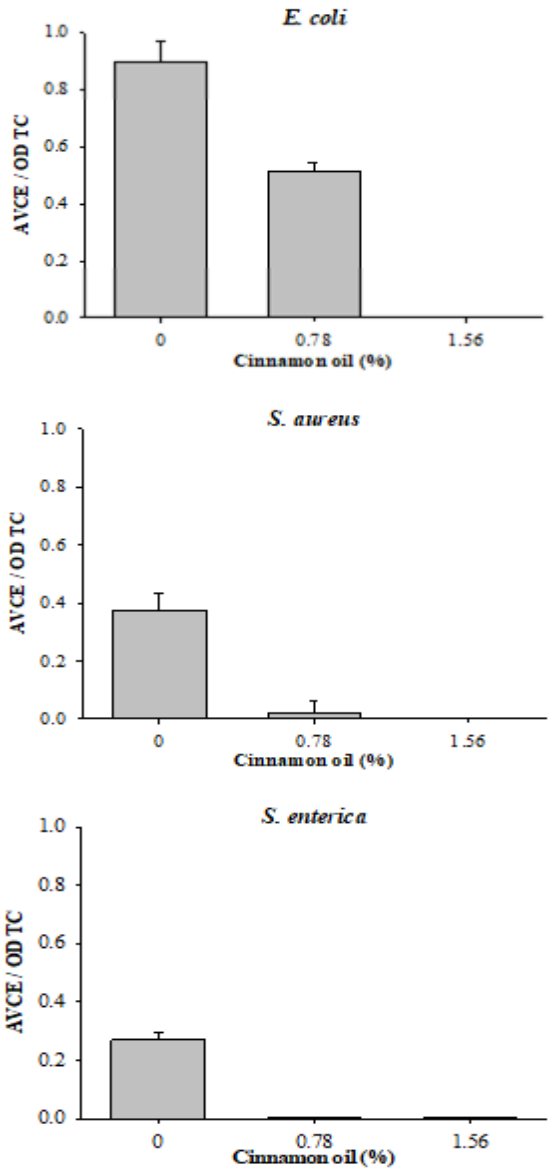

Figure 1. Ratio between the absorbance of violet crystal extract (AVCE) and the optical density of total cells (OD TC) of *E. coli, S. aureus* and *Salmonella* following cinnamon oil treatment. Bacteria were cultivated in nutrient broth for 48 h in the presence of different subinhibitory concentrations of cinnamon EO. Untreated control is shown.

dosages of cinnamon oil such as 0.5% and 1.0% were effective to reduce biofilm formation on polystyrene by *Staphylococcus epidermidis*, a notorious bacterium known for its high capability to form biofilms on medical devices (Nuryastuti *et al.*, 2009). Studies conducted by Szczepanski and Lipski (2014) showed that biofilm formation on polystyrene surface by the strain *Acinetobacter* F-Fü-04 Ibca was completely inhibited at 0.016% of cinnamon essential oil, and its MIC was 0.063%. Compared with commercial chemical sanitisers, cinnamon oil proved to be an efficient alternative in reduction or elimination of sessile cells of *E. coli* and *L. monocytogenes* (Oliveira *et al.*, 2012). Other studies have shown the inhibitory effect of natural antimicrobial compounds against biofilms formation by spoilage and pathogenic bacteria such as thyme oil, oregano oil and carvacrol (Soni *et al.*, 2013), coriander oil (Duarte *et al.*, 2013), the bacteriocin nisin (Cabo *et al.*, 2009; Davison *et al.*, 2010), bovicin HC5 (Pimentel-Filho *et al.*, 2014), and roselle calyx (*Hibiscus sabdariffa* L.) extract (Sulistyani *et al.*, 2016), among others.

Conclusion

The obtained data reinforce the idea that natural agents have potential applicability to prevent adhesion and to control biofilm formation. Essential oil can help to prevent the bacterial adhesion on surfaces, extending the shelf life and improving the sensory characteristics of foods. More studies evaluating the combination of antimicrobial substances must be performed to increase anti-adhesion effect and minimise the bacterial resistance, which is a common response of treated cells.

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